EPA and DHA Modulate Macrophage-Derived Inflammation and Subsequent Skeletal Muscle Inflammation

by

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A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Human Health and Nutritional Sciences

Guelph, Ontario, Canada

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ABSTRACT

EPA AND DHA MODULATE MACROPHAGE-DERIVED INFLAMMATION AND SUBSEQUENT SKELETAL MUSCLE INFLAMMATION

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Macrophage-derived inflammation contributes to chronic inflammation in adipose tissue in obesity and is also linked to the development of skeletal muscle (SM) insulin resistance. The long-chain n-3 PUFA have been shown to modulate cytokine secretion from macrophages, though subsequent effects on SM inflammation and function are unknown. A model of macrophage conditioned media (MCM) was used to examine effects of n-3 PUFA on macrophage inflammation and consequent effects on SM cells. Treatment of RAW 264.7 macrophages with long-chain n-3 PUFA decreased LPS-induced MCP-1 and IL-6 gene expression and MCP-1 secreted protein. In turn, MCM from n-3 PUFA-treated macrophages decreased TNF-α and IL-6 gene expression in LPS-stimulated L6 SM cells, but did not affect insulin-stimulated pAkt content. Long-chain n-3 PUFA did not affect gene expression of inflammatory signaling intermediates NF-κB and TLR4. Overall this thesis suggests that long-chain n-3 PUFA are important nutritional strategies for reducing macrophage-derived inflammation, with ensuing benefits in SM inflammation.
Acknowledgements

I would first like to thank my advisor, Dr. Lindsay Robinson, for her enthusiasm, support, and encouragement throughout my graduate studies. It has been wonderful to work with such a passionate researcher, who is so dedicated to her work and to her family. I aspire to be able to achieve such a balance in my own life. Thank you for your understanding and kindness throughout all of the significant life events I’ve seen during my time here at Guelph, and for your generous help in achieving my future goals.

I would also like to extend a thank you to my advisory committee, Dr. David Wright and Dr. David Dyck for their most helpful suggestions and insight as to the direction of my project. Meeting with such experienced and knowledgeable researchers can be intimidating, but you both made it a pleasant experience, full of constructive feedback, and I am grateful for that. Thank you also for the use of your laboratory equipment during my studies.

Thank you to the chair of my examination, Dr. David Ma for his engaging discussion with regards to my thesis. Thank you to Dr. Kelly Meckling and Dr. Jim Kirkland for the use of their cell culture room and related equipment during my time here at Guelph. Thank you also to Dr. David Mutch and Dr. David Ma for the use of their laboratory equipment, especially during the brief time when our own equipment was in for repair.

A big thank you to my fellow Robinson labmates, Dr. Justine Tishinsky, Anna De Boer, and Mary Cranmer-Byng for patiently teaching me the techniques I needed to succeed and for all your insightful discussion throughout my project. Thank you for always being available, even during your busiest times, and for making the lab environment such a fun place to work. A most sincere thank you to Dr. Jennifer Monk, who helped me to believe my goals were attainable and whose invaluable assistance with data analysis helped me to complete this project in a timely
manner. Thank you also to Danyelle Liddle, a future Robinson lab student, who was a very enthusiastic and meticulous assistant during the latter half of this project.

In addition to the Robinson lab, I have been fortunate to have met and worked with a number of amazing students and researchers who have helped to shape the researcher I am today. Thank you to you all. In particular, thank you to Dr. Ashley Patterson and Kristin Marks for being teachers, role models and friends as I have navigated my way through my undergraduate and graduate studies.

I must also extend a thank you to my “non-academic” friends and family. To my 5 high school girlfriends and the two Sarah’s, thank you for keeping me grounded in reality and for being a constant source of humour and encouragement. To my mom and dad and younger brother, thank you for your unrelenting support, despite not fully understanding what it is that I study. To my husband Peit, thank you for sticking with me throughout it all. Your belief in my abilities is sometimes stronger than my own and this achievement is both of ours.

Lastly, thanks be to God, without whom none of this would be possible.
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## List of Abbreviations

ACC acetyl-CoA carboxylase  
ALA alpha-linolenic acid  
AMPK AMP-activated protein kinase  
BSA bovine serum albumin  
CLS crown-like structures  
CPT-1 carnitine palmitoyltransferase-1  
CCR2 C-C chemokine receptor type 2  
COX-2 cyclooxygenase-2  
DHA docosahexaenoic acid  
DAG diacylglycerol  
DMEM Dulbecco’s Modified Eagle’s Medium  
EPA eicosapentaenoic acid  
ERK1/2 extracellular signal-regulated kinases 1/2  
FBS fetal bovine serum  
FFA free fatty acid  
GLUT4 glucose transporter type 4  
HFD high fat diet  
IFN-γ interferon-γ  
IκBα inhibitor kappa B α  
IKK-β inhibitor kappa B kinase β  
IL interleukin  
IMTG intramuscular triacylglycerol
iNOS inducible nitric oxide synthase (also known as Nos 2)
IR insulin resistance/insulin resistance
IRS-1 insulin receptor substrate-1
IRAK interleukin-1 receptor-associated kinase
JNK c-Jun N-terminal kinase
LA linoleic acid
LPL lipoprotein lipase
LPS lipopolysaccharide
MCM macrophage conditioned media
MCP-1 monocyte chemoattractant protein-1
mTOR mammalian target of rapamycin
MyD88 myeloid differentiation primary response protein 88
NF-κB nuclear factor-κB
p38 MAPK p38 mitogen-activated protein kinases
PA palmitic acid
PAI-1 plasminogen activator inhibitor-1
PBS phosphate buffered saline
PGC-1α peroxisome proliferator-activated receptor gamma co-activator 1α
PI3K phosphoinositide 3 kinase
PPARγ peroxisome proliferator-activated receptor γ
PUFA polyunsaturated fatty acids
S6K1 S6 kinase 1
SAT subcutaneous adipose tissue
SEM standard error of mean
SFA saturated fatty acid
SIRT1 sirtuin 1
SOCS-1 and 3 suppressor of cytokine signaling-1 and 3
TAG triacylglycerol
TIRAP toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR 2/4 toll-like receptor 2/4
TNF-α tumour necrosis factor-α
TRAF6 TNF receptor-associated factor 6
TZD thiazolidinedione
Chapter 1: Review of Literature

1.1: Introduction

Obesity, a condition characterized by an excess accumulation of fat, has become a global epidemic. In Canada, 23% of the population is obese, accounting for an estimated $1.6 billion in direct health care costs (1). Of particular concern is the strong relationship between obesity and associated pathologies such as insulin resistance and type 2 diabetes (2-4). There are a number of factors that may contribute to the development of insulin resistance in a state of obesity. The following section will focus on adipose tissue-derived inflammation and the roles of adipokines as communication signals with other cells and tissues, as these are most pertinent to this thesis.

1.2: Adipose Tissue-Derived Inflammation

Chronic inflammation present in adipose tissue in obesity may be involved in the development of insulin resistance in skeletal muscle (2-5). Adipose tissue is an active endocrine organ known to secrete a variety of soluble protein signaling molecules collectively called adipokines (2), which are involved in a number of physiological processes, including metabolism and energy homeostasis (6). In the lean state, such adipokines are secreted in appropriate amounts in response to various signals (7). In obesity, there is an increase in secretion of pro-inflammatory adipokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (8), contributing to a state of chronic low-grade inflammation. Cells of the innate and adaptive immune system that infiltrate the adipose tissue in obesity also contribute to this state of chronic inflammation, increasing secretion of pro-inflammatory factors, and decreasing secretion of anti-inflammatory factors such as IL-10 (9, 10). Adipokines derived from intra-muscular adipose tissue may interact in a paracrine fashion with surrounding skeletal muscle, while adipokines secreted from other depots, such as the
visceral fat depot, may act in an endocrine fashion, traveling through the circulation to exert
effects on peripheral skeletal muscle (2, 11). Further details regarding the association between
the IL-6, TNF-α, MCP-1 and IL-10 and obesity-associated insulin resistance can be found in the
following section.

1.2.1: Interleukin-6

Protein Structure and Evidence of Association with Obesity

Murine IL-6 is a 21710 Da protein formed of a sequence of 187 amino acids that is
similar in biological activity and structure to human IL-6 (12). Approximately one third of
circulating human IL-6 originates from adipose tissue, the majority from the stromal-vascular
fraction, with very little originating from adipocytes (6, 13). Circulating levels are correlated
with adiposity, insulin resistance, and type 2 diabetes (6, 13, 14). Macrophage infiltration into
the adipose tissue occurs in the obese state, and macrophages isolated from the adipose tissue of
ob/ob mice express IL-6 mRNA (4). Further, expression of IL-6 is higher in macrophages that
are recruited to the adipose tissue following a high fat diet, compared to the resident, non-
inflammatory macrophages (15). Treating 3T3-L1 adipocytes with macrophage conditioned
media (MCM, the media collected following the incubation of RAW 264.7 macrophages) also
increased expression of IL-6 in the adipocytes (3). IL-6 can be synthesized from skeletal muscle
that has been stimulated with inflammatory agents such as lipopolysaccharide (LPS), reactive
oxygen species or the inflammatory cytokines TNF-α and IL-1β (16).

Signaling, Functional Roles and Association with Insulin Resistance

IL-6 acts by binding to a Class I cytokine receptor, a complex consisting of two
membrane glycoproteins, which directly bind IL-6, and two signal-transducing glycoproteins
(gp130), required for high affinity binding (17). IL-6 is involved in the regulation of a number of
biological processes including haematopoiesis, immune responses and host defence mechanisms (12, 13), and inflammation (12, 18). Important to the study of obesity and related diseases, IL-6 also plays a role in lipid and carbohydrate metabolism (18). In humans and rodents, IL-6 was negatively associated with insulin sensitivity, and was shown to down-regulate lipoprotein lipase activity, and increase lipolysis (4, 6, 13). In rodents, IL-6 was also shown to increase serum TAG levels, specifically by increasing hepatic TAG secretion (19). Cell culture studies have identified the suppressor of cytokine secretion (SOCS)-3 as a protein target of IL-6, the activation of which inhibits insulin signaling (14). Interestingly, research involving knock-out mouse models has yielded conflicting results. Wallenius et al (2002) found that IL-6−/− mice developed mature-onset obesity while being fed a standard diet, while Di Gregorio et al (2004) found that IL-6−/− mice fed a high fat diet were in fact protected and gained less weight than their wild type controls. This discrepancy may be a result of the differing roles of IL-6 in the central nervous system and peripheral tissues (20).

There is also evidence that IL-6 may be anti-inflammatory and insulin sensitizing. IL-6 is synthesized and released from contracting muscle, when insulin responsiveness is enhanced, and IL-6 has been shown to stimulate the release of IL-1 receptor antagonist (IL-1ra) and IL-10, two anti-inflammatory cytokines (21). It has also been shown to inhibit the production of TNF-α in vitro (16). IL-6 has been shown to increase fatty acid oxidation and an acute treatment of L6 myotubes with IL-6 has also been shown to increase basal and insulin-stimulated glucose uptake and GLUT4 translocation (22). All of these effects were abolished in cells infected with an AMPK dominant-negative adenovirus (22), indicating that AMPK is involved in the signaling mechanism. This inconsistency with regards to IL-6 and insulin resistance may indicate different
effects of this cytokine depending on whether it is synthesized and released from skeletal muscle following a bout of exercise, or from adipose tissue in a state of chronic inflammation (16).

**Modulation**

Secretion of IL-6 is modulated by a number of factors including hormones, hypoxia, oxidative stress, inflammation, and diet and exercise (13, 18). Activation of nuclear factor (NF)-κB and c-jun N-terminal kinase (JNK)-1, both involved in the Toll-like receptor (TLR) inflammatory signaling pathway, increase the expression of the IL-6 gene (13, 23, 24). The state of chronic inflammation associated with obesity also increases secretion of IL-6. A component of Gram-negative bacteria, LPS, is often used to mimic the inflammatory response and has been shown to act through TLRs and NF-κB to increase adipose tissue levels of human IL-6 in vivo (13). Cell lines, which were established from the mesenteric fat of wild type, db/db, and ob/ob mice, and cultured with LPS, also showed increased production of IL-6 (25). TNF-α and IL-6, both elevated in obesity, can increase amounts of secreted IL-6 in vitro (13). Diet-induced weight loss in obese humans has been shown to reduce adipose tissue gene expression of IL-6 (26) and serum IL-6 concentration (27). IL-6 is also modulated by fatty acids. This will be discussed in a subsequent section.

**1.2.2: Tumour Necrosis Factor-α**

**Protein Structure and Evidence of Association to Obesity**

Mouse cachectin is a 17 kDa protein that is homologous to human TNF-α, with similar biological functions (28, 29). TNF-α has been shown to be a multifunctional cytokine, involved in the induction of insulin resistance, anorexia, and weight loss (6) and in the development of inflammation and obesity (30). In humans, TNF-α expression is associated with body adiposity (6, 14), and fasting insulin and TAG concentrations (6). TNF-α is primarily secreted from
macrophages (2, 4, 26, 31) and, in humans, it acts locally in the adipose tissue (26, 31). It is involved in increasing expression of pro-inflammatory cytokines, such as plasminogen activator inhibitor (PAI)-1, MCP-1 (30), IL-6 (3), and leptin (32).

**Signaling**

TNF-α exerts its effects through two receptors, TNFR1 (p55 in rodents, p60 in humans) and TNFR2 (p75 in rodents, p80 in humans), both of which are glycoproteins with a single transmembrane domain (33, 34). It binds with equal affinity to both receptors (33-35), however TNFR1 is thought to be the receptor most involved in LPS- and TNF-α- induced toxicity and development of insulin resistance, while TNFR2 is thought to be involved in the suppression of TNF-induced inflammatory actions and disruption of the insulin signaling pathway (33, 36).

**TNF-α and the Development of Insulin Resistance**

TNF-α has been shown to reduce insulin sensitivity (37). Intravenously treating male fa/fa rats with TNFR-IgG (a TNF-α neutralizer) increased the glucose infusion rate during hyperinsulinemic-euglycemic clamps (indicating improved insulin sensitivity) (37). More recent *in vitro* models have also provided evidence for the involvement of TNF-α in the development of insulin resistance (38, 39). TNF-α is able to activate S6 Kinase 1 (S6K1), a protein downstream from Akt and mammalian target of rapamycin (mTOR) (38). S6K1 phosphorylates IRS-1 at 4 different serine residues, inactivating insulin receptor substrate (IRS)-1 and preventing the insulin signaling cascade (38). Inhibitor kappa B kinase (IKK)2 was found to be an integral component of the signaling pathway (38). A separate study has also shown that TNF-α activates IKKβ and JNK, additional kinases capable of phosphorylating Serine 307 on IRS-1, inactivating it (40). The ability of TNF-α to disrupt insulin signaling at IRS-1 has also been shown to be mediated through phosphoinoside 3 kinase (PI3K), Akt and mTOR (41) and p38 mitogen
activated protein kinase (MAPK) (39). In addition, SOCS-3, a protein generally known to
suppress cytokine signaling, shows increased mRNA expression in the adipose tissue of obese
animals within one hour of a TNF-α injection (42). Though this study only looked at mRNA
expression of SOCS-3, this increased expression was associated with a reduced tyrosine
phosphorylation of IRS-1, thus preventing the interaction of IRS-1 with the insulin receptor and
PI3K (42). Lastly, TNF-α is capable of reducing the activity of proteins important in lipid
metabolism, as demonstrated by reduced levels of phosphorylated AMPK in mice treated for 24
hours with TNF-α, resulting in a reduction in fatty acid oxidation and the accumulation of toxic
lipids in peripheral tissues (43). Further discussion of the impact of fatty acid accumulation in
ectopic tissues can be found in a subsequent section.

Modulation

TNF-α gene expression is modulated by a number of factors, including stimulation by
LPS, viruses, and TNF-α itself, and down-regulation by drugs, such as phosphodiesterase
inhibitors, glucocorticoids, and thiazolidinediones (TZDs) (44). Important to the study of
obesity and insulin resistance are the anti-diabetic TZDs, namely troglitazone, pioglitazone, and
rosiglitazone (2, 11). Treatment of human skeletal muscle cells with 5 mmol/L of troglitazone
reversed the impairments in insulin signaling induced by 2.5 nmol/L of TNF-α (11). Similarly,
treating 24 subjects with impaired glucose tolerance for 10 weeks with pioglitazone reduced
plasma TNF-α levels (2). Both TZDs and the long-chain n-3 PUFA fatty acids are ligands for
peroxisome proliferator-activated receptor (PPAR)γ (45), suggesting a similar mechanism of
action. Discussion of fatty acid-induced modulation of TNF-α will be covered in a subsequent
section.
1.2.3: Monocyte Chemoattractant Protein-1

Protein Structure

Chemokines control chemotaxis, the movement of basophils, neutrophils and monocytes from the circulation to cells (46). Smooth muscle cell chemotactic factor (SMC-CF) and monocyte chemotactic and activating factor (MCAF), isolated from animal and human cells respectively, were both identified as chemokines that specifically attracted monocytes (47-50). Both SMC-CF and MCAF were later re-named monocyte chemoattractant protein (MCP)-1, a protein characterized as a C-C chemokine with its first two of four cysteine residues located adjacent to one another (49, 51-53). It is thought to be the human homologue to the product of the murine JE gene (49, 52). Rat MCP-1 has also been sequenced and found to be 148 amino acids in length, and similar to mouse MCP-1 (54). MCP-1 is produced by macrophages and endothelial cells (55), as well as human adipocytes, usually in response to IL-1, IL-4 or TNF-α (56).

Evidence of Association with Obesity and Insulin Resistance

Studies have shown that MCP-1 expression is increased in adipose tissue of both genetic and diet-induced obese mice (55-58) and that mice overexpressing MCP-1 have an even greater concentration of plasma MCP-1 than diet-induced obese mice (55). As mentioned previously, macrophages infiltrate the adipose tissue in obesity, and such infiltration has been shown in both genetic and diet-induced obese mice (55-57). Further, studies have shown the genetic deletion of MCP-1 in mice results in a reduction in macrophage infiltration and a protection from the development of insulin resistance (59). MCP-1 may also contribute to the development of obesity by disrupting lipid metabolism. MCP-1 has been shown to down-regulate the lipoprotein lipase (LPL) gene in 3T3-L1 adipocytes (56). Supporting this data, knock-out mice that did not express MCP-1 but who consumed a high fat diet had reduced adipose tissue fat pads, reduced
increases in adipocyte size, and reduced macrophage infiltration into adipose tissue (55). LPL is also present in skeletal muscle, however the effects of MCP-1 on LPL gene expression in this tissue are currently not known. Concerning the association between MCP-1 and insulin resistance, overexpression of MCP-1 in mice was found to induce insulin resistance, specifically by increasing the expression of hepatic gluconeogenic enzymes (55). Treatment of 3T3-L1 adipocytes or human skeletal muscle cells with MPC-1 also reduced insulin-stimulated Akt phosphorylation (60, 61) and insulin-stimulated glucose uptake (61). MCP-1 was shown to induce effects by activation of extracellular signal-regulated kinases (ERK)1/2 (61).

**Signaling**

All chemokines act by binding to similar 7-transmembrane spanning (7-TMS), G protein-coupled receptors and analysis of the signal transduction pathways has shown involvement of PI3K and MAPK (62). MCP-1 binds specifically to C-C chemokine receptor (CCR)2, of which two isoforms exist: CCR2A and CCR2B (62, 63). The CCR2 receptor has been shown to modulate inflammation. Adipose tissue macrophage content was reduced in CCR2<sup>−/−</sup> obese mice, as was expression of TNF-α (64). Further, CCR2<sup>−/−</sup> obese mice had lower fasting blood glucose and insulin concentrations, and were more insulin sensitive than obese wild type mice (64). Tamura et al (2010) also found improvements in insulin sensitivity in mice fed a high fat, high sugar diet supplemented with a CCR2 inhibitor compared to mice fed an un-supplemented diet. Mice fed the supplemented diet also had reduced adipose tissue macrophage infiltration and adipose tissue MCP-1 and TNF-α expression (5).

**Modulation**

Gene expression of MCP-1 is influenced by a number of factors, including LPS (54) and free fatty acids (FFAs) (57). TLR4 and NF-κB have been shown to be involved in the signal
cascade, both in the FFA-induced increase in MCP-1 gene expression and the LPS-induced increase in MCP-1 secretion (57, 65), linking the chronic inflammation in obesity to the regulation of MCP-1 expression and secretion. Anti-diabetic TZD drugs have also been shown to affect MCP-1, decreasing its expression in 3T3-L1 adipocytes and in the adipose tissue of ob/ob mice (57). MCP-1 is also modulated by fatty acids and this will be discussed in a subsequent section.

1.2.4: Interleukin-10

Protein Structure

IL-10 was first characterized as a cytokine released by murine T-helper-2 (Th2) cells that inhibited the mRNA expression and synthesis of IL-2 and interferon (IFN)-γ from murine Th1 cells (66). Human and murine IL-10 are similar in structure, composed of 160 and 157 amino acids respectively (67). IL-10 is also similar in structure to IFN-γ (67). IL-10 is now known to be synthesized from a variety of immune cells including Th2 cells, leukocytes, monocytes, macrophages, dendritic cells, B cells, cytotoxic T cells, natural killer cells, mast cells and neutrophilic and eosinophilic granulocytes (68).

Signaling

The IL-10 receptor is composed of two chains of trans-membrane glycoproteins, IL-10R1 and IL-10R2, and is part of the Class II cytokine receptor family (68). IL-10 first binds to IL-10R1, forming an IL-10 receptor complex that then binds to IL-10R2 (69). Binding of IL-10 leads to the downstream activation of signaling proteins Janus-activated kinase 1 (associated with IL-10R1) and tyrosine kinase 2 (associated with IL-10R2), which in turn phosphorylates
and activates signal transducer and activation of transcription (STAT)3, the activation of which is required to produce the anti-inflammatory actions of IL-10 (68, 70).

**Anti-Inflammatory Role of IL-10 and Association with Obesity and Insulin Resistance**

IL-10 is involved in regulating a number of immunological processes, most importantly inflammation (68) and this has been shown to positively affect insulin sensitivity. In macrophages, IL-10 has been shown to inhibit the mRNA expression and production of LPS-induced TNF-α and IL-6 (71). Treating RAW 264.7 macrophages with 500 μM palmitic acid in the presence of IL-10 also reduced the palmitic acid-induced increase in secreted TNF-α (72).

IL-10 has been shown to increase production of IL-1ra, which binds to the receptors for the pro-inflammatory cytokine IL-1, preventing any inflammatory response (73, 74). Treatment of 3T3-L1 adipocytes with IL-10 resulted in reduced MCP-1 secretion (59). Further to this, IL-10 prevented TNF-α-induced reductions in GLUT4 and insulin receptor protein, as well as prevented TNF-α-induced tyrosine phosphorylation of IRS-1 in these cells (59). Mice treated with a combination of IL-6 and IL-10 were protected from whole body insulin resistance induced by treatment with IL-6 alone (75). Including IL-10 in a triglyceride emulsion given to mice also prevented lipid-induced skeletal muscle insulin resistance (75). To further investigate the role of macrophage-specific IL-10 in obesity, mice with IL-10 deletion in haematopoietic cells were developed (72). Contrary to previous data indicating a modulatory role for IL-10 in macrophages, there were no differences in glucose and insulin intolerance between control mice and mice with IL-10 deletion in haematopoietic cells, nor were there any differences in gene expression of inflammatory signaling intermediates (72). The authors did find an increased expression and secretion of IL-10 from the adipose tissue of the mice with the IL-10 deletion,
and they hypothesized that this may be compensating for the IL-10 deletion in the haematopoietic cells (72).

**Modulation**

Macrophage secretion of IL-10 is induced by LPS and β-adrenergic agonists (68). NF-κB was found to be an important inflammatory signaling intermediate involved in the LPS-induced increase in IL-10 gene expression (76). IL-6 has also been shown to increase IL-10 expression in T cells, and STAT3 was found to be an important transcription factor involved in the up-regulation (76). Recently, a micro RNA (miRNA) known as has-miR-106a was found to be involved in regulation of IL-10 expression in Raji and Jurkat human lymphocyte cells (77).

Immune cell-derived IL-10 is also regulated by fatty acids, which is discussed in a subsequent section.

**1.3: Saturated Fatty Acids and the Development of Skeletal Muscle Insulin Resistance**

In addition to the increased secretion of pro-inflammatory adipokines from the adipose tissue in obesity, there is also an increase in circulating free fatty acids. Excess fatty acid spillover from the expanding adipose tissue mass into the blood results in subsequent storage of these fatty acids in ectopic tissues, such as skeletal muscle (78). Dietary fatty acids, in particular saturated fatty acids, are able to disrupt the insulin signaling pathway through inflammatory pathways. Saturated fatty acids are thought to activate inflammatory pathways by binding to TLR4 and its co-receptors MD2 and CD14 (24) which, in turn, activates the signal sequence of TIRAP, MyD88, IRAK, TRAF6, TAK1 and IKKβ, ultimately leading to the phosphorylation and degradation of inhibitor kappa B (IκB)-α and removing the inhibition on NF-κB, allowing NF-κB to enter the nucleus (24). The end result of this sequence of events is increased pro-inflammatory gene expression, the consequences of which have been previously mentioned.
Activation of TLR4 also activates the signal sequence of MAPKs, and subsequently JNK/ERK (24). Kinases involved in the TLR4/NF-kB and MAPK inflammatory pathways, in particular IKK-β and JNK, are able to phosphorylate the serine 307 residue on IRS-1, disrupting the insulin signaling pathway (79).

Multiple studies have provided evidence of saturated fatty acid-induced activation of inflammatory pathways, and of increased expression of pro-inflammatory cytokines in skeletal muscle. For example, C212 myotubes treated with 500 µM of palmitic acid (16:0) for 16 hours had increased expression of pro-inflammatory TNF-α, and both the ERK/MAPK and NF-κB inflammatory pathways were involved in the response (80). Treatment of primary human myotubes with 250 µM palmitic acid for up to 48 hours increased IL-6 mRNA and protein production (81). Similarly, palmitic acid was found to activate the degradation of IκB-α within one hour, liberating NF-κB and allowing it to bind to DNA in the nucleus of the cell (81). Stearic acid (18:0) has also been shown to increase the phosphorylation of IκB-α and the p65 subunit of NF-κB in L6 skeletal muscle cells (82). Stearic acid was shown to increase IL-6 and MCP-1 mRNA, and the effects were abolished if the cells were pre-treated with a TLR4 inhibitor (82). In separate experiments, inhibition of TLR4, NF-κB (83), or IKK (84) prevented palmitic acid-induced insulin resistance, providing further strong evidence for the involvement of this inflammatory pathway in saturated fatty acid-induced insulin resistance. Green et al (2011) also found that a dose of palmitic acid induced NF-κB in L6 rat skeletal muscle cells. A dose of 750 µM, however, for 16 hours was required to elicit the result (85). ERK was found to be the most involved MAPK in the pathway, and the results were blunted if an AMP-activated protein kinase (AMPK) activator was included in the treatment (85). Treating C2C12 myotubes with 0.75-1 mM of palmitic acid for 16 hours was also shown to induce the expression of cyclooxygenase
COX-2, an enzyme responsible for the formation of pro-inflammatory prostaglandins (86). NF-κB and p38 MAPK were found to be involved (86). Increased expression was abolished with the inclusion of a monounsaturated fatty acid in the incubation (86), emphasizing differential effects of dietary fatty acids in skeletal muscle inflammatory responses.

1.4: Long-chain n-3 Fatty Acids as a Therapeutic for Inflammation in Obesity

Contrary to the effects of saturated fatty acids, long-chain n-3 or omega-3 polyunsaturated fatty acids (PUFA) are generally thought to be anti-inflammatory and have the potential to reduce adipose tissue-associated inflammation and development of subsequent pathologies.

1.4.1: Health Effects and Metabolism of Long-Chain n-3 PUFA

Long-chain n-3 or omega-3 polyunsaturated fatty acids, specifically docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), are antiarrhythmic (87) and anti-inflammatory in nature, and have been shown to lower plasma triacylglycerol (TAG) levels and increase plasma HDL-cholesterol levels (88). In order to increase blood levels of EPA and DHA, α-linolenic acid (ALA), or EPA and DHA must be consumed, either from food sources or supplements (89). ALA is converted to EPA and DHA through a series of elongation and desaturation reactions (88). Competition exists for the enzymes (e.g. Δ6 desaturase) involved in this conversion pathway, as long chain n-6 fatty acids, derived from linoleic acid (LA), are synthesized through the same pathway (88). In addition, as synthesis of EPA and DHA from ALA is an inefficient process (88) direct consumption of EPA and DHA remains the best method to increase long-chain n-3 PUFA in vivo, in order to take advantage of their numerous health benefits discussed in this thesis.
1.4.2: Regulation of Metabolic Dysfunction in Adipose Tissue in Obesity

In addition to maintaining a healthy blood lipid profile, EPA and DHA can also modulate metabolic dysfunction in adipose tissue in a state of obesity (43, 89-91). In C57BL/J6 mice, a high fat diet supplemented with EPA and DHA was shown to reduce adiposity, likely as a result of the increased β-oxidation seen in the epididymal fat (92). EPA and DHA also increased expression of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α and nuclear respiratory factor (NRF)-1, two regulatory factors of mitochondrial biogenesis (92), which may also have contributed to the increase in fat oxidation. EPA and DHA have also been shown to increase β-oxidation through AMPK (88). AMPK acts as a metabolic sensor that, when activated, phosphorylates and deactivates acetyl-CoA carboxylase (ACC), leading to a decrease in malonyl-CoA content (93). Malonyl-CoA is involved in lipogenesis and also inhibits carnitine palmitoyltransferase-1 (CPT-1), the main transporter of fat into the mitochondria for oxidation (93). Thus, activation of AMPK reduces lipogenesis and increases β-oxidation. Interestingly, mice fed a high fat diet substituted with EPA and DHA had increased levels of total and phosphorylated AMPK, as well as an increased ratio of phosphorylated to total ACC in their epididymal fat, indicating a state favoured towards oxidation (88). Macrophage infiltration into the epididymal adipose tissue was also reduced by EPA and DHA (88) and will be further discussed in a subsequent section.

1.4.3: Modulation of Obesity-Associated Inflammation

EPA and DHA also modulate the expression and secretion of cytokines in obesity. Male Wistar rats fed a high fat, cafeteria style diet supplemented with 1 g/kg of body weight with EPA in ethyl ester form had reduced adipose tissue expression of TNF-α (94). A slightly lower dose of 0.5 g/kg of EPA fed to Goto-Kakizaki rats (spontaneously develop type 2 diabetes) reduced
adipose tissue gene expression of IL-6, and reduced skeletal muscle gene expression of IL-6 and TNF-α (95). Similarly, db/db mice fed a high fat diet supplemented with EPA and DHA had a significantly reduced adipose tissue gene expression of MCP-1 and near significant reduction in TNF-α compared to animals fed a high fat saturated fat diet (96). Macrophage infiltration into the adipose tissue was also reduced by the n-3 PUFA supplemented diet (96). Further discussion of the modulatory effects of n-3 PUFA on macrophage-derived inflammation can be found below.

In addition to prevention, supplementation with n-3 PUFA can also act to reverse existing inflammation. C57BL/6J mice fed a high fat diet for 6 weeks, followed by a high fat diet supplemented with EPA for a further 5 weeks had significantly lower levels of IL-6 in their epididymal fat than high fat fed controls (97). In overweight humans, serum TNF-α and IL-6 levels were reduced compared to control following consumption of 1.25 or 2.5 g of EPA and DHA per day for 4 months (98), while plasma levels of IL-6 were also decreased in individuals consuming 3.36 g of EPA and DHA per day for 8 weeks (99). Gene expression of MCP-1, IL-6 and CD40 (an M1 macrophage marker) was also reduced in the subcutaneous adipose tissue of n-3 PUFA supplemented patients (99).

1.4.4: Prevention of Insulin Resistance

As previously mentioned, inflammation may be involved in the development of insulin resistance, suggesting a potential therapeutic role for the anti-inflammatory n-3 PUFA (2-5). EPA and DHA have been shown to reduce insulin resistance, although conflicting data exists (100). One of the first studies to demonstrate the beneficial effects of n-3 PUFA was Storlien et al (1987). In this study, rats fed a high fat safflower oil (rich in n-6 PUFA) diet had a significantly reduced glucose infusion rate and decreased rate of glucose disposal during a
hyperinsulinemic euglycemic clamp (101). These effects were eliminated in rats fed a high fat diet supplemented with tuna oil (rich in n-3 PUFA) (101). Similar results were seen more recently in male C57BL/6 mice fed a high fat diet supplemented with EPA and DHA (102). n-3 PUFA have also been shown to reverse previous impairments in insulin sensitivity. For example, rats fed a high fat saturated fat diet for 4 weeks had reduced levels of insulin-stimulated glucose transport, while rats fed the same high fat saturated fat diet for 4 weeks, followed by a high fat saturated fat diet supplemented with 7.9% long-chain n-3 PUFA (4.1% EPA, 0.8% docosapentaenoic acid and 3.0% DHA) for an additional 4 weeks showed similar responses to insulin stimulation compared to control-fed animals (103). Transgenic restoration of n-3 PUFA has also provided evidence of the effectiveness of n-3 PUFA on prevention of the detrimental effects of a high fat diet. The fat-1 mouse expresses the fat-1 n-3 fatty acid desaturase enzyme and is capable of converting n-6 fatty acids into n-3 fatty acids, such that feeding these mice a diet rich in n-6 fatty acids results in a tissue n-6:n-3 ratio of 1:1 (104). Feeding wild type mice a high fat diet (55% kcal from fat, with an n-6:n-3 ratio of 18:1) increased fasting insulin levels and decreased glucose clearance, and such negative consequences were eliminated in the fat-1 mouse (104), suggesting a role for n-3 fatty acids.

While the benefits of n-3 PUFA in the prevention of insulin resistance have been shown in the above rodent studies, conflicting results exist for studies involving humans. For example, Crochemore et al (2012) found that insulin resistance, as evaluated by HOMA-IR actually increased with daily intake of 2.5 and 1.5 g of fish oil per day for 30 days. A separate study examining the effects of 2 g of fish oil per day for 2 months did not find any differences in insulin sensitivity as measure by HOMA-IR (105). Thus, although n-3 PUFA have been shown
to favourably alter the inflammatory secretory profile of adipose tissue and macrophages, their subsequent effects on skeletal muscle inflammation and function remain unresolved.

1.4.5: Mechanisms of Action

Numerous mechanisms have been proposed for the action of long-chain n-3 PUFA. Most relevant to this thesis is the TLR4/NF-κB signaling pathway.

**TLR4/NF-κB Signaling Pathway**

EPA and DHA have been shown to modulate inflammation through the TLR4 signaling pathway (24). TLR4 is primarily responsible for recognizing LPS in the cell wall of gram-negative bacteria and initiating an innate immune response (24, 106, 107). As mentioned earlier, evidence has shown that saturated fatty acids such as palmitic and lauric acid are also capable of binding to TLR4 and initiating a response (108). Once a ligand binds to TLR4 and its associated proteins CD14 and MD-2, myeloid differentiation factor 88 (MyD88) is recruited to the TIR domain of TLR4 (109). MyD88 then interacts with IRAK and TRAF-6, leading to the activation of IKK-β, which phosphorylates and degrades IκB-α, freeing NF-κB and allowing it to translocate to the nucleus to increase the gene expression of IL-6, TNF-α, and MCP-1 (24). Recruitment of MyD88 also activates MAPKs such as JNK and ERK1/2 (109). Lauric acid has been shown to activate NF-κB through this pathway in RAW 264.7 cells (109). Effects were inhibited by a mutant TLR4 (109). Pre-treatment of cells with DHA for 2 hours inhibited NF-κB activity and this inhibition was shown to be mediated by TLR4, not MyD88, indicating that DHA modulates TLR4 and its associated proteins and not the downstream signaling pathway (109). In a separate study by the same research group, DHA was shown to inhibit LPS-induced increases in COX-2, iNOS, and IL-1α in RAW 264.7 cells as well as prevent LPS-induced IκB-α degradation (108). In 3T3-L1 adipocytes, treatment with 200 μM of EPA also resulted in
significantly reduced NF-κB activation compared to both control and arachidonic acid-stimulated cells (97). Most recently, it has been suggested that n-3 PUFA reduce expression of pro-inflammatory genes by deacetylating NF-κB, through a pathway involving AMPK and sirtuin (SIRT)1 (110).

1.4: Macrophage Classification

A variety of cell types infiltrate the adipose tissue in obesity, including cells of the innate and adaptive immune system such as macrophages, CD+ T cells, CD4+ T-helper-1 and -2 cells, regulatory T cells, B cells, mast cells, neutrophils and eosinophils (9). These cells are capable of secreting cytokines and other pro-inflammatory factors and contribute to the development of insulin resistance (9). Though macrophages may be involved in the development of obesity-associated insulin resistance, they are also involved in a number of important processes, such as removal of apoptotic cells and tissue remodelling and repair (111, 112). The environment surrounding the macrophage is involved in determining its particular phenotype and function. Following exposure to certain cytokines and inflammatory agents, macrophages are generally characterized as one of two phenotypes (113). (59, 114)(59, 114)The M1 phenotype or “classically activated” macrophages are induced by LPS and interferon (IFN)-γ, and are generally considered pro-inflammatory as they secrete TNF-α, IL-6 and IL-12 (59, 113, 114). In contrast, the M2 phenotype or “alternatively activated” macrophages are induced by IL-4 and IL-13 and are considered anti-inflammatory due to lower expression of pro-inflammatory cytokines and higher levels of IL-10, an anti-inflammatory cytokine (59, 114). M2 macrophages also produce higher levels of arginase, an enzyme responsible for inhibiting iNOS activity (59). Most recently, a third subpopulation of infiltrating macrophages has been identified (115). These macrophages are recruited to the adipose tissue following a high fat diet, but express the genes
for arginase-1 and YM1, suggesting that these macrophages promote tissue repair and the resolution of inflammation in obesity (115).

Different macrophage phenotypes are associated with lean and obese-insulin resistant states. M2 macrophages are predominantly present in the adipose tissue of lean and CCR2<sup>-/-</sup> mice (59) and are found interspersed in the space around adipocytes (59). The macrophages recruited to adipose tissue following a high fat diet express higher levels of IL-6, iNOS and CCR2 compared to resident macrophages (116) and are located in clusters known as crown-like structures (CLS) around dead adipocytes (59). Overall, existing evidence suggests that high fat feeding results in a higher proportion of M1 pro-inflammatory macrophages in adipose tissue, potentially contributing to the increased secretion of pro-inflammatory cytokines in obesity (59, 116). Similarly, M1 macrophage marker genes in skeletal muscle biopsy samples were found to be positively associated with fasting plasma glucose and HbA1c levels in lean and obese normal glucose tolerant and type 2 diabetic individuals (117). Further, M2 macrophage marker genes in skeletal muscle biopsy samples were correlated with increased rates of glucose disposal (ie. improved insulin sensitivity) in patients who had undergone one year of a prescribed exercise program (117), providing further evidence of a difference in macrophage phenotype in individuals with differing insulin sensitivity.

**1.5: Macrophage Infiltration in Obesity and the Development of Insulin Resistance**

In addition to infiltrating the adipose tissue, macrophages also infiltrate skeletal muscle in obesity, making up 40% of total cells (4). Pro-inflammatory secretions from activated macrophages contribute to the state of inflammation within skeletal muscle, and reduce skeletal muscle response to insulin (118, 119).
Macrophage infiltration has also been proposed to contribute to the development of insulin resistance through the activation of inflammatory pathways in the macrophages (119-122). A number of studies using genetically modified macrophages have supported this hypothesis. Arkan et al (2005) first demonstrated this relationship using mice with specific IKK-β knock out in hepatocytes (IKK-β\textsuperscript{Δhep}) and myeloid cells (IKK-β\textsuperscript{Δmye}). IKK-β\textsuperscript{Δmye} mice had improved insulin sensitivity compared to controls as measured by glucose tolerance tests following 20 weeks of a high fat diet (121). IKK-β\textsuperscript{Δmye} mice also showed an increased peripheral glucose disposal rate following a euglycemic-hyperinsulinemic clamp (121) indicating specific effects of myeloid cell inflammation on skeletal muscle function. The presence of the protein kinase JNK1 in macrophages was also shown to contribute to the development of insulin resistance. Wild type mice reconstituted with JNK1\textsuperscript{−/−} mouse bone marrow had reduced expression of pro-inflammatory cytokines in the liver, and improved insulin sensitivity (123). Most recently, expression of SOCS-1 in macrophages was found to be an important regulator of insulin sensitivity and macrophage activation (124). Mice with a macrophage-specific deletion of SOCS-1 developed hepatic insulin resistance, and stimulation of the macrophages in the genetically altered mice with LPS or palmitic acid resulted in significantly greater increases in macrophage TNF-α and IL-6 gene expression (124). Thus, there is growing evidence of the effects of macrophage-specific inflammation in the development of insulin resistance.

In addition to the above research examining pro-inflammatory factors, studies have also examined the effects of anti-inflammatory proteins and transcription factors in macrophages and their subsequent effects on the development of insulin resistance. For example, mice with a specific macrophage deletion of PPARγ became significantly more insulin resistant compared to controls following 18 weeks of a high fat diet (122). In addition, though these mice had reduced
macrophage infiltration into the adipose tissue, macrophage gene expression of Nos2 and IL-6 was elevated, indicating the increased presence of classically activated macrophages (122). Induction of Arginase I, a gene associated with alternatively activated macrophages, was also reduced following IL-4 stimulation in these PPARγ KO macrophages (122). Studies such as this provide further evidence of the specific effects of macrophages in the development of insulin resistance.

1.6: Modulation of Macrophage-Derived Inflammation by n-3 PUFA

Similarly to adipose tissue-derived inflammation, macrophage-derived inflammation can be modulated by n-3 PUFA. Pre-treating RAW 264.7 macrophages with 114 μM EPA, followed by LPS, significantly reduced the LPS-induced TNF-α gene expression and secretion from the cells, as well as NF-κB activity (125). In a subsequent study, EPA was found to inhibit p44/p42 (ERK1/2) activation and activating protein (AP)-1 activity (126). A number of studies have also examined the use of a commercial emulsion of DHA and EPA on macrophage inflammation. Novak et al (2003) found that pre-treatment of 264.7 macrophages resulted in reduced LPS-induced TNF-α expression and secretion from the macrophages (127). Most recently, co-treating RAW 264.7 macrophages with 100 μM n-3 PUFA and LPS resulted in reduced expression of TNF-α, IL-6, IL-1β, and iNOS (110).

With regards to the anti-inflammatory IL-10, treating obese patients with dyslipidemia with 1.8 g of EPA daily resulted in reduced serum IL-10 and reduced monocyte IL-10 gene expression (128). Further work in THP-1 cells found that treatment with EPA resulted in a reduced expression and secretion of IL-10 that was PPARγ-dependent (128).

There have also been reports of differential effects of EPA and DHA. Oliver et al (2012) pre-treated J774.2 murine macrophages with either 50 μM EPA or 50 μM DHA prior to an LPS
stimulus and found that only pre-treatment with DHA was able to decrease secretion of TNF-α and increase secretion of IL-10 from LPS-stimulated cells. In another study from the same research group, DHA was also shown to have more potent effects than EPA, reducing IL-6 and IL-1β secretion from THP-1 human macrophages to a greater extent than reductions seen with EPA (129).

1.7: Models to Study the Effect of Fatty Acids on Macrophage-Muscle Communication

1.7.1: Macrophage Conditioned Media (MCM)

Various research groups have used a model of MCM to investigate the relationship between macrophage-derived inflammation, fatty acids and muscle function. Samokhvalov et al (2009) incubated L6 skeletal muscle cells for 24 hours in LPS- and palmitic acid-activated MCM and found that the muscle cells responded differently to each. LPS-MCM resulted in reduced expression of TNF-α, increased expression of IL-6 and MCP-1, and increased insulin-stimulated glucose uptake and GLUT4 translocation (118). These apparent beneficial effects of LPS-MCM were attributed to an increase in secretion of the anti-inflammatory IL-10 from the macrophages (118). Palmitic acid-MCM increased expression of TNF-α and MCP-1 in the L6 skeletal muscle cells, as well as increased protein levels of IκB-α and phosphorylated JNK and p38 MAPK (118). Palmitic acid-MCM also decreased insulin-stimulated glucose uptake and GLUT4 translocation (though the decrease was non-significant) (118). IL-10 added to the palmitic acid-MCM prevented this decrease (118). Most recently, the same research group has used this model to demonstrate the involvement of two novel protein kinase C isoforms, theta (θ) and epsilon (ε), in palmitic acid-MCM-derived impairments in insulin signaling and insulin-stimulated glucose uptake (130). Other research groups have used this model to show the effects of activation of various inflammatory pathways and kinases. MCM from fatty acid-activated murine bone
marrow-derived dendritic cells reduced the net increase in insulin-stimulated glucose uptake in L6 cells and this was prevented if the cells were harvested from TLR2/4 knockout mice (131), implicating the TLR2/4 signaling pathway in the immune cell-associated insulin resistance. Similarly, L6 skeletal muscle cells incubated in MCM from palmitic acid- and LPS-activated wild type peritoneal macrophages exhibited reductions in insulin-stimulated glucose uptake, and this was abolished if the cells were incubated in MCM from Jnk1−/− macrophages (123). Although previous research has shown that LPS-activated macrophages increase insulin-stimulated glucose uptake, the origin of the macrophages (cell line compared to primary cells) may account for the differences in results. While the effects of macrophage-derived inflammation on skeletal muscle inflammation and function have been examined in a number of studies using a model of MCM, the role of the long-chain n-3 PUFA in this model has not been well studied.

1.7.2: Model of Induced Inflammation Using LPS

Elevated levels of LPS have been hypothesized to contribute to inflammation and insulin resistance as obese patients with type 2 diabetes and genetically obese mice have elevated circulating levels of LPS (132-134), a condition known as metabolic endotoxemia (135). Given this, LPS has frequently been used to study metabolic endotoxemia and obesity-associated low-grade inflammation (82, 136, 137). For example, in L6 skeletal muscle cells, activation of the MAPK and NF-κB inflammatory pathways with LPS stimulation was associated with impaired glucose uptake into the cells and disrupted insulin signaling (82), while pre-treating the cells with a TLR4 inhibitor blocked the effects of LPS (82). LPS-induced inflammation has further been used to test the anti-inflammatory properties of certain dietary-related compounds. Flavonoids were shown in one study to decrease the production of TNF-α and IL-6 from LPS-treated L6 skeletal muscle cells (136). Tributyrin (a triacylglycerol composed of three butyrate
fatty acids) was also shown to be anti-inflammatory, reducing the increase in serum TNF-α following a LPS injection (138). Contrary to these dietary compounds, long-chain n-3 PUFA have not been well studied in this model, particularly in skeletal muscle cells.

1.8: Conclusion

Both pro- and anti-inflammatory cytokines are secreted by a variety of cells within adipose tissue in the obese state, most notably by infiltrating macrophages, contributing to an inflammatory environment that has been subsequently linked to the development of insulin resistance in skeletal muscle given that cytokines can act as communication signals between such tissues. Long chain n-3 PUFA have been shown to counteract macrophage inflammation in adipose tissue in obesity through modulation of cytokine production, however subsequent effects on skeletal muscle function are currently unknown. Investigation into the modulatory effects of n-3 PUFA on macrophage inflammation and subsequent effects on skeletal muscle inflammation and insulin response will increase our knowledge of the therapeutic potential of such dietary fatty acids to mitigate the negative effects of obesity and obesity-related pathologies.
Chapter 2: Aims of the Thesis

Immune cells, including macrophages, infiltrate adipose tissue in obesity (4). Interestingly, numerous studies have also shown significant differences in the number of infiltrated macrophages in skeletal muscle in obese compared to lean states (4, 119). Infiltrating macrophages are capable of secreting pro-inflammatory cytokines such as IL-6 and TNF-α, contributing to a chronic inflammatory state in adipose tissue and skeletal muscle in a state of obesity (118). Importantly, macrophage-derived cytokines such as TNF-α have been shown to contribute to the development of insulin resistance in skeletal muscle (37).

Recent work has examined the specific effects of macrophage-derived inflammation on skeletal muscle inflammation and function using a model of macrophage conditioned media (MCM) (118, 130, 139). Treatment of skeletal muscle cells with MCM from fatty acid- and LPS-activated macrophages resulted in increased gene expression of TNF-α, IL-6 and MCP-1 (118) and decreased insulin-stimulated glucose uptake (118, 130, 139). In contrast to saturated fatty acids, such as palmitic acid, which have been shown to activate pro-inflammatory pathways in macrophages (118, 131), long-chain n-3 PUFA, such as EPA and DHA, have been shown to attenuate the LPS-induced increases in inflammatory cytokine gene expression response in macrophages through modulation of signaling intermediates such as NF-κB (110) and TLR4 (109).

Currently, the ability of EPA and DHA to attenuate the LPS-induced TLR4/NF-κB inflammatory pathway in macrophages, and subsequent effects on skeletal muscle inflammation and function is not known. Additionally, it is not known if skeletal muscle will respond differently to varying levels of macrophage-derived inflammatory cytokines that represent an obese compared to a lean state. Further, underlying mechanisms, such as the roles of TLR4, NF-
κB and their respective signaling pathways, have not been fully investigated in the context of macrophage-derived inflammation and subsequent effects on skeletal muscle inflammation and function. Thus, the overall objective of this thesis was to examine the effects of long-chain n-3 PUFA on macrophage-derived inflammation, and consequent rat skeletal muscle inflammation and function, *in vitro*.

The specific objectives of the thesis were:

1. To confirm the effect of various fatty acids, including long-chain n-3 PUFA, on inflammatory mediator gene expression and secreted inflammatory proteins in RAW 264.7 macrophages stimulated, or not, with LPS.
2. To compare the effects of various fatty acid-modulated MCM (at various ratios to represent a lean versus obese state) on skeletal muscle cell inflammation and insulin-stimulated pAkt.
3. To investigate the involvement of the TLR4/NF-κB inflammatory pathway in the above relationships by measuring mRNA of NF-κB and TLR4 in RAW 264.7 macrophages and L6 skeletal muscle cells.

The overall hypotheses of the thesis were:

1. Treatment of RAW 264.7 macrophages with long-chain n-3 PUFA would attenuate the inflammatory response and cytokine secretion from LPS-activated macrophages, specifically through the TLR4/NF-κB pathway.
2. Treatment of L6 skeletal muscle cells with MCM derived from long-chain n-3 PUFA-treated macrophages would result in reduced inflammation and increased levels of insulin-stimulated pAkt in skeletal muscle cells.
3. Higher ratios of MCM, used to represent an obese state, would result in a more pronounced inflammatory response and reduced insulin responsiveness in skeletal muscle cells compared to lower ratios of MCM, used to represent a lean state.
Chapter 3: Investigation into the Effects of Fatty Acid-Treated Macrophage Conditioned Media on Skeletal Muscle Gene Expression and Function

3.1 Introduction

In obesity, macrophages infiltrate the adipose tissue and skeletal muscle, promoting a chronic inflammatory state (4). Research has shown a 40% (of total cells) infiltration of macrophages into the intra-muscular adipose tissue in the obese state, compared to a 10% infiltration in the lean state (4). Macrophages are capable of secreting various cytokines, such as TNF-α and IL-6 (118), contributing to the inflammatory signals sent to surrounding tissues. In addition, genetic deletion of macrophage-specific IKK-β and JNK-1 has provided evidence for a relationship between macrophage inflammation and the development of insulin resistance in skeletal muscle (121, 123).

Macrophages are responsive to fatty acids, providing a possible dietary strategy to modulate obesity-associated inflammation and ensuing effects on skeletal muscle function. For example, treatment of RAW 264.7 macrophages co-cultured with adipocytes with 125 μM DHA for 12 hours decreased expression of TNF-α, and NF-κB (140), confirming anti-inflammatory action of this long-chain n-3 PUFA. Further to this, co-treating RAW 264.7 macrophages with LPS and 100 μM of long-chain n-3 PUFA resulted in reduced mRNA expression of TNF-α, IL-6, IL-1β, and iNOS (110), possibly due to deacetylation and deactivation of NF-κB by the n-3 PUFA, through a pathway involving AMPK and SIRT1 (110). Contrary to n-3 PUFA, saturated fatty acids have been shown to increase the macrophage inflammatory response. For example, incubating RAW 264.7 macrophages with 500 μM palmitic acid for 6 hours increased secretion of TNF-α and IL-6 from the cells (118). Nguyen et al (2007) also found that incubating RAW 264.7 macrophages in a 500 μM saturated and unsaturated fatty acid mixture (including palmitic
acid) activated the JNK and IKK-β signaling pathways and increased secretion of IL-1β, IL-6, MCP-1 and TNF-α, possibly through mechanisms involving TLR-2 and TLR4.

The relationship between immune cell-derived inflammation, fatty acids, and muscle function has been further investigated using models of macrophage conditioned media (MCM). For example, L6 skeletal muscle cells incubated for 24 hours in LPS-activated MCM had reduced expression of TNF-α, increased expression of IL-6 and MCP-1, as well as increased insulin-stimulated glucose uptake and GLUT4 translocation (118). In the same study, palmitic acid-MCM increased TNF-α and MCP-1 expression in L6 skeletal muscle cells, as well as increased protein levels of IκB-α and phosphorylated JNK and p38 MAPK (118). Palmitic acid-MCM also decreased insulin-stimulated glucose uptake and GLUT4 translocation, while IL-10, a classic anti-inflammatory cytokine, added to the palmitic acid-MCM prevented this decrease in L6 muscle cells (118). In addition to demonstrating the effective use of MCM as a model to study the ability of macrophage-derived inflammation to modulate skeletal muscle inflammation and function, this work also showed that inclusion of an anti-inflammatory cytokine was able to modify responses to the MCM, suggesting that inclusion of other anti-inflammatory factors may also modulate this relationship.

While recent research has examined the modulatory effects of fatty acids on macrophage-derived inflammation, the role of long-chain n-3 PUFA in this response, as well as subsequent effects on skeletal muscle inflammation and function, have not been well studied. Thus, the overall objective of this work was to further understand the modulatory effects of long-chain n-3 PUFA on macrophage-derived inflammatory cytokine secretion, the inflammatory pathways involved, and the consequent effects on skeletal muscle inflammation and insulin responsiveness. It was hypothesized that treatment of skeletal muscle cells with MCM derived
from long-chain n-3 PUFA-treated macrophages will result in reduced skeletal muscle cell inflammation and increased insulin responsiveness.

3.2 Materials and Methods

3.2.1: Cell culture and differentiation

L6 myoblasts and RAW 264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to directions. Both cell types were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate (HyClone, Logan, UT), supplemented with 10% (v/v) fetal bovine serum (FBS, low endotoxin, Sigma, St. Louis, MO) and 1% (v/v) penicillin-streptomycin (HyClone, Logan, UT), in 5% CO2 at 37°C. L6 myoblasts were seeded in 6-well and 24-well plates at 15,000 cells/cm². At 70-80% confluence, the cells were incubated in DMEM supplemented with 2% (v/v) FBS and 1% (v/v) penicillin-streptomycin in order to facilitate differentiation (141). Myotubes were fully differentiated after 5 days (identified by a striated and multinucleated appearance under microscope), and were subsequently serum deprived with medium containing 0.25% FBS prior to experimental treatment. RAW 264.7 macrophages were seeded in T75 flasks and serum deprived overnight with medium containing 0.25% FBS prior to fatty acid treatment.

3.2.2: Fatty acids and LPS treatment of macrophages: collection of macrophage conditioned media (MCM)

Macrophages were pre-treated with fatty acids for 24 h, followed by treatment with fatty acids without or with LPS for a subsequent 4 h. For all fatty acid treatments, fatty acid stock solutions of EPA, DHA, and palmitic acid (Cayman Chemical, Ann Arbor, MI) were complexed with bovine serum albumin (BSA, ≤0.005% fatty acids, Sigma, St. Louis, MO Sigma) in serum-
reduced DMEM to a final concentration of 100 μM fatty acid:20 μM BSA. The fatty acid dose was based on previous work (110) that showed reduced LPS-induced gene expression of TNF-α, IL-6, IL-1β, and iNOS following a pre-treatment with 100 μM n-3 PUFA. Serum-reduced medium (without BSA) and BSA plus ethanol served as controls. For the combined treatment of fatty acids and LPS, 10 ng/mL LPS (Sigma, Oakville, Ontario, Canada) was added to the serum-reduced medium containing 100 μM fatty acid. This dose of LPS induced an inflammatory response in L6 myotubes and RAW 264.7 macrophages without toxic effects (data not shown). LPS and serum-reduced medium, and LPS and BSA plus ethanol served as controls. Following treatment, MCM were collected, filtered, and used fresh on the L6 myotubes.

3.2.3: L6 treatment with MCM

Myotubes were treated for 24 h with undiluted MCM or MCM diluted to the appropriate amount to reflect cytokine secretion from macrophages infiltrated in skeletal muscle in obese or lean individuals (40% and 10% infiltration, respectively). Undiluted MCM was considered a 100% solution, and was diluted to a 40% or 10% solution to reflect the percent of macrophage infiltration and their respective concentration of secreted cytokines (assuming that 40% of the macrophages infiltrating the tissue would secrete 40% of the total secretions). Since MCM contained LPS, FA and BSA, the diluent for the 40% and 10% MCM treatments was serum-reduced medium enriched with LPS, FA, and BSA so that all muscle cells were treated with the same amounts of all three components, such that only the amount of MCM differed between groups. A subset of muscle cells was also treated with media (subsequently referred to as non-MCM) containing LPS, FA and BSA alone as control. Previous gas chromatography pilot work demonstrated that both the n-3 PUFA and palmitic acid were taken up into the L6 skeletal muscle cells, increasing the cellular levels of these fatty acids (data not shown). All treatments
were performed in triplicate (3 wells) and all experiments repeated 3 times. Separate experiments were conducted in a similar manner in order to evaluate the effect of MCM on gene expression of inflammatory cytokines and signaling intermediates and phosphorylated-Akt (Ser 473).

3.2.4: Cytokines secreted from RAW 264.7 macrophages

Media was collected prior to and following fatty acid pre-treatment and fatty acid and LPS co-treatment. Secreted MCP-1, TNF-α, IL-6 and IL-10 were analyzed by Luminex xMAP technology (Bioplex-200 system; Mouse Cytokine/Chemokine Bio-Plex kit, Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer’s instructions. IL-6 was undetectable in the media and only data for secreted MCP-1, TNF-α and IL-10 is presented in the results section.

3.2.5: RNA isolation and quantitative PCR

Following treatments, RAW 264.7 macrophages and L6 myotubes were rinsed with 1x PBS (Sigma, St. Louis, MO) and RNA was isolated using an RNeasy kit (Qiagen, Toronto, ON), as per manufacturer’s instructions. Complimentary DNA (cDNA) was made from 1 µg of extracted RNA using a high capacity cDNA reverse transcription kit according to the manufacturer’s instructions (Applied Biosystems, Forest City, CA, USA). Primers for Il-6, Mcp-1, Tnfa, TLR4, and Nfkb, were designed using the Universal Probe Library Assay Design Center (Roche Applied Sciences, Penzberg, Germany) and their efficiencies were validated to confirm 90-105% primer efficiency. mRNA expression of all genes was analyzed in both skeletal muscle cells and macrophages. Samples were run in duplicate in 96-well plates, and each 20 µL reaction contained 5 µL cDNA, 0.4 uL of primer solution, 10 µL Power Sybr green 2x master mix (Applied Biosystems, Forest City, CA), and 4.6 µL of RNase free water. Real-time PCR analysis was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems,
Forest City, CA) using the default protocol: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, 60°C for 1 min, 15 s at 95°C and 15 s at 60°C for a total of 40 cycles. All results were normalized to Rplp0 mRNA expression, and the relative differences in gene expression between treatment groups were determined using the ΔΔCt method. Specifically, statistical analysis was performed on the ΔCt values (values normalized to Rplp0). Graphical representation of the results was presented as fold change relative to time zero.

3.2.6: Insulin-stimulated signaling proteins

Following 24 h treatment with MCM, L6 myotubes were serum-starved for 3-5 h and treated with a maximal dose of insulin (100 nM) for 10 min in 24-well plates. Cells were rinsed in 1x PBS, lysed using a supplied buffer, and analyzed for phosphorylated-Akt (Ser 473) using the InstantOne™ ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

3.2.7: Statistical Analysis

For all analysis, data from replicates was averaged and expressed as mean ± SEM. Statistical significance was set at p<0.05 and all statistical analysis was performed using SigmaPlot version 12.0 (San Jose, CA).

Macrophage Secreted Protein and Gene Expression Data

Secreted protein and gene expression data was analyzed using a two-way analysis of variance (ANOVA) for the effect of LPS stimulation and fatty acid treatment and their possible interactions. A Fisher LSD post-hoc test was used to identify all significant pairwise differences. Data that was not normally distributed was transformed prior to statistical analysis. Data that was not normally distributed following transformation (as determined by the Shapiro-Wilk test) was analyzed using a one-way ANOVA with Fisher LSD post-hoc test or a Kruskal-Wallis one-way
ANOVA on ranks with Dunn’s *post-hoc* test for the effect of LPS stimulation and fatty acid treatment.

**L6 Skeletal Muscle Cell Gene Expression Data**

Gene expression data for NF-κB, TLR4 and MCP-1 was analyzed using a two-way ANOVA for the effect of LPS stimulation and fatty acid treatment and their possible interactions. A Fisher LSD *post-hoc* test was used to identify all significant pairwise differences. Gene expression data for TNF-α and IL-6 were analyzed using a one-way ANOVA for the effect of fatty acid treatment. A Fisher LSD *post-hoc* test was used to identify all significant pairwise differences. Data that was not normally distributed or data that did not pass equal variance was transformed prior to analysis. Any data that was not normally distributed or data that did not pass equal variance following transformation was analyzed using either a one-way ANOVA with Fisher LSD *post-hoc* test or a Kruskal-Wallis one-way ANOVA on ranks with Dunn’s *post-hoc* test for the effect of LPS stimulation and fatty acid treatment. Comparisons between dilutions for all genes were analyzed using a one-way ANOVA and a Fisher LSD *post-hoc* test was used to identify all significant pairwise differences.

**L6 Skeletal Muscle Cell pAkt Data**

pAkt protein data was analyzed using a one-way ANOVA for the effect of fatty acid treatment. A Fisher LSD *post-hoc* test was used to identify all significant pairwise differences. Comparisons between dilutions for all treatments were also analyzed using a one-way ANOVA and a Fisher LSD *post-hoc* test was used to identify all significant pairwise differences.
4.3 Results

4.3.1 Fatty acid- and LPS- induced modulation of cytokine mRNA expression and secreted cytokine proteins in RAW 264.7 macrophages

a) MCP-1 mRNA Expression and Secreted Protein in RAW 264.7 Macrophages

In the absence of LPS, DHA significantly reduced MCP-1 gene expression compared to SR control (Table 1), while DHA and EPA+DHA significantly reduced MCP-1 gene expression compared to BSA+ethanol (Table 1). LPS stimulation increased expression of MCP-1 in SR control (p<0.001), DHA (p<0.05), EPA+DHA (p<0.001), and PA (p<0.001) compared to their respective non-LPS groups. Further, in the presence of LPS, macrophages treated with EPA and DHA exhibited lower mRNA expression of MCP-1 compared to SR control and PA-treated cells (p<0.05 compared to SR control, p<0.001 compared to PA, Figure 1A). Finally, MCP-1 mRNA expression was higher in PA-treated macrophages compared to BSA+ethanol (p<0.05, Figure 1A).

In the absence of LPS, DHA significantly reduced MCP-1 secreted protein compared with SR control (Table 1). LPS-stimulation increased MCP-1 protein secreted from SR control and macrophages treated with EPA+DHA (p<0.05) compared with their respective non-LPS groups. Interestingly, MCP-1 secreted protein was reduced in LPS-stimulated macrophages treated with EPA, DHA, and EPA+DHA compared with SR control macrophages (Figure 1B). EPA and DHA also reduced MCP-1 secreted protein compared to PA (p<0.05, Figure 1B).
Table 1: MCP-1 mRNA Expression and Secreted Protein in RAW 264.7 Macrophages in the Absence of LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA Expression (Fold Change)</th>
<th>Secreted Protein (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>4.85±0.59</td>
<td>1254.45±117.09</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>5.60±0.25</td>
<td>1638.94±135.04</td>
</tr>
<tr>
<td>EPA</td>
<td>2.61±0.34</td>
<td>489.85±39.06</td>
</tr>
<tr>
<td>DHA</td>
<td>1.39±0.15*</td>
<td>358.54±35.85*</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>1.55±0.03</td>
<td>505.77±42.51</td>
</tr>
<tr>
<td>PA</td>
<td>2.88±0.46</td>
<td>1128.59±96.41</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 1: Effect of various fatty acids on A) mRNA expression and B) secretion of MCP-1 from RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. In A, letters indicate differences
between treatments, p<0.05, except for EPA and DHA compared to PA, p<0.001. In B, letters indicate differences between treatments, p<0.05, except for DHA compared to SR control, p<0.001. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

b) TNF-α mRNA Expression and Secreted Protein in RAW 264.7 Macrophages

Fatty acids did not affect TNF-α mRNA expression in macrophages in either the absence of presence of LPS (Table 2 and Figure 2A). As expected, LPS stimulation increased TNF-α mRNA expression in all groups (p<0.001, except for EPA+DHA, p<0.05).

In the absence of LPS, EPA, DHA, and EPA+DHA significantly increased TNF-α secreted protein compared with SR control (Table 2). EPA and EPA+DHA also significantly increased secreted TNF-α protein compared to BSA+ethanol and PA-treated macrophages (Table 2). Incubation with LPS increased secreted TNF-α protein in the BSA+ethanol group, compared with its respective non-LPS group. In the presence of LPS, there were no differences between treatment groups (Figure 2B).
Table 2: TNF-α mRNA Expression and Secreted Protein in RAW 264.7 Macrophages in the Absence of LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA Expression (Fold Change)</th>
<th>Secreted Protein (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>4.45±1.02</td>
<td>52.59±7.08</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>4.04±0.87</td>
<td>79.48±11.16</td>
</tr>
<tr>
<td>EPA</td>
<td>2.74±0.10</td>
<td>334.78±27.72**</td>
</tr>
<tr>
<td>DHA</td>
<td>2.87±0.37</td>
<td>204.26±7.31*</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>3.30±0.65</td>
<td>387.60±17.57**</td>
</tr>
<tr>
<td>PA</td>
<td>3.18±0.29</td>
<td>192.24±33.59</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 2: Effect of various fatty acids on A) mRNA expression and B) secretion of TNF-α from RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR)
media+LPS and BSA with ethanol+LPS acted as controls. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

c) IL-6 mRNA Expression

In the absence of LPS, fatty acids did not affect IL-6 gene expression in macrophages (Table 3). LPS stimulation increased IL-6 mRNA expression in the SR control (p<0.001), BSA+ethanol (p<0.05) and PA-treated (p<0.001) macrophages compared to the un-stimulated condition. In the presence of LPS, EPA, DHA and EPA+DHA decreased IL-6 gene expression compared to PA (p<0.05 for DHA, p<0.001 for EPA and EPA+DHA, Figure 3). The decrease in IL-6 mRNA expression following incubation with EPA, DHA and EPA+DHA was also lower than the SR control (p<0.05, Figure 3).

**Table 3: IL-6 mRNA Expression in RAW 264.7 Macrophages in the Absence of LPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA Expression (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>8.61±3.77</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>8.48±3.75</td>
</tr>
<tr>
<td>EPA</td>
<td>2.47±1.18</td>
</tr>
<tr>
<td>DHA</td>
<td>3.77±1.63</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>1.01±0.45</td>
</tr>
<tr>
<td>PA</td>
<td>3.39±1.55</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 3: Effect of various fatty acids on mRNA expression of IL-6 in RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate differences between treatments, p<0.05, except for EPA and EPA+DHA compared to PA, p<0.001. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate.

EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

d) IL-10 Secreted Protein in RAW 264.7 Macrophages

In the absence of LPS, PA increased IL-10 secreted protein from macrophages compared to controls and all other fatty acids (p<0.001, Table 4). Incubation with EPA+DHA also significantly increased IL-10 secreted from macrophages compared to SR control (Table 4). LPS stimulation increased secreted IL-10 protein in SR control-treated macrophages compared with
their non-LPS control (p<0.05). Similar to the non-LPS condition, incubation with LPS and PA increased IL-10 secreted protein compared to controls and all other fatty acids (p<0.001, Figure 4). In the presence of LPS, EPA reduced secreted IL-10 protein compared to both controls (p<0.05, Figure 4).

**Table 4: IL-10 Secreted Protein in RAW 264.7 Macrophages in the Absence of LPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Secreted Protein (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>2.55±0.28</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>5.92±0.31*</td>
</tr>
<tr>
<td>EPA</td>
<td>3.38±0.18</td>
</tr>
<tr>
<td>DHA</td>
<td>3.63±0.20</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>4.81±0.22*</td>
</tr>
<tr>
<td>PA</td>
<td>11.00±0.41**</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 4: Effects of fatty acid treatment on secretion of IL-10 from RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate differences between treatments, p<0.05, except for PA compared to all other treatments, p<0.001. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
4.3.3: Fatty acid- and LPS-induced modulation of mRNA expression of inflammatory
signaling intermediates in RAW 264.7 macrophages

a) NF-κB mRNA Expression in RAW 264.7 Macrophages

NF-κB mRNA expression was not significantly affected by any fatty acids in the
presence or absence of LPS (Table 5 and Figure 5). LPS stimulation did not increase NF-κB
gene expression in any treatment groups.

Table 5: NF-κB and TLR4 mRNA Expression in RAW 264.7 Macrophages in the Absence of
LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NF-κB mRNA Expression (Fold Change)</th>
<th>TLR4 mRNA Expression (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>1.46±0.08</td>
<td>2.16±0.41</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>1.53±0.06</td>
<td>2.39±0.49</td>
</tr>
<tr>
<td>EPA</td>
<td>1.16±0.10</td>
<td>2.70±0.50</td>
</tr>
<tr>
<td>DHA</td>
<td>1.16±0.05</td>
<td>3.56±0.75</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>1.12±0.04</td>
<td>3.33±0.69</td>
</tr>
<tr>
<td>PA</td>
<td>1.77±0.17</td>
<td>1.49±0.45</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA:
docosahexaenoic acid, PA: palmitic acid.
**Figure 5:** Effect of various fatty acids on mRNA expression of NF-κB in RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

b) TLR4 mRNA Expression in RAW 264.7 Macrophages

TLR4 mRNA expression was not significantly affected by any fatty acids in the presence or absence of LPS (Table 5 and Figure 6). LPS stimulation decreased TLR4 mRNA expression in all groups (p<0.05 for all groups except SR control, p<0.001).
Figure 6: Effect of various fatty acids on mRNA expression of TLR4 in RAW 264.7 macrophages in the presence of LPS. Cells were treated for 24 h with 100 μM fatty acid and for a subsequent 4 h with fatty acid with 10 ng/mL LPS. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
4.3.4: Macrophage Conditioned Media (MCM) modulation of L6 skeletal muscle cell cytokine mRNA expression

a) MCP-1 mRNA Expression- Fatty Acid Treatment & LPS Stimulation Effects

In the absence of LPS, diluted (40% and 10%) EPA, DHA and EPA+DHA MCM increased MCP-1 gene expression compared to PA MCM, BSA+ethanol MCM and SR control MCM (Table 8 in Appendix). EPA non-MCM and DHA non-MCM also increased MCP-1 gene expression in skeletal muscle cells compared to control non-MCM (Table 8 in Appendix). Also, DHA non-MCM induced a greater increase in MCP-1 mRNA expression compared to PA non-MCM (Table 8 in Appendix).

As expected, LPS stimulation increased MCP-1 mRNA expression in most treatment groups. This was true of undiluted SR control, BSA+ethanol, DHA and PA MCM (p<0.05), 40% diluted SR control, BSA+ethanol and PA MCM (p<0.05), all 10% diluted treatment groups (p<0.001), and SR control, BSA+ethanol, EPA, and DHA non-MCM (p<0.05).

In the presence of LPS, differences between MCM treatments were only observed with the 10% diluted MCM and with non-MCM. More specifically, 10% diluted BSA+ethanol, EPA, DHA, and EPA+DHA MCM increased MCP-1 mRNA expression in skeletal muscle cells compared to SR control MCM (p<0.001 for EPA and DHA, p<0.05 for BSA+ethanol and EPA+DHA, Figure 19 in Appendix). Also, EPA MCM induced a greater increase in MCP-1 mRNA expression compared to PA MCM (p<0.05, Figure 19 in Appendix). BSA+ethanol, EPA and DHA non-MCM also increased MCP-1 gene expression compared to SR control and PA non-MCM (p<0.05, Figure 7B).
Figure 7: Effect of A) undiluted MCM with LPS and B) non-MCM with LPS on MCP-1 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM. Serum-reduced
(SR) media+LPS and BSA with ethanol+LPS acted as controls. In B, letters indicate significant
differences between treatments. Treatments sharing letters are not significantly different from
each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid,
DHA: docosahexaenoic acid, PA: palmitic acid.

b) MCP-1 mRNA Expression- MCM Dilution Effects

Overall, undiluted MCM significantly increased MCP-1 gene expression compared to
non-MCM. More specifically, in the absence of LPS, undiluted SR control MCM increased
MCP-1 gene expression compared to control non-MCM (p<0.05, Table 9 in Appendix).
Undiluted BSA+ethanol MCM increased MCP-1 gene expression compared to 40% and 10%
diluted MCM and BSA+ethanol non-MCM (p<0.05, Table 9 in Appendix). Undiluted EPA
MCM increased MCP-1 gene expression compared to 40% and 10% diluted MCM, and
undiluted PA MCM increased MCP-1 gene expression compared to 40% and 10% diluted MCM
and PA non-MCM (p<0.001, Table 9 in Appendix).

In the presence of LPS, there were also many differences between MCM dilutions. All
dilutions of BSA+ethanol and EPA+DHA MCM yielded different MCP-1 gene expression
levels, with the exception of the 10% diluted MCM and non-MCM (p<0.001, Figure 20 in
Appendix, Figure 8B). Undiluted SR control MCM increased MCP-1 gene expression
compared to 10% diluted MCM and non-MCM (p<0.05, Figure 20 in Appendix). Undiluted
EPA and DHA MCM resulted in higher MCP-1 gene expression in L6 cells compared to all
other dilutions (p<0.001 for EPA, p<0.05 for DHA, Figure 8A). All PA MCM dilutions resulted
in different levels of MCP-1 gene expression, with the undiluted PA MCM yielding the highest
level of MCP-1 gene expression (p<0.001 for all except 10% compared to non-MCM, p<0.05,
Figure 8B).
Figure 8: Effects of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on MCP-1 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate
significant differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.

EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

c) TNF-α mRNA Expression- Fatty Acid Treatment Effects

TNF-α mRNA expression was only measured in skeletal muscle cells that were treated with MCM in the presence of LPS or with non-MCM in the presence of LPS as non-LPS stimulated L6 cells had very low TNF-α mRNA expression.

In the presence of LPS, undiluted EPA, DHA and EPA+DHA MCM resulted in significantly lower TNF-α mRNA expression compared to BSA+ethanol MCM (p<0.05, Figure 9A). DHA and EPA+DHA MCM also induced lower TNF-α mRNA expression compared with SR control MCM (p<0.05, Figure 9A). For the 40% diluted MCM, BSA+ethanol MCM yielded higher (p<0.05) TNF-α mRNA expression, while EPA and EPA+DHA yielded lower (p<0.05 and p<0.001) TNF-α mRNA expression compared to SR control MCM (Figure 21 in Appendix). EPA MCM yielded higher TNF-α mRNA expression compared to PA MCM in the 10% dilution (p<0.05, Figure 21 in Appendix). Lastly, treatment of skeletal muscle cells with EPA, DHA and EPA+DHA non-MCM resulted in higher TNF-α mRNA expression compared to SR control non-MCM and PA non-MCM (p<0.001 for all except EPA+DHA compared to SR control, p<0.05, Figure 9B). The EPA- and DHA-induced increase in TNF-α mRNA expression was also higher compared to BSA+ethanol control (p<0.05, Figure 9B).
Figure 9: Effect of A) undiluted MCM with LPS B) non-MCM with LPS on mRNA expression of TNF-α in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate
significant differences between treatments. Treatment sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

d) TNF-α Gene Expression- MCM Dilution Effects

There was general trend for higher TNF-α mRNA expression following treatment of L6 cells with undiluted MCM compared to the other dilutions. More specifically, undiluted SR control MCM resulted in the greatest TNF-α mRNA expression in L6 cells compared with 10% and 40% diluted MCM and non-MCM (p<0.001 Figure 22 in Appendix). Similarly, undiluted BSA+ethanol MCM yielded higher TNF-α mRNA expression compared to the 10% diluted MCM and non-MCM (p<0.05, Figure 22 in Appendix). 40% diluted BSA+ethanol MCM also yielded higher expression levels than their respective non-MCM groups (p<0.05, Figure 22 in Appendix). Undiluted EPA, DHA and EPA+DHA MCM resulted in higher TNF-α mRNA expression compared to all other dilutions (p<0.05 for EPA and EPA+DHA, p<0.001 for DHA, Figure 10A and B). Finally, all dilutions of PA MCM resulted in different expression levels of TNF-α (p<0.001, Figure 10B).
Figure 10: Effect of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on mRNA expression of TNF-α in L6.
skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate significant differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.

EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

e) IL-6 mRNA Expression- Fatty Acid Treatment Effects

IL-6 mRNA expression was only measured in skeletal muscle cells treated with MCM in the presence of LPS, or with non-MCM in the presence of LPS, as non-LPS stimulated L6 cells had very low IL-6 mRNA expression.

In the presence of LPS, undiluted DHA MCM and EPA+DHA MCM resulted in lower IL-6 mRNA expression compared to SR control MCM and BSA+ethanol MCM (p<0.001 for all except DHA compared to SR control, p<0.05, Figure 11A). Undiluted SR control, EPA and PA MCM induced lower IL-6 mRNA expression compared to BSA+ethanol MCM (p<0.001, except for EPA compared to BSA+ethanol, p<0.05), while EPA+DHA MCM induced lower IL-6 mRNA expression compared to PA MCM (p<0.001, Figure 11A). For the 40% diluted MCM, SR control and EPA+DHA MCM resulted in lower IL-6 gene expression compared to BSA+ethanol MCM (p<0.05, Figure 23 in Appendix). Finally, for the 10% diluted MCM, all MCM yielded higher IL-6 mRNA expression compared to SR control (p<0.001 for all except PA compared to SR control, p<0.05, Figure 23 in Appendix). IL-6 mRNA expression induced by EPA or DHA MCM was also greater than that induced by EPA+DHA, PA and BSA+ethanol MCM (p<0.001, Figure 23 in Appendix). Lastly, all n-3 PUFA non-MCM and BSA+ethanol non-MCM resulted in higher IL-6 mRNA expression compared to SR control and PA non-MCM (p<0.001 for all except PA compared to BSA+ethanol, p<0.05, Figure 11B). IL-6 mRNA expression induced by EPA and DHA non-MCM was also greater compared to that induced by BSA+ethanol non-MCM (p<0.001, Figure 11B).
Figure 11: Effect of A) undiluted MCM with LPS and B) non-MCM with LPS on mRNA expression of IL-6 in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-
MCM. Letters indicate significant differences between treatments. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

f) IL-6 mRNA Expression- MCM Dilution Effects

All dilutions of SR control MCM and BSA+ethanol MCM resulted in different expression levels of IL-6, except for the 10% diluted MCM and non-MCM (p<0.001, Figure 24 in Appendix). Undiluted EPA, DHA and EPA+DHA MCM resulted in greater IL-6 gene expression compared to 10% diluted MCM and non-MCM (p<0.05, Figure 12A and B). Lastly, all dilutions of PA MCM yielded different IL-6 mRNA expression with undiluted PA MCM yielding the highest IL-6 mRNA expression (p<0.001 for all except 10% compared to non-MCM, p<0.05, Figure 12B).
Figure 12: Effect of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on mRNA expression of IL-6 in L6
skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate significant differences between dilutions. Dilutions sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

4.3.5: MCM modulation of mRNA expression of inflammatory signaling intermediates in L6 skeletal muscle cells

a) NF-κB mRNA Expression-Fatty Acid Treatment Effects

In the absence of LPS, differences in NF-κB mRNA expression were observed between treatment groups in the 40% diluted MCM, 10% diluted MCM and in the non-MCM. More specifically, 40% diluted DHA MCM significantly increased NF-κB gene expression compared to BSA + ethanol (Table 10 in Appendix). 10% diluted EPA, DHA and PA MCM significantly increased NF-κB gene expression compared to SR control (Table 10 in Appendix). EPA+DHA non-MCM induced lower NF-κB mRNA expression compared to EPA, DHA and PA, while DHA non-MCM induced higher NF-κB mRNA expression compared to controls (p<0.05, Table 10 in Appendix).

As expected, LPS stimulation increased NF-κB gene expression in many groups. Specifically, LPS stimulation increased gene expression in all undiluted treatments, except for SR control (p<0.05). LPS stimulation also increased gene expression in 40% diluted BSA+ethanol, 10% diluted SR control and BSA+ethanol and BSA+ethanol and EPA+DHA non-MCM (p<0.05). In the presence of LPS, differences between treatments were only observed in the 40% diluted MCM and non-MCM groups. More specifically, 40% diluted BSA+ethanol MCM and PA MCM increased NF-κB mRNA expression compared to SR control (p<0.05, Figure 25 in Appendix). In contrast, EPA MCM resulted in reduced NF-κB gene expression.
compared to BSA+ethanol and PA MCM (p<0.05, **Figure 25 in Appendix**). BSA+ethanol and DHA non-MCM increased NF-κB mRNA expression compared to PA non-MCM (p<0.05, **Figure 13B**). EPA and SR control non-MCM also significantly decreased NF-κB mRNA expression compared to BSA+ethanol non-MCM (p<0.05, **Figure 13B**).
Figure 13: Effect of A) undiluted MCM with LPS and B) non-MCM with LPS on NF-κB mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM. Serum-
reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. In B, letters indicate significant differences between treatments. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

**b) NF-κB mRNA Expression- MCM Dilution Effects**

In the absence of LPS, all dilutions of EPA MCM were different, except for 10% diluted EPA MCM and EPA non-MCM (p<0.05, Table 11 in Appendix). There were no other dilution effects on NF-κB mRNA expression.

In the presence of LPS, differences in NF-κB mRNA expression were observed in dilutions of all groups. For SR control and BSA+ethanol MCM, undiluted MCM resulted in greater NF-κB mRNA expression compared to 40% and 10% diluted MCM and control non-MCM (p<0.05 for all except BSA+ethanol undiluted compared to 10%, p<0.001, Figure 26 in Appendix). EPA, DHA and EPA+DHA undiluted MCM also induced higher NF-κB gene expression compared to all other dilutions (p<0.001 for DHA and EPA+DHA, p<0.05 for EPA, Figure 14A and B). Lastly, undiluted PA MCM resulted in higher NF-κB expression compared to 10% diluted MCM and PA non-MCM (p<0.05, Figure 14B).
Figure 14: Effect of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on mRNA expression of NF-κB in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate
significant differences between dilutions. Dilutions sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

c) TLR4 mRNA Expression- Fatty Acid Treatment Effects

In the absence of LPS, differences between treatments were observed in undiluted MCM, in the 10% diluted MCM and in non-MCM. Undiluted PA MCM decreased TLR4 gene expression compared to DHA and SR control MCM (Table 12 in Appendix). 10% diluted EPA+DHA MCM decreased TLR4 gene expression compared to BSA+ethanol, EPA and DHA MCM (Table 12 in Appendix). EPA+DHA non-MCM decreased TLR4 mRNA expression compared to BSA+ethanol, EPA, DHA and SR control (Table 12 in Appendix).

LPS stimulation significantly increased TLR4 gene expression in the undiluted BSA+ethanol, DHA and EPA+DHA groups (p<0.001, except for BSA+ethanol, p<0.05). LPS stimulation also increased TLR4 gene expression in 10% diluted EPA and DHA (p<0.05). In the presence of LPS, differences between treatment groups were detected in the undiluted and 10% diluted MCM and non-MCM. Undiluted EPA+DHA MCM decreased TLR4 gene expression compared to SR control (p<0.05, Figure 15A). For the 10% diluted MCM, EPA, DHA and EPA+DHA MCM induced lower TLR4 mRNA expression compared to SR control and BSA+ethanol MCM (p<0.05, Figure 27 in Appendix). For non-MCM, EPA, DHA and EPA+DHA induced lower TLR4 mRNA expression compared to BSA+ethanol (p<0.05, Figure 15B).
Figure 15: Effect of A) undiluted MCM with LPS and B) non-MCM with LPS on TLR4 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM.
Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. In A and B, letters indicate differences between treatments. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

d) TLR4 mRNA Expression- MCM Dilution Effects

In the absence of LPS, MCM dilutions did not affect TLR4 mRNA expression in L6 cells. In the presence of LPS, treatment with undiluted BSA+ethanol MCM induced lower TLR4 mRNA expression compared to all other dilutions (p<0.05 compared to 40% and 10% dilutions, p<0.001 compared to non-MCM, Figure 28 in Appendix). Undiluted EPA+DHA MCM and PA MCM induced lower TLR4 mRNA expression compared to their respective non-MCM (p<0.05 for EPA+DHA, p<0.001 for PA, Figure 16B).
Figure 16: Effect of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on mRNA expression of TLR4 in L6
skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate significant differences between dilutions. Dilutions sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

4.3.6: MCM modulation of pAkt in L6 skeletal muscle cells

a) Fatty Acid Treatment Effects

In the absence or presence of LPS, there were no fatty acid-induced differences in levels of pAkt in insulin-stimulated skeletal muscle cells treated with undiluted MCM (Table 14 in Appendix and Figure 17A). This was also true of L6 cells treated with various 40% diluted MCM and for cells treated with non-MCM in the presence of LPS (Table 14 and Figure 29 in Appendix and Figure 17B). In the absence of LPS, insulin-stimulated muscle cells treated with DHA non-MCM had significantly elevated levels of pAkt compared to cells treated with BSA+ethanol, EPA, EPA+DHA and PA non-MCM (Table 14 in Appendix).
Figure 17: Effect of A) undiluted MCM with LPS and B) non-MCM with LPS on levels of pAkt in insulin-stimulated L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-
MCM, serum-starved for 3 to 5 h, followed by treatment for 10 min with 100 nM insulin. Cells were rinsed in PBS and lysed in buffer prior to ELISA for determination of levels of pAkt. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.

a) MCM Dilution Effects

In the absence of LPS, there were no differences in levels of pAkt in insulin-stimulated skeletal muscle cells treated with the various dilutions of all MCM (Table 15 in Appendix). In the presence of LPS, only the EPA MCM exhibited any differences between dilutions. Treatment with 40% diluted EPA MCM and EPA non-MCM resulted in significantly decreased pAkt in insulin-stimulated skeletal muscle cells compared to treatment with undiluted MCM (Figure 18A).
Figure 18: Effect of various dilutions of A) EPA MCM with LPS and DHA MCM with LPS and B) EPA+DHA MCM with LPS and PA MCM with LPS on levels of pAkt in insulin-stimulated L6 skeletal muscle cells. Letters indicate significant differences between dilutions within the insulin-stimulated cells. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
4.4: Discussion

There is growing evidence indicating a role for macrophage-derived inflammation in the development of skeletal muscle insulin resistance in obesity. Dietary fatty acids have been shown to modulate such inflammation, and thus may play a role in preventing the consequent development of insulin resistance. Our study is the first to examine the effects of fish oil-derived long-chain n-3 PUFA EPA and DHA, and PA, a common dietary SFA, on macrophage-derived inflammation and subsequent effects on skeletal muscle inflammation and function. In order to study this relationship, we chose to pre-treat the RAW 264.7 macrophages with fatty acid for 24 hours and then co-treat with fatty acid and LPS. This model illustrates the preventative effects of n-3 PUFA when given in advance and alongside an inflammatory stimulus. The dose of LPS we chose was equivalent to ~5 endotoxin units, a level similar to that seen in plasma of high fat fed animals and obese, type 2 diabetic humans (132, 135). We demonstrated anti-inflammatory effects of long-chain n-3 PUFA through decreased macrophage MCP-1 and IL-6 gene expression and MCP-1 secreted protein in the presence of LPS, a known inflammatory stimuli. Surprisingly, we found that PA increased macrophage secretion of IL-10, a classical anti-inflammatory cytokine, in the presence or absence of LPS. As we found no effects of either long-chain n-3 PUFA or PA on any of the macrophage inflammatory signaling intermediates studied, more work is needed to better understand fatty acid-induced cytokine modulation and underlying mechanisms.

In order to study the influence of macrophage-derived cytokines on skeletal muscle inflammation and function, we treated L6 skeletal muscle cells with fatty acid-modulated macrophage-conditioned media (MCM) at varying concentrations to represent lean (10%) or obese (40%) macrophage infiltration into skeletal muscle. Overall, our results showed that, in
the presence or absence of LPS, undiluted MCM increased inflammatory cytokine gene expression in L6 skeletal muscle cells compared with either the lean (10% diluted) or obese (40% diluted) MCM, suggesting that the dilutions used in the current study led to loss of any macrophage cytokine effects seen with the undiluted MCM. It is important to consider that although we were trying to mimic macrophage infiltration into adipose tissue in a lean and obese state, in whole adipose tissue, cytokines are released from other cells (e.g. adipocytes) in addition to macrophages. Thus, cytokines secreted into media derived from macrophages alone (as in the current model) may not be sufficient to induce inflammation in skeletal muscle.

With regards to fatty acid effects, we demonstrated that treatment of L6 skeletal muscle cells with undiluted n-3 PUFA MCM in the presence of LPS decreased TNF-α and IL-6 expression in skeletal muscle cells, confirming anti-inflammatory effects of long-chain n-3 PUFA in this model. Contrary to this, incubation of L6 muscle cells with n-3 PUFA alone (i.e. not from MCM) increased expression of MCP-1, IL-6 and TNF-α in the presence or absence of LPS, indicative of an n-3 PUFA-induced pro-inflammatory response when n-3 PUFA are incubated directly with skeletal muscle cells. With regards to mechanisms, fatty acid-modulated MCM had little effect on any of the inflammatory signaling intermediates studied in L6 muscle cells and thus more work is needed to better understand fatty acid-induced mechanisms in this model. Lastly, we demonstrated that incubation of L6 skeletal muscle cells with EPA alone (i.e. not from MCM) decreased pAkt in some instances, but no other effects of MCM on pAkt were found. Overall, this thesis is the first to show that fish oil-derived long-chain n-3 PUFA favourably modulate macrophage inflammation with subsequent beneficial effects on skeletal muscle inflammation, but with no effect on pAkt, a key insulin signaling intermediate.
4.4.1: Methodology to study the lean compared to obese macrophage infiltration

An integral part of this study was to establish a model to investigate the influence of fatty acid-modulated MCM on skeletal muscle cell inflammation and function and, in particular, to study various dilutions of MCM designed to represent cytokine secretion from macrophages in skeletal muscle in either a lean or obese state. Existing literature has shown that lean and obese intramuscular adipose tissue in rodent skeletal muscle is composed of 10% and 40% macrophages (4), thus we elected to use these same percentages for our MCM dilutions. Our data showed consistently that treatment of L6 skeletal muscle cells with undiluted MCM resulted in the most significant increases in inflammatory cytokine gene expression, though undiluted MCM did not affect insulin-stimulated pAkt levels in skeletal muscle. Previous research has used undiluted MCM as a representative model of the effects of macrophage secretion on skeletal muscle cell inflammation and function (118), however undiluted MCM may not be the most physiologically relevant model as the cytokine secretion from macrophages only represents a portion of the total secretion from the adipose tissue. While our model attempted to more closely mimic macrophage infiltration into skeletal muscle in either the lean or obese state, our results indicate that it may be the combination of pro-inflammatory adipokine secretions from adipocytes and other immune cells within adipose tissue, in addition to secretions from macrophages per se, that contributes to the development of skeletal muscle insulin resistance in obesity given that the cytokine milieu from macrophages alone did not produce dramatic effects in our model.

4.4.2: Modulating effect of fatty acids on RAW 264.7 macrophage cytokines

Our finding that long-chain n-3 PUFA decreased MCP-1 and IL-6 gene expression and MCP-1 secreted protein in the presence of LPS is similar to other work (110) and generally
confirms the well-known anti-inflammatory effects of fish oil-derived n-3 fatty acids (97).

However, our findings with regards to lack of fatty acid effects on TNF-α do not agree with data from other studies. For example, Oliver et al (2012) found that treating J77.4 murine macrophages with 50 μM of EPA or DHA for 5 days, followed by LPS stimulation for 30 minutes decreased TNF-α secretion, which is in line with many other studies (110, 125, 127). In contrast, treatment of RAW 264.7 macrophages with 50 μM of EPA or DHA for 20 hours, followed by treatment with LPS for 12 hours increased secretion of TNF-α (142). Together with our own findings that long-chain n-3 PUFA had no influence on TNF-α produced by macrophages, current evidence is conflicting and further research is needed to confirm the response of macrophages to n-3 PUFA. Furthermore, while our data showed a trend for EPA-and DHA-induced reductions in TNF-α secreted protein, we found no fatty acid-induced differences in TNF-α mRNA expression in macrophages, possibly indicating that our data collection time point (4 h after LPS stimulation) was too late to observe any such changes. According to Park et al (2008), TNF-α mRNA has a half-life of 29 minutes, which is lengthened to 81 minutes in the presence of LPS. Chen et al (2006) also reported a longer half-life for TNF-α mRNA when it is exposed to LPS for 30 minutes compared to 1 or 2 hours. Thus, our collection of macrophages for mRNA analysis at 4 hours post LPS stimulation may not have been optimal to observe changes in TNF-α gene expression.

Saturated fatty acids are typically considered to exert pro-inflammatory effects by binding to TLR4 and increasing inflammatory cytokine gene expression and secretion (24, 109). Surprisingly, we found that PA significantly increased macrophage secretion of IL-10, a classic anti-inflammatory cytokine, in both the presence and absence of LPS. This finding was somewhat unexpected given that LPS-activated macrophages (particularly the M1 phenotype)
have been found to secrete more pro-inflammatory cytokines such as TNF-α and IL-6 whereas M2 polarized macrophages, are the phenotype associated with higher secretion of anti-inflammatory IL-10 (59, 114). However, our findings are similar to results showing that LPS-stimulated RAW 264.7 macrophages secreted significantly higher amounts of IL-10 than un-stimulated macrophages (118). Moreover, addition of IL-10 to PA-derived MCM prevented the decrease in insulin-stimulated glucose uptake in L6 skeletal muscle cells following treatment with PA MCM alone (118). Thus, elevated secretion of IL-10 may help to explain some of the results observed with our incubations of L6 skeletal muscle cells in MCM, discussed in a subsequent section.

4.4.3: Modulating effect of fatty acids on RAW 264.7 macrophage gene expression of inflammatory signaling intermediates

In the current study, neither long-chain n-3 PUFA nor PA affected macrophage gene expression of inflammatory signaling intermediates, which contradicts other work showing that pre-treating RAW 264.7 macrophages with EPA, followed by LPS stimulation, reduced gene expression of NF-κB, a key transcription factor in inflammatory signaling pathways (125). Previous work from our laboratory has also demonstrated EPA- and DHA-reduced gene expression of NF-κB in macrophages co-cultured with adipocytes (140). Martinez-Micaelo et al (2012) also found that treatment of THP-1 human monocytes with 25 μM of DHA, followed by LPS decreased nucleic p65 NF-κB and p65 NF-κB binding activity. Given the large amount of data showing a modulatory effect of n-3 PUFA on NF-κB, it is likely that NF-κB is involved and additional measurements may have shown this. For example, our study looked at gene expression data and did not find an effect of n-3 PUFA, however examining nucleic protein levels of p65 NF-κB, a component of the most abundant NF-κB heterodimer (143), or NF-κB
DNA binding activity may have provided further information as to the involvement of this transcription factor. Other signaling intermediates that may be modulated by n-3 PUFA include p44/p42 (ERK1/2) and TLR2, both of which have been implicated in an EPA-modulated response to LPS (126, 144). Previous work from our laboratory has also found that n-3 PUFA reduced TLR2 gene expression in inflamed adipocytes (140, 145), suggesting a role for other signaling intermediates, however this requires further study in the model used in the current study.

The inability of PA to modulate macrophage NF-κB and TLR4 gene expression in the current study also differs from other research (106, 146). PA has been shown to be a ligand for TLR4 (108), however our results do not indicate any significant PA-induced increase in NF-κB or TLR4 gene expression, possibly due to the dose of PA used. A dose of 200 μM of PA is still physiologically relevant (147) and has been shown to induce inflammation in macrophages (148), thus our dose may have been too low to elicit any changes. A less common dietary saturated fatty acid, lauric acid (12:0), has also been shown to be a ligand for TLR4 (108), and has been shown to activate NF-κB in RAW 264.7 macrophages (109). Lauric acid is one of two acylated saturated fatty acids in the lipid A portion of the LPS molecule that binds and activates TLR4 (24), perhaps indicating that lauric acid may be a more potent ligand for TLR4 compared to PA. Although we chose to study PA given its high prevalence in the diet, further work with lauric acid may help to discern the role of TLR4 as an inflammatory signaling intermediate.

4.4.4: Modulating effect of fatty acid-derived MCM on L6 skeletal muscle cell inflammatory gene expression and subsequent effects on insulin responsiveness

n-3 PUFA decreased macrophage-derived inflammation and importantly, this decrease resulted in reduced gene expression of TNF-α and IL-6 in L6 muscle cells treated with n-3
PUFA MCM. Decreased macrophage-derived inflammation did not, however, result in any significant differences in levels of insulin-stimulated pAkt in the skeletal muscle cells. We chose to measure pAkt as a representation of muscle function as it is an essential protein in the insulin signaling pathway (149). Differences between basal and insulin-stimulated glucose uptake are also not as significant in the L6 skeletal muscle cell line compared to whole muscle tissue (150) and a measurement of pAkt was chosen to provide a more clear indication of the effects of insulin in these cells. The results from the current study are not unlike other recent findings from our own laboratory. More specifically, Tishinsky et al (2013) examined the modulating effects of adipose conditioned media (ACM) generated from cultured adipose tissue explants obtained from rats fed diets differing in fatty acid composition. Incubation of soleus muscle in ACM from high fat saturated fat-fed rats and high fat saturated fat-fed rats supplemented with n-3 PUFA resulted in an increased gene expression of MCP-1, while incubation of soleus and extensor digitorum longus in ACM from low fat-fed rats resulted in an increased gene expression of IL-6 (151). Despite changes in inflammatory cytokine gene expression, there were no effects of the ACM on insulin-stimulated glucose uptake (151), which is in line with our current findings although the models used were quite different precluding more extensive comparison of the results in the absence of further work.

Interestingly, incubation of L6 cells with n-3 PUFA, particularly EPA, on its own (i.e. non-MCM), increased pro-inflammatory cytokine expression and also resulted in a decreased level of pAkt in skeletal muscle cells following insulin stimulation. More specifically, incubation of L6 muscle cells with EPA increased gene expression of both TNF-α and MCP-1, two key pro-inflammatory cytokines that have been shown to induce insulin resistance in rodent skeletal muscle and human skeletal muscle cells, respectively (37, 61). TNF-α has been previously
shown to activate IKKβ and JNK, two kinases capable of phosphorylating Serine 307 on IRS-1, inactivating it (40). The involvement of JNK1 in the relationship between macrophage-derived inflammation and insulin resistance was also shown in a study demonstrating that L6 skeletal muscle cells were protected from insulin resistance when incubated in CM from JNK1−/− macrophages (123). Interestingly, our results cannot be attributed to EPA-induced increases in NF-κB or TLR4 signaling intermediates, as treatment with EPA decreased gene expression of both in the presence of LPS. Other signaling intermediates such as JNK may be predominantly involved, but this requires further study. Finally, the pro-inflammatory effects of EPA on L6 cells may be related to the generation of reactive oxygen species in mitochondria, as long-chain PUFA can easily undergo oxidative peroxidation (152), but this also requires further study in our model.

Undiluted PA MCM and PA on its own (i.e. non-MCM) did not have an effect on L6 cell inflammatory cytokine gene expression or insulin-stimulated pAkt, disagreeing with data from a study by Samokhvalov et al (2009) which showed a reduced level of pAkt in L6 cells following incubation in PA MCM. As previously mentioned, this may be due to our observed increased secretion of IL-10, a classic anti-inflammatory cytokine, from macrophages treated with PA.

4.4.5: Study Strengths and Limitations and Future Directions

One strength of this study was the use of fatty acid-modulated MCM and in particular the inclusion of various dilutions of MCM designed to represent macrophage cytokine secretion in a lean and obese state. Using this model for the first time, we were able to identify specific fatty acid-induced effects, as well as to distinguish between possible effects of lean versus obese differences in macrophage-derived inflammation on skeletal muscle inflammation and insulin responsiveness. Compared to treatment of skeletal muscle cells with the undiluted MCM,
treatment with the 40% and 10% diluted MCM did not result in significant changes in cytokine gene expression. This suggests that cytokine secretion from other cells, in addition to macrophages, in adipose tissue may contribute to the development of skeletal muscle inflammation and insulin resistance and further study is required to determine which cells are involved and to what extent.

Another strength of this study was the inclusion of EPA and DHA alone, as well as in combination (EPA+DHA) as is found in fish and fish oil and many commercially available n-3 PUFA supplements, in order to identify any potential differences between EPA and DHA in terms of their ability to modulate inflammation in either macrophages or skeletal muscle cells. Interestingly, the combination dose of EPA+DHA was often less effective than an individual dose of EPA or DHA, particularly with regards to macrophage cytokine expression and secretion. Some studies have shown that EPA is a more potent anti-inflammatory agent compared to DHA (Sierra 2008), and perhaps some of this potency is lost when it is given in combination with DHA. With regards to the dose of fatty acid for this study, we chose to control for concentration-dependent effects of the fatty acids by using 100 μM of each fatty acid, as this dose of n-3 PUFA was effective in favourably altering inflammatory cytokines in our previous work and others (110, 145). However, 100 μM PA may not have been the most accurate physiological dose given that obese individuals can have plasma free fatty acid concentrations of up to 800 μM, 25% of which is palmitic acid (147). Thus, a higher dose (e.g. 200 μM) of PA might have more accurately represented an obese state and our lower dose in the current study may partly explain observed effects with PA in our model.

While our results indicated some inflammation-related modulatory effects of n-3 PUFA, future work is needed to determine the mechanisms involved in this model. We chose to measure
gene expression levels of NF-κB and TLR4 as previous research has shown that these signaling intermediates are up-regulated in LPS-activated macrophages, and modulated by n-3 PUFA (125, 140, 143). n-3 PUFA, however, had no effect on macrophage gene expression of NF-κB and TLR4, and incubation of skeletal muscle cells with n-3 PUFA on its own (ie, non-MCM), decreased gene expression of NF-κB and TLR4 in the skeletal muscle cells. As mentioned previously, there are many other signaling intermediates that may be involved in this relationship including TLR2, JNK, and ERK1/2, that require study in this model. In addition, n-3 PUFA have been shown to modulate the G-protein coupled receptor (GPR) 120 in RAW 264.7 macrophages, resulting in decreased LPS-induced phosphorylation of IKK and JNK, and prevention of LPS-induced IκB-α degradation (153). Data from our laboratory has recently shown n-3 PUFA modulation of GPR 120 in inflamed adipocytes (145) and this would be interesting to measure in the current model. Finally, it is possible that lipid mediators derived from n-3 PUFA, such as resolvins and protectins, may also be responsible for some of the beneficial effects observed. For example, db/db mice fed Resolvin-D1 (RvD1), a lipid mediator derived from DHA, for 16 days showed improved glucose tolerance and increased adipose tissue Akt phosphorylation compared to control-fed mice (154).

4.5: Conclusion

In conclusion, we found that long-chain n-3 PUFA decreased macrophage inflammatory cytokine expression and secretion in an inflammatory environment induced by the presence of LPS, thus confirming the anti-inflammatory action of these fish oil-derived fatty acids. This reduced macrophage inflammation consequently resulted in a reduced expression of inflammatory cytokines in skeletal muscle cells treated with n-3 PUFA MCM further confirming the anti-inflammatory actions of these fatty acids, although reduced macrophage inflammation
did not have any effects on insulin-stimulated pAkt in the L6 skeletal muscle cells. n-3 PUFA alone (i.e. not from MCM) increased expression of inflammatory cytokines in the presence or absence of LPS in the muscle cells and decreased pAkt in the absence of LPS. Although further study is required to determine the exact mechanisms involved in these relationships, our results highlight the potential involvement of n-3 PUFA in the modulation of macrophage-derived inflammation and the subsequent effects on skeletal muscle cell insulin responsiveness.
References


45. Tishinsky JM, Ma DW, Robinson LE. Eicosapentaenoic acid and rosiglitazone increase adiponectin in an additive and PPARgamma-dependent manner in human adipocytes. Obesity (Silver Spring) 2011; 19: 262-268.


145. Cranmer-Byng MM. Modulation of Lipopolysaccharide-Stimulated Adipokine Synthesis and Secretion by n-3 and n-6 Polyunsaturated Fatty Acids. 2013;.


151. Tishinsky JM, De Boer AA, Dyck DJ, Robinson LE. Modulation of visceral fat adipokine secretion by dietary fatty acids and ensuing changes in skeletal muscle inflammation. *Appl Physiol Nutr Metab* 2013;.


Appendix A: Real-time PCR Primer Sequences

Table 6: Mouse Primers for RAW 264.7 macrophages

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (3’-5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rplp0</td>
<td>actgtctaggacccgagaag</td>
<td>tcccacattgtctccagttct</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>gctgtctgtacacagttgc</td>
<td>caggtgatgggctttta</td>
</tr>
<tr>
<td>Il-6</td>
<td>aacgatagtcacttcgaga</td>
<td>gacatggaatggggtta</td>
</tr>
<tr>
<td>Tnfa</td>
<td>catcttcctatatgcggagcta</td>
<td>tgggagtagacaggtcactaacc</td>
</tr>
<tr>
<td>Nfkb</td>
<td>gagacctgcacactcaagac</td>
<td>ctcaggtccatctctggtggt</td>
</tr>
<tr>
<td>TLR4</td>
<td>agaaaattgccaggatgtgc</td>
<td>ctgatccatgcatttgaggt</td>
</tr>
</tbody>
</table>

Table 7: Rat Primers for L6 skeletal muscle cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (3’-5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rplp0</td>
<td>actgtctaggacccgagaag</td>
<td>tcccacattgtctccagttct</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>cgtgctgtctcagccagat</td>
<td>ggatcatctgtccagttgatg</td>
</tr>
<tr>
<td>Il-6</td>
<td>ccttcaggaacactatgaa</td>
<td>acaacatcagtcacaaggg</td>
</tr>
<tr>
<td>Tnfa</td>
<td>tgaacctgaggtgatcg</td>
<td>ggctgtctactcagtttt</td>
</tr>
<tr>
<td>Nfkb</td>
<td>caggagacacgagagaca</td>
<td>tgtgctggctatctgtcata</td>
</tr>
<tr>
<td>TLR4</td>
<td>agaaaattgccaggatgtgc</td>
<td>ctgatccatgcatttggtggt</td>
</tr>
</tbody>
</table>
**Appendix B: Extra L6 skeletal muscle cell cytokine mRNA data**

**Table 8: mRNA Expression of MCP-1 in L6 Skeletal Muscle Cells Incubated with Various MCM in the Absence of LPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCP-1 mRNA Expression in Undiluted MCM</th>
<th>MCP-1 mRNA Expression in 40% Diluted MCM</th>
<th>MCP-1 mRNA Expression in 10% Diluted MCM</th>
<th>MCP-1 mRNA Expression in non-MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>25.62±1.34</td>
<td>12.69±0.53</td>
<td>14.09±0.73</td>
<td>10.97±0.50</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>33.35±2.01</td>
<td>11.35±0.51</td>
<td>10.79±0.26</td>
<td>10.14±0.37</td>
</tr>
<tr>
<td>EPA</td>
<td>41.72±1.73*</td>
<td>21.17±0.44*</td>
<td>25.91±1.46**</td>
<td>31.22±1.60*</td>
</tr>
<tr>
<td>DHA</td>
<td>30.73±0.67</td>
<td>30.59±1.17**</td>
<td>40.80±1.55**</td>
<td>36.04±0.88*</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>34.26±0.88</td>
<td>26.69±0.95**</td>
<td>28.13±0.69**</td>
<td>24.40±0.95</td>
</tr>
<tr>
<td>PA</td>
<td>23.39±1.11</td>
<td>14.03±0.49</td>
<td>13.50±0.25</td>
<td>11.72±0.17</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control within each dilution. Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Figure 19**: Effect of 40\% diluted MCM with LPS and 10\% diluted MCM with LPS on MCP-1 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate significant differences between treatments. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Table 9: mRNA Expression of MCP-1 in L6 Skeletal Muscle Cells Incubated with various Dilutions of SR Control, BSA+Ethanol, EPA, DHA, EPA+DHA and PA MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Dilution</th>
<th>SR Control</th>
<th>BSA+Ethanol</th>
<th>EPA</th>
<th>DHA</th>
<th>EPA+DHA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>25.62±1.34a</td>
<td>33.35±2.01a</td>
<td>41.72±1.73a</td>
<td>30.73±0.67</td>
<td>34.26±0.88</td>
<td>23.39±1.11a</td>
</tr>
<tr>
<td>40% Diluted</td>
<td>12.69±0.53ab</td>
<td>11.35±0.51b</td>
<td>21.17±0.44b</td>
<td>30.59±1.17</td>
<td>26.69±0.95</td>
<td>14.03±0.49b</td>
</tr>
<tr>
<td>10% Diluted</td>
<td>14.09±0.73ab</td>
<td>10.79±0.26b</td>
<td>25.91±1.46b</td>
<td>40.80±1.55</td>
<td>28.13±0.69</td>
<td>13.50±0.25b</td>
</tr>
<tr>
<td>non-MCM</td>
<td>10.97±0.50b</td>
<td>10.14±0.37b</td>
<td>31.22±1.60ab</td>
<td>36.04±0.88</td>
<td>24.40±0.95</td>
<td>11.72±0.17b</td>
</tr>
</tbody>
</table>

Letters indicate differences between dilutions in each treatment group. Dilutions sharing letters are not significantly different from each other. Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Figure 20:** Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on MCP-1 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.
Figure 21: Effect of 40% diluted MCM with LPS and 10% diluted MCM with LPS on TNF-α mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate differences between treatment groups. Treatment groups sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Figure 22:** Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on TNF-α mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate differences between dilutions. Dilutions sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate.
**Figure 23:** Effect of 40% diluted MCM with LPS and in 10% diluted MCM with LPS on IL-6 mRNA expression in L6 skeletal muscle cells in. Cells were treated for 24 h with MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate differences between treatment groups. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Figure 24**: Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on IL-6 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.
Appendix C: Extra L6 skeletal muscle cell inflammatory signaling intermediate mRNA data

Table 10: mRNA Expression of NF-κB in L6 Skeletal Muscle Cells Incubated with Various MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NF-κB mRNA Expression in Undiluted MCM</th>
<th>NF-κB mRNA Expression in 40% Diluted MCM</th>
<th>NF-κB mRNA Expression in 10% Diluted MCM</th>
<th>NF-κB mRNA Expression in non-MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>1.71±0.03</td>
<td>1.49±0.05</td>
<td>1.31±0.05</td>
<td>1.42±0.05</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>1.22±0.02</td>
<td>1.17±0.03</td>
<td>1.42±0.04</td>
<td>1.52±0.06</td>
</tr>
<tr>
<td>EPA</td>
<td>1.27±0.03</td>
<td>1.29±0.03</td>
<td>1.80±0.06*</td>
<td>1.69±0.05</td>
</tr>
<tr>
<td>DHA</td>
<td>1.60±0.05</td>
<td>1.95±0.04</td>
<td>1.91±0.05*</td>
<td>1.99±0.04*</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>1.39±0.04</td>
<td>1.65±0.05</td>
<td>1.59±0.07</td>
<td>1.21±0.03</td>
</tr>
<tr>
<td>PA</td>
<td>1.49±0.12</td>
<td>2.15±0.14</td>
<td>1.98±0.09*</td>
<td>1.74±0.08</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control within each dilution. Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 25: Effect of 40% diluted MCM with LPS and 10% diluted MCM with LPS on NF-κB mRNA expression in L6 skeletal muscle cells in. Cells were treated for 24 h with MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate significant differences between treatments, p<0.05. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Table 11**: mRNA Expression of NF-κB in L6 Skeletal Muscle Cells Incubated with various Dilutions of SR Control, BSA+Ethanol, EPA, DHA, EPA+DHA and PA MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Dilution</th>
<th>SR Control</th>
<th>BSA+Ethanol</th>
<th>EPA</th>
<th>DHA</th>
<th>EPA+DHA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>1.71±0.03</td>
<td>1.22±0.02</td>
<td>1.27±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60±0.05</td>
<td>1.39±0.04</td>
<td>1.49±0.12</td>
</tr>
<tr>
<td>40% Diluted</td>
<td>1.49±0.05</td>
<td>1.17±0.03</td>
<td>1.29±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.95±0.04</td>
<td>1.65±0.05</td>
<td>2.15±0.14</td>
</tr>
<tr>
<td>10% Diluted</td>
<td>1.31±0.05</td>
<td>1.42±0.04</td>
<td>1.80±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.91±0.05</td>
<td>1.59±0.07</td>
<td>1.98±0.09</td>
</tr>
<tr>
<td>non-MCM</td>
<td>1.42±0.05</td>
<td>1.52±0.06</td>
<td>1.69±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.99±0.04</td>
<td>1.21±0.03</td>
<td>1.74±0.08</td>
</tr>
</tbody>
</table>

Letters indicate differences between dilutions in each treatment group. Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Figure 26:** Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on NF-κB mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.
Table 12: mRNA Expression of TLR4 in L6 Skeletal Muscle Cells Incubated with Various MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TLR4 mRNA Expression in Undiluted MCM</th>
<th>TLR4 mRNA Expression in 40% Diluted MCM</th>
<th>TLR4 mRNA Expression in 10% Diluted MCM</th>
<th>TLR4 mRNA Expression in non-MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>1.34±0.03</td>
<td>1.26±0.04</td>
<td>1.28±0.05</td>
<td>1.31±0.04</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>1.25±0.01</td>
<td>1.28±0.02</td>
<td>1.41±0.03</td>
<td>1.57±0.06</td>
</tr>
<tr>
<td>EPA</td>
<td>1.12±0.02</td>
<td>1.34±0.06</td>
<td>1.50±0.07</td>
<td>1.33±0.05</td>
</tr>
<tr>
<td>DHA</td>
<td>1.35±0.02</td>
<td>1.39±0.04</td>
<td>1.32±0.04</td>
<td>1.33±0.03</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>1.32±0.06</td>
<td>1.25±0.03</td>
<td>1.03±0.02</td>
<td>0.93±0.02*</td>
</tr>
<tr>
<td>PA</td>
<td>1.09±0.04*</td>
<td>1.46±0.06</td>
<td>1.30±0.04</td>
<td>1.25±0.04</td>
</tr>
</tbody>
</table>

* indicates p<0.05, ** indicates p<0.001 relative to serum-reduced (SR) control within each dilution. Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 27: Effect of 40% diluted MCM with LPS and 10% diluted MCM with LPS on TLR4 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM. Serum-reduced (SR) media+LPS and BSA with ethanol+LPS acted as controls. Letters indicate differences between treatments, p<0.05. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
**Table 13:** mRNA Expression of TLR4 in L6 Skeletal Muscle Cells Incubated with various Dilutions of SR Control, BSA+Ethanol, EPA, DHA, EPA+DHA and PA MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Dilution</th>
<th>SR Control</th>
<th>BSA+Ethanol</th>
<th>EPA</th>
<th>DHA</th>
<th>EPA+DHA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>1.34±0.03</td>
<td>1.25±0.01</td>
<td>1.12±0.02</td>
<td>1.35±0.02</td>
<td>1.32±0.06</td>
<td>1.09±0.04</td>
</tr>
<tr>
<td>40% Diluted</td>
<td>1.26±0.04</td>
<td>1.28±0.02</td>
<td>1.34±0.06</td>
<td>1.39±0.04</td>
<td>1.25±0.03</td>
<td>1.46±0.06</td>
</tr>
<tr>
<td>10% Diluted</td>
<td>1.28±0.05</td>
<td>1.41±0.03</td>
<td>1.50±0.07</td>
<td>1.32±0.04</td>
<td>1.03±0.02</td>
<td>1.30±0.04</td>
</tr>
<tr>
<td>non-MCM</td>
<td>1.31±0.04</td>
<td>1.57±0.06</td>
<td>1.33±0.05</td>
<td>1.33±0.03</td>
<td>0.93±0.02</td>
<td>1.25±0.04</td>
</tr>
</tbody>
</table>

Values are mean Fold Change ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 28: Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on TLR4 mRNA expression in L6 skeletal muscle cells. Cells were treated for 24 h with MCM or non-MCM. Letters indicate differences between dilutions. Values are means ± SEM, n=3 replicates in duplicate.
Appendix D: Extra L6 skeletal muscle cell pAkt data

Table 14: pAkt in Insulin-Stimulated L6 Skeletal Muscle Cells Incubated with Various MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pAkt Ser473 (A.U.) in Undiluted MCM</th>
<th>pAkt Ser473 (A.U.) in 40% Diluted MCM</th>
<th>pAkt Ser473 (A.U.) in non-MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Control</td>
<td>7.73±0.73</td>
<td>7.10±0.65</td>
<td>8.31±0.46&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSA+Ethanol</td>
<td>8.92±1.00</td>
<td>5.89±0.39</td>
<td>7.43±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EPA</td>
<td>9.99±0.65</td>
<td>7.22±0.63</td>
<td>6.22±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DHA</td>
<td>8.45±0.44</td>
<td>9.46±0.39</td>
<td>9.60±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EPA+DHA</td>
<td>7.82±0.50</td>
<td>7.27±0.24</td>
<td>6.67±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>8.25±0.95</td>
<td>7.62±0.63</td>
<td>6.99±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Letters indicate differences between treatments in each respective dilution. Treatments sharing letters are not significantly different from each other. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 29: Effect of 40% diluted MCM with LPS on levels of pAkt in insulin-stimulated L6 skeletal muscle cells. Cells were treated for 24 h with MCM, serum-starved for 3 to 5 h, followed by treatment for 10 min with 100 nM insulin. Cells were rinsed in PBS and lysed in buffer prior to ELISA for determination of levels of pAkt. Values are means ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Table 15: pAkt in Insulin-Stimulated L6 Skeletal Muscle Cells Incubated with various Dilutions of SR Control, BSA+Ethanol, EPA, DHA, EPA+DHA and PA MCM in the Absence of LPS

<table>
<thead>
<tr>
<th>Dilution</th>
<th>SR Control</th>
<th>BSA+Ethanol</th>
<th>EPA</th>
<th>DHA</th>
<th>EPA+DHA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>7.73±0.73</td>
<td>8.92±1.00</td>
<td>9.99±0.65</td>
<td>8.45±0.44</td>
<td>7.82±0.50</td>
<td>8.25±0.95</td>
</tr>
<tr>
<td>40% Diluted</td>
<td>7.10±0.65</td>
<td>5.89±0.39</td>
<td>7.22±0.63</td>
<td>9.46±0.39</td>
<td>7.27±0.24</td>
<td>7.62±0.63</td>
</tr>
<tr>
<td>non-MCM</td>
<td>8.31±0.46</td>
<td>7.43±0.65</td>
<td>6.22±0.39</td>
<td>9.60±0.21</td>
<td>6.67±0.09</td>
<td>6.99±0.25</td>
</tr>
</tbody>
</table>

Values are mean A.U. ± SEM, n=3 replicates in duplicate. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitic acid.
Figure 30: Effect of various dilutions of SR control MCM with LPS and BSA+ethanol MCM with LPS on levels of pAkt in insulin-stimulated L6 skeletal muscle cells. Cells were treated for 24 h with MCM, serum-starved for 3 to 5 h, followed by treatment for 10 min with 100 nM insulin. Cells were rinsed in PBS and lysed in buffer prior to ELISA for determination of levels of pAkt. Values are means ± SEM, n=3 replicates in duplicate.