Long-chain n-3 Polyunsaturated Fatty Acids Mitigate Inflammatory Adipokines Derived from Adipocyte-Macrophage Cross-talk and Ensuing Changes in M1-Macrophage Polarization Markers

by

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ABSTRACT

LONG-CHAIN N-3 POLYUNSATURATED FATTY ACIDS MITIGATE INFLAMMATORY ADIPOKINES DERIVED FROM ADIPOCYTE-MACROPHAGE CROSS-TALK AND ENSUING CHANGES IN M1-MACROPHAGE POLARIZATION MARKERS

Anna A. De Boer Adviser: University of Guelph, 2015 Dr. Lindsay E. Robinson

Macrophages are recruited into obese adipose tissue (AT) and interact with adipocytes to promote further macrophage recruitment and development of chronic AT inflammation, characterized by AT secreted proteins that play a role in development of pathologies, such as insulin resistance. High-fat diet-induced obesity rodent models have shown that the dietary long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may prevent excessive AT inflammation. However, the extent to which LC n-3 PUFA modify adipocyte-macrophage cross-talk and whether this occurs through signaling mechanisms involving peroxisome proliferator-activated receptor gamma (PPARγ) or the LC n-3 PUFA upregulated anti-inflammatory adipokine, adiponectin, is not known.

In this thesis, it was found that LC n-3 PUFA perturb inflammatory adipokine secretion resulting from adipocyte-macrophage cross-talk in an in vitro co-culture model designed to mimic the ratio of adipocytes:macrophages in obese AT. The addition of a potent PPARγ antagonist to co-cultures with EPA or DHA decreased adipocyte cellular adiponectin without affecting mRNA expression or protein secretion of inflammatory IL-6 and MCP-1 (CCL2). Anti-inflammatory effects of LC n-3 PUFA were also found in an in vitro co-culture model where
macrophages were isolated from low or high-fat-fed mice, with or without LC n-3 PUFA, suggesting that dietary LC n-3 PUFA also blunt inflammatory adipocyte-macrophage cross-talk. Additionally, when adiponectin was neutralized in co-cultures there was a partial loss of LC n-3 PUFA-mediated suppression of M1-macrophage polarization and associated cytokine secretion, as well as NLRP3 inflammasome gene expression. Finally, such adiponectin-dependent effects were further observed in an *ex vivo* model, wherein adiponectin-neutralizing antibody was added to AT conditioned media from LC n-3 PUFA-fed mice prior to incubation with a murine macrophage cell line. Here, neutralizing adiponectin partly reversed LC n-3 PUFA-induced anti-inflammatory effects, including macrophage lipid uptake, mRNA expression of M1-polarization markers and NLRP3 inflammasome genes, as well as secretion of CCL2. Together, the data in this thesis suggest that LC n-3 PUFA mitigate inflammatory adipocyte-macrophage cross-talk partly through an adiponectin-mediated mechanism. Ultimately, this work supports LC n-3 PUFA as a dietary strategy to mitigate obese AT inflammation.
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They say time flies when you’re having fun, and I can certainly say that I’ve had my share of fun experiences throughout my graduate degree thanks to a wonderful support network of people.

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List of Abbreviations

AA  arachidonic acid
ACM  adipose conditioned medium
Ad  adiponectin
AdipoR1/R2  adiponectin receptor 1/2
AIM2  absent in melanoma 2
ALA  alpha linolenic acid
AMPK  AMP-activated protein kinase
ANOVA  analysis of variance
Anti-Ad  Adiponectin-neutralizing antibody
AP-1  activator protein 1
APC  antigen presenting cell
ART  adipose resident T cell
AT  adipose tissue
ATM  adipose tissue macrophage
BSA  bovine serum albumin
CCL2 (MCP-1)  chemokine (c-c motif) ligand 2 (aka monocyte chemoattractant protein 1)
CCL5  chemokine (c-c motif) ligand 5 (aka regulated on activation, normal T cell (RANTES) expressed and secreted)
CCL7 (MCP-3)  chemokine (c-c motif) ligand 5 (aka monocyte chemoattractant protein 3)
CD  cluster of differentiation
cDNA  complementary DNA
CLS  crown-like structures
CON  control
DAMP  damage associated molecular pattern
DHA  docosahexaenoic acid
DIO  diet induced obesity
DMEM  Dulbecco's modified eagle medium
DPA  docosapentaenoic acid
ELISA  enzyme-linked immunosorbent assay
EPA  eicosapentaenoic acid
ER  endoplasmic reticulum
ERK  extracellular signal-regulated
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
FO  fish oil
GPR  G-protein coupled receptor
HF  high fat
IFNγ  interferon gamma
IL  interleukin
iNOS  inducible nitric oxide synthase
IRS-1  insulin receptor substrate-1
JNK  c-Jun N-terminal kinases
LA  linoleic acid
LC n-3 PUFA  long-chain n-3 polyunsaturated fatty acids
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>LF</td>
<td>low fat</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP1-IP</td>
<td>monocyte chemoattractant protein 1- induced protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLRP</td>
<td>NOD-Like Receptor family, Pyrin domain containing</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PA</td>
<td>palmitic acid</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<td>PCR</td>
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</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferative activation receptor</td>
</tr>
<tr>
<td>Px</td>
<td>polymyxin B</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SFA</td>
<td>saturated fatty acid</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signaling-3</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
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Chapter 1: Review of literature

1.1.1 Introduction: Obesity prevalence and etiology

Approximately 35% of the world’s adult population is overweight, with 11% classified as obese (5). Obesity is strongly associated with the development of metabolic diseases (e.g. type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease (43, 49, 167)), as well as autoimmune diseases (e.g. rheumatoid arthritis, psoriasis, multiple sclerosis, inflammatory bowel disease and type 1 diabetes (244)), and some cancers (e.g. breast (158)). Obesity-related pathologies stem partly from adipose tissue (AT) dysfunction, characterized by immune cell infiltration, which has prompted extensive immunometabolic research related to AT biology and the development of novel, immunomodulation strategies for the prevention and treatment of chronic disease.

1.1.2 Metabolic endotoxemia and AT dysfunction in obesity

The gut microbiota has a mutualistic relationship with the host that provides a number of benefits to the host including maintaining gut barrier function, modulating the immune response, and providing energy substrates (206). Additionally, it is now understood that the microbiota can regulate metabolism in a variety of tissues, including AT. In obesity, several studies have shown that the microbial population changes partly through dietary modification; habitual ‘Western’ diets high in fat and low in fermentable fiber may allow for greater expansion of opportunistic pathogens from Staphlococcus and Enterobacter genuses to thrive, especially certain strains of gram negative species (like E. coli) that produce endotoxin or lipopolysaccharide (LPS) (206). The resulting changes in the microbiome population can promote an increase in gut permeability
and subsequent LPS translocation into the systemic circulation (26). The increased systemic LPS leads to a sub-clinical form of inflammation termed metabolic endotoxemia which may exacerbate insulin resistance and metabolic dysfunction in obesity and type 2 diabetes (26). Notably, LPS has been shown to accumulate in AT during obesity (272); therefore, the contribution of LPS to AT dysfunction in obesity warrants further study.

### 1.1.3 White AT endocrine function and remodeling in obesity

White AT is now a well-appreciated endocrine organ (69). In addition to triglyceride storage, AT secretes proteins termed adipokines that play roles in systemic energy usage, food intake regulation and inflammation (173). Adipokines are secreted by adipocytes and cells of the stroma-vascular fraction including endothelial cells and immune cells like macrophages and T cells (224). Adipokine secretion becomes dysregulated in obesity due to AT remodeling (224) and subsequent cellular stress (e.g. ER stress, hypoxia, etc.) (38, 279). Remodeling is characterized by adipocyte hypertrophy (increased adipocyte size due to triglyceride accumulation) and hyperplasia (increased number of adipocytes), impaired angiogenesis, extracellular matrix overproduction (fibrosis), and immune cell infiltration (223) (see Figure 1.1). Notably, obese individuals exhibit increased plasma levels of inflammatory mediators, such as TNFα, IL-6, IL-1β, CCL2 (MCP-1), CCL5 (RANTES) and leptin, among others (reviewed by (11, 237)), while levels of the anti-inflammatory adipokine, adiponectin (Ad) decrease (97).
Figure 1.1: Changes in white AT immune cell and secretion profile during diet-induced obesity. Lean AT consists of resident T cells, notably Th2 and T reg cells, as well as innate cells, notably M2 macrophages. 1. Adipocytes from lean, insulin-sensitive AT secrete high amounts of the adipokine, Ad, which promotes M2 macrophage polarization. 2. With diet induced obesity, AT undergoes expansion and remodelling which causes inflammation and promotes immune cell chemotaxis. 3. Obese AT: Obese AT consists of an increased number of Th1 CD4+ T cells, CD8+ T cells and inflammatory M1 macrophages, while Th2, T reg and M2 macrophages populations tend to decrease. 4. CD8+ T cells and adipocytes secrete chemokines that promote monocyte recruitment into AT. 5. The local inflammatory microenvironment (e.g. increased LPS; decreased Ad) in obese AT tends to push monocytes to mature into inflammatory M1
macrophages. 6. AT resident adipocytes and immune cells secrete inflammatory cytokines which promote further immune cell chemotaxis and maturation, as well as impair adipocyte insulin sensitivity. Additionally, AT resident macrophages and adipocytes may present specific antigens to T cells which could cause further T cell activation and proliferation, thereby exacerbating inflammation and potentially generating an autoimmune response in obese AT.

1.1.4 Ad-mediated signaling in obesity

Of the many secreted adipokines that have been identified from AT, Ad has been well-studied due to its insulin sensitizing role in peripheral tissues, such as skeletal muscle and liver (reviewed by (232)). Ad belongs to the family of soluble defence collagens (283) and circulates in several isoforms, of which full-length and globular Ad have been most well studied. Full-length Ad is the most prominent isoform and is comprised of low, medium and high-molecular weight oligomers, and exerts its effects largely through the receptor, AdipoR2 on target cells (232). In contrast, the globular fragment represents <1% of circulating Ad; it may be cleaved by leukocytes in a local manner (245) where it binds to the receptor, AdipoR1 on target cells. Briefly, part of Ad’s insulin sensitizing effects are thought to occur by stimulating adenosine monophosphate-activated kinase activity (AMPK; a key enzyme involved in modulating cell energy status (214)) and subsequently increasing fatty acid oxidation in skeletal muscle and decreasing glucose production in the liver (275). Ad signals through its receptors AdipoR1 and AdipoR2 to exert these downstream insulin sensitizing effects in skeletal muscle and liver, respectively (232). Additionally, both AdipoR1 and R2 are present in macrophages (142), which will be further explored and discussed in this thesis.
With increased central adiposity, Ad secretion decreases (8), and likewise increases with weight loss (54, 112). It is not well understood why Ad secretion is downregulated with obesity, but it has been shown that TNFα downregulates Ad gene expression and subsequent protein secretion (81, 106). Other inflammatory cytokines, such as IL-6 (55) and IL-1β (117), have also been shown to decrease Ad secretion from adipocytes \textit{in vitro}. Interestingly, dietary long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) have been shown to increase circulating Ad in rodents (60, 230) and humans (63, 268). Specifically, LC n-3 PUFA, like eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) (178, 231), as well as LC n-3 PUFA-derived pro-resolving metabolites (37) (discussed in section 1.3.3) have been shown to increase the secretion of Ad from adipocytes, which could potentially serve to counteract the Ad decline that ensues in obesity.

1.2 Role of the immune system in obesity

1.2.1 Macrophage infiltration in obesity

In rodent models of obesity (101, 168, 250) and humans (25, 236) macrophage infiltration is markedly increased from about 3% to 20-30% of the cells in the AT stroma-vascular fraction, particularly in the visceral AT depots. This infiltration contributes to the obese inflammatory phenotype (250, 271, 286), and murine models of high-fat (e.g. 40-60% E) diet-induced obesity (DIO) have shown that this occurs concurrently with the development of systemic insulin resistance (168, 261). Specifically, resident endothelial cells secrete adhesion molecules, such as intracellular adhesion molecule-1 and platelet endothelial cell adhesion molecule-1 that promote diapedesis involved in the recruitment of macrophages and other immune cells into AT (40, 169). These AT macrophages (ATM) then form crown-like structures
where macrophages surround dying adipocytes (36), thereby facilitating a microenvironment where paracrine interactions occur. Importantly, macrophage phenotype plays a key role in moderating the degree of inflammatory cross-talk that ensues between adipocytes and macrophages in AT.

Macrophages display tremendous heterogeneity in tissues; whether tissue resident or recruited, they undergo specific differentiation programs in response to the local tissue microenvironment (160, 209) (Figure 1.2). There are two well defined macrophage phenotypes that can differentiate from circulating monocytes (160). These are generally referred to as M1 and M2 macrophage subsets (30, 160); however, polarization state is not absolute and can vary along the M1-M2 spectrum (160). M1 macrophages, or ‘classically activated’ macrophages are activated by the Th1 cytokine interferon-gamma (IFNγ) and microbial products, such as LPS (30). Following activation, M1 macrophages undergo a respiratory burst followed by production of reactive oxygen (ROS) and reactive nitrogen species (RNS) that promote microbicidal responses. This is followed by antigen presentation via the major histocompatibility complex II (MHCII) and the secretion of cytokines such as TNFα and IL-12 involved in promoting cell-mediated immunity (e.g. Th1/Th17 responses) (30, 209). M1 macrophages are essential for the clearance of intracellular pathogens; however, over-activation can promote chronic inflammation in autoimmune pathologies like rheumatoid arthritis, and inflammatory bowel disease (160). In contrast, the Th2 cytokines IL-4 and IL-13 secreted in response to parasitic infections, like helminth infections, promotes the maturation of M2 macrophages (30). M2 macrophages are characterized by the presence of scavenging receptors, CD206 (mannose receptor) and macrophage galactose-type C-type lectin 1 (MGL1) expression of antigen via MHCII and co-stimulatory molecules (209); and in mice, arginase activity (189), which diverts arginine away
from RNS production by the inducible nitric oxide synthase (iNOS) to metabolism favoring collagen synthesis (172). Although M2 macrophages are essential for the clearance of extracellular pathogens and wound healing, over activation can promote pathologies such as tissue fibrosis and allergies (209). Finally, IL-10, TGFβ, glucocorticoids (30), and ingestion of apoptotic cells (efferyctosis) (7) can promote the maturation of M2 macrophages with regulatory functions. Of note, regulatory macrophages are sometimes referred to as M2c macrophages by certain nomenclature (145), which highlights some inconsistencies in the field. These regulatory M2 macrophages are referred to as ‘deactivated’ macrophages since they no longer present antigen via MHCII (30). Macrophages can differentiate into the regulatory phenotype when they become satiated with apoptotic cells through efferyctosis; they secrete anti-inflammatory cytokines, like IL-10 and TGFβ; and travel to lymph nodes to promote immune tolerance and the resolution of inflammation (7).
Figure 1.2: An overview of stimuli that promote macrophage polarization to the M2 phenotype in lean AT or the M1 phenotype in obese AT, as well as their respective effector functions following polarization.

a. Macrophage phenotype and function in lean AT

In lean or healthy white AT, macrophages more closely resemble the alternatively activated M2 phenotype. These resident M2 macrophages express the anti-inflammatory cytokine IL-10 and contribute to the maintenance of insulin sensitivity in AT (136). Interestingly, IL-4 and IL-13 produced by resident eosinophils (265) and invariant natural killer T cells (96) contribute to AT M2 polarization (102). Moreover, in murine macrophages,
signaling through the IL-4/STAT6 axis via the nuclear receptor, peroxisome proliferative-activation receptor gamma (PPARγ) (174) attenuated AT inflammation, while disruption of such signaling decreased insulin sensitivity (194).

b. Macrophage phenotype and function in obese AT

In contrast to the lean or healthy state, circulating monocytes accumulate in obese AT, particularly in visceral depots, where they differentiate into macrophages in an effort to help with the tissue remodeling process and lipid homeostasis (113, 272). ATM also serve to promote angiogenesis (19) secreting platelet derived growth factor locally (180). Many ATM co-localize to CLS and exhibit an inflammatory M1 phenotype, characterized by increased lipid content, an ability to express high levels of anti-microbial iNOS (murine only) and secretion of inflammatory cytokines, such as TNFα and IL-6 (68, 136, 137, 254). However, evidence from recent studies (34, 159) strongly suggests that antigen presentation via MHCII, another key feature of the activated macrophage phenotype (30), warrants further consideration in ATM. Furthermore, Prier et al. (187) have shown that this shift from M2 to M1 macrophages occurs when lipid gets repartitioned from hypertrophic adipocytes to macrophages during obesity progression; M1 macrophages form in response to lipotoxicity and resemble triglyceride droplet laden foam-cells in AT. What is more, in DIO models, M1 ATM increasingly express the integrin/complement receptors 3 (CD11b) and 4 (CD11c) (136), which are involved in the clearance or apoptotic cells coated with complement protein C3bi (154). Specifically, this ATM subset (F4/80+CD11b+CD11c+) has a heightened inflammatory response when treated with fatty acids, which is abrogated when toll-like receptor (TLR)-4 is knocked out (208). Further, the CD11c+ subset seems to be crucial, as ablation of these cells in DIO murine models results in
normalization of insulin sensitivity and reduced systemic inflammation (184, 266). This follows since M1 macrophage-derived cytokines perturb adipocyte insulin sensitivity (139, 177), and the development of local AT insulin resistance plays a key role in promoting systemic insulin resistance (1, 271).

Interestingly, macrophage polarization is not absolute and may shift throughout the progression of obesity, or with weight-loss interventions (126). Macrophage populations exhibiting a mixed M1/M2 phenotype have been observed in obese AT in mice (126, 205) and humans (9, 19, 213, 254, 286); these cells express moderate levels of M2 markers such MGL1 or CD206 and the M1 marker CD11c, and express M2-associated genes involved in lipid metabolism and antigen presentation. Nevertheless, in future studies, it will be important to understand the contribution of different macrophage populations to AT inflammation and dysfunction. For example, over-activation of regulatory-like macrophages may be problematic; M2c (IL-10 polarized) macrophages increased the secretion of thrombopsin-1 in co-culture with primary human adipocytes (58), too much of which promotes excessive TGFβ signaling associated with decreased angiogenesis and excessive fibrosis (18, 120). Furthermore, other immune cell subsets including B cells (260), dendritic cells (14), neutrophils (50), eosinophils (265), mast cells (128), natural killer cells (171) and various T cell subsets (discussed below) have been reported to play key roles in AT tissue inflammation, possibly through direct or indirect modulation of resident ATM responses; however, this is beyond the scope of the current review (reviewed by (149, 262)).
1.2.2 T cell subsets in obese AT

Although human data is less resolved, work from murine in vivo studies has shown that T cells infiltrate AT (particularly visceral depots) prior to a large increase in the macrophage population during DIO (48, 109, 168, 261). AT-derived CCL5 (RANTES) plays a role in adipose recruited T cell (ART) chemotaxis (267) where CD3⁺ T cells then co-localize in between adipocytes or in CLS (186, 193). However, there are some discrepancies as to which T cell populations are associated with obesity and metabolic abnormalities, as well as the timeline of their recruitment. Some have shown that CD8⁺ T cells infiltrate AT (168, 186, 193), whereas others have shown that CD4⁺ T cells increase (109, 261), or a mixture of both (48, 135, 147, 159, 195, 241). Concomitant with this, CD4⁺ Foxp3⁺ T reg (44, 52, 56, 186, 261), and Th2 (261) cell populations get depleted in AT, while Th1 (46, 105, 219, 261) and Th17 (147) populations increase.

Although the stimuli that promote Th1/Th17 polarization in obese AT is not well defined, several groups have shown that the T cell receptor repertoire from AT isolated effector/memory T cells is restricted (44, 56, 159, 261). This suggests that specific antigens are driving the T cell response which could lead to autoimmune responses, including auto-antibody production. Specifically, such restriction occurs in the Vβ chain of the T cell receptor in ARTs compared to splenic T cells in obese mice (277). Interestingly, B cell-secreted IgM (6) followed by class switching to pathogenic IgG2c (260) in obese mice caused IgG to localize to CLS in visceral AT, drove IFNγ-producing CD4⁺ and CD8⁺ ARTs and M1 ATM responses, and impaired systemic insulin sensitivity, suggesting that auto-antibodies promote obesity-associated pathologies. Moreover, candidate antigens from insulin-resistant, obese male human subjects revealed several potential intracellular auto-antigens thought to be widely expressed in many tissues (262), as
well as bacterial antigens (155), possibly stemming from the obesity-driven increase in gut permeability (27, 64).

Regardless of the largely unknown antigens, recent research has shown that antigen presenting cell (APC)-mediated antigen presentation plays a key role in obesity-associated AT inflammation and systemic insulin resistance. Interestingly, knock-out of co-stimulatory molecules B7.1/2 (CD80/86; required for naïve T cell activation) in a murine DIO model worsened insulin resistance, while reducing tissue Foxp3+ T reg populations (29, 290). In contrast, blunting co-stimulatory signaling when T regs are not inherently impaired by antibody against CD80/86 (29), inhibiting co-stimulatory CD80/86 signaling by soluble cytotoxic T-lymphocyte-associated protein-4 antibody (66), or knock-out of other co-stimulatory molecules such as CD40 (186), improved DIO associated metabolic impairments including insulin sensitivity. Moreover, knock-out of whole-body MHCII (46) or B cell specific MHCI or II (260) in the DIO model resulted in decreased ATM recruitment with concomitant decreases in ARTs, and improvements in systemic insulin sensitivity; however, neutralization of MHCII after the onset of obesity does not show this improved metabolic phenotype (159). Similarly, using the DIO model, Yang et al. (277) showed that depletion of ART in visceral AT from young DIO mice improved insulin sensitivity, but not in older adult mice. Taken together, this data suggests that there may be an early window of time during which the adaptive immune response may be controlled in obesity, whereby AT inflammation and related pathologies such as systemic insulin resistance may be subdued or possibly all together reversed through immunotherapy.
a. Adipocyte-T cell interactions in obese AT: insight into monocyte recruitment and polarization

In obese AT, adipocytes interact with cells of the innate and adaptive immune systems through multiple signaling pathways, ultimately initiating an immune response that leads to monocyte recruitment. Like macrophages, LPS-treated adipocytes show increased NFκB activity (162, 211, 248), and increased TLR2 and 4 (2, 15, 106) in vitro and ex vivo, suggesting that LPS may prime further inflammatory cross-talk between adipocytes and immune cells infiltrating obese AT. For example, LPS-treated adipocytes secrete a plethora of cytokines, chemokines, and acute-phase proteins and result in CD4⁺ T-cell migration in vitro (151). Moreover, CD8⁺ T cells are recruited into AT as early as six weeks in DIO models, prompting CD8⁺ T cell-adipocyte paracrine interactions that lead to the secretion of chemokines involved in macrophage recruitment and M1 polarization (168).

Adipocyte MHCII-mediated antigen presentation to ARTs may play a role in ATM accumulation and polarization observed in DIO models (46). Interestingly, adipocytes themselves can interact with T cells, specifically through paracrine cross-talk and antigen presentation, whereby adipocytes activated CD4⁺ T cells in an antigen-specific, contact-dependent manner involving MHCII (46, 281). More specifically, adipocyte-secreted leptin promotes Th1 responses including IFNγ secretion (133). The IFNγ secreted from ARTs then prompts MHCII-mediated antigen presentation and subsequent IL-1β secretion from adipocytes (281). IL-1β then feeds back onto resident ARTs to promote continued IFNγ secretion, thereby creating a positive feed-back loop that may promote M1-macrophage polarization and AT inflammation (281). While adipocytes may initially express MHCII and present antigen to ARTs
in early stages of obesity (46), ATM MHCII-mediated antigen presentation may propagate inflammation once obesity is established, but this requires further study.

b. Macrophage-T cell interactions in obese AT

Macrophages have been shown to activate CD4\(^+\) effector/memory T cells in obese mice (34, 159) and humans (42) through a mechanism involving macrophage antigen presentation via MHCII. This novel research area will only be briefly explored in this thesis, but is an exciting new area of research for future studies. More specifically, the F4/80\(^+\)CD11b\(^+\)CD11c\(^+\) subset presented antigen via MHCII within fat-associated lymphoid tissue causing CD4\(^+\) effector/memory T cell proliferation within visceral AT (159). Of note, macrophage secreted IFN\(\gamma\) and IL-2 caused skewing to the Th1 phenotype in T cell co-cultures (159), which may partly explain the decreased ratio of T regs:Th1 cells observed in obese AT (34). While macrophage and adipocyte-mediated antigen presentation to T cells may drive AT inflammation in obesity, a more careful look into the innate signaling mechanisms that mediate antigen presentation and inflammatory cross-talk with immune cells is warranted.

1.3 Inflammatory signaling mechanisms in obese AT

1.3.1 Role of Toll-like receptors in AT inflammation

Toll-like receptors are innate pattern-recognition receptors involved in the recognition of pathogen-associated molecular patterns (PAMPS), as well as danger-associated molecular patterns (DAMPs). Specifically, TLR4 recognizes many PAMPS, most notably the lipid A portion of LPS from gram-negative bacteria (94), as well as DAMPs (71) such as saturated free fatty acids recognized through indirect association with the endogenous ligand, a secreted liver
glycoprotein called fetuin A (179). Additionally, lauric acid (C12:0), a saturated fatty acid, dimerizes with LPS and stabilizes the TLR4 protein complex within lipid raft signaling complexes in the plasma membrane, thereby facilitating more efficient receptor activation and downstream signaling (264). Further, metabolic endotoxemia causes LPS accumulation (272) and exacerbated TLR-mediated signaling in obese AT. Activation of TLR4-signaling occurs by both MyD88-dependent and -independent pathways, leading to activation of transcription factors, NFκB and AP-1, and production of many inflammatory cytokines (131).

Interestingly, TLR4 (and 2) are upregulated in obese AT in adipocytes, as well as cells of the stroma-vascular fraction, including macrophages (208). Along these lines, lipid infusion increased NFκB activation and nuclear activity in AT, but not in TLR4 KO mice (208), which may partly explain why TLR4 KO mice are partially protected from high fat diet-induced insulin resistance (185, 208, 239).

1.3.2 Role of the inflammasome in AT inflammation

Inflammasomes are also part of the innate immune system sensing endogenous PAMPS and DAMPS. Particularly, the NLRP3 inflammasome senses a wide array of DAMPS associated with metabolic dysfunction and tissue stress in obesity including high extracellular ATP, palmitic acid, cerimides, uric acid, and ROS among others (reviewed by (217, 253). NLRP-inflammasomes consist of a cytosolic multi-protein complex with nod-like protein receptors (NLPRs) along with the adaptor protein (ASC) which contains the caspase activation and recruitment domain (CARD) (201). Briefly, TLR ligands prime upregulation of inflammasome genes and assembly of the cytosolic protein signaling complex, while a second DAMP signal activates the inflammasome complex. Activation of this complex results in the proteolytic
cleavage and activation of caspase-1; mature caspase-1 then causes the proteolytic cleavage of pro-IL-1β and pro-IL-18 to their mature forms prior to secretion (Reviewed by (13, 201)). IL-1β acts at a local level binding to the IL-1 receptor on target cells, which promotes the expression of a self-amplifying cytokine network (13). Briefly, IL-1β signaling utilizes MyD88-dependent signaling to activate stress kinases, JNK, p38 MAPK and IKKβ culminating in the activation of AP-1 and NFκB transcription factors to increase the expression of several inflammatory cytokines and chemokines (13). Interestingly, a build-up of cellular ROS and inhibition of autophagy (207) (a cell survival mechanism to recycle nutrients or damaged proteins/organelles (228)) both promote persistent inflammasome activation, making these factors key targets for inflammasome activity mitigating therapies.

At the whole body level, blunting NLRP3 inflammasome activity may help to prevent the development of AT dysfunction and insulin resistance. Along these lines, NLRP3, PYCARD (ASC) and caspase-1 knock-out mice show improved insulin sensitivity in the DIO model (215, 241, 252). Strikingly, NLRP3 knock-out mice had decreased AT CD4+ and CD8+ T effector/memory cells, decreased macrophage infiltration and increased M2-macrophage marker expression (241). Likewise, in humans, mRNA markers of AT inflammasome activity are positively correlated with CD4+ Th1, Th17 (75) and CD8+ T cell (110) markers.

Evidence from murine (241, 252) and human studies (116, 122) suggests that macrophages (among other cell types) may propagate inflammation in obesity through activation of the NLRP3 inflammasome. Inflammasome activation in macrophages results in the formation of autophagolysosomes essential for pathogen processing and antigen presentation (228). Interestingly, macrophage AMPK activity preserves fatty acid oxidation, thereby preventing the build-up of ROS and reactive lipid species like cerimides that cause NLRP3 inflammasome
activity and M1 macrophage polarization (252). Finally, caspase-1 activity has also been observed in adipocytes (72, 216) where it impairs adipogenesis and insulin-stimulated glucose uptake (216). Taken together, inflammasome activation occurs in adipocytes and macrophages, thereby playing a key role in AT dysfunction and related pathologies. Given the potential role of dietary fatty acids in promoting AT inflammation and related pathologies through innate TLR and NLRP3 inflammasome signaling pathways, dietary strategies to mitigate such inflammation warrant further exploration.

1.3.3 Dietary lipids and obesity-associated pathologies

Dietary lipids comprise 20-35% of daily energy intake (kcal) with the majority of fats coming from saturated (SFA; 10% kcal) and polyunsaturated (PUFA; 6-11% kcal) sources and monounsaturated fatty acids (MUFA) making up the difference (291). Lipids play a wide variety of roles in the body beyond their role as energy substrates, including a structural role in membranes, as well as a role as signaling intermediates, interacting directly or indirectly with other signaling intermediates at the transcriptional and post-translational level (70).

Among the PUFA, the n-6 and n-3 classes are most well studied; the dietary parent compounds linoleic (18:2 n-6, LA) and alpha linolenic (18:3 n-3, ALA) acid are essential fatty acids in the human diet. Once provided in the diet, these essential fatty acids are then converted to other n-6 and n-3 fatty acids through a series of chain elongation and desaturation reactions (Figure 1.3). While these two pathways are independent, they share the same desaturation and elongation enzymes, meaning that the relative abundance of fatty acids (n-6 vs n-3) affect what type of fatty acids are produced (192). Humans consuming a Western diet tend to consume more n-6 fatty acids due to their wide distribution and abundance in vegetable oils (e.g. safflower, corn
and soybean oil) and animal products (meat, liver, and egg lipids), which favour the production of longer-chain n-6 fatty acids, such as arachidonic acid (20:4 n-6, AA) (192).

**Figure 1.3:** Shared n-6 and n-3 fatty acid desaturation and elongation pathways. Adapted from (192).

Recently, experts have argued that the decreased ratio of n-3:n-6 fatty acids consumed in the Western diet may contribute to the progression of chronic inflammatory diseases (70). The n-6 fatty acid, AA, serves as a substrate for the production of inflammatory eicosanoid mediators, which, although essential for an effective inflammatory response, could contribute to chronic inflammation in obesity-associated pathologies over time. In contrast, dietary LC n-3 PUFA like
EPA and DHA also serve as substrates for eicosanoid production but produce less inflammatory eicosanoids as well as pro-resolving, anti-inflammatory autocoids (e.g. resolvins) essential for the timely resolution of inflammation (203). Additionally, LC n-3 PUFA have anti-inflammatory effects at the transcriptional and translational level, which will be further explored and discussed in this thesis. Thus, increasing the consumption of LC n-3 PUFA may be advantageous to mitigate the progression of excessive inflammation, including the chronic inflammatory state characteristic of obesity. Unfortunately, the parent n-3 fatty acid, ALA, is poorly converted to highly unsaturated LC n-3 PUFA in humans, making this plant derived n-3 PUFA a poor precursor for subsequent in vivo production of EPA and DHA (192). Instead, LC n-3 PUFA are found in marine sources, like fatty fish (e.g. salmon, smelt and anchovies). However, these foods tend to be under consumed in the Western diet, and fish oil capsules and enriched foods (e.g. DHA-enriched milk and eggs) have become popular dietary strategies to meet the daily recommended adult n-3 PUFA intake of 0.5-2% kcal, including 0.25-2 g of LC n-3 PUFA (EPA+DHA) (291). Given the potential of dietary LC n-3 PUFA to mitigate inflammation, further exploration of LC n-3 PUFA-mediated anti-inflammatory effects within obese AT is necessary. Of particular importance, adipocyte-macrophage cross-talk could be a key inflammatory loop driving obese AT inflammation (221, 222) which could potentially be modulated by dietary fatty acids, including LC n-3 PUFA (discussed below).

1.3.4 Adipocyte-macrophage interactions in obese AT

Paracrine interactions or cross-talk between adipocytes and macrophages in obese AT play a key role in resident macrophage polarization and the adipokine profile, and can be influenced by dietary factors, such as fatty acids. Various in vitro studies have shown that
saturated fatty acids, such as lauric acid (C12:0) (124) and palmitic acid (C16:0) (222) released from adipocytes activates TLR2 and TLR4 signaling respectively, triggering NFκB-mediated inflammatory gene expression and subsequent cytokine secretion from macrophages. In fact, palmitic acid may exacerbate LPS-mediated inflammation seen in high-fat fed rodents exhibiting metabolic endotoxemia (119); therefore, the additive effects of specific fatty acids plus LPS on AT inflammation warrants further study. Along these lines, murine macrophages treated with palmitic acid plus LPS showed an additive increase in ROS levels (264) followed by NLRP3 inflammasome activation and IL-1β release (116, 252), suggesting that both TLR and inflammasome signaling contribute to M1-macrophage polarization. Consequently, M1-derived cytokines like TNFα and IL-6 feed back onto adipocytes through paracrine signaling to sustain adipocyte-derived inflammatory mediator secretion (e.g. MCP-1, IL-6) and lipolysis (221). This, in turn, may sustain the increased release of saturated free fatty acids and continued TLR/inflammasome signaling in macrophages. Thus, this cross-talk or paracrine signaling between adipocytes and macrophages may partly explain the increased proportion of M1 macrophages and the inflammatory adipokine secretion profile that characterizes obese AT.

1.3.5 Adipocyte-macrophage interactions: Role of dietary LC n-3 PUFA

Unlike saturated fatty acids, LC n-3 PUFA, namely EPA and DHA, are well known anti-inflammatory agents (22, 24) and can serve to abrogate excessive inflammatory adipocyte-macrophage paracrine interactions in obese AT. For example, in murine cellular models, co-culturing macrophages with adipocytes using macrophage conditioned media with DHA decreased secretion of IL-6 and TNFα while it increased secretion of IL-10 and increased insulin-stimulated glucose uptake (177). Ultimately, decreased secretion of inflammatory
adipokines could serve to decrease the ongoing recruitment of monocytes and further inflammatory adipocyte-macrophage paracrine interactions that exacerbate chronic inflammation in obesity. Supporting this concept, in murine models of obesity with the addition of LC n-3 PUFA (100, 135, 156, 235, 276) or resolvin D1 (82, 234) to the diet, or, endogenous production of LC n-3 PUFA in the fat-1 mouse (256), there were reductions in macrophage infiltration and a concomitant decrease in M1 macrophages in visceral AT. Further, these observations are associated with decreased plasma IL-6, MCP-1 and leptin; decreased AT mRNA expression of IL-6 and MCP-1 (10); as well as increased systemic insulin sensitivity (74, 82, 100, 135, 234, 256). Interestingly, the decreased macrophage recruitment may partly be attributable to decreased secretion of chemokines that were markedly decreased in adipocyte co-cultures with LC n-3 PUFA-enriched CD8\(^+\) T cells (157). The immunomodulatory effects of LC n-3 PUFA are largely attributable to signaling mechanisms involving the transcription factor PPAR\(\gamma\) and subsequent upregulation of Ad secretion; lipid rafts and antigen presentation; the G-protein coupled receptors, GPR120 and 40; as well as EPA and DHA-derived resolvins, protectins, maresins and ethanolamines (discussed below, overview given in Figure 1.4).
Figure 1.4: A simplified overview of TLR4 and NLRP3 inflammasome signaling that propagates inflammation within adipocytes and macrophages in obese AT. 1. TLR4 ligands such as LPS and SFAs in association with fetuin A can bind to the TLR4 receptor complex which initiates TLR4-mediated signaling. 2. This TLR4 signaling culminates in the activation of inflammatory signaling intermediates, namely NFκB, which translocates to the nucleus to promote the mRNA expression of inflammatory cytokines and chemokines, APC genes, and NLRP3 inflammasome genes. 3. Following translation, the NLRP3 inflammasome signaling complex is then assembled in the cytosol. 4. DAMPs like ROS and lipid metabolites like ceramides can cause activation of the NLRP3 inflammasome. 5. This culminates in the activation
of caspase-1 which then proteolytically cleaves pro-IL-1β and pro-IL-18 into mature proteins prior to secretion. 6. Secreted cytokines like IL-1β then promote the continual activation of inflammatory signaling cascades, which promotes the prolonged activation of inflammatory signaling pathways like NFκB and production of inflammatory intermediates. Interestingly, LC n-3 PUFA and Ad can mitigate excessive inflammatory signaling through interfering with the signaling cascade that leads to NFκB activation, NFκB-mediated transcription activity, as well as the build-up of metabolic by-products like ROS that cause NLRP3 inflammasome activation (discussed in more detail in the following sections).

a. LC n-3 PUFA and PPARγ dependent anti-inflammatory signaling mechanisms involving Ad

LC n-3 PUFA can also interfere with inflammatory signaling cascades through PPARγ-dependent signaling mechanisms. Many lipids, including LC n-3 PUFA, serve as ligands for PPARγ which heterodimerizes with the retinoic acid receptor (RXR) upon ligand binding to mediate the expression of genes involved in lipid metabolism and immunity (reviewed by (242)). Importantly, PPARγ antagonizes NFκB nuclear activity through trans-repression thereby decreasing the expression of NFκB responsive genes (reviewed by (73)). PPARγ is essential for IL-4 induced M2 macrophage polarization in mice (174) and M2-associated efferyctosis (28). Additionally, PPARγ-dependent mechanisms involve upregulation of the anti-inflammatory adipokine, Ad, specifically in murine (178) and human adipocytes (231), which is also potently upregulated by DHA metabolites, resolvin D1 and D2 (37).

Increasing the production of Ad in obesity may be a beneficial strategy to mitigate inflammation. Both full-length and globular Ad have anti-inflammatory properties in
macrophages, although some controversy exists, not all studies account for the LPS-binding capacity of Ad in vitro (reviewed by (51, 53)). Interestingly, leukocytes can cleave Ad via secreted elastase to release the bioactive globular isoform in a local manner (245); this may account for the enhanced Ad accumulation in the stroma-vascular fraction of AT in obese mice (163). Indeed, in a transgenic murine DIO model over-expression of Ad in macrophages improved insulin sensitivity and decreased systemic and AT inflammatory cytokines (140). Moreover, Ad can also bind to calreticulin receptors on macrophages which enhances M2-associated efferycytosis of apoptotic cells (226). Further, both full-length (62, 142, 175) and globular (238, 284) Ad have been shown to exhibit a response similar to endotoxin tolerance and macrophage polarization towards the M2 phenotype, or enhance M2 polarization in vitro (218) and in vivo (134), which could potentially serve to re-direct the cyclic inflammatory cross-talk between adipocytes and M1 macrophages in obesity. Notably, Ad-mediated anti-inflammatory effects involve inhibition of inflammatory signaling pathways including NFκB (62, 134, 269, 274), AP-1 (181), and ERK1/2 (182, 269, 289). Moreover, globular (182, 284) and full-length (111, 115, 142, 263) Ad promote IL-10 secretion from macrophages. IL-10 represses co-stimulatory molecule expression on APCs (78), suggesting that the Ad-IL-10 axis may affect APC functions. Finally, Ad may perturb M1 macrophage responses by inhibiting NLRP3 inflammasome activity. Ad decreased macrophage ROS (92, 175), increased autophagy (130), and decreased cellular ceramide by increasing ceramidinase activity (86), which may explain the Ad-mediated decrease NLRP3 inflammasome activity observed in myocytes (130), although this has yet to be directly shown in macrophages.
b. LC n-3 PUFA, lipid rafts and TLR signaling

Although it was not directly assessed in this thesis, it is worth noting that the anti-inflammatory effects of EPA and DHA may be partly due to their incorporation into the phospholipid fraction of cellular membranes, where they can act to decrease the signaling efficiency of protein complexes localized in small membrane microdomains called lipid rafts (220), including the TLR4 complex. More specifically, DHA has been shown to perturb LPS or lauric acid-stimulated TLR4 recruitment to lipid rafts, TLR4 homodimerization and MD2 (lymphocyte antigen 96) recruitment, and subsequent activation and downstream signaling in macrophages (264). This occurs because LC n-3 PUFA (like DHA) are stearically incompatible with the hydrophobic lipid species, like cholesterol concentrated in lipid rafts, thereby expanding the lipid raft size and decreasing the efficiency of raft localized protein-protein interactions and down-stream receptor signaling (reviewed by (240)). Secondly, as free fatty acids, LC n-3 PUFA antagonize saturated fatty acid binding to TLR4 in vitro, which could serve to further perturb TLR activation (124, 125) since TLR4 stimulation causes DHA and EPA pre-treated macrophages to secrete these PUFA as free fatty acids (170).

c. LC n-3 PUFA, antigen presentation and T cell responses

LC n-3 PUFA may prevent MHCII-driven auto-reactive T cell-mediated immune responses that contribute to chronic inflammation and related pathologies in obesity. Theoretically, this may be accomplished through 1) decreasing APC mediated antigen presentation, or 2) decreasing co-stimulatory molecule expression from professional APCs (like macrophages), the latter of which promotes a form of T cell anergy called adaptive tolerance in
which T cells remain anergic (or hypo-responsive) as long as the specific antigen persists (reviewed by (202)).

In support of the first idea, \textit{in vitro} treatment with EPA or DHA decreased MHCII expression in stimulated human (89) or murine (104) monocytes, and MHC1 expression in a murine B cell line (204). Moreover, \textit{in vitro} antigen presentation models, decreased T cell proliferation was observed with human (90, 91, 95, 287) or rodent T cells (67, 88, 197, 288). Complementary data from an \textit{in vivo} study in which mice were fed LC n-3 PUFA-enriched diets showed decreased MHCII expressing APCs in n-3 PUFA supplemented groups infected with the persistent pathogen, \textit{Lysteria monocytogenes} (88). Moreover, decreased T cell proliferation was observed \textit{in vivo} in antigen-specific n-3-enriched T cells in ovalbumin immunized mice (288). Further, these authors reported a specific decrease in Th1 cell proliferation following antigen presentation assays under Th1 polarizing conditions \textit{in vitro} in CD4\(^+\) T cells from mice fed a LC n-3 PUFA-enriched diet (288). This could not be attributed to enhanced T cell apoptosis and these T cell were hypo-responsive to IL-2 stimulation, which suggests that T cell anergy may also occur in LC n-3 PUFA-fed animals. Interestingly, the LC n-3 PUFA upregulated adipokine, Ad, also suppresses CD4\(^+\) (176) and CD3\(^+\) (total) T cell (257) responses in stimulated cells \textit{in vitro} and \textit{in vivo}; however, this field of study is in its infancy. Of note, LC n-3 PUFA also affect T cell activation and proliferation directly; \textit{in vitro} studies with LC n-3 enriched CD4\(^+\) T cells revealed decreased T cell activation and proliferation, which was partly attributed to suppression of lipid raft localized T cell receptor signaling following immunological synapse formation (107, 148, 282).

In support of the second idea, LPS-stimulated RAW 266.7 macrophages co-treated with DHA had decreased co-stimulatory CD86 mRNA and surface protein abundance (200). Along
these lines, DHA decreased LPS-induced CD86 promotor activity in RAW macrophages and decreased surface expression of CD80/86 and CD11c (a marker of dendritic cell maturation) in murine bone marrow derived dendritic cells (249), an effect shown to be largely dependent on PPARγ:RXR signaling (285). Of note, some reports have shown that LC n-3 PUFA mediated modulation of MHCII surface expression is not affected by cyclooxygenase or lipoxygenase inhibition (103, 104, 287), suggesting that DHA-derived pro-resolving metabolites are not acutely involved.

d. LC n-3 PUFA and GPR120/40-mediated inhibition of inflammatory signaling pathways

LC n-3 PUFA perturb several inflammatory signaling pathways through GPR120/40 mediated mechanisms by acting as ligands for these free fatty acid receptors (227). For one, DHA-mediated inhibition of inflammatory mediators, prostaglandin E2 and IL-6, in LPS-stimulated macrophages was dependent on phospholipase A2 regulation by GPR120 ((127, 129), suggesting that GPR120 signaling affects local eicosanoid production in macrophages. Most prominently, LC n-3 PUFA have been shown to interfere with LPS-stimulated NFκB (127, 129, 161, 165, 177, 227) and NLRP3 inflammasome (258, 276) activity in macrophages, which is largely dependent on GPR120 and its downstream adaptor, β-arrestin 2 (129, 227, 276). Interestingly, DHA has been shown to decrease caspase-1 activation and IL-1β secretion in AT (276) and macrophages (258). Specifically, DHA, more so than EPA (227), inhibits LPS-stimulated macrophage responses involving NFκB signaling in macrophages; a key step in inhibiting signal 1 in inflammasome priming (258). In addition, DHA increased autophagy, thereby targeting inflammasome complexes for degradation in phagolysosomes through a GPR120 dependent mechanism (258). Notably, DHA caused the GPR120 adaptor protein, β-
arrestin 2, to interact with NLRP3 and NLRP1b inflammasomes, but not AIM2 or NLRC4, suggesting that LC n-3 PUFA like DHA cause specific rather than general inflammasome inhibition (276). Finally, it is tempting to speculate that LC n-3 PUFA may also interfere with inflammasome activation in macrophages by limiting oxidative stress caused by excessive ROS (59, 247, 264), known to cause NLRP3 inflammasome activation (252). This may be partly mediated through LC n-3 PUFA induced upregulation of AMPK activity (273), although this relationship has yet to be proven. Overall, inhibition of phospholipase A2, NFκB and inflammasome activity by LC n-3 PUFA via GPR120/40 and β-arrestin 2 dependent signaling could serve to abrogate excessive inflammatory M1 macrophage responses by metabolic-induced stress in obesity.

e. LC n-3 PUFA, resolvins, maresins and macrophage phenotype

While it was not directly assessed in this thesis, it is worth noting that LC n-3 PUFA can exert anti-inflammatory effects promoting M2 or regulatory macrophage responses as lipid signaling mediators. LC n-3 PUFA promote the production of less inflammatory eicosanoid mediators, including prostaglandins, leukotrienes, and thromboxanes, compared to those derived from n-6 PUFA, namely AA, while increasing production of potent pro-resolving mediators like E and D series resolvins and protectins (23, 70), maresins (21) and EPA and DHA derived ethanolamines (12, 152). Pro-resolving lipid mediators promote M2 macrophage polarization and regulatory-like macrophage responses, characterized by downregulation of the integrin CD11b following satiating efferyctosis of apoptotic cells, whereby macrophages more efficiently emigrate to nearby lymph nodes to promote peripheral tolerance and resolution of chronic inflammation (7). Specifically, resolvin D1 (28, 82, 123), maresin 1 (41) and
docosahexaenoylethanolamine (153) derived from DHA have been shown to promote M2-associated pro-resolving effects in macrophages. Interestingly, production of these pro-resolving lipid mediators may be impaired in obese AT, thereby promoting the dysregulated adipokine profile, as well as peripheral insulin resistance (37, 74, 256). These observations may be partly attributable to the high ratio of n-6:n-3 fatty acids in the Western diet (233); however, increasing consumption of LC n-3 PUFA like DHA may serve to decrease this ratio and correct the aforementioned obesity-associated pathologies. Indeed, a shift to the M2 macrophage phenotype in ATM has been reported in DIO mice supplemented with resolvin D1 (234).

1.4 Summary

AT infiltration of immune cells, such as macrophages and T cells, plays a prominent role in driving obesity-associated inflammation and related metabolic dysfunction, including insulin resistance. Furthermore, paracrine interactions between adipocytes and immune cells, in obese AT play a key role in promoting inflammation and metabolic dysfunction. The key factors that drive this immune response in AT are not well understood but appear to involve adipocyte and/or macrophage-mediated TLR and NLRP3 inflammasome signaling, as well as antigen presentation. Pre-clinical studies have shown that saturated fatty acids and their metabolites can drive the aforementioned inflammatory pathways, while LC n-3 PUFA tend to inhibit them. Therapeutic strategies aimed at mitigating these inflammatory processes may valuable for alleviating obesity-associated pathologies. Modulation of diet, particularly dietary fat which contributes 20-30% of daily energy intake (291), may be a candidate strategy. Particularly, LC n-3 PUFA help to mitigate disease severity of other chronic inflammatory diseases (reviewed by (22, 210)); therefore, increasing consumption of these fatty acids may help to prevent or treat
obesity-associated pathologies characterized by chronic inflammation. Studies evaluating the role of LC n-3 PUFA in mediating adipocyte-macrophage cross-talk are lacking, which would potentially provide useful information regarding the role of LC n-3 PUFA in ameliorating obesity-associated pathologies.
Chapter 2: Rationale and aims of the thesis

Macrophage infiltration into obese adipose tissue (AT) occurs concurrently with the development of systemic insulin resistance (168, 261) and therefore macrophages may be a critical cell type and key driver in promoting inflammation, AT dysfunction, and insulin resistance. A few seminal in vitro studies co-culturing murine adipocytes and macrophages showed that adipocyte-macrophage paracrine interactions, or cross-talk, drive continuous inflammatory mediator secretion, particularly when the common dietary saturated fatty acid, palmitic acid (PA) was added to co-culture (221, 222). Further, such paracrine interactions promoted macrophage polarization to an inflammatory M1 phenotype (221, 222), and macrophages or soluble macrophage-derived inflammatory mediators decreased adipocyte insulin-sensitivity (139). However, these early co-culture studies (139, 177, 221, 270) did not consider the ratio of macrophages to adipocytes that occur in obese AT, and thus the physiological relevance of the data is less clear.

Another key aspect of obese AT inflammation is the driving role of elevated circulating lipopolysaccharide (LPS), termed metabolic endotoxemia, which further contributes to AT dysfunction, inflammation (272), and subsequent development of both local and systemic insulin resistance (26, 150). Thus, studies using LPS at a dose that is representative of the concentration observed in metabolic endotoxemia, and its subsequent impact on adipocyte-macrophage cross-talk are necessary.

Given the adverse effects of AT-derived inflammation in obesity, immunomodulatory strategies to alleviate this are needed. Interestingly, dietary fish oil-derived long-chain n-3 (LC n-3) PUFA, eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA),
have been shown to decrease macrophage infiltration and/or decrease the degree of inflammatory
M1 macrophage polarization in obese AT in murine obesity models (135, 156, 234). Thus, LC n-3 PUFA may be a candidate strategy to mitigate inflammatory adipocyte-macrophage cross-talk in co-culture; however, the mechanisms by which this occurs are not well understood, and therefore warrant further study. Notably, LC n-3 PUFA are known to upregulate secretion of Ad from adipocytes partly through a peroxisome proliferative activation receptor gamma (PPARγ) dependent mechanism (178, 231); therefore, it remains to be established if LC n-3 PUFA-mediated anti-inflammatory effects are dependent on PPARγ or Ad-mediated signaling in co-culture conditions that recapitulate key aspects of the obese AT microenvironment, such as cell ratio and low dose LPS. Ultimately, establishing whether inflammatory adipocyte-macrophage cross-talk can be blunted through dietary LC n-3 PUFA, as well as the underlying mechanisms by which this occurs, may be important in developing nutritional strategies to mitigate obesity-related AT inflammation.

The primary objective of this thesis was to evaluate if LC n-3 PUFA mitigate the inflammatory adipocyte-macrophage cross-talk that is central to obesity and obesity-related pathologies. To this end, three studies were conducted.

The specific aims of Study 1 were:

1. To establish murine in vitro co-culture models that represent the ratio of adipocytes:macrophages observed in obese AT and examine if adipocyte-macrophage cross-talk was driven primarily by soluble-mediators or cell contact using trans-well or contact co-culture systems, respectively.
2. To determine if *in vitro* inflammatory adipocyte-macrophage cross-talk was mitigated by EPA or DHA as compared to control or the common saturated fatty acid, PA.

3. To determine if the ability of EPA and/or DHA to mitigate *in vitro* inflammatory adipocyte-macrophage cross-talk was dependent on PPARγ signaling.

The hypothesis of Study 1 was:

1. The addition of LC n-3 PUFA (EPA or DHA) to adipocyte-macrophage co-cultures *in vitro* would decrease the secretion of inflammatory mediators relative to control or PA via a PPARγ dependent mechanism.

The specific aims of Study 2 were:

1. To determine if inflammatory adipocyte-macrophage cross-talk was mitigated by the incorporation of dietary fish oil-derived LC n-3 PUFA into splenic macrophages isolated from low or high-fat fed mice in an *in vitro* adipocyte-macrophage co-culture model.

2. To determine if the ability of dietary LC n-3 PUFA to mitigate inflammatory adipocyte-macrophage cross-talk was dependent on adiponectin (Ad)-mediated signaling.

The hypothesis of Study 2 was:

1. Compared to macrophages isolated from mice fed low or high-fat control diets, macrophages isolated from mice fed low or high-fat diets enriched with LC n-3 PUFA would result in decreased inflammatory mediators when co-cultured with adipocytes *in vitro*, and such anti-inflammatory effects would be mediated partly through Ad.
The specific aims of Study 3 were:

1. To determine if dietary fish oil-derived LC n-3 PUFA affected the intact AT secretory profile that, in turn, affected RAW 264.7 macrophage polarization and lipid uptake in *ex vivo* models representative of either acute or chronic inflammation.

2. To determine if dietary LC n-3 PUFA-mediated effects on macrophage polarization and lipid uptake were dependent on Ad-mediated signaling.

The hypothesis of Study 3 was:

1. Compared to control mice, the intact AT secretory profile from LC n-3 PUFA-fed mice would decrease markers of M1 macrophage polarization and lipid accumulation partly through an Ad-mediated mechanism.
Chapter 3: Docosahexaenoic Acid Decreases Pro-Inflammatory Mediators in an In Vitro Murine Adipocyte Macrophage Co-Culture independently of PPARγ

As published with minor revisions:


3.1 Abstract

Paracrine interactions between adipocytes and macrophages contribute to chronic inflammation in obese adipose tissue (AT). Dietary strategies to mitigate such inflammation include long-chain polyunsaturated fatty acids, docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, which act though PPARγ-dependent and independent pathways. We utilized an in vitro co-culture model designed to mimic the ratio of adipocytes:macrophages in obese AT, whereby murine 3T3-L1 adipocytes were cultured with RAW 264.7 macrophages in direct contact, or separated by a trans-well membrane (contact-independent mechanism), with 125 μM of albumin-complexed DHA, EPA, palmitic acid (PA), or albumin alone (control). Thus, we studied the effect of physical cell contact versus the presence of soluble factors, with or without a PPARγ antagonist (T0070907) in order to elucidate putative mechanisms. After 12 h, DHA was the most anti-inflammatory, decreasing CCL2 and IL-6 secretion in the contact system (-57%, -63%, respectively, p≤0.05) with similar effects in the trans-well system. The trans-well system allowed for isolation of cell types for inflammatory mediator analysis. DHA
decreased mRNA expression (p<0.05) of Ccl2 (-7.1 fold) and increased expression of the negative regulator, MCP-IP (+1.5 fold). In macrophages, DHA decreased mRNA expression of pro-inflammatory M1 polarization markers (p≤0.05), Nos2 (iNOS; -7 fold), Tnfa (-4.2 fold) and NfkB (-2.3 fold), while increasing anti-inflammatory Tgfβ1 (+1.7 fold). Interestingly, the PPARγ antagonist co-administered with DHA, EPA or PA in co-culture reduced (p≤0.05) Ad cellular protein, without modulating other cytokines (protein or mRNA). Overall, our findings suggest that DHA may lessen the degree of CCL2 and IL-6 secreted from adipocytes, and may reduce the degree of M1 polarization of macrophages recruited to AT, thereby decreasing the intensity of pro-inflammatory cross-talk between adipocytes and macrophages in obese AT.

3.2 Introduction

AT is an active endocrine organ that secretes many proteins collectively called adipokines, which play a role in obesity-associated pathologies, such as insulin resistance and type 2 diabetes (69). Various cells within AT, including adipocytes, macrophages, endothelial cells, and other immune cells within the stromal vascular fraction, contribute to the adipokine milieu to varying degrees (224). Adipokines include the adipocyte-derived hormones Ad and leptin, as well as cytokines, such as IL-6, TNFα, IL-10 and CCL2 (MCP-1) that are secreted from multiple cellular sources (69, 224). The chronic inflammatory state in obesity is partly attributable to increased macrophage infiltration into AT, followed by increased production of pro-inflammatory cytokines, such as TNFα, IL-6, and CCL2, as well as decreased secretion of adiponectin (Ad), an insulin-sensitizing adipokine (221). Paracrine interactions or cross-talk between adipocytes and
macrophages in obese AT play a key role in the generation of the adipokine profile and can be influenced by dietary factors, such as fatty acids (221, 222).

Interestingly, saturated fatty acids are known to exert pro-inflammatory effects (3, 222). More specifically, saturated free fatty acids like lauric acid (12:0) (124) and palmitic acid (PA, 16:0) (222) released from dysregulated adipocytes can activate toll-like receptor (TLR)-2 and TLR4 signaling respectively, which ultimately triggers NFκB-mediated pro-inflammatory gene expression and subsequent cytokine secretion from macrophages. Although negative feedback factors like suppressor of cytokine signaling 3 (SOCS3) (164) and monocyte chemoattractant 1-induced protein (MCP1-IP) (146) act to suppress pro-inflammatory cytokine signaling, these feedback factors may be dysfunctional in obese humans with type 2 diabetes (199). In turn, macrophages activated though TLR2 (124) or TLR4 (222) signaling have been shown to undergo polarization to a unique M1-like phenotype characterized by increased lipid content and secretion of pro-inflammatory cytokines, TNFα and IL-6 (138). These cytokines subsequently feed back onto adipocytes though paracrine signaling to sustain adipocyte-derived pro-inflammatory adipokine secretion and lipolysis (221). This in turn sustains the increased release of saturated free fatty acids and continued TLR-signaling in macrophages (222). Thus, in this cross-talk paradigm, dysfunctional adipocytes can be viewed as effectors secreting distress signals such as free fatty acids and chemokines, and macrophages can be viewed as the responders to these signals, which promotes their activation to the pro-inflammatory M1-like phenotype (221, 222) that characterizes obese AT (136). Moreover, the pro-inflammatory adipokine profile, generated in part though adipocyte macrophage cross-talk, is associated with decreased insulin sensitivity locally,
i.e. within adipocytes (139), and peripherally, in other metabolically active tissues such as skeletal muscle and liver (1). Thus, targeting paracrine interactions between adipocytes and macrophages as a mechanism to mitigate chronic inflammation in obesity can potentially be regarded as a therapeutic strategy.

In contrast to the effects of saturated fatty acids, long chain n-3 polyunsaturated fatty acids (PUFA), namely eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA), exert known anti-inflammatory effects (reviewed by (22, 240)). Thus, increased consumption of n-3 PUFA may represent a promising strategy to reduce the production of pro-inflammatory adipokines associated with obesity. Interestingly, mice fed an obesogenic high fat diet supplemented with EPA and DHA exhibited an improved adipokine profile characterized by elevated plasma Ad and decreased free fatty acid levels (60), as well as decreased macrophage infiltration into AT (235). Recently, we have shown that EPA and DHA increase Ad secretion and cellular protein in vitro, in part though activation of the nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ) (178, 231). Ad has been shown to drive macrophage polarization towards the anti-inflammatory M2 phenotype in vitro and ex vivo (142, 182), thereby potentially re-directing the cyclic pro-inflammatory cross-talk between adipocytes and macrophages. This suggests that n-3 PUFA may beneficially modulate the obesity-associated paracrine interactions between adipocytes and macrophages. Thus, the objectives of this study were to determine if 1) DHA and/or EPA decrease pro-inflammatory adipokine synthesis and secretion and 2) the effects of DHA and/or EPA are exerted though a PPARγ-dependent mechanism. To accomplish this, we used two in vitro co-culture models that cultured macrophages and adipocytes either in direct contact
(contact-dependent mechanism), or separated by a trans-well membrane (contact-
-independent mechanism), to mimic the ratio of adipocytes:macrophages reported in obese
AT (101). Here we showed that this model can be used to study the modulation of
adipokine synthesis and secretion in response to various fatty acids, with or without the
presence of a PPARγ antagonist.

3.3 Materials and Methods

3.3.1 Cell culture and differentiation

3T3-L1 pre-adipocytes (ATCC, CL-173, USA) and RAW 264.7 macrophages
(ATCC, TIB-71, USA) were grown and passaged according to the manufacturer’s
instructions. Both cell types were maintained separately in basic media containing
DMEM without sodium pyruvate (HyClone, USA), plus 10% v/v fetal bovine serum
(FBS, low-endotoxin, Canadian origin, Sigma, USA), and 1% v/v penicillin streptomycin
(HyClone, USA). Pre-adipocytes were seeded in 6-well plates (Corning, USA) at a
density of 3000 cells/cm², and 2 days post-confluence (designated as day 0),
differentiation was induced using basic media plus 1 µmol/L dexamethasone, 0.5 mmol/L
isobutyl-methylxanthine, and 5 µg/mL insulin (Sigma, USA) for 2 days (day 2) as
described previously (259). Media was replaced with basic media containing 5 µg/mL
insulin on days 2, 4, and 6 post-differentiation. On day 8, both cell types were serum
starved with basic media containing 0% FBS for 12 h to ensure quiescence prior to co-
culture experiments on day 9. After serum starving, macrophages were co-cultured with
adipocytes in direct cell contact or using a trans-well system on day 9 (described below).
3.3.2 Co-culture of adipocytes and macrophages

Co-culture of adipocytes and macrophages was performed using two different methods; a direct cell contact and trans-well (contact-independent) system, with modification of methods used by Suganami et al. (221). To co-culture, on day 9 three wells of mature adipocytes were counted using a hemocytometer and trypan blue exclusion, and then averaged to get an average adipocyte count. Following this, 3.5 mL of fresh basic media containing the various fatty acid treatments were added to the adipocyte cultures. For the trans-well experiments, 2.0 mL of media was placed on the adipocytes, a 0.4 µM polyester membrane trans-well was added to the well (Corning, USA), and then 1.5 mL of treatment media was added on top of the trans-well. Next, T-75 flasks (Sarstedt, Germany) containing macrophages at 80% confluence were counted using a hemocytometer and trypan blue exclusion, spun down at 335 x g at 24°C for 5 min, and then re-suspended in 2.0 mL of fresh basic media. Using the average adipocyte count, macrophages were added either directly on top of adipocytes (contact system), or indirectly onto the trans-well insert (trans-well system, contact-independent) at a dose of 17.1% of total cells; this dose represents the degree of macrophage infiltration as reported in the epididymal AT of db/db mice (101) and was confirmed to be pro-inflammatory relative to a lean dose of macrophages (3.8% of total cells; (101)) in pilot work (data not shown). Finally, the cells were co-cultured for 12 h since we observed that RAW 264.7 macrophages divide after this period in co-culture, thereby offsetting the ratio of adipocytes to macrophages we aimed to study.
3.3.3 Fatty acids and PPARγ antagonist treatments

For all co-culture experiments, fatty acid stock solutions of EPA, DHA and PA (≥98% pure, Cayman Chemicals, USA) were made using ethanol as vehicle and stored at -20°C purged with inert gas. The stock solutions were freshly complexed to bovine serum albumin (BSA, ≤0.1 ng/mg endotoxin, ≤0.02% fatty acids, Sigma, USA) at 37°C prior to each experiment, and then added to basic media to a final concentration of 125 µM fatty acid:25 µM BSA. Controls received an equal volume of ethanol vehicle. The fatty acid dose was chosen based on previous work (178) that showed 125 µM of DHA or EPA maximally increased Ad secretion from 3T3-L1 adipocytes. Macrophage viability (assessed by trypan blue exclusion) did not differ between fatty acid treatment and coculture conditions and exceeded 85% viability after a 12 h co-culture period (data not shown). BSA plus macrophages co-cultured with adipocytes acted as a positive control, while BSA with adipocytes alone (no macrophages) served as a negative control. For experiments with the PPARγ antagonist, T0070907 (Cayman Chemicals, USA), the antagonist was dissolved in dimethyl formamide according to the manufacturer’s instructions, and then added to fatty acid treated media to achieve a final concentration of 1 µM (IC₅₀= 1µM with 1nM Rosiglitazone; (121)) in the culture well. For treatments without the antagonist, an equal volume of dimethyl formamide vehicle was added to the treatment media (<0.1% v/v). Each treatment condition was run in triplicate, and the experiment was independently conducted 2 or 3 times (depending on the outcome measured, see figure legends), for a final sample size of n = 6-9.
3.3.4 Secreted and cellular cytokine analyses

Media was collected at 0 and 12 h for analysis of secreted cytokine protein concentrations. At 12 h, cells from the trans-well system (where adipocytes and macrophages could be isolated separately) were washed with 1x PBS (Sigma, USA), lysed using an All-prep Kit (Qiagen, Canada), and processed according to the manufacturer’s instructions. Extra protease and phosphatase inhibitors (Roche, Germany) were added to the lysis buffer prior to use at the recommended concentrations. Total cellular protein was quantified using the bichintrinsic assay (Fisher Scientific, Canada), so that cellular protein could be normalized to total protein. Secreted and cellular IL-6, CCL2, IL-10, and TNFα were analyzed by Luminex xMAP technology (Bioplex-200 system; Mouse Cytokine/Chemokine Bio-plex kit, Bio-Rad Laboratories, USA). Secreted and cellular Ad were measured by ELISA (Quantikine Mouse Ad/Acrp 30 ELISA, R & D Systems, USA) according to the manufacturer’s instructions. Cellular cytokine concentrations in picograms per milliliter (pg/mL) were normalized by dividing by the lysate total protein concentration in milligrams per milliliter (mg/mL), yielding a final concentration in picograms of analyte per milligram of adipocyte protein (e.g. pg CCL2/ mg total protein).

3.3.5 NFκB activity assessment

Since cellular protein could be isolated from each cell type in the trans-well system, cellular protein lysates (methods described above) from adipocytes co-cultured in the trans-well system for 12 h were used to measure NFκB activity assessed by ELISA measuring the ratio of phosphorylated-p65 NFκB (Ser 536) to total p65 NFκB as per the
manufacturer’s instructions (eBioscience, USA). An equal amount of protein was added to each well to normalize cellular protein between samples.

3.3.6 RNA isolation and quantitative PCR

At 12 h, cells were washed with 1x PBS (Sigma, USA), lysed using a RNeasy kit (Qiagen, Canada) and processed according to the manufacturer’s instructions. Only adipocytes and macrophages from the trans-well system were lysed because cells could be isolated separately in this system. cDNA was made from 1 µg of extracted RNA using a high capacity cDNA reverse transcription kit as per the manufacturer’s instructions (Applied Biosystems, USA). Real-time PCR analysis was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems, USA) 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. Primers were designed using the Universal Probe Library Assay Design Center (Roche Applied Sciences, Germany, Appendix A) and validated primer efficiencies were between 90-105%. Samples were run in triplicate in 96-well plates, and each 20 µL reaction contained 5 µL cDNA (50 ng), 0.4 µL of 10 µM primer solution, 10 µL Power Sybr green 2x master mix (Applied Biosystems, USA), and 4.6 µL of RNase free water. All results were normalized to Rplp0 mRNA expression, and the relative differences in gene expression between treatment groups and the pre-treatment (0 h) were determined using the ΔΔCt method.
3.3.7 Statistical analysis

All data are expressed as mean ± SEM. The predetermined upper limit of probability for statistical significance throughout this investigation was \( p \leq 0.05 \), and analyses were conducted using the SAS system (SAS Institute, USA) for Windows (version 9.2). Data were subjected to one-way ANOVA (fatty acid treatment main effect) followed, if justified, by testing using Tukey’s post-hoc test. For normally distributed data, Grubb’s test was used to detect and remove any outliers. Data sets not exhibiting a normal distribution, as assessed by the Shapiro-Wilk test for normality, were subjected to the Kruskal-Wallis test \( (\chi^2 \text{ approximation}) \) followed, if justified, by the statistical probability outcome \( (p \leq 0.05) \) using Wilcoxon two-sample testing. When two treatment factors were present, i.e. fatty acid treatment with either trans-well or contact co-culture effects on secreted cytokines (Figure 3.1C) and fatty acid treatment with or without the effect of the PPAR\( \gamma \) antagonist on Ad cellular protein (Figure 3.5D), data were analyzed by two-way ANOVA and followed, if justified, by testing using Tukey’s post-hoc test.

3.4 Results

3.4.1 Secreted adipokine profile in the contact versus trans-well co-culture systems

Secreted adipokines (CCL2, IL-6, TNF\( \alpha \) and IL-10) were measured in the culture supernatant in both the contact (Figure 3.1A) and trans-well (Figure 3.1B) co-culture systems. In the contact and trans-well co-culture systems the secretion of pro-inflammatory adipokines were increased when adipocytes were cultured with macrophages only (positive control) compared to the negative control (adipocytes alone treated with BSA), in contact (+327% CCL2, +147% IL-6), and trans-well (+101%
CCL2, +817% TNFα, p≤0.05), thereby demonstrating that pro-inflammatory cross-talk occurs between these cell types. Interestingly, significant increases in secreted IL-10 (p≤0.05, Figure 3.1A) relative to 0 h were dependent on macrophage contact since no IL-10 was detectable in the trans-well system (data not shown). Similarly, in all co-culture conditions, secreted CCL2 and IL-6 in the contact system was at least 1.5-fold higher than the level detected in the trans-well system under the same culture conditions/treatment (p≤0.05, Figure 3.1C). However, the reverse trend was seen for secreted TNFα in the positive control; there was a 2.0-fold increase in TNFα secretion in the trans-well system relative to the contact system (p≤0.05, Figure 3.1C).

3.4.2 Fatty acids differentially modulate CCL2, IL-6 and TNFα secretion in both the contact and trans-well co-culture systems

CCL2 secretion was reduced by DHA (-57% contact, -46% trans-well, p≤0.05) and EPA (-48% contact, -27% trans-well, p≤0.05) compared to the positive control (adipocytes plus macrophages only, no fatty acid treatment) in both the contact (Figure 3.1A) and trans-well (Figure 3.1B) co-culture systems. Interestingly, DHA decreased IL-6 secretion (-63% contact, -41% trans-well, p≤0.05) compared to the positive control, while EPA did not (Figure 3.1A & B). Moreover, only EPA increased TNFα secretion in the contact system relative to the negative control (+471%, p≤0.05, Figure 3.1A), whereas both DHA and EPA decreased TNFα secretion relative to the positive control in the trans-well system (-46%, p≤0.05, Figure 3.1B). Overall, PA was largely no more pro-inflammatory than the positive control, as assessed by CCL2, TNFα and IL-6 secretion,
with the exception of a small reduction in CCL2 secretion (-14%, p≤0.05) in the trans-well system (Figure 3.1B).

**Figure 3.1:** Adipokine secretion in the trans-well versus contact co-culture system. Adipokine secretion after the 12 h macrophage adipocyte co-culture incubation in the contact (A) and trans-well (B) treated co-culture conditions, and (C) a comparison of secreted CCL2, IL-6 and TNFα between the contact and trans-well co-culture systems. Note: no IL-10 was detected in the trans-well system (B). 0 h = serum starved adipocytes alone prior to co-culture and fatty acid treatment, (-) = negative control; adipocytes alone treated with 25 µM BSA, (+) = positive control; co-cultured adipocytes and macrophages
plus 25 µM BSA, DHA = co-cultured adipocytes and macrophages in the presence of 125 µM DHA, EPA= co-cultured adipocytes and macrophages in the presence of 125 µM EPA, and PA= co-cultured adipocytes and macrophages in the presence of 125 µM PA. Values are means ± SEM. The experiment was independently conducted 3 times for a final sample size of n= 6-9. A different letter or an asterisk (*) indicates treatments are significantly different from each other, p≤0.05.

3.4.3 Fatty acids do not affect cellular cytokine protein concentrations in co-cultured adipocytes in the trans-well system

Adipocyte cellular protein could only be collected from cells in the trans-well culture system and the cellular protein of all cytokines assessed were increased (p≤0.05) at 12 h compared to pre-treatment (0 h) without the addition of BSA (negative control) or macrophages (positive control; Appendix B). However, cellular adipocyte IL-6, IL-10, TNFα and CCL2 protein was not affected by any treatment (Appendix B).

3.4.4 Fatty acids differentially modulate adipokine and inflammatory mediator mRNA expression in co-cultured adipocytes in the trans-well system

Since the trans-well system allowed for isolation of RNA from each cell type used in co-culture (adipocytes and macrophages), mRNA expression of critical inflammatory adipokines (Il6, Ccl2), signaling intermediates (Nfkb, Tlr4, Tlr2) and negative feedback factors (Mcp1-IP, Socs3) were assessed in adipocytes exposed to the various treatments. mRNA expression of Il6 in fatty acid treatment groups was not significantly decreased relative to the positive control (adipocytes plus macrophages only, Figure 3.2A). In
contrast, relative to the positive control, mRNA expression of Ccl2 was decreased by DHA (-7.1 fold, \(p \leq 0.05\)), EPA (-4.9 fold, \(p \leq 0.05\)) and PA (-2.1 fold, \(p \leq 0.05\)). Moreover, both DHA (-3.9 fold, \(p \leq 0.05\)) and EPA (-2.7 fold, \(p \leq 0.05\)) further reduced Ccl2 mRNA expression compared to PA (Figure 3.2A). Of note, adipocyte mRNA expression of Il-10 and Tnfa was negligible under all treatment conditions in the trans-well system (data not shown). Similarly, adipocyte mRNA expression of Nfkb was not affected by fatty acid treatment (Figure 3.2B). Furthermore, only DHA prevented the increase (+2.3 fold, \(p \leq 0.05\)) in Tlr4 expression (Figure 3.2B) induced by the positive control relative to the pre-treatment at 0 h. In contrast, mRNA expression of Tlr2 was down-regulated by both DHA (-2.9 fold, \(p \leq 0.05\)) and EPA (-2.3 fold, \(p \leq 0.05\)) relative to the positive control and PA (-2.5 fold and -1.8 fold, DHA and EPA, respectively, \(p \leq 0.05\) Figure 3.2B). Intriguingly, relative to the positive control, only DHA up-regulated mRNA expression of Mcp1-IP (+1.5 fold, \(p \leq 0.05\), Figure 3.2C), a negative regulator of CCL2 signaling. In contrast, co-culture of macrophages plus adipocytes (positive control) led to an increase in mRNA expression of a negative regulator of IL-6, Socs3 (+1.9 fold, \(p \leq 0.05\)) relative to the negative control (adipocytes alone plus BSA), whereas other fatty acids did not (Figure 3.2C). However, Socs3 mRNA expression did not differ between fatty acid treatments (\(p > 0.05\)).
Figure 3.2: mRNA expression of inflammatory mediators in trans-well co-cultured adipocytes. The mRNA expression of key inflammatory (A) cytokines (Il6 and Ccl2), (B) signaling intermediates (Nfκb, Tlr4 and Tlr2) and (C) negative feedback factors (Mcp1-IP and Socs3) from adipocytes harvested from the trans-well system after 12 h of co-culture. 0 h = serum starved adipocytes alone prior to co-culture and fatty acid treatment, (-) =
negative control; adipocytes alone treated with 25 µM BSA, (+) = positive control; co-cultured adipocytes and macrophages plus 25 µM BSA, DHA = co-cultured adipocytes and macrophages in the presence of 125 µM DHA, EPA= co-cultured adipocytes and macrophages in the presence of 125 µM EPA, and PA= co-cultured adipocytes and macrophages in the presence of 125 µM PA. Values are mean fold change ± SEM. The experiment was independently conducted 3 times (in triplicate) for a final sample size of n= 6-9. A different letter indicates treatments are significantly different from each other, p≤0.05.

3.4.5 NFκB activity is not affected by fatty acid treatments in trans-well co-cultured macrophages or adipocytes

Both total (Figure 3.3A) and phosphorylated (i.e. activated) p65 NFκB (Figure 3.3B) were similar between treatments (p>0.05) in trans-well co-cultured macrophages and adipocytes. Additionally, NFκB activity, i.e. the ratio of phosphorylated/total p65 NFκB, was similar across treatments in trans-well co-cultured macrophages and adipocytes, regardless of fatty acid treatment (p>0.05; Figure 3.3C).
Figure 3.3: NFκB activity in trans-well co-cultured macrophages and adipocytes. The absorbance (at 450 nm) of (A) total p65 NFκB and phosphorylated- p65 NFκB, and (B) the ratio of phosphorylated- p65 NFκB: total p65 NFκB as a measure of NFκB activity in
adipocytes and macrophages harvested from the trans-well system at 12 h. 0 h = serum starved adipocytes alone prior to co-culture and fatty acid treatment, (-) = negative control; adipocytes alone treated with 25 µM BSA, (+) = positive control; co-cultured adipocytes and macrophages plus 25 µM BSA, DHA = co-cultured adipocytes and macrophages in the presence of 125 µM DHA, EPA= co-cultured adipocytes and macrophages in the presence of 125 µM EPA, and PA= co-cultured adipocytes and macrophages in the presence of 125 µM PA. Values are mean fold change ± SEM. The experiment was independently conducted 2 times (in triplicate) for a final sample size of n= 6. A different letter indicates treatments are significantly different from each other, p≤0.05.

3.4.6 DHA decreases mRNA expression of key M1 and M2 polarization markers but increases mRNA expression of regulatory cytokines Tgfβ1 and Il10

Macrophage specific mRNA expression of M1 (Nos2, Nfkb and Tnfα), M2 (Arg1, Cd206) polarization markers and regulatory cytokines (Tgfβ1 and Il10) were assessed from cells co-cultured in the trans-well system under all fatty acid treatment conditions. The addition of DHA to the trans-well co-culture system resulted in decreased macrophage mRNA expression of key M1 polarization markers including: Nos2 (-7 fold, p≤0.05), Nfkb (-2.3 fold, p≤0.05) and Tnfα (-4.2 fold, p≤0.05) relative to co-culture of adipocytes plus macrophages only (positive control; Figure 3.4A). Other fatty acids were not as potent as DHA, but still exerted some significant effects, namely, EPA-treated macrophages reduced Nfkb expression (-1.9 fold, p≤0.05), and PA decreased mRNA expression of Nfkb (-2.1 fold, p≤0.05) and Tnfα (-2.2 fold, p≤0.05) relative to the positive
control (Figure 3.4A). Macrophage mRNA expression of the M2 polarization marker Arg1 was reduced by DHA treatment (-8.5 fold, p≤0.05) (Figure 3.4B). Additionally, all co-culture conditions decreased mRNA expression of the mannose receptor Cd206, another M2 polarization marker; however, the magnitude of this effect was partially reversed by both DHA and EPA (by approximately +2.0 fold, p≤0.05) relative to both the positive control and PA (Figure 3.4B). With regard to anti-inflammatory regulatory cytokine expression, Tgfβ1 mRNA expression was increased by all fatty acid treatments (p≤0.05) relative to the positive control (Figure 3.4C). Conversely, DHA increased mRNA expression of Il10, relative to EPA and PA (+1.8 fold, p≤0.05), and exhibited a trend towards increased expression (+1.3 fold, p<0.07) relative to the positive control (Figure 3.4C).
Figure 3.4: M1 and M2 macrophage polarization marker mRNA expression in trans-well co-cultured macrophages. mRNA expression of key (A) M1 (Nos2, Nfkb and Tnfa), (B) M2 (Arg1, Cd206) polarization genes, and (C) regulatory cytokines (Tgfβ1 and Il10) from macrophages harvested from the trans-well system at 12 h. 0 h = serum starved
adipocytes alone prior to co-culture and fatty acid treatment, (+) = positive control; co-cultured adipocytes and macrophages plus 25 µM BSA, DHA = co-cultured adipocytes and macrophages in the presence of 125 µM DHA, EPA= co-cultured adipocytes and macrophages in the presence of 125 µM EPA, and PA= co-cultured adipocytes and macrophages in the presence of 125 µM PA. Values are mean fold change ± SEM. The experiment was independently conducted 2 times (in triplicate) for a final sample size of n= 6. A different letter indicates treatments are significantly different from each other, p≤0.05.

3.4.7 Acute PPARγ antagonism affects Ad cellular protein, but not other cytokine cellular protein or mRNA expression in trans-well co-cultured adipocytes

Adding the PPARγ antagonist did not affect CCL2, IL-6, TNFα or IL-10 mRNA expression, cellular or secreted protein in adipocytes co-cultured in the trans-well system relative to treatment conditions without the antagonist (representative data shown in Figure 3.5). Additionally, the PPARγ antagonist had no effect on macrophage polarization marker mRNA expression in trans-well co-cultured macrophages (data not shown). Interestingly, with or without the PPARγ antagonist, Ad protein was not significantly affected by any treatment (Figure 3.5A and B). However, when the PPARγ antagonist was present in the trans-well system, Ad cellular protein was decreased (p≤0.05) in co-cultured adipocytes in DHA or EPA-treated cultures (-27% and -38%, respectively, Figure 3.5C). In contrast, cellular CCL2 protein in adipocytes was not affected by the presence of the PPARγ antagonist (p>0.05, Figure 3.5D).
**Figure 3.5:** Effect of a PPARγ antagonist on Ad and CCL2 cellular protein in trans-well co-cultured adipocytes. The cellular protein concentrations of Ad harvested from adipocytes in the trans-well system at 12 h alone (A) or with the PPARγ antagonist, T0070907 added (B), and a comparison of Ad (C) or CCL2 (D) cellular protein with and without the PPARγ antagonist added. 0 h = serum starved adipocytes alone prior to co-culture and fatty acid treatment, (-) = negative control; adipocytes alone treated with 25
µM BSA, (+) = positive control; co-cultured adipocytes and macrophages plus 25 µM BSA, DHA = co-cultured adipocytes and macrophages in the presence of 125 µM DHA, EPA = co-cultured adipocytes and macrophages in the presence of 125 µM EPA, and PA = co-cultured adipocytes and macrophages in the presence of 125 µM PA. Values are means ± SEM. The experiment was independently conducted 2 times (in triplicate) for a final sample size of n= 6. A different letter or an asterisk (*) indicates treatments are significantly different from each other, p≤0.05.

3.5 Discussion

In obesity, paracrine interactions between adipocytes and adipose-infiltrating macrophages contribute to chronic inflammation characterized by abnormal secretion of pro-inflammatory adipokines (69, 224). Since long-chain n-3 PUFA like DHA and EPA can exert anti-inflammatory effects (22, 240), they may represent a dietary strategy to mitigate the harmful effects of pro-inflammatory adipokines. In the current study, we showed for the first time that co-culturing adipocytes and macrophages at a ratio of macrophages to adipocytes that is representative of obese AT (101), either in direct contact (contact-dependent mechanism) or separated by a porous trans-well insert (contact-independent mechanism), promoted a pro-inflammatory adipokine profile with elevations in secreted CCL2, IL-6 and TNFα (Figure 3.1). Interestingly, adipocytes and macrophages co-cultured in direct contact led to a relative doubling of secreted adipokines (with the exception of TNFα) as compared to the trans-well system, suggesting that direct cell to cell contact, and not just the presence of soluble mediators, preferentially drives pro-inflammatory cross-talk between adipocytes and macrophages
(Figure 3.1C). This trend has been shown elsewhere with IL-6 secretion in trans-well and contact co-culture conditions (270), but our study extends previous findings (177, 221) and, to our knowledge, is the first to demonstrate that cell co-culture conditions (contact-dependent versus contact-independent), in addition to the presence of DHA and EPA, modifies the secreted adipokine profile. Furthermore, we showed for the first time in co-culture conditions without a potent pro-inflammatory stimulus (e.g. lipopolysaccharide) or pre-co-culture incubation with n-3 PUFA (177), that DHA decreased mRNA expression of M1 (Tnfa and Nos2) and M2 associated genes (Cd206 and Arg1), while simultaneously increasing mRNA expression of the regulatory and anti-inflammatory cytokines, Tgfβ1 and Il10 in co-culture (Figure 3.4). Our findings support the idea that a possible mixed macrophage phenotype may exist in obese AT, characterized by expressing various levels of both M1 and M2 polarization markers (205, 209); however, additional characterization of the phenotype beyond gene expression of such markers (e.g. by flow cytometry) requires further study to confirm our findings. Finally, we showed that the selective PPARγ antagonist, T0070907, reduced Ad cellular protein in co-cultured adipocytes from DHA and EPA treated cultures (Figure 3.5C), without affecting other cytokine mRNA, cellular or secreted protein concentrations in this co-culture model (representative data in Figure 3.5D), thereby supporting the idea that antagonists that impair PPARγ transcriptional activity may not always promote pro-inflammatory responses (31, 225).
3.5.1 DHA more so than EPA decreases inflammatory mediator mRNA expression in adipocytes and cytokine secretion in co-culture

Analysis of secreted adipokine protein (Figure 3.1) and mRNA expression (Figure 3.2) in co-cultured adipocytes revealed further mechanistic insight into how DHA and EPA modulate inflammation in co-culture. DHA had the most potent anti-inflammatory effect as evidenced by a significant reduction in secreted CCL2 and IL-6 in both co-culture conditions. While EPA had somewhat similar effects to DHA in our model, it was unable to suppress the secretion of IL-6. These findings are consistent with a recent study showing that DHA can exert a more potent anti-inflammatory effect compared to EPA in co-culture (177). Interestingly, while n-3 PUFA exerted similar effects on both Ccl2 mRNA expression in adipocytes and secreted protein, this trend was not observed with respect to Il6. Unexpectedly, PA resulted in a more potent decrease in Il6 mRNA expression in adipocytes compared with DHA and EPA. However, the 12 h incubation used in our co-culture experiments may have been too short to see elevations in adipocyte Il6 mRNA expression since, in 3T3-L1 adipocytes alone, 125 µM of PA has been shown to increase Il-6 mRNA expression after 24 h (3). Although the adipocyte Il6 mRNA expression profile we observed was similar to what has previously been observed in co-culture (221), we did not see a PA-induced up-regulation of the classic IL-6 negative regulator, Socs3. Additionally, PA did not up-regulate mRNA expression of the negative regulator, Mcp1-IP in adipocytes compared to the positive control (adipocytes plus macrophages only); therefore, it does not appear that MCP1-IP, which can act as an RNase to degrade Il6 mRNA expression (146), was playing a key role in the PA co-culture treatment. In contrast, this negative feedback system involving MCP1-IP may
have been induced by DHA in co-culture (Figure 3.2C); however, the degree of DHA’s anti-inflammatory action elicited though MCP1-IP requires further investigation. Moreover, we observed that DHA and EPA reduce Tlr2 mRNA expression in co-cultured adipocytes, suggesting that these fatty acids more potently regulate Tlr2 expression than Tlr4. Indeed, TLR2 has been shown to be a key signaling pathway that EPA targets to downregulate Il-6 mRNA expression in AT stem cells (87); therefore, the TLR2 pathway also warrants further study in co-culture conditions. We did not detect substantial fatty acid-induced differences in adipocyte Nfκb or Tlr4 expression in our model. Furthermore, we observed that co-culture conditions (regardless of fatty acid treatment) did not increase NFκB activity, i.e. the ratio of phosphorylated/ total p65 NFκB in trans-well co-cultured adipocytes or macrophages (Figure 3.3), suggesting that measuring phosphorylated p65 NFκB (i.e. RelA; the transcriptionally active NFκB sub-unit), may not be a useful endpoint to measure the inflammatory status of cells after 12 h co-culture incubations. Finally, additional measurement of inflammatory signaling intermediates (e.g. JNK, ERK1/2, STAT3 etc.) is warranted in co-culture conditions to delineate if fatty acid treatments differentially modulate signaling though these inflammatory pathways in adipocytes or macrophages.

3.5.2 DHA decreases cocultured macrophage mRNA expression of M1 and M2-like macrophage markers

One of the challenges to mitigating chronic inflammation is decreasing the degree of M1-like macrophage polarization in macrophages infiltrated into obese AT, while increasing anti-inflammatory M2-like macrophages that secrete IL-10 and help resolve
inflammation (160). In our macrophage adipocyte co-culture model we examined the role of fatty acids in altering key inflammatory mediators implicated in the M1 (Nos2, Nfκb, Tnfa) and M2 (Arg1, Cd206, Tgfb1, Il10) macrophage polarization response. Similar to the effect of fatty acids on the secreted adipokine milieu, DHA was more anti-inflammatory than EPA, as evidenced by decreased mRNA expression of M1 markers Tnfa, iNOS (Nos2), and Nfκb, which are associated with the pro-inflammatory M1 macrophage phenotype. These phenomena may be partly attributed to the anti-inflammatory actions of PPARγ, including trans-repression of NFκB (183), since monocytes express PPARγ (77) and DHA is a potent PPARγ agonist (144). Interestingly, EPA and PA also decreased Nfκb mRNA expression to a similar degree as DHA. Since EPA (144) and even PA (99) to some extent may also act as PPARγ agonists, it is possible that trans-repression could occur here too, although this requires further investigation.

Collectively, the mRNA expression data in our model suggests that DHA-treated macrophages in co-culture with adipocytes display characteristics that are consistent with promoting a regulatory macrophage phenotype. The presence of such regulatory macrophages in obese AT may be ideal given their secretion of the anti-inflammatory cytokines, IL-10 and TGFβ1, and the lack of antigen presentation to T helper cells (160) that could exacerbate chronic inflammation in obesity (132). Finally, it is known that Ad may promote a regulatory macrophage phenotype in vitro and ex vivo (142, 182), and that DHA supplementation in a rodent high-fat diet induced obesity model promotes M2 macrophage polarization in AT (234). Therefore, further research into the role of fatty
acids in macrophage polarization is needed, particularly by PPARγ agonists, such as DHA, that promote Ad secretion after 24 h (178, 231).

3.5.3 DHA and EPA-mediated anti-inflammatory effects in co-culture do not acutely involve PPARγ

Since previous studies have shown the involvement of PPARγ in DHA and EPA-mediated effects (178, 231), we performed experiments using the selective PPARγ antagonist, T0070907. Interestingly, in our model the addition of the PPARγ antagonist in co-culture with adipocytes and macrophages plus DHA or EPA decreased Ad cellular protein concentrations (Figure 3.5C), without affecting other inflammatory mediators and cytokines (representative data in Figure 3.5D). These findings initially seemed counterintuitive since PPARγ can exert anti-inflammatory effects (183, 251), and plays an essential role in IL-4-induced M2 macrophage polarization (174). However, these anti-inflammatory effects may be context-dependent as PPARγ agonists, such as thiazolidinediones, are not always anti-inflammatory (229), and the use of PPARγ antagonists does not always promote a pro-inflammatory response (31, 225). Additionally, PPARγ agonists (e.g. rosiglitazone) and non-agonist ligands (e.g. SR1664; (35)) may decrease inflammatory signaling and preserve insulin sensitivity though non-canonical PPARγ signaling independent of transcriptional regulation (reviewed by (242)).

Overall, our data suggests that a more careful evaluation of PPARγ antagonists is needed before we can use them in experimental models to isolate the PPARγ-independent anti-inflammatory activities of n-3 PUFA.
**3.5.4 Methodological considerations**

In setting up this study we made decisions regarding fatty acid dose and incubation time that require further explanation. Firstly, previous findings determined that 125 µM DHA maximally increased secreted Ad in 3T3-L1 adipocytes (178) and therefore, we utilized this dosage for all fatty acids. Interestingly, 125 µM of PA did not induce additional pro-inflammatory cytokine secretion in co-culture relative to adipocytes and macrophages only (positive control). Although PA is thought to be pro-inflammatory due to its ability to act as a TLR4 agonist (3, 125, 222), and its ability to increase reactive oxygen species production and subsequent CCL2 secretion in adipocytes (79, 225), these effects may only be evident at higher doses (200-500 µM) and incubations longer than 12 h (3, 222, 225). Secondly, with regards to the timing of fatty acid exposure, we found fatty acid-induced differences in secreted cytokines (e.g. IL-6 and CCL2) after 12 h, which may have been too short to see changes in other secreted cytokines, such as IL-10, as previous reports have shown that DHA increases IL-10 secretion in 3T3-L1 adipocytes after 24 h (20).

**3.5.5 Summary**

In summary, our results demonstrate that fatty acids modulate the pro-inflammatory adipokine milieu generated in a co-culture model designed to represent the ratio of macrophages to adipocytes seen in obese AT (101). Overall, this data suggests that macrophage presence is necessary to induce pro-inflammatory cross-talk with adipocytes. Moreover, our study suggests that DHA may act to suppress inflammation concomitantly in both cell types. For one, DHA decreased the degree of M1 polarization
marker mRNA expression in macrophages while increasing expression of the potent anti-inflammatory cytokines, Tgfβ1 and Il10, thereby driving a gene expression profile that is consistent with promoting a regulatory macrophage phenotype. This data suggests that DHA may suppress macrophage activation in co-culture, which could then feedback to inhibit inflammatory cytokine release (e.g. CCL2 and IL-6) from adipocytes since DHA, and to a lesser extent EPA, decreased CCL2 and IL-6 secretion and Ccl2 mRNA expression in co-cultured adipocytes. Taken together, n-3 PUFA could decrease the intensity of pro-inflammatory cross-talk between adipocytes and macrophages, which may partly explain the decreased macrophage infiltration into obese AT observed in some rodent models (235). Thus, dietary n-3 PUFA, in particular DHA, may be a useful strategy to mitigate the effects of obesity-associated inflammation.
Chapter 4: Fish oil-derived n-3 polyunsaturated fatty acids reduce NLRP3 inflammasome activity and obesity-related inflammatory cross-talk between adipocytes and CD11b+ macrophages

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4.1 Abstract

Adipocyte-macrophage cross-talk plays a key role in obesity-associated inflammation by propagating innate immune responses in obese adipose tissue (AT). Long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) may mitigate inflammation, partly through up-regulation of the anti-inflammatory adipokine, adiponectin; however, specific mechanisms are not well understood. Thus, we determined if adipocyte-macrophage cross-talk could be mitigated by dietary LC n-3 PUFA, and if such effects were dependent on adiponectin-mediated signaling. We utilized an in vitro co-culture model designed to mimic the ratio of adipocytes:macrophages in obese AT, whereby murine 3T3-L1 adipocytes were co-cultured with splenic CD11b+-macrophages isolated from C57BL/6 mice fed high-fat control (HF-CON; 34% w/w fat) or fish-oil diets (HF-FO; 34% w/w fat containing 7.6% w/w FO), as well as mice fed low-fat control (LF-CON; 10% w/w fat) or FO diets (LF-FO; 10% w/w fat containing 3% w/w FO). Co-culture conditions tested the effects of soluble mediator-driven mechanisms (i.e. trans-well system), cell contact, and low-dose LPS on cell cross-talk (10 ng/mL LPS, acute: for 24h, or chronic: 24h
prior to co-culture to pre-inflame adipocytes mimicking chronic inflammation). HF-FO macrophages from acute LPS-stimulated trans-well co-cultures had decreased mRNA expression of *Casp1*, *Il1β* and *Il18*, and cellular caspase-1 activity compared to HF-CON macrophages (*p*≤0.05). Moreover, adipocytes from acute LPS-stimulated HF-FO co-cultures had decreased cellular caspase-1 activity and decreased IL-1β/IL-18 levels following chronic LPS pre-treatment compared to HF-CON co-cultures (*p*≤0.05). Additionally, in contact co-cultures with adiponectin-neutralizing antibody, the FO-mediated decrease in expression of NLRP3 inflammasome genes, M1-macrophage marker genes and inflammatory cytokine/chemokine secretion were controlled partly through adiponectin, while cellular caspase-1 activity and IL-1β/IL-18 protein levels were decreased independently of adiponectin (*p*≤0.05). Thus, LC n-3 PUFA may decrease the intensity of adipocyte-macrophage cross-talk, which supports the role of dietary LC n-3 PUFA as an immunomodulatory strategy to mitigate obesity-associated pathologies.

**4.2 Introduction**

Obesity-related pathologies, such as type 2 diabetes, stem partly from dysregulated adipose tissue (AT) function that is characterized by changes in immune cell content (e.g. macrophages (250), CD4⁺ (261) and CD8⁺ (168) T cells) and adipokine secretion profile, including increased TNFα, IL-6, IL-1β, CCL2 (MCP-1), CCL5 (RANTES) (11), and decreased adiponectin (Ad) (97). Notably, as obesity progresses, macrophage infiltration increases dramatically from 3% to approximately 20% of total non-adipocyte cells (101), contributing extensively to development of the obese inflammatory phenotype (250) and systemic insulin resistance (168, 261). Further, in obese AT, macrophages exhibit an inflammatory M1
phenotype, characterized by increased lipid content, an ability to express high levels of anti-
microbicidal iNOS (murine only), upregulation of integrins CD11b and CD11c, and secretion of
inflammatory cytokines such as TNFα and IL-6 (136, 187).

Further, an obesity-associated increase in gut permeability enhances lipopolysaccharide
(LPS) translocation into the systemic circulation, further promoting a sub-clinical form of
inflammation termed metabolic endotoxemia (26, 39). This, in turn, leads to LPS accumulation
and exacerbated TLR-mediated signaling in AT (272). Additionally, recent evidence suggests
that macrophages (52, 122, 241, 252) and adipocytes (216) may propagate inflammation in
obesity through activation of the NLRP3 inflammasome. More specifically, a priming signal
(signal 1, e.g. LPS) upregulates assembly of cytosolic NLRP3 inflammasome components and a
second activation signal [metabolic by-products e.g. reactive oxygen species (ROS), ceramides,
etc.] causes caspase-1 activation and caspase-1-mediated production of inflammatory cytokines,
IL-1β and IL-18 (253). Along these lines, LPS-primed murine macrophages treated with the
saturated fatty acid palmitic acid (16:0) showed increased ROS levels (264) followed by NLRP3
inflammasome activation and IL-1β secretion (116, 252), suggesting that both TLR and
inflammasome signaling contribute to M1 macrophage polarization. Consequently, M1-derived
TNFα and IL-6 feed back onto adipocytes through paracrine signaling to sustain adipocyte-
derived secretion of inflammatory mediators (e.g. MCP-1, IL-6), increased release of saturated
fatty acids and continued TLR/inflammasome signaling in macrophages (221). However, the
extent to which adipocyte-macrophage cross-talk perpetuates NLRP3 inflammasome activation
is not well understood, but may be an important target for nutritional intervention.

Long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), namely eicosapentaeonic
(20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are known to improve clinical
symptoms in obesity-associated chronic inflammatory diseases (70), and can mitigate immune cell-adipocyte paracrine interactions that exacerbate chronic inflammation in obesity. For example, we have recently shown that LC n-3 PUFA decrease macrophage chemotaxis resulting from CD8<sup>+</sup> T cell-adipocyte cross-talk (157), and secretion of inflammatory mediators like IL-6 and CCL2 in adipocyte-macrophage co-cultures (16). Moreover, LC n-3 PUFA upregulate secretion of Ad from adipocytes (178, 231), which has been shown to drive hypo-responsiveness to LPS in macrophages (142, 182); however, the extent to which Ad mediates the anti-inflammatory effects of LC n-3 PUFA in adipocyte-macrophage cross-talk is not well established.

The objective of the current study was to determine if splenic CD11b-enriched macrophages isolated from mice fed either a low or high-fat diet, with or without LC n-3 PUFA, would affect NLRP3 inflammasome gene expression and activation, M1 macrophage marker mRNA expression, or inflammatory mediator secretion when co-cultured with 3T3-L1 adipocytes under conditions designed to recapitulate aspects of the obese AT microenvironment. Specifically, using the dose of LPS observed in metabolic endotoxemia (26, 39) we mimicked acute and chronic LPS-driven inflammatory conditions by either acutely treating co-cultures with LPS or by using adipocytes that had been pre-treated with LPS for 24h to induce inflammation prior to co-culture with CD11b-enriched macrophages.

4.3 Materials and methods

4.3.1 Animals and diets

Mice were housed as described (141). For the low-fat diet, 8-wk-old male and female C57BL/6 mice (Charles River Laboratories, USA) were fed ad libitum either an AIN-93G
modified diet containing 10% w/w safflower oil diet (CON) or an isocaloric LC n-3 PUFA-enriched diet containing 3% w/w menhaden oil + 7% w/w safflower oil (LF-FO) for 4 wk (n=8 mice/diet), (Research Diets Inc, USA; Appendix C). For high-fat-fed obese mice, 4-5 wk-old male C57BL/6 mice were fed ad libitum one of two isocaloric high-fat (34% w/w) diets for 12 wk containing an equal amount of lard (23.4% w/w) plus either 10.6% w/w corn oil (HF), or 7.6% w/w menhaden oil + 3% w/w corn oil (HF-FO), (n= 6/diet), (Harlan, USA, Appendix D). All experimental procedures were approved by the University of Guelph Animal Care Committee.

4.3.2 Splenic CD11b+ macrophage isolation

Spleens were removed aseptically and CD11b+ cells were isolated by positive selection using magnetic CD11b microbeads according to the manufacturer’s protocol (Miltenyi Biotec, USA). CD11b+ cell purity (≥90%, representative histogram shown in Appendix E) was confirmed by staining with 0.5 µg of FITC-anti-CD11b antibody (clone M1-70, eBioscience) or isotype control and analyzed using a Becton-Dickinson FACSCalibur flow cytometer and BD CellQuest software. Purified CD11b+ cells were counted using a hemocytometer prior to co-culture. Using trypan blue exclusion, it was confirmed that cell viability exceeded 89%.

4.3.3 Adipocyte culture and differentiation

3T3-L1 pre-adipocytes (ATCC, USA) were grown and passaged according to the manufacturer’s instructions. Cells were maintained in basic media containing DMEM without sodium pyruvate (HyClone, USA), plus 10% v/v fetal bovine serum (FBS, low-endotoxin, Canadian origin, Sigma, USA) and 1% v/v penicillin streptomycin (HyClone, USA). Pre-
adipocytes were seeded in 6-well plates (Corning, USA) and differentiated into mature adipocytes as reported previously (16). On day 8 post-differentiation, media was changed to basic media for 24 h prior to co-culturing with purified splenic CD11b+ macrophages.

4.3.4 Co-culture of adipocytes and primary CD11b+-enriched macrophages

Co-culture of adipocytes and macrophages was performed using two different methods, namely a direct cell contact system (cell contact-dependent) and a trans-well (cell contact-independent, soluble mediator-driven) system. Macrophages were added either directly on top of adipocytes (contact system), or indirectly (trans-well system) onto a porous 0.4 µM polyester membrane trans-well insert (Corning, USA). Macrophages were added to each co-culture at a dose of 17.1% of total cells (3.4 x10^5 macrophages: 2.0 x10^6 adipocytes), which represents the ratio of adipocytes:macrophages reported in the epididymal AT of db/db mice (101). Cells were co-cultured under three stimulation conditions: i) unstimulated (culture media alone), ii) acute inflammatory conditions containing 10 ng/mL LPS from E. coli 055:B5 for 24 h (Sigma-Aldrich, USA), or iii) chronic inflammatory conditions where 3T3-L1 adipocytes were pre-treated with 10 ng/mL LPS for 24 h prior to co-culture and media was replaced with basic media for the 24 h co-culture period. The low dose of LPS used is representative of circulating endotoxin concentrations reported in obese humans and rodents (5-6 EU), (26, 39). 3T3-L1 adipocytes alone, treated with the aforementioned stimulation conditions served as negative controls. In some experiments, 10 µg/mL of Ad-neutralizing antibody (anti-Ad, AF1119, anti-mAcrp30, R&D Systems, USA) or isotype control anti-goat IgG (10 µg/mL, R&D Systems, USA) were added to culture wells with fresh basic media 10 min prior to adding macrophages. This antibody was chosen since it has been reported to suppress Ad-mediated signaling in adipocyte-
conditioned media (83, 191) and we confirmed the dose of antibody used suppressed Ad-
mediated signaling in pilot experiments with adipocyte conditioned media (Appendix F). After
24 h, supernatant was collected and cells were lysed using a RNA/Protein Isolation Kit (Norgen
Biotek, Canada) and processed according to the manufacturer’s instructions.

4.3.5 Gene expression

cDNA was made from 1 µg of extracted RNA using a high capacity cDNA reverse
transcription kit as per the manufacturer’s instructions (Applied Biosystems, USA). Real-time
PCR analysis was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems,
USA) using the default protocol described elsewhere (16). Primers were designed using the
Universal Probe Library Assay Design Center (Roche Applied Sciences, Germany, Appendix A)
and validated primer efficiencies were between 90–105%. All results were normalized to Rplp0
mRNA expression, and the relative differences in gene expression between treatment groups and
control were determined using the ΔΔCt method.

4.3.6 Caspase-1 activity

Total cellular protein was quantified using the bicinchoninic assay (Thermo Fisher
Scientific, USA). Caspase-1 activity was measured as previously reported (108) with minor
modification. Briefly, 60 µg of total purified cellular protein was added to each well of a 96-well
plate containing 400 µM of the synthetic caspase-1 substrate (Ac-YVAD-pNA, Sigma-Aldrich)
in 150 µL of protein elution buffer (with neutralizer added, pH 7.4, Norgen Biotek) containing
20% v/v glycerol. The reaction was conducted for 1 h at 37°C, after which cytosolic caspase-1
activity was detected by colourimetry and optical density (OD) was measured at 405 nm (108). OD was normalized to lysate total protein content and reported as OD/mg total protein.

4.3.7 IL-1β and IL-18 cellular protein analyses

Total cellular protein was quantified using the bicinchoninic assay (Thermo Fisher Scientific, Canada) so that cellular protein could be normalized to total protein. 10 µg of total cellular protein was added to each well to detect IL-1β and IL-18 by multiplex using the ProcartaPlex Mouse Basic kit (eBioscience, USA) and analyzed using the Bio-Plex 200 System (Bio-Rad, USA). Cellular protein concentrations in pg/mL were normalized to the lysate total protein concentration in mg/mL, yielding a final concentration of analyte in pg/mg total protein.

4.3.8 Secreted cytokine analyses

Supernatant was collected after 24 h of co-culture for analysis of secreted cytokine concentrations. IL-6, CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), TNFα and mature IL-18 and IL-1β were multiplexed using the ProcartaPlex Mouse Basic kit (eBioscience, USA) using undiluted supernatant and analyzed using the Bio-Plex 200 System (Bio-Rad, USA).

4.3.9 Fatty acid analysis

Total lipids were extracted from spleens as previously described (61), and the lipid-containing chloroform phase was processed as previously described (141). Fatty acid methyl esters were separated by gas chromatography using an Agilent 7890A gas chromatograph with DB-FFAP fused-silica capillary column (15 m, 0.1µm film thickness, 0.1 mm i.d.; Agilent Technologies, USA) and fatty acid peaks were identified by comparing retention times of
samples with those of known standards (Nu-Chek-Prep, USA) using the EZchrom Elite version 3.2.1 software.

4.3.10 Statistical analysis

All data are expressed as mean ± SEM. The predetermined upper limit of probability for statistical significance was set at $p \leq 0.05$ and analyses were conducted using SigmaPlot version 12.5 (USA). Data that were not normally distributed were transformed prior to statistical analysis, and normal distribution and equal variance was confirmed by Shapiro-Wilk test and Levene’s test, respectively. Data were subjected to two-way ANOVA followed, if justified, by testing using Fisher’s Least Squared Difference post-hoc test. Mouse physiological measurements comparing FO diets (Table 4.1, LF-FO or HF-FO) to their respective controls (LF-CON or HF-CON) were subjected to unpaired T-tests.

4.4 Results

4.4.1 Body weights, food intake and spleen fatty acid profile

Average food intake was monitored throughout the studies and did not differ between LF-CON and LF-FO diets or HF-CON and HF-FO diets ($p>0.05$). Body weight was also monitored throughout the studies and did not differ between LF-CON and LF-FO diets or HF-CON and HF-FO diets ($p>0.05$, final weights shown in Table 4.1). Additionally, compared to each respective LF or HF-CON diet, both LF-FO and HF-FO spleens were enriched in total n-3 PUFA, while total n-6 PUFA decreased ($p \leq 0.05$, Table 4.1).
Table 4.1: Mouse body weight measurements and spleen fatty acid profile

<table>
<thead>
<tr>
<th></th>
<th>LF-Cong</th>
<th>LF-FO</th>
<th>HF-Cong</th>
<th>HF-FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>20.9 ± 0.63</td>
<td>21.8 ± 0.52</td>
<td>17.9 ± 0.25</td>
<td>17.9 ± 0.68</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>26.1 ± 1.04</td>
<td>28.7 ± 1.82</td>
<td>38.3 ± 0.90</td>
<td>39.2 ± 1.33</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Total n-3 PUFA</td>
<td>1.26 ± 0.15</td>
<td>10.7 ± 1.17*</td>
<td>2.36 ± 0.35</td>
<td>11.6 ± 1.29*</td>
</tr>
<tr>
<td>% Total n-6 PUFA</td>
<td>42.0 ± 2.30</td>
<td>29.1 ± 0.68*</td>
<td>27.0 ± 1.39</td>
<td>18.6 ± 1.58*</td>
</tr>
</tbody>
</table>

1Values are means ± SEM (n=6-8/dietary group). Values with an asterisk are significantly different than their respective control (LF-FO vs LF-CON or HF-FO vs HF-CON, p≤0.05). Spleen n-3 PUFA (18:3n-3, 20:3n-3, 20:5n-3, 22:3n-3, 22:5n-3, 22:6n-3) and n-6 PUFA (18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6) are expressed as a percentage of total fatty acids.

4.4.2 Macrophage mRNA expression of NLRP3 inflammasome genes and cellular caspase-1 activity

To determine if macrophages from mice fed HF-CON had increased mRNA expression of key NLRP3 inflammasome genes (Nlrp3, Casp1, Il1β and Il18) relative to macrophages from LF-CON mice, as well as if these measurements were mitigated in co-cultures with FO-enriched macrophages, we used a trans-well co-culture model with 3T3-L1 adipocytes under various stimulation conditions that mimicked key aspects of the obese AT microenvironment. Relative to LF-CON macrophages for each co-culture stimulation condition, macrophages from HF-CON mice had increased mRNA expression of Nlrp3 (+1.5-fold acute inflammation), Casp1 (+4.9-fold unstimulated, +5.0-fold acute inflammation, +1.5-fold chronic inflammation), Il1β (+5.1-fold unstimulated, +2.3-fold acute inflammation) and Il18 (+3.0-fold unstimulated, +3.8-fold acute inflammation), (p≤0.05, Figure 4.1A). Moreover, relative to HF-CON macrophages, HF-FO macrophages had decreased mRNA expression of Nlrp3 (-1.8-fold chronic inflammation), Casp1 (-1.4-fold acute inflammation, -1.2-fold chronic inflammation), Il1β (-1.8-fold...
unstimulated, -2.4-fold acute inflammation, -1.6-fold chronic inflammation), and \textit{Il18} (-1.7-fold unstimulated, -1.6-fold acute inflammation), (p≤0.05, \textbf{Figure 4.1A}). Similarly, relative to LF-CON macrophages, LF-FO macrophages had decreased mRNA expression of \textit{Nlrp3} (-2.7-fold unstimulated, -1.4-fold acute inflammation, -2.7-fold chronic inflammation,), \textit{Casp1} (-1.5-fold chronic inflammation), \textit{Il1β} (-2.6-fold acute inflammation, -1.8-fold chronic inflammation) and \textit{Il18} (-3.8-fold unstimulated, -3.4-fold acute inflammation), (p≤0.05, \textbf{Figure 4.1A}).

Next, we aimed to determine whether changes in mRNA expression of NLRP3 inflammasome genes corresponded to changes in caspase-1 activity in trans-well co-cultured macrophages from mice fed HF diets (\textbf{Figure 4.1B}). Interestingly, relative to HF-CON macrophages, HF-FO macrophages had decreased caspase-1 activity in both acute (-2.7-fold) and chronic (-1.3-fold) stimulation conditions (p≤0.05, \textbf{Figure 4.1B}). Of note, IL-1β and IL-18 were undetectable in the cellular protein of co-cultured macrophages and the cell culture supernatant.
**Figure 4.1.** mRNA expression of inflammasome priming genes (A) and caspase-1 activity (B) in trans-well co-cultured macrophages. Unstimulated: co-culture conditions without additional inflammatory stimuli; acute inflammation: co-culture plus 10 ng/mL LPS for the 24 h co-culture period; and chronic inflammation: co-culture conditions where adipocytes were pre-treated with 10 ng/mL LPS for 24 h, then basic media for the 24 h co-culture period. Data shows the means ± SEM for LF-CON and FO (n=8/diet/co-culture condition), and HF-CON and FO (n=6/diet/co-culture condition). Bars not sharing a letter are significantly different (p≤0.05).

### 4.4.3 Adipocyte mRNA expression of NLRP3 inflammasome genes, cellular caspase-1 activity and IL-1β and IL-18 protein levels

We also determined whether adipocytes co-cultured with macrophages from HF-CON mice had increased mRNA expression of NLRP3 inflammasome genes relative to adipocytes co-cultured with macrophages from LF-CON mice, as well as if these measurements were mitigated in co-cultures with FO-enriched macrophages (LF-FO or HF-FO), (Figure 4.2A). Relative to adipocytes alone (negative controls), adipocytes in trans-well co-cultures with HF-CON macrophages had increased mRNA expression of *Nlrp3* (+2.7-fold unstimulated, +2.8-fold acute inflammation, +2.7-fold chronic inflammation) and *Il18* (+146.5-fold unstimulated, +110.2-fold acute inflammation, +112.8-fold chronic inflammation), (p≤0.05, Figure 4.2A). Similarly, relative to 3T3-L1 adipocytes in trans-well co-cultures with LF-CON macrophages, adipocytes co-cultured with HF-CON macrophages had increased mRNA expression of *Nlrp3* (+2.1-fold unstimulated, +2.5-fold acute inflammation, +3.1-fold chronic inflammation) and *Il18* (+111.4-fold unstimulated, +98.0-fold acute inflammation, +125.6-fold chronic inflammation), (p≤0.05, Figure 2A). Secondly, relative to adipocytes co-cultured with HF-CON macrophages, mRNA
expression of \textit{Nlrp3} increased (+1.5-fold unstimulated, +1.7-fold chronic inflammation) in adipocytes co-cultured with HF-FO macrophages, while expression of \textit{Il18} was not different between these two groups (p>0.05, Figure 4.2A). Finally, mRNA expression of \textit{Casp1} was not significantly altered under any stimulation condition, and adipocytes did not consistently express \textit{Il1β} (data not shown).

Next, we tested whether changes in mRNA expression of NLRP3 inflammasome genes corresponded to changes in caspase-1 activity (Figure 4.2B) or cellular IL-1β and IL-18 protein levels (Figure 4.2C) in trans-well co-cultured adipocytes. First, relative to adipocyte controls, caspase-1 activity in HF-CON adipocytes was increased in unstimulated (OD/ mg total protein= 0.2 in HF-CON group versus undetectable in adipocyte control) and acute inflammation (+4.8-fold) conditions (p≤0.05, Figure 4.2B). Moreover, relative to adipocytes co-cultured with HF-CON macrophages, adipocytes co-cultured with HF-FO macrophages had decreased caspase-1 activity in the unstimulated (-2.8-fold) and acute inflammation (-3.9-fold) conditions (p≤0.05, Figure 4.2B). Interestingly, relative to adipocytes co-cultured with HF-CON macrophages, adipocytes co-cultured with HF-FO macrophages had increased IL-1β (+9.9-fold) and IL-18 levels (602.4 pg/mg total protein versus undetectable in HF-CON in the acute inflammation condition), (p≤0.05, Figure 4.2C). However, the opposite trend was seen in the chronic inflammation condition; relative to adipocytes co-cultured with HF-CON macrophages, adipocytes co-cultured with HF-FO macrophages had decreased cellular protein levels of IL-1β (-1.3-fold) and IL-18 (-1.2-fold), (p≤0.05, Figure 4.2C).
**Figure 4.2:** mRNA expression of inflammasome priming genes (A), caspase-1 activity (B), and (C) cellular IL-1β and IL-18 protein levels in adipocytes co-cultured in trans-well with macrophages. Unstimulated: co-culture conditions without additional inflammatory stimuli; acute inflammation: co-culture plus 10 ng/mL LPS for 24 h co-culture period; and chronic inflammation: co-culture conditions where adipocytes were pre-treated with 10 ng/mL LPS for 24 h, then basic media for the 24 h co-culture period. Data shows the means ± SEM for adipocyte controls (n=4/stimulation condition), LF-CON and FO (n=8/diet/co-culture condition), and HF-CON and FO (n=6/diet/co-culture condition). Bars not sharing a letter are significantly different (p≤0.05).

### 4.4.4 Macrophage mRNA expression of M1 markers and secretion of IL-6 and TNFα in culture supernatant

In addition to confirming that NLRP3 inflammasome activation occurs in the trans-well co-culture model, we tested whether macrophages from HF-CON mice had increased mRNA expression of M1-associated macrophage polarization genes (*Itgax*; CD11c and *Nos2*; iNOS) relative to macrophages from LF-CON mice, as well as if expression of these markers were lessened in co-cultures with FO-enriched macrophages (Figure 4.3A). First, relative to LF-CON macrophages in each respective stimulation condition, HF-CON macrophages from trans-well co-cultures had increased mRNA expression of M1 marker genes, *Itgax* (+11-fold unstimulated, +1.7-fold acute inflammation, +3.8-fold chronic inflammation) and *Nos2* (+1.9-fold acute inflammation), (p≤0.05, Figure 4.3A). Moreover, relative to HF-CON macrophages, HF-FO macrophages had decreased mRNA expression of *Nos2* (-2.6-fold acute inflammation), (p≤0.05, Figure 4.3A). Similarly, relative to LF-CON macrophages, LF-FO macrophages had decreased
mRNA expression of M1 markers, Itgax (-3.0-fold acute inflammation, -1.5-fold chronic inflammation), and Nos2 (iNOS; -7.7-fold unstimulated, -2.8-fold acute inflammation, -8.0-fold chronic inflammation), (p≤0.05, Figure 4.3A).

Further, we examined if macrophages from HF-CON mice had increased secretion of M1-associated cytokines (IL-6 and TNFα) and chemokines (CCL2, CCL5 and CCL7) in the culture supernatant relative to macrophages from LF-CON mice, as well as if secretion of these mediators were lessened in co-cultures with FO-enriched macrophages (LF-FO, HF-FO; Figure 4.3B). Here, secretion of IL-6 and TNFα in co-culture with macrophages from all diet groups were elevated above the levels found in control adipocytes under acute inflammation stimulation conditions (p≤0.05, Figure 4.3B). Further, relative to co-cultures with LF-CON macrophages, co-cultures with HF-CON macrophages had increased secretion of IL-6 (+5.6-fold) and TNFα (+1.9-fold) in the acute inflammation condition, although IL-6 secretion decreased (-2.8-fold) in the chronic inflammation condition (p≤0.05, Figure 4.3B). Interestingly, relative to co-cultures with HF-CON macrophages, co-cultures with HF-FO macrophages had decreased levels of secreted IL-6 (-1.8-fold) and TNFα (-2.3-fold) in the acute inflammation condition (p≤0.05, Figure 4.3B). Additionally, relative to co-cultures with LF-CON macrophages, co-cultures with LF-FO macrophages had decreased levels of secreted IL-6 (-16-fold) and increased TNFα (+1.5-fold) in the acute stimulation condition (p≤0.05, Figure 4.3B). Finally, secretion of chemokines was increased above adipocyte control levels most consistently in co-cultures with macrophages from HF mice (CCL2: +1.7-fold acute inflammation; CCL5: +2.9-fold acute inflammation; CCL7: +1.9-fold unstimulated, +1.8-fold acute inflammation, +1.5-fold chronic inflammation (p≤0.05, Appendix G).
Figure 4.3: mRNA expression of M1 macrophage associated mRNA markers (A), and cytokines (B) in trans-well co-cultured macrophages. Unstimulated: co-culture conditions without additional inflammatory stimuli; acute inflammation: co-culture plus 10 ng/mL LPS for 24 h co-culture period; and chronic inflammation: co-culture conditions where adipocytes were pre-
treated with 10 ng/mL LPS for 24 h, then basic media for the 24 h co-culture period. Data shows the means ± SEM for adipocyte controls (n=4/stimulation condition), LF-CON and FO (n=8/diet/co-culture condition), and HF-CON and FO (n=6/diet/co-culture condition). Bars not sharing a letter are significantly different (p≤0.05).

4.4.5 mRNA expression of NLRP3 inflammasome genes, cellular caspase-1 activity and IL-1β and IL-18 protein levels in contact co-cultures with anti-Ad

Next, we added anti-Ad to contact co-cultures using the acute and chronic inflammation stimulation conditions with macrophages isolated from LF-CON or LF-FO mice to test whether Ad played a significant role in the anti-inflammatory effect of FO in co-culture (Figure 4.4). First, mRNA expression of \( Nlrp3 \) and \( Ili1β \) in contact co-cultures from CON or FO diets were elevated above the levels found in control adipocytes under acute inflammation stimulation conditions (p≤0.05, Figure 4.4A). Second, the anti-inflammatory effect of FO in mitigating mRNA expression of NLRP3 inflammasome genes (\( Nlrp3 \), \( Casp1 \) and \( Ili1β \); Figure 4.4A) was partly reversed when anti-Ad was added relative to FO contact co-cultures with IgG (\( Nlrp3 \): +1.6-fold acute inflammation, +1.8-fold chronic inflammation; \( Casp1 \): +1.3-fold acute inflammation, +1.4-fold chronic inflammation; and \( Ili1β \): +2.9-fold chronic inflammation, p≤0.05). Similar trends were observed in contact co-cultures with CON macrophages treated with anti-Ad relative to co-cultures with CON macrophages treated with IgG (\( Nlrp3 \): +2.1-fold chronic inflammation; \( Casp1 \): +1.6-fold acute inflammation, +1.3-fold chronic inflammation; and \( Ili1β \): +3.1-fold chronic inflammation), (p≤0.05, Figure 4.4A). Interestingly, relative to contact co-cultures with CON macrophages, co-cultures with FO macrophages had decreased mRNA expression of \( Nlrp3 \) (-2.5-fold acute inflammation) and \( Ili1β \) (-2.2-fold acute...
inflammation, -1.2-fold chronic inflammation), but some effects were subdued (e.g. Nlrp3: -1.3-fold acute inflammation) when anti-Ad was added to FO co-cultures (p≤0.05, Figure 4.4A). Finally, mRNA expression of Il18 did not significantly differ with treatment (data not shown).

We next investigated if changes in expression of NLRP3 inflammasome genes in contact co-cultures with anti-Ad corresponded to changes in caspase-1 activity (Figure 4.4B) or cellular protein levels of IL-1β and IL-18 (Figure 4.4C). First, caspase-1 activity was suppressed in contact co-cultures with FO macrophages in the acute inflammation stimulation condition relative to the adipocyte control (-3.3-fold) or co-cultures with CON macrophages (-4.0-fold); this held true regardless of adding anti-Ad (p≤0.05, Figure 4.4B). Moreover, co-cultures with CON or FO macrophages had decreased caspase-1 activity in the chronic inflammation condition relative to the adipocyte control (CON: -2.7-fold, FO: -3.3-fold) regardless of adding anti-Ad (p≤0.05, Figure 4.4B). Furthermore, in the acute stimulation condition, cellular protein levels of IL-1β and IL-18 were higher in co-cultures with FO macrophages relative to the adipocyte control (IL-1β: +3.3-fold, IL-18: +3.3-fold) and co-cultures with CON macrophages (IL-1β: +2.9-fold, IL-18: +2.2-fold), and this held true regardless of adding anti-Ad (p≤0.05, Figure 4.4C). However, relative to co-cultures with CON macrophages treated with IgG in the chronic stimulation condition, CON macrophage co-cultures with anti-Ad had decreased cellular IL-1β levels (-3.5-fold, p≤0.05, Figure 4.4C).
**Figure 4.4:** mRNA expression of NLRP3 inflammasome genes (A), caspase-1 activity (B), and cellular IL-1β and IL-18 protein levels (C) in contact co-cultures with macrophages from LF-CON or LF-FO diets ± anti-Ad. Acute inflammation: co-culture plus 10 ng/mL LPS for 24 h co-culture period, and chronic inflammation: co-culture conditions where adipocytes were pre-treated with 10 ng/mL LPS for 24 h, then basic media for the 24 h co-culture period. Data shows the means ± SEM, adipocyte controls (n=4/stimulation condition), and LF-CON and FO (n=6/diet/co-culture condition). Bars not sharing a letter are significantly different (p≤0.05).

4.4.6 mRNA expression M1 polarization genes and secretion of inflammatory cytokines in contact co-cultures with anti-Ad

We also examined if contact co-cultures with LF-CON or LF-FO macrophages had increased mRNA expression of M1-associated polarization markers when anti-Ad was added (Figure 4.5A). Here, the mRNA expression of *Itgax* (CD11c) in contact co-cultures from CON or FO diets was elevated above the levels found in control adipocytes under acute inflammation conditions (p≤0.05, Figure 4.5B). However, compared to the respective adipocyte chronic inflammation condition control (i.e. 24 h LPS pre-treatment), mRNA expression of *Nos2* (iNOS) was decreased in both CON and FO co-cultures (p≤0.05, Figure 4.5B). Further, the anti-inflammatory effect of FO in mitigating mRNA expression of M1 polarization genes was partly reversed when anti-Ad was added relative to FO contact co-cultures with IgG (*Itgax*: +1.4-fold chronic inflammation, *Nos2*: +1.6-fold acute inflammation, +2.0-fold chronic inflammation, p≤0.05, Figure 4.5A). A similar trend occurred in co-cultures with CON macrophages treated with anti-Ad relative to co-cultures with CON macrophages treated with IgG (*Nos2*: +1.7-fold acute inflammation, +4.3-fold chronic inflammation), (p≤0.05, Figure 4.5A). Interestingly,
relative to contact co-cultures with CON macrophages, co-cultures with FO macrophages had decreased mRNA expression of Nos2 (-2.5-fold acute inflammation), although this effect was subdued for Nos2 (-1.6-fold acute inflammation) when anti-Ad was added to FO co-cultures (p≤0.05, Figure 4.5A).

Finally, we examined if contact co-cultures with LF-CON or LF-FO macrophages had increased secretion of M1-associated cytokines (IL-6 and TNFα, Figure 4.5B) and chemokines (CCL2, CCL5 and CCL7, Figure 4.5C) in the culture supernatant when anti-Ad was added. Here, contact co-cultures with FO macrophages suppressed IL-6 secretion relative to co-cultures with CON macrophages in the acute inflammation stimulation condition (-3.4-fold) regardless of adding anti-Ad (Figure 4.5B). In contrast, relative to the respective IgG controls, when anti-Ad was added to both CON and FO co-cultures there was an increase in the secretion of IL-6 (CON: +2.6-fold, FO: +2.0-fold chronic inflammation), TNFα (CON: +1.4-fold, FO: +1.4-fold acute inflammation; CON: 6.7 pg/mL, FO: 6.4 pg/mL versus undetectable in adipocyte chronic inflammation control), (p≤0.05, Figure 4.5B), and chemokines CCL2 (CON: +2.4-fold, FO: +2.1-fold chronic inflammation) and CCL5 (CON: +2.9-fold, FO: +2.9-fold chronic inflammation), (p≤0.05, Figure 4.5C). Finally, CCL7 secretion did not vary with any treatment in contact co-culture (data not shown).
**Figure 4.5:** mRNA expression of M1-associated mRNA markers (A), cytokines (B), and chemokines (C) in contact co-cultures with macrophages isolated from LF-CON or LF-FO diets ± anti-Ad. Acute inflammation: co-culture plus 10 ng/mL LPS for 24 h co-culture period; and chronic inflammation: co-culture conditions where adipocytes were pre-treated with 10 ng/mL LPS for 24 h, then basic media for the 24 h co-culture period. Data shows the means ± SEM, adipocyte controls (n=4/stimulation condition), LF-CON and FO diet (n=6/diet/co-culture condition). Bars not sharing a letter are significantly different (p≤0.05).

### 4.5 Discussion

Adipocyte-macrophage paracrine interactions (or cross-talk) play a key role in promoting dysregulated adipokine secretion and the downstream development of systemic insulin resistance; however, the paracrine signals that mediate adipocyte-macrophage cross-talk are not well understood. In this study we showed for the first time that the expression of NLRP3 inflammasome genes and cellular caspase-1 activity were increased in trans-well co-cultured macrophages (Figure 4.1A, B) and adipocytes (Figure 4.2A, B), particularly in co-cultures with macrophages isolated from HF-fed mice, suggesting the importance of soluble mediators in this process. In particular, these effects were significantly decreased in co-cultures with FO-enriched macrophages most consistently in the acute LPS-induced inflammation condition, highlighting that FO exerts an anti-inflammatory effect when co-cultures were treated with the dose of LPS seen in obesity (26, 39). Additionally, we showed for the first time that some of the beneficial effects of FO, such as reducing expression of NLRP3 inflammasome-related genes (Figure 4.4A), M1 polarization marker genes (Figure 4.5A), and cytokine and chemokine secretion (Figure 4.5B,C) were controlled partly through Ad. In contrast, caspase-1 activity (Figure 4.4B)
and cellular IL-1β and IL-18 protein levels (Figure 4.4C) were modulated in co-cultures with FO-enriched macrophages independently of Ad, thereby providing mechanistic insight into the role of LC n-3 PUFA in mitigating inflammatory cross-talk.

While previous in vitro studies have shown that the saturated fatty acid palmitic acid can propagate inflammatory adipocyte-macrophage cross-talk (221, 222), and that EPA and DHA, well known FO-derived LC n-3 PUFA, can mitigate such cross-talk (16, 177), these studies administered albumin-complexed, purified free fatty acids, which lacks some physiological relevance. A novel aspect of the current study was our use of an in vitro model designed to endogenously enrich LC n-3 PUFA in macrophages from mice fed either a LF or HF FO-enriched diet. Additionally, we aimed to mimic key aspects of the obese AT microenvironment, such as low-dose LPS and reduced Ad, to study how adipocyte-macrophage cross-talk influences NLRP3 inflammasome priming and activation, M1 polarization marker mRNA expression and inflammatory cytokine and chemokine secretion, and if these factors were mitigated by the addition of FO to the diet.

4.5.1 Co-cultures with FO-enriched macrophages had decreased NLRP3 inflammasome activity and expression of M1 macrophage polarization markers

In trans-well co-cultured macrophages from HF-fed mice (Figure 4.1), as well as contact co-cultures with macrophages from LF-fed mice (Figure 4.4), FO enrichment significantly decreased mRNA expression of several key NLRP3 inflammasome genes, (e.g. Nlrp3, Casp1, Il1β and Il18) and caspase-1 activity under various inflammatory conditions. This data corresponded to decreased M1 marker genes, Itgax (CD11c) and/or Nos2 (iNOS), and secretion of M1-associated cytokines, IL-6 and TNFα, in trans-well co-culture with HF-FO-derived
macrophages (Figure 4.3) and contact co-culture with LF-FO-derived macrophages (Figure 4.5), suggesting that FO-derived LC n-3 PUFA critically affect both NLRP3 inflammasome and M1 macrophage polarization signaling. This data is in line with murine models of obesity showing that addition of LC n-3 PUFA to the diet (135, 156, 276) or endogenous production of LC n-3 PUFA in the fat-1 mouse (256), decreased the proportion of M1-like macrophages in visceral AT. Specifically, LC n-3 PUFA have been shown to interfere with LPS-stimulated NFκB (177, 227) and NLRP3 inflammasome (258, 276) activity in macrophages and adipocytes. Interestingly, DHA has been shown to decrease caspase-1 activation and IL-1β secretion in AT (276) and macrophages (258). More specifically, DHA, more so than EPA (227), inhibits LPS-stimulated macrophage responses involving NFκB signaling in macrophages; a key step in inhibiting signal 1 in inflammasome priming (258).

4.5.2 Some FO-mediated anti-inflammatory effects in co-culture are partly dependent on Ad

Mechanistically, the ability of FO to mitigate expression of NLRP3 inflammasome genes (Figure 4.4), M1 macrophage markers and associated secretion of inflammatory cytokines and chemokines (Figure 4.5) was mediated, in part, through Ad since the addition of Ad-neutralizing antibody to contact co-cultures with LF-FO-enriched macrophages significantly blunted this anti-inflammatory effect. This data suggests that Ad plays a role in mitigating NLRP3 inflammasome gene expression resulting from adipocyte-macrophage cross-talk. Interestingly, both full-length (142) and globular (284) Ad have been shown to drive LPS tolerance and macrophage polarization towards the M2 phenotype, which could potentially serve to re-direct the cyclic pro-inflammatory cross-talk between adipocytes and M1-like macrophages in obesity.
4.5.3 Co-culture conditions do not increase IL-1β or IL-18 secretion

Surprisingly, while FO suppressed caspase-1 activity in trans-well co-cultured macrophages from HF-FO fed mice (Figure 4.1) and adipocytes (Figure 4.2), mature IL-1β and IL-18 cellular proteins were only detected in adipocytes. This data supports other reports showing that NLRP3 inflammasome activation occurs in macrophages (241) and in adipocytes (216). Secondly, it was somewhat surprising to observe macrophage caspase-1 activity without observing significant increases in secreted IL-1β and IL-18; however, reports have shown that IL-1β and IL-18 are not efficiently secreted without strong inflammasome activating stimuli (33, 85), i.e. a priming signal such as LPS and a second activation signal (e.g. cytotoxic T cells (84)). This may be a safety mechanism to prevent unnecessary inflammatory responses and pyroptosis; a caspase-1-mediated form of cell death that results in membrane breakdown and secretion of inflammatory components (57). Further, adipocytes co-cultured with HF-FO macrophages exhibited elevated levels of cellular IL-1β and IL-18 compared to adipocytes co-cultured with HF-CON macrophages in the acute inflammation co-culture condition where LPS was present (Figure 4.2C), suggesting that FO can promote the accumulation of these cellular cytokines under this stimulation condition. Nevertheless, cellular IL-1β and IL-18 levels were decreased in adipocytes co-cultured with HF-FO macrophages in the chronic inflammatory condition; therefore, the timing of LPS exposure may affect the ability of HF-FO macrophages to suppress IL-1β and IL-18 protein levels in adipocytes.
4.5.4 Acute and chronic inflammation conditions evoke different responses in co-cultured adipocytes and macrophages

Interestingly, data from the acute inflammation condition differed significantly from the chronic inflammation condition, showing that the two models are mutually exclusive despite getting the same dose of LPS for 24 h. For example, mRNA expression of NLRP3 inflammasome genes (Figure 4.1A), the M1 marker, Nos2 (Figure 4.3A), and secretion of inflammatory cytokines IL-6 and TNFα (Figure 4.3B) in LF and HF trans-well co-cultured macrophages was highest in the acute inflammation condition where LPS was only present during the 24 h co-culture period. This is perhaps not surprising since LPS can prime Nos2 expression (198), inflammasome gene expression (258), and secretion of IL-6 in macrophages (45). Alternatively, for the M1 marker, Itgax (CD11c, Figure 4.1A), mRNA expression was highest in macrophages from HF fed mice in unstimulated and chronic inflammation conditions. This follows since CD11c is a marker of inflammatory M1 macrophages in obese AT (136) where chronic inflammation persists. Conversely, for adipocytes in trans-well co-cultures with macrophages from HF fed mice, LPS had a lasting effect; even in the chronic stimulation condition with LPS pre-treated cells (no LPS for 24 h co-culture period), mRNA expression of NLRP3 inflammasome genes, Nlrp3 and Il18, were similar to the acute inflammation condition (Figure 4.1A). Overall, these results show that the same dose of LPS found in obesity-associated metabolic endoxemia (10 ng/mL or 5-6 EU; (26, 39)) promotes inflammatory adipocyte-macrophage cross-talk in co-culture and has a lasting inflammatory effect, particularly in LPS pre-treated adipocytes. Thus, future work in in vivo obesity models should examine if dietary LC n-3 PUFA also play a role in maintaining gut barrier integrity, decreasing circulating
as well as AT accumulation of LPS, and subsequently decreasing inflammatory adipocyte-macrophage cross-talk.

**4.5.5 Methodological considerations and future directions**

Despite the novelty of our *in vitro* co-culture model, there are certain methodological aspects that could be improved in future studies. For one, a CD11b+ macrophage-enriched population of cells was obtained from the spleens of LF- and HF-fed mice, which may not be truly representative of macrophages derived from obese visceral AT. Nevertheless, the spleen is a rich source of macrophages that were clearly more pro-inflammatory in co-culture (compared to macrophages isolated from LF-fed mice) when isolated from HF-fed mice. Future studies will compare the effect of visceral AT-derived macrophages to splenic macrophages in co-culture to identify if there are local differences in macrophage phenotype that could affect downstream paracrine interactions with adipocytes. Secondly, it would be interesting to see if neutralizing Ad further exacerbated HF diet-induced expression of inflammatory mediators in co-culture; however, our initial question was aimed at identifying if Ad affected adipocyte-macrophage cross-talk without the influence of macrophages from HF-fed mice. Third, it is worth noting that the dose of LC n-3 PUFA in the HF-FO diet (4.5% kcal) appears somewhat supraphysiological given that human LC n-3 PUFA recommendations are 0.5-2% kcal (291); however, this dose corresponds to doses of approximately 9.4 g used in some human clinical trials showing favorable outcomes on different inflammatory markers (190). Ultimately, the current study aimed to provide evidence that feeding LC n-3 PUFA as part of a HF diet could prevent inflammatory responses in macrophages co-cultured with adipocytes.
4.5.6 Summary

Taken together, in adipocyte macrophage co-cultures recapitulating key aspects of the obese AT microenvironment, mRNA expression of NLRP3 inflammasome genes and cellular caspase-1 activity were increased in both macrophages and adipocytes, particularly in co-cultures with macrophages isolated from HF-fed obese mice. Notably, these effects were significantly decreased in co-cultures with FO-enriched macrophages. Here we showed for the first time that some of the beneficial effects of FO, such as decreasing expression of NLRP3 inflammasome and M1 marker genes, and secretion of inflammatory cytokines and chemokines, were modulated, in part, through Ad. Thus, our work emphasizes a beneficial role of LC n-3 PUFA in attenuating obesity-related inflammation through potential direct modulation of the innate immune response. Ultimately, this data supports further study of LC n-3 PUFA as an immunomodulatory strategy to mitigate obesity-associated pathologies.
Chapter 5: Fish oil-derived long-chain n-3 polyunsaturated fatty acids reduce expression of M1-associated macrophage markers in an ex vivo adipose tissue culture model, in part through adiponectin

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**De Boer AA**, Monk JM, Liddle DM, Power KA, Ma DWL and Robinson LE. Fish oil-derived long-chain n-3 polyunsaturated fatty acids reduce expression of M1-associated macrophage markers in an *ex vivo* adipose tissue culture model, in part through adiponectin.

5.1 Abstract

Adipose tissue (AT) macrophages (ATM) play a key role in obesity-associated pathologies, and their phenotype can be influenced by the local tissue microenvironment. Interestingly, long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) and the LC n-3 PUFA-upregulated adipokine, adiponectin (Ad), may mitigate excessive ATM inflammatory M1-polarization responses. However, to what extent LC n-3 PUFA and Ad work in concert to affect macrophage phenotype has not been examined. Thus, we used an established *ex vivo* AT organ culture model using visceral AT from mice fed a control (CON; 10% w/w safflower oil) n-6 PUFA-rich diet or an isocaloric fish-oil (FO; 3% w/w menhaden oil + 7% w/w safflower oil)-derived LC n-3 PUFA-rich diet to generate AT conditioned media (ACM). We then evaluated if CON or FO ACM affected macrophage polarization markers in a model designed to mimic acute (18 h ACM plus LPS for the last 6 h) or chronic (macrophages treated with LPS-challenged CON or FO ACM for 24 h) inflammation ± Ad-neutralizing antibody and the LPS-neutralizing agent, polymyxin B. In the acute inflammation model, macrophages treated with FO ACM had
decreased lipid uptake and mRNA expression of M1 markers (Nos2, Nfkb, Il6, Il18, Ccl2 and Ccl5) compared with CON ACM (p≤0.05); however, these effects were largely attenuated when Ad was neutralized (p>0.05). Further, in the chronic inflammation model, macrophages treated with FO ACM had decreased mRNA expression of M1 markers (Nos2, Tnfa, Ccl2 and Il1β) and IL-6 and CCL2 secretion (p≤0.05); however, some of these effects were lost when Ad was neutralized, and were further exacerbated when both Ad and LPS were neutralized. Taken together, this work shows that LC n-3 PUFA and Ad work in concert to suppress certain M1 macrophage responses. Thus, future strategies to modulate the ATM phenotype should consider the role of both LC n-3 PUFA and Ad in mitigating obese AT inflammation.

5.2 Introduction

In obesity, adipose tissue macrophages (ATM) play a key role in adipose tissue (AT) inflammation and subsequent development of obesity-associated pathologies, such as local and systemic insulin resistance (168, 261). More specifically, circulating monocytes accumulate in obese AT, particularly in visceral depots, wherein they differentiate into ATM to help with tissue remodeling and lipid homeostasis (47, 113, 272). Importantly, paracrine interactions, or cross-talk, between adipocytes and ATM play a key role in determining macrophage polarization status (i.e. M1 or M2) and the resultant AT secretory profile. Many ATM exhibit an inflammatory M1 phenotype, characterized by increased lipid content, NLRP3 inflammasome activation, expression of the integrin (CD11b) and high levels of anti-microbial iNOS (murine only), antigen presentation via MHCII, and secretion of inflammatory cytokines, such as TNFα and IL-6 (68, 136, 159, 254). Mechanistically, TNFα and IL-6 feed back onto adipocytes through paracrine signaling to sustain secretion of adipocyte-derived inflammatory mediators (e.g. CCL2,
IL-6) and release of fatty acids through lipolysis (221). Finally, some ATM exhibit a less inflammatory M2 phenotype, characterized by surface expression of scavenging receptors, like CD206, antigen presentation, and secretion of the anti-inflammatory cytokine, IL-10 (254).

Obesity is also characterized by increased circulating levels of gut bacteria-derived lipopolysaccharide (LPS), a condition termed metabolic endotoxemia (26), which leads to LPS accumulation in AT (272). While some macrophages can exhibit endotoxin tolerance or hyporesponsiveness when re-stimulated with LPS (255), it is unclear if this occurs in macrophages within obese AT. Interestingly, both the full-length (62, 142, 175) and globular (182, 238, 284) isoforms of the anti-inflammatory adipokine, adiponectin (Ad), have been shown to promote a response similar to endotoxin tolerance and macrophage polarization towards the M2 phenotype, or enhance M2 polarization in vitro (218) and in vivo (134). Therefore, Ad could potentially serve to re-direct the cyclic inflammatory cross-talk between adipocytes and M1 macrophages in obesity.

Given the impact of the ATM phenotype on subsequent inflammatory processes and AT dysfunction, strategies to alter ATM polarization status may be useful in mitigating obesity-related inflammation. Of interest, dietary long-chain polyunsaturated fatty acids (LC n-3 PUFA), such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA), are well-known anti-inflammatory agents (22, 70) and can serve to mitigate excessive inflammatory adipocyte-macrophage paracrine interactions in vitro (16, 177). While it has been shown that LC n-3 PUFA lessen inflammatory adipokine secretion (10) and M1 macrophage polarization in obese rodent AT (135, 156, 234, 276), the mechanisms by which this occurs are unclear. Furthermore, while LC n-3 PUFA upregulate secretion of Ad in murine (178) and human
adipocytes (231), it is unclear if Ad partly mediates the anti-inflammatory effects of LC n-3 PUFA.

Thus, to assess the potential role of Ad in LC n-3 PUFA-mediated anti-inflammatory effects on ATMs, we used an established *ex vivo* AT organ culture model (65) comprised of visceral AT from mice fed a control (CON) safflower oil-derived n-6 PUFA-rich diet or a fish-oil (FO)-derived LC n-3 PUFA-rich diet to generate AT conditioned media (ACM). We then evaluated if CON or FO ACM affected macrophage polarization markers in an acute inflammation model in the absence or presence of Ad-neutralizing antibody. Secondly, we used a low-grade chronic inflammation model whereby visceral AT organ cultures from CON and FO diet fed mice were challenged with LPS *ex vivo* for 24 h prior to incubation with macrophages to mimic the chronic inflammation of metabolic endotoxemia (26, 39). Additionally, we utilized both an Ad-neutralizing antibody and the LPS-neutralizing agent, polymyxin B, to assess if Ad or LPS driven mechanisms affect LC n-3 PUFA-mediated anti-inflammatory effects in macrophages treated with ACM in the chronic inflammation model.

5.3 Materials and methods

5.3.1 Animals and diets

All experimental procedures were approved by the University of Guelph Animal Care Committee. Mice were housed as described (141). Eight-week-old male C57BL/6 mice were fed *ad libitum* an AIN-93G modified diet containing either 10% w/w safflower oil (CON) or an isocaloric LC n-3 PUFA-enriched diet containing 3% w/w menhaden oil + 7% w/w safflower oil (FO) for 4 wk (n=5 mice/diet), (Research Diets Inc, USA; Appendix C). The FO diet contained
approximately 1.7% kcal from LC n-3 PUFA, which is in line with the human dietary recommended intake of 0.5-2% kcal for total n-3 PUFA (291).

5.3.2 Tissue collection and AT organ culture

Mice were terminated by CO2 asphyxiation followed by cervical dislocation. Epididymal fat pads were isolated and immediately placed into 50 mL sterile conical tubes containing 1x PBS (Sigma, USA). Fat pads were blotted dry, approximately cut into two equal portions, and then weighed. Tissue was divided into two per depot so that one sample could be challenged with low dose LPS (10 ng/mL) from *E. coli* serotype 055:B5 (Sigma, USA, cell culture-tested, one lot used) intended to mimic the 5-6 endotoxin units reported in metabolic endotoxemia (26, 39). Immediately after weighing, fat pads were moved to 15 mL sterile conical tubes containing 3 mL of DMEM high glucose (without sodium pyruvate, HyClone, USA) supplemented with 0.5% fetal bovine serum (sterile filtered, Canadian origin, Sigma, USA) and 1% penicillin–streptomycin (HyClone, USA). Each tissue sample was immediately minced using sterile scissors into ≤0.5 cm pieces to avoid tissue hypoxia. Under sterile conditions, media was then topped up so that the ratio of tissue to media was at the optimal ratio of 500 mg/15 mL media as reported elsewhere (65). To the tissues challenged with LPS, an LPS working solution was added to the culture media such that the final concentration was 10 ng/mL. AT samples were placed into 100 mm sterile culture dishes (Sarstedt, USA) and then cultures were placed in a humidified incubator for 24 h at 37°C with 5% CO₂. Twenty-four hours was chosen since secreted cytokines were elevated between 12-24 h (Figure 5.1). After the incubation period, the minced AT was removed using 70 µm sterile cell strainers (BD Biosciences, USA), and the AT-conditioned media (ACM) was aliquoted and stored at -80°C until further analysis.
5.3.3 Preparation of macrophage ACM treatments

The ACM was thawed once and then sterile filtered (0.22 µm, Sarstedt, USA) prior to use. Before incubation with macrophages, 5 µg/mL of Ad-neutralizing antibody (AF1119, anti-mAcrp30, R&D Systems, USA) or isotype control anti-goat IgG (5 µg/mL, R&D Systems, USA) was added to ACM. The samples were then incubated at 4°C as previously reported (83). This antibody was chosen since it has been reported to suppress Ad-mediated signaling in adipocyte-conditioned media (83, 191), and we confirmed the dose of antibody used suppressed Ad-mediated signaling in pilot experiments with adipocyte conditioned media (Appendix F). In the acute inflammation model (Figure 2-4), macrophages were pre-treated with ACM from CON or FO fed mice (no LPS) for 18 h, followed by the addition of LPS (10 ng/mL) to the ACM for the final 6 h (total 24 h). This time course was chosen since both globular (182) and full-length Ad (142) have previously been shown to promote a response similar to endotoxin tolerance using a comparable time course. In the chronic inflammation model (Figure 5-7), visceral AT organ cultures from CON and FO diet fed mice were challenged with LPS ex vivo for 24 h prior to incubation with macrophages for an additional 24 h. Ad-neutralizing antibody was added to ACM as described above. Additionally, the LPS neutralizing agent, polymyxin B (36 µM, 0.22 µm sterile filtered, cell-culture tested, Sigma, USA), was added to some ACM treatments with gentle vortexing every 10 min for a total of 30 min prior to administering ACM to macrophages at 37°C. For all experiments, macrophages treated with media containing 5 µg/mL IgG plus 10 ng/mL LPS served as the control.
5.3.4 Culture of RAW 264.7 macrophages

RAW 264.7 murine macrophages (ATCC, USA) were grown and passaged according to the manufacturer’s instructions. Macrophages were maintained in DMEM high glucose (without sodium pyruvate, HyClone, USA), plus 10% v/v fetal bovine serum (low-endotoxin, Canadian origin, Sigma, USA), and 1% v/v penicillin streptomycin (HyClone, USA). 4 h prior to experiments, macrophages were split using the manufacturer’s protocol, counted using trypan exclusion, spun down at 335 x g at ambient temperature for 5 min, and then re-suspended in DMEM high glucose plus 0.5% v/v FBS and 1% v/v penicillin streptomycin. Next, the cell density was adjusted to 1.0 x 10^6 cells/ mL and macrophages were seeded into 96-well (for oil red O procedure) or 24-well plates (for RNA/protein analysis) (Corning, USA). Without disturbing the adherent macrophages, media was replaced with the aforementioned control, CON or FO ACM treatments.

5.3.5 Gene expression

After 24 h, cells were washed with 1x PBS, lysed using a RNA/Protein Isolation Kit (Norgen Biotek, Canada), and processed according to the manufacturer’s instructions. cDNA was made from 1 µg of extracted RNA using a high capacity cDNA reverse transcription kit as per the manufacturer’s instructions (Applied Biosystems, USA). Real-time PCR analysis was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems, USA) using the default protocol described elsewhere (16). Primers were designed using the Universal Probe Library Assay Design Center (Roche Applied Sciences, Germany, Appendix A) and validated primer efficiencies were between 90–105%. All results were normalized to 18S mRNA
expression, and the relative differences in gene expression between treatment groups and the IgG control (no LPS) were determined using the ΔΔCt method.

5.3.6 Secreted adipokine analyses

Supernatant was collected after 12 and 24 h from ACM, and after 24 h following ACM culture with macrophages for analysis of secreted cytokine concentrations. CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), IL-4, IL-6, IL-10, TNFα and mature IL-1β and IL-18 were multiplexed using the ProcartaPlex Mouse Basic kit (eBioscience, USA) using undiluted supernatant and analyzed using the Bio-Plex 200 System (Bio-Rad, USA). To calculate the secretion of adipokines solely from macrophages after 24 h treatment with ACM, the concentration of cytokine measured in ACM prior to culture with macrophages was subtracted from the concentration of cytokine measured after the incubation. Importantly, cell protein and secreted levels of IL-1β and IL-18 were below the minimum detection threshold for all treatments. Additionally, secreted full-length Ad was diluted 100-fold and measured by ELISA (Quantikine Mouse Ad/Acrp 30 ELISA, R&D Systems, USA) according to the manufacturer’s instructions.

5.3.7 Macrophage fatty acid composition

Following 24 h, macrophages were scraped in 1x PBS and then centrifuged at 335 x g at ambient temperature for 10 min. The PBS was aspirated and the pellet was frozen at -80°C until further processing. Total lipids were extracted from cell lysates of macrophages and fatty acid methyl esters were prepared for analysis by gas liquid chromatography as described previously (61, 118). Fatty acids were identified by comparing the retention times of samples with those of a
known standard (GLC463; Nu-Chek Prep, USA). Fatty acid composition values are expressed as a percentage of total fatty acids. Within each ACM treatment, the lipid profile between macrophages treated with Ad-neutralizing antibody to those treated with IgG did not differ; therefore, only ACM treatments (IgG) are shown (Figure 5.2 and 5.5).

5.3.8 Macrophage oil red O procedure

Following the 24 h ACM treatment, neutral lipid deposition was assessed in macrophages with minor modification from the original procedure (76). Briefly, an oil red O working solution was made 6 h in advance by mixing 6 parts oil red O saturate (0.5 g in 100 mL isopropyl alcohol) in 4 parts ddH2O, and then filtered twice immediately before use. Next, media was aspirated and cells were then rinsed with 1x PBS. Cultures were fixed with 4% paraformaldehyde solution followed by light shaking at ambient temperature for 10 min. Cultures were then sequentially rinsed with 1x PBS and 70% ethanol before staining with oil red O working solution for 30 min (at ambient temp with light shaking). Following this, cultures were then rinsed again with 1x PBS, then 70% ethanol solution. The solutions were then aspirated and the plate was inverted to dry in a laminar flow hood for 30 min. Next, 200 µL of isopropyl alcohol was added to each well. The plate was sealed, shook lightly for 3 min, and then read immediately with a spectrophotometer at 520 nm. Wells without cells that received the aforementioned staining procedure served as the plate blank. Data is reported as the fold change in absorbance (per treatment) relative to the average absorbance for cells cultured in media without LPS or other additives (n=4).
5.3.9 Statistical analysis

All data are expressed as mean ± SEM. The predetermined upper limit of probability for statistical significance was p≤0.05 and analyses were conducted using SigmaPlot version 12.5 (USA). Data that were not normally distributed were transformed prior to statistical analysis, and normal distribution and equal variance was confirmed by Shapiro-Wilk test and Levene’s test, respectively. Data were analyzed using a two-way ANOVA followed, if justified, by testing using Fisher’s Least Squared Difference post-hoc test. Mouse parameters comparing CON and FO diets (Table 5.2) and macrophage lipid content between CON and FO ACM (Figure 5.2 and 5.5) were analyzed using unpaired t-tests.

5.4 Results

5.4.1 Mouse parameters

Mouse initial and terminal body weights, dietary food intake and epididymal fat pad weights did not differ between mice fed CON or FO diets (p>0.05, Table 5.1). Previous studies using these diets showed that LC n-3 PUFA are significantly enriched in liver and red blood cells (157).

Table 5.1: Mouse physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>21.0 ± 0.63</td>
<td>21.8 ± 0.52</td>
</tr>
<tr>
<td>Terminal Body weight (g)</td>
<td>25.0 ± 2.2</td>
<td>27.5 ± 1.2</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>2.24 ± 0.04</td>
<td>2.22 ± 0.03</td>
</tr>
<tr>
<td>Epididymal fat pad weight (g)</td>
<td>1.36 ± 0.27</td>
<td>1.45 ± 0.15</td>
</tr>
</tbody>
</table>

1Values are means ± SEM (n=5/dietary group)
5.4.2 ACM secretory profile after 24 h with or without LPS

In CON ACM, IL-6, TNFα, CCL2, CCL5 and CCL7 secretion increased in the presence of LPS at both 12 and 24 h (Figure 5.1). Similarly, in FO ACM, IL-6, CCL2 and CCL5 secretion increased when LPS was added at both 12 and 24 h; however, TNFα secretion only increased at 24 h (p≤0.05, Figure 5.1). Moreover, in FO ACM, secretion of CCL7 did not change over time or with LPS (p>0.05, Figure 5.1). Interestingly, in FO ACM without LPS, the secretion of IL-6, TNFα, CCL2 and CCL5 were decreased relative to CON ACM at both 12 and 24 h; however, CCL7 was only decreased at 24 h (p≤0.05, Figure 5.1). Similarly, relative to CON ACM with LPS, in FO ACM with LPS the secretion of IL-6 was decreased at both 12 and 24 h, although the secretion of other adipokines was only decreased at one time point (CCL2, CCL5 and CCL7 at 24 h; TNFα at 12 h), (p≤0.05, Figure 5.1). Furthermore, the secretion of full-length Ad in ACM did not vary with diet or LPS (p>0.05, Figure 5.1). Finally, IL-1β, IL-18, IL-4 and IL-10 concentrations were below the range of detection in all ACM treatments.
Figure 5.1. Time course secretion of adipokines from CON and FO ACM at 12 and 24 h. The secretion of M1-associated adipokines (IL-6, TNFα, CCL2, CCL5 and CCL7) from CON and FO ACM at 12 and 24 h time points ± LPS challenge. AT organ cultures from epididymal AT of
CON or FO fed mice were treated with or without low-dose LPS (10 ng/mL) for 24 h. Data shows the means ± SEM for CON and FO (n=5/diet/condition). Bars not sharing a letter are significantly different (p≤0.05).

5.4.3 Lipid profile in macrophages treated with ACM in the acute inflammation model

To examine if the Ad within ACM from CON or FO fed mice could promote a response similar to endotoxin tolerance, macrophages were pre-treated with ACM or media for 18 h with Ad-neutralizing antibody (anti-Ad) or control IgG, then LPS was added for 6 h for a total of 24 h. First, we measured total lipid uptake using oil red O staining to measure the relative abundance of neutral lipids, and then quantified the percent of total lipids (n-3 and n-6 PUFA, MUFA and SFA) within the macrophages treated for 24 h with CON or FO ACM (Figure 5.2). Here, without LPS, macrophages treated with CON ACM had increased neutral lipid relative to control (p≤0.05); however, no such increase occurred in macrophages treated with FO ACM (p>0.05, Figure 5.2). Second, adding LPS resulted in an increase in neutral lipid in control macrophages (p≤0.05, Figure 5.2). However, levels of neutral lipid did not change when LPS was added in macrophages treated with CON or FO ACM (p>0.05, Figure 5.2). Interestingly, when LPS was added to ACM with anti-Ad, levels of neutral lipid increased in macrophages treated with FO ACM (p≤0.05), but not CON ACM (p>0.05, Figure 5.2). Finally, the percent of n-3 PUFA (0.24% ±0.04), n-6 PUFA (0.33% ±0.03), MUFA (4.30% ±0.24) and SFA (95.2% ±0.22) did not differ in macrophages treated with CON or FO ACM in this model (p>0.05, Figure 5.2).
Figure 5.2: Neutral lipid and total lipid profile in macrophages in the acute inflammation model. Macrophages were treated with CON or FO ACM (no LPS) for 18 h, followed by the addition of low-dose LPS (10 ng/mL) for the last 6 h (24 h total). 5 µg/mL of anti-Ad or IgG were added to ACM treatments prior to culture with macrophages. Macrophages treated with media and 5 µg/mL IgG plus LPS served as the control. Data shows the means ± SEM (n=5/diet/condition). Bars not sharing a letter are significantly different (p≤0.05).

5.4.4 mRNA expression of M1 markers and associated secreted cytokines in macrophages treated with ACM in the acute inflammation model

First, treating macrophages with low dose LPS for 6 h (control) induced the expression of M1 markers and NLRP3 inflammasome-related genes shown in Figure 3 relative to macrophages treated with just IgG (data not shown, p≤0.05). Second, treating macrophages with either CON or FO ACM induced a response similar to endotoxin tolerance at the mRNA level as evidenced
by decreased expression of M1 markers, *Nfkb, Ccl2, Tnfa, Il6* and *Ccl5*, as well as NLRP3 inflammasome-associated genes *Nlrp3, Il18* and *Il1β* (**Figure 5.3A**, *p*≤0.05). In contrast, macrophages treated with CON ACM had increased mRNA expression of *Nos2* relative to control (**Figure 5.3A**, *p*≤0.05), while mRNA expression of *Casp1* in macrophages treated with CON or FO ACM did not differ from control (**Figure 5.3A**, *p*>0.05). Second, when anti-Ad was added, within each respective ACM group, the mRNA expression of M1 markers, *Nos2* (CON ACM only), *Tnfa, Il6* and *Ccl5*, as well as NLRP3 inflammasome-related genes, *Casp1, Nlrp3*, and *Il18* (**Figure 5.3A**), decreased (*p*≤0.05). In contrast, relative to each respective ACM treatment group, mRNA expression of *Il1β* increased with anti-Ad (*p*≤0.05, **Figure 5.3A**). Third, mRNA expression of M1 genes, *Nos2* (FO ACM only), *Nfkb* and *Ccl2* decreased in macrophages treated with ACM regardless of adding anti-Ad (**Figure 5.3A**). Additionally, relative to CON ACM, macrophages treated with FO ACM had decreased mRNA expression of *Nos2, Nfkb, Ccl2, Il6, Ccl2* and *Il18* (*p*≤0.05), although this effect was lost for *Nos2, Nfkb, Ccl2* and *Il18* with anti-Ad (*p*>0.05, **Figure 5.3A**). Fourth, secretion of IL-6 and CCL2 increased in both CON and FO ACM treated macrophages relative to control; however, with anti-Ad, secretion of IL-6 and CCL2 (CON ACM only) returned to control levels (*p*>0.05, **Figure 5.3B**). Interestingly, in FO ACM treated macrophages, secretion of CCL2 was elevated above all treatment groups, although this was partly subdued with anti-Ad (*p*≤0.05, **Figure 5.3B**).
**Figure 5.3:** mRNA expression of M1 markers (A: Nos2, Nfkb, Ccl2, Tnfa, Il6, Ccl5), NLRP3 inflammasome genes (Casp1, Il18 and Il1β) and secretion of M1-associated cytokines (B: IL-6 and CCL2) from macrophages treated with ACM ± anti-Ad in the acute inflammation model. Macrophages were treated with CON or FO ACM (no LPS) for 18 h, followed by the addition of low-dose LPS (10 ng/mL) for the last 6 h (24 h total). 5 µg/mL of anti-Ad or IgG were added to ACM treatments prior to culture with macrophages. Macrophages treated with media with 5 µg/mL IgG plus LPS served as the control. Data shows the means ± SEM (n=5/diet/condition). Fold changes are expressed relative to macrophages treated with IgG alone (not shown). Bars not sharing a letter are significantly different (p \leq 0.05).

### 5.4.5 mRNA expression of M2 markers and secreted IL-10 in macrophages treated with ACM in the acute inflammation model

Treating macrophages with low dose LPS for 6 h (control) induced mRNA expression of M2 markers, Il10 and Cd206, and the antigen presentation co-stimulatory molecule, Cd86, relative to macrophages treated with just IgG (data not shown, p \leq 0.05). Macrophages treated with CON ACM had decreased mRNA expression of Il10 relative to control, while macrophages treated with both CON and FO ACM had decreased Cd86 relative to control (p \leq 0.05, **Figure 5.4A**). However, when anti-Ad was added to CON and FO ACM, mRNA expression of both Il10 and Cd206 were decreased (p \leq 0.05, **Figure 5.4A**). Third, mRNA expression of Cd86 in macrophages treated with FO ACM was decreased relative to CON ACM, though this effect was lost with anti-Ad (p \leq 0.05, **Figure 5.4A**). Finally, IL-10 secretion was increased (p \leq 0.05) in macrophages treated with CON ACM relative to control regardless of adding anti-Ad (**Figure**...
5.4B. In contrast, IL-10 secretion decreased in macrophages treated with FO ACM relative to control and CON ACM (p≤0.05), and this occurred regardless of adding anti-Ad (Figure 5.4B).

**Figure 5.4:** mRNA expression of M2-associated markers (A: *Il10, Cd206* and *Cd86*) and B: secretion of IL-10 from macrophages treated with ACM ± anti-Ad in the acute inflammation model. Macrophages were treated with CON or FO ACM (no LPS) for 18 h, followed by the addition of low-dose LPS (10 ng/mL) for the last 6 h (24 h total). 5 µg/mL of anti-Ad or IgG were added to ACM treatments prior to culture with macrophages. Macrophages treated with
media and 5 µg/mL IgG +LPS served as the control. Data shows the means ± SEM (n=5/diet/condition). Fold changes are expressed relative to macrophages treated with IgG alone (not shown). Bars not sharing a letter are significantly different (p≤0.05).

5.4.6 Lipid profile in macrophages treated with ACM in the chronic inflammation model

To examine if the Ad within ACM from CON or FO fed mice could affect macrophage phenotype in a chronic inflammation model, macrophages were treated with ACM collected from LPS-challenged visceral AT organ cultures or control media for 24 h with anti-Ad or control IgG. Additionally, within ACM samples, LPS was neutralized by polymyxin B (Px) to test if Ad worked synergistically with LPS. First, in macrophages treated with CON or FO ACM, we measured the relative abundance of neutral lipids after 24 h using oil red O staining and then quantified the percent of total n-3 and n-6 PUFA, MUFA and SFA relative to total fatty acid content (only showing those above 0.2% trace amounts of total lipid) (Figure 5.5). Further, macrophages treated with CON ACM had similar neutral lipid relative to control; however, neutral lipid increased when Px, anti-Ad or anti-Ad plus Px were added to CON ACM (p≤0.05, Figure 5.5). In contrast, macrophages treated with FO ACM only had elevated levels of neutral lipid compared to control when anti-Ad or anti-Ad plus Px were added to FO ACM (p≤0.05, Figure 5.5). Second, compared to macrophages treated with CON ACM, macrophages treated with FO ACM had increased total n-3 PUFA, n-6 PUFA and MUFA, while total SFA decreased (p≤0.05, Figure 5.5). More specifically, macrophages treated with FO ACM had increased n-3 PUFA, including docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3); increased n-6 PUFA, including linoleic acid (LA, 18:2 n-6) and arachidonic acid (AA, 20:4 n-6); and increased MUFA, including palmitoleic (16:1 c9), oleic (18:1 c9) and vaccenic (18:1 c7).
c11) acid (p≤0.05, Figure 5.5). With regards to SFAs, macrophages treated with FO ACM also had increased myristic acid (14:0), while stearic acid (18:0) was decreased (p≤0.05, Figure 5.5).
**Figure 5.5:** Neutral lipid uptake and total lipid profile in macrophages treated with ACM ± anti-Ad and Px in the chronic inflammation model. Macrophages were treated with ACM from LPS-challenged CON or FO AT organ cultures for 24 h. 5 µg/mL of anti-Ad (or IgG) ± polymyxin B (36 µM; Px) were added to ACM treatments prior to culture with macrophages. Macrophages treated with media and 5 µg/mL IgG plus 10 ng/mL LPS ± Px served as the control. Data shows the means ± SEM (n=5/diet/condition). Bars not sharing a letter are significantly different (p≤0.05). An asterisk indicates that the CON group is significantly different than the respective FO group (p≤0.05).

### 5.4.7 mRNA expression of M1 markers in macrophages treated with ACM in the chronic inflammation model

Treating macrophages with low dose LPS for 24 h (control) induced the expression of M1 markers relative to macrophages treated with just IgG (data not shown, p≤0.05), and adding Px to media with LPS suppressed this expression (p≤0.05, **Figure 5.6A**). First, *Itgam* (CD11b) and *Nos2* were similarly expressed in control macrophages and macrophages treated with CON ACM; however, mRNA expression of *Nos2* decreased in FO ACM treated macrophages (p≤0.05, **Figure 5.6A**). Interestingly, with anti-Ad was added, mRNA expression of *Itgam* in CON ACM increased above control levels (p≤0.05, **Figure 5.6A**). Similarly, with anti-Ad in FO ACM, expression of *Nos2* increased to be similar to the control group (p>0.05, **Figure 5.6A**). Second, adding Px to ACM decreased mRNA expression of *Itgam* and *Nos2* in each respective ACM group; however, when both anti-Ad and Px were added to ACM, mRNA expression of *Itgam* and *Nos2* increased compared to each respective ACM group with Px (p≤0.05, **Figure 5.6A**). Intriguingly, in the FO ACM treatment with anti-Ad and Px, mRNA expression of *Itgam*
increased above all other treatment groups (p≤0.05, Figure 5.6A). Finally, with regard to differences between CON and FO ACM groups, mRNA expression of Nos2 was decreased in the FO ACM group even with Px or anti-Ad (p≤0.05, Figure 5.6A).

Moreover, macrophages treated with CON ACM had decreased mRNA expression of Tnfa relative to control, although expression of Il6 was similar to control, and expression of Ccl2 was greater than control (p≤0.05, Figure 5.6B). In contrast, macrophages treated with FO ACM had decreased mRNA expression of Tnfa and Il6 relative to control (p≤0.05), and expression of Ccl2 was similar between these two groups (p>0.05, Figure 5.6B). Second, when anti-Ad was added to each respective ACM group, increased mRNA expression of Il6 and Ccl2 was observed in the FO ACM group only (p≤0.05, Figure 5.6B). Third, adding Px to ACM decreased the mRNA expression of Tnfa, Il6, and Ccl2 in each respective ACM group; however, when both anti-Ad and Px were added to ACM, mRNA expression of these cytokines significantly increased in each respective ACM group with Px (with the exception of Tnfa expression in the CON ACM group), (p≤0.05, Figure 5.6B). Finally, with regards to differences between CON and FO ACM groups, mRNA expression of Tnfa and Ccl2 decreased in the FO ACM group, even with Px added (p≤0.05); however, this relationship was lost with the addition of anti-Ad and anti-Ad plus Px to FO ACM (p>0.05, Figure 5.6B).

5.4.8 mRNA expression of NLRP3 inflammasome genes in macrophages treated with ACM in the chronic inflammation model

Treating macrophages with low dose LPS for 24 h (control) induced the expression of NLRP3 inflammasome genes relative to macrophages treated with just IgG (data not shown, p≤0.05), and adding Px to media with LPS (with the exception of Il18) suppressed this
expression (p≤0.05, Figure 5.6C). First, macrophages treated with CON ACM had increased mRNA expression of \(\text{Il18} \) relative to the control (p≤0.05), while expression of \(\text{Nlrp3} \) was less than the control, and expression of \(\text{Il1β} \) was similar to control (p>0.05, Figure 5.6C). In contrast, macrophages treated with FO ACM had decreased mRNA expression of \(\text{Nlrp3} \) and \(\text{Il1β} \) relative to the control (p≤0.05), and expression of \(\text{Il18} \) did not differ from control (p>0.05, Figure 5.6C). In each respective ACM group, adding anti-Ad resulted in increased mRNA expression of \(\text{Nlrp3} \) in both CON and FO ACM groups (p≤0.05, Figure 5.6C). Adding Px in each respective CON or FO ACM group decreased mRNA expression of \(\text{Il18} \) and \(\text{Il1β} \), although \(\text{Nlrp3} \) expression only decreased in the CON ACM group (p≤0.05, Figure 5.6C). Interestingly, adding anti-Ad and Px to each respective CON or FO ACM group compared to ACM with just Px increased mRNA expression of \(\text{Nlrp3} \), \(\text{Il18} \) and \(\text{Il1β} \) (p≤0.05, Figure 5.6C). Finally, with regards to differences between CON and FO ACM groups, mRNA expression of \(\text{Il18} \) and \(\text{Il1β} \) decreased with FO ACM, even with anti-Ad was added (p≤0.05); however, this relationship was lost when anti-Ad plus Px were added to FO ACM (p>0.05, Figure 5.6C).

5.4.9 mRNA expression of MHCII-related antigen presentation genes in macrophages treated with ACM in the chronic inflammation model

Treating macrophages with low dose LPS for 24 h (control) induced the expression of MHCII-related antigen presentation genes relative to macrophages treated with just IgG (data not shown, p≤0.05), and adding Px to media with LPS (with the exception of \(\text{Cd74} \)) suppressed this expression (p≤0.05, Figure 5.6D). First, macrophages treated with CON ACM had increased mRNA expression of \(\text{Cd80} \) relative to the LPS control (p≤0.05), while expression of \(\text{Cd74} \) was similar to control (p>0.05), and expression of \(\text{Cd86} \) was less than control (p≤0.05, Figure 5.6D).
Moreover, macrophages treated with FO ACM had similar levels of \( \text{Cd74} \) and \( \text{Cd80} \) expression as the control (p>0.05), although expression of \( \text{Cd86} \) was less than the control (p≤0.05, Figure 5.6D). Second, when anti-Ad was added, expression of MHCII-related genes only differed within each respective ACM group in one scenario; mRNA expression of \( \text{Cd80} \) increased in the FO ACM group (p≤0.05, Figure 5.6D). Third, adding Px to each respective ACM group decreased mRNA expression of \( \text{Cd80} \) and \( \text{Cd86} \) in both CON and FO ACM (p≤0.05). Interestingly, when anti-Ad plus Px were added to each respective ACM group, mRNA expression of \( \text{Cd74}, \text{Cd80}, \) and \( \text{Cd86} \) increased compared to ACM with Px or anti-Ad alone (with the exception of \( \text{Cd80} \) expression in CON ACM with anti-Ad relative to CON ACM with anti-Ad and Px), (p≤0.05, Figure 5.6D). Finally, with regards to differences between CON and FO ACM groups, FO ACM groups had decreased mRNA expression of \( \text{Cd74} \) (between Px groups only), \( \text{Cd80} \) (between IgG and anti-Ad groups only), and \( \text{Cd86} \) (between IgG groups only), (p≤0.05, Figure 5.6D).
Figure 5.6: mRNA expression of M1 markers (A: *Itgam* and *Nos2*), associated cytokines (B: *Tnfa, Il6* and *Ccl2*), NLRP3 inflammasome genes (C: *Nlrp3, Il18* and *Il1β*) and key MHCII-
related antigen presentation genes (D: Cd74, Cd80 and Cd86) from macrophages treated with ACM ± anti-Ad and Px in the chronic inflammation model. Macrophages were treated with ACM from LPS-challenged (10 ng/mL) CON or FO AT organ cultures for 24 h. 5 µg/mL of anti-Ad (or IgG) ± polymyxin B (36 µM; Px) were added to ACM treatments prior to culture with macrophages. Macrophages treated with media and 5 µg/mL IgG and 10 ng/mL LPS ± Px served as the control. Data shows the means ± SEM (n=5/diet/condition). Fold changes are expressed relative to macrophages treated with IgG alone (not shown). Bars not sharing a letter are significantly different (p≤0.05).

5.4.10 Secretion of cytokines in macrophages treated with ACM in the chronic inflammation model

First, secretion of all of the aforementioned cytokines increased in LPS-treated macrophages after 24 h compared to the IgG control (p≤0.05, data not shown). Second, adding Px to the LPS control decreased secretion of all of the aforementioned cytokines (p≤0.05, Figure 5.7). Third, relative to control macrophages, those treated with CON ACM had increased secretion of IL-6 and CCL2 (p≤0.05), while TNFα was similar (p>0.05), and secretion of IL-10 was less than the control (p≤0.05, Figure 5.7). Moreover, compared to control, in FO ACM, secretion of CCL2 was similar, IL-6 was greater, and TNFα and IL-10 were decreased (p≤0.05, Figure 5.7). Fourth, when anti-Ad was added to each respective ACM group, the secretion of CCL2 increased in both CON and FO ACM, while secretion of TNFα increased in the CON ACM group only (p≤0.05, Figure 5.7). Fifth, when Px was added to each respective ACM group, secretion of TNFα increased in the FO ACM group, while IL-10 secretion decreased in both the CON and FO ACM group (p≤0.05), and secretion of IL-6 and CCL2 did not change.
Sixth, with regards to differences between CON and FO ACM groups, FO ACM groups had decreased secretion of IL-6 and CCL2 even when Px (with the exception of CCL2), anti-Ad or anti-Ad plus Px were added to ACM (p≤0.05, Figure 5.7). Interestingly, when anti-Ad was added to ACM there was a decrease in secretion of TNFα and IL-10 in the FO ACM group compared to the CON ACM group (p≤0.05); however, this effect was lost for TNFα when anti-Ad plus Px were added to FO ACM (p>0.05, Figure 5.7).

**Figure 5.7:** Secretion of M1-associated cytokines (IL-6, CCL2 and TNFα) and anti-inflammatory cytokine, IL-10 from macrophages treated with ACM ± anti-Ad and Px in the chronic inflammation model. Macrophages were treated with ACM from LPS-challenged (10
ng/mL) CON or FO AT organ cultures for 24 h. 5 µg/mL of anti-Ad (or IgG) ± polymyxin B (36 µM; Px) were added to ACM treatments prior to culture with macrophages. Macrophages treated with media and 5 µg/mL IgG and 10 ng/mL LPS ± Px served as the control. Data shows the means ± SEM (n=5/diet/condition). Bars not sharing a letter are significantly different (p≤0.05).

5.5 Discussion

While it was known that LC n-3 PUFA modulate adipocyte-macrophage paracrine interactions (16, 177), as well as the AT secretory profile in obese mice (10), the effect of LC n-3 PUFA on the intact AT secretory profile and how this subsequently affects macrophage polarization status was not known. Thus, we generated visceral ACM from mice fed a CON (n-6 PUFA-rich) or FO (LC n-3 PUFA-rich) diet and then examined its effects on macrophage phenotype under different stimulation conditions designed to mimic aspects of the AT microenvironment. Specifically, we utilized two models: an acute inflammation model to assess if pre-treating macrophages with ACM could promote a response similar to endotoxin tolerance in macrophages when stimulated with low dose LPS and a low-grade chronic inflammation model wherein AT organ cultures were challenged with LPS to make ACM prior to incubation with macrophages to mimic the inflammatory state that occurs in parallel with metabolic endotoxemia (26, 39). Here we report for the first time that in the acute inflammation model, macrophages treated with FO ACM had decreased lipid uptake (Figure 5.2) and mRNA expression of M1-associated markers (Nos2, Nfkb, Il6, Il18, Ccl2 and Ccl5, Figure 5.3) compared with CON ACM; however, these effects were largely attenuated when Ad was neutralized, indicating that Ad is an important factor in LC n-3 PUFA-mediated effects. Further, intact AT from mice fed CON or FO diets differed in the secretory response to a low-grade LPS
challenge, as evidenced by decreased IL-6, CCL2, CCL5 and CCL7 secreted from FO ACM (Figure 5.1). Additionally, in the chronic inflammation model, compared to CON ACM, macrophages treated with FO ACM had decreased mRNA expression of M1-associated markers (Nos2, Tnfa, Ccl2, Il1β, Cd80 and Cd86, Figure 5.6) and IL-6 and CCL2 secretion (Figure 5.7); some of these effects were lost when Ad was neutralized, and were further exacerbated when both Ad and LPS were neutralized. Taken together, this work provides evidence that LC n-3 PUFA and Ad may work in concert to suppress specific M1 macrophage responses in a microenvironment representative of AT.

5.5.1 Ad partly mediates the anti-inflammatory effects of FO ACM on macrophages

Intriguingly, the results suggest that the anti-inflammatory effects of LC n-3 PUFA in macrophages may be partly due to Ad-mediated signaling, which, to the best of our knowledge, has not been previously reported. Although previous studies have shown that LC n-3 PUFA increase circulating Ad in rodents (60, 230) and humans (63, 268), the current study did not find any changes in secreted levels of full-length Ad in ACM due to diet or LPS exposure (Figure 5.1B). This may have occurred because the 24 h ex vivo period used to generate ACM was not long enough to induce changes in the levels of Ad secreted from the AT organ cultures; however, this requires further study. Nonetheless, some of the anti-inflammatory effects of FO ACM on macrophages in both the acute and chronic inflammation models were lost when Ad was neutralized. For example, in the acute model, lipid uptake increased back to control level in FO ACM-treated macrophages when Ad was neutralized in LPS-challenged cells (Figure 5.2). Decreasing lipid uptake may be a mechanism by which LC n-3 PUFA act to decrease excessive M1-like macrophage responses in obese AT since lipotoxicity in macrophages is thought to be a
key driver of M1 macrophage polarization (187). Secondly, in the chronic inflammation model, compared to LPS control, the FO ACM-mediated decrease in mRNA expression of M1-associated markers (Nos2, Ccl2, Nlrp3 and Cd80, Figure 5.6) and secretion of CCL2 (Figure 5.7) was lost when Ad was neutralized. Taken together, this data suggests that Ad plays a role in LC n-3 PUFA-mediated anti-inflammatory effects in macrophages, such as buffering lipid uptake and mRNA expression of specific markers associated with the M1 macrophage phenotype.

5.5.2 Ad works synergistically with LPS to decrease inflammatory signaling in macrophages in the chronic inflammation model

Another novel finding in the current study was our data showing that Ad may work synergistically with LPS to decrease macrophage mRNA expression of M1 markers, particularly in FO ACM-treated macrophages in the chronic inflammation model. Specifically, M1 markers (Itgam, Il6, Figure 5.6A and B), NLPR3 inflammasome genes (Nlrp3, Il18, Il1β, Figure 5.6C), MHCII-related antigen presentation genes (Cd74, Cd80, Cd86, Figure 5.6D), as well as secretion of IL-6 and TNFα (Figure 5.7), further increased compared to FO ACM with anti-Ad when both anti-Ad plus the LPS neutralizing agent, polymyxin B, were added. Along these lines, Park et al. (181, 182), showed that globular Ad initially triggers signaling cascades that result in activation of NFκB and subsequent TNFα mRNA expression; however, 5 h post-induction, this triggered a compensatory anti-inflammatory response resulting in IL-10 secretion and subsequent desensitization when re-stimulated with LPS. Thus, Ad may have immunoregulating properties in response to an inflammatory stimulus such as LPS, which warrants greater characterization,
particularly in FO ACM-treated macrophages where some of the anti-inflammatory effects of Ad were negated when both Ad and LPS were blocked.

5.5.3 Neutralizing Ad within ACM in the acute inflammation model decreased M1 and M2 macrophage marker expression

Interestingly, in the acute inflammation model, neutralizing Ad in both CON and FO ACM led to a decrease in mRNA expression of several M1-associated markers (\textit{Tnfa, Il6, Ccl5, Figure 5.3A}; NLRP3 inflammasome genes \textit{Nlrp3, Casp1, Iil18, Figure 5.3A}; secretion of IL-6 and CCL2, \textit{Figure 5.3B}), as well as a decrease in expression of M2 markers (\textit{Il10 and Cd206, Figure 5.4}). This data suggests that Ad promotes mRNA expression of both M1 and M2 genes in ACM which is in line with a previous report showing that M1 macrophages treated with Ad showed more robust inflammatory cytokine secretion (e.g. IL-6), and M2 polarized macrophages treated with Ad showed more robust IL-10 secretion (218). This relationship requires further study \textit{in vivo} especially given that a recent study in obese mice showed Ad accumulation in AT stroma vascular cells, including macrophages (163), further suggesting that Ad could potentially promote more robust M1 and M2 macrophage polarization in obese AT.

5.5.4 FO ACM had anti-inflammatory effects in both the acute and chronic inflammation models compared to CON ACM

Our findings that FO ACM had marked anti-inflammatory effects in macrophages compared to CON ACM in both the acute and chronic inflammation models are in agreement with other studies showing that LC n-3 PUFA decrease the degree of M1 macrophage polarization in the chronically inflamed obese AT microenvironment \textit{in vivo} (135, 156, 234, 235,
Notably, compared to CON ACM generated from AT organ cultures, FO ACM had decreased secreted protein levels of IL-6, CCL2, CCL5 and CCL7 (Figure 5.1). Second, compared to CON ACM, FO ACM decreased mRNA expression of M1-associated markers in macrophages including Nos2, Nfkβ, Il6, Il18, Ccl2 and Ccl5 in the acute model (Figure 5.3A). Third, compared to CON ACM, FO ACM decreased mRNA expression of M1 markers (Nos2, Tnfa, Ccl2, Il18, Il1β, Cd80 and Cd86, Figure 5.6) and secretion of IL-6 and CCL2 in the chronic inflammation model (Figure 5.7), and this occurred concurrently with changes in the macrophage lipid profile. Notably, macrophage LC n-3 PUFA such as DPA and DHA increased, while SFA, such as stearic acid, decreased (Figure 5.5). This data shows that LC n-3 PUFA are released from AT within ACM and that these fatty acids are subsequently taken up by macrophages. This is not surprising since n-3 PUFA-fed animals and humans exhibit increased AT n-3 PUFA storage and mobilization (reviewed by (188)). Additionally, TLR4 stimulation causes DHA and EPA pre-treated macrophages to secrete these PUFA as free fatty acids (170), which represents a potential mechanism by which LC n-3 PUFA distribute themselves among macrophages to mitigate excessive inflammatory responses. Furthermore, this change in macrophage lipid profile could partly explain some of the anti-inflammatory effects observed after treating macrophages with FO ACM. After LC n-3 PUFA are taken up by macrophages, the subsequent anti-inflammatory effects may be partly due to their incorporation into the phospholipid fraction of cellular membranes where they can act to decrease the signaling efficiency of protein complexes localized in small, hydrophobic membrane microdomains called lipid rafts (220, 240), including the TLR4 complex (264); however, this relationship requires further study in ACM models.
5.5.5 FO ACM mitigates expression of co-stimulatory molecule genes in macrophages without increasing IL-10 mRNA expression or secretion

Treating macrophages with FO ACM also led to changes in macrophage mRNA expression of MHCII-related antigen presentation genes. Specifically, compared to CON ACM, macrophages treated with FO ACM had decreased mRNA expression of co-stimulatory molecules, including \( \text{Cd86} \) in the acute inflammation model (Figure 5.4), and \( \text{Cd80} \) and \( \text{Cd86} \) (Figure 5.6D) in the chronic inflammation model. This data complements previous research showing that LC n-3 PUFA decrease antigen presenting cell-mediated co-stimulatory molecule mRNA and surface expression (200, 249). Moreover, globular (182, 284) and full-length (111, 115, 142, 263) Ad promote IL-10 secretion from macrophages. IL-10 represses co-stimulatory molecule expression on antigen presenting cells (78), suggesting that the Ad-IL-10 axis may affect antigen presenting cell functions. In this study, FO ACM-treated macrophages did not upregulate IL-10 mRNA expression or secretion, suggesting that the FO-mediated decrease in mRNA expression of \( \text{Cd80} \) and \( \text{Cd86} \) occurs independently of IL-10 in this model. Overall, this data provides preliminary evidence that FO may mitigate antigen presentation in macrophages (key antigen presenting cells in obese AT (34, 42, 159)) by decreasing co-stimulatory molecule expression; however, this relationship requires further study.

5.5.6 Summary

In summary, we showed for the first time that ACM promotes a response similar to endotoxin tolerance in macrophages and that macrophages treated with FO ACM had decreased lipid uptake and expression of markers associated with an inflammatory M1 phenotype compared to CON ACM in both the acute and chronic inflammation models. Importantly, the
anti-inflammatory effect of FO ACM on suppressing lipid uptake and M1 marker expression in macrophages was partly lost when Ad was neutralized in FO ACM. Overall, our data suggests that Ad and LC n-3 PUFA work together to affect the macrophage phenotype in a microenvironment representative of obese AT. This relationship warrants further study in vivo to further understand the role of dietary LC n-3 PUFA in mitigating obese AT inflammation and related pathologies.
Chapter 6: Integrative Discussion

The primary objective of this thesis was to evaluate if LC n-3 PUFA mitigate the inflammatory adipocyte-macrophage cross-talk that occurs in obese AT. Studies using murine obesity models have shown that dietary LC n-3 PUFA lead to decreased macrophage infiltration or the degree of inflammatory M1 macrophage polarization in AT (135, 156, 234); however, the mechanisms by which this occurs at the level of cellular cross-talk had not been explored prior to this thesis. Further, research has shown that PPARγ is essential for IL-4-mediated M2 macrophage polarization (174), but the role of PPARγ in acutely modulating adipocyte-macrophage cross-talk in the presence or absence of fatty acids, such as EPA, DHA and PA, had not been previously explored. Second, while dietary LC n-3 PUFA have been shown to decrease macrophage or monocyte responsiveness to LPS or other inflammatory stimuli in vitro and ex vivo (227, 276), it was previously unknown if LC n-3 PUFA-enriched macrophages from lean or obese mice would mitigate inflammatory adipocyte-macrophage cross-talk. Finally, although it has been previously shown that Ad can promote a response similar to endotoxin tolerance and M2 polarization in macrophages (142, 182, 238, 284), it was unknown if Ad or LC n-3 PUFA as part of the intact AT secretory milieu could promote a response similar to endotoxin tolerance and affect other features of the M1 ATM phenotype, such as lipid uptake ex vivo.

6.1 Summary of results

In Study 1, LC n-3 PUFA perturbed pro-inflammatory adipokine secretion resulting from adipocyte-macrophage cross-talk in an in vitro co-culture model designed to mimic the ratio of adipocytes:macrophages reported in obese AT, partially confirming the first hypothesis of this
thesis. However, the hypothesis was partly refuted given that the addition of a potent PPARγ antagonist, in combination with EPA or DHA, to the novel adipocyte-macrophage co-culture led to decreased adipocyte cellular Ad levels without affecting mRNA expression, cellular or secreted protein levels of other pro-inflammatory adipokines (e.g. IL-6, TNFα).

After confirming that LC n-3 PUFA could mitigate pro-inflammatory adipocyte-macrophage cross-talk, Study 2 confirmed the hypothesis that the anti-inflammatory effects of LC n-3 PUFA were reproducible in an in vitro co-culture model wherein splenic-derived macrophages were enriched with dietary LC n-3 PUFA from both LF and HF-fed mice. Building on Study 1 to identify pathways involved in perpetuating inflammatory cross-talk and M1-macrophage polarization, co-cultures with macrophages from HF-fed mice led to NLRP3 inflammasome activation as evidenced by cellular caspase-1 activity in adipocytes and macrophages; however, caspase-1 activity was largely attenuated in LC n-3 PUFA enriched co-cultures under LPS-stimulated conditions. Additionally, when Ad was neutralized in adipocyte-macrophage co-cultures there was a partial loss of LC n-3 PUFA-mediated suppression of NLRP3 inflammasome and M1 polarization marker gene expression and inflammatory cytokine and chemokine secretion, while cellular caspase-1 activity and IL-1β and IL-18 protein levels were decreased independently of Ad neutralization. Collectively, the results of Study 2 suggest that Ad-mediated signaling underlies some of the protective effects of LC n-3 PUFA in mitigating inflammatory adipocyte-macrophage cross-talk.

Finally, Study 3 aimed to delineate dietary LC n-3 PUFA and Ad-mediated effects on M1 macrophage responses using ex vivo models of acute and chronic inflammation. Here, RAW 264.7 murine macrophages were treated with ACM derived from mice fed LF diets with or without LC n-3 PUFA. When Ad was neutralized in ACM in the acute inflammation model,
contrary to the hypothesis, macrophages displayed blunted upregulation of M1 and M2 mRNA markers in response to LPS regardless of diet. However, the hypothesis was partly supported in the chronic inflammation model. Similar to findings in Study 2, neutralizing Ad partly reversed LC n-3 PUFA-mediated mitigation of key M1-macrophage polarization markers, NLRP3 inflammasome, as well as MHCII-related antigen presentation genes in macrophages, and surprisingly, this was further exacerbated when both Ad and LPS were neutralized. In contrast to Study 2, macrophages treated with ACM in either model did not show full NLRP3 inflammasome activation as evidenced by detectable levels of cellular caspase-1 activity or secreted IL-1β and IL-18. Finally, ACM from LC n-3 PUFA-fed mice prevented increases in macrophage lipid uptake with LPS stimulation, but this effect was lost when Ad was neutralized in the ACM. Overall, the data suggests that Ad and LPS may play a role in buffering macrophage lipid uptake and M1 marker expression in LC n-3 PUFA-enriched macrophages; however, these relationships requires further study.

6.2 The development and use of adipocyte and AT-macrophage cross-talk models

Given that AT is a key source of mediators that contribute to obese AT inflammation and are thought to play a role in the development of obesity-associated pathologies, novel immunomodulating strategies are needed. Importantly, LC n-3 PUFA are bio-active fatty acids found in fish, fish oil and numerous widely-available supplements that may be one such nutrition strategy. Supporting this concept, the data in this thesis showed that LC n-3 PUFA modify adipocyte-macrophage cross-talk, a key pro-inflammatory loop driving obesity-related pathologies. This was demonstrated in this thesis using three different co-culture models: 1. with LC n-3 PUFA (as albumin-complexed free fatty acids) added to in vitro adipocyte-macrophage
co-culture, 2. with dietary LC n-3 PUFA incorporated into splenic-derived macrophages and used in co-culture with 3T3-L1 adipocytes, and 3. with dietary LC n-3 PUFA enriched in AT-derived conditioned media and used to incubate RAW macrophages.

Co-culture models are an important tool to study the convergence between cell cross-talk, inflammatory signaling and subsequent changes in cell phenotype and function. The studies in this thesis have provided foundational work showing that several key aspects of the obese AT microenvironment (e.g. adipocyte:macrophage cell ratio, low-dose LPS, and reduced Ad signaling), can be recapitulated in vitro. Secondly, these models were useful to further understand key signals that drive inflammatory cross-talk (e.g. soluble-mediator or cell contact driven mechanisms) and, importantly, if dietary anti-inflammatory nutrients, like LC n-3 PUFA, could mitigate inflammatory signaling in both adipocytes and macrophages. Although this model allowed for the study of specific mechanisms (e.g. PPARγ and Ad- dependent anti-inflammatory effects of LC n-3 PUFA), it did not fully account for the plethora of signals that macrophages and adipocytes encounter in vivo. Thus, the ex vivo ACM model used in Study 3 was a useful tool to show that LC n-3 PUFA still decrease outcomes associated with M1 macrophage polarization in the complex ACM milieu that contains a plethora of secreted factors from whole AT.

Building on the findings in this thesis, future work using the various co-culture models established herein could expand on functional changes in both macrophages and adipocytes following co-culture to show concretely that LC n-3 PUFA-mediated mitigation of inflammatory signaling intermediates at the mRNA, cellular and secreted protein level translate to functional changes in cell phenotype. For example, it should be confirmed that LC n-3 PUFA can also mitigate macrophage polarization following co-culture through flow cytometry to show that
decreases in surface expression of M1-marker surface receptors, CD11b and CD11c, correlate with the decrease in M1-macrophage polarization mRNA markers and the associated secreted cytokine profile reported in this thesis. Additionally, Study 3 suggested that LC n-3 PUFA and Ad affect mRNA expression of co-stimulatory molecules (CD80/86) associated with antigen presentation in macrophages treated with FO ACM, which should also be confirmed with flow cytometry to show changes in the surface expression of MHCII and co-stimulatory molecules CD80/86 associated with antigen presentation to CD4+ T cells. Indeed recent research has shown that antigen presentation in obese AT drives AT inflammation (34, 159) and that blocking this through various knock-out models improves systemic insulin sensitivity in murine DIO models (29, 46). Thus, it will be extremely valuable to evaluate if LC n-3 PUFA and Ad affect adipocyte or macrophage surface markers of antigen presentation following co-culture, as well as examining antigen presentation \textit{in vivo} within fat-associated lymphoid tissue during the course of DIO.

### 6.3 Adiponectin as a modulator of macrophage phenotype

The data in this thesis advanced our understanding that LC n-3 PUFA may exert part of their immunomodulating effects through upregulation of Ad. Supporting this, it was found for the first time in Study 2 and 3 that LC n-3 PUFA mitigate inflammatory adipocyte-macrophage cross-talk, partly through an Ad-dependent mechanism. Moreover, this research supports the idea that Ad plays a prominent immunometabolic role. Of importance, this research agrees with other recent research suggesting that Ad can have both a pro-inflammatory role (32, 80, 218) (as seen in the acute inflammation model in Study 3) and an anti-inflammatory role (134, 143, 175, 238, 257, 269, 289) (as seen in Study 2 in contact co-cultures and in Study 3 in the chronic inflammation model), depending on the circumstance.
In cases of acute inflammation, Ad may help promote a more robust immune response. For example, in Study 3, using macrophages treated with ACM in the acute inflammation model, neutralizing Ad in ACM suppressed mRNA expression of M1 polarization markers and inflammatory mediator secretion. As an abundant circulating soluble mediator, it is tempting to speculate that Ad could be part of innate AT immune defenses. AT may need to have innate defense mechanisms since it is an abundant tissue that is susceptible to bacterial infection, e.g. *M. tuberculosis* can hide in AT (166), and the lipid-soluble bacterial endotoxin (e.g. LPS) can build up in obese AT (272). Moreover, Ad is part of the soluble defense collagen family with homology to complement protein C1q, suggesting that it may affect macrophage function similar to complement proteins, like C1q (17). Additionally, both macrophages (142) and activated T cells (257) express surface AdipoR1 and 2, which suggests that they are poised to respond to Ad. Supporting these ideas, Ad has LPS binding ability (reviewed by (51)), and Ad can help macrophages endocytose apoptotic cells through interaction with macrophage calreticulin receptors (226).

In contrast, in situations where chronic, low-grade inflammation persists, like in obese AT, Ad may have an anti-inflammatory, pro-resolving effect. Supporting this idea, in Study 2 in contact co-culture and in Study 3 where macrophages were treated with LPS pre-treated AT organ cultures, neutralizing Ad increased mRNA expression of key M1 polarization markers and associated inflammatory mediator secretion. Notably, Ad has been shown to re-direct macrophage polarization away from the inflammatory M1 phenotype through a variety of mechanisms. Specifically, the relationship between Ad and macrophage energy metabolism may be an unexplored mechanism underlying changes in macrophage response to Ad. Moreover, a relatively new field of immunometabolic research has shown that macrophage energy
metabolism can subsequently affect polarization towards the M1 or M2 phenotype. Of note, fatty acid oxidation is the key energy (ATP) source for M2 macrophages while M1 macrophages require glycolysis and the pentose phosphate pathway for energy and the associated intermediates produced needed for the production of rapidly dividing cells (similar to the Warberg effect describing cancer cell metabolism, reviewed by (253)). Supporting this, previous research in macrophages has shown that inhibition of fatty acid oxidation through peroxisome proliferator-activated receptor gamma, coactivator-1β knockout (243) or impairing AMPK activity (196) prevents M2 macrophage responses. Mechanistically, macrophage AMPK activity may preserve fatty acid oxidation, thereby preventing the build-up of ROS and reactive lipid species like ceramides that cause NLRP3 inflammasome activity and M1 macrophage polarization (252). Overall, it seems that fuel type (lipids vs glucose) and abundance may regulate macrophage responses. Thus, LC n-3 PUFA (273) and Ad (275)-mediated promotion of fatty acid oxidation through AMPK may be useful strategies, particularly in combination, to modulate macrophage metabolism and potentially inhibit excessive M1 macrophage polarization; however, this relationship requires further study.

6.4 Are changes in AT inflammation an underlying mechanism by which LC n-3 PUFA affect systemic insulin sensitivity?

Given the decrease in inflammatory adipokines secreted from various adipocyte-macrophage co-culture conditions reported in all three studies of this thesis, it may be reasonable to speculate that these changes would coincide with increased adipocyte insulin sensitivity following co-culture. While inflammation may initially be an adaptive response to promote macrophage-mediated angiogenesis and AT remodeling (278), persistent inflammation may
promote the development of local and peripheral insulin resistance. Interestingly, \textit{in vitro} studies have shown that adipocyte insulin sensitivity is perturbed by co-culture with macrophages or macrophage conditioned media (139, 177). Arguably, AT macrophages and the ensuing production of inflammatory mediators produced from adipocyte-macrophage cross-talk could be part of underlying mechanisms by which AT becomes insulin resistant and, in turn, contributes to the development of systemic insulin resistance. Supporting this idea, several inflammatory signaling pathways inhibit the insulin signaling cascade through similar mechanisms. Briefly, with prolonged cell stress (e.g. oxidative and ER stress) various stress kinases such as JNK, IKKβ and ERK are activated, which lead to the activation of various downstream inflammatory signaling intermediates, like NFκB and AP-1, and subsequent production of inflammatory cytokines (e.g. TNFα and IL-6, reviewed by (280)). Following cytokine secretion, subsequent cytokine-mediated signaling in cells (e.g. via TNFα receptor-mediated signaling) could further sustain the downstream activation of the aforementioned stress kinases and inflammatory signaling intermediates (e.g. NFκB). Notably, activation of JNK, IKKβ, and SOCS3 are all known to impair the activity of insulin receptor substrate-1 (IRS-1) (280), thereby potentially decreasing insulin-stimulated glucose uptake and inhibition of lipolysis in adipocytes. Interestingly, both LC n-3 PUFA (59, 247, 264) and Ad (92, 175) act to decrease oxidative stress in macrophages, which could underlie our observed reductions in cytokine secretion in co-cultures with LC n-3 PUFA, although this requires further investigation. Ultimately, future research using the co-culture models established in this thesis will include measurements of adipocyte insulin sensitivity to elucidate if LC n-3 PUFA or Ad (either alone or in combination) play a role in preventing the loss of adipocyte insulin sensitivity following co-culture, and will
continue to elucidate the underlying mechanisms (e.g. mitigating oxidative stress, etc.) by which this could occur.

Overall, it will be important to go beyond the *in vitro* models used in this thesis to next show that LC n-3 PUFA can mitigate AT inflammation and the development of systemic insulin resistance using *in vivo* obesity models. Indeed, very recent work from our lab has shown that mice fed the same HF-control diets as in Study 2 had impaired glucose clearance as measured by an oral glucose tolerance test; however, mice fed the HF diet with LC n-3 PUFA showed significant improvements in glucose clearance, indicative of improved insulin sensitivity (Monk *et al.* (292)). Interestingly, the changes in insulin sensitivity observed in mice fed the HF diet with LC n-3 PUFA occurred concurrently with significant decreases in total body and visceral AT depot weight, as well as decreased mRNA expression of the M1 marker, iNOS, in the stroma-vascular fraction of visceral depot AT cells (non-adipocyte, macrophage-enriched) compared to HF-control mice. Collectively, this data suggests that there is an association between systemic insulin sensitivity and underlying changes in visceral AT depot size and macrophage phenotype in LC n-3 PUFA fed obese mice; however, the specific relationships require further investigation.

### 6.5 Consumption of dietary LC n-3 PUFA to combat obesity and insulin resistance

One key advantage of studies performed in this thesis was that most of the doses of LC n-3 PUFA used were within physiological concentrations. The concentrations of fatty acids used in Study 1 for *in vitro* administration of EPA and DHA in co-culture were in line with human plasma concentrations (246). However, the concentration of PA used in Study 1 was below concentrations of 2.5 mM observed in human plasma (246), which may explain why PA did not
induce pro-inflammatory effects at a dose of 125 µM. Secondly, most dietary LC n-3 PUFA are found in the plasma membrane of cells rather than as free fatty acids where they are thought to have immunomodulating effects through incorporation into membrane lipid rafts and perturbation of downstream protein complex signaling (220, 240), as well as through affecting local eicosanoid production (70). Therefore, Study 2 and Study 3 used diets enriched with fish oil-derived LC n-3 PUFA in order to enrich LC n-3 PUFA in cells and tissues of interest.

Of note, the percent of LC n-3 PUFA used in the LF diet (Study 2 and 3) was approximately 1.7% kcal, which is in line with the human n-3 PUFA recommended intake of 0.5-2% kcal recommended by the Food and Agriculture Organization/World Health Organization (291). Moreover, the dose of LC n-3 PUFA in the HF diet (Study 2, 4.5% kcal) corresponds to an approximate intake of 9.4 g/day which has been used in some human clinical trials showing favorable outcomes on different inflammatory markers (reviewed by (190)). Interestingly, several recent human clinical trials have examined the relationship between n-3 PUFA intake and AT inflammatory markers in various obese clinical populations (non-diabetic/diabetic, overweight/obese) consuming different doses and forms of LC n-3 PUFA or placebo for 12-14 weeks. Some studies have shown significant improvements in the n-3 PUFA supplemented group with regards to AT inflammatory markers (93, 98, 212), or improved insulin sensitivity (93), while one did not show improvements in AT or systemic inflammation markers (114). Taken together, further large-scale and long-term (>12 week) clinical trials are needed to establish the relationship between LC n-3 PUFA consumption and AT inflammation, as well as if LC n-3 PUFA can be used to mitigate obesity-associated pathologies, like insulin resistance. Supporting this idea, in a meta-analysis examining the relationship between fish oil supplementation and insulin sensitivity in various clinical obese or diabetic populations, fish oil
was only associated with improved insulin sensitivity using the HOMA-IR model (4), a longer term measure of insulin sensitivity.

6.6 Concluding remarks

In conclusion, the research in this thesis has contributed to the establishment of models that can be used for furthering our understanding of the downstream complications arising from adipocyte-macrophage cross-talk that may be integral to the development of obesity-related pathologies, e.g. the relationship between cross-talk, oxidative stress and adipocyte insulin sensitivity, as well as adipocyte-macrophage interactions with T cells, particularly through antigen presentation. Together, the findings of this thesis suggest that LC n-3 PUFA mitigate inflammatory adipocyte-macrophage cross-talk partly through an Ad-mediated mechanism. Ultimately, this work strengthens our understanding of LC n-3 PUFA-driven immunomodulatory effects in obese AT, and provides support for the further development of dietary LC n-3 PUFA-mediated therapies for the treatment of obesity-induced inflammation and related pathologies.
References


12. Balvers MGJ, Verhoeckx KCM, Plastina P, Wortelboer HM, Meijerink J, Witkamp RF. Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to


### Appendix A

**Primer sequences**

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1 All primers were desalted and used at a final concentration of 10 µmol/L
Appendix B

Adipocyte cellular protein levels of adipokines in trans-well co-cultured 3T3-L1 adipocytes after 12 h co-culture

The cellular protein concentrations of key cytokines (IL-6, CCL2, TNFα and IL-10) measured from adipocytes in the trans-well system at 12 hr. 0 hr = serum starved adipocytes alone prior to co-culture and fatty acid treatment, (-) = negative control; adipocytes alone treated with 25 µM
BSA, (+) = positive control; co-cultured adipocytes and macrophages plus 25 µM BSA, DHA =
cocultured adipocytes and macrophages in the presence of 125 µM DHA, EPA= co-cultured
adipocytes and macrophages in the presence of 125 µM EPA, and PA= co-cultured adipocytes
and macrophages in the presence of 125 µM PA. Values are means ± SEM. The experiment was
independently conducted 2 times (in triplicate) for a final sample size of n= 6. A different letter
indicates treatments are significantly different from each other, \( p \leq 0.05 \).
## Appendix C

Diet composition of the low-fat CON and FO diet

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1 Table adapted from (141).
## Appendix D

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Appendix E

The purity of the splenic CD11b⁺ macrophage-enriched population

A representative histogram showing that the purity of the splenic CD11b⁺ macrophage-enriched population. The stained population is subdivided to distinguish FITC-CD11b⁺ cells (M2 = ≥90%) from cells not expressing this marker (M1).
Appendix F

Optimization experiment for the dose of anti-Ad to use

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**Itgam (CD11b)**

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**Il1b**

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**Cd80**

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**Cd86**

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RAW 264.7 macrophages were treated with adipocyte conditioned media (from 3T3-L1 adipocytes containing approximately 1.0 µg/mL full-length Ad) for 18 h. 10 ng/mL LPS from E. coli 055:B5 was added for the last 6 h for a total of 24 h. In some treatment groups 20 µg/mL or 40 µg/mL of anti-Ad was added to ACM prior to incubation with macrophages such that the ratio of µg anti-Ad: µg full-length Ad was approximately 20:1 and 40:1, respectively. Adipocyte conditioned media plus LPS with IgG served as the control. Bars show the mean fold change and SEM. n=2/treatment condition.

For experiments in Study 2 and 3, the dose of anti-Ad added to cultures such that the µg anti-Ad: µg full-length ratio was ≥20:1 since the 20:1 dose was just as effective as the 40:1 dose at neutralizing Ad and subsequently increasing mRNA expression of M1 markers (Tnfa and Itgam), NLRP3 inflammasome genes (Nlrp3 and Il1b) and co-stimulatory molecule genes (Cd80 and Cd86) relative to control.

Research by Ho et al. (83) suggests that this specific Ad-neutralizing antibody (R&D systems, AF1119) blunts down steam signaling of both full length and globular Ad which primarily signal through AdipoR2 and AdipoR1, respectively. Specifically, adding this antibody to 3T3-L1 adipocyte conditioned media decreased glucose update in C2C12 myocytes and increased glucose production in HepG2 hepatocytes, suggesting that the antibody blunts signaling through AdipoR1 (globular Ad) and AdipoR2 (full length Ad), respectively.
Appendix G

Chemokine secretion in the trans-well co-culture system with macrophages isolated from low-fat and high-fat-fed mice ± FO
Levels of CCL2 (MCP1), CCL7 (MCP3) and CCL5 (RANTES) in adipocytes co-cultured with macrophages isolated from low fat or high fat fed mice (LF-CON, HF-CON) with the addition of FO in some diets (LF-FO, HF-FO). All co-cultures were set up using 3T3-L1 adipocytes co-cultured with macrophages at an obese ratio of 17% macrophages to adipocytes where macrophages were placed in the upper chamber in trans-well inserts. The stimulation conditions were as follows. Unstimulated: co-culture conditions without additional inflammatory stimuli; acute inflammation: co-culture plus 10 ng/mL LPS for 24 h co-culture period; and chronic inflammation: co-culture conditions where adipocytes were pre-treated with 10 ng/mL LPS for 24 h prior to co-culture, then basic media for the 24 h co-culture period. Adipocytes alone under the aforementioned stimulation conditions served as the negative control. Data shows the means ± SEM for adipocyte controls (n=4/stimulation condition), LF-CON and FO (n=8/diet/co-culture condition), and HF-CON and FO (n=6/diet/co-culture condition). An asterisk signifies that a treatment group is significantly different than the respective adipocyte control (unstimulated, acute inflammation or chronic inflammation, p≤0.05).