Fish-Oil–Derived n–3 PUFAs Reduce Inflammatory and Chemotactic Adipokine-Mediated Cross-talk between Co-cultured Murine Splenic CD8+ T Cells and Adipocytes

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Abstract

Background: Obese adipose tissue (AT) inflammation is characterized by dysregulated adipokine production and immune cell accumulation. Cluster of differentiation (CD) 8+ T cell AT infiltration represents a critical step that precedes macrophage infiltration. n–3 (ω-3) Polyunsaturated fatty acids (PUFAs) exert anti-inflammatory effects in obese AT, thereby disrupting AT inflammatory paracrine signaling.

Objective: We assessed the effect of n–3 PUFAs on paracrine interactions between adipocytes and primary CD8+ T cells co-cultured at the cellular ratio observed in obese AT.

Methods: C57BL/6 mice were fed either a 3% menhaden fish-oil + 7% safflower oil (FO) diet (wt:wt) or an isocaloric 10% safflower oil (wt:wt) control (CON) for 3 wk, and splenic CD8+ T cells were isolated by positive selection (via magnetic microbeads) and co-cultured with 3T3-L1 adipocytes. Co-cultures were unstimulated (cells alone), T cell receptor stimulated, or lipopolysaccharide (LPS) stimulated for 24 h.

Results: In LPS-stimulated co-cultures, FO reduced secreted protein concentrations of interleukin (IL)-6 (242.6%), tumor necrosis factor (TNF)-α (267%), macrophage inflammatory protein (MIP) 1α (252%), MIP-1β (262%), monocyte chemotactic protein (MCP) 1 (223%), and MCP-3 (219%) vs. CON, which coincided with a 74% reduction in macrophage chemotaxis toward secreted chemotaxins in LPS-enriched co-culture-conditioned media. FO increased mRNA expression of the inflammatory signaling negative regulators monocyte chemoattractant 1–induced protein (Mcpip; +9.3-fold) and suppressor of cytokine signaling 3 (Socs3; +1.7-fold), whereas FO reduced activation of inflammatory transcription factors nuclear factor (NF)-κB (p65) and signal transducer and activator of transcription 3 (STAT3) by 27% and 33%, respectively. Finally, mRNA expression of the inflammasome components Caspase1 (236.4%), Nod-like receptor family pyrin domain containing 3 (Nlrp3; 299%), and Il1b (268.8%) were decreased by FO compared with CON (P ≤ 0.05).

Conclusion: FO exerted an anti-inflammatory and antichemotactic effect on the cross-talk between CD8+ T cells and adipocytes and has implications in mitigating macrophage-centered AT-driven components of the obese phenotype.

Keywords: n–3 polyunsaturated fatty acids, CD8+ T cells, adipocytes, inflammatory cytokines, chemokines, paracrine cross-talk, macrophage chemotaxis, obesity, adipose tissue, mice

Introduction

Obesity is associated with chronic low-grade visceral adipose tissue (AT) inflammation driven by changes in circulating endotoxin and infiltrating immune cell populations, leading to increased inflammatory adipokine [e.g., IL-6, TNF-α, monocyte chemotactic protein (MCP) 1/chemokine (C-C motif) ligand (CCL) 2] secretion from multiple cellular sources, which, in part, contribute to the development of insulin resistance (IR) (1–4). Therefore, understanding paracrine interactions between adipocytes and specific AT immune cell populations that produce these inflammatory mediators is of importance and represents a
potentially target for intervention to mitigate obesity-associated inflammation before the development of IR.

The stromal vascular fraction (SVF) of AT is composed of multiple cell types, including preadipocytes, stem cells, endothelial precursor cells, and immune cells (5). In particular, among AT-infiltrating immune cell populations, macrophages have taken center stage as a hallmark of the obese phenotype, wherein the percentage of obese AT SVF macrophages increases by 20–30% (6, 7). Furthermore, macrophages undergo a phenotypic switch from the M2 phenotype to the M1 inflammatory phenotype (8, 9), characterized by increased lipid content and secretion of proinflammatory cytokines, such as TNF-α and IL-6, which promote local adipocyte IR (9–13). Interestingly, long-chain n-3 PUFAs, which have established anti-inflammatory action and improve the obese phenotype (14–17), were shown to decrease AT M1 macrophage accumulation (6) and increase M2 polarization in AT, with concomitant decreases in serum glucose (8). Furthermore, we showed that n-3 PUFAs blunt paracrine interactions (or cross-talk) between adipocytes and macrophages co-cultured at an obese AT cellular ratio, resulting in reduced inflammatory mediator production and M1 polarization (18).

Despite the macrophage-centric focus of the contribution of AT immune cells to the obese phenotype, other AT immune populations change during obesity. The proportion and function of visceral AT regulatory T cells [cluster of differentiation (CD) 4+ forkhead box P3 (Foxp3)+] markedly decrease in insulin-resistant obese mice (6, 7, 19, 20), as do classically defined CD4+ T helper 2 cells (6). Conversely, select obese AT-infiltrating B cells (21, 22), NK T cells (23, 24), CD4+ T helper 1 cells (6, 25, 26), and CD8+ T cells (7, 27, 28) are reported to increase. Collectively, the combined changes in the profile of AT immune cell populations will direct the development of the obese phenotype; however, how immune cells interact with adipocytes to modify the inflammatory AT milieu remains poorly understood (29). Of particular interest are CD8+ T cells, which accumulate in AT in advance of macrophage accumulation in obese humans and rodents (7, 28, 30) and co-localize to crown-like structures, clusters of lymphoid and myeloid cells around adipocytes (7). Importantly, in vivo depletion of CD8+ T cells (via neutralizing antibody injections) reduces macrophage chemotactic signal expression and subsequent AT macrophage infiltration, tissue inflammation, and systemic IR (7). Moreover, CD8-deficient mice (CD8a−/−) are resistant to the development of the high-fat diet (HFD) obese phenotype (glucose intolerance, macrophage AT infiltration, crown-like structure formation, and IL-6 and TNF-α AT production); however, adoptive transfer of CD8a+ T cells into HFD-fed CD8a−/− mice reversed the phenotypic and induced IR, thereby demonstrating that CD8+ T cells play an essential role in the development of the obese phenotype (7).

Our objective was to establish an ex vivo co-culture system that recapitulates the cellular inflammatory microenvironment that is reflective of early changes in the development of the obese AT phenotype (i.e., before inflammatory M1 macrophage AT infiltration) and to determine if this inflammatory microenvironment could be modified by diet. Therefore, n-3 PUFA–enriched CD8+ T cells were co-cultured in direct contact with adipocytes ex vivo at a cellular ratio that mimics the degree of CD8+ T cell infiltration in obese AT (7), and changes in inflammatory cytokine and chemotactic mediator production were assessed in unstimulated, T cell receptor (TCR)-stimulated, and LPS-stimulated co-cultures.

**Methods**

**Animals and diet.** Mice were housed as described previously (31). Five-week-old male and female C57BL/6 mice from an in-house breeding colony were fed ad libitum either an AIN-93G modified diet containing 10% wt: wt safflower oil control (CON) or an isocaloric n–3 PUFA–enriched diet containing 3% wt:wt menhaden oil + 7% wt:wt safflower oil [fish oil (FO)] for 3–4 wk (n = 10 mice/diet) (Research Diets), wherein n–3 PUFA membrane enrichment in mononuclear cells has been shown to reach near-plateau levels (32–34). Diets contained 200 g/kg casein, 3 g/kg l-cysteine, 336.7 g/kg corn starch, 132 g/kg maltodextrin, 10, 100 g/kg sucrose, 50 g/kg cellulose, 0.02 g/kg t-butylhydroquinone, 35 g/kg mineral mix S10022G, 10 g/kg vitamin mix V10037, 2.5 g/kg choline bitartrate, and either 97 g/kg safflower oil (CON) or 67.9 g/kg safflower oil + 29.1 g/kg menhaden oil (FO). The FA composition of each diet was as reported elsewhere (31). All experimental procedures were approved by the University of Guelph Animal Care Committee.

**Blood and liver collection.** Mice were killed by carbon dioxide asphyxiation followed by cervical dislocation, and blood was collected by cardiac puncture as described (31). Livers were aseptically removed and snap-frozen in liquid nitrogen, and all tissue samples were stored at −80°C.

**FA analysis.** Total lipids were extracted from RBCs and liver as described previously (35). Nitrogen-flushed samples were stored at 4°C overnight, and the lipid-containing chloroform phase was processed as described (36). FAMEs were separated by GC by using an Agilent7890A gas chromatograph (Agilent Technologies) with a DB-FFAP fused-silica capillary column (15 m, 0.1-µm film thickness, 0.1-mm i.d.; Agilent Technologies), and FAs were identified by comparing the retention times of the samples with those of known standards (Nu-Chek-Prep) by using EZChrom Elite version 3.2.1 software (Agilent Technologies).

**Splenic CD8+ T cell isolation.** Spleens were removed aseptically, and CD8+ T cells were isolated by positive selection with the use of magnetic CD8a (Ly-2) microbeads according to the manufacturer’s instructions and maintained in basic media containing DMEM without sodium pyruvate (HyClone) plus 10% FBS (HyClone) plus 10% FBS (HyClone) and 0.1-µm film thickness, 0.1-mm i.d.; Agilent Technologies), and FAs were identified by comparing the retention times of the samples with those of known standards (Nu-Chek-Prep) by using EZChrom Elite version 3.2.1 software (Agilent Technologies).

**Adipocyte cell culture.** Mouse 3T3-L1 preadipocytes (American Type Culture Collection CL-173) were grown and passed according to the manufacturer’s instructions and maintained in basic media containing DMEM without sodium pyruvate (HyClone) plus 10% FBS (HyClone) and 0.1-µm film thickness, 0.1-mm i.d.; Agilent Technologies), and FAs were identified by comparing the retention times of the samples with those of known standards (Nu-Chek-Prep) by using EZChrom Elite version 3.2.1 software (Agilent Technologies).

**Co-culture stimulation conditions.** Adipocytes alone (negative control) or adipocytes and primary CD8+ T cells were co-cultured...
in 6-well plates for 24 h in basic media at a ratio of 10% CD8+ T cells to adipocytes, a physiologic ratio designed to mimic the degree of CD8+ T cell AT infiltration reported in obese mice (7). Cells were co-cultured under 3 stimulation conditions: (1) unstimulated (culture media alone), (2) TCR stimulation consisting of 5 μg/mL soluble anti-CD3 (clone: 145-2C11; eBioscience) and 20 μg/mL soluble anti-CD28 (clone 37.51; eBioscience) (37), or (3) 10 μg/L LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich), which reproduces the level of endotoxin units reported in obese humans and rodents (5-6 endotoxin units/mL) (1, 38, 39). After 24 h, RNA (isolated from co-cultured adipocytes and CD8+ T cells) and culture supernatant were collected for analysis.

**Gene expression analysis.** RNA was isolated by using the RNeasy Mini Kit (Qiagen) or the RNA/protein purification kit (Norgen Biotek), and cDNA was made from 2 μg of extracted RNA by using a high-capacity cDNA Reverse Transcription kit as per the manufacturer’s instructions (Applied Biosystems/Life Technologies). Real-time PCR analysis was performed as described (18), and all primer sequences are shown in **Supplemental Table 1**. All results were normalized to the expression of the housekeeping gene ribosomal protein, large, P0 (Rplp0), and the relative differences in gene expression between groups were determined by using the ΔΔCt method.

**Secreted protein analysis.** The concentrations of the secreted adipokines and chemokines IL-6, IL-10, IFN-γ, TNF-α, MCP-1 (CCL2), MCP-3 (CCL7), macrophage inflammatory protein (MIP) 1α (CCL3), MIP-1β (CCL4), MIP-2 (chemokine (C-X-C motif) ligand (CXCL) 2), and regulated upon activation, normal T cell expressed and secreted (RANTES; CCL5) were multiplexed by using the ProcartaPlex Mouse Basic Kit (eBioscience) and analyzed by using the Bio-Plex 200 System and accompanying software, Bio-Plex Manager, version 6.0 (Bio-Rad).

**Macrophage chemotaxis assay.** RAW264.7 macrophages (American Type Culture Collection TIB-71) were maintained as described (18) in basic media containing DMEM (described above). Cell viability, which exceeded 95%, was assessed before both and after the chemotaxis assay by trypan blue exclusion and counted in a hemocytometer in a blinded manner. Macrophage chemotaxis was measured by using culture supernatant (300 μL) from LPS-stimulated CD8+ T cell/adipocyte co-cultures and 2.5 × 10⁵ RAW264.7 macrophages added to a 96-well ChemoTx Chemotaxis System microplate (Neuro Probe). Migrated cells were counted in a blinded manner. Detailed methods are provided in the Supplemental Methods.

**Transcription factor activation.** Cellular protein was collected by using the RNA/protein purification kit (Norgen Biotek), and protease and phosphatase inhibitors (Roche Applied Sciences) were added to the lysis buffer before use at the recommended concentrations. Total cellular protein was quantified by using the bichinchonic assay (Fisher Scientific). An equal amount of protein (10 μg/sample per assay) was used to determine the activity of both NF-κB p65 and signal transducer and activator of transcription 3 (STAT3) by measuring the ratio of activated (i.e., phosphorylated) NF-κB p65 (Ser 536) to total NF-κB p65 and phosphorylated STAT3 (Tyr 705) to total STAT3 by ELISA as per the manufacturer’s instructions (eBioscience).

**Statistical analysis.** The SAS system for Windows (version 9.0) was used to analyze all data with the use of Student’s *t* test for direct comparison of 2 means or 2-factor ANOVA for multiple treatment groups (main effects: diet and stimulation condition) with an upper limit of significance at *P* ≤ 0.05. Least-squares means was used for post hoc comparisons, and data sets not exhibiting a normal distribution (determined by using the Shapiro-Wilk test for normality) were subjected to the Kruskal-Wallis test (χ² approximation) followed by Wilcoxon 2-sample testing.

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**Results**

**Body weights, food intakes, and FA profile.** Body weights and daily food intakes during the experimental period are shown in **Table 1**. Initial and final mouse body weights and food intakes did not differ between dietary groups (*P* > 0.05). RBC and liver FA analysis showed an enriched percentage of total n-3 PUFAs (18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3) in mice fed the FO diet compared with CON mice (*P* = 0.04 and *P* = 0.05, respectively; **Table 1**). Conversely, the percentage of total n-6 FAs (18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:5n-6) detected was significantly higher in both tissues isolated from CON mice vs. FO mice (**Table 1**). SFA and MUFA species were also assessed in both tissue sites but did not differ between dietary groups (results not shown).

**Unstimulated CD8+ T cell/adipocyte co-culture gene expression.** The mRNA expression of critical inflammatory mediators, negative regulators, and inflammasome-related genes was measured from adipocytes and CD8+ T cells co-cultured in direct contact at a physiologically relevant cellular percentage as reported in the visceral AT of obese mice (i.e., mimics the observed 10% CD8+ T cell infiltration into the obese AT SVF) (7). Gene expression (irrespective of cellular source) was analyzed to address our initial aim of determining whether the combined inflammatory and chemotactic milieu within the CD8+ T cell/adipocyte co-culture microenvironment was altered by T cell dietary manipulation/membrane n-3 PUFA enrichment. In unstimulated co-cultures (cells in direct contact but without TCR or LPS stimulation as described below), mRNA expression of *Il1b, Il6, Rantes,* and *Mcp1* were reduced in FO-containing co-cultures compared with CON (*P* ≤ 0.05). Interestingly, mRNA expression of the MCP-1 negative regulator, monocyte chemotactic protein 1–induced molecule (Mcpip) (40) was upregulated (+5.7-fold; *P* ≤ 0.05) in FO-containing co-cultures vs. CON. Inflammatory components (41, 42) *Caspase1* and Nod-like receptor family pyrin domain containing 3 (*Nlrp3*) were downregulated by −57.6% and −76%, respectively (*P* ≤ 0.05) by FO compared with CON. mRNA expression of tumor necrosis factor α (*Tnfa*), interferon-γ (*Ifng*), *Il2*, and suppressor of cytokine signaling 3 (*Socs3*) did not differ (*P* > 0.05; **Table 2**). *Il18* mRNA expression did not differ between diet groups and *Il10* mRNA expression was undetectable in both groups. These data show that unstimulated inflammatory

**Table 1** Body weights, food intake, and representative tissue FA composition in CON and FO-fed mice

<table>
<thead>
<tr>
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<th>CON</th>
<th>FO</th>
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<tr>
<td>Initial body weight, g</td>
<td>16.8 ± 0.76</td>
<td>17.5 ± 1.28</td>
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<tr>
<td>Final body weight, g</td>
<td>21.8 ± 1.29</td>
<td>24.9 ± 1.00</td>
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<tr>
<td>Food intake, g/d</td>
<td>2.69 ± 0.11</td>
<td>2.84 ± 0.26</td>
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<tr>
<td>RBCs</td>
<td></td>
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<tr>
<td>n-6 FAs, % of total</td>
<td>42.7 ± 2.88</td>
<td>28.2 ± 1.47*</td>
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<tr>
<td>n-3 FAs, % of total</td>
<td>1.26 ± 0.17</td>
<td>7.08 ± 0.66*</td>
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<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>n-6 FAs, % of total</td>
<td>43.4 ± 2.95</td>
<td>28.4 ± 2.17*</td>
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<tr>
<td>n-3 FAs, % of total</td>
<td>0.36 ± 0.03</td>
<td>5.62 ± 0.62*</td>
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* Values are means ± SEMs; *n* = 10/dietary group for body weight and food intake data, *n* = 5/dietary group for all GC data. *Different from CON, *P* ≤ 0.05. Liver and RBC total n-6 FAs (18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:5n-6) and total n-3 FAs (18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3) are expressed as a percentage of total FAs. CON, control diet; FO, fish-oil-enriched diet.

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**Table 2** FA composition of liver and RBCs in CON and FO-fed mice

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<th>CON</th>
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</tbody>
</table>

* Values are means ± SEMs; *n* = 10/dietary group for liver and RBCs. *Different from CON, *P* ≤ 0.05.
FO reduces TCR-stimulated CD8\(^+\) T cell/adipocyte co-culture gene expression. In TCR-stimulated CD8\(^+\) T cell/adipocyte co-cultures, mRNA expression of all genes, except for \(\text{Mip}1\alpha\), was significantly decreased by FO, whereas FO reduced only MCP-1 concentrations compared with CON. Within LPS-stimulated co-cultures, FO reduced gene expression of \(\text{Tnf}a\), \(\text{Il}6\), \(\text{Il}2\), and \(\text{Mcp}1\), the negative regulators of MCP-1 and IL-6, respectively (40, 49), were significantly increased by FO compared with CON (+9.3-fold and +1.7-fold, respectively; \(P \leq 0.05\)), whereas FO reduced \(\text{Nlrp}3\) and \(\text{Caspase}1\) gene expression (Table 2; \(P \leq 0.05\)).

Unstimulated CD8\(^+\) T cell/adipocyte co-culture secreted protein. Secreted protein concentrations in the culture supernatant of unstimulated 10% CD8\(^+\) T cell/adipocyte co-cultures are shown in Figure 1. IFN-\(\gamma\), IL-10, TNF-\(\alpha\), MIP-1\(\alpha\), MIP-1\(\beta\), and MIP-2 were undetectable. In the unstimulated condition (CD8\(^+\) T cells and adipocytes alone, no stimulus), co-culture increased the secretion of IL-6, RANTES, MCP-1, and MCP-3 compared with the negative control (adipocytes alone), but there was no effect of diet (CON vs. FO; \(P > 0.05\)).

FO reduces TCR-stimulated CD8\(^+\) T cell/adipocyte co-culture secreted protein. Independent of diet, TCR-stimulated CD8\(^+\) T cell/adipocyte co-cultures increased secreted protein concentrations compared with unstimulated co-cultures and the negative control (adipocytes + aCD3/CD28) (\(P \leq 0.05\); Figure 2). In 10% CD8\(^+\) T cell/adipocyte TCR-stimulated co-cultures, only MCP-1 concentrations were reduced by FO compared with CON, and MIP-2 and IL-10 were undetectable.

FO reduces LPS-stimulated CD8\(^+\) T cell/adipocyte co-culture secreted protein. Secreted protein concentrations of inflammatory mediators and chemokines from LPS-stimulated
10% CD8+ T cell/adipocyte co-cultures are shown in Figure 3. As expected, LPS stimulation increased secreted protein concentrations compared with both unstimulated CD8+ T cell/adipocyte co-cultures and the negative control (adipocytes + LPS) (P ≤ 0.05). At the relevant obese AT cellular ratio of 10% CD8+ T cells to adipocytes, LPS-stimulated FO-enriched co-cultures reduced secreted protein concentrations of TNF-α, IL-6, MCP-1, MCP-3, MIP-1α, and MIP-1β compared with CON (Figure 3; P ≤ 0.05). Although secretions of MIP-1α and MIP-1β were significantly reduced in FO-enriched co-cultures compared with CON (P ≤ 0.05), they were expressed at a concentration that was an order of magnitude lower than MCP-1 and MCP-3. MIP-2, IL-10, and IFN-γ were not detected. Collectively, these data demonstrate a reproducible and potent anti-inflammatory and antichemotactic effect of FO on the secretory profile produced by LPS-mediated CD8+ T cell/adipocyte paracrine interactions.

As proof-of-concept, we increased the number of CD8+ T cells co-cultured with adipocytes (co-cultured at 1:1) and demonstrated that the magnitude of the secretory profile could be increased but is still blunted in the FO-enriched co-cultures compared with CON (P ≤ 0.05) in the unstimulated and TCR- and LPS-stimulated co-culture conditions (Supplemental Figures 2–4, respectively).

**Reduced macrophage chemotaxis in FO-LPS-stimulated co-culture supernatant.** The ability of chemotactic signals secreted from LPS-stimulated CD8+ T cell/adipocyte co-cultures to subsequently influence RAW264.7 macrophage migration is shown in Figure 4A. Macrophage migration toward chemotactic signals in conditioned media from FO-enriched CD8+ T cell/adipocyte co-cultures was reduced by 74% compared with CON (P ≤ 0.05), thereby demonstrating a functional outcome of the FO-reduced chemotactic secretory protein profile shown in Figure 3.

**Reduced inflammatory transcription factor activation in FO-LPS-stimulated co-cultures.** Mechanistically, changes in the activation of 2 critical inflammatory transcription factors, NF-κB p65 and STAT3, could help to explain the potent anti-inflammatory and antichemotactic effect of FO in LPS-stimulated CD8+ T cell/adipocyte co-cultures. Therefore, we assessed the activation (i.e., ratio of phosphorylated to total) of both transcription factors in the cellular protein isolated from LPS-stimulated CD8+ T cell/adipocyte co-cultures. Compared with CON, FO-enrichment significantly reduced both NF-κB p65 and STAT3 activation by 27% and 33%, respectively (Figures 4B, C, respectively; P ≤ 0.05).

**Discussion**

We examined the effect of co-culturing dietary FO-enriched splenic CD8+ T cells with 3T3-L1 adipocytes in direct cell contact that mimics the physiologically relevant degree of 10% CD8+ T cell infiltration reported in AT of HFD-fed obese mice (7). The seminal work by Nishimura et al. (7) definitively demonstrated the critical role of AT CD8+ T cells in local inflammatory and chemotactic mediator production, which subsequently drives macrophage AT recruitment (i.e., chemotaxis) and ultimately the development of systemic IR. Our novel co-culture system provides a direct means to recapitulate the cellular microenvironment of obese AT ex vivo in order to study inflammatory paracrine interactions between CD8+ T cells and adipocytes, which are poorly understood.

The LPS-stimulated CD8+ T cell/adipocyte co-culture condition used a physiologically relevant obese LPS dosage (1, 38, 39) and therefore most accurately recapitulates the obese AT cellular inflammatory microenvironment (7). An anti-inflammatory effect of FO was apparent, as evidenced by reduced mRNA expression and secreted protein concentrations of TNF-α, IL-6, and MCP-1 (Table 2, Figure 3) and reduced activation of the inflammatory transcription factors NF-κB p65 and STAT3 (Figure 4B, C). Furthermore, in LPS-stimulated co-cultures, FO upregulated mRNA expression of the negative regulators of IL-6 and MCP-1 signaling, Socs3 and Mcp1ip, respectively (40, 49) (Table 2). This may represent putative mechanisms of FO-induced anti-inflammatory and suppressive function, although further study is required. In addition, FO enrichment exerted an antichemotactic effect, decreasing secreted protein concentrations of macrophage chemokines (MIP-1α, MIP-1β, MIP-2, MCP-1, and MCP-3) compared with CON (Figure 3). Collectively, the FO-induced anti-inflammatory and antichemotactic
phenotype was associated with a relevant functional outcome, wherein macrophage chemotaxis was reduced by 74% compared with CON (Figure 4A). These data demonstrate that FO-induced changes in paracrine interactions mediated by CD8+ T cells and adipocytes can influence downstream macrophage recruitment, a critical step that connects changes in the obese AT cellular composition to the development of IR. Moreover, we provide direct evidence that other immune cell/adipocyte paracrine interactions, in addition to those previously shown in macrophage/adipocyte co-cultures (18), are susceptible to positive modulation by n–3 PUFAs.

In all co-culture conditions, FO reduced Mcp1 and increased Mcpip gene expression (Table 2), which correlated with reduced MCP-1 secretion in both TCR- and LPS-stimulated co-culture conditions (Figures 2 and 3). MCP-IP functions to control inflammatory responses by inhibiting NF-κB activation via its de-ubiquitinase activity or by degradation of mRNA encoding NFκB–induced inflammatory cytokines via its RNase activity (50, 51). Interestingly, NF-κB p65 activation was reduced in LPS-stimulated FO-enriched CD8+ T cell/adipocyte co-cultures (Figure 4B). This highlights a reproducible and potent inhibitory effect of FO on CD8+ T cell/adipocyte-driven Mcp1 expression, which is increased in obesity (7, 52–57) and signals through the MCP-1–C-C chemokine receptor 2 (CCR2) axis and promotes IR development via promoting AT macrophage recruitment (54, 55, 57). AT expression of other macrophage chemotaxins is elevated in obese rodents and humans, including MIP-1α (7, 12, 53), MIP-1β (53, 56), MIP-2 (53), MCP-3 (7, 52, 53), and RANTES (7, 56, 58), which contribute to macrophage AT infiltration and subsequent AT inflammation. Given that the cellular source of AT-derived chemokines (MCP-1, MCP-3, and MIP-1α) is reported to be the adipocyte and not the cells

**FIGURE 2** Secreted protein concentrations in culture supernatant from TCR-stimulated (anti-CD3/anti-CD28) CD8+ T cell/adipocyte co-cultures after 24 h containing 10% CD8+ T cells isolated from CON and FO-fed mice (panels A and B differ in magnitudes of secretion). Values are means ± SEMs, n = 8 (diet/co-culture condition) or 4 (adipocytes + anti-CD3/anti-CD28). For each variable, means without a common letter differ, P ≤ 0.05. CD, cluster of differentiation; CON, control diet; FO, fish-oil–enriched diet; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; TCR, T cell receptor.

**FIGURE 3** Secreted protein concentrations in culture supernatant from LPS-stimulated CD8+ T cell/adipocyte co-cultures after 24 h containing 10% CD8+ T cells isolated from CON and FO-fed mice (panels A, B, and C differ in magnitudes of secretion). Values are means ± SEMs, n = 4 (negative control; adipocytes + LPS) and 8 (diet/co-culture condition). For each variable, means without a common letter differ, P ≤ 0.05. CD, cluster of differentiation; CON, control diet; FO, fish-oil–enriched diet; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted.
comprising the SVF (53), it is likely that the chemokine cellular source in our co-culture system is the adipocyte. Our data support the finding that CD8$^+$ T cell/adipocyte-mediated paracrine interactions drive chemotactic signal expression, thereby facilitating macrophage AT recruitment (7). We have shown for the first time that this underappreciated component of the obese phenotype, when recapitulated in a CD8$^+$ T cell/adipocyte ex vivo co-culture model, is mitigated by FO intervention given that the adipocyte secretory profile was modified in a manner consistent with reduced chemotactic signaling to macrophages. We think our findings point to a critical obese AT immunoregulatory break point that is susceptible to dietary n–3 PUFA intervention, although further study is required.

The NLRP3 inflammasome is a critical signaling complex that functions as a regulator of innate immunity and inflammation in metabolic diseases (41, 59, 60). It is composed of a danger-sensing intracellular sensor, usually from the family of Nod-like receptors, e.g., NLRP3, the cysteine protease caspase 1, and the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (61). Inflammasome signaling leads to caspase-1 activation and subsequent release of IL-1β and IL-18, which promote an inflammatory microenvironment in obese AT (62). The NLRP3 inflammasome also exerts regulatory effects on glucose and insulin homeostasis, outcomes that are improved in obesity with inflammasome inhibition (63–65). Moreover, n–3 PUFA-mediated inhibition of the NLRP3 inflammasome underlies their anti-inflammatory and glucose-sensitizing effects in obesity (42). Obese NLRP3 inflammasome activation causes visceral AT leukocytosis and obese Nlrp3$^{-/-}$ mice exhibit reduced visceral AT expression of inflammatory cytokines (TNFa) and chemokines involved in lymphocyte recruitment (CCL20 and CXCL1), thereby changing the visceral AT immune cell composition and resulting in reduced CD8$^+$ and CD4$^+$ T cell numbers (64). Collectively, these data demonstrate that approaches to reduce the number of obese AT CD8$^+$ T cells results in reduced inflammation and improved insulin sensitivity (7, 28, 64). Moreover, antagonism of NLRP3 inflammasome activation/signaling may attenuate obesity-associated CD8$^+$ T cell AT infiltration. In CD8$^+$ T cell/adipocyte co-cultures (regardless of the stimulus), Caspase1, Nlrp3, and Il1b mRNAs were consistently reduced by FO, whereas II18 expression was unaffected by diet (Table 2). These outcomes, in particular in the LPS-stimulated co-cultures, wherein toll-like receptor signaling is linked to inflammasome activation (66), are indicative of FO-mediated reduced inflammasome activation, although further studies to assess the relation between FO and inflammasome activation in our system are required.

One mechanism through which FO may exert its anti-inflammatory and antichemotactic effects in our system may be by reducing CD8$^+$ T cell activation, although sustained CD8$^+$ T cell responses have been reported (67). CD8$^+$ T cell purity exceeded 90%; however, CD8$^+$-expressing dendritic cells could be present in our co-cultures and subsequently influence the outcome through suppression of T cell activation. In support of reduced T cell activation, II2 mRNA expression was decreased in FO-enriched TCR-stimulated co-cultures (Table 2). The cell-contact-mediated mechanism of CD8$^+$ T cell/adipocyte interaction would be through CD8 co-receptor stabilized TCR–major histocompatibility complex (MHC) class I adhesion (68, 69). Interestingly, CD8 was shown to localize in lipid rafts, a process that is dependent on the palmitoylation of the CD8 chain (70–72). Lipid rafts are liquid-ordered regions of the plasma membrane enriched in cholesterol and sphingolipids (73, 74). These dynamic, tightly packed lipid-protein assemblies can be induced to cluster to form a stable TCR signaling platform by compartmentalizing plasma membrane proteins and lipids and promoting efficient and prolonged signaling (75, 76). However, n–3 PUFA membrane incorporation disrupts lipid rafts and perturbs downstream signaling in T cells (77, 78), thereby potentially disrupting CD8$^+$ T cell/adipocyte contact via CD8/
MHC class I interaction and downstream signal transduction. In addition, TLR4 and members of the TLR4 signaling complex localize to and are assembled in lipid rafts (79–82), indicating that LPS responsiveness and downstream signaling may be perturbed in FO-enriched CD8^+ T cell/adipocyte co-cultures in a lipid raft-dependent manner. Collectively, these data provide a rationale supporting a lipid raft-mediated mechanism that underlies the reduced inflammatory and chemotactic signaling in FO-enriched CD8^+ T cell/adipocyte co-cultures, although future studies are required.

Gene expression was measured in samples composed of both CD8^+ T cells and adipocytes, and although, logically, the majority of gene expression is adipocyte-derived, it is impossible to definitively make that conclusion. Although the inability to ascertain the cellular source of inflammatory mediator and chemokine mRNA and/or secreted protein expression is a limitation, our initial research question was to determine if the inflammatory and chemotactic milieu within the cellular microenvironment composed of adipocytes and CD8^+ T cells [which populate the obese AT before macrophage AT infiltration (7)] could be modulated by FO in our system. Future studies discerning the cellular source of these critical inflammatory and chemotactic mediators will be conducted. The current study used splenic-derived CD8^+ T cells, as opposed to AT-derived CD8^+ T cells, from HFD-fed mice and a mixed population of CD8^+ T cells with unknown activation status (naive vs. effector/memory cells), because ~75% of obese AT CD8^+ T cells exhibit an activated effector phenotype (CD44^+, CD62L^-) (7). Despite these translational limitations, we were still able to recapitulate the obese cellular microenvironment in our co-culture system.

A beneficial effect of n-3 PUFAs on the obese inflammatory phenotype is reported (14, 83–86), but controversy exists (87, 88). Our data uniquely show that the early (i.e., premacrophage AT infiltration) obese cellular microenvironment (composed of CD8^+ T cells and adipocytes at a physiologically relevant obese cellular ratio) can be recapitulated ex vivo in a direct cell-contact co-culture model. Furthermore, we provide the first evidence, to our knowledge, that FO-enrichment of the CD8^+ T cell compartment can drive subsequent CD8^+ T cell/adipocyte paracrine interactions (i.e., cross-talk) in a reproductible anti-inflammatory and anti-chemotactic direction that reduces subsequent macrophage chemotaxis, providing a basis for further studies assessing the mechanisms that underlie CD8^+ T cell/adipocyte/macrophage paracrine interactions. In this connection, increased FO intake may be a useful strategy to mitigate the severity and/or development of later macrophage-driven aspects of the obese phenotype.

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