Using Immune Cell/Adipocyte Co-Culture Models to Identify Inflammatory Paracrine Signaling Mechanisms: A Process Attenuated by Long-Chain N-3 Polyunsaturated Fatty Acids

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Abstract

Obese adipose tissue (AT) is characterized by increased recruitment and infiltration of multiple immune cell populations, in particular T cells (CD4$^+$ or CD8$^+$ subsets) and macrophages, that interact with adipocytes through paracrine signaling (i.e., cross-talk). Adipocyte/immune cell cross-talk results in increased inflammatory and chemoattractant mediator production that contributes to local (i.e., AT) and systemic metabolic dysfunction. Therefore, co-culture models of adipocytes and immune cell populations represent an important experimental approach to study how paracrine interactions between cell types promote obese AT inflammation and dysfunction, and to identify intervention strategies to attenuate this cellular cross-talk. In this commentary, we will discuss the development of physiologically relevant adipocyte (differentiated and mature 3T3-L1 pre-adipocyte cell line) and primary immune cell population (namely CD4$^+$ T cells, CD8$^+$ T cells and CD11b$^+$ macrophages) co-culture models that recapitulate the critical features of the obese AT microenvironment via i) culturing cellular ratios that reproduce the cellular abundance of immune cells observed in obese AT, and ii) stimulation with a concentration of lipopolysaccharide (LPS) that mimics circulating endotoxin levels in obese humans and rodents. The co-culture models discussed are comprised of i) a cell contact-dependent model wherein the cells are in direct physical contact, ii) a cell contact-independent model, wherein cells are physically separated by trans-well semi-permeable membrane that prevents physical cell contact but permits soluble mediators to cross, and iii) a cell contact-independent model where conditioned media is generated from intact primary AT, or adipocyte/immune cell co-cultures to influence another cell type. Finally, we summarize the utility of these co-culture models by discussing recent findings demonstrating how n-3 polyunsaturated fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] derived from fish oil can attenuate inflammatory and chemotactic paracrine signaling between adipocytes and immune cell populations to improve AT function.

Keywords: Co-culture, Paracrine interactions, Cross-talk, Adipose tissue, Adipocyte, CD4$^+$ and CD8$^+$ T cells, Macrophages, n-3 polyunsaturated fatty acids

Introduction

This invited Commentary is on the methods paper entitled "*Studying adipocyte and immune cell cross talk using a co-culture system*" in Immunometabolism: Methods and Protocols [1]. Co-culturing individual immune cell populations (as primary cells or cell lines) with adipocytes represents a model system to study the paracrine interactions (or cross-talk) between cell types that can impact adipose tissue (AT) function. This is particularly relevant in obese AT, wherein paracrine interactions between cell types promotes the secretion of inflammatory mediators that contribute to increased local (i.e. within the AT) and systemic low-grade inflammation and metabolic dysfunction, including insulin resistance (IR) [2-5].

AT is comprised of adipocytes and multiple immune cell types within the stromal vascular cellular fraction, as reviewed elsewhere [6]. As lean AT converts into obese AT during prolonged periods of overnutrition, there is a change in both the number and activity of immune cell populations. In lean AT, regulatory T cells [Tregs; CD4$^+$, forkhead box P3 (FOXP3$^+$)] and M2-polarized macrophages (F4/80$^+$, CD11b$^+$, CD11c$^-$) have been shown to contribute to the

maintenance of insulin sensitivity, in part, via secretion of anti-inflammatory mediators [3,5,7]. Conversely, in obese AT, decreases in Treg and M2 cellular abundance combined with increased immune cell recruitment and infiltration changes the cellular composition of the AT stromal vascular fraction and contributes to inflammation and metabolic dysfunction of the tissue [8-17]. Specifically, the increased abundance of immune cells within obese AT includes macrophages exhibiting polarization to the inflammatory M1 phenotype (F4/80^{+}, CD11b^{+}, CD11c^{+}) [8-13], CD4^{+} T cells [4,9,14,18], CD8^{+} T cells [4,9,14], natural killer (NK) cells [19-21], B cells [22,23] and dendritic cells [24,25]. Thus, understanding the influence of the paracrine interactions between immune cell populations and adipocytes in obese AT that underlie obesity-associated inflammation and metabolic dysfunction (both locally and systemically) will help elucidate appropriate intervention strategies to attenuate inflammatory mediator production and improve AT function.

Our research group [26-32] and others [33-36] have demonstrated the anti-inflammatory mechanisms through which long-chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (20:5:n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), serve as an intervention strategy to improve obesity-associated AT inflammation and metabolic dysfunction. As such, high fat (HF) diet supplementation with fish oil derived LC n-3 PUFA can attenuate the severity of AT and systemic inflammation and associated metabolic dysfunction [26], which can be attributed, at least in part, to the paracrine interactions (or cross-talk) between immune cell subsets and adipocytes, as reviewed elsewhere [2]. Since research on adipocyte – immune cell cross-talk has centred on macrophages and T cell subsets, these immune cell populations are the focus of this commentary. We will discuss the development of the adipocyte-immune cell co-culture models and highlight our research findings demonstrating the ability of n-3 PUFA to mitigate paracrine signaling between co-cultured adipocytes and T cells (CD8^{+} and CD4^{+}) or macrophages with an emphasis on the secretory profile (inflammatory and chemotactic mediators), however, it is worth noting that we have also shown a beneficial impact of n-3 PUFA on the NLRP3 inflammasome and/or macrophage M1/M2 polarization status in these models [29,31,32,37].
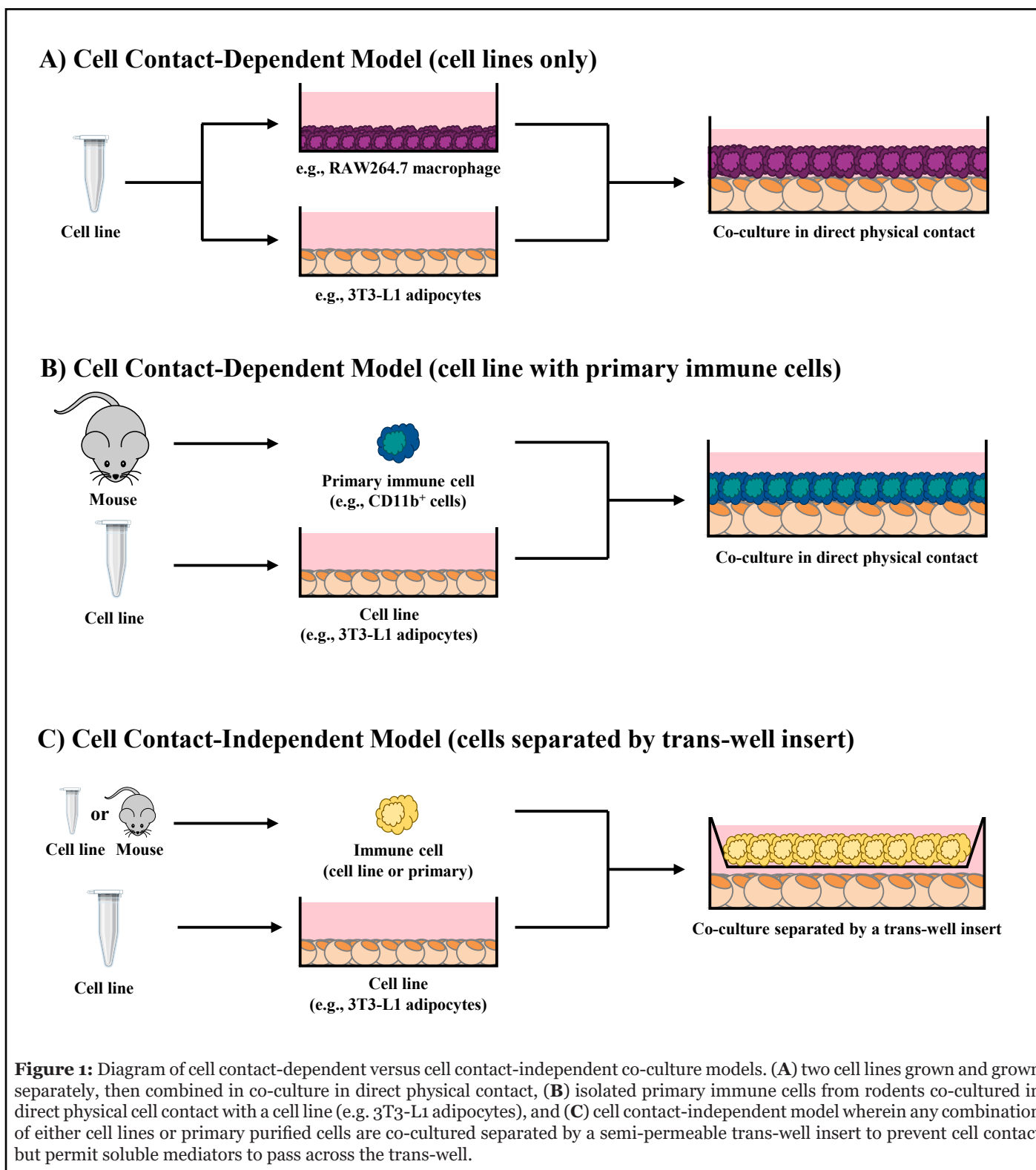
Adipocyte – Immune Cell Co-Culture Models

The adipocyte – immune cell (either macrophages, CD8^{+} or CD4^{+} T cells) co-culture model we developed [27-29,31,32,37-39], utilizes the murine 3T3-L1 pre-adipocyte cell line, which requires differentiation into lipid-laden mature adipocytes prior to co-culture with immune cells and provides a standardized component of the model between co-culture studies that is combined with varying immune cell populations [1]. To recapitulate a critical feature of the obese phenotype and provide an inflammatory stimulus, co-cultures are stimulated with a physiologically relevant dose of lipopolysaccharide (LPS, 10 ng/mL) derived from *Escherichia coli* serotype 055:B5, which reproduces the level of endotoxin units reported in obese humans and rodents (5-6 endotoxin units/mL) [40-42], that is not utilized in other adipocyte-immune cell co-culture models [43-47]. Moreover, we have conducted studies involving the pre-stimulation of adipocytes with LPS for 24 hr to recapitulate already inflamed AT prior to co-culture with either macrophages [27,28] or CD8^{+} T cells [31], as this would be the AT microenvironment that newly infiltrating immune cells would encounter *in vivo* [2,9]. Our initial co-culture work utilized the RAW264.7 macrophage cell line [38], which is commonly employed by other groups in adipocyte co-cultures treated with dietary bioactives [43-47]. More recently, we have utilized mouse primary splenic CD11b^{+} macrophages in co-culture with 3T3-L1 mature adipocytes [28], which increases the translational potential of this model compared to studies using primary splenocytes (comprised of multiple undefined cell types) [48], or immortalized cell lines (e.g. RAW264.7 macrophages) [43-47]. In this connection, our co-culture work with CD8^{+} and CD4^{+} T cells has exclusively utilized primary cells purified from the spleen of both lean and obese mice [29,31,32,37,39].
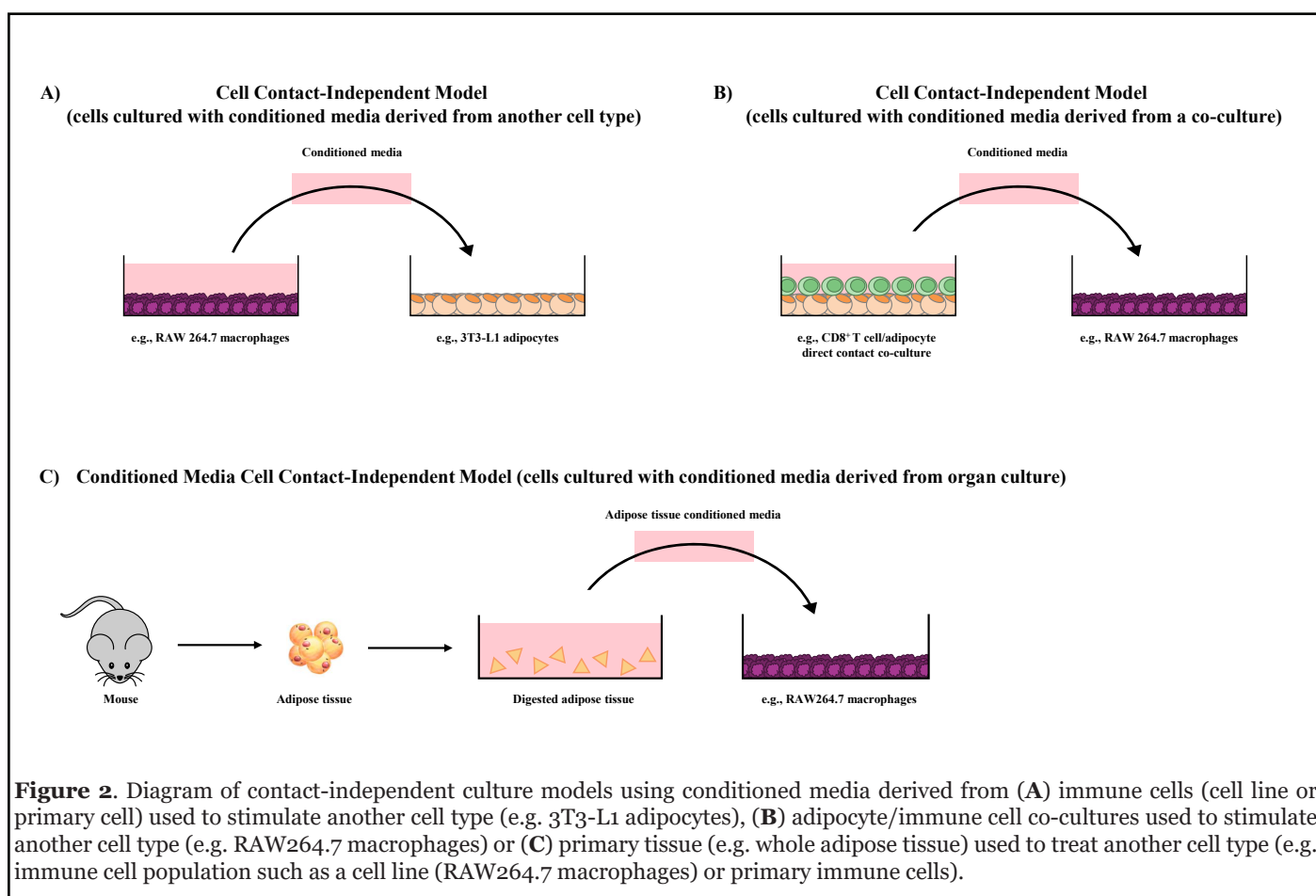
To further increase the translational relevance of the co-culture model, we have utilized a physiologically relevant cellular ratio of the immune cell population co-cultured with adipocytes to recapitulate their abundance within obese AT, which is in contrast to co-culture models utilizing equal numbers of adipocytes and macrophages (1:1 cellular ratio) [43-47]. In our model, macrophages are co-cultured with adipocytes at 17% of cells in culture [28,38], which is reflective of the level of macrophage cellular infiltration in epididymal AT of db/db mice and recapitulates the *in vivo* cellular ratio of macrophages:adipocytes [49]. Similarly, T cells are co-cultured with adipocytes to recapitulate the murine obese AT cellular ratio of adipocytes to T cells [9], with co-cultures comprised of 10% CD8^{+} T cells [31,37,39] and 5% CD4^{+} T cells [29,32].

Our research group has shown that cellular co-cultures can be established for 12 hr [38], 24 hr [28,31,32,37,39] or 48 hr [29] and can be utilized to discern the difference in outcomes resultant from i) direct cell contact (i.e., contact-dependent, Figure 1A and 1B), a combined outcome of both physical cellular interactions and paracrine signaling mechanisms, and ii) trans-well in direct cell contact (i.e., contact-independent, Figure 1C), wherein cells are separated by a semi-permeable 0.4 µM polyester membrane trans-well insert that precludes physical contact between cell types but permits the movement of

A) Cell Contact-Dependent Model (cell lines only)

Cell line

e.g., RAW264.7 macrophage

e.g., 3T3-L1 adipocytes

Co-culture in direct physical contact

B) Cell Contact-Dependent Model (cell line with primary immune cells)

Mouse

Cell line

Primary immune cell (e.g., CD11b+ cells)

Cell line (e.g., 3T3-L1 adipocytes)

Co-culture in direct physical contact

C) Cell Contact-Independent Model (cells separated by trans-well insert)

Cell line   **or**   Mouse

Cell line

Immune cell (cell line or primary)

Cell line (e.g., 3T3-L1 adipocytes)

Co-culture separated by a trans-well insert

Figure 1: Diagram of cell contact-dependent versus cell contact-independent co-culture models. (**A**) two cell lines grown and grown separately, then combined in co-culture in direct physical contact, (**B**) isolated primary immune cells from rodents co-cultured in direct physical cell contact with a cell line (e.g. 3T3-L1 adipocytes), and (**C**) cell contact-independent model wherein any combination of either cell lines or primary purified cells are co-cultured separated by a semi-permeable trans-well insert to prevent cell contact but permit soluble mediators to pass across the trans-well.

soluble mediators across the trans-well insert and the discernment of paracrine signaling effects [1]. To highlight a variation of the adipocyte/immune cell co-culture models, our research group has also utilized conditioned media (contact-independent) generated from i) one cultured cell line to another (Figure 2A), ii) adipocyte/CD8+ T cell co-culture conditioned media added to RAW264.7 macrophage cultures (Figure 2B) [31,37,39], and iii) intact primary AT conditioned media added to adipocyte/CD4+ T cell co-cultures [32] or macrophages [27] (Figure 2C).

Figure 2. Diagram of contact-independent culture models using conditioned media derived from (**A**) immune cells (cell line or primary cell) used to stimulate another cell type (e.g. 3T3-L1 adipocytes), (**B**) adipocyte/immune cell co-cultures used to stimulate another cell type (e.g. RAW264.7 macrophages) or (**C**) primary tissue (e.g. whole adipose tissue) used to treat another cell type (e.g. immune cell population such as a cell line (RAW264.7 macrophages) or primary immune cells).

CD8+ T cell and Adipocyte Co-Culture

CD8+ T cells have been shown to accumulate in obese AT prior to the accumulation of macrophages [9,50,51] and co-localize to crown-like structures in the AT [9]. *In vivo* depletion of CD8+ T cells via neutralizing antibody injections reduces the expression of macrophage chemotactic signals in AT and cellular trafficking, thereby reducing the magnitude of inflammation and systemic IR [9]. Similarly, the severity of the obese phenotype in CD8a-/- mice is attenuated but can be reversed through adoptive transfer of CD8+ T cells resulting in inflammatory mediator production and IR [9], thus, demonstrating the essential role of this cell type in the development and maintenance of obesity-associated AT dysfunction.

To highlight the utility of our co-culture models to study the paracrine signaling between adipocytes and immune cells, our initial contact-dependent co-culture studies used 3T3-L1 mature adipocytes cultured with purified splenic CD8+ T cells isolated from lean mice fed either a fish oil [37] or flaxseed oil [39] supplemented diet as the source of either marine- or plant-derived n-3 PUFA, respectively. Despite the difference in marine versus plant sources of n-3 PUFA that enriched the CD8+ T cells, in contact-dependent co-culture with adipocytes stimulated with LPS, the resultant anti-inflammatory and anti-chemotactic effect was consistent and characterized by reduced TNFα, IL-6, MCP-1, MCP-3 and MIP-1β secreted protein into the culture media compared to control [37,39]. Subsequently, the CD8+ T cell/adipocyte co-culture conditioned media was used in a follow-up experiment with RAW264.7 macrophages in a chemotaxis assay to demonstrate that the number of macrophages migrating towards the chemotactic signals in the conditioned media was reduced compared to control [37,39], indicating a functional outcome of reduced macrophage trafficking as a result of n-3 PUFA attenuating the paracrine interactions between CD8+ T cells and adipocytes. The anti-inflammatory and anti-macrophage chemotactic secretory profile of n-3 PUFA-enriched CD8+ T cells/adipocyte co-cultures was later confirmed in both the cell-contact dependent and cell contact-independent (trans-well) co-culture models, which was mechanistically attributed, in part, to the effects of TNFα [31]. The anti-inflammatory and anti-chemotactic secretory profile in n-3 PUFA co-cultures could be reproduced in control n-6 PUFA-enriched CD8+ T cell/ adipocytes co-cultures treated with a TNFα neutralizing antibody [31].

CD4$^+$ T cell and Adipocyte Co-Culture

CD4$^+$ T cells subsets have been shown to change in obese AT prior to the infiltration and accumulation of macrophages [14], wherein IFNγ-secreting Th1 cells have been shown to promote macrophage M1 polarization and contribute to AT metabolic dysfunction [14,18,50,52]. Additionally, IL-17-secreting Th17 cells have also been shown to contribute to obese AT inflammation [53].

In our co-culture model (using a physiologically relevant cellular ratio and LPS concentration) adipocytes were co-cultured in direct cell contact with primary splenic-derived purified CD4$^+$ T cells from lean mice fed isocaloric diets enriched with either n-3 or n-6 PUFA. Using this approach, n-3 PUFA increased mRNA expression and/or secreted protein of Th2 polarization markers (GATA3, IL-4) and reduced expression of Th1 polarization markers (Tbet, IFNγ), in addition to reducing the secretion of other inflammatory and macrophage chemotactic mediators (IL-1β, IL-6, MCP-1, MCP-3 and MIP-1α) [32]. These effects were reproduced in contact-dependent co-cultures containing adipocytes and splenic CD4$^+$ T cells from HF diet-induced obese mice consuming either an n-3 or n-6 PUFA-supplemented isocaloric diet (containing equal amounts of lard/saturated fatty acids) [29]. Collectively, our findings demonstrate the ability of adipocyte/CD4$^+$ T cell cross-talk to influence the local inflammatory and chemotactic microenvironment, which can impact subsequent macrophage chemotaxis and ultimately contribute to AT metabolic dysfunction. Future studies utilizing contact-independent conditioned media and the trans-well (cell contact-independent) models can help discern the inflammatory and chemotactic mediator cellular source to mechanistically identify the contribution of each cell type in co-culture to help direct targeted interventions to improve AT function.

Macrophages and Adipocyte Co-Culture

As obesity progresses, the infiltration of macrophages into the AT increases from 3% to approximately 20% of total non-adipocyte cells [54], wherein they exhibit an inflammatory M1 phenotype and increase inflammatory mediator production contributing substantially to obesity-associated inflammation and IR [2,9,12,14,55]. We [28,38] and others [43-47] have used 3T3-L1 adipocyte and RAW264.7 macrophage co-culture models [i.e., in cell contact-dependent and cell contact-independent (trans-well)] to study the paracrine signaling mechanisms that underlie AT dysfunction using different dietary bioactive interventions. Our initial co-culture work utilizing cell lines (3T3-L1 adipocytes and RAW264.7 macrophages) demonstrated that n-3 PUFA (EPA and DHA) could attenuate the intensity of the inflammatory cross-talk between cell types (e.g. reduced secretion of IL-6 and MCP-1) in both the cell contact-dependent and cell contact-independent (i.e., trans-well) co-culture models, which was associated with increased expression of non-inflammatory M2 macrophage markers [38]. Co-culture of 3T3-L1 adipocytes with primary splenic CD11b$^+$ macrophages from obese mice consuming a HF diet supplemented with or without n-3 PUFA confirmed the attenuated inflammatory mediator secretory profile and reduced expression of M1 macrophage markers in n-3 PUFA cultures [28]. This outcome was partially attributable to the effects of adiponectin [28], wherein n-3 PUFA have been shown to stimulate adiponectin secretion from adipocytes [56]. Finally, using a conditioned media contact-independent model in which RAW264.7 macrophage cultures were treated with conditioned media derived from intact primary AT from n-3 PUFA-fed mice, we demonstrated that macrophage-derived inflammatory and chemotactic mediator secretion (IL-6, MCP-1, MCP-3 and RANTES) and M1 macrophage markers were reduced, again in part, through an adiponectin-dependent mechanism [27].

Skeletal Muscle and Macrophage Co-Culture

Moving beyond co-culture studies focused on AT in obesity is relevant given that other tissues also contribute to systemic inflammation and whole-body IR in obesity, in part, through inflammatory immune cell-tissue cross-talk, thereby, providing other targets for the systemic impacts of n-3 PUFA on the obese phenotype [26]. Skeletal muscle is the primary site for insulin-simulated glucose uptake; however, in obesity, increased circulating free fatty acids and inflammatory cytokines interfere with insulin signaling in skeletal muscle to promote development of whole-body IR [57-59]. Similar to AT, obese skeletal muscle is characterized by the infiltration and accumulation of immune cells, particularly M1 macrophages, which contribute to local inflammation and IR through immune cell-myocyte cross-talk [60-63]. Therefore, our research group [64-66] and others [67-69], have utilized a contact-independent conditioned media co-culture model to examine the inflammatory macrophage-myocyte cross-talk that contributes to obese metabolic dysfunction. Importantly, attenuating macrophage-muscle inflammatory cross-talk represents another potential target for n-3 PUFA to improve obesity-associated insulin sensitivity. In this connection, RAW264.7 macrophages were stimulated with fatty acids [DHA (n-3 PUFA) versus palmitic acid (saturated fatty acid control)] and a physiologically relevant LPS dose (described above) to generate macrophage conditioned media (MCM), which contained secreted cytokines and chemokines that could impact muscle cell function in a cell contact-independent

manner. When MCM was collected and transferred to cultures of differentiated L6 myotubes we demonstrated that DHA-derived MCM improved insulin stimulated L6 myotube function by increasing the phosphorylation status of mediators in the insulin signaling cascade and subsequent glucose uptake [64]. A later co-culture study using MCM generated from RAW264.7 macrophages and L6 myotubes demonstrated that n-3 PUFA-mediated attenuation of inflammatory macrophage-myocyte cross-talk is attributable, in part, to a PPAR-γ-dependent mechanism [65].

Most recently, to increase the translational relevance of the macrophage-myocyte co-culture model to recapitulate more accurately the obese skeletal muscle microenvironment, rats were fed a HF diet enriched with either n-3 or n-6 PUFA and primary purified splenic CD11b+ macrophages were isolated and co-cultured in direct contact with L6 myotubes stimulated with LPS, wherein n-6 PUFA increased inflammatory cytokine production compared to n-3 PUFA co-cultures [66]. Subsequently, in a contact-independent experiment to determine the response of macrophages to mediators secreted from myocytes, purified CD11b+ cells from obese rats (consuming n-3 PUFA and n-6 PUFA-enriched HF diets) were cultured alone in conditioned media collected from LPS-stimulated L6 myocytes. This resulted in n-3 PUFA-enriched macrophages reducing expression of inflammatory cytokines and M1 polarization markers compared to n-6 PUFA [66]. Collectively, skeletal muscle cell (myotube)/macrophage co-culture models represent a relevant future direction to elucidate the underlying mechanisms contributing to obesity-associated IR.

Conclusion

Through the use of appropriately crafted adipocyte/immune cell (CD8+ and CD4+ T cell, macrophage) co-culture models [1], the critical features of obese AT can be recapitulated by including physiologically relevant cellular ratios of adipocytes to immune cell populations [9,49] and LPS stimulation conditions [40-42]. Using this approach, we have shown that n-3 PUFA can attenuate the severity of the inflammatory and chemotactic paracrine interactions between adipocytes and macrophages [27,28], CD8+ T cells [31,37,39] and CD4+ T cells [29,32], which collectively contribute to obese AT dysfunction [2]. Moreover, we have expanded this co-culture model to study paracrine interactions in another metabolically active tissue, namely skeletal muscle/immune cell cross-talk [64-66] to better understand the contribution of immune cells in various tissues towards the severity of the obese phenotype and identify immune-centric intervention strategies to improve obesity-associated metabolic dysfunction.

Conflicts of Interest

The authors state that there are no conflicts of interest.

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Author Contributions

J.M.M. – original draft preparation, concept development, review and editing; A.L.H – figures, review and editing; J.L.A.M. – review and editing; L.E.R. – concept development, review and editing.

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