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Crosstalk between adipocytes and immune cells in adipose tissue in an obese inflammatory state: role of contact-mediated signaling

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CROSSTALK BETWEEN ADIPOCYTES AND IMMUNE CELLS IN ADIPOSE TISSUE IN AN OBESE INFLAMMATORY STATE: ROLE OF CONTACT-MEDIATED SIGNALING

by

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DISSERTATION

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DEDICATION

I would like to dedicate this dissertation to my wonderful husband, Carlos Hiroshi Nitta, who is an indispensible part of my life. I appreciate all of the fruitful scientific conversations that encouraged me to strive for greatness. Thank you for your continued support and unlimited love.

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ABSTRACT

Obesity is defined as heightened fat accumulation leading to health impairments. It has been directly correlated to cardiovascular disease, type II diabetes mellitus, and cancer. Heightened cytokine levels are found in serum and adipose tissue of obese subjects, including TNFα (tumor necrosis factor alpha), IL-6 (interleukin-6), and MCP-1 (monocyte chemoattractant protein-1), being characterized as a chronic low-grade inflammatory disease. In this dissertation, I have generated a novel co-culture model between adipocytes and immune cells (derived from splenocytes) that mimics

inflammation seen in obese adipose tissue. This co-culture model allows for distinct evaluation between secreted paracrine factors (indirect cultures) and these factors plus direct cell contact. Paracrine signaling from both cell types increased the release of IL-6 and MCP-1, with a concomitant decrease of TNFα, whereas direct physical contact exacerbated the effects. The anti-inflammatory cytokine IL-10 (interleukin-10) did not play a role in the decreased secretion of TNFα. A time course study showed that direct and indirect co-cultures exhibited differential secretion rates, demonstrating cytokine-specific regulatory mechanisms. To determine specific cellular cytokine contributions, directly cultured cells were separated and analyzed showing both adipocytes and immune cells contribute significantly to inflammation. Adipocytes express MCP-1 and IL-6, whereas immune cells, TNFα and IL-6. Additionally, TNFα is necessary for this augmentation of IL-6 and MCP-1 secretion in direct contact. By use of non-toxic levels of signaling pathway inhibitors, I verified that the changes in cytokine secretions are mediated by NF-κB (nuclear factor kappa B) and MAPKs (mitogen-activated protein kinases). Specifically, NF-κB is the major signaling cascade for TNFα production, IL-6 is regulated by NF-κB, JNK (c-Jun N-terminal kinase), and p38, whereas MCP-1 by NF-κB, JNK, and MEK1. Analysis of the cell adhesion molecules on adipocytes identified 42 molecules. Direct contact with immune cells produced an up-regulation of Cadherin-1, claudin-4, -8, and -11, and down-regulation of Integrin alpha 6 in adipocytes. In conclusion, these results illustrate that direct contact and "crosstalk" between adipocytes and immune cells is paramount for exacerbation of inflammation in obesity. These changes are mediated by specific signaling cascades and cell adhesion molecules, which are important novel targets for this disease.

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36B4	60S acidic ribosomal protein PO
3T3-L1	Murine preadipocyte cell culture line
ACAM	Adipocyte Cell Adhesion Molecule
ACK	Ammonium-Chloride-Potassium
AP-1	Activator Protein-1
AT	Adipose Tissue
ATCC	American Type Culture Collection
Bay11	Bay11-7082
BMI	Body Mass Index
CAM	Cell Adhesion Molecule
CCL2	C-C Chemokine Ligand 2
CCR2	C-C Chemokine Receptor 2
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
Erk	Extracellular Signal-Related Kinase
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immune Sorbent Assay
FACS	Fluorescence Activated Cell Sorting

FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GLUT-4	Glucose Transporter type 4
HFD	High-fat Diet
IC_{50}	Inhibitory Concentration, 50%
ICAM-1	Intercellular Adhesion Molecule-1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IκB	Inhibitor of Nuclear Factor-Kappa B
IKK	Inhibitor of Nuclear Factor-Kappa B Kinase
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate-1
Itgα5	Integrin alpha 5
Itgα6	Integrin alpha 6
JAM	Junction Adhesion Molecule
JNK	c-Jun N-terminal Kinase
LD_{50}	Lethal Dose, 50%
LPS	Lipopolysaccharide
MAP2K	Mitogen-Activated Protein Kinase Kinase
MAP3K	Mitogen-Activated Protein Kinase Kinase Kinase
MAPK	Mitogen-Activated Protein Kinase
MAPKAP K2	MAPK-Activated Protein Kinase 2

MCP-1	Monocyte Chemoattractant Protein-1
MEK	Mitogen-Activated Protein Kinase Kinase
mRNA	Messenger Ribonucleic Acid
NCAM	Neural Cell Adhesion Molecule
NF-κB	Nuclear Factor-Kappa B
NKT	Natural Killer T cell
PI3K	Phosphatidyl-Inositol 3-Kinase
Ptprf	Protein Tyrosine Phosphatase, Receptor type, F
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RAW264.7	Murine macrophage cell line
RNA	Ribonucleic Acid
SGBS	Simpson–Golabi–Behmel syndrome preadipocyte cell line
Siglec1	Sialic acid binding Ig-like lectin
THP-1	Human monocytic cell line
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor alpha Receptor
VAP-1	Vascular Adhesion Protein-1
WHO	World Health Organization

CHAPTER 1

1. Introduction

1.1. Obesity

Obesity is defined as the excessive or abnormal accumulation of fat with detrimental health consequences (Garrow, 1988). According to the Centers for Disease Control and Prevention (CDC) more than one third of adults, over 72 million people, and 17% of children in the U.S.A. are obese (Flegal et al., 2010; Ogden et al., 2010). These rates have doubled among adults and tripled among children over the period of 1980-2008, demonstrating that this disease is rapidly approaching epidemic proportions. Obesity also has worldwide implications, and increasing body weight is encountered in many other countries (Caballero, 2007). In 2008, the World Health Organization (WHO) estimated that more than 1.4 billion adults were overweight (35% of the population) and 500 million, obese (11%). The body mass index (BMI) is the main tool used for determining if a person is overweight or obese. It is calculated by dividing body mass in kilograms by height in meters squared (kg/m^2). Overweight individuals have a BMI between 25 and 30, whereas obese individuals have a BMI of 30 or above. The major concern of obesity is the increased risk of other life-threatening health conditions, including cardiovascular diseases, type II diabetes mellitus, and cancer (endometrial, breast, and colon) (Bays et al., 2004; Grundy, 2002; Jensen, 2006; Renehan et al., 2008).

1.2. Inflammation in obesity

Adipose tissue (AT) is a dynamic organ and a tissue of great interest because it has now been established to play a role in the development of metabolic syndrome (Matsuzawa et al., 1999). The traditional view of AT focused on its function in fat storage and mobilization to accommodate energy needs and restore energy balance. It is now also known to be an important endocrine organ that plays a measurable role in regulating fatty acid metabolism and contributing greatly to an overall chronic inflammatory state, in obesity (Ferrante, 2007). Excessive adiposity leads to imbalances in fatty acid handling (Garrow, 1988) resulting in hyperlipidemia and elevated plasma cytokines, both of which contribute to the comorbidities of obesity.

Several recent investigations have shed light on inflammation in obesity. They collectively show that increases in adipose mass directly result in significant elevation in overall systemic inflammation, establishing obesity as a pro-inflammatory state (Fig. 1.1), contributing to the metabolic syndrome profile. A number of clinical observations have shown that the cytokines, TNFα (tumor necrosis factor alpha), IL-1β (interleukin-1β), and IL-6 (interleukin-6), are found within human arterial lesions (Tedgui and Mallat, 2006) and are significantly associated with increased cardiovascular risk and coronary artery disease (Harris et al., 1999; Libby and Ridker, 2004; Ridker et al., 2000a; Ridker et al., 2000b; Ridker et al., 2000c; Smith et al., 2004). Moreover, elevated cytokine levels in the circulation of obese individuals directly correlate with insulin resistance (Cottam et al., 2004; Grimble, 2002; Pickup and Crook, 1998). Direct administration of TNFα has been shown to specifically decrease insulin sensitivity and increase lipolysis in adipocytes (Hotamisligil et al., 1994; Zhang et al., 2002). Ultimately these studies

advance our knowledge regarding inflammation in obesity and its correlation with associated diseases.

1.2.1. Immune cell infiltration into adipose tissue

The inflammatory nature of AT in obese individuals is thought to occur as a result of "crosstalk" between infiltrating immune cells and lipid-laden adipocytes. Originally, only macrophages were thought to be the cause of the increased secretion of pro-inflammatory cytokines (Weisberg et al., 2003; Xu et al., 2003). Macrophages typically account for 5-10% of cells within AT obtained from non-obese donors whereas they can increase up to 40% in AT from individuals with diet-induced obesity (Weisberg et al., 2003). The recruitment of macrophages into obese AT has been linked to the AT-derived secretion of the chemokine MCP-1(monocyte chemoattractant protein-1, also known as CCL2, Chemokine (C-C motif) ligand 2) (Kamei et al., 2006; Kanda et al., 2006). More recently, further studies demonstrate that not only macrophages are increased in obese AT, but there is enhancement of other immune cells, such as T and B cells (Fig. 1.1) (Duffaut et al., 2009; Feuerer et al., 2009; McDonnell et al., 2012; Nishimura et al., 2009; Winer et al., 2011; Wu et al., 2007; Wu et al., 2012). The reason for this marked infiltration is currently unknown, although the effects these cells have on adipocyte physiology is just starting to be elucidated. Identifying the molecular details of immune cell-adipocyte "crosstalk" is pivotal for our understanding of abnormal fat handling and inflammation in obesity, as well as the development of rational intervention therapies for complications resulting from these abnormalities.

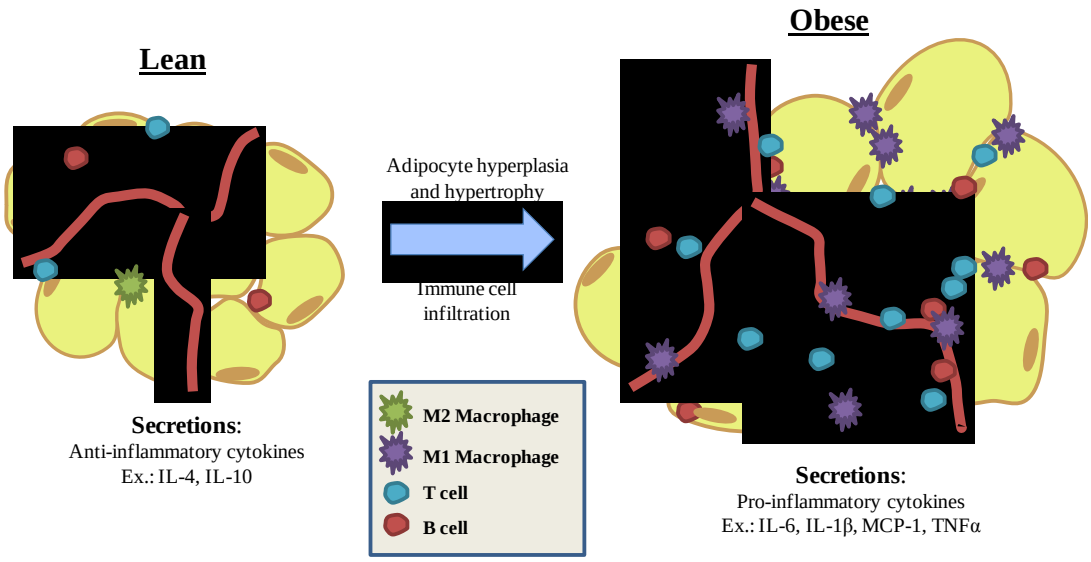

Figure 1.1 - Changes in adipose tissue structure, cell populations, and cytokine release in obesity. Lean adipose tissue is characterized by smaller adipocytes and residing immune cells (surveillance function). The macrophages here are the non-classical anti-inflammatory type M2 that secrete IL-10 and IL-4. With an overall increase in lipid load, adipocytes increase in number (hyperplasia) and size (hypertrophy), while immune cells infiltrate. Macrophages in obese AT are classically activated type M1. Secretions from obese AT are pro-inflammatory, such as IL-1, IL-6, MCP-1, and TNFα.

1.2.2. Inflammatory cytokines in obesity

Obese, hypertrophic AT produces several cytokines such as leptin, adiponectin, resistin, TNFα, IL-6, MCP-1, as well as others (Clement et al., 2004; Hotamisligil et al., 1993; Matsuzawa et al., 1999; Sartipy and Loskutoff, 2003; Takahashi et al., 2003). Many researchers have shown that this increase in cytokine production is due to the increase in immune cell infiltration (Fig. 1.1). Mutual cytokine expression by both immune cells and adipocytes in AT likely results in paracrine stimulation between them, aggravating the inflammatory changes seen in obesity (Suganami et al., 2005). I postulate that cell-cell contact between immune cells and adipocytes in obesity can mediate

"crosstalk" amongst them, leading to even more profound effects in cytokine expression than paracrine signaling alone.

Details below describe the importance of the specific cytokines, TNFα, IL-6, and MCP-1 in obesity.

1.2.2.1. TNFα

TNFα is a pro-inflammatory cytokine that can bind to two distinct cell surface receptors, which are ubiquitously expressed. Most of the effects in AT are mediated by its engagement to TNFR1 (TNFα receptor 1) (Uysal et al., 1998). It has a prominent role in obesity and is one of the major cytokines secreted in response to increased lipid load (Lumeng et al., 2007b; Ruan et al., 2002), together with IL-6. It has been shown to mediate insulin resistance in obesity (Hotamisligil et al., 1994) by directly decreasing insulin receptor phosphorylation, diminishing its activation, as well as phosphorylation of the serine residue S307 on IRS-1 (insulin receptor substrate-1), leading to its degradation (Hotamisligil et al., 1996). TNFα also influences insulin resistance indirectly through transcriptional events, mediated by a variety of downstream signaling events (Cawthorn and Sethi, 2008). Lack of TNFα by use of knock-out strategies results in a protective phenotype improving insulin sensitivity when fed a HFD (high-fat diet) (Uysal et al., 1997). TNFα can also exert pro-inflammatory effects by up-regulating the transcription of other cytokines, such as IL-6, as well as activate distinct signaling cascades, such as NF-κB (Nuclear Factor Kappa B) and the MAPKs (mitogen-activated protein kinases) (Jain et al., 1999; Ruan et al., 2002; Ryden et al., 2002).

1.2.2.2. IL-6

IL-6 is considered the second most important cytokine secreted in obesity, and its expression can be induced by TNFα treatment of adipocytes (Rotter et al., 2003; Yamashita et al., 2007). Prior studies, however, exhibit conflicting data regarding its role in inflammation. High systemic levels are encountered in obese and diabetic individuals (Kern et al., 2001), yet mice lacking IL-6 develop mature-onset obesity (Wallenius et al., 2002). It is found to be secreted in high levels in response to obesity, but at even higher levels in response to exercise (Febbraio and Pedersen, 2002; Steensberg et al., 2000), therefore appearing to exhibit pro-inflammatory and anti-inflammatory effects, dependent on stimulation, demonstrating different levels of regulation. Nonetheless, it has been shown to affect insulin sensitivity, although the exact mechanism by which this occurs has not been clearly elucidated (Rotter et al., 2003). Additionally, weight reduction in humans has been associated with a decrease in plasma IL-6 levels, reiterating its importance in obesity-driven inflammation (Bruun et al., 2003).

1.2.2.3. MCP-1

Monocyte chemoattractant protein-1 is a secreted chemokine responsible for recruitment of monocytes, neutrophils, and lymphocytes to sites of inflammation. It does so by directly binding to its receptor CCR2 (C-C chemokine receptor 2) present on recruited cells. Indeed, overexpression of MCP-1 in vessel walls leads to infiltration of macrophages and the generation of atherosclerosis (Namiki et al., 2002). Moreover, the importance of MCP-1 in recruiting immune cells into obese AT has generated conflicting results. Most researchers agree it is fundamental for macrophage recruitment into

expanding AT, leading to the generation of an inflammatory profile. Indeed, MCP-1 gene ablation in mice significantly reduces macrophage numbers in AT (Kanda et al., 2006) and a genetic deficiency in CCR2 is accompanied by a reduced inflammatory state as well as an improvement in insulin sensitivity in mice fed a HFD (Weisberg et al., 2006). Adipocyte-specific transgenic expression of MCP-1 increases recruitment of pro-inflammatory, classically activated macrophages (M1) into AT (Fig. 1.1) and potentiates insulin resistance (Kamei et al., 2006; Kanda et al., 2006). However, in contrast to these studies, two independent investigations report that MCP-$1^{-/-}$ mice on a HFD demonstrate no reduction in AT macrophage content (Inouye et al., 2007; Kirk et al., 2008). The controversy of these observations may be explained by the variations in the amount of time mice were fed a HFD, as well as the environment in which the animals were housed (pathogen-free or not). Mice maintained in a germ-free environment exhibit protection from obesity, even while consuming a HFD (Backhed et al., 2007).

1.2.2.4. Cell contributions to cytokine secretion in obesity

The cytokines that are specifically expressed by adipocytes or immune cells within AT continues to be a subject of controversy. Initially, since adipocytes were considered the predominant cell type in AT, they were thought to be the major source of inflammatory cytokines. Subsequently, inflammation in AT was linked directly to macrophage infiltration (Weisberg et al., 2003). Adipocyte cultures were incubated with macrophage-conditioned media resulting in increased mRNA expression and protein levels of inflammatory-related genes, including MCP-1 and IL-6 in adipocytes (Permana et al., 2006; Permana et al., 2009; Suganami et al., 2005). Reverse stimulation also occurs

wherein macrophages cultured with adipocyte-conditioned media increase their expression of IL-6 and TNFα (Suganami et al., 2005). These initial findings suggested that both cell types contribute to elevated cytokine expression by co-stimulating in a paracrine manner with secreted factors found in their respective culture media. More recently we learned that AT contains a variety of other cell types, a stromal vascular fraction including preadipocytes, endothelial cells, smooth muscle cells, fibroblasts, leukocytes, and macrophages, rendering the macrophage-adipocyte studies as merely a part of the puzzle. This diverse population of cells encountered in AT focused further studies to whole tissue, however led to mixed results regarding cytokine contributions. One study reports that the infiltrating macrophage population is responsible for almost all TNFα expression (Weisberg et al., 2003); whereas another suggests that the same cytokine is co-expressed by each cell type following macrophage infiltration (Suganami et al., 2005). Additional studies showed that adipocytes and macrophages both contribute to the expression of IL-6 (Samuvel et al., 2010; Weisberg et al., 2003), while others suggest it is mainly released by the stromal vascular fraction (Fain et al., 2003; Xie et al., 2010). The source of MCP-1 secretion in AT is also unknown (Bruun et al., 2005; Christiansen et al., 2005). Unfortunately the isolation procedure of primary adipocytes from AT can alter the cytokine profile of these cells, inducing inflammatory mediators and down-regulating adipocyte genes (Ruan et al., 2003), calling into question these whole tissue results with regards to cytokine contributions from each cell type.

1.2.3. Signaling in obesity

It has recently been postulated that immune cells and adipocytes "crosstalk" in AT by use of secreted paracrine factors, as well as direct cellular contact. Pro-inflammatory cytokine release in obese AT is a result of intracellular signaling in these cells. Previous studies regarding signaling in AT are able to give me insight as to which signaling cascades could potentially be important for adipocyte biology and inflammation (Gonzales and Orlando, 2008; Guha and Mackman, 2002; Hirosumi et al., 2002; Kotlyarov et al., 1999; Zhang et al., 2002). This allows me to determine which ones might play a role in this mediated "crosstalk". These major pathways include NF-κB, PI3K (phosphatidyl-inositol 3-kinase), and the MAPKs (Fig. 1.2).

1.2.3.1. NF-κB

NF-κB is a well-known transcription factor that is ubiquitously expressed, and plays a key role in metabolism and inflammation. In a non-stimulated state, NF-κB is localized to the cytoplasm, bound to the inhibitor of NF-κB (IκB) (Fig. 1.2C). The two major activators of NF-κB signaling include the binding of TNFα to its receptor (TNFR) and stimulation of toll-like receptor 4 (TLR4) via LPS (lipopolysaccharide) (Muzio et al., 2000). Both stimuli, present in obesity, result in activation of IKK (IκB kinase), which phosphorylates IκB, targeting it for ubiquitination and proteosomal degradation (Fig. 1.2C). This, in turn, releases NF-κB, which freely diffuses to the cell nucleus, leading to transcriptional activation of a variety of genes including cytokines, such as IL-1β, IL-6, and TNFα (Fig. 1.2C) (Solinas and Karin, 2010). This signaling pathway is active in the 3T3-L1 cell culture model, as previous work in our lab demonstrates that these cells

respond to TNFα stimulation through direct NF-κB stimulation of IL-1β, IL-6, and COX-2 (cyclooxygenase-2) (Gonzales and Orlando, 2008). Other cytokines, such as IL-1β are also capable of activating the NF-κB pathway (Martin and Wesche, 2002). Lack of the upstream activating kinase (IKK) of NF- κB protects mice from HFD-induced obesity, chronic inflammation, and insulin resistance (Chiang et al., 2009).

1.2.3.2. PI3K

One of the major hormones responsible for fat metabolism in adipocyte biology, insulin, mediates its effects through PI3K signaling. In functional (insulin sensitive) AT, insulin stimulates the phosphorylation of tyrosine residues of both the insulin receptor and IRS-1. This specific phosphorylation leads to downstream activation of PI3K signaling, and subsequent translocation of GLUT-4 (glucose transporter-4) to the cell membrane for glucose uptake (Fig. 1.2B) (Saltiel and Pessin, 2002). LPS binding to the TLR4 also results in PI3K activation (Guha and Mackman, 2002). Animals deficient in this signaling molecule exhibit impairment in immune cell migration confirming its role in inflammation (Hirsch et al., 2000).

1.2.3.3. MAPKs

Mammalian cells contain three mitogen-activated protein kinase pathways, JNK (c-Jun-N-terminal kinase), Erk (extracellular signal-related kinase), and p38 (Fig. 1.2A). TNFα and IL-1β can directly activate all MAPK pathways in adipocytes (Jager et al., 2007; Jain et al., 1999; Kumar et al., 2003; Martin and Wesche, 2002; Ryden et al., 2002). Signaling via these MAPK pathways is similar, in which a cascade of downstream

phosphorylation occurs culminating in activation of transcription factors. Sequentially, the MAP3K (mitogen-activated protein kinase kinase kinase) phosphorylates and activates its downstream molecule, the MAP2K (mitogen-activated protein kinase kinase), which in turn phosphorylates and activates the MAPK p38, JNK, or Erk. Each one of these signaling cascades contains their own specific kinases, as illustrated in Figure 1.2A.

JNK is considered the other major inflammatory signaling kinase, aside from NF-κB (Solinas and Karin, 2010), and is stimulated by stress signals. JNK activity is elevated in obesity, leading to insulin resistance (Sabio and Davis, 2010) by direct phosphorylation of IRS-1 (S307) in cellular models (Aguirre et al., 2000). The absence of this signaling pathway has been proven to exert protective effects regarding insulin sensitivity (Hirosumi et al., 2002). Additionally, signaling from JNK activates numerous proteins, including the transcription factor c-Jun, responsible for up-regulating the gene expression of inflammatory cytokines (Fig. 1.2A).

The signaling pathway MEK/Erk can be activated by several growth factors and cytokines, and is shown to be active in adipose tissue, liver, and muscles in obesity (Tanti and Jager, 2009). It also phosphorylates serine residues on IRS-1 resulting in insulin resistance (Tanti and Jager, 2009) and altered glucose transport (Jager et al., 2007). Its activation leads to up-regulation of the expression of TNFα and MCP-1 (Ito et al., 2007; Shi et al., 2002). Erk-1 deficient mice are protected from HFD induced obesity, and exhibit better glucose and insulin tolerance compared to their wild-type counterparts (Bost et al., 2005).

Stress signals such as cytokines and growth factors also activate the MAPK p38 (Fig. 1.2A). In adipocytes, stimulation by insulin results in p38 signaling (Sweeney et al., 1999). Its activation produces IL-1, IL-6, and TNFα, whereas its inhibition decreases their levels in human monocytes and macrophages (Kumar et al., 2003; Kumar et al., 1999). Because of its role in inflammation, p38 inhibitors have been used in clinical trials for rheumatoid arthritis and other inflammatory diseases (Kumar et al., 2003).

Of great interest to the understanding of inflammation in obesity is the interplay between cell signaling pathways (Gregor and Hotamisligil, 2011). Indeed, previous results show that the use of the MEK1 inhibitor PD98059 can block the translocation of NF-κB into the nucleus (Jain et al., 1999). Additionally, the NF-κB pathway can communicate with JNK (De Smaele et al., 2001; Jiao et al., 2012), which in turn can down-regulate PI3K (Aguirre et al., 2000; Lee et al., 2003). That signaling pathways can converge and communicate with each other is not a novel observation, however their relationship in obesity is not fully understood. Integration of these cascades and diverse cytokine stimulations will allow the identification of novel drug targets for reducing inflammation in obese adipose.

1.3. Cell Adhesion Molecules

The original concept of immune cell-adipocyte "crosstalk" was postulated from data showing that macrophage-conditioned media is able to induce a pro-inflammatory response in adipocytes (Permana et al., 2006; Permana et al., 2009). Direct cell contact between adipocytes and macrophages can elicit enhanced secretion of TNFα and MCP-1

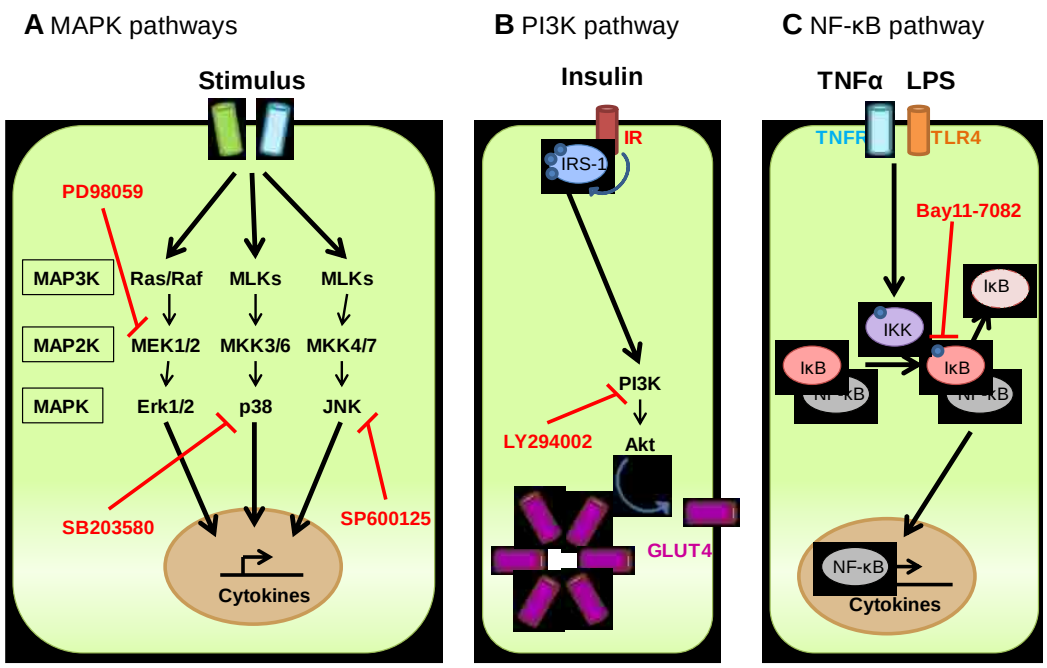

Figure 1.2 – Major signaling cascades in adipose tissue. (A) Mitogen-activated protein kinase (MAPK) pathways are activated by diverse stimuli, resulting in a cascade of downstream phosphorylation of signaling proteins. In MEK/Erk signaling the MAP3K (mitogen-activated protein kinase kinase kinase) Ras/Raf phosphorylates the MAP2K (mitogen-activated protein kinase kinase) MEK, which in turn phosphorylates and activates Erk. JNK and p38 activation initiate with MLK (mixed-lineage protein kinase) phsophorylation and subsequent MKK (MAP2K) activation. Specific chemical inhibitors of each of the MAPK pathways include PD98059, a MEK1 inhibitor; SB203580, which blocks p38; and SP600125, a JNK inhibitor. (B) PI3K singaling is activated by insulin stimulation of the insulin receptor (IR) in AT. This stimulus generates autophosphorylation of IR, recruiting and phosphorylating IRS-1 (insulin receptor substrate-1) tyrosine residues which activates PI3K and downstream Akt. This signal culminates with GLUT-4 (glucose transporter-4) translocation to the cell membrane for glucose uptake. The inhibitor LY294002 blocks PI3K activity. (C) NF-κB signaling is activated by stress signals, which include TNFα binding to its receptor (TNFR) and LPS stimulation of the Toll-like receptor 4 (TLR4), resulting in phosphorylation and activation of IKK (IκB kinase). IKK, in turn phosphorylates IκB (inhibitor of NF- κB) targeting it for degradation, allowing NF-κB to translocate into the nucleus. Bay11-7082 is a well established inhibitor of the phosphorylation of IκB, not allowing its dissociation from NF-κB.

(Lumeng et al., 2007a). Additionally, cell-cell contact, in conjunction with secreted cytokines, is a major contributing factor to the impairment of normal adipocyte function (Lumeng et al., 2007b). These data, along with the concept that immune cells need to infiltrate AT to elicit this inflammatory response suggests that these cell physically contact one another, and communicate by "crosstalk". In spite of this, there is no evidence describing which cell surface molecules on these cells could be responsible for this heightened inflammatory response when these cells are in contact. Adipocytes are typically globular structures containing a large fat droplet occupying a large area in the cell to such an extreme extent that it compresses the cytoplasm and nucleus up against the cell wall. Being that adipocytes are non-polarized cells and AT is a loose connective tissue, with few structural elements, it is not typically foreseen as a tissue requiring strong adhesion elements. Cell adhesion molecules (CAMs) are usually thought to be localized to cells that provide barrier functions, like epithelial and endothelial cells, however adipocytes seem to be engaging immune cells in such a manner leading to enhanced inflammation that one might question what could be on the surface of these cells. Unfortunately, little information is available as to the catalog of adhesion molecules expressed by adipocytes. Two studies make no direct correlation to obesity, but characterize a novel adipocyte cell adhesion molecule (ACAM) (Eguchi et al., 2005) and positively identify neural cell adhesion molecule (NCAM) (Yang et al., 2011) on the surface of these cells. Additional studies correlate specific CAMs (VAP-1, vascular adhesion protein-1; and ICAM-1, intercellular adhesion molecule-1) on adipocytes that are cleaved and enhance lymphocyte adhesion and extravasation into AT in a paracrine manner (Abella et al., 2004; Brake et al., 2006). To the best of my knowledge, only one

tight junction protein, claudin-6, is found to be expressed in adipose tissues and up-regulated in obesity (Hong et al., 2005), but its function is not elucidated. It is also not defined if this tight junction protein can engage other tight junction proteins on the surface of other cells. It is however clear that adipocytes most likely exhibit differential expression of adhesion molecules in non-obese compared to obese AT (Hong et al., 2005). Identification and characterization of CAMs on adipocytes will facilitate further studies in determining the importance of cellular "crosstalk" in obesity, and allow for novel target identification.

Rationale

Immune cells infiltrate obese AT and come into direct contact with resident adipocytes, leading to a heightened pro-inflammatory profile, ultimately causing cardiovascular disease, type II diabetes mellitus, and cancer. "Crosstalk" between these cells has been studied specifically among adipocytes and macrophages, failing to include other immune cells. We know that T and B cells are also extremely important in the exacerbation of inflammation in obesity. My goal is to elucidate the "crosstalk" between these cells and how it relates to cytokine release and signaling.

Hypothesis

Crosstalk established by direct contact between adipocytes and infiltrating immune cells in adipose tissue alters cytokine expression levels and/or patterns as a result of altered signaling pathways mediated through cellular adhesion molecules. This

crosstalk mechanism contributes to the development of a chronic, basal low-grade inflammatory response found in an obese state.

The specific aims to test this hypothesis are graphically depicted in Figure 1.3 and are as follows:

Project Aims

Aim 1: Determine if direct contact between adipocytes and immune cells affects the quantity and/or pattern of cytokine expression in these cell types.

Rationale: I chose a more physiologically relevant co-culture system, with lipid-laden differentiated 3T3-L1 cells, and a primary immune cell population derived from murine splenocytes. The spleen has been shown to be a reservoir of monocytes, T cells, and B cells (Avitsur et al., 2002; Swirski et al., 2009), representing the cell types encountered in obese adipose tissue. Cells are triggered to secrete varying amounts of cytokines, dependent on their stimuli, demonstrating different levels of regulation. I tested whether cells in my co-culture experiments can be distinctively activated dependent on solely paracrine factors or these factors in conjunction with cell contact (Fig. 1.3). To test the hypothesis I performed direct and indirect co-cultures of adipocytes and splenocytes and a time course study to determine the rates and levels of cytokine secretion.

Results: Chapter 2.

Aim 2: Identify the specific cytokines secreted by immune cells and adipocytes and determine the importance of TNFα in cellular crosstalk in AT.

Rationale: There is currently conflicting information regarding which cell types, adipocytes or immune cells, are responsible for expression and secretion of the pro-inflammatory cytokines in obese AT. Since many studies utilize procedures that have profound effects on cytokine secretion, I sought to determine each cell type contribution by developing a direct contact cell culture model (mimicking the obese phenotype) that does not significantly alter cytokine profiles. To do so, splenocytes derived from mice that ubiquitously express GFP (green fluorescent protein) were co-cultured with adipocytes and subsequently sorted by FACS (fluorescent-activated cell sorting), and their cytokine mRNA levels measured.

Of the cytokines secreted by either cell, TNFα is considered the major one secreted by AT in an obese setting. I wanted to determine its role in potentially enhancing the inflammatory profile of adipocyte and splenocytes (Fig. 1.3). Since my co-culture system allows for utilization of splenocytes from any genetically modified mouse, I was able to use splenocytes from mice that lacked TNFα as a tool to determine changes in IL-6 and MCP-1 secretion in direct and indirect co-cultures.

Results: Chapter 2

Aim 3: Identify the cell surface and/or adhesion molecules on adipocytes that are responsible for enhancing the obligatory cell-contact inflammatory crosstalk, as well as determine the signaling cascade(s) responsible for this differential cytokine secretion.

Rationale: Based on the differential cytokine secretion due to cell-cell contact, adhesion molecules on the surface of these cells are postulated to be responsible for this

phenomenon. To determine which molecules could potentially play a role in the contact-mediated effects on cytokine expression I compared adipocytes in direct contact with immune cells to ones co-cultured in the absence of contact. The identity of the cell adhesion molecules on adipocytes was determined by microarray analysis. These results also determined the major up- and down-regulated molecules on adipocytes when cells were in contact. Identification of these surface molecules is a fundamental first-step for understanding the "crosstalk" between these cells resulting in the changes seen in cytokine secretion in obesity (Fig. 1.3). The engagement of these adhesion molecules likely results in downstream signaling cascades. By using inhibitors for specific signaling pathways I can determine which ones are important for the heightened inflammatory profile seen in obesity.

Results: Chapter 3 (signaling) and Chapter 4 (adhesion molecules)

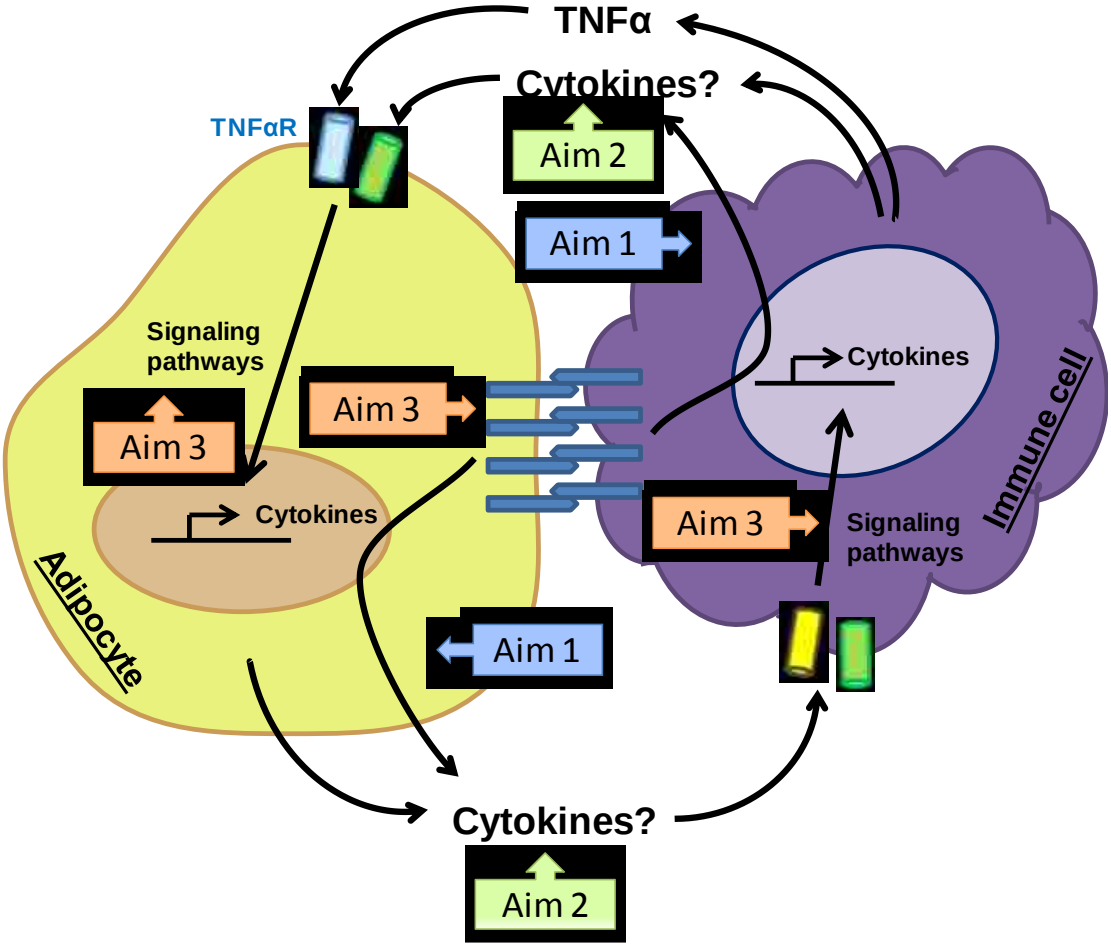

Figure 1.3 – Central hypothesis of crosstalk between adipocytes and immune cells. In obesity there is increased infiltration of immune cells into AT, allowing contact between these cells with resident adipocytes. Aim 1 determines if contact between these cells can alter their cytokine secretions, as well as how rapid and robust the response is. Aim 2 focuses on the distinct cytokines being expressed by each cell type following contact, as well as the importance of TNFα on enhancement of cytokine secretion. Aim 3 addresses the downstream signaling cascades important for cytokine secretion of adipocytes and immune cells, as well as determine which adhesion molecules are present on the surface of adipocytes that could be mediating these changes in cytokine secretion.

CHAPTER 2

Data contained within this chapter has been submitted to *PLOS One.*

Authors include: Carolina Franco Nitta and Robert A. Orlando

2. Crosstalk between immune cells and adipocytes requires both paracrine factors and cell contact to modify cytokine expression

2.1. Introduction

Obesity has reached epidemic proportions around the world and is now known to significantly contribute to the onset of atherosclerotic and hypertensive cardiovascular diseases, as well as type II diabetes mellitus (Garrow, 1988). Increases in adipose mass during the development of obesity are a result of increased adipocyte cell number, derived from pre-adipocyte stem cells, and increased cell mass (hypertrophy) resulting from excessive storage of dietary and endogenously synthesized fatty acids. Besides adipocytes, adipose tissue contains other cell types that participate in adipose function, including endothelial cells that supply proper oxygenation and nutrient delivery, fibroblasts that contribute to interstitial matrix deposition, and resident macrophages that provide an immunologic surveillance function.

Curiously, with the onset of obesity the cell type profile within growing adipose changes due to a substantial infiltration of inflammatory macrophages (Weisberg et al., 2003; Xu et al., 2003) and, as recently discovered, other immune cells such as T and B cells (Duffaut et al., 2009; Feuerer et al., 2009; McDonnell et al., 2012; Nishimura et al.,

2009; Winer et al., 2011; Winer et al., 2009; Wu et al., 2007). The precise role of these cell populations in obese adipose is not yet known. There is no evidence of tissue infection that would provide homing signals for circulating immune cells, although suggestions have been put forward that tissue injury due to anoxia and apoptosis or necrosis within rapidly expanding adipose may trigger macrophage recruitment (Cinti et al., 2005; Hosogai et al., 2007; Strissel et al., 2007; Ye et al., 2007). It has also been reported that inflammatory macrophages can account for up to 40% of the total cell population within obese adipose, making this a substantial physiological response (Weisberg et al., 2003).

Macrophage recruitment into obese adipose is mainly due to heightened secretion of MCP-1 (monocyte chemoattractant protein-1) (Fantuzzi, 2005; Kamei et al., 2006; Kanda et al., 2006; Wellen and Hotamisligil, 2003), which is followed by secretion of other cytokines, such as tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6). As a result, these secreted factors establish a low-level, chronic, systemic inflammation among obese individuals (Weisberg et al., 2003; Xu et al., 2003). This chronic inflammatory profile is thought to represent a mechanistic link between several multi-faceted metabolic diseases, such as hyperlipidemia, hypertension, obesity-dependent cardiovascular diseases and type II diabetes mellitus (Bays et al., 2004; Ford et al., 2002; Grundy et al., 2004; Jensen, 2006).

To better understand the contributions of chronic inflammation in obesity to these metabolic diseases, it is vital to define the cytokine expression profile among the various cell types within inflamed adipose and identify how paracrine and autocrine activities influence this profile. Although some reports have suggested that cytokine production is

limited to infiltrating macrophages, other studies have offered a more complex picture that involves intercellular communication between macrophages and adipocytes. For example, murine (3T3-L1 cells) or human (Simpson–Golabi–Behmel syndrome preadipocyte cell line – SGBS) adipocytes incubated with macrophage-conditioned media increases mRNA expression and protein levels of inflammation-related genes, including MCP-1 and IL-6 (Permana et al., 2006; Permana et al., 2009; Suganami et al., 2005). Reverse stimulation also occurs in which macrophages cultured with adipocyte-conditioned media increase their expression of IL-6 and TNFα (Suganami et al., 2005). These findings suggest that both cell types contribute to elevated cytokine expression by co-stimulating in a paracrine manner with secreted factors found in their respective culture media. We have recently confirmed and extended this paracrine communication activity by showing that cultured adipocytes can independently respond to TNFα stimulation by increasing IL-1β, IL-6, and COX-2 expression through nuclear factor kappa B (NF-κB) signaling (Gonzales and Orlando, 2008). Alternative to conditioned media, co-culture methods or whole adipose tissue explants have been used to explore macrophage-adipocyte intercellular communications. For the whole excised tissue approach, collagenase-treatment was used to disaggregate cells followed by differential centrifugation to separate buoyant adipocytes from more dense stromal vascular cells. This stromal vascular fraction contains a variety of cell types, including endothelial cells, fibroblasts, pre-adipocytes and macrophages; however, in an obese setting, it also contains a highly enriched inflammatory macrophage population (Weisberg et al., 2003; Xu et al., 2003). Unfortunately, the standard methodology for adipose tissue cell separation has led to mixed, sometimes conflicting results; one study reported that the

infiltrating macrophage population is responsible for almost all TNFα expression (Weisberg et al., 2003), with IL-6 being expressed by both populations (Samuvel et al., 2010; Weisberg et al., 2003), while other groups concluded that IL-6 was released mainly by non-fat cells (Fain et al., 2003; Xie et al., 2010). A third group reported that the stromal vascular fraction showed greater MCP-1 expression than the adipocyte fraction, concluding that macrophages are the main source of this chemokine in adipose tissue (Bruun et al., 2005; Christiansen et al., 2005). One likely explanation of these conflicting observations comes from a parallel study indicating that collagenase treatment, combined with the isolation procedure used for fractionating adipose tissue into macrophage and adipocyte cell populations can significantly alter cytokine expression profile by artificially inducing inflammatory mediators and down-regulating adipocyte-specific genes (Ruan et al., 2003).

In the present study, I have investigated the contributions of paracrine activities and cell-cell contact on pro-inflammatory cytokine production. To circumvent the problems associated with tissue disaggregation, I have developed a trans-well co-culture model using primary murine splenocytes and 3T3-L1 cell-derived mature adipocytes. Use of splenocytes provides a better representation of the immune cell population found in obese adipose (Avitsur et al., 2002; Sun et al., 2012) and ensures normal intracellular signaling patterns, rather than introducing possible complications from altered signal transduction events likely present in immortalized transformed monocytic cell lines such as THP-1 or RAW294.7. My findings provide novel evidence that when immune cells and adipocytes are cultured together, both diffusible, paracrine factors and cell-cell contact contribute synergistically in modifying TNFα, IL-6, and MCP-1 secretion. I also

provide evidence identifying which cytokine is expressed by each cell type and demonstrate a functional role for TNFα in providing signaling that potentiates the cell contact effects on cytokine secretion.

2.2. Materials and Methods

Animals and Animal Care

Male C57Bl/6J (Stock #000664), GFP (C57Bl/6-Tg(UBC-GFP) 30Scha/J; ubiquitous expression of Green Fluorescent Protein; Stock #004353), and TNFα $^{-/-}$ (B6.129S-Tnf ^{tm1Gkl}/J; Stock #005540) mice were purchased from Jackson Laboratories at 8 weeks of age. Animals were housed 2 per cage in a pathogen-free environment on a 12 h light/dark cycle and were provided free access to food and water. Mice were euthanized by CO_2 asphyxiation and processed immediately for spleen removal. All procedures in this study were approved by the Institutional Animal Care and Use Committee of the University of New Mexico.

Splenocyte isolation

Splenocyte isolation was performed according to Kruisbeek (Kruisbeek, 2001) using wild type C57Bl/6J, GFP-expressing C57Bl/6J, or TNFα $^{-/-}$ mice. After spleens were excised, they were placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin (complete DMEM). Spleens were homogenized into single cell suspensions by gently disaggregating tissue between frosted ends of two microscopy slides, filtered through a

100 μm cell strainer, and then centrifuged at 800 x g for 3 min at 4° C. Supernatants were discarded and cell pellets were resuspended in 1 mL ACK (Ammonium-Chloride-Potassium) Lysis Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2-EDTA; pH 7.4) for 5-10 min to remove contaminating red blood cells. Complete DMEM was then added and cells were centrifuged again at 800 x g for 3 min at 4° C. Supernatants were removed; cells were resuspended in complete DMEM and used for co-culture experiments after cell densities were determined by hemocytometer counting.

Cell Culture and Adipocyte Differentiation

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured and differentiated according to Gonzales and Orlando, 2008 (Gonzales and Orlando, 2008). Briefly, cells were grown in complete DMEM media at 37° C with 5% CO_2 and passaged twice weekly. For differentiation, cells were seeded into 6-well cell culture plates coated with 1% gelatin. When cells reached confluency, they were treated with 250 nM dexamethasone, 450 μM 3-isobutyl-1-methylxanthine, and 167 nM insulin for 3 days, followed by 167 nM insulin for an additional 3 days. Differentiation was confirmed by morphological changes, including intracellular lipid droplet accumulation.

Adipocyte and Splenocyte Co-cultures

3T3-L1 cells were grown to confluency in 6-well culture plates and differentiated to mature adipocytes as described above. Adipocytes were then co-cultured with isolated splenocytes from wild type C57Bl/6J, GFP-expressing C57Bl/6J, or TNFα $^{-/-}$ mice in

direct and indirect contact systems in the presence or absence of LPS (*E. coli* 0111:B4 – 1 μg/mL; Sigma Aldrich, St. Louis, MO) in complete DMEM. For controls, cells were cultured individually with or without LPS, or cultured together in the absence of LPS. For indirect co-cultures, cells were cultured in a transwell system, with differentiated 3T3-L1 cells in the lower chamber and splenocytes (1.5×10^6 cells) seeded in a 0.4 μm hanging cell insert (Millipore, Billerica, MA). The hanging insert is constructed with a membrane having pores that are large enough to permit the passage of small molecules, yet small enough to prevent the passage of even the most motile of cell types. For direct co-cultures, splenocytes (1.5×10^6 cells) were added to differentiated 3T3-L1 cells, allowing direct contact between the two cell types. For TNFα recovery studies (Fig 2.4C and D), adipocytes and splenocytes from TNFα $^{-/-}$ mice were co-cultured in direct contact with LPS (1 μg/mL) and supplemented with 0, 0.3, or 10 ng/mL purified murine TNFα (Cell Signaling, Danvers, MA).

Cytokine ELISAs

Murine IL-1β, IL-6, IL-10, MCP-1, and TNFα levels in co-culture supernatants were measured by ELISA Ready-Set-Go kit (eBioscience, San Diego, CA) according to the manufacturer's directions. Media samples were diluted accordingly to ensure samples were within the detection kit sensitivity, specifically for MCP-1 and IL-6.

Fluorescence-activated cell sorting

After 24 h of direct co-culturing of 3T3-L1 cells and splenocytes from GFP mice, cells were sorted by fluorescence-activated cell sorting (FACS). Co-cultures were

trypsinized and centrifuged at 800 x g for 3 min at 4° C. Supernatants were removed and cells were resuspended in complete low serum (0.5% FBS) DMEM media with 5 mM EDTA and passed through a 100 μm cell strainer (BD Falcon) to ensure single-cell suspensions. GFP-positive and negative cells were sorted using the Beckman Coulter Legacy MoFlo high-speed sorter into separate tubes for subsequent mRNA extraction.

RNA Isolation and Quantitative RT-PCR Analysis

Sorted 3T3-L1 cells and splenocytes were homogenized using the QIAshredder (Qiagen, Valencia, CA) and total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Levels of adiponectin, F4/80, IL-6, IL-10, MCP-1, and TNFα were measured by quantitative Real-Time PCR (qRT-PCR), which was carried out using the LightCycler 480 SYBR Green I Master Mix chemistry (Roche Diagnostics, Indianapolis, IN) and analyzed on the LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). Information on primer sequences, annealing temperatures, fragment sizes, and genes can be found in Table 2.1. The reaction cycling parameters for IL-10 were performed as a 3-step qRT-PCR with a pre-incubation step at 95° C for 5 min and amplification for 45 cycles at 95° C for 10 sec, 60° C for 15 sec, and 72° C for 1 sec. All other genes were amplified in a 2-step qRT-PCR reaction with pre-incubation at 95° C for 15 min, followed by 40 cycles of amplification at 95° C for 15 sec, and the annealing temperature (Table 2.1) for 1 min. A melting curve analysis was performed in each experiment for all genes to confirm specificity of single-target amplification. Gene expression changes were

calculated using the relative standard curve method (Livak, 1997) and 36B4 mRNA levels were used as a normalizer. All samples were amplified in triplicate.

Statistical Analysis

Statistical significance was determined by performing a t-test with Sigma Plot (Version 7.0) on replicate samples. Statistically significant differences were considered when $p < 0.05$.

Gene	Primer sequences	Annealing temperature	Fragment size	GenBank no.
F4/80	Forward – GCTGTGAGATTGTGGAAGCA Reverse – CTGTACCCACATGGCTGATG	66°C	136bp	NM_010130
IL-6	Forward – AGTTGCCTTCTTGGGACTGA Reverse – CAGAATTGCCATTGCACAAC	60°C	191bp	NM_031168
Adiponectin	Forward – GGAACTTGTGCAGGTTGGAT Reverse – CGAATGGGTACATTGGGAAC	63°C	293bp	NM_009605
MCP-1	Forward – TCACCTGCTGCTACTCATTCACCA Reverse – TACAGCTTCTTTGGGACACCTGCT	60°C	98bp	NM_011333
TNFα	Forward – ACGGCATGGATCTCAAAGAC Reverse – GTGGGTGAGGAGCACGTAGT	63°C	116bp	NM_013693
IL-10	Forward – ATGCAGGACTTTAAGGGTTACTTG Reverse - TAGACACCTTGGTCTTGGAGCTTA	60°C	254bp	NM_010548
36B4	Forward – AAGCGCGTCCTGGCATTGTCT Reverse – CCGCAGGGGCAGCAGTGGT	60-66°C	136bp	NM_007475

Table 2.1 – Gene and primer information for qRT-PCR. 36B4 annealing temperature varied according to target gene being analyzed. IL-6, interleukin-6; IL-10, interleukin-10; MCP-1, monocyte chemoattractant protein-1; TNFα, tumor necrosis factor alpha; and 36B4, 60S acidic ribosomal protein P0.

2.3. Results

Direct contact between splenocytes and adipocytes alters secreted inflammatory cytokine levels.

To first determine if cytokine secretion is regulated by paracrine effects (no cell contact) or if cell-cell contact between adipocytes and immune cells contributes to the inflammatory response, I performed co-culture studies using 3T3-L1-derived adipocytes and isolated murine splenocytes, and assessed the quantity and pattern of TNFα, IL-6, and MCP-1 secretions. Cells were co-cultured either in transwells to mimic indirect contact, permitting only paracrine communications through diffusible factors, or cultured together to allow for direct cell-cell contact communications along with paracrine effects. Control studies included adipocytes and splenocytes incubated alone with or without LPS, to measure the effect of LPS stimulation on adipokine or cytokine secretion in each cell type without influence of co-culture, and adipocyte-splenocyte co-cultures without stimulation by LPS, to determine if non-activated cells can affect adipokine and cytokine secretion. Significant differences in TNFα, IL-6, and MCP-1 secretions were found when comparing indirect and direct adipocyte-splenocyte co-cultures indicating that cell-cell contact does indeed contribute to adipokine and cytokine secretions (Fig. 2.1). For TNFα, co-culturing appears to dampen secretion. In individual cultures, LPS stimulates TNFα secretion in splenocytes (Fig. 2.1A, compare columns 3 and 4), but has no effect on adipocytes (Fig. 2.1A, compare columns 1 and 2). Co-culturing cells in transwells, to allow only paracrine communication, reduced TNFα secretion by 55% from that measured in LPS-stimulated splenocytes alone (Fig. 2.1A, compare columns 4 and 6). When cells were co-cultured with direct contact, TNFα secretion was reduced by an

additional 33% as compared to paracrine effects (Fig. 2.1A, compare columns 6 and 8; $p < 0.01$) demonstrating that paracrine factors and cell-cell contact both contribute to attenuate TNFα secretion. Also noteworthy, when adipocytes and splenocytes were co-cultured in the absence of LPS activation, whether indirect or direct culture, no measureable TNFα secretion was found (Fig. 2.1A, columns 5 and 7), confirming that activation of inflammatory immune cells is required to mediate the effects seen in obese adipose tissue.

For IL-6, co-culturing of adipocytes and LPS-activated splenocytes appears to enhance secretion (Fig. 2.1B). When each cell type was cultured individually in the presence of LPS, some IL-6 secretion was measured for both adipocytes (Fig. 2.1B, compare columns 1 and 2) and splenocytes (Fig. 2.1B, compare columns 3 and 4). In contrast to the effects seen on TNFα secretion, when cells were co-cultured in transwells to measure the effects of paracrine stimulation, IL-6 secretion was increased by ~3-fold over that measured for the summation of individual LPS-activated cultures (Fig. 2.1B, compare columns 2 + 4 with column 6). When cells were co-cultured with direct contact, IL-6 secretion was increased by an additional 36% over that measured for paracrine effects alone (Fig. 2.1B, compare columns 6 and 8; $p<0.01$). Similar to the effects seen on TNFα secretion, very little or no IL-6 secretion could be measured in the absence of LPS activation (Fig. 2.1B, columns 1, 3, 5, and 7).

In a similar manner as IL-6, co-culturing of adipocytes and LPS-activated splenocytes appears to have a substantial effect in enhancing MCP-1 secretion (Fig. 2.1C). In individual cultures, no measureable MCP-1 secretion could be detected in splenocytes with or without LPS stimulation (Fig. 2.1C, see columns 3 and 4). LPS

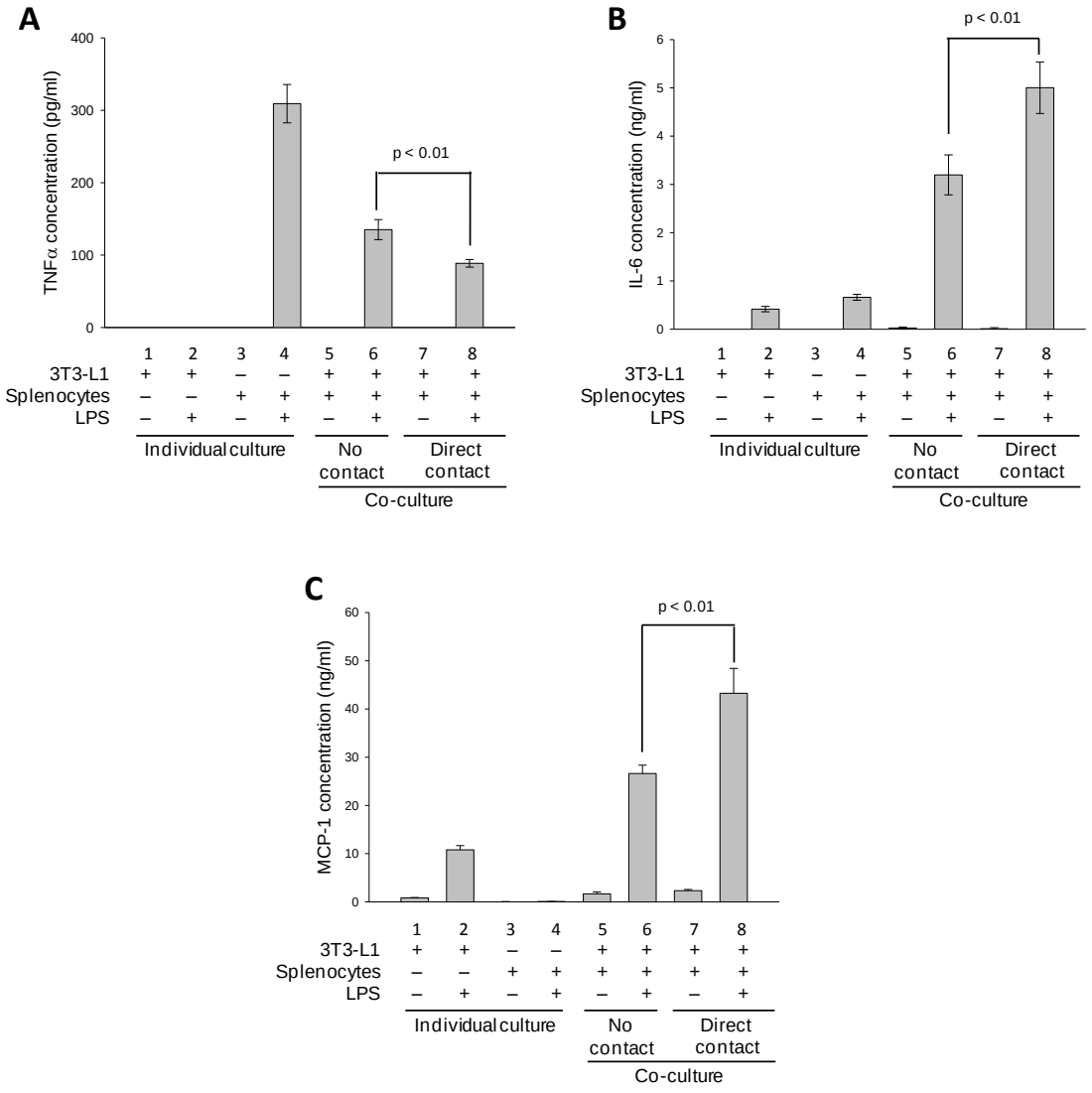

Figure 2.1 – Direct contact between splenocytes and adipocytes alters secreted levels of inflammatory cytokines. Differentiated 3T3-L1 adipocytes (columns 1 and 2) or isolated murine splenocytes (columns 3 and 4) were cultured alone or together with either direct contact (columns 7 and 8) or no contact (cells separated by a 0.4 μm transwell filter system; columns 5 and 6). Cells were additionally incubated in the absence (-) (columns 1, 3, 5, and 7) or presence (+) (columns 2, 4, 6, and 8) of LPS (1 μg/mL) for 24 h. Secreted cytokines, TNFα (A), IL-6 (B), and MCP-1 (C), were quantified by capture ELISA.

treatment of adipocytes alone was able to induce some MCP-1 secretion (Fig. 2.1C, compare columns 1 and 2) and this secretion level increased by 2.5-fold when co-cultured with splenocytes indirectly in transwells (Fig. 2.1C, compare columns 2 and 6). When adipocytes and splenocytes were co-cultured with direct contact, MCP-1 secretion increased by an additional 38% over that measured for just paracrine stimulation (Fig. 2.1C, compare columns 6 and 8; $p<0.01$).

As an additional note, there were no detectable levels of IL-1β in my co-culture system under any of the conditions tested (data not shown).

Paracrine stimulation and direct contact differentially effect cytokine secretion in a time-dependent manner.

To better define the changes in cytokine secretion patterns resulting from paracrine factors alone or these factors together with direct cell-cell contact, I chose to perform a time course study. Co-culturing of activated splenocytes and adipocytes without direct cell-cell contact led to a time-dependent increase in secreted TNFα levels, reaching a maximal effect after 24 h with a t½ of 5-6 h (Fig. 2.2A). When cells were cultured in direct contact, TNFα secretion was substantially reduced from levels measured from paracrine stimulation alone, achieving a maximal effect at <8 h with a t½ of 3-4 h.

Paracrine factors stimulated both IL-6 and MCP-1 secretion in co-cultures in a time-dependent manner, with maximal stimulation for IL-6 at approximately 15-20 h (t½ of 2-3 h) (Fig. 2.2B) and >48 h for MCP-1 (Fig. 2.2C). With direct cell-cell contact, stimulation of IL-6 secretion was greater than that measured for paracrine factors alone

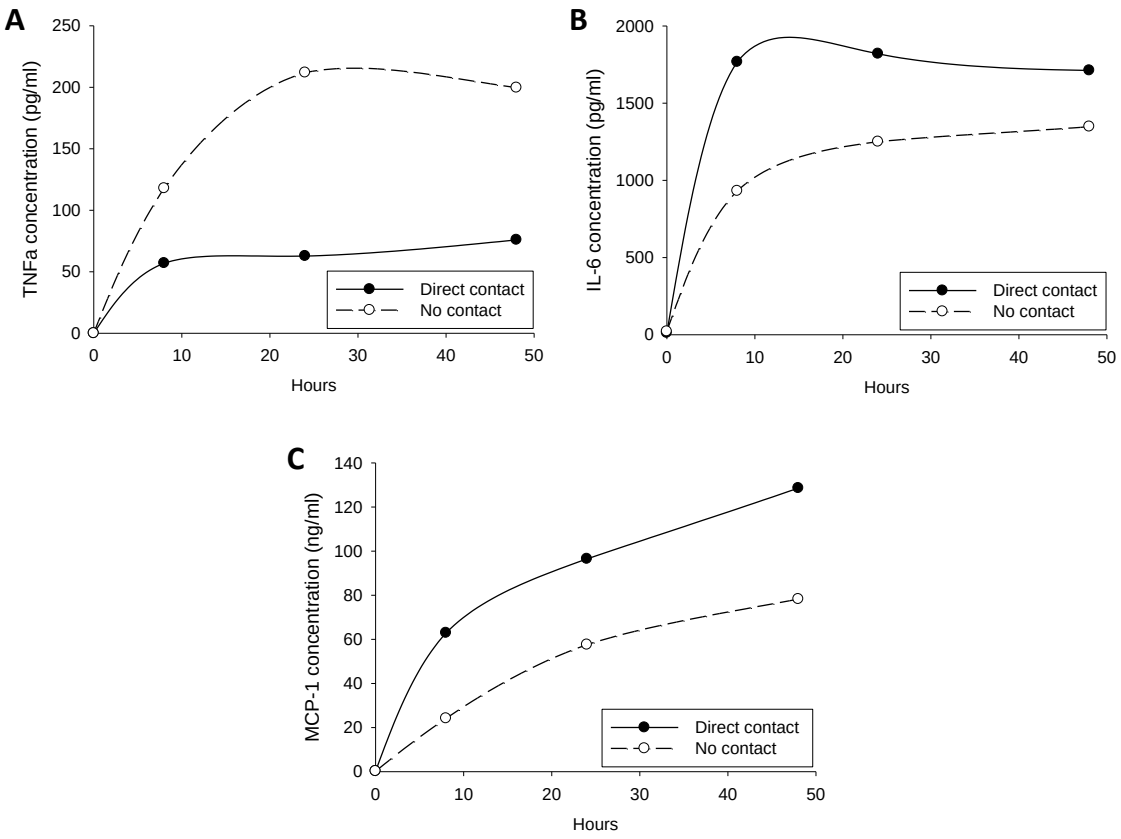

Figure 2.2 – Rates of cytokine secretion and overall levels are affected by direct contact between adipocytes and splenocytes. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were co-cultured with no contact (separated by a 0.4 μm transwell filter system) (dashed lines) or with direct contact (solid lines) and incubated with LPS (1 μg/mL) for 0, 8, 24, and 48 h. Cytokines, TNFα (A), IL-6 (B), and MCP-1 (C), were measured in culture media following these incubation times by capture ELISA.

with a maximal effect at <8 h and t½ of 4-5 h. The effect of cell-cell contact on increasing MCP-1 secretion was also rapid; however, the stimulatory effect did not reach a maximum even after 48 h of culture. These data demonstrate that paracrine factors, resulting from splenocyte-adipocyte co-culture, affect cytokine secretion in a time-dependent manner. Moreover, when cells were cultured under conditions allowing for

direct cell-cell contact, the effects on cytokine secretion were enhanced over what was measured for paracrine factors alone.

Pro-inflammatory cytokine expression profile for co-cultured splenocytes and adipocytes.

The next objective was to determine which cell type was responsible for expression of TNFα, IL-6, and MCP-1 following co-culturing. I chose to limit this evaluation to assessing mRNA expression levels because discriminating protein expression would require further individual culturing of the cells after co-culturing conditions and this extended incubation is known to artificially affect cytokine expression (Fain et al., 2004; Ruan et al., 2003). For this analysis, cells were co-cultured with direct cell-cell contact and then separated by fluorescence-activated cell sorting (FACS), followed by quantification of mRNA expression for TNFα, IL-6, and MCP-1 in each cell type. In order to separate splenocytes from adipocytes using FACS, I performed the co-culturing incubation with splenocytes isolated from mice constitutively expressing green fluorescent protein (GFP) in all cell types. The data shown in Figure 2.3A is representative of a FACS profile for adipocytes and GFP-splenocytes (gates R1 and R2, respectively). With this approach, I am able to clearly separate GFP-expressing splenocytes from non-fluorescent 3T3-L1 adipocytes following co-culturing. To confirm the relative purity of each cell population with a more sensitive assay, I examined mRNA expression for cell specific markers in each cell type: F4/80 for macrophages (splenocytes) and adiponectin for adipocytes. As seen in Figure 2.3B, F4/80 expression

was found only in the GFP-splenocyte population and adiponectin expression was found only in the adipocyte cell population.

Messenger RNA quantification for each cytokine revealed that, following their co-culture with direct cell-cell contact, adipocytes expressed relatively small quantities of TNFα mRNA, whereas splenocytes generated 4.5-fold greater levels than adipocytes (Fig. 2.3C). The opposite expression pattern was found for MCP-1; its expression was almost undetectable in splenocytes, whereas substantial expression was measured in adipocytes (Fig. 2.3C). Importantly, both adipocytes and splenocytes contribute equally to IL-6 expression following co-culture conditions (Fig. 2.3C), which is in contrast to previous studies which have suggested that the stromal vascular cells (largely macrophages) are the sole contributors of cytokines in inflamed adipose (Bruun et al., 2005; Fain, 2010; Fain et al., 2004; Fain et al., 2003).

TNFα signaling is necessary for cell contact-mediated increases in IL-6 and MCP-1 secretion.

The data presented above establish that crosstalk between splenocytes and adipocytes, in the form of paracrine factors and cell-cell contact, significantly influences cytokine secretion in my *in vitro* model of inflamed adipose. Of these cytokines, TNFα is known to be one of the major paracrine factors expressed in obese adipose (Hotamisligil et al., 1995; Hotamisligil et al., 1993). Based on this observation, I examined if the paracrine activity of TNFα influenced the cell-cell contact mediated changes to IL-6 and MCP-1 secretion measured in my splenocyte-adipocyte co-cultures. To determine this, I repeated the co-culture study shown in Figure 2.1 with splenocytes derived from

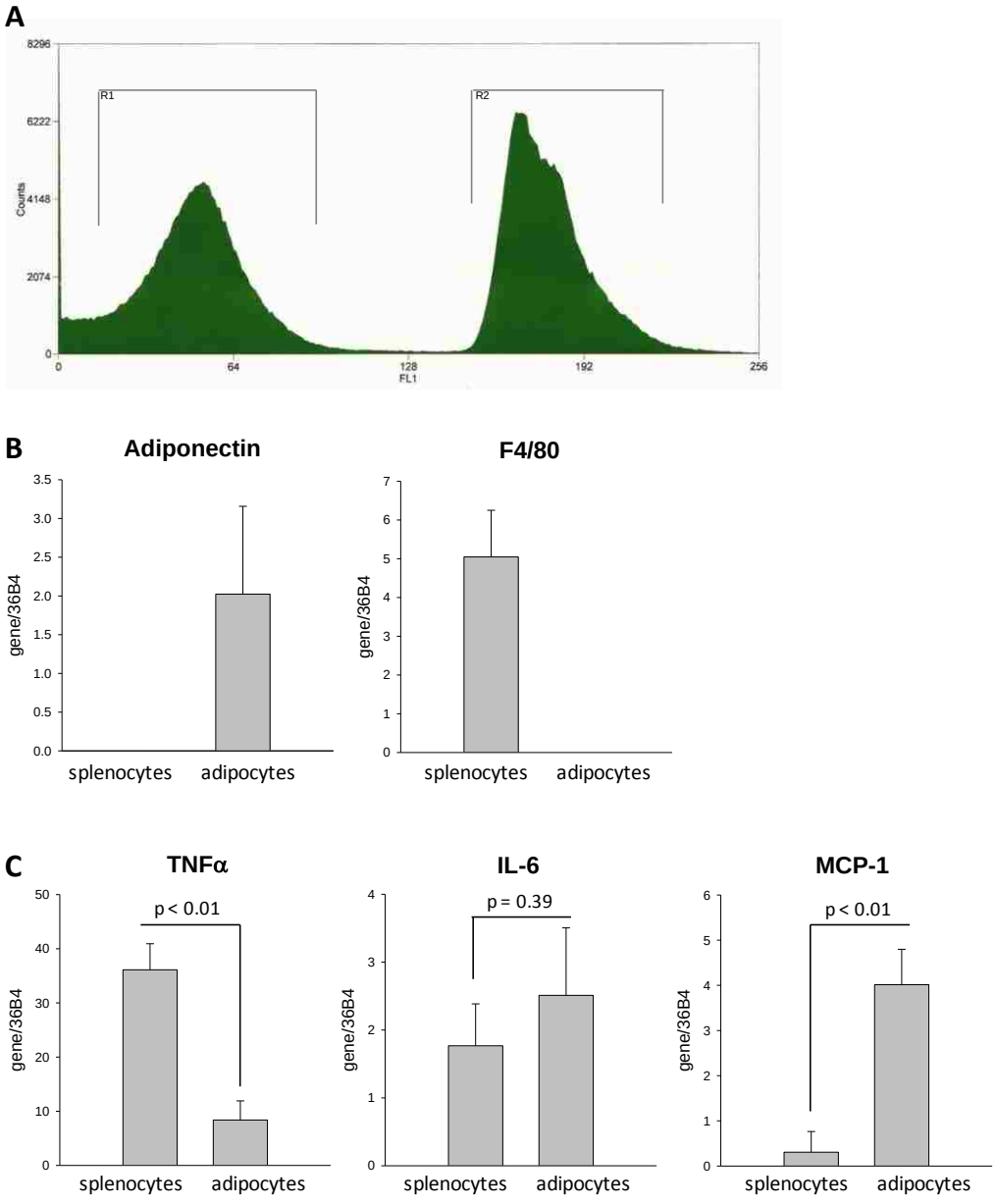

Figure 2.3 – Splenocytes and adipocytes differentially express pro-inflammatory markers. Differentiated 3T3-L1 adipocytes were co-cultured in direct contact with GFP-expressing murine splenocytes and activated by incubation with LPS (1 μg/mL) for 24 h. Cells were sorted for GFP-positive (splenocyte) and negative (adipocyte) cells by FACS. (A) Representative FACS is shown, with GFP-negative cells gated in R1 and GFP-positive cells gated in R2. (B) Quantitative real-time PCR (qRT-PCR) was used to measure adiponectin and F4/80 expression, specific markers for adipocytes and splenocytes, respectively, to confirm efficiency of cell sorting. (C) TNFα, IL-6, and MCP-1 mRNA expression levels were quantified by qRT-PCR in splenocytes and adipocytes following cell sorting to distinguish individual cytokine expression patterns. All qRT-PCR values were normalized to values obtained for 36B4, a ribosomal 60S subunit protein.

TNFα $^{-/-}$ mice. As expected, TNFα $^{-/-}$ splenocytes failed to express TNFα in the absence or presence of LPS (data not shown); however, with LPS-activation they did express IL-6 at levels similar to wild type splenocytes (Fig. 2.4A, column 2) confirming their functional response to LPS stimulation.

When TNFα $^{-/-}$ splenocytes were co-cultured with adipocytes in the absence of LPS activation, either with direct or no contact, little or no measureable IL-6 (Fig. 2.4A, compare columns 3 and 5) or MCP-1 (Fig. 2.4B, compare columns 3 and 5) could be detected (grey bars), similar to what was seen when using wild type splenocytes (black bars). With LPS activation, co-cultures of TNFα $^{-/-}$ splenocytes and adipocytes without direct contact demonstrated similar increases in IL-6 secretion as measured for wild type splenocytes (Fig. 2.4A, column 4, compare black and grey bars), indicating that TNFα paracrine activity is not required for enhancement of IL-6 secretion. For MCP-1, co-culture of LPS-activated TNFα $^{-/-}$ splenocytes and adipocytes without direct contact resulted in enhanced MCP-1 secretion when compared with individual adipocyte cultures (Fig. 2.4B, compare columns 1 and 4); however, this enhancement was somewhat less than what was measured using wild type splenocytes (Fig. 2.4B, column 4, compare black and grey bars), suggesting that TNFα paracrine activity contributes to some enhancement of MCP-1 secretion. Overall, these findings indicate that paracrine factors other than TNFα play a primarily role in enhancing IL-6 and MCP-1 secretion in co-cultures of splenocytes and adipocytes.

A very different effect on IL-6 and MCP-1 secretion was found when LPS-activated TNFα $^{-/-}$ splenocytes were co-cultured with adipocytes with direct cell-cell contact. The additional enhancement of IL-6 and MCP-1 secretion due to direct cell

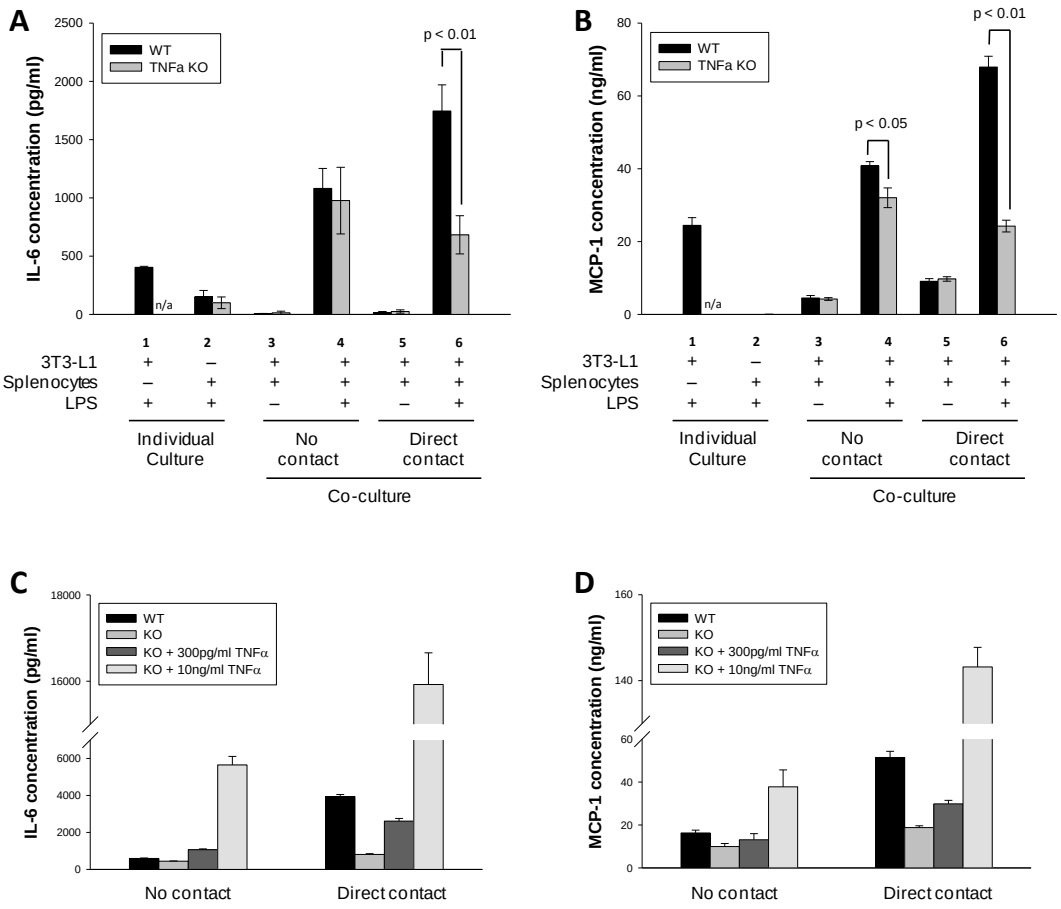

Figure 2.4 – Cell contact-mediated enhancement of IL-6 and MCP-1 secretion requires TNFα signaling. (A and B) Differentiated adipocytes or murine splenocytes (black bars, isolated from wild type mice; gray bars, isolated from TNFα $^{-/-}$ mice) were cultured alone (individual culture, columns 1 and 2) or in co-culture with no contact (columns 3 and 4) or direct contact (columns 5 and 6) as in Figure 2.1. Cells were incubated in the absence (-) or presence (+) of LPS (1 μg/mL) for 24 h as indicated. (C and D) Wild type or TNFα $^{-/-}$ splenocytes were incubated with adipocytes with direct contact in the presence of LPS (1 μg/mL) and co-cultures were supplemented with 0, 300 pg/mL or 10 ng/mL purified murine TNFα as indicated. Secreted IL-6 (A and C) and MCP-1 (B and D) were quantified by capture ELISA.

contact between TNFα $^{+/+}$ splenocytes and adipocytes is attenuated in co-cultures of TNFα $^{-/-}$ splenocytes and adipocytes (Fig. 2.4A and B, column 6, compare black and grey bars). The addition of exogenous TNFα to co-cultures of TNFα $^{-/-}$ splenocytes and adipocytes restored the contact-mediated enhancement in a dose-dependent manner for both IL-6 (Fig. 2.4C) and MCP-1 (Fig. 2.4D). These findings indicate that, although TNFα contributes little to the paracrine-mediated enhancement of IL-6 and MCP-1 secretion, its activity is necessary for cell contact-mediated augmentation of IL-6 and MCP-1 secretion.

Secretion of anti-inflammatory factor, IL-10, is unaltered by paracrine factors or direct cell contact.

Inflamed adipose is also known to express the anti-inflammatory cytokine, IL-10. Its secretion is thought to be in response to the elevated state of inflammation within obese adipose (Esposito et al., 2003; Juge-Aubry et al., 2005). Because of this, the anti-inflammatory properties of IL-10 may provide a counterbalancing effect to dampen the actions of inflammatory cytokines (Gotoh et al., 2012). Consequently, I examined the effects of paracrine factors and direct contact on IL-10 secretion in my co-culture system. In individual cultures, a small but measureable amount of IL-10 could be detected in non-stimulated and LPS-stimulated adipocytes (Fig. 2.5A, columns 1 and 2), while a 2.5-fold greater amount was found in cultures of LPS-stimulated splenocytes (Fig. 2.5A, column 4). When normal LPS-activated splenocytes were co-cultured with adipocytes in transwells to measure the effects of paracrine activity, elevated IL-10 levels were measured; however, the no contact co-culture levels were approximately the sum of what

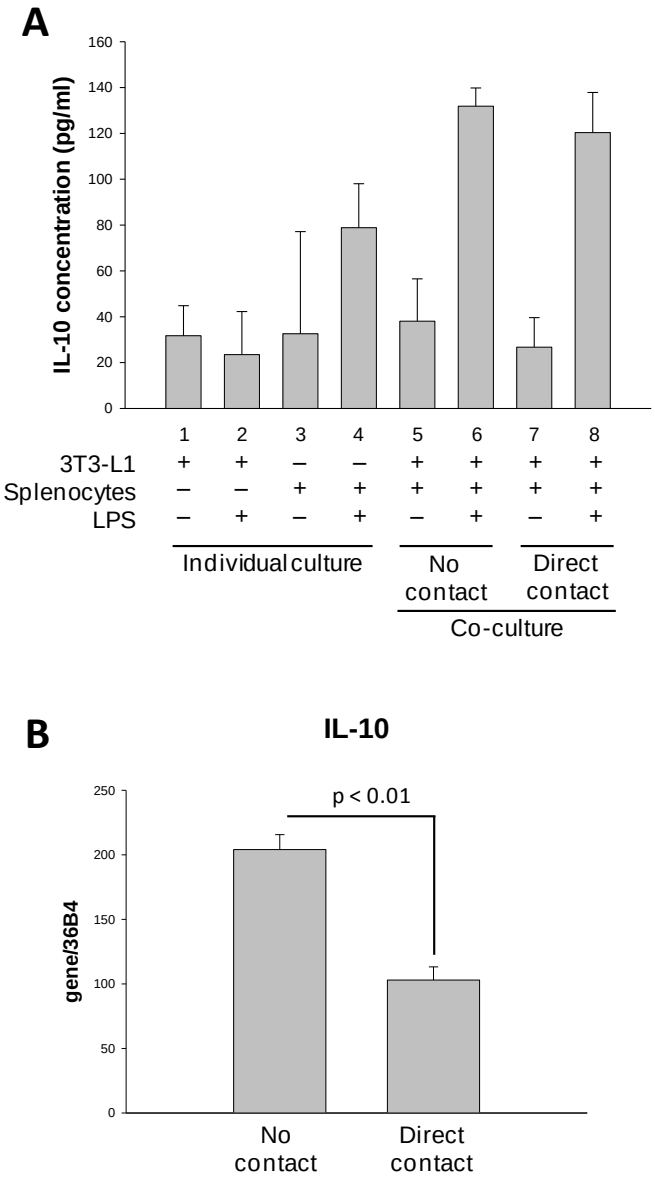

Figure 2.5 – Effects of paracrine factors and cell contact on IL-10 secretion and expression. (A) Differentiated 3T3-L1 adipocytes or wild type murine splenocytes were cultured alone (columns 1 and 2 or 3 and 4, respectively) or together with either no contact (columns 5 and 6) or direct contact (columns 7 and 8). Cells were incubated in the absence (-) (columns 1, 3, 5, and 7) or presence (+) (columns 2, 4, 6, and 8) of LPS (1 μg/mL) for 24 h as indicated. Interleukin-10 (IL-10) in culture media was quantified by capture ELISA. (B) Differentiated 3T3-L1 adipocytes were co-cultured with no contact or direct contact with GFP-expressing murine splenocytes as in Figure 2.3 and activated by incubation with LPS (1 μg/mL) for 24 h. Splenocytes were sorted as GFP-positive cells by FACS and IL-10 mRNA expression was measured by qRT-PCR. qRT-PCR values were normalized to values obtained for 36B4.

is being secreted by splenocytes and adipocytes in LPS-stimulated individual cultures (Fig. 2.5A, columns 2 + 4 compared with 6). Furthermore, unlike inflammatory cytokines examined above, no change in IL-10 levels was found when LPS-activated splenocytes and adipocytes were co-cultured with direct contact (Fig. 2.5A, compare columns 6 and 8). These data suggest that both paracrine factors and direct cell-cell contact have little or no influence on IL-10 secretion beyond what is stimulated by LPS treatment.

To further explore if cell-cell contact between splenocytes and adipocytes influences IL-10 expression, I next measured if its mRNA expression was altered following co-culture with direct contact. For this measurement, GFP-expressing splenocytes were once again used for co-culturing with adipocytes to permit FACS separation of the two cell types prior to RNA isolation. By qRT-PCR analyses, I found that direct contact of LPS-activated splenocytes and adipocytes reduced IL-10 splenocyte mRNA expression by 50% (Fig. 2.5B).

2.4. Discussion

It is now well established that excessive adiposity in obese individuals is accompanied by a low level, chronic systemic inflammatory state (Weisberg et al., 2003; Xu et al., 2003). This observation is clinically important in that chronic secretion of circulating cytokines may provide the mechanistic link between obesity and related cardiovascular and diabetic complications; that is, inappropriate cytokine signaling can potentiate atherosclerotic lesion development (Ross, 1999) and, in muscle and adipose, desensitize insulin responsiveness toward glucose clearance function (Guilherme et al.,

2008). Elevated circulating cytokines are thought to arise from pro-inflammatory macrophages that populate obese adipose (Weisberg et al., 2003; Xu et al., 2003). Recent investigations have also found increased presence of B cells (Duffaut et al., 2009; McDonnell et al., 2012; Winer et al., 2011), a significant accumulation of $CD8^+$T (effector) cells with a concomitant decrease of $CD4^+$ T (helper) cells (Duffaut et al., 2009; Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009; Wu et al., 2007), as well as activated natural killer T (NKT) cells (Ji et al., 2012; Wu et al., 2012). It has been postulated that B cells provide the initial trigger, leading to activation of $CD8^+$ T cells and monocytes/macrophages, which in turn leads to increased inflammation and insulin resistance (Winer et al., 2011). To obtain a more detailed understanding of the chronic inflammatory state in obese adipose, I determined if paracrine and cell contact-mediated intercellular communications between immune cells and adipocytes could establish a crosstalk that impacts both cytokine secretion patterns and levels. For this study, I have developed a novel co-culture model using isolated murine splenocytes and cultured murine adipocytes (3T3-L1). The immune cell population in murine splenocytes is known to include $CD8^+$ T cells (30%), $CD4^+$ T cells (16%), B cells (35%), NKT cells (7%), and a rich source of monocytes (8%) (Avitsur et al., 2002; Swirski et al., 2009). Most or all of these cells in the spleen have now been identified in obese adipose and likely contribute to some degree toward the pro-inflammatory response. The immune cell distribution in obese adipose tissue has been identified as of $CD8^+$ T cells (5%), $CD4^+$ T cells (7%), B cells (11%), NKT cells (6%) and macrophages (55%) (Sun et al., 2012). Although the percentage representation of each cell type varies between splenocytes and obese adipose, because of the similarity in immune cell identity, use of primary

splenocytes in a co-culture model provides a better representation of immune cells found in obese adipose as opposed to limiting my analyses to a pure population of monocytes/macrophages. 3T3-L1-derived adipocytes have some limitations due to their being an immortalized cell line, as indicated by storing lipids as multi-locular droplets, as opposed to primary adipocytes, which have uni-locular droplets, as well as very low levels of leptin secretion even when lipid laden. However, I believe these limitations are not significant enough to adversely influence the results of my study, since 3T3-L1-derived adipocytes do correctly respond to endocrine stimulation for fatty acid assimilation and mobilization indicating normal physiologic responses. In addition, these cells demonstrate gene expression profiles that mimic primary adipocyte cultures, especially with regard to metabolic genes needed for proper adipocyte function.

In the present study, I used this novel co-culture model to discriminate between the effects of paracrine signaling from effects mediated by direct cell-cell contact on cytokine secretion in immune cells and adipocytes. I found that when cells are cultured without direct contact, TNFα levels were significantly decreased indicating that soluble factors are secreted in the context of co-cultures that dampen TNFα secretion. Even greater attenuation of TNFα secretion was measured when splenocytes and adipocytes were cultured with direct cell-cell contact. While the maximal effect of paracrine activity reducing TNFα secretion was achieved by 24 h, cell-cell contact provided more rapid signaling to further reduce secretion within 8 h. Paracrine signaling and signaling derived from cell-cell contact appears to act sequentially to decrease TNFα secretion by splenocytes. This of course does not imply that TNFα levels decrease in obesity, rather these data refine the quantitative observations of immune cell-adipocyte crosstalk

influence on TNFα secretion patterns. Previous *in vivo* studies, whether in humans or rodents, measure plasma TNFα levels and relate these values to BMI or other obesity index, yet provide little or no information regarding the molecular effects of cell communications within adipose tissue depots. The data presented here define a novel communication signal mediated through cell surface contact that might represent a new target for small molecule inhibitors to reduce TNFα secretion in obese individuals.

In contrast to the effects measured on TNFα secretion, paracrine and direct cell-cell contact have a stimulatory effect on IL-6 and MCP-1 secretion. When splenocytes and adipocytes were co-cultured without direct cell-cell contact, secreted levels of both IL-6 and MCP-1 were significantly increased demonstrating that soluble factors are present that amplify secretion of these cytokines over what can be measured when the two cell types are cultured individually. Even greater increases in IL-6 and MCP-1 secretion were measured when splenocytes and adipocytes were cultured with direct cell-cell contact. While maximal effect of the paracrine activity on IL-6 secretion was achieved by 15-20 h, cell-cell contact maximally stimulated IL-6 secretion by <8 h. The effects of paracrine stimulation and cell-cell contact on elevating MCP-1 secretion were slower in that the combined effects were unable to reach a maximum even after 48 h of culture. Interpreting these differential effects on IL-6 and MCP-1 is complex. The time course of effects measured for both paracrine and cell-cell contact for IL-6 is similar to those measured for TNFα, although the responses are opposite: IL-6 secretion is increased, whereas TNFα is decreased. Having similar time courses may reflect that synergy between paracrine and cell contact signaling for IL-6 and TNFα secretion changes share overlapping pathways. The effect of paracrine and cell contact signaling on

MCP-1 secretion, however, is markedly different suggesting this synergy may require modulation of different regulatory pathways. This interpretation is also compelling when considering the overall level of MCP-1 secretion changes; the quantities of MCP-1 generated in co-cultures are substantially higher than IL-6 indicating a very robust effect on MCP-1 transcriptional/translational activation. Notably, TNFα signaling has been shown to target regulatory regions of the MCP-1 gene (Fasshauer et al., 2004; Ping et al., 1996) and, as I show in the present study, appears to contribute to at least some of the paracrine activity that modulates MCP-1 secretion in co-cultures.

Questions still linger as to which cell type within obese adipose tissue is responsible for the production of each cytokine (Yamashita et al., 2007). Previous attempts have been made to address these questions by disaggregating adipose tissue into adipocytes and stromal vascular cells with proteolysis, and culturing cells separately to characterize their cytokine expression profiles. Unfortunately, these attempts have often generated conflicting results, most likely due to disaggregation methods which are now known to alter cytokine expression (Ruan et al., 2003). Other macrophage-adipocyte co-culture studies have used immortalized macrophage-like cell lines, such as human (THP-1) or murine (RAW264.7) monocytic leukemia cell lines, each having questionable signal transduction pathways because of their transformed phenotype. For these reasons, I performed this study with cells obtained from murine spleens which express normal surface proteins and signaling responses to ensure that my results best represent the cytokine responses we would expect in obese adipose tissue. Following the co-culture of splenocytes obtained from constitutive GFP-expressing mice and adipocytes with direct cell-cell contact, cells were separated by FACS and cytokine expression was determined

immediately following sorting to prevent anomalous changes to cytokine profiles. My data show that the immune cells are the major contributors of TNFα expression, which is consistent with previous findings (Suganami et al., 2005); however, I also uniquely identified that adipocytes produce most or all of the MCP-1 and approximately one-half of the total IL-6. Together these findings indicate that both cell types make significant contribution towards establishing the chronic inflammatory state in obesity.

Because TNFα is one of the primary macrophage-derived, paracrine-acting cytokines involved in inflammation (Suganami et al., 2005), and considering its potency in increasing expression of other inflammatory mediators such as interleukins, prostaglandins and interferons, it is tempting to speculate that TNFα might have a significant role in establishing, or even augmenting, the chronic inflammation in obese adipose tissue. Some evidence has been reported addressing a role for TNFα in obesity (De Taeye et al., 2007) and insulin resistance (Hotamisligil et al., 1994; Uysal et al., 1997); however, few studies have examined the effects of TNFα activity on cytokine or adipokine secretion in adipose tissue. We have recently shown that TNFα activates cyclooxygenase-2 expression in adipocytes by activating the nuclear factor-kappaB (NF-κB) signaling pathway (Gonzales and Orlando, 2008). This study established that TNFα can activate signal transduction in adipocytes and that this signaling event proceeds through similar pathways as the innate immune response. These findings prompted me to next investigate if endogenously expressed TNFα from splenocytes is able to act in a paracrine manner and mediate the increases in IL-6 and MCP-1 secretion measured in my co-culture system. I also questioned if TNFα can mediate the additional increases I measured on cytokine secretion that result from direct cell-cell contact. By using

splenocytes obtained from TNFα deficient (-/-) mice in my co-culture model, I found that when these cells were cultured without direct contact, IL-6 secretion was increased to similar levels seen with wild type splenocytes. Comparable results were also found for MCP-1 secretion changes with TNFα contributing only a modest amount (~20%) of activity toward increased MCP-1 levels. These data demonstrate that soluble factors other than TNFα are largely responsible for driving the paracrine-mediated increases in IL-6 and MCP-1 secretion. Additionally, when TNFα $^{-/-}$ splenocytes are co-cultured with adipocytes in direct cell-cell contact, the additional increases measured for IL-6 and MCP-1 secretion, as seen with direct contact between wild type splenocytes and adipocytes, are absent. Addition of TNFα to these direct co-cultures restored the contact-dependent enhancement of IL-6 and MCP-1 secretion. Together, these data provide evidence that paracrine activity of TNFα contributes little or no function to the initial activation of IL-6 or MCP-1 secretion measured in co-cultures without direct contact; however, TNFα paracrine stimulation is required for cell contact-mediated augmentation of their secretion. These findings establish a novel role for TNFα in adipose inflammation and define a cellular mechanism whereby TNFα fuels the inflammatory response activated by immune cell-adipocyte contact.

The intracellular signaling events of this unique TNFα activity are unknown, but likely involve crosstalk between signaling pathways in adipocytes. Co-culture of splenocytes and adipocytes can engage cell surface molecules which could in turn activate intracellular signaling pathways that converge with TNFα receptor signaling. By this convergence, TNFα signaling may sustain or amplify signals initiated from surface receptor engagement and support elevated cytokine secretion levels. The link between

these cell surface events is likely to involve communication between known pathways; for example, use of specific inhibitors to block mitogen-activated protein kinase (MAPK) pathways (primarily ERK and JNK) and NF-κB activation was able to prevent the overall inflammatory response seen in co-culture studies of adipocytes and macrophages (Suganami et al., 2005; Suganami et al., 2007), or human adipose tissue (Lappas et al., 2005). However, these data were limited in that they only demonstrated global effects on cytokine production and did not address if inhibition of these inflammatory changes was due to inhibition of pathways in adipocytes, macrophages, or both. Other studies showed that stimulation of adipocytes with TNFα or cell-cell contact between adipocytes and immortalized macrophages can lead to up-regulation of the NF-κB pathway (Ruan et al., 2002; Suganami et al., 2007). The data gathered thus far suggest that TNFα receptor engagement could activate all or a combination of MAPK, NF-κB, Jak2, and p44/42 pathways to communicate in a synergistic manner with cell-cell contact to enhance a pro-inflammatory state in obese adipose. The data I present here shows that paracrine factors and direct cell-cell contact are at the center of coordinating the activities of these signaling pathways to direct the pattern and intensity of the inflammatory process.

Since increases in IL-10 secretion are responsive to the elevated state of inflammation within obese adipose (Esposito et al., 2003; Juge-Aubry et al., 2005), I also examined the effects of paracrine stimulation and direct contact on IL-10 secretion in my co-culture system. I found that co-culture of splenocytes and adipocytes without direct contact yielded IL-10 secretion levels that were approximately equal to the summation of individual cultures of LPS-stimulated splenocytes and adipocytes; suggesting that

paracrine factors have little or no influence on IL-10 levels, rather LPS activation is sufficient to maximize IL-10 secretion. Moreover, unlike inflammatory cytokines examined above, no change in IL-10 levels was found when splenocytes and adipocytes were co-cultured with direct contact. Interesting, direct contact of splenocytes with adipocytes was able to reduce IL-10 mRNA expression by 50% of what was measured for co-cultures without direct contact, indicating that cell contact does either dampen IL-10 transcription or reduce mRNA half-life. Clearly these data show that cell-cell contact affects IL-10 mRNA levels, but this effect is not reflected in secreted protein levels.

It is clear that immune cell infiltration into obese adipose tissue is fundamental for changes measured in cytokine secretions and that there is crosstalk between these cells and resident adipocytes. My findings here allow me to now postulate that there are specific, complementary cell surface molecules expressed on both cell types, that when engaged, can cause significant modifications to cytokine secretion profiles, in conjunction with the diffusible factors that are already being secreted into the local environment. From this novel identification, I anticipate that a more complete understanding of the cell surface and intracellular signaling events that mediate this effect might provide a novel therapeutic dimension targeted to reduce inflammation in obese adipose.

CHAPTER 3

Data contained within this chapter will be submitted for publication.

Authors include: Carolina Franco Nitta and Robert A. Orlando

3. NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways regulate cytokine secretion in crosstalk between adipocytes and immune cells

3.1. Introduction

Adipose tissue was originally known for its major fat storage capacity; however, in more recent years it has been characterized as an important endocrine organ. Recent studies have shown that obese individuals demonstrate an increased infiltration of immune cells such as macrophages, T, and B lymphocytes in their adipose tissue depots (Duffaut et al., 2009; Feuerer et al., 2009; McDonnell et al., 2012; Nishimura et al., 2009; Weisberg et al., 2003; Winer et al., 2011). These immune cells, in conjunction with adipocytes, secrete pro-inflammatory cytokines, mainly tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) (Fantuzzi, 2005; Wellen and Hotamisligil, 2003). TNFα and IL-6 are the major cytokines measured in response to obesity, with increased circulating levels and a direct correlation with insulin resistance (Cottam et al., 2004; Hotamisligil et al., 1994; Lumeng et al., 2007b; Pickup and Crook, 1998; Ruan et al., 2002). MCP-1 is considered a

monocyte/macrophage chemoattractant, responsible for recruitment of these immune cells into expanding adipose tissue (Fantuzzi, 2005; Kamei et al., 2006; Kanda et al., 2006; Wellen and Hotamisligil, 2003). In obesity, an enhanced lipid load and chronic low-grade pro-inflammatory phenotype has further detrimental and comorbid consequences, such as type II diabetes mellitus, cardiovascular diseases, and more recently some types of cancer (Ford et al., 2002; Garrow, 1988; Grundy et al., 2004; Renehan et al., 2008).

Immune cells infiltrating into adipose tissue come into direct contact with adipocytes, allowing "crosstalk" amongst them (Lumeng et al., 2007b). I have shown that the physical contact between these cell types is necessary for the exacerbation of the pro-inflammatory cytokine effects seen, whereas the diffusible factors alone cannot mimic that same profile (Nitta and Orlando, *submitted*, Chapter 2). The result of this interaction between these cells (whether diffusible or derived from contact) is downstream activation of important signaling cascades and ultimately transcription and secretion of pro-inflammatory cytokines, contributing to the obese inflammatory profile. Aside from traditional secreted cytokines, obese individuals have also recently been shown to exhibit endotoxemia, which are higher levels of circulating lipopolysaccharides (LPS) from gram-negative bacteria, due to changes in the gut microbiota as well as an increase in gut permeability (Burcelin et al., 2011; Cani and Delzenne, 2009). LPS is considered a potent stimulator of toll-like receptor 4 (TLR4), leading to downstream signaling and further transcription of pro-inflammatory cytokines.

Previous studies are able to give me insight in which signaling cascades are important for adipocyte biology and inflammation. TNFα plays a significant role in

insulin resistance and type II diabetes mellitus (Hotamisligil et al., 1993; Uysal et al., 1997), as well as directly activate NF-κB (Nuclear Factor-Kappa B) signaling (Ruan et al., 2002). Indeed, our group has demonstrated that cultured adipocytes increase IL-1β, IL-6, and cyclooxygenase-2 (COX-2) cytokine release in response to TNFα stimulation through signaling via NF-κB (Gonzales and Orlando, 2008). Treatment of 3T3-L1 cells or human adipocytes with LPS, and its subsequent binding to TLR4 leads to activation of NF-κB, and further downstream secretion of IL-6 and TNFα (Bès-Houtmann et al., 2007; Creely et al., 2007; Lin et al., 2000). TNFα activates the MAPK (mitogen-activated protein kinase) pathways, such as p38, MEK/Erk (p44/42 – Extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) in human fat cells (Jain et al., 1999; Ryden et al., 2002). Like other cytokines, IL-1β also has the potential to signal via NF-κB and the MAPKs (Jager et al., 2007; Kumar et al., 2003; Martin and Wesche, 2002). The activation of the JNK pathway can lead to insulin resistance (Sabio and Davis, 2010), whereas its deficiency in a murine model leads to the improvement of systemic insulin sensitivity (Hirosumi et al., 2002). From adipocyte and cell culture data, I have learned that MEK1/Erk1 and JNK are active signaling pathways in these cells (Ryden et al., 2002). Aside from cytokine signaling, insulin is considered the major PI3K (phosphatidyl-inositol 3 kinase) pathway activator in obesity (Li et al., 2012). Prior studies have shown that activation of PI3K pathway in monocytic cells can negatively regulate LPS-stimulated production of pro-inflammatory cytokines (Guha and Mackman, 2002; Schabbauer et al., 2004), potentially exerting an opposite effect than NF-κB and the MAPKs. Unfortunately, no data is available regarding the importance or specific

contributions of all of these major signaling cascades in a model system including adipocytes and infiltrating cells, mimicking an obese setting.

I have developed and characterized a novel in vitro cell-culture model to mimic, in a more physiological manner, the properties of immune cell infiltration into obese adipose tissue (Nitta and Orlando, *submitted*, Chapter 2). I further advance these studies by looking specifically at the signaling cascades involved in this setting, by using non-toxic levels of NF-κB, p38, MEK/Erk, JNK, and PI3K inhibitors to elucidate their importance in adipocyte and immune cell contact-mediated cytokine secretion.

3.2. Materials and Methods

Animals and Animal Care

Male C57Bl/6J (Stock #000664) mice were purchased from Jackson Laboratories at 8 weeks of age. Animals were housed 2 per cage in a pathogen-free environment on a 12 h light/dark cycle and had free access to food and water. Mice were euthanized by CO_2 asphyxiation and processed immediately for spleen removal. All procedures in this study were submitted and approved by the Institutional Animal Care and Use Committee of the University of New Mexico.

Splenocyte isolation

Mouse spleens were used as a source of immune cells in the co-culture experiments. Splenocyte isolation was performed according to Kruisbeek (Kruisbeek, 2001), from spleens removed from C57BL/6J mice. Briefly, after spleens were removed,

they were placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin (complete DMEM). Spleens were homogenized into single cell suspensions by gently disaggregating tissue between frosted ends of two microscopy slides, filtered through a 100 μm cell strainer, and then centrifuged at 800 x g for 3 min at 4° C. Supernatants were discarded and cell pellets were resuspended in 1 ml ACK Lysis Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2-EDTA; pH 7.4) for 5-10 min to remove contaminating red blood cells. Complete DMEM was added to stop red blood cell lysis and cells were centrifuged again at 800 x g for 3 min at 4° C. Supernatants were removed; cells were resuspended in complete DMEM medium, counted using a hemocytometer, and subsequently used for cell viability assays and co-culture experiments.

Cytotoxicity WST-1 Assay

The cytotoxic effects of the inhibitors used in this study were determined by the WST-1 assay (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's recommendations, measured by cell viability. Splenocytes were plated at a 4.5 x 10^4 density in 96-well plates with complete DMEM medium with varying concentrations of each inhibitor Bay11-7082 (Bay11, 0.625, 1.25, 2.5, 5, 10, 20, and 40 μM; Calbiochem, La Jolla, CA), SP600125 (0.625, 1.25, 2.5, 5, 10, 20, and 40 μM; Sigma Aldrich, St. Louis, MO), LY294002 (1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 μM; Promega, Madison, WI), PD98059 (1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 μM; Promega, Madison, WI), SB203580 (0.625, 1.25, 2.5, 5, 10, 20, and 40 μM; Promega,

Madison, WI), or vehicle alone (DMSO at 0.5% final concentration). Splenocytes were incubated for 24 h at 37° C with 5% CO_2. WST-1 was added to the cultures to a final concentration of 10% (vol/vol), and incubated at 37° C with 5% CO_2 for an additional 4 h. The plate was shaken and absorbance measured at 440 nm for the indicator color, subtracted from the 600 nm reference wavelength. Untreated splenocytes (DMSO alone) were set to 100% viability.

Cell Culture and Adipocyte Differentiation

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured and differentiated according to Gonzales and Orlando, 2008 (Gonzales and Orlando, 2008). Briefly, cells were cultured in complete DMEM media at 37° C with 5% CO_2 and passaged twice weekly. For differentiation, cells were seeded into 6-well cell culture plates coated with 1% gelatin. When cells reached confluency, they were treated with 250 nM dexamethasone, 450 μM 3-isobutyl-1-methylxanthine and 167 nM insulin for 3 days, followed by 167 nM insulin for an additional 3 days. Differentiation was confirmed by morphological changes, including intracellular lipid droplet accumulation.

Adipocyte and Splenocyte Co-cultures

Differentiated 3T3-L1 cells were co-cultured with isolated splenocytes from mice (1.5×10^6) in direct and indirect contact systems in the presence of LPS (*E. coli* 0111:B4 – 1 μg/ml; Sigma Aldrich, St. Louis, MO) for splenocyte activation. Individual cultures of 3T3-L1 cells or splenocytes with LPS were performed as controls. In direct co-

cultures, splenocytes were added to differentiated 3T3-L1 cells, allowing direct contact between the two cell types. For indirect co-cultures, cells were cultured in a transwell system, with differentiated 3T3-L1 cells in the lower chamber and the splenocytes seeded in a 0.4 μm hanging cell insert (Millipore, Billerica, MA). Cell culture experiments were performed in the presence of the cell pathway inhibitors (non-toxic concentrations, as determined by WST-1 assay) Bay11 (NF-κB inhibitor, 2 μM), SP600125 (JNK inhibitor, 2.5 μM), LY294002 (PI3K inhibitor, 1 μM), PD98059 (MEK1 inhibitor, 6.25 μM), SB203580 (p38 inhibitor, 5 μM), or vehicle alone (DMSO at 0.1% final concentration). Co-cultures were incubated for 24 h at 37° C with 5% CO_2 and media subsequently centrifuged and harvested for cytokine ELISA measurements.

Cytokine ELISAs

Murine TNFα, IL-6, and MCP-1 levels in co-culture experiment supernatants were measured by ELISA Ready-Set-Go! kit (eBioscience, San Diego, CA) and conducted as described by the manufacturer. Media samples were diluted accordingly to ensure samples were within the detection kit sensitivity, specifically for MCP-1 and IL-6.

Statistical Analysis

Statistical significance was determined by performing a t-test with Sigma Plot (Version 7.0) on replicate samples. Statistically significant differences were considered when $p < 0.05$.

3.3. Results

Cytotoxicity of inhibitors on splenocytes.

I determined the effects of each of the inhibitors used in this study on splenocyte viability by performing WST-1 cytotoxicity assays (Fig. 3.1). As such, I determined the maximum concentration of inhibitor for my adipocyte and splenocyte co-culture experiments without inducing cell death. I focused on the effects of the inhibitor alone on cytokine secretions, and not cell death-induced decreases in cytokine secretions. Bay11, a potent NF-κB inhibitor, demonstrated minimal cell death (~8%) at 2.5 μM concentration, however only 20% viability at 5 μM (Fig. 3.1A), and an LD_{50} of 4 μM. I have also determined the cytotoxicity of Bay11 on 3T3-L1 cells, and determined that a concentration of 2 μM does not lead to cell death (data not shown). PD98059 (MEK1 inhibitor) exhibited fairly low overall cytotoxic effects on splenocytes, with minimal death up to 6.25 μM concentration, whereas the highest concentration of PD98059 used, 100 μM, was responsible for only 40% death (Fig. 3.1B). The p38 inhibitor SB203580 showed low cytotoxicity with only 20% cell death at a concentration of 5 μM, but complete cell death at 40 μM (Fig. 3.1C). Lower concentrations of SP600125 (JNK inhibitor) did not exhibit cytotoxic effects, with only 10% cell death at 2.5 μM concentration, yet 60% cell death at a 40 μM concentration (Fig. 3.1D). LY294002 (PI3K inhibitor) demonstrated the highest cytotoxicity of the compounds tested, with 25% death at the lowest concentration of 1.5625 μM, and complete cell death at 12.5 μM (Fig. 3.1E). For the remainder of my studies, I used the following inhibitor concentrations, with minimal or no cell death: Bay11, 2 μM; PD98059, 6.25 μM; SB203580, 5 μM; SP600125, 2.5 μM; and LY294002, 1 μM.

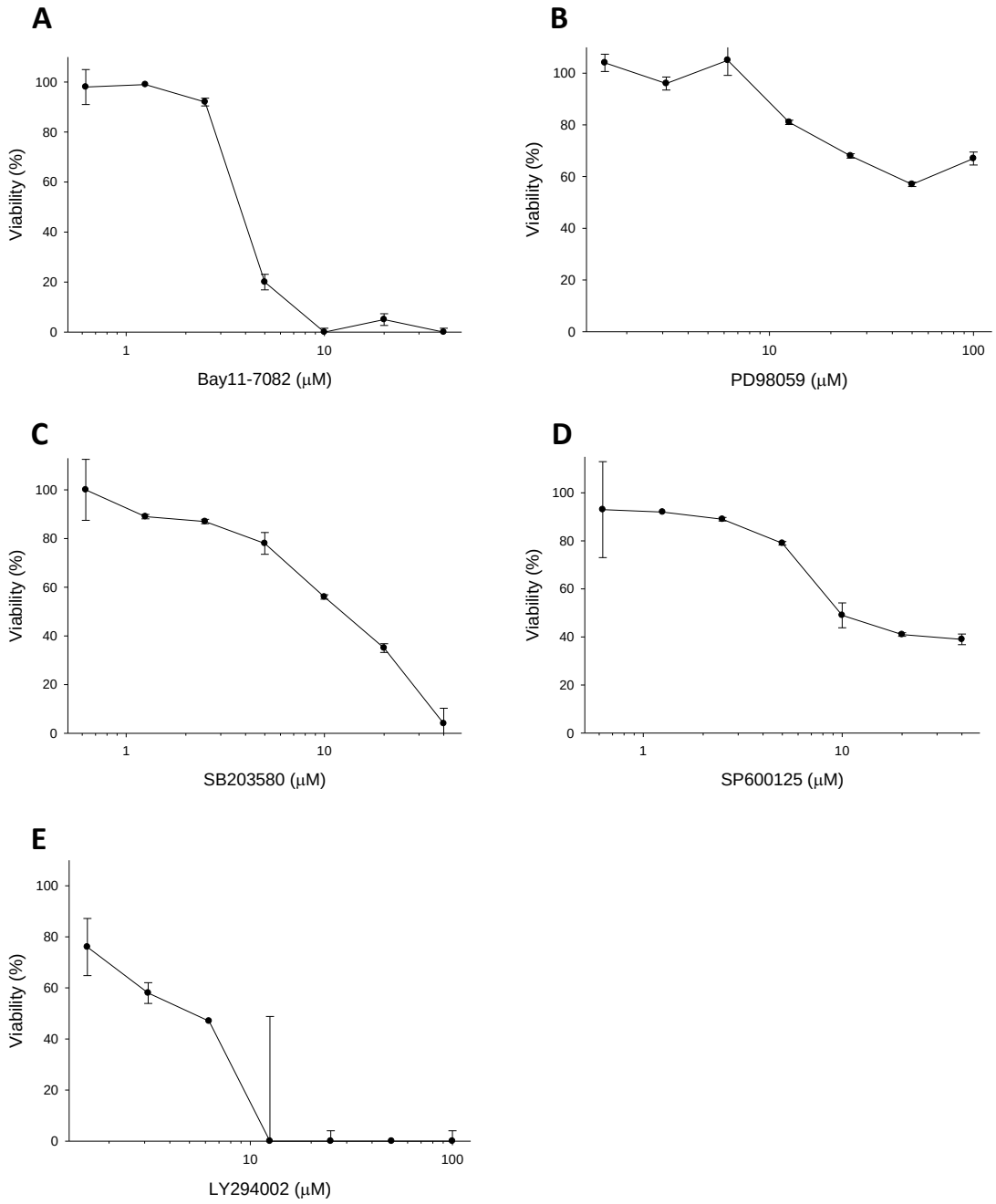

Figure 3.1 – Chemical signaling inhibitors affect splenocyte viability. Isolated splenocytes were incubated alone or with the indicated increasing concentrations of the chemical inhibitors Bay11-7082 (A), PD98059 (B), SB203580 (C), SP600125 (D), or LY294002 (E). Cell survival was determined by WST-1 assay and represented as a percentage of splenocyte viability treated with vehicle alone.

NF-κB regulates TNFα, IL-6, and MCP-1 cytokine secretion in co-cultures.

I examined the importance of the NF-κB pathway in a co-culture of adipocytes (differentiated 3T3-L1 cells) and immune cells (murine splenocytes), by using the chemical inhibitor Bay11. Cells were either cultured individually in the presence of LPS, as a control; or co-cultured with no contact between the cell types (in transwells) or in direct contact with each other, also in the presence of LPS. Results demonstrate that NF-κB plays a significant role in TNFα, IL-6, and MCP-1 cytokine secretions (Fig. 3.2).

Splenocytes cultured alone with LPS stimulation secrete high amounts of TNFα, whereas the co-culture of these cells with adipocytes leads to decreased secretion, and direct contact dampens this effect even further (Fig. 3.2A, black bars). Bay11 completely inhibits TNFα secretion of LPS-stimulated splenocytes (Fig. 3.2A, column 1), as well as co-cultures of adipocytes and splenocytes without contact (Fig. 3.2A, column 2). Nonetheless, when both cells are co-cultured in direct contact with each other in the presence of the NF-κB inhibitor a residual TNFα secretion is sustained (Fig. 3.2A, column 3).

Monocultures of adipocytes in the presence of LPS secrete low levels of IL-6 and MCP-1, with increasing levels in transwell co-cultures with adipocytes, and even further enhancement when cells are in direct contact (Fig. 3.2B and C, black bars). Bay11 does not influence the secretion of IL-6 or MCP-1 in monoculture of adipocytes with LPS (Fig. 3.2B and C, column 1). However, Bay11 decreases the secretion of IL-6 and MCP-1 in no contact co-cultures to the levels of the individual cultures of adipocytes with LPS (Fig. 3.2B and C, column 2, grey bar, compare to column 1). In a similar fashion, inhibition of NF-κB in direct contact co-cultures decreases IL-6 secretion approximately

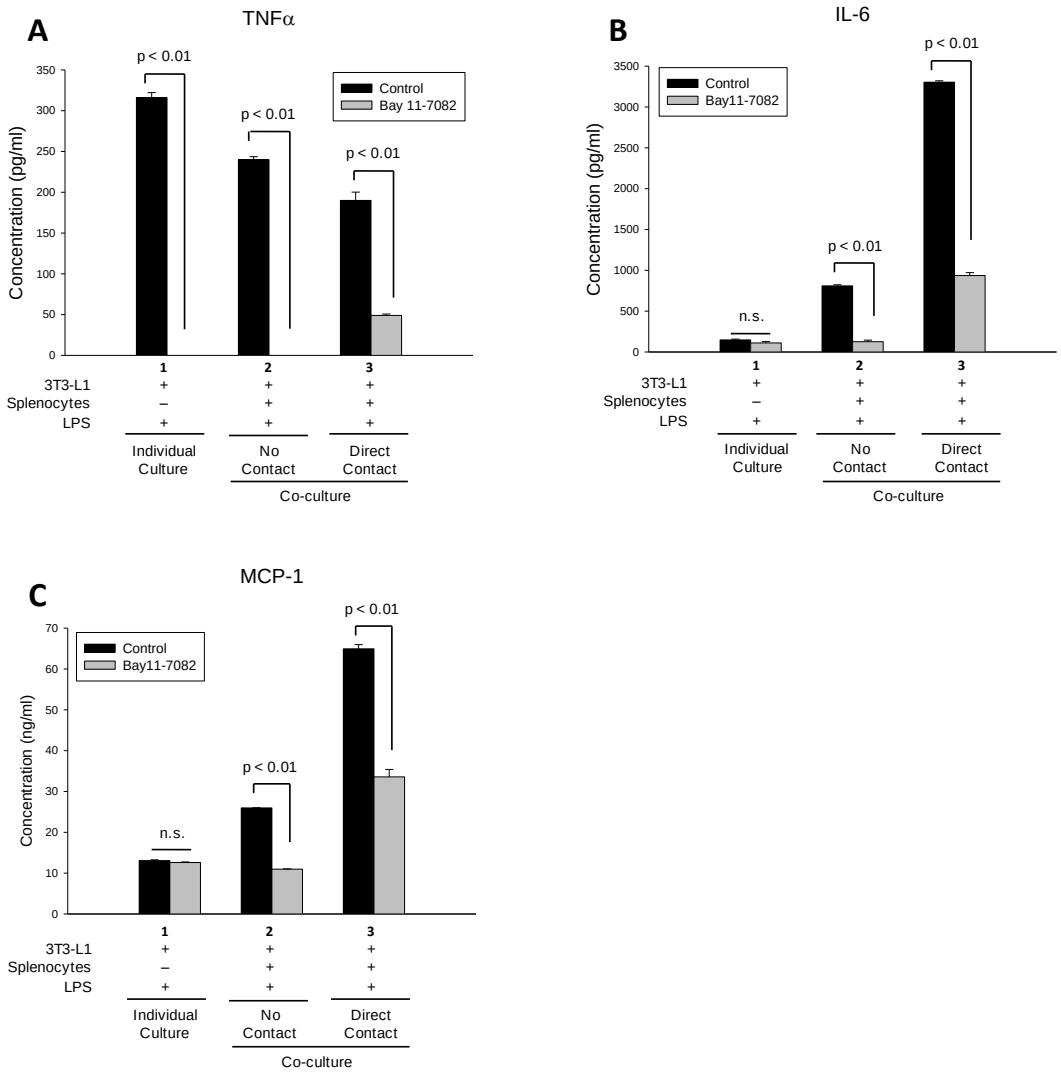

Figure 3.2 – NF-κB inhibitor Bay11-7082 alters TNFα, IL-6, and MCP-1 cytokine secretions from adipocyte and splenocyte co-cultures. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were cultured with LPS, individually or together in direct contact or no contact (separated by a 0.4 µm transwell filter), in the absence (DMSO control; black bars) or presence of the NF-κB inhibitor Bay11-7082 (2 µM; grey bars) for 24 h. Secreted TNFα (A), IL-6 (B), and MCP-1 (C) cytokines were quantified by capture ELISA.

70% (Fig. 3.2B, column 3), and MCP-1, 50% (Fig. 3.2C, column 3). Overall, NF-κB is the dominant signaling cascade for TNFα secretion, independent of cell type, and with an important role in IL-6 and MCP-1 secretion in co-cultures.

PI3K and MAPKs have a secondary role in signaling between adipocytes and immune cells

Individual cultures and co-cultures were further examined with regards to other important signaling pathways in these cells, such as PI3K and the MAPK pathways, MEK1/Erk1, p38, and JNK by use of chemical inhibitors. Given that NF-κB plays such a prominent role in TNFα secretion, I investigated the role of the other signaling pathways with regards to IL-6 and MCP-1 secretion. Addition of the PI3K inhibitor LY294002 to individual cultures and co-cultures of cells showed no changes in IL-6 and MCP-1 cytokine secretions, demonstrating that this pathway does not seem to play a role in these contact-mediated cytokine secretion enhancements in my co-cultures (data not shown).

IL-6 secretion is unaltered by PD98059 treatment (MEK1 inhibitor) when cells are in direct contact or no contact (Fig. 3.3A). Alternatively, the MEK1/Erk1 pathway is required for the secretion of MCP-1, since the specific inhibitor decreased the secretion of this chemokine by approximately 30% when cells are cultured without contact or in direct contact (Fig. 3.3B). I have demonstrated that the majority, if not all, of MCP-1 expression is derived from adipocytes (Nitta and Orlando, *submitted*, Chapter 2), therefore the inhibition of MEK1 on MCP-1 secretion can be attributed specifically to these cells. The p38 MAPK inhibitor SB203580 lowered the levels of IL-6 secretion in no contact and direct contact co-cultures by ~55% (Fig. 3.4A). Still, SB203580 had an

effect on MCP-1 secretion only in the co-culture where cells were in direct contact with each other, decreasing its secretion by 20% (Fig. 3.4B). Lastly, IL-6 and MCP-1 production was altered by treatment with the JNK inhibitor, SP600125 (Fig. 3.5). In co-cultures with no contact, SP600125 decreased IL-6 secretion by 20%, whereas in co-cultures with direct contact, the reduction was 30% (Fig. 3.5A). For MCP-1 secretion, the effects of SP600125 on MCP-1 were less prominent, with a 15% decrease in cells cultured with a transwell, and no significant effect on direct contact co-cultures (Fig. 3.5B). Altogether, these data demonstrate that p38 and JNK are activated in adipocyte and immune cell co-cultures resulting in IL-6 secretion, whether without contact (where only diffusible factors are present) or in direct contact (where diffusible factors are present plus cell-cell contact). For MCP-1 secretion, MEK1/Erk1 is a major signaling pathway in indirect and indirect contact of these cells, with p38 only impacting the contact-mediated induction, and JNK having a very minor role in this process.

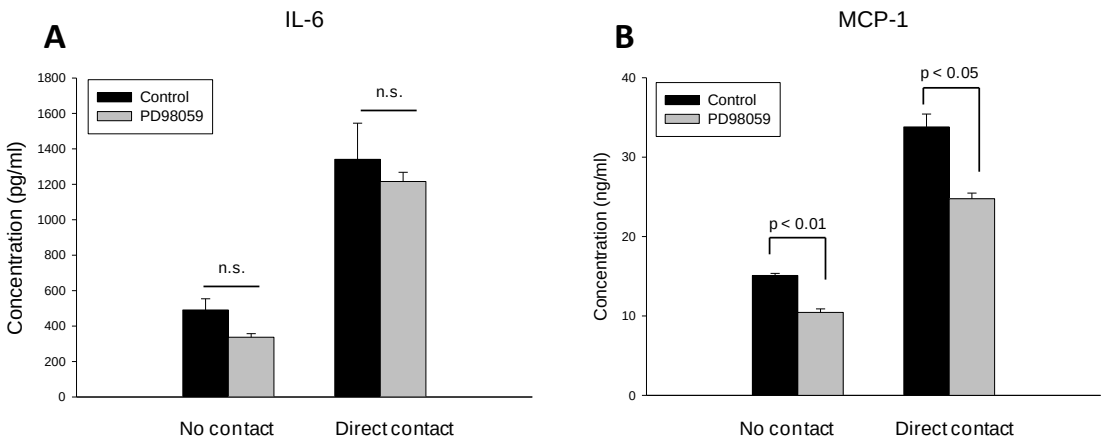

Figure 3.3 – MEK1 signaling is required for MCP-1 secretion in adipocytes. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were co-cultured with LPS together in direct contact or no contact (separated by a 0.4 µm transwell filter), in the absence (DMSO control; black bars) or presence of the MEK1 inhibitor PD98059 (6.25 µM; grey bars) for 24 h. Secreted IL-6 (A) and MCP-1 (B) cytokines were quantified by capture ELISA.

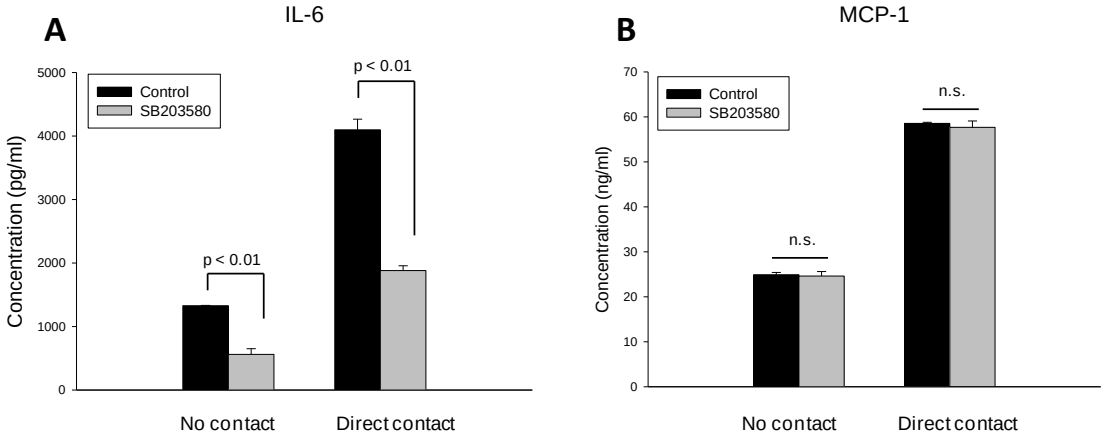

Figure 3.4 – Inhibition of p38 MAPK by SB203580 alters IL-6 secretion in adipocyte and splenocyte co-cultures. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were co-cultured with LPS together in direct contact or no contact (separated by a 0.4 µm transwell filter), in the absence (DMSO control; black bars) or presence of the MEK1 inhibitor SB203580 (5 µM; grey bars) for 24 h. Secreted IL-6 (A) and MCP-1 (B) cytokines were quantified by capture ELISA.

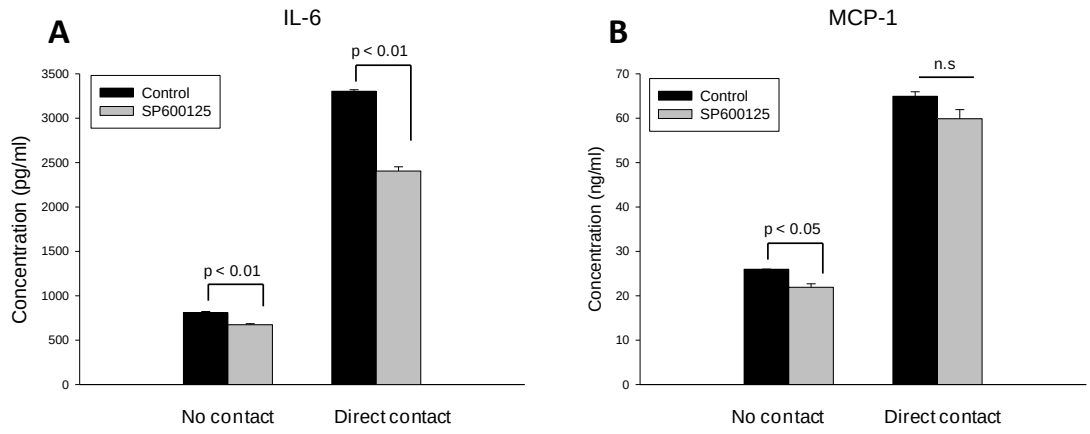

Figure 3.5 – JNK pathway is involved in IL-6 and MCP-1 secretion by adipocytes and splenocytes. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were co-cultured with LPS together in direct contact or no contact (separated by a 0.4 µm transwell filter), in the absence (DMSO control; black bars) or presence of the MEK1 inhibitor SP600125 (2.5 µM; grey bars) for 24 h. Secreted IL-6 (A) and MCP-1 (B) cytokines were quantified by capture ELISA.

3.4. Discussion

It is well established that NF-κB and its activator IKK are important signaling cascades in obesity, and their activation can lead to insulin resistance (Chiang et al., 2009; Gao et al., 2002; Hundal et al., 2002; Shoelson et al., 2003; Yin et al., 1998; Yuan et al., 2001). NF-κB activation is also paramount for generation of inflammation, such that a recent study in diet-induced obese mice with constitutively active IKK in adipose tissue exhibited enhanced mRNA levels of IL-6 and MCP-1 (Jiao et al., 2012). LPS is also responsible for potently activating this cascade in 3T3-L1 adipocytes, resulting in increased expression of TNFα and IL-6 (Song et al., 2006). Additionally, human adipose tissue treated with different NF-κB inhibitors can significantly reduce the release of TNFα and IL-6 (Lappas et al., 2005). My results demonstrate that TNFα secretion is

completely abrogated in individual splenocyte cultures or indirect co-cultures treated with Bay11, demonstrating the importance of the NF-κB pathway for transcription of this cytokine. NF-κB regulates the TNFα secretion mediated by diffusible factors in this co-culture system with adipocytes and immune cells. However, in direct contact co-cultures, treatment with Bay11 was unable to completely obliterate TNFα secretion. This residual TNFα secretion allows me to speculate that cell contact between adipocytes and splenocytes can lead to activation of additional signaling cascades. Indeed, the promoter region of the TNFα gene has been mapped, leading to the identification of the presence of seven differential transcription factor binding sites, all necessary for full activation of TNFα expression following LPS stimulation (Tsai et al., 2000; Yao et al., 1997). While NF-κB is the major transcription factor for TNFα secretion in no-contact co-cultures, consisting of an environment where only diffusible factors are available to stimulate it, alternative transcription factors can become activated when adipocytes and splenocytes engage one another. Furthermore, inhibition of NF-κB in co-cultures without contact resulted in a decrease of IL-6 and MCP-1 secretions to similar levels of individual adipocyte cultures, indicating that this pathway is important for the regulation of these cytokines when paracrine factors are analyzed. The levels of IL-6 and MCP-1 were also decreased in direct contact co-cultures treated with Bay11. I have recently shown that TNFα is responsible for the contact-mediated cytokine enhancement of IL-6 and MCP-1 in my model system (Nitta and Orlando, *submitted*, Chapter 2), and since direct contact co-cultures treated with Bay11 have limited secretion of TNFα, the decrease of IL-6 and MCP-1 seen could be due to the lower concentrations of TNFα in that system.

Since it has been demonstrated that insulin can activate PI3K in obesity (Li et al., 2012), I investigated its role in crosstalk between adipocytes and immune cells in my co-culture model. My results demonstrated that IL-6 and MCP-1 secretions were unaltered by the chemical inhibitor LY294002 when cells were either in direct contact or no contact. Prior studies have demonstrated that activation of the PI3K/Akt pathway in monocytic cells is responsible for inhibition of LPS-activated TNFα secretion (Guha and Mackman, 2002; Schabbauer et al., 2004), however the concentrations of the inhibitor LY294002 used in these experiments are 10 μM. This competitive inhibitor was highly toxic to splenocytes at low concentrations, so I chose to use minimal levels in my co-culture studies, minimizing cell death. LY294002 has been shown to be poorly selective for its target in vivo, however, the IC_{50} in vitro is reported between 0.5-1.5 μM (Stein, 2001). The concentration used in this study, therefore is sufficient for partial inhibition of this kinase (1 μM). However, its use did not alter any cytokine secretions, demonstrating that it most likely does not play a role in adipocyte or splenocyte IL-6 and MCP-1 production.

TNFα can distinctly activate the three mammalian MAPKs, namely Erk1/2, p38, and JNK in adipocytes, whereas LPS can activate these pathways in macrophages (Geppert et al., 1994; Hambleton et al., 1996; Han et al., 1994; Jain et al., 1999; Ryden et al., 2002; Shi et al., 2002). The importance of these pathways in my co-culture model was investigated with regards to their contributions in the secretions of IL-6 and MCP-1.

Erk1 deficient mice show that these animals are resistant to high fat diet (HFD) induced obesity and are protected from insulin resistance (Bost et al., 2005). Additionally, activation of the MEK/Erk pathway results in downstream up-regulation of

TNFα secretion (Ryden et al., 2002; Shi et al., 2002), whereas inhibition of this pathway, by use of PD98059, impedes TNFα-mediated Erk1 phosphorylation in human fat cells (Ryden et al., 2002). Unfortunately, there is a gap in knowledge regarding the importance of the Erk pathway in downstream IL-6 secretion in an obese setting. My studies demonstrate that the use of the MEK1 inhibitor PD98059 does not significantly influence IL-6 secretion, and most likely its regulation can be determined by other cell signaling pathways.

Several stimuli, including TNFα, IL-6, and insulin, are capable of inducing transcription of MCP-1 in 3T3-L1 cells (Fasshauer et al., 2004; Ito et al., 2007), which can have important implications in the exacerbation of the inflammatory profile in obese individuals. These studies looked at the relevance of certain downstream signaling cascades in the expression of MCP-1 and found that the MEK1/Erk1 MAPK pathway mediated this process, at least in part, with no involvement of p38 and PI3K (Fasshauer et al., 2004; Ito et al., 2007). My prior studies show that MCP-1 is a chemokine predominantly secreted by adipocytes (Nitta and Orlando, *submitted*, Chapter 2) and I wanted to investigate the signaling cascades responsible for MCP-1 secretion in a setting that more clearly mimics an obese phenotype. I demonstrate that MEK1 is important for MCP-1 protein production when immune cells and adipocytes communicate through paracrine factors as well as when they engage in direct contact with one another. Altogether, my results confirm that MCP-1 secretion by adipocytes is mediated by the NF-κB and MEK1 pathways, with minimal or no involvement of p38, JNK, and PI3K.

Current studies conducted with murine models deficient in the downstream substrate of p38, the MAPK-activated protein kinase 2 (MAPKAP K2) demonstrate

significant decrease in the production of IL-6 and TNFα (Kotlyarov et al., 1999). Direct activation of p38 leads to TNFα, IL-1, and IL-6 production, whereas its inhibition, by use of SB203580, leads to decreased levels of these cytokines in human monocytes and macrophages (Kumar et al., 2003; Kumar et al., 1999). It is important to note that my co-culture experiments were conducted with considerably smaller concentrations of SB203580 (5 μM) than prior studies, decreasing the chances of off-target inhibition and minimizing cell death, however still significantly suppressing its activity; this inhibitor has enhanced selectivity and potency for p38, with IC_{50} levels for cytokine release between 0.04 – 0.6 μM (Cuenda et al., 1995; Jain et al., 1999; Kumar et al., 1999). With these in mind, my adipocyte and splenocyte co-cultures treated with SB203580 resulted in decreased levels of IL-6 secretion, confirming previous reports (De Cesaris et al., 1998). Additionally, my data shows that p38 is important in both cell culture systems, in which splenocytes and adipocytes can communicate with each other via paracrine factors (no contact), or when these cells are in direct contact with each other, allowing for paracrine factors and cell-cell contact.

High fat diet (HFD)-fed and ob/ob mice have abnormally higher levels of JNK activation, and increased expression of IL-6 (Hirosumi et al., 2002; Sabio and Davis, 2010). TNFα binding to the TNFR1 or LPS binding to TLRs, as demonstrated in obesity, leads to the activation of the JNK pathway, causing downstream effects on the insulin receptor (IR), and ultimately insulin resistance, through phosphorylation of Ser307 on IRS-1 (Aguirre et al., 2000; Hirosumi et al., 2002; Medzhitov, 2001; Ryden et al., 2002). JNK is involved in the regulation of lipolysis (Ryden et al., 2002), and TNFα expression itself through activator protein-1 (AP-1) (Zagariya et al., 1998). Prior animal studies

have demonstrated that whole body JNK1 inhibition in HFD-fed or ob/ob mice results in protection from heightened adiposity, with reduced plasma levels of IL-6 and improved insulin sensitivity (Sabio et al., 2008). Furthermore, selective inactivation of JNK1 and JNK2 in adipose tissue and macrophages ameliorates the HFD-induced obesity dysfunctions, similar to whole-body depletions (Zhang et al., 2011). These tissue-specific knock-out mice also exhibit decreased IL-6 levels, as well as diminished expression of IL-6, TNFα, and MCP-1, with a decrease in macrophage infiltration, similar to their wild-type counterparts (Tuncman et al., 2006; Zhang et al., 2011). The JNK inhibitor used in this study, SP600125, affects all three gene products, JNK1, JNK2, and JNK3, as a competitor to the ATP-binding site, with an IC_{50} between 0.04 – 0.09 μM (Bennett et al., 2001). The concentrations used in my experiments are above the levels to inhibit 50% of the kinase, but still ensure cell health. It has been shown to block LPS-induced expression of TNFα (Bennett et al., 2001). My studies demonstrate that JNK plays a role in IL-6 secretion of adipocytes and immune cells, since addition of the specific inhibitor leads to decreased secretion of this cytokine, confirming the importance of this pathway in hindering cytokine production. Indeed, the therapeutic potential of specific JNK inhibitors has been demonstrated in type II diabetes mellitus and atherosclerosis (Kaneto et al., 2004; Ricci et al., 2004), making this a strong candidate for targeted drug therapy in obesity and its associated complications.

Cell signaling pathways can converge and cross-activate each other, making it even more difficult to understand the regulation of cytokine secretion in obesity (Gregor and Hotamisligil, 2011). Previous studies have identified overlap in these signaling cascades. In adipocytes, the use of PD98059 blocked the nuclear localization of NF-κB,

demonstrating that MEK1 is also responsible for downstream activation of this transcription factor (Jain et al., 1999). Additionally, NF-κB signaling decreases JNK cascade activation, establishing a link between these two pathways (De Smaele et al., 2001; Jiao et al., 2012). However, activation of JNK can lead to down-regulation of PI3K/Akt (Aguirre et al., 2000; Lee et al., 2003). In my adipocyte and splenocyte co-culture model, with paracrine stimulation alone or with paracrine stimulation in conjunction with cell contact, I investigated the importance of each signaling pathway individually, contributing to the understanding of the regulation of the pro-inflammatory cytokines TNFα, IL-6, and MCP-1 in an obese setting. Integration of these cascades and diverse cytokine stimulations will allow identification of novel drug targets for reducing inflammation in obese adipose.

CHAPTER 4

4. Expression changes of cell adhesion molecules on adipocytes that are in direct contact with immune cells

4.1. Introduction

Adipose tissue (AT) is a dynamic organ of great interest because of its now established role in the development of metabolic syndrome is obese individuals (Ford et al., 2002; Matsuzawa et al., 1999). Excessive adiposity has been positively associated with immune cell infiltration, namely B cells, T cells (Duffaut et al., 2009; Winer et al., 2011), and macrophages. Macrophage recruitment is due to secretion of AT-derived MCP-1 (monocyte chemoattractant protein-1) secretion (Kamei et al., 2006; Kanda et al., 2006; Weisberg et al., 2003). The invasion of immune cells into AT leads to an increase in TNFα (tumor necrosis factor alpha) and IL-6 (interleukin-6) cytokine production towards a pro-inflammatory state (Fantuzzi, 2005; Wellen and Hotamisligil, 2003). This chronic inflammation significantly increases an individual's risk for developing atherosclerotic lesions and insulin resistance (Grundy et al., 2004; Xu et al., 2003).

I have recently demonstrated that crosstalk between these infiltrating immune cells and lipid-laden adipocytes can lead to changes in inflammatory cytokines in a co-culture model (Nitta and Orlando, *submitted*, Chapter 2). I also clearly elucidated the importance of direct contact between these two cell types, whereas cells co-cultured without contact (only allowing exchange of diffusible paracrine factors) did not elicit the same response (Nitta and Orlando, *submitted*, Chapter 2). This observation led me to

speculate that these cells must exhibit cell adhesion molecules (CAMs) on their surfaces that give rise to this change in cytokine release. Currently, there is no evidence describing which cell surface molecules could be responsible for this heightened inflammatory response when there is cell-cell contact. Nonetheless, there are a few studies that identify CAMs on the surface of adipocytes that could shed some light on this subject. These cells display a novel adipocyte cell adhesion molecule, ACAM (also known by CLMP, CAR-like membrane protein) that is responsible for mediating cell-cell contact (Eguchi et al., 2005), and a neural cell adhesion molecule (NCAM), important for adipocyte differentiation (Yang et al., 2011). Vascular adhesion protein (VAP-1, also named semicarbazide sensitive amine oxidase, SSAO) and ICAM-1 (intercellular adhesion molecule-1) have also been detected on the surface of fat cells, both of which can be cleaved and lead to the shedding of a soluble form (Abella et al., 2004; Brake et al., 2006). These soluble proteins can enhance lymphocyte adhesion, and are important for immune cell infiltration in obese adipose tissue (Abella et al., 2004; Brake et al., 2006). Additionally, preadipocytes and adipocytes when stimulated with TNFα secrete factors responsible for enhancing leukocyte-endothelial cell adhesion (Mack et al., 2009). One study focused on a specific CAM on adipocytes and its role in obesity. The tight junction protein, claudin 6, was found to be expressed in four different adipose tissues and up-regulated in all of them when mice were fed a HFD (Hong et al., 2005). All of this compelling evidence led to the postulate that adipocytes, a non-polarized cell, can ultimately exhibit a variety of CAMs, which have the potential for cell-cell contact, or other novel functions, such as signaling, that can be regulated in obesity.

In this current study, I sought to determine the adhesion molecules present on the surface of adipocytes that could be mediating the effect of cellular crosstalk between adipocytes and infiltrating immune cells. To do so, I investigated the changes in cell adhesion molecules when adipocytes (differentiated 3T3-L1 cells) were co-cultured in contact with LPS-activated murine immune cells (splenocytes), mimicking an obese setting.

4.2. Materials and Methods

Animals and Animal Care

Male GFP (C57Bl/6-Tg(UBC-GFP)30Scha/J; ubiquitous expression of Green Fluorescent Protein; Stock #004353) mice were purchased from Jackson Laboratories at 8 weeks of age. Animals were housed 2 per cage in a pathogen-free environment on a 12 h light/dark cycle and were provided free access to food and water. Mice were euthanized by CO_2 asphyxiation and processed immediately for spleen removal. All procedures in this study were approved by the Institutional Animal Care and Use Committee of the University of New Mexico.

Splenocyte isolation

Mouse spleens were used as a source of immune cells in the co-culture experiments. The splenocyte population includes a majority of B cells, T cells, and monocytes (Avitsur et al., 2002; Swirski et al., 2009). Splenocyte isolation was performed according to Kruisbeek (Kruisbeek, 2001), from spleens removed from GFP

mice. Briefly, after spleens were removed, they were placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL streptomycin sulfate, and 100 units/ml penicillin (complete DMEM). Spleens were homogenized into single cell suspensions by gently disaggregating tissue between frosted ends of two microscopy slides, filtered through a 100 μm cell strainer, and then centrifuged at 800 x g for 3 min at 4° C. Supernatants were discarded and cell pellets were resuspended in 1 mL ACK Lysis Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2-EDTA; pH 7.4) for 5-10 min to remove contaminating red blood cells. Complete DMEM was added to stop red blood cell lysis and cells were centrifuged again at 800 x g for 3 min at 4° C. Supernatants were removed; cells were resuspended in complete DMEM medium, counted using a hemocytometer, and subsequently used for co-culture experiments.

Cell Culture and Adipocyte Differentiation

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured and differentiated according to Gonzales and Orlando, 2008 (Gonzales and Orlando, 2008). Briefly, cells were cultured in complete DMEM media at 37° C with 5% CO_2 and passaged twice weekly. For differentiation, cells were seeded into 6-well cell culture plates coated with 1% gelatin. When cells reached confluency, they were treated with 250 nM dexamethasone, 450 μM 3-isobutyl-1-methylxanthine and 167 nM insulin for 3 days, followed by 167 nM insulin for an additional 3 days. Differentiation was confirmed by morphological changes, including intracellular lipid droplet accumulation.

Adipocyte and Splenocyte Co-cultures

Differentiated 3T3-L1 cells were co-cultured with isolated splenocytes from GFP mice (1.5×10^6) in direct and indirect contact systems in the presence of LPS (*E. coli* 0111:B4 – 1 μg/mL; Sigma Aldrich, St. Louis, MO) for splenocyte activation. For indirect co-cultures, cells were cultured in a transwell system, with differentiated 3T3-L1 cells in the lower chamber and splenocytes seeded in a 0.4 μm hanging cell insert (Millipore, Billerica, MA). For direct co-cultures, GFP splenocytes were added to differentiated 3T3-L1 cells, allowing direct contact between the two cell types. Co-cultures were incubated for 24 h at 37° C with 5% CO_2 and cells immediately harvested for sorting.

Fluorescence-Activated Cell Sorting

After 24 h of direct co-culturing of 3T3-L1 cells and splenocytes from GFP mice, cells were sorted by fluorescence-activated cell sorting (FACS). Co-cultures were trypsinized and centrifuged at 800 x g for 3 min at 4° C. Supernatants were removed and cells were resuspended in complete low serum (0.5% FBS) DMEM media with 5 mM EDTA and passed through a 100 μm cell strainer (BD Falcon) to ensure single-cell suspensions. GFP-positive and negative cells were sorted using the Beckman Coulter Legacy MoFlo high-speed sorter into separate tubes. The GFP-negative population (adipocytes) and GFP positive (splenocytes) were subsequently used for mRNA extraction.

RNA Isolation

Sorted cells were homogenized using the QIAshredder (Qiagen, Valencia, CA) and total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and used for the cell adhesion molecule array and confirmatory quantitative real-time PCR.

Cell Adhesion Molecule Array

Expression levels of 88 CAMs on adipocytes (either cultured in direct contact or in a transwell system with splenocytes) were quantified using the PrimerArray® Cell Adhesion Molecule Array for mouse (Takara Bio Inc) according to the manufacturer's recommendations on the LightCycler 480 Instrument (Roche Diagnostics, Indianapolis, IN). The fold changes of genes up- and down-regulated of the adipocytes in direct contact were calculated with the accompanying software in comparison to the adipocytes cultured with no contact. Expression levels were normalized to the 8 housekeeping genes on the PrimerArray®.

Quantitative RT-PCR Analysis

To confirm the adipocyte expression changes of the array, I performed qRT-PCR on the genes that had the highest induction or repression after direct co-culturing. These experiments were carried out using the LightCycler 480 SYBR Green I Master Mix chemistry (Roche Diagnostics, Indianapolis, IN) and analyzed on the LightCycler 480 instrument. Primer sequences for claudin-11 (Holmes et al., 2006) and 36B4 (Nitta and

Orlando, *submitted*, Chapter 2) have been previously published. Information on the other primer sequences used in this study with their annealing temperatures and fragment sizes can be found in Table 4.1. The reaction cycling parameters for Cadherin-1 (also known as E-cadherin) and claudin-11 was performed as a 3-step qRT-PCR with a pre-incubation step at 95° C for 5 min and amplification for 45 cycles at 95° C for 10 sec, 60° C for 15 sec, and 72° C for 1 sec. All other genes were amplified as a 2-step qRT-PCR reaction with a pre-incubation at 95° C for 15 min, followed by 40 cycles of amplification at 95° C for 15 sec, and the annealing temperature (Table 4.1) for 1 min. A melting curve analysis was performed in each experiment for all genes to confirm specificity of single-target amplification. Gene expression changes were calculated using the relative standard curve method (Livak, 1997) and 36B4 mRNA levels were used as a normalizer. All samples were amplified in triplicate. Levels of F4/80 (splenocyte) and adiponectin (adipocyte) were measured by qRT-PCR confirming pure populations of cells following cell sorting (Fig. 3.3B).

Statistical Analysis

Statistical significance was determined by performing a t-test with Sigma Plot (Version 7.0) on replicate samples. Statistically significant differences were considered when $p < 0.05$.

Gene	Primer sequences	Annealing temperature	Fragment size
Cadherin-1	Forward – CAA GGA CAG CCT TCT TTT CG Reverse – TGG ACT TCA GCG TCA CTT TG	60°C	165bp
Claudin-4	Forward – TGG AAC CCT TCC GTT GAT TA Reverse – CAC TGG GCT GCT TCT AGG TC	60°C	156bp
Claudin-8	Forward – TCC CAA GGC GTA CAG ATT TC Reverse – CAC TCT CCA CTG AGG CAT GA	60°C	178bp
Integrin-α6	Forward – AGC CCC AGG GAC TTA CAA CT Reverse – CTC TTG GAG CAC CAG ACA CA	60°C	230bp
Ptprf	Forward – CTA CAG CCT CCG AGT CCT TG Reverse – GCT TCA GGT CCT CCA GAG TG	65°C	272bp
Siglec1	Forward – GCT GTC CTG TCT TCC TTT CG Reverse – CCC AGT GTA TTC TGG GCT GT	63°C	254bp

Table 4.1 – Gene and primer information for qRT-PCR. 36B4 sequence was published elsewhere (Nitta and Orlando, *submitted*, Chapter 2), and the annealing temperature of this gene varied according to target gene being analyzed. Ptprf, Protein tyrosine phosphatase, receptor type, F; and Siglec1, Sialic acid binding Ig-like lectin.

4.3. Results

Adipocytes express a variety of cell adhesion molecules.

A complete analysis of the CAMs on the surface of adipocytes has not currently been investigated. I have recently learned that immune cells, not only macrophages infiltrate into obese adipose tissue (Duffaut et al., 2009; Weisberg et al., 2003; Winer et al., 2011). I have demonstrated that the co-culturing of adipocytes and immune cells leads to changes in the secretion of pro-inflammatory cytokines, with the potential of altering downstream signaling (Nitta and Orlando, *submitted*,Chapter 2; and *in preparation*, Chapter 3). In this current study I have made use of a CAM array to determine the changes in expression level of these proteins on adipocytes, which have been in contact with splenocytes (immune cells) directly or indirectly. The results from this array are

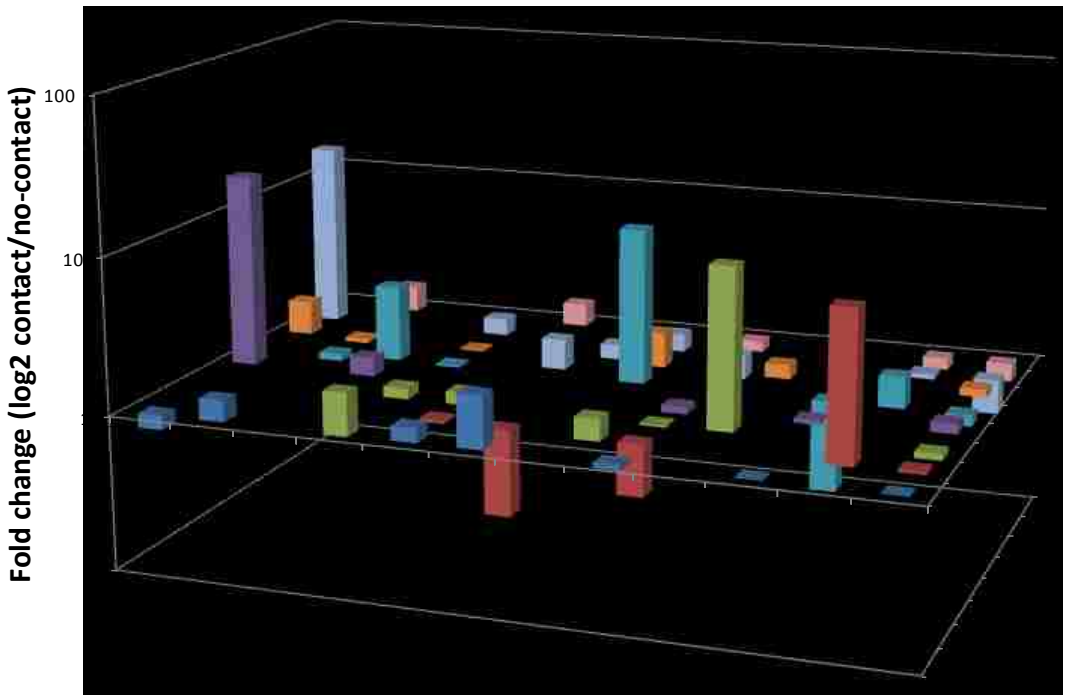

Figure 4.1 – Adipocytes differentially express cell adhesion molecules when in contact with immune cells. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were cultured in the presence of LPS in direct contact or no contact (separated by a 0.4 µm transwell) with GFP-expressing murine splenocytes. Co-cultures were incubated for 24 h incubation and then sorted by FACS. The mRNA from adipocytes was extracted and transformed into cDNA, and subsequently analyzed on the Takara PrimerArray® Cell Adhesion Molecule Array for mouse. Gene expression levels are depicted as fold-changes, in logarithmic scale. A list of the genes up- and down-regulated can be found in Table 4.2. Housekeeping genes are in column 12.

graphically depicted in Figure 4.1, with fold changes of all the genes tested in logarithmic scale, and housekeeping genes displayed in column 12. Table 4.2 compiles a list of all of the genes that are positively expressed in differentiated 3T3-L1 cells, and their respective fold changes from Figure 4.1. Most CAM genes on adipocytes had very little variation, or did not differ when these cells were cultured with splenocytes directly in comparison to no contact co-cultures (Table 4.2). Alternatively, seven genes had a larger than 3.5-fold induction or repression (Fig. 4.1 and Table 4.2), and warranted further investigation.

These were Cadherin-1; claudin 4, 8, and 11; Integrin alpha 6 (Itgα6); Protein tyrosine phosphatase, receptor type, F (Ptprf); and Sialic acid binding Ig-like lectin 1, sialoadhesin (Siglec1).

Adipocyte and immune cell contact leads to up-regulation of Cadherin-1 and Claudin 4, 8, and 11.

For additional confirmation of the CAM array, due to the possibility of false-positives, I chose to analyze the expressed genes with the most profound changes. After qRT-PCR analyses, with different primer sets than the Takara Array, I measured no significant change in the levels of Ptprf and Siglec1 (data not shown). From this, I concluded that these genes do not vary in my co-culturing conditions. On the other hand, the induction of Cadherin-1, claudin 4, 8, and 11 and the suppression of Itgα6 were further confirmed (Fig. 4.2). The down-regulation of Itgα6 are not as striking as the changes seen in the CAM array (-2.11 fold vs. -3.57 fold), however these findings are still significant ($p<0.05$).

4.4. Discussion

The identification of CAMs on the adipocytes has not been extensively studied, and is limited to mainly individual protein observations. Specific molecules have been identified as being a part of the surface of these cells, including ACAM, ICAM-1, NCAM, and VAP-1 (Abella et al., 2004; Brake et al., 2006; Eguchi et al., 2005; Yang et al., 2011). The current literature, however, lacks a more comprehensive list of CAMs

GenBank no.	Gene name	Description	Fold change	Location in Figure 1
NM_009655	Alcam	Activated leukocyte cell adhesion molecule	-1.39	C7
NM_133654	Cd34	CD34 antigen	-1.08	G11
NM_009855	Cd80	CD80 antigen	-1.03	C8
NM_021893	Cd274	CD274 antigen	-1.25	G6
NM_133983	Cd276	CD276 antigen	1.47	H2
NM_009864	Cdh1/E-cad	Cadherin 1 or E-cadherin	10.70	C9
NM_007664	Cdh2/N-cad	Cadherin 2 or N-cadherin	1.00	A10
NM_009903	Cldn4	Claudin 4	17.84	D1
NM_018778	Cldn8	Claudin 8	16.07	G1
NM_008770	Cldn11/OSP	Claudin 11 or oligodendrite-specific protein	8.94	B11
NM_021719	Cldn15	Claudin 15	-1.61	G5
NM_016782	Cntnap1	Contactin associated protein-like 1	1.24	F9
NM_172647	F11r/Jam	F11 receptor or junction adhesion molecule	1.41	H5
NM_009149	Glg1	Golgi apparatus protein 1	1.20	C5
NM_010380	H2-D1	Histocompatibility 2, D region locus 1	1.32	D3
NM_001001892	H2-K1	Histocompatibility 2, K1, K region	1.37	A2
NM_207648	H2-Q6	Histocompatibility 2, Q region locus 6	-1.19	H11
NM_010395	H2-T10	Histocompatibility 2, T region locus 10	-1.11	D8
NM_010398	H2-T23	Histocompatibility 2, T region locus 23	-1.06	D10
NM_008397	Itga6	Integrin alpha 6	-3.57	B6
NM_001001309	Itga8	Integrin alpha 8	-1.23	A1
NM_010578	Itgb1	Integrin beta 1 (fibronectin receptor beta)	-1.11	E2
NM_008404	Itgb2	Integrin beta 2	-2.12	B8
NM_013566	Itgb7	Integrin beta 7	1.69	F7
NM_023844	Jam2	Junction adhesion molecule 2	-1.41	G8
NM_023277	Jam3	Junction adhesion molecule 3	1.31	G7
NM_010758	Mag	Myelin-associated glycoprotein	3.10	E3
NM_001083897	Mpzl1	Myelin protein zero-like 1	-1.09	A8
NM_010875	Ncam1	Neural cell adhesion molecule 1	-1.03	E4
NM_177274	Negr1	Neuronal growth regulator 1	-1.18	H8
NM_001042752	Neo1	Neogenin	1.24	A5
NM_011213	Ptprf	Protein tyrosine phosphatase, receptor type, F	10.17	E7
NM_008984	Ptprm	Protein tyrosine phosphatase, receptor type, M	-1.96	C3
NM_008990	Pvrl2	Poliovirus receptor-related 2	1.18	C4
NM_021495	Pvrl3	Poliovirus receptor-related 3	1.29	G4
NM_011519	Sdc1	Syndecan 1	1.60	E11
NM_008304	Sdc2	Syndecan 2	1.05	B5
NM_011520	Sdc3	Syndecan 3	1.67	F1
NM_011521	Sdc4	Syndecan 4	-1.06	F2
NM_011426	Siglec1	Sialic acid binding Ig-like lectin 1, sialoadhesin	-3.85	E10
NM_011693	Vcam1	Vascular cell adhesion molecule 1	-1.02	F4
NM_001081249	Vcan	Versican	2.17	A6

Table 4.2 – Genes induced or repressed in 3T3-L1 adipocytes co-cultured in direct contact with splenocytes in comparison to cells co-cultured without direct contact. The genes affected by co-culturing of adipocytes and splenocytes in direct contact and their fold changes were determined as described in Materials and Methods. All adhesion molecule genes amplified in the Takara Cell Adhesion Molecule Array are shown, with respective fold changes, and are graphically depicted in Figure 4.1.

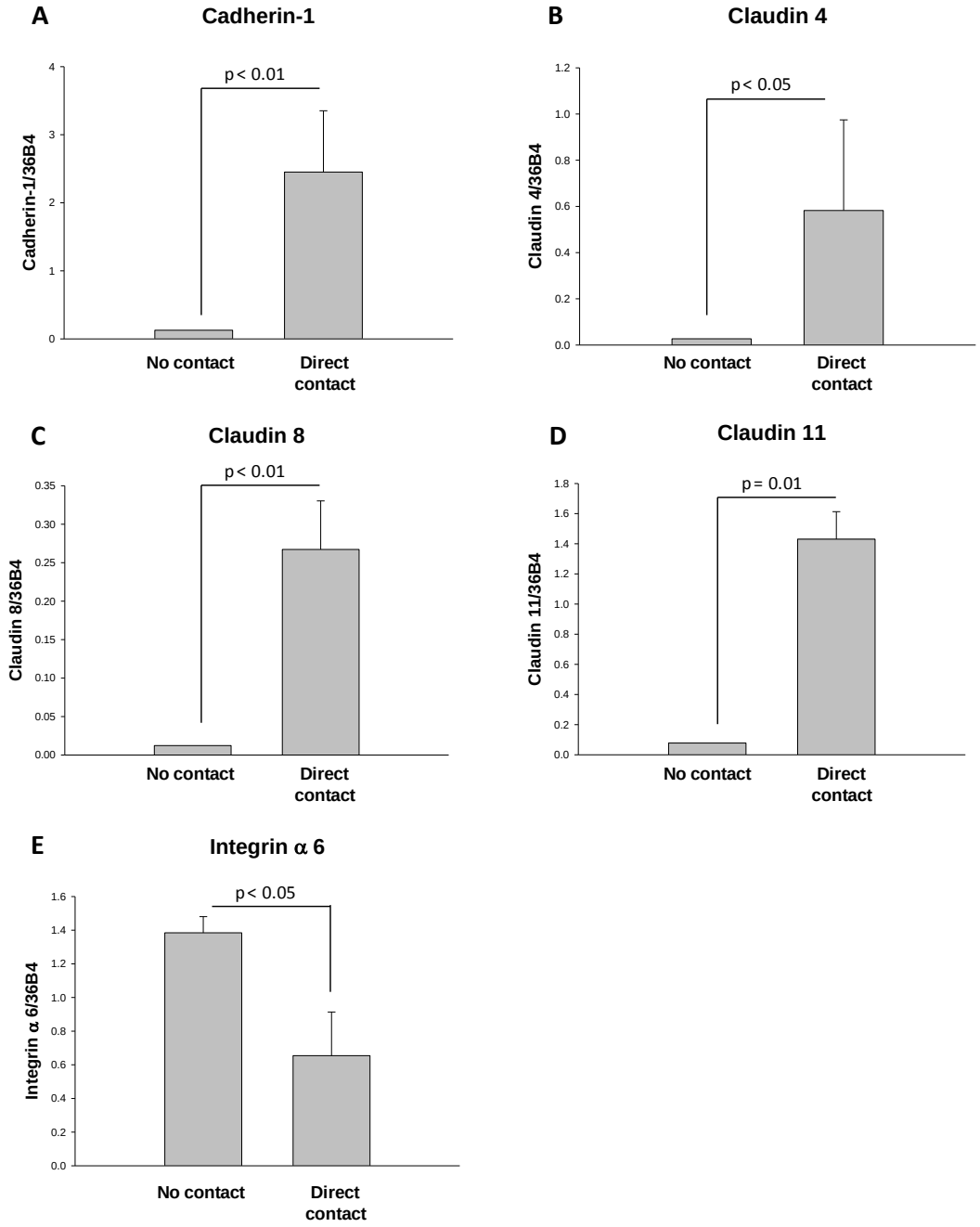

Figure 4.2 – Direct contact of adipocytes with immune cells up-regulates Cadherin-1, claudin 4, 8 and 11. Differentiated 3T3-L1 adipocytes and isolated murine splenocytes were cultured with LPS in direct contact or no contact (separated by a 0.4 μm transwell) with GFP-expressing murine splenocytes. Following 24 h incubation, cells were sorted by FACS and extracted and transformed into cDNA. Expression levels of Cadherin-1 (A), claudin 4 (B), claudin 8 (C), claudin 11 (D), and Integrin α 6 (E) was measured in adipocytes by qRT-PCR. Normalization was performed by amplification of the housekeeping gene 36B4.

present on adipocytes. I have positively identified 42 CAMs on the surface of differentiated 3T3-L1 cells by using a specific cell adhesion expression array in this study. These molecules most likely have some role in adipocyte differentiation, adhesion, signaling, or overall physiology. By knowing which molecules are present on the surface of these cells allows further investigation into their possible function, allowing for advances in the field of adipocyte biology.

The adhesion molecule NCAM-1 has been positively encountered on adipocytes in previous work (Yang et al., 2011) and my array results confirm this. Other CAMs previously identified on adipocytes did not constitute the genes evaluated on this Takara CAM array. My results elucidate that the field of CAMs on adipocytes is in its initial phase. Further work is warranted on CAMs to determine their importance and implications on obesity and inflammation. Currently, these studies are limited to increased leakiness of the gut wall after HFD feeding, due to changes in CAM expression (Lam et al., 2012; Suzuki and Hara, 2010), which is an indirect paracrine effect. A direct effect of adipocyte CAMs and their engagement to infiltrating immune cells in obese AT is still a topic of investigation.

Contact between two cells is an extremely important phenomenon in biological processes. In immunology, they become differentially activated or even change morphology and characteristics due to engagement of its receptors with its partner on an adjacent cell. Indeed, preadipocytes engaged with macrophages can become themselves activated macrophages (Charriere et al., 2003). The cell contact of lipid-laden adipocytes with immune cells in my co-culture setting generated an up-regulation of the tight junction proteins claudin 4, 8, and 11 in comparison to cells cultured without any direct

contact. Claudin 6 has been shown to be increased in adipogenesis and in adipose tissue of HFD-fed mice in comparison to normal chow (Hong et al., 2005). These proteins are originally known to be present on epithelial and endothelial cells, and function as a tight seal around them, creating an important barrier and selectively regulating paracellular solute transport (Anderson and Van Itallie, 2009). A variety of cytokines and growth factors, however, have the ability to modify cellular tight junctions through diverse mechanisms, including inside-out and outside-in signaling (Ahdieh et al., 2001; Amasheh et al., 2010; Capaldo and Nusrat, 2009; Ma et al., 2005; Oshima et al., 2001). In my co-culture system, I know that there are changes in cytokine release due to cellular contact (Nitta and Orlando, *submitted*, Chapter 2), which could account for the differential claudin expression seen here. Ultimately, TNFα treatment has the potential to up-regulate VCAM-1, integrin alpha 5 (Itgα5), and JAM (junction adhesion molecule), and decrease Itgα6 (Ruan et al., 2002). The importance of tight junction protein expression in adipocytes is also unknown, requiring further exploration.

In addition to up-regulation of claudins in adipocytes in contact with immune cells, there is a concomitant increase in Cadherin-1. Indeed, Cadherin-1 is initially required for the assembly of tight junctions, until its maturation, and then Cadherin-1 becomes part of the cellular adherens junction (Cereijido et al., 2000). Cadherins have also been shown to up-regulate the expression of claudins (Taddei et al., 2008), although I cannot determine if this is the case from the data presented.

During 3T3-L1 adipocyte differentiation a switch in integrin molecules is observed, with a decrease in Itgα5 and concomitant increase in Itgα6 (Liu et al., 2005). These results show that Itgα6 is observed on adipocytes, as my studies confirm.

Furthermore, I demonstrate changes in its expression when immune cells are in direct contact with fully differentiated adipocytes. Previous studies have shown that TNFα treatment of adipocytes decreases Itgα6 expression (Ruan et al., 2002). My direct co-culture experiment in comparison to indirect co-cultures led to dampened expression of Itgα6, further confirming a role for this adhesion molecule in these cells, although its role has not been elucidated.

CAMs are considered structural elements of cells, and are thought to be responsible for mediating mechanical cell-cell contact. However, recent studies are determining that a majority of these molecules have the capacity to function as *bona fide* receptors and lead to downstream signaling that can modify cellular architecture, differentiation, and growth (Balda and Matter, 2003; Ditlevsen et al., 2008). Certain CAMs even lose their adhesive properties, by cleavage, conformational changes, mutations, or shedding, but still maintain their signaling properties (Cavallaro and Dejana, 2011; Matter and Balda, 2003). Claudin proteins have been found in the cytoplasmic portion of the cell, and in some cases, vesicles, speculating that it may be involved in vesicle trafficking or cell-matrix engagement (Blackman et al., 2005; Matsuda et al., 2004). Tight-junction molecules are also now being identified on the surface of non-epithelial/endothelial cells and localized to different areas in the cell (D'Atri and Citi, 2002; Morita et al., 1999; Sanchez-Heras et al., 2006; Takai and Nakanishi, 2003).

These novel functions of CAMs on adipocytes can lead to potential beneficial solutions for obesity through new target identification. Novel therapeutics targeted at

blocking adipocyte-immune cell engagement and crosstalk could potentially reduce inflammation in obesity.

CHAPTER 5

5. Discussion and future directions

Obesity is a significant and current world-wide problem, with steadily increasing rates. Its comorbidities are vast, and include higher incidences of cardiovascular diseases, type II diabetes mellitus, and cancers (Bays et al., 2004; Grundy, 2002; Jensen, 2006; Renehan et al., 2008). The major link between obesity with cardiovascular diseases and type II diabetes is the presence of a characteristic low-grade chronic inflammation present in these individuals. Heightened lipid load, distinctive of obesity, results in immune cell infiltration into AT and increased cytokine secretions, such as TNFα, IL-6, and MCP-1 (Hotamisligil et al., 1995; Matsuzawa et al., 1999; Suganami et al., 2005; Takahashi et al., 2003). Current research continues to try to dissect out the mechanisms of why these cells become recruited to this tissue. The details succeeding cell infiltration resulting in inflammation and "crosstalk" between adipocytes and immune cells are the subjects of this dissertation.

5.1. Novel co-culture model of obesity

I have generated a novel cell culture system to mimic an obese phenotype that is physiologically more relevant. Initially, only one immune cell was thought to infiltrate obese AT and generate enhanced inflammation (Weisberg et al., 2003). Co-culture studies at that time were restricted to pure populations of adipocytes and macrophages (Suganami et al., 2005). Currently it is established that additional immune cells permeate obese AT, rendering prior studies incomplete. I have chosen splenocytes for my current

co-culture studies, which have comparable immune cell contributions to obese AT (Avitsur et al., 2002; Sun et al., 2012; Swirski et al., 2009). By using a system that includes a variety of cell types I am able to dissect out their "crosstalk". My studies elucidate that the communication between these cells is through paracrine factors as well as direct contact. Secreted paracrine factors include cytokines that are produced by one cell and signal by binding to the receptor on another cell. My splenocyte-adipocyte co-cultures without contact (allowing for only paracrine factors, by use of a transwell system) resulted in increased IL-6 and MCP-1 secretion, and a decrease in TNFα. I additionally show that contact between these cells in my direct contact co-cultures leads to further enhancement of the already paracrine-enhanced cytokines (Fig. 5.1). TNFα, which was decreased by paracrine factors is further dampened by direct contact. This novel system allows us to understand the importance of cell contact in cell communications in obesity.

To elucidate how these cytokines are regulated dependent on paracrine and contact-mediated factors, I performed a time course study of my co-cultures. I observed distinct release rates for each of the cytokines tested, which enabled me to determine that they are all regulated differentially. These studies also compared cytokine release from cells in direct contact or indirect contact in a time-dependent manner. They allowed me to speculate the involvement of distinct signaling pathways dependent on cell contact.

Determination of the respective cytokine contributions from adipocytes and immune cells in obesity has been a continued subject of controversy. Initial studies include use of immortalized cells, with potentially inadequate signaling, or purified

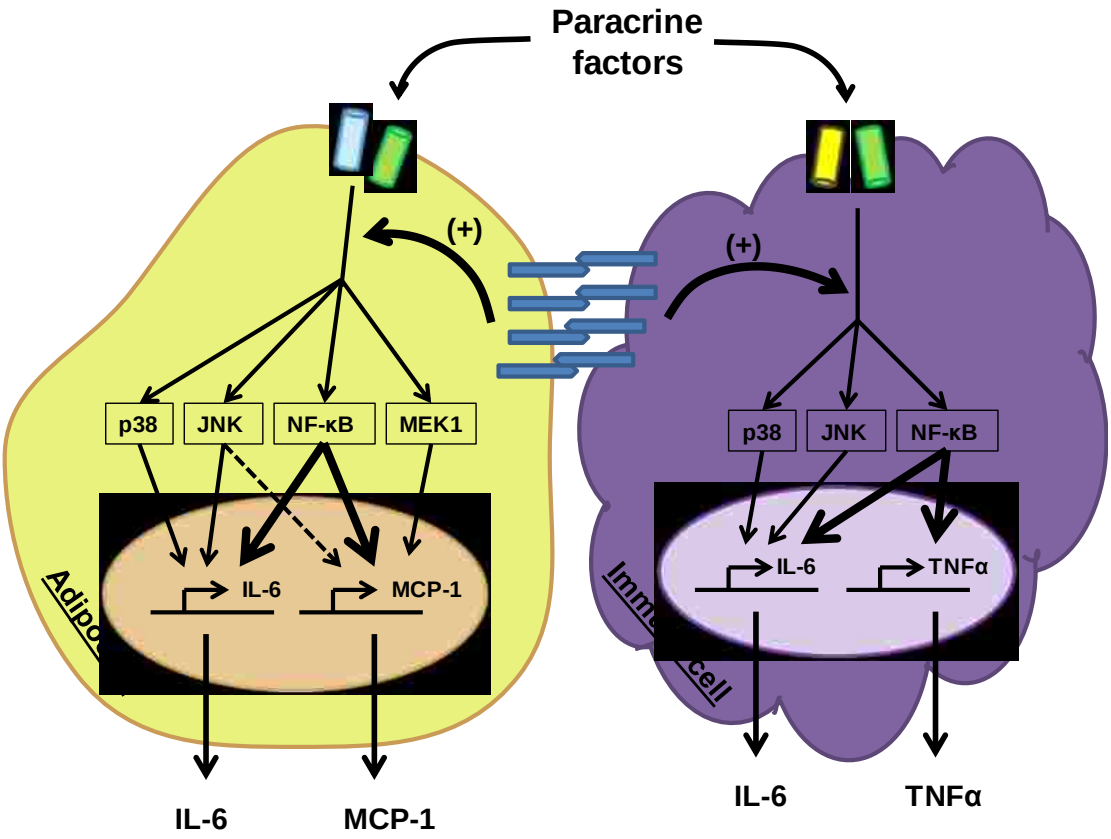

Figure 5.1 – "Crosstalk" between adipocytes and immune cells. In my co-culture system NF-κB and MAPK pathways are stimulated resulting in the secretion of inflammatory cytokines, which in turn, become paracrine factors themselves. In adipocytes, the NF-κB, p38, JNK, and MEK1 pathways are active and necessary for stimulation of IL-6 and MCP-1 production. Adipocytes, however, are not significant contributors of TNFα. NF-κB stimulation in adipocytes is a potent regulator of IL-6 and MCP-1, since blocking its activity with Bay11-7082 (a chemical inhibitor) decreases the levels of these cytokines significantly (thick arrows). MCP-1 expression is exclusive of adipocytes and is also regulated by MEK1, with minimal contribution from JNK (dashed arrow). IL-6 secretion is also mediated by activation of JNK and p38 MAPKs. Additionally, signaling in immune cells is highly regulated by NF-κB, whereas TNFα secretion is almost exclusive to this pathway (thick arrows). NF-κB, p38, and JNK are all responsible for IL-6 secretion in immune cells, as in adipocytes, in this system. When adipocytes and immune cells are additionally placed in direct contact with one another, there is an enhancement of downstream signaling occurring through these pathways (plus sign), exacerbating the paracrine response. IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; MCP-1, monocyte chemoattractant protein-1; MEK1, mitogen-activated protein kinase kinase 1; NF-κB, Nuclear Factor-Kappa B; and TNFα, tumor necrosis factor alpha.

macrophage cultures, failing to include other immune cells populations encountered in obese AT. Additional *ex vivo* studies that separate the stromal vascular fraction (including the immune cells) from the adipocytes in whole AT have also resulted in inconsistent findings. These conflicting results can be distinctively attributed to another study showing significant alteration in cytokine expression when these cell populations are isolated by collagenase digestion (Ruan et al., 2003). My novel co-culture system allows me to clarify these discrepancies by culturing immune cells derived from splenocytes, which are comparable to obese AT immune cell infiltration, in direct contact with adipocytes. To enable me to separate my cell populations after direct co-culture, I used splenocytes derived from ubiquitously expressing GFP mice, and sorted them by FACS. These two cell populations (adipocytes and splenocytes) were further analyzed for their expression of TNFα, IL-6, and MCP-1. By performing this procedure, I am ensuring that the cytokine contributions from these cells are not being altered due to additional manipulation or digestion. My results clearly show that both cell types have significant contributions to cytokine expression in this system, and are both required for increased inflammation in obese AT. Immune cells express TNFα and IL-6, whereas adipocytes contribute with expression of IL-6 and MCP-1 (Fig. 5.1).

Based on my observation that the expression of TNFα is derived mainly from splenocytes, I investigated the potential role that this cytokine could have on the enhancement of IL-6 and MCP-1 secretions. Prior studies have shown that TNFα is paramount in obesity inflammation (Hotamisligil et al., 1995). Here, I show that it is required for the contact-mediated enhancement of IL-6 and MCP-1. Without TNFα, direct co-cultures mimic the secretions of cells that are cultured lacking contact (only

allowing exchange of paracrine factors), demonstrating its importance in "crosstalk" and contact between these cells. Based on these observations, TNFα and its receptor, TNFR, continue to be interesting targets for therapy.

5.2. Signaling in crosstalk between adipocytes and immune cells

Dissecting the signaling cascades that are important in obesity is paramount for understanding how cytokines are regulated and secreted. I demonstrate the importance of the NF-κB and the MAPK pathways p38, JNK, and Erk in contact-mediated cytokine secretion. NF-κB signaling is responsible for the majority of TNFα secretion by splenocytes (Fig. 5.1). When adipocytes and immune cells are cultured in direct contact, residual TNFα secretion is encountered (Fig. 3.2A). To fully understand the signaling pathway responsible for the contact-mediated TNFα secretion, additional co-culture experiments using the NF-κB inhibitor Bay11-7082, in conjunction with another inhibitor from the MAPK pathway can elucidate this further. The promoter region of TNFα exhibits a c-Jun responsive sequence, implicating the contribution of JNK towards its regulation (Yao et al., 1997). Based on this observation, I can speculate that a direct co-culture of adipocytes and immune cells in the presence of Bay11-7082 and SP600125 (JNK inhibitor) is likely to fully abrogate TNFα secretion in my system.

The MAPKs are also important in downstream cytokine secretions in my co-cultures, specifically JNK and p38 for IL-6, and JNK and MEK1 for MCP-1 (Fig. 5.1). The importance of the signaling cascades involved in cytokine secretion is, however, restricted to inhibitor use and blocking of one particular protein in this cascade. Additional studies are needed to check for activation states of these proteins, as well as

upstream and downstream proteins involved in these signaling cascades. A positive phosphorylation pattern of specific MAPK signaling protein (using phospho-antibodies targeted for the proteins outlined in Figure 1.2A) will confirm activation of these kinases and their involvement in signaling. Phosphorylation patterns of IκB and IKK are also paramount for determination of the activation of the NF-κB pathway (Fig. 1.2C). Differential patterns encountered between co-cultures devoid of contact in comparison to direct contact co-cultures will determine how contact between these cells activates downstream signaling. Additional confirmation of the involvement of these pathways would include verification of the nuclear localization of downstream transcription factors which migrate towards the nucleus targeting gene transcription.

5.3. Cell adhesion molecules in obesity

My studies are novel in the characterization of CAMs in adipocytes. I have positively identified 42 CAMs expressed on differentiated 3T3-L1 cells. The procedure used for identification of these molecules in this study is restrictive to the genes contained on a commercially available specific gene array. Due to the limitation of this study, there is a high probability of identification of additional CAMs in adipocytes. Further studies would need to include a more comprehensive characterization of these molecules with potentially broader techniques. The use of other sources of adipocytes, from mouse and human, will also allow further identification, since the results contained here could be due to the cultured nature of the 3T3-L1 cells.

Up-regulation of Cadherin-1, claudin 4, 8, and 11 and down-regulation of Itgα6 in adipocytes with direct contact to immune cells demonstrates the involvement of these

CAMs in my cell culture system. Additional studies are necessary to confirm their increased expression in humans as well as mouse models of obesity. Western blot analysis of HFD-fed mice and obese individuals should result in increased protein levels in comparison to their lean counterparts, as well as expression levels measured by qRT-PCR. Furthermore, localization studies, by immunofluorescence of AT from HFD-fed mice or obese subjects will allow me to determine what area of the cell these molecules are confined to. These supplementary studies allow me to begin to elucidate the role the molecules play in these cells, as well as their importance *in vivo*. CAMs localized to cell membranes will play a different role in comparison to molecules localized to the cell nucleus, for example. Functional studies will ultimately determine their importance and role in obesity.

5.4. Use of *in vivo* transplantation model for future studies in signaling and cell adhesion molecules

The experiments conducted here are limited to *in vitro* studies, with cultured adipocytes and isolated splenocytes. Confirmatory studies using *in vivo* models are warranted, however whole animal ablation studies can lead to confounding results due to non-specificity of the knock-out. Additionally, not all knock-out animals are viable, limiting these studies. Tissue-specific ablation in mouse AT is an optimal alternative to whole-animal knock-out, and is generally performed using Cre-lox technology driven by an aP2 promoter. This aP2 gene (also known as fatty acid binding protein 4, FATP4) is also expressed by immune cells (data not shown), questioning the results of prior studies utilizing this technology, since both adipocytes and immune cells of these animals will

lack the targeted gene. Utilization of a tissue transplantation murine model is a viable solution without any major drawbacks.

Mouse transplantation of AT can be used to ensure that the mechanisms shown in the co-culture studies are confirmed *in vivo*, enabling further investigation of the importance of secreted cytokines, signaling pathways, and CAMs in obese AT. Whole mouse or human AT that is surgically implanted subcutaneously in mice exhibits normal physiological characteristics including graft vascularization and innervation (Bach-Mortensen et al., 1976; Gavrilova et al., 2000). A successful attempt of AT transplantation led to the restoration of insulin sensitivity in lipoatrophic A-ZIP/F-1 mice (devoid of AT) (Gavrilova et al., 2000). This procedure has two major advantages: (1) AT from any mouse model can be implanted into another, respecting murine backgrounds; and (2) control AT can be implanted into the same animal, allowing for direct comparison between both grafts.

To further characterize the importance of TNFα in obese AT, fat from $TNFR^{-/-}$ mice would be transplanted subcutaneously into a wild-type mouse and further fed a HFD for 12 weeks. Since TNFα expression is almost exclusive of the immune cell population, transplantation of AT lacking its receptor would allow for investigation of this cytokines' role in a normal murine environment. Immune cells from the recipient mouse would secrete higher levels of TNFα on a HFD, in comparison to lean animals, however the grafted AT will not be able to respond to that obese stimulus, since it lacks that specific receptor. If TNFα is responsible for contact-mediated enhancement of cytokine secretion, as shown in my cell culture model, I would expect to see a similar effect in these mice. Transplanted $TNFR^{-/-}$ AT should exhibit diminished IL-6 and MCP-

1 expression locally in comparison to its control (wild-type grafted AT) on the same mouse. Direct comparisons can additionally be made between knock-out tissue and wild-type transplanted AT in lean as well as HFD-fed mice. Measurements of inflammation can be determined by histological identification of immune cell infiltration (F4/80 staining) and expression levels of cytokines by qRT-PCR in all AT grafted conditions. Verification of the phosphorylation patterns of NF-κB and MAPK pathways will determine the impact of TNFα and its receptor on signaling in obesity. These experiments will address the importance of the crosstalk between both cells, and the importance of TNFα in this system. Due to the versatility of this procedure, it can also be used for additional studies focusing on immune cell recruitment by using AT devoid of the chemokine, MCP-1, for example.

Utilization of CAM-devoid AT transplantation can further define the importance of contact in adipocyte-immune cell "crosstalk" in an obese setting (HFD-fed animals). Claudin 11 knock-out animals are viable and can be transplanted into wild-type animals. Immune cells from the recipient mice are normal with regards to their ability to be recruited to this grafted AT lacking claudin 11. To determine the importance of claudin 11 on inflammation in adipocytes, cytokine levels would be measured by qRT-PCR analyses in comparison to wild-type grafted AT. To verify if claudin 11, aside from its already known tight-junction associated functions, has the potential to generate downstream signaling, phosphorylation patterns of the major signaling cascades (NF-κB and MAPKs) can be analyzed. Further identified CAMs from additional studies can utilize this transplantation model to verify their role in AT inflammation.

5.5. Summary

In conclusion, the data presented throughout this dissertation advances the current knowledge of adipose biology. It establishes that adipocytes and immune cells in AT both contribute to inflammatory cytokines in obesity but also significantly determines the importance of contact between these two cells in this process. These changes in cytokine secretions are carried out by the major signaling pathways NF-κB and MAPKs, and can be potentially mediated by CAMs on the surface of adipocytes. As such, if adipocytes and immune cells require direct engagement, it expands our knowledge and allows for additional target identification for addressing inflammation in obesity.

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