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MACROPHAGE-CONDITIONED MEDIUM INHIBITS ADIPOCYTE DIFFERENTIATION

By

Vanessa Auguste Constant

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Obesity is accompanied by a reduced adipogenic capacity that promotes adipocyte hypertrophy, low-grade inflammation, and insulin resistance. Macrophages infiltrate adipose tissue and may contribute to the low-grade inflammation associated with obesity and insulin resistance, suggesting that they could also play an anti-adipogenic role. I hypothesized that macrophage-secreted factors inhibit adipogenesis. My objectives were to assess if macrophage-conditioned medium (MacCM) inhibits adipocyte differentiation and to determine the mechanism by which the inhibition occurs. Murine J774 or human THP-1 MacCM was added to murine 3T3-L1 or human abdominal subcutaneous or omental preadipocytes. Either type of MacCM impaired murine and human adipogenesis as measured by triglyceride accumulation and protein expression of adipogenic markers. Time course studies revealed that THP-1-MacCM was required during the early phase of 3T3-L1 adipogenesis for its inhibitory effect. THP-1-MacCM stimulated the phosphorylation of ERK1/2 and IKKβ in 3T3-L1 preadipocytes. Pharmacological inhibition of ERK1/2, with the specific MEK1 inhibitor PD98059, alleviated the inhibitory effect of THP-1-MacCM on TG accumulation. In conclusion, MacCM inhibits adipocyte differentiation in culture. The anti-adipogenic effect depends on early exposure of THP-1-MacM to differentiating 3T3-L1 preadipocytes, and ERK1/2 is required for the inhibitory effect of THP-1-MacCM on TG accumulation.

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LIST OF ABBREVIATIONS

aP2	Fatty acid binding protein
ATCC	American Type Culture Collection
BMI	Body mass index
C/EBP	CCAAT/enhancer-binding protein
CS	Calf serum
dex	Dexamethasone
DMEM	Dulbecco's modified Eagle medium
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
GLUT4	Glucose transporter 4
IBMX	Isobutylmethylxanthine
IKK	IκB kinase
IL	Interleukin
I.O.D	Integrated optical density
IRS	Insulin receptor substrate
LPS	Lipopolyssacharide
MacCM	Macrophage-conditioned medium
MAPK	Mitogen activated protein kinase
MCE	Mitotic clonal expansion
MCP-1	Macrophage chemoattractant protein-1
MonCM	Monocyte-conditioned medium
NF-κB	Nuclear factor-κB
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PPAR	Peroxisome proliferator-activated receptor
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SD	Standard deviation
STAT	Signal transducer and activator of transcription
TG	Triglycerides
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TPA	12-*0*-tetradecanoyl phorbol-13-acetate
TZD	Thiazolidinedione

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INTRODUCTION

OBESITY

1. The prevalence of obesity

Obesity represents one of the leading and challenging health problems in the world. In the industrialized countries, obesity is rapidly reaching epidemic proportions. Over 1 billion adults are now classified as overweight with 300 million being obese (Haslam and James, 2005). In Canada approximately 23% of adults are obese and an additional 36% are overweight, whereas only 14% were obese in 1978 (Tjepkema, 2006). There is also an alarming rapid increase in the rate of childhood obesity and an associated early onset of metabolic complications, and this has been projected to lead to a decreased life expectancy (Lau et al., 2007). In the United States, the prevalence of obesity has more than doubled, from 13% in 1960 to 33% in 2004 (Ogden et al., 2007). Moreover, obesity is a risk factor for type 2 diabetes and cardiovascular diseases that are mediated by hypertension, dyslipidemia, and insulin resistance. Because of these serious complications, obesity was declared a chronic medical disease by the World Health Organization (Haslam and James, 2005).

2. Obesity as a complex disease

Although tremendous efforts in the field of obesity research have provided us with valuable information, the management of obesity remains an important medical challenge, mainly because obesity is a very complex disease. The World Health Organization defines overweight as a body mass index (BMI = weight in kg/height in m^2) of ≥ 25 and obesity as a BMI of ≥ 30 (Ogden et al., 2007). BMI is widely used as a standard for identifying patients at increased risk for obesity-related complications. However, BMI values can be misleading

because it cannot distinguish differences in anatomical body fat distribution that exists among individuals, and because lean body mass can also sometimes be the cause of an elevated BMI.

Anatomical distribution of fat is an independent predictor of health outcomes (Montague and O'Rahilly, 2000). Increased abdominal (upper body) or central obesity is associated with an increased risk of cardiometabolic diseases compared to femoral or gluteal (lowerbody) obesity (Krotkiewski et al., 1983; Langendonk et al., 2006). Central obesity can be further categorized into visceral central obesity (accumulation of fat around the internal organs) and subcutaneous central obesity (accumulation of fat under the abdominal skin). Visceral central obesity is more closely linked to adverse health outcomes compared to subcutaneous central obesity (Déspres and Lemieux, 2006; dos Santos et al., 2005). Patients with increased visceral fat are at higher risk of developing insulin resistance than those of equivalent weight but with a lower degree of visceral fat (Brochu et al., 2001). Waist circumference is therefore considered a better predictor of obesity-associated cardiometabolic diseases than BMI (Déspres and Lemieux, 2006).

Beyond total body fat, adipocyte size also seems to be important with respect to obesity-associated pathologies. Hypertrophied adipocytes predict type 2 diabetes independently of overall obesity and insulin resistance (Weyer et al., 2000). We can therefore surmise that not all obese individuals are created equally, and that obesity treatment needs to focus more on individuals who are at higher risk of developing cardiometabolic diseases.

3. How is obesity induced?

Energy balance is maintained when energy intake in the form of food is equal to energy expenditure. In the case of the development of obesity, energy intake is greater than energy expenditure. Interestingly, the homeostatic system regulating body weight in humans is prone toward weight gain and fat storage (Druce et al., 2004). Putting humans in an environment where excess calories are easily obtained with minimal physical effort appears to contribute to an increased prevalence of obesity and its associated pathologies. The positive energy balance causing obesity leads to expansion of the adipose tissue mass, and this is believed to be central to the related metabolic and vascular complications.

THE ADIPOSE TISSUE

1. Adipose tissue expansion

The increase in adipose tissue mass, allowing the storage of excess calories as triglyceride (TG), occurs via the coordination of two processes: 1) recruitment and differentiation of preadipocytes into adipocytes (hyperplasia); and 2) the increase in size of existing adipocytes (hypertrophy) (Gregoire, 2001; Hirsch et al., 1989). Differentiation of preadipocytes into adipocytes is also referred to as adipogenesis. Adipogenesis is under the tight control of secreted soluble factors from adipocytes and other cells. Those factors trigger intracellular signalling networks that in turn regulate specific transcription factors that activate the differentiation program. The current model of adipogenesis has been derived from cumulative and comparative data using both immortalized and primary preadipocyte cell lines (Avram et al., 2007).

2. The 3T3-L1 murine preadipocyte cell model

The murine 3T3-L1 cell line is one of the most extensively characterized and widely studied cell models for adipocyte differentiation (Cornelius et al., 1994; Gregoire et al., 1998). The murine 3T3-L1 cell line was originally isolated from Swiss 3T3 cells derived from disaggregated mouse embryos (Gregoire et al., 1998). Immortalized 3T3-L1 cells are fibroblast-like and can proliferate in culture indefinitely, but have a high capacity to differentiate into cells with the morphological and biochemical properties of adipose cells if allowed to reach growth arrest (Green and Meuth, 1974). The use of 3T3-L1 cells as an experimental model to study adipogenesis has led to the identification of many molecular and cellular events occurring during the conversion of preadipocyte into adipocyte (Figure 1).

3. Events occurring during 3T3-L1 adipogenesis

Growth arrest

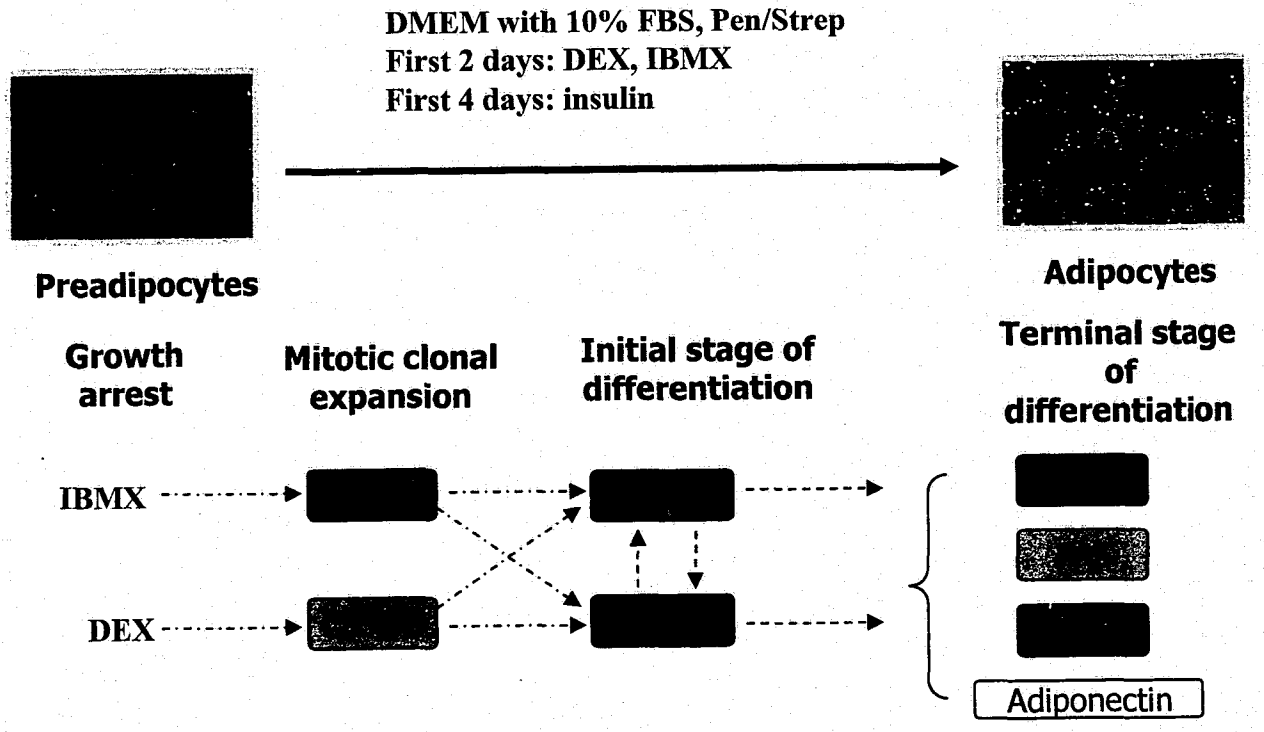
Upon reaching confluence, when cell-cell contact is maximized, proliferating preadipocytes in culture become growth-arrested (Avram et al., 2007). Growth arrest is a required first step for preadipocytes to reach terminal differentiation (Avram et al., 2007).

Hormonal induction

Growth-arrested 3T3-L1 preadipocytes can be induced to differentiate in a serum-supplemented medium containing a hormonal cocktail which is composed of the following adipogenic factors: dexamethasone (dex; a synthetic glucocorticoid agonist), a cAMP-elevating agent, isobutylmethylxanthine (IBMX; inhibits cAMP phosphodiesterase), and high levels of insulin that activate signalling pathways that control adipogenic gene

Figure 1. Overview of the differentiation of 3T3-L1 preadipocytes into adipocytes

Growth-arrested preadipocytes, induced to differentiate, undergo mitotic clonal expansion, exit the cell cycle, and proceed to terminal differentiation. Differentiation is under the control transcription factors which induce the expression of adipogenic genes in the mature adipocytes.


DMEM with 10% FBS, Pen/Strep
First 2 days: DEX, IBMX
First 4 days: insulin
Preadipocytes
Adipocytes
Growth arrest
Mitotic clonal expansion
Initial stage of differentiation
Terminal stage of differentiation
IBMX
DEX
Adiponectin

transcription (Rosen and MacDougald, 2006).

Mitotic clonal expansion

Growth-arrested preadipocytes induced to differentiate synchronously re-enter the cell cycle to undergo the mitotic clonal expansion (MCE) phase that involves at least one round of DNA replication and cell doubling (Gregoire, 2001). MCE is an early event that is required for adipocyte differentiation (Tang et al., 2003). MCE is different from preconfluent proliferation because it displays a different pattern of cell cycle gene expression (Gregoire et al., 1998). Chromatin re-organization during MCE has been speculated to be required for transcriptional activation of adipogenic genes (MacDougald and Mandrup, 2002). After the MCE phase, the cells enter a second growth-arrested state that is also required for terminal differentiation to proceed (Avram et al., 2007).

Terminal differentiation

Upon reaching terminal differentiation, the cells acquire the characteristics of mature adipocytes. They become spherical, accumulate lipid, and their ability to engage in *de novo* lipogenesis and lipolysis is increased (Avram et al., 2007). Terminal differentiation is accompanied by an increase in the expression of insulin receptors and glucose transporters (GLUT4) (Avram et al., 2007). Expression of genes involved in lipid metabolism such as fatty acid synthase (FAS) and fatty acid binding protein (aP2) is also increased (Rosen and Spiegelman, 2000). Synthesis of specific adipocyte-secreted products such as adiponectin also characterizes the mature adipocyte (Rosen and Spiegelman, 2000). Furthermore, the increase in β2- and the β3-adrenoreceptors render the cells sensitive to lipolytic stimuli (Avram et al., 2007).

Transcriptional regulation of adipogenesis

Adipogenesis is under the control of various genes encoding enzymes, transporters, transcription factors and other proteins whose patterns of expression change during the differentiation process. Adipogenesis can be viewed as a cascade of transcriptional events (Rosen and MacDougald, 2006). Two families of transcription factors, peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer binding proteins (C/EBPs) play a central role in the differentiation program of adipocytes (Rosen and MacDougald, 2006). C/EBPδ and C/EBPβ, activated by dex and IBMX respectively, are transiently expressed early during the MCE phase of differentiation. C/EBPδ and C/EBPβ induce the expression of C/EBPα and PPARγ which persists in the mature adipocyte (Rosen and MacDougald, 2006). C/EBPα and PPARγ, in addition to positively regulating each other, transactivate a profile of genes that characterize the adipocyte phenotype (Gregoire et al., 1998). The proteins encoded by these genes include: FAS, GLUT4, adiponectin, and aP2 (Rosen and Spiegelman, 2000).

PPARγ is considered to be an essential regulator of adipogenesis, and has been shown to be sufficient (Tontonoz et al., 1994) and necessary for adipocyte differentiation (Rosen et al., 1999). Thiazolidinediones (TZDs; antidiabetic insulin-sensitizing drugs) are PPARγ ligands and are very effective at promoting adipogenesis in culture and *in vivo* (Rosen and Spiegelman, 2000). The insulin-sensitizing action of TZD in type 2 diabetes can be explained by their proadipogenic action that enhances the ability of adipose tissue to expand thus preventing adipocyte hypertrophy and ectopic fat deposition that promote inflammation and impair insulin signalling. C/EBPα is necessary for the acquisition of insulin sensitivity of the mature adipocyte (Rosen and MacDougald, 2006).

4. Signalling pathways involved in 3T3-L1 adipogenesis

Transcription factors involved in adipogenesis function downstream of signalling pathways that communicate information regarding appropriate intracellular and extracellular conditions for differentiation (Rosen and MacDougald, 2006). The balance of pro- and anti-adipogenic signals determines whether or not preadipocytes will undergo adipogenesis. Many signalling pathways are involved in adipogenesis. For the purpose of this project, I focused on 3 signalling pathways. The extracellular signal-regulated kinase 1/2 (ERK1/2) and the signal transducer and activator of transcription (STAT) 3 were chosen because of their mitogenic role during MCE of 3T3-L1 adipogenesis (Deng et al., 2000; Prusty et al., 2002; Tang et al., 2005). The nuclear factor- κB (NF-κB) was chosen because it can mediate inflammatory responses, including in 3T3-L1 cells (Berg et al., 2004).

a. ERK1/2 signalling pathway

Extracellular signal-regulated kinase 1/2 (ERK1/2) is a subfamily of the mitogen activated protein kinases (MAPKs) which are serine/threonine kinases (Bost et al., 2005a). Upon stimulation with appropriate ligands, activation of Ras leads to the sequential activation of Raf, MEK1/2 and ERK1/2 (Bost et al., 2005a). MEK1/2 is the immediate upstream regulator of ERK1/2 (Bost et al., 2005a). Activation of MEK1/2 leads to the activation/phosphorylation of ERK1/2, and pharmacological inhibition of MEK1 with PD98059 blocks ERK1/2 activity (Bost et al., 2005a). Once activated, ERK1/2 is translocated to the nucleus to activate various transcription factors that, in turn, activate genes involved in proliferation and differentiation (Saltiel and Kahn, 2001).

The role of ERK1/2 in adipogenesis is controversial because different studies have

shown contradictory roles for its function in this process. In the first studies linking ERK1/2 to adipocyte differentiation, expression of an activated Ras construct (Benito et al., 1991), and oligonucleotide anti-sense reagents against ERK1/2 in 3T3-L1 preadipocytes (Sale et al., 1995) suggested a positive role for ERK1/2 in adipogenesis. A contradictory study showed that treatment of 3T3-L1 preadipocytes with the specific MEK inhibitor, PD98059, had no effect on or actually enhanced adipocyte differentiation (Font de Mora et al., 1997). Furthermore, expression of a constitutively activated MEK1 or overexpression of MAPK inhibited adipocyte differentiation; suggesting that ERK1/2 inhibits adipocyte differentiation (Font de Mora et al., 1997).

The function of ERK1/2 in adipogenesis seems to depend on the precise timing of its activation during the differentiation process (Prusty et al., 2002). ERK1/2 activity is required for proliferation during the MCE phase within the initial 12 hours after induction of differentiation, before significant upregulation of PPARγ (Prusty et al., 2002; Tang et al., 2005). During this time frame, ERK1/2 enhances the ability of C/EBPβ to activate the transcription of C/EBPα and PPARγ (Prusty et al., 2002; Tang et al., 2005). The inhibition of ERK1/2 pathway during MCE phase has been shown to prevent adipocyte differentiation (Tang et al., 2005). ERK1 knockout mice have decreased adiposity, and adipocytes isolated from these mice displayed impaired adipogenesis, further supporting a role for this isoform in adipocyte differentiation (Bost et al., 2005b). On the other hand, if the duration of ERK1/2 activity is inappropriately sustained, ERK1/2 can phosphorylate and reduce the transcriptional activity of PPARγ and, in this way, can inhibit adipocyte differentiation (Chan et al., 2001; Reginato et al., 1998) (Hu et al., 1996).

b. STAT3 signalling pathway

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of latent cytoplasmic transcription factors. In response to stimulation from various receptors, mainly those for cytokines, STATs are phosphorylated and translocated into the nucleus to modulate transcription of genes involved in development, cell growth, proliferation, and apoptosis (Stephanou and Latchman, 2005; Stephens et al., 1996). Each STAT family member has a unique tissue distribution and is activated by different groups of cytokines (Balhoff and Stephens, 1998). In 3T3-L1 adipocytes, STAT3 is activated by interferon-γ, IL-6, leukemia inhibitory factor, and platelet-derived growth factor (PDGF). STATs display unique expression patterns upon induction of human and murine adipocyte differentiation (Harp et al., 2001; Stephens et al., 1999), and there is evidence suggesting that these transcription factors may regulate adipogenesis.

STAT3 is highly tyrosine phosphorylated and bound to DNA in proliferating preadipocytes but not in growth-arrested preadipocytes or terminally differentiated adipocytes (Deng et al., 2000). Induction of differentiation is accompanied by an increase in STAT3 tyrosine phosphorylation levels that coincides temporally with postconfluent MCE (Deng et al., 2000). Inhibition of endogenous STAT3 expression with anti-sense oligonucleotides in postconfluent preadipocytes decreases cell proliferation. STAT3 has therefore been suggested to play a regulatory role in the proliferative phase of adipogenesis (Deng et al., 2000).

c. IKKβ-NF-κB signalling pathway

Nuclear Factor-κB (NF-κB) is the collective name given to a family of ubiquitous

transcription factors that induces the rapid expression of multiple genes involved in immune and inflammatory responses (De Bosscher et al., 2006). NF-κB is present as a latent and inactive dimer in the cytoplasm of most cells (Ghosh and Karin, 2002). It can be activated by many environmental stimuli including pro-inflammatory cytokines like tumor necrosis factor-α (TNFα) (Ghosh and Karin, 2002). The Toll-like receptors (TLRs) that sense lipopolysaccharide (LPS) found in pathogens also induce activation of NF-κB (Kawai and Akira, 2004). Upon activation, NF-κB is translocated to the nucleus where it induces the expression of genes regulating immune and inflammatory responses (Gilmore, 2006). The activity of NF-κB is regulated by the interaction with inhibitory IκB proteins which block the nuclear localization signal and interfere with sequences involved in DNA binding of a given NF-κB dimer (Gilmore, 2006). Activation of NF-κB involves a kinase cascade which begins with the activation of an IκB kinase (IKK) complex (Hoffmann et al., 2006). IKK mediates phosphorylation and proteasome-degradation of IκB which allows the nuclear translocation and accumulation of NF-κB dimer (Ghosh and Karin, 2002; Gilmore, 2006).

A potential role for NF-κB in adipogenesis has not been fully explored. However, the central role played by adipocytes in inflammatory responses suggests that NF-κB could possibly be involved in the regulation of adipogenesis. NF-κB activation has been shown to be obligatory for TNF-α-induced inhibition of adipogenesis (Ruan et al., 2002). Furthermore, one of the downstream targets of NF-κB was shown to be PPARγ, the expression of which was reduced by TNF-α treatment (Chae and Kwak, 2003).

5. Human abdominal primary preadipocyte cell model

Culture conditions for the study of primary human preadipocytes have also been

developed. The events occurring during the differentiation of primary human preadipocytes are similar to those that occur during the differentiation of murine 3T3-L1 preadipocytes. However, there are differences in the levels of expression of many adipogenic genes between primary preadipocytes and preadipocyte cell lines (MacDougald and Mandrup, 2002). Furthermore, in contrast to 3T3-L1 preadipocytes, primary preadipocytes do not undergo MCE in culture; rather, they are believed to have already proceeded through this phase *in vivo* (Entenmann and Hauner, 1996; Tomlinson et al., 2006).

Although 3T3-L1 preadipocytes represent the most widely used cell model for studying adipocyte differentiation (Gregoire et al., 1998), there are potential drawbacks associated with their exclusive use. Since they are aneuploid, they often possess characteristics that are different from those of primary preadipocytes (Cornelius et al., 1994). They do not allow the study of depot-specific differences in fat cell behaviour (Rosen and Spiegelman, 2000). Depot-specific differences in adipose tissue have very important clinical consequences. Preadipocytes isolated from subcutaneous and visceral depots have been shown to have different adipogenic potential in response to TZD-based differentiation protocol (Adams et al., 1997; Digby et al., 1998). This is further supported by *in vivo* studies which showed that PPARγ-induced weight gain resulted in increased accumulation of subcutaneous fat without any change in visceral fat (Akazawa et al., 2000; Mori et al., 1999). However, in the absence of TZD there is no difference in adipogenic potential between subcutaneous and visceral preadipocytes *in vitro* (Harmelen, et al., 2004; Shahparaki, et al., 2002). It is therefore helpful to confirm results obtained with a preadipocyte cell line to those from primary human preadipocytes (Cornelius et al., 1994).

Primary preadipocytes have several advantages over preadipocyte cell lines. Their

diploidy may better reflect the *in vivo* context and they can be derived from various adipose depots to allow depot specific studies (Gregoire et al., 1998). There are also drawbacks associated with the exclusive use of primary preadipocytes. They are already committed to the adipogenic lineage and cannot be used to study early adipogenic events. They can only undergo a limited number of passages and there is a need for an ongoing supply from donors (Avram et al., 2007). Donor heterogeneity is another challenge when working with primary cell culture.

6. Adipose tissue composition and functions

Adipose tissue has been reported to be composed of 50 to 70% adipocytes, 20 to 40% stromal vascular cells, such as preadipocytes, fibroblasts, non-differentiated mesenchymal cells, and 1 to 30% infiltrated macrophages (Hauner, 2005). The cellular composition of adipose tissue can vary substantially according to anatomical location and body weight (Hauner, 2005). The main function of adipose tissue is the maintenance of energy homeostasis, through storage of excess energy as TG, and release of fatty acids (FA) when energy is required. Energy provided by adipose tissue is required for various functions, including muscular movement, reproduction, growth, cellular maintenance, and maintenance of optimal body temperature (Avram et al., 2005).

7. Adipose tissue as an endocrine organ

Our view of adipose tissue biology has switched from the notion of storage organ to that of an endocrine organ with pleiotropic biological functions (Ailhaud, 2006). Adipose tissue sends out signals that regulate body weight homeostasis, insulin sensitivity, lipid

levels, blood pressure, coagulation, fibrinolysis, immune response and inflammation (Van Gaal et al., 2006). Adipose tissue factors that are secreted only by adipocytes are termed adipokines; they are leptin and adiponectin (Fantuzzi, 2005). Those secreted by adipocytes, preadipocytes, and macrophages are termed cytokines or chemokines. Cytokines can be anti-inflammatory such as interleukin-10 (IL-10) or pro-inflammatory such as interleukin-6 (IL-6) and TNF-α (Gimeno and Klaman, 2005). Adipose tissue also releases chemokines, such as macrophage chemoattractant protein (MCP-1), that attract immune cells (Fantuzzi, 2005).

Leptin and adiponectin are two very important adipokines. Leptin, secreted predominantly by adipocytes, is a signal for the amount of overall fat mass (Yildiz and Haznedaroglu, 2006). Leptin's primary role is the regulation of energy balance. It acts primarily in the arcuate nucleus of the hypothalamus to decrease food intake and increase energy expenditure, thus limiting weight gain (Munzberg et al., 2004). Leptin also modulates insulin action in the liver through attenuation of insulin-induced tyrosine phosphorylation of the insulin receptor substrate (IRS-1) (Cohen et al., 1996).

Adiponectin is an anti-inflammatory adipokine which is abundantly and exclusively secreted by adipocytes (Trayhurn and Beattie, 2001). Adiponectin is involved in the regulation of lipid oxidation and insulin sensitivity. It stimulates skeletal muscle fatty acid oxidation to improve insulin-stimulated glucose uptake, and increases hepatic oxidation to enhance the ability of insulin to suppress hepatic glucose production (Berg, 2001; Fruebis et al., 2001). Adiponectin is also important for cardiovascular health. It regulates the vascular response to lipid and inflammatory stimuli and inhibits the formation of foam cells that promotes the atherogenic process (Ouchi et al., 2000; Ouchi et al., 2001). High levels of serum adiponectin are associated with protection from myocardial infarction (Frystyk et al.,

2007; Pischon, 2004).

8. Too little adipose tissue can be as metabolically dangerous as too much adipose tissue

The expansion of adipose tissue during obesity is associated with the development of metabolic diseases (Ailhaud, 2006). Another important clinical problem is the lack of adipose tissue usually referred to as lipodystrophy. Interestingly, lipodystrophy, although rare in humans, is associated with the same metabolic complications as obesity (Grinspoon and Carr, 2005; Monajemi et al., 2007). HIV positive patients receiving highly active antiretroviral therapy can develop an acquired subcutaneous lipoatrophy (Grinspoon and Carr, 2005). They display altered fat distribution characterized by subcutaneous adipose tissue atrophy and increased central fat accumulation, and they are dyslipidemic and insulin-resistant (Grinspoon and Carr, 2005). Transgenic fatless mice display liver and muscle insulin resistance and eventually develop diabetes. Surgical implantation of adipose tissue in these mice improves insulin sensitivity of their liver and muscle (Gavrilova et al., 2000; Kim et al., 2000). The metabolic abnormalities seen in lipodystrophy could be caused by a disruption of adipokines and/or ectopic FA accumulation in muscle and liver cells (Rajala and Scherer, 2003). Therefore, functional adipose tissue is required for metabolic and vascular health.

DYSFUNCTIONAL ADIPOSE TISSUE

1. Characteristics of dysfunctional adipose tissue

Healthy or functional adipose tissue is important for maintaining both metabolic balance and vascular health; whereas dysfunctional adipose tissue is associated with insulin

resistance, inflammation, and cardiometabolic disease (Pausova, 2006). Dysfunctional adipose tissue is characterized by: 1) adipocyte hypertrophy that reflects an insufficient capacity of adipose tissue to form new adipocytes in order to store excess calories, 2) visceral fat accumulation, 3) excessive lipolysis, 4) aberrant production of bioactive molecules that creates a low-grade inflammatory state, and 5) macrophage infiltration (Heilbronn et al., 2004; Pausova, 2006). Many studies have assessed the relation between obesity, dyslipidemia, inflammation, and insulin resistance; however the sequence of events leading to adipose tissue dysfunction remains uncertain.

a. Adipogenic deficit

The development of obesity-related inflammation and insulin resistance is associated with an acquired deficit in adipogenesis that reduces the capacity of the adipose tissue to store excess calories (Danforth, 2000; Heilbronn et al., 2004) (Weyer et al., 2000). To compensate for this reduced adipogenic capacity, the balance is shifted toward adipocyte hypertrophy (Danforth, 2000; Heilbronn et al., 2004). However, adipocytes can only reach a critical finite size, and once this size is exceeded, the fat is redirected and stored in muscle and liver, a process commonly referred to as ectopic fat deposition (Heilbronn et al., 2004; Pausova, 2006) (Danforth, 2000). As described below, recent studies have provided information about how hypertrophied adipocytes and ectopic fat deposition contribute to obesity-associated cardiometabolic diseases.

b. Hypertrophied adipocytes

The correlation between adipocyte size and insulin sensitivity in humans was

observed over 30 years ago (Salans, 1968; Stern et al., 1972). Hypertrophied adipocytes display decreased membrane cholesterol content, accompanied by decreased insulin responsiveness and upregulation of genes encoding pro-inflammatory cytokines (Le Lay et al., 2001). Treatment of type 2 diabetic obese Zucker rats with the TZD, troglitazone, resulted in the remodelling of the adipose tissue (Okuno et al., 1998). The resulting adipose tissue consisted of smaller and more insulin-sensitive adipocytes, and fewer mature hypertrophied adipocytes, with the net result being an increase in whole body and adipose tissue insulin sensitivity (Okuno et al., 1998). In a more recent study, adipocytes isolated from patients were separated according to their size (Skurk et al., 2007). The fraction containing the very large adipocytes secreted the highest level of leptin, IL-6, IL-8, MCP-1 (Skurk et al., 2007). More interestingly the secretion of the anti-inflammatory cytokine, IL-10, decreased with the increasing adipocyte size (Skurk et al., 2007). This suggests that hypertrophied adipocytes contribute to insulin resistance and inflammation during obesity.

c. Ectopic fat deposition

Ectopic fat deposition occurs when hypertrophied adipocytes have exceeded their storage capacity. Adipocyte size has been shown to correlate positively with the rate of FFA efflux from the adipocyte (Reardon et al., 1973). Ectopic fat deposition in tissues such as muscle and liver has adverse health outcomes, because the uptake of FFAs leads to intracellular production of FA metabolites that can stimulate inflammation and inhibit insulin signalling (Suganami et al., 2005; Yu et al., 2002). Indeed, increased lipid accumulation reduces insulin-stimulated glucose uptake in muscle and impairs suppression of hepatic glucose production (Shulman, 2000). Moreover, lipid accumulation in the liver

induces hepatic steatosis, leading to overproduction of very low density lipoprotein (VLDL) that are converted to atherogenic low density lipoprotein (LDL), and predisposition to cardiovascular diseases (Van Gaal et al., 2006).

Interestingly, visceral fat accumulation itself can be viewed as a form of ectopic fat deposition which occurs when subcutaneous fat fails to expand appropriately (Déspres and Lemieux, 2006). Since visceral fat is less responsive to the anti-lipolytic effect of insulin, it was thought to cause adverse health outcomes mainly through the direct drainage of FFAs to the liver via the portal vein (Déspres and Lemieux, 2006; Zierath et al., 1998). However, more recent studies have shown that most portal FFAs originate from the systemic circulation with only 20 to 25% of FFAs originating from visceral fat (Jensen, 2006). The altered metabolic profile of obese patients with high visceral fat may be better explained by the inherent features of the visceral fat itself. Visceral fat is in close proximity with the interior organs. It secretes more pro-inflammatory and less anti-inflammatory cytokines than subcutaneous fat in obesity (Fontana et al., 2007; Maury et al., 2007).

d. Low-grade inflammation

Low-grade inflammation is a key feature of obesity, insulin resistance and type 2 diabetes. This inflammatory state within the adipose tissue is characterized by aberrant pro-inflammatory cytokine production, activation of inflammatory signalling pathways, and reduction of anti-inflammatory factors (Wellen and Hotamisligil, 2005). Interestingly, the regulation of metabolic and immune functions is mediated through common molecules and signalling pathways which are expressed in both adipocytes and macrophages (Hotamisligil, 2006). They include: NF-κB, TLR4, PPARγ, and TNFα (Hotamisligil, 2006).

Many lines of evidence have linked inflammation, insulin resistance and type 2 diabetes. Treatment of diabetic patients with high-dose aspirin, which inhibits the activity of IKKβ, an activator of NF-κB, reduced blood glucose, triglyceride, and C-reactive protein without weight loss (Hundal et al., 2002). Nutritional FAs activate TLR4 signalling in adipocytes and macrophages, and obese mice lacking TLR4 display reduced inflammatory gene expression in liver and adipose tissue (Shi et al., 2006). Treatment of cultured cells or animals with recombinant TNFα, known to be produced by hypertrophied adipocytes, impairs insulin action (Hotamisligil et al., 1996). Treatment of obese subjects with the PPARγ agonist pioglitazone results in increased insulin sensitivity and reduced inflammatory cytokine production (Di Gregorio et al., 2005). Those studies indicate that the link between inflammatory and metabolic signalling is a delicate balance and that chronic disturbance of metabolic balance, as seen in malnutrition and overnutrition could lead to aberrant immune responses (Hotamisligil, 2006). The infiltration of adipose tissue by macrophages during obesity is the perfect example of an aberrant immune response due to overnutrition.

MACROPHAGES IN ADIPOSE TISSUE

1. Infiltration of adipose tissue by macrophages

About 4 years ago, new evidence supporting obesity as low-grade inflammatory state was found. Infiltration of macrophages in adipose tissue of obese humans and rodents was shown by various groups (Curat et al., 2004; Weisberg, 2003) (Xu, 2003). The extent of this infiltration correlates positively with body mass index and adipocyte size (Weisberg, 2003; Xu, 2003) (Cancello et al., 2005). Xu *et al.* reported that macrophages represent 33% of

stromal vascular cells in genetically obese mice compared to only 2% in wild-type mice (Xu, 2003). Weisberg *et al.* estimated the percentage of macrophages in adipose tissue may vary from under 10% in lean mice and humans to over 50% in extremely obese, leptin-deficient mice and nearly 40% in obese humans (Weisberg, 2003). Bone marrow cell transplantation experiments suggested that macrophages infiltrating the adipose tissue are bone marrow-derived precursors which later differentiate into mature macrophages (Weisberg, 2003).

2. Understanding macrophages

Macrophages play an important role in the immune system. They are the first line of defence of innate immunity, and are important accessory cells in adaptive immunity (Ma et al., 2003). They recognize and phagocytose foreign organisms, release antimicrobial peptides, secrete molecules that attract other immune cells to areas of infection, and present antigen to lymphocytes (Gordon, 1998). Furthermore, they remove apoptotic cells in inflamed tissues (Duffield, 2003).

Macrophages are derived from differentiated myelogenic precursors in the bone marrow (Lehrke and Lazar, 2004). Everyday, 5 x 10^9 monocytes travel from the bloodstream to their target tissues, where the local microenvironment dictates their differentiation into tissue-specific macrophages (Ma et al., 2003). Tissue macrophages can proliferate to maintain the macrophage pool, or are activated to become functional macrophages (Ma et al., 2003; Xaus et al., 2001). Macrophages can be classically activated by lipopolyssacharide (LPS), IL-12, or interferon-γ (IFN-γ); or be alternatively activated by IL-4, IL-10, IL-13 or glucocorticoids (Duffield, 2003; Song et al., 2000). Classically activated macrophages are highly microbicidal and have enhanced pro-inflammatory cytokine production such as TNF-

α, IL-6, and IL-12 (Song et al., 2000). Alternatively activated macrophages are involved in wound healing and tissue remodelling and have enhanced production of anti-inflammatory cytokines such as IL-10 (Lumeng et al., 2007; Song et al., 2000). Macrophage activation is followed by deactivation (Duffield, 2003), and in the absence of cytokines or growth factors most macrophages die by apoptosis (Xaus et al., 2001). The half-life of tissue macrophages can be very long (years) (Xaus et al., 2001).

Macrophages are found within almost all tissues. Macrophages and their precursors are present in adipose tissue; however their functions have not been elucidated. In adipose tissue of lean mice, macrophages have been shown to be alternatively activated and expressing IL-10 which protected adipocytes from insulin resistance (Lumeng et al., 2007). However, in diet-induced obese mice the macrophages were classically activated which contributed to insulin resistance (Lumeng et al., 2007). In obese humans, the activation state of macrophages is not straightforward; although they express surface markers of alternatively activated macrophages, they can produce extensive amounts of pro-inflammatory cytokines (Zeyda et al., 2007).

3. The J774 murine macrophage cell model

J774 is a reticulum cell sarcoma line composed of a heterogeneous mixture of adherent and non-adherent cells that behave similarly to each other with regard to large amounts of lysozyme secretion and antibody-dependent phagocytosis (Ralph and Nakoinz, 1975). The J774 tumor arose in a female BALB/c/NIH mouse during a plasmacytoma induction program (Ralph et al., 1975). J774 cells have properties that are characteristic of macrophages. They are readily adherent, are phagocytic, express receptors for

immunoglobulin, and can specifically bind antibody-coated erythrocytes (Ralph and Nakoinz, 1975). Quantitative immunocytochemical characterization of four macrophage-like cell lines revealed that J774 is one of the two most mature cell lines (Nibbering and van Furth, 1988). The J774 cell line is therefore a good murine macrophage cell model. However, a limitation is that since J774 is a cancer cell line, it might not behave exactly like a normal macrophage.

4. The THP-1 human macrophage cell model

THP-1 cell line was isolated from the blood of a boy suffering from acute monocytic leukemia, and was shown to have functional characteristics of monocytes (Tsuchiya et al., 1980). This human monocytic leukemia cell line will stop proliferating and will differentiate into macrophage-like cells upon treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Differentiated THP-1 cells were characterized by their marked morphological change, expression of immunoglobulin receptor, their ability to phagocytose, and to adhere to tissue culture plastic (Tsuchiya et al., 1982). Differentiated THP-1 cells are known to behave more like native monocyte-derived macrophages compared to other human myeloid cell lines (Auwerx, 1991). Moreover, the THP-1 cell line offers the advantage of working with a homogenous population in contrast to the donor variability associated with native human monocytes (Auwerx, 1991). Therefore, the THP-1 cell line represents a valuable human macrophage model. However, as for the J774 model, THP-1 is a cancer cell line, and therefore might not behave exactly like human macrophages.

5. Mechanisms of macrophage infiltration in adipose tissue

Chemotactic factors induce macrophage recruitment in adipose tissue. Weight loss improves the inflammatory state seen in obese patients and is associated with a significant decrease in chemotactic gene expression with a concomitant decrease in subcutaneous depot macrophage number (Cancello et al., 2005; Christiansen et al., 2005). Macrophage chemoattractant protein-1 (MCP-1) is a chemokine that is highly produced by macrophages and endothelial cells (Baggiolini, 1998); and by adipocytes (Christiansen et al., 2005; Curat et al., 2004). Mice deficient in the MCP-1 receptor, C-C motif chemokine receptor-2 (CCR2) display reduced macrophage content in their adipose tissue (Weisberg et al., 2006). Moreover the levels of MCP1 are increased in obesity, decreased after weight loss, and this is also associated with a reduction of macrophage content in adipose tissue (Cancello et al., 2005; Christiansen et al., 2005). Interestingly, MCP-1 expression is higher in omental compared with subcutaneous adipose tissue (Bruun, 2005; Harman-Boehm et al., 2007). Consistent with this finding, visceral adipose tissue contains more macrophages than subcutaneous adipose tissue in obese subjects (Cancello et al., 2006; Harman-Boehm et al., 2007). The preferential macrophage infiltration in the omental depot is independent of obesity, since adipose tissue of lean subjects also displays more macrophages in the visceral depot compared to the subcutaneous depot (Harman-Boehm et al., 2007).

6. Role of macrophages in obesity-associated pathologies

Macrophage accumulation in adipose tissue has been shown to precede high-fat diet-induced insulin resistance, suggesting a causal role for macrophages in insulin resistance (Xu, 2003). Nevertheless, mechanisms by which macrophages contribute to obesity-associated insulin resistance and inflammation have not been fully elucidated. Adipose tissue

from obese subjects overexpresses MCP-1, derived mainly from macrophages within the adipose tissue. The overexpression of MCP-1 has been associated with insulin resistance and hepatic steatosis observed during obesity (Kamei et al., 2006; Kanda et al., 2006) (Di Gregorio et al., 2005). There is evidence suggesting that MCP-1 can alter metabolic functions independently from macrophage recruitment. Treatment of muscle cells with MCP-1 decreases insulin-stimulated glucose uptake (Kamei et al., 2006). Futhermore, addition of MCP-1 directly to differentiated adipocytes also decreased insulin-stimulated glucose uptake and the expression of adipogenic genes (Sartipy and Loskutoff, 2003). This suggests that MCP-1 also contributes to the reduction of the storage capacity of the adipose tissue and adipocyte hypertrophy, thereby leading to ectopic fat deposition in liver and skeletal muscle, which causes insulin resistance.

Macrophages in the adipose tissue of obese subjects have been shown to form crown-like structures completely surrounding single adipocytes (Cancello et al., 2005; Cinti et al., 2005). Weight loss is associated with a disappearance of the crown-like structures (Cancello et al., 2005). Adipocytes surrounded by macrophages display typical features of stressed, aged, inactive, and/or dying adipocytes (Cancello et al., 2005; Cinti et al., 2005). It has therefore been suggested that macrophages are recruited in adipose tissue to eliminate the increasing number of dying adipocytes observed with obesity (Cinti et al., 2005).

Increased macrophage infiltration in visceral but not in subcutaneous adipose tissue of morbidly obese individuals is also associated with hepatic fibroinflammatory lesions (Cancello et al., 2006). Interestingly severely obese women with normal glucose homeostasis do not exhibit the preferential visceral macrophage accumulation, whereas those with impaired glucose homeostasis had more macrophages in visceral compared to

subcutaneous fat (Harman-Boehm et al., 2007). This suggests that metabolically intact, severely obese women may be protected from the preferential macrophage accumulation into visceral fat (Harman-Boehm, et al., 2007). Therefore, obesity treatment targeting macrophages by either deactivating or eliminating them may be potentially useful in the treatment of cardiometabolic diseases.

7. Interaction of macrophages and adipose cells

The mechanisms by which macrophages and adipose cells influence each other are not clear. A few studies have addressed the interaction between macrophages and adipose tissue cells. 12 hour stimulation of J774 macrophage cells with adipocyte-conditioned medium induced a significant increase in the secretion of IL-6 and TNF-α from the J774 macrophage (Berg et al., 2004). Coculture of 3T3-L1 adipocytes and RAW264 macrophages resulted in the upregulation of pro-inflammatory cytokines like MCP-1, IL-6 and TNF-α in both the adipocytes and macrophages; and in the downregulation of the anti-inflammatory cytokine, adiponectin, in the adipocytes (Suganami et al., 2005). This group proposed a paracrine loop implicating FFAs derived from adipocytes and TNF-α derived from macrophages. FFAs induce a pro-inflammatory response in both adipocytes and macrophages by binding TLR4 and activating NF-κB; and TNF-α induces adipocyte lipolysis which increases the pool of FFAs (Suganami et al., 2005; Suganami et al., 2007b). Those *in vitro* studies were further supported by *in vivo* studies which showed that TLR4 mutant mice under a 16-week high-fat diet displayed an attenuation of adipose tissue inflammation (Suganami et al., 2007a).

RAW264.7-macrophage-conditioned medium (RAW-CM) induced greater NF-κB

activity and expression of several pro-inflammatory genes in 3T3-L1 adipocytes (Permana et al., 2006). Those studies have provided important data on the cross-talk between macrophages and adipocytes; however they focused on the interaction between macrophages and adipocytes. The effect of macrophages on the differentiation of preadipocytes had never been addressed before my studies. Furthermore, most of the data on the cross-talk between macrophages and adipocytes were published after my data was generated or while I was conducting my studies. Learning more about the effect of macrophages on preadipocytes is important because it might provide relevant information on how macrophages contribute to adipose tissue dysfunction.

OBJECTIVES

The purpose if this project was to assess the effect of macrophage-secreted factors on adipogenesis. I hypothesized that macrophage-secreted factors inhibits adipogenesis. The approach involved generating macrophage-conditioned medium (MacCM) using 2 macrophages cell lines, the murine J774 and the human THP-1. The effect of the MacCM on adipogenesis was tested on murine 3T3-L1 and primary human preadipocytes isolated from human abdominal subcutaneous and omental fat depots.

***Objective 1*:** Assess if MacCM inhibits the differentiation of preadipocytes.

***Objective 2*:** Assess the critical time point at which MacCM is required to inhibit differentiation.

***Objective 3*:** Identify signalling pathways implicated in the inhibitory effect of MacCM on the differentiation of preadipocytes into adipocytes.

MATERIALS AND METHODS

Culture of murine J774 macrophages

J774 macrophages from American Type Culture Collection (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics (Gibco) (100 U/ml penicillin and 0.1 mg/ml streptomycin). Culture medium was changed every 2-3 days. A cell scraper was used to detach the cells from the culture plates when subpassaging was required, split ratio never exceeded 1:6.

Preparation of medium conditioned by J774

Just prior to confluence, J774 cells were placed in fresh growth medium described above, and after 24 hours, the resulting J774-macrophage-conditioned medium (J774-MacCM) was collected and centrifuged (Megafuge 1.0R; Heraeus Instruments) at 200 x *g* for 5 minutes. The supernatants were frozen at -20°C and thawed in a 37° C bath prior to use for adipogenesis experiments. Neat medium (growth medium not exposed to the J774 cells) was assessed to ensure that processing (centrifugation and freezing) of the conditioned medium was not responsible for any observed effects on adipogenesis.

Culture of human THP-1 monocytes

THP-1 monocytes (ATCC) were grown in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 2 mmol/l L-glutamine, 1.5 g/l sodium bicarbonate (Fisher), 4.5 g/l glucose (Gibco), 10 mmol/l HEPES (Fisher), and 1 mmol/l sodium pyruvate (Gibco), 10% FBS, 0.05 mmol/l β-mercaptoethanol (Fisher), and antibiotics (100 U/ml

penicillin and 0.1 mg/ml streptomycin). Cell suspensions were centrifuged once a week at 200 x *g* for 5 minutes, then counted using a Neubauer hemacytometer and resuspended in fresh growth media. Every 2-3 days, cells were counted and fresh media was added to maintain the culture at a concentration lower than 1×10^6 cells/ml.

Preparation of medium conditioned by THP-1

THP-1-MacCM and THP-1 monocyte-conditioned medium (MonCM) were generated as follows. The monocytic cells, resuspended at 1×10^6 cells/ml, were either differentiated into macrophages with 100 nmol/l 12-O-tetradecanoyl phorbol-13-acetate (TPA, Sigma), or maintained as monocytes with vehicle (0.01% DMSO, Fisher), for 24 hours. The medium was then replaced with fresh growth medium (no TPA present), and after 24 hours, the conditioned medium was collected and centrifuged as described for the J774 cells. The supernatants were stored at −20°C and thawed in a 37° C bath prior to use for adipogenesis experiments. Neat medium (THP-1 growth medium not exposed to the THP-1 cells) was assessed to ensure that processing (centrifugation and freezing) of the conditioned medium was not responsible for any observed effect on adipogenesis.

Isolation of human abdominal subcutaneous and omental stromal preadipocytes

Paired samples of abdominal subcutaneous and omental adipose tissue were obtained from 8 consenting patients (5 women; 3 men) undergoing elective abdominal surgery (approved by the Research Ethics Committee of the Ottawa Hospital). Mean age was 49±14 years, and mean body mass index was 31±4 (±SD). Stromal preadipocytes were isolated as previously described (Artemenko et al., 2005; Hauner et al., 2001). Adipose tissue was

dissected away from fibrous connective tissue and capillaries, and digested with collagenase CLS type I (200 U/g of tissue) (Worthington) on a rotary shaker at 37° C for 1 hour. After the incubation time the digested tissue underwent filtration using a sterile 200μm nylon filter to eliminate connective tissue and other debris. The filtrated tissue was then centrifuged at 200g for 20 minutes in order to remove floating mature adipocytes. 10% FBS was added to the infranatant, which underwent progressive size filtration through sterile 100μm, 50μm, and 25μm nylon filters; and was then centrifuged at 200g for 20 minutes to ensure complete removal of mature adipocytes. Red cell lysis buffer (155 mM NH_4Cl, 5.7 mM, K_2HPO_4, 0.1 mM EDTA, pH 7.3) was added to the pellet for 5 minutes and then DMEM, supplemented with 20% FBS and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and (50 U/ml nystatin, Calbiochem), was added to the stromal cells and the mixture was centrifuged at 200g for 5 minutes. After staining with crystal violet, cells were counted using a Neubauer hemacytometer. The cell were seeded and grown in DMEM supplemented with 20% FBS and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 U/ml nystatin), and then expanded by further cell passages (maximum of 3) before being subjected to differentiation (Adams et al., 1997; Hutley et al., 2003). In some cases, the cells underwent cryopreservation once, prior to passaging, without any alteration in their ability to differentiate upon thawing (Ort et al., 2005).

Differentiation of human abdominal subcutaneous and omental stromal preadipocytes

Stromal preadipocytes (subcutaneous and omental, with matched passage number) were seeded at a density of 3×10^4 cells/cm^2 in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin. The following day, the

human stromal preadipocytes were placed in the appropriate neat medium, J774-MacCM, THP-1-MacCM, or THP-1-MonCM. The cells were either maintained as preadipocytes under these conditions, or differentiated with the addition of 5 μg/ml insulin (Sigma), 100 μmol/l indomethacin (Sigma) (a PPARγ agonist at this concentration), 0.5 μmol/l dexamethasone (Steraloids), and 0.25 mmol/l IBMX (Sigma) (Artemenko et al., 2005; Lehmann et al., 1997). After 12 to 15 days, cells were photographed with a Nikon Coolpix 995 digital camera mounted on a Nikon Eclipse TS-100 microscope. Cells were then lysed and processed for immunoblot analysis.

Culture and differentiation of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes (ATCC), kept at low passage, were grown in DMEM supplemented with 10% calf serum (CS; Invitrogen) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Culture medium was changed every 2-3 days. Except when subjected to differentiation, cultures were kept in a sub-confluent state, and passaged using trypsin-EDTA (Gibco) to detach the cells from the culture plates.

To test the effect of J774-MacCM on differentiation, post-confluent 3T3-L1 preadipocytes were placed in neat medium, in J774-MacCM, or in a mixture such that the percentage of neat medium or J774-MacCM ranged from 20% to 80% of the final volume for the dose response experiments. To test the effect of THP-1-MacCM and THP-1-MonCM, 3T3-L1 preadipocytes were placed in neat medium, in THP-1-MacCM, or in THP-1-MonCM on days 0, 2, 4, and 6 of differentiation, depending on the time course chosen. When indicated, post-confluent 3T3-L1 preadipocytes were pretreated for 15 minutes with 25 μM PD98059 (Calbiochem) or vehicle (DMSO) prior to induction of differentiation, then

studies I only measured ERK1/2 phosphorylation at a single time point of 15 minutes following stimulation. Since inappropriately prolonged ERK1/2 activation inhibits adipogenesis by phosphorylating and reducing the transcriptional activity of PPARγ (Chan et al., 2001; Reginato et al., 1998) (Hu et al., 1996), assessing the phosphorylation and transcriptional activity of PPARγ will also provide insight on the duration of ERK1/2 activity and the mechanism of inhibition of adipogenesis.

The reversal of the inhibitory effect of THP-1-MacCM of TG accumulation by PD98059 was not accompanied by any clear changes in FAS or PPARγ protein expression. The basis for this partial effect on TG accumulation is not known at present. Previous studies have reported the ability of PD98059 to potentiate 3T3-L1 adipogenesis indicated by an enhancement in the expression of adipogenic markers such as aP2, PPARγ (Boney et al., 2000; Font de Mora et al., 1997). Consistent with these studies, addition of PD98059 under my standard conditions of differentiation mildly enhanced differentiation as indicated by the increase in FAS protein expression and TG accumulation.

This mildly enhanced TG accumulation in the presence PD98059 suggests that the effect of reducing ERK1/2 activity may be primarily directed to regulatory factors that either inhibit lipogenesis or activate lipolysis. There are no published data to suggest that blocking ERK1/2 with PD98059 would enhance lipogenesis. However, in 3T3-L1 adipocytes, activation of ERK1/2 is known to be essential for maximal induction of lipolysis by TNFα, β3-adrenergic receptors agonists and the powerful vasoconstrictor endothelin-1 (Robidoux et al., 2006; Souza et al., 2003) (Juan et al., 2006). In these studies, inhibition of ERK1/2 with PD98059 decreased lipolysis (Robidoux et al., 2006; Souza et al., 2003) (Juan et al., 2006). It could be that a reduction of lipolysis might give rise to adipocytes containing more TG,

which would explain the enhanced TG accumulation I observed when PD98059 was added to THP-1-MacCM or to control medium during differentiation.

Previous studies indicate that macrophage-secreted factors can increase basal lipolytic rates. 3T3-L1 adipocytes treated with RAW264-MacCM or cocultured with RAW264 macrophages release more FFA (Permana et al., 2006; Suganami et al., 2007b) (Suganami et al., 2005). Future experiments need to be conducted in order to define the precise mechanism that permits a return of TG levels in the presence of PD98059. In my differentiation experiments, PD98059 was present throughout the entire time of differentiation, so it is difficult to isolate the effect of PD98059 on lipolysis on the mature adipocytes. The best approach will be to add PD98059 on day 4 of differentiation (i.e. after MCE is complete), and to assess if it reduces lipolysis of 3T3-L1 adipocytes.

The observation that ERK1/2 mediates the THP-1-MacCM effect only on TG accumulation suggested that more than one signalling pathway is required for the full anti-adipogenic effect of THP-1-MacCM. THP-1-MacCM did not induce phosphorylation of STAT3, suggesting it is not a candidate. STAT3 plays a mitogenic role during MCE. The reduced MCE that was observed also suggests that STAT3 is not a target of the THP-1-MacCM. However, my data do not completely rule out a role for STAT3 in the anti-adipogenic effect of THP-1-MacCM. I measured STAT3 phosphorylation at a single time point of 15 minutes following stimulation. It is possible that STAT3 is not phosphorylated by THP-1-MacCM at this particular time point. A more detailed time course will provide a more thorough analysis of the possibility of a STAT3 response. Since STAT3 is a transcription factor, it could be activating genes that have inhibitory effects on adipogenesis (Yoshimura, 2006).

Treatment of 3T3-L1 preadipocytes with THP-1-MacCM also phosphorylated IKKβ. In inflammatory conditions, serine kinases such as IKKβ are implicated in the etiology of insulin resistance and type 2 diabetes (Arkan et al., 2005). Mice heterozygous for a null mutation in IKKβ are partially protected from fat-induced insulin resistance (Yuan et al., 2001). Inhibition of IKKβ with salicylates improves insulin sensitivity (Kim et al., 2001; Yuan et al., 2001). IKKβ impairs insulin signalling through serine phosphorylation of the insulin receptor substrate-1 (IRS-1) (Shi et al., 2006), and this could interfere with 3T3-L1 adipocyte differentiation. It will therefore be relevant to assess insulin signalling target such as IRS-1 serine phosphorylation. IKKβ can also activate the NF-κB pathway (Gilmore, 2006). NF-κB is a transcription factor that induces the expression of anti-adipogenic genes such as TNF-α and IL-6 (De Bosscher et al., 2006). NF-κB could, in this way, inhibit 3T3-L1 adipocyte differentiation.

Further studies are required to determine if activation of IKKβ results in the activation of the NF-κB pathway. Western blot analysis of nuclear NF-κB subunits using nuclear protein extracts isolated from 3T3-L1 preadipocytes exposed to THP-1-MacCM would directly assess this. My attempts to investigate the role of the activation of IKKβ in the anti-adipogenic effect of THP-1-MacCM were unsuccessful. The IKKβ inhibitor I used, SC514, proved to be cytotoxic under my experimental conditions. As future alternatives, dominant negative of IκBα or small interfering RNA directed against NF-κB could help elucidate if this pathway is involved in the anti-adipogenic effect of THP-1-MaCM.

Others have shown that treatment of human preadipocytes with MaCM results in the activation of NF-κB (Lacasa et al., 2007). However, little is known about the contribution of preadipocytes to the low-grade inflammatory state observed in obesity. In my studies, the

activation of IKKβ by THP-1-MacCM suggests that macrophage-secreted factors give rise to inflamed preadipocytes. In inflammatory conditions, preadipocytes have been shown to synthesize more pro-inflammatory products than adipocytes (Chung et al., 2006; Poulain-Godefroy and Froguel, 2007). Therefore, inflamed preadipocytes also contribute to recruitment of macrophages in the adipose tissue and thereby contribute to the reduction of the adipogenic capacity of adipose tissue. Inflamed preadipocytes also contribute to adipose tissue dysfunction.

In this project, I tested the effect of MacCM on the differentiation of preadipocytes. To obtain preliminary information on the pathways that are involved in the anti-adipogenic effect of MacCM I conducted acute stimulation studies of 3T3-L1 preadipocytes with THP-1-MacCM in the absence of the usual differentiation inducers (dex, IBMX, and Insulin). The phosphorylation levels of ERK1/2, IKKβ, and STAT3 obtained in this context may be different from what would be observed if the stimulation had been conducted in the presence of the differentiation inducers. Therefore, it will be important to assess the effect of acute stimulation of preadipocyte with THP-1-MacCM in the presence of differentiation inducers. Nevertheless, the signalling data in preadipocytes are supported by a recent published study showing that the protein expression and the phosphorylation levels of ERK1/2 and IKKβ are higher in omental compared to subcutaneous adipose tissue of severely obese individuals (Bashan et al., 2007). This could provide more understanding into how increased macrophage infiltration into omental compared to subcutaneous depot is linked to obesity-associated comorbidities (Cancello et al., 2006; Harman-Boehm et al., 2007).

At present, it is not known which factor(s) secreted by murine J774 or human THP-1 macrophages is mediating the anti-adipogenic effect. The strength of our approach relies on

the fact that MacCM provides a more integrated system to study the net effect of macrophage-derived cytokines on adipocyte differentiation. Previous studies have assessed the effect of individual c ytokines known to be secreted by macrophages on adipogenesis. Cytokines studied in such isolated fashion yield pro- and anti-adipogenic effects. TNF-α was recognized early as an inhibitor of 3T3-L1 and human adipocyte differentiation (Torti, FM. 1989; Petruschke, T. 1993). Interleukin 6 may have a similar role in subcutaneous adipose tissue differentiation (Sopasakis, VR. 2004). In contrast, macrophage colony-stimulating factor promotes rabbit subcutaneous adipocyte hyperplasia, raising the possibility of enhanced adipogenesis (Levine, JA. 1998). Similarly, the human ortholog of mouse resistin, hFIZZ3, is expressed by macrophages, and stimulates human subcutaneous preadipocyte proliferation, yielding more adipocytes (Ort,T. 2005).

J774 macrophages are known to secrete IL-1β. THP-1 macrophage-secreted products include TNF-α, IL-1α, and IL-1β (Auwerx, J. 1991). One or more of these factors could be mediating the anti-adipogenic effect. Enzyme-linked immunosorbent assay (ELISA) can be used to investigate the factors that are present in the MacCM. Preliminary ELISA testing indicates that the THP-1-MacCM generated in our laboratory does not contain IL-6. MacCM can also be fractionated by molecular weight cut-offs, and the effects of the different fractions can be tested with respect to their ability to inhibit adipocyte differentiation. This will give insight into the molecular weight of the candidate factor(s). Furthermore, fractions obtained from different molecular weight cut-offs can be combined and their effect can be tested on adipocyte differentiation. This will indicate if the anti-adipogenic effect of MacCM requires the combined effect of different factors. Another strategy could be immunodepletion studies of candidate factors, or the use of neutralizing antibodies targeting specific

candidates.

Conclusions

My results demonstrate that J774- or THP-1-MacCM impairs 3T3-L1 and human adipogenesis *in vitro*. The results also suggest that adipose tissue function may be altered by two distinct effects of MacCM. 1) By an impairment of MCE resulting in reduced access of transcription factors to DNA regulatory elements and in that way inhibiting the induction of genes associated with the mature phenotype of the adipocyte. 2) By decreased lipid storage of differentiated adipocytes. These *in vitro* studies suggest a dual attenuation of adipogenic capacity by MacCM. They provide a basis for future experiments to understand the mechanism of the loss of adipogenic capacity *in vivo,* and the resulting pro-inflammatory and insulin-resistant state of obesity.

It should be noted that the underlying molecular mechanisms implicated in the anti-adipogenic effect of macrophage-secreted factors *in vivo* are much more complex. The nature of paracrine interactions between macrophages and adipose cells are likely to be influenced by their environment. Infiltrating macrophages could represent a special subpopulation of circulating monocytes, and it has even been suggested that adipose tissue macrophages may arise from adipose stromal cells (Charriere et al., 2003). Furthermore, the profile of cytokines secreted by resident macrophages may be influenced by adipose cells. It will therefore be important to test the anti-adipogenic effect of MacCM produce by using adipose tissue-derived macrophages. Nevertheless, our data provide new information which can be viewed as a framework for future studies. This framework is important because learning more about the action of macrophage-secreted factors on the adipogenic process

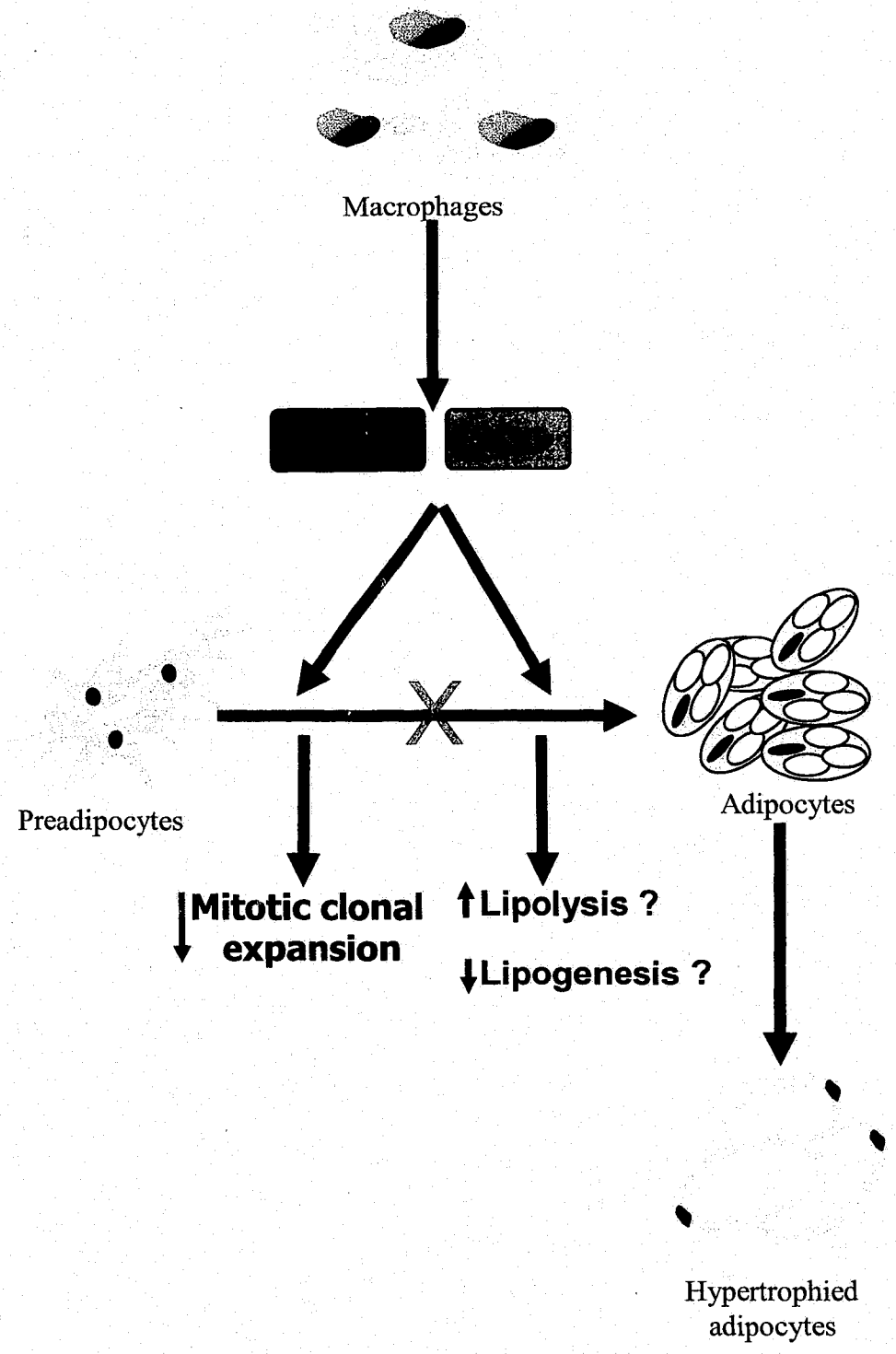
may lead to new approaches to modulate adipocyte differentiation and thereby manage the pro-inflammatory profile of hypertrophied adipocytes within adipose tissue.

Proposed model

The results obtained from these *in vitro* studies suggest a dual attenuation of adipogenic capacity by MacCM (Figure 21). MacCM can act at an early stage of differentiation to impair adipogenesis. During this time frame, macrophage-secreted factors impair MCE, an early obligatory event for differentiation. The impairment of mitotic clonal expansion may interfere with access of transcription factors to DNA regulatory elements and in that way inhibit the induction of genes associated with the mature phenotype, thereby reducing the pool of differentiated adipocytes. This would lead to compensatory hypertrophy of pre-existing adipocytes. MacCM may not only be targeting MCE, since the differentiation of human preadipocytes, which do not proceed through MCE in culture, was also inhibited. The phosphorylation of ERK1/2 and IKKβ, based on the 3T3-L1 preadipocyte data, suggests their involvement in the anti-adipogenic effect of MacCM. However we only confirmed a role for ERK1/2 in the inhibitory effect of MacCM on TG accumulation. A late effect of MacCM on differentiated adipocyte can not be excluded. MacCM could increase lipolysis and/or decrease lipogenesis. The overall effect of macrophage-secreted factors is a reduction in the lipid storage capacity of the adipose tissue.

Figure 21. Proposed model for the anti-adipogenic effect of MacCM

MacCM act at an early stage of differentiation to impair adipogenesis. During this time frame, MacCM impairs MCE, an early obligatory event for differentiation. The impairment of MCE may lead to compensatory hypertrophy of pre-existing adipocytes. MacCM induced phosphorylation of ERK1/2 and IKKß in 3T3-L1 preadipocytes. A role for ERK1/2 was confirmed in the inhibitory effect of MacCM on TG accumulation. MacCM may also have a late effect on differentiated adipocytes, such as increasing lipolysis and/or decreasing lipogenesis.


Macrophages
Preadipocytes
Adipocytes
Mitotic clonal expansion
Lipolysis ?
Lipogenesis ?
Hypertrophied adipocytes

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CONTRIBUTIONS OF COLLABORATORS

Thanks to Anne Landry for assessing the effect of medium conditioned by J774 macrophages on the differentiation of human abdominal subcutaneous and omental adipocyte differentiation. Thanks to Michelle Yarmo for assessing the effect of medium conditioned by THP-1 macrophages on the MCE phase during differentiation of 3T3-L1 preadipocytes.

Vanessa Auguste Constant

Summary of qualifications

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- Fluent in both official languages

Education

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Degree to be awarded Fall 2007

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Supervisor: Supervisor: Dr. Alexander Sorisky, Professor, University of Ottawa

Work experience

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Research technician Summers 2005 and 2004
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French Monitor Fall 2004-Spring 2005
Ottawa-Carleton district school board, Ottawa, Ontario

Sales associate Summers 2002-2004
Parliament Hill Boutique, Ottawa, Ontario

Caregiver Summer 2001-Winter 2004
Central Health Services, Ottawa, Ontario

Volunteer Work

Lets Talk Science, Ottawa, Ontario — Fall 2005 -Winter 2006

Canadian National Institute for the Blind, Ottawa, Ontario — Summer 2003

Franco-Ontarian Festival, Ottawa, Ontario — Summer 2002

Canadian Multiple Sclerosis Foundation, Ottawa, Ontario — Summer 2001

Scientific Publications

Constant, VA., Gagnon, A., Yarmo, M., Sorisky, A. (2007) The anti-adipogenic Effect of macrophage-conditioned medium depends on ERK1/2 activation. In revision - judged to be potentially acceptable for publication after addressing concerns of reviewers.

Constant, VA., Gagnon, A., Landry, A., Sorisky, A. (2006) Macrophage-conditioned medium inhibits the differentiation of 3T3-L1 and human abdominal preadipocytes. *Diabetologia* 49(6):1402-11

Other Contributions

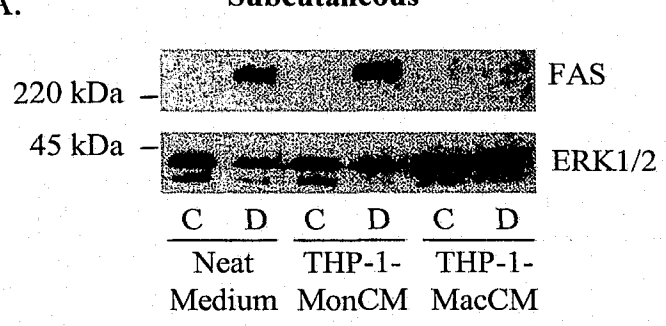
Poster presentation at the Endocrine Society's 89th annual meeting in Toronto, Canada in June, 2007. Title: Role for ERK1/2 pathway in the inhibitory effect of THP-1-macrophage-conditioned medium on 3T3-L1 adipogenesis. Authors: Constant, VA., Gagnon, A., Yarmo, M., Sorisky, A.

Poster presentation at the 66th Scientific Sessions-American Diabetes Association meeting in Washington DC, U.S.A in June, 2006. Title: The differentiation of 3T3-L1 and human abdominal preadipocytes is inhibited by macrophage-conditioned medium. Authors: Constant, VA., Gagnon, A., Landry, A., Sorisky, A.

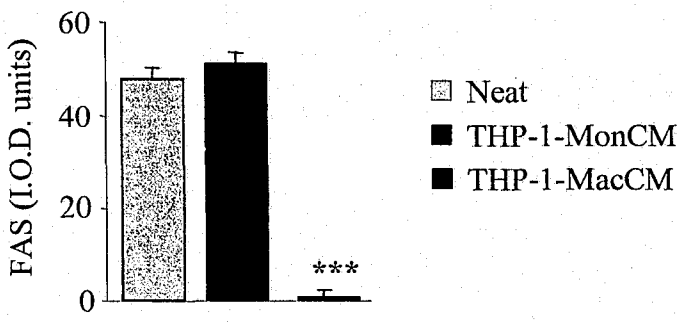
Figure 12. Medium conditioned by THP-1 macrophages inhibits differentiation of human abdominal subcutaneous and omental preadipocytes as assessed by FAS expression levels

Human subcutaneous (A and B) and omental (C and D) preadipocytes were induced to differentiate in neat medium, THP-1-MonCM, or THP-1-MacCM for 14 days. **A and C.** Solubilised protein from control and differentiated cultures was immunoblotted with antibodies against FAS, or ERK1/2 (loading control). Representative immunoblots from a single patient sample are shown. **B and D.** Densitometric data for the differentiated samples from the 4 patients are expressed as mean +/- SD. *** indicates $p<0.001$ compared with differentiation in neat medium and THP-1-MonCM.

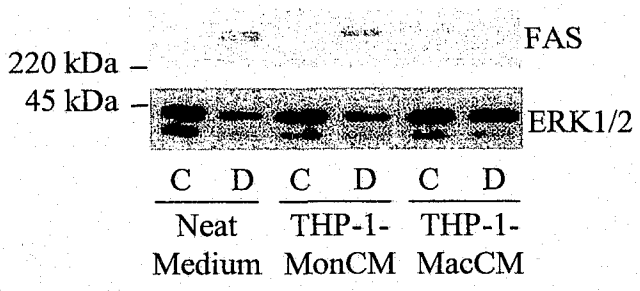
A.


Subcutaneous
220 kDa
45 kDa
FAS
ERK1/2
C D C D C D
Neat Medium
THP-1-MonCM
THP-1-MacCM

B.


FAS (I.O.D. units)
60
40
20
0
***
Neat
THP-1-MonCM
THP-1-MacCM

C.


Omental
220 kDa
45 kDa
FAS
ERK1/2
C D C D C D
Neat Medium
THP-1-MonCM
THP-1-MacCM

D.

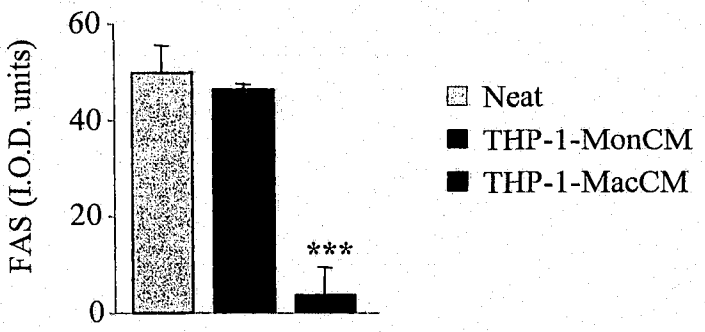
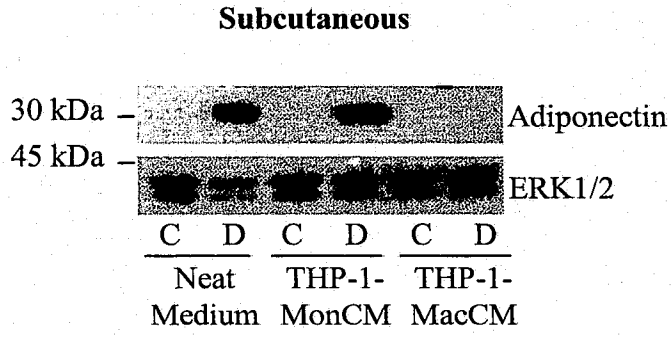

FAS (I.O.D. units)
60
40
20
0
***
Neat
THP-1-MonCM
THP-1-MacCM

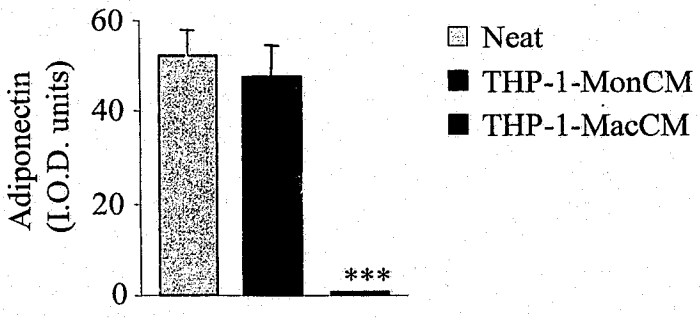
Figure 13. Medium conditioned by THP-1 macrophages inhibits differentiation of human abdominal subcutaneous and omental preadipocytes as assessed by adiponectin expression levels

Human subcutaneous (A and B) and omental (C and D) preadipocytes were induced to differentiate in neat medium, THP-1-MonCM, or THP-1-MacCM for 14 days. **A and C.** Solubilised protein from control and differentiated cultures was immunoblotted with antibodies against adiponectin, or ERK1/2 (loading control). Representative immunoblots from a single patient sample are shown. **B and D.** Densitometric data for the differentiated samples from the 4 patients are expressed as mean +/- SD. *** indicates $p<0.001$ compared with differentiation in neat medium and THP-1-MonCM.

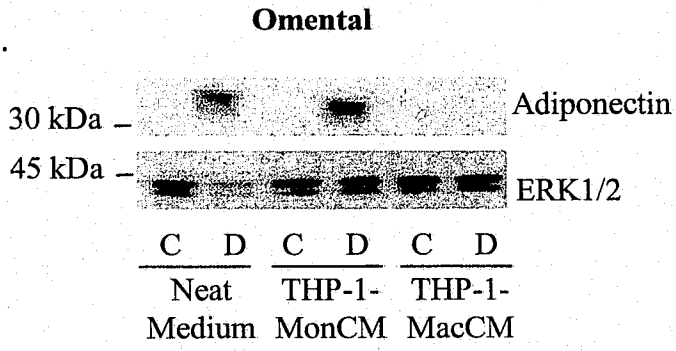
A.


Subcutaneous
30 kDa
45 kDa
Adiponectin
ERK1/2
C D C D C D
Neat Medium
THP-1-MonCM
THP-1-MacCM

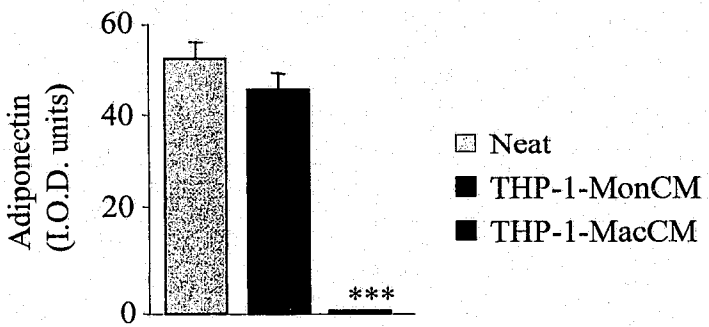
B.


Adiponectin (I.O.D. units)
60
40
20
0
***
Neat
THP-1-MonCM
THP-1-MacCM

C.


Omental
30 kDa
45 kDa
Adiponectin
ERK1/2
C D C D C D
Neat Medium
THP-1-MonCM
THP-1-MacCM

D.


Adiponectin (I.O.D. units)
60
40
20
0
***
Neat
THP-1-MonCM
THP-1-MacCM

accumulation (Figure 14A). However when exposure of the differentiating 3T3-L1 preadipocytes to THP-1-MacCM was delayed to either days 2, 4, or 6, there was no longer any effective inhibition of lipid droplet formation. These results were confirmed with a maximal 59% ($p<0.001$) reduction in TG accumulation when THP-1-MacCM was present from day 0 to 8, but only a 14% ($p<0.05$) reduction when THP-1-MacCM was added on day 2, and no significant reduction at all, when THP-1-MacCM was added on days 4 or 6 (Figure 14B). The protein expression of FAS and PPARγ was reduced by 47% and 29% ($p<0.01$) respectively, when THP-1-MacCM was added from day 0 to 8 (Figures 15A-D). However, THP-1-MacCM had no significant effect on PPARγ and FAS expression when added after the first two days of differentiation. The neat medium had no effect on 3T3-L1 adipocyte differentiation.

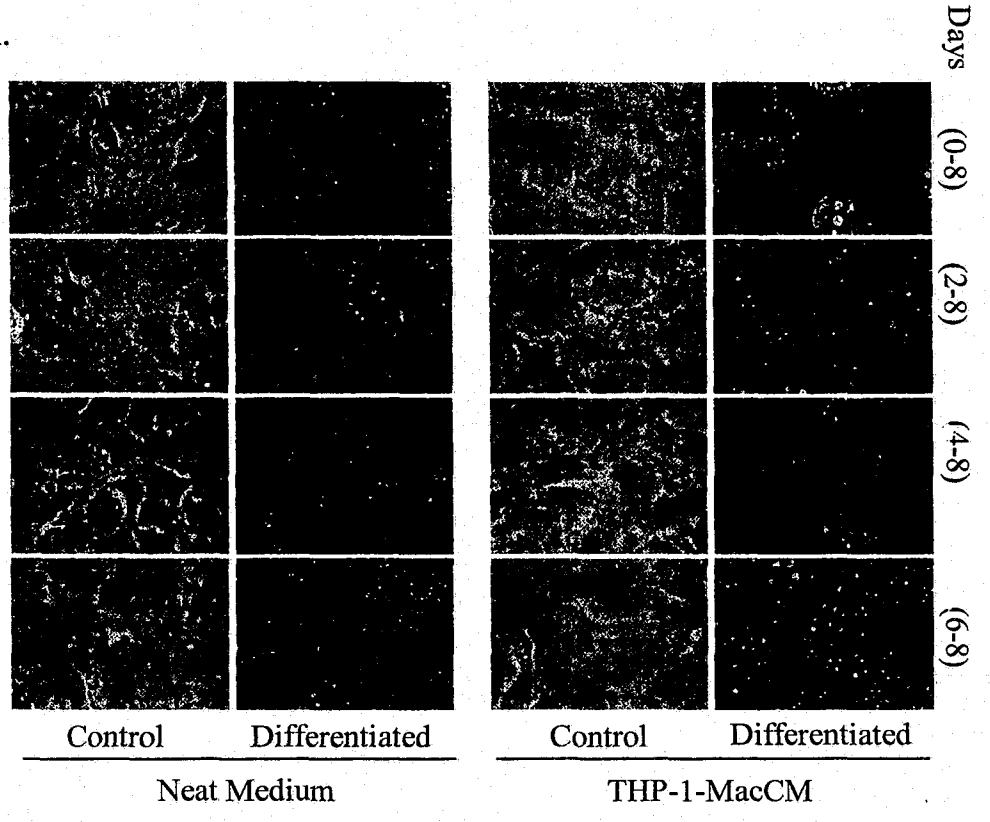
Medium conditioned by THP-1 macrophages impairs MCE during 3T3-L1 adipocyte differentiation

Having shown that THP-1-MacCM is required at the induction of differentiation to impair 3T3-L1 adipogenesis, we investigated if MCE, an early event that is required for differentiation to proceed (Farmer, 2006; Tang et al., 2003), was impaired by THP-1-MacCM (these experiments were conducted by Michelle Yarmo, a fourth-year Honours student under my supervision in Dr. Sorisky's laboratory). 3T3-L1 preadipocytes induced to differentiate in the presence of neat medium underwent the expected clonal expansion over the first 4 days, from 0.8 x 10^6 cells/dish to 2.4 x 10^6 cells/dish a 3 fold (n=3, $p<0.001$ vs. day 0) (Figure 16). However, in the presence of THP-1-MacCM, MCE was attenuated by 42% with a rise from 0.8 x 10^6 cells/dish to only 1.7 x 10^6 cells/dish (n=3, $p<0.01$; compared to

Figure 14. Medium conditioned by THP-1 macrophages is required at the induction of differentiation to impair adipogenesis as assessed by morphology and triglyceride accumulation

3T3-L1 preadipocytes were induced to differentiate in the presence of neat medium or THP-1-MacCM on indicated days for up to 8 days. **A.** Cultures were photographed at 400x magnification. Pictures representative of 4 independent experiments are shown. **B.** Triglyceride (TG) was extracted, quantified, and normalised to protein content. Results are expressed as the mean±SD of 4 independent experiments. *** indicates $p<0.001$; * indicates $p< 0.05$ compared with differentiation in neat medium.

A.


Days
(0-8)
(2-8)
(4-8)
(6-8)
Control
Differentiated
Control
Differentiated
Neat Medium
THP-1-MacCM

B.

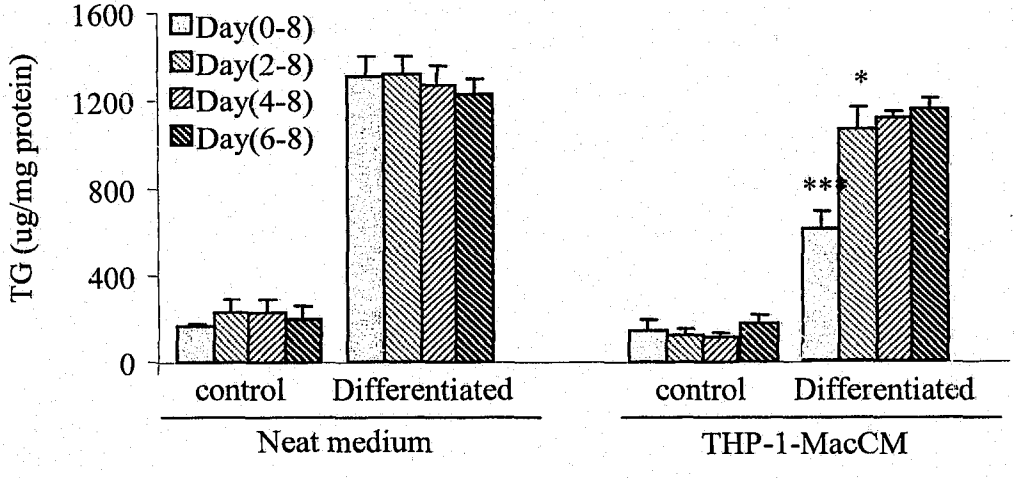
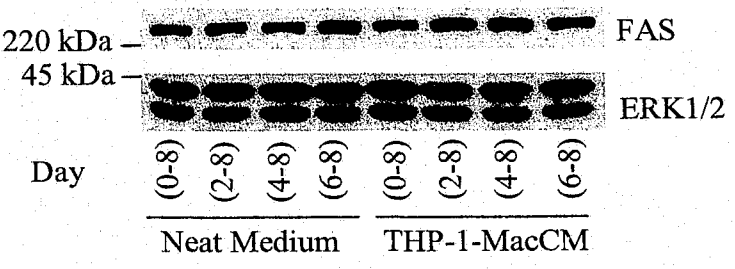

TG (ug/mg protein)
1600
1200
800
400
0
Day(0-8)
Day(2-8)
Day(4-8)
Day(6-8)
**
*
control
Differentiated
control
Differentiated
Neat medium
THP-1-MacCM

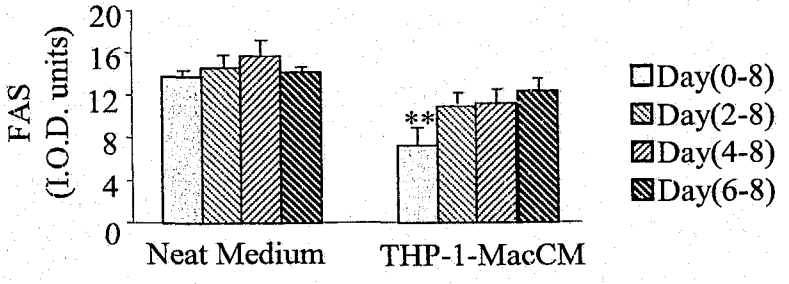
Figure 15. Medium conditioned by THP-1 macrophages is required at the onset of differentiation to inhibit adipogenesis as assessed by FAS and PPARγ expression levels.

3T3-L1 preadipocytes were induced to differentiate in the presence of neat medium or THP-1-MacCM on indicated days for up to 8 days. **A and C.** Solubilised protein from differentiated cultures was immunoblotted with antibodies against FAS, PPARγ, or ERK1/2 (loading control). Immunoblots shown are representative of 4 independent experiments. **B and D.** Densitometric data for the differentiated samples from the 4 experiments are expressed as mean±SD. ** indicates $p<0.01$ compared with differentiation in control medium.

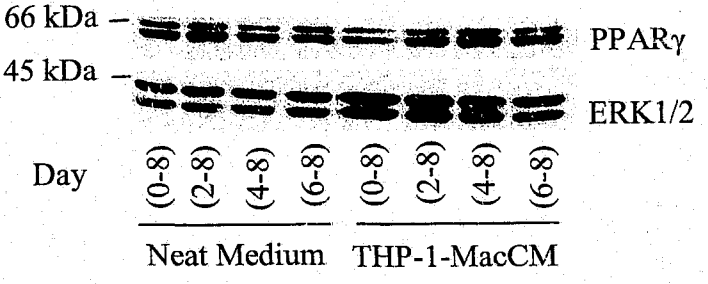
A.


220 kDa
45 kDa
FAS
ERK1/2
Day
(0-8)
(2-8)
(4-8)
(6-8)
(0-8)
(2-8)
(4-8)
(6-8)
Neat Medium
THP-1-MacCM

B.


FAS
(I.O.D. units)
20
16
12
8
4
0
**
Neat Medium
THP-1-MacCM
Day(0-8)
Day(2-8)
Day(4-8)
Day(6-8)

C.


66 kDa
45 kDa
PPARγ
ERK1/2
Day
(0-8)
(2-8)
(4-8)
(6-8)
(0-8)
(2-8)
(4-8)
(6-8)
Neat Medium
THP-1-MacCM

D.

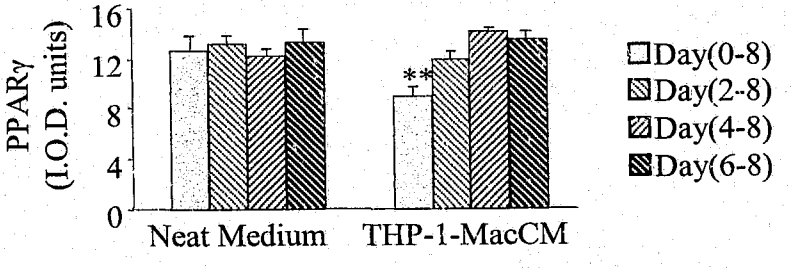
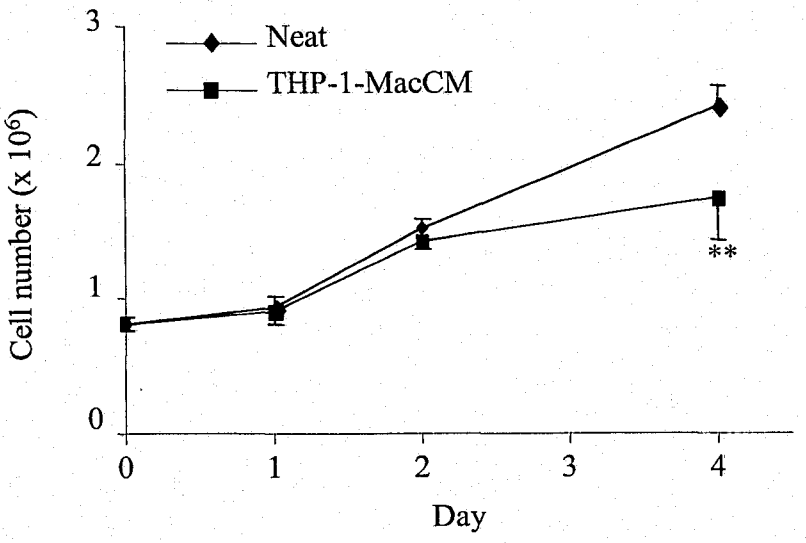

PPARγ
(I.O.D. units)
16
12
8
4
0
**
Neat Medium
THP-1-MacCM
Day(0-8)
Day(2-8)
Day(4-8)
Day(6-8)

Figure 16. Medium conditioned by THP-1 macrophages inhibits the clonal expansion phase of 3T3-L1 adipogenesis.

3T3-L1 preadipocytes were induced to differentiate in neat medium or THP-1-MacCM. On the indicated days after induction of differentiation, cells were trypsinized, and counted using a hemacytometer. Results are the mean±SD of duplicate dishes from 3 independent experiments. ** indicates $p<0.01$ compared with differentiation in neat medium.


Neat
THP-1-MacCM
Cell number (x 10^6)
3
2
1
0
0
1
2
3
4
Day
**

neat medium).

Medium conditioned by THP-1 macrophages phosphorylates ERK1/2 and IKKβ in 3T3-L1 preadipocytes

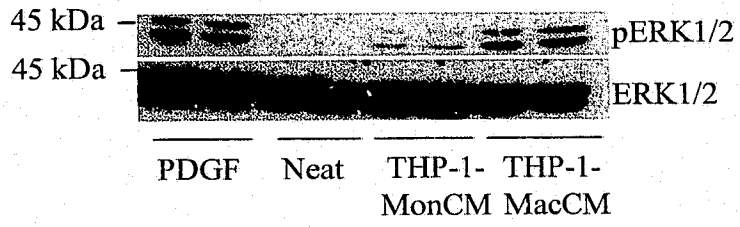
Given the observed alterations in MCE, I measured the acute effect of THP-1-MacCM on the activation of two mitogenic kinases, ERK1/2 and STAT3, both of which have been implicated in the clonal expansion phase of 3T3-L1 adipogenesis (Prusty et al., 2002; Tang et al., 2005) (Deng et al., 2000). Acute stimulation of confluent 3T3-L1 preadipocytes with THP-1-MacCM significantly increased phosphorylation of ERK1/2 by 6.5 fold (n=3, $p<0.01$; Figure 17A, B) compared to neat medium. Medium conditioned by THP-1 monocytes (THP-1-MonCM), which does not impair adipogenesis, caused only a minimal increase in ERK1/2 phosphorylation that did not reach statistical significance. THP-1-MacCM did not promote phosphorylation of another mitogenic target, STAT3, suggesting some degree of selectivity in its intracellular targets (Figures 17C, D).

Knowing that macrophages secrete pro-inflammatory factors, I investigated the inflammatory response of 3T3-L1 preadipocytes to the acute effect of THP-1-MacCM. I measured the phosphorylation of IKKβ, which is involved in the activation of NF-κB, the master regulator of inflammatory responses. THP-1-MacCM but not THP-1-MonCM induced a significant 9 fold (n=3, $p<0.001$) increase in IKKβ phosphorylation levels (Figures 18A, B) compared to neat medium. PDGF serves as a positive control for the phosphorylation of ERK1/2, STAT3, and IKKβ.

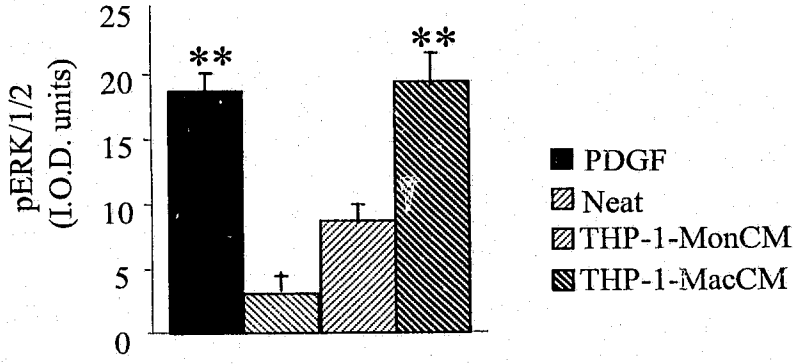
Figure 17. Medium conditioned by THP-1 macrophages phosphorylates ERK1/2 in 3T3-L1 preadipocytes

3T3-L1 preadipocytes were stimulated for 15 minutes with control medium, THP-1-MonCM, THP-1-MacCM, or 10 ng/ml PDGF dissolved in THP-1 growth medium (positive control). **A and C.** Solubilised protein were immunoblotted with antibodies directed against phospho-ERK1/2 (pERK1/2), ERK1/2, phospho-STAT3 (pSTAT3), or STAT3. Immunoblots shown are representative of 3 independent experiments. **B and D.** Densitometric data are expressed as mean±SD of the 3 independent experiments, each performed in duplicate. ** indicates $p < 0.01$ compared with stimulation with neat medium.

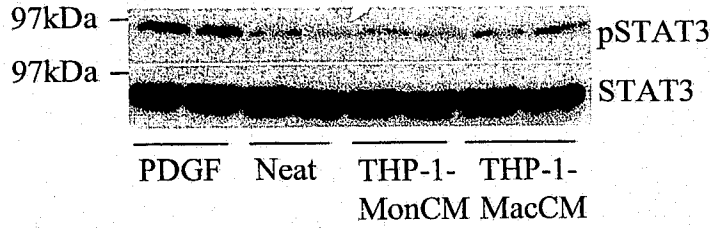
A.


45 kDa
45 kDa
pERK1/2
ERK1/2
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

B.


pERK/1/2 (I.O.D. units)
25
20
15
10
5
0
**
**
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

C.


97kDa
97kDa
pSTAT3
STAT3
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

D.

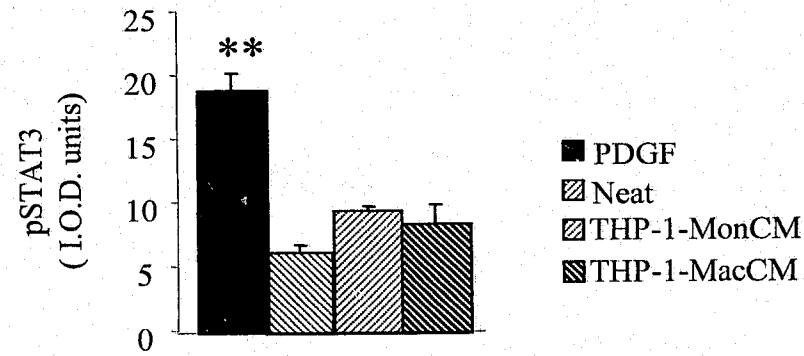
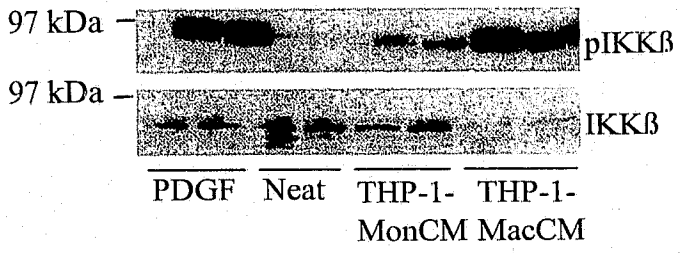

pSTAT3 (I.O.D. units)
25
20
15
10
5
0
**
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

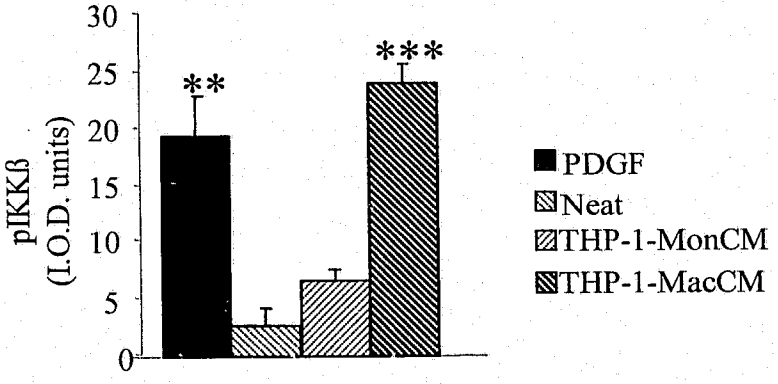
Figure 18. Medium conditioned by THP-1 macrophages phosphorylates IKKß in 3T3-L1 preadipocytes

3T3-L1 preadipocytes were stimulated for 15 minutes with control medium, THP-1-MonCM, THP-1-MacCM, or 10 ng/ml PDGF dissolved in THP-1 growth medium (positive control). **A**. Solubilised protein was immunoblotted with antibodies directed against phospho-IKKß (pIKKß) or IKKß. Immunoblots shown are representative of 3 independent experiments. **B.** Densitometric data are expressed as mean±SD of the 3 independent experiments, each performed in duplicate. *** indicates $p<0.001$; ** indicates $p<0.01$ compared with stimulation with neat medium.

A.


97 kDa
pIKKß
97 kDa
IKKß
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

B.


30
25
20
15
10
5
0
pIKKß (I.O.D. units)
**
***
PDGF
Neat
THP-1-MonCM
THP-1-MacCM

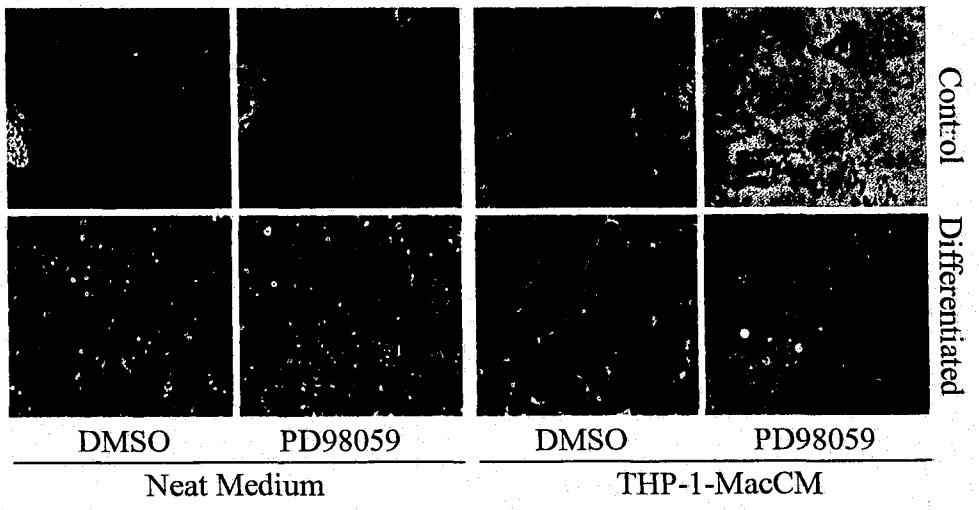
The inhibitory effect of medium conditioned by THP-1 macrophages on TG accumulation in differentiating 3T3-L1 is alleviated by PD98059, a MEK1 inhibitor

Since acute stimulation of confluent 3T3-L1 preadipocytes with THP-1-MacCM phosphorylated ERK1/2, it was important to determine if this protein plays a role in the anti-adipogenic effect of THP-1-MacCM. 3T3-L1 preadipocytes were pretreated with 25 μmol/L PD98059, then induced to differentiate with THP-1-MacCM or neat medium with or without PD98059. THP-1-MacCM impaired 3T3-L1 differentiation; this was indicated by a reduction in lipid droplet accumulation. However, the presence of PD98059 prevented this reduction as assessed morphologically (Figure 19A). The 69% (n=3, $p<0.001$) suppression of TG accumulation caused by THP-1-MacCM during 3T3-L1 adipogenesis was substantially weakened by the addition of PD98059 during adipogenesis, resulting in only a 25% (n=3, $p<0.01$) decrease in TG accumulation (Figure 19B). In contrast, PD98059 did not alter the inhibitory effect of THP-1-MacCM on the protein expression of FAS, and had only a minor effect on the inhibition of PPARγ expression (Figures 20A-D). Addition of PD98059 under control conditions of differentiation mildly enhanced differentiation, as assessed by a 16% (n=3, $p<0.01$) more TG accumulation (Figure 19B) and 35% higher FAS protein expression (n=5; $p<0.05$; Figures 20A, B).

Figure 19. The inhibitory effect of medium conditioned by THP-1 macrophages on lipid accumulation during adipogenesis is reversed by PD98059, a MEK1 inhibitor

3T3-L1 preadipocytes were pretreated for 15 minutes with PD98059 (25μM) or vehicle (DMSO), then induced to differentiate in the presence of neat medium or THP-1-MacCM with PD98059 (25μM) or vehicle for 6 days. **A.** Cultures were photographed at 400X magnification. Pictures representative of duplicate of 5 independent experiments are shown. **B.** TG was extracted, quantified, and normalised to protein content. Results are expressed as the mean±SD of 3 independent experiments. *** indicates $p<0.001$; ** indicates $p<0.01$ between indicated pairs.

A.


Control
Differentiated
DMSO
PD98059
DMSO
PD98059
Neat Medium
THP-1-MacCM

B.

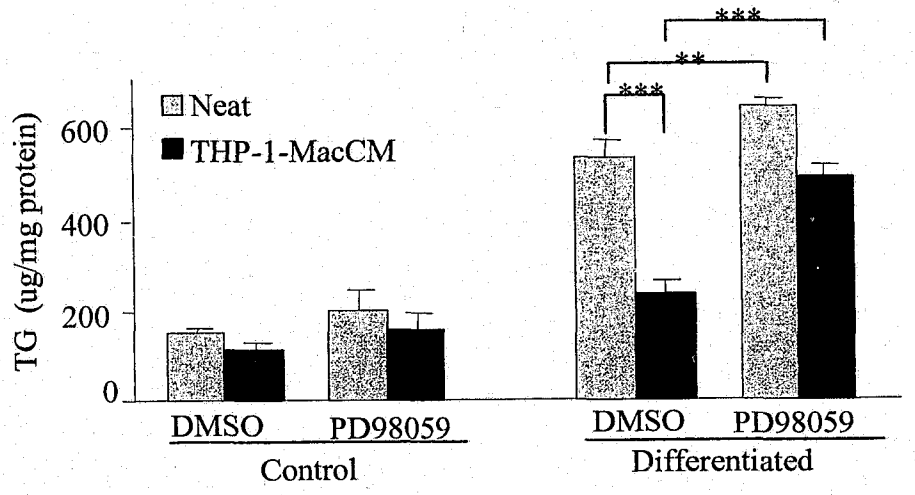
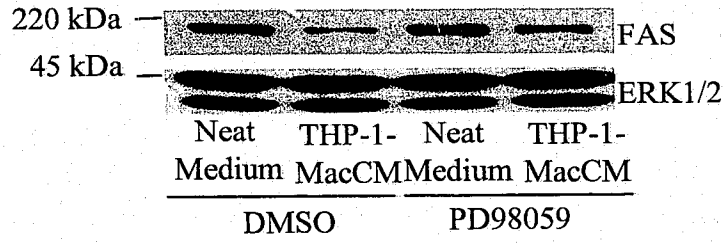

***
**
***
Neat
THP-1-MacCM
TG (ug/mg protein)
600
400
200
0
DMSO
PD98059
DMSO
PD98059
Control
Differentiated

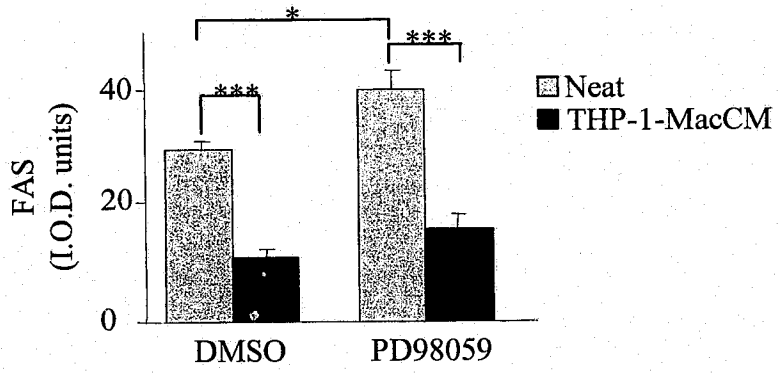
Figure 20. The inhibitory effect of medium conditioned by THP-1 macrophages on FAS and PPARγ during adipogenesis is not reversed by PD98059

3T3-L1 preadipocytes were pretreated for 15 minutes with PD98059 (25μM) or vehicle (DMSO), then induced to differentiate in the presence of Neat medium or THP-1-MacCM with PD98059 (25μM) or vehicle for 6 days. **A and C.** Solubilised protein from differentiated cultures were immunoblotted with antibodies directed against FAS, PPARγ, or ERK1/2 (loading control). Immunoblots shown are representative of 5 independent experiments. **B and D.** Densitometric data are expressed as mean±SD of 5 independent experiments. Statistical significance is indicated as follows: *** indicates $p<0.001$; * indicates $p<0.05$ between indicated pairs.

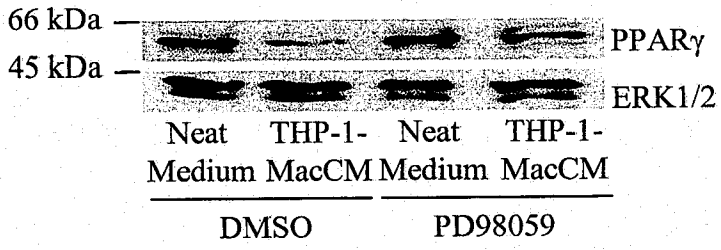
A.


220 kDa
45 kDa
FAS
ERK1/2
Neat Medium
THP-1-MacCM
Neat Medium
THP-1-MacCM
DMSO
PD98059

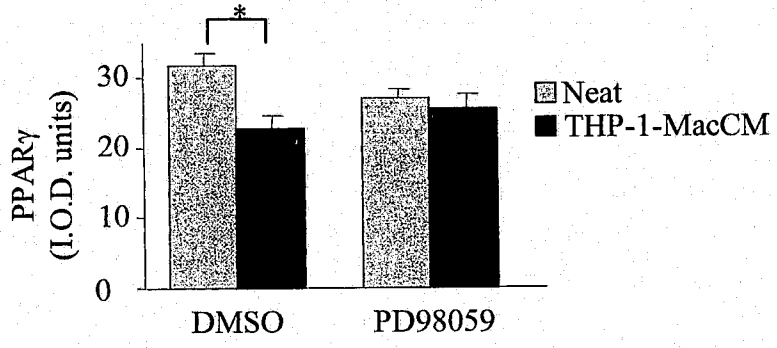
B.


*
***
***
40
20
0
FAS (I.O.D. units)
Neat
THP-1-MacCM
DMSO
PD98059

C.


66 kDa
45 kDa
PPARγ
ERK1/2
Neat Medium
THP-1-MacCM
Neat Medium
THP-1-MacCM
DMSO
PD98059

D.


*
30
20
10
0
PPARγ (I.O.D. units)
Neat
THP-1-MacCM
DMSO
PD98059

DISCUSSION

The inflammatory state observed with obesity is characterized as a deregulated production of cytokines and acute-phase reactants, leading to activation of inflammatory signalling pathways (Wellen and Hotamisligil, 2005). Interestingly, there is evidence of an increased infiltration of macrophages in adipose tissue of obese humans and rodents (Curat et al., 2004; Weisberg, 2003) (Xu, 2003). Inflamed macrophages release a large number of pro-inflammatory cytokines, some of which are also produced by adipocytes. Together, adipose tissue macrophages and adipocytes contribute to the low-grade inflammatory response associated with obesity and insulin resistance (Weisberg, 2003).

To gain insights into how macrophage-secreted factors contribute to adipose tissue dysfunction, I generated MacCM using the murine J774 and the human THP-1 macrophages. The effect of MacCM was subsequently tested on the differentiation of 3T3-L1 murine preadipocytes and human stromal preadipocytes isolated from the abdominal subcutaneous and omental fat depots. The results demonstrated that MacCM from either murine J774 or human THP-1 macrophages impairs murine 3T3-L1 and human adipocyte differentiation.

To obtain mechanistic information on how MacCM impairs adipocyte differentiation, I used the human THP-1 macrophages and murine 3T3-L1 preadipocytes as model systems. The data shows that the critical time point for the inhibition of 3T3-L1 adipogenesis by THP-1-MacCM is within the first 2 days of the 8-day differentiation process, which is the time period associated with MCE. THP-1-MacCM reduced preadipocyte proliferation during the MCE phase. THP-1-MacCM increased ERK1/2 phosphorylation, and pharmacological inhibition of ERK1/2 weakened the ability of THP-1-MacCM to block TG accumulation.

Functional adipose tissue is required to maintain normal physiological processes such

as metabolic, vascular, and immune function; whereas dysfunctional adipose tissue is associated with insulin resistance, inflammation, and cardiometabolic diseases (Pausova, 2006). One of the proposed causes of dysfunctional adipose tissue is an insufficient capacity to accumulate excess energy intake through adipogenesis (Pausova, 2006). In the context of obesity, this deficit in adipogenesis may favour the development of hypertrophied adipocytes that are insulin-resistant, thereby upregulating the production of pro-inflammatory adipokines (Danforth, 2000; Heilbronn et al., 2004) (Le Lay et al., 2001). Furthermore, limitation of adipose tissue capacity to store excess energy intake will result in ectopic fat deposition in tissues not designed for lipid storage, such as in liver and skeletal muscle, and this will lead to insulin resistance and inflammation in those tissues (Danforth, 2000; Heilbronn et al., 2004).

Factors that impair adipogenesis are only just beginning to be defined. Since there is more macrophages infiltration reported in the adipose tissue in obese state, I hypothesized that macrophage-secreted factors inhibit adipogenesis. Furthermore, weight loss improves the insulin-resistant and inflammatory state seen in obese state, and this was concomitant with a significant decrease in subcutaneous adipose tissue macrophage number (Cancello et al., 2005; Christiansen et al., 2005) (Clement et al., 2004). Weight loss also enhances adipogenic C/EBPα protein expression in human stromal abdominal subcutaneous preadipocytes (Aubin et al., 2004).

My data reveal a significant inhibition of 3T3-L1 adipocyte differentiation is caused by either murine J774 or human THP-1 MacCM, without any evidence of cytotoxity. The inhibitory effect of THP-1-MacCM on 3T3-L1 adipogenesis was less potent compared to effect of J774-MacCM. A possible explanation for this could be the more robust 3T3-L1

adipocyte differentiation in RPMI medium than in DMEM used for the J774-MacCM studies, which might have weakened the effect of the THP-1-MacCM. The use of either RPMI or DMEM medium was dictated by the growth requirements of the two types of macrophages. Another possible explanation could be a species-specific effect of murine J774-MacM acting on murine 3T3-L1 adipocyte differentiation, allowing a more potent inhibition.

The immortalised murine 3T3-L1 preadipocyte is one of the most widely used cell models to study adipocyte differentiation (Cornelius et al., 1994; Gregoire et al., 1998). They have a high capacity to differentiate into cells with the morphological and biochemical properties of adipose cells (Green and Meuth, 1974). However, they are aneuploid and therefore possess characteristics that are different from those of tissue preadipocytes (Cornelius et al., 1994). It was important to confirm the results obtained with the 3T3-L1 cell line with stromal preadipocytes isolated from human abdominal subcutaneous and omental fat depots. To minimise inter-subject variability, the subcutaneous and omental adipose tissue for these studies were paired samples from each donor.

The maximal differentiation response of the omental preadipocytes was somewhat lower than that of subcutaneous preadipocytes. This has been observed by others who have also used an adipogenic protocol containing PPARγ agonists (Adams et al., 1997; Digby et al., 1998). J774-MacCM induced a moderate inhibition of differentiation of abdominal subcutaneous preadipocytes, whereas the differentiation of omental stromal preadipocytes was less affected. In contrast, no depot-related response was observed with THP-1-MacCM, because differentiation of both omental and subcutaneous preadipocytes was completely suppressed by THP-1-MacCM.

The reason for the apparent depot-related susceptibility to J774-MacCM effect, but not that of THP-1-MacCM is not known. There may be a variation in the profile of secreted products between these two macrophages models. The more potent inhibition of human THP-1-MacCM on human adipocytes may be due to the same species of cells being used. Nevertheless, the depot-related susceptibility to J774-MacCM is consistent with other depot-specific properties of adipose tissue (Montague and O'Rahilly, 2000). The release of the pro-inflammatory cytokines IL-6 and IL-8 and the chemokine MCP-1 is higher in visceral compared to subcutaneous adipose tissue (Maury et al., 2007), suggesting that visceral adipose tissue is more pro-inflammatory. Furthermore, omental adipose tissue contains more macrophages than does subcutaneous adipose tissue (Cancello et al., 2006; Harman-Boehm et al., 2007).

My findings demonstrating an inhibition of adipocyte differentiation by MacCM were supported by findings from another study published after our report. This group showed that conditioned medium obtained from macrophages differentiated from human blood monocytes, as well as conditioned medium obtained from adipose tissue-derived macrophages, inhibited the differentiation of subcutaneous preadipocytes (Lacasa et al., 2007). The effect of their conditioned medium was only tested on subcutaneous preadipocytes. Therefore, there were no data on depot-related differences. There results validate the human THP-1 and murine J774 macrophages models, given that they mimic the effect of the primary human macrophages on human adipocyte differentiation

The underlying molecular mechanisms by which macrophage-conditioned medium inhibits human and 3T3-L1 adipocyte differentiation are unknown. I initiated mechanistic studies analyzing the effect of MacCM from human THP-1 macrophages on murine 3T3-L1

adipocyte differentiation. This choice was dictated by an interest in characterizing the effect of a human macrophage cell line on adipogenesis and because THP-1 cell line offers the advantage of working with a homogenous population in contrast to native human monocytes (Auwerx, 1991). The 3T3-L1 preadipocyte offers the advantage of obtaining large supplies of preadipocytes, in short periods of time, that differentiate reliably and homogeneously, compared to primary human preadipocyte.

I investigated the critical time point required for the addition of THP-1-MacCM to impair adipogenesis. The data indicate that the initial 2 day phase of differentiation is the critical time period during which preadipocytes are susceptible to the inhibitory effect of THP-1-MacCM. Since this is when MCE occurs during 3T3-L1 adipogenesis, I assessed whether THP-1-MacCM interferes with this early obligatory step of differentiation. The negative effect on cell proliferation during this time period suggested that it may be a primary event perturbed by THP-1-MacCM, and might explain why exposure to THP-1-MacCM beyond day 2 of differentiation is without significant effect. Further analysis of cell-cycle regulators such as p27 and retinoblastoma protein (Rb) during MCE would be helpful to understand more about how MCE is altered. Transcription factors expressed early during differentiation might also be perturbed by THP-1-MacCM, which could consequently impair MCE. The transcription factor C/EBPβ which is expressed early during adipocyte differentiation initiates MCE (Tang et al., 2005). Since C/EBPβ must acquire DNA-binding activity before initiating clonal expansion and transcription of C/EBPα and PPARγ genes, (Tang et al., 2005). THP-1-MacCM might inhibit the expression or the DNA-binding activity of C/EBPβ. This will also require further investigation.

Human preadipocytes do not proceed through MCE upon induction of adipogenesis

in vitro. They are believed to have already proceeded through this phase *in vivo* (Entenmann and Hauner, 1996; Tomlinson et al., 2006). Nevertheless, conditioned medium, derived from several different macrophage models, inhibits human adipogenesis (Lacasa et al., 2007). This suggests that other anti-adipogenic targets, apart from MCE, must exist.

The preadipocyte intracellular signalling pathways that are activated or blocked by MacCM, and that may participate in the inhibition of differentiation, have not been defined. The anti-adipogenic effects we observed led us to examine ERK1/2 phosphorylation. Under my conditions, phosphorylation of ERK1/2 by THP-1-MacCM appears to be inhibitory to the development of the fully differentiated adipocyte, since treatment with PD98059 significantly weakened the inhibitory effect on TG accumulation. This is consistent with the response of 3T3-L1 preadipocytes to TNF-α in which ERK1/2 is phosphorylated; PD98059 blocks ERK1/2 activation by TNF-α and reverses the inhibitory effect of TNF-α on adipogenesis (Font de Mora et al., 1997). Inappropriately prolonged ERK1/2 activation in 3T3-L1 preadipocytes, for example by overexpression of ERK1/2 or MEK (Font de Mora et al., 1997), by stretch activation (Tanabe et al., 2004), or by treatment with the environmental contaminant 2,3,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hanlon et al., 2003) has been shown to inhibit adipogenesis. Furthermore, the inhibition of adipogenesis in all these studies was reversed by treatment with PD98059 (Font de Mora et al., 1997; Tanabe et al., 2004) (Hanlon et al., 2003). IL-6, which impairs 3T3-L1 adipocyte differentiation, increased and sustained phosphorylation of ERK1/2 for 4-6 days after induction of differentiation (Gustafson and Smith, 2006). In my studies, THP-1-MacCM may maintain a prolonged activation of ERK1/2 pathway. This possibility needs to be evaluated by conducting time course stimulation of confluent 3T3-L1 preadipocytes with THP-1-MacCM, since in my