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FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

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The Anti-Adipogenic Effect of Macrophage-Conditioned Medium on 3T3-L1 and Human Adipogenesis

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THE ANTI-ADIPOGENIC EFFECT OF MACROPHAGE-CONDITIONED MEDIUM ON 3T3-L1 AND HUMAN ADIPOGENESIS

Michelle Nada Yarmo

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the M.Sc. degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology
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ABSTRACT

Macrophages accumulate in the adipose tissue of obese rodents and humans. We and others have reported that macrophage-secreted factors inhibit adipogenesis. This study aims to investigate the molecular mechanisms underlying this inhibitory effect. Murine 3T3-L1 preadipocytes were differentiated with medium conditioned by murine J774 macrophages (J774-MacCM) or human THP-1 macrophages (THP-1-MacCM). Clonal expansion, an early required adipogenic event, was inhibited by both MacCMs. Rb phosphorylation, required for cell cycle progression, was impaired by J774-MacCM. To expand our studies to a more physiological setting, human abdominal subcutaneous preadipocytes were differentiated with THP-1-MacCM or with conditioned medium from blood monocyte-derived macrophages (MDM-CM) activated with LPS. The IKKβ/NF-κB pathway appeared to be required for the THP-1 MacCM anti-adipogenic effect. Furthermore, human preadipocytes differentiated in MDM-CM (LPS) displayed a distinct morphology, altered fibronectin expression, as well as reduced lipid accumulation and expression of adipogenic markers. These studies suggest that macrophage-secreted factors impair proximal events in the adipogenic program.

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LIST OF ABREVIATIONS

aP2	Adipocyte fatty acid binding protein
ATCC	American Type Culture Collection
ATM	Adipose tissue macrophage
ATP	Adenosine triphosphate
BMI	Body mass index
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAK	Cdk-activating kinase
CCL2	C-C motif chemokine ligand 2
C/EBP	CCAAT/enhancer binding protein
cdk	Cyclin-dependent kinase
cki	cdk inhibitor
CS	Calf serum
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FoxO1	Forkhead box O1
GLUT4	Glucose transporter protein 4
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDAC3	Histone deacetylase 3
HRP	Horseradish peroxidase
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor-1
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IOD	Integrated optical density
IR	Insulin receptor
IRS	Insulin receptor substrate
LPS	Lipopolysaccharide
MacCM	Macrophage-conditioned medium
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDM	Monocyte-derived macrophage
MDM-CM	Monocyte-derived macrophage-conditioned medium
MonCM	Monocyte-conditioned medium
mTOR	Mammalian target of rapamycin
Na_3VO_4	Sodium orthovanadate

NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor-kappa B
NLS	Nuclear localization signal
PBMC	Peripheral-blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PDGFR	PDGF receptor
PDK	Phosphoinositide-dependent protein kinase
PH	Pleckstrin homology
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-trisphosphate
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
RHD	Rel-homology domain
RPMI	Roswell Park Memorial Institute
SH2	Src homology 2
T2D	Type 2 diabetes mellitus
TAZ	Transcriptional coactivator with PDZ-binding motif
TG	Triglyceride
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
WAT	White adipose tissue
WHO	World Health Organization
WHR	Waist-to-hip ratio

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INTRODUCTION

I. Obesity

Obesity is a global epidemic that has heightened the demand for effective prevention and treatment methods. In Canada, the overall age-standardized prevalence of obesity has risen from 10% in 1970 to 23% in 2004, with a 15% increase observed in men and a 9% increase observed in women (1). Obesity results from a chronic positive energy balance during which energy intake is greater than energy expenditure (2, 3). Obesity is a major risk factor for a wide array of health problems and chronic diseases in westernised societies, including hypertension, type 2 diabetes mellitus (T2D), dyslipidemia, fatty liver, cardiovascular disease, Alzheimer's disease and even some cancers (1, 4, 5). It is believed that the positive energy balance that occurs in obesity leads to an accumulation of adipose tissue mass and a dysfunctional state, which is central to the pathogenesis of metabolic disorders (2, 3).

The World Health Organization (WHO) considers a body mass index (BMI = weight in kg/height in m^2) of ≥ 25 as overweight and a BMI of ≥ 30 as obese (6). BMI is currently one of the fundamental classification systems used to identify obese individuals who are at risk of developing obesity-associated complications (7). Obese and overweight individuals can be further subdivided based on waist-to-hip ratio (WHR), which determines their body fat distribution. A WHR > 0.8 in females and > 0.95 in males is classified as central obesity (8).

Anatomical distribution of fat plays a central role in metabolic risk. Whereas increased subcutaneous fat in the thighs and hips (peripheral obesity) has little to no risk, increased intra-abdominal/visceral fat (central obesity) is associated with a high risk of

developing metabolic diseases (4). Visceral central obesity (fat accumulation around the internal organs) in particular is more closely associated with adverse health problems than subcutaneous central obesity (fat accumulation under the abdominal skin) (4, 9). A subset of metabolically healthy but obese individuals exist that show a normal metabolic profile due at least in part to lower visceral fat (10). The subset of obese individuals who are at higher risk of developing metabolic and cardiovascular diseases are those with higher visceral fat, lower insulin sensitivity and an unfavourable lipid profile, defined by lower high density lipoprotein cholesterol and higher fasting triglycerides . Therefore, in order to develop effective treatments, we need to target these at risk obese individuals.

II. The Adipose Tissue

It is estimated that the human adipose tissue is composed of 50-70% mature adipocytes, 20-40% stomal preadipocytes and 1-30% adipose tissue macrophages. However, this cellular composition varies according to body weight and anatomical location (11). The main function of adipose tissue is the maintenance of energy homeostasis, by storing excess energy as triglyceride (TG) and releasing free fatty acids (FFAs) when energy is needed (12). However, it is now recognized that, in addition to storing energy, the adipose tissue is a multifunctional organ that plays an active endocrine role in regulating whole body metabolism and homeostasis by secreting various endocrine and paracrine factors (11, 12). These factors regulate insulin sensitivity, lipid levels, coagulation, fibrinolysis, inflammation and immune response as well as body weight homeostasis (13).

The term adipokine refers to any factor produced and secreted primarily by the adipose tissue. The hormones leptin and adiponectin are adipokines that are mainly produced by adipocytes in the adipose tissue (14, 15). Cytokines are regulatory proteins that are

involved in many biological processes, including immunity, inflammation, differentiation, growth, cell proliferation and apoptosis (16, 17). Chemokines are a superfamily of low-molecular weight chemotactic cytokines that stimulate the recruitment and activation of inflammatory cells by binding to specific G-protein coupled cell-surface receptors on their target cells (16, 18). Cytokines and chemokines are produced and secreted by adipocytes or neighbouring cells, including preadipocytes and macrophages (19). They include anti-inflammatory cytokines, such as interleukin (IL)-10; pro-inflammatory cytokines, such as IL-6 and tumor necrosis factor (TNF)-α; and pro-inflammatory chemokines, such as monocyte chemoattractant protein-1 (MCP-1) (20, 21).

Adipose tissue expansion occurs in response to a need for additional fat mass stores, when caloric intake surpasses energy requirements (22). Excess calories are stored as TG in adipocytes (12, 23). Expansion involves two coordinated processes: hypertrophy and hyperplasia. Hypertrophy is the enlargement of existing adipocytes via the accumulation of triglycerides in their lipid droplets. Hyperplasia is the formation of new adipocytes via the recruitment, proliferation and differentiation of preadipocytes. The differentiation of preadipocytes to adipocytes is also known as adipogenesis (22, 24). Adipogenesis is regulated by various endocrine and paracrine factors from adipocytes and other cells. These factors trigger multiple intracellular signaling cascades, which result in the expression of downstream adipocyte-specific and adipocyte-associated genes as well as the activation of the differentiation program. Studies using clonal and/or primary preadipocyte cell lines have provided cumulative and comparative data that have been used to establish the current model of adipogenesis (22).

a. Murine 3T3-L1 preadipocyte cell model

The 3T3-L1 cell line was clonally isolated from Swiss 3T3 cells derived from disaggregated 17 to 19 day mouse embryos. These cells can be maintained as preadipocytes or can be differentiated into adipocytes, as they have already undergone determination and are committed to the adipocyte lineage (22, 25). The 3T3-L1 cell line is one of the most extensively characterized and widely studied cell models for preadipocyte differentiation (26). The use of this *in vitro* cell model has made it possible to characterize and identify the molecular and cellular events that occur in adipogenesis (Figure 1) (25).

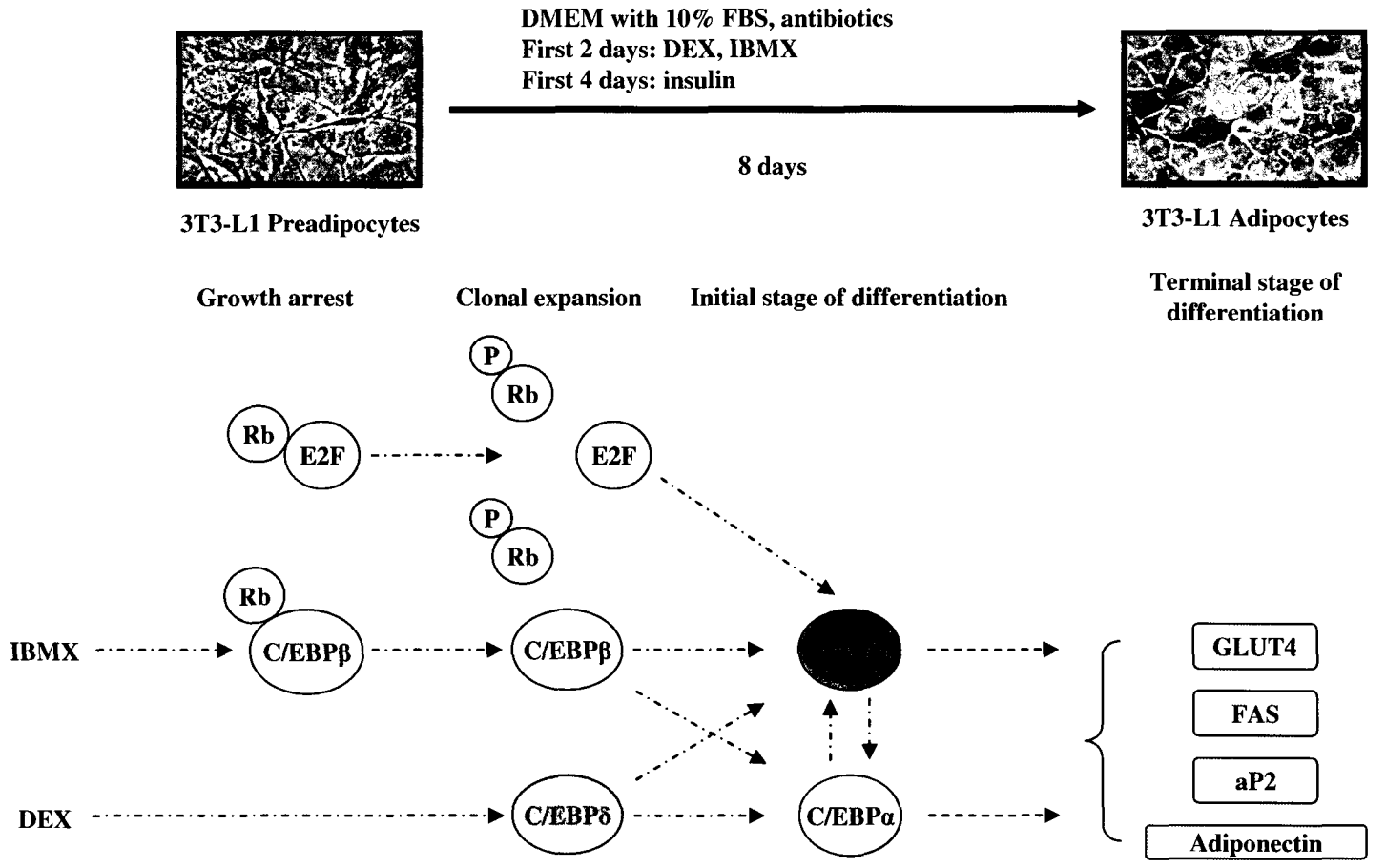
Immortalized murine 3T3-L1 cells are morphologically similar to fibroblastic preadipocytes found in the adipose tissue stroma and can be maintained indefinitely in a growing state in culture (26, 27). When the cells become growth arrested, they have the ability to differentiate into cells with the morphological and biochemical characteristics of mature adipocytes (25, 27). However, as an immortalized cell line, 3T3-L1 cells are aneuploid and therefore possess characteristics that are different from primary preadipocytes (26). These cells also do not allow for the study of depot-specific differences in fat cell behaviour (28).

b. Human primary preadipocyte cell model

Primary human preadipocyte culture models have been established and used to gain a greater understanding of human adipogenesis. These preadipocytes are isolated from the stromal-vascular fraction of adipose tissue by collagenase digestion and centrifugation (22). Although the events occurring during the differentiation of preadipocytes from clonal cell lines are similar to primary cultures, there are still important differences in adipocyte-specific gene expression levels between the two cell models (12).

Figure 1. Summary of 3T3-L1 preadipocyte differentiation.

Growth-arrested preadipocytes are induced to differentiate when exposed to adipogenic inducers comprised of insulin, DEX, IBMX and FBS. These cells synchronously undergo mitotic clonal expansion, exit the cell cycle and then proceed to terminal differentiation. Cell-cycle proteins control clonal expansion and promote adipogenesis. A cascade of transcription factors oversee the terminal stages of differentiation, which culminate in the expression of adipocyte-specific genes in the mature adipocytes. See text for further details and abbreviations.


DMEM with 10% FBS, antibiotics
First 2 days: DEX, IBMX
First 4 days: insulin
8 days
3T3-L1 Preadipocytes
3T3-L1 Adipocytes
Growth arrest
Clonal expansion
Initial stage of differentiation
Terminal stage of differentiation
P
Rb
Rb
E2F
E2F
P
Rb
Rb
IBMX
C/EBPβ
C/EBPβ
DEX
C/EBPδ
C/EBPα
GLUT4
FAS
aP2
Adiponectin

Compared to immortalized cell lines, these diploid primary preadipocytes provide a better representation of a physiological system. The ability to isolate these cells from various anatomic locations has provided information regarding regional differences between adipose tissue depots *in vivo*, which can have important metabolic consequences (22). However, unlike clonal cell lines, these preadipocytes cannot be used to study early adipogenic events. It is believed that these primary preadipocytes represent a later stage of differentiation, beyond that of clonal cell lines, since they do not require mitotic clonal expansion for differentiation (25, 29). Primary preadipocytes are also difficult to harvest, can only undergo a limited number of passages, comprise a small fraction of total adipose tissue and thus require an ongoing supply from donors (22). Inter-donor variability, such as preadipocyte differentiation capacity, also provides a challenge in term of standardization (25). However, the use of primary cell lines has helped to confirm and verify results obtained with clonal cell lines (26).

III. Adipogenesis

a. Growth arrest

The first step required for preadipocyte differentiation is growth arrest. Preadipocyte cell lines and primary preadipocytes must initially become growth arrested in order to commit to terminal differentiation (22, 25). In culture, when proliferating preadipocytes reach confluence they become growth arrested at the G_0/G_1 cell cycle boundary by contact inhibition (cell-cell contact) (22, 30).

b. Hormonal induction

When maintained in culture with fetal bovine serum (FBS) for several weeks, 3T3-L1 preadipocytes will spontaneously differentiate into fat cell clusters (25). However, after growth arrest, most cell culture systems require a cocktail of inducing agents to accelerate differentiation. Differentiation protocols vary based upon the specific cell model used. 3T3-L1 cells can be induced to differentiate in serum supplemented medium containing a hormonal cocktail of adipogenic factors (22). The commonly used adipogenic cocktail is composed of dexamethasone (DEX; a synthetic glucocorticoid agonist), isobutylmethylxanthine (IBMX; a cAMP-elevating agent that acts by inhibiting phosphodiesterases and blocking the inhibitory regulatory protein G_i) and high (supraphysiological) concentrations of insulin, which act through the insulin-like growth factor-1 (IGF-1) receptor pathway and activates signal transduction pathways that regulate adipogenic gene transcription (22, 26). Our laboratory and others have shown that in some cases, insulin can also act through the insulin receptor (IR) to induce differentiation (31-33). For clonal cell lines, high levels of insulin accelerate the rate of lipid accumulation but do not affect the number of differentiated cells. For primary preadipocytes, which undergo low levels of spontaneous differentiation, insulin increases the fraction of differentiated cells (22, 25). The differentiation of primary preadipocyte cultures also requires the adipogenic factor indomethacin, which acts as a ligand that binds and activates the adipogenic transcription factor peroxisome proliferator-activated receptor (PPAR) γ when used at a concentration of 100 μM. Indomethacin also blocks prostaglandin synthesis by inhibiting cyclooxygenase activity when used at a concentration 2-3 orders of magnitude lower than that required for induction of differentiation (2, 26, 34).

c. Mitotic clonal expansion

Mitotic clonal expansion is a prerequisite for 3T3-L1 adipogenesis (30, 35). Growth arrested 3T3-L1 preadipocytes synchronously re-enter the cell cycle when induced to differentiate. The cells undergo one to two round of postconfluent mitosis (DNA replication) and clonal expansion (cell doubling) (22, 36). This results in the clonal amplification of committed cells (25). These mitoses are thought to facilitate chromatin reorganization, enabling access of transcription factors to their target sequences in the promoters of adipocyte-specific genes (22, 37). Differences in cell cycle protein expression imply that clonal expansion and preconfluent cell growth are regulated differently in 3T3-L1 preadipocytes (22).

Cell cycle progression (from G_1 to S phase) is regulated by the phosphorylation status of the retinoblastoma tumor suppressor gene product, Rb, a nuclear phosphoprotein (30, 38, 39). The pocket proteins, which consist of Rb, p107 and p130, are negative regulators of cell cycle progression (38, 40). At the G_1 phase of the cell cycle, active, hypophosphorylated Rb inhibits growth by binding to and sequestering critical regulatory proteins, such as members of the E2F and CCAAT/enhancer binding protein (CEBP) family of transcription factors (30, 39, 41). E2F transcription factors regulate the transcription of many genes involved in cell cycle regulation/progression and differentiation, including thymidine kinase gene and PPARγ (37, 38, 42). On the other hand, C/EBP transcription factors act in a signaling cascade to initiate adipogenesis. Phosphorylation of Rb is mediated by members of the family of proline-directed, serine/threonine cyclin-dependent kinases (cdks) (43). These cdks become activated once they associate with their regulatory cyclin subunit and are subsequently phosphorylated by a cdk-activating kinase (CAK) (44-46). Furthermore, cdk inhibitors (ckis) negatively regulate the activity of cdks (38, 47). For instance, the cki $p27^{Kip1}$ binds to cyclinA/cdk2, cylinE/cdk2 and cyclinD/cdk4/6 complexes and inhibits their activity

(35, 45). Therefore, as preadipocytes progress through clonal expansion, the downregulated expression of $p27^{Kip1}$ facilitates activation of cyclin/cdk complexes (37, 45). At the G_1/S boundary, Rb is phosphorylated sequentially by cyclinD/cdk4/6 complex, during early G_1 phase, and by cyclinE/cdk2 and cyclinA/cdk2 complexes, during late G_1 phase and S phase entry respectively (35, 38, 40). The phosphorylation of Rb leads to the release and activation of E2Fs, which can then activate the transcription of genes that are required for progression through the S phase (40, 44). An *in vitro* study demonstrated that hypophosphorylated Rb negatively regulates C/EBPβ DNA-binding activity during the early stages of 3T3-L1 preadipocyte differentiation. Furthermore, this study showed that phosphorylated Rb does not associate with C/EBPβ and allows for maximal C/EBPβ DNA-binding activity. This study therefore provided a link between mitotic clonal expansion and the early stages of adipogenesis in 3T3-L1 adipose cells (30). Rb is also capable of recruiting histone deacetylase 3 (HDAC3) to the promoters of PPARγ target genes. This leads to the formation of a PPARγ-Rb-HDAC3 repressor complex which can then terminate PPARγ's ability to drive gene expression. In order to stimulate adipogenesis, the inhibition of HDAC3 activity or the dissociation of the repressor complex through Rb phosphorylation is required (48).

Unlike the aneuploid 3T3-L1 clonal cell line, human primary preadipocytes induced to differentiate in culture do not proceed through mitotic clonal expansion. It is postulated that the diploid human preadipocytes isolated from fat depots of adult patients have already undergone the critical mitoses *in vivo* and may represent a later stage of adipocyte development (22, 25, 29, 36).

d. Transcription factors regulating adipogenesis

Adipogenesis involves a temporally regulated cascade of genetic events, which culminates in the transcriptional activation of a large number of adipocyte-specific genes (12, 35, 49). The C/EBPs and PPARγ transcription factors are crucial members of this genetic cascade that promote adipogenesis (49). C/EBPs are members of the basic-leucine zipper family of transcription factors (28). PPARγ is a member of the nuclear-receptor superfamily and is considered as the master regulator of adipogenesis, as it is required and sufficient to induce differentiation (37, 49, 50). For instance, thiazolidinediones, anti-diabetic drugs that increases insulin sensitivity and act as ligands for PPARγ, were shown to be very effective at promoting adipogenesis *in vitro* and *in vivo* (28, 51).

Within 2 to 4 hours after hormonal induction of differentiation, C/EBPβ and C/EBPδ are quickly synthesized and translocated to the nucleus (30, 35). The adipogenic inducers IBMX and DEX trigger the expression of C/EBPβ and C/EBPδ respectively (22). Both transcription factors however, do not acquire DNA-binding activity until 12 to 16 hours after hormonal induction (30, 35). This delay in DNA-binding activity appears to be essential in order to allow mitotic clonal expansion to proceed and prevent the early expression of both C/EBPα and PPARγ, which are antimitotic (52-56). Although the mechanisms involved in this delay are not precisely known, Rb and ERK1/2 may be potential candidates implicated in this lag period. As previously mentioned, the phosphorylation of Rb is required during the early stages of 3T3-L1 adipogenesis in order to release C/EBPβ and E2F transcription factors (30, 40). In addition, it appears that the sequential phosphorylation of C/EBPβ by extracellular signal-regulated kinase (ERK) 1/2 and by glycogen synthase kinase 3β is also a prerequisite for C/EBPβ DNA-binding function (53, 57). Once C/EBPβ and C/EBPδ acquire DNA-binding activity, they become localized to the centromeres and bind to consensus

C/EBP-binding sites in centromeric satellite DNA (58). Acquisition of DNA-binding ability allows C/EBPβ and C/EBPδ to activate the transcription of both C/EBPα and PPARγ genes by binding to C/EBP regulatory elements in their proximal promoters (35, 58). Both C/EBPα and PPARγ facilitate the termination of the mitotic clonal expansion phase. C/EBPα and PPARγ maintain their expression through cross-regulation and by a positive feedback loop mechanism to induce their own expression (12, 59). These critical adipogenic transcription factors coordinately activate the transcription of genes that produce the adipocyte phenotype (35, 59). The product of adipocyte-specific genes include: glycerophosphate dehydrogenase, fatty acid synthase (FAS), glucose transporter protein 4 (GLUT4) and fatty acid binding protein (aP2) (28). C/EBPα is also required for the acquisition of insulin sensitivity of the mature adipocyte (12, 49, 60).

e. Growth arrest and terminal differentiation

C/EBPα and PPARγ mediate the second and final growth arrest phase known as G_D. This step is required for commitment to terminal differentiation. After permanent withdrawal from the cell cycle, the committed cells begin to express late adipogenic markers and actively transcribe genes involved in glucose and lipid metabolism (22). The cells undergo changes in morphology (from fibroblastic to spherical), engage in *de novo* lipogenesis, accumulate lipid droplets and acquire the characteristics and functions of mature adipocytes. The adipocytes acquire insulin sensitivity through increased expression of the IR and glucose transporters (e.g. GLUT4). The adipocytes also acquire a greater sensitivity to lipolytic stimuli due to an increase in β2- and β3-adrenergic receptors (22, 25).

IV. Signaling Pathways in Adipogenesis

As previously discussed, the complex transcriptional cascade which regulates adipogenesis involves the sequential activation of a variety of transcription factors. Signaling pathways upstream of these transcription factors are responsible for communicating the intracellular and extracellular conditions that determine the preadipocyte's fate for differentiation (49). Whether or not preadipocytes undergo adipogenesis is determined by the balance of pro-adipogenic and anti-adipogenic signals (12). Although there are several signaling pathways involved in adipogenesis, my project has focused on the proteins involved in the following four pathways: the PI3K/Akt signaling pathway, the ERK1/2 signaling pathway, the IKKβ/NF-κB signaling pathway and the PDGF signaling pathway.

a. PI3K/Akt pathway

Insulin initiates a signaling cascade that regulates several complex processes, such as glucose uptake, lipogenesis and the synthesis of glycogen and other proteins (61, 62). In adipogenesis, insulin mediates its effect through the phosphoinositide 3-kinase (PI3K)/Akt pathway (63). The binding of insulin to the IR leads to the intramolecular trans-autophosphorylation of the tyrosine kinase domains and subsequent activation of the intrinsic substrate kinase activity of IR (61). Well known downstream targets of the insulin receptor tyrosine kinase are members of the insulin receptor substrate (IRS) family, namely IRS-1 and IRS-2. The phosphorylation of IRS members at their tyrosine residues creates docking sites for Src homology 2 (SH2)-domain containing proteins, including the p85 regulatory subunits of the PI3K (61, 62). This results in the activation of the p110 catalytic subunit of PI3K and the subsequent production of phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] from phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] at the plasma membrane (62). The primary

lipid product of PI3K, PI(3,4,5)P_3, regulates the activity of downstream effectors (61, 62). The intracellular second-messenger PI(3,4,5)P_3 recruits and activates atypical protein kinase C (PKC) isoforms and serine/threonine Akt protein kinases (also known as protein kinase B) to the plasma membrane (61, 64). The binding of Akt to PI(3,4,5)P_3 by its pleckstrin homology (PH) domain induces a conformational change in Akt which exposes its regulatory serine 473 and threonine 308 residues (65). Phosphoinositide-dependent protein kinase (PDK) is also recruited to the plasma membrane by its PH domain (64). Activation of Akt results from its phosphorylation at serine 473 and threonine 308, which is mediated by the mammalian target of rapamycin (mTOR) and PDK1, respectively (61, 66, 67). Once activated, Akt can phosphorylate multiple substrates and transduce signals that control many cellular functions (64, 66). For instance, Akt has been shown to impair the nuclear translocation of forkhead box O1 (FoxO1) and GATA2/3, which function as anti-adipogenic transcription factors by negatively regulating the activity of PPARγ and C/EBPβ respectively (37, 49, 68). Expression of a constitutively activated Akt in 3T3-L1 preadipocytes was shown to be sufficient to induce spontaneous adipogenesis in the absence of insulin (69). Furthermore, mouse embryo fibroblasts cultured from Akt1/Akt2 double-knockout mice also displayed impaired adipogenesis (70).

b. ERK1/2 pathway

The serine/threonine kinases known as ERKs are a subfamily of the mitogen-activated protein kinases (MAPKs) (71, 72). ERKs are involved in signaling cascades that control cellular functions, such as proliferation and differentiation (72). The binding of insulin/IGF-1 or extracellular growth factors, such as epidermal growth factor and platelet derived growth factor (PDGF), to their specific transmembrane receptor tyrosine kinases

induces this signaling cascade (57, 71, 73). This leads to the activation of Ras (a small GTPase) and the sequential recruitment and activation of the kinases Raf, MEK1/2 and ERK1/2 (71, 73). ERK1/2 is found in an inactive state in the cytoplasm, where it is bound by MEK1/2. The phosphorylation of MEK1/2 by Raf results in its activation. Active MEK1/2 can then phosphorylate ERK1/2 at its threonine and tyrosine residues. Upon activation, ERK1/2 is released from MEK1/2 and this is followed by its dimerization and translocation to the nucleus (73).

In the regulation of adipogenesis, ERK1/2 appears to have a complex role and its activity seems to be temporally regulated (71, 74). ERK1/2 activity is required for proliferation during the early mitotic clonal expansion phase. Within the initial 12 hours after induction of differentiation, ERK1/2 phosphorylation of C/EBPβ leads to the acquisition of DNA-binding function and the subsequent transactivation of C/EBPα and PPARγ genes (53, 57). Inhibition of ERK1/2 during clonal expansion inhibited terminal differentiation, reflecting the functional importance of this phosphorylation step (53). The activity of ERK1/2 however, must be terminated prior to PPARγ up-regulation, since phosphorylation of PPARγ by ERK1/2 negatively regulates its transcriptional activity and inhibits differentiation (53, 57, 75, 76). Studies that prolonged the activation of ERK1/2, by overexpression of ERK1/2 or MEK, resulted in impaired differentiation (76, 77). ERK1 also appears to have an essential role in differentiation, as ERK1 knockout mice had decrease adiposity and preadipocytes isolated from these mice exhibited impaired adipogenesis (72).

c. IKKβ/NF-κB pathway

The pleiotropic nuclear factor-kappa B (NF-κB) transcription factors are critical regulators of many cellular processes such as cell survival, inflammation, adaptive- and innate- immune responses, cell adhesion, proliferation, tissue remodelling and apoptosis (78, 79). Members of the NF-κB family in mammalian cells include RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), which can form homo- and hetero-dimers producing various NF-κB complexes (79). In most unstimulated mammalian cells, the NF-κB complex is found in an inactive state in the cytoplasm where it is bound to a member of the inhibitors of NF-κB (IκB) family of proteins by its Rel subunit (79, 80). In mammalian cells, the members of the IκB family include IκBα, IκBβ and IκBε. These inhibitors function in part by masking the conserved nuclear localization signal (NLS) found in the N-terminal Rel-homology domain (RHD) of the NF-κB subunits. IκBα also contains a nuclear export signal that leads to the rapid export of any nuclear NF-κB complexes. Although NF-κB can be activated through several, different pathways, the most frequently observed pathway is the classical or canonical pathways (79). This pathway activates the most studied NF-κB complex, a heterodimer composed of p50 and RelA/p65 subunits, which generally promotes gene expression (80). Activation of this pathway occurs in response to several inflammatory stimuli, such as the pro-inflammatory cytokines TNF-α and IL-1, engagement of the T-cell receptor or exposure to bacterial products, such as lipopolysaccharide (LPS) (79, 80). The inflammatory cytokines are recognized by specific membrane receptors, including TNF receptor, while microbial pathogens and bacterial products are recognized by the Toll-like receptors (TLRs) (78).

Stimulation of the classical pathway leads to the phosphorylation of IκBα at serine 32 and serine 36 predominantly by IκB kinase (IKK) β (78, 80). IKKβ belongs to the IKK

complex, which commonly consists of two catalytic subunits, IKKβ and IKKα (also known as IKK2 and IKK1 respectively), and several copies of the regulatory subunit NF-κB essential modifier (NEMO or IKKγ). The NEMO subunit is responsible for activation of the IKK complex (79). A pharmacological selective inhibitor of IKKβ is sc-514, which inhibits IKKβ activity by binding specifically at its adenosine triphosphate (ATP)-binding site (81, 82). The site-specific IKKβ-mediated phosphorylation results in the subsequent ubiquitinylation of IκBα, which is then targeted by the 26S proteasome for degradation (78, 79). The degradation of this cytoplasmic NF-κB inhibitor leads to the release of the NF-κB complex and unmasks the NLS. This promotes the rapid nuclear translocation of the NF-κB complex (78-80). The RHD of NF-κB mediates its binding to discrete DNA sequences, known as κB elements, found in the promoters and enhancers of its target genes. NF-κB subunits are subject to phosphorylation by nuclear kinases and/or modifications by acetylases and phosphatases, which can lead to promoter-specific effects, influence their ability to interact with co-activators or co-repressors and result in transcriptional activation or repression. Also, in response to distinct stimuli, NF-κB complexes can be targeted to specific promoters and enhancers through cooperative interactions with DNA-bound transcription factors, which results in selective gene transcription. The complex induces gene transcription through its C-terminal transcriptional activation domain, which is found in all Rel subunits (79). Activation of the classical pathway results in enhanced expression of inflammatory cytokines and chemokines (83).

The role of NF-κB in adipogenesis is not yet fully understood. However, due to the central role of adipose cells in the proinflammatory state of the adipose tissue in obesity, the NF-κB pathway may be involved in the regulation of adipogenesis (84-86). Previous studies

have shown that IL-1β and TNFα significantly suppress adipogenesis in murine adipose cell lines through inhibition of PPARγ expression and activation of the NF-κB pathway (87-89). It has also been suggested that IKKβ and NF-κB are associated with the development of insulin resistance and T2D (90). IKKβ can inhibit insulin signaling through phosphorylation of IRS-1 at serine 307 (rodent) and serine 312 (human) (79, 91). IKKβ has also been shown to phosphorylate proteins involved in adipose tissue function, such as14-3-3β (79, 92). IKKβ can phosphorylate and inhibit the 14-3-3β protein, which has been shown to bind and sequester the transcriptional coactivator with PDZ-binding motif (TAZ) in the cytoplasm. TAZ was recently characterized as a PPARγ suppressor in mesenchymal stem cell differentiation. Thus, by disrupting 14-3-3β binding, TAZ could translocate to the nucleus and suppress PPARγ-mediated adipogenic gene expression (79, 93, 94).

d. PDGF pathway

Growth factors stimulate cell proliferation and influence many other cellular functions (95). PDGF, a serum growth factor, is a major protein in the blood that regulates cell attachment, survival and proliferation in many cell types (95, 96). The major storage site for PDGF is the α-granules of platelets however, it can be synthesized by a variety of different cells, including macrophages and fibroblasts (97). PDGF is a dimeric molecule composed of disulfide-linked related polypeptide chains that assembles into heterodimers or homodimers (98). The PDGF family consists of five proteins, namely PDGF-AA, PDGF-BB, PDGF-AB PDGF-CC and PDGF-DD (96). The PDGF receptor (PDGFR) gene encodes a 120 kDa polypeptide, which undergoes further modifications to become a 180 kDa mature, activated PDGFR (99). The PDGFR consists of two structurally related protein tyrosine

kinase receptors, namely the α- and β-receptors. The dimeric PDGF isoforms therefore bind to the two receptors simultaneously, resulting in receptor dimerization (97). Upon dimerization, PDGFR undergoes intramolecular trans-autophosphorylation of the tyrosine residues (97, 100). A pharmacological selective tyrosine kinase inhibitor of PDGFR is imatinib mesylate, which prevents downstream signaling by competing with ATP for the ATP-binding site of this kinase (101, 102). In addition to PDGFR, imatinib mesylate inhibits several other tyrosine kinases (102-104). Autophosphorylation of the PDGFR tyrosine residues creates docking sites that recruit SH2-domain containing downstream signal transduction proteins, such as PI3K and a GTPase activating protein for Ras. The signal transduction pathways downstream of PDGFR therefore include the PI3K/Akt pathway and the ERK1/2 pathway, both previously described (97, 100).

In contrast to insulin and IGF-1, which are pro-adipogenic factors, PDGF is an anti-adipogenic factor that negatively regulates adipogenesis (105). Plasma and adipose tissue levels of PDGF are elevated in ob/ob mice (106). Previous studies have shown that mRNA and protein level of PDGFR decline during 3T3-L1 differentiation (107-109). In addition to the stage of differentiation, PDGFR expression was shown to correlate with adipocyte cell size (110). Published results from our laboratory have shown that in human preadipocytes, PDGF, but not insulin, activates the pro-inflammatory kinase IKKβ and that this is a required signaling event for the inhibition of differentiation by PDGF. However, the PDGF-stimulated IKKβ activity was not associated with IκB degradation or NF-κB activation (81). PDGF has also been shown to mediate its anti-adipogenic effect through conventional PKCs in 3T3-L1 and human preadipocytes (105). At present, the precise mechanisms and signaling networks that regulate the anti-adipogenic effect of PDGF are not yet known.

V. Adipose Tissue Dysfunction and Inflammation

The importance of functional adipose tissue is appreciated and highlighted by metabolic disorders that result from too much (obesity) or too little (lipodystrophy) white adipose tissue (WAT). The inability of WAT to store FFAs leads to their redistribution to nonadipose tissues, primarily the liver, muscles and pancreas, and subsequently, the development of metabolic diseases that contribute to the insulin-resistant state (111). An example of an extreme metabolic disorder is lipodystrophy, which is characterized by the selective loss of adipose tissue. Although it has a lower prevalence than obesity, lipodystrophy shares common metabolic complications with obesity, such as insulin resistance (112). Patients undergoing anti-retroviral HIV treatment can develop lipodystrophy and its associated-complications, namely peripheral lipoatrophy (localized adipose tissue reduction), central adiposity, systemic insulin resistance and hyperlipidemia (112, 113). Dysregulation of adipokines and/or accumulation of FFAs in nonadipose tissues are believed to be the cause of lipodystrophy-associated metabolic abnormalities (111, 112). Therefore, functional adipose tissue is essential for maintaining metabolic homeostasis and vascular health.

Adipose tissue dysfunction is characterized by: insufficient capacity to store excess energy due to impaired adipocyte formation, ectopic storage of FFAs, atypical adipocyte production of bioactive molecules and macrophage infiltration into the adipose tissue. Dysregulation of the adipose tissue thus results in obesity-associated disorders such as insulin resistance, inflammation, cardiovascular and metabolic diseases (114).
The molecular mechanisms underlying adipose tissue dysfunction are not yet known. In obesity, the inability of the adipose tissue to accommodate excess energy intake through proliferation and/or differentiation of preadipocytes leads to a reduction in energy storage

capacity and compensatory adipocyte hypertrophy (115). However, the enlargement of preexisting adipocyte can only occur to a certain extent. Once these adipocytes are filled to maximum capacity, they become very insulin resistant and are characterized by a hyperlipolytic state (9, 114, 116, 117). The redistribution of cholesterol in hypertrophied adipocytes from the plasma membrane to the lipid-droplets leads to increased expression of angiotensinogen and pro-inflammatory cytokines (118). The increased FFA release, which correlates directly with adipocyte size, also promotes inflammation in a paracrine manner (119, 120). Thus, hypertrophied adipocytes contribute to obesity-associated inflammation and insulin resistance.

Adipocytes and immune cells, such as macrophages, share common mediators and signaling pathways that regulate metabolic, inflammatory and innate immune responses, including NF-κB, TLRs and TNF-α (84). The first molecular link between obesity, diabetes and chronic inflammation was TNF-α. It was found that TNF-α was overproduced in adipose tissue of obese humans and mice and, when administered exogenously, resulted in insulin resistance (86). Since then, many other inflammatory mediators and cytokines overexpressed in adipose tissue of obese rodents and humans have been shown to have an effect on insulin action, in addition to regulating immune response and metabolic function (84, 86).

As mentioned, the increased FFA flux that results from impaired adipogenesis is redirected to the visceral adipose tissue, liver, skeletal muscles and other nonadipose tissues, a phenomenon known as ectopic fat deposition (9, 116). Ectopic fat deposition in the liver impairs its metabolism and leads to hepatic insulin resistance, impaired suppression of hepatic glucose production by insulin and hepatic steatosis (9, 13, 114) In the muscle, excess lipid accumulation leads to muscle insulin resistance and downregulates insulin-stimulated glucose uptake (114, 115). The inability of the insulin-sensitive subcutaneous adipose tissue

to expand and store the surplus of energy results in ectopic accumulation of fat in the visceral adipose tissue. This is associated with an altered pro-inflammatory profile of the expanded visceral adipose tissue, which may contribute to the abnormal metabolic profile of viscerally obese individual (9).

VI. Adipose Tissue Macrophages

Recent studies have shown that obesity, in both humans and rodents, induces the accumulation of macrophages in adipose tissue and that this macrophage content correlates positively with BMI and adipocyte size (121-123). The percent macrophage content in adipose tissue was estimated to be fewer than 10% of cells in lean mice and humans to more than 50% of cells in obese, leptin-deficient mice and almost 40% of cells in obese humans. Bone marrow transplant studies suggest that adipose tissue macrophages (ATMs) are derived from bone marrow-derived precursors, which infiltrate the adipose tissue depot and later differentiate into mature macrophages (122). It is now recognized that these infiltrated macrophages are a significant source of adipose tissue-derived pro-inflammatory factors and are involved in the development and maintenance of adipose tissue inflammation in obesity (121-123).

a. Macrophages

Macrophages are mononuclear phagocytes that are present in almost all tissues (122). They are important immune effector cells that primarily mediate the innate immune response and participate in adaptive immunity (122, 124). Some of their other functions include: secreting factors that attract other immune cells to the site of injury or infection; presenting antigens to lymphocytes; releasing anti-microbial peptides; recognition and phagocytosis of

foreign organisms (125). They are also involved in the removal of cellular debris and apoptotic cells (124). Macrophages differentiate from peripheral-blood mononuclear cells (PBMCs), which develop from a common myeloid progenitor cell in the bone marrow. Monocytes released from the bone marrow into the bloodstream migrate into tissues and replenish tissue macrophage populations (126). Resident tissue macrophages can also proliferate to maintain the macrophage population or can become activated in response to innate or adaptive immune signals (124, 127). Classically activated, or M1, macrophages are produced in response to engagement of TLRs by interferon-γ or microbial products, such as LPS (21, 124). They have enhanced microbicidal capacity and produce high levels of pro-inflammatory cytokines and mediators (124). Alternatively activated, or M2, macrophages are produced in response to anti-inflammatory products such as glucocorticoids, IL-4, IL-13 and IL-10 (21). They are involved in wound healing and produce high levels of anti-inflammatory cytokines such as IL-10 (21, 124). In healthy, non-obese humans, ATMs appear to function similar to M2 macrophages as they produce little to no pro-inflammatory cytokines and seem to support adipocyte function and maintain insulin sensitivity (124). In obese humans however, ATMs are described to have an M2 phenotype, based on cell surface markers but have the potential to produce excessive amounts of pro-inflammatory cytokines (128).

b. Murine J774 macrophage cell model

J774 is a murine reticulum cell sarcoma line. This heterogenous population is composed of adherent and non-adherent cells that have similar properties, such as antibody-dependent phagocytosis and synthesis of lysozymes (129). The cell line is derived from a tumor that arose in a female BALB/c/NIH mouse during a plasmacytoma induction program

(130). J774 macrophage-like cells have properties that are characteristic of macrophages, such as adherence, morphology, receptors for immunoglobin and mediate antibody-dependent lysis of target cells (129). Based on the expression of cell surface-antigens, the J774 macrophage cell line was found to be more mature compared to two other cell lines, WEHI-3 and P388-D1 (131). The J774 cell line therefore provides a good macrophage model system. However, as a cancer cell line, J774 cells may differ in their gene expression profiles, phenotype and behaviour compared to normal macrophages.

c. Human THP-1 macrophage cell model

THP-1 is a human leukemic cell line derived from a heterogeneous population of tumor cells cultured from the blood of a boy with acute monocytic leukemia (132, 133). It is a cell line that has retained its monocytic properties, including immunological functions (134). When treated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), this human monocytic leukemia cell line will stop proliferating, undergo morphological changes and differentiate into mature macrophage-like cells. These differentiated THP-1 cells display the characteristics of activated macrophages, such as adherence to culture dish substratum and the ability to phagocytose (135, 136). As it is difficult to obtain a pure population of native human monocytes, this cell line offers the advantage of providing a homogenous population of cells. In addition, compared to other human myeloid cell lines, these differentiated THP-1 cells behave more like native monocyte-derived macrophages (132). This THP-1 cell line therefore provides a valuable human macrophage cell model. However, as a cancer cell line, THP-1 cells may still differ in their gene expression profiles, phenotype and behaviour compared to native human macrophages (137).

d. Human blood monocyte-derived macrophage cell model

It is believed that ATMs are derived from bone marrow progenitor cells, which differentiate into blood monocytes, circulate in the blood and infiltrate tissues where they become macrophages (21). Compared to THP-1 cells, human blood monocyte-derived macrophages (MDMs) more accurately represent the population of infiltrated macrophages in the adipose tissue. In addition, MDMs stimulated with the bacterial endotoxin LPS are more likely to resemble the *in vivo* phenotype of macrophages in the presence of inflammatory stimuli compared to macrophage cell lines (83). Although MDMs represent a more physiological system, they do not offer the advantage of being readily available, easily standardized and able to undergo unlimited number of passages like clonal cell lines. As well, MDMs are subject to inter-donor variability due to factors such as purity, degree of adherence to culture dishes and the presence of contaminating lymphocytes, human serum or exogenous cytokines (137). Thus, the use of clonal cell lines, in addition to this cell model, provides a complementary and useful strategy to investigate the effect of macrophage-secreted factors on preadipocyte differentiation.

e. Role of macrophages in adipose tissue

The mechanism of macrophage recruitment to adipose tissue is not fully understood. However, it is believed that peripheral blood monocytes infiltrate the adipose tissue following a gradient of chemokines secreted by the adipose tissue (138). One candidate is the monomeric polypeptide C-C motif chemokine ligand 2 (CCL2), also previously known as MCP-1 (124, 139, 140). CCL2 is produced by macrophages, endothelial cells and adipocytes (141). It is a major ligand for C-C motif chemokine receptor-2, which is expressed by several cell types, including adipocytes (139). This chemotactic molecule is essential for the

recruitment of monocytes/macrophages in many inflammatory models and is highly expressed in proportion to adiposity in obese rodents and humans (140-143). CCL2 levels also appeared to be higher in omental human adipose tissue depots compared to subcutaneous adipose tissue depots (141, 144). This was consistent with the finding that macrophage infiltration was greater into omental adipose tissue compared to subcutaneous adipose tissue in both lean and obese humans. Although this phenomenon was found to be more exaggerated in obese subjects, it appears that the preferential recruitment of macrophage to omental adipose depots is independent of obesity (144). In addition to increased macrophage infiltration, overexpression of CCL2 in adipose tissue was associated with insulin resistance and hepatic steatosis that occurs with obesity in mice (145). *In vitro* studies have also shown that addition of CCL2 to differentiated 3T3-L1 adipocytes decreased the expression of adipogenic genes and impaired insulin-stimulated glucose uptake. It has also been suggested that CCL2 contributes to the development of insulin resistance and may cause adipocyte dedifferentiation (140). By altering adipocyte function and metabolism, it is possible that CCL2 may be contributing to the reduction in lipid storage capacity and adipocyte hypertrophy that occurs in obesity, resulting in ectopic fat deposition and insulin resistance.

Obesity is also associated with extensive adipocyte necrosis, possibly due to the cytotoxic effects of compensatory adipocyte hypertrophy. It was estimated that in WAT of obese mice and humans, more than 90% of all infiltrated macrophages were localized to dead adipocytes (146). These macrophages form crown-like structures, or synctia, around individual degenerating or dead adipocytes in order to sequester and ingest residual lipid droplets and adipocyte debris. Local hypoxia and the secretion of activators of immune cells

by these necrotic cells have therefore also been suggested to lead to the recruitment, accumulation and persistence of ATMs in obese individuals (139, 146).

Obesity-induced macrophage accumulation appears to be causative of insulin resistance, as it precedes high-fat diet induced insulin resistance (123). *In vivo* studies have shown that insulin resistance, hepatic steatosis and macrophage accumulation, induced by a high-fat diet, are significantly reduced in CCL2 homozygous knockout mice. Furthermore, acute expression of a dominant-negative mutant of CCL2 also improved insulin resistance and hepatic steatosis in obese mice (145). The mechanisms by which macrophages contribute to obesity-associated inflammation and systemic insulin resistance have yet to be elucidated.

f. Cross-talk and interactions of macrophages and adipose cells

In vitro co-culture systems composed of macrophages and adipocytes have led to the suggestion that a paracrine loop implicating adipocyte-derived FFAs and macrophage-derived TNF-α may exacerbate inflammatory changes in adipose tissue (120). Co-culture of 3T3-L1 adipocytes and the murine macrophage cell line RAW264 resulted in increased expression of pro-inflammatory chemokines/cytokines, such as MCP-1, IL-6 and TNF-α, in both the adipocytes and macrophages. Furthermore, this also resulted in decreased expression of the anti-inflammatory cytokine adiponectin in the adipocytes (120). It is also postulated that macrophage-induced adipocyte lipolysis, by TNF-α for instance, results in increased secretion of FFAs by hypertrophied adipocytes. These FFAs are naturally occurring ligands for TLR4, which is expressed by both adipocytes and macrophages. They can therefore induce a pro-inflammatory response in both cell types by binding TLR4 and activating the NF-κB pathway (120, 147). These studies were further supported by *in vivo* studies that showed that TLR4 deficiency protects against saturated-fat induced obesity and

is associated with reduced macrophage infiltration in the adipose tissue, improved insulin sensitivity and altered obesity-associated adipose tissue inflammatory responses (148). These and other studies have provided useful insights into the cross-talk and interactions occurring between macrophages and adipocytes. However, the mechanisms through which these interactions occur are not clear.

In recent years, our laboratory, as well as others, has examined the effect of macrophages on the differentiation of preadipocytes. These studies have revealed that macrophage-secreted factors impair adipogenesis in both human and mouse *in vitro* cell models (2, 83, 149). This would suggest a possible role for macrophages in limiting adipose tissue expansion in addition to its contribution to obesity-associated inflammation. The mechanisms implicated in the macrophage's anti-adipogenic effect are not known. It is therefore important that we further investigate these mechanisms in order to understand the role of macrophages in adipose tissue dysfunction.

HYPOTHESIS AND OBJECTIVES

The overall hypothesis of this research project is that macrophages play an anti-adipogenic role by secreting factors that inhibit adipogenesis. More specifically, I hypothesize that:

a) Mitotic clonal expansion in 3T3-L1 adipogenesis is a target of MacCM.

b) The IKKβ/NF-κB pathway in human preadipocytes is a target of THP-1-MacCM.

The purpose of this study was to investigate the mechanism(s) involved in the anti-adipogenic action of macrophage-conditioned medium (MacCM). Two macrophage cell lines, murine J774 and human THP-1 macrophages, were used to generate MacCM. Murine

3T3-L1 and primary human preadipocytes, isolated from abdominal subcutaneous fat depots, were used to examine the mechanism(s) involved in the anti-adipogenic effect of MacCM. In addition, a macrophage cell model was established using human primary blood MDMs to generate MacCM. The effect of this MacCM on adipogenesis was tested on primary human preadipocytes.

Objective 1:

Investigate the mechanism(s) for the anti-adipogenic effect of J774-MacCM on 3T3-L1 preadipocyte differentiation.

Objective 2:

Identify the signaling pathway(s) activated in human preadipocytes by THP-1-MacCM and examine its (their) role in the anti-adipogenic effect.

Objective 3:

Establish a model system to study the effect of MacCM from human primary blood MDMs on human preadipocyte differentiation.

MATERIALS AND METHODS

Materials

Unless specified otherwise, all reagents used were of the highest grade commercially available.

PREPARATION OF CONDITIONED MEDIUM

Cell culture of murine J774 macrophages and preparation of conditioned medium

J774 macrophages, purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) (J774 growth medium). Culture medium was changed every 2 to 3 days. A cell scraper was used to detach the J774 macrophages from the culture plates for passaging. The split ratio never surpassed 1:6.

At confluence, J774 cells were exposed to fresh J774 growth medium (described above), which was collected 24 hours later. This conditioned medium (J774-MacCM), as well as J774 growth medium not exposed to J774 cells (J774 control medium), was centrifuged (Megafuge 1.0R; Heraeus Instruments) at 150 × g for 5 minutes. The supernatants were stored at –20 °C and thawed in a 37°C water bath prior to use for adipogenesis experiments. J774 control medium was similarly prepared and assessed to ensure that processing (centrifugation and freezing) of the MacCM was not responsible for any observed effects on adipogenesis.

Cell culture of human THP-1 monocytes and preparation of conditioned medium

THP-1 monocytes, purchased from ATCC, were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate ($NaHCO_3$), 4.5 g/L glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 10% FBS, 0.05 mM β-mercaptoethanol, and antibiotics (THP-1 growth medium). Culture medium was changed every 2 to 3 days. Cells were counted using a Neubauer hemacytometer prior to the addition of fresh growth medium to maintain the culture at a concentration lower than 1×10^6 cells/ml. Once a week, cell suspensions were centrifuged at 150 x g for 5 minutes, counted using a Neubauer hemacytometer and resuspended in fresh growth medium.

The THP-1-MacCM and THP-1 monocyte-conditioned medium (THP-1-MonCM) were generated as follows. The THP-1 monocytic cells, resuspended at 1×10^6 cells/ml, were either maintained as monocytes with 0.01% dimethylsulfoxide (DMSO) or differentiated into macrophages with 100 nM TPA for 24 hours. The medium was then replaced with fresh THP-1 growth medium (no TPA present) and, after 24 hours, the conditioned media were collected and centrifuged at 150 x g for 5 minutes. THP-1 growth medium not exposed to THP-1 cells (THP-1 control medium) was similarly prepared and assessed to ensure that processing (centrifugation and freezing) of the MacCM was not responsible for any observed effects on adipogenesis. The supernatants were stored at –20 °C and thawed in a 37°C water bath prior to use for adipogenesis experiments.

Isolation/culture of human peripheral blood mononuclear cells and preparation of conditioned medium

In collaboration with Dr. Jonathan Angel's laboratory, 10 to 60 ml of blood (depending on the amount of MDM-CM required) was drawn from healthy adult volunteers

into heparinised syringes (100 U/ml) and processed to isolate PBMCs, as previously described (150-152). Briefly, PBMCs were isolated by density gradient centrifugation (400 x g for 30 minutes) over Ficoll-Paque PLUS (2:1 ratio blood:Ficoll) (GE Healthcare, Baie d'Urfe, QC). The cell layer, consisting mainly of mononuclear cells, was collected, washed with sterile, warm (37°C) phosphate-buffered saline (PBS) and centrifuged at 400 x g for 5 minutes (153, 154). The supernatant was discarded and the cell pellet was washed once again with sterile, warm PBS and centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, 2 g/L $NaHCO_3$ and antibiotics. Cells were then seeded and allowed to adhere for 1 hour at 37°C and 10% CO_2.

Adherent blood monocytes were washed with sterile, warm PBS. The cells were then incubated for 24 hours at 37°C with RPMI-1640 supplemented with 2 mM L-glutamine, 2 g/L $NaHCO_3$, 10% FBS and antibiotics (MDM growth medium). To test the effect of macrophage activation, the cells were incubated in MDM growth medium so that they differentiated in the absence (untreated MDMs) or presence of 1 μg/ml LPS (Sigma) (LPS-treated MDMs) (152, 155). After 24 hours, the conditioned media (Day 1-MDM-CM (untreated) and Day 1-MDM-CM (LPS)) were collected and centrifuged at 400 x g for 5 minutes. The MDMs were subsequently incubated in fresh MDM growth medium (no LPS present) for 24 hours at 37°C. After 24 hours, the conditioned media (Day 2-MDM-CM (untreated) and Day 2-MDM-CM (LPS)) were collected and centrifuged at 400 x g for 5 minutes. MDM growth media not exposed to MDM cells (MDM control (untreated) medium and MDM control (LPS) medium) were similarly prepared and assessed to ensure that processing (centrifugation and freezing) of the MDM-CM and the presence of LPS were not responsible for any observed effects on adipogenesis. The supernatants were stored at –20 °C and thawed in a 37°C water bath prior to use for adipogenesis experiments.

3T3-L1 CELL STUDIES

Cell culture and differentiation of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes (ATCC), kept at low passage, were grown to confluence in DMEM supplemented with 10% calf serum (CS) and antibiotics. Culture medium was changed every 2 to 3 days. When sub-passaging, cultures were kept in a sub-confluent state, and passaged using trypsin-EDTA to detach the cells from the culture plates.

Preadipocytes were induced to differentiate two days post-confluence, either in J774 control medium or in J774-MacCM. To induce differentiation, the media were supplemented with 0.25 μM DEX and 0.5 mM IBMX for the first 2 days, and 1 μM insulin for the first 4 days. Non-differentiating preadipocytes were maintained in the corresponding medium without the adipogenic inducers. Culture medium was changed every 2 days. Depending on the experiment, 3T3-L1 preadipocytes were induced to differentiate for the indicated amount of time.

Assessment of mitotic clonal expansion during differentiation of 3T3-L1 preadipocytes

For enumeration studies, 3T3-L1 preadipocytes were plated in duplicate at 1 x 10^5 cells/cm^2 (day 0) in 35 mm plates. Preadipocytes were induced to differentiate on day 0 in either J774 control medium, J774-MacCM, THP-1 control medium or THP-1-MacCM. Non-differentiating preadipocytes, maintained in DMEM with 10% CS and antibiotics, were counted on day 0. Cells were rinsed, trypsinized, and counted on day 0, 1, 2, 4, 6 and 8 of the differentiation process using a Neubauer hemacytometer.

Bromodeoxyuridine (BrdU), an analogue of thymidine, was used to as an indicator of DNA synthesis (156). BrdU incorporation was assessed in confluent 3T3-L1 preadipocytes grown in duplicate on glass coverslips, placed in J774 control medium or J774-MacCM

(with or without adipogenic inducers) for 18 h, and incubated with 10 μM BrdU for 2 hours. Cells were washed and then fixed with ice-cold methanol for 10 minutes. DNA was denatured in 2 M HCl for 1 hour at 37 °C, then neutralized in 0.1 M borate buffer, pH 8.5. BrdU incorporation was visualized after incubation with FITC-conjugated BrdU antibodies (0.5 ng/ml; Roche), and fluorescence was visualized using a Zeiss Axioplan 2 imaging microscope equipped with an Axiocam digital camera (Carl Zeiss, Toronto, Canada). BrdU-positive cells in 10 random fields from duplicate coverslips were counted by two independent observers.

Preparation of 3T3-L1 and human adipose cell lysates

Cells were washed twice with ice-cold PBS. The cells were then lysed and proteins solubilized in Laemmli buffer containing 1 mM sodium orthovanadate (Na_3VO_4), β-mercaptoethanol, 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM sodium pyrophosphate, and 50 mM sodium fluoride (NaF) (157). Cell plates were subsequently scraped and the cell lysates were passed through a 26 ½ gauge syringe, boiled for 5 minutes and then stored at -20°C. The protein concentrations were quantified using the modified Lowry assay with bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA). Colorimetric assays were quantified using the Ultraspec 3000 UV/Visible spectrophotometer from Pharmacia Biotech or the FLUOstar Galaxy spectrophotometer from BMG.

Immunoblot analysis

Equal amounts of solubilized protein (30 to 50 μg, depending on the experiment) were resolved by SDS-PAGE (7.5% or 12.5% acrylamide) at 150 volts for 1 hour and

transferred to a nitrocellulose membrane at 70 volts for 40 minutes to 2 hours, depending on the molecular weight of the protein being assessed (Bio-Rad Apparatus). Non-specific binding sites on the membranes were blocked by incubating the membranes for 1 hour in PBS/0.1% Tween20/5% skim milk. After blocking, the membranes were incubated at 4°C overnight with the indicated primary antibodies: $p27^{Kip1}$ (1:500) and phospho-Rb (Ser807/811) (1:500), both from Cell Signaling (Danvers, MA); C/EBPβ (1 μg/ml), from Santa Cruz Biotechnology (Santa Cruz, CA); Rb (1:250), from BD Biosciences (Mississauga, ON); ERK1/2 (0.5 μg/ml), from Upstate Biotechnology (Charlottesville, VA). After overnight incubation, membranes were washed twice for 15 minutes with PBS/0.1% Tween20 to remove any unbound primary antibody. The membranes were then incubated for 1 hour in PBS/5% skim milk containing the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Following incubation with the secondary antibody, the membranes were washed to remove any unbound secondary antibody. Immunoreactivity was detected by placing chemiluminescence HRP substrate solution (GE Healthcare, Baie d'Urfe, QC or Millipore, Billerica, MA) onto the membranes for 5 minutes. For chemiluminescence detection, membranes were exposed to Bioflex scientific imaging film (Clonex). The relative intensity of the bands was assessed by Molecular Analyst imaging software (version 1.4; Bio-Rad) or AlphaImager imaging system (Alpha Innotec Co., San Leandro, CA), and expressed as integrated optical density (IOD) units. The anti-rabbit, anti-goat and anti-mouse HRP-conjugated secondary antibodies were purchased from Amersham Biosciences or Jackson Laboratories, West Grove, PA. The prestained protein ladder used was purchased from Fermentas, Burling, ON.

Immunocytochemistry of C/EBPβ nuclear localization

3T3-L1 preadipocytes were grown to confluence. Cells were induced to differentiate in J774 control medium or J774-MacCM for 6 or 24 hours, as previously described. Non-differentiating preadipocytes were maintained in DMEM with 10% CS and antibiotics for time 0. For nuclear localization, cells were plated in duplicate and seeded on coverslips in a 12-well plate. The coverslips were rinsed twice in ice-cold PBS and fixed for 20 minutes in 4% paraformaldehyde at room temperature. Cells were washed and then permeabilized at room temperature with PBS/0.02% BSA/0.075% Triton X-100 for 30 minutes. The permeabilized cells were then washed and antigenic sites were subsequently blocked with PBS/0.02% BSA for 1 hour at room temperature. After washing, the coverslips were incubated for 1 hour at room temperature with antibodies against C/EBPβ (1 μg/ml) (Santa Cruz, CA). Non-specific controls were incubated with blocking solution only. The coverslips were then washed and incubated for 1 hour at room temperature with AlexaFluor488-labeled anti-rabbit IgG (1 μg/ml) (Molecular Probes). Permeabilized cells were further stained for 10 minutes at room temperature with Hoechst solution (1 μg/ml in PBS) and then washed. The coverslips were mounted on glass slides with Mowiol and allowed to dry overnight. Fluorescence was visualized with a Zeiss Axioplan 2 microscope (at 400X magnification) equipped with an Axiocam digital camera.

Measurement of C/EBPβ DNA binding activity

3T3-L1 preadipocytes were grown to confluence in 100 mm plates. Cells were induced to differentiate in J774 control medium or J774-MacCM for 24 hours, as described previously. Cell plates were rinsed twice in ice-cold PBS. The cells were subsequently lysed,

scraped and nuclear extracts were prepared as previously described by Shreiber, *et al* (1989) (158). Briefly, cells were lysed in 400 µL cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF). Cells were scraped, allowed to swell for 15 minutes on ice and 25 µL of 10% NP-40 was added to the suspension, which was then vortexed for 10 seconds. The homogenates were then centrifuged at 2 500 x g for 1 minute at 4°C. Supernatants were discarded, and the nuclear pellets were resuspended in 50 µL ice-cold buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated for 30 minutes at 4°C on a rotary shaker for continuous mixing. Samples were centrifuged at 2 500 x g for 5 minutes at 4°C, and the supernatants were collected. Protein concentrations were quantified using the modified Lowry assay using BSA as a standard, as described previously. Nuclear lysates were stored at –80 °C and thawed on ice prior to use. C/EBPβ-specific DNA binding was determined using the TransAM ELISA kit (Active motif, Carlsbad, CA), according to the manufacturer's instructions. For the DNA binding assay, 2.5 µg of the nuclear extracts was used. Rat liver nuclear extract, provided with the kit, was used as a positive control for the assay.

HUMAN SUBCUTANEOUS ABDOMINAL ADIPOSE CELL STUDIES

Isolation of human abdominal subcutaneous stromal preadipocytes

Subcutaneous adipose tissue samples were obtained from 13 consenting patients (all women) undergoing elective abdominal surgery (approved by The Ottawa Hospital Research Ethics Board). Mean age was 46 ± 3 years, and mean BMI was 28.0 ± 2.6 kg/m^2 (± SD; range from 21.7 to 48.4 kg/m^2). These patients were weight-stable and not acutely ill. Only 2 of the 13 patients were smokers. Preadipocytes were isolated as previously described (159,

160). Briefly, adipose tissue was separated from connective tissue and capillaries by dissection and then digested with collagenase CLS type 1 (600 U/g of tissue) on a rotary shaker for 1 hour at 37°C. The digested tissue was subjected to progressive size filtration and centrifugation. Filtration of the digested tissue, to eliminate connective tissue and other debris, was performed using a sterile 200 μm nylon filter. The filtered tissue was then centrifuged at 200 x g for 20 minutes to remove floating mature adipocytes. This was followed by the addition of 10% FBS to the infranatant, which underwent progressive size filtration (100 μm, 50 μm and 25 μm sterile nylon filters) and was centrifuged at 200 x g for 20 minutes to remove any remaining mature adipocytes. The pellet was incubated for 5 minutes in erythrocyte lysis buffer (155 mM ammonium chloride, 5.7 mM potassium phosphate, 0.1 mM EDTA (pH 7.3)). DMEM supplemented with 10% FBS, antibiotics and 50 U/ml nyastatin was added to the stromal cells, which was then centrifuged at 200 x g for 5 minutes. Cells were counted using a Neubauer hemacytometer. The stromal preadipocytes were subsequently seeded at 6 000 cells/cm^2 and grown in DMEM supplemented with 10% FBS and antibiotics. Upon reaching 80-90% confluence, cells were expanded for a maximum of three passages (161, 162). In some cases, stromal preadipocytes were cryopreserved before passaging and, once thawed, were grown in DMEM supplemented with 10% FBS and antibiotics. It has been shown that this did not alter their ability to differentiate into adipocytes (163).

Differentiation of human abdominal subcutaneous stromal preadipocytes

For differentiation, the passaged stromal preadipocytes were seeded at a density of 3 x 10^4 cells/cm^2 and grown to confluence in DMEM supplemented with 10% FBS, antibiotics and 50 U/ml nystatin. Confluent human stromal preadipocytes were then treated with the

appropriate THP-1 control medium, THP-1-MonCM or THP-1-MacCM. To induce differentiation, the media were supplemented with adipogenic inducers, namely 5 μg/ml insulin, 100 μM indomethacin (a PPARγ agonist at this concentration), 0.5 μM DEX, 0.25 mM IBMX (Cambrex Bio Science, East Rutherford, NJ, USA) (159, 164). Non-differentiating preadipocytes were maintained in the corresponding medium without the adipogenic inducers. For some experiments, confluent human preadipocytes were pre-treated for 15 minutes with 100 μM sc-514 (Calbiochem) or vehicle, 0.1% DMSO, prior to induction of differentiation (165). After 12 to 15 days, cultures were photographed with a digital camera (Coolpix 995; Nikon, Mississauga, ON, Canada) mounted on a microscope (Eclipse TS-100; Nikon). Cells were washed and TG was extracted and quantified spectrophotometrically (described below). Cellular remains were solubilised in Laemmli buffer, and processed for immunoblot analysis (described below) (166).

Previous experiments conducted by V. Constant (M.Sc. student) in our laboratory suggested that using undiluted MDM-CM to induce differentiation resulted in preadipocyte cell death. Therefore, to reduce the potential toxicity of the conditioned media collected from MDMs, the MDM-CM and MDM control media were further diluted (1:5) in RPMI-1640 supplemented with 2 mM L-glutamine, 2 g/L $NaHCO_3$, 10% FBS, antibiotics and 50 U/ml nyastatin prior to use for adipogenesis experiments. Differentiation of confluent human preadipocytes was induced as previously described, by supplementing the media with 5 μg/ml insulin, 100 μM indomethacin, 0.5 μM DEX, 0.25 mM IBMX. Non-differentiating preadipocytes were maintained in the corresponding media, without the adipogenic inducers. After 12 to 15 days, cultures were photographed with a digital camera mounted on a microscope, as previously described. Cells were processed for TG measurement and immunoblot analysis (described below).

Signaling studies with human abdominal subcutaneous stromal preadipocytes

For time course experiments, confluent human preadipocytes were treated for 5, 15, 30 or 60 minutes with THP-1 control medium or THP-1-MacCM, both supplemented with 5 μg/ml insulin, 100 μM indomethacin, 0.5 μM DEX, 0.25 mM IBMX. Confluent preadipocytes were maintained in DMEM with 10% FBS, antibiotics and 50 U/ml nyastatin for time 0. After treatment, cells were lysed and processed for immunoblot analysis (described below).

For other experiments, duplicate plates of confluent human preadipocytes were pre-treated for 90 minutes with 10 μM imatinib mesylate (kindly provided by Novartis, Basel, Switzerland) or vehicle, 0.1% DMSO (167). These cells were then treated for 15 minutes with THP-1 control medium or THP-1-MacCM, both supplemented with 5 μg/ml insulin, 100 μM indomethacin, 0.5 μM DEX, 0.25 mM IBMX in the presence of 10 μM imatinib mesylate or 0.1% DMSO. After 15 minutes, cells were lysed and processed for immunoblot analysis (described below).

Cells lysates were prepared as described in the 3T3-L1 Cell Studies. The protein concentrations were quantified using the modified Lowry assay with BSA as a standard (Bio-Rad, Hercules, CA). Colorimetric assays were quantitated using the Ultraspec 3000 UV/Visible spectrophotometer from Pharmacia Biotech or the FLUOstar Galaxy spectrophotometer from BMG.

Immunoblot analysis

Equal amounts of solubilized protein (5 to 10 μg, depending on the experiment) were resolved by SDS-PAGE (10%, 12.5% or 15% acrylamide) at 150 volts for 1 hour and

transferred to a nitrocellulose membrane at 70 volts for 1 hour to 1 hour 20 minutes, depending on the molecular weight of the protein being assessed (Bio-Rad Apparatus). Non-specific binding sites on the membranes were blocked by incubating the membranes for 1 hour in PBS/0.1% Tween20/5% skim milk. After blocking, the membranes were incubated at 4°C overnight with the indicated primary antibodies: PPARγ (1:1000), FAS (1:500), phospho-tyrosine (1:1000), phospho-Akt (Ser473) (1:1000), Akt (1:1000), phospho-IKKα (Ser180)/IKKβ (Ser181) (1:250), IκBα (1:500), phospho-ERK1/2 (Thr202/Tyr204) (1:2000), all from Cell Signaling (Danvers, MA); PPARγ (2 μg/ml) and Akt (clone N-19) (2 μg/ml), both from Santa Cruz Biotechnology (Santa Cruz, CA); fibronectin (1:10 000), from BD Biosciences (Mississauga, ON); ERK1/2 (0.5 μg/ml), IKKβ (2 μg/ml), both from Upstate Biotechnology (Charlottesville, VA); aP2 (0.1 μg/ml), from R&D Systems (Minneapolis, MN). Membranes were washed and detected as described in 3T3-L1 Cell Studies. The relative intensity of the bands was assessed by Molecular Analyst imaging software (version 1.4; Bio-Rad) or AlphaImager imaging system (Alpha Innotec Co., San Leandro, CA), and expressed as IOD units. The anti-rabbit, anti-goat and anti-mouse HRP-conjugated secondary antibodies were purchased from Amersham Biosciences or Jackson Laboratories, West Grove, PA. The prestained protein ladder used was purchased from Fermentas, Burling, ON.

TG assay

Cells were washed twice with ice-cold PBS. Cellular TG was extracted with isopropanol:heptane (2:3) and spectrometrically measured as previously described (168, 169). Briefly, lipid was extracted for 30 minutes at room temperature with 1 ml of isopropanol:heptane solution. A second extraction was performed for 15 minutes at room

temperature with 0.5 ml of isopropanol:heptane solution. Lipid extracts were then combined, dried in a Savant Speed Vac Plus SC110A and stored at -20°C. After TG extraction, the cellular remains were solubilised in Laemmli buffer containing 1 mM Na_3VO_4 and β-mercaptoethanol (157). Cellular remains were subsequently scraped and the cell lysates were passed through a 26 ½ gauge syringe, boiled for 5 minutes and then stored at -20°C. The protein concentrations were quantified using the modified Lowry assay using BSA as a standard, as described previously. The TG assay was performed as described by Neri and Frings (1973) using Triolein as a standard (170). Colorimetric assays were quantified using the Ultraspec 3000 UV/Visible spectrophotometer from Pharmacia Biotech or the FLUOstar Galaxy spectrophotometer from BMG. TG was expressed as μg of TG per well divided by mg of soluble cell protein (168).

Measurement of NF-κB DNA binding activity

Human abdominal subcutaneous preadipocytes were grown to confluence in duplicate 60 mm plates. Cells were induced to differentiate in the presence or absence of THP-1-MacCM as previously described. After 30 or 60 minutes, cells were washed twice with ice-cold PBS containing phosphatase inhibitors (125 mM NaF, 250 mM β-glycerophosphate, 250 mM phenylphosphate, and 25 mM Na_3VO_4), and nuclear extracts were prepared according to the manufacturer's instructions for the TransAM NF-κB p65 Activation kit (Active Motif, Carlsbad, CA). Briefly, cells were lysed in 1 ml hypotonic buffer (20 mM HEPES (pH 7.5), 5 mM NaF, 10 μM sodium molybdate and 0.1 mM EDTA). Cells were scraped and allowed to swell for 15 minutes on ice. NP-40 was added to a final concentration of 0.5%. The homogenates were then centrifuged at 2 500 x g for 1 minute at 4°C. Supernatants were discarded, and nuclear pellets were resuspended in 50 μL of ice-cold

complete lysis buffer and incubated for 30 minutes at 4°C on a rotary shaker for continuous mixing. Samples were centrifuged at 2 500 x g for 10 minutes at 4°C, and the supernatants were collected. Protein concentrations were quantified using the modified Lowry assay using BSA as a standard, as described previously. Nuclear lysates were stored at –80 °C and thawed on ice prior to use. NF-κB-specific DNA binding was determined using the TransAM ELISA kit (Active motif, Carlsbad, CA), according to the manufacturer's instructions. For the DNA binding assay, 2.5 μg of the nuclear extracts was used. A nuclear fraction prepared from activated Jurkat cells (a human acute T-lymphoblastic leukemia cell line), provided with the kit, was used as a positive control for the assay (171).

Statistical Analysis – 3T3-L1 and Human Cell Studies

For all experiments, repeated measures of ANOVA followed by the Newman–Keul's post-test, (for comparison of multiple means) was used (Instat, version 3.05; GraphPad, San Diego, CA), and differences with p values <0.05 were considered significant.

RESULTS

PART I

Medium conditioned by J774 macrophages and THP-1 macrophages impairs the mitotic clonal expansion phase of 3T3-L1 adipogenesis.

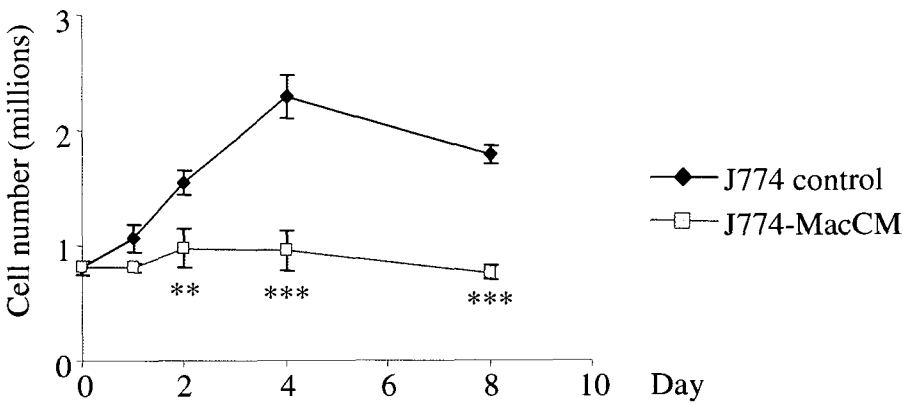
Our laboratory has shown that THP-1-MacCM and J774-MacCM are required at the onset of differentiation (first 2 days) for maximal inhibition of 3T3-L1 adipogenesis (74, 172). Mitotic clonal expansion is a required, early event that occurs during 3T3-L1 preadipocyte differentiation (22). To evaluate the effect of macrophage-derived factors on this early event, I used the established murine 3T3-L1 preadipocyte cell line and generated macrophage-conditioned medium from murine J774 macrophages and human THP-1 macrophages. 3T3-L1 preadipocytes were induced to differentiate in the presence of J774 control medium, J774-MacCM, THP-1 control medium, or THP-1-MacCM over a period of 8 days. The cells were counted on indicated days. Published results from our laboratory have previously shown that exposure of 3T3-L1 preadipocytes to either J774-MacCM or THP-1-MacCM for 8 days completely inhibited 3T3-L1 adipogenesis, as assessed morphologically by cell rounding and lipid droplet accumulation (2). My results show that 3T3-L1 cell number increased from 0.81 x 10^6 to 2.3 x 10^6 cells/dish within 4 days of induction of adipogenesis in J774 control medium, a ~3-fold increase ($n=3$; $p<0.001$ compared to day 0; Figure 2A). This increase was inhibited by 77% on day 2 ($n=3$; $p<0.01$) and 89% on day 4 ($n=3$; $p<0.001$) when 3T3-L1 preadipocytes were induced to differentiate in J774-MacCM.

In THP-1 control medium, 3T3-L1 cell number increased from 0.80 x 10^6 to 2.4 x 10^6 cells/dish within 4 days of induction of adipogenesis, a ~3-fold increase ($n=3$; $p<0.001$ compared to day 0; Figure 2B). This increase was inhibited by 42% on day 4 ($n=3$; $p<0.01$) when 3T3-L1 preadipocytes were induced to differentiate in THP-1-MacCM.

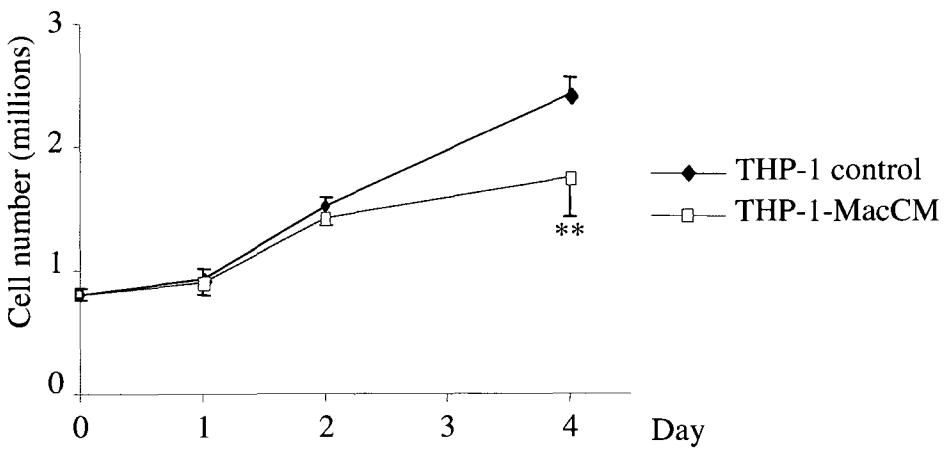
Figure 2. Medium conditioned by J774 macrophages and THP-1 macrophages impairs the mitotic clonal expansion phase of 3T3-L1 adipogenesis.

Confluent 3T3-L1 preadipocytes were induced to differentiate in J774 control medium, J774-MacCM, THP-1 control medium or THP-1-MacCM. Non-differentiated preadipocytes, maintained in growth medium, were counted on day 0. **A-B.** At indicated times, cells from duplicate dishes were trypsinized and enumerated using a hemacytometer. Results are expressed as the mean +/- SE of 3 independent experiments, each performed in duplicate. ** indicates $p<0.01$ and *** indicates $p<0.001$, compared to values obtained from cells in control medium on the same day.

A.


Cell number (millions)
3
2
1
0
0
2
4
6
8
10
Day
**
***
***
J774 control
J774-MacCM

B.


Cell number (millions)
3
2
1
0
0
1
2
3
4
Day
**
THP-1 control
THP-1-MacCM

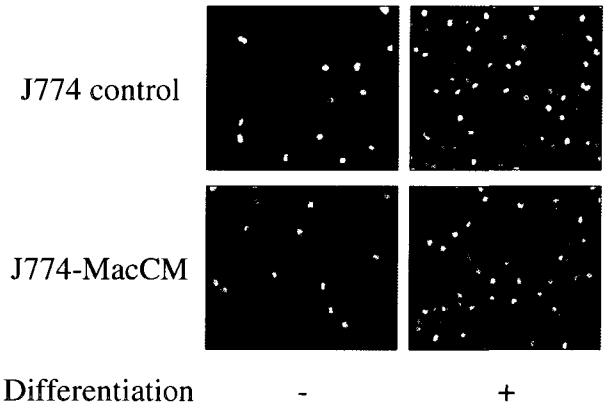
Although both J774-MacCM and THP-1-MacCM had negative effects on 3T3-L1 cell proliferation, J774-MacCM was a more potent inhibitor of clonal expansion. The differences in potency between both MacCM was also observed with regards to their ability to inhibit 3T3-L1 adipogenesis (2). This may be due to the different growth requirements of the two types of macrophages. While DMEM was used for the J774-MacCM studies, RPMI medium was used for the THP-1-MacCM studies. RPMI medium appears to result in more robust 3T3-L1 preadipocyte differentiation, since results from our laboratory have shown a greater accumulation of TG upon differentiation in RPMI medium compared to DMEM (2). Therefore, it may be more difficult to inhibit 3T3-L1 differentiation in the presence of RPMI medium. Another explanation could be the species-specific effect of murine J774-MacCM acting on murine 3T3-L1 adipose cells, allowing a more potent inhibition. There may be species differences with respect to the potency of similar secreted molecules as well as differences in how these molecules engage receptor/kinase activation in 3T3-L1 cells. The profile of secreted products may also differ between the two macrophage cell models. Therefore, in all of my other studies, I have paired cell models according to their species of origin in order to obtain a more potent and uniform inhibition and to minimize inter-species variability.

Cell number can be affected by both proliferation and cell death. BrdU incorporation, an indicator of DNA synthesis, was thus assessed to confirm these results (156). BrdU-positive cells were photographed and counted after 3T3-L1 preadipocytes were induced to differentiate for 18 hours (Figure 3A). Upon induction of adipogenesis in J774 control medium, there was a 6-fold increase in the number of BrdU-positive cells ($n=4$; $p<0.001$; Figure 3B). However, only a 2.7-fold increase was observed when 3T3-L1 preadipocytes

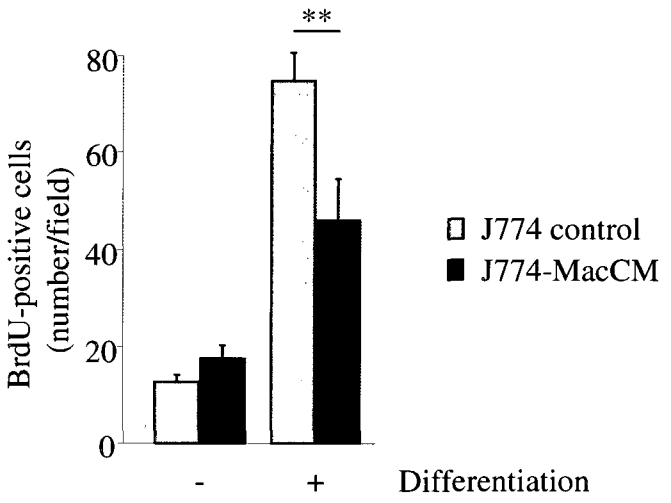
Figure 3. Medium conditioned by J774 macrophages impairs active DNA synthesis in differentiating 3T3-L1 preadipocytes.

Confluent 3T3-L1 preadipocytes were placed in J774 control medium or J774-MacCM (with or without adipogenic inducers of differentiation) for 18 hours, then labeled with 10 μM BrdU for 2 hours. Cells were fixed and stained with fluorescein-tagged BrdU antibodies. **A.** Photomicrographs taken at 200X magnification are representative of 4 independent experiments, each performed in duplicate. NS, non-specific controls were immunostained in the absence of the antibody. **B.** BrdU-positive cells were counted in 10 fields for each sample, by two independent observers, and results represent the mean +/- SE of 4 independent experiments, each performed in duplicate. ** indicates $p<0.01$ between indicated pairs.

A.


J774 control
J774-MacCM
Differentiation
-
+

B.


**
80
60
40
20
0
BrdU-positive cells
(number/field)
J774 control
J774-MacCM
-
+
Differentiation

were induced to differentiate in J774-MacCM, a 55% reduction compared to J774 control medium (n=4; $p<0.01$).

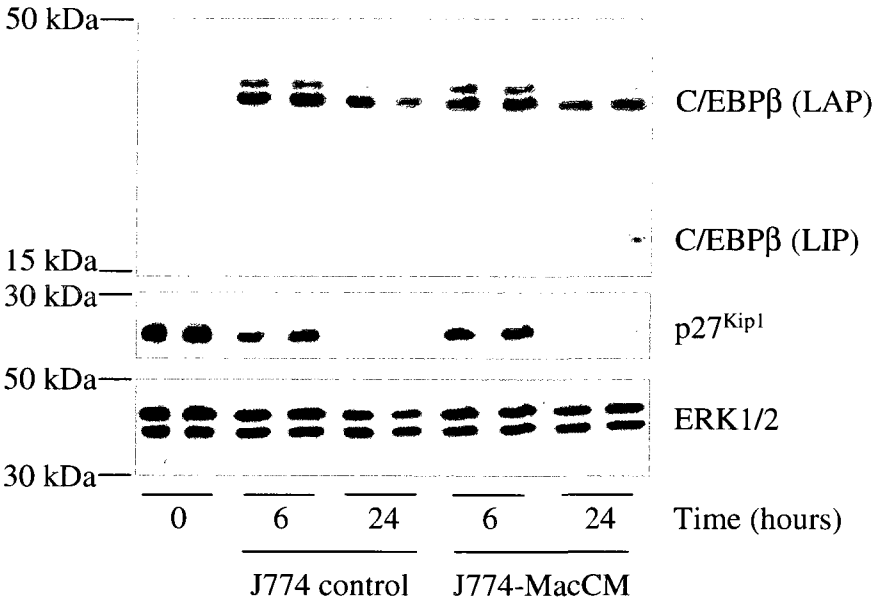
Medium conditioned by J774 macrophages inhibits the differentiation-induced phosphorylation of Rb in 3T3-L1 preadipocytes.

The expression of key regulators of cell cycle progression is tightly governed during the clonal expansion phase of adipogenesis (37). Given the observed alterations in mitotic clonal expansion, I examined the effect of J774-MacCM on the expression of proteins involved in the cell cycle, namely C/EBPβ, $p27^{Kip1}$ and Rb (30, 39, 41). 3T3-L1 preadipocytes were induced to differentiate in the presence of J774 control medium or J774-MacCM for 6 or 24 hours. Non-differentiated preadipocytes were maintained in growth medium for time 0. The expression of both isoforms of C/EBPβ (LAP and LIP) was strongly increased at 6 hours of induction of adipogenesis in J774 control medium and remained elevated at 24 hours, although the level of LAP drifted downwards by this time (173) (n=3; Figure 4A-B). The presence of J774-MacCM resulted in a trend of lower levels of LAP and higher levels of LIP, but this did not reach significance. The protein expression of $p27^{Kip1}$ was appropriately down-regulated in 3T3-L1 preadipocytes differentiated either in J774 control medium or J774-MacCM (n=3; Figure 4A-B). ERK1/2 protein expression served as a loading control, since it does not change upon adipogenesis (57, 174). As previously reported, Rb phosphorylation, assessed using a phospho-specific antibody as well as by mobility shift, was stimulated 5-fold in cells induced to differentiate in J774 control medium (n=3; Figure 5A-B) (30, 38, 45). However, a 94% inhibition in Rb phosphorylation was

Figure 4. Medium conditioned by J774 macrophages does not affect C/EBPβ and $p27^{Kip1}$ expression in differentiating 3T3-L1 preadipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in J774 control medium or J774-MacCM for 6 or 24 hours, as indicated. Non-differentiated preadipocytes were maintained in growth medium for time 0. **A.** Solubilized protein from duplicate cultures was immunoblotted with antibodies against C/EBPβ, $p27^{Kip1}$ or ERK1/2 (loading control). Immunoblots representative of 3 independent experiments are shown. **B.** Densitometric data from the 3 experiments are expressed as mean +/- SE. IOD, integrated optical density.

A.


50 kDa
C/EBPβ (LAP)
C/EBPβ (LIP)
15 kDa
30 kDa
p27Kipl
50 kDa
ERK1/2
30 kDa
0
6
24
6
24
Time (hours)
J774 control
J774-MacCM

B.

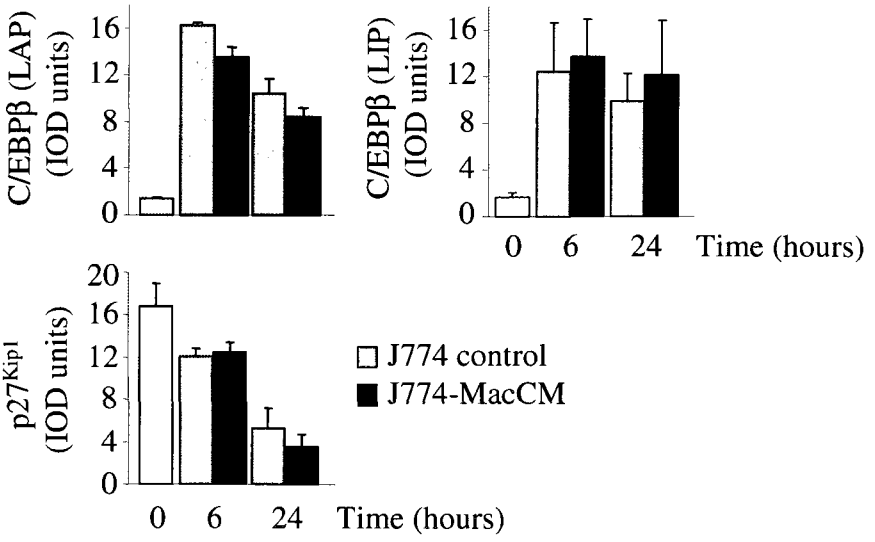
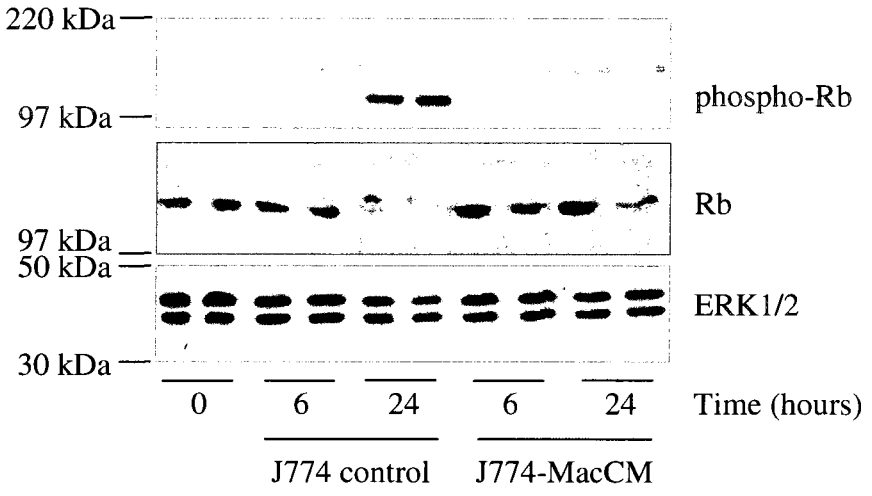

C/EBPβ (LAP) (IOD units)
16
12
8
4
0
C/EBPβ (LIP) (IOD units)
16
12
8
4
0
0
6
24
Time (hours)
p27Kipl (IOD units)
20
16
12
8
4
0
J774 control
J774-MacCM
0
6
24
Time (hours)

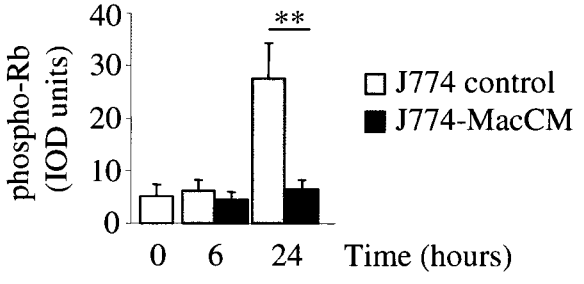
Figure 5. Medium conditioned by J774 macrophages impairs Rb phosphorylation in differentiating 3T3-L1 preadipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in J774 control medium or J774-MacCM for 6 or 24 hours, as indicated. Non-differentiated preadipocytes were maintained in growth medium for time 0. **A.** Solubilized protein from duplicate cultures was immunoblotted with antibodies against phospho-Rb, Rb or ERK1/2 (loading control). Immunoblots representative of 3 independent experiments are shown. **B.** Densitometric data from the 3 experiments are expressed as mean +/- SE. ** indicates $p<0.01$ between indicated pairs. IOD, integrated optical density.

A.


220 kDa
97 kDa
phospho-Rb
Rb
97 kDa
50 kDa
ERK1/2
30 kDa
0
6
24
6
24
Time (hours)
J774 control
J774-MacCM

B.


40
30
20
10
0
**
phospho-Rb (IOD units)
J774 control
J774-MacCM
0
6
24
Time (hours)

observed in cells exposed to adipogenic inducers in J774-MacCM (n=3; $p<0.01$). These results suggest that regulation of Rb downstream targets may be impaired by J774-MacCM.

C/EBPβ function may be altered, as it has been reported to be influenced by the state of Rb phosphorylation (30, 37, 41). Although C/EBPβ is expressed within 2-4 hours after hormonal induction of differentiation, it does not aquire DNA-binding activity until 12 to 16 hours from the time of induction (30, 35). The binding of C/EBPβ to consensus C/EBP-binding sites in centromeric satellite DNA is reflected by a shift from a diffuse to a punctate nuclear immunofluorescent staining pattern (58). Upon addition of adipogenic inducers under J774 control conditions, centromeric localization of C/EBPβ, indicative of DNA binding, was observed at 24 hours (n=3; Figure 6A). This was unaffected by the presence of J774-MacCM (n=3). Cell nuclei were counterstained with Hoechst dye to verify the localization of the nuclei. Quantitative assessment of DNA binding showed that J774-MacCM does not inhibit, but actually increases, C/EBPβ DNA-binding activity compared to cells differentiated in presence of J774 control medium (1.6-fold increase; n=5; $p<0.05$; Figure 6B).

PART II

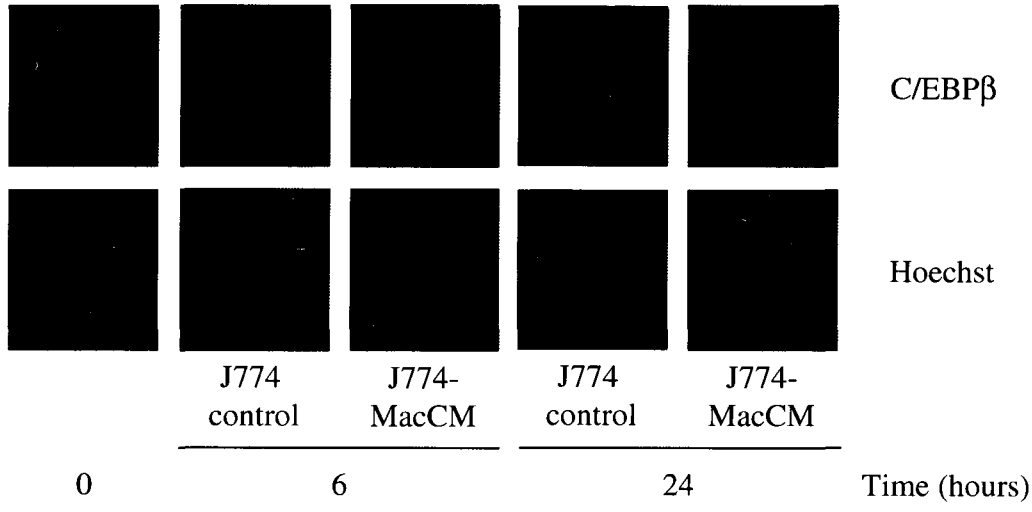
Medium conditioned by THP-1 macrophages stimulates time-dependent changes in signal transduction pathways in human abdominal subcutaneous preadipocytes.

As previously shown by our laboratory, THP-1-MacCM strongly inhibited the differentiation of human subcutaneous preadipocytes (2). As THP-1-MacCM appears to be more potent than J774-MacCM with respect to inhibiting human adipogenesis, THP-1 macrophages were used to generate conditioned medium in these human adipose cell studies

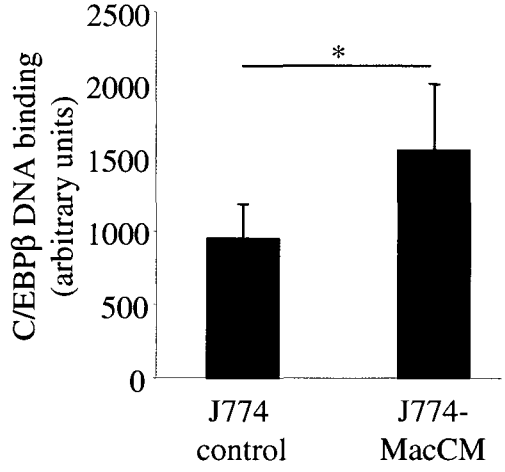
Figure 6. Medium conditioned by J774 macrophages does not impair C/EBPβ centromeric localization or DNA binding activity in differentiating 3T3-L1 preadipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in J774 control medium or J774-MacCM for 6 or 24 hours, as indicated. Non-differentiated preadipocytes were maintained in growth medium for time 0. **A.** 3T3-L1 preadipocytes were seeded on glass coverslips prior to induction of differentiation. Cells were fixed, permeabilized and stained with antibodies against C/EBPβ and Hoechst dye. Immunoreactivity was detected with AlexaFluor488-conjugated IgGs. Photomicrographs taken at 400X magnification are representative of 3 independent experiments, each performed in duplicate. NS, non-specific controls were immunostained in the absence of the primary antibody. **B.** Nuclear fractions were prepared 24 hours after induction of differentiation, and assessed for DNA-binding as described. Results are expressed as the mean +/- SE of 5 independent experiments. * indicates $p<0.05$ between indicated pairs.

A.


C/EBPβ
Hoechst
J774 control
J774-MacCM
J774 control
J774-MacCM
0
6
24
Time (hours)

B.


C/EBPβ DNA binding (arbitrary units)
2500
2000
1500
1000
500
0
*
J774 control
J774-MacCM

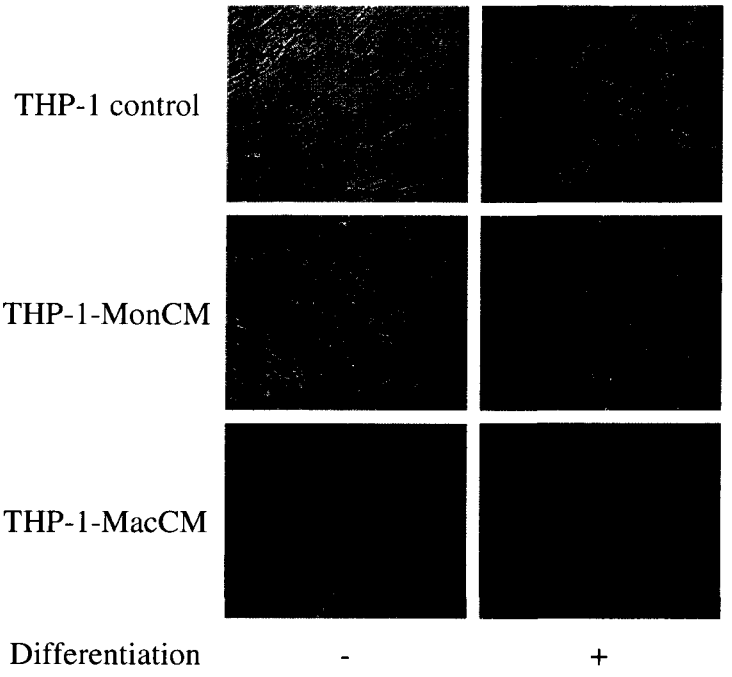
(2). In contrast to THP-1 control medium and THP-1-MonCM, exposure of human preadipocytes to THP-1-MacCM for ~14 days strongly impaired preadipocyte differentiation, as assessed morphologically by cell rounding and lipid droplet accumulation (Figure 7A). These results were consistent with TG accumulation and protein expression of adipogenic markers. Compared to THP-1 control medium, THP-1-MacCM significantly reduced TG accumulation by 86% (n=3; $p<0.01$; Figure 7B). This was accompanied by a significant decrease in the expression of PPARγ, aP2 and FAS, by 91%, 98% and 92% respectively (n=3; $p<0.01$ compared to THP-1 control; Figure 8A-B). Human adipogenesis was unaffected by both THP-1 control medium and THP-1-MonCM, as assessed by cell rounding (Figure 7A), increased TG accumulation (n=3; $p<0.05$; Figure 7B) and increased expression of adipogenic markers upon differentiation (n=3; $p<0.01$; Figure 8A-B). The apparent decrease in levels of adipogenic markers in Figure 8A associated with THP-1-MonCM may be due to variation in lane loading considering the ERK1/2 signal. However, this was not significant after considering the data from all 3 experiments (n=3; Figure 8B).

Given that human primary preadipocytes induced to differentiate in culture do not proceed through mitotic clonal expansion, I identified early signaling pathways that are activated in human preadipocytes by macrophage-derived factors in order to examine their role in the anti-adipogenic effect on human adipogenesis (22, 25, 36). To identify these pathways, I performed a time-course study, whereby human abdominal subcutaneous preadipocytes were exposed for 5, 15, 30 and 60 minutes to THP-1 control medium or THP-1-MacCM in the presence of adipogenic inducers. Acute treatment of confluent human preadipocytes with THP-1 control medium did not induce significant changes in cellular protein tyrosine phosphorylation (n=3; Figure 9A-B) or Akt phosphorylation (n=3; Figure 10A-B) compared to time 0. The expression levels of IκBα also did not significantly change

Figure 7. Medium conditioned by THP-1 macrophages inhibits the differentiation of human abdominal subcutaneous preadipocytes, as assessed by morphology and triglyceride accumulation.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in THP-1 control medium, THP-1-MonCM or THP-1-MacCM for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** After ~14 days, cultures were photographed at 200X magnification. Pictures representative of 3 separate patient samples are shown. **B.** Triglyceride (TG) was extracted, quantified and normalized to protein content. Results are expressed as the mean ± SE of 3 separate patient samples. a indicates $p<0.05$ compared to matched non-differentiated (-) conditions. b indicates $p<0.01$ compared to differentiated (+) THP-1 control and THP-1-MonCM conditions.

A.


THP-1 control
THP-1-MonCM
THP-1-MacCM
Differentiation
-
+

B.

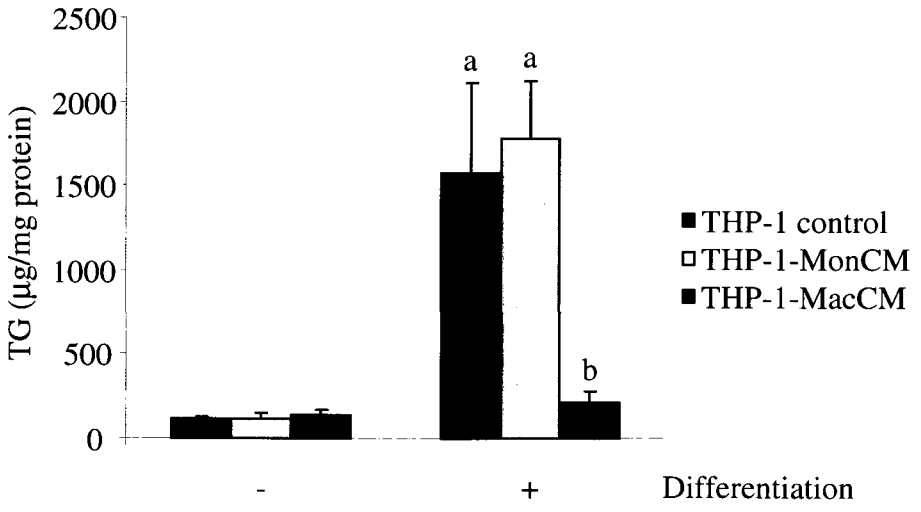
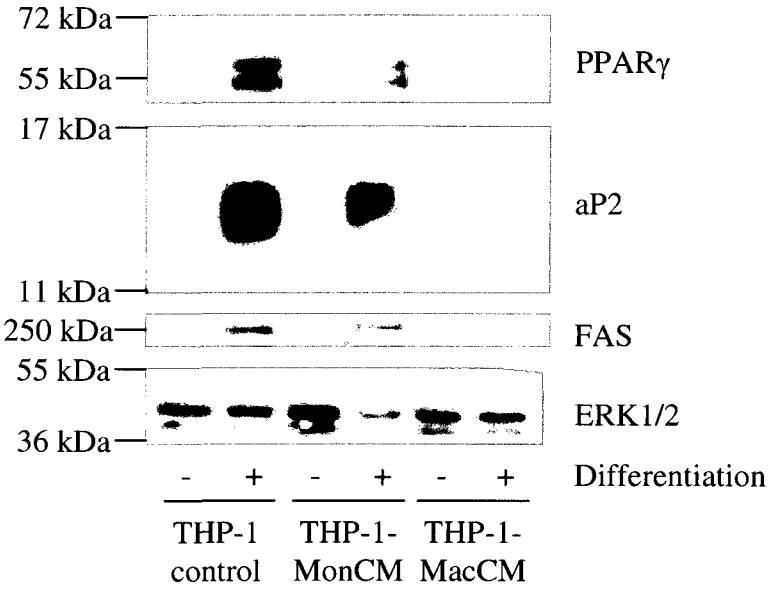

2500
2000
1500
1000
500
0
TG (µg/mg protein)
a
a
b
THP-1 control
THP-1-MonCM
THP-1-MacCM
-
+
Differentiation

Figure 8. Medium conditioned by THP-1 macrophages inhibits the differentiation of human abdominal subcutaneous preadipocytes, as assessed by PPARγ, aP2 and FAS expression levels.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in THP-1 control medium, THP-1-MonCM or THP-1-MacCM for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** Solubilized protein from each culture was immunoblotted with antibodies against PPARγ, aP2, FAS or ERK1/2 (loading control). Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. a indicates $p<0.01$ compared to matched non-differentiated (-) conditions. b indicates $p<0.01$ compared to differentiated (+) THP-1 control and THP-1-MonCM conditions. IOD, integrated optical density.

A.


72 kDa
55 kDa
17 kDa
11 kDa
250 kDa
55 kDa
36 kDa
PPARγ
aP2
FAS
ERK1/2
- + - + - +
Differentiation
THP-1 control
THP-1-MonCM
THP-1-MacCM

B.

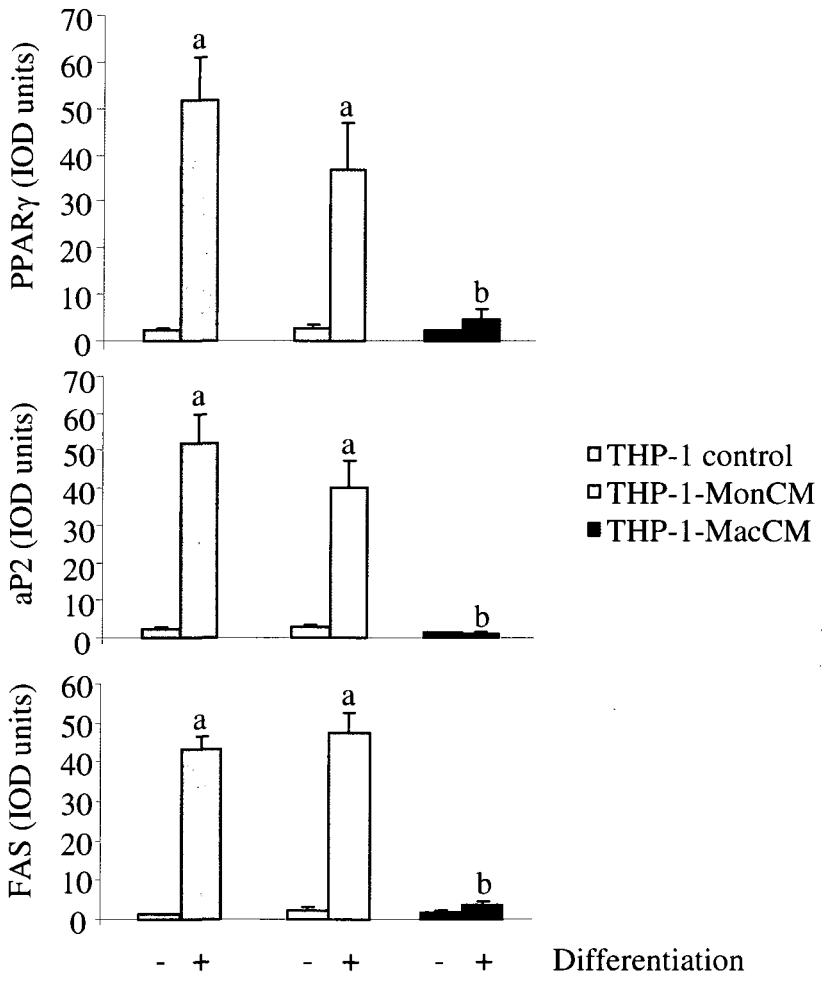
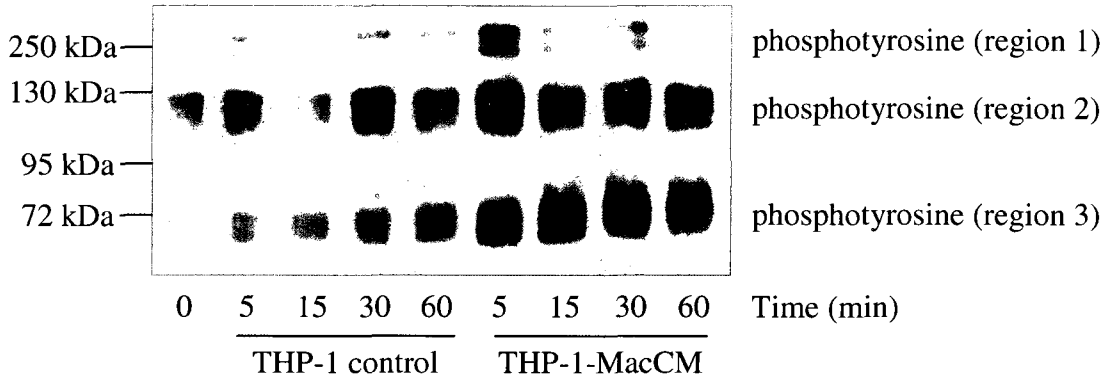

PPARγ (IOD units)
aP2 (IOD units)
FAS (IOD units)
0
10
20
30
40
50
60
70
a
b
THP-1 control
THP-1-MonCM
THP-1-MacCM
- + - + - +
Differentiation

Figure 9. Medium conditioned by THP-1 macrophages stimulates time-dependent increases in protein tyrosine phosphorylation in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 5, 15, 30 or 60 minutes with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. Non-differentiated preadipocytes were maintained in growth medium without adipogenic inducers of differentiation for time 0. **A.** Solubilized protein from each culture was immunoblotted with antibodies against phosphotyrosine. A representative immunoblot from one patient sample is shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. * indicates $p<0.05$ compared to value obtained from cells in THP-1 control medium for the same time point. IOD, integrated optical density.

A.


250 kDa
130 kDa
95 kDa
72 kDa
phosphotyrosine (region 1)
phosphotyrosine (region 2)
phosphotyrosine (region 3)
0 5 15 30 60 5 15 30 60
Time (min)
THP-1 control
THP-1-MacCM

B.

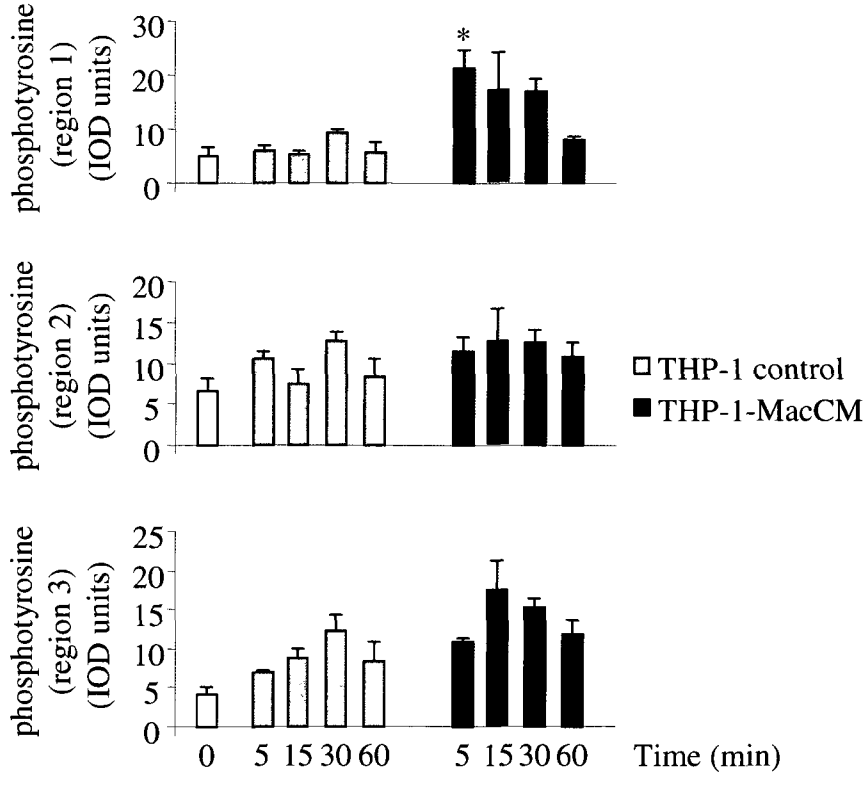
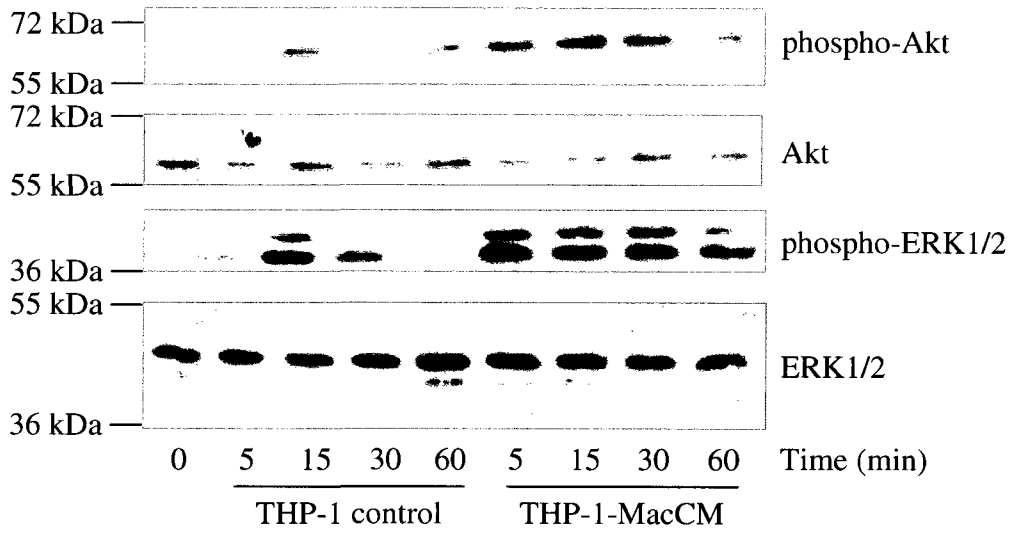

phosphotyrosine (region 1) (IOD units)
30
20
10
0
*
phosphotyrosine (region 2) (IOD units)
20
15
10
5
0
THP-1 control
THP-1-MacCM
phosphotyrosine (region 3) (IOD units)
25
20
15
10
5
0
0 5 15 30 60 5 15 30 60
Time (min)

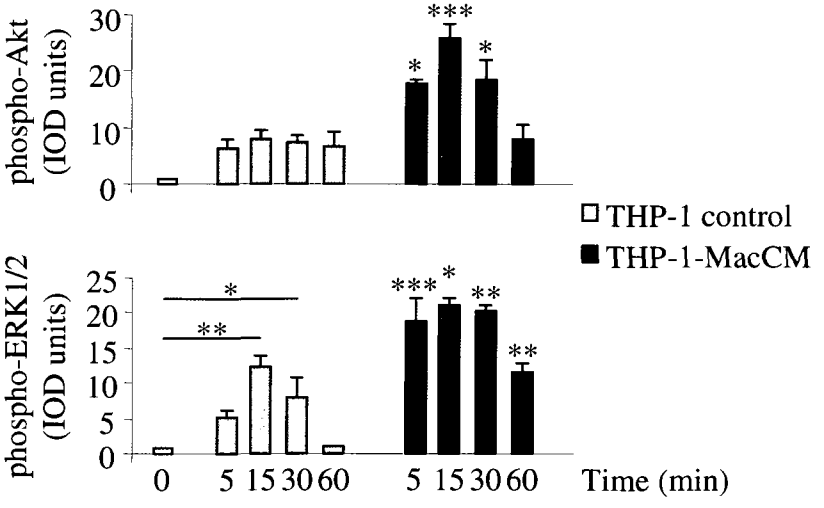
Figure 10. Medium conditioned by THP-1 macrophages stimulates time-dependent increases in Akt and ERK1/2 phosphorylation levels in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 5, 15, 30 or 60 minutes with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. Non-differentiated preadipocytes were maintained in growth medium without adipogenic inducers of differentiation for time 0. **A.** Solubilized protein from each culture was immunoblotted with antibodies against phospho-Akt, Akt (loading control), phospho-ERK1/2 or ERK1/2 (loading control). Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. For THP-1-MacCM, * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to values obtained from cells in THP-1 control medium for the same time point. For THP-1 control medium, * and ** are between indicated pairs. IOD, integrated optical density.

A.


72 kDa
55 kDa
72 kDa
55 kDa
36 kDa
55 kDa
36 kDa
phospho-Akt
Akt
phospho-ERK1/2
ERK1/2
0 5 15 30 60 5 15 30 60
Time (min)
THP-1 control
THP-1-MacCM

B.


phospho-Akt (IOD units)
30
20
10
0
*
***
*
phospho-ERK1/2 (IOD units)
25
20
15
10
5
0
*
**
***
*
**
**
0 5 15 30 60 5 15 30 60
Time (min)
THP-1 control
THP-1-MacCM

upon exposure to THP-1 control medium compared to time 0 (n=3; Figure 11A-B). However, temporal increases in ERK1/2 (n=3; $p<0.01$ and $p<0.05$ compared to time 0; Figure 10A-B) and IKKβ (n=3; $p<0.05$ compared to time 0; Figure 11A-B) phosphorylation, that reached statistical significance, were observed in the presence of THP-1 control medium. The protein tyrosine phosphorylation levels (region 1) (n=3; $p<0.05$; Figure 9A-B) as well as Akt (n=3; $p<0.05$ and $p<0.001$; Figure 10A-B), ERK1/2 (n=3; $p<0.001$, $p<0.05$ and $p<0.01$; Figure 10A-B) and IKKβ (n=3; $p<0.05$, $p<0.001$, $p<0.01$; Figure 11A-B) phosphorylation levels were significantly increased over time upon exposure to THP-1-MacCM compared to THP-1 control medium. As well, THP-1-MacCM also significantly reduced the expression levels of IκBα compared to THP-1 control medium (n=3; $p<0.001$ and $p<0.05$; Figure 11A-B). These results suggest that, in addition to increasing the tyrosine phosphorylation of high molecular weight proteins, THP-1-MacCM activates the Akt, ERK1/2 and IKKβ signaling pathways, which could be potentially implicated in its anti-adipogenic effect.

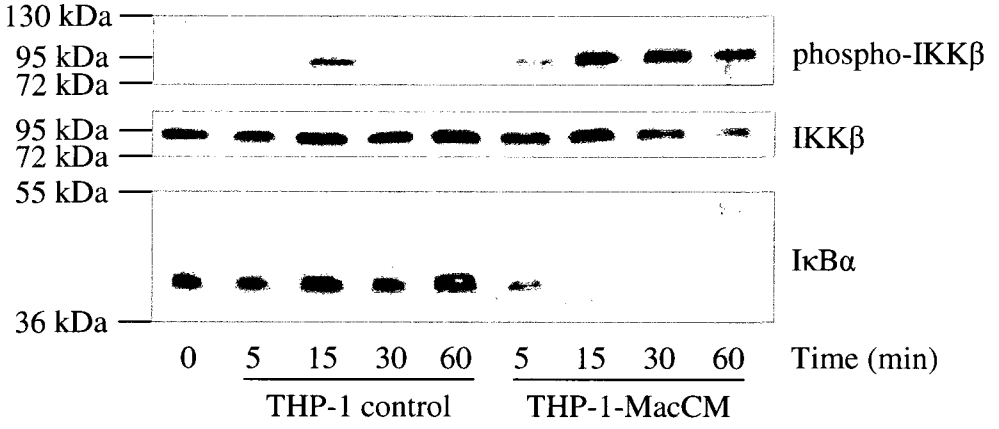
Medium conditioned by THP-1 macrophages increases NF-κB p65 subunit activation in human abdominal subcutaneous preadipocytes.

Since macrophages are known to secrete pro-inflammatory factors, I investigated the inflammatory response of human preadipocytes to the acute effect of THP-1-MacCM (83). Given that THP-1-MacCM induced time-dependent increases in IKKβ phosphorylation levels and time-dependent decreases in IκBα, I measured the activation of NF-κB, a major downstream target and a master regulator of inflammatory responses (79). Compared to THP-1 control medium (in the presence of adipogenic inducers), human abdominal

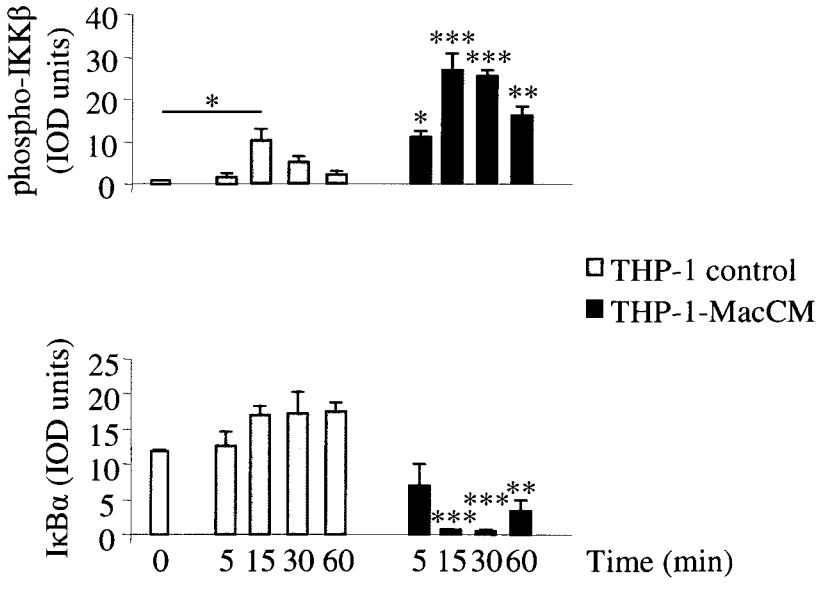
Figure 11. Medium conditioned by THP-1 macrophages stimulates time-dependent increases in IKKβ phosphorylation levels and time-dependent decreases in IκBα protein levels in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 5, 15, 30 or 60 minutes with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. Non-differentiated preadipocytes were maintained in growth medium without adipogenic inducers of differentiation for time 0. **A.** Solubilized protein from each culture was immunoblotted with antibodies against phospho-IKKβ, IKKβ (loading control) or IκBα. Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. For THP-1-MacCM condition, * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to values obtained from cells in THP-1 control medium for the same time point. For THP-1 control medium, * is between the indicated pair. IOD, integrated optical density.

A.


130 kDa
95 kDa
72 kDa
phospho-IKKβ
95 kDa
72 kDa
IKKβ
55 kDa
IκBα
36 kDa
0 5 15 30 60 5 15 30 60
Time (min)
THP-1 control
THP-1-MacCM

B.


phospho-IKKβ (IOD units)
40
30
20
10
0
*
*
***
***
**
THP-1 control
THP-1-MacCM
IκBα (IOD units)
25
20
15
10
5
0
***
***
**
0 5 15 30 60 5 15 30 60
Time (min)

subcutaneous preadipocytes exposed for 30 or 60 minutes to THP-1-MacCM (in the presence of adipogenic inducers) significantly increased NF-κB p65 subunit DNA binding activity by 18.7-fold and 11.5-fold, respectively (n=3; $p<0.05$; Figure 12). Nuclear fractions prepared from activated Jurkat cells served as a positive control for NF-κB DNA-binding activity. Thus, the IKKβ/NF-κB pathway may play a role in the anti-adipogenic effect of THP-1-MacCM, given that this pathway was activated in response to this conditioned medium.

The increase in IKKβ phosphorylation levels and decrease in IκBα protein levels, stimulated by medium conditioned by THP-1 macrophages, is not affected by imatinib, a PDGFR inhibitor, in human abdominal subcutaneous preadipocytes.

It is not known which factor(s) within the complex MacCM is (are) responsible for the anti-adipogenic effect. PDGF has been shown to negatively regulate adipogenesis (105). Results from our laboratory have shown that PDGF inhibits human preadipocyte differentiation (81). Kosaka *et al.* have also previously shown that PMA-treated THP-1 macrophages secrete PDGF (175). The increased tyrosine phosphorylation of high molecular weight proteins (region 1), observed in the presence of THP-1-MacCM (Figure 9A-B), prompted me to investigate if this band was PDGFR. To investigate this, confluent human abdominal subcutaneous preadipocytes were pre-treated for 90 minutes with 10 μM imatinib, then exposed for 15 minutes to THP-1 control medium or THP-1-MacCM in the presence of adipogenic inducers. As previously observed, in the absence of imatinib, THP-1-MacCM significantly increased the tyrosine phosphorylation of the high molecular weight proteins (region 1) by 1.9-fold compared to THP-1 control medium (n=3; $p<0.01$; Figure 13A-B). In the presence of THP-1 control medium, imatinib did not induce significant changes in the

Figure 12. Medium conditioned by THP-1 macrophages increases NF-κB p65 subunit activation in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 30 or 60 minutes with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. Nuclear fractions were prepared from each culture and assessed for DNA-binding as described. Nuclear fractions prepared from activated Jurkat cells were used as a positive control. Results are expressed as the mean +/- SE of 3 independent experiments, each performed in duplicate. * indicates $p<0.05$ between indicated pairs. milli-OD, optical density.

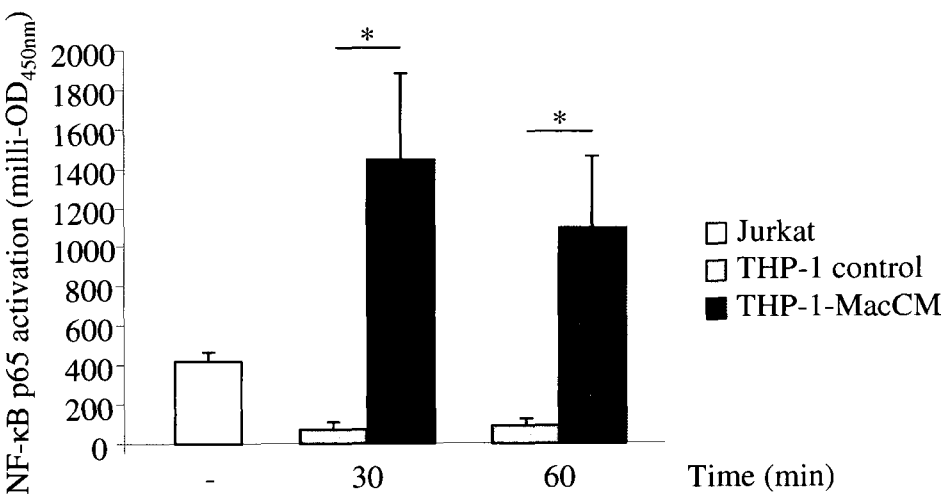
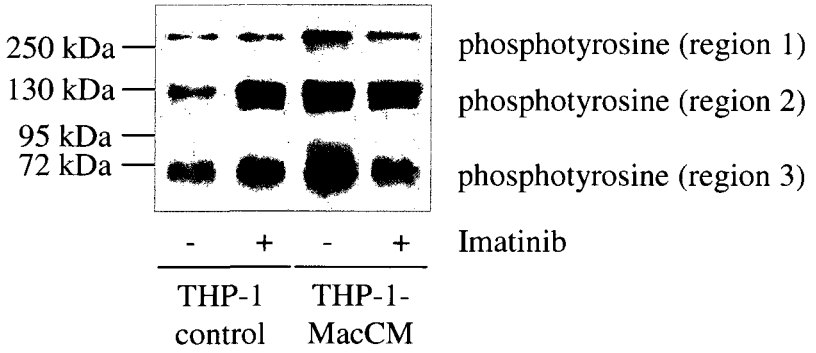

NF-κB p65 activation (milli-OD450nm)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
-
30
60
Time (min)
Jurkat
THP-1 control
THP-1-MacCM

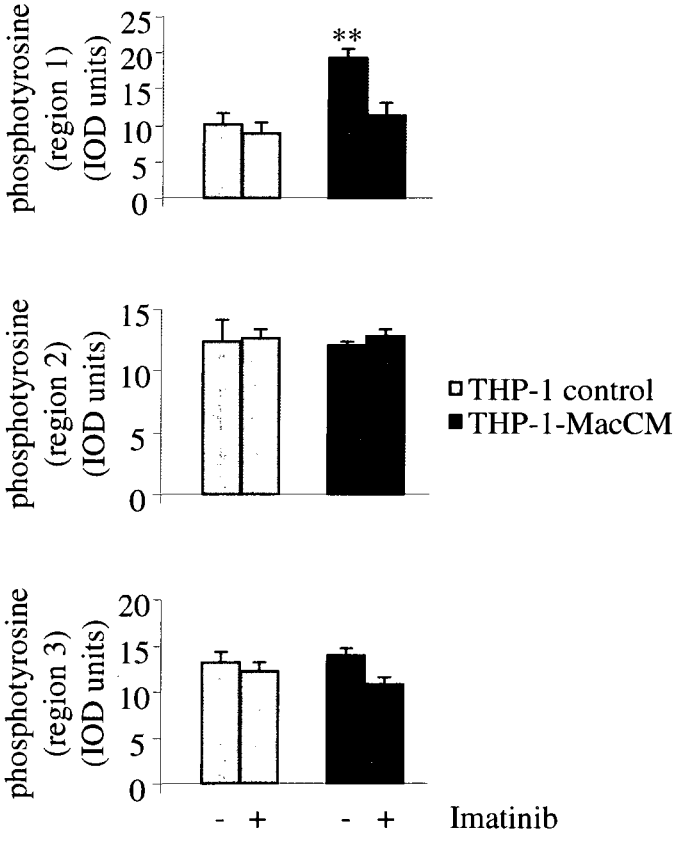
Figure 13. The increase in protein tyrosine phosphorylation, stimulated by medium conditioned by THP-1 macrophages, is impaired by imatinib, a PDGFR inhibitor, in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 90 minutes with imatinib (10 μM), followed by 15 minutes treatment with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. **A.** Solubilized protein from duplicate cultures was immunoblotted with antibodies against phosphotyrosine. A representative immunoblot from one patient sample is shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. ** indicates $p<0.01$ compared to imatinib (-) THP-1 control and imatinib (+) THP-1-MacCM conditions. IOD, integrated optical density.

A.


250 kDa
130 kDa
95 kDa
72 kDa
phosphotyrosine (region 1)
phosphotyrosine (region 2)
phosphotyrosine (region 3)
- + - +
Imatinib
THP-1 control
THP-1-MacCM

B.


phosphotyrosine (region 1) (IOD units)
25
20
15
10
5
0
**
phosphotyrosine (region 2) (IOD units)
15
10
5
0
THP-1 control
THP-1-MacCM
phosphotyrosine (region 3) (IOD units)
20
15
10
5
0
- + - +
Imatinib

cellular protein tyrosine phosphorylation (n=3; Figure 13A-B). However, imatinib significantly reduced the tyrosine phosphorylation of the high molecular weight proteins (region 1) by 73% when human preadipocyte were exposed to THP-1-MacCM (n=3; $p<0.01$; Figure 13A-B). These results suggest that this high molecular weight protein may be PDGFR.

Recent results published by our laboratory have showed that IKKβ phosphorylation, that occurred in response to PDGF treatment, of human preadipocytes was not associated with IκBα degradation or NF-κB activation (81). As previously observed, in the absence of imatinib, THP-1-MacCM significantly increased IKKβ phosphorylation levels and significantly decreased IκBα expression levels compared to THP-1 control medium (n=3; $p<0.01$ and $p<0.001$, respectively; Figure 14A-B). In the presence of either THP-1 control medium or THP-1-MacCM, imatinib did not induce significant changes in IKKβ phosphorylation levels or IκBα expression levels (n=3; Figure 14A-B). These results suggest that PDGF is not relevant to THP-1-MacCM induced NF-κB activation, observed in human abdominal subcutaneous preadipocytes, as imatinib did not affect IKKβ phosphorylation levels or IκBα expression levels.

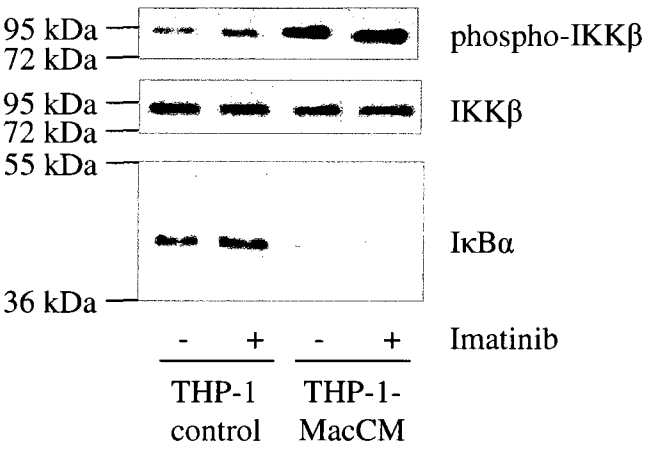
The anti-adipogenic effect of medium conditioned by THP-1 macrophages on human abdominal subcutaneous preadipocyte differentiation is impaired by sc-514, an IKKβ inhibitor.

Since acute treatment of human abdominal subcutaneous preadipocytes with THP-1-MacCM resulted in activation of the IKKβ/NF-κB pathway, it was important to further investigate if this pathway plays a role in the anti-adipogenic effect of THP-1-MacCM. To

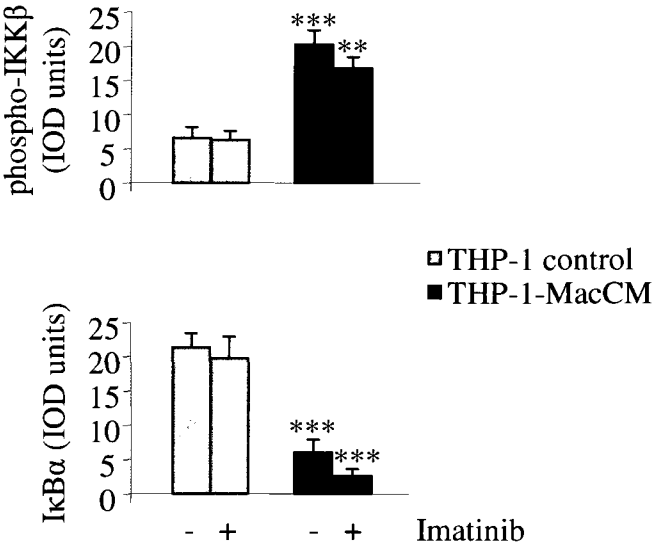
Figure 14. The increase in IKKβ phosphorylation levels and decrease in IκBα protein levels, stimulated by medium conditioned by THP-1 macrophages, is not affected by imatinib, a PDGFR inhibitor, in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were treated for 90 minutes with imatinib (10 μM), followed by 15 minutes treatment with THP-1 control medium or THP-1-MacCM, in the presence of adipogenic inducers of differentiation. **A.** Solubilized protein from duplicate cultures was immunoblotted with antibodies against phospho-IKKβ, IKKβ (loading control) or IκBα. Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to matched THP-1 control condition. IOD, integrated optical density.

A.


95 kDa
72 kDa
phospho-IKKβ
95 kDa
72 kDa
IKKβ
55 kDa
IκBα
36 kDa
- + - +
Imatinib
THP-1 control
THP-1-MacCM

B.

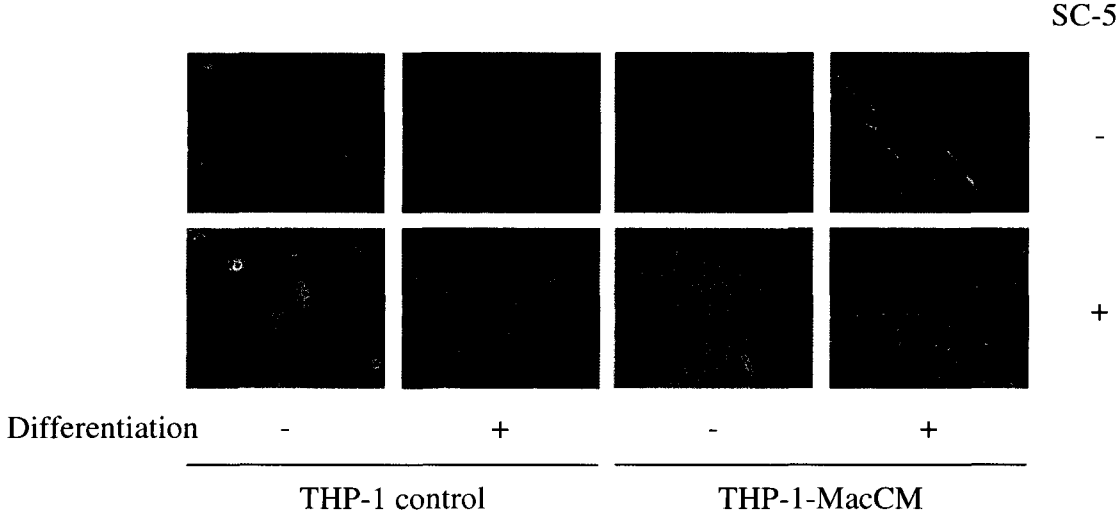

phospho-IKKβ (IOD units)
25
20
15
10
5
0
***
**
THP-1 control
THP-1-MacCM
IκBα (IOD units)
25
20
15
10
5
0
***
***
- + - +
Imatinib

examine this, confluent human preadipocytes were pre-treated for 15 minutes with 100 μM sc-514, then induced to differentiate in the presence of THP-1 control medium or THP-1-MacCM for ~14 days. In the presence of THP-1 control medium, preadipocyte differentiation was unaffected by sc-514, as assessed by cell rounding (Figure 15A), increased TG accumulation (n=4; $p<0.001$; Figure 15B) and increased expression of adipogenic markers upon differentiation (n=4; $p<0.001$; Figure 16A-B). As previously observed, in the absence of sc-514, THP-1-MacCM strongly inhibited the differentiation of human preadipocytes, as assessed morphologically by cell rounding and lipid droplet accumulation (Figure 15A). These results were consistent with reduced TG accumulation (n=4; $p<0.001$; Figure 15B) and impaired expression of adipogenic markers (n=4; $p<0.001$; Figure 16A-B) upon differentiation in THP-1-MacCM. However, the presence of sc-514 appeared to weaken and/or reverse the anti-adipogenic effect of THP-1-MacCM. This effect was characterized morphologically by cell rounding and lipid accumulation under THP-1-MacCM conditions in the presence of sc-514 (Figure 15A). The 86% reduction in TG accumulation caused by THP-1-MacCM was significantly weakened by the addition of sc-514, resulting in only a 32% decrease in TG accumulation compared to THP-1 control medium conditions (n=4; $p<0.05$; Figure 15B). This resulted in a 5-fold recovery in TG accumulation with sc-514 under THP-1-MacCM conditions (n=4; $p<0.05$; Figure 15B). The 63% decrease in PPARγ expression levels with THP-1-MacCM was substantially weakened by sc-514, resulting in only a 3% decrease (n=4; Figure 16A-B) or a 3-fold recovery (n=4; $p<0.001$; Figure 16A-B) in PPARγ expression levels. Similarly, the 75% decrease in FAS expression levels was substantially weakened by sc-514, resulting in only a 9% decrease (n=4; Figure 16A-B) or a 3.5-fold recovery (n=4; $p<0.001$; Figure 16A-B) in FAS expression

Figure 15. The anti-adipogenic effect of medium conditioned by THP-1 macrophages on human abdominal subcutaneous preadipocyte differentiation is impaired by sc-514, an IKKβ inhibitor, as assessed by morphology and triglyceride accumulation.

Confluent human abdominal subcutaneous preadipocytes were treated for 15 minutes with sc-514 (100 μM; +) or DMSO vehicle (-), followed by induction of differentiation in THP-1 control medium or THP-1-MacCM for ~14 days. Non-differentiated preadipocytes were similarly treated with sc-514 (+) or vehicle (-), but maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** After ~14 days, cultures were photographed at 200X magnification. Pictures representative of 4 separate patient samples are shown. **B.** Triglyceride (TG) was extracted, quantified and normalized to protein content. Results are expressed as the mean ± SE of 4 separate patient samples. a indicates $p<0.001$ compared to matched non-differentiated (-) THP-1 control condition. b indicates $p<0.05$ compared to sc-514 (+), differentiated (+) THP-1 control, sc-514 (+), non-differentiated (-) THP-1-MacCM and sc-514 (-), differentiated (+) THP-1-MacCM conditions. c indicates $p<0.001$ compared to sc-514 (-), differentiated (+) THP-1 control condition.

A.


SC-514
-
+
Differentiation - + - +
THP-1 control
THP-1-MacCM

B.

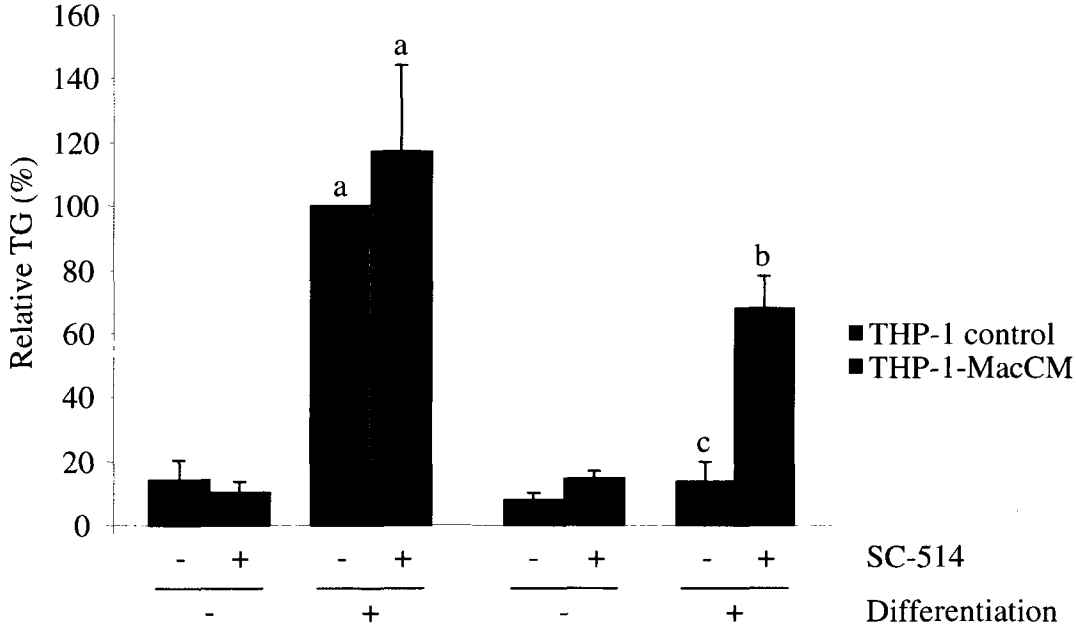
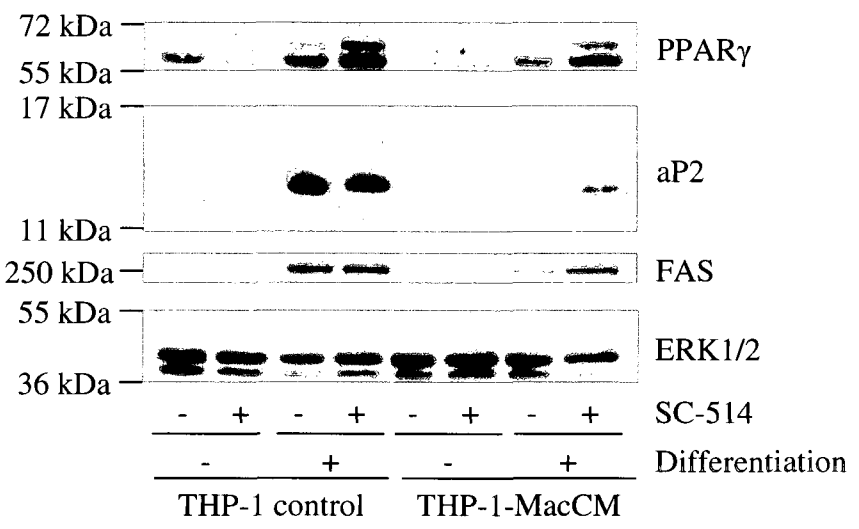

160
140
120
100
80
60
40
20
0
Relative TG (%)
a
a
b
c
THP-1 control
THP-1-MacCM
- + - + - + - +
SC-514
- + - +
Differentiation

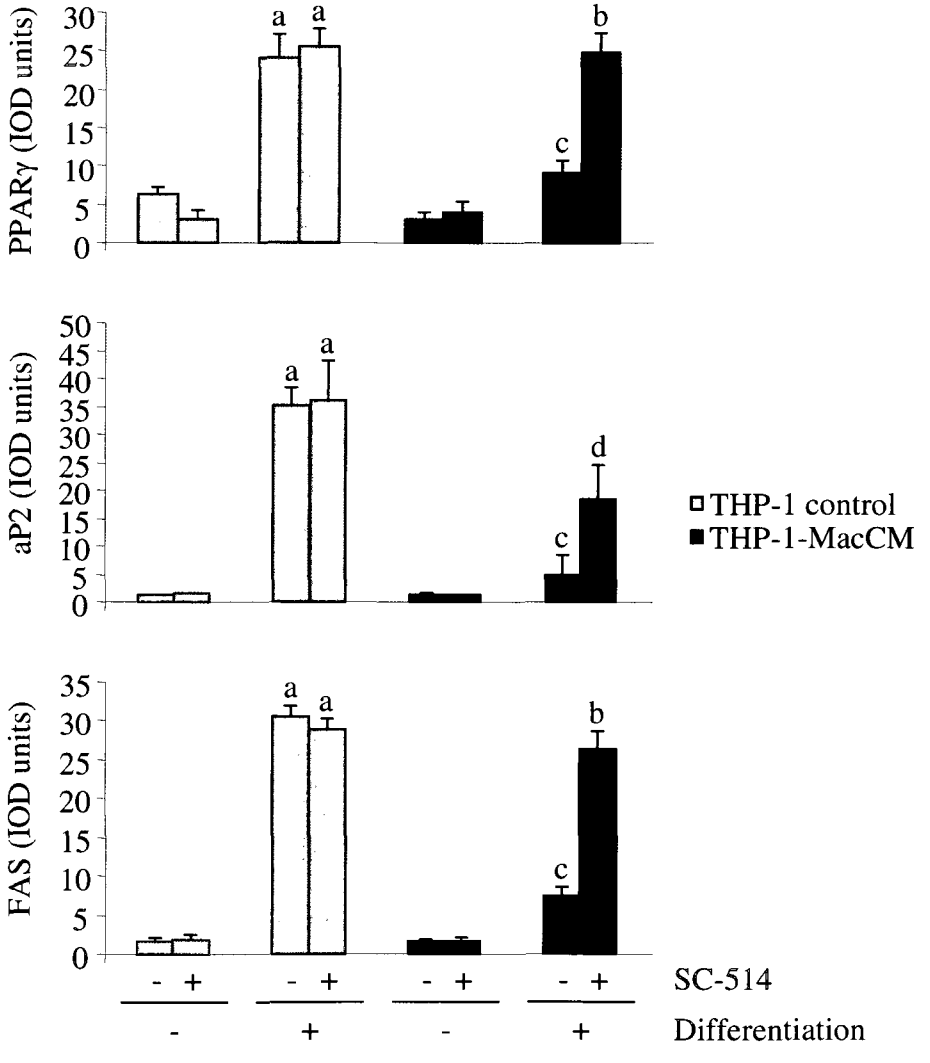
Figure 16. The anti-adipogenic effect of medium conditioned by THP-1 macrophages on human abdominal subcutaneous preadipocyte differentiation is impaired by sc-514, an IKKβ inhibitor, as assessed by PPARγ, aP2 and FAS expression levels.

Confluent human abdominal subcutaneous preadipocytes were treated for 15 minutes with sc-514 (100 μM; +) or DMSO vehicle (-), followed by induction of differentiation in THP-1 control medium or THP-1-MacCM for ~14 days. Non-differentiated preadipocytes were similarly treated with sc-514 (+) or vehicle (-), but maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** Solubilized protein from each culture was immunoblotted with antibodies against PPARγ, aP2, FAS or ERK1/2 (loading control). Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 4 separate patient samples are expressed as mean +/- SE. a indicates $p<0.001$ compared to matched non-differentiated (-) THP-1 control condition. b indicates $p<0.001$ compared to sc-514 (+), non-differentiated (-) THP-1-MacCM and sc-514 (-), differentiated (+) THP-1-MacCM condition. c indicates $p<0.001$ compared to sc-514 (-), differentiated (+) THP-1 control condition. d indicates $p<0.05$ compared to sc-514 (+), differentiated (+) THP-1 control condition. IOD, integrated optical density.

A.


72 kDa
55 kDa
17 kDa
11 kDa
250 kDa
55 kDa
36 kDa
PPARγ
aP2
FAS
ERK1/2
- + - + - + - +
SC-514
- + - +
Differentiation
THP-1 control
THP-1-MacCM

B.


PPARγ (IOD units)
aP2 (IOD units)
FAS (IOD units)
a
b
c
d
THP-1 control
THP-1-MacCM
- + - + - + - +
SC-514
- + - +
Differentiation

levels. The 86% decrease in aP2 expression levels was weakened less substantially by sc-514, resulting in only a 49% decrease (n=4; $p<0.05$; Figure 16A-B) or a 4-fold recovery that did not reach significance (n=4; Figure 16A-B). Taken together, these results suggest that IKKβ is required for the anti-adipogenic effect of THP-1-MacCM.

PART III

Medium conditioned by LPS-activated human blood monocyte-derived macrophages inhibits the differentiation of human abdominal subcutaneous preadipocytes.

A previous study that generated conditioned medium from MDMs stimulated with LPS showed that the products secreted from this macrophage cell model significantly impaired the differentiation of human subcutaneous preadipocytes. Furthermore, this study demonstrated that this conditioned medium reproduced the effects of conditioned medium generated from human ATMs on human preadipocyte differentiation (83). To expand upon our previous observations, I have established a similar model system, which better represent a physiological setting, to study the effect of macrophage-secreted factors from MDMs on human abdominal subcutaneous preadipocyte differentiation. In collaboration with Dr. Angel's laboratory, blood samples were collected from volunteers and processed to isolate PBMCs. The monocytes that were isolated were treated for 24 hours with or without 1 μg/ml LPS prior to the collection of the first batch of conditioned media, identified as Day 1-MDM-CM (untreated) and Day 1-MDM-CM (LPS). The MDMs were subsequently placed in fresh growth medium for 24 hours without LPS prior to the collection of the second batch of conditioned media, identified as Day 2-MDM-CM (untreated) and Day 2-MDM-CM (LPS). Human abdominal subcutaneous preadipocytes were induced to differentiate in the

presence of MDM control medium (untreated or LPS), Day 1-MDM-CM (untreated or LPS) or Day 2-MDM-CM (untreated or LPS) over a period of 12-15 days.

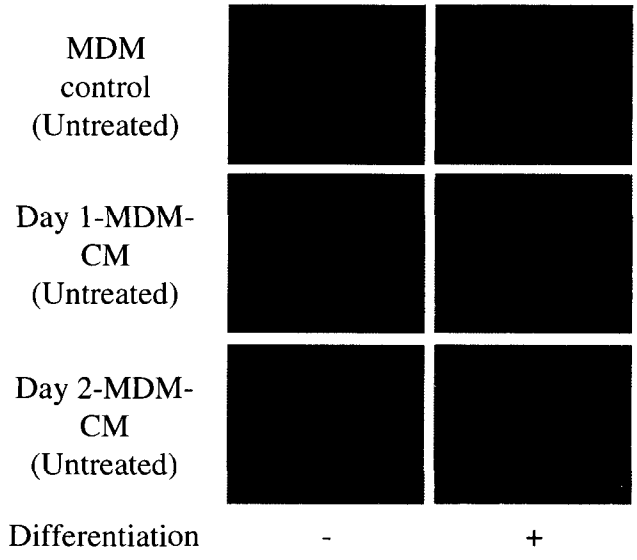
Exposure of human preadipocytes to Day 1-MDM-CM (untreated) or Day 2-MDM-CM (untreated) for ~14 days did not result in inhibition of preadipocyte differentiation, as assessed morphologically by cell rounding and lipid droplet accumulation (Figure 17A). The TG accumulation observed upon induction of differentiation in either Day 1-MDM-CM (untreated) or Day 2-MDM-CM (untreated) did not differ significantly from the TG accumulation following differentiation in MDM control medim (n=3; Figure 17B). Although a mild inhibitory effect on TG accumulation was observed for Day 1-MDM-CM (untreated) and Day 2-MDM-CM (untreated), it did not reach statistical significance. Fibronectin expression levels decreased significantly following induction of differentiation, regardless of the conditioned medium used (n=3; Figure 18A-B). There was also no significant difference in the differentiation-dependent expression levels of PPARγ and aP2 in the presence of either Day 1-MDM-CM (untreated) or Day 2-MDM-CM (untreated) compared to MDM control medium (n=3; Figure 18A-B). Both PPARγ and aP2 expression levels increased upon differentiation, regardless of the conditioned medium used. However, a mild, non-significant inhibitory effect on PPARγ and aP2 expression levels was observed following differentiation in Day 1-MDM-CM (untreated) and Day 2-MDM-CM (untreated) compared to MDM control medium.

In contrast to conditioned medium from untreated MDMs, exposure of human preadipocytes to Day 1-MDM-CM (LPS) or Day 2-MDM-CM (LPS) for ~14 days inhibited preadipocyte differentiation, as assessed morphologically by impaired cell rounding and lipid accumulation (n=4; Figure 19A). Furthermore, this inhibition appeared to be more potent for Day 1-MDM-CM (LPS) compared to Day 2-MDM-CM (LPS). Compared to MDM control

Figure 17. Medium conditioned by untreated human blood MDMs does not inhibit the differentiation of human abdominal subcutaneous preadipocytes, as assessed by morphology and triglyceride accumulation.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in MDM control medium (untreated), Day 1-MDM-CM (untreated) or Day 2-MDM-CM (untreated) for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** After ~14 days, cultures were photographed at 200X magnification. Pictures representative of 3 separate patient samples are shown. **B.** Triglyceride (TG) was extracted, quantified and normalized to protein content. Results are expressed as the mean ± SE of 3 separate patient samples. * indicates $p<0.05$ between indicated pairs.

A.


MDM control (Untreated)
Day 1-MDM-CM (Untreated)
Day 2-MDM-CM (Untreated)
Differentiation
-
+

B.

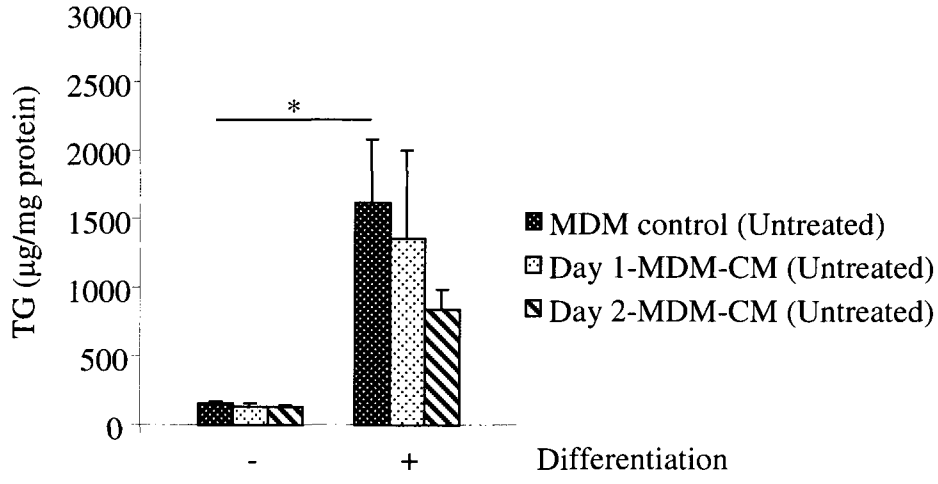
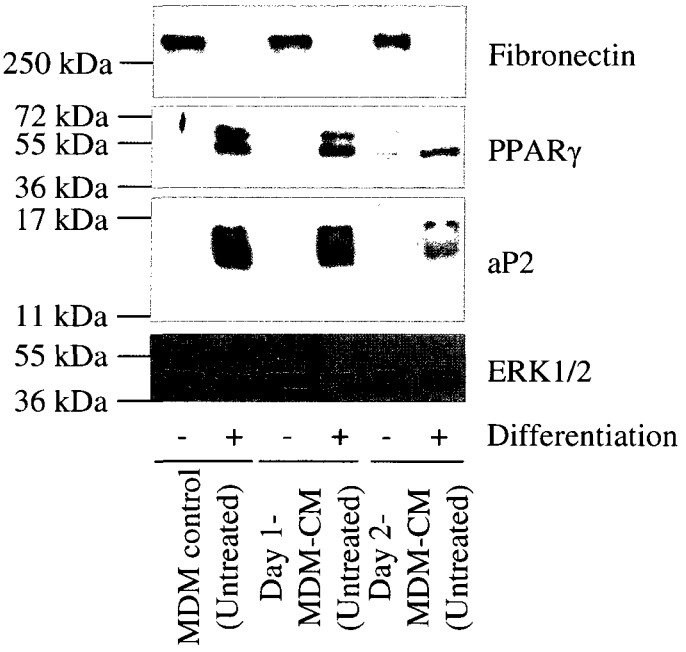

3000
2500
2000
1500
1000
500
0
TG (µg/mg protein)
*
-
+
Differentiation
MDM control (Untreated)
Day 1-MDM-CM (Untreated)
Day 2-MDM-CM (Untreated)

Figure 18. Medium conditioned by untreated human blood MDMs does not prevent the differentiation-dependent changes in fibronectin, PPARγ and aP2 expression levels in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in MDM control medium (untreated), Day 1-MDM-CM (untreated) or Day 2-MDM-CM (untreated) for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** Solubilized protein from each culture was immunoblotted with antibodies against fibronectin, PPARγ, aP2 or ERK1/2 (loading control). Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 3 separate patient samples are expressed as mean +/- SE. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ between indicated pairs. IOD, integrated optical density.

A.


250 kDa
72 kDa
55 kDa
36 kDa
17 kDa
11 kDa
55 kDa
36 kDa
Fibronectin
PPARγ
aP2
ERK1/2
- + - + - +
Differentiation
MDM control (Untreated)
Day 1-MDM-CM (Untreated)
Day 2-MDM-CM (Untreated)

B.

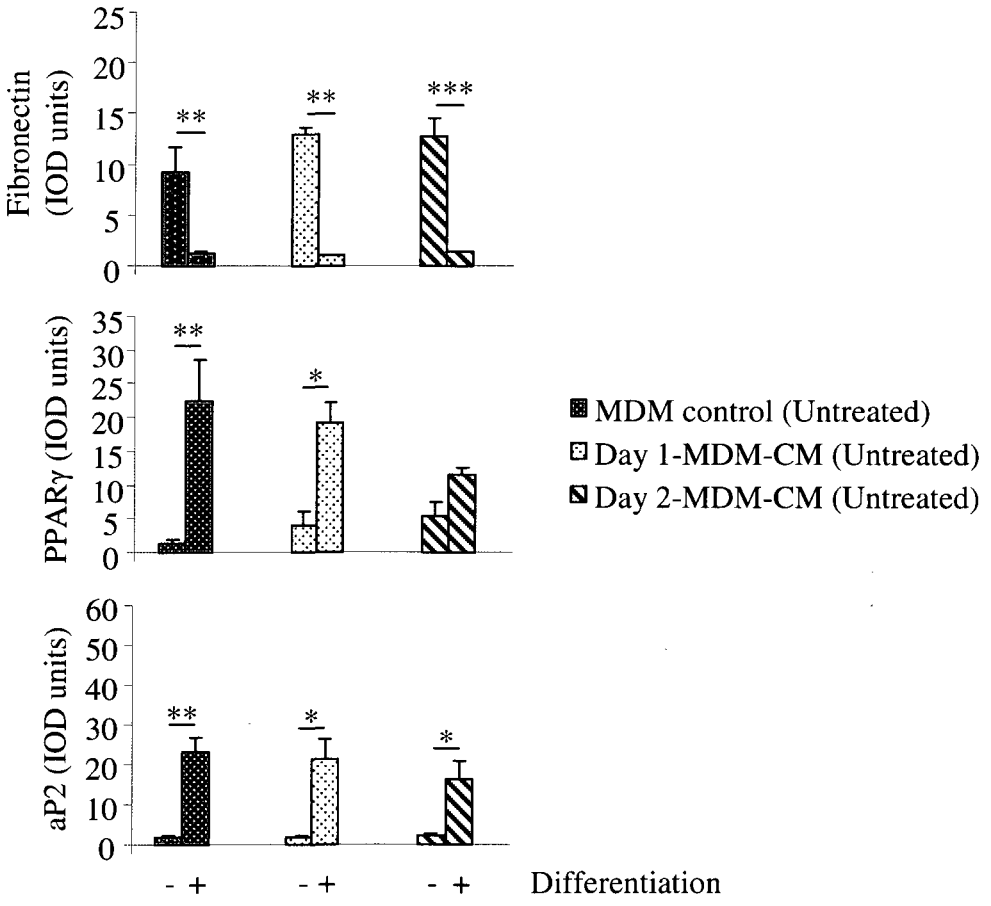
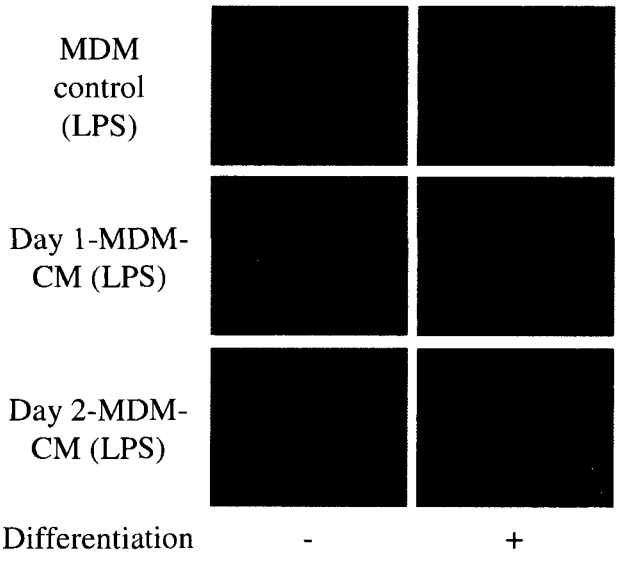

Fibronectin (IOD units)
PPARγ (IOD units)
aP2 (IOD units)
MDM control (Untreated)
Day 1-MDM-CM (Untreated)
Day 2-MDM-CM (Untreated)
- + - + - +
Differentiation

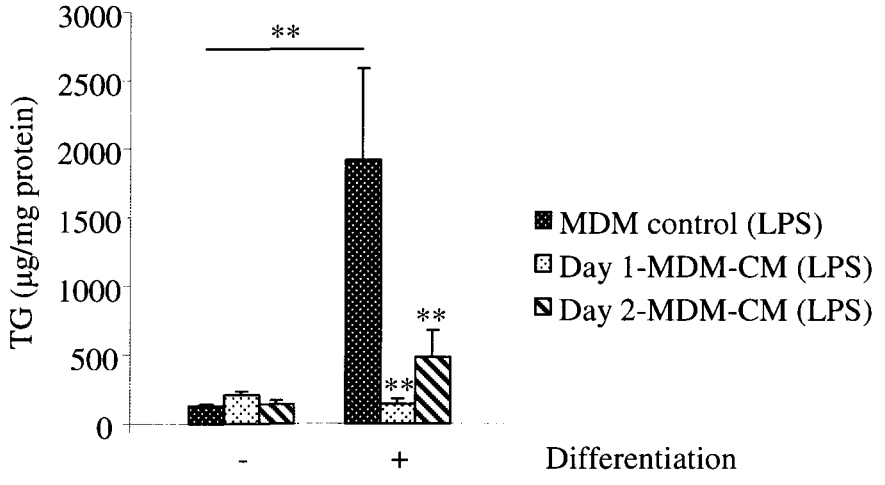
Figure 19. Medium conditioned by LPS-activated human blood MDMs inhibits the differentiation of human abdominal subcutaneous preadipocytes, as assessed by morphology and triglyceride accumulation.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in MDM control medium (LPS), Day 1-MDM-CM (LPS) or Day 2-MDM-CM (LPS) for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** After ~14 days, cultures were photographed at 200X magnification. Pictures representative of 4 separate patient samples are shown. **B.** Triglyceride (TG) was extracted, quantified and normalized to protein content. Results are expressed as the mean ± SE of 4 separate patient samples. ** indicates $p<0.01$ between indicated pairs. For Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS) conditions, ** are compared to differentiated (+) MDM control condition.

A.


MDM control (LPS)
Day 1-MDM-CM (LPS)
Day 2-MDM-CM (LPS)
Differentiation
-
+

B.


3000
2500
2000
1500
1000
500
0
TG (µg/mg protein)
**
**
**
-
+
Differentiation
MDM control (LPS)
Day 1-MDM-CM (LPS)
Day 2-MDM-CM (LPS)

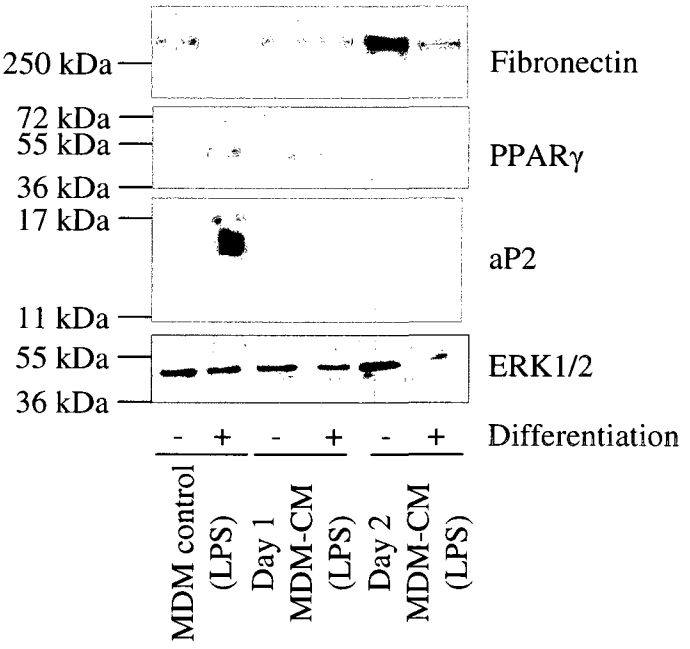
medium (LPS), Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS) significantly reduced the differentiation-induced TG accumulation, by 92% and 75% respectively ($n=4$; $p<0.01$; Figure 19B). Fibronectin expression levels decreased significantly, by 93% and 78%, following induction of differentiation in MDM control medium (LPS) and Day 2-MDM-CM (LPS), respectively ($n=4$; $p<0.001$; Figure 20A-B). A non-significant, 41% decrease in fibronectin expression was also observed for Day 1-MDM-CM (LPS) upon induction of differentiation. Compared to cell differentiated in MDM control medium (LPS), a 7.4-fold increase in fibronectin expression was observed following differentiation in Day 1-MDM-CM (LPS). PPARγ expression levels significantly decreased by 71% and 69% following differentiation in Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS), respectively ($n=4$; $p<0.01$; Figure 20A-B). aP2 expression levels also significantly decreased by 93% and 86% following differentiation in Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS), respectively ($n=4$; $p<0.01$; Figure 20A-B). These results suggest that MDMs must be activated by LPS in order to secrete factors that inhibit adipogenesis, since there was no significant effect on preadipocyte differentiation caused by untreated MDMs.

The inhibition of human preadipocyte differentiation observed with Day 1-MDM-CM (LPS) or Day 2-MDM-CM (LPS) was not due to LPS alone. A significant increase in TG accumulation as well as PPARγ and aP2 expression levels was observed upon induction of differentiation in MDM control medium containing LPS ($n=4$; $p<0.01$, $p<0.05$ and $p<0.01$ respectively). Furthermore, the mean TG levels following differentiation in MDM control medium (untreated) and MDM control medium (LPS) were found to be 1623.30 μg/mg protein and 1918.45 μg/mg protein, respectively.

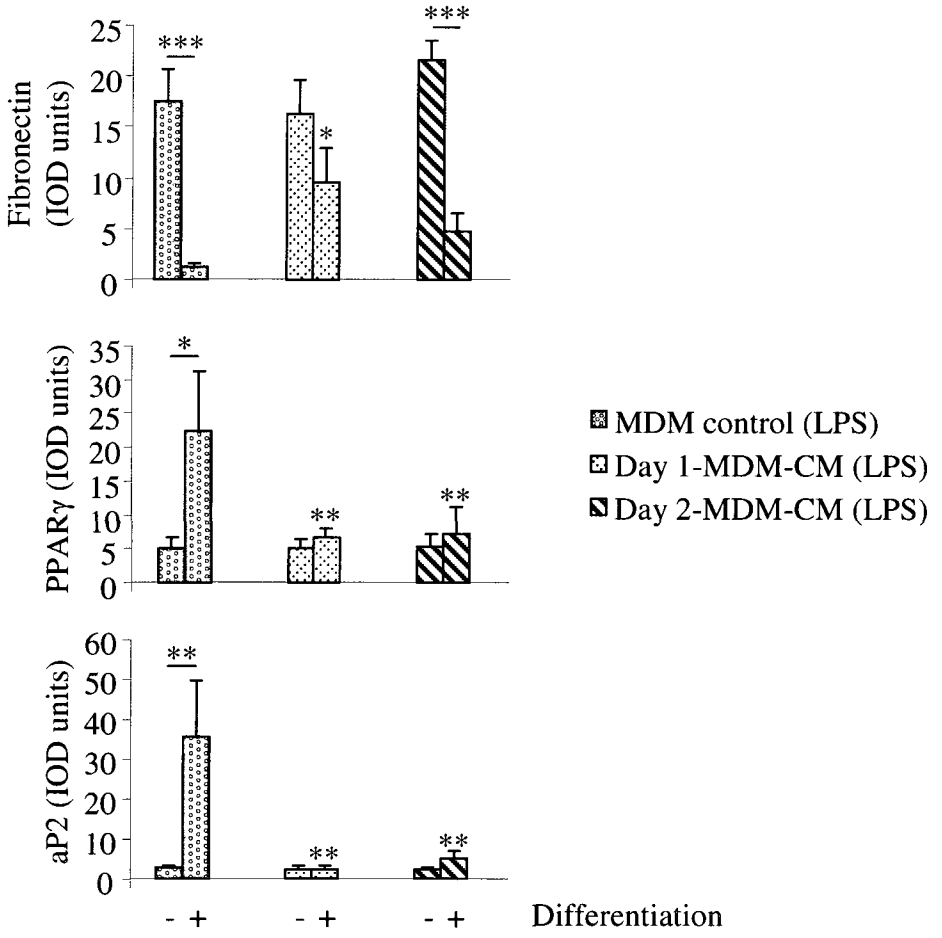
Figure 20. Medium conditioned by LPS-activated human blood MDMs inhibits the differentiation-dependent changes in fibronectin, PPARγ and aP2 expression levels in human abdominal subcutaneous preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate in MDM control medium (LPS), Day 1-MDM-CM (LPS) or Day 2-MDM-CM (LPS) for ~14 days. Non-differentiated preadipocytes were maintained in the corresponding medium without adipogenic inducers of differentiation for ~14 days. **A.** Solubilized protein from each culture was immunoblotted with antibodies against fibronectin, PPARγ, aP2 or ERK1/2 (loading control). Representative immunoblots from one patient sample are shown. **B.** Densitometric data from 4 separate patient samples are expressed as mean +/- SE. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ between indicated pairs. For Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS) conditions, * and ** are compared to differentiated (+) MDM control condition. IOD, integrated optical density.

A.


250 kDa
72 kDa
55 kDa
36 kDa
17 kDa
11 kDa
55 kDa
36 kDa
Fibronectin
PPARγ
aP2
ERK1/2
- + - + - +
Differentiation
MDM control (LPS)
Day 1 MDM-CM (LPS)
Day 2 MDM-CM (LPS)

B.


Fibronectin (IOD units)
PPARγ (IOD units)
aP2 (IOD units)
MDM control (LPS)
Day 1-MDM-CM (LPS)
Day 2-MDM-CM (LPS)
- + - + - +
Differentiation

DISCUSSION

Adipocytes and ATMs both contribute to the chronic systemic low-grade inflammation associated with obesity, insulin resistance and T2D (85, 122). This inflammatory state is characterized by a deregulated production of pro-inflammatory cytokines and the activation of various inflammatory signaling pathways (86). Interactions between macrophages or macrophage-secreted products and adipose cells may cause adipose tissue dysfunction. Recent evidence has suggested a possible role for macrophages in limiting adipose tissue expansion in addition to its contribution to obesity-associated inflammation. This deficit in adipogenesis could favour the development of insulin-resistant hypertrophied adipocytes, thus upregulating the production of pro-inflammatory molecules. In addition, limitations on adipose tissue capacity to store excess energy intake would lead to ectopic fat deposition as well as insulin resistance and inflammation in these tissues (115, 116, 118). Our laboratory, as well as other research groups, has recently shown macrophage-secreted factors impairs adipogenesis in both human and mouse *in vitro* cell models (2, 83, 149). However, the mechanisms and signaling pathways by which macrophages inhibit adipogenesis have not yet been defined.

Results from our laboratory have shown that macrophage-secreted factors are required at the onset of differentiation for maximal inhibition of preadipocyte differentiation (172). The data show that the critical time point for the inhibition of 3T3-L1 adipogenesis by both J774-MacCM and THP-1-MacCM is within the first 2 days of the 8-day differentiation process (74, 172). This time period coincides with mitotic clonal expansion, an early required event of 3T3-L1 adipogenesis (22, 35, 36). My results show that both J774-MacCM and THP-1-MacCM diminish clonal expansion in 3T3-L1 preadipocytes induced to differentiate. These results suggest that clonal expansion may be a primary target of MacCM

and could explain why exposure to MacCM beyond day 2 of differentiation is without significant effect in 3T3-L1 adipogenesis.

To further confirm that the change in cell number was due to an effect on proliferation, BrdU incorporation was assessed in 3T3-L1 preadipocytes induced to differentiate in J774-MacCM. Compared to the effect assessed by cell enumeration, a weaker inhibition was observed for BrdU incorporation in the presence of J774-MacCM. This would suggest that some cells were entering the S phase of the cell cycle. However, FACS analysis, performed by Dr. AnneMarie Gagnon in our laboratory, showed that 3T3-L1 preadipocytes induced to differentiate with J774-MacCM are severely restrained from entering the S phase of the cell cycle, compared to the cells differentiated in J774 control medium (172). It is possible that the BrdU antibody signal observed may have been somewhat non-specific. Furthermore, the percentage of apoptotic cells, which was assessed by Hoechst staining, was minimal in all culture conditions, indicating that changes in apoptosis cannot be used as an explanation for the reduced cell counts in J774-MacCM (172).

To understand more about how clonal expansion is altered by J774-MacCM, I examined the expression of cell cycle regulators as potential targets of J774-MacCM. The protein expression of $p27^{Kip1}$, a cyclin dependent kinase inhibitor, was appropriately down-regulated in 3T3-L1 preadipocytes differentiated either in J774 control medium or J774-MacCM (37, 38, 47). Furthermore, the expression of both isoforms of C/EBPβ (LAP and LIP) did not significantly change in the presence of J774-MacCM. However, almost a complete abrogation of Rb phosphorylation occurred with J774-MacCM. In adipogenesis, the role of Rb appears to be complex. As previously mentioned, the phosphorylation-dependent inactivation of Rb allows the release of members of the E2F family of transcription factors and permits cell cycle progression (176). Based on this information, it

may be assumed that the deletion of Rb would promote preadipocyte differentiation. However, it was found that the complete deletion of Rb is not associated with increased adipogenesis (177). This could be due to the important role of Rb in cell cycle exit and its interaction with members of the C/EBP family of transcription factors (30, 41, 177). In cell cycle regulation, Rb acts as a transcriptional co-repressor by associating with E2F and recruiting HDAC, resulting in transcriptional repression which impairs cell cycle G_1 exit (41). Furthermore, *in vitro* studies have demonstrated that Rb interacts with all three C/EBP family members, namely C/EBPβ, C/EBPα and C/EBPδ, possibly affecting the transcriptional activation of their downstream targets, which are required for terminal differentiation (30, 41, 177).

Transcription factors expressed early during differentiation may also be perturbed by J774-MacCM, which could consequently have a negative effect on clonal expansion. C/EBPβ is expressed early upon preadipocyte differentiation and initiates mitotic clonal expansion. C/EBPβ must acquire DNA-binding activity prior to initiating clonal expansion and transactivating the C/EBPα and PPARγ genes (53). As previously mentioned in the introduction, C/EBPβ DNA-binding function was reported to be influenced by the state of Rb phosphorylation (30, 37). I investigated the effect of J774-MacCM on the DNA-binding activity of C/EBPβ in 3T3-L1 preadipocytes induced to differentiate (30, 37). The inhibition of Rb phosphorylation by J774-MacCM did not impair C/EBPβ DNA-binding, assessed by centromeric localization and ELISA-based specific DNA-binding. However, it is possible that the binding of C/EBPβ to DNA does not necessarily correlate with the transactivation of its target genes (178, 179). It has been suggested that C/EBPβ acts as a transcriptional switch and that its activity depends on the integration of signals from the intra- and extracellular environment (180). In addition to post-translational modifications and changes in subcellular

localization, the interaction of C/EBPβ with other coregulatory molecules can also modulate its transcriptional activity (179, 181-183). Although it is not known how frequently this uncoupling occurs, it raises the possibility that C/EBPβ transcriptional activity could potentially still be affected by J774-MacCM. To examine this possibility, a reporter gene assay could be performed in the presence of J774-MacCM using constructs in which C/EBPβ-specific promoters drive the expression of luciferase in response to C/EBPβ activity (63).

As previously mentioned, growth-arrested preadipocytes re-enter the cell cycle through activation of G1 cyclins/cdks and regulation of pocket proteins (38, 40, 42). The cdks, which are activated upon association with cyclins, phosphorylate Rb (43-45). Specifically, cyclinD/cdk4/6, cyclinE/cdk2 and cyclinA/cdk2 complexes are involved in the sequential phosphorylation of Rb (38, 40). The expression and/or activities of these upstream regulators of Rb phosphorylation may be impaired by MacCM. To investigate this possibility, immunoblotting could be used to examine whether there are any changes in the temporal expression of cyclins by MacCM. In addition, kinase assays could be performed to look at possible changes in the activities of these cdks upon treatment with MacCM.

The phosphorylation of Rb is needed for the dissociation of its downstream target, E2F, and the transactivation of E2F-dependent genes, such as PPARγ (37, 40, 44). I have attempted to examine the DNA-binding function of E2F in the presence of MacCM using an EMSA gel shift assay. However, due to technical difficulties and limitations with the E2F antibody used, I was unable to determine if there was any change in E2F DNA-binding ability in response to MacCM. The effect of MacCM on the expression and DNA-binding function of E2F will therefore require further research in order to characterize the mechanism underlying the inhibition of clonal expansion by MacCM.

ERK1/2 has also been shown to be a regulator of mitotic clonal expansion (53). Recent data published by our laboratory showed that under non-adipogenic conditions, THP-1-MacCM acutely stimulates ERK1/2 phosphorylation in 3T3-L1 preadipocytes. However, adipogenic stimulation of ERK1/2 phosphorylation in 3T3-L1 preadipocytes was unaffected by J774-MacCM, suggesting that macrophage-secreted factors do not inhibit clonal expansion by interfering with ERK1/2 activation (172).

Mitotic clonal expansion is required for 3T3-L1 adipogenesis (30, 35). My data suggest that J774-MacCM and THP-1-MacCM are anti-proliferative during the clonal expansion phase of adipogenesis that occurs in the embryonic 3T3-L1 cell model. Unlike 3T3-L1 preadipocytes, isolated human preadipocytes are thought to have already proceeded through a clonal expansion phase *in vivo*, and differentiation of these cells in culture is not associated with proliferation (22, 25, 29, 36). Regardless of this, conditioned medium from several different macrophage models is capable of inhibiting human adipogenesis (2, 83).

Published results from our laboratory have shown that THP-1-MacCM completely suppresses human adipogenesis. Furthermore, our laboratory has demonstrated that THP-1-MacCM is a more potent and uniform inhibitor of human adipogenesis compared to J774-MacCM (2). The precise targets of MacCM in human adipogenesis are not known. I have therefore examined the human preadipocyte intracellular signaling pathways that are activated or blocked by MacCM, and that may be involved in its anti-adipogenic effect. My results show that upon acute induction of differentiation, THP-1-MacCM appears to induce tyrosine phosphorylation of high molecular weight proteins as well as activate a variety of signaling pathways, including Akt, ERK1/2 and IKKβ/NF-κB pathways. Lacasa *et al.* have previously shown that conditioned medium from LPS-activated MDMs induces an inflammatory state characterized by NF-κB activation in fully differentiated human

adipocytes. Their results showed a 2-fold increase of the active form of the NF-κB subunit and a 70% decrease in IκBα in differentiated human adipocytes treated with conditioned medium from LPS-activated MDMs (83).

The macrophage-secreted factor(s) implicated in the regulation of adipogenesis is (are) still unidentified. As mentioned previously, PDGF is an anti-adipogenic growth factor that is secreted by both J774 and PMA-treated THP-1 macrophages (81, 175, 184). PDGFR downstream signal transduction pathways include the PI3K/Akt pathway, the ERK1/2 pathway and the IKKβ/NF-κB pathway (81, 97, 100). Using the selective tyrosine kinase inhibitor imatinib, I observed a significant decrease in tyrosine phosphorylation of high molecular weight proteins, which was consistent with the possibility that this is PDGFR. However, the presence of imatinib did not alter the levels of phospho-IKKβ or IκBα in the presence of THP-1-MacCM. This suggests that PDGF is not relevant to THP-1-MacCM induced NF-κB activation. These results are consistent with recent data published by our laboratory that showed that PDGF treatment of human preadipocytes was not associated with IκBα degradation or NF-κB activation (81).

The serine/threonine kinase IKKβ is involved in the etiology of insulin resistance and T2D in inflammatory conditions (90, 185, 186). Insulin sensitivity was shown to be improved in insulin-resistant models by heterozygous gene deletion (*Ikkβ* +/-) or by salicylates, which work as IKKβ inhibitors (187, 188). IKKβ is known to activates NF-κB, which induces the expression of anti-adipogenic genes such as TNFα and IL-6 (185, 189). Recent results from our laboratory have revealed that activation of IKKβ in PDGF-treated human stromal preadipocytes is associated with the inhibition of adipogenesis (81). Since treatment with sc-514 significantly weakened the inhibitory effect of THP-1-MacCM on TG

accumulation and expression of adipogenic markers, this would suggest that IKKβ is required for the anti-adipogenic effect of THP-1-MacCM. The involvement of the NF-κB pathway in the anti-adipogenic effect of THP-1-MacCM will still need to be investigated. This could be accomplished using an inhibitor of NF-κB, such as the inhibitory peptide SN50, or using small interfering RNA directed against NF-κB (190, 191). As previously discussed IKKβ has been shown to phosphorylate and inhibit proteins involved in adipose tissue function, such as 14-3-3β, and inhibit insulin signaling through phosphorylation of IRS-1 at serine 307 (rodent) and serine 312 (human) (79, 91, 92). It would therefore be relevant to assess the role of these candidate targets of IKKβ in the anti-adipogenic effect of THP-1-MacCM.

The precise relationship between the anti-adipogenic effect of MacCM and the different signaling events activated in response to MacCM in human preadipocytes is not known. It is possible that the ERK1/2 pathways may also play a role in the inhibition of human preadipocyte differentiation by THP-1-MacCM. I have shown that ERK1/2 phosphorylation was significantly enhanced in human preadipocytes induced to differentiate in THP-1-MacCM. Results published by our laboratory have also shown that ERK1/2 activation is implicated in the inhibitory effect of THP-1-MacCM on TG accumulation in 3T3-L1 preadipocyte differentiation (74). Experiments using PD98059, a pharmacological inhibitor of the ERK1/2 pathway, could be performed with human preadipocytes to establish whether the ERK1/2 pathway is involved in the inhibitory effect of THP-1-MacCM (74, 81).

The contribution of preadipocytes to the inflammatory state associated with obesity is not well understood. It has been reported that under inflammatory conditions, preadipocytes synthesize more pro-inflammatory products compared to adipocytes (192, 193). Since my

results suggest that THP-1-MacCM induces the activation of the IKKβ/NF-κB pathway, this would suggest that macrophage-secreted factors produce inflamed preadipocytes. It is thus possible that these inflamed preadipocytes contribute to the recruitment of macrophages to the adipose tissue as well as contribute to adipose tissue dysfunction in obesity.

It was previously reported that LPS activated MDMs significantly impaired the differentiation of human subcutaneous preadipocytes and reproduced the effects of human ATMs on adipogenesis (83). Since the phenotype of macrophage cell lines could, in part, contribute to their anti-adipogenic effect, I have established an experimental cell model using human primary MDMs. This cell model more accurately represents an *in vivo* system, since it is the most likely source for infiltrated macrophages in the adipose tissue (83, 122). In contrast to the previously established MDM cell model by Lacasa *et al.*, the isolated PBMCs were allowed to differentiate for 24 to 48 hours rather than 7 to 8 days (83). An important initial step in the transition from a circulating monocyte to a tissue macrophage is adherence (194). It is believed that the cell's transcriptional activity and cellular reactivity is modified through adherence (194). Monocyte adherence to capillary endothelium and different extracellular matrix components triggers monocyte activation in extravascular sites of infection, chronic inflammatory disorders and tissue damage (195). It has been reported that adherence selectively activates the expression of numerous genes including growth factors (such as PDGF), cytokines (such as IL-1α, IL-1β, IL-8 and TNF) and macrophage-colony stimulating factor (195-200). Adherence is thus sufficient to induce high, steady-state levels of mRNA expression of various genes (194). However, exposure to a second signal, such as LPS endotoxin, is required for the secretion of these factors, most notably IL-1β and TNF (194, 201, 202).

My results show that MDM-CM from untreated MDMs did not significantly inhibit human adipogenesis or the differentiation-dependent expression of fibronectin. However, MDM-CM from LPS-activated MDMs significantly impaired human adipogenesis and inhibited the differentiation-dependent changes in fibronectin. Furthermore, the inhibition appeared to be more potent with Day 1-MDM-CM (LPS) compared to Day 2-MDM-CM (LPS). Using their experimental MDM cell model, Lacasa *et al.* have previously reported that conditioned medium from both untreated and LPS-activated MDM inhibit human preadipocyte differentiation, as assessed by lipid accumulation and mRNA expression levels of adipogenic markers, namely PPARγ, C/EBPα and aP2. In addition, this study showed that fibronectin protein expression was 3-fold higher with LPS-activated MDM-CM compared to control or untreated MDM-CM in preadipocytes differentiated for 12 to 16 days. These results are in agreement with the data I obtained using my Day 1-MDM-CM (LPS). I did not however, observe the significant inhibition of human adipogenesis with untreated MDM-CM that was reported by Lacasa *et al.* However, my results with untreated MDM-CM showed mild, non-significant inhibitory tendencies towards human adipogenesis.

The results published by Lacasa *et al.* suggest that, in response to LPS-activated MDM-CM, fibronectin expression increases with treatment (83). However, this study did not show basal fibronectin protein expression levels in undifferentiated preadipocytes in the presence of this MDM-CM. My results showed that following induction of differentiation, fibronectin expression decreased significantly in the presence of both MDM control medium (LPS) and Day 2-MDM-CM (LPS). A smaller, non-significant decrease in fibronectin expression was also found upon induction of differentiation in Day 1-MDM-CM (LPS). Compared to the Lacasa *et al.* paper, my results provide a different interpretation as to the effect of LPS-activated MDM-CM on fibronectin expression upon differentiation of human

preadipocytes. It would appear that this MDM-CM does not simply increase fibronectin expression but rather prevents the differentiation-dependent decrease in fibronectin expression.

The fact that my untreated MDM-CM did not have a significant inhibitory effect on human adipogenesis contrasts the significant inhibitory effect of untreated MDM-CM obtained by Lacasa *et al.* This could be due to the different cell phenotypes obtained upon differentiation of PBMCs for different lengths of time. The differences in potency between Day 1-MDM-CM (LPS) and Day 2-MDM-CM (LPS) could be due to the removal of LPS from the growth medium. The exacerbated production of inflammatory and anti-adipogenic biomolecules in response to LPS could be downregulated with the removal of this inflammatory stimulus. It is possible that the activated state is dependent upon LPS and that the cascade of inflammatory events triggered by LPS is not self-perpetuating. Continued or repeated exposure to LPS may be required to maintain or increase the production of these various biomolecules. Further research will therefore be required to characterize the inhibitory effect of MDM-CM on human preadipocyte differentiation and to identify intracellular signaling pathways activated by MDMs in order to reproduce previous findings with THP-1-MacCM.

At the present time, it is not known which macrophage-secreted factor(s) is (are) mediating the anti-adipogenic effect. Due to the large array of biomolecules secreted by macrophages, including growth factors, cytokines, proteolytic enzymes and metabolites, it is possible that various factors, working individually or cooperatively, are required for their effects on adipogenesis (203). Our method of using MacCM provides an integrated approach that allows our laboratory to estimate the overall effect of macrophage-derived products on preadipocyte signaling pathways (74). Previous studies have reported that some pro-

inflammatory cytokines, known to be produced by macrophages, inhibit adipogenesis. For instance, TNF-α is known to inhibit both 3T3-L1 and human adipogenesis (87, 204, 205). It has also been shown that TNF-α disrupts the regulation of pocket proteins, namely p130 and p107, resulting in a complete block in mitotic clonal expansion during 3T3-L1 preadipocyte differentiation (206). IL-1β has also been described as a strong suppressor of adipogenesis (89, 205, 207). Published results have shown that IL-1 or TNF-α induce cell cycle arrest in human melanoma cells through the hypophosphorylation of Rb (208). A study reported IL-6 as an inhibitor of human subcutaneous adipogenesis (209). While J774 macrophages are known to produce the cytokine IL-1β, THP-1 macrophages are known to secrete the cytokines TNF-α, transforming growth factor-β, IL-6, IL-1β and IL-1α (132, 210, 211). Similarly, LPS-activated MDMs produce high levels of TNF-α and IL-6 (83). Therefore, one or more of these factors could be mediating the anti-adipogenic effect.

To identify the cytokines secreted and found in common between the various MacCMs, further studies would need to be performed using a cytokine antibody array kit. Another approach would involve the fractionation of the MacCM, using molecular weight as a cut-off. Each fraction could then be tested individually or in combination for their ability to inhibit adipogenesis, compared to similar fractions obtained from control medium. This would allow us to determine if the anti-adipogenic effect of MacCM requires the individual or combined effect of different factors. Mass spectrometry could then be performed on the fraction(s) that inhibits preadipocyte differentiation in order to identify the candidate factor(s). To further confirm the anti-adipogenic effect of these candidate factor(s), immunodepletion or immunoneutralization studies targeting the candidate factor(s) could be performed.

CONCLUSION

The *in vivo*, paracrine interactions between macrophages and adipose cells are complex. My results offer new insights on the nature of these interactions and provide preliminary information on the signaling pathways involved in the anti-adipogenic effect of MacCM. First, I have shown that J774-MacCM or THP-1-MacCM impairs the mitotic clonal expansion phase in 3T3-L1 adipogenesis. Furthermore, this was accompanied by an inhibition in the differentiation-induced phosphorylation of Rb in the presence of J774-MacCM. Second, THP-1-MacCM was found to activate Akt, ERK1/2 and IKKβ/NF-κB pathways in human preadipocytes induced to differentiate. In addition, it appears that a pro-inflammatory signaling response, not associated with PDGFR, is a required element for the anti-adipogenic effect of THP-1-MacCM on human preadipocytes. Lastly, I have established an experimental cell model using human primary MDMs that better represents an *in vivo* system in order to further our investigations of the effect of macrophage-secreted factors on human preadipocyte differentiation. These findings provide a framework for future studies in order to understand the underlying molecular mechanisms and cellular interactions that lead to adipose tissue dysfunction *in vivo*. This area of research will therefore be important in order to identify therapeutic targets for the treatment of adipose tissue dysfunction associated with obesity and its complications.

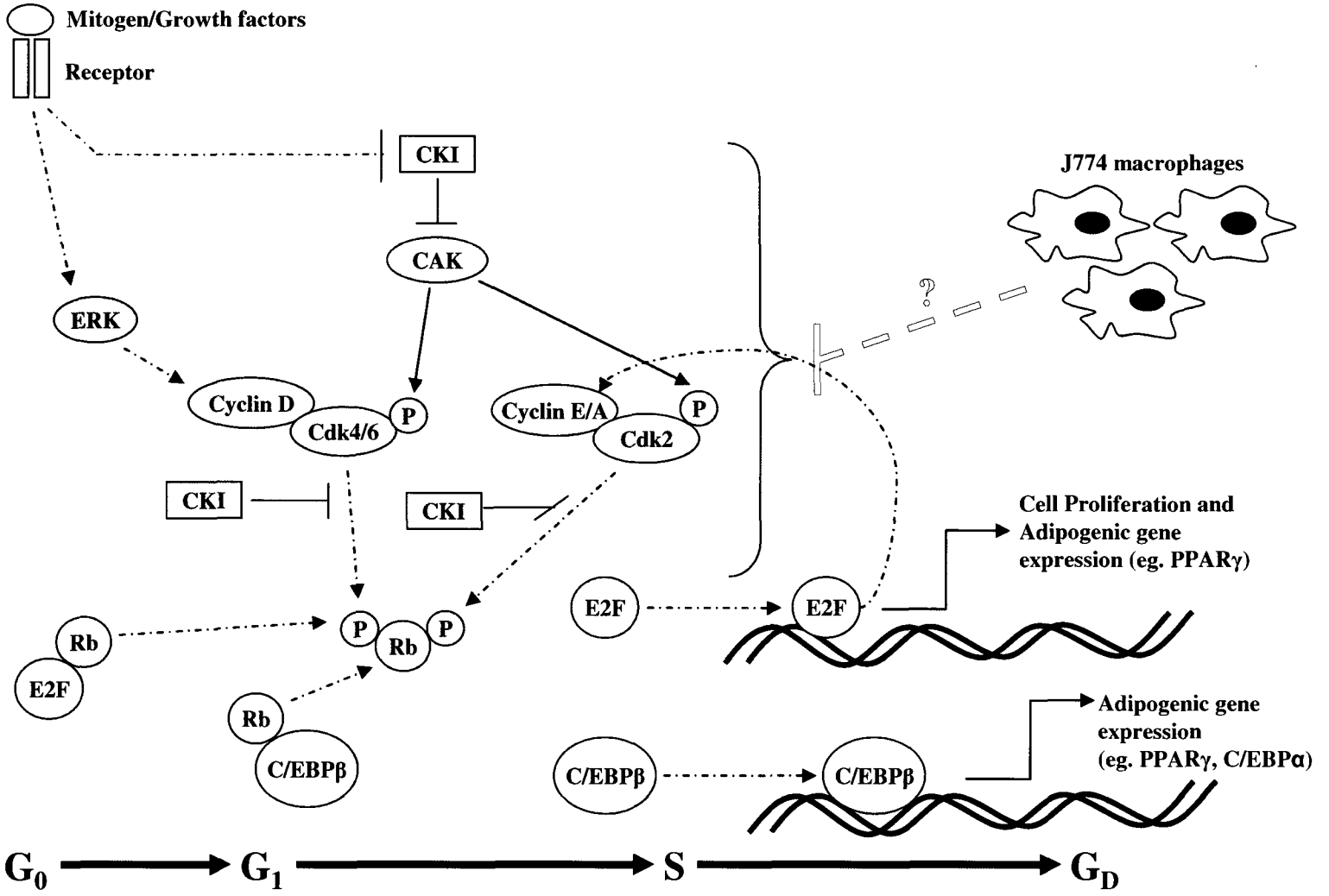
PROPOSED MODELS

The results from my *in vitro* studies suggest that both J774-MacCM and THP-1-MacCM act at an early stage of differentiation in order to impair adipogenesis. In 3T3-L1 preadipocytes, J774 macrophage secreted-factors impair mitotic clonal expansion and Rb phosphorylation, which is required for cell cycle progression (Figure 21). It is possible that the inhibition of clonal expansion interferes with access of transcription factors to DNA regulatory elements, thereby preventing the expression of adipogenic genes associated with the phenotype of mature adipocytes. The presence of J774-MacCM may interfere with upstream regulators of Rb phosphorylation, such as cyclinD/cdk4/6, cyclinE/cdk2 and cyclinA/cdk2, as well as their ckis. Impaired Rb phosphorylation in the presence of J774-MacCM could block the mitotic clonal expansion phase by inhibiting the activation of cell cycle regulators, such as E2F, and their downstream targets. In addition, hypophosphorylated Rb could negatively regulate adipogenesis through its interaction with early adipogenic transcription factors, such as the C/EBPs. This would potentially reduce the number of differentiated adipocytes and lead to compensatory adipocyte hypertrophy.

Human preadipocytes appear to differentiate without proceeding through mitotic clonal expansion in culture. MacCM may therefore be targeting other signaling pathways in order to inhibit human adipogenesis. It is also possible that MacCM is targeting the same signaling pathways as in 3T3-L1 preadipocytes but that these pathways are directed to different cellular responses in human preadipocytes. I have shown that THP-1-MacCM activates the IKKβ/NF-κB pathway (Figure 22). Furthermore, I have established that IKKβ plays a role in the anti-adipogenic effect of THP-1-MacCM on human preadipocytes. However, this pro-inflammatory signaling response in human preadipocytes did not appear to be associated with PDGFR signaling. It is possible that THP-1-MacCM acts via a cytokine

Figure 21. Proposed model for the anti-adipogenic effect of J774-MacCM on 3T3-L1 preadipocyte differentiation.

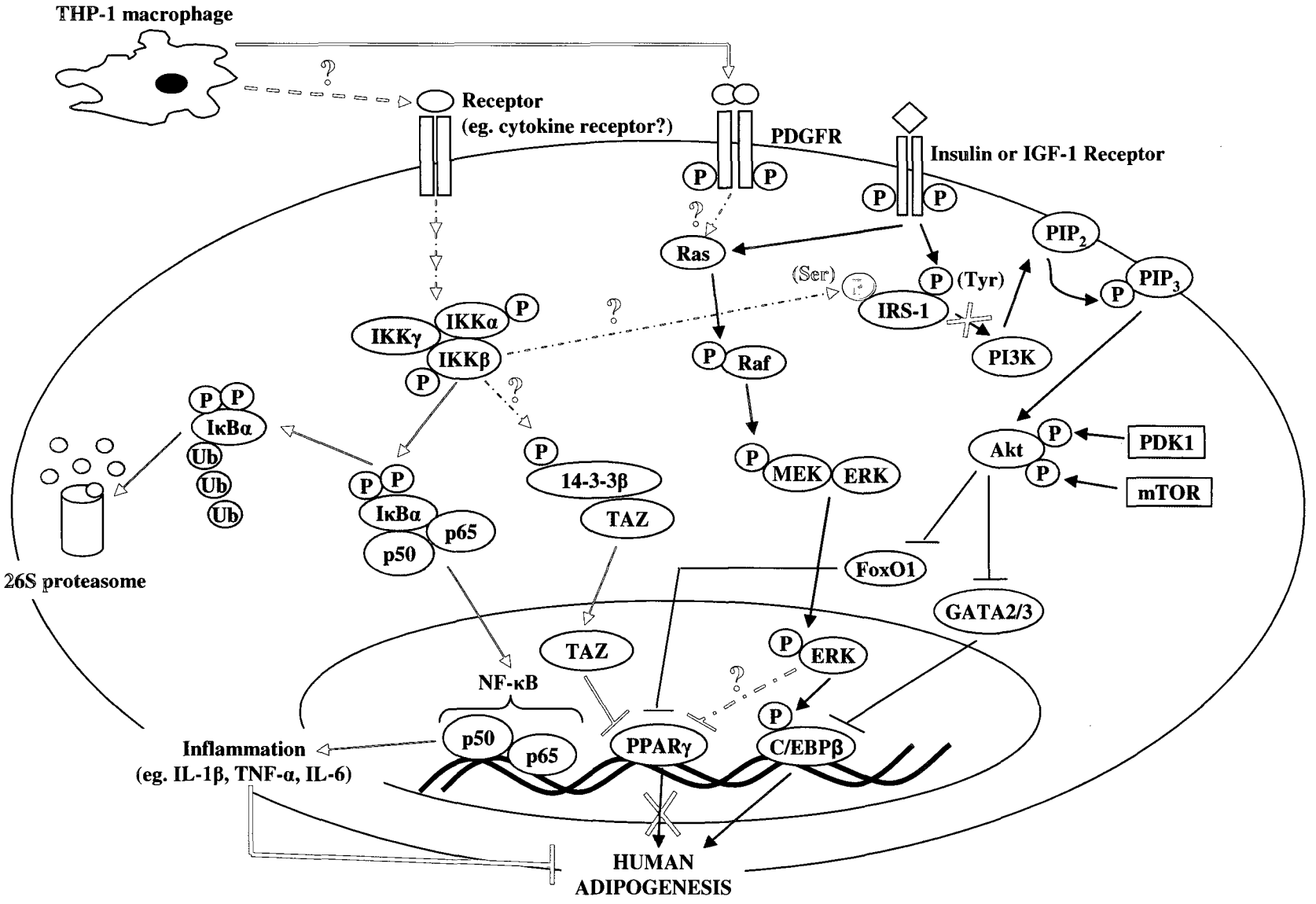
J774-MacCM impairs 3T3-L1 adipogenesis by acting on proximal events in the adipogenic program. Mitotic clonal expansion, an early required event in 3T3-L1 preadipocyte differentiation, is induced by exposure to mitogens/growth factors present in the adipogenic medium. G_0, G_1 and S indicate phases of the cell cycle, while G_D corresponds to the growth-arrested state of terminally differentiated cells. The expression of cyclin D has been linked to signaling by the ERK subfamily of MAP kinases. Mitogenic signaling negatively regulates ckis, which impair cell cycle progression. These ckis can interfere with CAK-mediated activation of cdks or can inhibit the catalytic activity of the assembled, CAK modified cyclin-cdk complexes. The subsequent phosphorylation of Rb by cyclin-cdk complexes leads to the release of E2F and C/EBPβ, which then activate the transcription of genes required for cell cycle progression and terminal differentiation. Furthermore, cyclin E has been shown to be an E2F target gene and can thus reinforce its own expression through a positive feedback loop. J774-MacCM inhibited clonal expansion and impaired Rb phosphorylation, which is required for cell cycle progression. It is possible that the presence of J774-MacCM interferes with upstream regulators of Rb phosphorylation, such as cyclinD/cdk4/6 and cyclinE/A/cdk2, as well as their ckis. J774-MacCM did not appear to interfere with the regulation and activity of C/EBPβ. However, impaired Rb phosphorylation, induced by J774-MacCM, may inhibit the activation of cell cycle regulators, such as E2F, and their downstream targets. See text for further details and abbreviations.



3T3-L1 Mitotic Clonal Expansion

Figure 22. Proposed model for the anti-adipogenic effect of THP-1-MacCM on human preadipocyte differentiation.

THP-1-MacCM acts on early events of differentiation in order to impair human adipogenesis. Pro-adipogenic pathways, which are activated in response to insulin or IGF-1, include the ERK1/2 and PI3K/Akt pathways. Activation of the ERK1/2 pathway results in the phosphorylation, DNA-binding function and activation of C/EBPβ-transcriptional activity. Activation of the PI3K/Akt pathway impairs the nuclear translocation of the anti-adipogenic transcription factors FoxO1 and GATA2/3, thereby preventing the negative regulation of PPARγ and C/EBPβ activity respectively. THP-1-MacCM activates the IKKβ/NF-κB pathway independent of PDGFR signaling. It is possible that THP-1-MacCM acts via a cytokine receptor in order to activate the IKKβ/NF-κB pathway. The enhanced expression of inflammatory cytokines that results from activation of IKKβ/NF-κB pathway could inhibit adipogenesis. IKKβ plays a role in the anti-adipogenic effect of THP-1-MacCM on human preadipocytes. It is possible that IKKβ phosphorylates and inhibits the 14-3-3β protein, which binds and sequesters the anti-adipogenic protein TAZ in the cytoplasm. THP-1-MacCM-induced activation of IKKβ could lead to the release and translocation of TAZ to the nucleus, where it can suppress PPARγ-mediated adipogenic gene expression. THP-1-MacCM-mediated IKKβ activation could also inhibit insulin signaling through the serine phosphorylation of IRS-1. Activation of the anti-adipogenic PDGF pathway by THP-1-MacCM could lead to the activation of the ERK1/2 pathway. It is possible that PDGFR signaling may lead to the prolonged activation of ERK1/2, which could then phosphorylate and negatively regulate PPARγ transcriptional activity. See text for further details and abbreviations.


THP-1 macrophage
Receptor
(eg. cytokine receptor?)
PDGFR
Insulin or IGF-1 Receptor
Ras
IKKα
IKKγ
IKKβ
IRS-1
(Ser)
(Tyr)
PI3K
PIP2
PIP3
Raf
MEK
ERK
Akt
PDK1
mTOR
IκBα
Ub
26S proteasome
p65
p50
14-3-3β
TAZ
FoxO1
GATA2/3
NF-κB
Inflammation
(eg. IL-1β, TNF-α, IL-6)
PPARγ
C/EBPβ
HUMAN
ADIPOGENESIS

receptor in order to activate the IKKβ/NF-κB pathway. Once activated, IKKβ could phosphorylate and impair the function of transcription factors involved in adipose tissue function, such as 14-3-3β, and/or insulin signaling, such as IRS-1. Further studies will be required to determine the precise upstream regulators and downstream targets of IKKβ, which mediate the anti-adipogenic effect of THP-1-MacCM. It is possible that other signaling pathways may play a role in the inhibition of human preadipocyte differentiation by THP-1-MacCM. The THP-1-MacCM-mediated activation of PDGFR could be linked to the enhanced activation of the ERK1/2 pathway, which occured in the presence of THP-1-MacCM. Additional research will therefore be essential to determine if one or more of these signaling pathways are involved in the inhibitory effect of THP-1-MacCM on human preadipocyte differentiation.

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CURRICULUM VITAE

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Competencies:

- Language: Fluently bilingual (both oral and written) in English and French
- Computer Skills: Microsoft Word, PowerPoint, Excel, EndNote X1, GraphPad InStat Version 3.05, AlphaEaseFC Version 4.0 and Outlook

Education:

Master of Science, Biochemistry September 2007-Present
- University of Ottawa, Ontario
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Honours, Baccalaureate in Biochemistry January 2003-April 2007
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Academic Achievements:

Master of Science, Biochemistry
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Abstracts/Publications:

Manuscript in Preparation

Yarmo MN, Gagnon A, Sorisky A. 2009. The anti-adipogenic effect of macrophage-conditioned medium requires the IKKβ/NF-κB pathway.

Peer Reviewed Publications

Yarmo MN, Landry A, Molgat AS, Gagnon A, Sorisky A. 2009. Macrophage-conditioned medium inhibits differentiation-induced Rb phosphorylation in 3T3-L1 preadipocytes. *Exp Cell Res.* 315:411-418.

Constant VA, Gagnon A, Yarmo M, Sorisky A. 2008. The anti-adipogenic effect of macrophage-conditioned medium depends on ERK1/2 activation. *Metabolism.* 57:465-472.

Abstracts

Yarmo MN, Molgat ASD, Landry A, Gagnon A, Sorisky A. June 2008. The inhibition of 3T3-L1 preadipocyte differentiation by macrophage-conditioned medium. 90th Annual Meeting – The Endocrine Society, San Francisco, California.

Constant VA, Gagnon A, Yarmo M, Sorisky A. June 2007. Role for the ERK1/2 pathway in the inhibitory effect of THP-1-Macrophage conditioned medium on 3T3-L1 adipogenesis. 89th Annual Meeting –The Endocrine Society, Toronto, Canada.

Work Experience:

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Summer Research Studentship May 2006-August 2006
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Biochemistry Laboratory Demonstrator (French) May 2006
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Tutoring - Grade 12 Biology and Chemistry September 2005-June 2006
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Canadian Blood Services - Summer Student May 2005-August 2005
- Epidemiology and Surveillance Department
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Casual File Clerk - Summer Student August 2001
- Ottawa-Carleton District School Board, Ottawa, Ontario

Community Involvement:

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- October 2007-April 2008
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- August 2006-March 2007
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Health Promotion Peer Educator Volunteer
- September 2005-April 2006; September 2006-April 2007
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CHEO Playroom Monitor Volunteer
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Patient Services – Neurology Department Volunteer
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