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The antiadipogenic effect of macrophage-conditioned medium depends on ERK1/2 activation

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Abstract

The proatherogenic state of obesity is associated with hypertrophied adipocytes that may arise because of deficient adipogenesis. Macrophages infiltrate adipose tissue as a function of obesity and may release factors that attenuate adipogenesis. Macrophage-conditioned medium inhibits human and 3T3-L1 adipocyte differentiation in culture, but underlying molecular mechanisms have yet to be defined. Exposure of 3T3-L1 cells throughout the 8-day period of differentiation to medium conditioned by THP-1 macrophages (THP-1-MacCM) blocked adipogenesis. Triacylglycerol (TG) accumulation and induction of peroxisome proliferator–activated receptor γ and fatty acid synthase protein levels were inhibited by 59% (n = 4, $P < .001$), 29% (n = 4, $P < .01$), and 47% (n = 4, $P < .01$), respectively. THP-1-MacCM had no effect when added after the first 2 days of differentiation, indicating that early exposure of its targets must be needed to inhibit 3T3-L1 adipogenesis. Cell enumeration revealed a 44% decrease in clonal expansion compared with standard differentiation (n = 3, $P < .01$). Addition of THP-1-MacCM to 3T3-L1 preadipocytes increased ERK1/2 phosphorylation by 6.5-fold (n = 3, $P < .01$). PD98059 (an inhibitor of the ERK1/2 pathway) impaired the negative effect of THP-1-MacCM on TG accumulation, indicated by an inhibition of 25% vs 69% (n = 3, $P < .001$), without altering fatty acid synthase or peroxisome proliferator–activated receptor γ levels. Our data implicate ERK1/2 as an important signaling mediator for the inhibitory effect of THP-1-MacCM on TG accumulation during 3T3-L1 adipogenesis.

1. Introduction

Obesity is now considered to be a proinflammatory, proatherogenic state [1]. Macrophages reside within adipose tissue as a function of the degree of obesity [2,3]. They are hypothesized to contribute to adipose tissue dysfunction, but little is known about the nature of interactions between macrophages and adipose cells.

We have recently reported that conditioned medium from 2 experimental macrophage models (MacCM), murine J774 and human THP-1 cells, strongly inhibit both murine 3T3-L1 and human preadipocyte differentiation [4]. Similar findings were obtained by another group using medium conditioned by human blood monocyte-derived macrophages or by adipose tissue–derived macrophages [5]. This antiadipogenic effect may have pathophysiological relevance. Obesity-associated inflammation and insulin resistance may arise because of a relative deficiency in adipogenesis when accompanied by compensatory adipocyte hypertrophy, in the context of a chronically positive energy balance [6,7]. Learning more about how macrophage-secreted factors alter the adipogenic process may lead to new approaches to modulate adipocyte differentiation and thereby assuage the proinflammatory profile of adipose tissue.

The proximal preadipocyte intracellular signaling molecules that are activated by MacCM and that may participate in the inhibition of differentiation have not been defined. To start to address this objective, we have continued to use MacCM because it is a robust model that reflects the in vivo exposure of preadipocytes. Furthermore, although isolated factors produced by the macrophage may have specific effects, the integrated signaling responses of preadipocytes to the entire output of factors in MacCM will be more informative to identify relevant antiadipogenic pathways. We report here that the early mitotic clonal expansion phase of 3T3-L1 adipogenesis is targeted by THP-1-macrophage–

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conditioned medium (THP-1-MacCM). Furthermore, THP-1-MacCM leads to phosphorylation of ERK1/2. When PD98059, a pharmacological inhibitor of the ERK1/2 pathway, was added to THP-1-MacCM, the usual inhibition of adipose cell lipid accumulation was substantially reversed.

2. Materials and methods

2.1. Culture of human THP-1 monocytes and preparation of conditioned medium

THP-1 monocytes (ATCC, Manassas, VA) were resuspended at 1×10^6 cells per milliliter in Roswell Park Memorial Institute 1640 medium with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mmol/L sodium pyruvate, supplemented with 10% fetal bovine serum, 0.05 mmol/L β-mercaptoethanol, and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). The THP-1-MacCM and THP-1 monocyte–conditioned medium (THP-1-MonCM) were generated as follows. The monocytic cells were either differentiated into macrophages with 100 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate or maintained as monocytes with 0.01% dimethylsulfoxide (DMSO) as vehicle for 24 hours. The medium was then replaced with fresh growth medium (no 12-*O*-tetradecanoylphorbol-13-acetate present); and after 24 hours, medium was collected and centrifuged (200*g*, 5 minutes). Growth medium not exposed to cultured cells (control medium) was also prepared as above to ensure that processing (centrifugation and freezing) was not responsible for any observed effect on adipogenesis. The supernatants were stored at −20°C and thawed before use for adipogenesis experiments.

2.2. Culture and differentiation of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes (ATCC), kept at low passage, were grown to confluence in Dulbecco's modified Eagle medium supplemented with 10% calf serum and antibiotics. Confluent 3T3-L1 preadipocytes were induced to differentiate in the presence of THP-1-MacCM or control medium that was added on the indicated days of differentiation. Differentiation was induced by supplementing the medium with 0.25 μmol/L dexamethasone and 0.5 mmol/L isobutylmethylxanthine for the first 2 days and 1 μmol/L insulin for the first 4 days. Nondifferentiating preadipocytes were kept in the corresponding medium without adipogenic inducers. When indicated, 3T3-L1 preadipocytes were pretreated for 15 minutes with 25 μmol/L PD98059 (Calbiochem, San Diego, CA) or vehicle (DMSO) before induction of differentiation, with continued exposure throughout the differentiation protocol. After 6 to 8 days, cultures were photographed with a digital camera (Coolpix 995; Nikon, Mississauga, Ontario, Canada) mounted on a microscope (Eclipse TS-100, Nikon). Cells were washed, and triacylglycerol (TG) was extracted and quantified spectrophotometrically [8]. Cellular remains were solubilized in Laemmli buffer [9], and protein was quantified with the modified Lowry method using bovine serum albumin as a standard (Bio-Rad, Hercules, CA) and processed for immunoblot analysis. For clonal expansion studies, duplicate plates of 3T3-L1 preadipocytes undergoing differentiation were rinsed, trypsinized, and counted on the indicated days using a Neubauer hemacytometer.

2.3. Acute stimulation of 3T3-L1 preadipocytes

Confluent 3T3-L1 preadipocytes were stimulated with control medium, THP-1-MonCM, THP-1-MacCM, or 10 ng/mL platelet-derived growth factor (PDGF) in THP-1 growth medium for 15 minutes. Cells were lysed in Laemmli buffer [9] containing 1 mmol/L sodium orthovanadate, 5 mmol/L ethylene glycol tetraacetic acid (EGTA), 5 mmol/L sodium pyrophosphate, and 50 mmol/L sodium fluoride. Solubilized protein was quantified as described above and processed for immunoblot analysis.

2.4. Immunoblot analysis

Equal amounts of solubilized proteins (10-60 μg, depending on the experiment) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked; and membranes were incubated with antibodies specific for ERK1/2 (1.0 μg/mL; Upstate Biotechnology, Charlottesville, VA), fatty acid synthase (FAS; 1 μg/mL; BD Biosciences, Mississauga, Ontario, Canada), peroxisome proliferator–activated receptor γ (PPARγ; 2 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK1/2 (Thr202/Tyr204; pERK1/2; 1:1000; Cell Signaling Technology, Beverly, MA), signal transducer and activator of transcription 3 (STAT3; 1:1000; Cell Signaling Technology), or phospho-STAT3 (Ser727 pSTAT3; 1:1000; Cell Signaling Technology) followed by incubation with the appropriate horseradish peroxidase–conjugated secondary antibodies. Immunoreactivity was detected by chemiluminescence (GE Healthcare, Baie d'Urfe, Quebec, Canada, or Millipore, Billerica, MA). Relative intensity of the bands was assessed by Molecular Analyst imaging software (version 1.4, Bio-Rad) or AlphaImager imaging system (Alpha Innotec, San Leandro, CA) and expressed as integrated optical density units.

2.5. Statistical analysis

Analysis of variance, followed by the Newman-Keul test, was used to assess differences between means (Instat, version 3.05; GraphPad, San Diego, CA), with P values $< .05$ considered significant.

3. Results

3.1. THP-1-MacCM is required at the onset of differentiation to impair 3T3-L1 adipogenesis

We have previously demonstrated that the continuous presence of THP-1-MacCM during differentiation of 3T3-L1

preadipocytes inhibits lipid accumulation and induction of specific adipocyte markers, without evidence of cytotoxicity [4]. To determine the critical time point required for the addition of THP-1-MacCM to impair adipogenesis, 3T3-L1 preadipocytes were induced to differentiate in the presence of either THP-1-MacCM or control medium at specific times during the 8-day process. The THP-1-MacCM impaired 3T3-L1 differentiation when present from days 0 to 8 as assessed morphologically, with a reduction in lipid droplet accumulation (Fig. 1A). However, when exposure of the differentiating 3T3-L1 preadipocytes to THP-1-MacCM was delayed to days 2, 4, or 6, there was no longer any inhibition on lipid droplet formation.

These results were confirmed quantitatively with a maximal 59% (n = 4, $P < .001$) reduction in TG accumulation when THP-1-MacCM was present from days 0 to 8, but only a 14% (n = 4, $P < .05$) reduction when THP-1-MacCM was added on day 2, and no significant decreases occurred when exposure started on day 4 or 6 (Fig. 1B). The induction of FAS and PPARγ protein expression was reduced by 47% and 29% (n = 4, $P < .01$), respectively, when THP-1-MacCM was present from days 0 to 8 (Fig. 1C, D); however, no significant inhibition of these adipogenic markers was observed when exposure to the conditioned medium occurred on day 2, or later. ERK1/2 serves as a control for loading. Control medium did not affect 3T3-L1 adipocyte differentiation.

3.2. THP-1-MacCM impairs clonal expansion during 3T3-L1 adipocyte differentiation

Upon induction of adipogenesis, 3T3-L1 preadipocytes undergo 1 to 2 rounds of DNA replication and cell doubling referred to as clonal expansion, an event that is necessary for differentiation to proceed [10,11]. When differentiating in the presence of the control medium, 3T3-L1 cells underwent the expected clonal expansion over the first 4 days, from 0.8×10^6 to 2.4×10^6 cells per dish, a 3-fold increase (n = 3, $P < .001$ vs day 0). However, in the presence of THP-1-MacCM, the clonal expansion response was attenuated by 44%, with a rise from 0.8×10^6 to 1.7×10^6 cells per dish (n = 3, $P < .01$; compared with standard differentiation).

3.3. THP-1-MacCM stimulates ERK1/2 phosphorylation in 3T3-L1 preadipocytes

Many intracellular signaling pathways have been implicated in the positive as well as negative regulation of adipogenesis. Given the mitotic clonal expansion results, we measured the acute effect of THP-1-MacCM on the activation of 2 proteins implicated in proliferation, the protein kinase ERK1/2 and the transcription factor STAT3, both of which have been implicated in the clonal expansion phase of 3T3-L1 adipogenesis [12-14]. Stimulation of confluent 3T3-L1 preadipocytes with THP-1-MacCM significantly increased phosphorylation of ERK1/2 by 6.5-fold (n = 3, $P < .01$; Fig. 2A). Medium conditioned by THP-1 monocytes (THP-1-MonCM), which does not impair adipogenesis [4], caused only a minimal increase in ERK1/2 phosphorylation that was not statistically significant. These data suggest that the secreted factor(s) promoting ERK1/2 activation is relatively specific to the macrophage phenotype. The THP-1-MacCM did not promote phosphorylation of another mitogenic target, STAT3, suggesting selectivity for intracellular targets (Fig. 2B). The PDGF-treated samples serve as a positive control for the phosphorylation of both ERK1/2 and STAT3.

Given that THP-1-MacCM stimulated ERK1/2 phosphorylation acutely, we investigated whether the MEK1 inhibitor PD98059 (prevents ERK1/2 phosphorylation) would alter the ability of THP-1-MacCM to inhibit the 3T3-L1 clonal expansion (Fig. 2C). The 37% inhibition of clonal expansion by THP-1-MacCM ($P < .05$ compared with control differentiation conditions) was not significantly altered by PD98059, although there was a nonsignificant trend suggesting a more potent inhibition. PD98059 on its own also reduced clonal expansion.

3.4. PD98059 alleviates the inhibitory effect of THP-1-MacCM on TG accumulation in differentiating 3T3-L1 preadipocytes

To investigate the role of the ERK1/2 pathway in the antiadipogenic effect of THP-1-MacCM, 3T3-L1 preadipocytes were pretreated with PD98059 and then induced to differentiate with THP-1-MacCM or control medium with or without 25 μmol/L PD98059. Addition of PD98059 under standard conditions of differentiation mildly enhanced the response, as assessed by 16% more TG accumulation (n = 3, $P < .01$; Fig. 3B) and 35% higher FAS protein expression (n = 5, $P < .05$; Fig. 3C). These results are consistent with previous reports on the ability of PD98059 to potentiate 3T3-L1 adipogenesis [15,16]. The suppression of lipid accumulation caused by THP-1-MacCM during adipogenesis (69% inhibition of TG accumulation) was substantially weakened by the addition of PD98059 during adipogenesis (25% inhibition of TG accumulation; Fig. 3A, B). In contrast, PD98059 did not alter the inhibitory effect of THP-1-MacCM on the protein expression of FAS and had only a minor effect on the inhibition of PPARγ expression (Fig. 3C, D).

4. Discussion

Our data reveal that the critical time point for the inhibition of 3T3-L1 adipogenesis by THP-1-MacCM is within the first 2 days of the 8-day differentiation period, the period associated with the mitotic clonal expansion phase. Cell proliferation during this event was reduced by THP-1-MacCM. Acute phosphorylation of the mitogenic kinase ERK1/2 was stimulated by THP-1-MacCM and was required for the full antiadipogenic effect.

The seminal observation that macrophages infiltrate and reside within adipose tissue in obese animal models and

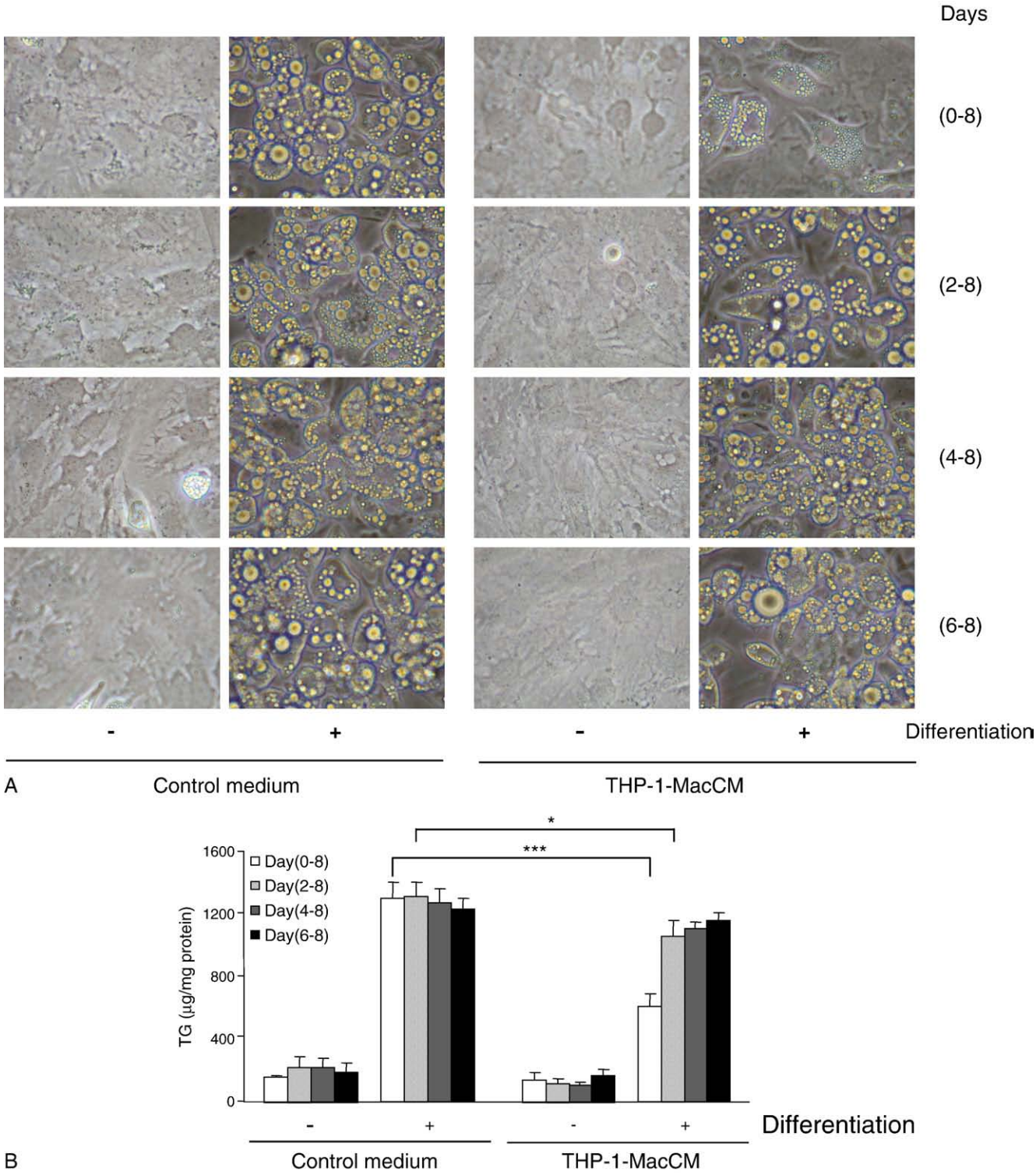

Fig. 1. Medium conditioned by THP-1 macrophages is required at the onset of differentiation to inhibit adipogenesis. 3T3-L1 preadipocytes were differentiated or kept under nonadipogenic conditions in the presence of control medium or THP-1-MacCM on indicated days for up to 8 days. A, Cultures were photographed at 400× magnification. Pictures representative of 4 independent experiments are shown. B, Triacylglycerol was extracted, quantified, and normalized to protein content. Results are expressed as the mean ± SEM of 4 independent experiments. *** $P < .001$ or * $P < .05$ compared with differentiation in control medium. C and D, Solubilized protein from differentiated cultures were immunoblotted with antibodies against FAS, PPARγ, or ERK1/2 (loading control). Immunoblots shown are representative of 4 independent experiments. Densitometric data for the differentiated samples from the 4 experiments are expressed as mean ± SEM. ** $P < .01$ compared with differentiation in control medium.

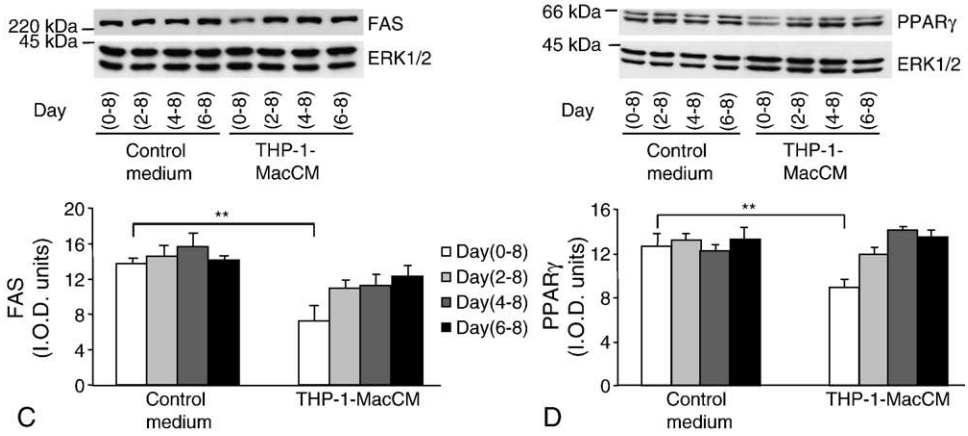

Fig. 1 (*continued*).

humans suggests novel routes toward adipose tissue inflammation. These professional inflammatory cells secrete a wide variety of cytokines that might be released into the circulation or that might act in a paracrine fashion on neighboring adipose cells.

We have explored the hypothesis that secreted macrophage factors within adipose tissue can inhibit adipogenesis. An acquired deficit in adipogenesis has been postulated to be an important event because it favors the development of hypertrophied adipocytes that are inflamed and insulin-

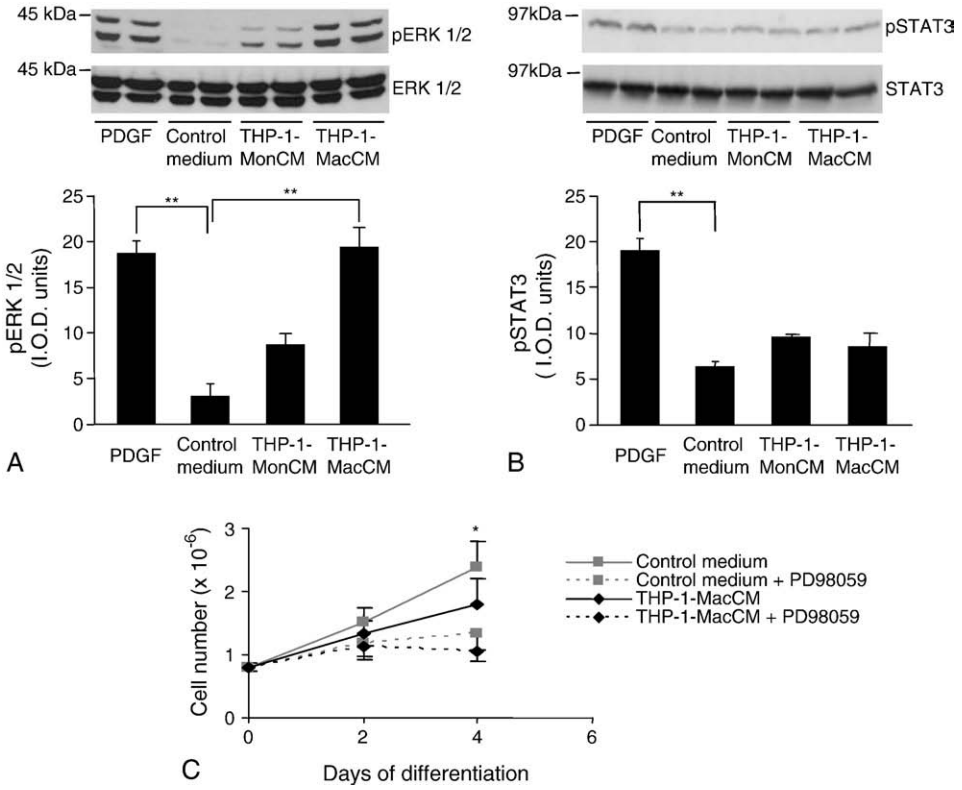

Fig. 2. Medium conditioned by THP-1 macrophages stimulates ERK1/2 phosphorylation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were stimulated for 15 minutes with control medium, THP-1-MonCM, THP-1-MacCM, or 10 ng/mL PDGF (positive control). A and B, Solubilized protein was immunoblotted with antibodies directed against phospho-ERK1/2 (pERK1/2), ERK1/2, phospho-STAT3 (pSTAT3), or STAT3. Immunoblots shown are representative of 3 independent experiments. Densitometric data are expressed as mean ± SEM of the 3 independent experiments, each performed in duplicate. ** $P < .01$ compared with stimulation with control medium. C, 3T3-L1 preadipocytes were pretreated for 15 minutes with 25 μmol/L PD98059 and then induced to differentiate or not in control or THP-1-MacCM supplemented with 25 μmol/ PD98059 or DMSO (vehicle). On indicated days, cells were trypsinized and enumerated. Results are expressed as number of cells per dish and represent the mean ± SEM of the 3 independent experiments, each performed in duplicate. * $P < .05$ compared with other conditions on day 4.

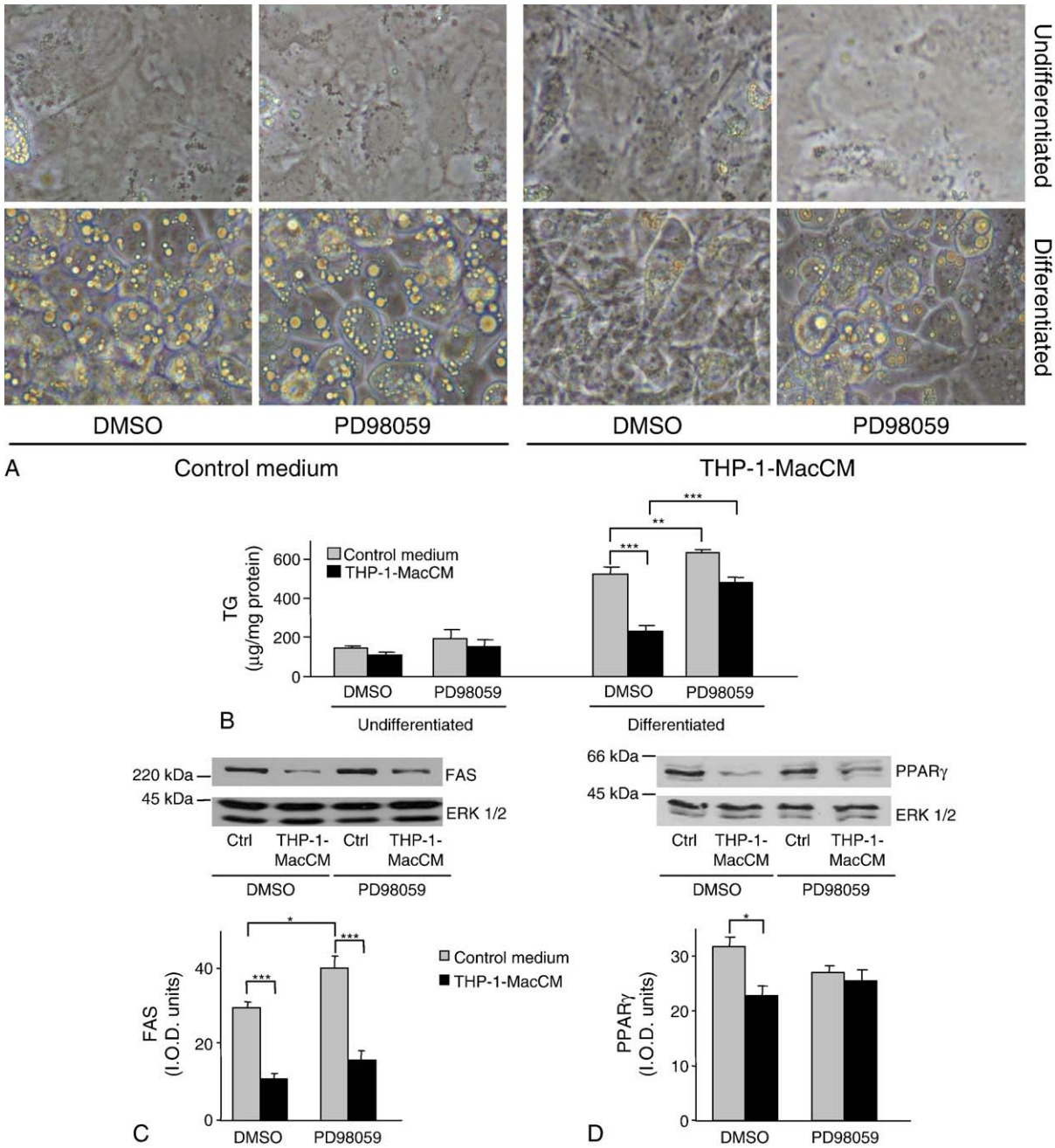

Fig. 3. The inhibitory effect of THP-1-MacCM on lipid accumulation during adipogenesis is reversed by PD98059. 3T3-L1 preadipocytes were pretreated for 15 minutes with PD98059 (25μmol/) or vehicle (DMSO) and then induced to differentiate in the presence of control medium or THP-1-MacCM with PD98059 (25μmol/) or vehicle for 6 days. A, Cultures were photographed at 400× magnification. Pictures representative of 5 independent experiments are shown. B, Triacylglycerol was extracted, quantified, and normalized to protein content. Results are expressed as the mean ± SEM of 3 independent experiments. *** $P <$ .001 or ** $P <$ .01 between indicated pairs. C and D, Solubilized protein from differentiated cultures were immunoblotted with antibodies directed against FAS, PPARγ, or ERK1/2 (loading control). Immunoblots shown are representative of 5 independent experiments. Densitometric data are expressed as mean ± SEM of 5 independent experiments. *** $P <$ .001, ** $P <$ .01, or * $P <$ .05 between indicated pairs. Ctrl indicates control.

resistant [6,7]. Our previous work documented that MacCM from 2 macrophage models, murine J774 and human THP-1 cells, inhibits murine 3T3-L1 and human adipocyte differentiation in culture [4]. A subsequent report by another group demonstrated that conditioned medium of human macrophages derived from blood monocytes or isolated from adipose tissue also produced the antiadipogenic effect [5].

Our new data indicate that the initial 2-day phase of differentiation is the critical period during which preadipocytes are susceptible to the inhibitory effect of THP-1-MacCM. Because this is when the mitotic clonal expansion occurs during 3T3-L1 adipogenesis, we evaluated whether THP-1-MacCM interferes with this early obligatory step of differentiation. The negative effect we observed on cell

proliferation during this period suggests it may be the primary event perturbed by THP-1-MacCM and might explain why exposure to THP-1-MacCM beyond day 2 of differentiation is without significant effect. The initial 2-day period is also critical for the ability of several cytokines to inhibit the adipocyte differentiation response of BMS2 cells, a bone marrow–derived stromal cell line [17].

The antiadipogenic effects observed led us to examine ERK1/2 phosphorylation. The role of ERK1/2 in the regulation of adipogenesis is complex. There appears to be a requirement for an early but finite duration of ERK1/2 activity during the mitotic clonal expansion, within the initial 12 hours after induction of differentiation, before significant up-regulation of PPARγ [12,13]. Targets of ERK1/2 during this time frame, such as C/EBPβ, are thought to be important to induce full expression of C/EBPα and PPARγ [18,19]. Genetic deletion of the ERK1 isoform generates a lean phenotype in the mouse, also supporting an essential role for at least this isoform [20]. However, if ERK1/2 activation is inappropriately prolonged, for example, by overexpression of ERK1/2 or MEK or by stretch activation, adipogenesis is inhibited [15,21,22]. In this scenario, ERK1/2 phosphorylation of PPARγ is thought to negatively regulate its transcriptional activity [21,23,24].

Under our conditions, the increase in ERK1/2 phosphorylation induced by THP-1-MacCM appears to inhibit the fully differentiated state because blockade by PD98059 significantly weakened the inhibitory effect of MacCM on TG accumulation. The reversal of the inhibition of TG accumulation by PD98059 was not accompanied by any clear changes in FAS or PPARγ protein expression. There was also no effect of PD98059 on the THP-1-MacCM–dependent inhibition of clonal expansion. The underlying basis for these observations is not known at present, but it appears that ERK1/2 inhibition of the THP-1-MacCM effect on adipogenesis acts primarily on the regulation of lipogenesis or lipolysis. Interestingly, activation of ERK1/2 is essential for maximal induction of lipolysis by tumor necrosis factor α and β3-adrenergic receptors [25,26]. Indeed, another study suggests that MacCM increases basal lipolytic rates [27]. Future studies will be needed to define the precise mechanism that permits a return of TG levels when PD98059 is added to MacCM.

The reduced proliferation we observed contrasts with the previously described increased proliferation of human preadipocytes induced to differentiate in the presence of lipopolysaccharide (LPS)-activated monocyte-derived macrophage-conditioned medium [5]. However, the presence of LPS remaining in the conditioned medium could have independently influenced proliferation in this model. Furthermore, human preadipocytes do not proceed through a clonal expansion phase upon induction of adipogenesis in culture; they are believed to have already proceeded through this phase in vivo [28]. Nevertheless, conditioned medium, derived from several different macrophage models, inhibits human adipogenesis [4,5], suggesting that the effect of MacCM may not be solely directed to the clonal expansion phase.

Much remains unknown about the role of macrophages residing within adipose tissue in obesity. Examining signaling responses of human preadipocytes to human macrophages will avoid species differences related to secreted factors and receptor/kinase activation. The effects of individual cytokines known to be secreted by macrophages on adipocyte differentiation have been reported to be either anti- or proadipogenic [29-33]. A strength of our study is that MacCM represents a more integrated approach to gauge the overall impact of macrophage-derived products on preadipocyte signaling networks. Nevertheless, as more is learned about the preadipocyte response profile, it may become advantageous to examine macrophage candidate factors (or combinations thereof) to assess their contribution. For example, the THP-1 cell line is known to secrete interleukin 1β, tumor necrosis factor α, and monocyte chemoattractant protein 1 [34]. Tumor necrosis factor α and interleukin 1β are known to inhibit adipogenesis [35], whereas data supporting an antiadipogenic effect for monocyte chemoattractant protein 1 are inconsistent [36,37].

Our results provide new information on the nature of interactions between macrophages and preadipocytes and describe one of the signaling pathways by which adipogenesis may be impaired. Our results also suggest that adipose tissue function may be altered by 2 distinct effects of MacCM: impairment of clonal expansion, reducing the number of adipocytes, as well as decreased lipid storage of differentiated adipocytes. This dual attenuation of adipogenic capacity may be a critical determinant of the proatherogenic and insulin-resistant state of obesity.

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