The role of interleukin 1 β in the anti-adipogenic action of macrophages on human preadipocytes

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Abstract

When adipose tissue accumulates in obesity, the ability of preadipocytes to differentiate permits a hyperplastic expansion of functional adipocytes that preserves insulin sensitivity. Adipose infiltration by macrophages is associated with an adipogenic deficit and the appearance of inflamed, insulin-resistant hypertrophied adipocytes. Interleukin 1 β (IL1 β) has been reported to account for the anti-adipogenic action of macrophages in a mouse model. Using the THP-1 human macrophage cell line and human primary preadipocytes, our objective was to determine whether IL1 β was necessary for the ability of conditioned medium from THP-1 macrophages (THP-1-MacCM) to: i) stimulate human preadipocyte inhibitor of κ B kinase β (IKK β) and ii) inhibit human adipocyte differentiation. IL1 β is present in THP-1-MacCM, and THP-1-MacCM or IL1 β (500 pg/ml; its concentration in THP-1-MacCM) acutely stimulated IKK β phosphorylation and inhibitor of κ B (I κ B) degradation in preadipocytes. IL1 β was sufficient to inhibit adipogenesis on its own, and this was blocked by SC-514, an IKK β inhibitor, as has been reported for THP-1-MacCM. I κ B degradation by IL1 β -immunodepleted THP-1-MacCM was attenuated, whereas IKK β phosphorylation and the inhibition of adipocyte differentiation were unchanged. Therefore, in contrast to what has been suggested for mouse cell models, IL1 β is not required for the ability of MacCM to inhibit adipogenesis in human cell models.

Key Words

- preadipocyte
- differentiation
- macrophage
- interleukin 1 β
- cytokine
- inflammation

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Introduction

Obesity-associated insulin resistance is related to adipose tissue inflammation, characterized by macrophage infiltration and altered adipose tissue remodeling, including adipocyte hypertrophy (Weisberg *et al.* 2003, Xu *et al.* 2003, Sun *et al.* 2011). Hypertrophied adipocytes display upregulated inflammatory gene expression and decreased responsiveness to insulin, leading to inappropriate lipolysis and the deleterious deposition of fatty acids in the liver and muscle (Danforth 2000, Heilbronn *et al.* 2004, Arner *et al.* 2010, 2011). A number of studies have

demonstrated that macrophages can release factors that impair adipogenesis (Constant *et al.* 2006, 2008, Lacasa *et al.* 2007, Stienstra *et al.* 2008, Keophiphath *et al.* 2009, Yarmo *et al.* 2009, 2010, Lu *et al.* 2010, Ide *et al.* 2011, Gagnon *et al.* 2012), thus predisposing to adipocyte hypertrophy and its consequences.

Determining the precise identity of anti-adipogenic macrophage factors has been challenging. One candidate is interleukin 1 β (IL1 β), a member of the IL1 family of cytokines. It is synthesized in an inactive form, and

becomes active as an inflammatory cytokine when processed by caspase 1 within the inflammasome, as recently reviewed (Tack *et al.* 2012). IL1 β expression is increased in obese mouse models, and it is associated with metabolic inflammation. It is sufficient to induce insulin resistance in adipocytes, interference with its action improves insulin sensitivity, and its genetic deletion results in a more favorable metabolic phenotype. Within adipose tissue, IL1 β is produced by macrophages and, to a lesser extent, by adipose cells (Tack *et al.* 2012).

IL1 β binds to the IL1 type 1 receptor to activate pro-inflammatory intracellular signaling molecules, such as inhibitor of κ B kinase β (IKK β), that can block the adipocyte differentiation program (Gregoire *et al.* 1998, Tanti & Jager 2009). IKK β is also activated in human preadipocytes by medium conditioned by human THP-1 macrophages (THP-1-MacCM), and this medium also suppresses the adipogenic response (Yarmo *et al.* 2010). Furthermore, IL1 β was recently proposed to be the factor underlying the anti-adipogenic action of MacCM in a mouse model (Lu *et al.* 2010). Therefore, our objective was to determine whether IL1 β was responsible for the ability of human THP-1-MacCM to: i) stimulate human preadipocyte IKK β and ii) inhibit human adipocyte differentiation.

Materials and methods

Culture of THP-1 macrophages and preparation of THP-1-MacCM

THP-1 monocytes (ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium (Life Technologies) containing 2 mM L-glutamine, 4.5 g/l HEPES, 1 mM sodium pyruvate, 0.05 mM β -mercaptoethanol, and supplemented with 10% fetal bovine serum (FBS; Life Technologies) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; Life Technologies), designated THP-1 growth medium. Conditioned medium was prepared as described previously (Constant *et al.* 2006). Briefly, cells were resuspended at a density of 1×10^6 cells/ml in THP-1 growth medium and differentiated into macrophages with the addition of 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 24 h. The medium was replaced with serum-free THP-1 growth medium (without PMA), and conditioned by THP-1 macrophages for 24 h. The conditioned medium (THP-1-MacCM) was collected and centrifuged at 150 g for 5 min. The supernatant was collected and either used directly for preadipocyte studies or frozen in aliquots at -20°C for future use.

The anti-adipogenic effect of THP-1-MacCM was the same in either case. This standardized approach yielded THP-1-MacCM with stable properties from batch to batch. Individual batches of THP-1-MacCM were used for each preadipocyte experiment. The medium never exposed to THP-1 cells (control medium) was similarly processed to control for medium manipulations. The heat treatment of the control medium and THP-1-MacCM consisted of boiling for 15 min, followed by centrifugation (12 000 g, 5 min) of the cooled medium. The supernatants were collected and frozen for future use.

The control medium and THP-1-MacCM were fractionated by filtration through Amicon 3 or 50 kDa molecular-weight cut-off filters (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The filtrates and retentates were collected (<3 , ≥ 3 or <50 kDa, ≥ 50 kDa). Unfractionated media and pooled fractions were also prepared and used as controls.

For IL1 β immunodepletion experiments, the control medium and THP-1-MacCM were incubated for 1 h at room temperature in the presence or absence of 10 $\mu\text{g/ml}$ mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or human IL1 β antibody (R&D Systems, Minneapolis, MN, USA). After incubation for 30 min at room temperature with protein G-agarose, antibody-antigen complexes were removed from the medium by centrifugation. The supernatants were used in adipogenesis and cell stimulation studies.

Isolation and culture of human abdominal subcutaneous preadipocytes

Abdominal subcutaneous adipose tissue samples were obtained from 21 weight-stable patients (14 females and seven males) undergoing elective abdominal surgery (approved by the Ottawa Hospital Research Ethics Board), aged 53 ± 10 years (mean $\pm$ s.d.), with a BMI of $30 \pm 9 \text{ kg/m}^2$ (mean $\pm$ s.d.; range 22–45), and were processed as described previously (Constant *et al.* 2006). There was no detectable effect of BMI, gender, or age on the anti-adipogenic effect of THP-1-MacCM on preadipocytes. The types (and numbers) of surgery were: abdominal liposuction (10) for cosmetic reasons; repair of incisional hernia (2); gastric bypass (1) for weight management; hysterectomy (3) for endometrial hyperplasia, fibroids, and ovarian cancer; abdomenoplasty (1) for cosmetic reasons; pelvic pouch repair (1) originally constructed for previous ulcerative colitis; ileostomy closure (1) post-resolution of previous diverticulitis; rectosigmoid resection (1) for colon cancer; and partial liver resection (1) for benign

hepatic cyst. Medications used include: statins (2); proton pump inhibitors (3); escitalopram (2); L-thyroxine replacement (1); estrogen/progesterone replacement (1); allopurinol (1); enalapril (1); hydrochlorothiazide (1); carbamazepine (1); olanzapine (1); and lithium (1).

Patients on insulin or glucocorticoid steroids were excluded. Briefly, adipose tissue was removed, dissected to remove blood vessels, and digested with collagenase CLS type 1 (600 U/g tissue; Worthington Biochemical Corporation, Lakewood, NJ, USA). Stromal preadipocytes, obtained following progressive size filtration and centrifugation, were grown in DMEM (Life Technologies) supplemented with 10% FBS, antibiotics, and 50 U/ml nystatin (EMD Millipore) (designated preadipocyte growth medium). They were either cryopreserved until use, or directly passaged, up to a maximum of three times. Preadipocytes were seeded at a density of 3×10^4 cells/cm² and grown to confluence prior to adipocyte differentiation or acute stimulation.

Adipocyte differentiation

Preadipocytes were placed in the control medium or THP-1-MacCM (with or without heat treatment, fractionation, or immunodepletion) supplemented with 10% FBS and the following adipogenic inducers: 5 μ g/ml insulin (Roche); 0.5 μ M dexamethasone (Steraloids, Newport, RI, USA); 0.25 mM isobutylmethylxanthine; and 100 μ M indomethacin (both from Sigma), a peroxisome proliferator-activated receptor γ (PPAR γ) agonist at this concentration (Lehmann *et al.* 1997), for 2 weeks. This protocol for human primary preadipocytes was based on that for Poietics Human Subcutaneous Preadipocytes, in which there were no subsequent medium changes during the duration of differentiation (Lonza Group Ltd., Allendale, NJ, USA). Where indicated, 50 μ g/ml SN-50 (#481480; EMD Millipore) or water vehicle was present. For some experiments, preadipocyte growth medium was supplemented with adipogenic inducers and with indicated doses of recombinant human IL1 β (#201-LB-005; R&D Systems) in the presence or absence of 100 μ M SC-514 (#401485; EMD Millipore) or vehicle (0.1% DMSO), or with the indicated doses of recombinant human tumor necrosis factor α (TNF α ; #210-TA-010; R&D Systems). After 2 weeks of differentiation, 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 triacylglycerol (TG) was extracted and quantified spectrophotometrically, as described previously (Constant *et al.* 2006). The remaining

protein was solubilized in Laemmli buffer (Laemmli 1970) and processed for the determination of protein concentration by the modified Lowry method (Bio-Rad), with BSA as the standard, followed by immunoblot analysis.

Acute cell stimulation

Preadipocytes were stimulated for 30 min with or without 500 pg/ml IL1 β in preadipocyte growth medium, or with the control medium or THP-1-MacCM, immunodepleted of IL1 β or not, and supplemented with 10% FBS; all media contained adipogenic inducers. Cellular protein was solubilized in Laemmli buffer (Laemmli 1970) containing 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 5 mM EGTA, and processed for the determination of protein concentration and immunoblot analysis, as described.

Quantification of macrophage-secreted factors

IL1 β and platelet-derived growth factor-BB (PDGF-BB) were measured in the control medium and THP-1-MacCM using ELISA Kits, according to the manufacturer's instructions (R&D Systems). Wnt-5A was assessed by immunoblotting the concentrated control medium and THP-1-MacCM, using an Amicon 3 kDa molecular-weight cut-off filter unit (EMD Millipore).

Immunoblot analysis

Equal amounts of solubilized cellular protein (10–20 μ g depending on the experiment), or concentrated control medium or THP-1-MacCM (for wnt-5A), were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Non-specific binding sites were blocked by 5% non-fat milk, and the membranes were probed with the following antibodies directed against: adipocyte fatty acid-binding protein (aP2; 0.1 μ g/ml; R&D Systems); PPAR γ (1:1000); IKK β (1:1000); inhibitor of κ B (I κ B) α (1:1000); phospho-IKK α / β (Ser176/180) (all from Cell Signalling, Danvers, MA, USA); ERK1/2 (0.25 μ g/ml; EMD Millipore; serves as a loading control); fatty acid synthase (FAS; 1:1000; BD Biosciences, Mississauga, ON, Canada); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000; Abcam, Cambridge, MA, USA; serves as a loading control); or wnt-5A (1 μ g/ml; Santa Cruz Biotechnology). The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies. Signal intensity was visualized by chemiluminescence (EMD Millipore) and quantified using the AlphaImager imaging system

(Alpha Innotec Co., San Leandro, CA, USA). Intensity was expressed as integrated optical density units.

Statistical analysis

One- or two-way ANOVA was used to compare multiple means (Instat, version 3.05; GraphPad, San Diego, CA, USA and Microsoft Excel 2003). Student–Newman–Keuls (one-way ANOVA) or Tukey's (two-way ANOVA) *post hoc* tests were used to determine significance between samples, with $P < 0.05$ considered as significant.

Results

THP-1-MacCM was heat-treated to determine whether this would alter its ability to inhibit the differentiation of primary human preadipocytes into adipocytes. Following the heat treatment, THP-1-MacCM no longer inhibited the accumulation of TG nor the induction of PPAR γ and aP2, indicating that the anti-adipogenic factor(s) is heat-sensitive (Fig. 1A, B and C). The size fractionation of THP-1-MacCM at a 3 kDa molecular-weight cut-off demonstrated no anti-adipogenic activity at all in the < 3 kDa fraction (0% inhibition of TG accumulation, $n = 4$), and complete inhibitory activity remaining in the ≥ 3 kDa fraction ($90 \pm 2\%$ inhibition of TG accumulation, $n = 4$, $P < 0.01$). Further fractionation suggested that there was some anti-adipogenic activity in the < 50 kDa range, based on a consistent trend of lower levels of TG, PPAR γ , and aP2, although this did not reach significance (Fig. 1D, E and F). Together, these data on heat sensitivity and molecular size separation are consistent with the factor being a cytokine.

We investigated whether IL1 β at a concentration of 500 pg/ml could activate IKK β (Fig. 2). This is the IL1 β concentration observed in our THP-1-MacCM (Fig. 5B). This value is very stable across the batches of THP-1-MacCM that we have produced, and the same applies to TNF α levels (Molgat *et al.* 2012). IL1 β strongly stimulated IKK β phosphorylation from undetectable basal levels and also resulted in complete I κ B degradation in human preadipocytes. Both responses were comparable with what is observed with THP-1-MacCM stimulation (Fig. 2).

Having established that IKK β is responsive to IL1 β in the human preadipocyte model, we examined the effect of the IKK β inhibitor SC-514 on the IL1 β -mediated inhibition of adipocyte differentiation (Fig. 3). IL1 β at 500 pg/ml blocked adipocyte differentiation significantly, reducing TG accumulation by 73%, FAS levels by 81%, and aP2 levels by 80%. In the presence of SC-514 (100 μ M), the

inhibition of these adipogenic markers was completely prevented. The effect of IL1 β on PPAR γ levels was a more modest 38% reduction that did not reach significance.

We conducted a dose–response study of IL1 β on human adipocyte differentiation. The inhibition of TG accumulation was not evident at 10 pg/ml, but was noted at 1000 and 10 000 pg/ml (Fig. 4A). A similar effect of IL1 β was seen with the inhibition of the adipogenic markers PPAR γ and aP2 at 1000 and 10 000 pg/ml, but not at 10 pg/ml (Fig. 4B). These results, together with the data in Fig. 3, demonstrate that the concentration of IL1 β in THP-1-MacCM is in the range that is sufficient to inhibit human adipocyte differentiation.

We immunodepleted IL1 β from THP-1-MacCM to determine its importance for THP-1-MacCM signaling and for the anti-adipogenic effect. The concentration of IL1 β in THP-1-MacCM is in the range of 500 pg/ml, and was reduced effectively following immunodepletion (Fig. 5A). With respect to preadipocyte signal transduction, the IL1 β -depleted medium still increased IKK β phosphorylation, but the previously observed degradation of I κ B was attenuated (Fig. 5B). However, despite this alteration in the signaling response, there was no change in the anti-adipogenic action of IL1 β -depleted THP-1-MacCM on human adipocyte differentiation, assessed by the accumulation of TG as well as by the levels of aP2 and PPAR γ (Fig. 5D and E). The observation that the complete anti-adipogenic effect could occur without complete I κ B degradation suggests that NF- κ B activation may not be critical, which is consistent with studies that we performed showing that the NF- κ B inhibitor SN-50 (50 μ g/ml) has no effect on the anti-adipogenic action of THP-1-MacCM ($89 \pm 5\%$ inhibition of TG accumulation without SN-50 vs $91 \pm 3\%$ inhibition with SN-50, $n = 3$, $P = 0.48$).

We investigated whether three other macrophage factors that have been implicated in the blockade of adipocyte differentiation might be present in THP-1-MacCM. PDGF and wnt-5a levels were undetectable in THP-1-MacCM ($n = 3$). TNF α , at 20 pg/ml, the concentration present in THP-1-MacCM, had no significant effect on adipocyte differentiation ($5 \pm 4\%$ inhibition of TG accumulation, $n = 3$, $P = 0.35$). As a positive control, TNF α was inhibitory at a higher concentration of 25 000 pg/ml ($95 \pm 3\%$ inhibition of TG accumulation, $n = 3$, $P < 0.01$).

Discussion

Adipose tissue levels of IL1 β are elevated in obesity-associated insulin resistance, and are produced principally by macrophages and, to a lesser extent, by adipocytes

Figure 1

Heat sensitivity and size fractionation of the anti-adipogenic activity of THP-1-MacCM. Human preadipocytes were induced to differentiate for 13–15 days in the control medium (Ctrl) or THP-1-MacCM (MacCM) heat-treated or not (A, B and C), and fractionated or not with a 50 kDa MW cut-off filter (D, E and F). (A and D) Representative photomicrographs from one patient sample are shown. (B and E) TG was extracted, quantified, and normalized to protein content. Data are mean $\pm$ S.E.M. of three (B) or five (E)

separate patient samples. (C and F) Solubilized cellular proteins were immunoblotted with the indicated antibodies. ERK1/2 serves as a loading control. Immunoblots from one patient sample are shown. Densitometric data are the mean $\pm$ S.E.M. of three (C) or five (F) separate patient samples, and are normalized to the loading control (ERK1/2). Statistical significance (two-way ANOVA) is denoted as $*P < 0.05$ or $**P < 0.01$, between the indicated pairs.

Figure 2

IL1 β stimulates IKK β phosphorylation and I κ B degradation in differentiating human preadipocytes. Preadipocytes were stimulated for 30 min with 500 pg/ml IL1 β , control medium (Ctrl), or THP-1-MacCM (MacCM), all in the presence of adipogenic inducers. Solubilized cellular proteins were immunoblotted with the indicated antibodies. Immunoblots from one patient sample are shown. Densitometric data are the mean $\pm$ S.E.M. of three separate patient samples, and are normalized to the loading control (IKK β). Statistical significance (one-way ANOVA) is denoted as * P < 0.05 or *** P < 0.001, compared with the other conditions.

(Tack *et al.* 2012). Infiltrating macrophages, which prevent preadipocyte differentiation, lead to adipocyte hypertrophy and inflammation. IL1 β was recently proposed to be responsible for the anti-adipogenic action, based on studies using mouse J774A.1 macrophages and mouse 3T3-L1 preadipocytes (Lu *et al.* 2010). IL1 β itself, at a very low concentration of 5 pg/ml, inhibited 3T3-L1 adipocyte differentiation in that study, as assessed by reduced Oil Red O staining. Furthermore, GH, which attenuated the anti-adipogenic effect of J774A.1 macrophages, reduced IL1 β expression and release. However, there were no direct attempts to block or remove IL1 β in that experimental model to verify whether it was required for macrophage-mediated inhibition.

This led us to evaluate whether IL1 β might be responsible for the anti-adipogenic effect of THP-1-MacCM on human primary preadipocytes that we observed previously (Yarmo *et al.* 2010). Heat treatment and size fractionation studies were consistent, with the inhibitory factor being a heat-sensitive factor that was >3 and <50 kDa. The activity in the <50 kDa fraction was weaker than the intact medium or the pooled fractions, and the observed trend of inhibition did not quite reach significance. It is possible that other factors in the $\geq$ 50 kDa fraction, not active on their own, are required to work synergistically with those in the <50 kDa fraction to exert the full anti-adipogenic effect.

We investigated whether IL1 β was able to inhibit human adipocyte differentiation. At a concentration matching what we have measured in THP-1-MacCM

(500 pg/ml), IL1 β on its own strongly reduced the differentiation response, assessed by the levels of TG, PPAR γ , FAS, and aP2. Furthermore, as was the case for the anti-adipogenic effect of THP-1-MacCM (Yarmo *et al.* 2010), the inhibitory effect of IL1 β was nullified with the use of SC-514, a selective IKK β inhibitor (Kishore *et al.* 2003, Karin *et al.* 2004). We have previously reported that THP-1-MacCM stimulates IKK β phosphorylation (an indicator of activation) in human preadipocytes (Yarmo *et al.* 2010), and we now demonstrated that IL1 β similarly stimulates IKK β phosphorylation and I κ B degradation.

In contrast to the studies of Lu *et al.* (2010), our dose-response curve shows that a much higher concentration of IL1 β , 500 pg/ml or greater, is required to inhibit human adipocyte differentiation. Our indicators to assess the differentiation response were more detailed than Oil Red O staining, and included the quantification

Figure 3

Anti-adipogenic effect of IL1 β in human preadipocytes is reduced by SC-514. Human preadipocytes were induced to differentiate for 13–15 days with 500 pg/ml human recombinant IL1 β , in the presence or absence of 100 μ M SC-514 or vehicle (DMSO). (A) Representative photomicrographs from one patient sample are shown. (B) TG was extracted, quantified, and normalized to protein content. Data are the mean $\pm$ S.E.M. of three separate patient samples. (C) Solubilized cellular proteins were immunoblotted with the indicated antibodies. ERK1/2 serves as a loading control. Immunoblots from one patient sample are shown. Densitometric data are the mean $\pm$ S.E.M. of three separate patient samples, and are normalized to the loading control (ERK1/2). Statistical significance (two-way ANOVA) is denoted as * P < 0.05 between the indicated pairs.

Figure 4

IL1 β inhibits human adipogenesis in a dose-dependent manner. Human preadipocytes were induced to differentiate for 13–15 days in the absence or presence of human recombinant IL1 β at the indicated doses. (A) TG was extracted, quantified, and normalized to protein content. Data are the mean $\pm$ S.E.M. of three separate patient samples. (B) Solubilized cellular proteins were immunoblotted with the indicated antibodies. GAPDH serves as a loading control. Immunoblots from one patient sample are shown. Densitometric data are the mean $\pm$ S.E.M. of three separate patient samples, and are normalized to the loading control (GAPDH). Statistical significance (two-way ANOVA) is denoted as $^{**}P < 0.01$ between the indicated pairs.

of TG accumulation, as well as the induction of PPAR γ , aP2, and FAS. We used human IL1 β for our studies, but Lu *et al.* (2010) did not provide information on the type of IL1 β that they used, so it is difficult to comment on whether species specificity is an issue. It may be that differences between mouse 3T3-L1 vs primary human stromal preadipocytes contribute to these contrasting results.

There has actually been very little investigation on the effects of IL1 β on human preadipocytes. One study has reported an inhibition of the differentiation of subcutaneous abdominal preadipocytes (Simons *et al.* 2005), but used only one concentration of IL1 β that was much higher at 10 000 vs the 500 pg/ml range in our study. Another group, studying orbital human preadipocytes, also used only one dose of IL1 β at 10 000 pg/ml to demonstrate its anti-adipogenic effect (Yoon *et al.* 2011). Measurements of IL1 β in human adipose tissue in healthy lean individuals by microdialysis are in the 70 pg/ml range (Clausen *et al.* 2009), and 30 pg/ml in healthy obese women (Siklova-Vitkova *et al.* 2009). It is possible that higher concentrations within the tissue are found in the setting of obesity-associated insulin resistance with the accompanying influx of pro-inflammatory macrophages (Fain 2010), but microdialysis data in this setting have not been reported.

To determine the importance of IL1 β in THP-1-MacCM, we immunodepleted the cytokine and studied the resulting MacCM to determine the consequences on preadipocyte inflammatory signaling and the adipogenic response. IL1 β immunodepletion reduced the I κ B degradation response, although there was no significant difference in the extent of IKK β phosphorylation. This result suggests that IL1 β , in particular, contributes to the complete I κ B degradation in human preadipocytes induced by THP-1-MacCM, whereas residual factors in THP-1-MacCM remain that are capable of elevating IKK β phosphorylation.

However, despite this altered signaling response to IL1 β -depleted THP-1-MacCM, the anti-adipogenic effect of this medium was not affected. A limitation in drawing this conclusion is the low number of adipose tissue samples obtained from a somewhat heterogeneous patient population, and therefore the possibility that an alteration in the differentiation response was missed. Yet, our data (levels of TG, PPAR γ , and aP2) show no trend in that direction, and a power analysis indicates that a change of 25% in the extent of inhibition would have been detected at an acceptable type II error of 0.8.

The weaker extent of I κ B degradation suggests that NF- κ B activation is not required for this medium in order to inhibit adipocyte differentiation. Our studies showing no effect of SN-50 were consistent with this. BY contrast, IKK β phosphorylation is critical, since the IKK β inhibitor SC-514 abrogated the anti-adipogenic action of THP-1-MacCM (Yarmo *et al.* 2010). It is known that IKK β phosphorylation does not always lead to I κ B degradation and NF- κ B activation, and that other

Figure 5

IL1 β is not required for the stimulation of IKK β phosphorylation and the anti-adipogenic effect of THP-1-MacCM. (A) IL1 β protein in the control medium (Ctrl) or THP-1-MacCM (MacCM), immunodepleted with IL1 β antibodies (Ab), mouse IgG, or no Ab. Data are the mean $\pm$ S.E.M. of four separate experiments. Human preadipocytes were treated for 30 min (B) or 14 days (C, D and E) with the control medium (Ctrl) or THP-1-MacCM (MacCM) immunodepleted with or without the IL1 β Ab or mouse IgG, all supplemented with adipogenic inducers. Solubilized cellular proteins were immunoblotted with the indicated antibodies. Immunoblots from one

patient sample are shown (B and E), with IKK β and ERK1/2 serving as loading controls, respectively. Densitometric data are the mean $\pm$ S.E.M. of three separate patient samples, and normalized to the loading control (IKK β for (B) and ERK1/2 for (E)). Representative photomicrographs from one patient sample are shown (C). TG was extracted, quantified, and normalized to protein content. Data are the mean $\pm$ S.E.M. of three separate patient samples (D). Statistical significance (two-way ANOVA) is denoted as * P < 0.05 or ** P < 0.01, between the indicated pairs.

established IKK β cellular targets could potentially mediate the anti-adipogenic action, including IRS-1, FOXO3, and 14-3-3b (Chariot 2009). Another group has reported the same response pattern of IKK β phosphorylation without I κ B degradation in human bronchial epithelial cells stimulated with interferon- γ (Tudhope *et al.* 2007). We have also described this pattern in PDGF-stimulated human preadipocytes (Gagnon *et al.* 2009). However, our analysis of THP-1-MacCM revealed that there was no detectable PDGF.

TNF α has been investigated as an anti-adipogenic macrophage-secreted factor for human adipocyte differentiation. However, in one study, immunoneutralization of TNF α barely altered the anti-adipogenic effect, although it did result in a major reduction in preadipocyte inflammatory gene expression (Lacasa *et al.* 2007). Furthermore, the amount of TNF α was not actually quantified in their conditioned medium. At 20 pg/ml, the concentration measured in THP-1-MacCM (Molgat *et al.* 2012), TNF α had no effect on human adipocyte differentiation. As a positive control, TNF α at 25 000 pg/ml was anti-adipogenic.

More recently, wnt-5a was demonstrated to be expressed by human blood monocytes and adipose tissue macrophages (Bilkovski *et al.* 2011). The evidence of wnt-5a secretion and its importance for inhibiting adipocyte differentiation was based on mouse J774A.1 macrophage cultures and mouse 3T3-L1 cells. It is not known whether wnt-5a would have a similar role in human adipocyte differentiation. We were not able to detect wnt-5a in our human THP-1-MacCM.

Investigations concerning the anti-adipogenic effect of MacCM have used multiple macrophage and preadipocyte cell culture models. The data accumulated are now sufficient to reveal that different anti-adipogenic factors may operate in the various systems. For example, using mouse J774A.1 macrophages and 3T3-L1 cells, an anti-adipogenic factor was found to be heat stable and <3 kDa (Ide *et al.* 2011), vs the larger, heat-sensitive factor noted here using human THP-1 macrophages and primary human preadipocytes. Future studies should carefully specify the experimental models used as this important question is pursued.

In the *in vivo* setting, it may be expected that anti-adipogenic effects of macrophages would constrain adipose tissue growth and favor hypertrophy, and thus lead to inflamed, insulin-resistant adipocytes and associated metabolic complications. There will also be additional complexity in that adipose macrophages may exist in a variety of polarized states, including M1

pro-inflammatory, M2 anti-inflammatory, and mixed phenotypes, and they may interact with preadipocytes by direct contact as well as through paracrine signals.

In summary, our results demonstrate that IL1 β is able to inhibit human adipocyte differentiation, but at concentrations much higher than reported for 3T3-L1 adipocyte differentiation. However, our immunodepletion strategy shows that it is not a critical component for the anti-adipogenic activity of THP-1-MacCM. Identifying key anti-adipogenic factors in the future will allow them to be evaluated as potential targets to improve adipose tissue remodeling and function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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