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Adipocytes Modulate the Phenotype of Human Macrophages through Secreted Lipids

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Previous studies have shown accumulation and an enhanced proinflammatory profile of macrophages in adipose tissue of obese mice, indicating the presence of an interaction between adipocytes and macrophages in this tissue. However, the consequences of this interaction in humans are yet incompletely understood. In this study, we explored the modulating effects of adipocytes on the phenotype of macrophages in humans and studied the possible molecular pathways involved. Adipocyte-conditioned media (ACM) treatment of macrophages for 48 h strongly reduced the LPS-induced IL-12p40 secretion by macrophages, whereas the production of TNF- α and other cytokines remained largely unaffected. This effect was independent of the source of adipocytes. Interestingly, the level of inhibition correlated directly with body mass index (BMI) of the adipocyte donor. Because adipocytes release many different cytokines, adipokines, and lipids, we have separated the protein and lipid fractions of ACM, to obtain insight into the molecular nature of the soluble mediators underlying the observed effect. These experiments revealed that the inhibitory effect resided predominantly in the lipid fraction. Further studies revealed that PGE₂ and linoleic and oleic acid were potent inhibitors of IL-12p40 secretion. Interestingly, concentrations of these ACM-derived lipids increased with increase in BMI of the adipocyte donor, suggesting that they could mediate the BMI-dependent effects of ACM. To our knowledge, these results provide first evidence that obesity-related changes in adipose tissue macrophage phenotype could be mediated by adipocyte-derived lipids in humans. Intriguingly, these changes appear to be different from those in murine obesity. *The Journal of Immunology*, 2013, 191: 1356–1363.

Obesity is associated with a low-grade inflammation, characterized by increased serum concentrations of acute-phase proteins and other metabolic changes such as insulin resistance and dyslipidemia (1, 2). Moreover, adipose tissue in obesity appears to fulfill a pivotal role as a site and source of inflammation (2–4). Besides obesity-related changes in adipocytes, alterations in the composition of the adipose tissue stromal

vascular cell fraction are also considered important factors contributing to adipose tissue inflammation. The stromal vascular cell fraction is composed of various cell types, including progenitor cells, fibroblasts, endothelial cells, nerve cells, and immune cells (3, 5). Among the immune cells characterized in adipose tissue, macrophages are not only the most abundant, but have also been shown to accumulate with increase in adipose tissue mass (3, 6).

Macrophages can express a wide range of polarization states depending on their environment and tissue localization (7, 8). The phenotype of macrophages in adipose tissue has been most extensively studied in murine models of obesity. Approximately two types of adipose tissue macrophages (ATMs) can be distinguished, as follows: resident ATMs and infiltrating or crown-like structure-associated ATMs (6). Adipose tissue-resident macrophages are dispersed throughout the adipose tissue and are described to have an anti-inflammatory phenotype (6). The crown-like structure-associated ATMs, however, have a proinflammatory phenotype and are thought to infiltrate with increase in adiposity (9, 10). Additionally, previous data indicated that, due to the infiltration of proinflammatory macrophages in obesity, the balance of macrophage polarization switches in favor of the proinflammatory phenotype (6, 10, 11). Although macrophages have been shown to infiltrate the adipose tissue also in human obesity, changes in ATM phenotype are less clear as human ATMs in obesity express a mixed phenotype with both pro- and anti-inflammatory characteristics (12, 13). Nevertheless, some evidence exists that crown-like structure-associated ATMs in humans have a more proinflammatory phenotype compared with ATMs dispersed throughout the adipose tissue (14). Still, the exact mechanisms involved in the accumulation and polarization of ATMs in humans remain incompletely understood.

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Abbreviations used in this article: AA, arachidonic acid; ACM, adipocyte-conditioned media; ATM, adipose tissue macrophage; BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; IFP, infrapatellar fat pad; MTBE, methyl-*tert*-butyl-ether; PD1, protectin D1; Sc, subcutaneous adipose tissue.

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Several mechanisms have been proposed to contribute to changes in the phenotype of ATMs in obesity. Among these, obesity-induced alterations in adipose tissue-secreted soluble factors, such as increased MCP-1 release, could contribute to preferential recruitment of proinflammatory monocytes to the adipose tissue (10, 15–17). Likewise, a direct crosstalk between ATMs and adipocytes could initiate metabolic changes in both cell types, contributing to ATM phenotype modifications in obesity (3, 18, 19). The majority of studies, however, have focused on the effect of macrophages on adipocyte metabolism. In these studies, soluble factors secreted by macrophages have been indicated to influence metabolic functions both in human and murine adipocytes [reviewed in Keuper et al. (20)]. Studies on the effect of adipocytes on macrophage function are scarce and generally limited to the murine system (19, 21). Most studies with human macrophages have explored the effect of individual adipokines such as leptin and adiponectin on macrophage phenotype (22–25). However, adipocytes can release large numbers of soluble mediators with different biological functions, and their global effect on macrophages is difficult to predict.

In the current study, we investigated the hypothesis that soluble mediators secreted by adipocytes are able to modulate the phenotype of macrophages in humans, thereby contributing to obesity-related changes in adipose tissue. By using adipocytes derived from different adipose tissue depots, we show that soluble mediators and especially lipids secreted by adipocytes are able to inhibit LPS-induced IL-12p40 secretion by macrophages. Interestingly, this effect is independent of the source adipocytes and is enhanced when adipocytes are derived from obese individuals. Analyses of the lipids secreted by adipocytes indicated that fatty acids are likely candidates to mediate this effect.

Materials and Methods

Human subjects

Patients with osteoarthritis undergoing joint replacement surgery were recruited into the study, and infrapatellar fat pad (IFP) adipose tissue samples were obtained after informed consent. Subcutaneous adipose tissue (Sc) was acquired as rest material from aesthetic abdominoplastic surgeries. This study was approved by the local medical ethical committee.

Generation of adipocyte-conditioned medium and isolation of different adipocyte-conditioned medium fractions

The isolation of adipocytes and subsequent generation of adipocyte-conditioned medium (ACM) were performed, as previously described (26). Proteins and lipids in ACM were separated using methyl-*tert*-butyl-ether (MTBE). Briefly, 5 ml MTBE was added to 1 ml ACM, and the samples were vortexed for 30 min at room temperature before centrifugation for 10 min at 1000 × *g*. The lipid-containing upper layer was carefully removed, and the isolation procedure was repeated. After combining both MTBE fractions, this lipid fraction was subsequently dried under a gentle stream of N₂ at 37°C overnight before reconstitution to the original volume in DMEM/F12 4.5 g/L glucose/15 mM HEPES/glutamax/pen/strep (Life Technologies) supplemented with 2% fatty acid-free BSA (Sigma-Aldrich) by sonication at 37°C. The remaining MTBE was evaporated from the aqueous bottom layer under a gentle stream of N₂. All obtained fractions were directly used in experiments.

Isolation and culture of monocyte-derived macrophages

PBMCs were isolated from buffy coats of healthy blood donors (Sanquin Blood Bank, The Netherlands). Subsequently, CD14⁺ monocytes were isolated using magnetic-labeled anti-CD14 beads (Miltenyi Biotec), according to the manufacturer's instructions (purity >90%). Next, cells were cultured for 7 d in RPMI 1640/glutamax/pen/strep (Invitrogen) supplemented with 10% FCS (medium) at 37°C in 5% CO₂ atmosphere in the presence of 5 ng/ml GM-CSF (R&D Systems). Culture medium and GM-CSF were refreshed on days 3 and 5. ACMs of different donors were added to the cells in a final ratio of 1:5 on day 5. Stock solutions of oleic, linoleic, and palmitic acid (all Sigma-Aldrich) and PGE₂ (Cayman Chemicals) were dried

under a gentle stream of N₂ before reconstitution by sonication at 37°C in DMEM/F12 4.5 g/L glucose/15 mM HEPES/glutamax/pen/strep (Life Technologies) supplemented with 2% fatty acid-free BSA (Sigma-Aldrich). The reconstituted lipids were then added to the cells on day 5. To block the uptake of PGE₂, the PGE₄ antagonist L-161,982 (Cayman Chemicals) was added 1 h prior to PGE₂ or ACM treatment on day 5. On day 6, all cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich), before collecting supernatants on day 7. Cell viability was evaluated by lactate dehydrogenase assay (Bioconnect), according to the manufacturer's instructions.

Detection of cytokines

Concentrations of IL-12p40 (R&D Systems) and TNF-α (OptEIA kit; BD Biosciences) were measured by ELISA in culture supernatants, according to the manufacturer's instructions. Levels of PGE₂ were determined in ACM using a competitive ELISA (Cayman Chemicals), according to the manufacturer's instructions. By Luminex technology, 44 cytokines (Merck Millipore) were measured in culture supernatants of a selective number of experiments, according to the manufacturer's instructions.

Flow cytometric analysis

Approximately 50,000 ACM-treated or control cells were stained with surface Ab solution containing mixes of the following Abs: PE-conjugated CD16, DC-SIGN; FITC-conjugated HLA-DR and PE-Cy-7-conjugated CD14 and the appropriate isotype controls (all Abs were from BD Biosciences). Exclusion of dead cells was performed in experiments when possible using the dead cell discrimination kit (Miltenyi Biotec), according to the manufacturer's specifications. All incubations were performed on 4°C, for 30 min. Cells were fixed with paraformaldehyde and analyzed with a LSR II flow cytometer using Diva 6 software (BD Biosciences).

Oil red O

Cells were stained for neutral lipids with Oil red O. Briefly, ACM-treated or control-treated macrophages were washed once and subsequently fixated in 4% formalin (Merck). After a brief dip in 60% 2-propanol (Merck), lipids were visualized with a working solution of Oil red O (Sigma-Aldrich). After 15 min, slides were briefly dipped into the 60% 2-propanol again and washed 3 min under tap water. After drying, cells were imbedded in Kaiser's glycerine (Merck), and cells were visualized using a Leica microscope and Leica software (original magnification ×40).

Lipid mediator lipidomics

Lipid mediators were isolated from ACM by solid-phase extraction and analyzed by liquid-chromatography mass spectrometry (LC-MS/MS), as described elsewhere (27). Briefly, deuterated lipid mediators were used as internal standards and were added to ACM before solid-phase extraction. Lipids were extracted from 250 μl ACM after protein precipitation with 1 ml MeOH and subsequently analyzed on a QTrap 5500 mass spectrometer (AB Sciex, Boston, MA) coupled to a quaternary Agilent 1100 pump (Agilent, Waldbronn, Germany) and a manual injection valve (Rheodyne, Oak Harbor, WA). The following binary gradient of water (A) and MeOH (B) with 0.01% acetic acid was used: 0 min 55% B, held for 2 min, then ramped to 65% at 3 min; 88% at 15 min and 100% B at 16.5 min, held for 3.5 min. The injection volume was 20 μl. Products were identified according to published criteria, including retention times and ≥6 diagnostic ions present in the full MS-MS spectrum to match those of synthetic standards (28). Lipid mediators were considered detectable upon a signal to noise ratio >10.

Fatty acid determination

Lipids were isolated from ACM and analyzed by LC-MS/MS, as described elsewhere, with some modifications (27). Briefly, odd-numbered fatty acids FA13:0 and FA19:0 were used as internal standards and were added to ACM before lipid extraction. Lipids were extracted from 50 μl ACM by adding 3 μl internal standards solution (FA13:0 and FA19:0 50 μg/ml in acetonitrile), 180 μl MeOH, and 600 μl MTBE, and shaking for 30 min. Then, 200 μl H₂O was added and samples were spun for 3 min at 13,200 × *g*. Extraction was repeated with 100 μl MeOH and H₂O and 300 μl MTBE. The obtained organic layers were combined and subsequently dried under a gentle stream of N₂ before final drying in a Eppendorf concentrator for 1 h. The dry residue was dissolved in 75 μl reconstitution solution (65% acetonitrile, 30% isopropanol, 5% H₂O) by vortexing for 10 s, followed by 10 s of sonication. Subsequently, 75 μl H₂O was added and samples were analyzed by LC-MS/MS. The HPLC system was a Dionex Ultimate 3000, consisting of a binary pump, connected to an autosampler equipped with a 1.0 μl injection loop and a column oven, which was maintained at 50°C.

The column was an Ascentis express C-18, 5 cm × 300 μm, 2.7 μm (Sigma-Aldrich), and the flow rate was 9.0 μl/min. The gradient program started at 65% eluent A (water:acetonitrile 80:20, containing 5 mM ammonium formate and 0.05% formic acid) and 35% eluent B (isopropanol:acetonitrile:water 90:9:1, containing 5 mM ammonium formate and 0.05% formic acid) kept constant for 2 min, then linearly increasing to reach 95% B at 30 min and held for 5 min. The IT-MS was a Bruker amaZon speed, which was operated in the ultrascan mode (Bruker Daltonics). The dry temperature was set to 180°C. Nitrogen 99.9990% was used as dry gas (8 ψ) and nebulizer gas (4 L/min). The capillary voltage was set to ±3.5 kV. The MS was operated in the electrospray ionization± mode, and auto MSn spectra collection was applied. The auto MSn settings were as follows: collision energy-enhanced fragmentation mode ramping from 80 to 120%, fragmentation time 30 ms, isolation width 1 amu, precursor exclusion after 3 collected spectra for 1 min. Fatty acids were considered detectable upon a signal to noise ratio >10.

Statistical analyses

The body mass index (BMI) was calculated by dividing the body weight (Kg) by height squared (m). Correlations were calculated using Spearman's rank correlation test. Overall differences between ACM treatment and control were evaluated using a linear mixed model, to correct for differences between the 20 macrophage donors. Differences between treatment and control were evaluated using the Student's *t* test.

The statistical package for the social sciences (SPSS) version 17.0 was used (SPSS, Chicago, IL) to analyze the data. Bonferroni's adjustment for multiple testing was performed when assessing the significance of the studied associations.

Results

ACM treatment of macrophages inhibits LPS-induced IL-12p40 secretion

First, we studied the effect of soluble factors secreted by adipocytes on the cytokine production of activated macrophages (Fig. 1). Incubation of macrophages with ACM derived from two different adipose tissue depots, the IFP of osteoarthritic patients or s.c. adipose tissue (Sc) derived from aesthetic surgeries, resulted in macrophage lipid loading (visualized by Oil red O; Supplemental Fig. 1) and a strong reduction in IL-12p40 secretion in 91 of 94 ACMs tested (32 IFP- and 62 Sc-derived ACMs; Fig. 1A, 1C, 1E). This inhibition of IL-12p40 secretion appeared independent of the adipose tissue depot type (Fig. 1E). In contrast, the secretion of TNF-α was not significantly affected (Fig. 1B, 1D, 1F), indicating a cytokine-specific effect of ACM. No differences in cell viability between treatments and controls were detected by lactate dehydrogenase assay (data not shown). Intriguingly, the percentage of inhibition of IL-12p40 secretion increased with increase in BMI of the ACM donors (Fig. 1G, 1H). To further characterize the polarization of macrophages, flow cytometric analyses were performed for CD163, CD206, HLA-DR, DC-SIGN, and CD16. These analyses revealed a significant upregulation of CD6 and HLA-DR expression and a trend for upregulation of DC-SIGN upon ACM treatment (Supplemental Fig. 2 and data not shown).

To obtain an indication whether ACM treatment influenced the secretion of other cytokines, 43 cytokines were measured in a restricted number of experiments (four different Sc-derived ACMs) using luminex technology. These experiments revealed that ACM treatment also significantly inhibited the secretion of macrophage-derived chemoattractant and platelet-derived growth factor arachidonic acid (AA) by macrophages in four of four ACMs tested, whereas RANTES, IL-10, G-CSF, MIP-1β, MCP-3, and IFN-α secretion was only modulated by some ACMs (data not shown). In contrast, ACM treatment of macrophages significantly increased TGF-α secretion by macrophages in four of four ACMs analyzed. The remaining cytokines were either unchanged upon treatment or undetectable (IL-3, IL-5, IL-9, IL-13, and IL-23) in culture

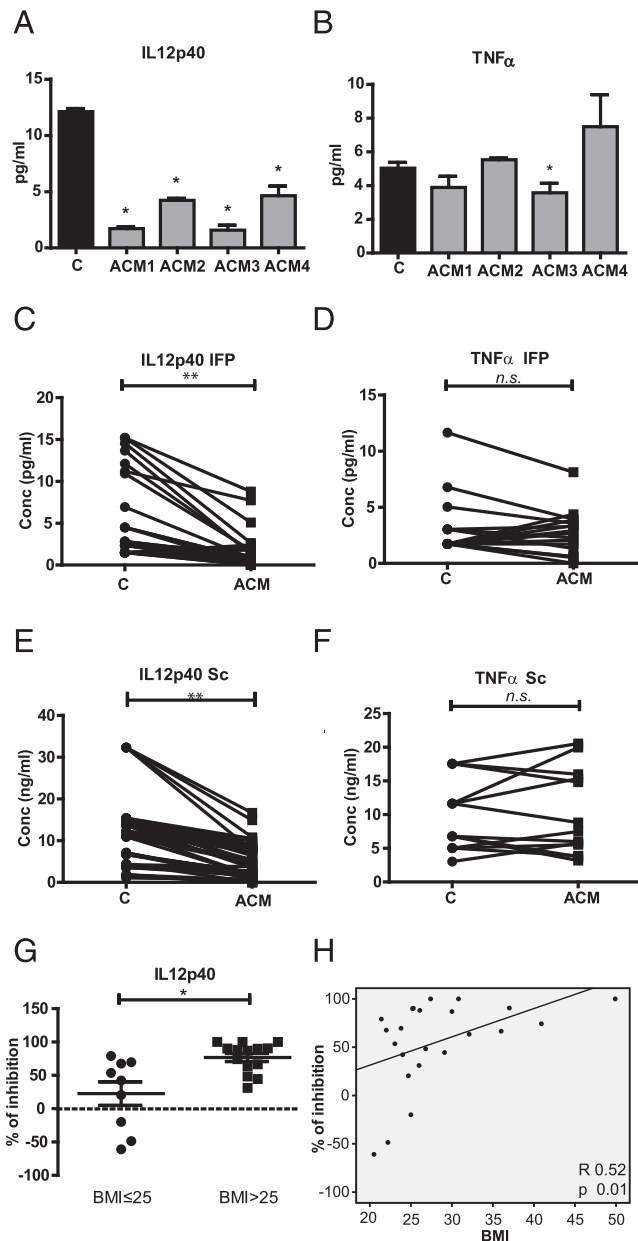


FIGURE 1. Soluble factors secreted by adipocytes modulate IL-12p40 secretion by LPS-stimulated macrophages. IL-12p40 and TNF-α concentrations were measured in culture supernatants by ELISA. IFP (A–D), s.c. (Sc) (E, F) ACM, or control media (C) were added to monocyte-derived macrophage cultures the last 48 h, and 100 ng/ml LPS was added the last 24 h of culture. (A and B) One representative experiment; bars represent the mean with SD of triplicate values; *p* values <0.05, determined by Student *t* test, were considered significant. (C–F) Summary of all experiments performed with IFP (32)- and Sc (62)-derived ACM; each dot represents the mean of triplicate values, and each line indicates an ACM-treated macrophage with its control; *p* values were determined using a linear mixed model, and *p* values <0.025 were considered significant. (G) Percentage of inhibition of IL-12p40 secretion (defined as [the concentration of IL-12p40 in the untreated sample – the concentration of IL-12p40 in the ACM-treated sample]/untreated sample × 100) upon treatment with IFP-derived ACM of donors with a BMI ≤ 25 and BMI > 25 (all IFP-derived ACMs); *p* values were determined by Mann-Whitney *U* test (H). Spearman's rank correlation of the percentage of IL-12p40 inhibition and BMI of the ACM donor. **p* < 0.05, ***p* < 0.0001.

supernatants (data not shown). The effects of ACM on IL-12p40 were most pronounced, and therefore this cytokine was selected as readout for further experiments.

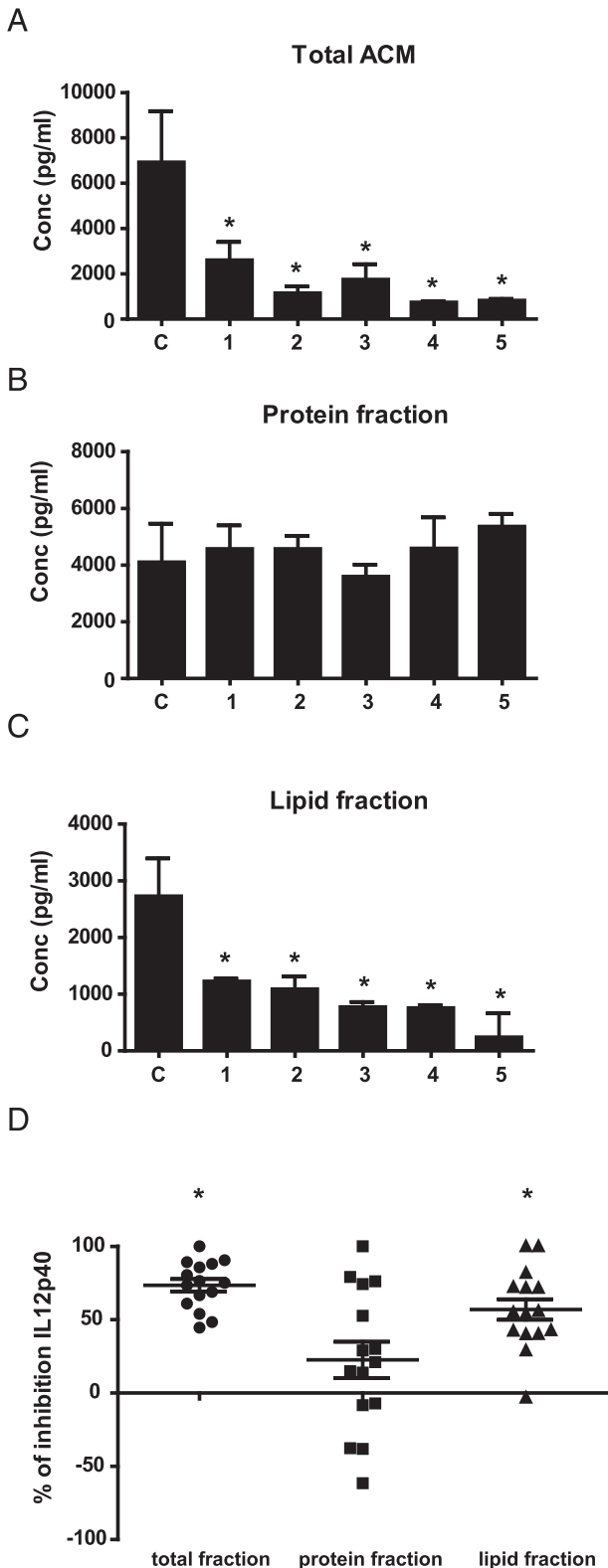


FIGURE 2. The lipid fraction of ACM inhibits LPS-induced IL-12p40 secretion. IL-12p40 concentrations were measured in culture supernatants by ELISA. Monocyte- derived macrophages were treated for 48 h with (A) total ACM, (B) the total ACM protein fraction, or (C) the total ACM lipid fraction of five different ACM samples (1–4 Sc-derived and sample 5 IFP-derived ACMs) or medium control (C). One representative experiment is shown (A–C); bars represent mean with SD of triplicate values. (D) Summary of all performed experiments (both IFP-derived and Sc-derived ACMs). Each dot indicates the percentage of change in IL-12p40 secretion upon ACM treatment compared with its control (assigned the value 0),

The lipid fraction of ACM inhibits LPS-induced IL-12p40 secretion

Because adipocytes can secrete proteins and lipids, we wished to determine the fraction responsible for the observed effect. Therefore, we separated the lipid and protein fraction of ACM to get more insight into the molecular nature of the inhibitor(s). LPS-induced IL-12p40 secretion was measured in culture supernatants of macrophages activated in the presence of total ACM, the ACM protein, or the ACM lipid fraction. A representative experiment is shown in Fig. 2A–C, and a summary of all experiments performed is depicted in Fig. 2D. The strongest inhibition of IL-12p40 was observed with the total and lipid fraction of ACM, whereas IL-12p40 was not significantly modulated by the ACM protein fraction.

ACM contains several lipids

Because the lipid fraction appears to be a potent inhibitor of activation-induced IL-12p40 production, we next aimed at characterizing the lipids present in ACM in more detail. Several lipid mediators and fatty acids were detected using LC-MS/MS (Fig. 3). Of the fatty acids present in ACM, oleic acid (detected in 10 of 10 ACM samples analyzed) and palmitic acid (detected in 7 of 10 samples) were the most abundant, followed by lower concentrations of linoleic acid (detected in 9 of 10 samples), palmitoleic acid (detected in 7 of 10 samples), γ -linolenic acid (detected in 7 of 10 samples), DHA (detected in 7 of 10 samples), AA (detected in 7 of 10 samples), docosapentaenoic acid (detected in 5 of 10 samples), myristic acid (detected in 2 of 10 samples), and eicosapentaenoic acid (EPA) (detected in 1 of 10 samples) (Fig. 3A). Interestingly, we could also detect the presence of several oxylipins in ACM, such as the AA-derived eicosanoid precursors 5-/12-/15-hydroxyeicosatetraenoic acid (HETE) (detected in 10 of 10; 4 of 10; 3 of 10 analyzed ACM samples) and PGE₂ (detected in 5 of 10 samples). Moreover, we could detect the EPA derivatives 5-/12-/15/18-hydroxyeicosapentaenoic acid (HEPE) (detected in 3 of 10; 1 of 10; 1 of 10; 1 of 10 samples), precursors of proresolving lipid mediators and the DHA-derived protectin D1 (PD1; 3 of 10) (Fig. 3B) [reviewed in Serhan et al. (29)]. An example of the detection of PD1 in ACM is shown in Supplemental Fig. 3.

Several lipids can inhibit IL-12p40 secretion

The data described above indicate the presence of several lipids, such as PGE₂ and fatty acids in ACM. PGE₂ has been described to inhibit IL-12 secretion by monocytes and macrophages (30–33). Because PGE₂ was readily detectable in ACM, we next investigated the possibility that its presence in ACM is responsible for the modulation of macrophage IL-12p40 secretion. As expected, synthetic PGE₂ was able to inhibit IL-12p40 secretion in seven of nine experiments (an example is shown in Fig. 4A and summarized in Supplemental Fig. 4A), whereas the secretion of TNF- α was not affected in three of three replicative experiments (an example is shown in Fig. 4B). This effect could be completely reversed by the PGE₄ receptor antagonist L-161,982 (Fig. 4C, and summarized in Supplemental Fig. 4B), but not by antagonists of other PG receptors (data not shown). However, the inhibitory effect of ACM could not be reversed by blocking the interaction of PGE₂ with the PGE₄ receptor, using the L-161,982 antagonist

obtained by dividing the means of triplicate values per condition; mean change with SD is depicted; **p* < 0.05, determined by Student *t* test (A–C) or Wilcoxon rank sum test (D), was considered significant.

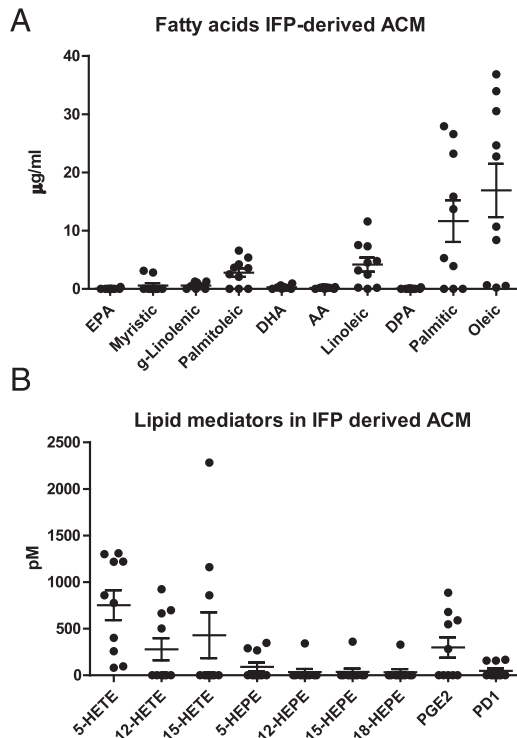


FIGURE 3. ACM contains several lipids. Lipids were measured in 10 IFP-derived ACM samples by LC-MS/MS. Concentration of fatty acids (**A**) and lipid mediator precursors and lipid mediators (**B**) in ACM; each dot represents one ACM. Indicated are means \pm SEM.

(Fig. 4C), suggesting that PGE₂ is not the main contributing lipid to the inhibitory effect of ACM.

Therefore, we next investigated whether other lipids or fatty acids present in ACM could induce IL-12p40 inhibition. To this end, we explored the effect of the three most abundant fatty acids detectable in ACM, as follows: oleic, palmitic, and linoleic acid on macrophage IL-12p40 secretion. Although these fatty acids have been implicated in immune modulation (34–38), it is not known whether they can inhibit IL12 production by macrophages. As shown in Fig. 4D–F, several fatty acids could modulate IL-12p40 secretion: oleic acid and linoleic acid were able to inhibit IL-12p40 secretion in six of six and in four of six experiments. The addition of palmitic acid did not affect IL-12p40 secretion in four of six experiments. These data provide insight into the nature of the lipids involved in the effects observed and, more importantly, indicate the presence of multiple lipids and fatty acids that can potentially modulate macrophage function.

Some lipids display BMI-related abundance

Because we observed the inhibitory effect to be dependent on the BMI of the adipocyte donor, we next examined whether the ACM-derived lipids also display a BMI-dependent concentration range. Indeed, we detected higher concentrations of all fatty acids in ACM of the overweight/obese adipocyte donors compared with lean donors (Fig. 5). Additionally, the concentration of PGE₂ in ACM correlated positively with BMI of the adipocyte donor, and the same trend was observed for 5-/12-HETE and 5-HEPE in ACM (data not shown). These data strengthen the previous findings supporting the hypothesis that various lipids such as fatty acids could mediate the suppressive effect of ACM on IL-12p40 secretion by activated macrophages.

Discussion

Previous studies in murine models of obesity revealed an accumulation of proinflammatory macrophages in adipose tissue, im-

plying the presence of an interaction between adipocytes and macrophages in this tissue. In the current study, we have shown that adipocyte-secreted soluble factors are able to modulate the phenotype of human macrophages by inhibiting the secretion of IL-12p40. This effect resided predominantly in the ACM lipid fraction and was BMI dependent, being more pronounced when ACM was derived from overweight/obese individuals. Further studies indicated that among the lipids identified in ACM, PGE₂, and linoleic and oleic acid were potent inhibitors of IL-12p40 secretion. Concentrations of these ACM-derived lipids increased with increase in BMI of the adipocyte donor, supporting the hypothesis that they could mediate the BMI-dependent effects of ACM. To our knowledge, these results provide first evidence that obesity-related changes in macrophage phenotype could be mediated by adipocyte-derived lipids in humans.

To our knowledge, our study is the first to investigate the interplay between mature tissue-derived adipocytes and primary macrophages in an in vitro human system and to show obesity-related changes in macrophage phenotype. Our results are rather unexpected, in view of previously published data (mostly) in the murine system, which indicate that obesity has a proinflammatory effect on adipose tissue macrophages. In addition to enhanced secretion of TNF- α and IL-6 by ATMs, also increased IL-12p40 expression in obese adipose tissue has been described, which coincided with the development of insulin resistance (6, 10, 11, 39). These data are supported by findings showing that adipocyte-secreted factors mainly induced the upregulation of proinflammatory cytokines such as IL-6 and TNF- α in murine macrophages in vitro (19, 21). Taken together, these data suggest that the interaction between adipocytes and macrophages is a possible mechanism for obesity-related changes in the phenotype of adipose tissue macrophages. In our system, however, we generally observed a reduction in IL-12p40 secretion upon macrophage ACM treatment, whereas TNF- α secretion was not affected. In addition, we observed increased surface expression of the M1 markers CD16 and HLA-DR, whereas the percentages of cells positive for the M2 markers CD206 and CD163 were not affected (Supplemental Fig. 2 and data not shown), indicating a possible discrepancy between the immunomodulatory effects of ACM in humans compared with mice (3, 40). Furthermore, this observed effect was independent of the source of the adipocytes, because similar results were obtained when macrophages were treated with ACM derived from different adipose tissue depots. Interestingly, our observations are in line with previous studies indicating a mixed phenotype of ATMs in several different adipose tissue depots (12, 13, 26).

Several factors could underlie these differences. For instance, the genetic background of the mice could influence immune responses. Most studies use C57BL/6 mice, which are Th1 prone and more inflammatory than other genetic backgrounds. This could skew and exaggerate the observed effects in mice. Dissimilarities in observed effects in mice and humans could be explained not only by increased genetic variation in humans, but also with differences in duration of obese condition, as in humans effects of obesity are studied years after it has developed, but mouse studies are usually performed shortly after obesity has been induced. This could be relevant in the light of a recent publication indicating that variation in nutrient availability, rather than BMI alone, are important regulators of ATM recruitment and proinflammatory changes (41). Finally, whereas most authors use adipocyte cell lines for their studies, we have used exclusively tissue-derived mature adipocytes. These could be different from in vitro differentiated adipocytes, secreting different soluble mediators. All together, these differences in experimental conditions could account for discrepancies between our data and previously published findings.

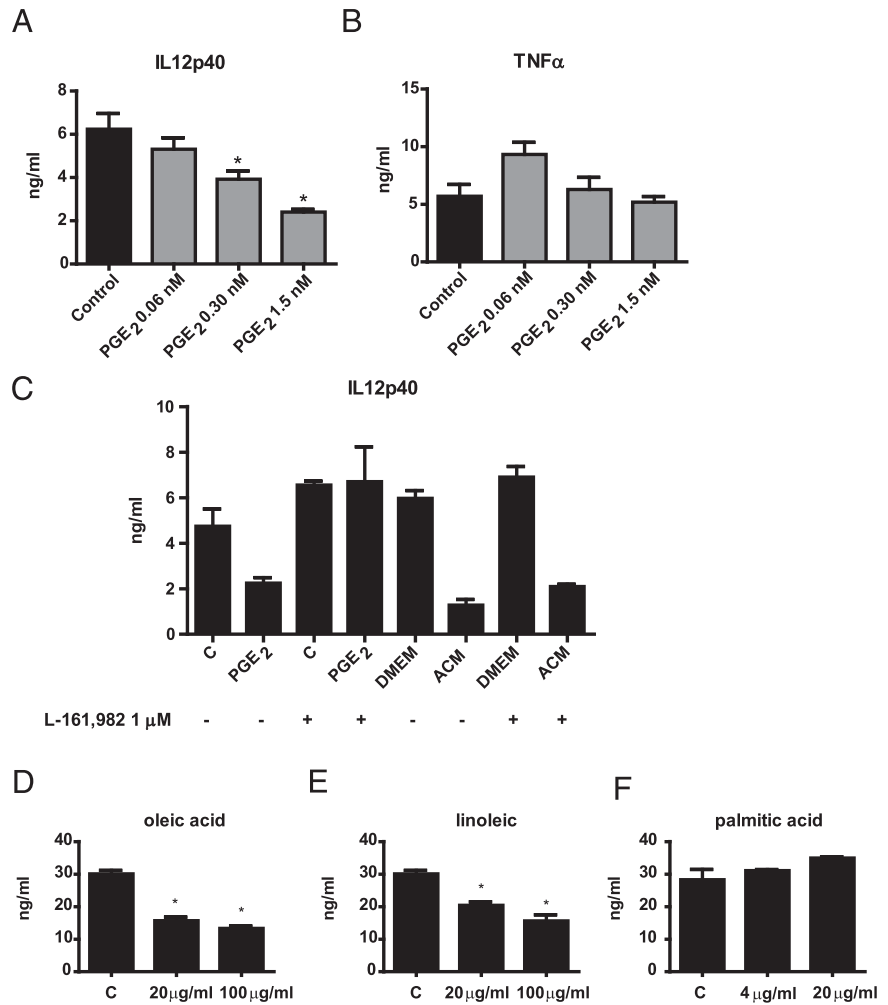


FIGURE 4. Several lipids can inhibit LPS-induced IL-12p40 secretion. IL-12p40 and TNF- α concentrations were measured in culture supernatants by ELISA. Monocyte-derived macrophages were treated for 48 h with (A–C) PGE₂, (D) oleic (OA), (E) linoleic (LIN), and (F) palmitic (PA) acid. Alternatively, (C) macrophages were preincubated for 60 min in the presence or absence of the PGE₄ receptor antagonist L-161,982, before addition of either PGE₂, (IFP-derived) ACM, or control medium (C, DMEM) for 48 h. A total of 100 ng/ml LPS was added during the last 24 h of culture. (A–F) One representative experiment is shown; each bar represents the mean with SD of triplicate values; **p* < 0.05, determined by Student *t* test, was considered significant.

Although the secretion of several cytokines could be modulated upon macrophage ACM treatment, most of the 43 cytokines tested remained unaffected. Moreover, the inhibitory effect on IL-12p40 secretion seemed to be specific for this subunit as ACM treatment did not affect the secretion of IL-12p70, nor of IL-23 (not detectable). The lack of IL-23 secretion might be explained by the presence of IL-10 in our culture as it was previously shown in a murine study that LPS-induced IL-23 secretion by macrophages can be inhibited by IL-10 (42).

In line with previously published data, in our samples IL-12p40 is secreted in excess of IL-12p70 (43). Therefore, it is likely that our data reflect a modulation of IL-12p40 monomer or dimer (IL-12p80) secretion. The biological consequences of this observed

downregulation of IL-12p40 subunits are not yet clear as both pro- and anti-inflammatory functions have been ascribed to the IL-12p40 subunit [reviewed in Cooper and Khader (44)]. Among the functions described for IL-12p40/p80 is its capacity to act as a chemoattractant for macrophages (45, 46) and its capacity to promote IFN- γ in CD8⁺ T cells (47). Because macrophages and CD8⁺ T cells in adipose tissue have been associated with insulin resistance (14, 48), it can be speculated that reduced IL-12p40 production could be associated with an improvement in insulin sensitivity. However, the IL-12p40 homodimer has also been shown to antagonize IL-12p70 activity in murine (49) cells and human cell lines (50), which would argue for a rather anti-inflammatory function for this subunit. Because associations have been described between increased serum levels of IL-12p70 and insulin resistance in humans (51, 52), a reduction of IL-12p40 in serum could result in increased action of IL-12p70 and subsequent insulin resistance. In an attempt to translate our findings to systemic IL-12p40 levels, we measured serum IL-12p40 levels in 12 lean and 12 obese individuals. However, we found detectable levels of IL-12p40 of ~100 pg/ml only in two healthy lean controls (data not shown). Therefore, further studies are necessary to make firm conclusions regarding the biological consequences of the inhibition of IL-12p40.

The exact factors mediating the inhibitory effect remain to be established. Several studies have reported an effect of adipokines on macrophage polarization (22–25). Addition of the most abundant adipokines present in ACM, which are leptin, adiponectin, and adipisin, could not mimic the inhibition of macrophage IL-

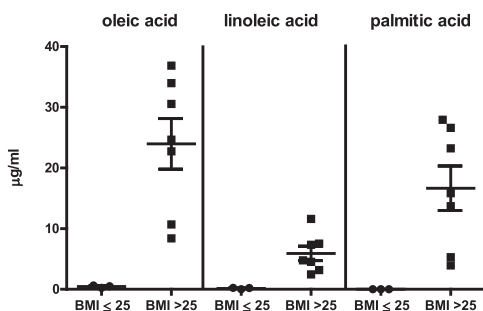


FIGURE 5. Some lipids display BMI-dependent concentrations. Concentrations of oleic, linoleic, and palmitic acid in IFP-derived ACM of donors with a BMI \leq 25 and BMI > 25, measured by LC-MS/MS.

12p40 secretion in our system (data not shown). Although we cannot exclude an effect of ACM-derived proteins on macrophage cytokine secretion, the inhibitory effect on IL-12p40 secretion was most consistently observed in the ACM lipid fraction. This is interesting in the light of recent studies in murine models of obesity, in which the phenotypic modulation of adipose tissue macrophages has been attributed to an increase in macrophage lipid content (53). In line with these observations, our results also indicate an accumulation of lipids in ACM-treated macrophages (Supplemental Fig. 1), indicating it as a possible underlying mechanism for the observed effect.

Our data suggest that several groups of lipids present in ACM could contribute to the effects observed. In line with previous observations, we have shown that oleic acid, the most predominant unsaturated fatty acid in ACM, potentially inhibited IL-12p40 secretion in the concentrations found in ACM, in contrast to palmitic acid, the most abundant saturated fatty acid in ACM.

Moreover, we have identified several polyunsaturated fatty acid metabolites. Some of these, such as 5-/12-/15-HETE and the eicosanoid PGE₂, have been previously shown to be secreted by adipocytes (54, 55). We could confirm and expand these findings by detecting the presence of the DHA-derived PD1 and the EPA-derived markers for lipoxygenase activity, the 5-/12-/15-/HEPEs, in ACM (Fig. 3B). Next to DHA derivatives, these EPA derivatives constitute biomarkers and precursors for anti-inflammatory and proresolving lipid mediators such as lipoxins and resolvins. These recently described proresolving mediators are known to have potent immunomodulatory functions even in nM range, including regulation of adipokines and adipose tissue inflammation (56). Secretion of lipid mediator precursors by adipocytes indicates a novel mechanism by which adipocytes could regulate inflammation and resolution of inflammation in adipose tissue.

Collectively, our data indicate that lipids secreted by adipocytes are able to modulate the phenotype of macrophages by inhibiting IL-12p40 secretion. This modulation is dependent on the BMI of the adipocyte donor and appears different from murine models of obesity. To our knowledge, these results provide first evidence that obesity-related changes in macrophage phenotype could be mediated by adipocyte-derived lipids in humans and indicate an important immunomodulatory function of adipocytes in humans.

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Disclosures

The authors have no financial conflicts of interest.

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