Pro-inflammatory CD11c⁺CD206⁺ adipose tissue macrophages are associated with insulin resistance in human obesity

Running Title: Adipose tissue macrophages in human obesity


1. Autoimmunity & Transplantation Division, Walter & Eliza Hall Institute of Medical Research, 1 G Royal Parade, Parkville 3050, Victoria, Australia
2. Burnet Clinical Research Unit, Royal Melbourne Hospital, Grattan Street, Parkville 3050, Victoria, Australia
3. Centre for Obesity Research and Education, Monash University, Commercial Road, Prahran 3181, Victoria, Australia
4. Bioinformatics Division, Walter & Eliza Hall Institute of Medical Research, 1 G Royal Parade, Parkville 3050, Victoria, Australia
5. Department of Pediatrics and Adolescent Medicine, University of Ulm, Eythstr. 24, 89075 Ulm, Germany

Contact:
Leonard C. Harrison
Email: harrison@wehi.edu.au


Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Objective: Insulin resistance and other features of the metabolic syndrome have been causally linked to adipose tissue macrophages (ATMs) in mice with diet-induced obesity. We aimed to characterize macrophage phenotype and function in human subcutaneous and omental adipose tissue in relation to insulin resistance in obesity.

Research Design and Methods: Adipose tissue was obtained from lean and obese women undergoing bariatric surgery. Metabolic markers were measured in fasting serum and ATMs characterized by immunohistology, flow cytometry and tissue culture studies.

Results: ATMs comprised CD11c^+CD206^+ cells in ‘crown’ aggregates and solitary CD11c^-CD206^+ cells at adipocyte junctions. In obese women, CD11c^+ ATM density was greater in subcutaneous than omental adipose tissue and correlated with markers of insulin resistance. CD11c^+ ATMs were distinguished by high expression of integrins and antigen presentation molecules, interleukin (IL)-1β, -6, -8 and -10, tumor necrosis factor-α and CC chemokine ligand-3, indicative of an activated, pro-inflammatory state. In addition, CD11c^+ ATMs were enriched for mitochondria and for RNA transcripts encoding mitochondrial, proteasomal and lysosomal proteins, fatty acid metabolism enzymes and T-cell chemoattractants, whereas CD11c^-ATMs were enriched for transcripts involved in tissue maintenance and repair. Tissue culture medium conditioned by CD11c^+ ATMs, but not CD11c^-ATMs or other stromovascular cells, impaired insulin-stimulated glucose uptake by human adipocytes.

Conclusions: These findings identify pro-inflammatory CD11c^+ ATMs as markers of insulin resistance in human obesity. In addition, the machinery of CD11c^+ ATMs indicates they metabolize lipid and may initiate adaptive immune responses.
The metabolic syndrome associated with obesity is characterized by insulin resistance, hyperglycaemia, hypertension and dyslipidaemia, reversible by weight loss (1). Studies in obese mice indicate that adipose tissue inflammation, centred on macrophages recruited to and activated by an expanding adipose tissue mass, is a mechanistic link between obesity and insulin resistance. The density of adipose tissue macrophages (ATMs) correlates with adipose tissue inflammatory markers and insulin resistance (2) but, more importantly, mice which lack the pro-inflammatory enzyme IKK-β in macrophages do not develop diet-induced insulin resistance (3) and mice which lack the anti-inflammatory transcription factor PPARγ in macrophages develop insulin resistance (4; 5).

Two ATM populations have been described in mice. In lean animals, solitary ‘resident’ ATMs predominate. These ATMs have an ‘alternative’ (M2) macrophage phenotype characterized by increased expression of interleukin-10 (IL-10) and arginase (6) and may facilitate adipogenesis (7). Obese mice also exhibit ‘crown’ macrophages aggregated around necrotic adipocytes (8). These ATMs have increased expression of the integrin CD11c and markers of ‘classical’ (M1) macrophages, including IL-6 and inducible nitric oxide synthase (INOS) (6; 9; 10). The ‘phenotypic switch’ from M2 to M1 could be an important determinant of insulin resistance in obese mice because CD11c promoter-dependent conditional deletion of ATMs improves insulin sensitivity (11).

A role for ATMs in the pathophysiology of human insulin resistance is less well established. As in mice, adipose tissue of obese humans exhibits increased expression of genes encoding pro-inflammatory cytokines (12) and contains increased numbers of ATMs compared to adipose tissue from lean controls (13; 14). However, ATM density, determined by histology or CD68 mRNA expression, was found to correlate weakly or not at all with insulin resistance (15-17). In addition, the existence of M1 and M2 ATM subsets has not been confirmed. A role for ATMs in human insulin resistance is also confounded by several differences between mice and humans. These include increased expression of resistin (18) and reduced expression of INOS (19) and arginase (20) in human macrophages, and reduced expression of CD11c by ATMs (21) and a relative paucity of crown macrophages in human adipose tissue (17; 21; 22). To better understand the role of ATMs in human obesity and insulin resistance, we enumerated and characterized ATMs from lean and obese women.

RESULTS

Crown ATMs express CD11c - Subcutaneous and omental adipose tissue was obtained from obese women (BMI range 39-56) undergoing bariatric surgery. In tissue sections stained for the monocyte/macrophage marker CD68, the majority of monocytes/macrophages appeared as slender, ‘resident’ cells at the junctions of two or more adipocytes (Figure 1a). Less frequently, ATMs were surrounded adipocytes in heterogeneously distributed ‘crown’ aggregates. Crown ATMs were larger, ovoid cells that occasionally coalesced to form syncytial giant cells (Figure 1a). Scattered monocytes were also observed within arterioles and venules and, infrequently in omental adipose tissue, lymphoid aggregates. Serial sections were stained for CD11c, a marker of mouse pro-inflammatory ATMs (6). CD11c was predominantly expressed by crown ATMs, although many resident ATMs showed weak CD11c staining (Figure 1b). Preferential expression of CD11c by crown ATMs was confirmed by fluorescence microscopy (Figure 1c). CD11c immunoreactivity was
Adipose tissue macrophages in human obesity

exclusive to CD68+ cells. Sections were also stained for CD206, a marker of ATMs that does not stain monocytes (21). By light microscopy, CD206 staining intensity was similar in resident and crown ATMs (Figure 1d), but by fluorescence microscopy staining was weaker in crown ATMs (Figure 1e).

Analysis of crown and resident ATMs by flow cytometry - To analyse ATMs in more detail, flow cytometry was performed on stromovascular cells isolated from digested subcutaneous and omental adipose tissue. Because CD68 is an intracellular antigen, anti-CD14 and -CD45 antibodies were used to identify ATMs, preadipocytes (PA) (23) and CD14-CD45+ cells; the latter were designated LYM because most also expressed the T-cell marker, CD3 (Figure 2a). By immunohistochemistry (Figure 2b), CD68 expression was specific to ATMs. We determined the phenotypes of stromovascular cells with antibodies to CD11c and CD206 (Figure 2c). CD206 was expressed by all CD11c- ATMs and not by PA or LYM. However, only a subset of CD11c+ ATMs expressed CD206. As with fluorescence microscopy (Figure 1e), these CD11c+ ATMs had a lower CD206 fluorescence intensity than CD11c- ATMs. In addition, staining with Oil Red O revealed that they contained more intracellular lipid than CD11c- ATMs (Figure 2d). CD11c-CD206+ ATMs had a similar profile to blood monocytes (Figure 2c). Together with immunohistochemistry, flow cytometry demonstrated that CD11c-, CD11c+CD206+ and CD11c+CD206- ATM populations represent, respectively, resident ATMs, crown ATMs and adipose tissue monocytes. While CD11c-CD206- cells were rarely observed in adipose tissue sections (Figure 1e), they were readily detected by flow cytometry (Figure 2c). In addition, due to the presence of lymphoid aggregates, omental samples sometimes contained very high numbers of LYM cells and CD11c-CD206- ATMs.

Crown ATMs are markers of insulin resistance - To determine the relationship between ATM populations and insulin resistance, ATM density was quantitated in subcutaneous and omental adipose tissue from three groups of women: formerly obese (FOb), obese (Ob) and obese with metabolic syndrome (ObMS), (Table 1). The Ob and ObMS groups were comparable in age and BMI, two independent determinants of ATM density in humans (15; 17). Histologically, in both adipose tissue depots, crown ATM density was usually zero in FOb and higher in ObMS than Ob (Figure 3a). In addition, in ObMS, crown density was higher in subcutaneous than omental adipose tissue. The differences between Ob and ObMS adipose tissue were not explained by differences in adipocyte size (Figure 3b). ATM density was then measured by flow cytometry, expressed as percent viable cells within the stromovascular population. None of the omental samples from all 29 women studied contained lymphoid aggregates. Crown density and CD11c+CD206+ ATM density were significantly correlated (Figure 3c), validating flow cytometric assessment. In both subcutaneous and omental adipose tissue, obesity was associated with a significant increase in densities of CD11c+CD206- (monocyte) (not shown), CD11c+CD206+ (crown) (Figure 3d) and CD11c- (resident) (Figure 3e) ATMs. Insulin resistance was associated with increased numbers of CD11c+CD206+ ATMs (Figure 3d), but similar numbers of CD11c- ATMs (Figure 3e). Accordingly, the CD11c+CD206+/CD11c- ATM ratio was greater in ObMS compared to Ob (Figure 3f). Although the differences in subcutaneous and omental ATM densities between FOb, Ob and ObMS were qualitatively similar, crown and CD11c+CD206+ ATM densities were lower in omental adipose tissue. LYM and PA densities were increased and decreased, respectively, in obesity but were not
significantly different between Ob and ObMS (not shown).

In the 24 obese women, CD11c⁺CD206⁺/CD11c⁻ ATM ratio correlated significantly with insulin resistance (HOMA-IR) (Figure 3g). In contrast, there was no correlation between HOMA-IR and either CD11c⁻ ATM density, age or BMI. A similar and more significant correlation between HOMA-IR and CD11c⁺CD206⁺/CD11c⁻ ATM ratio was confirmed in another 89 obese women, particularly in subcutaneous adipose tissue (Figure 3h). These results, together with the comparison of ATM densities between Ob and ObMS above, implicate CD11c⁺CD206⁺ ATM in the pathogenesis of insulin resistance.

Crown ATMs have an M1 surface phenotype

To further characterize ATMs, cell surface marker expression was determined by flow cytometry. Results were similar for subcutaneous and omental ATMs and are shown therefore only for subcutaneous ATMs and blood monocytes (Figure 4). Expression of the innate immune molecules CD14, TLR2, TLR4 and CCR2 was highest on blood monocytes, with variable expression by ATM subtypes. Expression of integrins was highest on blood monocytes and progressively declined from CD11c⁺CD206⁻ to CD11c⁺CD206⁺ to CD11c⁻ ATMs. In contrast, CD11c⁻ ATMs expressed the highest levels of CD163, a marker of alternative macrophage activation (24), and CD34, a marker of adipogenic/angiogenic ATMs (7). Finally, CD11c⁺CD206⁺ ATMs expressed the highest levels of CD45, the antigen presenting molecules CD1c and HLA-DR, and the T-cell co-stimulatory molecule CD86. This was most striking for CD1c, known to bind and present lipid antigens.

Crown ATMs are the major source of pro-inflammatory cytokines and chemokines

To determine their potential contribution to adipose tissue inflammation, stromovascular cells were isolated and cultured overnight, and secreted cytokines and chemokines measured in conditioned medium. Insufficient CD11c⁺CD206⁺ adipose tissue monocytes could be isolated for these experiments. ATMs from subcutaneous adipose tissue of three different donors secreted at least 50-fold higher amounts of IL-1β, IL-6, IL-8, IL-10, TNF-α and CCL3 compared to PA and LYM populations. In each donor, comparison of the two ATM subtypes revealed that unstimulated CD11c⁺CD206⁺ ATMs secreted more IL-1β, IL-6, IL-8, IL-10, TNF-α and CCL3 than CD11c⁻ ATMs, with IL-8 most highly and IL-10 most differentially secreted (Figure 5). LPS induced a non-selective increase in cytokine/chemokine secretion by both ATM subtypes. No clear differences in cytokine/chemokine secretion were detected between subcutaneous and omental ATMs (data not shown).

Crown ATMs are enriched for mitochondria and T-cell chemoattractants

CD11c⁺CD206⁺ and CD11c⁻ ATM transcriptomes were compared by microarray. RNA was prepared from subcutaneous ATMs isolated from six obese women. 3825 differentially expressed genes were identified at a false discovery rate of 5% (Supplemental Table 1, available in the online appendix at http://diabetes.diabetesjournals.org). Genes encoding CD1c, CD11a, CD11c, CD49d, CD86, CD163 and HLA-DR were differentially expressed in a pattern consistent with the flow cytometry findings. In addition, differential expression of CD11c (ITGAX), the fatty acid metabolism genes APOE and FABP4 and the collagen genes COL1A2 and COL6A3 was validated by RT-PCR in independently prepared ATMs (Supplemental Figure 1).

Functional annotation clustering analysis (25), comparing the 1756 genes up-regulated in CD11c⁺CD206⁺ ATMs to all human genes, revealed a striking enrichment for genes encoding mitochondrial proteins. It was then
demonstrated that CD11c⁺CD206⁺ ATMs contain greater numbers of mitochondria, by immunohistochemical staining for the mitochondrial voltage-dependent anion channel (VDAC1) (Figure 6a) and by determining mitochondrial DNA copy number in sorted stromovascular cell populations (Figure 6b). Staining with Mitotracker Red demonstrated that CD11c⁺CD206⁺ ATMs, together with preadipocytes, have the highest mitochondrial activity within the stromovascular population (Figure 6c). Clustering analysis also revealed that CD11c⁺CD206⁺ ATMs were enriched for transcripts encoding glucose and fatty acid metabolism proteins, integrins, proteosomal and lysosomal proteins and T-cell activation proteins. In contrast, of the 2069 genes up-regulated in CD11c⁻ ATMs, scavenger receptors, the transforming growth factor-β (TGF-β) family of cytokines, components of the extracellular matrix and platelet-derived growth factor-β (PDGFB), were over-represented.

Crown ATM-conditioned medium inhibits insulin action - To determine whether products of ATMs could inhibit insulin action, insulin-stimulated glucose uptake by human SGBS adipocytes was measured in the presence of 20% v/v serum-free medium conditioned by stromovascular cells or flow-sorted CD11c⁺CD206⁺ ATMs, CD11c⁻ ATMs, LYM or PA cells. In four independent experiments, medium conditioned by CD11c⁺CD206⁺ crown ATMs consistently inhibited glucose uptake at 1 and 10nM insulin (Figure 7).

DISCUSSION
This study documents for the first time two ATM subsets in human obesity with distinct anatomical and functional properties. CD11c⁺CD206⁺ ATMs localise to crowns, express higher levels of integrins, antigen presentation molecules and pro-inflammatory cytokines, and one or more secreted factors that impair insulin action. These are features of classically activated macrophages (24; 26), yet CD11c⁺CD206⁺ ATMs also have features of alternatively activated macrophages, namely high mitochondrial copy number (27) and high levels of IL-10 mRNA and protein, both basally and in response to LPS (24; 28). CD11c⁻ ATMs on the other hand occur as solitary cells and express high levels of scavenger receptors and genes implicated in tissue maintenance and repair. These are features of alternatively activated macrophages (24). CD11c⁻ ATMs also express higher levels of CD34, a marker of ATMs within angiogenic cell clusters (7), as well as PDGFB, a putative mitogen for adipocyte stem cells (29), implying a role for CD11c⁻ ATMs in adipogenesis. Thus, the M1/M2 paradigm of mouse CD11c⁺/CD11c⁻ ATMs (6) may not be entirely applicable to humans, as previously suggested (21). Causality is virtually impossible to establish in humans but, by analogy with mice in which CD11c⁺ macrophages or key macrophage genes have been targeted (3-5; 11), pro-inflammatory ATMs may have a primary role in mediating insulin resistance. This is supported by the relationship between the CD11c⁺/CD11c⁻ ATM ratio and insulin resistance, the overall pro-inflammatory profile of CD11c⁺CD206⁺ ATMs and their ability to impair insulin action in our studies. CD11c⁺CD206⁺ ATMs and/or cells with which they could interact such as T cells may release specific factor(s) that act locally in adipose tissue and/or systemically in key target organs such as liver, muscle and pancreas.

In age- and BMI-matched obese women, insulin resistance was associated with an increased CD11c⁺CD206⁺ ATM density and CD11c⁺/CD11c⁻ ATM ratio; in contrast, CD11c⁻ ATM density was similar in subcutaneous and omental adipose tissue. Because CD11c⁺CD206⁺ ATMs are a minor ATM subpopulation, it is not surprising that
previous studies found no association between total ATM density and insulin resistance after correcting for age, sex and BMI (12; 15-17). Our findings highlight the importance of correcting for clinical heterogeneity and quantitating the CD11c⁺CD206⁺ ATM subpopulation, and are consistent with a recent report that crown macrophage density in subcutaneous abdominal adipose tissue correlated with insulin resistance in an obese, predominately female population (30). Crown aggregate and CD11c⁺CD206⁺ ATM density was lower in omental than subcutaneous adipose tissue, implying that the latter is an important determinant of insulin resistance, but this is at odds with clinical studies which show that visceral adipose tissue correlates better with insulin resistance (31). However, it is consistent with the greater size of subcutaneous versus omental adipocytes and with a recent study in lean and obese men that found subcutaneous not visceral fat mass correlated with insulin resistance (32), and would support the view (33) that visceral adiposity is a consequence rather than a cause of insulin resistance. On the other hand, immunohistochemistry with antibodies to HAM56 and CD40, although semi-quantitative, identified higher a crown ATM density in omental adipose tissue of obese men and women (15; 34). This difference may be methodological or explained by our exclusive study of women or other differences in the study populations.

The relatively high secretion of IL-8 by CD11c⁺CD206⁺ ATMs raises the possibility that CD11c⁺ ATM-derived IL-8 could promote metabolic complications of obesity, consistent with earlier reports that serum IL-8 is increased in obesity and type 2 diabetes (35; 36) and that adipocytes are not the predominant source of adipose tissue-derived IL-8 (37; 38). While IL-8 is a potent neutrophil chemoattractant (39), neutrophils were not evident on tissue sections or in stromovascular preparations, suggesting another role for CD11c⁺ ATM-derived IL-8. Further investigation of IL-8 in human obesity will require specific antagonists, as homologues of human IL-8 and its receptor CXCR1 are absent in mice and rats (39). Phenotyping of ATMs revealed a progressive decrease in expression of integrins from blood monocytes to CD11c⁺CD206⁺, CD11c⁺CD206⁻ and CD11c⁻ ATMs, and absent expression of the CCL2 chemokine receptor CCR2 on CD11c⁻ ATMs. This suggests a developmental pathway from blood monocytes to resident ATMs, in accord with mouse studies reporting that ATMs are derived mostly from blood (10; 13; 14) and that blockade of monocyte recruitment to adipose tissue with a CCL2 receptor antagonist (40) or by genetic ablation of CD49d (41) protects mice against metabolic complications of obesity. Our findings indicate that, in addition to CCR2 antagonists, agents that block the integrins CD11a, CD11b, CD11c, CD31 or CD49d might also be therapeutic in this setting.

By microarray, CD11c⁺CD206⁺ ATMs were enriched for lipid-rich vacuolar and mitochondrial RNAs and transcripts encoding the lipid-binding proteins APOE and FABP4 and enzymes for fatty acid metabolism. This profile is consistent with a role for CD11c⁺CD206⁺ ATMs in converting potentially toxic lipid from dead adipocytes into non-toxic lipoproteins for disposal from adipose tissue into the circulation (8). CD11c⁺CD206⁺ ATMs were also enriched for transcripts encoding components of the lysosome and proteasome, and for proteins implicated in T-cell attraction and activation. These features, together with their pro-inflammatory phenotype, raise the possibility that CD11c⁺CD206⁺ ATMs could initiate adaptive immune responses to adipose tissue antigens, analogous to presentation of oxidised low-density lipoprotein to T cells by CD1 on foamy macrophages in atherosclerotic lesions (42). Recent studies in
obese mice implicate antigen-specific adipose tissue T cells in insulin resistance (43-45) and suggest that cross-talk between T cells and CD11c^+CD206^+ ATMs could promote insulin resistance and perhaps other complications of obesity in humans. Of the few genes definitely associated with insulin resistance in obesity identified from genome-wide scans, PPAR-γ was the only one overrepresented in CD11c^-CD206^+ ATMs.

In summary, we characterize distinct CD11c^- and CD11c^+ subsets of human ATMs and present evidence that CD11c^+CD206^+ ATMs form crowns, are enriched in the adipose tissue of insulin-resistant women, are pro-inflammatory and impair insulin action. It will be important to determine whether CD11c^+CD206^+ ATMs are also associated with features of the metabolic syndrome in other ethnic groups and in men, whose ATM density is reported to be higher than that of BMI-matched women (15). Finally, identifying the factors elaborated by CD11c^+CD206^+ ATMs that impair insulin action may lead to novel anti-inflammatory therapies for the complications of obesity.

**EXPERIMENTAL PROCEDURES**

**Subjects and tissue:** Initially, tissues were obtained from 29 Caucasian women undergoing laparoscopic surgery for insertion or revision of a gastric band. Subsequently, tissues were obtained from a further 89 Caucasian women to confirm initial findings and undertake mechanistic studies. Subcutaneous and omental adipose tissues were resected from the peri-umbilical region and omentum near the Angle of His, respectively, placed in DME medium (Sigma, Australia) supplemented with 20mM Hepes (Sigma) and transported to the laboratory within 2 hours. Approval was given by the Human Research and Ethics Committees of The Avenue Hospital and Walter and Eliza Hall Institute of Medical Research.

**Biochemistry:** Analyses were performed on fasting blood samples provided within 3 months of surgery. Insulin resistance was determined using the HOMA2 calculator (46). Adiponectin and leptin Luminex assays (Linco Research, MI) and high molecular weight (HMW) adiponectin ELISA (Fujirebio, Japan) were performed on sera collected immediately prior to anaesthesia. Cytokine/chemokine concentrations in cell culture supernatants were determined by Luminex assays (Linco Research).

**Immunohistochemistry:** Formalin-fixed 5µm adipose tissue sections were de-waxed in xylene and boiled in antigen unmasking solution (Vector, CA) for 15 minutes. Frozen 12µm sections cut from adipose tissue embedded in OCT compound (Tissue-Tek, CA) were fixed in acetone. Primary antibodies used were: CD68 (PG-M1; Dako, Denmark), CD11c (563; Novocastra, UK), CD206 (551135; BD Biosciences, Australia) and VDAC1 (ab14734; Abcam, UK). For single antibody stains, sections were treated with 1% H_2O_2 then washed in PBS before overnight incubation with primary antibody. Antigen signal was detected by further incubation with biotinylated anti-mouse IgG antibody (Dako) followed by streptavidin-HRP (Vector) and then DAB+ liquid (DAKO). For immunofluorescence, anti-mouse Alexa488 (Invitrogen, CA), biotinylated anti-mouse IgG3 (BD Biosciences) and streptavidin-Alexa594 (Invitrogen) were used as secondary antibodies. Images were obtained using an Axioskop2 microscope (Zeiss, Germany) and AxioVision v4.6 software (Zeiss). Oil Red O staining was performed on cells fixed in formalin vapour by incubation in 0.6% w/v Oil Red O (Sigma) in 60% v/v isopropanol for 15 minutes followed by counterstaining with hematoxylin.

Crowns were defined as three or more CD68-positive cells surrounding an adipocyte and crown density as the number of crowns per
high power field. A minimum of four low-power (40x) fields was counted on two separate occasions by one observer (JW), blinded to the adipose tissue donor. Mean adipocyte size in pixels was determined from three 100x fields using ImageJ software (NIH, MD).

**Adipose tissue digestion and flow cytometry:** Adipose tissue was minced with sterile scissors, centrifuged at 755g to remove blood cells and digested in DME/Hepes (10mL/2g) supplemented with 10mg/mL fatty acid-poor BSA (Calbiochem, CA), 35µg/mL liberase blendzyme 3 (Roche, IN) and 60U/mL DNase I (Sigma) in a 37º waterbath. Samples were minced every 5 minutes for 50 minutes then passed through a sterile strainer before centrifuging at 612g. The stromovascular cell pellet was treated with red cell lysis solution (155mM NH₄Cl, 10mM KHCO₃ and 90µM EDTA), applied to a 70µm filter and suspended in FACS buffer (PBS/1mM EDTA/5mg/mL fatty acid-poor BSA). For cytokine release and gene microarray experiments, stromovascular cells were initially separated into CD14⁺ and CD14⁻ fractions with anti-CD14 magnetic beads and MS separation columns (Miltenyi, Australia). Flow cytometry was performed on a FACSAria (weekly CV<7%) and analysed with FacsDiva v 6.0 software (BD Biosciences). Cells were stained in the presence of 10%v/v fluorochrome-conjugated antibodies and suspended in FACS buffer containing 0.05µg/mL propidium iodide (PI). FSc-A/FSc-H and PI gates were used to identify single, live cells. Fluorescence intensity was normalised by subtracting the fluorescence intensity of PE-conjugated isotype-control antibody or of cells incubated in the absence of Mitotracker Red. For cell sorting, CD14⁺, CD14⁻ or stromovascular preparations were stained with CD11c-APC, CD14-FITC, CD45-PC7 and CD206-PE antibodies. The purity of all isolations was >90% (Supplemental Figure 2). All antibodies were obtained from BD Biosciences with the exception of TLR2-PE and TLR4-PE (BioLegend, CA), CCR2-PE (R&D Systems, MN) and CD14-PC7 and CD45-PC7 (Beckman Coulter, France). Mitotracker red mitochondrial dye was from Molecular Probes (OR).

**Conditioned medium:** For measurement of secreted cytokines, ATMs were sorted from the CD14⁺ stromovascular population, suspended at 20,000 in 100µL RPMI medium (Sigma) supplemented with 10% FCS (Thermo Electron, Melbourne) and cultured±20 ng/mL LPS in 96-well flat-bottom tissue culture plates (BD Labware, NJ) for 24 hr. Cell viability after 24 hr, determined by ethidium bromide/acridine orange uptake (BDH Chemicals, UK), was 60-90%.

To condition medium to screen for inhibition of insulin action, individual cell types were sorted directly from the stromovascular population, suspended in serum-free F3 medium (DME/HAMF12 containing 8mg/L biotin, 4mg/L pantothenate, 2mg/L gentamicin, 2mM Glutamax, 10mg/L transferrin, 200pM triiodothyronine and 100nM hydrocortisone; all from Sigma) and cultured in 96-well round-bottom plates for 48 hr.

**Insulin-stimulated glucose uptake:** Freshly isolated human adipocytes rapidly lose viability and sensitivity to insulin. The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell line, which is neither transformed nor immortalized, can be induced to differentiate in vitro, providing a unique tool for the study of human adipocytes and their response to insulin (47). SGBS preadipocytes were first differentiated in 96-well plates as previously described (47) and then cultured for 48h in serum-free F3 medium conditioned (20% v/v) by medium from total stromovascular cells, CD11c+CD206+ ATMs, CD11c- ATMs, LYM or PA cells. After 48h, adipocyte
morphology and LDH activity of the supernatant was not significantly different between groups. Adipocytes were then washed in KRP buffer (136mM NaCl, 4.5mM KCl, 1.25mM CaCl₂, 1.25mM MgCl₂, 0.6mM Na₂HPO₄, 0.4mM NaH₂PO₄, 10mM HEPES, 0.1% BSA), incubated at 37º C for 20 minutes in 40µL KRP ± insulin before addition of 10µL KRP containing 0.25mM unlabeled 2-deoxyglucose and 0.1µCi tritiated 2-deoxyglucose (PerkinElmer) for a further 10 minutes. Cells were then washed with ice-cold PBS containing 80mg/L phloretin (Sigma) before lysis in 0.1M NaOH, transfer into UltimaGold scintillant (PerkinElmer) and β-scintillation counting. Counts (cpm) were corrected for cell-free blanks. The intra-assay CV for replicate control wells was 5-18%.

Quantitative PCR and gene microarray: RNA was prepared from cell pellets using a Picopure RNA kit (Arcturus, CA). DNA was prepared by proteinase K digestion and ethanol precipitation. Primer sequences are shown (Supplemental Table 2). Amplification efficiencies of primer pairs were not significantly different over the concentration ranges measured. For RT-PCR, total RNA was reverse-transcribed using Superscript III (Invitrogen) and amplified on an ABI Prism 7700 platform using the Sybergreen reporter (Qiagen, CA). Gene expression relative to β-actin was determined using the comparative Cₜ method.

Gene microarray of CD11c⁺ and CD11c⁺CD206⁻ ATMs was performed using 50ng total RNA and HumanWG-6 v2 BeadChips (Illumina, CA). Microarray analysis used the lumi, limma and annotation packages of Bioconductor (48). Expression data was background-corrected using negative control probes followed by a variance stabilising transformation (49) and quantile normalisation. Gene-wise linear models were fitted to determine differences between cell populations, taking into account subject-to-subject variability. Significant differentially expressed genes were identified using empirical Bayes moderated t-tests and a false discovery rate of 5% (50). Functional annotation clustering analysis (25) of differentially expressed genes was performed online (www.david.abcc.ncifcrf.gov) using default settings.

Statistical analysis: Statistical analyses were performed with Prism v5.0a software for Macintosh (Graphpad, San Diego). Pairs of groups were compared by the Mann-Whitney test or Wilcoxon matched pairs test as appropriate. Multiple groups were analysed by ANOVA, with Neuman-Keuls post-test comparisons of group pairs. Correlation was determined by Spearman rank-log test. p<0.05 was considered significant.

ACKNOWLEDGMENTS

We thank the women who participated in this study, the staff of The Avenue Hospital who assisted with adipose tissue collection and Jennifer Carden and Anthony Burn for blood sampling. Jon Whitehead arranged transfer of SGBS cells to our laboratory and Catherine McLean provided secretarial assistance. This work was funded by Program (516700) and Infrastructure (361646) grants from the National Health and Medical Research Council of Australia (NHMRC), a Victorian State Government Operational Infrastructure Support Grant, the Diabetes Australia Research Trust and the Royal Australian College of Physicians Research Foundation. JMW is a Doherty Fellow and LCH a Senior Principal Research Fellow of the NHMRC. The authors declare no relevant conflict of interest.
REFERENCES
Adipose tissue macrophages in human obesity


33. Miles JM, Jensen MD. Counterpoint: visceral adiposity is not causally related to insulin resistance. Diabetes Care 2005;28:2326-2328

FIGURE LEGENDS

Figure 1. Immunohistochemistry of subcutaneous adipose tissue in obesity a. Formalin-fixed adipose tissue section stained for CD68 showing a crown (asterisk) and several resident ATMs (arrowheads). b. Serial section stained for CD11c showing crown ATMs preferentially expressing CD11c. c. Formalin-fixed adipose section stained for CD11c and CD68 or with non-specific isotype control antibodies confirming that crown ATMs (upper panel) but not resident ATMs (middle panel) express CD11c. d. Frozen adipose tissue section stained for CD206 showing a crown (asterisk) and several resident ATMs. e. Frozen section stained for CD206 and CD11c (upper panel) or with isotype control antibodies (lower panel) showing crown ATMs expressing both CD11c and CD206 but resident ATMs expressing only CD206. Scale bar in all images=50µm.

Figure 2. Flow cytometry of subcutaneous adipose tissue stromovascular cells a. After excluding doublets and dead cells, a CD14 versus CD45 contour plot identified three stromovascular cell populations (PA, LYM and ATM). b. Cytospins of each population stained for CD68 confirmed that the CD14+CD45+ population represents ATMs. Scale bar=20µm. c. Flow cytometry phenotype of gated PA, LYM and ATM populations stained for CD11c and CD206 reveals that PA cells do not express either marker while a minority of LYM cells express low levels of CD11c. ATM cells could be separated into three distinct subpopulations when stained for CD11c and CD206; one of these, CD11c+CD206− ATMs, had a similar phenotype to blood monocytes (MONO). Background staining by isotype control antibodies is indicated by grey lines. d. CD11c+CD206− and CD11c− ATMs were isolated by flow cytometry cell sorting and stained with Oil Red O. Representative images show CD11c+CD206+ ATMs contain more lipid than CD11c− ATMs. Scale bar=10µm.

Figure 3. ATM quantitation in subcutaneous and omental adipose obtained from FOb, Ob and ObMS women a, b. Crown density and mean adipocyte size determined by histology. c. Significant correlation between histological and flow cytometry measures of crown ATM density. d, e. CD11c+CD206+ (crown) and CD11c− (resident) ATM densities determined by flow cytometry. f. The CD11c+CD206+/CD11c− ratio is increased in ObMS women, confirming enrichment of crown ATMs in the metabolic syndrome. g, h. Relationship between insulin
Adipose tissue macrophages in human obesity

resistance and CD11c⁺CD206⁻/CD11c⁻ ratio in subcutaneous and omental adipose in the initial cohort of 24 obese women and in a subsequent cohort of 89 obese women. *, **, ***; p< 0.05, 0.005, 0.0005, respectively (Mann Whitney t test).

Figure 4. Cell surface phenotype of subcutaneous ATMs and blood monocytes a. Innate immune molecules; b. Integrins; c. CD34; d. Scavenger receptors; e. Adaptive immune molecules. Monocytes and ATM subsets were identified using anti-CD206FITC, -CD11cAPC and -CD14PC7 antibodies. PE-conjugated antibodies were used to determine the expression of cell surface markers. Results are mean±sem of three independent experiments. *, significantly different (P<0.05) in ANOVA by post-test comparison.

Figure 5. Cytokine secretion by cultured ATMs Subcutaneous CD11c⁻ and CD11c⁺CD206⁺ ATMs (20,000 cells/well) were isolated by flow cytometry cell sorting and cultured in duplicate for 24 hr in 100µL RPMI/10%FCS±20ng/mL LPS. Cytokine concentrations in the supernatant are shown. Results are mean±sem of three independent experiments. Differences between CD11c⁻ and CD11c⁺CD206⁺ ATMs for all cytokines except CCL2 were significant (p<0.05 by paired t-test) following normalisation.

Figure 6. Crown ATMs and preadipocytes are enriched for mitochondria a. Formalin-fixed adipose section stained with antibodies against VDAC1 and CD68 or non-specific antibodies. VDAC1 staining intensity was greatest for crown ATMs and less intense for resident ATMs and CD68-negative cells. Scale bar = 50µm. b. Mitochondria per cell was calculated as the relative amount of mitochondrial versus genomic DNA, determined by qPCR of DNA isolated from sorted stromovascular cells using CYTB and LEP primers. Results are mean±sem of three independent experiments. *, significantly different (P<0.05) in ANOVA by post-test comparison. c. Stromovascular cells were incubated in RPMI/10% FCS with or without 50nM Mitotracker Red for 15 minutes in a 37º waterbath and then incubated on ice with CD206FITC, CD11cPE and CD45PC7 antibodies before analysis using flow cytometry. Results are mean±sem of three independent experiments. *, significantly different (P<0.05) in ANOVA by post-test comparison.

Figure 7. Crown ATMs release factors that inhibit insulin action Serum-free F3 medium was conditioned for 48 hr with 50,000 total stromovascular cells, CD11c⁺CD206⁺ ATMs, CD11c⁻ ATMs, LYM or PA cells. Differentiated human SGBS adipocytes were then incubated in conditioned medium (20% v/v) in fresh F3 medium for 48 hr, before measurement of insulin-stimulated ³H-deoxyglucose uptake. Data (mean±sem) are from four independent experiments using cells isolated from subcutaneous (n=3) and omental (n=1) adipose tissue from three obese women with metabolic syndrome. Glucose uptake was normalised to the cpm values from total stromovascular cell-conditioned medium and 10nM insulin (100%). *, significantly different (P<0.05) in ANOVA by post-test comparison.
Table 1. Clinical characteristics of the formerly obese (FOb), obese (Ob) and obese with metabolic syndrome\(^\#\) (ObMS) women studied

<table>
<thead>
<tr>
<th></th>
<th>Obese</th>
<th>FOb, n=5</th>
<th>Lean</th>
<th>Ob, n=12</th>
<th>ObMS, n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35±5 (20-52)</td>
<td>39±6 (21-54) *</td>
<td>46±2 (28-55)</td>
<td>47±3 (30-61) *</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>40±3 (34-46)</td>
<td>26±1 (23-30) **</td>
<td>46±1 (39-53)</td>
<td>44±1 (39-56)</td>
<td></td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.85±0.01 (0.82-0.87)</td>
<td>0.80±0.01 (0.76-0.82) **</td>
<td>0.85±0.02 (0.75-0.98)</td>
<td>0.93±0.02 (0.86-1.1) ** ††</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>4.6±0.3 (3.8-5.5)</td>
<td>4.5±0.2 (4.1-5.0)</td>
<td>5.0±0.1 (4.7-5.5)</td>
<td>6.4±0.5 (5.0-10.0) * ††</td>
<td></td>
</tr>
<tr>
<td>Fasting insulin (mIU/L)</td>
<td>12±1 (8-15)</td>
<td>4±0.4 (3-5) **</td>
<td>13±2 (4-26)</td>
<td>24±3 (13-47) * †</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.5±0.2 (1.0-2.0)</td>
<td>0.46±0.05 (0.4-0.6) **</td>
<td>1.6±0.3 (0.5-3.3)</td>
<td>3.2±0.4 (1.7-6.3) ** ††</td>
<td></td>
</tr>
<tr>
<td>Fasting triglycerides (mmol/L)</td>
<td>1.4±0.3 (0.8-2.5)</td>
<td>1.0±0.2 (0.7-1.7)</td>
<td>1.4±0.1 (0.6-2.5)</td>
<td>2.4±0.4 (1.1-6.2) * †</td>
<td></td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.5±0.1 (1.4-1.7)</td>
<td>1.4±0.1 (1.1-1.7)</td>
<td>1.5±0.1 (1.2-2.0)</td>
<td>1.1±0.1 (0.7-1.7) * †</td>
<td></td>
</tr>
<tr>
<td>Alanine transaminase (IU/L)</td>
<td>25±7 (12-53)</td>
<td>17±2 (14-22)</td>
<td>20±2 (14-35)</td>
<td>44±10 (16-144) ††</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>1/5</td>
<td>1/5</td>
<td>2/12</td>
<td>5/9</td>
<td></td>
</tr>
<tr>
<td>Type 2 Diabetes</td>
<td>0/5</td>
<td>0/5</td>
<td>0/12</td>
<td>3/12</td>
<td></td>
</tr>
<tr>
<td>Metabolic Syndrome Score</td>
<td>1.4±0.2 (1-2)</td>
<td>0.2±0.2 (0-1)**</td>
<td>1.3±0.5 (1-2)</td>
<td>3.4±0.7 (3-5) ** ††</td>
<td></td>
</tr>
<tr>
<td>Fasting adiponectin (mg/L)</td>
<td>np</td>
<td>19±3 (11-26)</td>
<td>13±1 (8-25)</td>
<td>11±1 (7-22)</td>
<td></td>
</tr>
<tr>
<td>Fasting HMW adiponectin (mg/L)</td>
<td>np</td>
<td>15±3 (7-25)</td>
<td>9±1 (5-21)</td>
<td>7±1 (4-15)</td>
<td></td>
</tr>
<tr>
<td>Fasting leptin (µg/L)</td>
<td>np</td>
<td>47±5 (35-61)</td>
<td>64±7 (34-100)</td>
<td>65±6 (34-130)</td>
<td></td>
</tr>
</tbody>
</table>

\(^\#\) Metabolic syndrome is defined as three or more of: waist >88cm, BP >130/85, fasting glucose >5.6mmol/L, HDL <1.2 mmol/L and triglyceride >1.7mmol/L.

Continuous data are presented as mean±s.e. (range) *: p<0.05 cf obese FOb, **: p<0.005 cf obese FOb, †: p<0.05 cf Ob, ††: p<0.005 cf Ob, np: not performed.
Figure 1

A

B

C

D

Adipose tissue macrophages in human obesity
Figure 2

A

B

C

D

CD11c^+CD206^+ATM  CD11c^-ATM
Figure 3

A

B

C

D

E

F

G

H

Adipose tissue macrophages in human obesity
Figure 4

Adipose tissue macrophages in human obesity

Figure 4

A

B

C

D

E

Blood monocyte
CD11c+CD206+ ATM
CD11c+CD206- ATM
CD11c+ ATM

Mean fluorescence intensity

CD14

TLR2

TLR4

CCR2

P<0.0001
P=0.3
P=0.2
P=0.004

Mean fluorescence intensity

CD11a

CD11b

CD31

CD49d

P<0.0001
P=0.08
P=0.008
P<0.0001

P<0.01

Mean fluorescence intensity

CD34

P=0.01

Mean fluorescence intensity

CD16

CD64

CD36

CD163

P=0.03
P=0.19
P=0.5
P=0.02

Mean fluorescence intensity

CD45

HLA-DR

CD1c

CD86

P=0.03
P<0.0001
P=0.02
P=0.02
Figure 6

A

VDAC1  CD68  Merge

B

C

Mitochondrial/cell

Mitotracker red (MFI)

CD11c-CD206+ ATM  CD11c- ATM  LYM  PA

CD11c-CD206+ ATM  CD11c- ATM  LYM  PA
Figure 7

[Graph showing Relative \(^3\)H-deoxy glucose uptake (%) against Insulin concentration (nM) for different cell types: Stromovascular cells, CD11c\(^+\)CD206\(^+\) ATM, CD11c\(^-\) ATM, LYM, and PA. Different concentrations are shown on the x-axis (0, 0.1, 1, 10 nM), and the y-axis shows the percentage uptake. Significant differences are indicated by asterisks (*) for some conditions.]