Macrophage Infiltration into Omental *Versus* Subcutaneous Fat across Different Populations: Effect of Regional Adiposity and the Comorbidities of Obesity

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Context: Macrophage infiltration into adipose tissue has been demonstrated to accompany obesity, with a potential preferential infiltration into intraabdominal *vs.* sc fat.

Objective: Our objective was to determine whether this occurs across different populations with a range of body mass indexes and to assess the relationship with regional adiposity and comorbidity of obesity.

Setting and Patients: In two independent cohorts, we used paired omental (OM) and sc fat biopsies from lean controls or predominantly sc or intraabdominally obese persons with minimal comorbidity (n = 60, cohort 1), or from severely obese women with a significant rate of comorbidity (n = 29, cohort 2).

Results: Elevated macrophage infiltration into OM *vs.* sc fat was observable in lean subjects and exaggerated by obesity, particularly if predominantly intraabdominal. This was paralleled by increased monocyte chemoattractant protein-1 (MCP1) and colony-stimulating factor-1 (CSF1) mRNA levels. Level of CSF1 and MCP1 mRNA correlated with the number of OM macrophages (r = 0.521, $P < 0.0001$ and r = 0.258, $P < 0.051$, respectively). In severely obese women (mean body mass index = $43.0 \pm 1.1 \text{ kg/m}^2$), higher protein expression of both MCP1 and CSF1 was detected in OM *vs.* sc fat. Number of OM macrophages, but not of sc macrophages, correlated with waist circumference (r = 0.636, $P = 0.001$ *vs.* r = 0.170, $P = 0.427$) and with the number of metabolic syndrome parameters (r = 0.385, $P = 0.065$ *vs.* r = −0.158, $P = 0.472$, respectively). Preferential macrophage infiltration into OM fat was mainly observed in a subgroup in whom obesity was associated with impaired glucose homeostasis.

Conclusions: Preferential macrophage infiltration into OM fat is a general phenomenon exaggerated by central obesity, potentially linking central adiposity with increased risk of diabetes and coronary heart disease. (*J Clin Endocrinol Metab* 92: 2240–2247, 2007)

MACROPHAGE INFILTRATION into adipose tissue was recently shown to accompany obesity (1, 2), suggesting that a low-grade inflammatory state develops in fat tissue (3). Initial studies in rodents implied that infiltrating macrophages are of bone-marrow origin (1) and may be recruited in response to death of hypertrophied fat cells (4) and/or because of excessive secretion from adipose tissue of proinflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP1) (5, 6). A major unanswered question is whether macrophage infiltration plays a causal role in the comorbidity of obesity or is merely a consequence of the obese state. Causality was suggested in mice by the observation that an increase in macrophage-specific gene expression in fat tissue precedes, or coincides with, a decrease in insulin sensitivity after initiation of high-fat feeding (2).

When a genetic approach was used to interfere with MCP1 action (by knockout of either MCP1 or its receptor CC chemokine receptor 2), protection against insulin resistance was observed (5, 6). In human obesity, directly establishing a causative role for macrophage infiltration into adipose tissue in the comorbidity of human obesity is difficult. Nevertheless, infiltration of fat by macrophages has been reported in humans (1, 4). Moreover, the extent of macrophage infiltration into sc fat tissue was shown to decrease upon weight loss (7) or after insulin infusion in insulin-sensitive (but not -resistant) subjects (8). These studies demonstrate a dynamic responsiveness of fat tissue macrophage infiltration to metabolic and/or nutritional cues. However, the contribution of fat tissue macrophages to the pathophysiology of human obesity and its related morbidities are still largely unknown. An intriguing hypothesis would be that macrophage infiltration can be mechanistically linked to the increased pathogenicity of intraabdominal (IA) fat (9, 10). Indeed, several indirect lines of evidence exist for increased macrophage infiltration into omental (OM) fat (11). Very recently, macrophage infiltration into OM fat, but not into sc fat, was shown to be associated with clinical parameters of obesity comorbidity, as well as with the severity of histological changes in liver biopsies (12). In the present study, we aimed at expanding on these observations by addressing the fol-

lowing questions. 1) Can these findings be generalized to other cohorts? 2) Is the increased macrophage infiltration into OM (*vs.* sc) fat affected by fat distribution (sc *vs.* IA adiposity)? 3) What is the occurrence of depot-specific macrophage infiltration in the full range of body mass index (BMI), from the lean to the morbidly obese? To address these questions, we present data from two complementary but independent cohorts for which paired OM and abdominal-sc fat tissue biopsies have been obtained.

Subjects and Methods

Experimental subjects

Cohort 1. The Leipzig (Germany) cohort fat-tissue bank consists of paired samples of OM and sc adipose tissue obtained from Caucasian men (n = 30) and women (n = 30) who underwent open abdominal surgery for gastric banding, weight reduction surgery, or explorative laparotomy (with negative findings). The cohort was established to characterize alterations in the two fat depots in response to obesity *per se* rather than to its associated comorbidities.

　　Therefore, included were lean, healthy controls and obese subjects, which fulfilled the following inclusion criteria: 1) absence of any acute or chronic inflammatory disease as determined by a leukocyte count of less than 6000 per mm², C-reactive protein less than 3 mg/dl, and absence of any clinical sign of infection; 2) glycated hemoglobin A1c less than 7.5% and fasting plasma glucose less than 180 mg/dl (four individuals in the sc obese and six subjects in the IA-obese group had type 2 diabetes and were treated with metformin); 3) systolic blood pressure less than 160 mm Hg and diastolic blood pressure less than 95 mm Hg; 4) low-density lipoprotein cholesterol less than 100 mg/dl and high-density lipoprotein cholesterol more than 35 mg/dl; 5) no clinical evidence for either cardiovascular or peripheral artery disease; 6) no concomitant medication intake, except for metformin in patients with type 2 diabetes; 7) no alcohol or drug abuse; and 8) no pregnancy. Patients with malignant diseases were also excluded from the study.

　　BMI was calculated as weight divided by squared height. Waist circumference was measured in the standing position in the midline between iliac crest and the lowest rib, or at the maximum trunk circumference (when different from that location, particularly in severely obese persons). Percent body fat was measured by dual x-ray absorptiometry. To determine predominantly IA and sc adipose tissue areas, computed tomography (CT) scans were taken at the L4–L5 level of the lumbar spine with an attenuation range of −30 to −190 Hounsfield units.

Using a combination of BMI and the IA to sc fat ratio, we stratified the participants into lean (BMI < 25 kg/m²; n = 20) and two obese groups (BMI > 28.5 kg/m²), with predominant IA (CT ratio > 0.5; n = 20) or predominant sc (CT ratio < 0.5; n = 20) adipose tissue accumulation. Basal, fasting blood samples were taken after an overnight fast to determine glucose, insulin, and standard laboratory parameters. Plasma insulin was measured with a two-site chemiluminescent enzyme immunometric assay for the IMMULITE automated analyzer (Diagnostic Products, Los Angeles, CA). Patients gave written informed consent to all aspects of the study, and all procedures were approved by the ethics committee of the University of Leipzig. The clinical characteristics of patients in cohort 1 are shown in Table 1.

Cohort 2. The Beer-Sheva (Israel) cohort fat tissue bank includes severely obese women undergoing elective laparoscopic abdominal surgery (mostly gastric banding). The cohort was established to study biochemical differences between the two depots in severe obesity, frequently associated with significant comorbidity. Clinical parameters were obtained in the preoperative outpatient clinic as described above for cohort 1, and all biochemical and endocrinological determinations (after overnight fast) were performed in the central laboratories of the Soroka University Medical Center using Olympus analytical equipment and reagents. All procedures in this study have been conducted in accordance with the guidelines in the Declaration of Helsinki and were approved in advance by the Soroka's University Medical Center institutional review committee. Patients gave written informed consent to all procedures.

　　Insulin resistance was determined in cohort 1 by the euglycemic-hyperinsulinemic clamp (13), and in cohort 2 by the homeostatic model assessment for insulin resistance (HOMA-IR); HOMA-IR = [fasting insulin (μU/ml) $\times$ fasting glucose (mmol/liter)/22.5], and insulin resistance was defined as HOMA-IR greater than 2. In both centers, fat tissue biopsies were immediately transferred from the operating room to the lab, where they were rinsed, fixed in 4% formaldehyde, or frozen in liquid nitrogen and stored in −80 C freezers until processed.

Histological examination

　　The sc and OM fat samples were fixed at room temperature in 4% formaldehyde and embedded in paraffin. Five-micrometer sections were mounted on glass slides, deparaffinized in xylol, and stained for CD68 using anti-CD68 monoclonal mouse antihuman antibody (Dako, Glostrup, Denmark; close PGM1 M0876, dilution 1:100), using standard immunohistochemistry methods. Macrophages were identified in the adipose parenchyma (CD68 within blood vessels were excluded) when cytoplasmic staining for CD68 was present along with an identifiable

TABLE 1. Clinical and biochemical characteristics (age-adjusted): Leipzig cohort (cohort 1, n = 60)

	Lean (n = 20)		sc obese (n = 20)		IA obese (n = 20)	
	Male (n = 10)	Female (n = 10)	Male (n = 10)	Female (n = 10)	Male (n = 10)	Female (n = 10)
Age (yr)	48 ± 13	43 ± 14	58 ± 11	53 ± 15	55 ± 7[a]	69 ± 13[c,d]
BMI (kg/m²)	23.9 ± 1.9	24.2 ± 2.2	37.2 ± 1.8[b]	36.4 ± 1.7[b]	35.7 ± 1.7[c]	36.4 ± 2.6[c]
Waist circumference (cm)	87.2 ± 5.2	70.5 ± 6.0	140.6 ± 4.9[b]	135.1 ± 4.7[b]	138.0 ± 4.7[c]	129.6 ± 7.1[c]
Diastolic blood pressure	77.5 ± 2.0	75.1 ± 2.3	79.8 ± 1.9	82.6 ± 1.9	84.7 ± 1.8	86.4 ± 2.8[c]
Systolic blood pressure	123.9 ± 3.4	117.9 ± 3.9	142.7 ± 3.2[b]	146.8 ± 3.1[b]	141.0 ± 3.1[c]	145.2 ± 4.6[c]
Fasting plasma glucose (mmol/liter)	5.4 ± 0.4	5.2 ± 0.5	5.3 ± 0.4	6.6 ± 0.4	5.9 ± 0.4	5.2 ± 0.6
Fasting plasma insulin (μU/ml)	4.0 ± 4.4	4.0 ± 5.1	17.3 ± 4.2	33.0 ± 4.1[b]	26.6 ± 4.1[c]	23.1 ± 6.1
Triglycerides (mg/dl)	204.7 ± 29.8	204.3 ± 34.4	255.4 ± 28.3	184.2 ± 27.3	148.1 ± 27.2	161.1 ± 40.9
HDL-cholesterol (mg/dl)	56.3 ± 4.7	55.1 ± 5.4	46.0 ± 4.5	56.0 ± 4.3	53.6 ± 4.3	52.4 ± 6.5
GIR (ml/min)	94 ± 7	85 ± 8	88 ± 8	39 ± 7[a,b]	40 ± 8[c,d]	59 ± 9
No. of metabolic syndrome parameters	0.9 ± 0.1	1.2 ± 0.3	1.8 ± 0.3	1.9 ± 0.4	2.4 ± 0.5	2.6 ± 0.6
% fat and distribution						
CT ratio	NA	NA	0.26 ± 0.1	0.21 ± 0.1	0.73 ± 0.2	0.89 ± 0.1
Body fat (%)	22.2 ± 2.1	22.4 ± 2.4	43.8 ± 2.0[b]	41.6 ± 1.9[b]	35.3 ± 1.9[c]	38.2 ± 2.9[c]

　　Age adjustment was performed for all variables, except age and the composite measures (no. of metabolic syndrome parameters and CT ratio). Metabolic syndrome parameters were according to the NCEP ATP III criteria. Data are means ± SE. GIR, Glucose infusion rate during the steady state of a euglycemic-hyperinsulinemic clamp; HDL, high-density lipoprotein.

[a] $P < 0.05$ between males and females in each group.
[b] $P < 0.05$ for sc obese group *vs.* lean.
[c] $P < 0.05$ for IA obese *vs.* lean.
[d] $P < 0.05$ for sc obese *vs.* IA obese (for [b], [c], and [d], all comparisons are in the same gender only).

mononuclear nucleus and presented as the number per 100 adipocytes (percent macrophages) or as number of cells per 12 ×400 fields, as indicated. Counting was performed blinded to sample data and was confirmed by a certified pathologist (N.S.-V.).

MCP1 and colony-stimulating factor-1 (CSF1) measurements

For protein determination of MCP1 and CSF1, 100 and 200 mg total tissue homogenate protein (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL) was used to determine in duplicate human macrophage-CSF and human chemokine (C–C motif) ligand 2/MCP1, respectively, using commercial ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Final concentration was calculated based on an internal standard curve.

MCP1, CD68, and CSF1 mRNA expression was determined by quantitative real-time RT-PCR in a fluorescent temperature cycler (TaqMan; Applied Biosystems, Darmstadt, Germany). Total RNA was extracted using QIAGEN RNeasy MiniKit (including the RNase-free DNase set; QIAGEN, Hilden, Germany), and 1 μg RNA was reversed transcribed with standard reagents (Life Technologies, Grand Island, NY). Expression of MCP1, CD68, and CSF1 mRNA was quantified relative to 18S rRNA gene expression using the second-derivative maximum method of the TaqMan Software (Applied Biosystems). The following primer pairs were used: human MCP1, 5'-CTCCAAGGGCCTCCTCTAC-3' (sense) and 5'-AAGGGCGAGATGACTCTGAA-3' (antisense); human CSF1, 5'-CCCAGTGTCATCCTGGTCTT-3' (sense) and 5'-CTCCCACG-GAGAGACTGTTC-3' (antisense); and human CD68, 5'-AGTGGACAT-TCTCGGCTCAG-3' (sense) and 5'-TGATGAGAGGCAGCAAGATG-3' (antisense). Human 18S rRNA mRNA expression was measured with a premixed assay on demand for human 18S rRNA (PE Biosystems, Weiterstadt, Germany).

Statistical analyses

We computed age-adjusted means and SEM of the characteristic values of the study population and compared the adjusted means between the subgroups in uni-ANOVA tests (Bonferroni model). Partial (age-adjusted) Pearson's coefficients for correlations between clinical parameters and macrophage infiltration in sc or OM adipose tissue were determined. The variables that were not normally distributed, *e.g.* MCP1, CSF1, and CD68 were natural log-transformed before analysis of correlations, as indicated. Linear regression analysis models was performed to explore the partial independent β-coefficients of sc macrophage infiltration and waist circumference with macrophages in the OM and the partial independent β-coefficients of OM macrophage and sc macrophage counts with number of metabolic syndrome parameters, given the limited size of the sample. To compare means within groups, we performed nonparametric Wilcoxon signed rank pairwise *t* test. All analyses were performed using the SPSS-14 software package. A *P* value of 0.1 was considered the borderline for trend, whereas 0.05 was the cutoff value for statistical significance.

Results

The effect of regional adiposity on macrophage infiltration

To determine the association between regional adiposity and macrophage infiltration, we used data from the Leipzig cohort (cohort 1, Table 1). We compared a lean control group with two obese subgroups characterized by predominantly IA or sc adipose tissue accumulation, as assessed by CT scan measurements of fat distribution. Both obese groups were insulin resistant compared with the lean controls, based on glucose infusion rate and fasting insulin levels. Within the obese groups, insulin resistance was more pronounced in the female subgroup in sc-obese and in the male IA-obese. Triglyceride levels were relatively high, but not statistically different between groups, consistent with other German cohort studies (14–16). Nevertheless, the mean number of positive metabolic syndrome parameters in the various subgroups was 2.6 or lower. Infiltrating macrophages (CD68$^+$ cells with morphological characteristics of macrophages) were readily detectable in the tissue as discrete cells and occasionally were gathered in clusters of four to 10 cells (Fig. 1, A–C). The total number of infiltrating macrophages was directly counted and presented as the number per 100 adipocytes (percent macrophages). CD68-positive macrophages were more highly abundant in OM compared with sc fat in all groups, including lean controls (Fig. 1D). Yet, lean con-
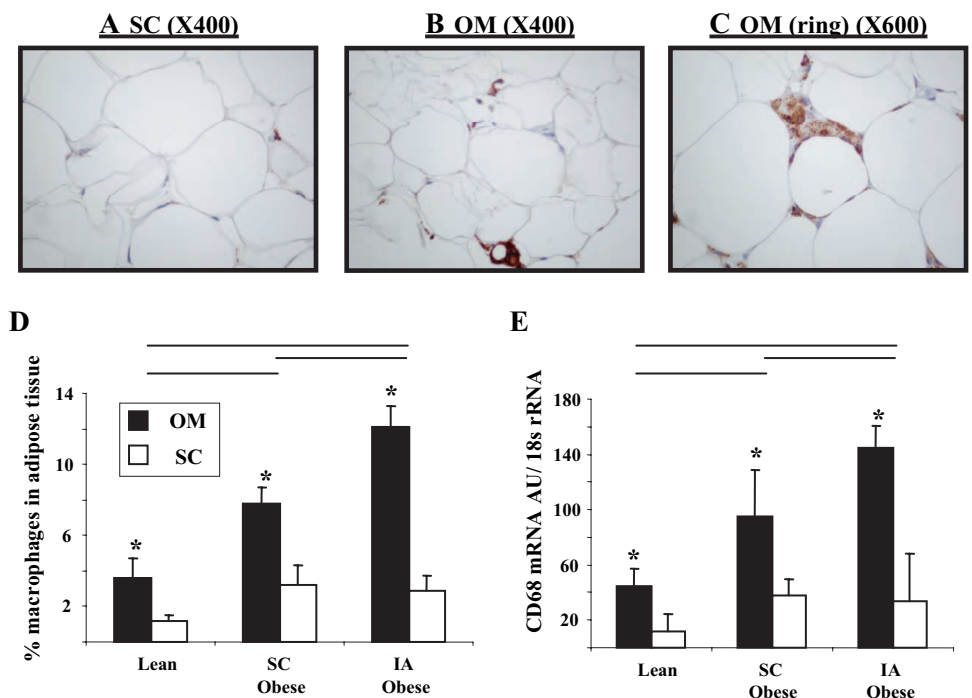

FIG. 1. Increased macrophage infiltration into OM compared with sc fat in lean and predominantly sc- or IA-obese persons (cohort 1). A–C, The sc (A) or OM (B and C) fat biopsies were subjected to immunohistochemistry using anti-CD68 antibodies. D, The number of CD68-positive macrophages per 100 adipocytes is presented for lean controls and persons with predominantly sc or IA obesity of cohort 1, as determined by CT scans. E, Quantitative real-time PCR was used to measure CD68 mRNA in fat tissue samples and was controlled for the abundance of the 18S rRNA in each sample. Results are means ± SE of 20 persons (10 males, 10 females) in each group. *, Significant (*P* < 0.05) differences between two groups (also shown by *lines above bars*).

trols had lower macrophage counts in each depot compared with the respective depot in both obese groups. Intriguingly, IA-obesity was associated with higher macrophage infiltration compared with sc-obese subjects, particularly in OM. The immunohistological results highly correlated with the adipose tissue abundance of CD68 mRNA as measured by quantitative real-time PCR (Fig. 1E). To corroborate the observation that IA-obesity was associated with increased macrophage infiltration particularly into OM, correlation analysis between macrophage count and the CT ratio (as a measure of fat tissue distribution) was performed. Intriguingly, whereas increased CT ratio did not significantly correlate with percent sc macrophages (r = 0.120; P = 0.460), increased IA adiposity was associated with a higher degree of macrophage infiltration into OM (r = 0.535; P < 0.0001).

Macrophage infiltration into adipose tissue is believed to be at least partly mediated by increased expression and secretion of inflammatory cytokines involved in macrophage recruitment and activation, such as MCP1 and CSF1. We therefore determined the relative expression of these cytokines at the mRNA level in the different study groups of cohort 1. Both MCP1 and CSF1 mRNA were more highly abundant in OM compared with sc in the three groups (Fig. 2, A and B, respectively). Obesity, particularly if IA, was associated with increased expression of these cytokines in OM. Because a significant association between age and percent OM macrophages was found (r = 0.39; P = 0.001), age-adjusted correlation analyses were performed. Macrophage infiltration into sc or OM correlated (P < 0.001) with BMI (r = 0.660 and 0.559, respectively), waist circumference (r = 0.746 and 0.680), fasting insulin levels (r = 0.503 and 0.501), and systolic blood pressure (r = 0.583 and 0.531), without displaying a differential relationship between the two depots. In contrast, highly significant correlations were observed between the number of infiltrating macrophages

and CSF1 mRNA expression in OM, whereas only a tendency for a weaker correlation was observable in sc (Fig. 2C). MCP1 mRNA levels tended to correlate weakly with macrophage infiltration only into OM. Collectively, the results demonstrate in a cohort of lean, sc- or IA-obese patients with minimal comorbidity that increased macrophage infiltration occurs in OM *vs.* sc and is exaggerated by obesity particularly if predominantly IA.

Depot-differential macrophage infiltration: association with obesity comorbidity

To characterize macrophage infiltration into OM *vs.* sc in the severely obese BMI range associated with comorbidity, we used data from cohort 2 (Table 2). Twenty-nine severely obese women (BMI range, 32–57 kg/m^2; mean, 43.0 kg/m^2), all of whom met the National Cholesterol Education Panel Adult Treatment Panel III (NCEP ATP III) criteria for central obesity (waist circumference range of 94–145 cm) were included. As a group, the patients were insulin resistant (HOMA-IR = 4.1 ± 2.6). Twenty-seven percent of the cohort exhibited abnormalities in glucose homeostasis (*i.e.* were overtly diabetic or had fasting glucose levels ≥ 100 mg/dl). This subgroup exhibited a significantly higher mean number of metabolic syndrome parameters compared with the severely obese women with normal glucose homeostasis.

The mean macrophage counts assessed by counting 12 ×400 fields per fat depot per person were 19.3 ± 4.1 and 28.8 ± 4.9 CD68-positive cells in sc and OM sections, respectively (Fig. 3A), representing a mean 2.5 ± 0.5-fold higher number of macrophages in OM *vs.* sc fat depots. This difference remained statistically significant (P = 0.046) even when presented as percent macrophages (Fig. 3B), suggesting that increased numbers of OM macrophages could not be attributed to smaller adipocytes in this depot compared with

Fig. 2. MCP1 and CSF1 mRNA expression level in lean and predominantly sc- or IA-obese persons (cohort 1). A and B, Quantitative real-time PCR was used to measure the relative expression (compared with 18S rRNA) of MCP1 (A) or CSF1 (B) in OM or sc fat biopsies from persons in cohort 1. *, Significant (P < 0.05) differences between two groups (also shown by *lines above bars*). C, Age-adjusted Pearson's correlation coefficients were determined between the mRNA level of MCP1, CSF1, and CD68 and the percent macrophages in each fat depot across the entire population of cohort 1 (n = 60).
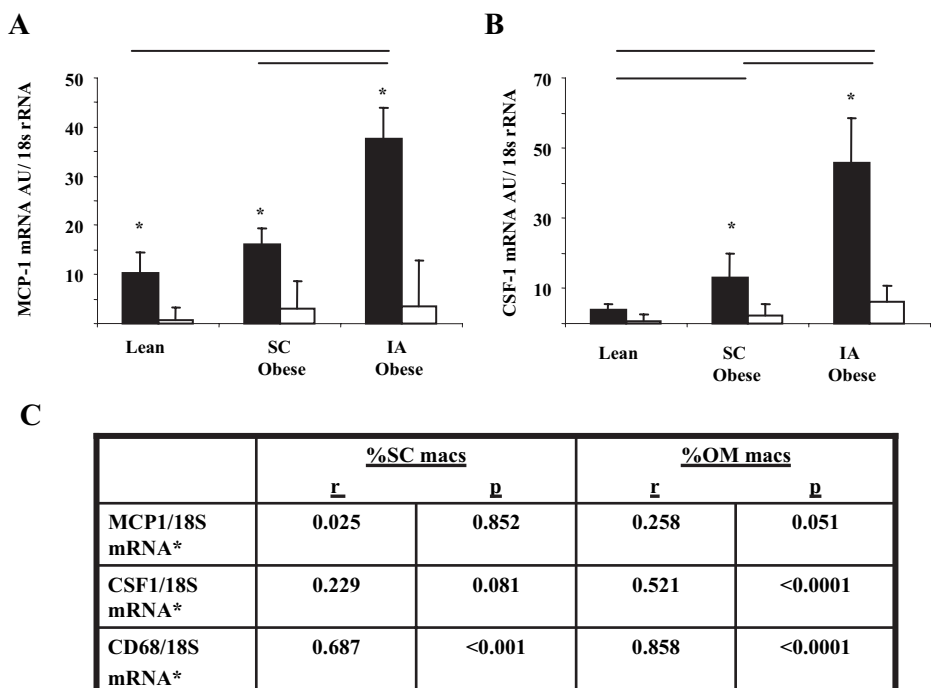
C

	%SC macs		%OM macs	
	r	**p**	**r**	**p**
MCP1/18S mRNA*	0.025	0.852	0.258	0.051
CSF1/18S mRNA*	0.229	0.081	0.521	<0.0001
CD68/18S mRNA*	0.687	<0.001	0.858	<0.0001

TABLE 2. Clinical and biochemical characteristics (age-adjusted): Beer-Sheva cohort (cohort 2, n = 29)

	Entire cohort (n = 29)		Normal glucose homeostasis (n = 19),[a] mean ± SE	Impaired glucose homeostasis or diabetic (n = 8), mean ± SE	P value (normal *vs.* abnormal glucose homeostasis)
	Mean ± SE	Range			
Age (yr)	45.1 ± 2.0	25–75	42.4 ± 2.7	51.6 ± 2.2	0.044
BMI (kg/m^2)	43.0 ± 1.1	32.0–57.8	41.5 ± 1.4	49.0 ± 3.2	0.042
Waist circumference (cm)	120.5 ± 2.2	94–145	117.5 ± 2.7	129.4 ± 7.3	0.143
Diastolic blood pressure	82.8 ± 2.1	64–110	82.0 ± 2.8	79.4 ± 5.9	0.693
Systolic blood pressure	141.0 ± 3.5	109–210	144.0 ± 4.7	138.4 ± 10.0	0.619
Fasting plasma glucose (mmol/liter)	5.2 ± 0.2	3.9–7.8	4.8 ± 0.1	6.8 ± 0.3	<0.001
Fasting plasma insulin (μU/ml)	17.9 ± 1.7	8–45	14.6 ± 2.1	22.3 ± 6.0	0.235
Triglycerides (mg/dl)	139.9 ± 8.6	59–236	134.2 ± 10.0	178.5 ± 30.7	0.186
HDL-cholesterol (mg/dl)	53.2 ± 2.1	32–82	55.9 ± 2.6	43.7 ± 6.3	0.086
HOMA-IR	4.1 ± 0.5	1.7–13.9	3.2 ± 0.3	6.7 ± 1.4	0.001
No. of metabolic syndrome parameters	2.7 ± 0.2	1–5	2.2 ± 0.2	4.1 ± 0.3	<0.001

Age adjustment was performed for all variables, except age and the composite measures (no. of metabolic syndrome parameters and HOMA-IR). Metabolic syndrome parameters were according to the NCEP ATP III criteria. HDL, High-density lipoprotein.
[a] Two missing fasting glucose values in cohort 2.

sc fat. Notably, cohort 2 displayed a lower percent macrophage count in both fat depots (Fig. 3B compared with 1D). We confirmed that this difference was not attributable to technical variations between the laboratories by repeating macrophage counts of slides from cohort 2 in the Leipzig labs (counts of these slides correlated between the laboratories with r = 0.600). Protein levels of MCP1 and of CSF1 were significantly increased in OM compared with sc (Fig. 3, C and D, respectively), in keeping with the findings in cohort 1 at the mRNA level (Fig. 2, A and B).

We next assessed whether macrophage infiltration into OM or sc correlates with clinical and metabolic indices relevant to the comorbidity of obesity in cohort 2. Anthropometric measures such as BMI (r = 0.557; P = 0.003) and waist circumference (Fig. 4A) were significantly correlated with
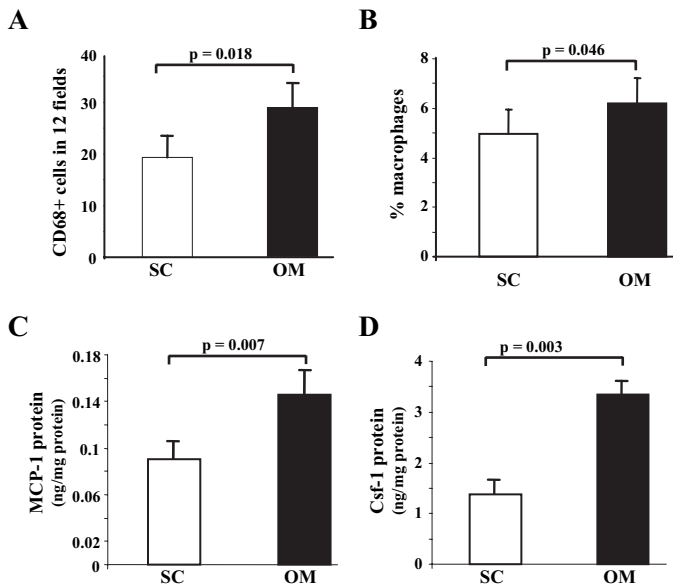

FIG. 3. Macrophage infiltration and MCP1 and CSF1 protein levels in OM and sc fat samples of severely obese women (cohort 2). A and B, Macrophage infiltration, expressed as the number of CD68-positive macrophage cells per 12 ×400 histological fields (A) or as percent macrophages (B) was determined as described in the legend for Fig. 1. C and D, Protein levels of MCP1 (C) and CSF1 (D) were measured using specific ELISA kits, as detailed in *Results*.

the number of macrophages infiltrating OM but not sc. Remarkably, the number of positive metabolic syndrome parameters (as per the NCEP ATP III criteria) tended to correlate (r = 0.382; P = 0.065) with the number of macrophages infiltrating the OM but not the sc, reaching a borderline significance (Fig. 4B). When regression analysis was performed with OM macrophages as the dependent variable, both sc macrophage infiltration (β = 0.408; P = 0.021) and waist circumference (β = 0.495; P = 0.007) were independently associated with the number of infiltrating macrophages in the OM. When combined, these two variables could account for 48.5% of the variation (R^2) in the OM macrophage count predictive model. When additional regression analysis was performed with the number of metabolic syndrome parameters as the dependent variable, OM macrophages remained associated with this parameter independent of sc macrophages counts (β = 0.551; P = 0.029). These results suggest a stronger association of macrophage infiltration into OM *vs.* sc depot with overall obesity-related morbidity risk. To further investigate this observation, we compared the women with normal glucose homeostasis [fasting plasma glucose < 100 mg/dl, a cutoff largely excluding persons with impaired glucose tolerance (17), and no previous diagnosis of diabetes or use of antidiabetic drugs], with those with impaired glucose homeostasis (Table 2). The latter group was significantly (P < 0.05) older, heavier, and more insulin resistant and had a higher number of positive metabolic syndrome parameters compared with women with no apparent abnormalities in glucose homeostasis. In the normal glucose-tolerant women, an average 2.3-fold higher CD68-positive cell count was observed in OM *vs.* sc fat, but this difference did not reach statistical significance by paired *t* test (Fig. 4C). In contrast, among the eight women with impaired glucose metabolism, CD68-positive cells were 3.5-fold more abundant in OM compared with sc, and this difference was statistically significant (Fig. 4D).

Discussion

In the present study, we assessed whether the higher macrophage infiltration into OM (*vs.* sc) fat in obesity is an intrinsic characteristic of this fat depot, how regional adiposity affects it, and what is the relation to comorbidities
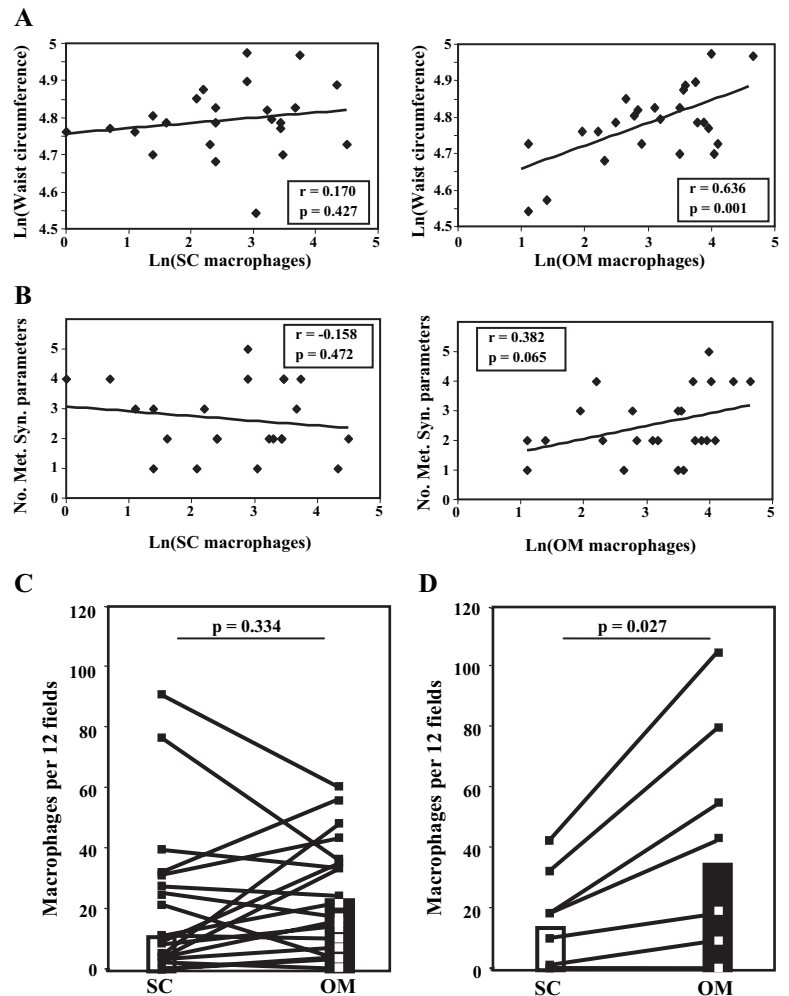
FIG. 4. Relationship between OM and sc macrophage counts and obesity comorbidity (cohort 2). A and B, Using cohort 2, Pearson's correlations were performed between sc macrophage counts (*left*) or OM macrophage counts (*right*) and waist circumference (A) and the total number of positive parameters of the metabolic syndrome (NCEP ATPIII criteria) (B). Macrophage counts and waist circumference were natural-log transformed before correlation analyses. C and D, Macrophage counts were determined as described in the legend for Fig. 3 and presented in two subgroups of cohort 2: severely obese women with no signs of impairment in glucose homeostasis (C) or with impaired fasting plasma glucose or overt diabetes (D).

accompanying obesity. Using the Leipzig cohort (cohort 1) we show that: 1) increased macrophage infiltration into OM fat is observable in lean controls, suggesting that it is an inherent trait of this depot compared with sc; 2) obese people have higher levels of macrophages, mainly in OM, and particularly if fat is accumulated IA *vs.* sc; and 3) although adipose tissue macrophage counts correlated with clinical parameters of obesity and its comorbid manifestations, this was not restricted to the OM fat *vs.* sc depot. The Beer-Sheva cohort of severely obese women (cohort 2) revealed that: 1) absolute macrophage counts were relatively low compared with cohort 1, but increased macrophage infiltration into OM *vs.* sc was still discernable in the entire group; 2) OM but not sc macrophage counts correlated with BMI and waist circumference and tended to correlate with the number of features comprising the metabolic syndrome; and 3) the subgroup of severely obese women with normal glucose homeostasis did not significantly exhibit the preferential OM macrophage accumulation. In contrast, severely obese women with impaired glucose homeostasis had on average a 3.5-fold higher number of macrophages in OM compared with sc fat, consistent with the Leipzig cohort and the published French cohort. Collectively, our findings (summarized in Table 3) demonstrate preferential macrophage infiltration

into IA fat as a common phenomenon extending from the lean to the obese. Moreover, OM macrophage infiltration is exaggerated by obesity particularly if centrally distributed and when associated with the comorbid states that accompany severe obesity. Yet, metabolically intact, severely obese women may represent a unique subgroup relatively protected from the preferential macrophage accumulation into OM.

Generalization of the higher macrophage infiltration into OM vs. sc fat

Of the various subgroups studied herein, severely obese women with normal glucose homeostasis did not seem to display strong preferential OM *vs.* sc macrophage infiltration. This subgroup, which by definition demonstrates lower association between obesity and its comorbidity, may represent a unique "fat and fit" phenotype, potentially attributable to genetic and/or environmental-nutritional factors (such as Mediterranean diet). Moreover, this apparent resistance to the deleterious effects of obesity may in and of itself etiologically contribute to the extent of excessive fat accumulation; if obesity leads to less pronounced insulin resistance and/or if compensatory hyperinsulinemia does not

TABLE 3. Summary of findings: macrophages, CSF1, MCP1, and macrophage correlation with clinical parameters in OM *vs.* sc fat

	OM/sc macrophages	OM/sc CSF1	OM/sc MCP1	Correlation between clinical parameters and macrophages	
				OM	sc
Cohort 1 (n = 60)				⇑⇑	⇑⇑
Lean	⇑	⇑ mRNA	⇑ mRNA		
sc obese	⇑⇑	⇑⇑ mRNA	⇑ mRNA		
IA obese	⇑⇑⇑	⇑⇑⇑ mRNA	⇑⇑ mRNA		
Cohort 2 (n = 29), severely obese women	⇑	⇑⇑ protein	⇑ protein	⇑	↔
NGT	↔				
IGT	⇑				

IGT, Impaired glucose tolerant; NGT, normal glucose tolerant.

lead to β-cell failure, the anabolic effects of insulin on fat tissue are less restricted, thus promoting excessive lipid storage. Moreover, adipocyte hypertrophy was suggested as an underlying mechanism for macrophage recruitment, because it precipitated necrotic cell death (4). In our subgroup of severely obese women, the absolute number of OM macrophages seemed to be lower than that found in the German cohort or reported in the published French cohort (12). It is therefore plausible that in this subgroup, adipose tissue may respond to hypernutrition by a higher tendency to hyperplastic rather than hypertrophic response of fat cells. Although these speculations need to be further challenged experimentally, the relative paucity of parameters of obesity comorbidity in this subgroup (Table 2) along with a less pronounced preferential OM fat macrophage infiltration (Fig. 4C) contribute to the notion that inflammation of OM fat may link IA fat accumulation to its metabolic consequences.

Potential mechanisms for preferential macrophage infiltration into OM fat

Two inflammatory cytokines involved in monocyte/macrophage chemotaxis and activation, MCP1 and CSF1, were more highly expressed at the mRNA or protein level in OM *vs.* sc in all subsets of participants, including lean controls. This finding implies that an inherent difference in these proinflammatory cytokines exists between the two depots, which is exaggerated by the degree of obesity, particularly if predominantly IA. Consistent with our findings, levels of MCP1 secreted into the medium from human visceral fat explants were higher compared with sc explants (18), but this was largely attributed to the excess in residential tissue macrophages rather than being the cause for macrophage infiltration. However, obesity was shown to be associated with higher expression and secretion of MCP1 from isolated adipocytes, although this was suggested to function mainly as a local factor (19). Our study detects a trend for a weak correlation between age-adjusted OM macrophage counts and MCP1 mRNA expression. Interestingly, secretion of MCP1 was suggested to directly mediate muscle insulin resistance, suggesting macrophage-dependent as well as macrophage-independent roles for this inflammatory cytokine in obesity comorbidity (20). It would seem that human studies that modulate MCP1 expression, secretion, or receptor binding are required before its role in human obesity can be fully confirmed.

The role of CSF1 expression for macrophage accumulation in adipose tissue is less studied. Although mice lacking functional CSF1 have been used to demonstrate the bone-marrow origin of macrophages infiltrating adipose tissue (1), a role for local production of CSF1 in macrophage infiltration and/or activation is not established. Adipose tissue fibroblasts and endothelial cells may be a source of CSF1 expression and secretion in this tissue, although adipocytes, particularly in rapidly growing adipose tissue (21), may also present a cellular source of adipose tissue CSF1. Remarkably, the difference in level of expression between OM and sc fat was more pronounced for CSF1 protein than for MCP1 (Fig. 3, C and D). Moreover, a highly significant correlation was observed between the level of expression of CSF1 mRNA and percent macrophage counts in OM, elucidating a possible role for CSF1 in the inflammatory state accompanying obesity.

In conclusion, this study supports the notion that macrophage infiltration into OM is an inflammatory feature of this fat depot that is exaggerated by central obesity and may play a role in linking central adiposity with associated conditions such as diabetes and coronary heart disease.

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