Roles of Chemokine Ligand-2 (CXCL2) and Neutrophils in Influencing Endothelial Cell Function and Inflammation of Human Adipose Tissue

Christine Rouault, Vanessa Pellegrinelli, Raphaela Schilch, Aurélie Cotillard, Christine Poitou, Joan Tordjman, Henrike Sell, Karine Clément, and Danièle Lacasa

Institut National de la Santé et de la Recherche Médicale (C.R., V.P., A.C., C.P., J.T., K.C., D.L.), Unité 872 Nutriomique; Université Pierre et Marie Curie-Paris 6 (C.R., V.P., A.C., C.P., J.T., K.C., D.L.), Centre de Recherche des Cordeliers, Unité Mixte de Recherche (UMR) 872; and Université Paris Descartes (C.R., V.P., A.C., C.P., J.T., K.C., D.L.), UMR S 872, F-75006 Paris, France; Paul-Langerhans-Group (R.S., H.S.), German Diabetic Center, 40225 Düsseldorf, Germany; and Institut Cardiométabolisme et Nutrition (C.R., C.P., J.T., K.C.), Pitié-Salpêtrière Hospital, F-75013 Paris, France

The hypertrophied white adipose tissue (WAT) during human obesity produces inflammatory mediators, including cytokines (IL-6 and TNFα) and chemokines ([C-C motif] chemokine ligand 2 and IL-8). These inflammatory factors are preferentially produced by the nonadipose cells, particularly the adipose tissue infiltrating macrophages. We identified the chemokine (C-X-C motif) ligand 2 (CXCL2) by a transcriptomic approach. Because CXCL2 could represent a WAT-produced chemokine, we explored its role in obesity-associated inflammation. CXCL2 levels in serum and mRNA in WAT were higher in obese subjects compared with lean ones. CXCL2 secretions were higher in sc and visceral (vis) WAT from obese compared with lean subjects. In vis WAT, CXCL2 mRNA expression was higher in macrophages compared with other WAT cells and positively correlated with the inflammatory macrophage markers TNFα and IL-6. CXCL2 triggered the in vitro adhesion of the neutrophils, its selective cell targets, to endothelial cells (ECs) of vis WAT (vis WAT-ECs). Immunohistological analysis indicated that activated neutrophils were adherent to the endothelium of vis WAT from human obese subjects. Blood neutrophils from obese subjects released high levels of proinflammatory mediators (IL-8, chemokine motif ligand 2 [CCL2], matrix metalloproteinase [MMP] 9, and myeloperoxidase [MPO]). Visceral WAT-ECs, treated by neutrophil-conditioned media prepared from obese subjects, displayed an increase of the expression of inflammatory molecules associated with senescence and angiogenic capacities. To conclude, CXCL2, a WAT-produced chemokine being up-regulated in obesity, stimulates neutrophil adhesion to vis WAT-ECs. Activated neutrophils in obesity may influence vis WAT-ECs functions and contribute to WAT inflammation. **(*Endocrinology* 154: 1069–1079, 2013)**

Obesity is associated with auto-inflammation, which is an important determinant shared by other associated metabolic and vascular pathologies, such as type 2 diabetes and atherosclerosis (1, 2). White adipose tissue (WAT) from obese subjects produces inflammatory mediators, including cytokines (IL-6 and TNFα) and (C-C motif) and (C-X-C motif) chemokines ([C-C motif] chemokine ligand [CCL]2 and IL-8). These factors are produced preferentially by the nonadipose cell fraction, particularly macrophages, which accumulate in human WAT in proportion to fat mass (3, 4). Importantly, visceral (vis) WAT, which hypertrophy is linked to obesity complications such as

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Abbreviations: BMI, body mass index; CCL, (C-C motif) chemokine ligand; CD, cluster of differentiation; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; DAPI, 4′,6-diamidino-2-phenylindole; EC, endothelial cell; ECBM, EC basal medium; ICAM, intercellular adhesion molecule; MMP, matrix metalloproteinase; MPO, myeloperoxidase; SA-β-gal, senescence-associated-β-galactosidase activity; SASP, senescent-associated secretory phenotype; VCAM, vascular adhesion molecule; vis, visceral; WAT, white adipose tissue; WAT-M, WAT macrophage.

insulin resistance (5), is more infiltrated by macrophages than sc WAT in human obesity (6). WAT macrophages (WAT-Ms) mainly originate from blood monocytes (7), which are in a proinflammatory state in obesity (8) and prone to migrate in hypertrophied WAT. The mechanism by which immune cells accumulate in human WAT still remains poorly defined.

Leukocyte recruitment in tissues through endothelium proceeds in sequential steps, such as integrin-dependent arrest and transmigration, during which chemokines exert specialized roles. Chemokine (C-X-C motif) ligands (CXCLs) have been shown in mice models to mediate leukocyte arrest, whereas monocyte chemoattractant protein 1/CCL2 and regulated on activation, normal T cell expressed and secreted/CCL5 preferentially function in the subsequent transmigration (9, 10).

A key role has been proposed for monocyte chemoattractant protein 1/CCL2, because mice deficient for CCL2 and its major receptor CCR2 show decreased macrophage accumulation in adipose tissue (11, 12), whereas CCL2 overexpression causes macrophage accumulation and insulin resistance (13). We identified CCL5 as another adipose-produced chemokine that is overproduced in human obese WAT and showed that CCL5 is involved in the recruitment of WAT-Ms (14). However, a question remains as to whether chemokines with the (C-X-C motif) also participate in the initiation and maintenance of inflammatory phenomena in obese WAT. To search for candidate chemokines, we developed a transcriptomic approach to characterize changes in gene expression of preadipose cells in the presence of inflammatory secretions of WAT-Ms (data deposited in Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, series accession GSE28445). We identified CXCL2 among the most overexpressed genes in these conditions. This chemokine triggers leukocyte infiltration in inflamed tissues, but its role and target receptors in human WAT are unknown. The selective cellular targets of CXCL2 are neutrophils. WAT from mice fed a high-fat diet is infiltrated by neutrophils in the early steps of obesity development and precedes macrophage accumulation (15, 16). In human obesity, neutrophil activation in sc WAT positively correlates with body mass index (BMI) and blood pressure. Strikingly, these activated neutrophils localize inside WAT vessels where they are proposed to participate in vascular inflammation (17, 18). Interestingly, endothelial cells (ECs) from vis WAT of obese subjects exhibited an inflamed and senescent phenotype overexpressing adhesion molecules, which play a crucial role in leukocyte recruitment (19).

We tested the hypothesis that CXCL2 could contribute to WAT inflammation by recruiting neutrophils and that these selective cell targets of CXCL2 could deeply alter the function of ECs in obese WAT. To address these issues, we combined a series of tissue and cellular experiments using exclusively human samples.

Materials and Methods

The following is the list of the antibodies used in the study: antihuman CXCL2 and IL-8 polyclonal antibodies (Peprotech, Rocky Hill, New Jersey); antihuman CD66b, CD15, and vascular cell adhesion molecule (VCAM)-1 mouse monoclonal antibodies and intercellular adhesion molecule (ICAM)-1 polyclonal antibody (Abcam, Cambridge, United Kingdom); antiphospho-histone γ-H2A.X monoclonal antibody (Millipore, Billerica, Massachusetts); and antihuman CD15 monoclonal antibody (Beckman Coulter GmbH, Krefeld, Germany).

The human recombinant proteins CXCL2 and IL-8 were purchased from Peprotech. The chemokine (C-X-C motif) receptor (CXCR)2 antagonist (SB225002) was purchased from Tocris (Ellisville, Missouri).

Subjects and biochemical analyses

In an accepted protocol related to the pathophysiology of low-grade inflammation in obesity (Assistance Publique/Hôpitaux de Paris, Clinical Research Contract), the obese subjects are candidates for gastric surgery programs. The subjects had not been on diet before the surgery. Their weights had been stable at least 3 months before the surgery. A group of 14 morbidly obese women was recruited for gene expression analysis of various inflammatory markers. Subcutaneous and vis WAT samples were obtained from these subjects during the surgery procedure. Needle biopsies of abdominal sc were also obtained in lean and morbidly obese subjects (during a preoperative medical examination). Biochemical variables were measured after an overnight fast. The clinical and biochemical parameters of the 14 morbidly obese and 9 lean women (age-matched counterparts participating to clinical studies), in whom CXCL2 blood measurements (ELISA tests described below) were performed, are presented in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Plasma glucose, triglycerides, total cholesterol, and high-density lipoprotein levels (mM) were measured enzymatically. Insulinemia (μU/mL) was measured with immunoradiometric assay (Bi-INSULINE IRMA; CIS Bio International, Bagnols-sur-Cèze, France). For these studies, all the participants gave written and informed consent. All clinical investigations were performed according to the Declaration of Helsinki and approved by the ethics committees of Pitié-Salpêtrière or by the local ethic committee of Heinrich Heine University (Düsseldorf, Germany) (explant studies).

Cultures of human adipose tissue explants

CXCL2 secretion from paired sc and vis WAT from 8 lean (age, 62.6 ± 6.7 y; BMI, <25 kg/m^2) and 7 obese (age, 55.3 ± 7.1 y; BMI, >30 kg/m^2) subjects was evaluated by ELISA. After extensive washing to remove cell and tissue debris, WAT explants (100 mg/mL) were incubated in smooth muscle cell hunger medium (Promocell, Heidelberg, Germany) under aseptic con-

ditions. After a 24-hour incubation period, supernatant was collected and stored at −80°C until measurements.

RNA preparation and real-time PCR

RNA extraction, reverse transcription, and real-time PCR were conducted as described previously (20). Primers that were used are listed in Supplemental Table 2. Values were normalized to 18S expression.

Preparation of WAT-Ms and WAT-ECs

Stromal vascular fraction cells were obtained from sc WAT biopsies (20). Isolation of WAT-Ms and WAT-ECs from human WAT stromal vascular fraction was performed as described (3). These cells were suspended in PBS/2% fetal bovine serum/1 mmol/L EDTA and incubated with CD34-positive selection cocktail, followed by an incubation with magnetic nanoparticles (Stemcell Technologies, Grenoble, France). The CD34-negative cell fraction was incubated with CD14-positive selection cocktail to give WAT-M. The WAT-ECs were obtained after CD31-positive selection from the CD34-positive cell fraction (Stemcell Technologies). The phenotype of WAT-ECs was verified by 1) expression of an endothelial specific marker, the von Willebrand Factor; and 2) tube formation assay on Matrigel-GF reduced (BD Biosciences, Franklin Lakes, NJ). The purity of the different cell preparation was about 90%.

Immunochemistry in human vis WAT

Immunochemistry analyses were performed on serial sections of vis WAT from 3 lean and 3 obese subjects as described in Ref. 14. The lean subjects had abdominal programmed surgery (inguinal hernia, cholecystectomy, hysterectomy, gastro-oesaphageal reflux). They had no inflammatory state and accepted that the vis WAT sample was collected in the same location as that of obese subjects. CD66b and CD15 were detected with the corresponding monoclonal antibodies. Negative controls were performed by omitting the primary antibody. Nuclei were counterstained with Mayer's hematoxylin.

Isolation of human circulating neutrophils

Human neutrophils were isolated from EDTA (0.5%)-treated venous blood of healthy human volunteers and morbidly obese subjects (age matched) by the 1-step purification on polymorphprep gradient dextran (Nycomed, Oslo, Norway) as previously described in Ref. 21. The purity and viability of the preparations were more than 90%.

Adhesion assays

Human blood neutrophils were labeled for 30 minutes with 10μM calcein acetoxymethyl ester as described (3). Visceral WAT-ECs were grown to confluence for 5–6 days on fluoroblok insert system of 8-μm pore size coated with fibronectin (Costar, Cambridge, MA) as described (3).

For adhesion assays, confluent WAT-ECs were incubated with recombinant proteins (100 ng/mL) or with control media. Then, labeled neutrophils were added to WAT-ECs for 1 hour at 37°C, and the numbers of adherent fluorescent cells were counted in 5 random fields with counting after blind lectures by 2 different investigators.

Preparation of conditioned media from human neutrophils and angiogenesis arrays

Neutrophils (1×10^6) were suspended in 1-mL EC basal medium (ECBM) with 1% BSA. Neutrophils were treated or not by CXCL2 (100 ng/mL) or IL-8 (100 ng/mL) for 1 hour at 37°C. Then, after washing, neutrophils were placed in 1-mL ECBM with 1% BSA for 18 hours at 37°C, after which conditioned media were collected and stored at −80°C until measurements. Control medium corresponds to ECBM with 1% BSA incubated without neutrophils for 18 hours at 37°C. Screening for angiogenesis-related proteins, secreted by human neutrophils, was performed by hybridizing-conditioned media with antibody-coated membranes according to company's instructions (human angiogenesis array ARY007; R&D Systems, Minneapolis, Minnesota). Briefly, 1 mL of conditioned media was preincubated with the detection antibody cocktail for 1 hour at room temperature and then incubated with the membranes overnight at 4°C. After washing, the membranes were incubated with the streptavidin-horseradish peroxidase for 30 minutes at room temperature. Specific signals were detected with the enhanced chemoluminescence detection solution (GE Healthcare, Little Chalfont, United Kingdom) and immediately exposed to x-ray films. Background was very low, and only significant signals obtained after a 5-minute exposition were taken into account.

ELISA and multiplex immunoassay

IL-8 and IL-6 levels were measured in plasma and also in explant media from sc, and ELISA kits were used to determine the concentrations of CXCL2 in explant media and sera according to the company's instructions (900-K120; Peprotech). Samples were tested in duplicates and in the same run for explants media (1/40 dilution). Sera were tested in duplicates separately (dilution 1/4 for lean and 1/8 for obese subjects). We measured the intra- and interassay coefficients that were 4.95% and 10.5%, respectively.

Conditioned media from unstimulated neutrophils were analyzed diluted (1:1) in ECBM with 1% BSA. Concentrations of human cytokines were determined using human cytokine/chemokine Panel I 39plex and human CVD1 7plex kits from Millipore according to the company's instructions: 25 μL of standard cytokines or samples were dispensed into the wells, followed by 25 μL of primary antibody-bead mixture, which were added into each well and incubated overnight at 4°C. The next day, 25 μL of biotinylated detection antibodies and then 25 μL of streptavidin-phycoerythrin were added and incubated at room temperature for 1 hour. Each step was followed by 3 washes with kit wash buffer and vacuum manifold. Multianalyte profiling was performed on the Luminex-200 system and the Xmap Platform (Luminex Corp, Austin, TX). Calibration was performed with microspheres for classification. Reporter readings and sheath fluid were also purchased from Luminex Corp. Acquired fluorescence data were analyzed by the Xponent software version 3 using standard curves obtained with serial dilutions of standard cytokines mixtures. The following factors were measured: IL-8, CXCLs, CXCL4, CCL2, CCL3, CCL4, matrix metalloproteinase (MMP-8, MMP-9), and myeloperoxidase (MPO).

Tube formation assay by vis WAT-ECs

Visceral WAT-ECs (1×10^4 cells per well) were seeded onto Matrigel-GF reduced (BD Biosciences) in μ-slide angiogenesis (Ibidi, Madison, WI) and cultured in ECBM in the presence or not of the conditioned media prepared from neutrophils of lean and morbidly obese subjects. After 6 hours of incubation, 3 phase-contrast images were recorded on a digital camera (Olympus, Tokyo, Japan). The total tube length, branching points, and total loops of the network structures were measured and quantified in 3 random fields for each experimental condition using Wimasis Software (Ibidi).

Senescence of sc WAT-ECs

ECs isolated from sc WAT of lean and healthy women were treated or not by neutrophil-conditioned media prepared from lean and morbidly obese subjects for 48 hours. After cell fixation, senescence-associated-β-galactosidase activity (SA-β-gal) was assessed according to the company's instructions (Sigma, St Louis, Missouri). After 2 days, 5 phase-contrast images were recorded on a digital camera (Olympus). The number of SA-β-gal positive cells was normalized to the total number of cells (counterstaining by hematoxylin).

Subcutaneous WAT-ECs cultured on coverslips were treated or not by neutrophil-conditioned media prepared from lean and morbidly obese subjects for 48 hours. After fixation, coverslips were incubated with phospho-γ-H2A.X antibody and then the corresponding Cy2-conjugated antimouse IgG. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Negative controls were performed by omitting the primary antibody. Coverslips were examined with an Olympus BX41 fluorescence microscope (Olympus). Three phase-contrast images were recorded on a digital camera (Olympus). The number of phospho-γ-H2A.X positive nuclei was normalized to the total number of nuclei (DAPI staining).

Western blot analysis

Cell extracts were prepared in buffer containing a cocktail of protease and phosphatase inhibitors (complete mini and phosphostop; Roche Diagnostics, Mannheim, Germany). The membranes were probed overnight at 4°C with the corresponding primary antibodies. Specific signals were detected with the ECL detection solution (GE Healthcare) and immediately exposed to x-ray films. Signals were quantified by densitometry.

Statistical analysis

Data are expressed as mean ± SEM. Differences in clinical and biochemical parameters between lean and obese women were determined using the Mann-Whitney unpaired nonparametric test. Spearman coefficients were computed to examine correlations. The cellular experiments were performed at least 5 times, and statistical analyses were performed using Student's t test. Comparisons between more than 2 groups were carried out using a 1-way ANOVA with post hoc tests, in which $P < .05$ was considered statistically significant.

Results

CXCL2 is increased in obesity

Circulating levels of CXCL2 were approximately 3-fold higher in 14 obese subjects than in 9 lean subjects of the same age ($P = .003$) (Figure 1A). In agreement with the serum inflammatory profile, mRNA levels of CXCL2 were significantly increased in sc WAT from obese subjects compared with lean subjects ($P = .025$). The same profile was observed for mRNA levels of IL-6 and 2 other CXCL chemokines, IL-8 and CXCL5 ($P = .006$) (Figure 1B).

Next, we investigated CXCL2 secretion in paired sc and vis WAT samples from 8 lean and 7 obese subjects (Düsseldorf group). As depicted in Figure 1C, in vis samples, CXCL2 secretion was higher in obese compared with lean subjects. We found a correlation between CXCL2 secretion and BMI ($r = 0.59$, $P = .0054$). The same profile of CXCL2 secretion was observed for sc samples (correlation with BMI; $r = 0.51$, $P = .02$). In another group of 5 obese women among the 14 women, for which clinical parameters are presented in Supplemental Table 1, sc and vis WAT secretions of CXCL2, IL-6, and IL-8 were measured. The data clearly show that CXCL2 was secreted at comparable levels of IL-6 and IL-8 (Supplemental Table 3).

CXCL2 and IL-8 expression in adipocytes, preadipocytes, macrophages, and ECs isolated from vis WAT from 6 obese subjects was determined by reverse transcription PCR to evaluate the contribution of the different cell types to CXCL2 expression in the specific WAT depot. CXCL2, as IL-8, was expressed more in macrophages than other cell types ($P < .05$, n = 6) (Figure 1D). An identical expression profile was observed for adipose cells from lean vis WAT ($P < .05$, n = 6) (Supplemental Figure 1).

Because CXCL2 was preferentially secreted by the vis depot and by macrophages, we explored the association between the expression of CXCL2 and several macrophage markers. In vis WAT from 14 obese subjects, CXCL2 mRNA positively correlated with M1 markers, such as TNFα ($r = 0.78$, $P = .017$) and IL-6 ($r = 0.93$, $P = .0003$), but not with M2 markers, such as CD206 and alternative macrophage activation-associated CC chemokine-1 (Table 1) (22). In addition, the secretion of CXCL2 was stimulated more by in vitro M1 (lipopolysaccharide treated) monocyte-derived macrophages than M2 cells (dexamethasone treated; $P < .05$, n = 4) (Supplemental Table 4).

CXCL2 stimulates the adhesion of human neutrophils

Because CXCL2 has been shown to trigger neutrophils in inflamed tissues (23), we proposed that this chemokine could contribute to neutrophil adhesion to ECs of human WAT. First, because the presence of activated neutrophils detected by the specific marker CD66b was previously reported in sc WAT vessels from obese women (BMI, >30

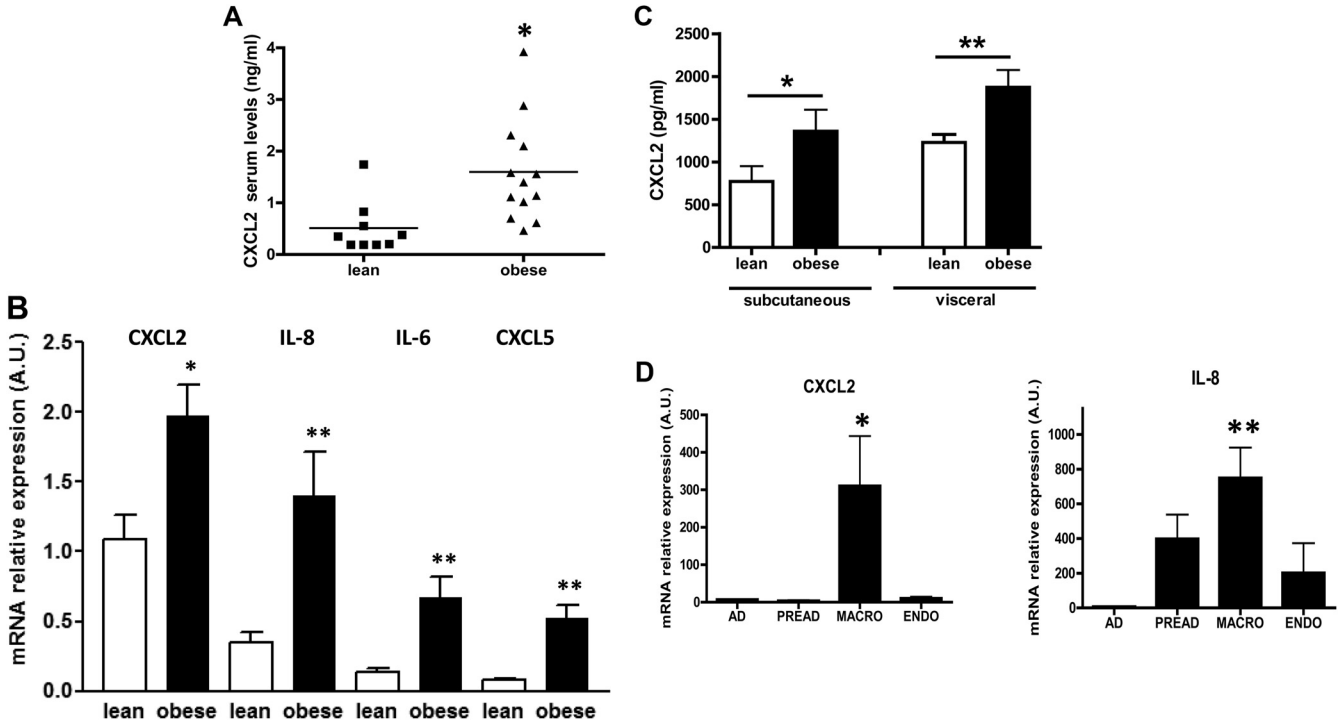

Figure 1. CXCL2 and human obesity. (A) Serum levels of CXCL2 in 9 lean (BMI, 21.3 ± 0.4 kg/m^2; age, 36.5 ± 2.4 y) (black square) and 14 obese (BMI, 46.2 ± 3.5 kg/m^2; age, 36.1 ± 2.7 y) (black triangle) women. *P = .003 Mann-Whitney test. Horizontal bar represents the mean value. (B) CXCL2, IL-8, IL-6, and CXCL5 expressions were quantified by real-time PCR and normalized to 18S in needle biopsies of sc WAT from 7 lean (white bar) and 16 age-matched obese (black bar) individuals. *P = .025 vs lean, **P = .006 vs lean Mann-Whitney test. (C) CXCL2 secretions were measured by ELISA assay in paired sc and vis WAT from 8 lean (white bar) and 7 obese (black bar) individuals. *$P < .05$, vs lean, **$P < .001$ vs lean. The t values were 2.20 (sc WAT) and 2.15 (vis WAT). (D) Adipocytes (AD), preadipocytes (PREAD), macrophages (MACRO), and ECs (ENDO) were isolated from vis WAT from obese individuals (n = 6). CXCL2 and IL-8 expressions were quantified by real-time PCR and normalized to 18S. *$P < .05$, vs AD, PREAD, and ENDO (CXCL2); **$P < .01$ vs AD, PREAD, and ENDO (IL-8). The F values were 4.98 (CXCL2) and 5.35 (IL-8). A.U., arbitrary units.

kg/m^2) (18), we extended these observations to vis depots by staining for CD66b inside the vessel walls of vis WAT from 3 morbid obese and lean subjects. In marked contrast to monocytes/macrophages, very few neutrophils were observed in the parenchyma of vis WAT. These data were confirmed by immunostaining for another marker of neutrophils, CD15 (Figure 2).

Considering these observations, we examined the adhesion of neutrophils to ECs of vis WAT in response to the CXCL chemokines. The recombinant proteins CXCL2 and IL-8 significantly increased the adhesion of labeled neutrophils to vis WAT-ECs (each ~3.5-fold, $P < .05$ for both, n = 5) (Figure 3A). CXCL2 and IL-8 share the same receptor, CXCR2, which was highly expressed in neutrophils compared with monocytes and different cell types isolated from vis WAT of obese subjects (Supplemental Table 5). We here observed that CXCR2 antagonist (SB225003) totally blocked the CXCL2-induced adhesion of neutrophils to vis WAT-ECs ($P < .05$, n = 5) (Figure 3B).

TABLE 1. Spearman correlations between CXCL2 and macrophage marker expression in vis adipose tissue from obese subjects

	CXCL2 expression	
	R	***P* value**
M1 markers		
IL-6	0.93	.0003
TNFα	0.78	.017
M2 markers		
CD206	0.29	N.S.
AMAC-1	0.05	N.S.

AMAC-1, Alternative macrophage activation-associated CC chemokine-1; N.S., not significant.

Taken together, these observations suggest that, in vis WAT, CXCL2 and IL-8 may contribute to the chemotaxis of neutrophils, which are another type of circulating cells greatly activated in obese subjects (18, 24). Because we observed a specific adhesion of neutrophils to vis WAT-ECs, we hypothesized that neutrophils impact the phenotype of ECs in morbid obesity. The contribution of CXCL2 was examined in this context.

Neutrophil secretions in human obesity

To gain more information on the phenotype of human neutrophils in obesity, we compared the levels of

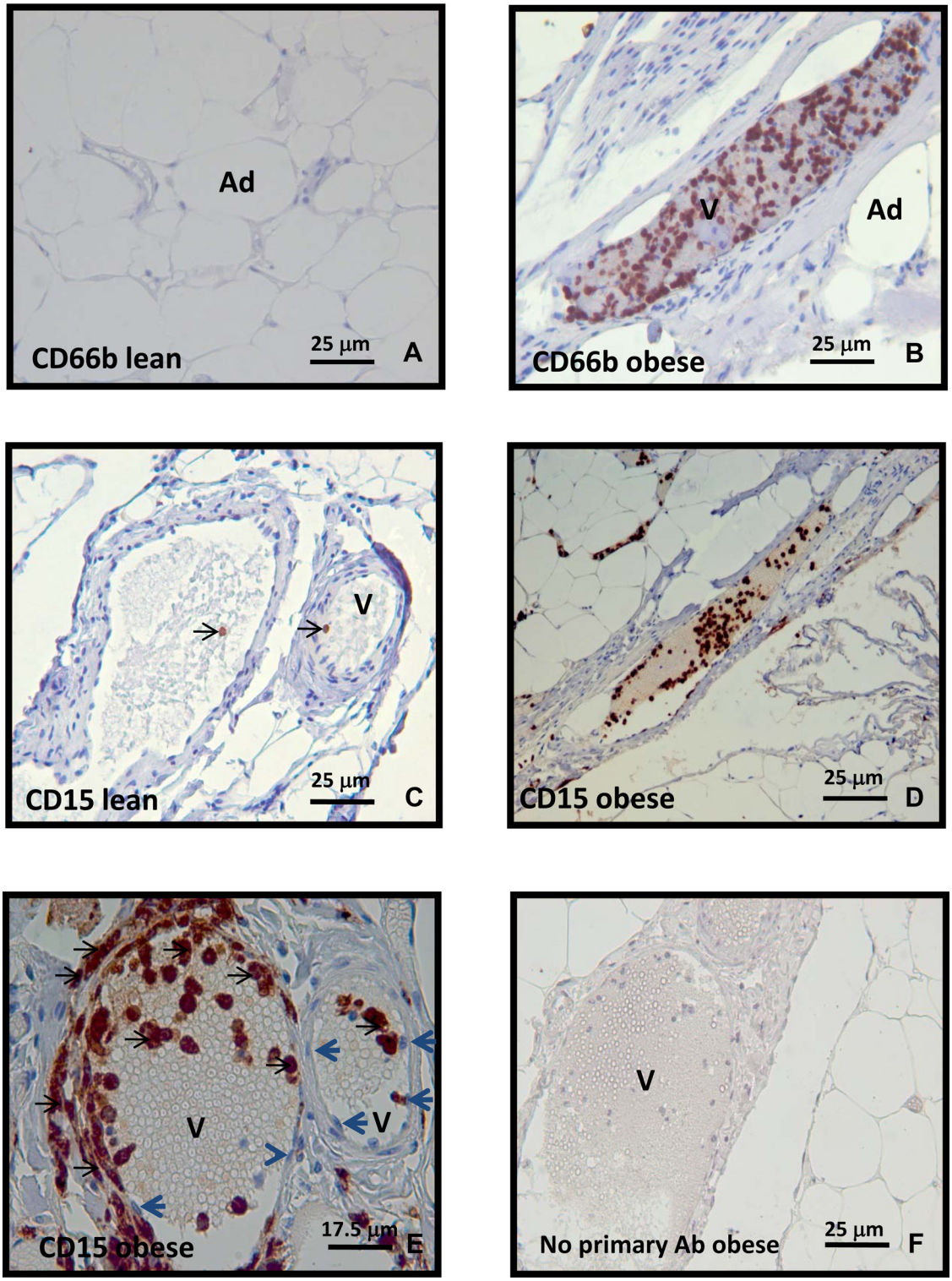

Figure 2. Immunochemistry in human vis WAT. Serial sections of vis WAT from 3 lean (A) and 3 obese (B) subjects were stained for CD66b. Staining for CD15 of vis WAT from lean (C) and obese subjects (D and E). Negative control (no primary antibody) in F. Ad, Adipocyte; V, vessel; black arrows, activated neutrophils; blue arrows, ECs.

inflammatory mediators secreted by circulating neutrophils from 7 lean and obese subjects. Among these mediators, MMP-9, MPO, and chemokines, such as IL-8, CXCL1/3, and CCL2, were secreted more by neutrophils from 7 obese subjects than neutrophils from 7 lean subjects ($P < .02$, n = 7) (Table 2). In contrast, CXCL2 secretion from neutrophils was not detected in this assay.

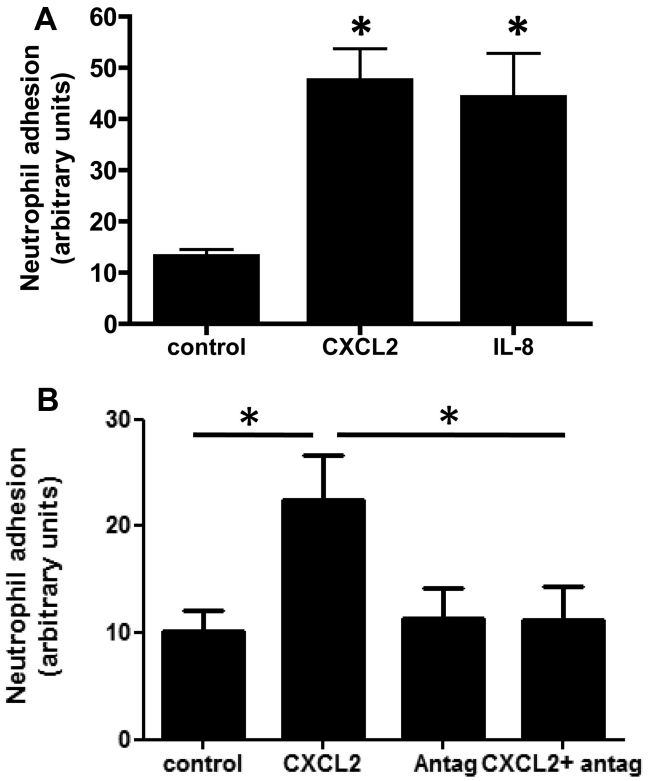

Figure 3. CXCL2 and adhesion of human neutrophils. (A) Adhesion of human neutrophils labeled with calcein to vis WAT-ECs either pretreated or not (control) with recombinant proteins (CXCL2 or IL-8, 100 ng/mL) for 1 hour at 37°C. (B) Adhesion of human neutrophils labeled with calcein to vis WAT-ECs either pretreated or not (control) with CXCL2 (100 ng/mL), CXCR2 antagonist (100nM), or both CXCL2+ CXCR2 antagonist (antag) for 1 hour at 37°C. Data are the mean ± SEM of 5 separate experiments each using different neutrophils and WAT-EC preparations. *$P < .05$. The F values were 9.08 (A) and 3.48 (B).

Neutrophils, chemokines, and WAT-EC phenotype

Angiogenesis is led by proliferation, migration, and capillary morphogenesis of ECs. In human obesity, EC biology is profoundly altered with an inflammatory and senescent phenotype (19). We examined whether neutrophils contribute to these biological EC alterations and whether CXCL2 has an impact on these relationships.

TABLE 2. Comparison of the basal secretory profile of neutrophils from lean and obese subjects

Inflammatory mediators	Lean	Obese
IL-8 (pg/mL)	90.2 ± 21.7	285 ± 44[a]
CXCL1/3 (pg/mL)	14.7 ± 5.1	85.6 ± 10[b]
CXCL4 (pg/mL)	635 ± 163	1510 ± 860 N.S.
CCL2 (pg/mL)	2.9 ± 2	56.6 ± 22.6[b]
CCL3 (pg/mL)	6.8 ± 3.8	28.2 ± 9.3 NS
CCL4 (pg/mL)	45.5 ± 17.7	59.3 ± 20.4 N.S.
MMP-8 (ng/mL)	7.3 ± 1.7	9.4 ± 2.3 N.S.
MMP-9 (ng/mL)	38.3 ± 5.8	80.6 ± 20.1[a]
MPO (ng/mL)	218.3 ± 57.9	764 ± 166[b]

Mann-Whitney test. N.S., Not significant.
[a] $P < .05$.
[b] $P < .02$.

We first identified angiogenesis-related factors from neutrophils using membrane arrays, a nonquantitative approach. Among the factors secreted by neutrophils in lean and obese subjects IL-8, MMP-8, MMP-9, and tissue inhibitor of metalloproteinase 1, we also detected CXCL4 as a neutrophil chemokine (Supplemental Figure 2A).

Next, we compared the angiogenic capacities of neutrophils from lean and obese subjects toward vis WAT-ECs. The ability to form capillary-like structures in Matrigel was enhanced approximately 2-fold ($P < .05$, n = 3) in the presence of conditioned media from neutrophils from obese subjects (Figure 4 and Supplemental Figure 2B). However, the conditioned media of neutrophils from obese subjects treated with CXCL2 or IL-8 did not increase tube formation by vis WAT-ECs (Supplemental Figure 2C).

Finally, we examined the contribution of activated neutrophils to the senescence of ECs originating from lean sc WAT. Conditioned media of neutrophils from morbidly obese subjects clearly increased the number of senescent ECs according to SA-β-gal activity (~2.5-fold, $P < .05$, n = 4) (Figure 5A) and γ-H2A.X staining, a marker of senescent nuclei (~2.5-fold, $P < .05$, n = 4) (Figure 5B). However, conditioned media of neutrophils treated with CXCL2 or IL-8 did not further increase the senescence of ECs (data not shown).

Senescent ECs displayed increased expression of inflammatory adhesion molecules. We observed an overproduction of ICAM-1 and VCAM-1 by vis WAT-ECs in response to factors secreted by neutrophils from obese subjects (~3-fold, $P < .01$, n = 5) (Figure 5C). Senescent cells are also characterized by a secretory phenotype (senescent-associated secretory phenotype [SASP]) with high expression of CXCL2 and other proteins (CXCL1, CXCL3, IL-6, IL-8, plasminogen activator inhibitor 1, and MMP-3) (25). As depicted in Figure 5D, the gene expression in WAT-ECs of several of these SASP molecules (CXCL1, CXCL2, CXCL3, IL-6, and IL-8) is increased to a greater extent by neutrophil-conditioned medium from obese compared with lean subjects ($P < .05$, n = 5).

Discussion

Several studies have shown that (C-X-C motif) chemokines influence WAT biology. CXCL5 was proposed as a link between obesity and insulin resistance (26). IL-8, also called CXCL8, is overexpressed by human WAT during obesity (27) with a precise role remaining to be identified.

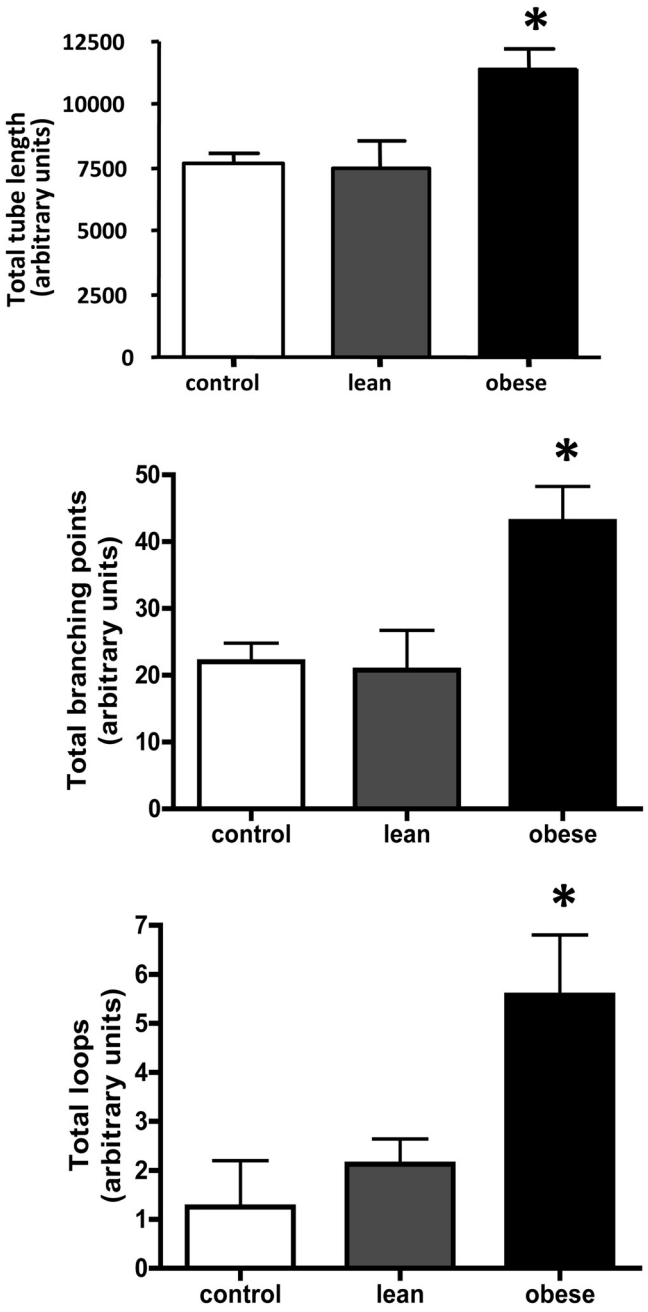

Figure 4. Human neutrophils from obese subjects and vis WAT-EC functions. Assays of tube formation by vis WAT-ECs either pretreated or not (control, white bar) with conditioned media prepared from lean (gray bar) or obese (black bar) neutrophils for 6 hours. Graphs show the quantification of total tube length, branching points, and total loops. Quantifications in 3 random fields for each experimental condition were performed using Wimasis Software. Data are the mean ± SEM of 3 separate experiments each using different WAT-EC preparations. $*P < .05$ vs control. The F values were 5.41 (total tube length), 5.19 (total branching points), and 5.71 (total loops).

Our study extends the contribution of another C-X-C chemokine (CXCL2) in the pathological alterations observed in obese WAT and in systemic inflammation found in obesity. Interestingly, these chemokines share the same CXCR2 receptor, which deficiency in mice models protects from obesity and insulin resistance (26, 28). In human obesity, CXCR2 is expressed in some cell types of vis WAT, although at very low levels compared with the CXCL2 main target, the neutrophils.

For the first time, we showed, using in vitro systems, that CXCL2 contributes to the adhesion of neutrophils to ECs and that could promote inflammation and senescence of ECs of obese vis WAT. However, although promoting neutrophil adhesion, CXCL2 per se does not seem to contribute to EC alteration induced by activated neurophils in our cell models. CXCL2 may nevertheless maintain the increased neutrophil adhesion to ECs, because it is overproduced by obese vis WAT and singularly by inflamed and senescent ECs.

We found that human obesity is associated with increased CXCL2 levels, CXCL2 expression in WAT, and CXCL2 secretion, particularly in the vis WAT, a tissue depot shown to be deleterious for many obesity comorbidities. This latter finding is consistent with the increased macrophage accumulation and vessel density observed in vis WAT compared with sc WAT (6, 19). Compared with IL-8 and IL-6, CXCL2 displays comparable levels of secretion by obese WAT, thus arguing in favor of a putative role in obesity-associated inflammation.

Macrophages have been mainly classified into proinflammatory (M1) or antiinflammatory (M2) states (29, 30). CXCL2 was observed mainly expressed by vis WAT-Ms and correlated with some proinflammatory markers, such as IL-6 and TNFα. Our findings are supported by the fact that in vitro-induced M1 macrophages secreted high levels of CXCL2 compared with M2 macrophages. However, the precise phenotype of human WAT-Ms is still a matter of debate and probably varies according to the degree of obesity and its stage of progression. For example, human WAT-Ms have been reported to exhibit a mixed M2 phenotype accompanied by high production of proinflammatory mediators in overweight and moderately obese subjects (31, 32) .

Neutrophils are the selective targets of CXCL2 and IL-8, which promote their activation with surface exposition of adhesion molecules as CD11b and CD66b (33). During tissue infiltration, leukocyte interactions with the endothelium develop in sequential steps triggered by specialized chemokines: selectin-mediated rolling under the specific control of CXCL1 and CXCL2, integrin-dependent arrest, and transendothelial diapedesis (9, 10). Our immunohistological analysis clearly indicated that activated neutrophils, as assessed by CD66b detection, were adherent to the endothelium of vis WAT. Here, we show that CXCL2 stimulates the in vitro adhesion of neutrophils to the ECs of vis WAT. However, activated neutrophils are not present in adipose tissue parenchyma, sug-

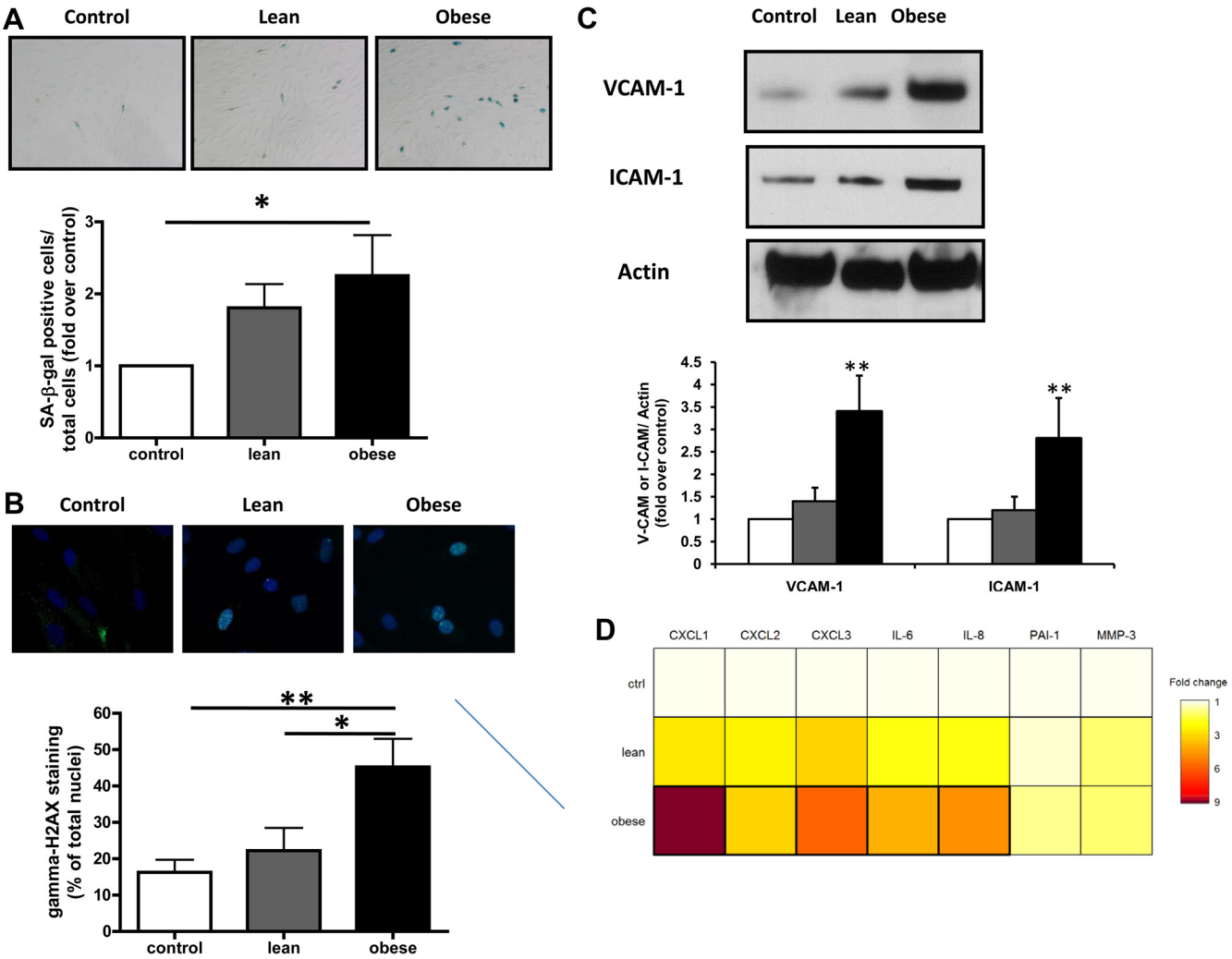

Figure 5. Human neutrophils from obese subjects induced WAT-EC senescence. WAT-ECs isolated from lean sc WAT were either pretreated or not (control, white bar) with conditioned media prepared from lean (gray bar) or obese (black bar) neutrophils for 48 hours. (A) SA-β-gal activity was measured after fixation of the WAT-ECs. F value was 2.92. (B) WAT-ECs were immunostained against phospho-γ-H2AX (green, Cy2-conjugated antimouse IgG) and nuclei (blue, DAPI) after cell fixation. Representative microphotographs and graph quantifications are presented (magnification, ×10 [A] and ×63 [B]). F value was 7.26. (C) WAT-EC lysats were immunoblotted to detect VCAM-1, ICAM-1, and actin. Graphs show quantifications of the immunoblots. The F values were 6.97 (V-CAM-1) and 3.65 (I-CAM-1). (D) Heat map representation of the expression of SASP molecules by WAT-ECs either pretreated or not (control) with conditioned media prepared from lean or obese neutrophils for 48 hours. Gene expression of the SASP molecules was quantified by real-time PCR and normalized to 18S. Graded shades from yellow to red represent fold changes in gene expression. Bold represents significant variations compared with control ($P < .05$). Data are mean ± SEM of 4–5 separate experiments each performed with different preparations of WAT-ECs and neutrophil-conditioned media. *$P < .05$, **$P < .01$ vs control. The F values were 12.54 (CXCL1), 2.41 (CXCL2), 3.88 (CXCL3), 5.69 (IL-6), and 9.31 (IL-8).

gesting that, in vivo, they might not transmigrate into the tissue like macrophages, or if they do, that might be a transient phenomenon. In another situation of "low-grade inflammation," such as nonalcoholic steatohepatitis, a common complication of obesity, neutrophils surround steatotic and necrotic hepatocytes in crown-like structures (34). Necrotaxis cues, such as formyl peptide released from necrotic cells, guide neutrophils to the final injury site (35). Although in hypertrophied WAT necrotic adipocytes are surrounded by macrophages in crown-like structures (6), the necrotactic factors could not be adequately released to promote neutrophil localization to necrotic sites in WAT.

Interestingly, the activated form of blood neutrophils occurs in a greater proportion in obese, presenting increased circulating CXCL2 levels (shown in the present study), than in lean individuals (24). We extended these observations by showing that neutrophils isolated from blood of obese subjects release high amounts of proinflammatory mediators (several chemokines, as well as MMP-9 and MPO) compared with neutrophils from lean subjects. Some of these proinflammatory mediators are stocked in neutrophil granules (primary for MPO and secondary/tertiary for MMP-9) and release upon exocytosis (36). These findings suggest that neutrophils from obese subjects could be prone to degranulation, provoking and/or exacerbating auto-inflammation.

Neutrophils are involved in tissue damage via the production of MMP-9 and reactive oxygen species (37). In nonalcoholic fatty liver disease, a precursor state of nonalcoholic steatohepatitis, neutrophil infiltration, driven by CXCL1 and IL-8 in association with increased MPO activity, may contribute to the progression of this disease (34). In human atherosclerosis, a pathology strongly associated with obesity, activated neutrophils, the accumulation of which is possibly driven by CXCL1/2 and IL-8, are potential contributors to plaque rupture through the production of MMP-9 (38, 39). Here, we highlighted that neutrophils could contribute to pathological alterations of WAT via their impact on EC dysfunctions. We showed enhanced angiogenic capacities of neutrophils from obese subjects towards ECs from vis WAT. In addition to reactive oxygen species, activated neutrophils release many angiogenic molecules, such as IL-8, CXCL1/3, and MMP-9. In particular, MMP-9 and IL-8, by degrading the basement membrane and promoting the vis ECs migration, respectively, are probably important contributors to the proangiogenic effects of neutrophils. Because angiogenesis and inflammation are interrelated processes, these observations could be relevant in the context of a neovascularization for the repair of hypertrophied WAT.

An important functional alteration of ECs is their senescence. Obesity induces the senescence of mouse aortic ECs (40) and microvascular ECs from human vis WAT (19). A major finding of our in vitro experiments was that neutrophils from obese subjects are important players in the initiation/perpetuation of the senescence of WAT-ECs. Cellular senescence is associated with an increased production of inflammatory adhesion molecules (41). Secretions from activated mouse neutrophils have been demonstrated to contribute to monocyte recruitment in tissues (42). In agreement with those findings, we showed that neutrophils from obese subjects induce the overproduction of adhesion molecules (VCAM and ICAM) by ECs, favoring thus monocyte adhesion to these cells.

We also observed the secretion of proatherogenic CXCL4 from neutrophils. This chemokine may form heterodimers with CCL5 and then enhance monocyte adhesion to inflamed endothelium (43). Interestingly, CCL5, which is up-regulated during obesity, induces monocyte chemotaxis in vis WAT, as we previously reported (14).

Although we showed that CXCL2 stimulates neutrophil adhesion, this chemokine did not appear to directly mediate the proangiogenic and senescent effect of neutrophils on ECs. However, senescent cells develop a specific secretory phenotype with particularly high expression of the chemokines CXCL2 and IL-8 (25). In response to factors secreted by neutrophils from obese subjects, WAT-ECs overproduced CXCL2, which could lead to increased adhesion of neutrophils and perpetuation of vascular inflammation in vis WAT during obesity (Supplemental Figure 3).

Thus, it is tempting to speculate that, during obesity, the senescence of ECs, along with decreased vessel density (44), could be involved in the modified oxygen tension and inflammation of the hypertrophied WAT.

Our experimental study presents some limitations. Although being performed with human primary cells isolated from blood or WAT, our in vitro experiments did not reproduce the complex inflammatory environment of WAT from obese individuals. However, our results suggest that neutrophils, which are selective targets of CXCL2, could be positioned as active actors in WAT inflammation by their impact on EC functions and thus could contribute to the perpetuation of WAT inflammation.

Finally, considering therapeutic options, whether targeting CXCL2 signaling and neutrophil activation can reduce WAT inflammation and ameliorate obesity-associated complications, remains to be determined.

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Address all correspondence and requests for reprints to: Danièle Lacasa, Institut National de la Santé et de la Recherche Médicale, Unité 872, Team 7, Nutriomique, 15 Rue de l'École de Médecine, F-75006 Paris, France. E-mail: daniele.lacasa@crc.jussieu.fr.

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