Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans

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Abstract Macrophage infiltration of white adipose tissue (WAT) is implicated in the metabolic complications of obesity. The precipitating event(s) and function(s) of macrophage infiltration into WAT are unknown. We demonstrate that >90% of all macrophages in WAT of obese mice and humans are localized to dead adipocytes, where they fuse to form syncytia that sequester and scavenge the residual "free" adipocyte lipid droplet and ultimately form multinucleate giant cells, a hallmark of chronic inflammation. Adipocyte death increases in obese (*db/db*) mice (30-fold) and humans and exhibits ultrastructural features of necrosis (but not apoptosis). These observations identify necrotic-like adipocyte death as a pathologic hallmark of obesity and suggest that scavenging of adipocyte debris is an important function of WAT macrophages in obese individuals. The frequency of adipocyte death is positively correlated with increased adipocyte size in obese mice and humans and in hormone-sensitive lipase-deficient ($HSL^{-/-}$) mice, a model of adipocyte hypertrophy without increased adipose mass. WAT of $HSL^{-/-}$ mice exhibited a 15-fold increase in necrotic-like adipocyte death and formation of macrophage syncytia, coincident with increased tumor necrosis factor-α gene expression. These results provide a novel framework for understanding macrophage recruitment, function, and persistence in WAT of obese individuals.—Cinti, S., G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, S. Wang, M. Fortier, A. S. Greenberg, and M. S. Obin. **Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* 2005.** 46: **2347–2355.**

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Adipose tissue inflammation is now recognized as an important early event in the development of obesity complications, especially type 2 diabetes (1–7). Recent studies suggest that adipose tissue inflammation is attributable in large part to the proinflammatory actions of bone marrow-derived white adipose tissue (WAT) macrophages. These studies demonstrate that *1*) macrophage numbers and/or macrophage inflammatory gene expression in WAT are positively correlated with adipocyte size and body mass index (BMI) in mice and negatively correlated with weight loss in obese humans (1, 5, 8); *2*) macrophages are the predominant source of tumor necrosis factor-α (TNF-α) and a significant source of interleukin-6 and nitric oxide in WAT of obese (*ob/ob*, *db/db*) mice and humans (2, 5, 7, 9); *3*) a "spike" in macrophage inflammatory gene expression in WAT immediately precedes or is coincident with the onset of hyperinsulinemia in murine diet-induced obesity (7); and *4*) obese mice with genetically ablated macrophage inflammatory (i.e., nuclear factor κB) signaling are protected from insulin resistance (10). Together, these observations implicate macrophage activation in the development of obesity-associated WAT inflammation and insulin resistance.

Despite these advances, we have yet to elucidate the underlying cause and function of macrophage infiltration into WAT of obese subjects. Macrophages are monocytic phagocytes that function in innate immunity and wound healing by sequestering and clearing pathogens, dead cells, and cell debris in an activation-dependent manner (11). Macrophage activation consists of biochemical, morphological, and functional changes that result in the secretion of preformed and/or newly synthesized constituents, such as cytokines and chemokines. These, in turn, switch on the inflammatory response (11). Macrophage activation at sites of inflammation is typically transient, giving way to repair processes that reestablish local tissue function. However, at sites of resistant infectious agents (e.g., tuberculosis) or poorly bio-

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Abbreviations: ADRP, adipocyte differentiation-related protein; BMI, body mass index; CLS, crown-like structures; $HSL^{-/-}$, hormone-sensitive lipase-deficient; MGC, multinucleate giant cell; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

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degradable tissue irritants (i.e., foreign bodies), macrophages remain activated and fuse to form multinucleate giant cells (MGCs) that can persist for weeks or months surrounding the unresolved site. At these sites, MGCs actively phagocytose debris and can acutely produce proinflammatory cytokines (interleukin-1α, TNF-α) until the insult is either cleared by phagocytosis or encapsulated (12).

MGCs have been reported surrounding individual adipocytes in WAT of obese (but not lean) mice and humans (1, 5, 7), suggesting that in the obese state, individual adipocytes become focal and persistent sites of macrophage activation. Macrophage infiltration and MGC formation in adipose tissue are also observed in conjunction with adipocyte ("fat") necrosis, which occurs in various human diseases (e.g., Weber-Christian disease, Rothmann-Makai syndrome, pancreatic panniculitis) (13) and follows the failure of autologous adipose implants (14). These observations suggest the potential involvement of adipocyte cell death in macrophage recruitment and activation in WAT of obese individuals.

The present work demonstrates that *1*) the overwhelming predominance (>90%) of WAT macrophages in obese mice and humans are localized selectively to sites of necrotic-like adipocyte death, where they form syncytia that sequester and ingest adipocyte debris, in particular the residual lipid droplet; and *2*) the frequency of adipocyte death is increased dramatically in obese mice and humans and in a mouse model of adipocyte hypertrophy without obesity. These results identify adipocyte death as an important modulator of obesity-associated macrophage responses in WAT.

MATERIALS AND METHODS

Mice

All animal procedures were in accordance with National Institute of Medical Research guidelines. *db/db* and C57BL/6 mice were purchased from Charles River Italy at 5 weeks of age and used for experimental procedures at 12 weeks of age. Mice were individually caged and maintained on a 12 h/12 h light/dark cycle with free access to standard pellet food (Harlan) and water. Obese male *db/db* mice (n = 5) and lean wild-type (C57BL/6) control mice (n = 5) were used to investigate obesity-associated adipocyte necrosis. The generation of hormone-sensitive lipase-deficient ($HSL^{-/-}$) mice has been described previously (15). $HSL^{-/-}$ mice were backcrossed to C57BL/6 mice for eight generations and used at 8 weeks of age for gene expression analysis and at 12 weeks of age for histological analysis.

Human adipose tissue

Fat biopsies were obtained from male (n = 12) and female (n = 9) patients aged 44.7 ± 16.2 years (mean ± SD) undergoing institutionally approved elective surgery at Ancona General Hospital (Ancona, Italy). Fully informed and written consent was obtained in all cases. The subjects did not suffer from any ongoing disease (infection or cancer). A total of 28 human biopsies from subcutaneous (abdominal and gluteal) and visceral (omental) depots were examined from 12 obese patients (BMI ⩾ 30; 5 male, 7 female), 2 overweight patients (BMI 25–29.9; 1 male, 1 female), and 7 lean subjects (BMI 20–24.9; 5 male, 2 female). The BMI (mean ± SD) of male subjects (31.3 ± 8.7) was not significantly different from that of female subjects (35.1 ± 9.1) ($P = 0.39$).

Light microscopy

Mice were euthanized in the fed state with an overdose of anesthetic (xylazine-ketamine) and immediately perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2–5 min. Subcutaneous and visceral WAT depots were dissected using a Zeiss OPI1 surgical microscope and assessed by light microscopy, transmission electron microscopy, immunohistochemistry, and morphometry. The following WAT depots were examined: subcutaneous (inguinal) and visceral (perivesicular) WAT depots in *db/db* and lean control mice, and visceral (perirenal) depots in $HSL^{-/-}$ and $HSL^{+/+}$ mice. After dissection, WAT depots were further fixed by immersion in the perfusion fixative (overnight, 4°C), dehydrated, cleared, and then embedded in paraffin. Five micrometer thick serial sections were obtained, the first stained by hematoxylin and eosin to assess morphology and the rest processed for immunohistochemistry (see below).

Electron microscopy

Small fragments of tissue were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h, postfixed in 1% osmium tetroxide, and embedded in an Epon-Araldite mixture. Semithin sections (2 μm) were stained with toluidine blue, and thin sections were obtained with an MT-X ultratome (RMC, Tucson, AZ), stained with lead citrate, and examined with a CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Immunohistochemistry

Immunohistochemistry was performed on dewaxed 5 μm serial sections using 3% hydrogen peroxide to inactivate endogenous peroxidases followed by normal goat or horse serum to reduce nonspecific staining. Consecutive serial sections were incubated overnight (4°C) with the following primary antibodies: anti-MAC-2/galectin-3 (1:3,800; Cedarlane Laboratories), anti-mouse F4/80 (1:100; Serotec), anti-perilipin (PREK; 1:300) (16), anti-S-100B (1:250; Dako), and a polyclonal antibody raised against a C-terminal peptide (TEVKNKASLKVQQEVKAQ) of mouse adipocyte differentiation-related protein (ADRP; 1:100). Primary antibody was omitted from negative controls. Appropriate positive controls were also used to test the specificity of all antibodies. Biotinylated, HRP-conjugated second antibodies included goat anti-rabbit IgG (ADRP-C, PREK, S-100B), rat anti-goat IgG (F4/80), and horse anti-mouse IgG (MAC2/galectin-3) (Vector Laboratories, Burlingame, CA). Histochemical reactions were performed using the Vectastain ABC Kit (Vector Laboratories) and Sigma Fast 3,3′-diaminobenzidine as substrate (Sigma, St. Louis, MO). Sections were counterstained with hematoxylin.

Morphometry

Tissue sections were observed with a Nikon Eclipse E800 light microscope using a ×20 objective, and digital images were captured with a DXM 1200 camera. Crown-like structure (CLS) density (CLS per 400 adipocytes), adipocyte surface area, and adipocyte volume were determined using a drawing tablet and the Nikon Lucia IMAGE version 4.61 morphometric program. The mean cell volume was derived from the mean surface area, and, positing that adipose cell density is the same as trioline (0.915 μg/μl), mean cell weight (μg lipid per cell) was mathematically derived from mean cell volume (17).

Gene expression analysis

Total WAT RNA was extracted from frozen perirenal fat of 2 month old male wild-type or $HSL^{-/-}$ mice using the RNeasy Lipid Tissue kit (Qiagen, Mississauga, Canada). Total bone marrow macrophage RNA was extracted after washing in 5 ml of PBS and direct lysis of confluent cells in the Petri dish, according to the

manufacturer's protocol (RNeasy kit; Qiagen). RNA served as a template for first-strand synthesis using poly(dT) primers and Superscript II reverse transcriptase (Invitrogen, Burlington, Canada). For quantitative real-time PCR (SmartCycler; Cepheid), we used the QuantiTect SYBR green PCR kit (Qiagen). Primer sets used were as follows: Mac-2a (5′-ATGAAGAACCTCCGGGAAAT-3′ and 3′-GCTTAGATCATGGCGTGGTT-5′), TNF-α (5′-CCAGTGTGGGAAGCTGTCTT-3′ and 3′-AAGCAAAAGAGGAGGCAACA-5′), and β-actin (5′-CGTTGACATCCGTAAAGACCT-3′ and 3′-GCAGTAATCTCCTTCTGCATCC-5′). Each reaction yielded amplicons of ~100 bp. PCR conditions were as follows: 15 s at 94°C, 20 s at 60°C, and 20 s at 72°C, for 45 cycles. After amplification, a melting curve (0.01°C/s) was used to confirm product purity. Results are expressed relative to β-actin.

Statistical analysis

Results are presented as means ± SD. Data analysis was performed using Systat (version 10) or Instat GraphPad. Differences between groups were assessed by full interaction general linear models and protected post-hoc tests, paired or unpaired *t*-tests, or Fisher exact tests, as appropriate. Differences between groups were considered statistically significant at $P \leq 0.05$.

RESULTS

WAT macrophages localize almost exclusively to sites of necrotic-like adipocyte death in obese *db/db* mice and obese humans

We initially assessed the frequency and distribution of mature macrophages in fixed WAT of lean (wild-type) and obese *db/db* mice, an established model of obesity-associated WAT inflammation (7). Macrophages were identified based on immunoreactivity for both F4/80 (data not shown) and MAC-2 (also known as galectin-3), a lectin expressed by activated macrophages that mediates macrophage phagocytic and inflammatory responses (18–20). Surprisingly, macrophages were nonrandomly distributed in WAT of both lean and obese mice. Rather than being dispersed throughout the tissue, macrophages were instead aggregated in CLS, which contained up to 15 macrophages surrounding what appeared to be individual adipocytes (**Fig. 1**). CLS formation was a rare event in lean wild-type mice (0.34 ± 0.28 CLS per 100 adipocytes) (Fig. 1A) but was increased ~30-fold (10.50 ± 1.05 CLS per 100 adipocytes) in obese *db/db* mice ($P < 0.001$) (Fig. 1B). Notably, the preponderance of MAC-2-positive macrophages in WAT of lean wild-type and obese *db/db* mice were localized to CLS (lean, 82.3 ± 5.3%; obese, 90.6 ± 1.8%). CLS contained only an occasional (<1%) neutrophil or lymphocyte and no preadipocytes, based on electron microscopic morphology (21) and the absence of immunoreactivity for the preadipocyte marker S-100B (22, 23) (data not shown). CLS macrophages formed MAC-2-positive MGCs (Fig. 1C, D), indicating that individual adipocytes act as chronic sites of macrophage activation (12, 24). Together, these observations demonstrate that *1*) the vast majority of WAT macrophages are aggregated in CLS that form around individual adipocytes; and *2*) the number of such "inflammatory" adipocytes increases dramatically in obese *db/db* mice.

We next used electron microscopy to examine the ultrastructural features of those adipocytes that were surrounded by CLS. Remarkably, in all visceral and subcutaneous WAT depots of multiple obese mice, CLS were localized exclusively to dead adipocytes (**Fig. 2A**). Adipocyte death in obese *db/db* mice exhibited none of the classical ultrastructural features of apoptosis, such as chromatin condensation, plasma membrane blebbing (zeiosis), or membrane-bound apoptotic bodies with nuclear fragments (25–27) (Fig. 2A, B). However, in all instances, obesity-associated adipocyte death exhibited features of necrosis, including ruptured plasma membranes, dilated endoplasmic reticulum, cell debris in the extracellular space, and the appearance of small lipid droplets in the cytoplasm

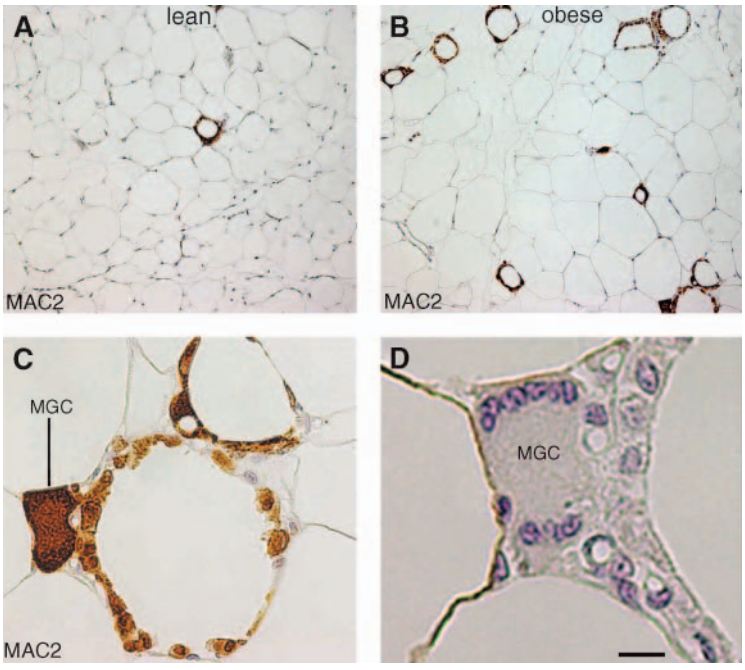

Fig. 1. White adipose tissue (WAT) macrophages localize to crown-like structures (CLS) around individual adipocytes, which increase in frequency with obesity. Light microscopy of visceral WAT of lean (A) and obese *db/db* (B) mouse showing MAC-2 immunoreactive macrophages (brown color) aggregated to form rare (A; lean) or numerous (B; obese) CLS among unilocular adipocytes. Note that almost all MAC-2 immunoreactive macrophages are organized to form CLS. C: Enlargement of the bottom right corner of B showing that almost all mononuclear cells in CLS are MAC-2 immunoreactive (i.e., activated macrophages). Note the multinucleate giant cell (MGC), which stains intensely for MAC-2. D: Serial section consecutive to that shown in C confirming the presence of multiple nuclei (blue) in the MGC. Bar = 100 μm for A, B, 28 μm for C, and 10 μm for D.

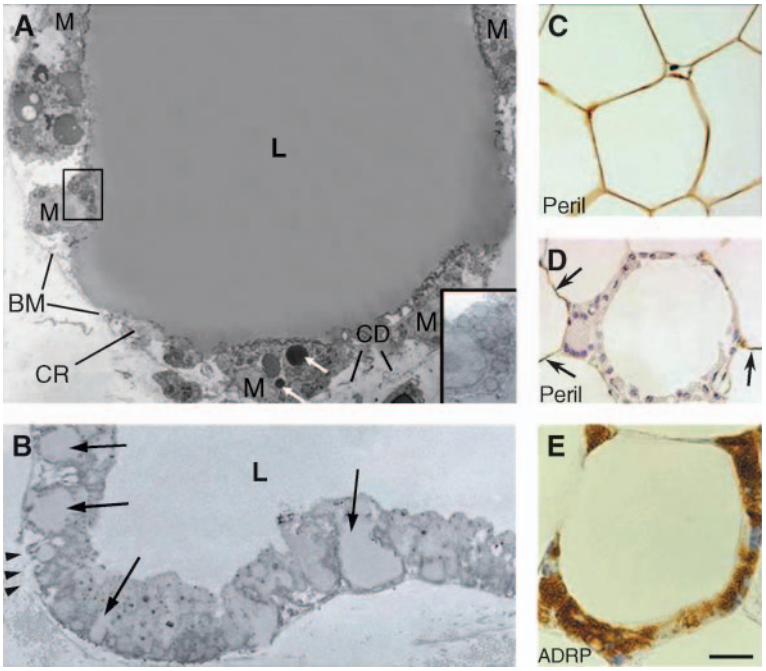

Fig. 2. CLS form exclusively at sites of adipocyte death and scavenge the residual adipocyte lipid droplet. A: Electron micrograph showing CLS macrophages (M) surrounding the residual lipid droplet (L) of a dead adipocyte. Evidence of necrosis includes disrupted basal membrane (BM), cytoplasmic remnants (CR), cell debris (CD) in the interstitium, and the apparent degeneration of the unilocular lipid droplet into small droplets. White arrows indicate lipid-laden phagolysosomes. The inset shows an enlargement of the squared area showing small adipocyte lipid droplets engulfed by a macrophage. B: Necrotic features in *db/db* mouse adipocyte before recruitment of CLS macrophages. Note the rupture of basal membrane (arrowheads) and the loss of lipid droplet (L) integrity manifest as small lipid droplets in the cytoplasm (arrows). C, D: Lipid droplet degeneration in necrotic adipocytes can be detected at the light microscope level by the loss of lipid droplet-associated proteins. All adipocytes of lean mice (C) are perilipin (Peril) immunoreactive (brown), whereas adipocytes surrounded by CLS in obese mice (D) are not immunoreactive for perilipin [or adipocyte differentiation-related protein (ADRP); not shown]. Note that viable adipocytes (not surrounded by CLS) in obese mice (D) retain perilipin immunoreactivity (arrows). E: Scavenging of residual adipocyte lipid droplet by CLS macrophages is associated with upregulated ADRP expression (brown) and the formation of ADRP-positive lipid droplets in CLS macrophages. Bar = 5.3 μm for A, 2.7 μm for A, inset, 0.8 μm for B, 50 μm for C, 43 μm for D, and 16 μm for E.

(26–29) (Fig. 2A, B). Disrupted plasma membranes, small cytoplasmic lipid droplets, and interstitial cell debris were recently noted as in vivo features of experimentally induced adipocyte necrosis in mice (26). We also detected degenerating adipocytes that were not surrounded by CLS macrophages (Fig. 2B), indicating that the sequestration of adipocytes within CLS is secondary to adipocyte demise. These adipocytes exhibited ultrastructural features of necrosis (i.e., ruptured basal membranes, organelle degeneration, and small cytoplasmic lipid droplets) but no detectable features of apoptosis (Fig. 2B).

The appearance of small cytoplasmic lipid droplets suggested that the unilocular adipocyte lipid droplet degenerated during the process of obesity-associated adipocyte death. Lipid droplet degeneration was confirmed by immunohistochemistry for the essential lipid droplet-associated protein perilipin, a key regulator of adipocyte lipolysis (Fig. 2C, D) (16, 30). No perilipin immunoreactivity was detected on lipid droplets of adipocytes surrounded by CLS (Fig. 2D). In contrast, perilipin was detected in adjacent, viable adipocytes (Fig. 2C, D). These results confirm that nonviable adipocytes are foci of macrophage localization and syncytia formation in WAT of obese *db/db* mice. In conjunction with ultrastructural studies of obesity-associated adipocyte death and CLS formation (Fig. 2A, B), these observations indicate that each CLS designates a site of necrotic-like adipocyte death. Given that >90% of macrophages in WAT of obese *db/db* mice are present in CLS (Fig. 1B), our results additionally demonstrate that the preponderance of macrophages in WAT of obese mice are selectively localized to individual dead adipocytes.

CLS macrophages persist at sites of obesity-associated adipocyte death to scavenge the exposed "free" lipid in the interstitium (Fig. 2A, E). The scavenging of free lipid by CLS macrophages is likely to be a protective response, at least initially (see Discussion). Internalization of adipocyte lipid (or its reesterified derivatives) is associated with lipid droplet formation and intense macrophage immunoreactivity for ADRP (Fig. 2E), a lipid droplet-associated protein that increases in cells in response to the accumulation of neutral lipid (31). Upregulated ADRP expression in CLS macrophages provides one explanation for the previ-

ous report of upregulated ADRP gene expression in WAT of obese *db/db* mice (7). The persistence of lipid-scavenging macrophages and the formation of MGCs at free lipid droplets of dead adipocytes (Fig. 1C, D) suggest that these droplets act as chronic sites of macrophage activation in WAT. However, we currently do not know whether or how scavenging of adipocyte lipid and/or MGC formation by WAT macrophages promotes or prolongs the proinflammatory macrophage gene expression that characterizes obesity and its complications (5, 7).

We next investigated the relationship between WAT macrophage localization and obesity-associated adipocyte death in human subjects. We used CLS formation around perilipin-negative adipocytes as a marker of adipocyte death (**Fig. 3A–D**) and electron microscopy to assess necrotic and apoptotic features (Fig. 3E). As in *db/db* mice, CLS present in human WAT were localized exclusively to degenerate adipocytes, based on the absence of perilipin staining (Fig. 3B, D). Again, adipocyte death exhibited only necrotic features, including plasma membrane rupture, the presence of numerous small lipid droplets in the cytoplasm, and dilated endoplasmic reticulum and mitochondria (Fig. 3E).

Adipocyte death in human subjects was positively correlated with obesity, with adipocyte death detected in 13 of 19 biopsies from obese subjects (BMI $>$ 30–45) but in only 2 of 9 biopsies from lean or overweight individuals (BMI $<$ 30) ($P = 0.04$ by Fisher's exact test). Adipocyte death was also correlated with mean adipocyte size, which increases in obesity (see below). Adipocyte size in biopsies containing one or more dead adipocytes (0.37 ± 0.08 μg lipid per cell; n = 15) was twice that of biopsies containing no dead adipocytes (0.17 ± 0.09 μg lipid per cell; n = 13) ($P < 0.001$). This association was observed in both subcutaneous and visceral WAT depots (Fig. 3F). Interestingly, adipocyte death was detected in a lean subject (BMI = 24) with large adipocytes (average = 0.40 μg lipid per cell), whereas no dead adipocytes were detected in an obese subject (BMI = 34) with relatively small adipocytes (average = 0.23 μg lipid per cell). These observations suggest that rates of adipocyte death increase as adipocytes enlarge.

Adipocyte hypertrophy in the absence of obesity is associated with necrotic-like adipocyte death and CLS formation

Increases in WAT mass reflect adipocyte hypertrophy, adipocyte hyperplasia, or both (32). The correlation of adipocyte death with adipocyte size in humans (see above) and associations of adipocyte hypertrophy with dysregulated adipocyte metabolism and increased adipocyte stress (1, 5, 33, 34) suggested that adipocyte hypertrophy might promote obesity-associated adipocyte death. To test this

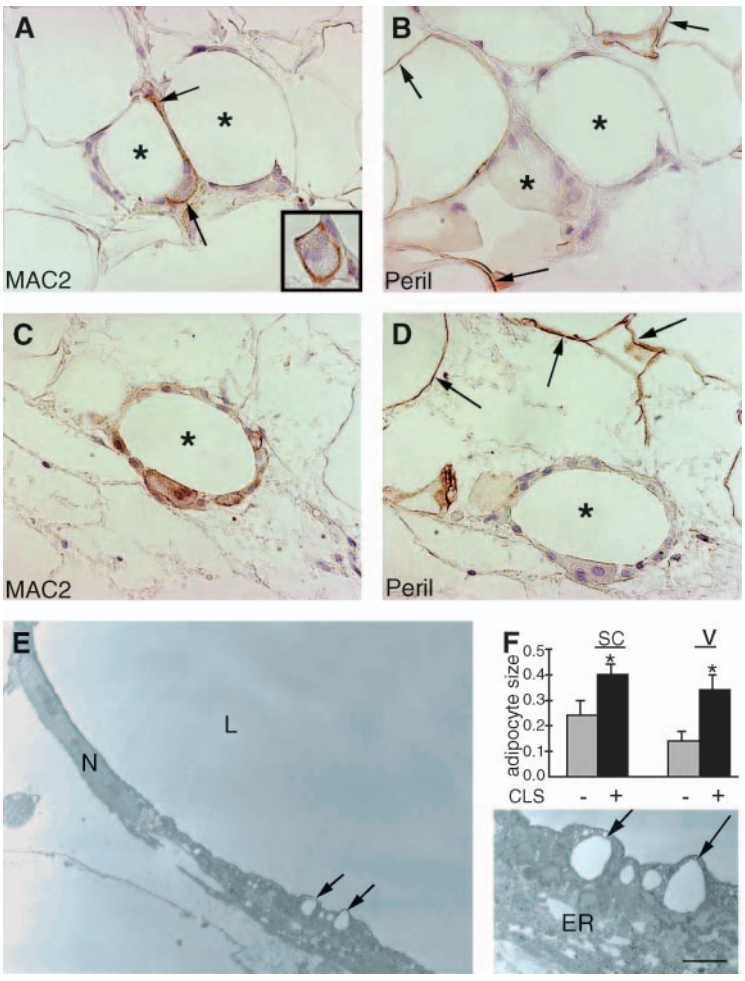

Fig. 3. CLS formation at sites of adipocyte death in human WAT and correlation of adipocyte death and adipocyte size. Human subcutaneous (A, B) and visceral (omental; C, D) fat depots demonstrating MAC-2-positive (brown) macrophages (A, C) organized exclusively in CLS surrounding degenerate (perilipin-free; B, D) lipid droplets (asterisks). Arrows in A highlight MAC-2 immunoreactivity. The inset in A more clearly shows surface staining for MAC-2 in a CLS macrophage from the same patient. Arrows in B and D denote perilipin immunoreactivity of lipid droplets in viable adipocytes. E: Representative electron micrograph showing necrotic degenerative features of obesity-associated adipocyte death in human WAT. Evidence of necrosis includes dilated endoplasmic reticulum (ER) and mitochondria (not shown) and the presence of numerous small lipid droplets (arrows; enlarged at bottom right). Note that the nucleus (L) exhibits no signs of apoptosis (i.e., chromatin condensation, pyknosis). F: Adipocyte death is correlated with mean adipocyte size in human subcutaneous (SC) and visceral (V) adipose tissue. Fat biopsies from human subjects (body mass index range of 20–45) were scored for the presence (+) or absence (−) of CLS (i.e., MAC-2-positive cells surrounding a perilipin-negative lipid droplet). Values shown are means ± SD. * $P < 0.05$ for within-depot comparison. Bar = 27 μm for A–D, 3.7 μm for E, and 1.5 μm for E, inset.

hypothesis, we assessed the frequency of adipocyte death and CLS formation in HSL$^{-/-}$ mice. HSL is a major lipase in mature adipocytes, and HSL abrogation results in increased adipocyte lipid storage and, thus, adipocyte hypertrophy. Adipocyte hypertrophy in HSL$^{-/-}$ mice is not associated with increased adipose mass (15, 35–37). Thus, HSL$^{-/-}$ mice provide a genetic model with which to examine the association of adipocyte hypertrophy and adipocyte death in the absence of obesity.

In the present study, mean cell size of viable (perilipin-positive) adipocytes in HSL$^{-/-}$ mice (0.51 ± 0.14 μg lipid per cell) was >3-fold that of wild-type controls (0.15 ± 0.05 μg lipid per cell) ($P < 0.001$). As early as 8 weeks of age, increased adipocyte size in HSL$^{-/-}$ mice was associated with macrophage infiltration and inflammation, manifested as increased levels of transcripts for MAC-2 (7-fold) and TNF-α (5-fold) in perirenal WAT (P9 0.05) (**Table 1**) (31). TNF-α gene expression was not increased in HSL$^{-/-}$ macrophages that were differentiated in vitro from bone marrow-derived precursors (data not shown). Thus, increased TNF-α gene expression is not an intrinsic property of nonactivated macrophages in HSL$^{-/-}$ mice. These data indicate that adipocyte hypertrophy in HSL$^{-/-}$ mice is coincident with macrophage recruitment and activation and with WAT inflammation in the absence of increased adipose mass.

As in obese *db/db* mice and obese humans, the overwhelming preponderance (98 ± 0.7%) of macrophages (F4/80, MAC-2-positive cells) in WAT of lean HSL$^{-/-}$ mice were aggregated in CLS that selectively surrounded nonviable (perilipin-negative) adipocytes (**Fig. 4A**). By 12 weeks of age, the frequency of adipocyte death (CLS formation around perilipin-negative adipocytes) in WAT of HSL$^{-/-}$ mice was ~15-fold greater (29.0 ± 2.0 CLS per 100 adipocytes) than the frequency of adipocyte death in wild-type controls (2.0 ± 0.4 CLS per 100 adipocytes). Adipocyte death in lean HSL$^{-/-}$ mice exhibited ultrastructural features of necrosis, including basal membrane rupture and endoplasmic reticulum dilatation (Fig. 4B). As observed in WAT of obese mice and humans (Figs. 2C, D, 3E), lipid droplet degeneration was a characteristic early feature of adipocyte death in HSL$^{-/-}$ mice (Fig. 4B). Moreover, as with adipocyte death in obese mice and humans, we detected no chromatin or nuclear condensation (Fig. 4B), consistent with a previous report that the frequency of apoptotic [i.e., terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive] cells is not increased in WAT of HSL$^{-/-}$ mice (37). CLS macrophages in WAT of HSL$^{-/-}$ mice actively ingested exposed adipocyte lipid (Fig. 4C), elaborated numerous lipid droplets, and expressed intense ADRP immunoreactivity (Fig. 4D). Thus, adipocyte hypertrophy in the absence of increased adipose mass is associated with *1*) increased rates of necrotic-like adipocyte death; *2*) selective macrophage recruitment to sites of adipocyte death; and *3*) the formation of lipid-scavenging macrophage syncytia around free adipocyte lipid droplets. This is identical to the events observed in WAT of obese mice and humans (Figs. 1–3).

TABLE 1. Evidence of macrophage infiltration and inflammatory activation in WAT of HSL$^{-/-}$ mice

	Genotype			
mRNA	HSL$^{-/-}$	HSL$^{+/+}$	Ratio $^{-/-}/^{+/+}$	P
MAC-2	105.2 ± 26.7	14.8 ± 5.1	7.1	0.02
TNF-α	0.9 ± 0.3	0.2 ± 0.1	4.5	0.04

HSL$^{-/-}$, hormone-sensitive lipase-deficient; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue. Relative levels of MAC-2 and TNF-α mRNA in HSL$^{-/-}$ and HSL$^{+/+}$ mice were determined by real-time PCR of perirenal WAT from 2 month old male mice (n = 6) of each genotype. Gene expression is expressed relative to that measured for β-actin (see Materials and Methods). Ratios comparing HSL$^{-/-}$ and HSL$^{+/+}$ mice are shown.

DISCUSSION

Bone marrow-derived macrophages accumulate in WAT of obese mice and humans, where they promote adipose and systemic inflammation and the development of obesity-related metabolic complications (5, 7, 38). However, neither the underlying cause nor the function of macrophage recruitment in WAT of obese subjects is known. In the present study, we demonstrate that *1*) the preponderance of WAT macrophages in lean and obese mice and humans is localized to individual dead adipocytes; *2*) at these sites, macrophages express activation markers (MAC-2), form syncytia (CLS) that surround and scavenge residual adipocyte lipid, and ultimately form MGCs, a hallmark of chronic inflammatory states; and *3*) the frequency of adipocyte death is increased >30-fold in a mouse (*db/db*) model of obesity-associated WAT inflammation (7) as well as in obese humans (BMI > 30). These observations suggest that adipocyte death promotes macrophage recruitment, accumulation, and persistence in WAT of obese individuals.

The mechanism of adipocyte death has potentially significant implications for the development of obesity-associated WAT inflammation. Broadly speaking, apoptosis is noninflammatory, reflecting the packaging of cell constituents into inflammation-suppressive apoptotic bodies (26, 27, 39). In contrast, during necrosis, cell contents are released into the extracellular space, where they evoke inflammatory responses (27, 39, 40). Electron microscopy is the most reliable technique for assessing classical apoptosis, which is defined by stereotypic morphological changes, including chromatin condensation, plasma membrane blebbing, and the formation of membrane-bound apoptotic bodies containing nuclear fragments (26, 27, 39). In the present study, obesity-associated adipocyte death exhibited none of these apoptotic hallmarks. Rather, moribund and dead adipocytes invariably displayed features of necrosis, most notably, rupture of the plasma membrane (Figs. 2A, B, 4B), dilatation of the endoplasmic reticulum (Figs. 3E, 4B), and release of cell contents to the extracellular space (Fig. 2A) (26, 27, 40). These observations suggest that obesity-associated adipocyte death occurs by necrosis.

Arguing against this conclusion and supporting an apop-

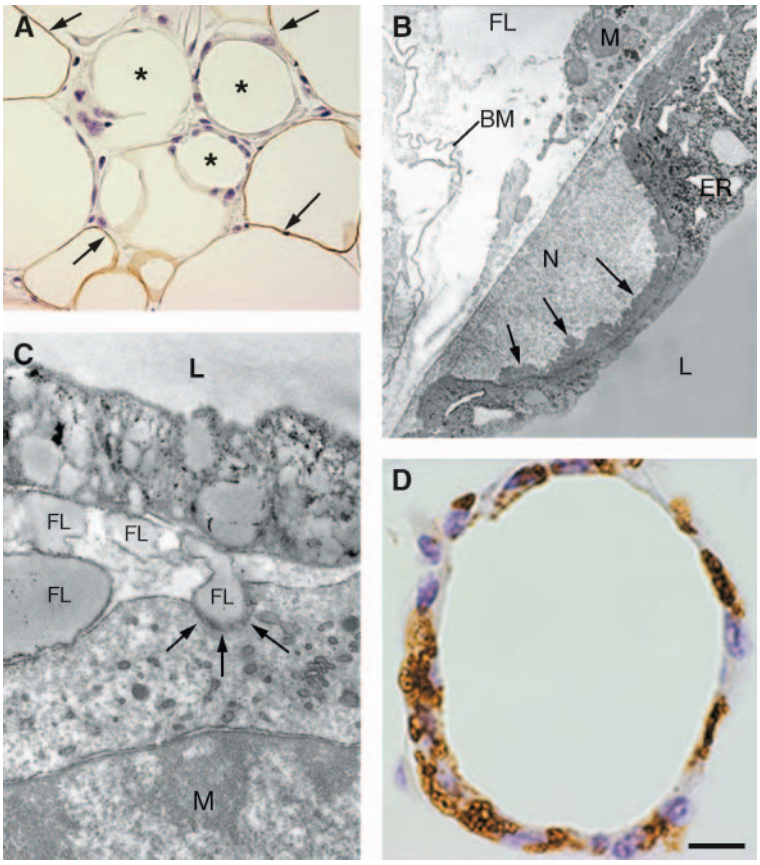

Fig. 4. Adipocyte death, CLS formation, and lipid scavenging in hormone-sensitive lipase-deficient ($HSL^{-/-}$) mice, a model of adipocyte hypertrophy without obesity. A: Perilipin immunohistochemistry demonstrating CLS formation selectively around perilipin-free adipocyte-like structures (asterisks) but not around viable adipocytes expressing perilipin (arrows). B: Electron micrograph demonstrating degenerative aspects (early signs of necrosis) and necrosis in an $HSL^{-/-}$ mouse. Degenerative aspects include dilatation of the endoplasmic reticulum (ER) and lipid droplet (L) infiltration into the nucleus (arrows). Cell death is indicated by the ruptured basal membrane (BM) and free lipid droplets (FL) in the interstitium. The absence of chromatin or nuclear condensation suggests degeneration in the absence of "classical" apoptosis. L, adipocyte lipid droplet; M, macrophage. C: High-power electron micrograph showing internalization of free lipid (FL) derived from the degenerating adipocyte lipid droplet (L) by CLS macrophage (M). D: CLS macrophages of $HSL^{-/-}$ mice elaborate numerous intracellular lipid droplets and are intensely immunoreactive for the lipid droplet-associated protein, ADRP. Bar = 36 μm for A, 1.25 μm for B, 0.45 μm for C, and 15 μm for D.

totic mechanism of obesity-associated adipocyte death is the relative absence of neutrophils at sites of adipocyte death (this study) and in adipose tissue of obese mice and humans (7). Whereas apoptosis can activate macrophages without the participation of other proinflammatory cells (i.e., neutrophils), the inflammatory response to necrosis is typically a sequential process in which neutrophils initially participate. Neutrophil infiltration into WAT is observed in response to adipocyte necrosis in human pathologic conditions as well as acutely in response to experimentally induced adipocyte necrosis in mice (13, 14, 29, 41). Thus, obesity-associated adipocyte death appears to occur by an alternative death pathway sharing features of both necrosis and apoptosis. A number of such alternative death pathways (e.g., "necrosis-like programmed cell death," "paraptosis") have recently been described in which apoptotic signaling pathways and proteolytic cascades may participate, but the morphology of cell execution is predominantly necrotic (27, 40). These necrosis-like forms of apoptotic death typically lack chromatin condensation and apoptotic bodies (as in the present study). A critical unresolved question is whether this alternative pathway of adipocyte death promotes proinflammatory activation of CLS macrophages and MGCs, thereby contributing to the chronic proinflammatory tone in WAT of obese individuals (5, 7).

The development of obesity is associated with the release by adipocytes of macrophage chemoattractants, arrestants, and activators (5, 38, 42, 43). Increased release of these substances by metabolically dysregulated or otherwise "distressed" adipocytes or by adjacent cells could provide one mechanism to account for the preferential localization of WAT macrophages to dead adipocytes. However, it is conceivable that events unrelated to obesity-associated adipocyte death induce initial macrophage extravasation from the blood into WAT and that these macrophages are subsequently recruited to moribund adipocytes. Irrespective of the factors promoting initial macrophage recruitment to WAT, the relative paucity of macrophages that were not localized to dead adipocytes in the present study suggests that macrophages are recruited to degenerating or dead adipocytes soon after extravasation into WAT.

Our data for human subjects and for the $HSL^{-/-}$ mouse model of adipocyte hypertrophy without obesity suggest that adipocyte hypertrophy per se promotes adipocyte death, macrophage aggregation, and CLS formation. Therefore, hypertrophy-induced adipocyte death is a plausible mechanism underlying the reported association between mean adipocyte size and the magnitude of macrophage infiltration and inflammatory gene expression in WAT (7). In addition, the association between adipocyte size and adipocyte death provides a potential explanation for the fact that, during the development of obesity, adipocytes get only "so large," after which adipocyte hypertrophy gives way to adipocyte hyperplasia (32). The present study suggests that the initial hypertrophic response of adipocytes renders them prone to necrotic-like death, after which only adipocyte hyperplasia can efficiently maintain or increase the lipid-storage capacity of adipose tissue.

The mechanism(s) by which hypertrophy potentially promotes adipocyte death is unclear, but cell stress is im-

plicated. Hypertrophic adipocytes are subjected to multiple cytotoxic stressors, including endoplasmic reticulum stress, hypoxia, increased TNF-α, reactive oxygen species, and free fatty acids (1, 4, 33, 34, 44, 45). These cytotoxic stresses activate inflammatory signaling cascades that regulate stress-induced cell death (46, 47) and downregulate adipocyte insulin signaling and peroxisome proliferator-activated receptor γ (PPARγ)-regulated gene expression (34, 48, 49). Interestingly, experimental depletion of systemic insulin levels or targeted disruption of PPARγ gene expression in mature adipocytes has been shown to induce adipocyte necrosis and subsequent inflammatory cell infiltration in mouse WAT (29, 41). Thus, compromised insulin signaling and/or downregulated expression of PPARγ-regulated genes in hypertrophic adipocytes may contribute to obesity-associated adipocyte death. Stress-induced mechanisms of adipocyte death may be complemented and locally amplified by cytotoxins released from activated WAT macrophages (i.e., TNF-α, nitric oxide, reactive oxygen species). Thus, WAT macrophages could conceivably participate in adipocyte "execution"(7), thereby contributing to a feed-forward mechanism of adipocyte death and macrophage recruitment.

MAC-2 expression in CLS macrophages and MGCs is consistent with the lectin's roles in leukocyte recruitment, phagocytosis, lipid scavenging, and inflammatory progression (18–20, 50, 51). MAC-2 expression at sites of adipocyte necrosis may be functionally linked to macrophage aggregation and CLS formation, as MAC-2 is a potent macrophage chemoattractant that promotes macrophage aggregation (19). MAC-2 also possesses antiapoptotic activity that is implicated in macrophage survival at sites of inflammation (51). The chemoattractant and antiapoptotic activities of MAC-2 likely facilitate and prolong macrophage recruitment, aggregation, and function at sites of adipocyte death.

Clearance of free lipid appears to be an important function of MAC-2-expressing macrophages in WAT (this study) as well as in the arterial wall (52) and in the regenerating peripheral nervous system after injury (53). In WAT, free lipid droplets released from necrotic adipocytes must be sequestered and removed from the interstitium, because they are insoluble and provide an unprotected source of cholesterol and cytotoxic fatty acids that could damage WAT cells (44, 54, 55). In addition, clearance of free lipid (and other adipocyte debris) must precede remodeling of the extracellular matrix and the recruitment of adipocyte precursors required for new fat cell differentiation at sites of adipocyte loss (56). This and other remodeling functions of WAT macrophages (11) may be particularly important in maintaining and expanding the lipid-storage capacity of obese individuals despite dramatically increased rates of adipocyte death. However, it is currently undetermined whether and how lipid-laden macrophage syncytia are "cleared" from WAT to make room for new adipocytes.

In summary, we demonstrate for the first time that *1*) the preponderance of macrophages in WAT of lean and obese mice and humans is selectively localized to dead adipocytes; *2*) rates of adipocyte death increase dramatically in obesity, potentially reflecting cytotoxic effects of adipocyte hypertrophy; *3*) obesity-associated adipocyte death appears to involve an alternative death pathway exhibiting morphological features of necrosis and the leukocyte-eliciting profile of apoptosis; and *4*) free lipid droplets of dead adipocytes act as persistent sites of macrophage fusion, lipid uptake, and MGC formation. These observations provide a novel framework for understanding macrophage recruitment and function in WAT and for elucidating the underlying causes of obesity-associated WAT inflammation.

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