Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding

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Abstract **Chronic inflammation of adipose tissue in obesity is by now an established phenomenon, but the initiating event(s) of the inflammatory cascade are still unknown. We hypothesized that neutrophil infiltration into adipose tissue may precede macrophage infiltration as in classical immune responses. Here we demonstrate that early (3 and 7 days) after initiating high-fat feeding of C57BL/6J mice, neutrophils transiently infiltrate the parenchyma of intra-abdominal adipose tissue. Mean periepdidymal fat myeloperoxidase expression (representing neutrophils) was significantly increased 3.5-fold ($P < 0.01$) and 2.9-fold ($P < 0.03$), at days 3 and 7 compared with day 0. Immunohystochemistry analysis demonstrated a physical binding between neutrophils and adipocytes, which was supported by in vitro adherence assay: mouse peritoneal neutrophils adhered to a monolayer of 3T3-L1 mouse adipocytes, in a manner dependent on their activation state, 41.9 ± 3.7% or 29.5 ± 2%, by PMA or the IL-8 analog CXCL1 (KC), respectively, compared with 24.8 ± 1.5% in unstimulated neutrophils, respectively. The degree of surface exposure of CD11b (Mac-1) corresponded to the percentage of adhered neutrophils. The adherence was prevented by preincubating neutrophils or adipocytes with anti-CD11b or anti-ICAM-1 antibodies. Furthermore, immunoprecipitation of CD11b from lysates of a mixed neutrophil-adipocyte cell population resulted in coimmunoprecipitation of ICAM-1, indicating that the interaction is mediated by neutrophil CD11b and adipocyte ICAM-1.—** Elgazar-Carmon, V., A. Rudich, N. Hadad, and R. Levy. **Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J. Lipid Res.* 2008.** 49: **1894–1903.**

Supplementary key words inflammation • obesity • adipocytes

Obesity is increasingly accepted as a condition characterized by low-grade chronic inflammation (1). Systemically, this is evidenced by elevated levels of various inflammatory markers including C-reactive protein, TNFα, and IL-6, and by an activated state of circulating leukocytes (2–4). Adipose tissue, the most clearly physically altered tissue in obesity, has become recognized in recent years as an important source and as a target of inflammatory processes. Many of the secreted products of adipose tissue found to be elevated in the obese state are well-characterized chemoattractants and/or activators of monocytes or polymorphonuclear cells, such as monocyte chemoattractant protein 1 and interleukin 8 (IL-8), respectively (5). Consistently, adipose tissue in obesity has been shown to be infiltrated by macrophages, at least some of which are of bone marrow origin (6, 7). Several studies now suggest that this infiltration of immune cells plays a role in the pathogenesis of metabolic alterations that accompany obesity: pharmacologically or genetically antagonizing monocyte chemoattractant protein 1 attenuated monocyte/macrophage infiltration into adipose tissue in obese mice, and protected against the development of insulin resistance (8, 9). In humans, the degree of macrophage infiltration into the omental fat depot correlates with histological alterations in the liver and with clinical parameters of comorbidity (10, 11). Adipocyte-macrophage coculture approaches revealed a putative functional paracrine loop between the two cell types, in which TNFα secretion from macrophages results in enhanced adipocyte lipolysis, and the ensuing secretion of fatty acids further stimulates inflammatory cytokines, creating a vicious cycle (12). Collectively, adipose tissue may be infiltrated by immune cells, and this may be a significant contributing process to the pathogenesis of obesity comorbidity.

Time-course analysis of adipose tissue infiltration by macrophages was assessed in the high-fat fed mouse model of obesity, which demonstrated increased macrophage infiltration beginning at 8 weeks of diet-induced obesity (6, 13). In various infectious and noninfectious inflammatory processes, "chronic" inflammatory infiltration of tissues characterized by predominant mononuclear cell infiltrate is usually preceded by infiltration of the tissue by circulat-

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ing neutrophils ("acute infiltrate") (14). This phase may be transient, or more persistent. Based on the finding that in an established (chronic) state of obesity, adipose tissue may be infiltrated by immune cells of the monocyte/macrophage lineage, and on the fact that adipose tissue can also secrete cytokines that predominantly attract neutrophils (IL-8), we hypothesized that neutrophil infiltration of adipose tissue may precede the macrophage infiltration. Here, we demonstrate, for the first time, that neutrophils transiently infiltrate adipose tissue as early as 3 days after initiating a high-fat diet in mice. We then use in vitro cellular systems to characterize adipocyte-neutrophil adherence molecularly, demonstrating that a protein complex formation between neutrophil CD11b and adipocyte ICAM-1 mediates this interaction.

METHODS

Materials and reagents

Chromium 51 ($Na^{51}CrO_4$; PerkinElmer Life and Analytical Sciences Inc., Waltham, MA), $MnCl_2$, phorbol-12-myristate-13-acetate (PMA), diphenyleneiodonium (DPI), sodium dodecyl sulfate (SDS), percoll (Sigma, Israel), RPMI 1640, phosphate buffered saline (PBS), Hank's Balanced Salt Solution in the presence or absence of 1 mM Ca^{2+} ($HBSS^{2+}$ or $HBSS^{2-}$ respectively; Beth-Haemek, Biological Industries, Israel), ECL detection kit (Amersham-Biosciences Uppsala, Sweden), recombinant murine CXCL1 (KC) (Cytolab/Peprotech Asia), anti-mouse CD54, PE-conjugated rat IgG2b, PE-conjugated anti-mouse CD54, anti-mouse CD54 (YN 1/1.7), anti-mouse CD11b (Biolegend), Integrin α_M goat polyclonal antibody (Santa Cruz, CA), FITC-conjugated rat IgG2b, FITC-conjugated rat anti-mouse CD11b (Serotec), rat monoclonal (NIMP-R14) to neutrophils (Novus Biological Inc.), goat anti-mouse myeloperoxidase (Santa Cruz, CA), anti-mouse Mac2 (Cedarlane Laboratories Limited, Ontario, Canada), anti-pSer473-Akt and anti-Akt (cell signaling, Beverly, MA) Avidin-biotin VECTA-STAIN Kit Elite PK 6105 (Vector Laboratories, Burlingame, CA).

Animals and diets

The study was approved by Ben-Gurion University Institutional Animal Care and Use Committee, and was conducted according to the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animal (National Research Council, 1996). Male C57BL/6J mice (Jackson, ME) at 6 weeks of age were fed either a low-fat diet (6% calories from fat; Harlan Teklad 2018sc) or a high-fat diet (60% calories from fat; research diets #12492), as previously performed (13). At 0, 3, 7, 14, 21, 28 days and 8 and 16 weeks mice were euthanized by CO_2 and periepddidymal fat, and fat depots in the subcutaneous inguinal area were dissected out, and immediately fixed in 4% formaldehyde, or snap frozen and stored in liquid nitrogen until further analyzed. At 16 weeks animals received an intravenous bolus of insulin (0.5 U/Kg) or PBS 7 min prior to tissue collection.

3T3-L1 cell culture and treatment

3T3-L1 preadipocytes were grown to confluence and were induced to differentiate into adipocytes in Dulbeco's Modified Eagle's Medium, as we previously described (15). Cells were used 12–14 days following the induction of differentiation, when exhibiting >85% adipocyte morphology by light microscopy.

Neutrophil or monocyte purification

CD-1 mice (about 2 months of age) were injected intraperitoneally with 4% thioglycollate (5 ml) to induce chemical peritonitis. Following 16 to 20 h, peritoneal cavities were lavaged with 15 ml sterile RPMI 1640 culture medium. Neutrophils were separated by Ficoll/Hypaque centrifugation and hypotonic lysis of erythrocytes resulting in 85–90% purity (16). Cells were counted by hemocytometry and their viability was determined by trypan blue exclusion. Macrophages were obtained from mice 4 days after induction of peritonitis and washed two times with PBS, yielding 80–95% macrophages.

Phagocyte cells-adipocytes adherence assay

Neutrophils or macrophages were labeled with 1 μCi $Cr^{51}/10^6$ cells at 37°C during 1 h of gentle shaking (17). Following two washes with cold PBS the phagocytes were resuspended in $HBSS^{2+}$ or $HBSS^{2-}$ at 7.5×10^5 cells/ml. Cr^{51} radiolabeled neutrophils or macorphages were added onto 6- or 12-well plates of confluent, fully differentiated 3T3-L1 adipocytes, and allowed to adhere for 30 min (optimal conditions) in a 5% CO_2 incubator (37°C). Where indicated, monocytes or neutrophils were pretreated for 3 min with 50 ng/ml PMA or 100 ng/ml KC (mouse IL-8 analog). Following the adherence period, cells were thoroughly washed twice with PBS, and the remaining adhered prelabeled phagocytic cells together with the adipocytes were lyzed in 500 μl lysis buffer containing of 1% Triton-X 100, 50 mM tris-HCl (pH, 7.5), 1 mM EDTA and 1 mM EGTA. Radioactivity was then measured in the lysates by γ counter (Diagnostic Products Corp., Biermann, Germany), along with a sample of prelabeled phagocytic cells to calculate the 100% count, based on which the proportional radioactivity in the adherence assay was calculated as the relative percent.

Superoxide anion measurement

The production of superoxide anion (O_2^-) by neutrophils was measured as the superoxide dismutase-inhibitable reduction of acetyl ferricytochrome c by the microtiter plate technique, as previously described with modifications (18). An aliquot of radiolabeled neutrophils or macrophages (5×10^5 cells/well) used for the adherence assay was taken and suspended in 100 μl incubation medium containing ferricytochrome c (150 m*M*), and left unstimulated, or were stimulated, as indicated, with PMA (50 ng/ml), or KC (100 ng/ml). The reduction of ferricytochrome c was followed by a change of absorbance at 550 nm at 2 min intervals for 30 min on a Thermomax Microplate Reader (Molecular Devices, Melno Park, CA). The maximal rates of superoxide generation were determined and expressed as nanomoles $O_2^-/10^6$ cells/10 min using the extinction coefficient $E_{550} = 21\ mM^{-1}\ cm^{-1}$.

Surface expression of Mac-1 (CD11b) in neutrophils

Resting or stimulated neutrophils were incubated with FITC-conjugated anti-CD11b antibody or with a control FITC-conjugated IgG2b- antibody for 40 min in 4°C in $HBSS^{2+}$ or $HBSS^{2-}$. After two washes with the same buffer the cells were fixed with 2.5% formaldehyde, and surface CD11b was detected by FACS analysis (Becton Dickinson, Mountain View, CA).

Surface expression of ICAM-1 in adipocytes

Differentiated adipocytes or nondifferentiated preadipocytes (3T3-L1 fibroblasts) were trypsinized and washed once with PBS. PE-conjugated anti-ICAM-1 antibody or a control PE-conjugated IgG- antibody were added to the cells for 40 min at 4°C. After two washes with PBS, the cells were fixed with

2.5% formaldehyde, and ICAM-1 was detected by FACS analysis (Becton Dickinson).

Immunoblot analysis

Epididymal and subcutaneous fat were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% NP-40, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 0.25% sodium deoxycholate, 0.1% (v/v) 2-mercaptoethanol, and inhibitors (a 1:1,000 dilution of protease inhibitor mixture; Sigma). The lysates were gently shaken for 15 min at 4°C, centrifuged (12,000 × *g*, 15 min at 4°C), and the fraction between the pellet and adipose fat was collected. Purified peritoneal neutrophils were lyzed by 1% Triton × 100 lysis buffer containing 50 mM Hepes (pH, 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 10 μM $MgCl_2$, and inhibitors (a 1:1,000 dilution of protease inhibitor mixture; Sigma), incubated in 4°C for 15 min and centrifuged (13,000 × *g*, 30 min at 4°C) to obtain the supernatant. Protein concentration was determined using the Bio-Rad Bradford method procedure (Munich, Germany). Samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (for each sample 100 μg and for the neutrophils 3 μg). The resolved proteins were electrophoretically transferred onto a nitrocellulose membrane, blocked in 5% milk in TBS-T (TBS containing 0.1% Tween-20), and incubated overnight at 4°C with either goat anti-myeloperoxidase (MPO) (1:100), anti-mouse β-actin (1:10,000), anti-pSer473 Akt (1:1000) or anti-Akt (1:1000). After being washed with TBS-T (four washes for 15 min each) the membranes were incubated with 1:10,000 dilutions of the respective HRP-conjugated secondary antibodies for 1.5 h at room temperature, and proteins were quantified using video densitometry analysis (ImageGauge version 4.0 Fuji) as described previously (15, 19). In each experiment the intensity of the band from each animal was expressed as a fold value of the amount of MPO in 3 μg of neutrophils, and expressed in arbitrary units.

Co-immunoprecipitation of ICAM-1 with CD11b in mixed adipocyte-neutrophils lysates

After the adherence assay between neutrophils and adipocytes, the mixed populations were lyzed in 1% Triton lysis buffer. The lyzed samples were incubated overnight on ice with either no additional antibody or with 5 μg/ml anti-CD11b antibody (Santa-Cruz Biotechnology). Thirty μl of recombinant protein G agarose were added to the samples and they were tumbled end-over-end for 1 h and washed three times with lysis buffer. The samples were boiled in SDS sample buffer for detection of CD11b the lysis buffer was devoid of β-mercaptoethanol. Proteins were separated on 7% SDS-polyacrylamide gel electrophoresis (PAGE) and the resolved proteins were electrophoretically transferred onto a nitrocellulose membrane, blocked in 5% milk in TBS-T (TBS containing 0.1% Tween-20) and incubated overnight at 4°C with goat anti-CD11b (1:500) or rat anti-ICAM-1 (1:200). After being washed with TBS-T (four washes for 20 min each), the membranes were incubated with 1:10,000 dilutions of the respective HRP-conjugated secondary antibodies for 1.5 h at room temperature, and proteins were quantified using the enhanced chemiluminescence detection system (Amersham).

Immunocytochemistry and immunohistochemistry

Neutrophils were detected by mouse monoclonal antibody against neutrophils (NIMP-R14) as described (20), and by cell size and nuclear morphology. Preadipocytes were differentiated into mature adipocytes on cover slips and the adhesion assay was preformed with unstimulated or activated neutrophils. The adhered cells were fixed with 4% formaldehyde for 25 min at room temperature. Nonspecific reactivity was blocked by incubating the slides with 20% normal rabbit serum for 20 min (avidin-biotin VECTA-STAIN Kit Elite PK 6105; Vector Laboratories, Burlingame CA). The cells were then incubated in 1:10 dilution anti NIMP-R14 antibodies in PBS for 1 h at room temperature, washed, and further incubated with 1:200 dilution of biotinylated anti-rat IgG, followed by avidin-biotin complex/HRP –DAB, which resulted in brown staining of neutrophils. For each treatment a negative control was prepared without the primary antibody.

For immunohistochemistry, epididymal and subcutaneous fat tissues were embedded in paraffin and sectioned using a Leica microtome (Agentek, Canada). Neutrophil staining by NIMP-R14 was analyzed in sections of control mice or high fat-fed mice. Macrophages were detected using Mac2 antibody (21). The sections were incubated with rabbit serum (1:75) for 30 min and then incubated in 1:3800 dilution of anti-Mac2 antibodies in PBS over night at 4°C. The sections were washed and further incubated with 1:200 dilution of biotinylated anti-rat IgG, followed by avidin-biotin complex/HRP –DAB, which resulted in brown staining of macrophages. For each treatment, a negative control was prepared by omitting the primary antibody. In addition, the sections were histologicaly counter-stained with hematoxylin and analyzed in a blinded fashion using an Olympus BX-60 microscope. Analysis of macrophage crown-like structures (CLS) was performed by counting the mean number per field (10 fields per mouse from four individual mice per condition) of Mac2 positive aggregates.

Statistical analysis

Data are presented as means ± SEM. Significance of differences between two experimental conditions was assessed by Student's *t*-test. A *P* value of 0.05 was considered the cut-off for significance.

RESULTS

To assess whether neutrophils infiltrate adipose tissue prior to macrophage infiltration, we used C57BL/6J mice, a mouse strain known to be prone to obesity when fed a high-fat diet. As compared with mice fed control chow, mice on a high-fat diet exhibited an accelerated rate of weight gain (**Fig. 1A**). The difference in body weight was statistically significant at all time points, even as early as 3 days after initiation of high-fat feeding (22.8 ± 0.05 g and 24.4 ± 0.12 g for normal chow or high-fat fed mice, respectively, $P < 0.001$). At 16 weeks, marked decrease in insulin-stimulated phosphorylation of Akt was observed in adipose tissue (Fig. 1A, inset), confirming the development of expected metabolic changes. To detect adipose tissue neutrophils, we utilized two approaches: *i*) Western blotting of total adipose tissue lysates for the neutrophil-specific protein MPO; *ii*) immunohistochemistry using the neutrophil marker NIMP-R14. Periepidydimal adipose tissue MPO protein expression was very low on day 0, elevated on days 3 and 7, and was indistinguishable from day 0 at all subsequent time points up to 28 days (Fig. 1B) and at 8 and 16 weeks (data not shown). Densitometry analysis of adipose tissue MPO expression of 4–5 individual mice in each time point is shown in Fig. 1C. Mean periepdidymal fat MPO expression was significantly increased 3.5-fold

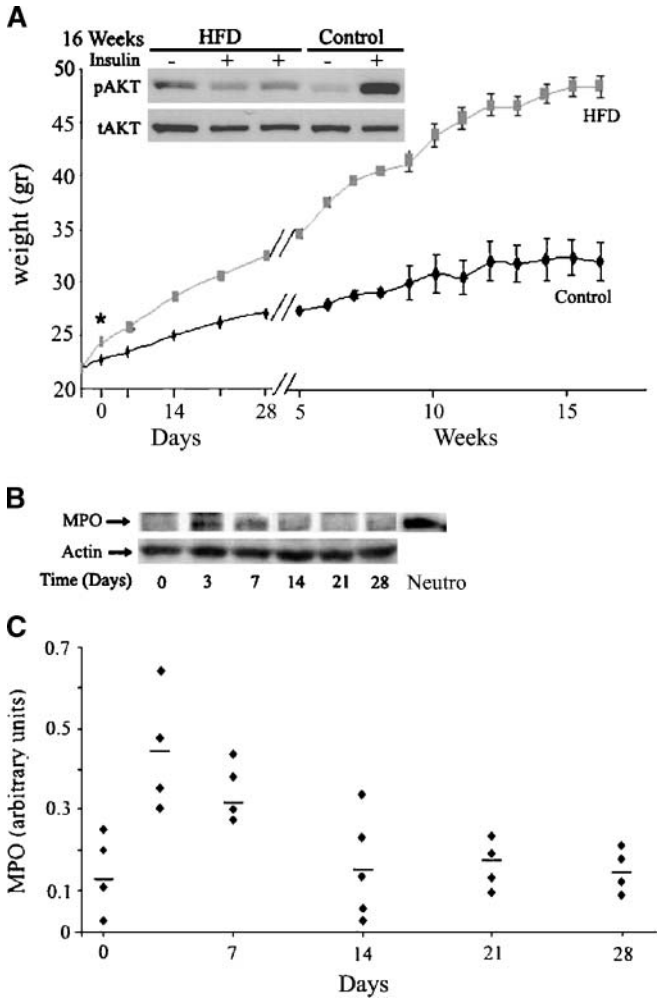

Fig. 1. Early increase in adipose tissue myeloperoxidase (MPO) expression in response to a high-fat diet. A: Mouse weight gain during 16 weeks of a high-fat diet (HFD) or normal (low fat) diet (control). Inset: immunoblot analysis of pSer473-Akt and total Akt in adipose tissue at 16 weeks of HFD or control diet. * $P < 0.001$ for the difference between the two groups from day 3 onwards. B: Representative immunoblot analysis of MPO and the corresponding β-actin in total adipose tissue lysates (100 μg). Peritoneal neutrophils lysate (3 μg) was used as positive control (neutro). C: Densitometry analysis of adipose tissue MPO expression of 4–5 individual mice at each time point. Horizontal lines indicate the average of the densitometry value in each time point.

and 2.9-fold at days 3 and 7 compared with day 0 ($P < 0.01$ and $P < 0.03$, respectively). No increase in MPO expression could be detected in normal chow-fed mice (data not shown). Moreover, there was no detectable increase in MPO expression in subcutanoues adipose tissue (data not shown), and circulating neturophil counts were not significantly different at 3 days between high-fat and normal chow-fed mice ($0.57 \pm 0.19 \times 10^3$ and $0.47 \pm 0.05 \times 10^3$ neutrophils per micro-liter, respectively, $P = 0.26$), suggesting that that MPO represented extravascular neutrophils.

Histological examination of adipose tissue samples were also consistent with the MPO findings, demonstrating the presence of neutrophils within the parenchyma of the adipose tissue in between adipocytes (**Fig. 2A**). Whereas neutrophils determined by NIMP-R14-positive staining could not be detected in adipose tissue at day 0, multiple neutrophils were visible between adipocytes in periepididymal fat sections of mice fed a high-fat diet for 3 and 7 days, suggesting direct neutrophil-adipocyte adherence. By 16 weeks on high-fat diet, adipocyte size was markedly increased, but discrete NIMP-R14-positive cells could not be observed (Fig. 2B). Rather, at this established obese state, adipose tissue was heavily infiltrated by macrophages, as assessed by Mac2 immunostaining revealing multiple CLS, consistent with previous reports (21). CLS could not be detected at days 3 and 7 and were observable only from day 21 and later (Fig. 2C). In fact, comparing the time-course analysis of MPO expression (Fig. 1C) to that of Mac2-positive cells (Fig. 2B) in intra-abdominal fat revealed highly distinct patterns of tissue infiltration. Collectively, these results demonstrate that neutrophils transiently infiltrate intra-abdominal adipose tissue early in the course of diet-induced obesity, preceding by weeks the well-described infiltration of adipose tissue by macrophages.

To study the molecular determinants of adipocyte-neutrophil adherence, we utilized an in vitro cellular approach to quantitatively assess the adherence of peritoneal mouse neutrophils to the murine 3T3-L1 adipocyte cell line. We first assessed if morphologically this system can recapitulate the in vivo findings. By light microscopy analysis, neutrophils seemed to form a physical association with the 3T3-L1 differentiated adipocytes (**Fig. 3A**), reminiscent of the in vivo histological findings. We next assessed neutrophil-adipocyte adherence by adding Cr^{51} radiolabeled peritoneal neutrophils onto a monolayer of differentiated mouse 3T3-L1 adipocytes. After 30 min at 37°C, approximately 25% of the neutrophils remained adhered to the adipocytes, and this percentage of adherence was not influenced by the presence or absence of 1 mM Ca^{2+} (Fig. 3B). Activating neutrophils with 50ng/ml PMA for 3 min before addition to the adipocyte monolayer significantly increased the adherence capacity to adipocytes to 37.0% ± 3.0% in the presence of Ca^{2+} ($P < 0.005$ compared with unstimulated neutrophils). When neutrophils were activated and allowed to adhere in the absence of Ca^{2+}, no effect of PMA was discernable. This Ca^{2+} dependency suggested the involvement of integrins in the PMA-stimulated neutrophil-adipocyte adherence. To further corroborate this possibility, we assessed the effect of Mn^{2+} ions, as this condition has been shown to increase the adherence process by enhancing integrins affinity (22). Remarkably, cells exposed to Mn^{2+} (3 mM) exhibited increased adherence to adipocytes compared with unstimulated neutrophils, reaching similar levels to those observed in PMA-stimulated cells (Fig. 3B). To verify that these findings using radioactive Cr^{51}-based adherence assay represented direct adherence of neutrophils to the adipocytes grown in the cell culture well, a mixed neutrophil-adipocyte cell population was immunostained by the neutrophil marker NIMP-R14 (**Table 1**). While neutrophil adherence to areas devoid of adipocytes was not significantly altered by PMA or Mn^{2+}, both conditions increased the number of neutrophils directly adhered to adipocytes,

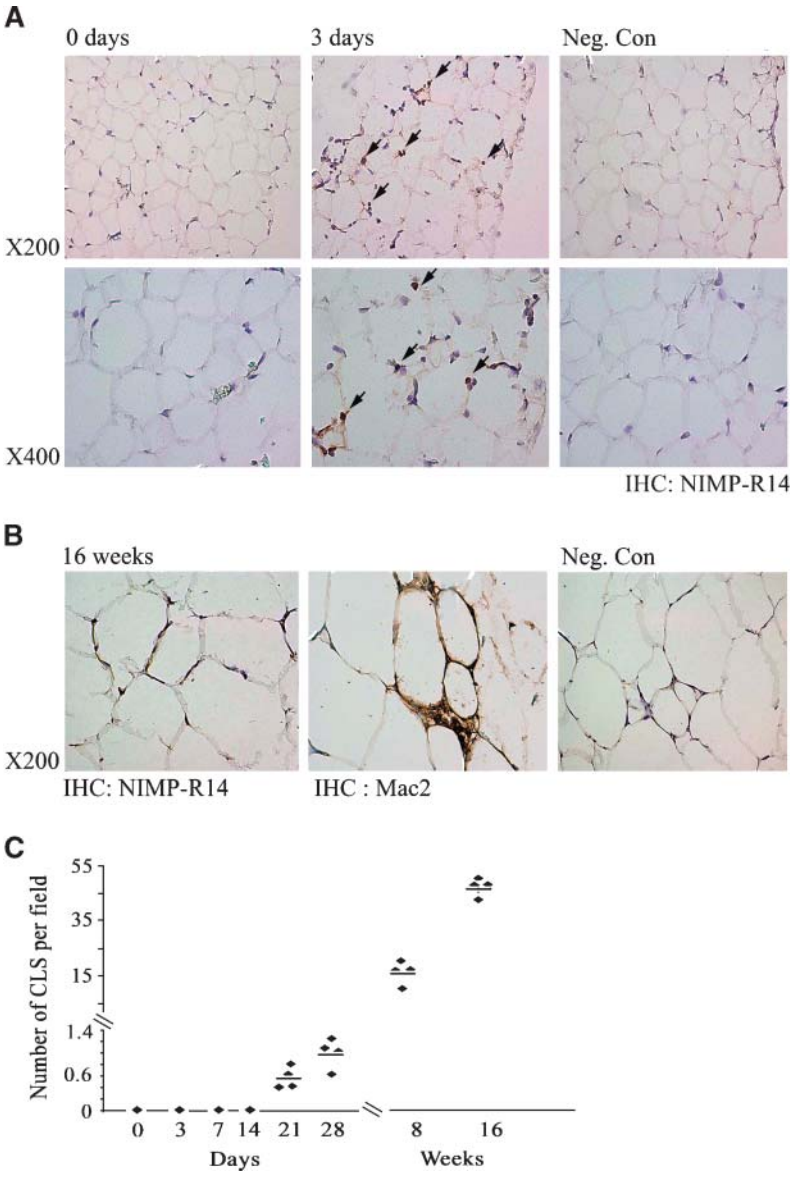

Fig. 2. Neutrophil infiltration of adipose tissue in vivo. A: Immunolocalization of neutrophils (shown by arrows) in periepydidimal fat using anti-NIMP-R14 antibodies and hematoxylin counter-stating in mice fed a high-fat diet (3 days, middle panel) compared with control mice at time 0 (left panel). Negative controls of sections from mice on a high-fat diet for 3 days are presented in the right panel. Immunostaining results are representative of the mice after 3 and 7 days of high-fat feeding whose MPO expression is shown in Fig. 1C. B: Light microscopy of periepidydimal fat from obese mice (16 weeks on a high-fat diet) showing Mac2 immunoreactive macrophages (middle panel). Negative control for mice fed a high-fat diet for 16 weeks (right panel). No staining for neutrophils was observed at this time point using NIMP-R14 antibody (left panel). C: The mean number of Mac2-positive crown-like structures (CLS) per field was assessed as described in Methods.

consistent with the radioactive Cr^{51}-based assay. The adherence capacity of primed or PMA-stimulated neutrophils to adipocytes was only slightly lower than that of peritoneal monocyte/macrophages (Fig. 3C), and was significantly ($P < 0.05$) higher compared with their capacity to adhere to nondifferentiated 3T3-L1 preadipocytes (fibroblasts, Fig. 3D). Hence, an in vitro cell system can recapitulate the neutrophil-adipocyte interaction observed early after initiating high-fat feeding in mice. Furthermore, it suggests that increased adherence of activated neutrophils involves integrins and depends on the adipocyte differentiation state.

Because neutrophil adherence to endothelial cells is mediated by surface exposure of CD11b (Mac-1) (23), we next assessed whether neutrophil adherence to adipocytes involves this integrin. As shown in **Fig. 4A** by FACS analysis, PMA, and to a lesser degree KC (the mouse equivalent of IL-8), increased surface exposure of CD11b (median: 265.5 ± 4.2, 156.6 ± 0.69, respectively, compared with 115 ± 5.6 in unstimulated cells). There was a correspondence between the degree of surface exposure of CD11b by PMA and KC and the percentage of adherence of neutrophils to adipocytes (41.9% ± 3.7% and 29.5% ± 2%, respectively, compared with 24.8% ± 1.5% in unstimulated cells (Fig. 4B). When PMA stimulation was performed in the absence of Ca^{2+}, the degree of CD11b (Mac-1) exposure was similar to that observed in the presence of Ca^{2+} (data not shown), consistent with the finding that the binding is a calcium-dependent adherence process (Fig. 3B). To confirm the functional role of neutrophil CD11b in the interaction with adipocytes, we utilized neutralizing anti-CD11b antibodies. Incubation of neutrophils with a neutralizing CD11b antibody prior to the adherence assay significantly prevented adherence in the unstimulated and the PMA-stimulated conditions, whereas a control antibody had no effect (Fig. 4C).

Because neutrophil activation robustly increases superoxide generation by the neutrophil NADPH oxidase, we

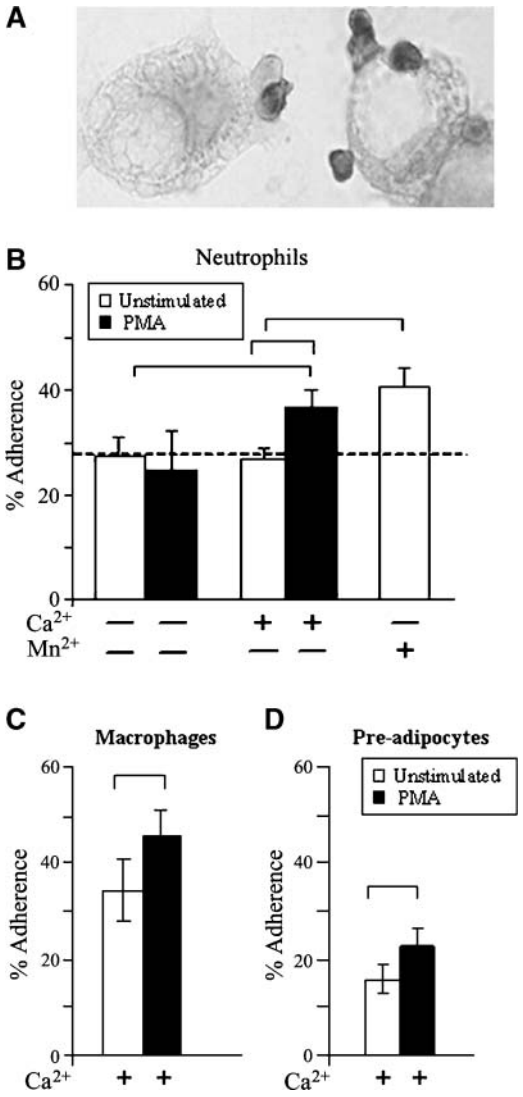

Fig. 3. Adherence of neutrophils to adipocytes depends on their activation state and on the presence of calcium. A: Adhered neutrophils to a monolayer of 3T3-L1 adipocytes were visualized using immunostaining with anti-NIMP-R14 antibody. Adherence of neutrophils (B) or macrophages (C), unstimulated or stimulated by PMA (50 ng/ml), to a monolayer of 3T3-L1 adipocytes was assessed by preradiolabelling cells as detailed in Methods in the presence or absence of 1 mM calcium. Shown are means ± SEM of % adherance of neutrophils (10 independent experiments) or macrophages (three independent experiments). Lines connecting bars indicate $P < 0.005$ and $P < 0.05$ between treatments for B and C, respectively. D: Nondifferentiated 3T3-L1 preadipocytes (fibroblasts) were used for the adherence assay with unstimulated or stimulated neutrophils (n = five independent experiments, and lines connecting bars indicate $P < 0.05$ between treatments).

TABLE 1. Adherence of neutrophils to adipocytes

Neutrophils Treatment	Number of Neutrophils Adhered to Adipocytes	Total number of Neutrophils in the Microscopic Field
Unstimulated	9.5 ± 1.9	10.8 ± 1.6
PMA stimulated	*13.4 ± 1.5	*14.8 ± 2.1
Mn^{2+}	*15.9 ± 1.6	*17.1 ± 1.8

Unstimulated or PMA stimulated neutrophils were adhered for 30 min in 37°C to the adipocytes in the presence of Ca^{2+}. When indicated, adherence of unstimulated neutrophils was performed in the presence of 3 mM Mn^{2+}. After the adherence assay, cells were fixed with 4% formaldehyde, and neutrophils stained using mouse anti-neutrophils antibody (NIMP-R14). Results are the mean ± SEM number per filed, derived from three independent experiments, with 10 individual fields per experiment. * $P < 0.001$ compared with unstimulated neutrophils.

next determined whether the surface exposure of CD11b and the resulting adherence to adipocytes depends upon superoxide generation using DPI, an inhibitor of NADPH oxidase. PMA markedly enhanced the rate of superoxide generation by neutrophils (**Fig. 5A**), along with the increases in the adherence to adipocytes (Fig. 5B) and in CD11b surface exposure (Fig. 5C). Remarkably, although DPI completely prevented PMA-induced superoxide generation, it had no effect on neutrophil-adipocyte adherence or on the surface exposure of CD11b. These observations demonstrate that CD11b exposure and neutrophil-adipocyte adherence are independent of superoxide ion generation through the activation of the phagocyte NADPH oxidase complex.

ICAM-1 is a known counter-molecule for complex formation with CD11b. To investigate if a CD11b-ICAM-1 complex formation mediates neutrophil-adipocyte adherence, we first assessed the presence of ICAM-1 on adipocytes. By FACS analysis, adipocytes exhibited much higher florescence when reacted with anti-ICAM-1 antibody compared with control IgG (**Fig. 6A**). In contrast, nondifferentiated 3T3-L1 preadipocytes (fibroblasts) did not react with anti-ICAM-1 above the control antibody (Fig. 6B). These findings suggest that upon adipogenesis ICAM-1 is upregulated, consistent with recent reports (24, 25). We utilized neutralizing anti-ICAM-1 antibodies to assess its functional requirement for neutrophil-adipocyte adherence. When adipocytes were incubated with anti-ICAM-1 antibody prior to the adherence assay (but not with nonimmune IgG), the percentage of adhered neutrophils was diminished, both in unstimulated and in PMA-stimulated conditions (Fig. 6C). This result resembled the observations obtained by incubating neutrophils with anti-CD11b antibodies (Fig. 4C). Next, we utilized a coimmunoprecipitation approach to demonstrate an ICAM-1-CD11b complex formation between adipocytes and neutrophils, respectively. After allowing neutrophil-adipocyte to adhere, lysates were made from the mixed cell population, and immunoprecipitation of CD11b was performed. Immunoprecipitates were then subjected to SDS-PAGE, followed by immuno detection of CD11b and of ICAM-1. Consistent with the observation that PMA stimulation of neutrophils increased the percentage of adherence to adipocytes (Fig. 3B), PMA increased the recovery of CD11b in the immunoprecipitate (Fig. 6D, upper panel) and of the coimmunoprecipitated ICAM-1 (Fig. 6D, lower panel) compared with unstimulated neutrophils. Because CD11b was not detected in lysates of adipocytes and ICAM-1 was not detected in lysates of neutrophils (data not shown), we deduce that coprecipitates of ICAM-1 with CD11b indicates the formation of a protein complex contributed by

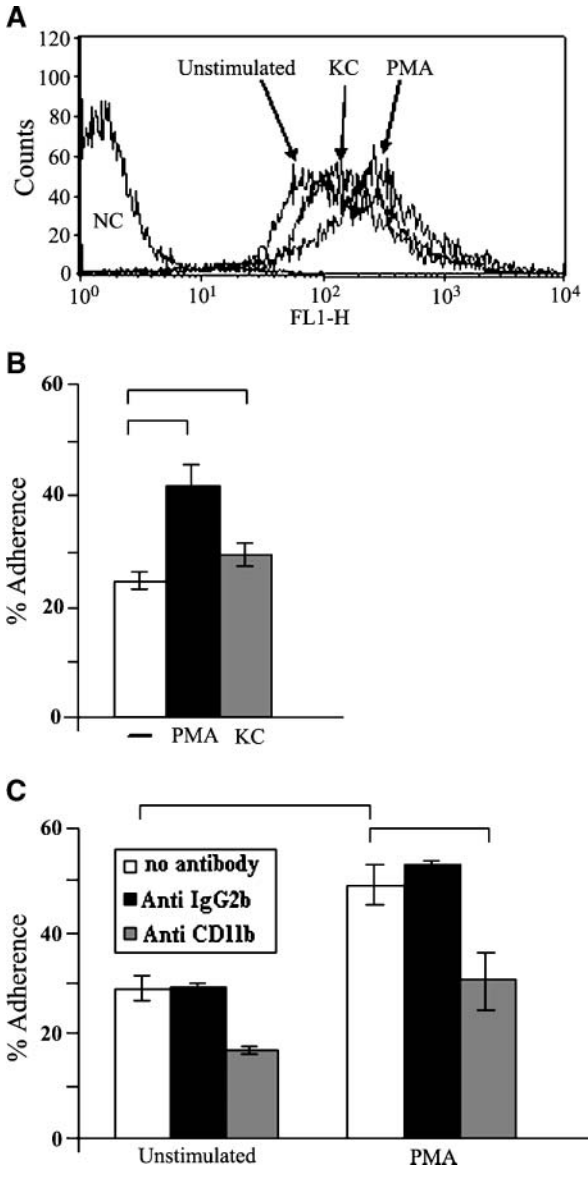

Fig. 4. Adherence of neutrophils to adipocytes corresponds to the surface exposure of Mac-1 (CD11b) and neutralizing antibody attenuates the adherence. A: A representative FACS analysis of three experiments analyzing surface Mac-1 (CD11b) on neutrophils left untreated, or stimulated with PMA (50 ng/ml) or KC (CXCL1) (100 ng/ml) for 3 min. B: Neutrophil adherence to adipocytes was determined in neutrophils unstimulated or stimulated by PMA or KC (n = six independent experiments; lines connecting bars indicate $P < 0.001$ for PMA and $P < 0.005$ for KC versus unstimulated cells). C: Nonstimulated or PMA-stimulated neutrophils were incubated without (white bars) or with anti-CD11b monoclonal antibody (5 μg/ml, gray bars) or a control nonspecific mouse IgG2b monoclonal antibody (black bars) for 10 min before the adherence assay to 3T3-L1 adipocytes. Results are means ± SEM of % adhered neutrophils in three independent experiments (lines connecting bars indicate $P < 0.05$).

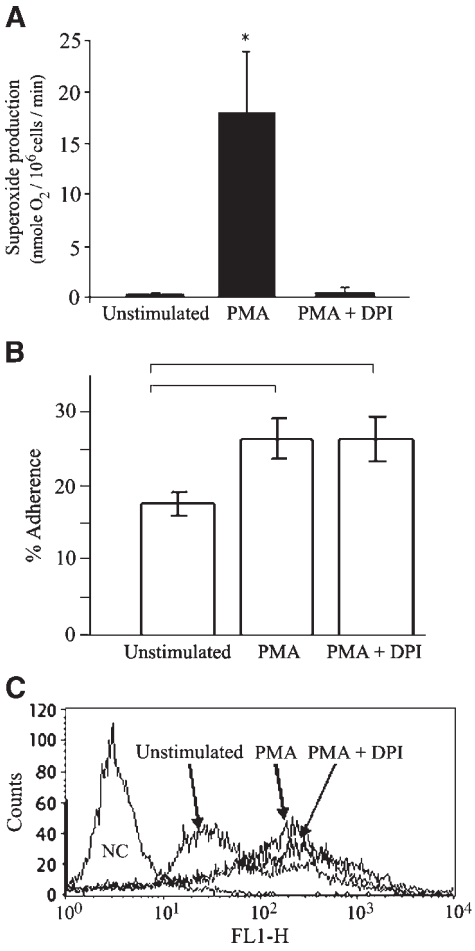

Fig. 5. Neutrophil adherence to adipocytes and surface Mac-1 exposure are independent of superoxide generation. Superoxide production by ferricytochrome C reduction analysis (A), adherence to adipocytes (B) and surface Mac-1 (CD11b) exposure by FACS analysis of nonstimulated or PMA-stimulated neutrophils in the presence or absence of the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (10 μg/ml) (C). Median fluorescence of 10,000 cells was: unstimulated = 30.06; PMA-treated = 296.7; PMA + DPI = 276.7. Results are means ± SEM of 10 experiments in A. Lines connecting bars in B indicate statistically significant differences between groups ($P < 0.05$) among three independent experiments. C is representative results of three independent experiments. * $P < 0.001$ for the effect of PMA compared to unstimulated cells or cells treated with DPI.

the two cell types. Collectively, these results support the hypothesis, that the direct adherence between neutrophils and adipocyte is mediated by the protein complex formation between CD11b and ICAM-1, contributed by neutrophils and adipocytes, respectively.

DISCUSSION

The obesity pandemic and its unprecedented health toll underlie the enormous effort to understand the mechanisms connecting excessive adipose tissue accumulation with disease. Several lines of evidence suggest that increased fat mass per se is not sufficient for obesity-induced metabolic dysregulation because clinically approximately 20% of obese persons seem to escape the common health effects of obesity despite an increase in fat mass (7). Moreover, surgical removal of large amounts of fat by liposuction provides no metabolic benefit (26). Along the same line, in mice whose intra-abdominal fat mass was artificially increased by transplantation of fat from a lean donor

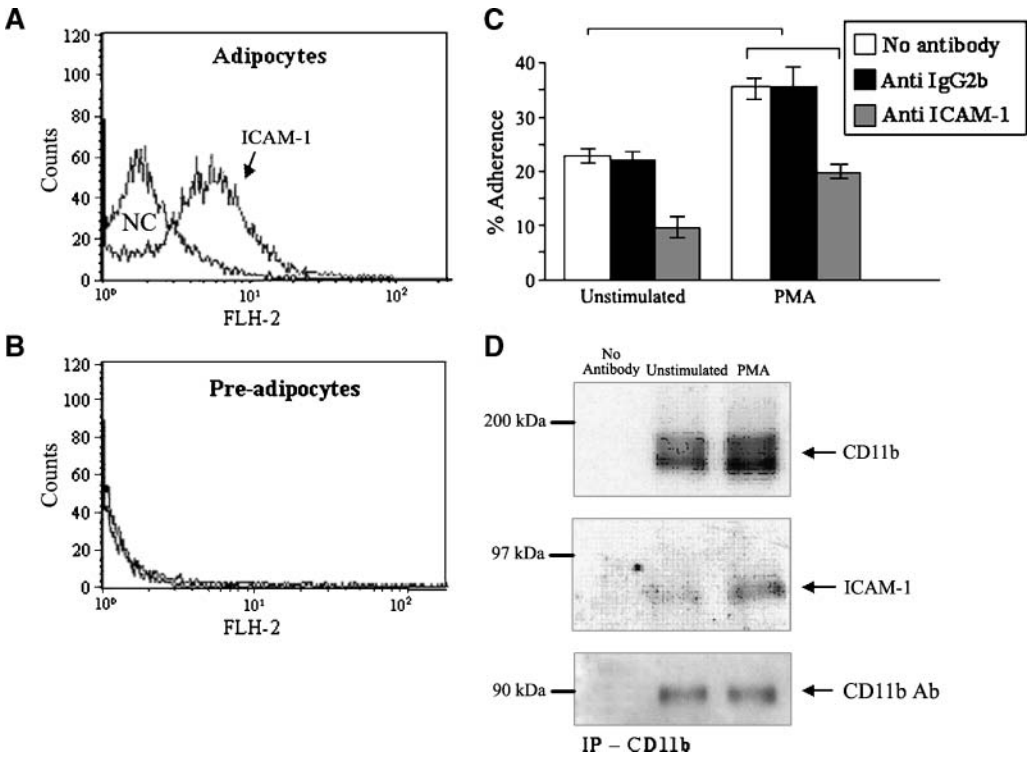

Fig. 6. Adipocyte ICAM-1 coimmunoprecipitates with neutrophil CD11b and mediates neutrophil-adipocyte adherence. Surface expression of ICAM-1 in 3T3-L1 adipocytes (A) or in nondifferentiated preadipocytes (B) by FACS analysis using PE-conjugated anti-ICAM-1 antibody or control PE-conjugated IgG. The median fluorescence of 10,000 cells was in adipocytes: IgG = 1.4 ± 0.17; ICAM-1 = 6.32 ± 0.75. Shown are representative results of four independent experiments. C: Nonstimulated or PMA-stimulated neutrophils were incubated without (white bars) or with anti-ICAM-1 monoclonal antibody (10 μg/ml, gray bars) or a control nonspecific mouse IgG2b monoclonal antibody (black bars) for 1 h before the adherence assay to 3T3-L1 adipocytes. Results are means ± SEM of % adhered neutrophils in three independent experiments (lines connecting bars indicate $P < 0.05$). D: Coimmunoprecipitation of CD11b and ICAM-1 from a mixed cell-type lysates. Unstimulated or PMA (50 ng/ml)-stimulated were allowed to adhere to adipocytes, and lysates were subjected to anti CD11b immunoprecipitation, followed by separation on nonreducing conditions and detection of CD11b or ICAM-1 by Western blot analysis using the respective antibodies. Equal amounts of anti-CD11b IgG (CD11b Ab) were added to lysates of unstimulated or stimulated mixed adhered neutrophil-adipocyte. Results shown are representative of three independent experiments.

littermate exhibited improved, rather than impaired, glucose tolerance (27). Thus, adipose tissue should be altered in obesity in order to mediate the pathophysiological metabolic-endocrine consequences of excessive fat accumulation. Three major conclusions can be deduced from the present study regarding adipose tissue alterations: *i*) early in the course of high-fat feeding of a commonly used mouse strain prone to diet-induced obesity, neutrophils transiently infiltrate the parenchyma of intra-abdominal adipose tissue, and directly interact with adipocytes; *ii*) in vitro neutrophils can directly adhere to adipocytes in a process that is affected by cations and by the neutrophil activation state, but is independent of superoxide production; *iii*) neutrophil-adipocyte adherence is mediated by the formation of a molecular complex, contributed by neutrophil CD11b/Mac-1 and adipocyte ICAM-1.

The notion that "chronic inflammatory infiltrate" is preceded by a transient "acute inflammatory infiltrate" dominated by neutrophils is a well-established paradigm in inflammation (28). Our finding that this paradigm also holds true for adipose tissue in response to high-fat feeding provides additional support for the notion of adipose tissue inflammation in obesity. This concept was initially raised by the discovery that the prototypic proinflammatory TNF-α is upregulated in adipose tissue in obesity and contributes to obesity-induced insulin resistance (29, 30). Subsequently, adipose tissue in obesity was shown to secrete increased levels of additional inflammatory cytokines such as IL-6, IL-8 (2, 4, 5), as well as adipokines having an immunoregulatory function, such as adiponectin (12, 31). More recently, augmented infiltration of macrophages into obese adipose tissue was reported (6), and adipocytes and macrophages were shown to engage in a vicious cycle mediated by inflammatory cytokines and lipids (12). Together, these findings suggest that adipose tissue inflammation may constitute a major pathological process augmenting systemic inflammation and contributing to the increased cardiometabolic risk associated with obesity. However, how the inflammatory cascade is initiated in obesity largely remains unknown. Our present

findings are the first to demonstrate that high-fat feeding causes a significant recruitment of neutrophils to intra-abdominal adipocyte tissue. This transient "acute inflammatory infiltrate" occurs as an early response to high-fat feeding, peaking at 3–7 days and subsiding thereafter.

The signals for inflammatory cell infiltration into adipose tissue have been a major focus of interest. Macrophages were proposed to infiltrate adipose tissue secondary to hypertrophy-induced adipocyte cell death (13, 21). Yet, neutrophils are unlikely to respond to this alteration in adipose tissue, because their infiltration occurs within the first 1–2 weeks of initiating a high-fat feeding (Fig. 1B and 2A), while adipocyte cell death occurs only weeks later (13). A potential chemoattractant for neutrophil infiltration into adipose tissue is IL-8. This cytokine is a potent neutrophil chemokine [as neutrophils migrate along a concentration gradient of IL-8 (32, 33)], which was shown to be secreted by adipose tissue in obesity (2). Other factors may include more general proinflammatory cytokines, as well as fatty acid metabolites (PGE_2 and LTB_4) and complement (C3b). Regardless of the exact adipose tissue-derived chemoattractant(s) that mediate neutrophil recruitment, our results are consistent with a recent study demonstrating increased leukocyte-endothelial cells platelet interaction in the microcirculation of obese visceral adipose tissue (34). Moreover, pregnant obese women with preeclampsia have been shown to exhibit extensive vascular inflammation characterized predominantly by neutrophils, and this was apparent in adipose tissue blood vessels (35, 36). Here, our immunohistochemsitry analyses clearly reveal that in response to high-fat feeding neutrophils are not restricted to the adipose tissue vasculature. Rather, they infiltrate the parenchyma and are found in close contact with adipocytes (Fig. 2A), consistent with our findings in an isolated cellular system demonstrating direct adherence of neutrophils to adipocytes (Fig. 3A, Table 1). This cell-cell interaction is mediated by complex formation between neutrophil CD11b/Mac1 and adipocyte ICAM-1, reminiscent of neutrophil adherence to endothelial cells (28, 37). This conclusion relies on the strong association between the degree of exposure of CD11b on the surface of neutrophils and percent adherence to adipocytes (Fig. 4A and B), the attenuation of this adherence by neutralizing anti-CD11b antibodies (Fig. 4C) or anti-ICAM antibodies (Fig. 6C), and on the physical binding of CD11b to ICAM-1 exclusively contributed by neutrophils and adipocytes, respectively (Fig. 6D).

The potential contribution of inflammatory cell infiltration into adipose tissue to the pathophysiology of obesity is by far the most important question issue in this field. Several lines of evidence support a role for adipose tissue macrophages in the induction of insulin resistance, based on time-course analyses (13) and on interference strategies with macrophage infiltration (8). In humans, the degree of macrophage infiltration into intra-abdominal adipose tissue correlated with liver histological changes (10) and with markers of cardiometabolic risk (10, 11). However, recent studies still question the roles and underling mechanisms for adipose tissue inflammation. Studies in mice in which the MCP1-CCR2 system was inhibited questioned its role in adipose tissue inflammation and insulin resistance (38), macrophages were proposed to exert a role in adipose tissue growth and angiogenesis (39), and human studies provide associations between phenomena, but cannot prove cause-effect relationships. Assuming adipose tissue inflammation is a process with relevant physiological consequences, what could be the role of neutrophils? Circulating neutrophils are activated in obese persons (unpublished results), suggesting that infiltrating neutrophils in adipose tissue actively release various substances including reactive oxygen species, TNF-α, thromboxane, MPO, and matrix metalloproteinase-8, all of which have the capacity to cause cellular damage and dysfunction. Moreover, there is a growing body of evidence demonstrating that leukocyte recruitment out of blood vessels and into tissues is essential not only for the development of an appropriate inflammatory response to injury or infection, but also to the debilitating sequence of events leading to inflammatory diseases, such as asthma and allergy (40–42). Consistently, interfering with leukocyte recruitment might reduce the sequel of the inflammatory cascade in various inflammatory processes (40, 41). Thus, whereas the pathophysiological relevance of adipose tissue inflammation as a whole has yet to be fully understood, neutrophil infiltration could constitute a key event in initiating the inflammatory cascade in response to high-fat feeding.

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