Original Article

Diet-Induced Increases in Adiposity, but Not Plasma Lipids, Promote Macrophage Infiltration Into White Adipose Tissue

Kimberly R. Coenen,[1] Marnie L. Gruen,[1] Alan Chait,[2] and Alyssa H. Hasty[1]

Obesity, hyperlipidemia, and insulin resistance are cardinal features of the metabolic syndrome and individually increase the risk for developing diabetes and cardiovascular disease, a risk that is amplified when they are simultaneously present. It is becoming increasingly clear that macrophages can infiltrate white adipose tissue (WAT) in the obese state, and their presence is associated with pathophysiological consequences of obesity, such as inflammation and insulin resistance. To determine whether hyperlipidemia could potentiate macrophage infiltration into WAT in the presence of obesity, obesity-prone agouti yellow mice (A^y/a) on a hyperlipidemia-prone LDL receptor (LDLR)–deficient ($LDLR^{-/-}$) background were placed on chow or Western diet. In addition, A^y/a mice that were LDLR sufficient were also placed on Western diet. Both genetics and diet increased the degree of adiposity; however, plasma lipids were elevated only in the Western diet–fed $LDLR^{-/-}$ mice. The extent of macrophage accumulation in WAT correlated with the degree of adiposity. However, hyperlipidemia did not impact macrophage recruitment to WAT or the downstream metabolic consequences of macrophage accumulation in WAT, such as inflammation and insulin resistance. These data have important implications for the pathogenesis of diet-induced obesity in humans, even when plasma lipid abnormalities are not present. *Diabetes* 56:564–573, 2007

Obesity is a major health care concern because of its association with other pathological conditions related to the metabolic syndrome. Some of the risk factors associated with obesity include dyslipidemia, a pro-inflammatory state, hypertension, and insulin resistance (1,2). The dyslipidemia seen in obesity is characterized by elevated levels of nonesterified fatty acids (NEFAs), triglycerides, and small dense LDL particles, along with reduced levels of HDL (1–3). The individual components of the metabolic syndrome are independent risk factors for diabetes and cardiovascular disease; however, when these elements are concurrent, they have additive or even synergistic effects (2). In fact, individuals with familial combined hyperlipidemia are more insulin resistant compared with normolipidemic relatives, even after correcting for BMI (4). Moreover, insulin sensitivity is significantly lower in obese hyperlipidemic subjects compared with nonobese hyperlipidemic subjects (5). Therefore, the presence of both obesity and hyperlipidemia can exert an additive effect on insulin resistance.

Recent studies have explored various forms of obesity and found increased levels of macrophages in white adipose tissue (WAT) of obese rodents and humans compared with lean controls (6–13). In addition, multiple studies have established that adipose tissue resident macrophages (ATMs) are responsible for much of the inflammatory cytokine production in WAT in the obese state (8,10,14,15). Furthermore, it has been shown that macrophage infiltration into WAT precedes or is coincident with insulin resistance in obese mice (10). Weight loss has been shown to result in a decrease in ATM accumulation, and improved inflammatory status in humans, solidifying the association of obesity with macrophage infiltration of WAT (6,12,16). In addition, treatment of insulin-resistant humans with the insulin-sensitizing peroxisome proliferator–activated receptor γ agonist, pioglitazone, to improve insulin sensitivity also reduced ATM accumulation (13). Thus, ATMs appear to play a key role in the pathophysiological conditions associated with obesity and the metabolic syndrome.

Although research on macrophage infiltration into WAT is prominent at this time, the potential effects of other components of the metabolic syndrome, such as hyperlipidemia, on ATM content in the obese state remain to be elucidated. Fasting hypertriglyceridemia is often associated with increased visceral adiposity, linking hyperlipidemia with adiposity-related abnormalities (17,18). Plasma triglyceride levels are independently linked to inflammation and can be increased during acute infection or upon exposure to multiple inflammatory cytokines (19). In addition, pharmaceutical lowering of plasma lipid levels with statins has been shown to reduce plasma inflammatory cytokine levels (20). Finally, a recent report from Cancello et al. (7) demonstrated a strong positive correlation between ATM number and plasma triglyceride levels and a negative correlation with HDL cholesterol in obese humans. These observations led us to hypothesize that

From the [1]Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee; and the [2]University of Washington, Department of Medicine, Seattle, Washington.

Address correspondence and reprint requests to Alyssa H. Hasty, Vanderbilt University Medical Center, Room 702 Light Hall, Nashville, TN 37232-0615. E-mail: alyssa.hasty@vanderbilt.edu.

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ATM, adipose tissue resident macrophage; LDLR, LDL receptor; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; NEFA, nonesterified fatty acid; NMR, nuclear magnetic resonance; RIA, radioimmunoassay; SAA, serum amyloid A; TBO, Toluidine Blue O; TNF-α, tumor necrosis factor-α; VDDRC, Vanderbilt Digestive Diseases Research Center; WAT, white adipose tissue.

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TABLE 1
Body composition and plasma parameters of LDLR$^{-/-}$ groups

	Body wt (g)	Total fat (g)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	NEFAs (mEq/l)	Glucose (mg/dl)
a/a;LDLR$^{-/-}$ chow diet	26.7 ± 0.5	2.7 ± 0.1	208 ± 6	81 ± 6	0.35 ± 0.04	126 ± 5
Ay/a;LDLR$^{-/-}$ chow diet	33.0 ± 0.9*	7.8 ± 0.9*	241 ± 10	124 ± 11	0.41 ± 0.03	143 ± 7
a/a;LDLR$^{-/-}$ Western diet	36.6 ± 0.9*†	11.9 ± 0.7*‡	1,284 ± 68*‡	482 ± 36*‡	0.94 ± 0.07*‡	136 ± 6
Ay/a;LDLR$^{-/-}$ Western diet	47.3 ± 0.4*‡§	20.3 ± 0.3*‡§	1,570 ± 62*‡§	1,025 ± 56*‡§	1.45 ± 0.12*‡§	123 ± 6

Data are means ± SE from 18 to 22 mice per group. Body composition and plasma parameters of mice were measured after 12 weeks on chow diet or Western diet. *$P < 0.001$ compared with a/a;LDLR$^{-/-}$ chow diet. †$P < 0.01$ compared with A^y/a;LDLR$^{-/-}$ chow diet. ‡$P < 0.001$ compared with A^y/a;LDLR$^{-/-}$chow diet. §$P < 0.001$ compared with a/a;LDLR$^{-/-}$ Western diet.

hyperlipidemia could also promote macrophage infiltration into WAT in the obese state and that a portion of the relationship between inflammation and hyperlipidemia could be attributed to increased ATM accumulation.

To test this hypothesis, we used a series of mouse models with increasing obesity and hyperlipidemia to determine the contribution of hyperlipidemia to ATM accumulation. Analysis of these mice revealed that macrophage infiltration is correlated with the degree of adiposity; however, these effects were independent of the degree of hyperlipidemia. These data indicate that hyperlipidemia does not increase macrophage recruitment to WAT in obesity in this model system. In addition, our data highlight the fact that excessive dietary fat intake can promote adipose tissue dysfunction, leading to local and systemic inflammation as well as hyperinsulinemia and decreased adiponectin levels, irrespective of plasma lipid levels.

RESEARCH DESIGN AND METHODS

Mice and diets. The mice used in these studies were purchased from the Jackson Laboratories (Bar Harbor, ME) and are on a C57BL/6 background. Like wild-type mice, LDL receptor (LDLR)$^{-/-}$ mice are susceptible to diet-induced obesity and insulin resistance. In addition, they also develop hyperlipidemia (21,22), making them a useful model in which to study the physiological consequences of combined obesity and hyperlipidemia. The A^y/a;LDLR$^{-/-}$ mice were produced by intercrossing obesity-prone agouti yellow mice (A^y/a) with LDLR$^{-/-}$ mice. Mice were fed ad libitum and given free access to water. The Western diet was purchased from Harlan-Teklad (TD88137) and is set at a caloric density of 4.8 kcal/g with 42% of the calories from a fat source of anhydrous milk fat and 0.15% added cholesterol. All animal care and experimental procedures were performed with approval from and according to the regulations of the Institutional Animal Care and Usage Committee of Vanderbilt University.

Body composition. Total lean tissue mass, fat, and free fluid were determined by nuclear magnetic resonance (NMR) using the Minispec (Bruker, The Woodlands, TX) in the Mouse Metabolic Phenotyping Center at Vanderbilt University.

Plasma collection and measurements. Mice were fasted for 5 h before all blood collections and when killed. Mice were bled from the retro-orbital venous plexus using heparinized capillary collection tubes. Plasma was separated by centrifugation, aliquoted, and stored at −80°C. Lipid and glucose analyses were performed on all samples (Tables 1 and 2). Total plasma cholesterol and triglyceride levels were measured using enzymatic kits from Raichem (San Diego, CA) according to the manufacturer's instructions. NEFA measurements were performed using the NEFA-C kit by Wako (Neuss, Germany). Glucose levels were determined using Lifescan's OneTouch glucometer (Johnson and Johnson, Northridge, CA). Insulin and leptin measurements were performed on samples with sufficient volumes using radioimmunoassays (RIAs). Serum amyloid A (SAA) analyses were performed by enzyme-linked immunosorbent assay using a polyclonal antibody from R&D Systems (Minneapolis, MN) against recombinant mouse SAA1 (1:2,000 dilution). Secondary anti-goat antibody was used at a 1:4,000 dilution (23).

Adiponectin measurement. Plasma samples were electrophoresed through nondenatured, nonreduced 4–12% SDS gels. Protein was transferred to nitrocellulose membranes and probed with rabbit anti-mouse adiponectin (ABCAM, Cambridge, MA) at a 1:2,000 dilution. Secondary antibody was used at a 1:5,000 dilution, and signal was detected using ECL reagents (Amersham, U.K.). Bands were quantified using the Quantity One software (Bio-Rad).

Adipose tissue histochemistry. Excised abdominal WAT was stored in 10% formalin and then embedded in paraffin. Paraffin sections were fixed and stained with Toluidine Blue O (TBO) according to the manufacturer's instructions (Newcomer Supply, Middleton, WI). Immunohistochemical staining for F4/80 was performed with antibody from Serotec (Raleigh, NC) at a 1:100 dilution. Secondary antibody (Dako, Glostrup, Denmark) was used at a 1:100 dilution.

Gene expression. RNA was isolated from visceral WAT using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time, quantitative RT-PCR was performed using the ABI Prism7700 sequence detection system (Applied Biosystems, Foster City, CA). Primer-probe sets (Assays-on-Demand) were purchased from Applied Biosystems. Expression of genes were compared with 18S controls using the $\Delta\Delta$CT method as described previously (24). To eliminate the potential for interplate variability, all six groups were amplified on one plate for each gene (limiting the number of samples from each group).

Statistics. Statistical analyses were performed using GraphPad Prism 4 software (San Diego, CA) and JMP software from SAS (Cary, NC). Data are expressed as means ± SE and were considered significant at $P < 0.05$ based on ANOVA analyses with Bonferroni post hoc test or Student's t test. Correlative relationships were determined using linear regression analyses. The Shapiro-Wilks W test was performed on the standard residual values to test for normality.

RESULTS

Obesity, adiposity, and plasma lipids in chow diet–fed and Western diet–fed LDLR$^{-/-}$ mice. To determine whether hyperlipidemia contributes to macrophage infiltration into WAT in obesity, genetic and dietary manipulations were used. Obesity-prone mice, ubiquitously and ectopically expressing the agouti protein (A^y/a), were crossed with hyperlipidemia-prone LDLR-deficient (LDLR$^{-/-}$) mice. At 4 months of age, male A^y/a;LDLR$^{-/-}$ mice and their control (a/a) littermates, a/a;LDLR$^{-/-}$, were either maintained on

TABLE 2
Body composition and plasma parameters of LDLR$^{+/+}$ groups

	Body wt (g)	Total fat (g)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	NEFAs (mEq/l)	Glucose (mg/dl)
a/a;LDLR$^{+/+}$ Western diet	42.6 ± 1.2	15.2 ± 0.6	235 ± 12	58 ± 3	0.37 ± 0.02	157 ± 7
Ay/a;LDLR$^{+/+}$ Western diet	50.0 ± 0.6*	20.3 ± 0.3*	353 ± 8*	70 ± 4†	0.41 ± 0.02	130 ± 8†

Data are means ± SE from 12 mice per group. Body composition and plasma parameters of mice were measured after 12 weeks on Western diet. *$P < 0.0001$. †$P < 0.05$.

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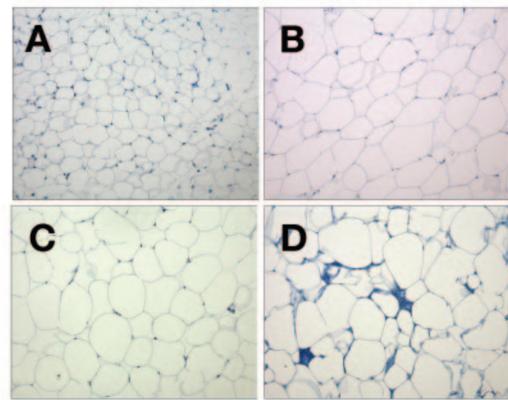
chow diet or fed a high-fat, Western diet for 12 weeks. The resulting four experimental groups were as follows: *1*) a/a;LDLR$^{-/-}$ mice on chow diet, *2*) A^{y}/a;LDLR$^{-/-}$ mice on chow diet, *3*) a/a;LDLR$^{-/-}$ mice on Western diet, and *4*) A^{y}/a;LDLR$^{-/-}$ mice on Western diet.

At the end of the diet feeding, body weight, total body fat, total plasma cholesterol, plasma triglycerides, and NEFAs were measured (Table 1). In every case, the A^{y}/a mice were heavier than their a/a controls, and the Western diet–fed mice were heavier than their chow diet–fed controls. This resulted in a stepwise increase in body weight going from the a/a;LDLR$^{-/-}$ chow diet mice to the A^{y}/a;LDLR$^{-/-}$ Western diet mice. The increase in weight gain was attributed to a gain in total body fat, a measure of adiposity, as determined by NMR analysis.

After 12 weeks, total plasma cholesterol levels were dramatically increased in the LDLR$^{-/-}$ groups fed a Western diet, with the a/a;LDLR$^{-/-}$ Western diet and A^{y}/a;LDLR$^{-/-}$ Western diet groups displaying six- and eightfold increases compared with lean a/a;LDLR$^{-/-}$ chow diet mice, respectively ($P < 0.001$). Diet and genetics resulted in a synergistic increase in plasma triglyceride levels in the A^{y}/a;LDLR$^{-/-}$ Western diet group (1,059 ± 51 compared with 456 ± 36 mg/dl in the a/a;LDLR$^{-/-}$ Western diet group, $P < 0.001$). NEFA levels were also increased in the Western diet–fed a/a;LDLR$^{-/-}$ and A^{y}/a;LDLR$^{-/-}$ mice ($P < 0.001$). Thus, these four groups of mice provide a gradation of obesity and hyperlipidemia.

Adipose tissue morphology in lean, moderately obese, and severely obese mice. When mice were killed, abdominal WAT was collected and used for histochemical staining. TBO staining was performed on WAT from the four groups and provided evidence of unique morphological differences between WAT from lean (a/a;LDLR$^{-/-}$ chow diet), moderately obese (A^{y}/a;LDLR$^{-/-}$ chow diet and a/a;LDLR$^{-/-}$ Western diet), and severely obese (A^{y}/a;LDLR$^{-/-}$ Western diet) mice (Fig. 1*A–D*). Adipocytes from moderately obese groups were hypertrophied compared with those from lean mice. Although adipose tissue from the A^{y}/a;LDLR$^{-/-}$ Western diet group exhibited larger adipocytes, there were also a number of smaller adipocytes present. Quantification of adipocyte size confirmed a normal distribution for the lean and moderately obese groups with mean diameters increasing from 53 ± 5 μm in the a/a;LDLR$^{-/-}$ chow diet group, to 99 ± 5 and 102 ± 3 μm in the A^{y}/a;LDLR$^{-/-}$ chow diet and a/a;LDLR$^{-/-}$ Western diet groups, respectively (Fig. 1*E*, $P < 0.0001$ for A^{y}/a;LDLR$^{-/-}$ chow diet and a/a;LDLR$^{-/-}$ Western diet vs. a/a;LDLR$^{-/-}$ chow diet group). Although the mean adipocyte size in the severely obese A^{y}/a;LDLR$^{-/-}$ Western diet group did not differ from the moderately obese groups (103 ± 4 μm), the distribution of cell size varied widely, demonstrating the heterogeneous nature of the tissue. The smaller adipocytes appeared to be surrounded by ATMs in this group, similar to what has been reported for WAT from other obese models (Fig. 1*D*) (8,10,16,25). These data suggest that individual adipocytes are altered upon macrophage infiltration into adipose tissue, especially in the presence of severe obesity.

Qualitative and quantitative analysis of macrophage markers in WAT. Based on the TBO images, it appeared that the A^{y}/a;LDLR$^{-/-}$ Western diet group had significantly greater ATM content than the other groups, suggesting that hyperlipidemia could increase macrophage infiltration into WAT. However, these mice were also more obese than the other groups. Thus, two additional groups were added: *1*) a/a;LDLR$^{+/+}$ mice fed Western diet; and *2*) A^{y}/a;LDLR$^{+/+}$ mice fed Western diet. Body weight and total body fat were slightly higher in the Western diet–fed LDLR$^{+/+}$ groups compared with the Western diet–fed LDLR$^{-/-}$ groups; however, plasma lipid levels were not elevated by the Western diet feeding because of the presence of LDLRs (Table 2).



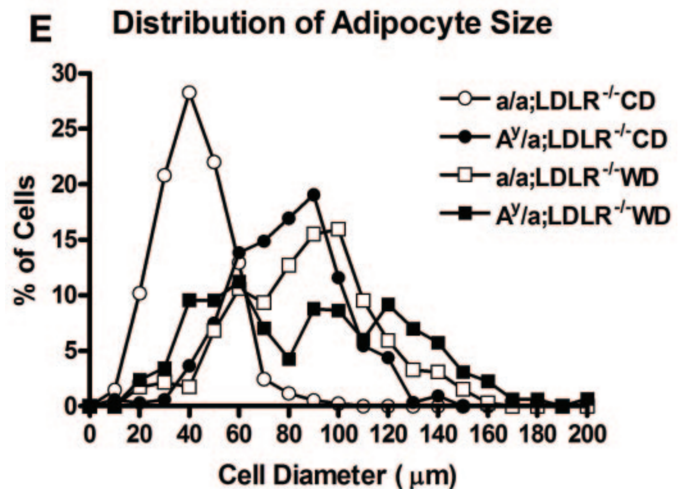


FIG. 1. Histological analysis of WAT morphology. Sections of abdominal WAT were collected when mice were killed, fixed, paraffin embedded, and stained with TBO. *A–D*: Sections from a/a;LDLR$^{-/-}$ chow diet, A^{y}/a;LDLR$^{-/-}$ chow diet, a/a;LDLR$^{-/-}$ Western diet, and A^{y}/a;LDLR$^{-/-}$ Western diet mice, respectively; magnification, ×10. *E*: Adipocyte diameters were measured from two random fields from three to four mice per group. White circles, a/a;LDLR$^{-/-}$ chow diet; black circles, A^{y}/a;LDLR$^{-/-}$ chow diet; white squares, a/a;LDLR$^{-/-}$ Western diet; and black squares, A^{y}/a;LDLR$^{-/-}$ Western diet. The data are expressed as the percentage of cells found in a given diameter size range.

After TBO staining of WAT from the initial four groups, more specific immunohistochemical characterization of abdominal WAT from all six groups was performed using an antibody to the macrophage surface marker F4/80 (Fig. 2*A–L*). Similar morphological differences and degree of macrophage infiltration were detected in the A^{y}/a;LDLR$^{+/+}$ Western diet mice as was noted in the A^{y}/a;LDLR$^{-/-}$ Western diet (Fig. 2, compare *F* and *L* with *E* and *K*).

Abdominal WAT from all six groups was also collected for the evaluation of gene expression of macrophage markers F4/80 and CD68 using quantitative RT-PCR. Mirroring the stepwise increase in body weight and total body fat (Tables 1 and 2), there was an incremental increase in F4/80 and CD68 expression (Fig. 2*M* and *N*).

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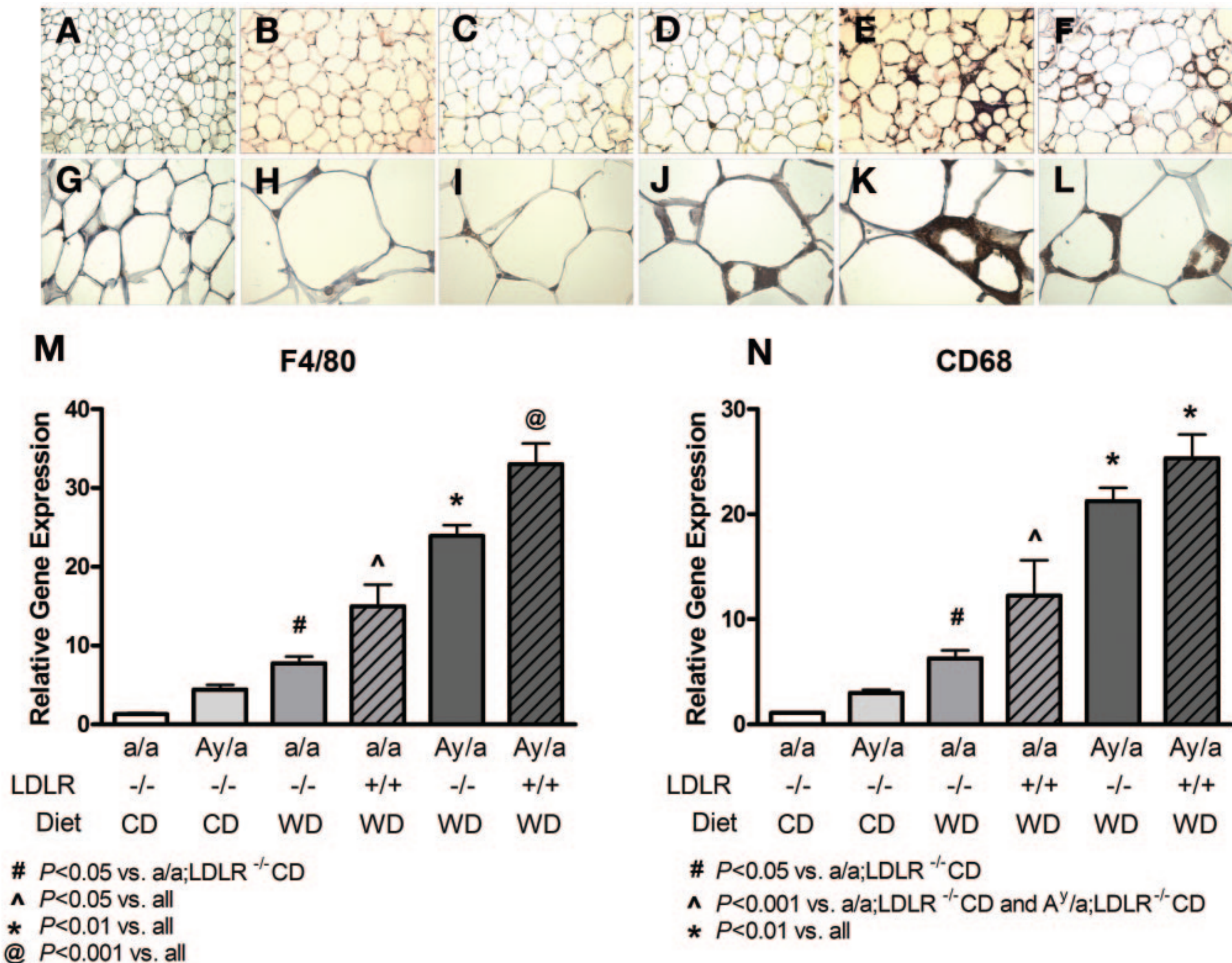

FIG. 2. ATM accumulation in obese, hyperlipidemic mice. *A–L*: WAT sections from mice in all six groups were immunostained with antibody to F4/80, a macrophage surface marker. Genotypes from *left* to *right* are as follows: a/a;LDLR$^{-/-}$ chow diet, A^y/a;LDLR$^{-/-}$ chow diet, a/a;LDLR$^{-/-}$ Western diet, a/a;LDLR$^{+/+}$ Western diet, A^y/a;LDLR$^{-/-}$ Western diet, and A^y/a;LDLR$^{+/+}$ Western diet. F4/80 staining of WAT with images taken at magnification ×10 (*A–F*) and ×40 (*G–L*). *M* and *N*: RNA was extracted from abdominal WAT, and quantitative real-time RT-PCR analysis was used to determine the mRNA expression of the macrophage markers F4/80 and CD68. Data are expressed as the means ± SE from 6 to 20 mice per group. Genotypes and statistics are indicated below graphs.

Because of the slightly higher body weight and adiposity in the Western diet–fed LDLR$^{+/+}$ mice compared with the LDLR$^{-/-}$ mice, subsets of mice were selected based on their total body fat mass so that there were no significant differences in the mean total body fat masses between each subset. The mean body fat mass of the moderately obese hyperlipidemic a/a;LDLR$^{-/-}$ Western diet group was matched to the normolipidemic a/a;LDLR$^{+/+}$ Western diet group. Likewise, the mean body fat mass of the severely obese hyperlipidemic A^y/a;LDLR$^{-/-}$ Western diet group was matched to the normolipidemic A^y/a;LDLR$^{+/+}$ Western diet group (Fig. 3*A* and *B*; a/a;LDLR$^{-/-}$ chow diet and A^y/a;LDLR$^{-/-}$ chow diet are included for reference). By controlling for adiposity, the specific contribution of hyperlipidemia to ATM accumulation in settings of moderate and severe obesity could be determined. Evaluation of F4/80 and CD68 expression in the adiposity-matched groups revealed that hyperlipidemia did not increase ATM accumulation in the LDLR$^{-/-}$ mice compared with their normolipidemic LDLR$^{+/+}$ controls (Fig. 3*C* and *D*). In fact, F4/80 expression was elevated in the normolipidemic A^y/a;LDLR$^{+/+}$ Western diet group compared with the hyperlipidemic A^y/a;LDLR$^{-/-}$ Western diet group (Fig. 3*C*).

Correlation of ATM content with plasma lipid levels. To further address our hypothesis that hyperlipidemia can potentiate macrophage accumulation in WAT in obesity, we performed correlative analyses using F4/80 and CD68 gene expression in relation to total plasma cholesterol, NEFA, and triglyceride levels for each of the six groups individually. Neither F4/80 nor CD68 expression correlated with total plasma cholesterol or NEFA levels in any of the groups (data not shown). There was a positive correlation between F4/80 expression (but not CD68) and plasma triglyceride levels only in the a/a;LDLR$^{-/-}$ Western diet group (F4/80, $r^2 = 0.24$, $P < 0.05$; CD68, $r^2 = 0.16$, $P = 0.07$). However, there was also an association between plasma triglycerides and body fat mass in this group ($r^2 = 0.24$, $P < 0.05$). Taken together, these data do not support a role for hyperlipidemia aggravating ATM accumulation in obesity in this model.

Correlation of ATM accumulation with moderate and severe obesity. Comparison of adipose tissue F4/80 expression with body weight and total body fat in all six

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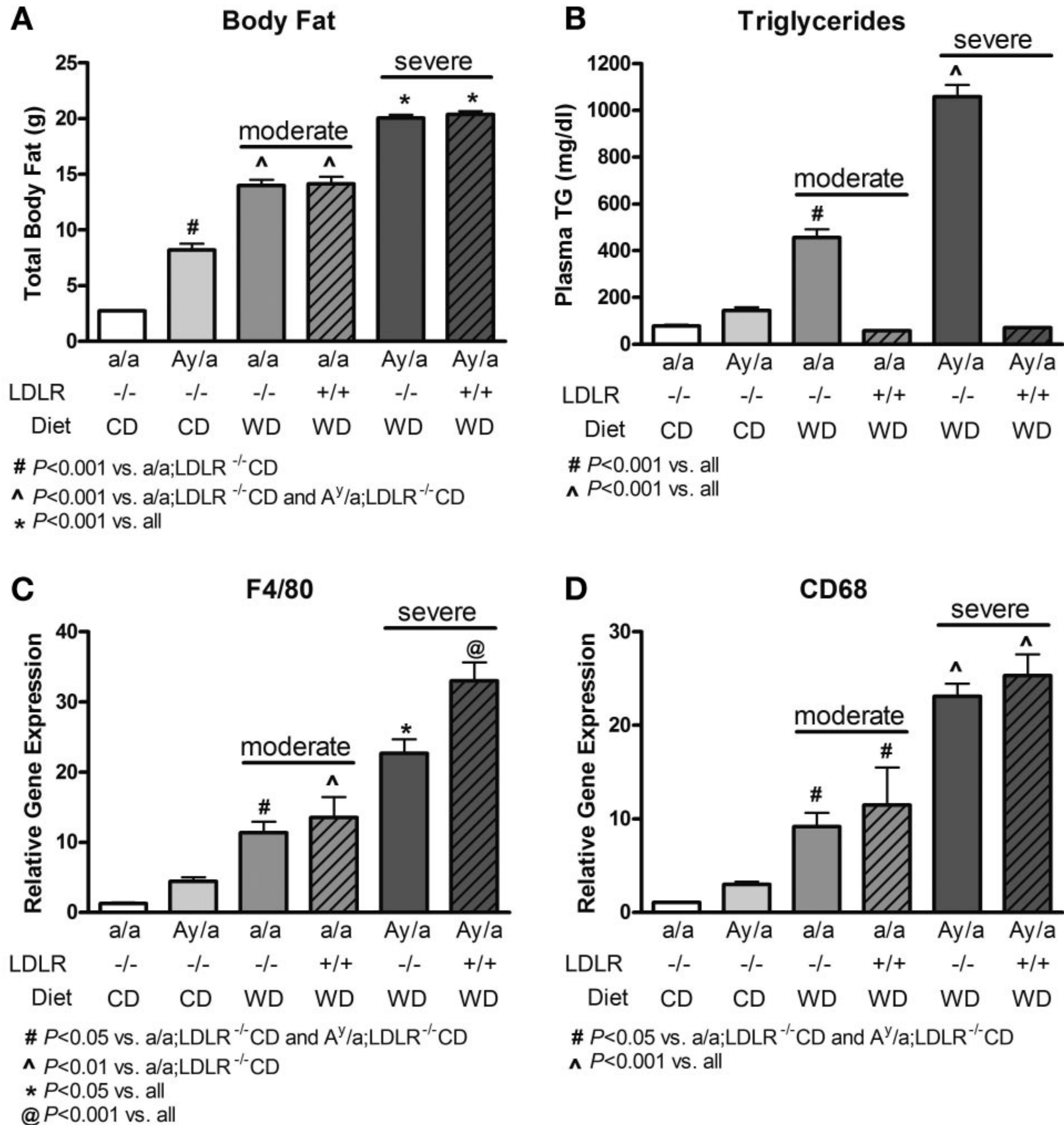

FIG. 3. Macrophage gene expression in adiposity-matched moderately and severely obese groups. F4/80 and CD68 expression were compared in adiposity-matched moderately obese and severely obese groups (normolipidemic a/a;LDLR$^{+/+}$ Western diet matched to hyperlipidemic a/a;LDLR$^{-/-}$ Western diet and normolipidemic A^y/a;LDLR$^{+/+}$ Western diet matched to hyperlipidemic A^y/a;LDLR$^{-/-}$ Western diet). *A*: Total body fat. *B*: Plasma triglycerides. *C*: F4/80 expression. *D*: CD68 expression. Data are expressed as means ± SE from 5 to 17 mice per group. Genotypes and statistics are indicated below graphs.

groups combined confirmed a correlation of ATM content with obesity and adiposity (r^2 = 0.79 and 0.76, respectively, $P < 0.0001$; Fig. 4*A* and *B*). These correlations also held true for CD68 expression (data not shown).

Data from Fig. 2 demonstrate that severe obesity is associated with a greater degree of macrophage infiltration of WAT; however, these data do not indicate whether ATM content correlates with obesity in both moderate and severe conditions. Thus, we performed correlative analyses on the moderately obese a/a;LDLR$^{-/-}$ Western diet and a/a;LDLR$^{+/+}$ Western diet groups combined and on the severely obese A^y/a;LDLR$^{-/-}$ Western diet and A^y/a;LDLR$^{+/+}$ Western diet mice combined. Body weight was significantly correlated with WAT macrophage content in both moderately and severely obese mice. Surprisingly, the correlation between body weight and F4/80 expression was stronger in the moderately obese mice than in the severely obese mice, where more macrophages were detected ($r^2 = 0.53$, $P < 0.0001$ and $r^2 = 0.24$, $P = 0.01$, respectively; Fig. 4*C*). In addition, ATM numbers correlated with total body fat only in the moderately obese group ($r^2 = 0.55$, $P < 0.0001$; Fig. 4*D*). These data suggest that adiposity contributes to macrophage accrual in moderate obesity, but other factors may play a more significant role in the continued recruitment of macrophages to WAT in severe obesity.

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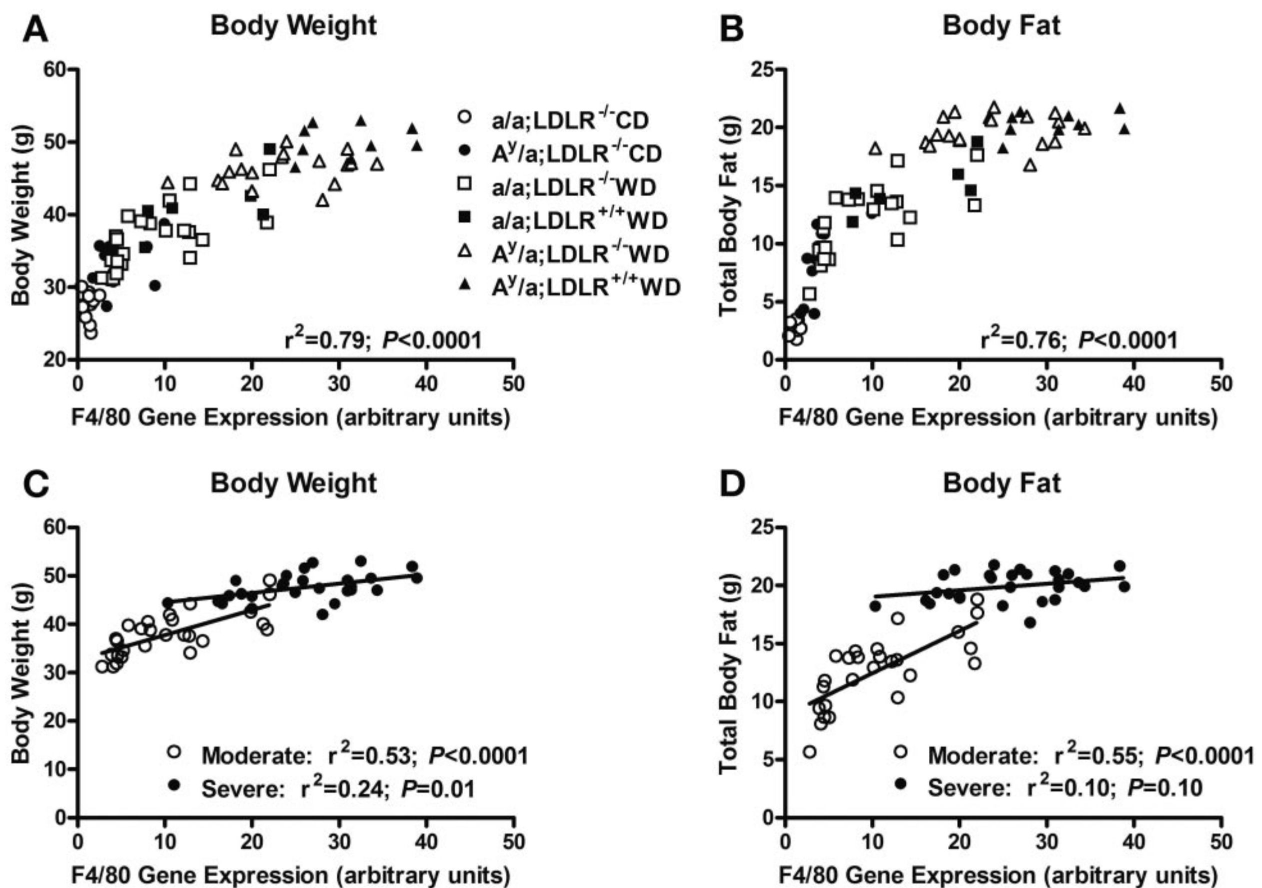

FIG. 4. Correlation of F4/80 gene expression with body composition. Correlative analyses were performed on mice from all six groups using F4/80 real-time RT-PCR data compared with body weight, r^2 = 0.79, P < 0.0001 (*A*) and total body fat, r^2 = 0.76, P < 0.0001 (*B*). Correlative analyses were performed on moderately obese (a/a;LDLR$^{-/-}$ Western diet and a/a;LDLR$^{+/+}$ Western diet) and severely obese (A^y/a;LDLR$^{-/-}$ Western diet and A^y/a;LDLR$^{+/+}$ Western diet) groups comparing F4/80 expression with body weight (*C*) and total body fat (*D*). Data represent 6–20 mice per group. Genotypes are as indicated in legends.

Effects of obesity and hyperlipidemia on inflammation in WAT. It has been suggested that macrophage infiltration into WAT can contribute to local inflammation (10,26–32). Accordingly, we determined the degree of WAT inflammation by using quantitative RT-PCR to measure gene expression of inflammation-related genes (Fig. 5). Similar to the F4/80 and CD68 expression analyses shown in Fig. 3*C* and *D*, these assays were performed on adiposity-matched a/a;LDLR$^{-/-}$ Western diet and a/a;LDLR$^{+/+}$ Western diet groups and on A^y/a;LDLR$^{-/-}$ Western diet and A^y/a;LDLR$^{+/+}$ Western diet groups, so that the specific contribution of hyperlipidemia to local WAT inflammation could be determined. Gene expression of the inflammatory cytokine tumor necrosis factor-α (TNF-α) was increased in parallel with body weight and ATM content but was not affected by hyperlipidemia (Fig. 5*A*). Interleukin 6 displayed a trend toward increased mRNA expression upon Western diet feeding (Fig. 5*B*). Expression of the inflammatory receptor toll-like receptor 4 (TLR4) was equally increased in all four Western diet–fed groups compared with the two chow diet–fed groups ($P <$ 0.05; Fig. 5*C*). Expression of the chemoattractants monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) displayed a trend toward being increased in the A^y/a;LDLR$^{-/-}$ chow diet, a/a;LDLR$^{-/-}$ Western diet, and a/a;LDLR$^{+/+}$ Western diet mice compared with the lean a/a;LDLR$^{-/-}$ chow diet group (Fig. 5*D* and *E*) and were significantly increased in the severely obese A^y/a;LDLR$^{-/-}$ Western diet and A^y/a;LDLR$^{+/+}$ Western diet groups compared with all other groups ($P < 0.05$). Finally, the expression of the inflammatory gene SAA3 demonstrated impressive 81-, 64-, and 69-fold increases in the a/a;LDLR$^{+/+}$ Western diet, A^y/a;LDLR$^{-/-}$ Western diet, and A^y/a;LDLR$^{+/+}$ Western diet groups compared with the lean a/a;LDLR$^{-/-}$ chow diet group (P<0.001; Fig. 5*F*). Thus, the increased ATM numbers resulted in dramatic increases in local inflammation. However, the addition of hyperlipidemia to obesity did not increase any measures of local inflammation in WAT.

Effects of obesity and hyperlipidemia on circulating levels of SAA, insulin, leptin, and adiponectin. In addition to examining the effects of obesity and hyperlipidemia on macrophages in WAT, we also sought to determine whether systemic abnormalities commonly linked with the metabolic syndrome, such as a pro-inflammatory state, insulin resistance, and leptin resistance (33–35), were impacted by the severe obesity seen in A^y/a;LDLR$^{-/-}$ Western diet and A^y/a;LDLR$^{+/+}$ Western diet mice. To assess the degree of systemic inflammation in our mouse model, we measured circulating levels of SAA (Fig. 6*A*). SAA levels increased according to the extent of obesity, with a/a;LDLR$^{-/-}$ chow diet mice having the lowest level (1.4 ± 0.3 μg/ml); A^y/a;LDLR$^{-/-}$ chow diet, a/a;LDLR$^{-/-}$ Western diet, and a/a;LDLR$^{+/+}$ Western diet groups showing two-, six-, and fivefold increases (2.7 ± 0.7, 7.9 ± 0.7, and 6.4 ± 0.8 μg/ml, respectively); and severely obese

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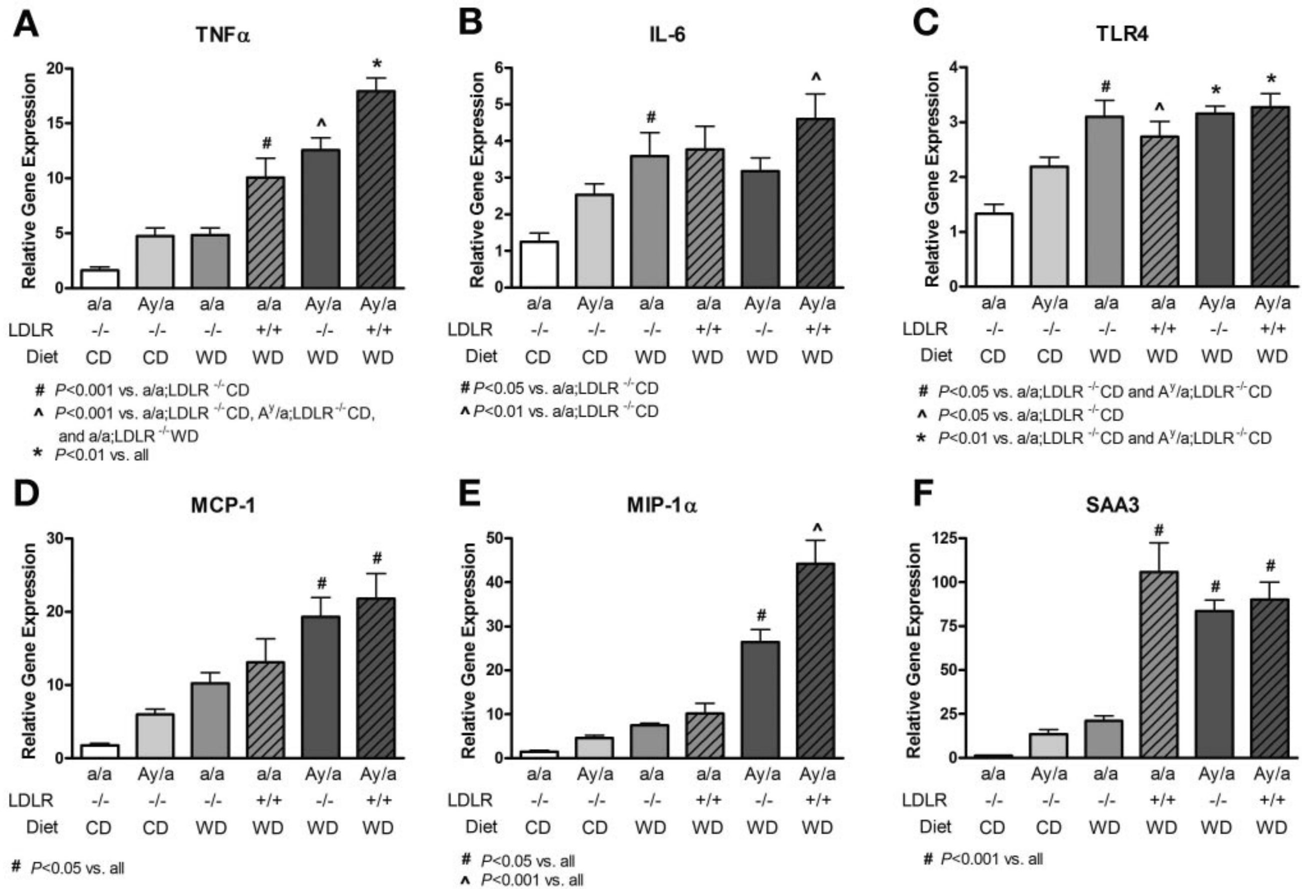

FIG. 5. Local inflammation in obese WAT. Real-time RT-PCR of abdominal WAT was used to compare gene expression of inflammatory-related markers. *A*: TNF-α. *B*: IL-6. *C*: TLR4. *D*: MCP-1. *E*: MIP-1α. *F*: SAA3. Data are expressed as means ± SE from 5 to 17 mice per group. Samples are from all mice in the a/a;LDLR$^{-/-}$ chow diet and Ay/a;LDLR$^{-/-}$ chow diet groups and from adiposity-matched mice from the moderately obese (a/a;LDLR$^{-/-}$ Western diet and a/a;LDLR$^{+/+}$ Western diet) and severely obese (A^{y}/a;LDLR$^{-/-}$ Western diet and A^{y}/a;LDLR$^{+/+}$ Western diet) groups. Genotypes and statistics are indicated below graphs.

A^{y}/a;LDLR$^{-/-}$ Western diet and A^{y}/a;LDLR$^{+/+}$ Western diet groups demonstrating 15- and 11-fold increases over lean mice (20.6 ± 1.9 and 15.6 ± 2.3 μg/ml, respectively, $P < 0.01$; Fig. 6*A*). Plasma insulin levels increased with obesity and mirrored the extent of macrophage infiltration into WAT (Fig. 6*B*). The coincidence of severe obesity with hyperlipidemia caused an increase in plasma leptin levels in the A^{y}/a;LDLR$^{-/-}$ Western diet group compared with the equally obese but normolipidemic A^{y}/a;LDLR$^{+/+}$ Western diet group (122 ± 6 and 94 ± 5 ng/ml, respectively, $P < 0.001$; Fig. 6*C*). Plasma adiponectin levels were dramatically decreased by Western diet feeding and were lower in LDLR$^{+/+}$ groups compared with LDLR$^{-/-}$ groups. Thus, the increased hyperinsulinemia and decreased adiponectin levels suggest a loss of insulin sensitivity in the more obese groups that display increased ATM accumulation.

DISCUSSION

Obesity is an integral part of the metabolic syndrome and is commonly associated with other metabolic disturbances such as dyslipidemia, inflammation, and insulin resistance. Although hyperlipidemia is not always present with obesity, the combination of the two has been shown to have an increased effect on the abnormalities linked with the metabolic syndrome (2,5). Much attention has been focused recently on the observation that macrophages infiltrate WAT in obesity; however, the impact of hyperlipidemia on this process has yet to be established. In our current study, we used a series of mouse models in which body weight and plasma lipid levels were manipulated such that the contribution of hyperlipidemia to ATM accumulation could be evaluated in both moderate and severe obesity. Contrary to expectations, hyperlipidemia did not increase macrophage infiltration into WAT in either moderate or severe obesity. In addition, our current data demonstrated the following novel findings: *1*) ATM accrual correlated with adiposity in moderate but not severe obesity; yet *2*) ATM numbers, as well as local and systemic inflammation, were disproportionately increased in severe obesity; and *3*) dramatic morphological changes occurred in WAT from severe compared with moderately obese mice.

There is some evidence in the literature suggesting that hyperlipidemia could contribute to ATM accumulation. Hyperlipidemia is intimately associated with obesity and the metabolic syndrome and may contribute to or be caused by inflammation (1,19). In fact, plasma triglyceride levels have been shown to positively correlate, and HDL inversely correlate, with ATMs in humans (7). Thus, it is reasonable to hypothesize that hyperlipidemia could also promote macrophage migration to WAT. Although our current study comparing moderately and severely obese mice with and without hyperlipidemia does not support this hypothesis, the scope of our findings is limited by the

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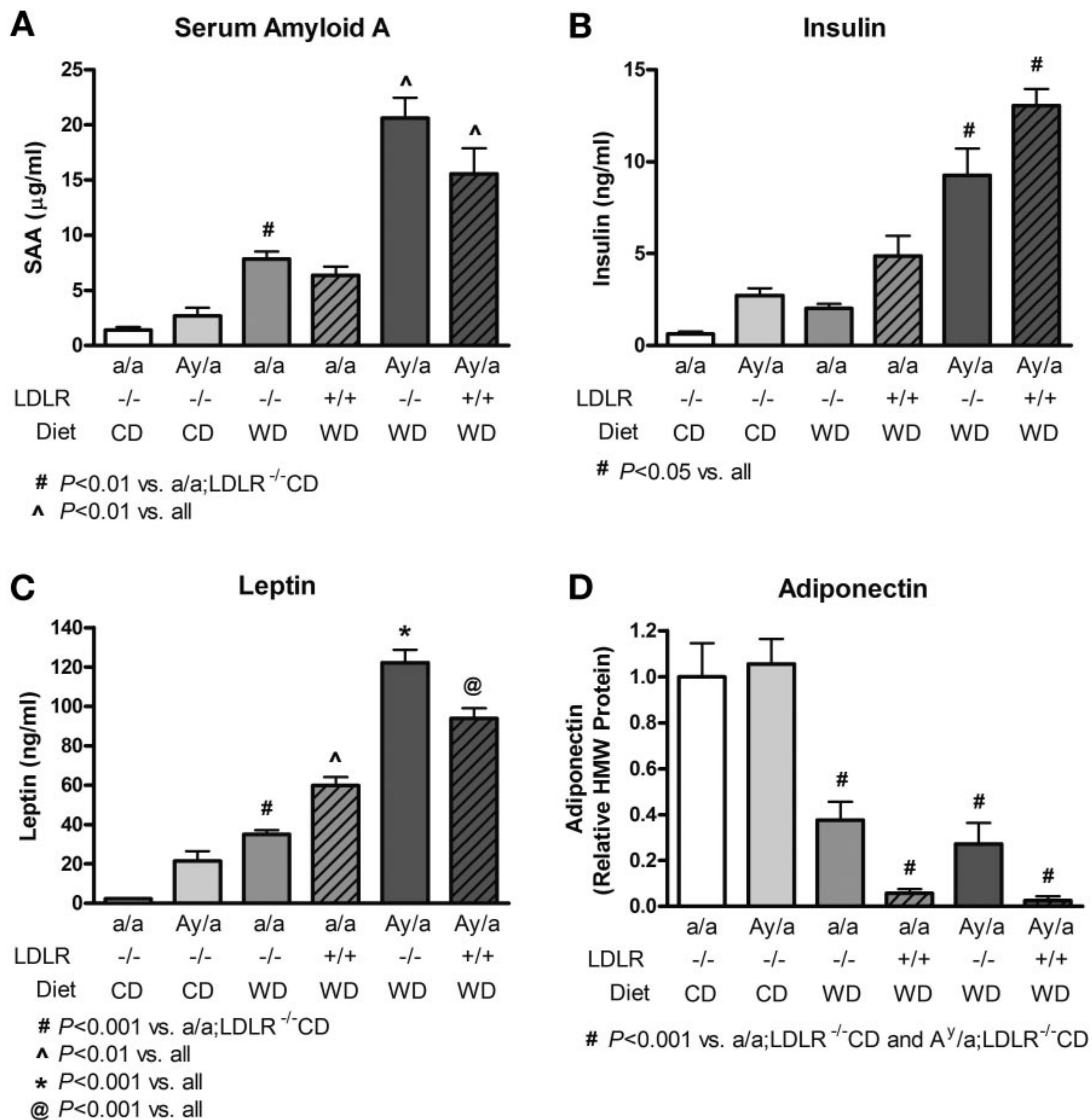

FIG. 6. Circulating levels of SAA, insulin, leptin, and adiponectin. Plasma was collected from mice at 12 weeks after diet. *A*: SAA levels were measured in 12–15 mice from each group using enzyme-linked immunosorbent assay. *B*: Insulin levels were measured in 9–14 mice from each group using RIA. *C*: Leptin levels were measured in 9–11 mice from each group using RIA. *D*: Adiponectin levels were measured in six mice from each group by SDS-PAGE as described in RESEARCH DESIGN AND METHODS. The high–molecular weight (HMW) form of the protein was quantified using densitometric analysis and is expressed relative to the a/a;LDLR$^{-/-}$ chow diet levels. Data are presented as means ± SE. Genotypes and statistics are indicated below graphs.

use of a single cause of hyperlipidemia, i.e., LDLR deficiency. We cannot rule out the possibility that the lack of LDLRs directly affects lipid deposition within WAT, with potential implications to the attraction of macrophages to WAT. In our studies, the a/a;LDLR$^{-/-}$ Western diet group did display a positive correlation between plasma triglycerides and ATM numbers similar to what was found in humans (7). However, in this group, plasma triglyceride levels were also correlated with total fat mass. Thus, in the studies by Cancello et al. (7), it is possible that positive and negative correlations of WAT macrophages with plasma triglyceride and HDL levels, respectively, were reflective of increased obesity (7). Therefore, plasma lipids may correlate with ATM numbers when their levels are causally affected by the increased adipose tissue. Finally, our studies were performed in mouse abdominal WAT, which may respond differently to hyperlipidemia than human visceral WAT.

In this model system, severely obese mice clearly had more macrophages in their WAT than moderately obese mice, but the strong correlation between adiposity and ATM accumulation seen in moderate obesity was not present. These data suggest that there is no upper limit in the extent to which macrophages can accrue in WAT; however, the factors contributing to their accrual may differ depending on the degree of obesity. It is likely that early macrophage recruitment, seen in moderate obesity, is driven by the adipocytes, hence the strong correlation between ATM and obesity. In fact, unpublished data from our laboratory demonstrates that the adipokine leptin is a potent monocyte chemoattractant. In contrast, the exaggerated increase in ATMs in severely obese mice, relative

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to the increase in adiposity, suggests that macrophages themselves become the dictating factor in propagating the recruitment of additional macrophages.

We also observed that the pattern of local and systemic inflammation mirrored the extent of macrophage infiltration into WAT more closely than plasma lipid levels. Previous studies have shown that ATMs secrete the majority of inflammatory cytokines found in WAT (8,10,14,36). In our Western diet–fed A^y/a;LDLR$^{-/-}$ and A^y/a;LDLR$^{+/+}$ animals, we detected dramatic increases in TNF-α, MCP-1, MIP-1α, and SAA3 expression. In fact, mRNA expression of chemokines MCP-1 and MIP-1α was at least 11- and 18-fold elevated, respectively, in WAT from these groups compared with the lean a/a;LDLR$^{-/-}$ chow diet group. Gene expression level of the acute-phase reactant SAA3 was at least 64-fold increased in the severely obese mice. These data are consistent with the idea that ATMs are sending out distress signals, thus amplifying macrophage recruitment to WAT. Expression of the inflammation-related receptor TLR4 was also increased in WAT from the Western diet–fed mice. Together, these data provide evidence that Western diet–fed mice were in a heightened state of inflammation, both by releasing and perceiving inflammatory stimuli. The relevance of WAT inflammatory status pertains to its diverse downstream effects such as insulin resistance.

Macrophage accumulation in WAT is closely associated with insulin resistance and leads to the question of whether macrophage infiltration into WAT precedes insulin resistance or whether insulin resistance precedes ATM accumulation. Xu et al. (10) demonstrated that the presence of macrophages in WAT temporally preceded the development of hyperinsulinemia in mice. Conversely, Di Gregorio et al. (13) have shown that thiazolidinedione treatment of insulin resistance in humans results in reduced macrophage infiltration into WAT. Thus, macrophage accumulation in WAT may promote insulin resistance, and insulin resistance may then aggravate macrophage infiltration, creating a vicious cycle. The obese mice in our studies show a loss of protection against insulin resistance as evidenced by their decreased adiponectin levels (Fig. 6*D*). In addition, plasma insulin levels (Fig. 6*B*) increased in a fashion that mirrored ATM content (Fig. 2*M* and *N*). Together, these data provide support for the relationship between insulin resistance and macrophage infiltration into WAT.

One very clear difference in WAT from the group with both severe obesity and elevated ATM content was a change in adipocyte morphology (Fig. 1). The WAT of these mice not only consisted of the hypertrophied adipocytes traditionally found in obesity, but also contained smaller adipocytes, creating a heterogeneous mixture of adipocyte sizes. These smaller adipocytes were always surrounded or crowned by macrophages. The explanation for size heterogeneity in these mice is not known. Cinti et al. (25) have suggested that macrophages are attracted to necrosing adipocytes to engulf the lipid core, resulting in what appears to be smaller adipocytes. Other studies have suggested that macrophages are attracted to adipocytes that are undergoing apoptosis (10). It has long been believed that hypertrophied adipocytes behave differently than normal-sized adipocytes. In support of this, a recent study from Jernas et al. (37) demonstrated a change in inflammatory status of large versus small adipocytes. In this context, our study raises the question of whether the homogeneity of adipocyte size is also important. We speculate that WAT consisting of a normal, homogeneous population of small adipocytes is able to function properly. In moderate obesity, when adipocytes have hypertrophied yet remain in a homogeneous population, WAT physiology is mildly impaired. However, in severe obesity, when both small and large adipocytes exist in a heterogeneous population, WAT becomes dysfunctional.

The impact of dietary manipulations in this mouse model should not be overlooked. The fat source of the Western diet used in this study consists of 69% saturated fatty acids, 27% monounsaturated fatty acids, and 4% polyunsaturated fatty acids. Saturated fatty acids have been traditionally known to have deleterious effects on health, but more recently, they have been specifically implicated in activating TLR4, leading to the expression of inflammatory gene products (38,39). Not only can saturated fatty acids activate TLR4, but polyunsaturated fatty acids can impede the activation of TLR4 (39,40). Interestingly, the TLR4 expression was more closely related to high-fat diet feeding than adiposity (Fig. 5*C*). During the preparation of this manuscript, Flier and colleagues (41) demonstrated a key role for TLR4 to ATM accumulation in high-fat diet–fed mice. Thus, it is possible that TLR4 present on ATMs is activated by an increase in saturated fatty acid flux in the Western diet–fed mice.

Collectively, our data suggest that hyperlipidemia does not contribute to obesity-driven ATM accumulation and its downstream pathophysiological consequences such as inflammation and insulin resistance. These data may have important implications to the pathogenesis of diet-induced obesity, even when plasma lipid abnormalities are not present.

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