Original Article

Absence of CC Chemokine Ligand 2 Does Not Limit Obesity-Associated Infiltration of Macrophages Into Adipose Tissue

Karen E. Inouye,[1] Hang Shi,[1] Jane K. Howard,[1] Christine H. Daly,[2] Graham M. Lord,[3] Barrett J. Rollins,[2] and Jeffrey S. Flier[1]

Macrophage recruitment to adipose tissue in obesity contributes to enhanced adipose tissue inflammatory activity and thus may underlie obesity-associated metabolic dysfunction. Obese adipose tissue exhibits increases in CC chemokine ligand 2 (CCL2, or monocyte chemoattractant protein-1), an important macrophage-recruiting factor. We therefore hypothesized that elevated CCL2 may contribute to obesity-associated adipose tissue macrophage recruitment. Male 6-week-old CCL2$^{-/-}$ and wild-type mice ($n =$ 11–14 per group) were fed standard and high-fat diets until 34 weeks of age. At 12–16 and 25–29 weeks of age, blood was collected for plasma glucose and hormone measurements, and glucose tolerance and insulin tolerance tests were performed. Adipose tissue was collected at 34 weeks for analysis of macrophage infiltration. Surprisingly, CCL2$^{-/-}$ mice on high-fat diet showed no reductions in adipose tissue macrophages. CCL2$^{-/-}$ mice on standard and high-fat diet were also glucose intolerant and had mildly increased plasma glucose and decreased serum adiponectin levels compared with wild-type mice. On high-fat diet, CCL2$^{-/-}$ mice also gained slightly more weight and were hyperinsulinemic compared with wild-type mice. Because macrophage levels were unchanged in CCL2$^{-/-}$ mice, the phenotype appears to be caused by lack of CCL2 itself. The fact that metabolic function was altered in CCL2$^{-/-}$ mice, despite no changes in adipose tissue macrophage levels, suggests that CCL2 has effects on metabolism that are independent of its macrophage-recruiting capabilities. Importantly, we conclude that CCL2 is not critical for adipose tissue macrophage recruitment. The dominant factor for recruiting macrophages in adipose tissue during obesity therefore remains to be identified. *Diabetes* 56: 2242–2250, 2007

Obesity is a major risk factor for type 2 diabetes, insulin resistance, and cardiovascular disease (1,2). There is increasing evidence that these disorders are characterized by enhanced inflammatory activity (3–6). Obese adipose tissue produces increased amounts of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) (3,7), interleukin (IL)-6 (8), transforming growth factor-β (9), and inducible nitric oxide synthase (iNOS) (10). These have been implicated in the pathogenesis of insulin resistance and cardiovascular disease (3,4,6,11). Recent studies have shown that obese adipose tissue exhibits increased infiltration by macrophages and, moreover, that macrophages may be a primary source of proinflammatory factors (12–16). Thus, macrophage infiltration into adipose tissue may be an important contributor to the enhanced inflammatory activity in obesity.

A factor that may be important for macrophage infiltration into fat is the chemokine CC chemokine ligand 2 (CCL2, or monocyte chemoattractant protein [MCP]-1). CCL2 is crucial for macrophage recruitment in several inflammatory states (17–21). Moreover, its levels in adipose tissue and plasma are highly elevated in obesity (15,22–24). Recent studies have shown that obese mice deficient in CCL2 (25) or its receptor, CC chemokine receptor 2 (CCR2) (26), have decreased adipose tissue macrophage infiltration and improved metabolic function. Conversely, mice overexpressing CCL2 in fat show the opposite phenotype (25,27). In the current study, we also examined CCL2$^{-/-}$ mice to study the role of CCL2 in macrophage recruitment and metabolic function. Surprisingly, CCL2$^{-/-}$ mice on high-fat diet did not show any reductions in adipose tissue macrophages. Moreover, the absence of CCL2 in mice on standard and high-fat diets did not improve metabolic control, and even appeared to worsen it. Thus, CCL2 does not appear to be critical for macrophage recruitment in obesity, and it may have effects on metabolism that are independent of its macrophage-recruiting capabilities.

RESEARCH DESIGN AND METHODS

Male CCL2$^{-/-}$ and wild-type mice on a C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) were housed two to four per cage and maintained on a 14/10-h light/dark cycle. We placed 6-week-old mice ($n =$ 11–14 per group) on standard diet (rodent diet no. 8664, 4.05 kcal/g, 17.4% kcal fat; Harlan Teklad, Madison, WI) or high-fat diet (no. D12331, 5.56 kcal/g, 58% kcal fat; Research Diets, New Brunswick, NJ). All groups, regardless of diet, had

the same caloric intake (~14 kcal/day). At 12 weeks of age, fasting glucose, insulin, leptin, and adiponectin levels were measured. The same animals then underwent glucose tolerance tests at 14 weeks (glucose at 1.5 mg/g body wt i.p. after overnight fast) and insulin tolerance tests (ITTs) at 16 weeks (Humulin R, 1 mU/g body wt i.p. after 4-h fast; Eli Lilly, Indianapolis, IN). Fasting glucose and hormones, glucose tolerance tests, and ITTs were again performed at 25, 27, and 29 weeks of age, respectively. Insulin sensitivity was also assessed by homeostasis model assessment of insulin resistance, which was calculated by multiplying fasting insulin levels (μl/ml) by fasting glucose levels (mmol/l) divided by 22.5. At 23 weeks, body fat composition was determined by dual-energy X-ray absorptiometry (MEC Lunar, Minster, OH). All procedures were approved by the Beth Israel Deaconess Medical Center institutional animal care and use committee.

Adipose tissue collection. At 34 weeks of age, mice were killed by CO_2 inhalation, and fat depots were collected. We placed 0.4–1.2 g of perigonadal and subcutaneous fat in Krebs-Ringer HEPES (KRH) buffer containing 10 mg/ml fatty acid–poor BSA (Sigma-Aldrich, St. Louis, MO) for isolation of adipocytes and stromal vascular cells. A portion of the perigonadal fat was snap-frozen for RNA analysis. Perigonadal and subcutaneous fat samples were also placed in zinc formalin fixative for immunohistochemical analysis.

Isolation of adipocytes and stromal vascular cells from adipose tissue. Adipose tissue in KRH buffer was minced and centrifuged to remove blood cells. It was then digested in 0.12 units/ml of low-endotoxin collagenase (Liberase 3; Roche Applied Science, Indianapolis, IN) by shaking (80 Hz) at 37°C for 45 min. Samples were filtered through 300-μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA), and the resulting suspension was centrifuged at 500g for 10 min to separate stromal vascular cells from adipocytes. Adipocytes were stored in phenol-guanidine thiocyanate reagent (Qiagen, Valencia, CA) at −80°C for subsequent RNA isolation. Stromal vascular cells were incubated in erythrocyte lysis buffer (0.154 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA) for 2 min and then washed with KRH-BSA. Cells were then suspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 5 mmol/l EDTA and 0.2% fatty acid–poor BSA). A portion of the cells was counted with a hemacytometer. Based on trypan blue exclusion, the percentage of live cells per sample was 70 ± 3 and 70 ± 2% for perigonadal and subcutaneous stromal vascular cells, respectively. The remaining stromal vascular cells underwent FACS analysis, as described below.

Determination of adipose tissue macrophage levels and isolation of macrophages from stromal vascular cells by FACS. Stromal vascular cells were incubated in the dark on a shaker with FcBlock (20 μg/ml; BD Pharmingen, San Jose, CA) for 30 min at 4°C. Perigonadal stromal vascular cells were incubated for 50 min with allophycocyanin-conjugated F4/80 antibody (5 μg/ml) and phycoerythrin-conjugated CD11b (Mac1) antibody (2 μg/ml; Caltag Laboratories, Burlingame, CA), both cell surface markers of mature macrophages that are not detected on preadipocytes (27,28). Subcutaneous stromal vascular cells were incubated only with F4/80 antibody. After incubation, cells were washed with FACS buffer. Perigonadal stromal vascular cells were sorted into F4/80-positive, CD11b-positive, F4/80/CD11b double-positive, and F4/80/CD11b double-negative populations with a MoFlo cell sorter (Dako Cytomation, Fort Collins, CO). Summit version 4 software (Dako Cytomation) was used to determine the percentages of the cell populations in the stromal vascular fraction. F4/80/CD11b$^{+/+}$ and F4/80/CD11b$^{-/-}$ cells were stored in phenol-guanidine thiocyanate reagent (Molecular Research Center, Cincinnati, OH) at −80°C for subsequent RNA isolation. Subcutaneous stromal vascular cells were fixed in 4% paraformaldehyde in PBS, and the percentage of F4/80-positive cells was determined with a FACSCalibur machine and CellQuest software (BD, Franklin Lakes, NJ). We estimated the number of macrophages per depot according to the following formula: number of F4/80-positive cells per depot = % F4/80-positive cells in stromal vascular fraction × number of live stromal vascular fraction cells isolated per gram of adipose tissue × mass of depot in grams. For perigonadal stromal vascular cells, we used the combined percentage of F4/80$^+$, CD11b$^+$, and F4/80/CD11b$^{+/+}$ cells to determine the number of macrophages in perigonadal adipose tissue.

RT-PCR on adipose tissue, adipocyte, and stromal vascular cell RNA. Total RNA was isolated from whole perigonadal adipose tissue, adipocytes, F4/80/CD11b$^{+/+}$ cells, and F4/80/CD11b$^{-/-}$ cells and then reverse-transcribed to cDNA (Retroscript; Ambion, Austin, TX). TaqMan (Applied Biosystems, Branchburg, NJ) RT-PCR was then performed for measurement of mRNA for the macrophage markers F4/80, CD11b, and CD68, as well as TNF-α, IL-6, IL-1β, IL-10, iNOS, suppressor of cytokine signaling 3 (SOCS-3), and hypoxia-inducible factor-1α (HIF-1α). Relative expression levels of the mRNAs were determined using standard curves. Samples were adjusted for total RNA content by comparison with cyclophilin or 18S expression. TNF-α, IL-1β, iNOS, SOCS-3, cyclophilin, and 18S primer (Invitrogen, Carlsbad, CA) and probe (Biosearch Technologies, Novato, CA) sequences can be provided on

request. For the remaining genes, primer-probe mixtures from Applied Biosystems were used.

Adipose tissue immunohistochemistry. Paraffin sections of adipose tissue were analyzed for macrophage content by staining with antibody against F4/80. Sections underwent trypsin antigen retrieval, followed by blocking with avidin, biotin (Vector Laboratories, Burlingame, CA), and rabbit serum. Sections were then incubated with rat anti-mouse F4/80 antibody (1:75; Serotec, Raleigh, NC) overnight at 4°C. F4/80 staining was detected by incubating the slides with biotinylated rabbit anti-rat antibody, followed by treatment with peroxidase-conjugated streptavidin (Vector Laboratories) and 3-amino-9-ethylcarbazole (Dako, Carpinteria, CA). Slides were counterstained with hematoxylin. For determination of macrophage infiltration, two 10×-magnified fields from each of two adipose sections per mouse were analyzed. The percentage of macrophages was determined by counting the number of nuclei of F4/80-positive cells relative to the total number of cell nuclei per field.

Serum and plasma measurements. Serum insulin (CrystalChem, Downers Grove, IL), leptin (CrystalChem), adiponectin (Linco Research, St. Charles, MO), and CCL7 (Bender MedSystems, Burlingame, CA) concentrations were determined by enzyme-linked immunosorbent assay. Serum CCL2 levels were measured with a mouse serum adipokine LincoPlex kit (Linco Research). Plasma glucose levels were measured with a glucometer (Lifescan, Milpitas, CA).

Statistical analysis. Data are the means ± SE. Measurements were analyzed by one-way ANOVA followed by Duncan's post hoc test for multiple comparisons. Significance was set at $P < 0.05$. Statistical analysis was performed with Statistica software (Statsoft, Tulsa, OK).

RESULTS

Serum CCL2 and CCL7 levels are increased in wild-type mice on high-fat diet but are undetectable in CCL2$^{-/-}$ mice. Wild-type mice on high-fat diet had markedly increased ($P < 0.001$) serum CCL2 levels compared with mice on standard diet (39 ± 11 pg/ml, $n = 9$, and 167 ± 34 pg/ml, $n = 9$, for standard and high-fat diet, respectively). CCL2 was very low or undetectable in CCL2$^{-/-}$ mice on either diet (0 pg/ml, $n = 8$, and 4.0 ± 4.0 pg/ml, $n = 9$, respectively). To rule out whether compensatory increases in other CCR2 ligands might be occurring in CCL2$^{-/-}$ mice, we measured serum levels of another CCR2 ligand, CCL7 (MCP-3). CCL7 was also increased ($P < 0.001$) in high-fat diet–fed wild-type mice (1.3 ± 0.7 pg/ml, $n = 11$, and 15.7 ± 4.3 pg/ml, $n = 11$, for standard and high-fat diet, respectively). Surprisingly, CCL7 was also undetectable in CCL2$^{-/-}$ mice (0 pg/ml, $n = 10$, and 0 pg/ml, $n = 12$, for standard and high-fat diet, respectively), suggesting that CCL7 levels may be regulated by CCL2.

CCL2$^{-/-}$ mice show mildly enhanced weight gain in response to high-fat diet compared with wild-type mice. Unexpectedly, CCL2$^{-/-}$ mice gained more weight on high-fat diet than wild-type mice ($P < 0.05$) (Fig. 1A). From 18 to 34 weeks of age, CCL2$^{-/-}$ mice weighed on average 13% more than high-fat diet–fed wild-type mice. This was associated with 15% increases in percent body fat and fat pad weights and elevated serum leptin levels ($P < 0.05$) (Fig. 1C–E). Lean mass was unaltered (data not shown). CCL2$^{-/-}$ mice on standard diet weighed slightly less than wild-type mice ($P < 0.05$ at some ages) (Fig. 1B). However, there were no differences in body fat, serum leptin, and lean mass. Food intake for CCL2$^{-/-}$ mice on high-fat and standard diets did not differ from wild-type mice (data not shown).

Absence of CCL2 does not limit macrophage infiltration into adipose tissue. Immunohistochemical analysis of perigonadal adipose tissue stained with antibody against the macrophage marker F4/80 showed that high-fat diet in wild-type mice induced a fivefold increase in the percentage of macrophages (Fig. 2A, C, and E). Messenger RNA levels of the macrophage markers F4/80, CD11b, and CD68 were also increased ($P < 0.05$) after high-fat diet
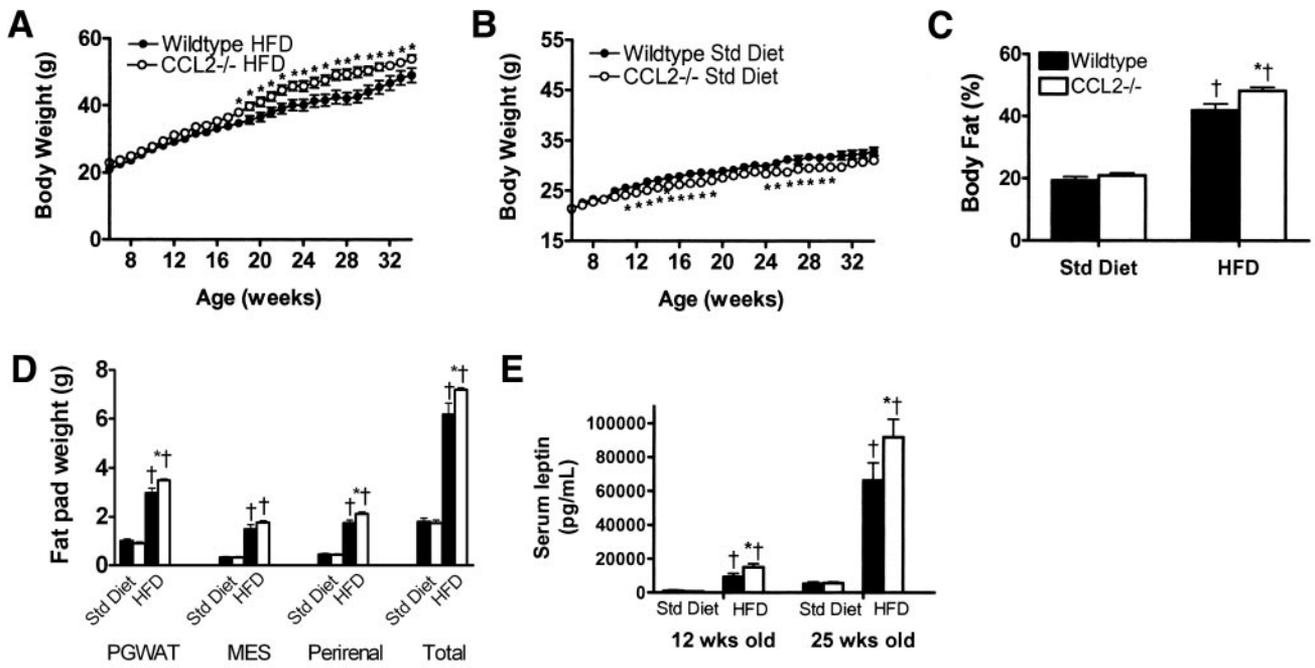
FIG. 1. Body weights for high-fat diet (HFD) (*A*) and standard (Std) diet (*B*), percent body fat as measured by dual-energy X-ray absorptiometry at 23 weeks of age (*C*), perigonadal (PGWAT), mesenteric (MES), and perirenal fat pad weights at 34 weeks of age (*D*), and fasting serum leptin levels (*E*) of wild-type (*n* = 12) and CCL2$^{-/-}$ (*n* = 12) mice fed standard or high-fat diet. Values are the means ± SE. *$P < 0.05$ vs. wild-type; †$P < 0.05$ vs. standard diet of same genotype.
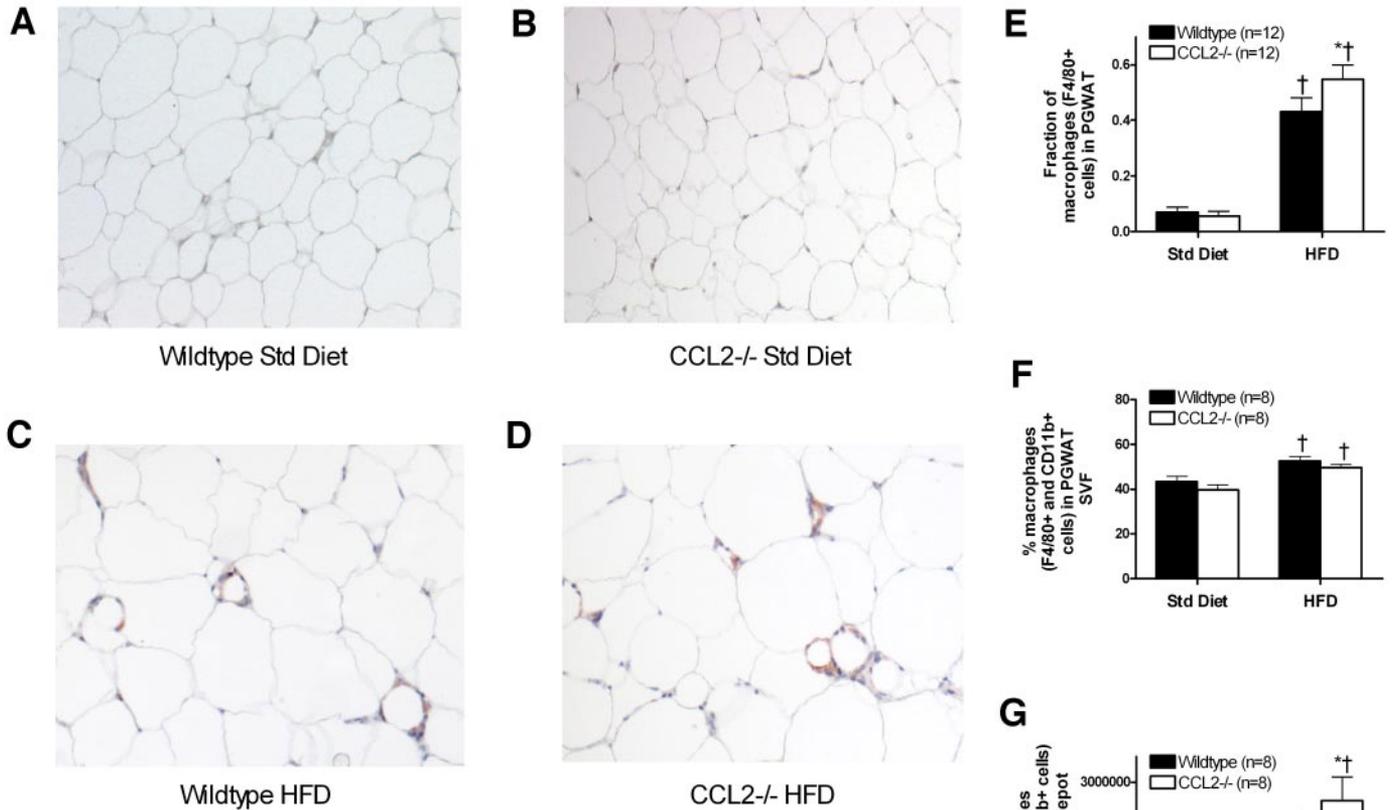
FIG. 2. Perigonadal adipose tissue of wild-type (*A* and *C*) and CCL2$^{-/-}$ (*B* and *D*) mice fed standard (Std) or high-fat diets (HFD) stained with antibody against the macrophage marker, F4/80 (red), 10× magnification. Immunohistochemical analysis of the fraction of F4/80-positive cells relative to total number of cells within perigonadal adipose tissue (PGWAT) (*E*). FACS analysis of the percentage of F4/80- and/or CD11b-positive cells within the perigonadal stromal vascular fraction (SVF) (*F*) and per perigonadal depot (*G*). Values are expressed as the means ± SE. *$P < 0.05$ vs. wild type; †$P < 0.05$ vs. standard diet of same genotype.

TABLE 1

	Wild-type standard diet	CCL2$^{-/-}$ standard diet	Wild-type high-fat diet	CCL2$^{-/-}$ high-fat diet
n	12	11	12	12
F4/80	0.33 ± 0.02	0.37 ± 0.04	$0.86 \pm 0.12*$	$0.94 \pm 0.10*$
CD11b	0.68 ± 0.03	0.66 ± 0.01	$0.91 \pm 0.05*$	$0.85 \pm 0.05*$
CD68	0.42 ± 0.03	0.42 ± 0.01	$1.15 \pm 0.11*$	$1.21 \pm 0.17*$

F4/80, CD11b, and CD68 mRNA expression in perigonadal adipose tissue of 34-week-old wild-type and CCL2$^{-/-}$ mice fed standard or high-fat diet starting at 6 weeks of age. Gene expression was normalized relative to cyclophilin. *$P < 0.05$ vs. standard diet.

(Table 1). By FACS, there was a small increase ($P < 0.05$) in the percentage of macrophages within the stromal vascular fraction (Fig. 2F) and a fivefold increase ($P < 0.05$) in macrophage numbers per depot in high-fat–fed diet mice (Fig. 2G).

Surprisingly, high-fat diet–fed CCL2$^{-/-}$ mice did not show any reductions in the percentage of macrophages in adipose tissue stained for F4/80 (Fig. 2D and E). There was even a very mild, but significant ($P < 0.05$), increase in the percentage of macrophages. By FACS, the percentage of macrophages within the stromal vascular fraction was similar to wild-type mice (Fig. 2F). However, macrophage numbers per depot were also mildly increased ($P < 0.05$) (Fig. 2G). This may in part have been attributable to the increased size of the perigonadal depot in CCL2$^{-/-}$ mice on high-fat diet. Nonetheless, RT-PCR for F4180, CD11b, and CD68 mRNA showed no differences between wild-type and CCL2$^{-/-}$ mice on high-fat diet (Table 1). Taken together, the data show that absence of CCL2 did not decrease obesity-associated infiltration of macrophages into adipose tissue. In CCL2$^{-/-}$ mice on standard diet, macrophage numbers and macrophage marker mRNA levels also did not differ from those in wild-type mice (Fig. 2B and E–G, Table 1). Macrophage numbers were also unaltered in subcutaneous fat of CCL2$^{-/-}$ mice (not shown).

CCL2 absence does not improve metabolic function. CCL2 absence did not improve metabolic function in CCL2$^{-/-}$ mice fed either diet. Unexpectedly, metabolic control even appeared to be slightly worsened in CCL2$^{-/-}$ mice. At 14 and 27 weeks of age, CCL2$^{-/-}$ mice on both diets were glucose intolerant compared with wild-type mice ($P < 0.05$) (Figs. 3A–D). The glucose intolerance grew worse with age in CCL2$^{-/-}$ mice on standard diet. At 27 weeks, peak glucose levels in standard diet–fed CCL2$^{-/-}$ mice were 6 mmol/l higher than in wild-type mice. For high-fat diet–fed mice, the difference in glucose tolerance at 27 weeks was smaller than at 14 weeks because of the worsened glucose tolerance of wild-type mice. CCL2$^{-/-}$ mice on standard and high-fat diet also showed mild increases ($P < 0.05$) in fasting glucose and decreases ($P < 0.05$) in serum adiponectin (Figs. 3E and F). Fasting insulin levels and values for homeostasis model assessment of insulin resistance in 25-week-old high-fat diet–fed CCL2$^{-/-}$ mice were nearly double those of wild-type levels ($P < 0.05$) (Figs. 4A and B), suggesting that CCL2 absence worsened obesity-associated insulin resistance. However, these results are inconclusive because insulin sensitivity as assessed by ITT did not appear to differ (Figs. 4C and D).

CCL2 absence does not alter inflammatory marker mRNA expression in whole adipose tissue. We also investigated the effect of obesity and CCL2 absence on adipose tissue inflammatory marker mRNA. As with macrophage marker mRNA, perigonadal TNF-α mRNA levels were low in both wild-type and CCL2$^{-/-}$ mice on standard diet and increased similarly in response to high-fat diet (wild-type standard diet: 0.45 ± 0.03, $n = 12$; CCL2$^{-/-}$ standard diet: 0.42 ± 0.04, $n = 10$; wild-type high-fat diet: 0.95 ± 0.07, $n = 12$; CCL2$^{-/-}$ high-fat diet: 1.03 ± 0.06, $n = 10$; TNF/cyclophilin expression; $P < 0.001$ vs. standard diet). In wild-type and CCL2$^{-/-}$ mice, high-fat diet also led to similar increases in CCR2 mRNA, indicating that CCL2 absence did not lead to compensatory increases in expression of its receptor (wild-type standard diet: 0.62 ± 0.04, $n = 12$; CCL2$^{-/-}$ standard diet: 0.49 ± 0.02, $n = 11$; wild-type high-fat diet: 0.86 ± 0.06, $n = 12$; CCL2$^{-/-}$ high-fat diet: 0.79 ± 0.06, $n = 12$; CCR2/cyclophilin expression; $P < 0.05$, high-fat diet vs. standard diet). This finding, combined with the reduced serum CCL7 levels in CCL2$^{-/-}$ mice, suggests that the phenotype of CCL2$^{-/-}$ mice was unlikely to have been caused by increased signaling by other CCR2 ligands. There were no effects of high-fat diet or CCL2 absence on IL-6, IL-1β, iNOS, and SOCS-3 mRNA levels (data not shown).

Absence of CCL2 alters inflammatory gene expression in adipose tissue cells. We also examined the effects of high-fat diet and CCL2 absence on expression of inflammatory markers in adipocytes, macrophages (F4/80 and CD11b$^{+/+}$ cells), and nonmacrophage stromal vascular cells (F4/80 and CD11b$^{-/-}$ cells) isolated by FACS. In wild-type mice on standard diet, F4/80 mRNA was primarily restricted to the macrophage fraction (Fig. 5A). TNF-α, IL-1β, and IL-10 mRNA were also highest ($P < 0.05$) in the macrophages (Figs. 5B–D). High-fat diet in wild-type mice increased ($P < 0.05$) TNF-α mRNA in adipocytes and IL-1β and -10 mRNA in adipocytes and nonmacrophage stromal vascular cells. Unexpectedly, F4/80 mRNA was also increased ($P < 0.05$) in obese adipocytes. This was possibly attributable to residual macrophages in the adipocyte fraction. TNF-α and IL-10 mRNA levels in obese adipocytes strongly correlated with F4/80 expression (TNF-α vs. F4/80, $R^2 = 0.78$, $P < 0.05$; IL-10 vs. F4/80, $R^2 = 0.78$, $P < 0.05$), suggesting that residual macrophages contributed to the increases in adipocyte TNF-α and IL-10 mRNA. Conversely, adipocyte IL-1β and F4/80 mRNA levels were not significantly correlated ($R^2 = 0.44$, $P = $ NS), suggesting that the increase in IL-1β mRNA may have been adipocyte specific.

Obesity in wild-type mice increased ($P < 0.05$) macrophage F4/80 mRNA, perhaps indicating enhanced macrophage activity. Obesity also increased ($P < 0.05$) IL-6 mRNA in nonmacrophage stromal vascular cells and SOCS-3 mRNA in macrophages, but it decreased ($P < 0.05$) SOCS-3 mRNA in adipocytes (Fig. 5E and F). Messenger RNA for HIF-1α, a controller of the cellular response to hypoxia that is increased in adipose tissue of obese humans (13), was increased ($P < 0.05$) in nonmacrophage stromal vascular cells, but it was decreased ($P < 0.05$) in adipocytes in response to high-fat diet (Fig. 5G).

CCL2$^{-/-}$ mice on standard diet had higher ($P < 0.05$) TNF-α and IL-10 mRNA levels across all cell populations compared with their wild-type counterparts. There was higher ($P < 0.05$) IL-1β mRNA expression in adipocytes and nonmacrophage stromal vascular cells and higher ($P < 0.05$) IL-6 and HIF-1α mRNA in adipocytes, but there

FIG. 3. Glucose tolerance tests (A–D), fasting plasma glucose (E), and serum adiponectin levels (F) in wild-type and CCL2$^{-/-}$ mice fed standard (Std) (A and B) or high-fat diet (HFD) (C and D). Values are the means ± SE. *$P < 0.05$ vs. wild type; †$P < 0.05$ vs. standard diet of same genotype.
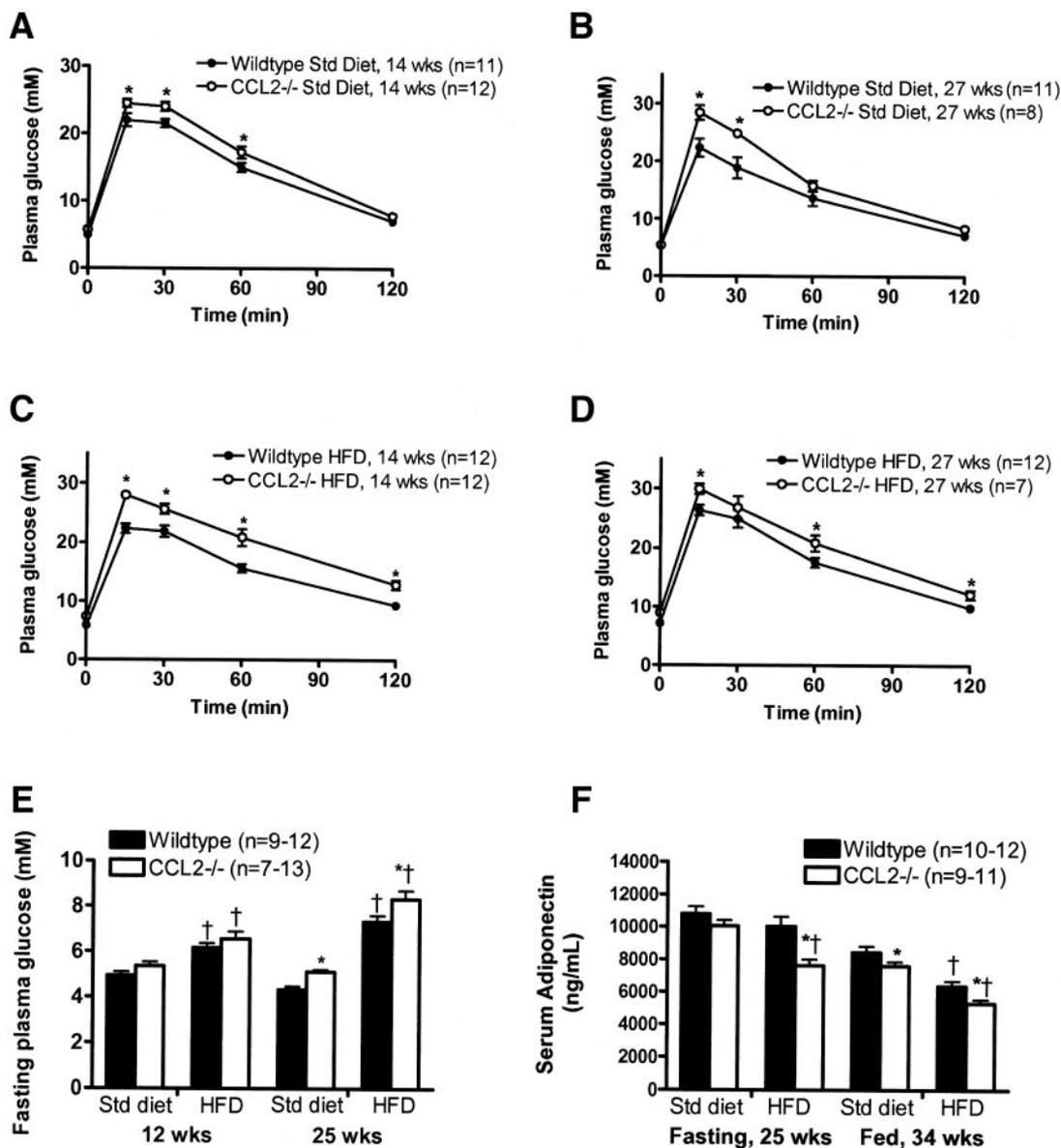
was lower ($P < 0.05$) SOCS-3 mRNA in adipocytes. Although these data contrast with the similar inflammatory gene mRNA levels in whole adipose tissue, they suggest that there may have been some enhancement of inflammatory activity in adipose tissue of CCL2$^{-/-}$ mice on standard diet. In high-fat diet–fed mice, CCL2 deficiency enhanced ($P < 0.05$) the obesity-associated increases in macrophage F4/80 and SOCS-3 mRNA and nonmacrophage stromal vascular cell IL-10 and HIF-1α mRNA. However, adipocyte TNF-α and IL-1β mRNA levels in high-fat diet CCL2$^{-/-}$ mice were lower ($P < 0.05$) than in wild-type mice. Adipocyte F4/80 mRNA was also lower, suggesting that the decreased TNF-α and IL-1β mRNA levels may have been caused by the presence of fewer residual macrophages in the adipocyte fraction.

DISCUSSION

Obesity is associated with increased macrophage infiltration of adipose tissue (12–16). Moreover, there is evidence to suggest that macrophages may be largely responsible for the enhanced inflammatory activity of obese adipose tissue (12,15). The mechanisms underlying macrophage recruitment to adipose tissue are not known. The chemokine CCL2 is a critical chemoattractant for macrophages in several inflammatory models (17–21). In obesity, levels of CCL2 in serum and adipose tissue are markedly increased (15,22–24), suggesting that CCL2 may be an important macrophage-recruiting factor.

We studied mice deficient in CCL2 to examine the role of CCL2 in macrophage recruitment and metabolic function. Surprisingly, despite having nearly undetectable serum CCL2 levels, immunohistochemical and FACS analysis of adipose tissue showed that CCL2$^{-/-}$ mice on standard and high-fat diets did not have reduced macrophage numbers compared with wild-type mice. RT-PCR also showed identical increases in adipose tissue gene expression of the macrophage markers F4/80, CD11b, and CD68 in CCL2$^{-/-}$ and wild-type mice after high-fat diet. Our results contrast with the recent reports of reduced adipose tissue macrophages in obese CCL2$^{-/-}$ (25) and
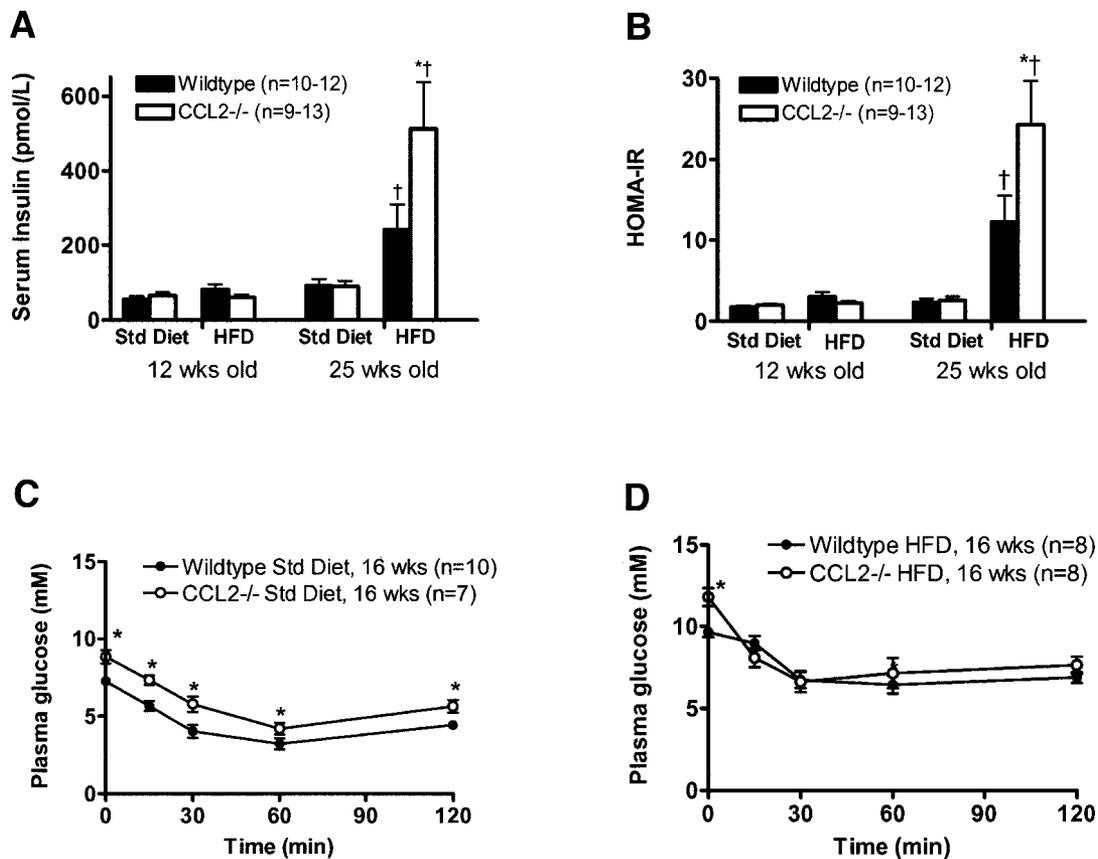
FIG. 4. Serum insulin (A), homeostasis model assessment of insulin resistance (B), and ITTs (at 16 weeks of age) (C and D) in wild-type and CCL2$^{-/-}$ mice fed standard (Std) (C) or high-fat diet (HFD) (D). Values are the means ± SE. *$P < 0.05$ vs. wild type; †$P < 0.05$ vs. standard diet of same genotype.

CCR2$^{-/-}$ (26) C57BL/6J mice. Notably, however, the reductions in macrophage numbers in these studies were small. By immunohistochemistry, the fraction of macrophages in perigonadal fat was only decreased by 20–30% (25,26). Furthermore, another study in high-fat diet–fed CCR2$^{-/-}$ mice on a DBA1/J background showed no decreases in adipose tissue macrophages (30). At the time of tissue collection, our mice were only a few weeks older than the previously studied CCL2$^{-/-}$ and CCR2$^{-/-}$ mice (25,26). Thus, it is unlikely that the fact we collected adipose tissue at 34 weeks of age was the reason for the lack of reductions in macrophages in our mice.

Transgenic mice overexpressing CCL2 in adipose tissue have also been studied (25,27). In one study, CCL2 transgenic mice had nearly double the serum CCL2 levels seen in wild-type mice on high-fat diet, but they had 40% fewer macrophages in fat (25). Other CCL2 transgenic mice had 2.5-fold higher plasma CCL2 levels than wild-type mice but only 25% more macrophages (27). These and our data suggest that CCL2 may not be the primary macrophage-recruiting factor in obese adipose tissue. Although CCL2 is an important macrophage-recruiting factor in several inflammatory conditions (18–21), there are circumstances in which CCL2 is elevated but does not contribute to macrophage recruitment (31,32). For example, macrophage infiltration into skin incision wounds is not reduced in CCL2$^{-/-}$ mice, even though CCL2 levels at wound sites are high in normal animals (31). Given the mild effects of CCL2 absence on adipose tissue macrophages in the previous studies, it is possible that differences in experimental

conditions contributed to the discrepancies between our findings. In the earlier study, CCL2$^{-/-}$ and wild-type mice were from different colonies (25). We studied the same CCL2$^{-/-}$ mice used in the previous study (25). However, our controls were wild-type mice from the colony to which our CCL2$^{-/-}$ mice had been backcrossed. Variations in genetic background due to being from different colonies may have contributed to the differences between wild-type and CCL2$^{-/-}$ mice in the earlier study. With regards to the CCR2$^{-/-}$ mice, it cannot be ruled out that the phenotype in CCR2$^{-/-}$ mice was also attributable to decreased action of the other CCR2 ligands CCL7 (MCP-3) (33), CCL8 (MCP-2) (34), CCL12 (MCP-5) (35), and CCL13 (MCP-4) (36).

CCL2 absence did not improve metabolic function and, unexpectedly, seemed to slightly worsen it. CCL2$^{-/-}$ mice on high-fat diet had slightly increased body fat and higher serum insulin levels than wild-type mice. The increased adiposity may have been an effect of CCL2 absence to enhance adipose tissue growth. Interestingly, the obese CCR2$^{-/-}$ mice studied previously had larger adipocytes (25). Earlier reports also showed that CCL2 induces adipocyte dedifferentiation in 3T3-L1 adipocytes by reducing triglyceride content and expression of genes expressed in mature adipocytes, such as lipoprotein lipase, adipsin, GLUT-4, aP2, β3-adrenergic receptor, and peroxisome proliferator–activated receptor-γ (24,37). Surprisingly, CCL2$^{-/-}$ mice on high-fat and standard diet were also glucose intolerant and had mildly increased glucose levels. Given that macrophage numbers were unchanged, this phenotype appears to have been caused by absence of
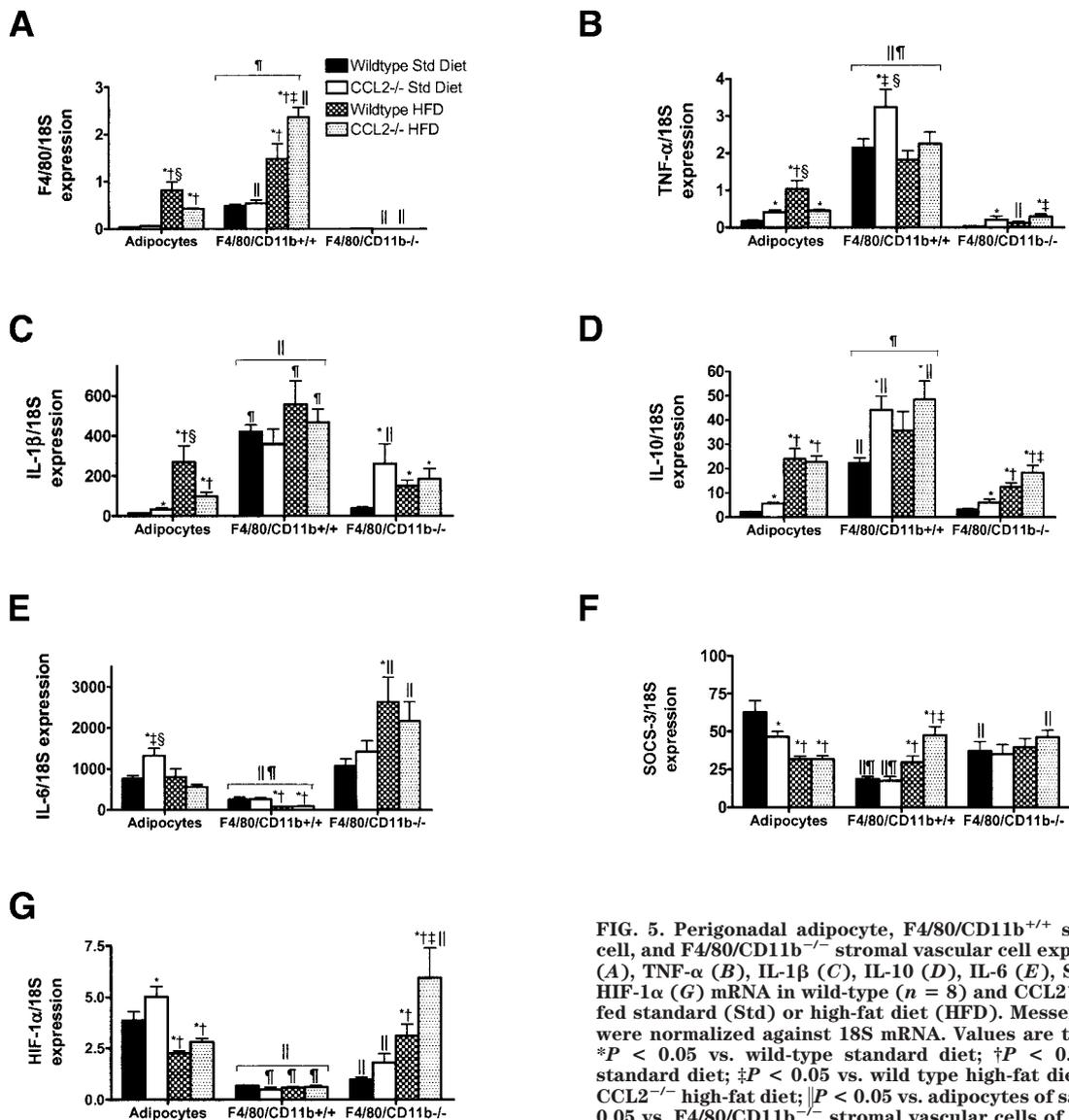
A

B

C

D

E

F

G

FIG. 5. Perigonadal adipocyte, F4/80/CD11b$^{+/+}$ stromal vascular cell, and F4/80/CD11b$^{-/-}$ stromal vascular cell expression of F4/80 (A), TNF-α (B), IL-1β (C), IL-10 (D), IL-6 (E), SOCS-3 (F), and HIF-1α (G) mRNA in wild-type (n = 8) and CCL2$^{-/-}$ (n = 8) mice fed standard (Std) or high-fat diet (HFD). Messenger RNA levels were normalized against 18S mRNA. Values are the means ± SE. *P < 0.05 vs. wild-type standard diet; †P < 0.05 vs. CCL2$^{-/-}$ standard diet; ‡P < 0.05 vs. wild type high-fat diet; §P < 0.05 vs. CCL2$^{-/-}$ high-fat diet; ‖P < 0.05 vs. adipocytes of same group; ¶P < 0.05 vs. F4/80/CD11b$^{-/-}$ stromal vascular cells of same group.

CCL2 itself. Previous studies showed unaltered metabolic function in lean CCL2$^{-/-}$ and CCR2$^{-/-}$ mice and improved glucose tolerance and insulin sensitivity in CCL2$^{-/-}$ and CCR2$^{-/-}$ mice on high-fat diet (25,26). As mentioned above, these differences from our data may have been related to background variations in the CCL2$^{-/-}$ and wild-type mice used in the earlier study, as well as loss of action of other CCR2 ligands in CCR2$^{-/-}$ mice. Nonetheless, our findings also contrast with the glucose intolerance and insulin resistance seen in mice overexpressing CCL2 in fat under the ap2 promoter (25,27). The reason for the worsened metabolic regulation in our animals is not known. It should be noted, however, that deficiency of CCL2 has complex effects on immune function, causing both upregulation and downregulation of inflammatory factors (32,38,39). In endotoxin-treated mice, neutralization of CCL2 increases serum TNF-α and IL-12 levels (39), and in CCL2$^{-/-}$ or CCL2-neutralized mice, others have found higher levels of TNF-α and IL-10 (32,38). In our isolated adipocytes, macrophages, and nonmacrophage stromal vascular cells from adipose tissue of lean and obese CCL2$^{-/-}$ mice, there was some enhancement of inflammatory gene expression. Although this did not translate into increased inflammatory gene expression in the

whole tissue, we cannot rule out that increased inflammatory activity in fat or elsewhere could have contributed to the defects in our mice.

Because CCR2 has several ligands, we explored whether the metabolic defects in our CCL2$^{-/-}$ mice were caused by compensatory increases in other chemokines that act at CCR2 or increases in CCR2 itself. We did not observe any increases in serum CCL7 or adipose tissue CCR2 mRNA in CCL2$^{-/-}$ mice. In fact, serum CCL7 was undetectable in these animals. In agreement, a previous study in CCL2$^{-/-}$ mice reported 50% decreases in kidney levels of CCL7 and CCL2 mRNA (21). The decrease in CCL7 expression does not appear to be attributable to inadvertent targeting of CCL7 during the generation of CCL2$^{-/-}$ mice (18). Thus, the worsened metabolic control in CCL2$^{-/-}$ mice was not likely caused by compensatory increases in CCL7 and other CCR2 ligands or by enhanced sensitivity to these ligands because of increased CCR2 expression.

In addition to examining the role of CCL2 in obesity-associated macrophage recruitment, we examined the contributions of different adipose tissue cell populations to the inflammatory activity of adipose tissue. The available data suggest that macrophages are the primary source of adipose tissue inflammatory cytokines, such as TNF-α

and iNOS (12). In mice on standard diet, we found that TNF-α, IL-1β, and IL-10 mRNA were most prominently expressed in the macrophage fraction, suggesting that macrophages may be the main source of these cytokines. Conversely, SOCS-3 mRNA was highest in adipocytes, whereas IL-6 mRNA was lowest in macrophages. Obesity increased IL-1β mRNA in adipocytes and IL-10 and -1β mRNA in nonmacrophage stromal vascular cells. TNF-α and IL-10 mRNA were increased in adipocytes, but it is unclear whether this was caused by the presence of residual macrophages. IL-6, SOCS-3, and HIF-1α mRNA were increased in some populations but decreased in others. Overall, our data suggest that enhanced inflammatory activity in adipose tissue is caused not only by increased macrophage infiltration, but also by inflammatory activation of adipose tissue cells.

In summary, we report that absence of CCL2 does not attenuate obesity-associated macrophage recruitment into adipose tissue or improve metabolic function. In this study, CCL2 absence appeared to even slightly worsen obesity and cause mild metabolic dysregulation, even in mice fed low-fat diets, indicating that CCL2 has effects on metabolism that are independent of its macrophage-recruiting capabilities. Though CCL2 is an important macrophage-recruiting factor in several inflammatory conditions (18–21), our data and the previous reports showing little or no effect of CCL2 or CCR2 absence to reduce adipose tissue macrophages in obesity (25,26,30) lead us to conclude that CCL2 is not critical for obesity-associated macrophage recruitment to adipose tissue. The dominant factor for drawing macrophages into adipose tissue in obesity therefore remains to be identified.

REFERENCES

1. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 289:76–79, 2003
2. Hubert HB, Feinleib M, McNamara PM, Castelli WP: Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation* 67:968–977, 1983
3. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
4. Lyon CJ, Law RE, Hsueh WA: Minireview: adiposity, inflammation, and atherogenesis. *Endocrinology* 144:2195–2200, 2003
5. Sjoholm A, Nystrom T: Endothelial inflammation in insulin resistance. *Lancet* 365:610–612, 2005
6. Wellen KE, Hotamisligil GS: Inflammation, stress, and diabetes. *J Clin Invest* 115:1111–1119, 2005
7. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993
8. Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B: Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* 85:3338–3342, 2000
9. Porreca E, Di Febbo C, Vitacolonna E, Baccante G, Di Castelnuovo A, Angelini A, Febo F, Di Nisio M, Cuccurullo F: Transforming growth factor-beta1 levels in hypertensive patients: association with body mass index and leptin. *Am J Hypertens* 15:759–765, 2002
10. Perreault M, Marette A: Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 7:1138–1143, 2001
11. Bastard JP, Maachi M, Van Nhieu JT, Jardel C, Bruckert E, Grimaldi A, Robert JJ, Capeau J, Hainque B: Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J Clin Endocrinol Metab* 87:2084–2089, 2002
12. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003
13. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clement K: Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54:2277–2286, 2005
14. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Greenberg AS, Obin MS: Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46:2347–2355, 2005
15. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003
16. Cousin B, Andre M, Casteilla L, Penicaud L: Altered macrophage-like functions of preadipocytes in inflammation and genetic obesity. *J Cell Physiol* 186:380–386, 2001
17. Gunn MD, Nelken NA, Liao X, Williams LT: Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *J Immunol* 158:376–383, 1997
18. Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, Rollins BJ: Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 187:601–608, 1998
19. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ: Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2:275–281, 1998
20. Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM: Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J Exp Med* 193:713–726, 2001
21. Tesch GH, Schwarting A, Kinoshita K, Lan HY, Rollins BJ, Kelley VR: Monocyte chemoattractant protein-1 promotes macrophage-mediated tubular injury, but not glomerular injury, in nephrotoxic serum nephritis. *J Clin Invest* 103:73–80, 1999
22. Christiansen T, Richelsen B, Bruun JM: Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes* (Lond) 29:146–150, 2005
23. Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, Kotani H: Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. *J Biol Chem* 278:46654–46660, 2003
24. Sartipy P, Loskutoff DJ: Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 100:7265–7270, 2003
25. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M: MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116:1494–1505, 2006
26. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW Jr: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116:115–124, 2006
27. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T: Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 281:26602–26614, 2006
28. McKnight AJ, Macfarlane AJ, Dri P, Turley L, Willis AC, Gordon S: Molecular cloning of F4/80, a murine macrophage-restricted cell surface

glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. *J Biol Chem* 271:486–489, 1996

29. Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L, Penicaud L: A role for preadipocytes as macrophage-like cells. *FASEB J* 13:305–312, 1999

30. Chen A, Mumick S, Zhang C, Lamb J, Dai H, Weingarth D, Mudgett J, Chen H, Macneil DJ, Reitman ML, Qian S: Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res* 13:1311–1320, 2005

31. Low QE, Drugea IA, Duffner LA, Quinn DG, Cook DN, Rollins BJ, Kovacs EJ, DiPietro LA: Wound healing in MIP-1alpha(-/-) and MCP-1(-/-) mice. *Am J Pathol* 159:457–463, 2001

32. Panoskaltsis-Mortari A, Hermanson JR, Taras E, Wangensteen OD, Charo IF, Rollins BJ, Blazar BR: Post-BMT lung injury occurs independently of the expression of CCL2 or its receptor, CCR2, on host cells. *Am J Physiol Lung Cell Mol Physiol* 286:L284–L292, 2004

33. Combadiere C, Ahuja SK, Van Damme J, Tiffany HL, Gao JL, Murphy PM: Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptors 1 and 2B. *J Biol Chem* 270:29671–29675, 1995

34. Gong X, Gong W, Kuhns DB, Ben Baruch A, Howard OM, Wang JM: Monocyte chemotactic protein-2 (MCP-2) uses CCR1 and CCR2B as its functional receptors. *J Biol Chem* 272:11682–11685, 1997

35. Sarafi MN, Garcia-Zepeda EA, MacLean JA, Charo IF, Luster AD: Murine monocyte chemoattractant protein (MCP)-5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. *J Exp Med* 185:99–109, 1997

36. Garcia-Zepeda EA, Combadiere C, Rothenberg ME, Sarafi MN, Lavigne F, Hamid Q, Murphy PM, Luster AD: Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *J Immunol* 157:5613–5626, 1996

37. Gerhardt CC, Romero IA, Cancello R, Camoin L, Strosberg AD: Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Mol Cell Endocrinol* 175:81–92, 2001

38. Matsukawa A, Hogaboam CM, Lukacs NW, Lincoln PM, Strieter RM, Kunkel SL: Endogenous MCP-1 influences systemic cytokine balance in a murine model of acute septic peritonitis. *Exp Mol Pathol* 68:77–84, 2000

39. Zisman DA, Kunkel SL, Strieter RM, Tsai WC, Bucknell K, Wilkowski J, Standiford TJ: MCP-1 protects mice in lethal endotoxemia. *J Clin Invest* 99:2832–2836, 1997