Monocyte Chemoattractant Protein-1 Deficiency Fails to Restrain Macrophage Infiltration Into Adipose Tissue

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OBJECTIVE—Monocyte chemoattractant protein-1 (MCP-1), a CC-motif chemokine, has been proposed to play critical roles in insulin resistance and recruitment of monocytes into adipose tissue. We hypothesized that the absence of MCP-1 would improve the former and diminish the latter.

RESEARCH DESIGN AND METHODS—We investigated these two hypotheses by quantifying glucose metabolism and the accumulation of macrophages in adipose tissue of control and MCP-1–deficient (*Mcp1*$^{-/-}$) mice after feeding the animals a high-fat diet for 10 or 16 weeks.

RESULTS—We first established that the two strains were in the same genetic background and that macrophage recruitment into inflamed peritoneum was markedly reduced in the MCP-1–deficient animals. In striking contrast, independent studies at two different facilities at either an early or late time point failed to detect any impairment in macrophage accumulation in adipose tissue of fat-fed *Mcp1*$^{-/-}$ mice. Immunoblot analysis revealed higher levels of Mac2, a macrophage-specific protein, in multiple fat depots of *Mcp1*$^{-/-}$ mice fed a high-fat diet. These mice also had significantly more adipose tissue than control mice, but their glucose metabolism was similar.

CONCLUSIONS—Our observations suggest that MCP-1 does not play a prominent a role in promoting macrophage recruitment into adipose tissue or in systemic insulin resistance. ***Diabetes*** **57:1254–1261, 2008**

Obesity, an underlying feature of metabolic diseases, affects >30% of adults in the U.S. (1). Moreover, visceral obesity associates with chronic inflammation and is a major risk factor for diabetes and the metabolic syndrome, which is a constellation of insulin resistance, hypertension, and lipid abnormalities that greatly increases the risk for atherosclerosis (2–5). Adipose tissue itself may promote inflammation and insulin resistance by secreting cytokines, such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) (6,7). Because recent studies demonstrated that macrophages accumulate in adipose tissue of obese mice and humans (8–10), these cells might play a previously unrecognized role in obesity-induced inflammatory changes. Importantly, recent studies of mice deficient in inflammatory proteins strongly support the proposal that macrophages make a major contribution to obesity, systemic inflammation, and insulin resistance (11–13).

Monocyte chemoattractant protein-1 (MCP-1; *SCYA2*, also known as CC chemokine ligand 2 [CCL2]), a C-C motif chemokine, is important for recruiting macrophage precursors, monocytes, into atherosclerotic tissue and other chronic inflammatory lesions (14–16). A central role in inducing insulin resistance in adipocytes and skeletal muscle cells has also been proposed (17–20). MCP-1 interacts with its receptor, CC chemokine receptor 2 (CCR2), on the surface of monocytes to initiate signaling, which ultimately leads to cell adhesion and tissue infiltration (21,22). In mice and humans, MCP-1 production increases in plasma and adipose tissue in both diet-induced and genetic forms of obesity (10,18,23–25). This suggests that monocytes are recruited to and infiltrate expanding adipose tissue, where they differentiate into macrophages.

Recent studies have produced conflicting results, however (26,27). One study examined CCR2-deficient (*Ccr2*$^{-/-}$) mice fed a high-fat diet (27). These mice gained less weight and showed greater insulin sensitivity than control mice, and their adipose tissue contained fewer macrophages and expressed lower levels of inflammatory markers. Two other studies found that overexpression of MCP-1 in adipose tissue promoted macrophage accumulation and insulin resistance (17,26). Moreover, MCP-1–deficient (*Mcp1*$^{-/-}$) mice fed a high-fat diet accumulated fewer macrophages in their adipose tissue and were more insulin sensitive than control mice (26). Taken together, these observations suggest that MCP-1 is of central importance for promoting both macrophage accumulation in adipose tissue and insulin resistance in mice.

In contrast, Inouye et al. (28) recently detected similar levels of macrophages in adipose tissue of control and *Mcp1*$^{-/-}$ mice fed a high-fat diet. They also found that *Mcp1*$^{-/-}$ mice on that diet were hyperinsulinemic, suggesting that MCP-1 exerts metabolic effects that are independent of the accumulation of macrophages in adipose tissue. Therefore, the precise role of MCP-1 in obesity remains to be determined.

We used *Mcp1*$^{-/-}$ mice to further explore the hypothesis that MCP-1 plays a central role in insulin resistance and monocyte recruitment into adipose tissue. Because of the differing results in previous studies, it was important to document that the control and *Mcp1*$^{-/-}$ mice had the same genetic background and that *Mcp1*$^{-/-}$ mice accumulated fewer macrophages in inflamed tissue. Using animals fed a

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CCL, CC chemokine ligand; CCR2, CC chemokine receptor 2; CX3CL1, CX3C chemokine ligand 1 (fractalkine); ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; MCP, monocyte chemoattractant protein; NMR, nuclear magnetic resonance; PAI-1, plasminogen activator inhibitor-1; SPF, specific pathogen free; TNF-α, tumor necrosis factor-α.

high-fat diet, we collected data from an early time point at one facility and a late time point at another. Our results suggest that macrophages accumulate in adipose tissue in response to diet-induced obesity even in the absence of MCP-1. Because compelling evidence implicates MCP-1 in monocyte recruitment into atherosclerotic lesions, our observations also support the proposal that different pathways promote macrophage accumulation in different types of inflamed tissue.

RESEARCH DESIGN AND METHODS

All studies were approved by the Animal Care and Use Committee of the University of Washington. Male control and *Mcp1*$^{-/-}$ mice (aged 3–5 weeks) in the C57BL/6 genetic background were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were housed two to five per cage in a temperature-controlled room with a 12-h light/dark cycle and given free access to food and water. Two separate studies of diet-induced obesity were performed. In the first, mice were maintained in a specific pathogen–free (SPF) facility and fed either a low- or a high-fat diet for 10 weeks. In the second, mice were maintained in a non-SPF vivarium and fed the diets for 16 weeks. Siblings derived from separate colonies of control and *Mcp1*$^{-/-}$ mice bred at the University of Washington (founder mice obtained from The Jackson Laboratories) were used for the first study, and The Jackson Laboratories mice were used in the second. Food was removed 4 h before blood was collected from the retroorbital sinus into tubes containing EDTA. The genetic backgrounds of the control and *Mcp1*$^{-/-}$ mice were determined by genome scanning, using 150 polymorphic markers that differentiate the C57BL/6 and 129Sv genomes (The Jackson Laboratories).

Diets. Mice were fed a pelleted, low-fat diet containing 4.5% fat by weight (LabDiet 5053; PMI Nutrition International) until they were 6–8 weeks of age. They were then maintained on the low-fat diet or fed a high-fat diet containing 35% fat by weight (D12492; Research Diets, New Brunswick, NJ). The animals were weighed weekly. Food intake was determined by weighing food daily for 3 to 5 days after 5 weeks of feeding.

Body composition. Body composition was determined by nuclear magnetic resonance (NMR) by the Rodent Energy Metabolism and Body Composition Core (University of Washington) (Whole Body Composition Analyzer; Echo Medical Systems, Houston, TX).

Biochemical assays. Plasma glucose (Pointe Scientific), cholesterol (Diagnostic Chemicals Limited), and triglycerides (after removal of free glycerol) (diagnostic kit no. 450032; Roche Molecular Biochemicals) were determined biochemically. Plasma insulin, leptin, adiponectin (Linco, St. Charles, MO), and MCP-1 levels (R&D Diagnostics) were determined by enzyme-linked immunosorbent assay (ELISA). Plasma cytokine levels were measured using a customized LincoPlex kit (Linco).

Hepatic lipids. Triglycerides were measured in lipids extracted from frozen mouse liver (29) and solubilized with Triton X-100 (30).

Peritoneal macrophages. Peritoneal macrophages were harvested by lavage from mice 3 days after intraperitoneal injection of sterile 4% thioglycollate solution (Sigma-Aldrich) (31). Cells were cytospun onto microscope slides, fixed, and stained for quantification.

mRNA analysis. Total RNA and protein were extracted from adipose tissue, using TRIzol reagent (Invitrogen). Quantitative RT-PCR was performed by the Diabetes Endocrinology Research Center Molecular and Genetics Core (University of Washington; Applied Biosystems primers and reagents).

Fractionation of adipose tissue. Briefly, dissected fat depots were rinsed in PBS, minced, and then digested for 1 h at 37°C in Krebs-Ringer bicarbonate (pH 7.4) containing 4% BSA and 1.5 mg/ml type I collagenase (Sigma-Aldrich). Digested tissue was filtered through 250-μm nylon mesh and separated into adipocyte and stromal vascular fractions by centrifugation (32). Total RNA was extracted from the adipocyte fraction as described for mRNA analysis.

Immunoblot analysis. Adipose tissue was homogenized in 50 mmol/l Tris, pH 7.4; 1% Triton X-100; 0.2% SDS; 0.2% sodium deoxycholate; and 1 mmol/l EDTA. After centrifugation (16,000*g* for 10 min), the supernatant was collected, and its protein concentration was determined (BCA kit; Pierce). Protein extract was subjected to SDS-PAGE, transferred to a polyvinylidine fluoride membrane (Bio-Rad Laboratories), and incubated with a macrophage-specific primary antibody (Mac2; Cedarlane, Burlington, Ontario, Canada). Signals were detected using ECL (Pierce) after incubation with the appropriate secondary antibody conjugated with horseradish peroxidase.

Insulin sensitivity and glucose tolerance testing. Insulin sensitivity and glucose tolerance tests were performed after a 6-h fast. Mice were injected intraperitoneally with human insulin (1 unit/kg body wt) or sterile 20% glucose in PBS (2 g/kg body wt). Plasma glucose levels were monitored at specified times after injection (Lifescan One Touch Glucometer).

Immunohistochemistry. Sections of formalin-fixed, paraffin-embedded tissue were incubated overnight at 4°C with the macrophage-specific antibody Mac2 (1:2,000 dilution; Cedarlane). Sections were washed three times in 0.05% Tween-20 in PBS and then incubated with a biotinylated goat anti-rat secondary antibody (Sigma-Aldrich), followed by an avidin-biotin-peroxidase conjugate (ABC Elite; Vector Laboratories, Burlingame, CA) for 30 min and then peroxide and peroxidase substrate. Macrophages were quantified by counting Mac2-positive cells. Adipocyte area was determined from four high-power (×200) fields/animal ($n = 5$ per group), using a digital imaging system (ImagePro Plus; Media Cybernetics, Bethesda, MD). All studies were performed by an observer blinded to animal genotype.

Statistical analysis. The normality of the data was assessed by the Shapiro-Wilk test. For nonparametric data and normally distributed data, differences between groups were assessed with the Mann-Whitney *U* test and Student's *t* test, respectively. For multiple comparisons between groups, ANOVA with Bonferroni's correction was used. $P < 0.05$ was accepted as significant. Statistical analyses were performed using SPSS for Windows (SPSS 11.0, Chicago, IL). Results represent means ± SE.

RESULTS

MCP-1 expression, genetic background, and recruitment of peritoneal macrophages. We first confirmed that the *Mcp1*$^{-/-}$ mice were deficient in MCP-1 protein and that the animals were in the same genetic background as the control C57BL/6J mice. MCP-1 was undetectable at both the mRNA (by quantitative PCR) and protein (by immunoblot analysis) level in multiple tissues of *Mcp1*$^{-/-}$ mice (data not shown). A genome-wide scan of DNA isolated from four different *Mcp1*$^{-/-}$ mice indicated a genetic background that was >99% C57BL/6.

In response to the high-fat diet, MCP-1 mRNA levels increased threefold in epididymal adipose tissue of the control mice (Fig. 1*A*; $P = 0.05$), and there was a nonsignificant trend toward higher levels of CCR2 expression in control mice but not in *Mcp1*$^{-/-}$ (Fig. 1*B*). Circulating MCP-1 levels and levels of MCP-1 and CCR2 in epididymal adipose tissue have been reported to increase in response to weight gain and a high-fat diet (9). In control mice, plasma MCP-1 levels exhibited a nonsignificant increase on the high-fat diet (Fig. 1*C*). As expected, MCP-1 was not detected in plasma from *Mcp1*$^{-/-}$ mice on either diet.

Compared with control mice, *Mcp1*$^{-/-}$ mice recruit fewer macrophages to the peritoneum in response to an intraperitoneal injection of thioglycollate (31). We confirmed this observation (Fig. 1*D*). MCP-1 deficiency reduced the total number of inflammatory cells recruited to the peritoneum by 26%, primarily by reducing the number of macrophages by 47%. This reduction in macrophage number was less than originally described (31), perhaps because the earlier studies used F1 mice in a mixed genetic background (129Sv/JxC57BL/6). Collectively, these observations indicate that the mice used in our current studies were deficient in MCP-1, were in the C57BL/6 genetic background, and were less able than control mice to recruit monocytes into an inflamed tissue.

Weight gain and body composition. To determine whether MCP-1 contributes to obesity and macrophage accumulation in adipose tissue, we monitored the weight of control and *Mcp1*$^{-/-}$ mice in two different animal facilities (studies 1 and 2). Mice were fed either a high-fat diet (35% by weight; 60% of calories) or a low-fat diet (4.5% by weight; 12% of calories) for 10 weeks (study 1; $n = 6–7$ mice) or 16 weeks (study 2; $n = 10$ mice), beginning at 6–8 weeks of age. All of the mice appeared healthy and consumed similar quantities of food. All of the mice fed the low-fat diet developed similar body weights (data not

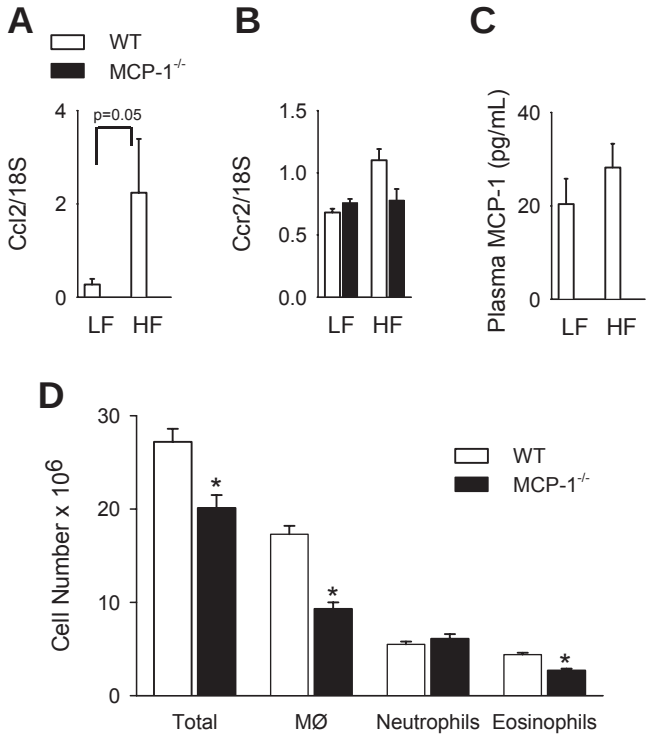

FIG. 1. MCP-1 expression and recruitment of peritoneal macrophages in *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. *A* and *B*: mRNA levels of MCP-1 (CCL2) and CCR2 in epididymal adipose tissue from control (WT) and *Mcp1*$^{-/-}$ mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks were determined by quantitative RT-PCR ($n = 3$). Levels were normalized to 18S expression levels. *C*: Circulating MCP-1 was quantified by ELISA ($n = 3$–7), using plasma from mice fed the diets for 10 weeks. *D*: Peritoneal inflammatory cells elicited by thioglycollate were quantified by immunohistochemistry ($n = 4$–5). *$P < 0.05$, control vs. *Mcp1*$^{-/-}$ cells by Student's *t* test. MØ, macrophages.

shown). In both studies, *Mcp1*$^{-/-}$ mice fed the high-fat diet gained slightly more weight than control mice (Fig. 2*A*).

We analyzed body composition in study 1 by performing NMR on mice 4–6 weeks after the diets were initiated. We chose subsets of mice of similar body weight so that any differences in body composition could be attributed solely to MCP-1 status. On the low-fat diet, *Mcp1*$^{-/-}$ and control mice did not differ in fat mass or lean mass (Fig. 2*B*). However, on the high-fat diet, despite having similar body weights as control mice, *Mcp1*$^{-/-}$ mice had significantly more fat mass ($P = 0.008$) and less lean mass ($P = 0.021$; Fig. 2*B*; $n = 3$–5 mice).

At the completion of the studies, adipose depots were removed from the mice and weighed (Fig. 2*C*). Adipose depot mass increased in both strains on the high-fat diet. Every depot of fat isolated from *Mcp1*$^{-/-}$ mice weighed more than the corresponding one from control mice on either the low-fat or high-fat diet; this difference reached significance in perirenal fat isolated from *Mcp1*$^{-/-}$ mice fed the high-fat diet ($P = 0.035$).

Accumulation of macrophages in adipose tissue. We quantified macrophages in adipose tissue by immunohistochemistry and by immunoblotting tissue proteins separated by SDS-PAGE. After 10 weeks on the high-fat diet, epididymal fat from *Mcp1*$^{-/-}$ and control mice had similar percentages of cells that reacted with Mac2, a marker specific for mature macrophages (Fig. 3*A*; study 1). After 16 weeks, however, *Mcp1*$^{-/-}$ mice on the high-fat diet had significantly more immunoreactive macrophages in their epididymal adipose tissue than did control mice (Fig. 3*B*).

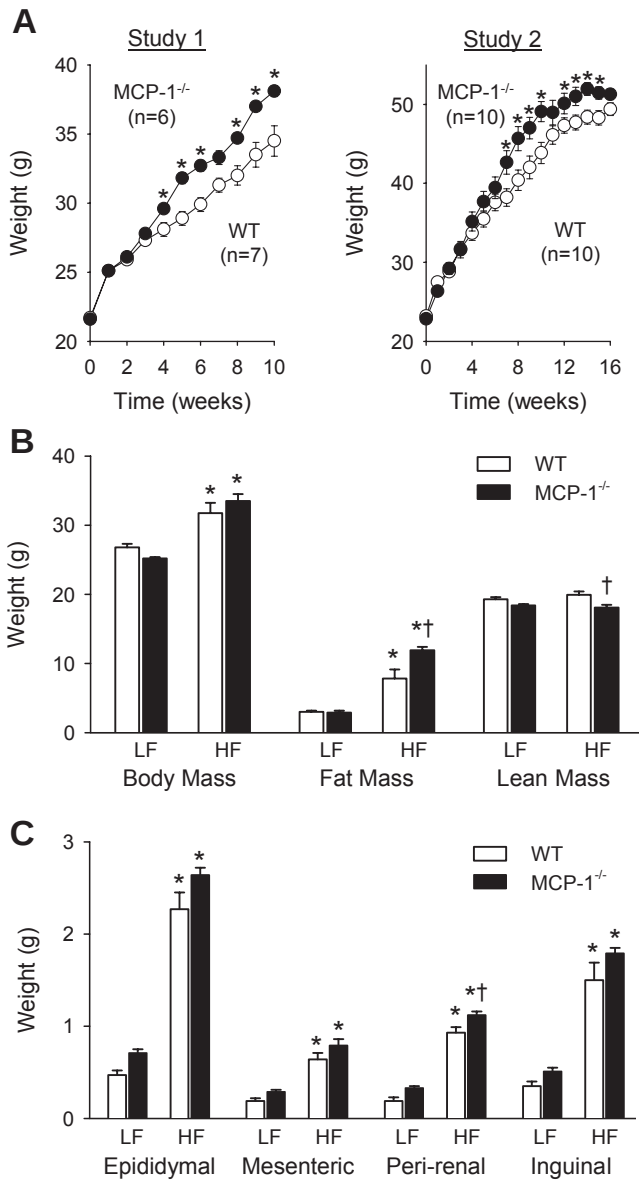

FIG. 2. Body weight and body composition of *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. *A*: Control (WT; ○) and *Mcp1*$^{-/-}$ (●) male mice were fed a low- or high-fat diet for either 10 weeks (study 1) or 16 weeks (study 2). The two strains gained similar amounts of weight on the low-fat diet (data not shown). *B*: Body composition was determined in individual control ($n = 4$ per diet) and *Mcp1*$^{-/-}$ ($n = 5$ per diet) mice by NMR after the low-fat (LF) or high-fat (HF) diet was provided for 6 weeks. *C*: Fat pads were weighed immediately after dissection of control ($n = 8$ low-fat fed, $n = 5$ high-fat fed) and *Mcp1*$^{-/-}$ mice ($n = 6$ low-fat fed, $n = 7$ high-fat fed) fed the diets for 10 weeks. Results represent means ± SE. *$P < 0.05$, low-fat vs. high-fat diet. †$P < 0.05$, control vs. *Mcp1*$^{-/-}$ mice by ANOVA.

Immunoblot analysis of epididymal, inguinal, mesenteric, and perirenal fat pads also detected higher levels of Mac2 in the *Mcp1*$^{-/-}$ mice fed the high-fat diet for 10 weeks (Fig. 3*C*; $n = 3$).

We quantified mRNA levels for two macrophage proteins (F4/80 and CD68) in the epididymal, mesenteric, perirenal, and inguinal fat pads of *Mcp1*$^{-/-}$ and control mice fed the high-fat diet for 10 weeks. In both strains, F4/80 and CD68 levels in epididymal, mesenteric, and inguinal tissue were approximately twice as high on the high-fat diet as on the low-fat diet (Fig. 3*D*). Moreover, perirenal levels of CD68 were higher in the fat-fed

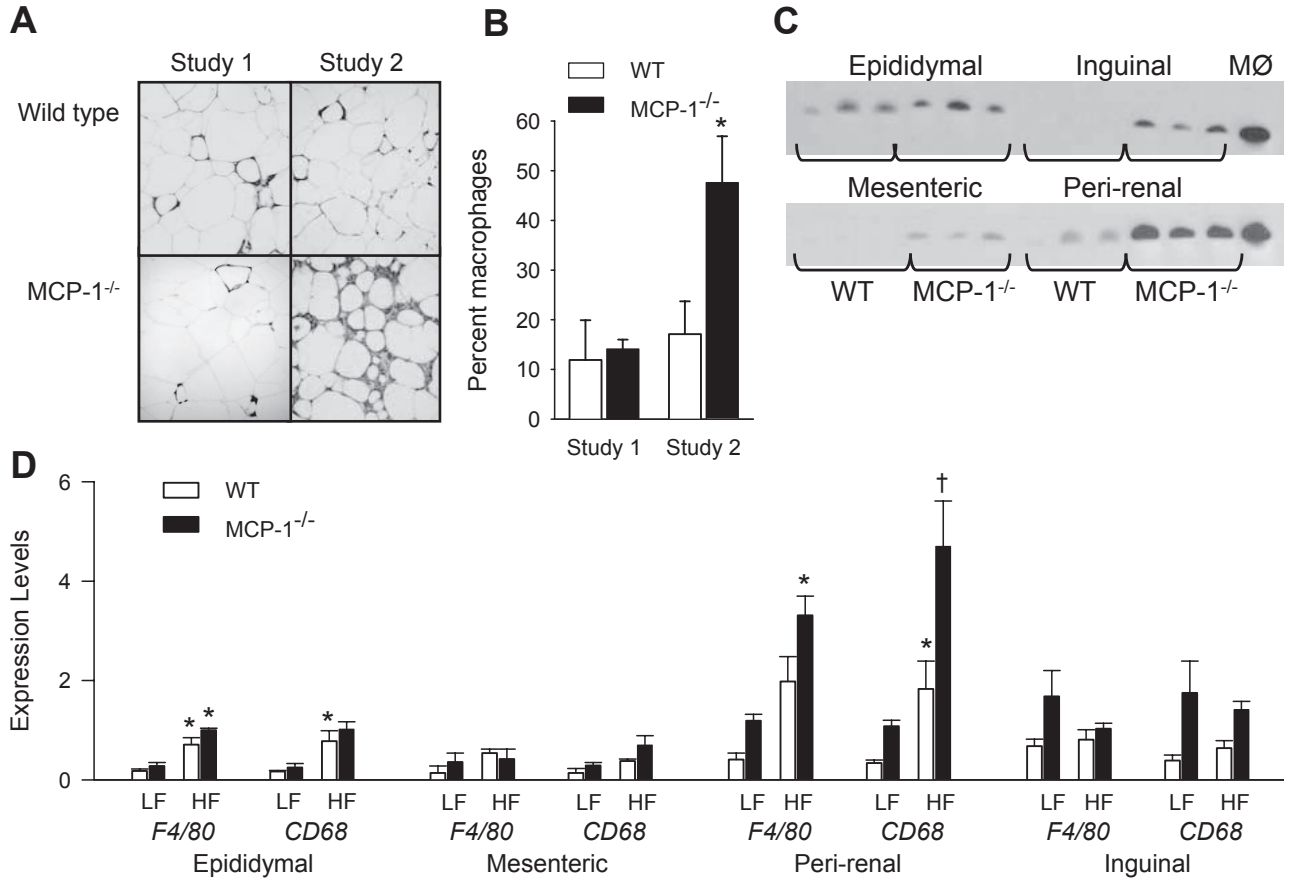

FIG. 3. Macrophage accumulation in adipose tissue of *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. *A* and *B*: Macrophage accumulation in epididymal adipose tissue of wild type (WT; n = 4 and 5 per diet group, studies 1 and 2) and *Mcp1*$^{-/-}$ (n = 6 and 5, studies 1 and 2) mice was quantified after immunohistochemical staining with the macrophage-specific antibody Mac2. *C*: Immunoblot analysis (using Mac2) of adipose tissue proteins from mice fed the high-fat diet for 10 weeks. The proteins were separated by SDS-PAGE. Elicited peritoneal macrophages (MØ) were used as a positive control. *D*: Quantitative RT-PCR analysis of macrophage markers F4/80 and CD68 in adipose tissues from mice fed the high-fat diet for 10 weeks. Levels were normalized to 18S expression levels. *P < 0.05, high-fat vs. low-fat diet. †P < 0.05, high-fat–fed *Mcp1*$^{-/-}$ vs. control mice.

Mcp1$^{-/-}$ mice than in the control mice. Because recent studies suggest that a high-fat diet promotes the accumulation of CD11c^{+} macrophages in adipose tissue (33), we quantified mRNA levels for this macrophage/dendritic cell marker in adipose tissue. CD11c levels rose to a similar extent in both the control and *Mcp1*$^{-/-}$ mice fed the high-fat diet (data not shown). Taken together, these observations strongly suggest that MCP-1 is not required for monocyte recruitment into adipose tissue in response to a high-fat diet. Both immunohistochemical and immunoblot analyses suggested that MCP-1 deficiency might cause more macrophages to accumulate in adipose tissue after 16 weeks on a high-fat diet.

Adipocyte size and adipokine expression. Adipocyte size was measured histochemically in epididymal adipose samples of mice fed the high-fat diet. In study 1 (10 weeks), the two strains had adipocytes of similar size. In study 2 (16 weeks), the *Mcp1*$^{-/-}$ mice had significantly larger adipocytes than the control mice (Fig. 4*A*). These findings suggest that MCP-1 may help restrict lipid accumulation in adipocytes if a high-fat diet persists.

To determine whether MCP-1 plays any role in adipokine secretion and expression, we measured circulating levels of leptin and adiponectin. Circulating leptin levels increased 8- to 10-fold in both strains in response to the high-fat diet (Fig. 4*B*), but there was little change in adiponectin levels. Previous studies have demonstrated that obesity and insulin resistance are not always associated with low levels of adiponectin in mice (34).

Expression levels of leptin mRNA in epididymal adipose tissue mirrored circulating leptin levels, increasing ~threefold when both strains were fed the high-fat diet for 10 weeks (Fig. 4*C*). There was a trend toward lower levels of adiponectin expression in control mice fed the high-fat diet, but adiponectin expression did not respond to diet in the *Mcp1*$^{-/-}$ mice (Fig. 4*C*). Thus, leptin expression in adipose tissue and circulating levels of leptin increased in response to diet-induced weight gain in both strains of mice, whereas circulating adiponectin levels were unchanged, despite a possible reduction in mRNA levels.

Cytokine levels. Plasminogen activator inhibitor-1 (PAI-1) was readily detected in plasma from low-fat–fed control and *Mcp1*$^{-/-}$ mice (464 ± 94 and 554 ± 82 pg/ml, respectively), and the levels doubled when the strains were fed the high-fat diet (1,074 ± 125 and 1,378 ± 280 pg/ml, respectively). In contrast, IL-6 and TNF-α were undetectable in plasma from the two strains of mice, regardless of the diet's fat content. We also found no differences in expression levels of TNF-α in epididymal tissue from the mice (Fig. 4*D*). These findings appear to rule out major differences in levels of systemic inflammation in control and *Mcp1*$^{-/-}$ mice fed the two different diets.

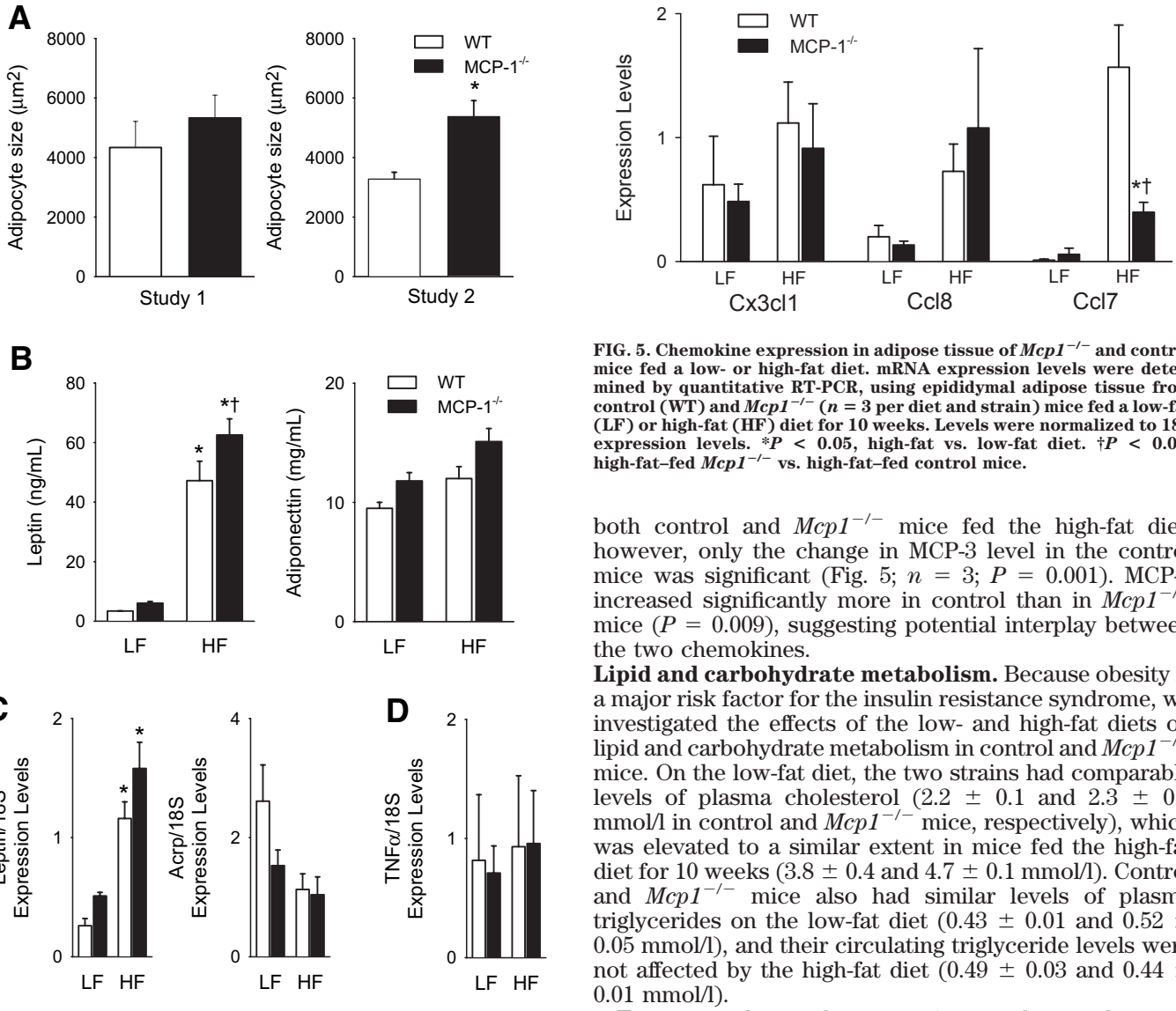

FIG. 4. Adipocyte size and adipokine secretion and expression in *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. *A*: The cross-sectional area of adipocytes was determined in four high-power fields in epididymal adipose tissue sections from control (WT) and *Mcp1*$^{-/-}$ mice (n = 5 per group) fed a low-fat (LF) or high-fat (HF) diet for 10 or 16 weeks. *P < 0.05, high-fat–fed *Mcp1*$^{-/-}$ vs. control mice. *B*: Circulating leptin and adiponectin levels were determined by ELISA in plasma from mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks. *P < 0.05, high-fat vs. low-fat diet. †P < 0.05, high-fat–fed *Mcp1*$^{-/-}$ vs. high-fat–fed control (WT) mice. *C*: Quantitative RT-PCR was used to determine levels of leptin and adiponectin mRNA in the adipocyte fraction of epididymal adipose tissue from control (WT; n = 3 and 5, low- and high-fat diets) and *Mcp1*$^{-/-}$ (n = 6 per diet) mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks. Levels were normalized to 18S expression levels. *P < 0.05, high-fat vs. low-fat diet. *D*: TNF-α expression levels in epididymal adipose tissue from WT (n = 3 per diet) and *Mcp1*$^{-/-}$ (n = 3 per diet) mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks were determined using quantitative RT-PCR. Levels were normalized to 18S expression levels.

Expression of other chemokines in adipose tissue. To determine whether other chemokines and their receptors might influence the accumulation of macrophages in adipose tissue, we quantified expression levels of MCP-2 (CCL8), MCP-3 (CCL7), and fractalkine (CX_3C chemokine ligand 1 [CX_3CL1]) in epididymal adipose tissue harvested from mice fed the high-fat diet for 10 weeks. Expression levels of all three chemokines tended to be elevated in both control and *Mcp1*$^{-/-}$ mice fed the high-fat diet; however, only the change in MCP-3 level in the control mice was significant (Fig. 5; n = 3; P = 0.001). MCP-3 increased significantly more in control than in *Mcp1*$^{-/-}$ mice (P = 0.009), suggesting potential interplay between the two chemokines.

FIG. 5. Chemokine expression in adipose tissue of *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. mRNA expression levels were determined by quantitative RT-PCR, using epididymal adipose tissue from control (WT) and *Mcp1*$^{-/-}$ (n = 3 per diet and strain) mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks. Levels were normalized to 18S expression levels. *P < 0.05, high-fat vs. low-fat diet. †P < 0.05, high-fat–fed *Mcp1*$^{-/-}$ vs. high-fat–fed control mice.

Lipid and carbohydrate metabolism. Because obesity is a major risk factor for the insulin resistance syndrome, we investigated the effects of the low- and high-fat diets on lipid and carbohydrate metabolism in control and *Mcp1*$^{-/-}$ mice. On the low-fat diet, the two strains had comparable levels of plasma cholesterol (2.2 ± 0.1 and 2.3 ± 0.1 mmol/l in control and *Mcp1*$^{-/-}$ mice, respectively), which was elevated to a similar extent in mice fed the high-fat diet for 10 weeks (3.8 ± 0.4 and 4.7 ± 0.1 mmol/l). Control and *Mcp1*$^{-/-}$ mice also had similar levels of plasma triglycerides on the low-fat diet (0.43 ± 0.01 and 0.52 ± 0.05 mmol/l), and their circulating triglyceride levels were not affected by the high-fat diet (0.49 ± 0.03 and 0.44 ± 0.01 mmol/l).

To assess glucose homeostasis, we chose subsets of mice of similar body weight so that any differences could be attributed solely to MCP-1 status. The weights of wild-type and *Mcp1*$^{-/-}$ mice used for the study were 27.4 ± 0.8 and 27.9 ± 0.2 g, respectively, on the low-fat diet and 36.9 ± 0.6 and 37.7 ± 0.6 g, respectively, on the high-fat diet. Low-fat–fed control and *Mcp1*$^{-/-}$ mice had comparable levels of fasting plasma insulin and glucose; those levels became elevated to a similar extent when both strains were fed the high-fat diet for 10 weeks (Fig. 6*A* and *B*). The insulin-to-glucose ratio (a measure of insulin resistance [35]) increased similarly and significantly in mice fed the high-fat diet (Fig. 6*C*). As expected, glucose disposal was impaired in high-fat–fed mice of either strain compared with low-fat–fed mice (data not shown). However, the two strains exhibited similar glucose tolerance and insulin sensitivity on the high-fat diet (Fig. 6*D* and *E*).

Hepatic steatosis associates with insulin resistance syndrome and hepatic insulin resistance (36,37). Therefore, we determined whether MCP-1 deficiency affects hepatic triglyceride content. The latter increased in both strains in response to the high-fat diet, but no differences were found between control and *Mcp1*$^{-/-}$ mice fed the low- or high-fat diet for 10 weeks (low fat, 13.2 ± 2.8 and 10.1 ± 1.1 mg/g; high fat, 28.0 ± 2.5 and 28.7 ± 3.2 mg/g for

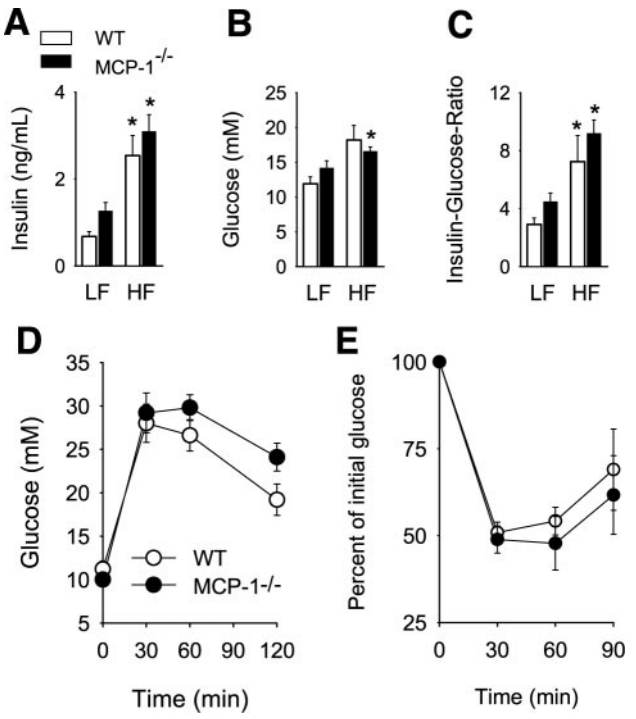

FIG. 6. Glucose homeostasis in *Mcp1*$^{-/-}$ and control mice fed a low- or high-fat diet. *A–C*: Insulin levels were determined by ELISA, and glucose levels were determined biochemically in control (WT; $n = 4–7$ per group) and *Mcp1*$^{-/-}$ ($n = 6–7$ per group) mice fed a low-fat (LF) or high-fat (HF) diet for 10 weeks. The insulin-to-glucose ratio was calculated {[insulin (ng/ml)/glucose (mg/dl)] × 1,000} for $n = 5–8$ mice in each strain and diet group. *D* and *E*: Glucose ($n = 3–7$ per strain) and insulin tolerance tests ($n = 5–6$ per strain) for control (WT; ○) and *Mcp1*$^{-/-}$ (●) mice fed the high-fat diet. On the low-fat diet, the two strains did not differ on either test (data not shown).

control and *Mcp1*$^{-/-}$ mice, respectively). These findings are consistent with the cytokine results, which appeared to rule out major differences in levels of systemic inflammation in control and *Mcp1*$^{-/-}$ mice fed the two different diets. Taken together, these data indicate that MCP-1 is unlikely to directly influence glucose or lipid homeostasis or hepatic steatosis in mice. Overall (Table 1), our data suggest that diet-induced macrophage accumulation in adipose tissue and metabolic changes were similar in both strains.

DISCUSSION

Many lines of evidence indicate that MCP-1 recruits monocytes into atherosclerotic lesions and the inflamed peritoneum of mice, where the cells differentiate into macrophages (14–16). The observation that macrophages accumulate in adipose tissue of obese mice and humans (9,10) coupled with the finding that obese humans have elevated MCP-1 expression in their adipose tissue and increased circulating levels of MCP-1 (24,38,39) led to the proposal that MCP-1 might promote macrophage accumulation in adipose tissue and enhance diet-induced obesity (40,41). Studies from two groups using male mice deficient in CCR2 or MCP-1 appeared to support this suggestion (26,27), although the macrophage content of adipose tissue changed only modestly. In contrast, we found no evidence that macrophage accumulation is impaired in adipose tissue of male *Mcp1*$^{-/-}$ mice fed a high-fat diet for either 10 or 16 weeks. Immunoblot analysis demonstrated higher levels of Mac2, a macrophage-specific protein, in epididymal, mesenteric, perirenal, and inguinal fat pads harvested from *Mcp1*$^{-/-}$ mice after 10 weeks, suggesting increased macrophage accumulation. Thus, our observations do not support the proposal that MCP-1 is required for macrophage infiltration into adipose tissue in fat-fed mice.

We also found that *Mcp1*$^{-/-}$ mice fed the high-fat diet gained slightly more weight than control mice due to increased expansion of adipose tissue. Their plasma leptin levels were also higher. In contrast, plasma levels of adiponectin were similar in the two strains of animals fed the high-fat diet, raising the possibility that MCP-1 deficiency does not affect insulin sensitivity. Consistent with this proposal, glucose homeostasis (as assessed by fasting glucose and insulin levels as well as by glucose tolerance and insulin sensitivity tests) was not altered by MCP-1 deficiency in a subset of the animals whose weights resembled those of control mice. We also detected similar levels of PAI-1 and TNF-α in plasma of the control and *Mcp1*$^{-/-}$ mice, suggesting that MCP-1 does not promote systemic inflammation in fat-fed mice.

Because of the conflicting conclusions about the ability of MCP-1 to promote macrophage accumulation in adipose tissue, we documented that our mice were truly deficient in MCP-1 at both the mRNA and protein level. Importantly, we also demonstrated that our *Mcp1*$^{-/-}$ mice were less able than control mice to accumulate monocytes in inflamed peritoneum (31). Because a mixed genetic background can confound the interpretation of differences in mouse phenotypes, we also confirmed that the *Mcp1*$^{-/-}$ mice were in the same genetic background as the control mice used for our studies. Our observations strongly support the proposal that the mechanisms underlying the

TABLE 1
Metabolic features of *Mcp1*$^{-/-}$ versus control mice fed a low- or high-fat diet

Parameter	*Mcp1*$^{-/-}$ vs. control mice	
	High-fat diet	Low-fat diet
MCP-1 mRNA and protein	Undetectable	Undetectable
Macrophage recruitment into the peritoneal cavity	Not examined	Decreased
Recruitment of macrophages into adipose tissue	No change (study 1) or increased (study 2)	No change (study 1 and study 2)
CCR2 and MCP-3 mRNA	Decreased	No difference
Weight gain	Increased	No difference
Fat mass	Increased	No difference
Lean mass	Decreased	No difference
Plasma leptin	No difference	No difference
Plasma PAI-1, TNF-α, lipids, and adiponectin	No difference	No difference
Hepatic lipids	No difference	No difference
Glucose tolerance	No difference	No difference

accumulation of macrophages in adipose tissue differ from those that promote the accumulation of macrophages in the peritoneum or atherosclerotic vascular tissue.

Our findings are consistent with recent observations by Inouye et al. (28), who detected similar levels of macrophages in adipose tissue of male control and *Mcp1*$^{-/-}$ mice fed a high-fat diet. Those investigators also found that *Mcp1*$^{-/-}$ mice became hyperinsulinemic on a high-fat diet, suggesting that MCP-1 exerts metabolic effects that are independent of macrophages in adipose tissue. Indeed, MCP-1 impairs insulin signaling in cultured cells (17,18). In contrast, we found little evidence of impaired insulin sensitivity in *Mcp1*$^{-/-}$ mice. Our failure to observe differences in insulin resistance between MCP-1–deficient and control mice might reflect the failure of plasma levels of MCP-1 to increase in the control animals fed the high-fat diet. In future studies, it will clearly be important to examine further the role of MCP-1 in directly mediating insulin resistance in vivo.

Weisberg et al. (27) found that mice deficient in CCR2, the monocyte receptor for MCP-1, were protected from diet-induced obesity, hepatic steatosis, and macrophage infiltration into adipose tissue. However, CCR2 binds other monocyte chemoattractants, including MCP-2 (CCL2) and MCP-3 (CCL7) (22). Thus, it is possible that chemokines distinct from MCP-1 are important in monocyte attraction and macrophage accumulation in the adipose tissue of fat-fed mice. Alternatively, MCP-1 may act through other chemokine receptors in the absence of CCR2.

We investigated the latter possibility by examining expression levels in adipose tissue of the CX_3CL1 (fractalkine) and the C-C chemokines MCP-2 and MCP-3. Although we observed no differences in fractalkine, MCP-2, or MCP-3 levels, there were differences between control and *Mcp1*$^{-/-}$ mice. On the high-fat diet, control mice dramatically increased their MCP-3 expression, whereas the increase was significantly muted in *Mcp1*$^{-/-}$ mice. These findings suggest that MCP-3 may also participate in macrophage accumulation in control mice. Moreover, they suggest that MCP-1 may facilitate MCP-3 expression.

Our results clearly differ from those of Kanda et al. (26), who found that macrophage accumulation in adipose tissue was reduced in male *Mcp1*$^{-/-}$ mice fed a high-fat diet. These investigators also demonstrated that *Mcp1*$^{-/-}$ mice were protected from insulin resistance and hepatic steatosis and that mice engineered to overexpress MCP-1 in adipose tissue exhibited insulin resistance, steatosis, and adipose macrophage accumulation. Similar results were reported by Kamei et al. (17), who used the aP2 promoter to drive MCP-1 expression in fat. In the latter study, there was a 2.5-fold increase in plasma levels of MCP-1 but a modest 25% increase in the macrophage content of adipose tissue. However, it is unclear whether expression of high levels of MCP-1 in adipose tissue under the control of an unnatural promoter is a physiologically relevant model.

A number of other factors might explain the conflicting results in the various studies, including differences in genetic background, sampling time, age, environment, and sex. All studies examining the impact of MCP-1 and CCR2 deficiency on insulin sensitivity and the accumulation of macrophages in adipose tissue have used male mice; thus, sexual dimorphism does not account for the discrepant results. In our studies, we used siblings derived from control and *Mcp1*$^{-/-}$ mice bred at the University of Washington for the first study, whereas The Jackson Laboratories provided the two strains of mice used in our second study. It is thus possible that maternal and perinatal effects of MCP-1 could account in part for the differences observed between our first study and those reported by Kanda et al. (26). However, differences in the genetic backgrounds of the animals are unlikely to explain the differences of either study. Another important factor may be the environment, which is known to have powerful effects on mouse phenotypes. For example, mice housed individually or in groups exhibit clear differences in adiposity and weight loss in response to leptin (42,43).

In summary, our observations (Table 1) do not support the hypothesis that MCP-1 mediates insulin resistance and diet-induced accumulation of macrophages in adipose tissue. Instead, they indicate that the chemokine may restrain macrophage accumulation in this tissue. While these findings do not eliminate the MCP-1/CCR2 axis as a mechanism for monocyte recruitment into adipose tissue, they strongly suggest that alternative pathways are important during diet-induced obesity. Importantly, our observations indicate that the mechanisms underlying the accumulation of macrophages are not the same in adipose tissue as in the inflamed peritoneum or atherosclerotic vascular tissue.

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