CXCR3 Controls T-Cell Accumulation in Fat Inflammation

Viviane Zorzanelli Rocha, Eduardo J. Folco, Cafer Ozdemir, Yuri Sheikine, Thomas Christen, Galina K. Sukhova, Eva H.C. Tang, Marcio Sommer Bittencourt, Raul D. Santos, Andrew D. Luster, David E. Cohen, Peter Libby

Objective—Obesity associates with increased numbers of inflammatory cells in adipose tissue (AT), including T cells, but the mechanism of T-cell recruitment remains unknown. This study tested the hypothesis that the chemokine receptor CXCR3 participates in T-cell accumulation in AT of obese mice and thus in the regulation of local inflammation and systemic metabolism.

Approach and Results—Obese wild-type mice exhibited higher mRNA expression of CXCR3 in peripipidymal AT-derived stromal vascular cells compared with lean mice. We evaluated the function of CXCR3 in AT inflammation in vivo using CXCR3-deficient and wild-type control mice that consumed a high-fat diet. Peripipidymal AT from obese CXCR3-deficient mice contained fewer T cells than obese controls after 8 and 16 weeks on high-fat diet, as assessed by flow cytometry. Obese CXCR3-deficient mice had greater glucose tolerance than obese controls after 8 weeks, but not after 16 weeks. CXCR3-deficient mice fed high-fat diet had reduced mRNA expression of proinflammatory mediators, such as monocyte chemotactant protein-1 and regulated on activation, normal T cell expressed and secreted, and anti-inflammatory genes, such as Foxp3, IL-10, and arginase-1 in peripipidymal AT, compared with obese controls.

Conclusions—These results demonstrate that CXCR3 contributes to T-cell accumulation in peripipidymal AT of obese mice. Our results also suggest that CXCR3 regulates the accumulation of distinct subsets of T cells and that the ratio between these functional subsets across time likely modulates local inflammation and systemic metabolism. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: fats ● inflammation ● obesity

During obesity, adipose tissue (AT) accumulates inflammatory cells, including macrophages and T cells, which interact with endothelial cells and adipocytes in a local inflammatory network. This cellular cross talk augments local production of multiple chemokines and cytokines, such as monocyte chemotactant protein-1 (MCP-1) and tumor necrosis factor-α, key elements in maintaining and propagating the inflammatory response in AT.

Various studies have examined the mechanisms of macrophage accumulation in AT of obese mice. Production of large amounts of MCP-1 by adipocytes in culture and by fat from obese mice suggested that this chemokine participates in the increased local macrophage traffic. Indeed, obese animals with deficiency of MCP-1 or its receptor, CCR2, have fewer macrophages in AT, less local inflammation, and improved insulin sensitivity compared with obese controls. We and others have shown increased T-cell numbers in AT from obese mice, relative to lean controls. Nishimura et al demonstrated that immunologic and genetic depletion of CD8+ T cells lowered macrophage accumulation and AT inflammation and improved systemic insulin resistance. Interferon-γ (IFN-γ), a prototypical cytokine of the Th1 subpopulation and an established orchestrator of the inflammatory response in atherosclerosis and other immune conditions, regulates fat inflammation, suggesting that adaptive immunity participates in the pathophysiology of obesity.

The exact mechanism of lymphocyte accumulation in AT remains unknown. Increased expression of regulated on activation, normal T cell expressed and secreted (RANTES) and its receptor (CCR5) in AT of obese mice and humans suggests that this duo participates in local T-cell migration, a conjecture.

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not yet supported by definitive evidence. Moreover, RANTES recruits not only T cells but also dendritic cells, natural killer cells, mast cells, eosinophils, and basophils to sites of inflammation and infection.\textsuperscript{11}

In contrast to the broad specificity of RANTES, the CXCR3 chemokine ligands—monokine induced by IFN-\(\gamma\) (MIG), IFN-\(\gamma\)–inducible protein 10 (IP-10), and IFN-inducible T-cell \(\alpha\) chemoattractant—selectively induce chemoattraction of T cells.\textsuperscript{12} Overexpression of CXCR3 and its ligands occurs in a wide array of infectious and inflammatory diseases, including atherosclerosis.\textsuperscript{13} Indeed, apolipoprotein E–deficient mice with deletion of either CXCR3 or IP-10 have significantly less atherosclerosis than do control apolipoprotein E–deficient mice.\textsuperscript{14,15} Treatment with a CXCR3 antagonist (NBI-74330) mitigates plaque development in association with reduced accumulation of effector T cells and macrophages in lesions of low-density lipoprotein receptor–deficient mice.\textsuperscript{16} Recently, we showed that the adipocyte-derived mediator adiponectin, which diminishes in the plasma of obese subjects, inhibits the production of CXCR3 ligands by macrophages and reduces T-cell accumulation in atheromata, suggesting a link between hypoadiponectinemia and T-cell accumulation via CXCR3.\textsuperscript{17}

These observations led us to test the hypothesis that CXCR3 participates in T-cell accumulation in AT of obese animals. Our results unveil CXCR3 as a crucial player in this process, demonstrating that it regulates local inflammation and affects systemic metabolic pathways that operate in obesity.

**Figure 1.** Periepididymal adipose tissue–derived stromal vascular cells (SVCs) from obese mice express more CXCR3 than SVCs from lean mice. RNA was extracted from adipose tissue–derived SVCs isolated from lean (low-fat diet, LFD) or obese (high-fat diet, HFD) C57BL/6 mice. The graph represents relative mRNA levels of CXCR3, IFN-\(\gamma\)–inducible protein 10 (IP-10), and monokine induced by IFN-\(\gamma\) (MIG) in the LFD group (gray bars) and HFD group (black bars) after 8 weeks of diet, normalized to GAPDH mRNA expression. Data are expressed relative to LFD. *\(P<0.05\) vs LFD; \(n=5\)/group.

**Figure 2.** Obese CXCR3-deficient mice and controls presented similar body weight after 8 weeks and after 16 weeks of high-fat diet (HFD). CXCR3-deficient mice and C57BL/6J controls were fed ad libitum standard low-fat diet after weaning until 8 weeks of age. Mice were then switched to an HFD and were kept on it for 8 or 16 additional weeks. Body weight of each animal was measured at baseline and at 1, 2, 4, 6, 8, 10, 14, and 16 weeks after starting HFD. Graphs A and B represent the mean body weight of CXCR3-deficient mice (black bars) and their respective controls (gray bars) after 8 and 16 weeks of HFD, respectively.
Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Obese Wild-Type Mice Exhibit Higher CXCR3 Expression in Periepididymal AT Than Lean Wild-Type Mice

Periepididymal AT-derived stromal vascular cells (SVCs) from obese C57BL6 mice had significantly higher levels of CXCR3 mRNA than SVCs from lean controls after 8 weeks of high-fat diet (HFD) or low-fat diet, respectively. Levels of mRNAs encoding the T-cell chemoattractants and the CXCR3 ligands IP-10 and MIG did not differ between the lean and obese animal groups at this time point (Figure 1).

Obese CXCR3-Deficient Mice Accumulate Fewer T Cells in Periepididymal AT Than Obese Wild-Type Mice

CXCR3-deficient mice and C57BL6 controls began receiving HFD at 8 weeks of age. With the exception of the first week of HFD feeding, body weights between the 2 groups were not different (Figure 2).

CXCR3-deficient mice and controls showed no consistent differences in VO₂, VCO₂ production, or RER before or 4 weeks after the initiation of HFD (Figure 1 in the online-only Data Supplement). Physical activity was lower, and a small but statistically significant decrease occurred in food intake among the CXCR3-deficient mice compared with controls on HFD.

After 8 weeks of HFD, both groups of mice had similar mean body weights (Figure 2) but different periepididymal fat weights (not shown). The number of SVCs isolated from the periepididymal AT of obese CXCR3-deficient mice compared with respective controls after 8 weeks of HFD did not differ: $2.41 \times 10^6 \pm 1.4 \times 10^6$ SVCs and $2.76 \times 10^6 \pm 1.2 \times 10^6$ SVCs, respectively ($P=0.5; n=11–13$ in each group). This lack of significant difference persisted even when the cell count was adjusted for body weight or the amount of fat used in the experiment (not shown).

Obese CXCR3-deficient animals contained fewer CD3⁺ T lymphocytes in their periepididymal AT (represented as % of

![Figure 3](http://atvb.ahajournals.org/)

Figure 3. Periepididymal adipose tissue from obese CXCR3-deficient mice contains fewer T cells than obese controls after 8 weeks of high-fat diet (HFD), as detected by flow cytometry. Stromal vascular cells from 1 g of adipose tissue of CXCR3-deficient mice and wild-type control mice fed an HFD for 8 weeks were labeled with conjugated antibodies to CD3, CD4, CD8, B220, F4/80, and CD11c and analyzed by flow cytometry. The graphs represent percentages of gated cells in the CXCR3-deficient group (black bars) and the control group (gray bars). Data are shown as mean±SD. *$P<0.02$; n=9/group. VAT indicates XXX.
16-week HFD-fed CXCR3-deficient mice had significantly fewer CD4+ and CD8+ T-cell subsets were also decreased in the same AT depot of obese CXCR3-deficient mice compared with obese wild-type counterparts (1.5±1 versus 2.6±0.7; P<0.02 and 0.8±0.3 versus 1.9±0.4; P<0.001, respectively; Figure 3). Proportions of B220+ B cells, F4/80+ macrophages, and CD11c+ dendritic cells did not differ between the 2 groups (Figure 3). Consistent with the flow cytometry results, quantitative immunohistochemistry also showed fewer CD3+ T cells in periepididymal AT from obese CXCR3-deficient mice compared with obese controls (Figure 4). Complete and differential blood counts did not reveal any difference in cell subsets between obese CXCR3-deficient mice and obese wild-type controls in peripheral blood (Table II in the online-only Data Supplement). Likewise, both groups of mice had similar proportions of splenic cells expressing F4/80, CD3, CD4, CD8, and B220 (Figure II in the online-only Data Supplement).

Both groups had similar mean body weight (Figure 2) and periepididymal fat weight (not shown) after 16 weeks of HFD. CXCR3-deficient mice yielded significantly fewer AT-derived SVCs compared with controls: 3.43×10⁶ (±3.4×10⁵) SVCs and 5.30×10⁶ (±1.7×10⁶) SVCs, respectively (P=0.045; n=5–6 in each group). This difference persisted after adjustment of cell count for body weight but was attenuated after correction of the cell count for the weight of fat used in the experiment (not shown).

Similar to the results at the 8-week time point, AT from 16-week HFD-fed CXCR3-deficient mice had significantly fewer CD3+ T cells (7±0.9 versus 13±2.4; P<0.01), including both subsets—CD4+ (4±0.3 versus 10±3.5; P<0.02) and CD8+ (3.4±0.4 versus 8.9±1.8; P<0.001)—compared with control mice on the same diet (Figure 5). Fat of CXCR3-deficient mice had fewer CD25+ cells (panel not shown), both stimulated T cells and T regulatory cells (Tregs), than control mice (3.5±0.44 versus 4.4±0.58; P=0.02). AT from CXCR3-deficient mice had more F4/80+ macrophages than did control mice at the same time point (Figure 5). The proportion of cells positive for CD11c or MHC II did not differ between the 2 groups.

When consuming low-fat diet, the body weight of CXCR3-deficient and wild-type mice showed a small yet statistically significant difference up to 9 weeks (not shown). At the time of harvesting, after ≈20 weeks of low-fat diet, the body weights of the CXCR3-deficient and control groups did not differ, although the periepididymal fat weighed less in the CXCR3-deficient mice than in controls (not shown).

**Obese CXCR3-Deficient Mice Exhibit Decreased Expression of Inflammation-Related Genes in Periepididymal AT Compared With Obese Wild-Type Mice**

CXCR3 deficiency led to a decrease in expression of several genes related to inflammation. After 8 weeks of HFD, obese CXCR3-deficient mice exhibited lower mRNA expression of chemokines such as MCP-1 and RANTES in AT compared with their wild-type counterparts (Figure 6A). Compared with controls, CXCR3-deficient mice also had lower levels of interleukin-10 (IL-10) (Figure 6A), a cytokine with anti-inflammatory functions. Tregs and M2 macrophages can elaborate IL-10 in several inflammatory conditions and seem to exert a metabolic protective effect in obesity. This finding prompted us to measure mRNA levels of the Treg marker Foxp3 (a forkhead family transcription factor) and arginase-1, one of the signature products of M2 macrophages in mice. The expression of these markers did not differ significantly between CXCR3-deficient mice and wild-type controls after 8 weeks of HFD (Figure 6A). After 16 weeks of HFD, CXCR3-deficient mice had significantly reduced expression of IL-10, Foxp3, and arginase-1 mRNAs in periepididymal AT compared with controls (Figure 6B). IFN-γ, a signature of T-helper 1 cells, showed a trend toward decreased mRNA expression at both 8 weeks and 16 weeks of HFD, but the differences did not reach statistical significance (Figure 6). The mRNA expression levels of tumor necrosis factor-α, interleukin-6, IP-10, MIG, and MHC II did not differ between the CXCR3-deficient and control groups at both time points (not shown).

In agreement with the reduced mRNA expression of Foxp3 in the periepididymal fat of CXCR3-deficient mice compared with controls after 16 weeks of diet, immunohistochemistry also showed significantly fewer Foxp3-positive cells in the AT of CXCR3-deficient animals (Figure 7).

Obese CXCR3-deficient mice exhibited improved glucose tolerance and decreased plasma levels of leptin and total cholesterol compared with obese wild-type mice.
Despite having comparable body weights, CXCR3-deficient mice had greater glucose tolerance in response to intraperitoneal glucose load than did control mice after 8 weeks of high-fat diet (HFD) (according to the area under the curve and all the individual time points on the curve) (Figure 8A and 8C). Mice that consumed HFD for 16 weeks showed a similar trend, but the difference was not statistically significant; although there was a difference at baseline and at 20 minutes after glucose loading, all other time points on the glucose tolerance curve did not differ significantly between the groups, resulting in a nonsignificant difference between the areas under the curve (Figure 8B and 8D).

CXCR3-deficient mice had lower concentrations of plasma leptin than their obese wild-type counterparts after both 8 and 16 weeks of HFD (Figure III in the online-only Data Supplement). Similarly, total plasma cholesterol levels, which increased over time in both groups of mice, were lower in CXCR3-deficient mice compared with controls (Figure III in the online-only Data Supplement). Plasma concentrations of adiponectin and insulin did not differ significantly between the 2 groups at either time point (Figure III in the online-only Data Supplement).

Discussion

Despite the increasing recognition of the participation of T lymphocytes in the pathophysiology of obesity, the mechanism by which they accumulate in fat under obese conditions remains unclear. MCP-1 and RANTES, chemokines abundantly expressed in AT of obese mice, can induce migration of several cell types (including T lymphocytes) during inflammatory processes. Indeed, both of these chemokines seem to participate pivotally in macrophage accumulation in AT of obese mice and humans.3,9,18 The finding that T-cell accumulation in AT of obese mice precedes the appearance of macrophages,5,6 however, suggests the operation of a chemokine/receptor system with higher selectivity for T cells than that of RANTES/CCR5 or MCP1/CCR2. Our results demonstrate that CXCR3 participates in T-cell accumulation in AT in the context of obesity. The exclusive ligation of CXCR3 with the T-cell chemoattractants IP-10, MIG, and IFN-inducible T-cell α chemoattractant, and its abundant expression in activated T cells, make this chemokine system an ideal candidate to initiate T-cell recruitment in AT of obese animals and, therefore, to orchestrate fat inflammation.
subtypes—CD4+ and CD8+ cells. This reduction occurred pads, represented by reduced numbers of both T lymphocyte the reduction of the Treg cellular pool in periepididymal fat ing suggests that changes in specific T-cell subsets, such as weeks of HFD, macrophages were more numerous in the CXCR3-deficient mice compared with controls. After 16 degree of selectivity among them and support a crucial role pathways in fat inflammation, these results indicate a high the diversity and potential importance of other chemotactic Figure 6. Obese CXCR3-deficient mice exhibit decreased expression of inflammation-related genes in their periepididymal adipose tissue, compared with obese wild-type mice. mRNA levels of monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), interferon-γ (IFN-γ), ARG1, forkhead family transcription fac- tor (Foxp3), and interleukin-10 (IL-10) in adipose tissue from CXCR3-deficient mice (black bars) and control mice (gray bars) at 8 weeks (A) and at 16 weeks of high-fat diet (HFD; B) were quantified by reverse transcription–quantitative polymerase chain reaction and normalized to GAPDH mRNA expression. Fold change was calculated relative to wild-type controls. *P<0.05 vs wild-type controls; n=6/group.

Our data show a significant increase of CXCR3 mRNA lev- els in periepididymal AT-derived SVCs from obese C57BL6 mice compared with lean controls. Because CXCR3 expression associates with T-cell activation, this finding suggests that obesity enhances accumulation of activated T cells in AT. CXCR3 deficiency in HFD-fed obese mice is associated with significantly fewer CD3+ cells in periepididymal fat pads, represented by reduced numbers of both T lymphocyte subtypes—CD4+ and CD8+ cells. This reduction occurred at both early (8 weeks) and late (16 weeks) time points, with a more marked fall at 16 weeks. Therefore, despite the diversity and potential importance of other chemotactic pathways in fat inflammation, these results indicate a high degree of selectivity among them and support a crucial role of CXCR3 in the accumulation of T cells in AT of obese animals across time. Unlike T-cell numbers, macrophage and dendritic cell numbers were not lower in AT of obese CXCR3-deficient mice compared with controls. After 16 weeks of HFD, macrophages were more numerous in the fat of CXCR3-deficient mice relative to controls. This finding suggests that changes in specific T-cell subsets, such as the reduction of the Treg cellular pool in periepididymal fat of CXCR3-deficient mice compared with controls, impact the local number of macrophages later. Indeed, Treg cells can have suppressive effects on macrophages and T-effector cells. The similar numbers of T cells and other leukocyte populations in the spleen and blood of obese CXCR3-deficient mice and control mice further support a local effect of CXCR3 on T-cell accumulation in AT of obese animals, independent of the circulating cell number.

T lymphocytes display functional heterogeneity, and different T-cell subsets can exert proinflammatory or anti-inflam- matory actions on other lymphocyte subpopulations and other cells of the innate immune system.19 CXCR3 deficiency in the context of obesity is associated with reduction of the mRNA expression levels of RANTES (a product of cytotoxic T lymphocytes) and MCP-1, predominantly secreted by activated macrophages. Conversely, obese CXCR3-deficient mice also had decreased expression of IL-10 (a prototypical anti-inflam- matory cytokine) and Foxp3 (a Treg marker). These results indicate that CXCR3 participates in the accumulation of various T-cell subsets and thus helps define the expression profiles of proinflammatory and anti-inflammatory molecules present in AT of obese mice. CXCR3 deficiency also reduces the expression of arginase-1, a marker of alternatively activated macrophages (M2) induced by Th2 cytokines such as IL-10,20 typically involved in tissue repair and inflammation blockade.21,22 This finding suggests that the CXCR3/IP-10–MIG–IFN-inducible T-cell α chemoattractant chemokine system indirectly regulates macrophage function in AT of obese mice.

CXCR3 deficiency correlated with significant changes in several systemic metabolic variables. Obese CXCR3-deficient mice had reduced levels of plasma leptin and cholesterol. In most atherosclerosis-susceptible mouse strains (eg, C57BL/6), plasma high-density lipoprotein cholesterol levels decrease and total cholesterol levels increase substantially after initiating an HFD. The majority of the increase in
plasma cholesterol derives from increase of very-low-density lipoprotein and low-density lipoprotein fractions. But, considering that in normal mice the high-density lipoprotein fraction carries most of cholesterol and that high-density lipoprotein cholesterol levels fall under HFD, we cannot exclude a contribution of high-density lipoprotein cholesterol decrease among the CXCR3-deficient mice to our findings.

Obese CXCR3-deficient mice also had greater glucose tolerance than their obese wild-type counterparts after 8 weeks of HFD. But differences in glucose tolerance curves between the 2 groups became nonsignificant after 16 weeks of HFD, coinciding with a substantial fall in the expression of anti-inflammatory markers—including IL-10 (also significantly decreased after 8 weeks of HFD), arginase-1, and Foxp3—in the fat tissue of CXCR3-deficient mice compared with controls. In agreement with this finding, the AT of CXCR3-deficient mice had significantly fewer Foxp3-positive cells than did that of controls, as determined by immunohistochemical analysis, suggesting that CXCR3 plays a role in Treg accumulation in AT. Several studies have shown that reduced numbers of anti-inflammatory cells (such as M2 macrophages and Tregs) and their products in AT associate with deterioration of metabolic homeostasis. Feuerer et al demonstrated that AT of obese mice contains fewer Tregs than AT of lean mice, and this diminished pool of cells may cause excessive inflammation and its metabolic consequences in obesity. Moreover, treatment with a CD3-specific antibody reduced the predominance of Th1 cells over Foxp3 cells in obese mice, reversing insulin resistance.

Our findings suggest that CXCR3 deficiency interferes with the accumulation of distinct T-cell subsets with antagonistic functions, and temporal changes in the size of distinct T-cell pools may alter the balance of expression of proinflammatory and anti-inflammatory molecules. The net effect of this duality between proinflammatory and anti-inflammatory forces within the AT likely impacts local and systemic metabolism. Understanding the roles of both the innate and adaptive immune arms in the pathophysiology of obesity may pave the road toward novel therapeutic alternatives against this condition.

Figure 8. Obese CXCR3-deficient mice exhibit improved glucose tolerance compared with obese wild-type mice. Average glucose tolerance curves of CXCR3-deficient mice (black curves) and wild-type control mice (gray curves) after 8 weeks (A) and after 16 weeks (B) of high-fat diet (HFD). Areas under the glucose tolerance curves (AUC) were calculated for each mouse, and the average of each group is represented in C and D. Data are shown as mean±SD; *P<0.01; n=19 to 22/group. GTT indicates XXX.

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Acknowledgments

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Disclosures

None.

References


Significance

Obesity associates with macrophages and T cells in adipose tissue, and these inflammatory cells likely contribute to the metabolic consequences of obesity. Although the mechanisms of macrophage traffic in adipose tissue have undergone extensive exploration, the mechanism of local T-cell accumulation remains unknown.

This study demonstrates that the chemokine receptor CXCR3 contributes importantly to T-cell accumulation in peripancreatic adipose tissue of obese mice. Our results also suggest that CXCR3 mediates the accumulation of distinct subsets of T cells, including T-regulatory cells, and therefore may influence local expression of pro- and anti-inflammatory mediators. The ratio between these functional T-cell subsets across time modulates local inflammation and systemic metabolism.
CXCR3 Controls T-Cell Accumulation in Fat Inflammation
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Materials and Methods

Diet-induced obesity in mice

Male C57BL/6J mice (The Jackson Laboratory) and CXCR3-deficient mice (CXCR3\(^{-/-}\)) in the C57BL/6J background\(^{(1)}\) (kindly provided by Dr. Andrew Luster) were fed *ad libitum* a standard low-fat diet (LFD) (PicoLab Rodent Chow 5053; 13% kcal from fat) after weaning. At 8 weeks of age, mice were switched to a high-fat diet (HFD) (D12108 from Research Diets; 40% kcal from fat, 1.25% cholesterol, 0% cholate) and were kept on this diet for 8 or 16 additional weeks. Lean controls were maintained on the LFD throughout the experiment. Mouse genotyping was performed by PCR on DNA extracted from tails.

On the day of harvesting, mice were weighed, and received anesthesia with 2,2,2-tribromoethanol (2.5 mg/10 g body weight [BW]) and an injection of heparin, both intraperitoneally. Heparinized blood was collected by pericardiocentesis. The animals were then perfused with normal saline by cannulation of the left ventricle with drainage through the right atrium, the appendage of which was amputated. After perfusion, peri-epididymal AT was collected, weighed, and divided into different samples for various analyses (see below).

All experiments involving animals were performed according to a protocol approved by the Standing Committee on Animals of Harvard Medical School.

Analysis of AT-derived stromal vascular cells (SVCs) by flow cytometry

Peri-epididymal fat from diet-induced obese C57BL/6J and CXCR3\(^{-/-}\) mice was enzymatically digested, as described previously\(^{(2)}\). Briefly, approximately 1 g of peri-epididymal fat was minced, digested in PBS containing 2% bovine serum albumin and 250 U/ml collagenase II (Worthington), and incubated at 37° C for 1 hour. The digested tissue was passed through a 70-\(\mu\)m cell strainer, and the flow-through was centrifuged. After aspirating the supernatant and lysing red blood cells with ACK buffer (Gibco) for 2 minutes, the remaining cells (denoted SVCs) were washed with media, counted, and labeled with conjugated
antibodies to F4/80 (Caltag), CD3, CD4, CD8, CD11c, B220, IAIE, and CD25 (BD Pharmingen), or their respective isotype controls, before analysis by a FACScan.

**Analysis of inflammatory cells in AT by immunohistochemistry**
Peri-epididymal fat from diet-induced obese C57BL/6J and CXCR3⁻/⁻ mice was fixed as described previously (3), embedded in paraffin, and stained by the avidin-biotin-peroxidase method. Briefly, after pretreatment with Heat Retrieval Solution (Dako, cat# S1699), 5-mm sections were stained for CD3 (Abcam, cat# ab16669) and then incubated with appropriate biotinylated secondary antibodies, followed by incubation with avidin-biotin complex (Vector) and visualization with 3-amino-9-ethyl carbazole (DAKO). Sections were counterstained with Gill’s hematoxylin solution (Sigma). Positive cells were counted under the microscope in 10 consecutive high-power fields.

The staining for Foxp3 (eBioscience, San Diego, CA) followed treatment with 10mM citrate buffer (pH 6.0) under high-pressure conditions, and all positive nuclei per section were counted.

**Quantification of gene expression by reverse transcription-quantitative PCR (RT-qPCR)**
Total RNA was isolated with RNeasy Lipid Tissue Midi Kit (Qiagen) from up to 500 mg of AT, and equal amounts were reverse-transcribed by Superscript II (Invitrogen), according to the manufacturer’s instructions. We assessed RNA concentration and purity using a Nanodrop 1000 UV spectrophotometer (Thermo Scientific). Quantitative PCR was performed in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). The sequences of the primers used are described in Supplementary Table 1. The mRNA levels of the various genes tested were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as an internal control in all experiments.

**Measurements of cytokines and metabolic parameters**
Adiponectin was measured using a QuantiKine ELISA kit (R&D Systems). Plasma levels of leptin and insulin were also measured by ELISA (Crystal Chem). Total plasma cholesterol and glucose were determined by enzymatic colorimetric methods (Wako).

For glucose tolerance tests, mice were deprived of food for 12 hours and then injected intraperitoneally with glucose (1 mg/g of body weight). Blood from the tail vein was used for glucose determination in a blood glucose meter (OneTouch Ultra, LifeScan) at 0, 20, 40, 60, 90, and 120 minutes after glucose administration.

**Indirect calorimetry, physical activity, and food intake**

Mice were individually housed in metabolic cages under a 12-hour light/dark cycle, with access to diet and tap water ad libitum. The cages were enclosed in a temperature-controlled Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH, USA). Mice were subjected to non-invasive monitoring of gas exchange, physical activity, and food intake (4). Rates (ml/kg/h) of O₂ consumption (VO₂) and CO₂ production (VCO₂) were determined at 11-minute intervals and normalized to body weight. Values of respiratory exchange ratio (RER) were calculated as VO₂/VCO₂. Physical activities were measured by beam breaks detected by light sensors built into the bottom of the cages.

**Peripheral blood cell count**

Peripheral blood was collected at sacrifice in EDTA-coated vials (Sarstedt, Germany), and complete and differential blood counts were performed using a Hemavet 950FS multispecies automated hematology analyzer (Drew Scientific, Inc.).

**Statistical analysis**

Statistical analysis was performed with Excel (Microsoft Office) and GraphPad Prism 4.0 (GraphPad Software) software packages. Means of several variables
(BMI, cell counts, and plasma measurements) were compared between CXCR3-deficient mice and respective controls with the two-tailed, unpaired Student’s t-test.

Calculation of the relative expression ratios of genes in peri-epididymal fat of CXCR3-deficient mice to those of wild-type mice was performed with the Relative Expression Software Tool version 2 (http://rest.gene-quantification.info/) and the Pair Wise Fixed Reallocation Randomization Test, according to the method described by Pfaffl et al. (5) For the analysis of serial data (glucose tolerance test), areas under the curves were compared with the unpaired Student’s t-test. All p values ≤ 0.05 were considered statistically significant.

Supplement Material

Supplemental table I

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<th>Gene</th>
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<tr>
<td>CXCR3</td>
<td>5'-GCCAAGCCATGTACCTTGAG-3'</td>
<td>5'-GGAGAGGTTGCTGTGTTTTCCAG-3'</td>
</tr>
<tr>
<td>IP - 10</td>
<td>5'-GCTGGCGTCACTTTTCTGC-3'</td>
<td>5'-TCTCAGTGGCCTCACTCATC-3'</td>
</tr>
<tr>
<td>MIG</td>
<td>5'-GCCATGCGATCTCCTTATCA-3'</td>
<td>5'-TCCTGAGCTTTGGTGACAAAAAC-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-AGCAGCAAGTGCTCCAATC-3'</td>
<td>5'-GGGAAGGCTATACAGGGTC-3'</td>
</tr>
<tr>
<td>RANTES</td>
<td>5'-AGCAGCAAGTGCTCCAATC-3'</td>
<td>5'-GGGAAGGCTATACAGGGTC-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-GCCATGCGATCTCCTTATCA-3'</td>
<td>5'-TCCTGAGCTTTGGTGACAAAAAC-3'</td>
</tr>
<tr>
<td>IL -10</td>
<td>5'-AGCAGCAAGTGCTCCAATC-3'</td>
<td>5'-GGGAAGGCTATACAGGGTC-3'</td>
</tr>
<tr>
<td>FOXP3</td>
<td>5'-AGAAGGCTGGGAGAGCTATGAG-3'</td>
<td>5'-AGCAGCAAGTGCTCCAATC-3'</td>
</tr>
<tr>
<td>ARG1</td>
<td>5'-AGAAGGCTGGGAGAGCTATGAG-3'</td>
<td>5'-AGCAGCAAGTGCTCCAATC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GCCAAGCCATGTACCTTGAG-3'</td>
<td>5'-GGAGAGGTTGCTGTGTTTTCCAG-3'</td>
</tr>
</tbody>
</table>

Supplemental table II

<table>
<thead>
<tr>
<th>Blood leukocytes</th>
<th>C57BL6 (n=4)</th>
<th>CXCR3KO (n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total white cell count, K/ul</td>
<td>8.3</td>
<td>2.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Neutrophils, K/ul</td>
<td>2.6</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Lymphocytes, K/ul</td>
<td>5.0</td>
<td>4.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Monocytes, K/ul</td>
<td>0.6</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Eosinophils, K/ul</td>
<td>0.07</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Basophils, K/ul</td>
<td>0.01</td>
<td>0.01</td>
<td>0.4</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>33.6</td>
<td>26.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>58.6</td>
<td>66.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>7.1</td>
<td>6.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0.7</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Basophils, %</td>
<td>0.1</td>
<td>0.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Comment:** The table shows means of total and differential leukocyte counts in CXCR3-deficient and control mice. The values were compared by Student’s t test.

Supplemental figure I. CXCR3-deficient mice and controls presented similar energy expenditure and slightly different food intake and movement.
Mice were individually housed in metabolic cages and subjected to non-invasive monitoring of gas exchange, physical activity, and food intake. Rates (ml/kg/h) of O₂ consumption (VO₂) and CO₂ production (VCO₂) were determined at 11 min intervals and were normalized to body weight. Food intake was measured gravimetrically. Activities were measured by beam breaks along the bottom of the cages. Measurements were performed in CXCR3-deficient mice (KO) and controls (WT) before (A, B, C, D, E, F) and after 4 weeks of high-fat diet (G, H, I, J, L, M).
Statistical significance of metabolic data were analyzed by using two-tailed unpaired Student's t test.*p <0.05; n= 6 in each group.
RER, respiratory exchange ratio (RER = VO₂/VCO₂)

Supplemental figure II. Spleens of diet-induced obese CXCR3-deficient mice and controls have similar proportions of macrophages and lymphocytes. Splenocytes from CXCR3-deficient mice and wild-type control mice fed a high-fat diet were labeled with conjugated antibodies to F4/80, CD3, CD4, CD8, and B220, and were analyzed by flow cytometry. The graphs represent the percentages of gated cells in the CXCR3-deficient group (black bars) and the control group (gray bars). Means were compared by Student’s t test.*p <0.05.

Supplemental figure III. Obese CXCR3-deficient mice exhibit decreased leptin and total cholesterol levels, compared to obese wild-type mice. Plasma levels of cholesterol, leptin, adiponectin, and insulin were measured in heparinized blood collected at sacrifice from CXCR3-deficient mice and wild-type control mice fed a high-fat diet for 8 and 16 weeks. Means were compared by Student’s t test. *p <0.05.

Supplemental figure I.
B

VCO₂
Low-fat diet

\[ \text{VCO}_2, \text{ml/kg/h} \times 10^3 \]

WT
KO

Light  Dark  Light  Dark
D

Total food intake in 48 hrs
Low-fat diet

E

Ambulatory Activity
Low-fat diet

p=0.217

p=0.048
Total Activity
Low-fat diet

WT

KO

p=0.023
G

VO2
4 weeks of high fat feeding

H

VCO2
4 weeks of high fat feeding
I

RER
4 weeks of high fat feeding

[Graph showing RER over time with two lines for WT and KO groups.]

J

Food intake in 48 hrs
4 weeks of high fat feeding

[Bar graph showing food intake with error bars for WT and KO groups, p=0.024.]
Ambulatory Activity
4 weeks of high fat feeding

Total Activity
4 weeks of high fat feeding
Supplemental figure II.

Spleens of diet-induced obese CXCR3-deficient mice and controls have similar proportions of macrophages and lymphocytes.
Supplementary figure III.

8 weeks HFD

![Graph showing plasma cholesterol levels for C57BL6 and CXCR3KO mice after 8 weeks of HFD.]({image1.png})

16 weeks HFD

![Graph showing plasma cholesterol levels for C57BL6 and CXCR3KO mice after 16 weeks of HFD.]({image2.png})

![Graph showing plasma leptin levels for C57BL6 and CXCR3KO mice after 8 weeks of HFD.]({image3.png})

![Graph showing plasma leptin levels for C57BL6 and CXCR3KO mice after 16 weeks of HFD.]({image4.png})