Visualized macrophage dynamics and significance of S100A8 in obese fat

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Chronic low-grade inflammation of adipose tissue plays a crucial role in the pathophysiology of obesity. Immunohistological microscopic analysis in obese fat tissue has demonstrated the infiltration of several immune cells such as macrophages, but dynamics of immune cells have not been fully elucidated and clarified. Here, by using intravital multiphoton imaging technique, to our knowledge for the first time, we analyzed and visualized the inflammatory processes in adipose tissue under high-fat and high-sucrose (HF/HS) diet with lysozyme M-EGFP transgenic (LysMEGFP) mice whose EGFP was specifically expressed in the myelomonocytic lineage. Mobility of LysMEGFP-positive macrophages was shown to be activated just 5 d after HF/HS diet, when the distinct hypertrophy of adipocytes and the accumulation of macrophages still have not become prominent. Significant increase of S100A8 was detected in mature adipocytes just 5 d after HF/HS diet. 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visualized time-dependent changes of immune cell dynamics during the development of obesity, and succeeded in identifying the early triggering event of chronic inflammation in obese adipose tissue.

**Results**

**Visualization of LysMEGFP-Positive Macrophages in Adipose Tissues.** To analyze visually the dynamic behaviors of macrophages within adipose tissue of living mice, we used lysozyme M-EGFP transgenic (LysMEGFP) mice whose EGFP was specifically expressed in the myelomonocytic lineage to visualize immune cell dynamics (22). To analyze immune cell dynamics in adipose tissue in the process of obesity, intravital imaging in epididymal white adipose tissue (WAT) of LysMEGFP mice was carried out under a normal chow (NC) or a high-fat and high-sucrose (HF/HS) diet (23). To analyze the cellular dynamics in early and late stages of obesity, we obtained images from mice fed with HF/HS diet for 5 d and 8 wk, respectively (Fig. 1 A and B). A few moving LysMEGFP-positive cells could be detected in adipose tissues in mice fed an NC diet (Fig. 1, Left, and Movie S1). On the contrary, it was of note that cell migration was apparently enhanced in mice fed with an HF/HS diet for just 5 d (Fig. 1, Middle, and Movie S2). Because histological changes such as enlargement of adipocyte sizes were not obvious at this time point, this early change in cellular dynamics could be detected by the intravital imaging technique. In 8 wk feeding with HF/HS diet, a number of LysMEGFP-positive cells were not only continuously recruited and vigorously migrating, and some were surrounding dead adipocytes, forming crown-like structures (CLSs) (6, 7) (Fig. 1, Right, and Movie S3), when the obesity-induced histological changes were prominent. Quantitative image data analyses showed that cell tracking velocity was significantly increased, by approximately twofold, after just 5 d of HF/HS diet feeding, and its increase was maintained at 8 wk after HF/HS diet feeding (Fig. 1B). Histological analysis of WAT revealed that adipocyte size was not changed at day 5 under HF/HS diet compared with NC diet, and adipocytes were significantly larger at 8 wk after HF/HS diet (Fig. 1C).

**Identification of LysMEGFP-Positive Cell Population in Adipose Tissues.** Flow cytometry analysis was next performed to identify the cell population of LysMEGFP-positive cells in adipose tissues. Fig. 2 A presents the comparison of LysMEGFP-positive macrophages in WAT and circulating blood. In contrast to the cell population in blood, the frequency of LysMEGFP-positive neutrophils was very low in WAT, whereas 60–70% of LysMEGFP-positive adipose cells were identified to be macrophages (Fig. 2A). As shown in Table S1, the proportions of adipose EGFP-positive cells in neutrophils (CD11b<sup>+</sup> Ly-6C<sup>+</sup>), macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>), and inflammatory monocytes (CD11b<sup>+</sup> Ly-6C<sup>+</sup>Ly-6G<sup>+</sup>) were 95.3%, 91.8%, and 56.6%, respectively, under NC diet.

Fig. 2B shows representative changes of LysMEGFP-positive cell population in WAT under HF/HS diet study. The population of neutrophils (CD11b<sup>+</sup> Ly-6G<sup>+</sup>) was not changed on day 5, but it was rather decreased on 8 wk after HF/HS diet feeding. The total macrophage population (CD11b<sup>+</sup> F4/80<sup>+</sup>) was not altered during HF/HS diet feeding. However, the population of CD11c<sup>+</sup> macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>CD11c<sup>+</sup>) was increased after just 5 d of HF/HS diet feeding, and the increase was maintained at 8 wk after HF/HS diet. Population of inflammatory monocytes (CD11b<sup>+</sup> Ly-6C<sup>+</sup>) was not obviously increased during HF/HS diet. Fig. 2C demonstrates the number of LysMEGFP-positive cells in WAT. Concordant with the data shown in Fig. 2B, total LysMEGFP-positive cells were not altered on day 5 after HF/HS diet and were slightly but significantly increased at 8 wk after HF/HS diet. Neutrophils (CD11b<sup>+</sup> Ly-6G<sup>+</sup>) were not altered on day 5 but tended to be decreased at 8 wk after HF/HS diet. The number of macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>) was not changed during HF/HS diet, but CD11c<sup>+</sup> macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>CD11c<sup>+</sup>) were gradually increased on day 5 after HF/HS diet and were significantly augmented at 8 wk after HF/HS diet. Inflammatory monocytes (CD11b<sup>+</sup> Ly-6C<sup>+</sup>) were not altered on day 5, but they tended to increase at 8 wk after HF/HS diet. The proportion of adipose EGFP-positive cells in macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>) was unchanged during HF/HS diet feeding (Table S1). These results indicate that the highly mobile LysMEGFP-positive cells in the very early stage of obesity would be macrophages, which would be the initial phenomenon observed during the course of obesity-induced chronic inflammation.

**Up-Regulation of Adipocyte S100A8 mRNA Expression in the Very Early Stage of Obesity.** Metabolic parameters (Fig. 3A) and adipose tissue mRNA expression levels (Fig. 3B) were examined to understand the mechanism for the increased mobility of adipose macrophages at day 5 after HF/HS diet. Body weights of the HF/HS diet group were similar to those of the NC diet group at day 5, whereas they were significantly elevated at 8 wk after HF/HS diet. Weights of WAT were slightly but significantly increased in the HF/HS group compared with the NC group at day 5 and markedly increased at 8 wk after HF/HS diet. Plasma glucose concentrations were not changed at day 5 after HF/HS diet, but were elevated at 8 wk after HF/HS diet. There were no significant differences in plasma triglyceride levels between NC and HF/HS groups. Levels of plasma total cholesterol were significantly elevated in the HF/HS group at day 5, and the increase was maintained at 8 wk after HF/HS diet. These results suggest that metabolic status is largely unchanged at day 5 after HF/HS diet, although there are marked differences observed between NC and HF/HS groups at 8 wk.

We next examined the inflammatory gene expressions in WAT to explore the early response leading to enhanced migration of macrophages and resultant adipose chronic inflammation (Fig. 3B). Among a large number of molecules we tested, only one molecule, S100A8, one of the alarmins, was significantly up-regulated in mRNA level (Fig. 3B) just 5 d after HF/HS diet feeding, whereas the other alarmin molecules such as S100A9, HMGB1, and IL-1α were not altered at day 5 after HF/HS diet. Several chemokines and proinflammatory cytokines, such as CCL2, CCL3, and IL-1α (Fig. 3B), which already have been reported to be critically involved in obesity-induced chronic inflammation of adipose tissues, were all elevated in the later phase of obesity (8 wk) but essentially unaltered in the very early stage (day 5). These results suggest the possibility that S100A8 contributes to the very early pathological event in adipose inflammation. To examine the cell type expressing S100A8, WAT was next fractionated into mature adipocyte fraction (MAF) and stromal vascular fraction (SVF; Fig. 3C). A significant increase of S100A8 mRNA level was observed in the MAF but not in the SVF on day 5 after HF/HS diet. Moreover, similar to the mRNA changes, S100A8 protein level was increased on day 5 after HF/HS diet and was further augmented at 8 wk after HF/HS diet (Fig. 3D), suggesting that adipose S100A8 immediately responded to the HF/HS diet.

**Inflammatory Responses to S100A8 in RAW264.7 Macrophages and 3T3-L1 Adipocytes.** To clarify the functional role of S100A8 in obese adipose tissue, chemotaxis assay was next performed by using a ready-made chemotaxis assay chamber, EZ-TAXISCAN, which is useful to assess chemotactic activity visually and quantitatively (Fig. 4A and Movies S4–S6). As a result, a macrophage cell line, RAW264.7, exhibited a positive chemotaxis toward recombinant S100A8 proteins in a concentration-dependent manner, as previously reported (24).

Treatment with recombinant S100A8 protein increased TNF-α and MCP-1 mRNA levels in RAW264.7 cells (Fig. 4B, lanes 1–3). To rule out the effect of endotoxin contamination in recombinant S100A8 protein, RAW264.7 cells were treated with the LPS inhibitor polymyxin B. S100A8-induced increases of TNF-α and...
MCP-1 mRNA levels were partly reduced by polymyxin B (Fig. 4B, lanes 4–6), whereas polymyxin B efficiently blocked the LPS-induced increases of TNF-α and MCP-1 mRNA levels (Fig. 4B, lanes 7–12). In addition, also in 3T3-L1 adipocytes, recombinant S100A8 protein induced mRNA expression levels of serum amyloid A3, MCP-1, chemokine (C-X-C motif) ligand 1 (CXCL1), and CXCL5 in the presence of polymyxin B (Fig. 4C, lanes 4–6), whereas such increases were not observed in LPS treatment under polymyxin B (Fig. 4C, lanes 10–12). These results suggest that adipose S100A8 acts not only as a chemoattractant to...
macrophages but also as a proinflammatory factor on macrophages and adipocytes.

**In Vivo Effect of S100A8 on the Mobility of LysMEGFP-Positive Cells in Adipose Tissues.** We next examined whether S100A8 can also accelerate macrophage mobility in adipose tissues in vivo by using LysMEGFP mice. Similar to the experiments shown in Fig. 1A and B, intravital imaging of adipose tissue was conducted by adding recombinant S100A8 protein or an equal amount of LPS directly onto WAT. The mobility of adipose LysMEGFP-positive cells was gradually increased at 1 h after S100A8 administration (Fig. 5A and Movie S7), whereas such increased mobility was not observed in the LPS administration group (Movies S8 and S9). Tracking velocity was significantly increased by the treatment with recombinant S100A8 protein after 1 h (Fig. 5B), suggesting that S100A8 is indeed functional for mobilizing adipose tissue-resident macrophages in vivo.

**In Vivo Effect of S100A8 Antibody on the Mobility of LysMEGFP-Positive Cells in Adipose Tissues and Insulin Sensitivity.** To examine whether enhanced macrophage mobility at an early stage of obesity was caused by adipose S100A8, we tested the effect of inhibition of endogenous S100A8 by its antibody on the mobility of adipose LysMEGFP-positive cells on day 5 after HF/HS diet. The intravital imaging was performed until 150 min after the administration of S100A8 antibody or IgG isotype-matched control antibody (Fig. 5C–E). As a result, the S100A8 antibody treatment almost completely blocked the HF/HS diet-induced mobility of adipose

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**Fig. 2.** Populations of LysM<sup>EGFP</sup>-positive cells in adipose tissue and blood. (A) Percent population of neutrophils, macrophages, and inflammatory monocytes in LysM<sup>EGFP</sup>-positive cells in epididymal adipose tissue (Left) and blood (Right) from LysM<sup>EGFP</sup> mice fed with NC. (B) Representative flow cytometry dot plots showing CD11b<sup>+</sup> Ly6G<sup>+</sup> (neutrophils), CD11b<sup>+</sup> F4/80<sup>+</sup> (macrophages), CD11b<sup>+</sup> F4/80<sup>+</sup> CD11c<sup>+</sup> (M1 macrophages), and CD11b<sup>+</sup>Ly6C<sup>hi</sup> (inflammatory monocytes) in epididymal adipose tissue of LysM<sup>EGFP</sup> mice fed an NC diet or HF/HS diet for 5 d (5d) or 8 wk (8w). (C) Number of LysM<sup>EGFP</sup>-positive cells in epididymal adipose tissue. Data are expressed as means ± SEM. Dots represent values from individual mice (n = 6; *P < 0.05 and ***P < 0.001).

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induced activation of adipose LysMEGFP-positive cell mobility at mean tracking velocity integrating every 30 min and indicates LysMEGFP-positive cells was significantly suppressed later than C(Fig. 5 observed when IgG isotype control antibody was administered diet, before the increase of adipose LysMEGFP-positive cells. On the contrary, it should be noted that LysMEGFP-positive cells in adipose tissues contain some undefined fractions (ii) S100A8 mRNA and protein levels were increased in adipose tissues just 5 d after HF/HS diet. (iii) S100A8 accelerated migration of RAW264.7 cells and activated inflammatory response in macrophages and adipocytes. (iv) S100A8 activated the mobility of adipose LysMEGFP-positive cells whereas its antibody effectively suppressed the HF/HS diet-induced activity of adipose LysMEGFP-positive cell mobility. Finally, (v) Administration of S100A8 antibody improved insulin sensitivity under HF/HS diet.

Obesity is recognized as a state of chronic low-grade inflammation (5, 6). Accumulating evidence demonstrates that the progression of adipose tissue inflammation is strongly associated with overreactive innate and adaptive immune responses (6–11). However, it is difficult to predict the balance of immune responses by conventional research using immunohistological and flow cytometric procedures because cellular responses to inflammation are particularly dynamic and complicated. To analyze the dynamic processes underlying adipose inflammation may require intravital imaging of intact tissues in living animals. Here, for the first time to our awareness, we succeeded in visualizing immune cell dynamics from the beginning of the development of obesity. Strikingly, the mobility of LysMEGFP-positive cells infiltrating in adipose tissues was increased in the very early stage of obesity without the prominent hypertrophy of adipocytes and the increase in the number of macrophage accumulation. However, it remains to be elucidated where such activated LysMEGFP-positive cells relocate to different positions in adipose tissues. The present study also could not provide evidence whether LysMEGFP-positive cells interact with other cell types in adipose tissues. There are several technical limitations at present, including that intravital imaging in adipose tissues cannot be performed more than 3 h to obtain a live image. Further technical advances may provide novel insights into the activated macrophages and other immune cells in adipose tissues.

Discussion

The major findings of the present study of intravital imaging of adipose tissues during development of obesity were as follows. (i) Adipose LysMEGFP-positive cells mainly consisted of macrophages, and their mobility was activated just 5 d after HF/HS diet, before the increase of adipose LysMEGFP-positive cells. (ii) S100A8 mRNA and protein levels were increased in adipose tissues just 5 d after HF/HS diet. (iii) S100A8 accelerated migration of RAW264.7 cells and activated inflammatory response in macrophages and adipocytes. (iv) S100A8 activated the mobility of adipose LysMEGFP-positive cells whereas its antibody effectively suppressed the HF/HS diet-induced activity of adipose LysMEGFP-positive cell mobility. Finally, (v) Administration of S100A8 antibody improved insulin sensitivity under HF/HS diet.
cells in adipose tissues under NC diet. Generation of monocyte/macrophage-specific fluorescent protein-transgenic mice would be necessary to better understand the visible activity of macrophages in adipose tissue. Eosinophils have been shown to migrate into adipose tissue and maintain metabolic homeostasis in mice (12). Talukdar et al. clearly demonstrated that neutrophils infiltrated into adipose tissue just 3 d after 60% high-fat diet, and deletion of neutrophil elastase decreased adipose tissue inflammation in their diet-induced obesity mice (11). However, the present study shows that the proportion of adipose neutrophils to stromal vascular cells (SVCs) was not increased after 5 d of HF/HS diet feeding (Fig. 2C). The reason for such a difference remains uncertain, although the age of mice starting high-fat diet feeding and the fat composition of high-fat diet in the present study (30% fat) were not similar to those in the previous study (11). It might be possible that immune response may vary with murine age and fat composition of meals.

Increases of chemokines and their related molecules may be involved in the development of adipose tissue inflammation. Several potential modulators of inflammatory and immune responses have been identified in the development of obesity, e.g., CCL2 (MCP-1), CCL3 [macrophage inflammatory protein-1α (MIP-1α)], CCL5 (MCP-2), CCL7 (MCP-3), CCL5 (RANTES), and CXCL14 (13, 25, 26), and accumulating evidence shows the crucial role of the CCL2/CCR2 pathway in adipose inflammation (13, 14), although the initial factors triggering the inflammation in this context had still been unknown. The present study suggested that S100A8 is a critical component regulating the initial step of inflammatory cascade in obese adipose tissue. Importantly, S100A8 was increased in the very early stage of obesity, whereas no changes in CCL2 (MCP-1) and CCL3 (MIP-1α) mRNA expression levels were observed in this stage (Fig. 3 B and C). However, we cannot completely exclude the possibility that MCP-1 or other chemokines would be secreted locally with limited concentrations that were undetectable by quantitative PCR of whole adipocyte fractions. The mobility of adipose LysM<sup>EGFP</sup>-positive cells and the migration of macrophages were increased by S100A8 administration (Figs. 4 A and 5A and B), whereas the mobility of adipose LysM<sup>EGFP</sup>-positive cells was suppressed by treatment with S100A8 antibody under HF/HS diet (Fig. 5 D and E). However, the present study could not fully show whether S100A8 directly or indirectly induced mobility of adipose LysM<sup>EGFP</sup>-positive cells. The possibility could not be excluded that S100A8 induces the local production of MCP-1 and/or other chemotactic factors that mobilize adipose tissue-resident LysM<sup>EGFP</sup>-positive cells. LysM<sup>EGFP</sup> mice with macrophage-specific ablation of MCP-1 and/or other chemotactic factors will be needed in the future to determine whether S100A8 possesses a direct effect on mobility of adipose LysM<sup>EGFP</sup>-positive cells.

As shown in Fig. 5 C and D, S100A8 antibody-mediated suppressive effect on the HF/HS diet-induced mobility of adipose LysM<sup>EGFP</sup>-positive cells was not observed immediately (it took approximately 90 min), which may reflect the time course for penetrating S100A8 antibody into the tissues or that necessary for S100A8 antibody to block the release of other chemokines locally. In vitro examinations also showed the proinflammatory effect of S100A8 on macrophages and adipocytes (Fig. 4 B and C).
Fig. 5. Effect of S100A8 and S100A8 antibody on the mobility of LysMEGFP-positive cells and diet-induced insulin resistance. Intravital two-photon imaging (A) and mean tracking velocity (B) in epididymal adipose tissues of LysMEGFP mice in the absence (Left) or presence (Right) of recombinant S100A8 protein (Movie S7). Colored lines show the associated trajectories of LysMEGFP-positive cells. (Scale bar: 100 μm.) Data points (n = 469 from four mice for control; n = 555 from five mice for S100A8) and bars represent the values for individual cells and averages, respectively (***P < 0.001). (C) Intravital two-photon images after treatment of IgG isotype control antibody (Left) or S100A8 antibody (Right) in epididymal adipose tissues of LysMEGFP mice fed with HF/HS diet for 5 d (Movie S10). Colored lines show the associated trajectories of LysMEGFP-positive cells. (D) Line graph of percent changes in mean tracking velocity of LysMEGFP-positive cells after antibody administration under HF/HS diet for 5 d. The mobility of LysMEGFP-positive cells was set in 100% at pretreatment (n = 4 per group). Data are expressed as means ± SEM (**P < 0.01 and ***P < 0.001 vs. IgG control group at the same period). (E) Line graph of plasma glucose under insulin tolerance test. C57BL6 mice were treated with IgG isotype control antibody or S100A8 antibody and fed with HF/HS diet from 11 wk of age. For insulin tolerance test, mice received 0.75 U/kg insulin i.p. on day 21 (n = 4 per group). Data are expressed as means ± SEM (**P < 0.05 vs. IgG control group). (G) Area under the curve (AUC) of insulin tolerance test calculated from 0 min to 60 min (n = 4 per group). Data are expressed as means ± SEM (**P < 0.05).
A series of the present data may provide the possibility that adipose S100A8 increases in the very early stage of obesity, recruits macrophages into adipose tissue, and induces local inflammation by its effect on adipocytes and macrophages. S100A8 antibody treatment also enhanced insulin sensitivity under HF/HS diet (Fig. 5 F and G), suggesting that increased mobility of adipose macrophages via S100A8 impact on the development of insulin resistance and diabetes in obesity. Generation and analysis of adipose-specific S100A8 transgenic and/or KO mice would be required to confirm the significant role of S100A8 in adipose inflammatory response and insulin resistance.

Two members of the S100 protein family, S100A8 and S100A9, have been identified as important endogenous alarmins that are released from the activated phagocytes and are recognized by TLR4 on monocytes (16). Significance of S100A8 has not been clarified in adipose tissue, but our previous CDN microarray analysis identified, to our knowledge, for the first time, the increased expression levels of S100A8 in obese adipose tissue (27), and we recently reported that adipose S100A8 mRNA level was highly expressed in MAF in obesity (28). Moreover, circulating S100A8/A9 heterodimer complex (calprotectin) positively correlated with adiposity in human subjects (28, 29). Interestingly, PPAR-γ agonists significantly reduced adipose S100A8 mRNA level and plasma S100A8/A9 complex (calprotectin) concentration in obese diabetic mice (27) and human subjects (30). Furthermore, we have demonstrated, to our knowledge, for the first time, that blocking against S100A8 ameliorated diet-induced insulin resistance. These results suggest that S100A8 plays an essential role in the inflammatory responses of obese adipose tissue and contributes to the pathogenesis of obesity from the very early stage. Chronic inflammation usually takes a long course, and intricate inflammatory chain reactions will occur when it has happened. In light of the treatment, it would be desirable if we can block the initial trigger and completely halt the very early event of this vicious cycle. From the point of view, S100A8 would become a good target for a new line of therapeutics against obesity-induced chronic inflammation.

Materials and Methods

Animals. LysM<sup>GFP</sup> mice (22) were maintained with NC diet. At 10 wk of age, LysM<sup>GFP</sup> mice were continued with NC diet or were changed to HF/HS diet (F2HHSD; Oriental Yeast) (31). LysM<sup>GFP</sup> mice were analyzed at 5 d and 8 wk after NC or HF/HS diet. Mice were anesthetized with an i.p. injection of a mixture of medetomidine (0.3 mg/kg body weight), midazolam (4 mg/kg body weight), and butorphanol tartrate (5 mg/kg body weight). Blood samples were collected from the inferior vena cava after 16 h of fasting, and erythrocytes were separated for RNA extraction. For immunohistological analysis, plasma glucose, triglyceride, and cholesterol concentrations were measured with glucose Cl, triglyceride E, and cholesterol E tests (Wako Pure Chemical Industries), respectively. To analyze adipocyte size, low-power field images were acquired from five animals in each group, after which the area of 35 adipocytes in each field were measured. The histogram shown was constructed from 175 cells from each group. For insulin tolerance test study, C57BL/6 mice were injected i.p. with Igl IgG control antibody or S100A8 antibody at 50 μg per mouse three times per week and started feeding HF/HS diet from 11 wk of age. Mice were subjected to insulin tolerance test by i.p. administration of 0.75 U/kg insulin on day 21. Mice were kept in rooms set at 22 °C with a 12:12 h dark/light cycle (lights on from 800 h to 2000 h).

Multiphoton Intravital Adipose Tissue Imaging. The imaging system was composed of a multiphoton microscope (A1-MP; Nikon) driven by a laser (Chameleon Vision II Ti: Sapphire; Coherent) tuned to 800–840 nm and an inverted microscope equipped with a 20× multimmersion objective lens (Plan Fluor; N.A., 0.75; Nikon). Fluorescent signals were detected through band-pass emission filters at 500–550 nm (for GFP), at 563±25 nm [for 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)], and at 601±6 nm (for Qtracker). For adipose tissue imaging, mice were anesthetized with isoflurane, and epididymal WAT was carefully exposed and incubated with BODIPY (Life Technologies). Vessels were visualized by the i.v. injection of Qtracker (Life Technologies) just before imaging. Epididymal WAT was covered with saline solution-moistened sheets of paper, and mice were set in a heating box to maintain body temperature during intravital imaging. Sequential images were taken every 30 s for 1 h. Image stacks were collected at a 5-μm vertical step size. To quantify leukocyte dynamics, raw imaging data were processed as follows: EGFP-positive cells in sections were tracked, and mobility was calculated as the mean tracking velocity and mean tracking length by using IMARIS 3D and 4D real-time image processing and analysis software (Bitplane) (32, 33).

Evaluation of the Effect of S100A8 on Adipose Tissue by Intravital Imaging. For the recombinant S100A8 protein treatment study, the epididymal WAT of LysM<sup>GFP</sup> mice was exposed, and human recombinant S100A8 protein (10 μg per mouse; GIOTTO) was injected directly onto the epididymal WAT after staining. Sequential images of WAT were taken every 30 s from 1 to 30 min to 150 min in the course of S100A8 antibody treatment. The mean tracking velocity of LysM<sup>GFP</sup>-positive cells before antibody treatment was set as 100%. Continuous time-lapse imaging after antibody treatment was divided into five sections of 30-min intervals and quantified.

Isolation of the SVF and Flow Cytometry. To isolate the SVF, minced adipose tissue in Krebs-Ringer bicarbonate Hepes buffer (120 mmol/L NaCl, 4 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L MgSO<sub>4</sub>, 1 mmol/L CaCl<sub>2</sub>, 10 mmol/L NaHCO<sub>3</sub>, 30 mmol/L Hepes, 20 μmol/L adenosine, and 4% [wt/vol] BSA (Calbiochem)] was centrifuged to remove blood cells and then incubated in a collagenase solution (2 mg/mL) at 37 °C for 30 min under continuous shaking. The tissue was then centrifuged, and the resultant pellet containing the SVF was filtered through 100-μm mesh and finally resuspended in PBS solution. Cells were first incubated with anti-mouse CD16/CD32 (BioLegend) for 15 min at 4 °C, then incubated with labeled monoclonal or isotype control antibody. Flow cytometric analysis was performed by using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (Tree Star). The antibodies used were as follows: anti-CD11b (clone M1/70; BioLegend), anti-ly-6G (clone 1A8; BioLegend), anti-F4/80 (clone BMB; BioLegend), anti-ly-6-C (clone Al-21, BD Pharmingen), and anti-CD11c (clone N418; eBioscience).

Quantitative Real-Time PCR. Total RNA was isolated by using RNA STAT-60 (Tel-Test) according to the protocol supplied by the manufacturer. The quality and quantity of total RNA were determined by using an ND-1000 Spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time quantitative PCR amplification was conducted with a VIAPlex one PCR system (Life Technologies) using Thunderbird quantitative PCR mix (Toyobo) according to the protocol recommended by the manufacturer. The results for each sample were normalized to the respective 36B4 mRNA levels. The primer sets were as follows: mouse 36B4, 5′-GGCCAATAGGGCTTGCAG-3′ (forward) and 5′-TGTGACCGGAAAAAGGAGAAG-3′ (reverse); mouse S100A8, 5′-GACAATGAAATTACCTGGAGGAG-3′ (forward) and 5′-TGGTGCTCTTGGTGTA-GATG-3′ (reverse); mouse S100A9, 5′-ACAACGGGAACACCTCCATTG-3′ (forward) and 5′-CAGCCTGATTGTCCGGTCT-3′ (reverse); mouse HKRAG1, 5′-TGGGCAAAG-5′GCTGAAACGCT-3′ (forward) and 5′-GGATGCTGGCTCATTGATTTGG-3′ (reverse); mouse CCL2, 5′-CAACTCACCTGCTGCTACTAC-3′ (forward) and 5′-TGGGTACACCTCTGACTC-3′ (forward); mouse CCL3, 5′-CAACATTCCAGAAGGAGGAC-3′ (forward) and 5′-GAGATTTCCTCGAGGAGGAGG-3′ (reverse); and mouse IL-1α, 5′-CAACAGGTTGAAGCAGCTGC-3′ (forward) and 5′-TCTCCCT-GAGGCCTGCAGGGA-3′ (reverse).

Immunoblotting. Epididymal adipose tissues were frozen in liquid nitrogen immediately and lysed in RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor mixture (Cell Signaling). Lysates were incubated in lysis buffer for 30 min on ice before centrifugation and collection of the supernatant. Total protein concentration was determined by using the BCA-200 Protein Assay Kit (Pierce). For immunoblotting, protein samples were incubated with a reducing sample buffer [2% (wt/vol) SDS, 50 mmol/L Tris HCl, pH 6.8, and 10% (vol/vol) glycerol with 2-mercaptoethanol] and separated by 4–20% gradient SDS/PAGE. Human recombinant S100A8 protein (GIOTTO) was used as a positive control. Immunoblot analysis was performed first with antibody S100A8 (Santa Cruz), followed by incubation with secondary
antibody conjugated with horseradish peroxidase. The ECL Prime system (GE Healthcare) was used for detection of the protein signal.

Cell Culture. RAW264.7 cells were maintained in the regular medium (DMEM supplemented with 10% FCS and 1% penicillin/streptomycin (P/S)). RAW264.7 cells were plated in 12-well plates (2 x 10^5 cells per well) and were cultured for 24 h before the experiment. RAW264.7 cells were treated with or without 1 and 10 μg/mL of S100A8 (Giottio) or 1 and 10 ng/mL of LPS (Sigma-Aldrich) in the presence or absence of 25 μg/mL of polymyxin B (Sigma-Aldrich), and cells were harvested after the indicated treatment for 24 h. 3T3-L1 cells were maintained and differentiated, as described previously (34). Briefly, cells were grown to confluence and differentiated by induction medium (DMEM supplemented with 10% FCS and 1% P/S) containing 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin. After incubation with the induction medium for 48 h, the medium was changed to maintenance medium (DMEM supplemented with 10% FCS and 1% P/S). On day 7 after differentiation, 3T3-L1 adipocytes were incubated with or without 1 and 10 ng/mL of S100A8 (Giottio) or 1 and 10 ng/mL of LPS (Sigma-Aldrich) in the presence of 25 μg/mL of polymyxin B (Sigma-Aldrich), and cells were harvested after the indicated treatment for 24 h.

Chemotaxis Assay. Chemotaxis experiments were conducted in an EZ-TAXIScan chamber according to the manufacturer’s protocol (GE Healthcare), as described previously (32, 33). Briefly, EZ-TAXIScan is a visually accessible chemotactic chamber in which one compartment contains the ligand (S100A8) and another compartment contains the cells (RAW264.7 cells), and these compartments are connected by a microchannel. A stable concentration gradient of chemotaxtractant can be reproducibly formed and maintained through the channel without medium flow. Images of RAW264.7 cells undergoing chemotaxis in response to S100A8 were obtained at 1-min time-lapse intervals for 3 h (37 °C). Sequential image data were processed by using the ImageJ program (National Institutes of Health) with an add-on program, MT Track J.

Statistical Analysis. The statistical significance of differences between groups was determined by two-tailed t tests or Dunnett test for Gaussian-like distributions. The Wilcoxon rank-sum test or the Steel test was used to calculate the P values for highly skewed distributions. P values less than 0.05 were considered significant. All analyses were performed with the JMP Statistical Discovery Software (version 11.0; SAS Institute).

Study Approval. All animal studies were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine and also conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

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Supporting Information
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Table S1. Proportions of adipose EGFP-positive cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>NC</th>
<th>5 d</th>
<th>8 wk</th>
</tr>
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<tbody>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; Ly6G&lt;sup&gt;+&lt;/sup&gt; (neutrophils), %</td>
<td>95.3 ± 4.5</td>
<td>98.4 ± 1.8</td>
<td>97.3 ± 3.0</td>
</tr>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; F4/80&lt;sup&gt;+&lt;/sup&gt; (macrophages), %</td>
<td>91.8 ± 3.8</td>
<td>93.6 ± 5.6</td>
<td>92.4 ± 1.6</td>
</tr>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; Ly6C&lt;sup&gt;hi&lt;/sup&gt; (inflammatory monocytes), %</td>
<td>56.6 ± 16.2</td>
<td>63.9 ± 11.5</td>
<td>85.2 ± 6.3</td>
</tr>
</tbody>
</table>

Movie S1. Intravital two-photon images in epididymal adipose tissues of LysM<sup>EGFP</sup> mice fed with NC diet. For adipose tissue imaging, adipocytes (blue) were stained with BODIPY and blood vessels (red) were stained by i.v. injection of nontargeted Q-dots just before imaging. LysM<sup>EGFP</sup>-positive cells appeared in green.

Movie S1
**Movie S2.** Intravital two-photon images in epididymal adipose tissues of LysM\textsuperscript{EGFP} mice fed with HF/HS diet for 5 d. For adipose tissue imaging, adipocytes (blue) were stained with BODIPY and blood vessels (red) were stained by i.v. injection with nontargeted Q-dots just before imaging. LysM\textsuperscript{EGFP}-positive cells appear in green.

**Movie S3.** Intravital two-photon images in epididymal adipose tissues of LysM\textsuperscript{EGFP} mice fed with HF/HS diet for 8 wk. For adipose tissue imaging, adipocytes (blue) were stained with BODIPY and blood vessels (red) were stained by i.v. injection with nontargeted Q-dots just before imaging. LysM\textsuperscript{EGFP}-positive cells appear in green.
Movie S4. In vitro dynamic chemotaxis of RAW264.7 cells visualized by EZ-TAXIScan. RAW264.7 cells were loaded onto the lower chamber. The upper chambers were filled with medium (control).

Movie S5. In vitro dynamic chemotaxis of RAW264.7 cells visualized by EZ-TAXIScan. RAW264.7 cells were loaded onto the lower chamber. The upper chambers were filled with medium containing 1 μg/mL of S100A8.
**Movie S6.** In vitro dynamic chemotaxis of RAW264.7 cells visualized by EZ-TAXIScan. RAW264.7 cells were loaded onto the lower chamber. The upper chambers were filled with medium containing 10 μg/mL of S100A8.

**Movie S7.** Intravital two-photon imaging in epididymal adipose tissues of LysM<sup>EGFP</sup> mice in the absence (Left) or presence (Right) of S100A8. The epididymal fat of LysM<sup>EGFP</sup> mice was exposed, and recombinant S100A8 was administered locally onto the epididymal fat after staining.
**Movie S8.** Intravital two-photon imaging in epididymal adipose tissues of LysM<sup>EGFP</sup> mice under LPS administration. The epididymal fat of LysM<sup>EGFP</sup> mice was exposed, and LPS was administered locally onto the epididymal fat after staining.

**Movie S9.** Intravital two-photon imaging in epididymal adipose tissues of LysM<sup>EGFP</sup> mice under LPS administration. The epididymal fat of LysM<sup>EGFP</sup> mice was exposed, and LPS was administered locally onto the epididymal fat after staining.
Movie S10. Intravital two-photon images after treatment of IgG isotype control antibody (Upper) or S100A8 antibody (Lower) in epididymal adipose tissues of LysM<sup>EGFP</sup> mice fed with HF/HS diet for 5 d. Control or S100A8 antibody was administered directly onto the epididymal fat tissues after staining. Sequential images of fat tissues were taken every 30 s from −30 min to 150 min in the course of antibody treatment. Shown are representative movies at 0–60 min (Left) and 90–150 min (Right) after administration of indicated antibodies.

Movie S10