Research Article

Intraperitoneal Injection of Clodronate Liposomes Eliminates Visceral Adipose Macrophages and Blocks High-fat Diet-induced Weight Gain and Development of Insulin Resistance

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Abstract. Macrophage infiltration in adipose tissue is strongly correlated with obesity. The exact role of macrophage in the development of obesity, however, has not been fully understood. In this study, using intraperitoneal injection of clodronate liposomes, we tissue-specifically depleted visceral adipose tissue macrophages (VATMs) and explored their roles in initiation and progression of obesity. Two sets of experiments were conducted, using mice on a high-fat diet as the animal model. Mice were injected with clodronate liposomes at the beginning of high-fat diet feeding to investigate the role of VATMs in the initiation of obesity. Treatment starting on week 5 was designed to explore the function of VATMs in the progression of weight gain. The results show that intraperitoneal injection of clodronate liposomes effectively depleted VATMs, which blocked high-fat diet-induced weight gain, fat accumulation, insulin resistance, and hepatic steatosis. Similarly, clodronate liposomes suppressed progression of weight gain in mice after being fed with a high-fat diet for 4 weeks and improved insulin sensitivity. Gene expression analysis showed that depletion of VATMs was associated with downregulation of the expression of genes involved in lipogenesis and gluconeogenesis including *accl*, *fas*, *scd1*, and *pepck*, decreased expression of genes involved in chronic inflammation including *mcp1* and *tnfa*, and suppressed expression of macrophage specific marker genes of *f4/80* and *cd11c* in adipose tissue. Depletion of VATMs was associated with prevention of the formation of crown-like structures in white adipose tissue and the maintenance of a low level of blood TNF- α . Collectively, these data demonstrate that VATMs appeared to play a crucial role in the development of obesity and obesity-associated diseases and suggest that adipose tissue macrophages could be regarded as a potential target for drug development in prevention and therapy of obesity and obesity-associated complications.

KEY WORDS: high-fat diet-induced obesity; inflammation; insulin resistance; liposomes; visceral adipose tissue macrophage.

INTRODUCTION

The prevalence of obesity has increased globally due to changes in lifestyle and diet preference. It is estimated that more than 60% of Americans are overweight (BMI > 25 kg/m²), of which half are clinically obese with a BMI greater than 30 kg/m² (1). In addition, studies have linked obesity to more than 50 human diseases including insulin resistance, fatty liver, cardiovascular diseases, cancers, and other diseases (2). Weight loss by dietary restriction and exercise, although proven effective, is not sustained in obese patients, which makes treatment using antiobesity drugs essential for reducing and maintaining body weight. Many factors are linked to obesity including diet, inherited genetic traits, behavior, energy

consumption, psychology, environment, and socioeconomic status (3). However, the lack of a full understanding of the underlying mechanism of obesity remains a major hurdle for the development of new and effective strategies in coping with this epidemic.

Recent studies show that obesity is related to chronic inflammation and activation of adipose tissue macrophages (ATMs). Existing results also suggest that the ATM-induced chronic inflammation plays central roles in the development of obesity and its associated diseases (4–6). ATM infiltration is a typical feature of obesity caused by high-fat diet or genetic deficiency of leptin (7). Previous studies by Canello *et al.* and Wellen *et al.* clearly showed that ATM accumulation in adipose tissue closely correlated with body weight gain and insulin resistance in both rodents and humans (4,8). However, weight loss can lead to reduction in both inflammatory and ATM content. Notably, ATMs are a prominent source of proinflammatory cytokines that affect the action of insulin in peripheral tissues (9), providing a potential link between obesity and insulin resistance. Although this correlation has been established, the exact role of ATM in initiating and

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progressing obesity and especially in development of obesity-related diseases such as insulin resistance and fatty liver is not yet fully understood.

In this study, through the use of intraperitoneal injection of clodronate liposomes, we tissue-specifically depleted visceral ATMs to explore their role in obesity development. Our data clearly show that mice without visceral adipose tissue macrophages (VATMs) are protected from high-fat diet-induced obesity, insulin resistance, and hepatic steatosis. In addition, obese mice treated with clodronate liposomes are protected from further weight gain and show marked alleviation in metabolic disorders. Collectively, these data revealed the crucial roles of VATMs in the initiation and progression of obesity and indicated that these macrophages could be regarded as a potential target for the development of antiobesity drugs.

MATERIALS AND METHODS

Materials

Clodronate and empty liposomes were purchased from clodronateliposomes.org (Vrije University, Netherlands). Free clodronate was purchased from Sigma (St. Louis, MO). High-fat diet (60% kJ/fat, 20% kJ/carbohydrate, 20% kJ/protein) was purchased from Bio-Serv (Frenchtown, NJ, catalog number F3282). RNeasy Tissue kit was from Qiagen (Valencia, CA). The SuperScript III First-Strand Synthesis System was purchased from Invitrogen (Carlsbad, CA). Oil Red O solution was obtained from Electron Microscopy Science (Hatfield, PA). The glucometer and test strips were purchased from LifeScan (Milpitas, CA). The F4/80 antibody was purchased from Spring Bioscience (Pleasanton, CA). ELISA kits for measurements of IL-6 and TNF- α were purchased from eBioscience (San Diego, CA). Serum insulin level was determined using ELISA kits from Mercodia (Winston Salem, NC). Free fatty acids level was determined using kits from Infinity (Middletown, VA).

Animals and Treatment

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Three sets of experiments were conducted. In set 1, two groups of mice were fed with a high-fat diet for 10 days. Group 1 animals in set 1 experiments were intraperitoneally injected with clodronate liposomes (110 mg/kg) when fed with a high-fat diet on day 0. The control animals in group 2 were injected with an equal volume of empty liposomes. Three mice were killed on day 0, 2, 4, and 6, respectively. In set 2 experiments, four groups of mice were fed with high-fat diet. Group 1 animals in set 2 experiments were intraperitoneally injected with clodronate liposomes (110 mg/kg) twice weekly. Groups 2, 3, and 4 were used as control and injected with an equal volume of phosphate-buffered saline (PBS), free clodronate, or empty liposomes, respectively. In experiment set 3, two groups of mice were fed with a high-fat diet for the first 4 weeks, and starting on week 5, these animals were injected with either clodronate liposomes or empty liposomes twice weekly.

Animals in experiment sets 2 and 3 were kept on high-fat diet continuously for 9 weeks. Body weight and food intake were measured weekly, as well as body composition using EchoMRI-100TM from Echo Medical Systems (Houston, TX). After the last intraperitoneal injection of clodronate liposomes, empty liposomes, free clodronate, or PBS, the mice were fasted overnight, blood collected, and then killed for tissue collection. The use of animals was approved by the Institutional Animal Care and Use Committee of the University of Georgia (protocol number, A2011 07-Y2-A3).

Intraperitoneal Glucose Tolerance Test

For intraperitoneal glucose tolerance test (IPGTT), mice were fasted overnight and intraperitoneally injected with glucose at 2 g/kg body weight. Blood glucose levels were measured using a glucometer at 0, 30, 60, 90, and 120 min after injection of glucose. Based on fasting glucose and insulin levels, we calculated the value of Homeostasis Model of Assessment-Insulin Resistance (HOMA-IR) using the following formula: $\text{HOMA-IR} = [\text{fasting insulin (ng/ml)} \times \text{fasting plasma glucose (mg/dl)}] / 405$ (10).

Histochemical Study

For hematoxylin and eosin (H&E) staining, selected tissue was freshly collected and immediately fixed overnight in 10% neutral buffered formalin. Tissue samples were dehydrated using gradient ethanol and were embedded in paraffin. Tissue sections were cut at 6 μm in thickness and dried at 37°C for 2 h. H&E staining were performed using a commercial kit from BBC Biochemical (Washington, DC). For frozen sections and Oil Red O staining, liver samples were frozen in liquid nitrogen and sectioned at 8 μm in thickness using a Cryostat. Tissue sections were placed on slides and washed with 60% isopropanol before being stained with Oil Red O from Electron Microscopy Sciences for 30 min and counterstained using hematoxylin. Tissue slides were examined using a Nikon ECLIPSE-Ti optical microscope, and pictures were taken and analyzed using Nikon NIS-Elements AR software.

Biochemical Analysis of Blood Samples

Blood samples from animals that were fasted overnight were collected and centrifuged to collect plasma. Plasma concentrations of glucose, insulin, and free fatty acids were determined, respectively (11).

Gene Expression Analysis

Liver and white adipose tissue samples were freshly collected and immediately frozen at -80°C until use. Total RNA was isolated using an RNeasy kit from Invitrogen. Two micrograms of total RNA were used for first-strand cDNA synthesis as recommended by the manufacturer. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green as an indicator on the ABI StepOne Plus Real-Time PCR system. The PCR was carried out for 40 cycles at 95°C

Table I. Primer Sequences for qRT-PCR Analysis of Gene Expression

Name	Forward sequence	Reverse sequence
<i>F4/80</i>	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
<i>Cd11c</i>	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACCTC
<i>Cd36</i>	CCTTAAAGGAATCCCCGTGT	TGCATTTGCCAATGTCTAGC
<i>Pepck</i>	AAGCATTCAACGCCAGGTTC	GGGCGAGTCTGTCAAGTTCAAT
<i>Acc-1</i>	GCCTCTTCCTGACAAACGAG	TGACTGCCGAAACATCTCTG
<i>Fas</i>	AGAGATCCCGAGACGCTTCT	GCCTGGTAGGCATTCTGTAGT
<i>Scd-1</i>	TTCTTACACGACCACCACCA	CCGAAGAGGCAGGTGTAGAG
<i>Il6</i>	GCTTCTTAGCGCTAGCCTCAATG	TGGGGCTGATTGGAAACCTTATTA
<i>Tnf-α</i>	TCCTGCATCCTGTCTGGAAG	GTCTTCTGGGCCACTGACTG
<i>Mcp-1</i>	CTGGATCGGAACCAATGAG	CGGGTCAACTTCACATTCAA
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

for 15 s and 60°C for 1 min. The final reaction mixture contained 20 ng of cDNA, 250 nM of each primer, 10 μ l of 2 $\times$ SYBR Green PCR Master, and RNase-free water to compensate the reaction mixture volume to 20 μ l. Fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. The data were normalized to GAPDH mRNA as an internal control. Primer sequences employed are summarized in Table I.

Statistical Analysis

Statistical analysis was done by one-way ANOVA. All data are reported as mean $\pm$ standard deviation (SD) with statistical significance set at $p < 0.05$.

RESULTS

Effect of Intraperitoneal Injection of Clodronate Liposomes on Macrophages in the Epididymal and Subcutaneous White Adipose Tissues and the Liver

Using F4/80 mRNA levels as a marker for macrophages, the effectiveness and time course of macrophage elimination by clodronate liposomes were determined in mice fed with a high-fat diet. Three types of tissue were examined including

visceral adipose tissue (VAT) present in the abdominal cavity, adipose tissue present in subcutaneous areas, and the liver. Results in Fig. 1(a) showed that VATMs decreased to approximately 15% of the original level on day 2, rose to $\sim$ 73% on day 4, and returned to their original level on day 6. Macrophages in the liver were less sensitive to the treatment exhibiting 70% of the original level on day 2 and 100% on day 4, (Fig. 1(c)). In contrast, the F4/80 level of subcutaneous adipose tissue remained unchanged in clodronate liposome-treated mice compared to those treated with empty liposomes (Fig. 1(b)). Figure 1(d) verified that clodronate liposomes had no effect on animal growth. These results suggested that clodronate liposomes were effective in depleting VATM, exhibited some activity against liver macrophages and had no effect on macrophages in subcutaneous adipose tissue. The temporary effect of clodronate liposome on VATM indicated that a schedule of twice weekly injections of clodronate liposomes was appropriate and it was adopted for the remainder of the study.

Effect of Chronic Depletion of VATMs on Mice Fed with a High-Fat Diet

Two sets of experiments were performed to examine the effect of depletion of VATM on high-fat diet-induced weight

Fig. 1. Effect of clodronate liposomes on elimination of macrophage in epididymal white adipose tissue, subcutaneous white adipose tissue, and liver of mice on high-fat diet. Mice were intraperitoneally injected with either clodronate liposomes (black circles) or equal volume of empty liposomes (white circles) on day 0 and kept on a high-fat diet. Three mice in each group were killed on days 0, 2, 4, and 6, respectively. Total RNA was prepared from the epididymal and subcutaneous adipose tissues and the liver, and qRT-PCR was performed to determine *f4/80* mRNA level. Plots show time-dependent change of *f4/80* mRNA levels in epididymal adipose tissue (a), subcutaneous adipose tissue (b); and in the liver (c). Mice body weight was measured every 2 days before killing (d). The value represents mean $\pm$ SD, $n = 3$. $^{##}p < 0.01$ and $p < 0.05$ between clodronate liposome-treated animals and controls

Fig. 2. Effect of VATM elimination on mice fed with a high-fat diet. Two sets of experiment were performed. In the first set, mice fed with a high-fat diet were injected twice weekly with PBS (white square), free clodronate (white triangles), empty liposomes (white circles), or clodronate liposomes (black circles) for 9 weeks and mouse body weight (a) and daily food intake (b) were measured with time. At the end of the experiments, mouse fat and lean mass (c, white bar, PBS-treated; gray bar, free clodronate-treated; dark gray bar, empty liposome-treated; and black bar, clodronate liposome-treated), H&E staining of adipocytes (d: a, PBS-treated; b, free clodronate-treated; c, empty liposome-treated; d, clodronate liposome-treated), and average size of white adipocytes (e) of the same groups of animals were examined. For experiment set 2, mice were fed with a high-fat diet for 4 weeks followed by twice weekly injection of clodronate liposomes (black circles) or empty liposomes (white circles) for 5 weeks. Mouse body weight (f) and daily food intake (g) were monitored with time. At the end of the 5-week treatment, mouse fat and lean mass (h), H&E staining of adipocytes (i: a, empty liposome-treated; b, clodronate liposome-treated), and average size of white adipocytes (j) of the same groups of animals were examined. The value represents mean $\pm$ SD, $n=5$. $^{*}p<0.01$ and $^{*}p<0.05$ between clodronate liposome-treated animals and controls. Bars in d and i represent 100 μ m

gain. The first set was designed to examine the preventive effect of VATM deletion on high-fat diet-induced obesity when clodronate liposome treatment started as animals were placed on a high-fat diet. The second set of experiments was designed to examine the effect of eliminating VATMs from animals preexposed with high-fat diet for 4 weeks. For the preventive effect, data in Fig. 2a showed that mice treated with clodronate liposomes suppressed weight gain. Nine weeks after high-fat diet feeding, mice treated with clodronate liposomes had an average body weight of 32 g, $\sim 30\%$ lower than that of control mice treated with PBS, free clodronate, or empty liposomes, and no significant differences were observed among the later three groups. Mice treated with clodronate liposomes, but not free clodronate or empty liposomes, were protected from obesity, demonstrating that this protective effect was solely due to a deficiency of VATMs rather than other factors. Chronic administration of clodronate liposomes did not alter food intake (Fig. 2b) compared to treatment with free clodronate, free liposomes, or PBS. Fat mass of clodronate liposome-treated animals was around 2 g compared to approximately 11 g in control animals, suggesting an importance of VATMs in fat accumulation. Similarly, the average size of adipocytes in clodronate liposome-treated animals was around 50–40 μ m smaller than the cells in control animals (Fig. 2d and e).

A similar study was conducted to examine the effect of eliminating VATMs in animals preexposed with a high-fat diet for 4 weeks. Results in Fig. 2f showed that clodronate liposome treatment suppressed the continuous gain of body weight compared to those treated with empty liposomes. On average, control animals treated with empty liposomes gained about 7.4 g from weeks 5 to 9, compared to 2.5 g for animals receiving twice weekly injections of clodronate liposomes. No difference in food intake was seen (Fig. 2g). A slightly lower fat mass and lean mass were observed in clodronate liposome-treated animals (Fig. 2h). Histochemical staining of VAT revealed no statistical difference in size of adipocytes (Fig. 2i, j). These results collectively suggested that elimination of VATMs blocked progression of obesity development.

Impacts of VATM Elimination on Blood Chemistry

Disorders in glucose and lipid metabolism are typical features of high-fat diet-induced obesity. To assess the impacts of elimination of VATMs on glucose/lipid homeostasis, we collected blood samples from mice fasted overnight at the end of treatment and measured glucose, free fatty acid, and insulin levels. Results of these measurements (Fig. 3a and c) showed that depletion of VATMs was associated with low levels of fasting glucose, insulin, and free fatty acids in animals chronically treated with clodronate liposomes for the entire 9 weeks. Interestingly, the same treatment in animals preexposed to a high-fat diet for 4 weeks did not result in a change of glucose and free fatty acid levels, but insulin level reduced from 9 ng/ml in empty liposomes-treated animals to approximately 2 ng/ml in animals treated with clodronate liposomes (Fig. 3d, f), indicating that VATM depletion reversed high-fat diet-induced insulin resistance.

To further investigate the impact of VATM depletion on glucose homeostasis, we performed IPGTT and calculated its area under the curve. Results of these tests and calculations showed that mice with VATM depletion also exhibited better glucose homeostasis compared to controls (Fig. 4a, d). The area under curve derived from IPGTT, as shown in Fig. 4b, e, showed more than a 60% reduction in animals treated with clodronate liposomes for 9 weeks, compared to approximately 20% in animals treated with clodronate liposomes for only the last 5 weeks of the 9-week time period. HOMA-IR (Fig. 4e, f) confirmed that mice treated with clodronate liposomes had higher insulin sensitivity.

Depletion of VATMs Prevented High-Fat Diet-Induced Hepatic Steatosis and Decreased Expression of Genes Involved in Gluconeogenesis and Lipogenesis in Mouse Liver

In addition to body weight gain, hepatic steatosis is also a common problem associated with high-fat diet. To investigate the impact of the depletion of VATMs on fat aggregation in the mouse liver, we measured liver weight and examined its morphological change using H&E and Oil Red O staining. Results of the liver weight measurement showed that mice treated with clodronate liposomes for 9 weeks had a lower ratio of liver to body weight (Fig. 5a). A similar trend was observed in animals preexposed with high-fat diet for 4 weeks, although the difference was not statistically significant (Fig. 5e). Results of H&E staining showed that depleting VATMs was associated with protection of mice from developing hepatic steatosis, as evidenced by less vacuole in tissue sections (Fig. 5b, f), compared to the empty liposome-treated controls. This conclusion was further confirmed via the results of Oil Red O staining showing less red dots in mouse liver treated with clodronate liposomes (Fig. 5e, g).

To explore the underlying mechanism, we next determined mRNA levels of genes involved in lipogenesis and gluconeogenesis using qRT-PCR. Results of the gene expression analysis showed that treatment with clodronate liposomes greatly decreased expression of genes involved in lipogenesis, including *accl*, *fas*, and *scd1* (Fig. 5d, h). Similar results were observed with

pepck (Fig. 5d, h), a key gene for gluconeogenesis. Collectively, this set of data demonstrated that depleting VATMs was associated with protection of mice from high-fat diet-induced hepatic steatosis, and this effect was partially mediated through downregulating expression of genes involved in lipogenesis and gluconeogenesis in the liver.

Depleting VATMs Prevented Formation of Crown-Like Structures in Adipose Tissues and Blocked Development of Chronic Inflammation

Crown-like structures are composed of macrophages and dead adipocytes, which are typical signs of chronic inflammation. To investigate the effect of clodronate liposomes on the

formation of crown-like structures, we conducted tissue section and performed immunohistochemical staining using an antibody against F4/80 protein, a specific marker for macrophages. As expected, mice treated with clodronate liposomes completely lacked crown-like structures (Fig. 6a, e). Consistent with this observation, results of gene expression analysis in animals treated for 9 weeks showed that treatment with clodronate liposomes resulted in a striking loss of gene marker for macrophages, including *f4/80* downregulation by ~95.3% and *cd11c* by ~93.1% (Fig. 6c). Similar data were obtained from preobese mice (Fig. 6f). We also measured the expression of genes involved in inflammation, including *tnf-α* and *mcp1*. Results of these measurements showed that injections of clodronate liposomes greatly

Fig. 3. Effect of VATM elimination on serum biochemistry. Fasting glucose, insulin, and free fatty acid levels were determined at the end of the 9-week treatment (a, b, and c) or at the end of 4-week no treatment followed by 5-week twice weekly treatment (d, e, and f). The values represent mean ± SD, n=5. ^{##}*p* < 0.01 and [#]*p* < 0.05 between clodronate liposome-treated animals and controls

Fig. 4. Effect of VATM elimination on insulin sensitivity. Glucose response curves (a, d), area under curve (b, e), and HOMA-IR (c, f) were generated by performing intraperitoneal glucose tolerance test and by determining serum concentration of insulin at the end of 9-week treatment (a, b, c) or at the end of the 4-week no treatment followed by 5-week twice weekly treatment (d, e, f). The values represent mean $\pm$ SD, $n=5$. ## $p<0.01$ and # $p<0.05$ between clodronate liposome-treated animals and controls

downregulated expression of these two genes (Fig. 6c, g). We next determined the blood levels of the TNF- α protein using ELISA. Consistent with gene expression data, treatment with clodronate liposomes significantly decreased the blood levels of TNF- α (Fig. 6d, h). These data clearly revealed that depletion of VATMs using clodronate liposomes completely prevented formation of crown-like structures and markedly suppressed high-fat diet-induced inflammation.

DISCUSSION

Macrophage infiltration and chronic inflammation are tightly correlated with fat accumulation in adipose tissue. In this study, we demonstrated that depleting VATMs (Fig. 1)

blocked initiation of obesity and suppressed weight gain without apparent influence on food intake (Fig. 2). Meanwhile, VATM depletion was associated with protection of mice from high-fat diet-induced insulin resistance, glucose intolerance (Figs. 3 and 4), and hepatic steatosis (Fig. 5). Mechanistically, results in Fig. 6 showed that depleting VATMs was linked to the lack of development of crown-like structures in adipose tissue and downregulated expression of genes involved in lipogenesis, gluconeogenesis, and chronic inflammation.

Obesity, characterized as a state of low level of inflammation, is a critical determinant leading to the development of insulin resistance, type-2 diabetes, and progression to fatty liver (8,12). A previous study shows

Fig. 5. Effect of VATM elimination on hepatic lipid content and expression of genes involving in lipid/glucose metabolism in the liver. Relative liver weight (**a**, **e**), H&E staining (**b**: *a*, PBS-treated; *b*, free clodronate-treated; *c*, empty liposome-treated; *d*, clodronate liposome-treated; **f**: *a*, empty liposome-treated; *b*, clodronate liposome-treated) and Oil Red O staining (**c**: *a*, PBS-treated; *b*, free clodronate-treated; *c*, empty liposome-treated; *d*, clodronate liposome-treated; **g**: *a*, empty liposome-treated; *b*, clodronate liposome-treated), and relative mRNA levels of selected genes in the liver were analyzed at the end of the 9-week treatment (**a–d**) or at the end of the 4-week no treatment followed by 5-week treatment (**e–h**). The values represent mean $\pm$ SD, $n=5$. $^{##}p<0.01$ and $^{#}p<0.05$ between clodronate liposome-treated animals and controls. The bars represent 20 μ m

that there is a four- to fivefold increase in adipose tissue macrophage content from the lean to the obese state (5), indicating an underlying relationship between macrophage-mediated inflammation and obesity. Other studies demonstrated that macrophages infiltrate adipose tissue at the onset of weight gain and directly contribute to and perpetuate the inflammatory state of fat in both mouse models and humans (7,13).

Liposomes can be used as vehicles for intracellular delivery of drugs into phagocytic cells. Clodronate delivered into macrophages in this way will kill these cells as a result of intracellular accumulation and irreversible metabolic damage. The so-called liposome-mediated macrophage “suicide” approach, which is based on this principle, is now frequently applied in studies aimed at macrophage function (14,29). By intraperitoneal injection of clodronate liposomes, we demonstrated that depletion of VATMs was linked to improvement in the inflammatory nature of the adipose microenvironment and protection of mice from high-fat diet-induced obesity (Figs. 2 and 6).

It is commonly accepted that macrophages play a pivotal role in adipose tissue remodeling, which requires coordinated regulations of angiogenesis (15) and modulations of the extracellular matrix (16). Accumulating evidence shows that modulating angiogenesis can affect adiposity in rodents by regulating the growth of adipose tissue vasculatures (17). The role of macrophages in regulating angiogenesis has been extensively studied. Through releasing a set of factors including TNF- α (18) and MCP-1 (19), macrophages actively initiate and accelerate angiogenesis. Consequently, the macrophages have been identified as a potential target for antiangiogenic therapies (20). In line with these previous studies, we believed that low fat mass in clodronate liposome-treated animals (Fig. 2) at least partially resulted from impaired angiogenesis.

Besides angiogenesis, the extracellular matrix is also considered important for adipose tissue remodeling. Recent studies demonstrate that the protein composition and dynamics of the extracellular matrix significantly affect adipose tissue function (21). The extracellular matrix not only functions to provide mechanical support for adipose tissue but also regulates the physiological and pathological events of adipose tissue remodeling through a

variety of signaling pathways. Interestingly, emerging evidence also shows there is a complicated crosstalk between macrophages and extracellular matrix scaffolds. The extracellular matrix is important for differentiating and activating macrophages. The activated macrophages in turn promote inflammation and extracellular matrix destruction, leading to tissue remodeling (22). Depleting VATMs, as shown in this study might also attenuate extracellular matrix destruction, thereby restricting visceral fat remodeling and growth.

Visceral adiposity is correlated with accumulation of excess lipids in the liver. Ectopic fat accumulated in the liver further activates resident macrophages, called Kupffer cells, and triggers an inflammatory response, which exacerbates hepatic steatosis. Depleting Kupffer cells by gadolinium chloride results in a beneficial effect on hepatic lipid accumulation in mice (23). Huang *et al.* (24) also reports similar results in rats, but obesity did not develop in the model used. Our results revealed that depleting VATMs was linked to prevention of high-fat diet-induced hepatic steatosis (Fig. 5). In addition, ectopic fat often results in cell autonomous impairment in insulin signaling (25). In the liver, insulin resistance is selective in that insulin fails to suppress gluconeogenesis, but continues to stimulate lipid synthesis (26). Coinciding with their study, our data showed VATMs depletion was associated with decreases in the expression of genes involved in gluconeogenesis and lipogenesis in the mouse liver, resulting in improved insulin resistance and alleviated glucose intolerance (Figs. 4 and 5).

In addition to its role in ectopic fat accumulation, visceral adipose tissue is also prone to inflammation and inflammatory cytokine production, which also contributes to insulin resistance (27). TNF- α , a prototypical inflammatory cytokine originally found from macrophages infiltrating into adipose tissue in obese mice, provides the first clues between inflammation and insulin resistance (28). A previous study focusing on short-term effect of eliminating VAT macrophage demonstrates that two times intraperitoneal injection of clodronate liposomes significantly decreases serum proinflammatory cytokines and mildly improves insulin sensitivity (29), however, does not affect body weight. Our data further demonstrate that long-term VATM depletion was associated with significant reduced systemic and local TNF- α level and improvement in insulin sensitivity. Besides, VATM depletion was also linked to a significant decrease in body weight (Fig. 3).

In summary, in this study, we demonstrate that VATM depletion was linked to protection of mice against high-fat diet-induced obesity, insulin resistance, and hepatic steatosis. These results suggest that macrophage infiltration into visceral adipose tissue might be a key feature of the inflammatory responses to high-fat diet-induced obesity. As far as prevention and treatment of obesity are concerned, VATMs may represent a potential target for drug development.

Fig. 6. Effect of VATM elimination on local and systemic inflammatory response. Immunohistochemistry using antibodies against F4/80 (**a**: *a*, PBS-treated; *b*, free clodronate-treated; *c*, empty liposome-treated; *d*, clodronate liposome-treated; **e**: *a*, empty liposome-treated; *b*, clodronate liposome-treated), relative mRNA levels of IL-6, TNF- α , and MCP-1 (**c**, **g**) in epididymal adipose tissues, and plasma concentration of TNF- α were analyzed in mice at the end of the 9-week treatment (**a–d**) or at the end of the 4-week no treatment followed by 5-week treatment (**e–h**). The values represent mean $\pm$ SD, $n=5$. $^{##}p<0.01$ and $^{#}p<0.05$ between clodronate liposome-treated animals and controls. Original magnification in **a** and **e** is $\times 40$, inset magnification is $\times 100$. Arrows in **a** and **e** point the crown-like structure

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Conflict of interest The authors claim no conflicts of interest.

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