Regulatory Mechanisms for Adipose Tissue M1 and M2 Macrophages in Diet-induced Obese Mice

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Objective: To characterize the phenotypic changes of adipose tissue macrophages (ATMs) under different conditions of insulin sensitivity.

Research design and methods: The number and the expressions of marker genes for M1 and M2 macrophages from mouse epididymal fat tissue were analyzed using flow cytometry after the mice had been subjected to a high-fat diet and pioglitazone treatment.

Results: Most of the CD11c-positive M1 macrophages and the CD206-positive M2 macrophages in the epididymal fat tissue were clearly separated using flow cytometry. The M1 and M2 macrophages exhibited completely different gene expression patterns. Not only the numbers of M1 ATMs and the expression of M1 marker genes, such as TNFα and MCP-1, but also the M1 per M2 ratio were increased by a high-fat diet (HFD) and decreased by subsequent pioglitazone treatment (Pio), suggesting the correlation with whole body insulin sensitivity. We also found that the increased number of M2 ATMs after an HFD was associated with the up-regulated expression of IL-10, an anti-inflammatory Th2 cytokine, in the adipocyte fraction as well as in adipose tissue. The systemic overexpression of IL-10 by an adenovirus vector increased the expression of M2 markers in adipose tissue.

Conclusions: M1 and M2 ATMs constitute different subsets of macrophages. Insulin resistance is associated with both the number of M1 macrophages and the M1/M2 ratio. The increased expression of IL-10 after an HFD might be involved in the increased recruitment of M2 macrophages.
Obesity and insulin resistance are closely associated with a state of low-grade inflammation in adipose tissue, where resident macrophages play important roles (1-9). Adipose tissue macrophages (ATMs) consist of at least two different phenotypes, i.e., classically activated M1 macrophages and alternatively activated M2 macrophages. A recent report proposed that M1 or M2 ATMs are distinguished by the presence or the absence of CD11c, an M1 macrophage marker (10). M1 ATMs produce proinflammatory cytokines, such as TNFα, IL-6 and MCP-1, thus contributing to the induction of insulin resistance (11-13). On the other hand, M2 ATMs, which are the major resident macrophages in lean adipose tissue, are reported to have a different gene expression profile, characterized by the relatively high expression of CD206, arginase-1, Mgl1 and IL-10, which are involved in the repair or remodeling of tissues (10-14).

Recent studies have demonstrated the involvement of M1/ M2 ATMs in the regulation of insulin sensitivity. The deletion of M1 marker genes such as TNFα (15) and CCR2 (8) or the ablation of CD11c-positive cells resulted in the normalization of insulin sensitivity (16). On the other hand, the mice with the macrophage-specific knockout of PPARγ or PPARβ/δ displayed insulin resistance with reduced number and impaired function of M2 macrophages (17-20). The latter studies also indicated that IL-4 or IL-13 from adipocytes or hepatocytes promotes the expression of PPARγ and PPARβ/δ in monocytes, resulting in the differentiation of M2 macrophages. Although it is generally accepted that M1 ATMs induce insulin resistance by secreting a variety of proinflammatory cytokines, how M2 ATMs contribute to the amelioration of insulin resistance is currently unknown.

Recently, Lumeng et al. used CD11c as an M1 marker in a flow cytometry analysis and reported that high fat diet-induced obesity causes a shift in ATMs from an M2 polarized state in lean animals to an M1 proinflammatory state (10). However, the precise mechanism of how the increased ratio of M1 macrophages is induced in diet-induced obese mice is not fully understood, e.g., are M1 macrophages newly recruited from circulating monocytes? Or do M2 macrophages present in lean adipose tissue differentiate into M1 macrophages?

To evaluate the changes in the number as well as the gene expression of M1 and M2 markers more precisely, we analyzed mouse ATMs by flow cytometry using CD206 as an M2 marker in addition to using CD11c as an M1 marker. Here we show that the CD11c-positive/CD206-negative M1 ATMs and the CD206-positive/CD11c-negative M2 ATMs constituted distinct populations. The number of M1 macrophages and the M1/M2 ratio are related to the development of insulin resistance. Interestingly, the overexpression of IL-10 by adenovirus vector increased the markers of M2 ATMs in adipose tissue, suggesting that enhanced IL-10 expression by a high-fat diet and/or pioglitazone treatment may be involved in a phenotypic switch in ATMs.

**RESEARCH DESIGN AND METHODS**

**Materials.** Pioglitazone was kindly provided by Takeda Pharmaceutical Company (Japan). A rat monoclonal antibody against mouse F4/80, a hamster monoclonal antibody against mouse CD11c, a rat CD206 antibody conjugated with Alexa 647 and a rat Mgl1 antibody conjugated with Alexa 488 were purchased from AbD-Serotec (Oxford, UK). A rat F4/80 antibody conjugated with FITC and a hamster CD11c antibody conjugated with phycoerythrin were purchased from eBiosciences (CA, USA), BD Biosciences
Regulation of adipose tissue M1/M2 macrophages (CA, USA) respectively.

Maintenance of mice. Six-week-old male C57BL/6J mice were purchased from CLEA Japan (Japan). The mice were maintained under a standard light cycle (12 h light/dark) and were allowed free access to water and food. They were fed on normal chow (NC) containing 10% calory from fat (Nosan Corporation, Japan) or a high fat diet (HFD) containing 60% fat (Research Diets, NJ) for 17 weeks. During the last 5 weeks of the diet intervention, half of the mice receiving the HFD were switched to HFD supplemented with pioglitazone (10mg/kg/day). The animal care policies and procedures for the experiments were approved by the animal studies committee at the University of Toyama.

Infection of Adenovirus Vector. A cDNA encoding human IL-10 was inserted into an adenovirus vector using the Adenovirus Expression Vector Kit (Ad-hIL-10) (Takara Bio, Japan) as described previously (21). Ad-X-DsRed2 (Ad-R2), an adenovirus vector encoding Discosoma sp. red fluorescent protein, was used as a control vector (Clontech Lab., Inc., CA). Ad-hIL-10 or Ad-R2 was injected, intraperitoneally.

Immunohistochemistry of Macrophages in White Adipose Tissues. F4/80-staining was performed using an amino acid polymer detection system and Histofine Mouse Stain Kit (Nichirei, Japan). The sections were incubated with anti-F4/80 antibody (dilution, 1:100) overnight at 4°C and were incubated with a secondary antibody of Histofine Simple Stain Max PO (Rat) for 30 min. For CD11c-staining, the sections were incubated with hamster anti-mouse CD11c antibodies (dilution, 1:20) for 3 h, then with goat anti-hamster IgG antibody (dilution, 1:100) for 1 h, and finally with Histofine Simple Stain Max PO (goat) for 30 min. Negative control studies were also performed without using these first antibodies. All sections were counterstained with hematoxylin. The total number of cells and crown-like structure were counted in ten different high-power fields from each section.

Isolation of Adipocytes and Stromal-vascular Fractions. Epididymal adipose tissues from mice were rinsed in saline minced into fine pieces, and digested with collagenase (Sigma-Aldrich, MO) with Krebs-Henseleit-HEPES buffer, pH7.4, supplemented with 20mg/mL of bovine serum albumin and 2 mmol/L glucose at 37°C using a shaker for 45 min. Then, the samples were passed through a mesh and fractionated by brief centrifugation (1000 rpm). The pellets or the floating cells were collected as the stromal-vascular fraction (SVF) or as the adipocyte fraction, respectively. Cells in each fraction were used for RNA extraction or flow cytometry analysis.

Flow Cytometry Analysis. Cells in the SVF were incubated in Pharm Lyse (BD Biosciences, CA) for 15 min at 4°C and resuspended in Pharmingen stain buffer (BD Biosciences, CA). The cells were incubated with 2.4G2 (BD Biosciences, CA) for 10 min, and then with primary antibodies or the matching control isotypes for 30 min at 4°C. Then, the cells were rinsed twice and resuspended in Pharmingen stain buffer. After incubating with 7-AAD (BD Biosciences, CA), the cells were analyzed using a FACS Aria cell sorter (BD Biosciences, CA). The data analysis was performed using FlowJo (Tree Star, OR). M1 or M2 macrophages were identified as F4/80-positive/CD11c-positive/CD206-negative or F4/80-positive/CD11c-negative/CD206-positive cells, respectively. The numbers of M1 or M2 macrophages were calculated by multiplying the number of trypan blue-negative cells by the ratio of F4/80-positive/CD11c-positive/CD206-negative cells or that of F4/80-positive/CD11c-negative/CD206-positive cells.

Quantitative RT-PCR. Total RNA was extracted from epididymal fat tissue or sorted
cells using the RNeasy kit (Qiagen, Germany). cDNA was synthesized with Multiscribe reverse transcriptase (Applied Biosystems, CA). Quantitative RT-PCR for each gene was performed using the TaqMan method (50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min) with Premade Primer sets (Applied Biosystems, CA). The relative abundance of transcripts was normalized according to the expression of 18S mRNA and was analyzed using a standard curve method.

**Statistical Analysis.** Results were presented as the means ± SEM. The data were analyzed using unpaired t-tests. Differences were considered statistically significant at p<0.05.

**RESULTS**

M1 macrophage markers were increased by high fat feeding and decreased by pioglitazone treatment, while M2 macrophage markers were not. Before testing the effects of high fat treatment or pioglitazone treatment on adipose tissue macrophages (ATMs), the effect of these treatments on glucose metabolism and adipose tissues were examined. High fat treatment for 17 weeks increased the glucose levels, in an intraperitoneal insulin tolerance test (ITT) and a glucose tolerance test (GTT) in C57BL/6 mice. Pioglitazone treatment for the last 5 weeks of 17 week-HFD treatment significantly lowered the glucose profile suggesting an improvement in insulin sensitivity and glucose metabolism (data not shown). The average adipocyte size in epididymal fat tissue was significantly larger in high fat-fed mice than in mice fed normal chow. Pioglitazone treatment decreased the adipocyte size by ~36 % (data not shown). We next examined the changes in the marker genes for macrophages in epididymal fat tissue with or without high fat treatment and pioglitazone treatment. The expression of F4/80 mRNA was increased by high fat feeding and was markedly reduced by pioglitazone treatment (Fig. 1A). ATMs were recently reported to consist of at least two subclasses, referred to as M1 and M2 macrophages (12; 13). The expressions of all the M1 markers examined were regulated very simply by high fat feeding and pioglitazone treatment, i.e., the mRNAs of CD11c, TNFα and MCP-1 were all up-regulated by high fat feeding and were down-regulated by pioglitazone treatment (Fig. 1B-D). On the other hand, the M2 markers, such as CD206, CD163, CD209a and IL-10, were not as simply regulated as those for the M1 markers (Fig. 1E-H). Some of the M2 markers were reduced but others were increased or not altered by high fat feeding and pioglitazone treatment. Interestingly, the expression of IL-10 was increased by high fat feeding and was further increased by pioglitazone treatment, although insulin sensitivity was deteriorated by the former and ameliorated by the latter. These changes in the M2 markers may have arisen as a result of the selective induction or suppression of such genes.

M1 and M2 macrophage fractions were clearly separated by flow cytometry using CD11c and CD206 as M1 and M2 markers, respectively. Recently, Lumeng et al. reported that adipose tissue M1 or M2 macrophages are defined by the presence or the absence of CD11c, an M1 marker (10). In this study, we extended Lumeng’s report and tried to separate M2 ATMs using CD206 as an M2 marker. After first sorting of the F4/80-positive cells from the stromal vascular fraction (SV fraction) of epididymal fat tissue from high fat-fed mice, the cells were then analyzed by flow cytometry using CD11c and CD206 as markers of M1 and M2 macrophages, respectively. As shown in Fig. 2A, clearly separated cell fractions were observed, i.e., most of the CD11c-positive cells were CD206-negative, whereas most of the CD11c-negative cells were CD206-
positive. As expected, the fractions showed very different gene expression profiles. CD11c, TNFα and IL-6 were highly expressed in the CD11c-positive/CD206-negative fraction but not in the CD11c-negative/CD206-positive fraction. Conversely, CD206, CD163, CD209a, IL-10 and Mgl2 were highly expressed in the CD11c-negative/CD206-positive fraction but not in the CD11c-positive/CD206-negative fraction (Fig. 2B). Accordingly, we identified the CD11c-positive/CD206-negative fraction as the M1 fraction and the CD11c-negative/CD206-positive fraction as the M2 fraction in this study.

Recently, Lumeng et al. identified Mgl1-positive F4/80 macrophages as M2a fraction (14). Thus, we examined whether the expression of CD206 was correlated with that of Mgl1. Flow cytometry analysis showed the expressions of these two markers were well correlated as shown in Fig. 2C. Thus, we believe that CD206 can be as useful a marker as Mgl1 for detecting the M2 fraction.

Numbers of M1 and M2 macrophages were increased by high fat feeding and decreased by pioglitazone treatment. Using this useful cell sorting system, we further examined whether the changes in M1 and M2 markers observed in whole epididymal fat tissue (Fig. 1) could be explained by changes in the number of M1 and M2 macrophages or by changes in the gene expression profiles of each cell. First, the cell numbers in the M1 and M2 fractions were evaluated in samples obtained from mice with or without high fat treatment or pioglitazone treatment (Fig. 3). The ratio of the M1 fraction in F4/80-positive cells was increased by high fat feeding and decreased by pioglitazone treatment, while the ratio of the M2 fraction was conversely decreased by high fat feeding and increased by pioglitazone treatment (Fig. 3C). However, it should be noted that the number of M2 ATMs normalized according to the weight of the epididymal fat tissue was increased by high fat feeding and decreased by pioglitazone treatment, similar to the changes in M1 ATMs (Fig. 3D), because the total number of ATMs normalized according to the weight of the epididymal fat tissue was remarkably increased by high fat feeding and decreased by pioglitazone treatment (Fig. 3B). These results suggest that the changes in the M1 markers in the whole epididymal fat tissue shown in Fig. 1 can be explained, at least in part, by the changes in the number of M1 ATMs, while the various changes in the M2 markers cannot be explained simply by the changes in the number of M2 ATMs. The changes in ATMs were also examined by immunostaining. As previously reported (22), F4/80-positive cells formed crown-like structures (CLS) in the epididymal fat tissue of high fat-fed mice, which were rarely observed in that of normal chow-fed mice. Pioglitazone treatment significantly decreased the number of CLS (Fig. 3E). Similar changes of CD11c-positive CLS by these treatments were also observed, indicating that the number of M1 ATMs was increased by high fat feeding and decreased by pioglitazone treatment (Fig. 3F, G).

Expression of M1 markers in each cell was not altered, but the expression of some M2 markers was up-regulated by pioglitazone treatment. We next evaluated the changes in the gene expression profiles of each cell in the M1 and M2 fractions. After sorting the cells into M1 and M2 fractions, the expressions of several M1 and M2 markers were measured. Because the cell count in the M1 fraction from normal chow-fed mice was very low, the expressions of M1 marker genes could not be measured by our usual methods in these mice. Thus, we compared the expressions of M1 and M2 markers in the M1 and M2 fractions normalized according to the number of macrophages only in high fat-fed mice. The expressions of the M1 markers in the M1 fraction were not altered by pioglitazone treatment, whereas some M2
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markers, such as IL-10 and CD209, were selectively increased in the M2 fraction (Fig. 4A-C). Taken together, these results suggest that the expressions of M1 markers in whole epididymal fat tissue is mainly regulated by the number of macrophages, while the expressions of M2 markers in whole tissue strongly reflects the gene expressions in each cell.

High fat feeding upregulated IL-10 expression in the adipocyte fraction. We next investigated the expression of cytokines in the adipocyte fraction, which may regulate the differentiation or recruitment of ATMs. The expression of TNFα or IL-6 in the adipocyte fraction was significantly increased or tended to be increased by high fat feeding, but the expression of MCP-1 was not (Fig. 5A-C). These data suggest the involvement of adipocyte-derived TNFα or IL-6 in the regulation of the profiles of ATMs, but the involvement of MCP-1 was less likely. Kang et al. recently reported that adipocyte-derived IL-4 and IL-13 upregulate myeloid PPARδ to promote the alternative activation of M2 macrophages (19). In contrast to Kang’s report, we could barely detect the gene expressions of IL-4 or IL-13 in the adipocyte fractions, although they were detected in the spleen (data not shown). On the other hand, the expression of IL-10 was significantly increased by a high-fat diet and decreased by pioglitazone treatment (Fig. 5D).

Systemic overexpression of IL-10 by adenovirus vector increased M2 macrophages in epididymal fat tissue. IL-10 has also been reported to promote the differentiation of M2 macrophages (13). To determine the impact of the enhanced expression of IL-10 in adipose tissue by a high fat diet and/or pioglitazone treatment (Fig. 1H, 4F), we examined whether IL-10 is involved in M2 macrophage recruitment to adipose tissue. IL-10 was systemically overexpressed in both normal chow-fed and high fat-fed mice through the intraperitoneal injection of adenovirus vectors. Serum IL-10 levels increased transiently and peaked at a serum level of ~300 pg/ml 4 days after injection (data not shown). Thus, the effects of IL-10 overexpression on the ATM profiles in epididymal fat tissue were evaluated on day 4. IL-10 overexpression increased the F4/80 mRNA in both normal chow-fed and high fat-fed mice (Fig. 6A). Unexpectedly, IL-10 overexpression did not suppress the expression of M1 macrophage markers, such as CD11c, MCP-1, TNFα, and IL-6 (Fig. 6B-E). Consistent with the data of M1 markers, IL-10 overexpression did not affect the number of F4/80-positive crown-like structures, which are reportedly formed mainly by M1 macrophages (Fig. 6F). Rather, the expression of all the M2 markers, e.g., CD206, Ym1, CD163, and CD 209a, were markedly up-regulated by the treatment (Fig. 6G-J). These results suggest that the enhanced expression of IL-10 is involved in the recruitment of M2 macrophages into adipose tissue.

DISCUSSION

Many previous studies have reported that the number of adipose tissue macrophages (ATMs) is elevated in both the subcutaneous fat and epididymal fat tissues of obese human subjects or rodents (1-3; 10). Recently, Lumeng et al. reported that CD11c-positive ATMs express proinflammatory cytokines such as TNFα and IL-6, while CD11c-negative ATMs express M2 markers such as Ym-1, arginase1 and IL-10. They defined the former as M1 ATMs and the latter as M2 ATMs (10). We extended their report and analyzed the SV fraction of adipose tissue using flow cytometry and CD11c and CD206 as M1 and M2 markers, respectively. We found that most of the CD11c-positive ATMs were CD206-negative and that most of the CD206-positive ATMs were CD11c-negative, indicating that these two subsets of ATMs constitute distinct populations (Fig. 2A). As
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previously reported (10), our current data also showed that CD11c-positive cells expressed several proinflammatory cytokines, such as TNFα, IL-6, and that CD206-positive cells expressed very different gene markers, including CD163, CD209, IL-10 and Mgl2 (Fig. 2). These results demonstrate that M1 and M2 macrophages can be more clearly distinguished using CD206 as an M2 marker in addition to using CD11c as an M1 marker. Recently, Lumeng et al. used Mgl1 as a surface marker for M2 macrophages and identified M2 macrophage population that expresses IL-10 at high levels (14). Our flow cytometry analysis showed that the expression of CD206 and Mgl1 were strongly correlated (Fig. 2C). We believe that CD206 is as good a marker as Mgl1 for detecting M2 populations. As for double negative cells for CD206 and CD11c, these cells expressed lower levels of M1 markers such as TNFα and IL-6 than CD11c-single positive F4/80-positive cells. In addition, they expressed lower levels of M2 markers such as Mgl2, IL-10 and CD163 than CD206-single positive F4/80-positive cells (data not shown). It seems that they are less mature or less polarized type of macrophages.

Similar to previous reports (10), our present study showed that diet-induced obesity increased the number of CD11c-positive/ CD206-negative M1 macrophages by 65-fold, when normalized according to the weight of the epididymal fat (Fig. 3D). On the other hand, the number of CD11c-negative/ CD206-positive M2 macrophages per weight basis was also increased by 6 fold (Fig. 3D), although the ratio of M1/M2 macrophages was increased (Fig. 3C). The primary trigger for the recruitment of M1 macrophages is thought to be the secretion of TNFα from enlarged adipocytes (3). This cytokine is presumed to stimulate preadipocytes and endothelial cells to produce MCP-1, thus recruiting monocytes into the adipose tissue and stimulating M1 ATM differentiation (6; 10). In contrast, the molecular signals that trigger the recruitment of M2 macrophages in obese adipose tissues are not fully understood. The activation of PPARγ signaling in macrophages has also been reported to be necessary for the alternative activation of ATMs (17; 23). It was recently demonstrated that in response to adipocyte-derived Th2 cytokines, including IL-4 and IL-13, the expression of PPARβ/δ was induced, thus leading to the differentiation or recruitment of the alternative M2 phenotype in Kupffer cells and ATMs in mice (19; 20). We found that the gene expression of IL-10 was elevated in both adipose tissue and adipocyte fraction in obese mice (Fig 1H, 5D). Furthermore, the systemic overexpression of IL-10 increased M2 macrophages in epididymal fat tissue (Fig. 6). Thus, we speculated that the activation of IL-10 signaling as well as IL-4/IL-13 in monocytes may contribute to the increase in M2 ATMs.

Since IL-10 gene expression is increased in both the adipocyte fraction and the macrophage fraction, we speculated that IL-10 might be involved in M2 macrophage recruitment under HFD conditions. Adipocyte enlargement is known to be observed earlier than the accumulation of macrophages in adipose tissue (1; 2; 6). Thus, the increased expression of IL-10 in larger adipocytes might trigger the recruitment of M2 macrophages at an earlier stage.

In contrast, pioglitazone reduced the number of M2 macrophages while up-regulating the expression of IL-10. We presume that factors other than IL-10, which should be reduced by pioglitazone treatment, are responsible for the reduced number of M2 macrophages.

We also compared the expression of the IL-10 gene in the adipocyte fraction and the M2 macrophage fraction. We found that the expression of IL-10 in M2 macrophages was around 100-fold higher than that in adipocytes
Regulation of adipose tissue M1/M2 macrophages (data not shown), suggesting that M2 macrophage-derived IL-10 constitutes a major portion of the IL-10 in adipose tissue. Thus, the pioglitazone-induced increase in the whole adipose tissue can be explained by the increased expression of IL-10 in the M2 macrophages. Meanwhile, the reduced expression of IL-10 in the adipocytes had a minimal effect on the whole adipose tissue after pioglitazone treatment.

Then, what is the role of M2 macrophages in terms of insulin resistance? IL-10 is reported to improve insulin-stimulated glucose uptake in TNF-α treated 3T3-L1 adipocytes (10). In fact, our preliminary results indicated that IL-10 overexpression using adenovirus vector improves insulin sensitivity in normal chow-fed and high fat-fed mice. Thus, it may be possible that adipose tissue-M2 ATM-derived IL-10 may contribute not only to anti-inflammatory effects in adipose tissue but also to improving insulin signaling in adipocytes.

Whether the increase in the M1/M2 ratio in obese mice is due to the transdifferentiation of M2 macrophages into M1 macrophages or the de novo recruitment of monocytes into the adipose tissue has been a topic of investigation. Because our data showed that the numbers of both M1 and M2 macrophages increased, we presumed that the phenotypic shift of ATMs toward an M1-dominant state cannot be explained simply by the conversion of M2 macrophages to M1 macrophages, but rather by the de novo recruitment of circulating monocytes to adipose tissue followed by their differentiation into M1 and M2 macrophages. Consistent with this scenario, a recent report by Lumeng et al also demonstrated that pulse-labeled ATMs were recruited from spatially distinct regions to the adipose tissue of diet-induced obese mice (14).

Pioglitazone reduced the number of both M1 and M2 ATMs. We previously hypothesized that the size of adipocytes is inversely related to insulin sensitivity based on the observation that a thiazolidinedione increases the number of smaller adipocytes and decreased the number of larger adipocytes (24; 25). Since larger adipocytes produce more ROS, ROS might be one of the triggers that recruit macrophages into adipose tissue (26). Once a thiazolidinedione is administered, the decreased ROS production by smaller adipocytes may result in the reduction in the total number of ATMs. The marked reduction of M1 ATMs may explain, at least in part, the hypothesis that smaller adipocytes are associated with insulin sensitivity. As for M2 ATMs, the increased expression of IL-10 after pioglitazone treatment may explain the observation that the reduction of the M2 number was less than that of the M1 ATM number. Pioglitazone may directly bind to the PPRE sites in the IL-10 gene promoter and stimulate the IL-10 gene expression within macrophages (23; 27). The increased expression of IL-10 by pioglitazone may contribute to the amelioration of insulin resistance (28-30).

In this study, we identified two clearly separate macrophage fractions in flow cytometry analysis using CD206 as a marker for M2 macrophages in addition to CD11c, a well established M1 marker. These two fractions exhibited completely different gene expression patterns. We found that the number of M1 ATMs and M1/M2 ratio were closely associated with insulin resistance in an HFD+pioglitazone group as well as an HFD group. Furthermore, we also suggested that IL-10 upregulated under HFD conditions might be involved in the M2 macrophage recruitment, thus contributing to reducing inflammation and improving insulin signal.

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REFERENCES
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Figure Legends

Figure 1. Effect of high fat diet and pioglitazone on gene expressions of epididymal fat tissue. Total RNA was isolated from the whole epididymal fat tissue and real time RT-PCR for the indicated genes was conducted as described in the Research Design & Methods section. Each data was normalized according to 18S mRNA level and presented as a value relative to that for normal chow-fed mice. The results are shown as mean ± SEM for 5 to 8 animals per group. *p<0.05, **p<0.01

Figure 2. Gene profiles of macrophages positive or negative for CD11c and CD206 in epididymal fat tissue. Cells in the stromal vascular fraction of epididymal fat tissue from mice fed a high fat diet (HFD) for 12 weeks were analyzed using flow cytometry. F4/80-positive cells were further analyzed with anti-CD11c and anti-CD206 antibodies. Representative flow cytometry results are shown in (A). Blue dots show isotype control. Total RNA was isolated from F4/80-positive/ CD11c-positive/ CD206-negative cells or F4/80-positive/ CD11c-negative/ CD206-positive cells. Real time RT-PCR for the indicated genes was conducted as described in the Research Design & Methods section (B). Each data was normalized according to the 18S mRNA level. The results are shown as the mean ± SEM for 4 to 7 animals per group. *p<0.05, **p<0.01. The correlation between CD206 expression and Mgl1 expression was examined. Representative results are shown in (C).

Figure 3. Effect of high fat diet and pioglitazone treatment on the number of M1/M2 macrophages in epididymal fat tissue. Cells in the stromal vascular fraction of epididymal fat tissue were analyzed using flow cytometry as described in Fig. 2. The cell numbers in the F4/80-positive fraction (B), F4/80-positive/ CD11c-positive/ CD206-negative fraction (black bars) or F4/80-positive/ CD11c-negative/ CD206-positive fraction (white bars) (C, D) were counted as described in the Research Design & Methods section. The cell numbers in the CD11c-positive/ CD206-negative and CD11c-negative/ CD206-positive fractions are shown according to the percentage of each fraction per total F4/80-positive cells (C), the cell number in each fraction from 1 g of epididymal fat tissue (D). Epididymal fat tissues were stained with anti-F4/80 (E) or anti-CD11c antibody (F, G). (E, F) The number of crown-like structures (CLS) was counted as described in the Research Design & Methods section. The results are shown as the mean ± SEM for 5 to 8 animals per group. *p<0.05, **p<0.01. Representative images with anti-CD11c antibody are shown in (G). Bar = 200 µm.

Figure 4. Effect of pioglitazone on gene expressions in CD11c-positive and negative fractions. Macrophages in the F4/80-positive/ CD11c-positive/ CD206-negative fraction or the F4/80-positive/ CD11c-negative/ CD206-positive fraction of epididymal fat tissue were sorted using flow cytometry as described in Fig. 2. Total RNA was isolated, and real time RT-PCR for the indicated genes was conducted as described in the Research Design & Methods section. Each data was normalized according to the 18S mRNA level. The results are shown as the mean ± SEM for 6 animals per group. *p<0.05.

Figure 5. Effect of high fat diet and pioglitazone on gene expressions of adipocyte fraction in epididymal fat tissue. Total RNA from the adipocyte fraction was isolated, and real time RT-PCR for the indicated genes was conducted as described in the Research Design & Methods section. Each data was normalized according to the 18S mRNA level. The results are shown as
the mean ± SEM for 6 animals per group. *p<0.05.

**Figure 6. Effect of IL-10 overexpression on macrophage genes in epididymal fat tissue.** Mice were fed with a normal chow (NC) or a high-fat diet (HFD) and were subsequently injected with either Ad-hIL-10 (IL-10) or a control vector (R2), then sacrificed 4 days after injection. Purification of the mRNA and real-time RT-PCR for the indicated genes were performed as described in the Research Design & Methods section. (F) Epididymal fat tissues were stained with anti-F4/80 antibody. Then the number of crown-like structures (CLS) was counted, as described in the Research Design & Methods section. Results are shown as the mean ± SEM of at least 4 mice per group. *p<0.05; **p<0.01.
Figure 2

A

B

C

Regulation of adipose tissue M1/M2 macrophages
Figure 3

A

B

C

D

E

F

G

Regulation of adipose tissue M1/M2 macrophages
Figure 4

**F4/80, CD11c, CD206:**

A

[Graph showing relative expression of CD11c in HFD and HFD+Pi0 groups]

B

[Graph showing relative expression of TNFα in HFD and HFD+Pi0 groups]

C

[Graph showing relative expression of MCP-1 in HFD and HFD+Pi0 groups]

D

[Graph showing relative expression of CD206 in HFD and HFD+Pi0 groups]

E

[Graph showing relative expression of CD163 in HFD and HFD+Pi0 groups]

F

[Graph showing relative expression of IL-10 in HFD and HFD+Pi0 groups]

G

[Graph showing relative expression of CD209a in HFD and HFD+Pi0 groups]

Figure 5

**A**

[Graph showing relative expression of TNFα in NC, HFD, and HFD+Pi0 groups]

**B**

[Graph showing relative expression of IL-6 in NC, HFD, and HFD+Pi0 groups]

**C**

[Graph showing relative expression of MCP-1 in NC, HFD, and HFD+Pi0 groups]

**D**

[Graph showing relative expression of IL-10 in NC, HFD, and HFD+Pi0 groups]
Figure 6

A. F4/80
B. CD11c
C. MCP-1
D. TNF-α
E. IL-6
F. FA7B Receptor
G. CD206
H. Ifn1
I. CD163
J. CD206s

Regulation of adipose tissue M1/M2 macrophages