Loss of myeloid lipoprotein lipase exacerbates adipose tissue fibrosis with collagen VI deposition and hyperlipidemia in leptin-deficient obese mice

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During obesity, tissue macrophages increase in number and become proinflammatory, thereby contributing to metabolic dysfunction. Lipoprotein lipase (LPL), which hydrolyzes triglyceride in lipoproteins, is secreted by macrophages. However, the role of macrophage-derived LPL in adipose tissue remodeling and lipoprotein metabolism is largely unknown. To clarify these issues, we crossed leptin-deficient Lepob/ob mice with mice lacking the Lpl gene in myeloid cells (Lplm−/−;Lepob/ob mice) to generate Lplm−/−;Lep−/−;Lepob/ob mice. We found the weight of perigonadal white adipose tissue (WAT) was increased in Lplm−/−;Lep−/−;Lepob/ob mice compared with Lep−/−;Lepob/ob mice due to substantial accumulation of both adipose tissue macrophages and collagen that surrounded necrotic adipocytes. In the fibrotic epidydimal WAT of Lplm−/−;Lep−/−;Lepob/ob mice, we observed an increase in collagen VI and high mobility group box 1, while α-smooth muscle cell actin, a marker of myofibroblasts, was almost undetectable, suggesting that the adipocytes were the major source of the collagens. Furthermore, the adipose tissue macrophages from Lplm−/−;Lep−/−;Lepob/ob mice showed increased expression of genes related to fibrosis and inflammation. In addition, we determined Lplm−/−;Lep−/−;Lepob/ob mice were more hypertriglyceridemic than Lep−/−;Lepob/ob mice. Lplm−/−;Lep−/−;Lepob/ob mice also showed slower weight gain than Lep−/−;Lepob/ob mice, which was primarily due to reduced food intake. In conclusion, we discovered that the loss of myeloid Lpl led to extensive fibrosis of perigonadal WAT and hypertriglyceridemia. In addition to illustrating an important role of macrophage LPL in regulation of circulating triglyceride levels, these data show that macrophage LPL protects against fibrosis in obese adipose tissues.

Obesity, which is pandemic in most parts of the world, is characterized by chronic and low-grade inflammation (1). Within white adipose tissue (WAT) from obese mice and humans, crown-like structures are created by adipose tissue macrophages (ATMs) (2), which scavenge lipid droplets released by dying adipocytes. ATMs, which produce proinflammatory cytokines, might also contribute to insulin resistance (3). Obesity is also accompanied by excessive deposition of extracellular matrix within adipose tissues (4). As adipose tissue expands, local hypoxia induces the expression of hypoxia-inducible factor 1α in adipocytes, which conceivably mediates the fibrotic response in obesity (5). ATMs are also involved in the development of adipose tissue fibrosis (6). Obese mice develop hepatic steatosis with increased number of recruited hepatic macrophages (7).

Lipoprotein lipase (LPL) hydrolyzes triglycerides (TGs) in chylomicrons and very-low-density lipoprotein (VLDL), thereby liberating free fatty acids (FFAs) (8). This lipolytic reaction takes place at the luminal side of capillary endothelial cells, on which LPL is tethered to proteoglycans and glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 after translocation from its sites of synthesis. LPL is synthesized by various cells including adipocytes, cardiomyocytes, skeletal muscle cells, and macrophages. LPL activity requires correct folding, which is mediated by lipase maturation factor 1. Macrophages also synthesize LPL, where its actions might be exclusive of regulation of circulating TG levels (9).

In vitro studies showed that LPL secreted from macrophages stimulates foam cell formation by converting VLDL to lipoproteins with high affinity for lipoprotein receptors (10), increasing uptake of lipoproteins by acting as a receptor ligand, or by increasing the approximation of lipoproteins to the cell surface (11). Not surprisingly, macrophage LPL associates with greater atherosclerosis. Indeed, several laboratories including ours showed that mice lacking Lpl in bone marrow or due to a macrophage specific knockout had reduced atherosclerosis (12–14). Consistently, mice overexpressing Lpl in macrophages (15) or with LPL activity increased due to a deficiency of its inhibitor angiopoietin-like protein 4 (16) showed increased atherosclerosis. Moreover, the amount of LPL activity within an artery directly correlates with the extent of

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atherosclerosis (17), and this LPL is primarily expressed in macrophages (18, 19).

Because ATMs comprise as much as 50% of the cells in adipose tissue in severe obesity (20), we hypothesized that myeloid cell Lpl deficiency would decrease lipid accumulation in adipocytes by limiting the supply of lipoprotein-derived FFA. It is also possible that myeloid cell Lpl deficiency would ameliorate adipose tissue inflammation, because FFA generated during lipolysis might be proinflammatory (21). Together with the increased number of ATMs, the increased number of recruited hepatic macrophages in obese mice can substantially affect plasma lipoprotein metabolism via changing tissue LPL activity.

However, our previous study using Apoe−/− and Apoe-deficient mice lacking Lpl in myeloid cells (Lpl−/−;Apoe−/−) fed a Western-type diet for 12 weeks revealed no differences in body weight, weights of gonadal fat pad, or expression of genes specific for macrophages or inflammation in WAT (14). Similarly, a combined adipose and macrophage knockout had only minor effects on white adipose development and histology (22). This caused us to question whether the obesogenic effects of the Western-type diet were insufficient to see alterations in adipose tissue, especially in the setting of apoE deficiency (23).

To circumvent the limitation of the use of Western-type diet feeding, we used leptin-deficient Lepob/ob mice in the present study. Because Lepob/ob mice are more obesogenic than the Western-type diet, the use of Lepob/ob mice can allow us to detect the effects of myeloid cell Lpl deficiency on obesity-related phenotypes. We found that Lepob/ob mice lacking Lpl in myeloid cells (Lpl−/−;Lepob/ob) fed a regular laboratory diet developed more extensive fibrosis in epidydymal WAT (eWAT) and hypertriglyceridemia than Lepob/ob mice, while they had lower body weight. The ATMs of Lpl−/−;Lepob/ob mice showed increased expression of fibrogenic genes compared with those of Lepob/ob mice. Thus, myeloid cell-derived Lpl has a critical role in the regulation of adipose tissue fibrosis during the development of obesity.

**Results**

**Loss of myeloid Lpl exacerbated fibrosis in WAT in Lepob/ob background**

By gross inspection, eWAT from Lpl−/−;Lepob/ob mice was more brownish compared with that from Lepob/ob mice at the age of 24 weeks (Fig. 1A). Female perigonadal fat from Lpl−/−;Lepob/ob mice also showed the same appearance (data not shown). Adipocytes were significantly smaller in Lpl−/−;Lepob/ob mice than in Lepob/ob mice by 27% at the age of 24 weeks (Fig. 1, B and C), while adipocyte number per area did not differ between Lepob/ob and Lpl−/−;Lepob/ob mice (Fig. 1D). Masson’s trichrome and Sirius red staining revealed that the fibrotic areas were increased in Lpl−/−;Lepob/ob mice compared with Lepob/ob mice by 3.7- and 7.3-fold, respectively (Fig. 1, E–H). Consistent with these results, eWAT collagen content was increased in Lpl−/−;Lepob/ob mice compared with Lepob/ob mice by 1.8-fold (Fig. 1I). eWAT TG content per gram of tissue in Lpl−/−;Lepob/ob mice was reduced compared with Lepob/ob by 36% (Fig. 1, J). On the other hand, eWAT TG content per whole tissue was not different between the two groups (Fig. 1, K). The number of necrotic perilipin-negative adipocytes surrounded by Mac-2-positive macrophages was increased in Lpl−/−;Lepob/ob mice compared with Lepob/ob mice by 4.7-fold (Fig. 2).

Similarly, perirenal fat from Lpl−/−;Lepob/ob mice was more brownish compared with that from Lepob/ob mice (Fig. 3A). Moderate but significant interstitial changes were also observed in the perirenal fat from Lpl−/−;Lepob/ob mice (Fig. 3B). Furthermore, inguinal subcutaneous fat from Lpl−/−;Lepob/ob mice was a little more brownish compared with that from Lepob/ob mice (Fig. 3D). Mild interstitial changes were also observed in the subcutaneous fat from Lpl−/−;Lepob/ob mice (Fig. 3E). Unlike eWAT, perirenal and subcutaneous adipocyte sizes were not different between Lepob/ob and Lpl−/−;Lepob/ob mice (Fig. 3, C and F).

The expression of fibrosis-related genes and collagen VI (Col6) is increased in WAT in Lpl−/−;Lepob/ob mice

To determine whether the brownish appearance of eWAT from Lpl−/−;Lepob/ob mice reflected browning (enrichment of beige adipocytes) or inflammatory and fibrotic changes in the eWAT from Lpl−/−;Lepob/ob mice, we measured mRNA levels of uncoupling protein 1 (Ucp1), macrophage marker genes, and genes involved in inflammation and fibrosis in the eWAT at the age of 24 weeks. The expression of Ucp1 was not increased in Lpl−/−;Lepob/ob compared with Lepob/ob mice (Fig. 4A), indicating that browning, an increase in the number of beige adipocytes, did not cause brownish coloring of the eWAT of Lpl−/−;Lepob/ob mice. We did note an increase in expression levels of macrophage markers in these mice. Cd68 and F4/80 were expressed at much higher levels with the ob/ob background (Fig. 4B). Cd68 and Cd11b were 81% and 4.4-fold higher in Lpl−/−;Lepob/ob than in Lepob/ob mice, respectively, although the absence of Lpl did not markedly affect the expression levels of F4/80 or Cd11b on the wildtype background (Fig. 4B).

We next checked the expression levels of proinflammatory M1 macrophage markers (Cd11c, Tnfα, monocyte chemotactic protein-1 (Mcp1), Il6, and Nos2) (Fig. 4C). Cd11c and Tnfα were expressed at much higher levels with the ob/ob background. The mRNA expression of Cd11c and Il6 were 3.2-fold and 5.8-fold higher in Lpl−/−;Lepob/ob than in Lepob/ob mice, respectively, while there were no significant differences in the mRNA expression of Tnfα, Mcp1, and Nos2 between the two groups.

With regard to the expression levels of anti-inflammatory M2 macrophage markers (Fig. 4D), Il10 was expressed at higher levels, while Fizz1 (also known as resistin like alpha [Retnla]) was expressed at lower levels compared with ob/ob background. Lpl−/−;Lepob/ob mice had significantly higher mRNA levels of Cd206 than Lepob/ob mice, while Lpl−/−;Lepob/ob mice had significantly lower levels of Fizz1 than Lepob/ob mice. There was no difference in the expression of either Il10 or Arg1 between Lepob/ob and Lpl−/−;Lepob/ob mice.
Figure 1. Loss of myeloid Lpl augments adipose tissue fibrosis in Lepob/ob mice. (A) gross appearance of eWAT at the age of 24 weeks. Two mice fat pads are shown in each genotype. The scale bar represents 2 cm. (B) histology of eWAT stained with H&E. (C, D) the average adipocyte size and number of adipocytes per area in eWAT were quantified (n=5–7). (E, F) Masson’s trichrome staining in eWAT and quantification of Masson’s trichrome–positive areas (indicated by an arrow) (n = 6). (G, H) Sirius red staining in eWAT and quantification of Sirius red–positive areas (indicated by an arrow) (n = 6). (I) collagen content in eWAT. (J) eWAT TG content per gram of tissue (n = 8). (K) eWAT TG content per whole tissue was calculated by multiplying the whole tissue weight (n = 8). The scale bar represents 200 μm (B, E, G). Values are expressed as means ± SD. *p < 0.05, ***p < 0.001. eWAT, epidydimal white adipose tissue; TG, triglyceride.
Finally, we checked the expression levels of the genes related to fibrosis, Hif1a, Tgb1, tissue inhibitor of metalloproteinase 1 (Timp1), Col1a1, Col3a1, Col6a3, and Acta2 (Fig. 4E). Tgb1, Timp1, Col1a1, Col3a1, and Col6a3 were significantly higher in Lpm/m−;Lepob/ob than in Lepob/ob mice by 36%, 186%, 104%, 58%, and 74%, respectively. The expression of Acta2 (also known as α-smooth muscle actin, a marker of fibrogenic myofibroblasts (24), was significantly decreased in Lpm/m−;Lepob/ob mice.

To confirm the findings in the mRNA expression at the protein level, we performed immunoblot analyses of Acta2, Timp1, Col1, Col3, and Col6 in the eWAT (Fig. 5A). Consistent with the gene expression, Acta2 protein was markedly decreased in Lpm/m−;Lepob/ob mice. Timp1 protein was not significantly different between four groups of mice. Among the three types of collagen, the changes in Col6 were most striking; Lpm/m−;Lepob/ob mice had a 5.5-fold higher content of Col6 in eWAT than Lpl/fl/fl mice (Fig. 5B). Similar to the expression pattern of Col6, Lpm/m−;Lepob/ob mice had a 2.9-fold higher content of high mobility group box 1 (HMGB1) protein, a nuclear nonhistone DNA-binding protein that is implicated in inflammation and efferocytosis (25), in eWAT than Lepob/ob mice (Fig. 5, A and B). Although the protein contents of the eWAT of Lpm/m−;Lepob/ob mice were 2- to 3-fold higher than those of Lpl/fl/fl, Lpm/m−/m−, or Lepob/ob mice (Lpl/fl/fl, 11.0 ± 2.3 mg/g; Lpm/m−/m−, 13.1± 1.7 mg/g; Lepob/ob, 11.4 ± 1.9 mg/g; Lpm/m−/m−;Lepob/ob, 30.8 ± 4.2 mg/g wet tissue, n = 6), we loaded the same amounts of proteins (30 μg) to each lane of the gels, therefore, the increases in the protein levels of collagens, Timp1, and HMGB1 should be much higher than the above values when expressed per wet tissue weight.

We also measured mRNA levels of the genes in subcutaneous fat (Fig. 6), which was less fibrotic than eWAT in Lpm/m−;Lepob/ob mice (Fig. 3, D and E). The mRNA expression patterns were essentially similar to those in eWAT, except that the induction of the expression of Cd206, Col1a1, Col3a1, and Col6a3 in Lpm/m−/m−;Lepob/ob mice compared with in Lepob/ob mice was not observed, corroborating the morphological findings that there was no significant difference in the extent of fibrosis in subcutaneous fat between Lepob/ob and Lpm/m−/m−;Lepob/ob mice (Fig. 3E).

The expression of fibrosis-related genes is increased in ATMs from Lpm/m−/m−;Lepob/ob mice

To determine whether the changes in fibrotic genes were due to macrophages or other cells within the eWAT, we collected F4/80-positive cells from the stromal vascular fraction (SVF) of the eWAT using a magnetic activated cell sorting system and compared the mRNA expression (Fig. 7). Because
the extensive fibrosis prohibited us to isolate SVF from 24-week-old Lpl<sup>m<sup>-/m<sup>-;Lep<sup>ob<sub>ob</sub></sup> mice, we used 16-week-old mice whose fibrotic changes in the adipose tissue were milder (Fig. S1A). The eWAT mRNA expression levels of macrophage markers and the genes related to tissue fibrosis were essentially similar to those in eWAT of 24-week-old mice with some exceptions (Fig. S1, B and C): the expression of F4/80 was increased, but the expression of Timp1 and Col6a3 was not increased in Lpl<sup>m<sup>-/m<sup>-;Lep<sup>ob<sub>ob</sub></sup> mice compared with Lep<sup>ob<sub>ob</sub></sup> mice.

ATMs from Lpl<sup>m<sup>-/m<sup>-;Lep<sup>ob<sub>ob</sub></sup> mice had negligible expression of Lpl mRNA compared with those from Lep<sup>ob<sub>ob</sub></sup> mice (Fig. 7A), confirming that ATMs were successfully isolated and that Lpl was successfully ablated in ATMs. The expression levels of genes related to tissue fibrosis (Hif1a, Tgfb1, and Timp1) were higher in ATMs from Lpl<sup>m<sup>-/m<sup>-;Lep<sup>ob<sub>ob</sub></sup> compared with those from Lep<sup>ob<sub>ob</sub></sup> mice by 44%, 37%, and 91%, respectively (Fig. 7D). As had been noted in WAT, the expressions of M1 and M2 markers did not change in a constant manner (Fig. 7, B and C).

**Figure 3.** Gross appearance and histology of perirenal and subcutaneous adipose tissues. (A, D) perirenal and subcutaneous (inguinal) fats were taken from 24-week-old male mice for gross inspection. Two mice fat pads are shown in each genotype. The scale bar represents 2 cm. (B, E) H&E staining. The scale bar represents 200 μm. (C, F) the average adipocyte size was quantified (n = 5–6). Values are expressed as means ± SD.
Despite the presence of greater numbers of necrotic cells (Fig. 2), the mRNA expression of the genes involved in efferocytosis, Mer tyrosine kinase (MerTK), Low-density lipoprotein receptor-related protein 1 (Lrp1), and growth arrest-specific 6 (Gas6), was higher in ATMs from \( \text{Lpl}^{-/-}\text{m}^{-/-}\text{Lep}^{ob/ob} \) compared with those from \( \text{Lep}^{ob/ob} \) mice by 138%, 25%, and 181%, respectively (Fig. 7E). This suggests that greater gene expression was reactive, the receptors were not functional, or the creation of necrotic cells overwhelmed the uptake process.

**Figure 4. mRNA expression profiles in epidydimal white adipose tissue.** Total RNA was extracted from epidydimal white adipose tissue of male mice with four genotypes at the age of 24 weeks. The mRNA expression levels were determined by real-time quantitative PCR. (A) Ucp1, (B) macrophage markers, (C) inflammatory M1 macrophage markers, (D) M2 macrophage markers, (E) tissue fibrosis related genes (n = 7). \( \text{Lpl}^{ob/ob} \) (white bars), \( \text{Lpl}^{-/-}\text{m}^{-/-}\text{Lep}^{ob/ob} \) (white hatched bars), \( \text{Lep}^{ob/ob} \) (gray bars), \( \text{Lpl}^{-/-}\text{m}^{-/-}\text{Lep}^{ob/ob} \) (gray hatched bars). Values are expressed as means ± SD. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
To examine the effect of the loss of myeloid Lpl on plasma lipoprotein metabolism, we measured plasma levels of total cholesterol (TC), TG, and FFA (Table 1). Lplm−/m−;Lepob/ob mice had 2.1-fold higher levels of plasma TG than Lepob/ob mice but 39% lower levels of plasma TC. The FFA levels were not different between the two groups. There were no significant differences in plasma levels of TC, TG, or FFA between Lplfl/fl and Lplm−/m− mice. As is known to occur with the ob/ob background, Lepob/ob mice had 3.3-fold higher levels of plasma TC than Lplfl/fl mice.

High-performance liquid chromatography (HPLC) analyses of plasma lipoproteins showed that plasma levels of total cholesterol (TC), TG, and FFA between Lplfl/fl and Lplm−/m− mice. As is known to occur with the ob/ob background, Lepob/ob mice had 3.3-fold higher levels of plasma TC than Lplfl/fl mice.

High-performance liquid chromatography (HPLC) analyses of plasma lipoproteins showed that VLDL-TG was increased (Fig. 8B) and low-density lipoprotein and high-density lipoprotein cholesterol were decreased (Fig. 8A) in Lplm−/m−;Lepob/ob mice compared with Lepob/ob mice. LPL activity in post-heparin plasma was 45% less in Lplm−/m−;Lepob/ob mice than in Lepob/ob mice (Fig. 8C). mRNA expression levels of Lpl in Lplm−/m−;Lepob/ob mice were 78% lower in eWAT, 45% lower in subcutaneous fat, and 37% lower in quadriceps than in Lepob/ob mice (Fig. 8D). These results suggest that the reduced synthesis of macrophage-derived LPL in both adipose tissues and skeletal muscles contributed to the reduced activities of post-heparin lipolytic activity. Hepatic VLDL-TG production assessed by Triton WR1339 inhibition of lipolysis showed that production was increased in Lplm−/m−;Lepob/ob mice compared with that in Lepob/ob mice (Fig. 8E). Therefore, both impaired clearance and overproduction of VLDL-TG contribute to the increased plasma level of VLDL-TG. To clarify how loss of Lpl increased hepatic VLDL production in ob/ob background, we measured mRNA expression of genes related to VLDL secretion (Fig. 8F). There were no significant differences in the expression of the genes involved in VLDL secretion (Mttp or Apob), de novo lipogenesis (Srebp1c, Fas, and Acc1), or FFA β-oxidation (Cpt1a and Acox1). On the
contrary, the increased hepatic expression of Lpl in Lepob/ob mice was abrogated in Lplm−/m−;Lepob/ob mice.

Next, we evaluated hepatic steatosis (Fig. 8F). Lepob/ob mice showed a 1.8-fold increase in hepatic TG content compared with Lplfl/fl mice. There was no difference in hepatic TG content between Lepob/ob and Lplm−/m−;Lepob/ob mice. Consistently, there was no discernable difference in the pathological features of fatty liver disease such as ballooning of hepatocytes (Fig. S3A) and the extent of oil red O staining (Fig. S3B).

Loss of myeloid Lpl reduces body weight in Lepob/ob mice

We measured body weight every week and weighed the organs at 24 weeks of age (Fig. 9, A–D). Both male and female Lplfl/fl and Lplm−/m− mice gained weight at similar rates over the entire observation period (Fig. 9, A and B). The weight of perigonadal, perirenal, mesenteric, and subcutaneous (inguinal) WAT was not different between the two groups (Fig. 9, C and D). Lepob/ob mice gained significantly more weight than Lplfl/fl and Lplm−/m− mice throughout the study period (from 6 to 24 weeks of age) (Fig. 9, A and B). Beginning
at 15 to 16 weeks, both male and female \( Lpl^{−/m} - Lepob/ob \) mice gained significantly less weight than \( Lepob/ob \) mice. At the age of 24 weeks, \( Lpl^{−/m} - Lepob/ob \) mice were 17% lighter than \( Lepob/ob \) mice of the same sex (Fig. 9, A and B). \( Lpl^{−/m} - Lepob/ob \) mice also had less subcutaneous fat than \( Lepob/ob \) mice; the decrease was 34% in males and 28% in females. By contrast, \( Lpl^{−/m} - Lepob/ob \) perigonadal fat pads (also termed eWAT in males) were 44% heavier than those from male

Figure 7. mRNA expression profiles in adipose tissue macrophages. F4/80-positive adipose tissue macrophages were collected from epidydimal white adipose tissue of mice with two genotypes at the age of 16 weeks by magnetic sorting. The mRNA expression levels were determined by real-time quantitative PCR: (A) \( Lpl \), (B) inflammatory M1 macrophage markers, (C) M2 macrophage markers, (D) tissue fibrosis-related genes, (E) efferocytosis-related genes \((n = 6)\). \( Lpl^{−/m} \) (gray bars), \( Lpl^{−/m} - Lepob/ob \) (gray hatched bars). Values are expressed as means ± SD.* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).

Table 1

| Blood glucose and lipids                  | \( Lpl^{−/ft} \) | \( Lpl^{−/m} − \) | \( Lpl^{−/ob} \) | \( Lpl^{−/m}−Lep^{−/ob} \) |
|------------------------------------------|-----------------|-----------------|-----------------|--------------------------|
| FBG (mmol/l)                             | 2.94 ± 0.58     | 2.98 ± 0.56     | 6.64 ± 1.84     | 4.99 ± 1.07              |
| Insulin (ng/ml)                          | 0.36 ± 0.28     | 0.24 ± 0.12     | 4.60 ± 2.30     | 3.70 ± 0.64              |
| HOMA-IR                                  | 1.2 ± 0.8       | 0.8 ± 0.3       | 32.5 ± 10.4     | 21.1 ± 4.8               |
| \( ad \) lib BG (mmol/l)                 | 6.63 ± 0.69     | 6.25 ± 0.87     | 8.70 ± 1.89     | 6.16 ± 1.76              |
| Total cholesterol (mmol/l)              | 2.43 ± 0.25     | 2.26 ± 0.27     | 8.11 ± 0.87     | 4.92 ± 0.80              |
| Triglyceride (mmol/l)                    | 1.01 ± 0.41     | 0.99 ± 0.41     | 1.44 ± 0.46     | 3.03 ± 1.81              |
| FFA (mmol/l)                             | 2.0 ± 0.2       | 1.8 ± 0.5       | 1.2 ± 0.3       | 1.1 ± 0.1               |

FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of insulin resistance.

Blood samples were collected after fasting for 16 h at the age of 22 to 24 weeks (n=7–11) or \( ad \) libitum fed (at the beginning of light cycle) mice at the age of 24 weeks (n=5–10). HOMA-IR was calculated as FBG (mg/dl) x fasting plasma insulin (μIU/ml) divided by 405.

* \( p < 0.05 \) compared with \( Lpl^{−/ft} \) mice.

** \( p < 0.01 \) compared with \( Lpl^{−/m}− \) mice.

*** \( p < 0.001 \) compared with \( Lpl^{−/ob} \) mice.

* \( p < 0.05 \) compared with \( Lpl^{−/m}− \) mice.

** \( p < 0.01 \) compared with \( Lpl^{−/ob} \) mice.

*** \( p < 0.001 \) compared with \( Lpl^{−/m}− \) mice.

Values are expressed as means ± SD.
Lepob/ob mice, while they were 34% heavier in females. There were no significant differences in the weight of mesenteric fat, brown adipose tissue, and liver between the two groups (Fig. 9, C and D). Computed tomography (CT) scan of the whole bodies revealed that subcutaneous fat mass was decreased in Lplm−/m−;Lepob/ob mice compared with Lepob/ob mice by 42% (Fig. S4). However, visceral fat mass was not different between the two groups. Therefore, the smaller gain of body weight in Lplm−/m−;Lepob/ob mice can be ascribed to the smaller weight gain in subcutaneous fat.

Lplm−/m−;Lepob/ob mice eat less food than Lepob/ob mice

To understand why Lplm−/m−;Lepob/ob mice were lighter than Lepob/ob mice, we measured food intake, core body temperatures, energy expenditure, and locomotor activity (Fig. 9, E–I). Cumulative food intake was not different between Lpffl/fl and Lplm−/m−;Lepob/ob (Fig. 9E). However, cumulative food intake in Lplm−/m−;Lepob/ob mice was significantly reduced from 19 weeks of age compared with intake by Lepob/ob mice (Fig. 9E). Daily food consumption, which was calculated from the food intake from 9 to 24 weeks of age, was reduced by 11% in Lplm−/m−;Lepob/ob mice compared with Lepob/ob mice (Fig. 9F). Core body temperatures were not different between Lepob/ob and Lplm−/m−;Lepob/ob mice (35.4 ± 0.8°C versus 35.2 ± 0.7°C, n=8–10). Oxygen consumption (VO2) and carbon dioxide production (VCO2) during light, dark, and 24-h periods and locomotor activity were not different between the two groups at 9 to 10 and 23 weeks of age (Fig. 9, G–I). The results were essentially the same when the data were analyzed for light
and dark phases separately (Fig. S5). In a pair feeding experiment, we did not find a significant difference in the changes of body weight when fed the same amount of diet (4.3 g) every day for 8 weeks from 12 to 20 weeks of age (Fig. 9J), supporting that the difference in the body weight gain reflected the difference in food intake but not that in energy expenditure.
We explored the reasons for the change in eating. Expression of orexigenic neuropeptides neuropeptide Y (Npy) and Agouti-related peptide (Agrp), which were increased in Lep−/− mice compared with Lpfl−/− mice, were not different between Lpl−/−/−;Lep−/−/− and Lpl−/−/− mice (Fig. S6). The expression of pro-opiomelanocortin (Pomc), an anorexigenic neuropeptide, which was reduced in Lep−/−/− mice compared with Lpfl−/− mice, was also not different between Lpl−/−/−;Lep−/−/− and Lep−/−/− mice (Fig. S6).

Discussion

In the present study, we investigated the effects of the loss of Lpl in myeloid cells on the morphology and gene expression in the WAT and other obesity-related phenotypes in a Lep−/− background. The most striking phenotypes were worsening of interstitial fibrosis with Col6 deposition and increased infiltration of ATMs with both proinflammatory and anti-inflammatory characteristics, which surrounded perilipin-negative (dead) adipocytes and disappearance of Acta2 expression in the perigonal WAT, but not in the subcutaneous WAT. We also found that Lpl−/−/−;Lep−/−/− mice had hypertriglyceridemia likely due to both reduced LPL activity and increased VLDL production, and decreased overall adiposity conceivably due to the decreased food intake.

First, we would like to discuss the phenotype in the WAT. The eWAT of Lpl−/−/−;Lep−/−/− mice contained substantial amounts of necrotic adipocytes (Fig. 2), as detected by perilipin-negative lipid droplets, with an increase in the number of ATMs, and extensive fibrosis detected using Mason’s trichrome and Sirius red staining (Fig. 1, E–H). The mRNA expression patterns of the eWAT from 24-week-old mice were largely consistent with the pathology. The loss of Lpl upregulated some of M1 (Cd11c) and M2 markers (Cd206 and Tgfb1) (Fig. 4, B–D). Concomitantly, genes related to fibrosis (Timp1, Col1a1, Col3a1, and Col6a3) increased (Fig. 4E). The changes in Cd11c, Cd206, Fizz1, Tgfb1, and Timp1 in the eWAT were also observed in the ATMs (Fig. 7). It is of note that some M2 markers were decreased (Fizz1) or unchanged (Arg1). In general, M2 macrophages are thought to be associated with fibrosis (26). Although we did not confirm these findings in flow cytometric analysis, which was difficult to perform because of the extensively fibrotic eWAT, we could conclude that the loss of Lpl polarized the ATMs toward both proinflammatory M1 (Cd11c) and anti-inflammatory M2 phenotypes (Cd206 and Tgfb1). Probably, M1 and M2 phenotypes are not mutually exclusive, as exemplified by adipose tissues that have both CD11c and CD206 in obese humans (27), and M2-like CD11c+ ATMs in obese mice (28).

In support of the results of mRNA expression, the protein contents of Col6, but not Timp1, Col1, and Col3, were significantly increased in the eWAT of Lpl−/−/−;Lep−/−/− mice compared with that of Lep−/−/− mice (Fig. 5). The remarkable accumulation of Col6 might have resulted from both increased synthesis and decreased degradation due to the increased Timp1, an inhibitor of many matrix metalloproteinases. The amounts of Col6 in WAT are reported to be correlated with WAT fibrosis, inflammation, and insulin resistance in mice (29) and humans (30, 31). Since leptin is known to inhibit Col6a3 expression (32), its deficiency might accentuate the Col6 overproduction, leading to WAT fibrosis in the current model of ob/ob background. However, a similar extensive fibrosis with Col6 deposition was observed in eWAT of Lpl−/−/− mice fed a high-fat diet containing 60 kcal% fat for 24 weeks (data not shown), indicating that obesity itself exacerbated WAT fibrosis in Lpl−/−/− mice irrespective of the presence of leptin signaling.

In adipose tissue, collagens are synthesized by various cell types such as adipocyte progenitors, adipocytes, fibroblasts, and myofibroblasts (4). mRNA and protein expressions of Acta2 were significantly reduced in the eWAT of Lpl−/−/−;Lep−/−/− mice compared with that of Lep−/−/− mice (Fig. 4E and 5). Consistently, Jager et al. reported a similar decrease in the expression of Acta2 in SVF from fibrotic eWAT of mice fed a high-fat diet compared with that from mice fed a low-fat diet (33). Since Acta2 is normally expressed in myofibroblasts (24) or platelet-derived growth factor receptor-α-positive adipocyte progenitors (34), it is unlikely that an increase in the number of these cells mediated the fibrosis in the eWAT of Lpl−/−/−;Lep−/−/− mice. Together with the finding that Col6, which is specifically secreted by adipocytes (29), is significantly increased in the eWAT of Lpl−/−/−;Lep−/−/− mice (Fig. 5), it is more conceivable that adipocytes are the origin of the accumulated collagens.

It is of note that adipose tissue fibrosis was not observed in Apoe−/− mice lacking myeloid Lpl (Lpl−/−/−;Apoe−/−) fed Western-type diet for 12 weeks in our preceding study (14). It is possible that the obesity attained by feeding Western-type diet for 12 weeks was not sufficient to cause adipose tissue fibrosis due to resistance to obesity of Apoe−/− mice (23). Another possible explanation is that changes in white blood cells due to leptin deficiency (35) or greatly accelerated adipose development promotes fibrosis when these changes are combined with the effects of Lpl deficiency.

The ATMs from Lpl−/−/−;Lep−/−/− mice showed high expression of efferocytosis-related molecules compared with the ATMs from Lep−/−/− mice (Fig. 7E). Similar upregulation of the efferocytosis-related genes was reported in mice deficient in mannose-binding lectin (36). These mice had an increased number of crown-like structures and apoptotic cells in adipose tissue due to defective clearance of dying adipocytes, together with enhanced expression of efferocytosis-related molecules. It is interesting to note that macrophage LPL enhanced Fc receptor-mediated phagocytosis especially under low glucose conditions in vitro (9). Indeed, LPL is a ligand for low-density lipoprotein receptor-related protein 1 (LRP1), which is an efferocytosis-related molecule (37). These results suggest that LPL in ATMs is required for the effective clearance of apoptotic cells, which, in this case, would be dying adipocytes. It is conceivable that the dying adipocytes induce ATM efferocytosis pathways, but the clearance inefficiency caused by LPL deficiency leads to release of alarmins or damage-associated molecular patterns such as HMGB1, which subsequently stimulate tissue fibrosis (38). Indeed, HMGB1 protein
content was markedly increased in the eWAT of Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) mice (Fig. 5). Although it is not known whether the accumulated HMGB1 is originated from undisposed dead adipocytes or from stimulated adipocytes and/or immune cells, it is reasonable to speculate that the HMGB1 accumulated in WAT stimulates fibrosis and inhibits efferocytosis of the dead adipocytes.

How did the loss of Lpl change the phenotype of ATMs? Fatty acids produced by lipolysis stimulate the expression of some pro- or anti-inflammatory markers in macrophages (39, 40). Moreover, M1 macrophages depend on glycolysis and oxidative phosphorylation of pyruvate, while M2 macrophages depend on fatty acid oxidation (41). Therefore, loss of Lpl might modulate macrophage phenotype by restricting the supply of fatty acids. Moreover, the alarmins produced from dying adipocytes might make the ATMs more fibrogenic, as discussed above.

LPL expressed in myocytes and adipocytes is thought to be responsible for regulation of circulating TG levels. For this reason, we did not expect Lpl\(^{m−/m−}\); Lep\(^{ob/ob}\) mice to have higher level of TG (Table 1) and VLDL (Fig. 8B). Because the LPL activity in the post-heparin plasma was decreased (Fig. 8C), the impaired clearance of VLDL likely contributed to the hypertriglyceridemia. In the eWAT and subcutaneous fat, the mRNA expression of Lpl was significantly decreased. This might reflect either the reduction in macrophage or adipose LPL expression. Given that ATMs comprise as much as 50% of cells in adipose tissue in severe obesity (20), loss of Lpl in ATMs likely contributes to the reduced Lpl expression in the WAT. However, the immunostaining for perilipin suggested most of the adipocytes were not alive in the eWAT of Lpl\(^{m−/m−}\); Lep\(^{ob/ob}\) mice (Fig. 2). Therefore, reduction of LPL secretion from adipocytes might also contribute to the reduced Lpl expression in the WAT. Skeletal muscle also contains macrophages whose numbers are increased with obesity (42). Therefore, it is possible that macrophages are also responsible for the reduced Lpl in muscle. In addition to the impaired clearance of VLDL, VLDL overproduction might contribute to the hypertriglyceridemia. Because there were not significant differences in plasma NEFA levels and the gene expression of VLDL assembly, de novo lipogenesis, and FFA β-oxidation, it is unlikely that VLDL secretion rate per hepatocyte was increased. We would rather ascribe the reduced hepatic expression of Lpl due to the lack of LPL secretion from hepatic macrophages, which are increased in ob/ob mice, to the VLDL overproduction.

Finally, we would like to discuss the obesity-related phenotype. How did the loss of myeloid Lpl decrease body weight gain and food intake in the setting of leptin deficiency? We previously reported that body weight of Apoe\(^{−/−}\) mice lacking myeloid Lpl (Lp\(^{m−/m−}\); Apoec\(^{−/−}\)) fed Western-type diet for 12 weeks and Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) mice fed a high-fat diet containing 45 kcal% fat for 10 weeks were not different from that of control groups (14). This initially suggested that leptin deficiency led to the reduction of body weight of Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) mice. Since there were no differences in energy expenditure, locomotor activity, and body weight gain in a pair feeding experiment, the attenuation of body weight gain in Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) mice should be attributable to the reduced food intake. Interestingly, similar attenuation of body weight gain was observed in Lep\(^{ob/ob}\) mice lacking hormone-sensitive lipase (Lipe) globally (43).

Because lysozyme is expressed in the brain cells such as microglia and neurons (44), it is possible that loss of Lpl in these cells modulated the appetite. Although deletion of Lpl in neurons under the control of NEX (MATH2, NeuroD6) made mice hyperphagic and obese (45), deletion of Lpl in microglia might have an opposite effect on appetite via modulating the activation status of microglia (46). Further studies are warranted to clarify the underlying mechanism.

In conclusion, the loss of myeloid Lpl led to extensive fibrosis of perigonadal WAT, hypertriglyceridemia, and reduced food intake in leptin-deficient mice. Moreover, we show that macrophage LPL assists in modulating circulating TG levels under conditions of metabolic stress. Increased adipose content of dead adipocytes suggests that, in some tissues, macrophage LPL is needed for efficient efferocytosis. Greater adipocyte synthesis of Col6 in mice deficient in both LPL and leptin suggests a secondary fibrotic response to impaired clearance of dead cells. These results should advance our understanding of the mechanisms by which ATMs control adipose tissue fibrosis in obesity and illustrate additional functions for LPL exclusive of lipid uptake.

Experimental procedures

The mice and antibodies used for the experiments were summarized in Table S1.

Mice

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at Jichi Medical University. Lp\(^{m−/m−}\) mice were generated by crossing floxed Lpl mice (47) with transgenic mice on a C57BL/6J background in which Cre recombinase was expressed in myeloid cells under the control of the lysozyme promotorm (Lys—Cre) (Jackson Laboratory) (14). Thereafter, Lp\(^{m−/m−}\) mice were crossed with mice heterozygous for leptin deficiency to generate Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) mice, which were then recrossed to produce Lp\(^{m−/m−}\); Lep\(^{ob/ob}\) and Lep\(^{ob/ob}\) littermates. Mice were fed a normal laboratory diet containing 4.8% (w/w) fat and 25.1% (w/w) protein (CE-2, Japan CLEA). All mice were maintained at controlled temperature (23°C) and humidity (50%) under a light 12 h/dark 12 h cycle in a room kept under a condition equivalent to specific pathogen free and given free access to food. No criteria except for age, gender, and genotypes were set for inclusion and exclusion. Mice were randomly assigned to each experimental group from littermates. If necessary, the mice were euthanized by cervical dislocation. All efforts were made to minimize potential co-founders and suffering.

M.T. was aware of group allocation. No adverse events were observed during the study period.
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**CT scans**

The body fat composition was analyzed by CT scans using LaTheta LCT-200 (Hitachi Aloka Medical) and LaTheta software (Hitachi Aloka Medical) according to the manufacturer’s protocol. Anesthetized mice were scanned at 1-mm intervals from nose to anus.

**Food intake**

Weekly food intake and body weight of female mice were measured after the mice were housed in an individual cage at 9 weeks of age.

**Pair-feeding experiment**

Based on the results of food intake, we calculated the average daily food intake from 12 to 20 weeks to be 4.3 g. We fed that amount of food every day for 8 weeks as described (48).

**Locomotor activity and indirect calorimetry**

After female mice were acclimated in an individual metabolic cage for 3 days, VO₂ and VCO₂ were measured using ARCO-2000 (ARCO System) for 4 days as described (49). Locomotor activity was measured using the ACTIMO-100 activity monitoring system (Shinfactory). The mice were used at 9 to 10 and 23 weeks of age.

**Measurements of glucose, insulin, lipids, and lipoproteins**

Blood glucose was determined by a FreeStyle blood glucose monitoring system (NIPRO). Plasma insulin was determined by mouse ELISA kits (Morinaga). TC, TG, and FFA concentrations were determined enzymatically using Determiner NEFA C-test Wako (Fuji fi), L TCII (Kyowa Medex), and NEFA C-test Wako (Fujifilm). Plasma lipoproteins were analyzed by HPLC (50). After the lipids were extracted from the liver and eWAT by methanol and chloroform, TG contents were measured with an enzymatic kit.

**Measurements of LPL activity**

Plasma was obtained 10 min after 300 U/kg of heparin was intravenously injected to the fasted mice for measurements of LPL activity in triplicates (14).

**Measurements of VLDL-TG production**

Hepatic VLDL-TG production was estimated by intravenous injection of 500 mg/kg of Triton WR1339 (50). Plasma TG concentrations were measured at 1, 2, 3, and 4 h after injection.

**Histology**

The tissues of 24-week-old mice were fixed with 4% paraformaldehyde. Frozen sections of the liver were stained with Oil red O. Paraffin-embedded sections of the adipose tissue and liver were stained with hematoxylin and eosin, Masson’s trichrome, or Sirius red. More than 200 cells were analyzed for determination of the average adipocyte size using ImageJ software (National Institutes of Health). Masson’s trichrome– or Sirius red–positive areas were used to estimate the extent of fibrosis. For immunofluorescence, sections were incubated with Mac-2 antibody (Cedarlane) and perilipin antibody (Sigma-Aldrich) overnight. The sections were incubated with Alexa Fluor 488 (Thermo Fisher Scientific) for Mac-2 and/or Alexa Fluor 568 (Thermo Fisher Scientific) for perilipin. Nucleus was stained with 4’,6-diamidino-2-phenyldiolone (DAPI). Images of sections were captured using a confocal laser microscope (FV1000; Olympus). Perilipin staining was used to identify necrotic adipocytes. More than 400 cells were analyzed for determination of the necrotic adipocytes.

**Collagen content in adipose tissue**

Collagen content in adipose tissue was measured with a collagen assay kit (QuickZyme Biosciences).

**Isolation of F4/80-positive cells by magnetic sorting**

F4/80-positive cells were isolated from the SVF of eWAT of male mice using MS columns (Miltenyi Biotec) for magnetic activated cell sorting system (51). The primer-probe sets for real-time quantitative PCR are listed in Table S2.

**Immunoblot analyses**

Aliquots of frozen eWAT (~150 mg) were homogenized in 200 μl of a buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM sodium EDTA, 1% NP-40) supplemented with a protease inhibitor cocktail (Sigma). Protein, 30 μg, was subjected to 3 to 8% SDS-PAGE for immunoblot analyses of collagen I(Col1), Col3, and Col6 or 10% SDS-PAGE for immunoblot analyses of Acta2, Timp1, and HMGB1. After the membranes were blocked for 30 min in a blocking one solution (Nacalai Tesque), they were incubated with primary antibodies for Timp1 (R&D systems), Col1 (Bios), Col3 (Bios), Col6 (Novus Biologicals), Acta2 (abcam), or HMGB1 (abcam) overnight at 4°C. The membranes were incubated with anti-rabbit (GE healthcare) or anti-goat (Proteintech) IgG horseradish peroxidase–conjugated secondary antibody for 1 h in TBS-T solution at room temperature. An enhanced chemiluminescent substrate ECL prime (GE Healthcare) was used to visualize the horseradish peroxidase. Image Quant Las 4000mini (Cyiva) was used to detect the enhanced chemiluminescence. All membranes were stained with Ponceau S after protein transfer for protein normalization.

**Statistics**

Data are presented as means ± SD. GraphPad Prism version 6.0 (GraphPad Software) and EZR version 1.52 (Saitama Medical Center, Jichi Medical University), graphical user interface for R version 4.0.2 (R Foundation for Statistical
Computing), were used for data analyses. The Shapiro–Wilk test was used to test normality; F or Brown–Forsythe test was used to test equal variance. If the data were consistent with normal distribution and equal variance, Student’s t test was used to compare the mean values between two groups, and the one-way ANOVA was used for multiple comparison. If the variances were not equal, Welch t test was used for comparison between two groups. When ANOVA results were statistically significant (i.e., $p < 0.05$), individual comparisons were made with the Tukey post hoc test. If the data were not distributed normally, nonparametric tests such as the Mann–Whitney U or Kruskal–Wallis test were used. When Kruskal–Wallis test results were statistically significant (i.e., $p < 0.05$), individual comparisons were made with the Steel–Dwass post hoc test. Body weights were compared by repeated-measures ANOVA with Bonferroni’s post hoc analysis.

**Data availability**

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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