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# HIF-1α in Myeloid Cells Promotes Adipose Tissue Remodeling Toward Insulin Resistance

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**Adipose tissue hypoxia is an important feature of pathological adipose tissue expansion. Hypoxia-inducible factor-1α (HIF-1α) in adipocytes reportedly induces oxidative stress and fibrosis, rather than neoangiogenesis via vascular endothelial growth factor (VEGF)-A. We previously reported that macrophages in crown-like structures (CLSs) are both hypoxic and inflammatory. In the current study, we examined how macrophage HIF-1α is involved in high-fat diet (HFD)–induced inflammation, neovascularization, hypoxia, and insulin resistance using mice with myeloid cell–specific HIF-1α deletion that were fed an HFD. Myeloid cell–specific HIF-1α gene deletion protected against HFD-induced inflammation, CLS formation, poor vasculature development in the adipose tissue, and systemic insulin resistance. Despite a reduced expression of *Vegfa* in epididymal white adipose tissue (eWAT), the preadipocytes and endothelial cells of HIF-1α–deficient mice expressed higher levels of angiogenic factors, including *Vegfa*, *Angpt1*, *Fgf1*, and *Fgf10* in accordance with preferable eWAT remodeling. Our in vitro study revealed that lipopolysaccharide-treated bone marrow–derived macrophages directly inhibited the expression of angiogenic factors in 3T3-L1 preadipocytes. Thus, macrophage HIF-1α is involved not only in the formation of CLSs, further enhancing the inflammatory responses, but also in the inhibition of neoangiogenesis in preadipocytes. We concluded that these two pathways contribute to the obesity-related physiology of pathological adipose tissue expansion, thus causing systemic insulin resistance.**

Chronic inflammation of adipose tissue is strongly associated with systemic insulin resistance (1). The pathological expansion of adipose tissue is advocated as a theory explaining the mechanisms of adipose tissue remodeling and inflammation observed in obesity (2,3). The pathological expansion of adipose tissue is characterized by macrophage infiltration and local hypoxia caused by a relative insufficiency of neoangiogenesis (3). As obesity progresses, the degree of neovasculature becomes inadequate to compensate for the rapid adipose tissue expansion, causing hypoxia-mediated dysfunction of the adipose tissue and subsequent systemic insulin resistance (1,2,4–6). In both

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humans and rodents, the oxygen concentrations in adipose tissue decrease in accordance with the progression of obesity, which is accompanied by reduced capillary densities and blood flow (7). However, how hypoxia in adipose tissues, especially in adipose tissue macrophages (ATMs), interrelates with other features of pathological expansion is poorly understood.

Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of hypoxic responses. Under hypoxic environments, the HIF-1α subunit is translocated from the cytoplasm to the nucleus, where it dimerizes with the HIF-1$\beta$ subunit and activates the transcription of genes that are required for hypoxic biological responses, including neoangiogenesis and adaptive glucose metabolism (8,9). HIF-1α is activated in both adipocytes and macrophages in adipose tissues during obesity progression (8,10,11). In the early stages of obesity, hypoxic areas are created via the hypertrophy of adipocytes per se (12). HIF-1α activation in adipocytes also causes metabolic dysfunction and subsequent proinflammatory responses (11,13–17). In later stages of obesity, crown-like structures (CLSs), which are formed by an accumulation of HIF-1α–activated ATMs, become the major site of hypoxia (18). HIF-1α activation in macrophages induces not only proinflammatory responses (18,19), but also neoangiogenesis by secreting proangiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF) (20–22), in tumors. However, despite a number of studies on HIF-1α in adipocytes, it remains an open question whether HIF-1α in ATMs mediates proinflammatory or neoangiogenic responses, thus contributing to healthy or pathological expansion, respectively.

When growing adipose tissues maintain their normal functions (healthy expansion), neoangiogenesis is properly accomplished by various proangiogenic factors released from adipocytes and other cells in the stromal vascular fraction (SVF), including macrophages, preadipocytes, and endothelial cells (23). Adipocyte-derived VEGF reportedly plays a role in the accomplishment of healthy adipose tissue expansion (24), and VEGF creates vascularized white adipose tissue, providing improved or maintaining adipocyte metabolism (3,25,26). However, we recently showed that *Vegfa* expression is unexpectedly downregulated in adipocytes under obese conditions, although it is upregulated in the SVF, indicating that *Vegfa* expression is differentially regulated by HIF-1α in adipocytes and macrophages under obese conditions. The reduction in *Vegfa* expression in the adipocytes of obese mice (18) can be explained, at least in part, by the fact that adipocyte HIF-1α is not a strong inducer of *Vegfa* gene expression (13). In addition, differentiating preadipocytes also produce proangiogenic factors, including FGF2, VEGF-A, hepatocyte growth factor, and platelet-derived growth factors (10,23). The differentiation of preadipocytes into mature adipocytes is reportedly linked to a concomitant increase in the production of angiogenic factors (27). However, how those angiogenic factors released from macrophages and preadipocytes are regulated by HIF-1α–dependent pathways remains elusive.

In the current study, we analyzed how HIF-1α in macrophages influences glucose metabolism and vascular development, hypoxia, and inflammation in adipose tissue during the development of obesity using myeloid-specific HIF-1α knockout (KO) mice fed a high-fat diet (HFD). The KO mice exhibited improved glucose metabolism with less inflammation, less hypoxia, and better vasculature development in adipose tissue despite reduced *Vegfa* expression in adipose tissue. The improved vasculature was associated with the augmented expression of angiogenic genes in preadipocytes and endothelial cells. Here, we demonstrated that HIF-1α in myeloid cells plays an important role in the pathophysiology of obesity-related pathological expansion of adipose tissue and systemic insulin resistance.

## RESEARCH DESIGN AND METHODS

### Generation and Maintenance of Mice

HIF-1α$^{flox/flox}$/LysM-cre (KO) mice were generated by crossing a C57BL/6J mouse containing loxP sequences on either side of the Hif-1α gene with a C57BL/6J mouse expressing cre recombinase from the lysozyme M promoter, which is found only in myeloid lineage cells, thereby yielding a homozygous mouse with HIF-1α–deficient monocytes and macrophages (28). Mice containing the floxed Hif-1α allele that did not express the cre recombinase gene (HIF-1α$^{flox/flox}$) were used as the control group. The mice were maintained under standard light conditions (12-h light/dark cycle) and were allowed free access to water and food. Male 6-week-old KO mice and control mice were fed ad libitum either a normal chow diet that contained 10% of calories from fat (CLEA Japan, Tokyo, Japan) or an HFD that contained 60% of calories from fat (D12492; Research Diets Inc.) for 18 weeks. The animal care policies and procedures/protocol used in the experiments were approved by the Animal Experiment Ethics Committee of the University of Toyama (Toyama, Japan).

### Glucose Tolerance Test and Insulin Tolerance Test

In the intraperitoneal glucose tolerance test (IPGTT), mice were subjected to a 12-h fast and were then injected with glucose (1 mg/g body wt) intraperitoneally. In the intraperitoneal insulin tolerance test (IPITT), mice were injected intraperitoneally with 1.2 units/kg body wt human insulin after a 4-h fast. Blood samples were then collected from the tail vein. The blood glucose levels were measured using the STAT STRIP Express 900 (Nova Biomedical, Waltham MA). The serum insulin levels were determined using the Mouse Insulin ELISA KIT (Shibayagi, Shibukawa, Japan).

### Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen). Quantitative RT-PCR was performed, as described previously (29). For each gene, mRNA expression was calculated relative to the *β-actin* expression level or relative to the *Gtf2b* expression level and was presented as a value relative to that for control mice.

### Immunoblotting

Immunoblotting of mouse tissues was performed as described previously (18,30), using anti-AKT and antiphosphorylated AKT antibodies (Cell Signaling Technology).

### Immunohistochemistry and Hypoxia Probe Administration

Tissues were processed as histological sections or whole-mount tissues and were subjected to immunohistochemical analysis, as described previously (18,31). A rabbit polyclonal antibody against mouse F4/80 was purchased from Santa Cruz Biotechnology, and a hamster monoclonal antibody against mouse CD11c was purchased from AbD-Serotec (Kidlington, U.K.). The vasculature was detected by incubating paraffin sections with anti–platelet endothelial cell adhesion molecule-1 (CD31) antibody (25–0311–81; eBioscience) for 1 h and then with Histofine Simple Stain Max-PO (rat polyclonal antibodies) for 30 min. The total numbers of CD31-positive cells were counted in high-power fields from each section. Immunofluorescence was performed by preparing 30- to 40-μm-thick frozen sections; and incubating them overnight with anti–platelet endothelial cell adhesion molecule-1 (CD31) antibody (Clone 2H8; Merck Millipore) and rat anti-mouse CD13 (Bio-Rad), and then overnight with the respective secondary antibodies (32,33). Goat anti-Armenian hamster IgG was from Jackson ImmunoResearch (West Grove, PA), and CF555 goat anti-rat IgG (H+L) was purchased from Biotium (Fremont, CA). The CD13- and CD31-positive cells were visualized under a Leica TCS-SP5 microscope (oil objective, ×63).

Immunohistostaining for the hypoxia probe was performed using the Hypoxyprobe-1 plus kit (Hypoxyprobe, Burlington, MA) according to the manufacturer instructions. Pimonidazole, a hypoxia probe, was intraperitoneally injected at a dose of 60 mg/kg body wt at 30 min before tissue collection.

### Quantification of Perfusion Capacity Using Yellow Dye Microspheres

Microspheres (100,000 beads/100 μL; Triton Technologies, Tempe, AZ) were injected into mice, as described previously (5). Tissue samples were collected and dissolved in 4 mol/L KOH. After overnight hydrolysis, the tissue samples were washed with 10% Triton X-100 reagent and acidified ethanol reagent, and the reagents were then allowed to evaporate at room temperature. The fluorescent dye was recovered with acidified cellosolve acetate. Fluorescence was measured using a plate reader and was normalized to the weight of each tissue.

### Cell Fractionation Using a Magnetic Activated Cell Sorting System

Epididymal white adipose tissue (eWAT) was collected from mice and was then dissociated into SVF and adipocytes, as described previously (18). The cells were subjected to magnetic-activated cell sorting (MACS) purification, which was performed according to the manufacturer instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The SVF was preceded by MACS with anti-CD31 and anti–platelet-derived growth factor receptor-α (PDGFRα [CD140a]) microbeads. Then we obtained the fractions enriched in CD31-positive endothelial cells and PDGFRα-positive preadipocytes. PDGFRα-positive cells were purified after the removal of CD31/CD45-positive endothelial and hematopoietic cells.

### Flow Cytometry Analysis

Flow cytometry was performed as described previously (29). Cells in SVF were incubated with 2.4G2 (BD Biosciences), followed by the primary antibodies or matching control isotypes. A rat CD45 antibody conjugated with fluorescein isothiocyanate (eBioscience), a rat F4/80 antibody conjugated with allophycocyanin (Bio Legend), and 7-aminoactinomycin D (BD Biosciences) were used as the primary antibodies. The cells were analyzed and sorted using a FACSAriaII (BD Biosciences).

### Preparation and Culture of Bone Marrow–Derived Macrophages

Bone marrow–derived macrophages (BMDMs) were collected and cultured as described previously (18,29). Bone marrow was collected from femurs and tibias of 8- to 12-week-old mice. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 55 μmol/L β-mercaptoethanol, and 100 ng/mL recombinant murine macrophage colony-stimulating factor (R&D Systems) for 7 days.

### Coculture of 3T3-L1 Preadipocytes and BMDMs

The coculture of preadipocytes and macrophages was performed as described previously (34,35), with some modifications. 3T3-L1 cells (American Type Culture Collection) were cultured in six-well plates. BMDMs were pretreated with 10 ng/mL lipopolysaccharide and 10 ng/mL interferon-γ (IFN-γ) for 24 h to induce inflammatory M1 polarity. In the direct coculture experiment, $1.0 \times 10^5$ BMDMs were plated onto 3T3-L1 preadipocytes after 48 h of confluency. The cells were cultured in contact with each other for 24 h and then harvested. In the indirect coculture experiment, transwell inserts with a 0.4-μm porous membrane (Corning) were prepared to separate the 3T3-L1 cells from the BMDMs. M1-induced BMDMs ($1.0 \times 10^6$ cells) were plated in the upper chamber, and 3T3-L1 preadipocytes were plated in the lower chamber. The preadipocytes were harvested after 24 h of incubation.

### Statistical Analysis

The statistical analysis was performed using the Student *t* test or a two-factor ANOVA and post–Tukey-Kramer test. A nonrepeated ANOVA followed by the Tukey-Kramer test was used for the statistical analysis of three groups. Differences were considered statistically significant at $P < 0.05$. The results were presented as the mean ± SEM.

## RESULTS

### HIF-1α Deficiency in Macrophages Improves Glucose Metabolism in HFD-Fed Mice

In this study, we generated KO mice to clarify how HIF-1α in macrophages affects adipose tissue expansion and

glucose metabolism during the development of obesity. The deletion of >90% of HIF-1α protein in BMDMs was confirmed by Western blot (data not shown). Then we examined the expression levels of genes downstream of HIF-1α in BMDMs. The expressions of genes encoding inflammatory markers, including *Il-6*, *Il-1b*, and *Nos2*, as well as hypoxia-related genes, including *Vegfa* and *Glut1*, which were markedly induced by in vitro hypoxia treatment, were significantly decreased by HIF-1α deficiency (Supplementary Fig. 1*A*). In fact, the expressions of hypoxia-related genes downstream of HIF-1α were markedly downregulated in the eWAT of KO mice (Supplementary Fig. 1*B*). These results indicate that HIF-1α was successfully deleted and functionally inactive in the macrophages of KO mice.

KO mice and control mice were fed an HFD from 6 weeks of age for 18 weeks. During that period, there were no significant differences in body weight or food intake between the two groups (Supplementary Fig. 2*A* and *B*). To determine the effects of HIF-1α deficiency in myeloid cells on glucose homeostasis, we compared glucose tolerance and insulin response between KO mice and control mice in the HFD-fed condition. As shown in Fig. 1, HIF-1α deficiency lowered the glucose profile in the IPGTT and IPITT results, and the insulin concentration in the IPGTT results (Fig. 1*A*–*C*). Consistent with the improved glucose tolerance and insulin sensitivity in the KO mice, insulin-stimulated AKT phosphorylation was increased in the eWAT, liver, and skeletal muscle (Fig. 1*D*). The expressions of metabolic genes, including *adiponectin*, *Glut4*, *Pparg*, and *Pgc1a*, were increased in eWAT (Fig. 1*E*). The expressions of genes involved in gluconeogenesis and β-oxidation were downregulated in the liver (Fig. 1*F*). In addition, the expressions of some mitochondrial genes and genes involved in β-oxidation were upregulated in skeletal muscle (Fig. 1*G*). These results suggest that myeloid-specific HIF-1α deletion improves glucose tolerance and insulin sensitivity without affecting body weight and food intake in HFD-induced obese mice. When KO mice and control mice were fed a normal chow diet, no significant differences in the glucose profiles seen in the IPGTT and IPITT results or in body weight were observed between the two groups (Supplementary Fig. 3*A*–*C*).

### HIF-1α Deficiency in Macrophages Ameliorates Inflammatory Profile of Adipose Tissue in HFD-Fed Mice

We also investigated the inflammatory status of eWAT. Fewer CLSs were observed in KO mice than in controls (Fig. 2*A* and *B*), indicating less macrophage infiltration in response to the HFD challenge. This decrease in macrophage infiltration was confirmed using quantitative real-time RT-PCR, which revealed a lower expression of *F4/80*, a macrophage marker, as well as a reduced expression of *Cd11c*, an M1 macrophage marker, in KO mice, whereas the M2 macrophage markers remained unchanged (Fig. 2*C*). The reduced expression of the M1 macrophage marker was supported by a significant downregulation of inflammatory genes, especially *Tnf*, *Mcp-1*, and *Il-1b*, and of oxidative stress-related genes in KO mice, compared with the controls (Fig. 2*C*). These results indicate that an improved metabolic phenotype was associated with a low inflammatory profile in the adipose tissue of KO mice.

### HIF-1α Deficiency in Macrophages Increases the Vasculature and Reduces the Hypoxic Area in Adipose Tissues of HFD-Fed Mice

Vasculature development is a critical factor distinguishing healthy expansion from the pathological expansion of adipose tissue. Consequently, we examined the vascular status of eWAT in KO and control mice. The expressions of *Cd31* and *VE-cad*, of endothelial cell markers, and of *Cspg4* (NG2), a pericyte marker, were upregulated in the eWAT of the KO mice (Fig. 3*A*). Consistent with the results of the gene expression study, an immunohistochemical analysis revealed a significant increase in CD31-positive cells in the eWAT of KO mice, compared with their control counterparts (Fig. 3*B* and *C*). Double staining for eWAT with antibodies against CD31 and CD13, a marker for pericytes, also revealed a significant increase in CD31- or CD13-positive cells in the KO mice (Fig. 3*D*). In normal chow–fed KO mice, CD31-positive cells and CD13-positive pericytes were similar to those in the control mice (Supplementary Fig. 4). In addition, we performed a colored microsphere analysis in eWAT that allowed us to evaluate tissue perfusion by determining whether the vasculature was actually better developed (5). The number of microspheres recovered in the eWAT of KO mice was significantly larger than that in the control group, indicating greater tissue perfusion. In contrast, there were no significant differences between the two groups in the numbers of microspheres recovered from other tissues, including the lung, liver, and skeletal muscle (Fig. 3*E*). We further investigated whether the better vascular development in the KO group was related to the levels of hypoxia in the eWAT using pimonidazole, a hypoxia probe. Immunostaining of eWAT with antipimonidazole antibody showed a reduction in signaling in the KO group, compared with that in the controls (Fig. 3*F*).

### HIF-1α Deficiency in Macrophages Upregulates the Expression of Angiogenic Factors in the Preadipocyte Fraction of eWAT

To investigate the mechanism responsible for the increase in angiogenesis in KO mice, we measured the expression levels of angiogenic factor genes in eWAT. As we recently reported (18), the expression of *Vegfa*, a strong inducer of angiogenesis that lies downstream of HIF-1α (36–38), was markedly reduced in the KO group. Interestingly, consistent with the better developed vasculatures in eWAT, the expressions of other angiogenic factor genes, including *Angpt1*, *Fgf1*, and *Fgf10*, were significantly upregulated in the eWAT of KO mice, compared with their floxed littermates (Fig. 4*A*). To identify the cells responsible for the high expression levels of angiogenic factor genes, we purified a PDGFRα-positive preadipocyte fraction and a

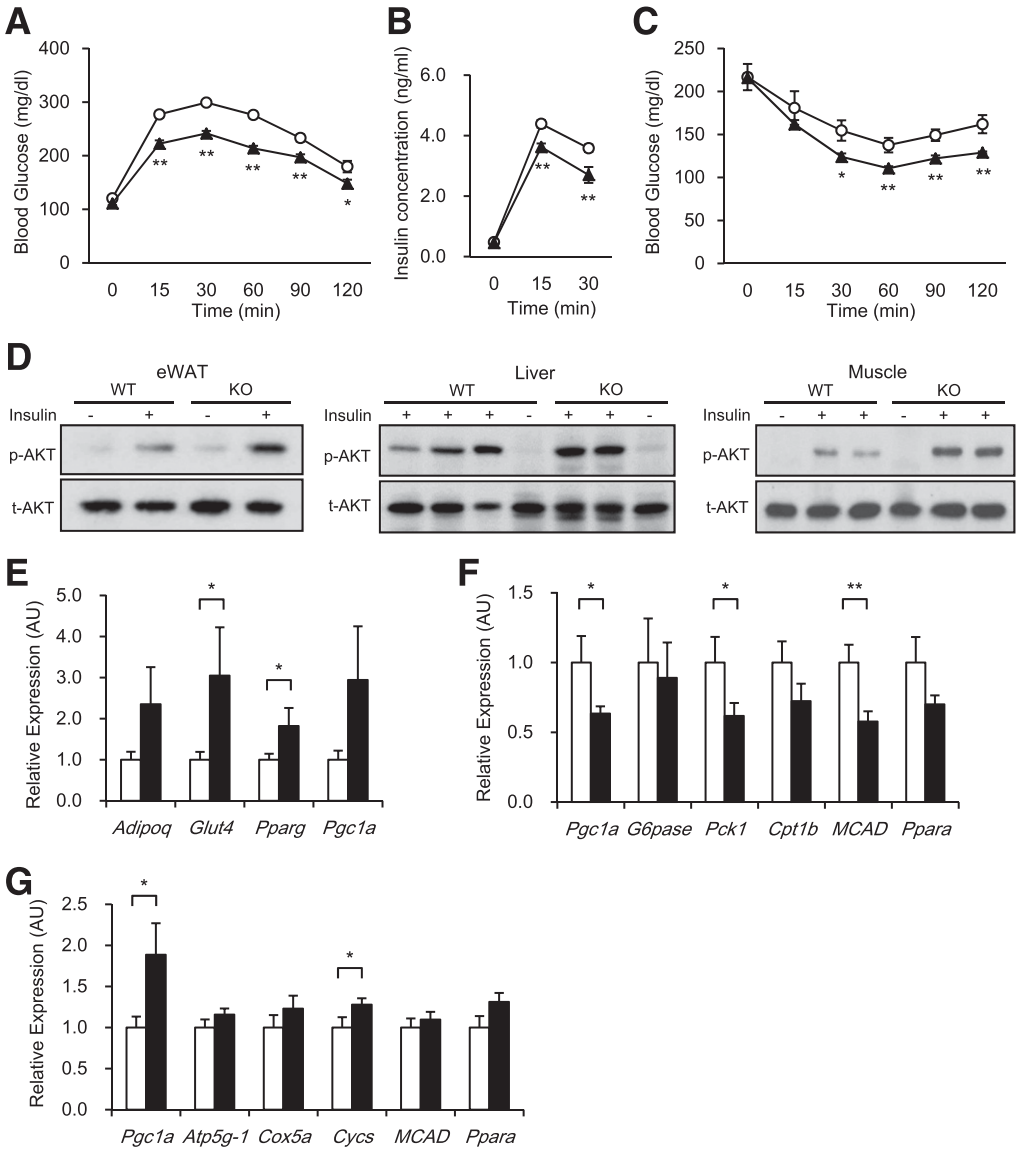


**Figure 1**—Deletion of HIF-1α gene in myeloid cells protects against diet-induced insulin resistance. IPGTT: Glucose concentration (*A*) and insulin concentration (*B*); $n = 5$. *C*: IPITT: $n = 5$. *D*: Western blotting for AKT phosphorylation in the eWAT, liver, and skeletal muscle. mRNA expression of metabolism-related genes in eWAT (*E*; $n = 12$), the liver (*F*; $n = 9$), and skeletal muscle (*G*; $n = 8$). White circles, WT; black triangles, KO; white bars, WT; black bars, KO. The results are shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, compared with the WT control group. AU, arbitrary units.

CD31-positive endothelial cell fraction from the SVF using a MACS system, as shown in Supplementary Fig. 5 (39–41). We found that the expressions of angiogenic factor genes including *Vegfa*, *Angpt1*, *Angpt2*, *Fgf1*, *Fgf2*, and *Fgf10* were significantly upregulated in PDGFRα-positive cells from KO mice, compared with that in wild-type (WT) control mice (Fig. 4*B*). We also found an increased expression of *Vegfa*, *Angpt1*, *Fgf1*, and *Fgf10* in the CD31-positive endothelial fraction, and *Angpt2* in the adipocyte fraction in the eWAT of KO mice (Fig. 4*C* and *D*). We also sorted the F4/80-positive macrophages using flow cytometry. HIF-1α deficiency did not affect the expression of angiogenic factor genes in macrophages in eWAT (Supplementary Fig. 6). These findings suggest that macrophage HIF-1α may negatively regulate vessel formation by suppressing the expression of angiogenic factors not only in preadipocytes, but also in endothelial cells and adipocytes.

### HFD Reduced the Expression of Angiogenic Factors in Adipose Tissues

Next, we compared the expressions of vascular markers and angiogenic factors in the adipose tissue of C57BL/6J mice fed normal chow or HFD for 12 weeks. The expressions of endothelial markers, including *Cd31*, and those of angiogenic factors, including *Vegfa*, *Angpt1*, and *Fgf10*, were markedly decreased in the HFD-fed mice (Fig. 5). These data suggested that HFD-induced adipose tissue expansion is associated with the reduced expression of angiogenic factor genes.

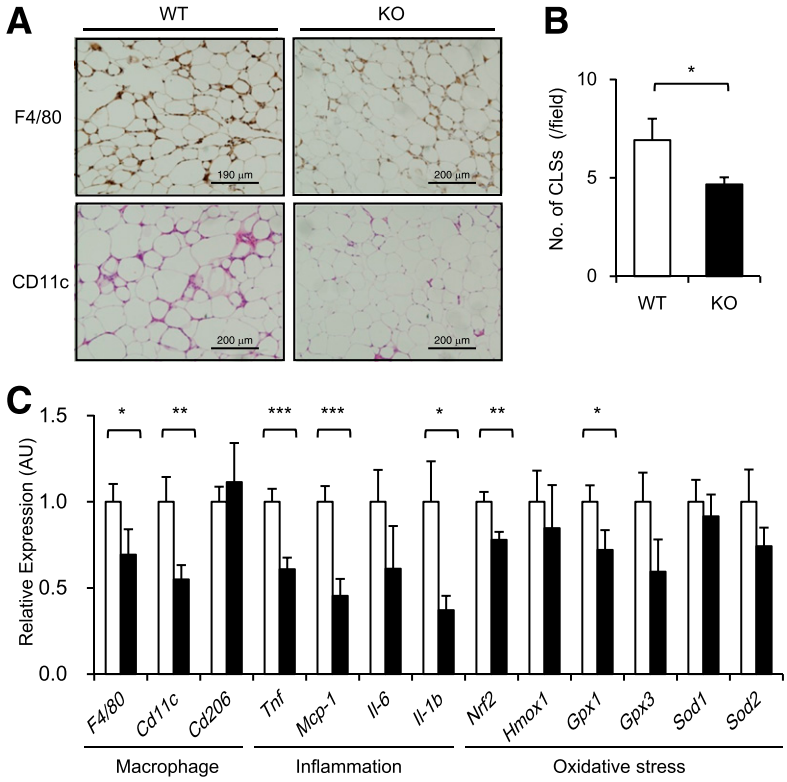


**Figure 2**—Deletion of HIF-1α gene in myeloid cells decreases the inflammatory profile in eWAT. *A*: Immunostaining of eWAT with anti-F4/80 and anti-CD11c antibodies. Representative images are shown from four independent experiments. Scale bars: top left panel, 190 μm; remaining panels, 200 μm. *B*: Quantification of CLSs. $n$ = 4 mice in each group. *C*: mRNA expression of genes related to M1/M2 macrophage, inflammation, or oxidative stress in eWAT. $n$ = 10. White bars, WT; black bars, KO. The results are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. AU, arbitrary units.

### Inflammatory Macrophages Inhibit Proangiogenic Factors in Preadipocytes

Because we presumed that macrophages expressing HIF-1α somehow inhibit proangiogenic gene expression in preadipocytes through the secretion of inflammatory cytokines, we performed coculture studies using BMDMs and 3T3-L1 preadipocytes. BMDMs were treated with lipopolysaccharide and IFN-γ to induce inflammatory M1 polarity, and then BMDMs and 3T3-L1 preadipocytes were indirectly cocultured within the upper and lower chambers, respectively. Unexpectedly, inflammatory BMDMs failed to inhibit the expression of angiogenic factors in 3T3-L1 preadipocytes (Supplementary Fig. 7*A*). To exclude a possibility that the concentration of humoral factors secreted by BMDMs was not high enough, 3T3-L1 preadipocytes were treated with a high concentration of inflammatory cytokines, such as tumor necrosis factor-α (10 ng/mL) and interleukin-β (IL-1β) (10 ng/mL). Neither of these treatments affect the expression of angiogenic factors, excluding *Fgf2* (Supplementary Fig. 7*B*). These results suggest that the expression of angiogenic factors was not inhibited by humoral factors from BMDMs.

We next performed the direct coculture studies of 3T3-L1 preadipocytes and BMDMs with or without inflammatory M1 induction. When inflammatory BMDMs from control mice were directly cocultured, the expression of proangiogenic factors, excluding *Vegfa*, in the cell mixture was markedly suppressed compared with those in a cell mixture of 3T3-L1 preadipocytes and naive BMDMs (Fig. 6*A*). M1-induced BMDMs from KO mice failed to inhibit some proangiogenic factors, such as *Fgf1*, *Fgf2*, and *Fgf10* (Fig. 6*A*). Because the deletion of HIF-1α did not alter the expression of proangiogenic factors in M1-induced BMDMs, when they were cultured separately from 3T3-L1 cells (Fig. 6*B*), as well as in ATMs sorted from eWAT (Supplementary Fig. 6), the expression of angiogenic factors shown in Fig. 6*A* was not altered in BMDMs, but was altered in 3T3-L1 preadipocytes. These results suggest that the inflammatory BMDMs inhibit proangiogenic gene expression through direct contact with preadipocytes.

## DISCUSSION

The pathological expansion of adipose tissue observed in obesity is characterized by enlarged adipocyte dysfunction, macrophage infiltration, insufficient neoangiogenesis, and tissue hypoxia. In particular, the chronic inflammation of adipose tissue with infiltrated inflammatory macrophages is well known to be involved in the pathophysiology of insulin resistance and to be deeply associated with systemic insulin resistance (1,2).

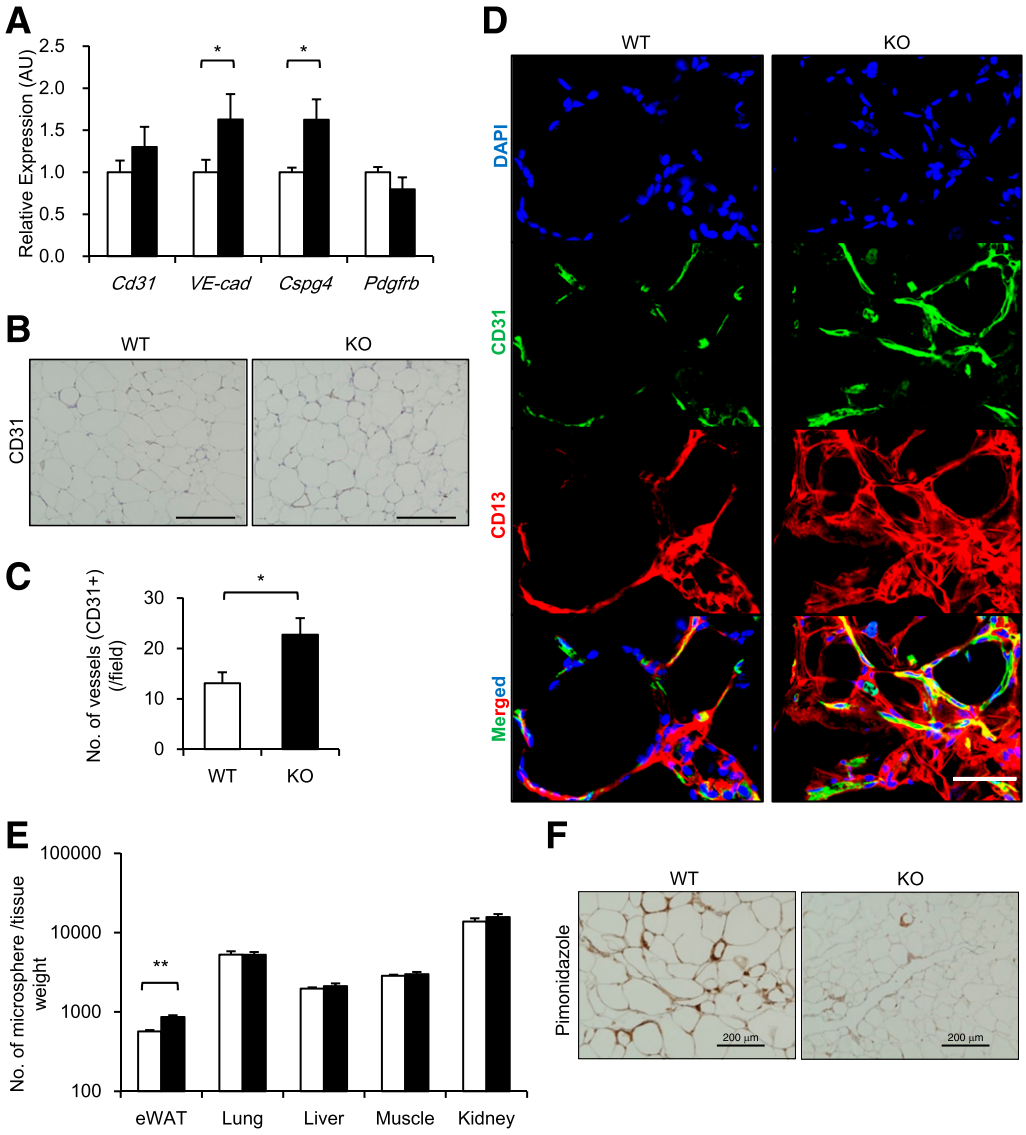


**Figure 3**—Deletion of HIF-1α gene in myeloid cells increases angiogenesis in eWAT. *A*: mRNA expression of genes related to angiogenesis in eWAT. $n = 10$. *B* and *C*: Immunostaining of eWAT with anti-CD31 antibody. The numbers of CD31-positive vessels were counted and are shown in *C*. $n = 9$. *D*: Immunofluorescence labeling of eWAT with anti-CD31 (green) and anti-CD13 (red) antibodies. Scale bar, 50 μm. *E*: Numbers of microspheres in eWAT, lung, liver, skeletal muscle, and kidney. $n = 3$. *F*: Immunostaining of eWAT with antipimonidazole antibody. White bars, WT; black bars, KO. The results are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (for *A*, *C*, and *E*). Representative images are shown from four independent experiments (*B*, *D*, and *F*). AU, arbitrary units. Scale bars for *B* and *F*, 200 μm.

It has been a matter of debate for the past decade whether the series of hypoxic reactions caused by HIF-1α is a healthy adaptive reaction or, rather, is part of a pathological process. Although adipose tissue hypoxia caused by inadequate angiogenesis is postulated to be an important element in the pathological expansion of adipose tissue, the role of macrophage HIF-1α in hypoxic responses, including inflammatory responses, the persistence of hypoxia, and limited vessel growth in adipose tissue, has not been fully investigated. The results of the current study showed that the adipose tissue of KO mice exhibited a healthy expansion characterized by a low inflammatory profile (Fig. 2), a well-developed vasculature despite reduced *Vegfa* gene expression (Figs. 3*A*–*E* and 4*A*), and reduced hypoxia (Fig. 3*F*). These findings suggested that macrophage HIF-1α is a key molecule in the induction of pathological adipose tissue expansion.

Hypoxia affects both adipocytes and macrophages during the development of obesity. In an early stage of obesity, adipocyte hypoxia occurs first, and adipocyte HIF-1α is activated. Several reports have demonstrated that adipocyte HIF-1α does not induce VEGF or angiogenic programs (11,13–15) and instead causes metabolic dysfunction and/or fibrosis, followed by inflammatory responses. Adipocyte HIF-1α mediates the production of reactive oxygen species that activate the nuclear factor-κB

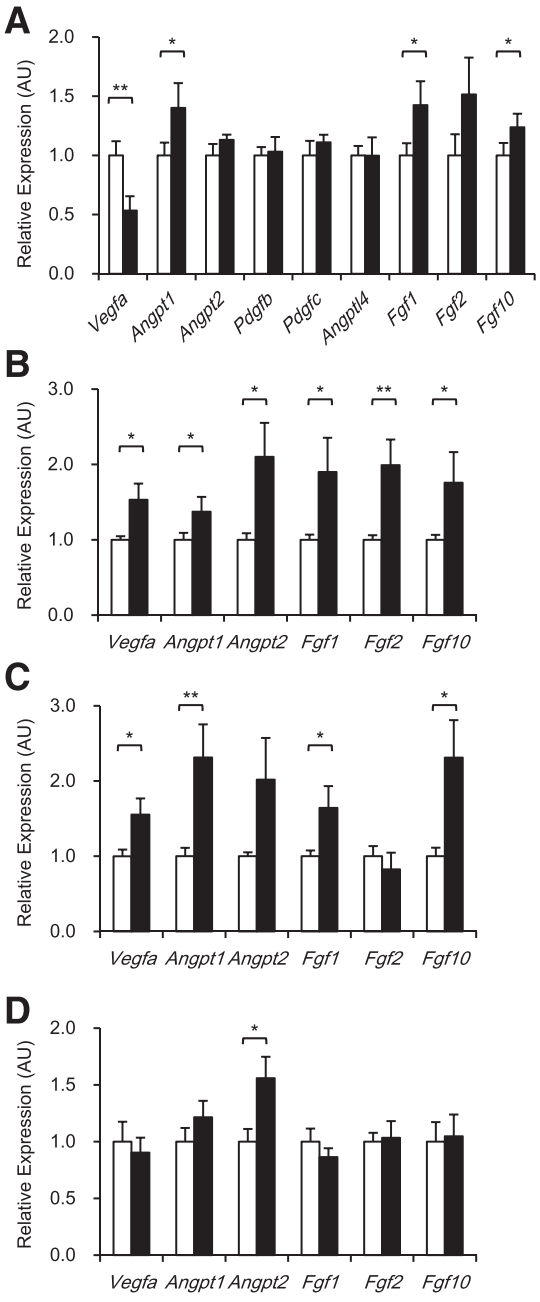


**Figure 4**—HIF-1α deficiency in myeloid cells upregulates the expression of angiogenic factors in preadipocyte fractions and endothelial cell fractions of eWAT. *A*: mRNA expression of genes related to angiogenic factors in eWAT. $n = 12$. mRNA expression of angiogenic factors in PDGFRα-positive preadipocyte fraction (*B*; $n = 14$–16), in a CD31-positive endothelial cell fraction (*C*; $n = 10$), and in an adipocyte fraction (*D*; $n = 13$). Each data point was normalized to the *β-actin* mRNA level and is presented as a value relative to that for WT control mice. White bars, WT; black bars, KO. The results are shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$. AU, arbitrary units.

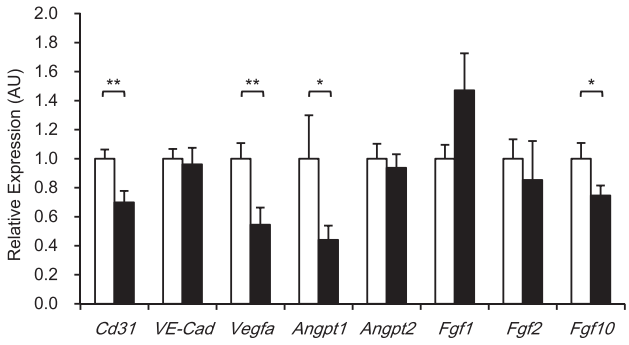


**Figure 5**—HFD reduced the expression of angiogenic factors in adipose tissue. mRNA expression of genes related to angiogenic factors in eWAT from normal chow–fed (white bars) and HFD-fed (black bars) mice. $n = 8$ mice per group. Each data point was normalized to the *Gtf2b* mRNA level and presented as a value relative to that for normal chow–fed control mice. The results are shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$. AU, arbitrary units.

pathway, inducing the expressions of several chemokines and the recruitment of ATMs into adipose tissue (11,13–15). It also stimulates the production of extracellular matrix from adipocytes and promotes fibrosis (13). Such inflammation and fibrosis induce insulin resistance. This hypothesis is supported by several reports (11,13,14) on adipocyte-specific HIF-1α KO mice. During the later stage of HFD-induced obesity, CLSs are formed and create additional hypoxic areas in adipose tissues. Macrophages aggregated around dead adipocytes are remarkably hypoxic, with activated HIF-1α (18). These hypoxic macrophages express high levels of inflammatory cytokines via HIF-1α activation (Supplementary Fig. 1), further worsening insulin resistance. Thus, macrophage HIF-1α links hypoxia and inflammation in the CLSs and contributes to the development of pathological expansion.

The development of vasculature is a hallmark of the healthy expansion of adipose tissue. How does the adipose vasculature develop during the HFD-induced growth of adipose tissue? One possible candidate is macrophage-derived VEGF-A–induced angiogenesis. In hypoxic environments in tumors, macrophage HIF-1α is involved in angiogenesis, supplying blood to tumor cells. Interestingly, our study showed that the myeloid cell–specific deletion of HIF-1α resulted in an increased development of vasculature, despite the reduction in VEGF-A gene expression in the adipose tissue (Fig. 3*B*–*D*), suggesting that macrophage HIF-1α somehow inhibits vasculature development in adipose tissue in HFD-induced obesity. Our data also showed that preadipocytes and endothelial cells, rather than macrophages, were important sources of angiogenic factors during the HFD-induced expansion of adipose tissue (Fig. 4*B* and *C*). We speculated that the upregulated gene expression of angiogenic factors, including VEGF-A, angiopoietin 1, and FGFs, in preadipocytes and endothelial cells contributed to the well-developed vasculature in the adipose tissue of KO mice despite a reduction in *Vegfa* (Fig. 4*A*–*C*). The ability of preadipocytes to express several angiogenic factors may be a major mechanism determining whether the healthy or pathological expansion of adipose tissue occurs.

Another interesting point in this study is that macrophages expressing HIF-1α inhibited angiogenic gene expression in preadipocytes through direct contact, rather

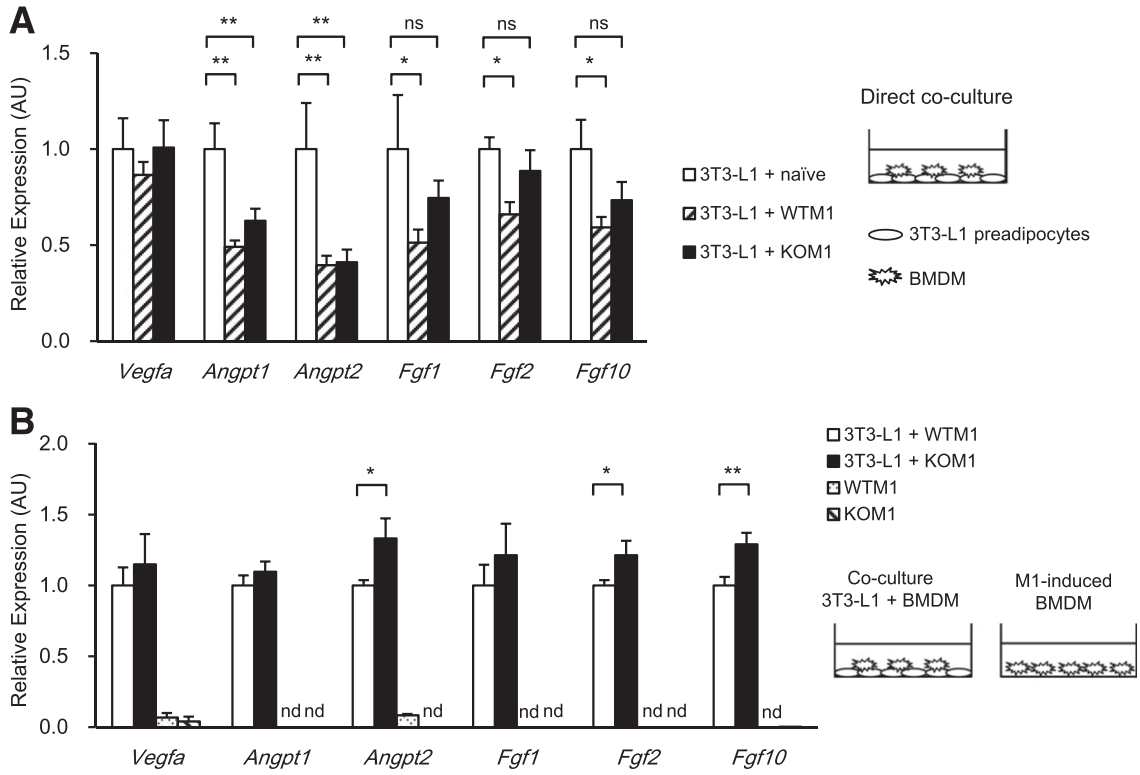


**Figure 6**—Inflammatory macrophages inhibited the expression of angiogenic factors in 3T3-L1 preadipocytes and HIF-1α deficiency in myeloid cells attenuated the inhibition of some angiogenic genes. *A*: Gene expression of angiogenic factors in cocultured cells. M1-induced BMDMs from WT or KO mice ($1.0 \times 10^5$ BMDMs/well) were plated onto confluent 3T3-L1 cells in a six-well plate. BMDMs from WT mice without the induction of M1 macrophages (naive BMDMs) were cocultured with 3T3-L1 preadipocytes as a control. The cells were incubated for 24 h in contact with each other and harvested to extract mRNA. $n = 10$. White columns, 3T3-L1 with naive BMDMs; striped columns, 3T3-L1 with BMDMs from WT mice; black columns, 3T3-L1 with BMDMs from KO mice. *B*: mRNA expression of angiogenic factors in cocultured samples ($n = 14$) and M1-induced BMDMs without coculture ($n = 8$). White columns, 3T3-L1 with BMDMs from WT mice; black columns, 3T3-L1 with BMDMs from KO mice; dotted columns, BMDMs from WT mice; striped columns, BMDMs from KO mice. Each data point was normalized to the *Gtf2b* level. The results are shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, compared with a cocultured sample of 3T3-L1 preadipocytes and M1-induced BMDM from WT mice. AU, arbitrary units; nd, not detected; ns, not significant.

than via humoral inflammatory cytokines (Fig. 6 and Supplementary Fig. 7). Thus, HIF-1α in ATMs suppresses preadipocyte-related angiogenesis. The molecular mechanisms underlying this phenomenon should be elucidated in the future.

Recently, Choe et al. (42) reported that macrophage HIF-2α ameliorates adipose tissue inflammation and insulin resistance in obese mice. Because HIF-1α and HIF-2α are major transcription factors that share a structural and functional homology enabling them to mediate similar hypoxic responses, such as angiogenesis and glycolysis, the current study and its results might seem contradictory and confusing. However, despite their extensive homology, it is well known that HIF-1α and HIF-2α both have their own functions in macrophages that determine their functional polarity. IFN-γ and IL-4/IL-13 promote the selective accumulation of HIF-1α and HIF-2α, which in turn enhances the expression of M1 and M2 genes, respectively (43). Taken together, these findings suggest that the activation of HIF-1α in macrophages is involved in the induction of insulin resistance via the expression of M1 genes, including proinflammatory cytokines, whereas the activation of HIF-2α in macrophages is involved in the maintenance or enhancement of insulin sensitivity via the expression of M2 genes.

Insulin-stimulated AKT phosphorylation was also enhanced in the liver and skeletal muscle (Fig. 1*D*) with increased mitochondrial gene expression in skeletal muscle and reduced gluconeogenic gene expression in liver of KO mice (Fig. 1*F* and *G*). We speculated that the mechanisms by which macrophage HIF-1α affects insulin signaling and metabolism in the liver and skeletal muscle were as follows. First, elevated ATM-derived serum inflammatory cytokines inhibit insulin signaling and metabolism in the liver and the skeletal muscles. Second, insulin signaling and mitochondrial metabolism in the skeletal muscle and liver are further disturbed by resident macrophages as well as locally recruited myeloid cells. In fact, the mRNA expressions of M1 macrophage marker and proinflammatory cytokines were decreased in the liver of KO mice (Supplementary Fig. 8).

In the current study, we demonstrated the mechanisms through which macrophage HIF-1α is involved in the induction of insulin resistance; specifically, macrophage

HIF-1α plays a crucial role in the suppressed angiogenesis in adipose tissue and the production of inflammatory cytokines. Angiogenic genes in preadipocytes are inhibited via direct contact with preadipocytes and inflammatory M1 macrophages. Our current findings provide a new approach to the development of therapeutic interventions for insulin resistance and obesity.

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**Author Contributions.** A.T., A.M., and A.N. performed the in vitro and in vivo experiments, analyzed the data, and prepared the article. T.K., K.O., A.A., and Y.I. performed the in vitro and in vivo experiments. S.Y. and K.Ts. acquired the immunostaining data and revised the manuscript. S.S. and T.N. helped to perform the experiments and provide animal care (experiments on knockout mice) and revised the manuscript. M.I. acquired and analyzed the data (flow cytometry and cell sorting). Y.W. performed the coculture experiment. Y.N., K.Ta., K.K., J.I., and M.S. analyzed and interpreted the data. N.G. provided the knockout mice and advised on performance of the animal experiments. M.M., K.S., S.F., and I.U. analyzed the data and wrote the manuscript. K.To. conceived, supervised, and designed the entire study; analyzed the data; and wrote the manuscript. All authors approved the final version of the manuscript for publication. K.To. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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