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Role of macrophages in depot-dependent browning of white adipose tissue

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Abstract Sympathetic stimulation induces beige adipocytes in white adipose tissue (WAT), known as browning of WAT. In this study, exposure of mice to cold ambient temperature (10 °C) for 24 h induced the mRNA expression of uncoupling protein 1 (UCP1), a marker for beige adipocytes, in inguinal WAT, but not in perigonadal WAT. Thus, we examined the role of macrophages in depot-dependent WAT browning in mice. Flowcytometric analysis showed that total number of macrophages was higher in perigonadal WAT than in inguinal WAT. Cold exposure failed to change the expression of macrophage marker genes in inguinal WAT; however, it increased the mRNA expression of CD11c and tumor necrosis factor-α in perigonadal WAT, indicating that proinflammatory M1 macrophage is activated. The removal of macrophages using clodronate significantly enhanced cold-induced UCP1 mRNA expression in perigonadal WAT. These results suggest that M1 macrophages are involved in the phenotype of perigonadal WAT that hardly undergo browning.

Keywords Beige adipocyte · Uncoupling protein 1 · White adipose tissue · Browning · Macrophage · Cold exposure

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Introduction

Brown adipose tissue (BAT) is a tissue specialized for thermogenic energy expenditure, in contrast to white adipose tissue (WAT) that stores excessive energy as triglycerides [1, 2]. BAT thermogenesis depends on uncoupling protein 1 (UCP1), a mitochondrial protein abundantly expressed in brown adipocytes, which dissipates the proton gradient that normally drives the synthesis of cellular ATP. The thermogenic activity of UCP1 is controlled by the sympathetic nervous system: norepinephrine (NE) released from nerve endings activates the β-adrenergic receptors (β-AR) on brown adipocytes, resulting in the activation of UCP1 as well as induction of UCP1 mRNA expression. Prolonged sympathetic activation, such as cold exposure, changes WAT to BAT-like thermogenic phenotype; this phenomenon is called browning of WAT [3, 4]. In the browning process, UCP1-expressing beige adipocytes emerge in WAT. Although beige adipocytes seem to originate from a distinct cell lineage from brown adipocytes [5], they show similar thermogenic function as brown adipocytes [6, 7]. Because human brown fat decreases with aging [8], browning of WAT is expected to increase the number of thermogenic adipocytes, which can be applied for the treatment of obesity.

Sympathetic nervous system, the β3-AR pathway in particular, is the most important in the regulation of browning processes because continuous injection of β3-AR agonist mimics the effect of cold exposure [9, 10]. Additionally, surgical denervation of sympathetic nerves to inguinal WAT or knockout of β3-AR in mice results in attenuated cold-induced beige adipocyte induction [11, 12], although there is a report showing that β3-AR is dispensable for browning [13]. On the other hand, macrophages

have been reported to play bidirectional roles in the induction of beige adipocytes. In obese animals, pro-inflammatory M1 macrophages migrate into WAT, particularly into abdominal WAT [14, 15], and suppress the cold-induced beige adipocyte induction [16]. In contrast, anti-inflammatory resident M2 macrophages have been reported to induce beige adipocytes by synthesizing NE in response to cold stimulation [17, 18].

It is known that browning of WAT differs depending on the WAT depot in mice: subcutaneous WAT easily undergoes browning than abdominal WAT [19]; however, its underlying mechanism is still unclear. In this study, we examined the role of macrophages in the depot-dependent difference in browning of WAT using C57BL/6 mice.

Methods

Animals and tissue sampling

The experimental procedures and care of animals were approved by the Animal Care and Use Committee of Hokkaido University (Hokkaido, Japan). All experiments using mice were performed in accordance with the guidelines of Hokkaido University Manual for Implementing Animal Experimentation, in the animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. C57BL/6J mice were purchased from Japan SLC Inc. (Shizuoka, Japan), housed in plastic cages in an air-conditioned room at 23 °C with a 12:12 h light:dark cycle, and given free access to laboratory chow (Oriental Yeast, Tokyo, Japan). Male 6–8-week-old mice were used in the experiments. For the cold exposure experiment, each mouse was housed in a plastic cage placed in a cold room at 10 °C for 4–72 h. For the experiment of macrophage removal, mice were intraperitoneally injected with clodronate- or saline-containing liposomes (65 mg/kg; XYGIEIA BIO SCIENCE, Osaka, Japan) 20 h before the cold exposure for 4 h. The mice were then euthanized with carbon dioxide, and inguinal and perigonadal WATs were quickly removed and transferred into RNAlater storage solution (Thermo Fisher Scientific, Gaithersburg, MD, USA) for quantitative PCR analysis, 10% phosphate-buffered formalin for histological examination, or used for the isolation of stromal–vascular (SV) fraction and flow cytometry analysis.

Isolation of the SV fraction

Adipose tissue fragments were cut into small pieces and incubated in DMEM containing 2% fatty acid-free bovine serum albumin (Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) and 2 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for 2 h with shaking at 100 cycles/min. The suspension was filtered through a 200-μm nylon filter and centrifuged at $120 \times g$ for 5 min at room temperature. The pellet was suspended in ACK erythrocyte lysis buffer (150 μM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA-2Na). The sample was centrifuged at $120 \times g$ for 5 min at room temperature. The pellet was resuspended in PBS containing 2% fetal calf serum (FCS) and used for flow cytometry analysis.

Flow cytometry analysis

The SV fraction was incubated with a mixture of antibodies containing either anti-CD31-PE-Cy (BioLegend, San Diego, CA, USA), anti-CD34-PE (BioLegend), anti-Sca1-PerCP/Cy5.5 (BioLegend), and anti-PDGFRα (CD140a)-APC (BioLegend) or anti-CD11c-FITC (BD Biosciences, San Jose, CA, USA), anti-CD206-PerCP/Cy5.5 (BioLegend), and anti-F4/80-APC (BioLegend) for 30 min on ice. After centrifugation at $1000 \times g$ for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in PBS containing 2% FCS. The suspension was filtered through a 40-μm nylon filter and analyzed using a flow cytometer (BD FACSVerse, BD Biosciences) with singlet discrimination to detect APC, PE-Cy, PE, and PerCP/Cy5.5-stained cells.

mRNA analysis

Total RNA was extracted using the RNAiso reagent (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Total RNA (2 μg) was reverse-transcribed using a 15-mer oligo(dT) adaptor primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed on a fluorescence thermal cycler (LightCycler system, Roche Diagnostics, Mannheim, Germany) using FastStart Essential DNA Green Master (Roche Diagnostics). Absolute expression levels were determined using a standard curve method with respective cDNA fragments as standards. The mRNA levels are expressed as relative values compared to β-actin mRNA levels. The primers used in this study are as follows: 5′-TCGTTAC CACAGGCATTGTGAT-3′ and 5′-TGCTCGAAGTCTA GAGCAAC-3′ for β-actin, 5′-CTTTGGCTATGGGCTT CCAGT-3′ and 5′-GCAAGGAGGACAGAGTTTATCGT G-3′ for F4/80, 5′-ACCCTCACACTCAGATCATCTTC-3′ and 5′-TGGTGGTTTGCTACGACGT-3′ for TNF-α, 5′-GGCCTCTACGACTCAGTCCA-3′ and 5′-TAAGCCGGC TGAGATCTTGT-3′ for UCP1, 5′-CTGGATAGCCTTTC TTCTGCTG-3′ and 5′-GCACACTGTGTCCGAACTC-3′ for CD11c, 5′-CCACAGCATTGAGGAGTTTG-3′ and 5′-ACAGCTCATCATTTGGCTCA-3′ for mannose receptor

C-type 1 (Mrc1), 5′-TCCTTCTACCACCTCAGCGAG-3′ and 5′-CCGGATGGTCACTCTTTAGGAAG -3′ for platelet-derived growth factor receptor (PDGFR) α.

Histology

Tissue specimens were fixed in 10% formalin, then embedded in paraffin, and cut into 4-μm-thick sections that were stained with hematoxylin and eosin. The stained samples were examined under a light microscope.

Data analysis

Values are expressed as mean ± SE. Statistical analysis was performed using the Student's t test or analysis of variance followed by Tukey–Kramer post hoc test.

Results

To confirm the depot-dependent difference in the browning of WAT, C57BL/6J mice were exposed to cold temperature (10°C) for 24 or 72 h. In a control group, no specific difference was observed between the histological appearances of inguinal and perigonadal WAT; both displayed white adipocytes containing large unilocular lipid droplets mainly composed of tissues (Fig. 1a). In inguinal WAT, beige adipocytes containing multilocular lipid droplets appeared after cold exposure for 24 h. The multilocular lipid droplets reduced in size and the area of beige adipocytes increased after cold exposure for 72 h. In contrast, beige adipocytes were not observed in perigonadal WAT after cold exposure for 24 and 72 h. In accordance with these histological observations, mRNA expression of UCP1 was very low in the control group, both in inguinal and perigonadal WAT (Fig. 1b). In inguinal WAT, UCP1 expression significantly increased after cold exposure for 24 h (16-fold), and it was sustained until 72 h. In perigonadal WAT, UCP1 expression was not detected after cold exposure for 24 and 72 h. These results confirm the depot-dependent browning and indicate that the browning process starts within 24 h after cold exposure.

To examine the population of macrophages in each depot, we conducted flowcytometric analysis using SV cells isolated from inguinal and perigonadal WAT (Table 1). F4/80+ macrophages consisted approximately 14.6 ± 1.7% of SV cells in inguinal WAT, which was significantly lower than that in perigonadal WAT (24.3 ± 2.3%). In contrast, the number of CD31− CD34+ Sca1+ PDGFRα+ progenitor cells, which have been reported to potentially differentiate into beige adipocytes [20], tended to be higher in inguinal WAT (37.6 ± 11.3%) than in perigonadal WAT (28.9 ± 8.6%), but this

difference was not statistically significant. The number of CD31+ endothelial cells did not differ between inguinal (16.5 ± 5.6%) and perigonadal (18.6 ± 5.4%) WAT.

Next, we examined the effect of cold exposure on the population of each cell type. Cold exposure for 24 h showed no effect on the number of SV cells both in inguinal WAT ($19.2 \pm 4.0 \times 10^5$ cells/depot in the control group and $20.2 \pm 4.5 \times 10^5$ cells/depot in the cold group) and perigonadal WAT ($10.7 \pm 1.8 \times 10^5$ cells/depot in the control group and $11.7 \pm 2.0 \times 10^5$ cells/depot in the cold group). When we counted F4/80+ macrophages separately as M1 macrophages (F4/80+ CD11c+ CD206−) and M2 macrophages (F4/80+ CD11c− CD206+), the number of M1 macrophages was much lower than that of M2 macrophages in both inguinal WAT ($0.031 \pm 0.009 \times 10^5$ cells for M1 and $1.7 \pm 0.6 \times 10^5$ cells for M2 macrophages) and perigonadal WAT ($0.054 \pm 0.026 \times 10^5$ cells for M1 and $1.5 \pm 0.4 \times 10^5$ cells for M2 macrophages) in the control group (Fig. 2). Cold exposure did not affect the number of M1 and M2 macrophages in inguinal and perigonadal WAT. Also, the number of endothelial and progenitor cells showed no change after cold exposure. These results indicate that gonadal WAT contain a higher percentage, but not the actual number, of macrophages than inguinal WAT, and cold exposure has no effect on the number of each cell type.

We then measured the expression of the macrophage marker genes. Consistent with the results of flowcytometric analysis, F4/80 mRNA expression was not affected by cold exposure in both inguinal and perigonadal WAT (Fig. 3). In inguinal WAT, cold exposure caused no change in the expression of M1 macrophage markers TNF-α and CD11c and M2 macrophage marker Mrc1, although CD11c expression tended to be lower in the cold group. In perigonadal WAT, cold exposure significantly increased the expression of TNF-α to 3.1-fold and CD11c to 1.9-fold, but showed no effect on the expression of Mrc1. These results suggest that cold exposure activates M1 macrophages in perigonadal-specific manner.

To evaluate the role of macrophages in WAT browning, especially in the initial step of UCP1 induction, the effect of macrophage removal by clodronate injection on UCP1 expression was examined after the short-term cold exposure. Cold exposure for 4 h was sufficient to induce significant increase in UCP1 expression in inguinal WAT (20.7-fold) but not in perigonadal WAT (Fig. 4). In agreement with the results in Fig. 3, cold exposure showed no effect on gene expressions of F4/80 and Mrc1 both in inguinal and perigonadal WAT, whereas this treatment significantly increased TNF-α expression only in perigonadal WAT. To confirm the removal of macrophages by clodronate injection, F4/80 mRNA expression was measured, and it was found to be reduced in the spleen of the

Fig. 1 Effect of cold exposure on morphological features and uncoupling protein 1 (UCP1) expression in inguinal and perigonadal white adipose tissue (WAT). C57BL/6J mice were exposed to cold temperature (10 °C) for 24 or 72 h. **a** Sections of inguinal and perigonadal WAT were stained with hematoxylin and eosin. **b** UCP1 mRNA expression was measured by quantitative real-time PCR. Data were normalized to β-actin expression and expressed as relative to the value of inguinal WAT of the control group. Values are expressed as mean ± SE for 4 mice. *p < 0.05
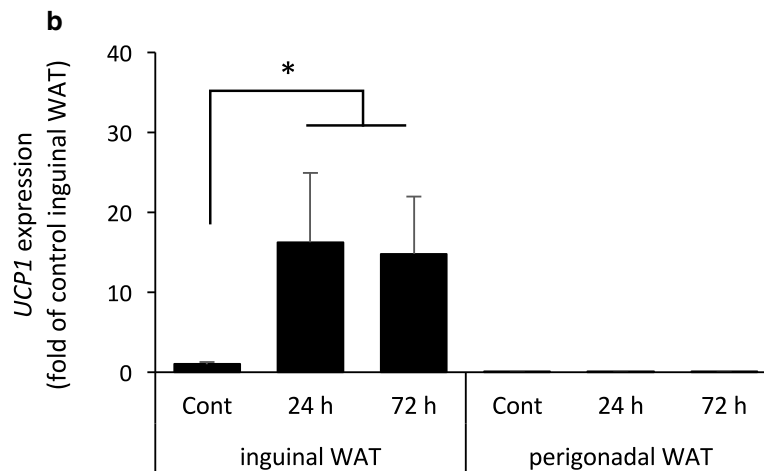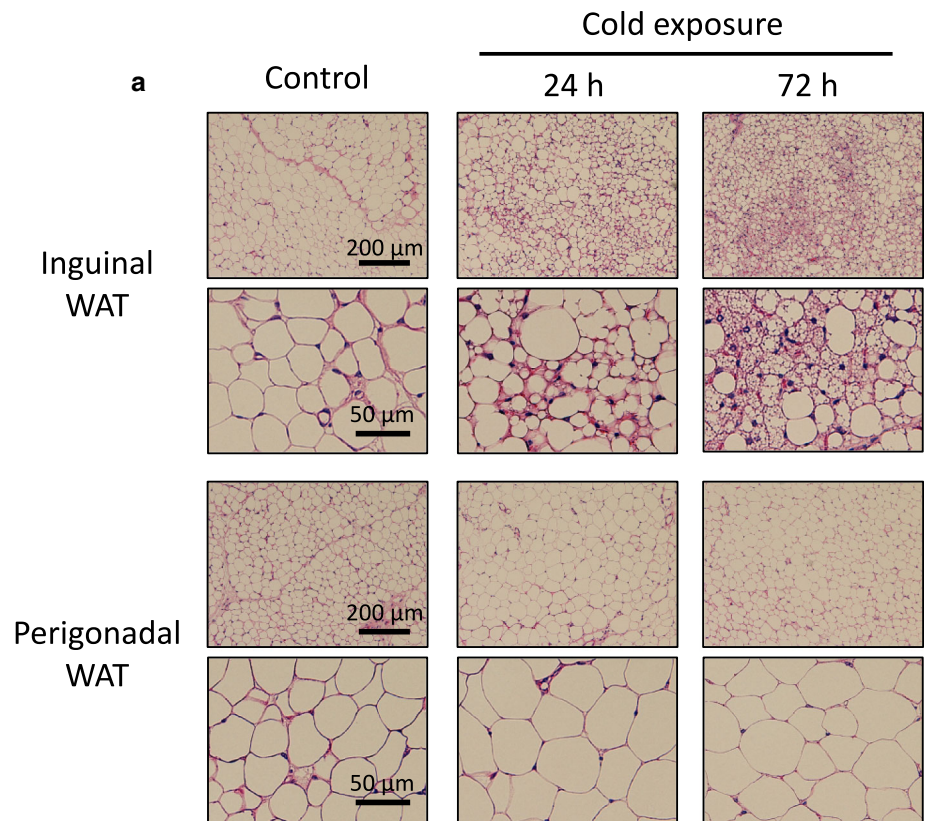

Table 1 Percentage of each type of cells in stromal-vascular fraction of inguinal and perigonadal WAT

	Inguinal WAT (%)	Perigonadal WAT (%)
F4/80+ cells	14.6 ± 1.7	24.3 ± 2.3[*]
CD31+ cells	16.5 ± 5.6	18.6 ± 5.4
CD31−, CD34+, Sca1+, PDGFRα+ cells	37.6 ± 11.3	28.9 ± 8.6

Data are mean ± SE

* $p < 0.05$ vs. inguinal WAT

Fig. 2 Effect of cold exposure on cellular composition of stromal–vascular (SV) fraction in inguinal and perigonadal WAT. C57BL/6J mice were exposed to cold temperature (10 °C) for 24 h. SV fraction was isolated from inguinal and perigonadal WAT and the population of M1 (F4/80+, CD11c+, CD206−) and M2 (F4/80+, CD11c−, CD206+) macrophages, endothelial cells (CD31+), and PDGFRα progenitor cells (CD31−, CD34+, Sca1+ and PDGFRα+) were measured by flow cytometry. The number of each type of cell per depot were estimated by multiplying the cell number in SV fraction by the percentage determined by flowcytometric analysis. Values are expressed as mean ± SE for 4 mice
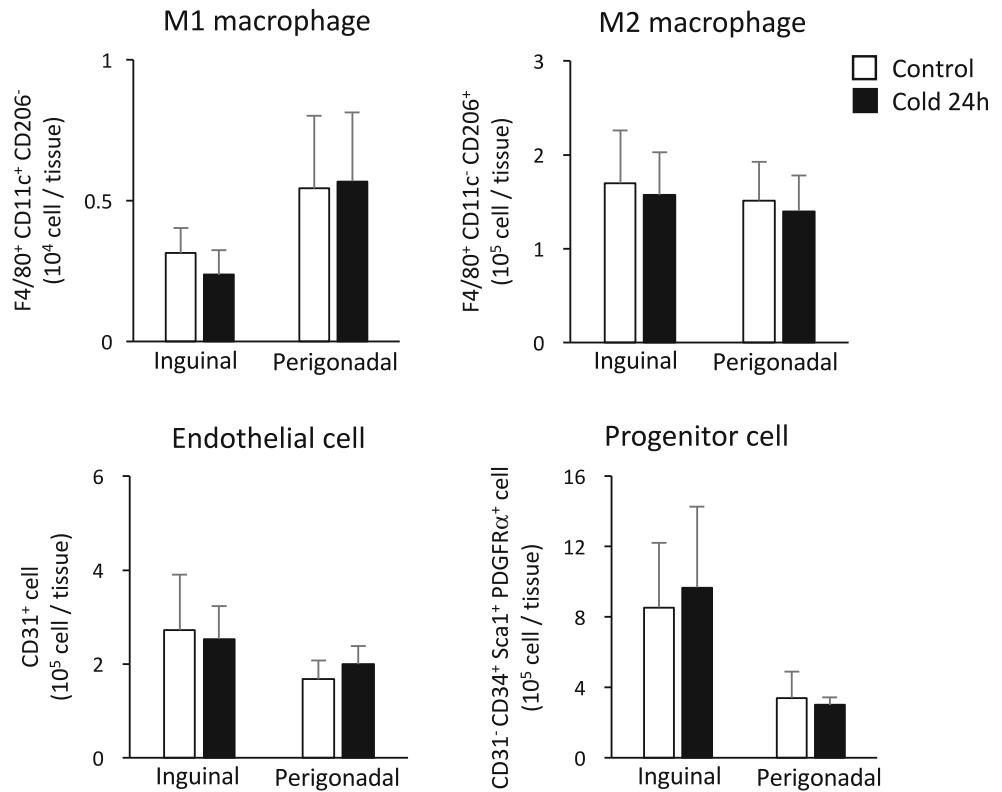

clodronate group to 17.7% of that in the saline group (Fig. 4a). However, unexpectedly, clodronate injection showed no effect on gene expression of F4/80, as well as Mrc1, TNF-α, PDGFRα, and UCP1 in inguinal WAT. On the other hand, in perigonadal WAT, F4/80 mRNA expression was significantly reduced by clodronate injection to 12.2% of that in the saline group. In addition, Mrc1 and TNF-α expression was decreased (4.4 and 21.4% of that in the saline group, respectively), indicating that both M1 and M2 macrophages were removed by clodronate injection. Clodronate treatment significantly increased cold exposure-induced UCP1 expression to 3.7-fold of that in the saline group. The expression of PDGFRα tended to be lower in the clodronate group than in the saline group, but the difference was not statistically significant.

Discussion

It is known that WAT browning induced by sympathetic stimulation differs depending on the WAT depot: subcutaneous WAT easily undergoes browning than abdominal WAT [19]. In this study, we examined the role of macrophages in the depot-dependent difference in browning of WAT using C57BL/6 mice. As reported [21], cold exposure induced marked browning in inguinal WAT but not in perigonadal WAT in this strain of mice. We found that cold exposure activated M1 macrophages specifically in perigonadal WAT, and the removal of macrophages enhanced cold exposure-induced UCP1 expression in perigonadal WAT. These results suggest the possible involvement of M1 macrophages in the browning-resistant phenotype of perigonadal WAT.

In obese animals, proinflammatory M1 macrophages infiltrate into WAT, abdominal WAT in particular, and produce cytokines such as TNF-α and IL-1β, which induce insulin resistance [14, 15, 22]. It has been reported that cold-induced browning of WAT is suppressed in diet-induced obese mice [16]. In addition, the injection of IL-1β suppressed cold-induced browning [23], indicating that M1 macrophages suppress WAT browning by secreting inflammatory cytokines. Thus, we assumed that the low browning ability of abdominal WAT may be due to the abundance of proinflammatory macrophages. In fact, the number of macrophages tended to be higher in perigonadal WAT than in inguinal WAT. However, when M1 and M2 types were analyzed separately, we found that the populations of both type of macrophages were not different
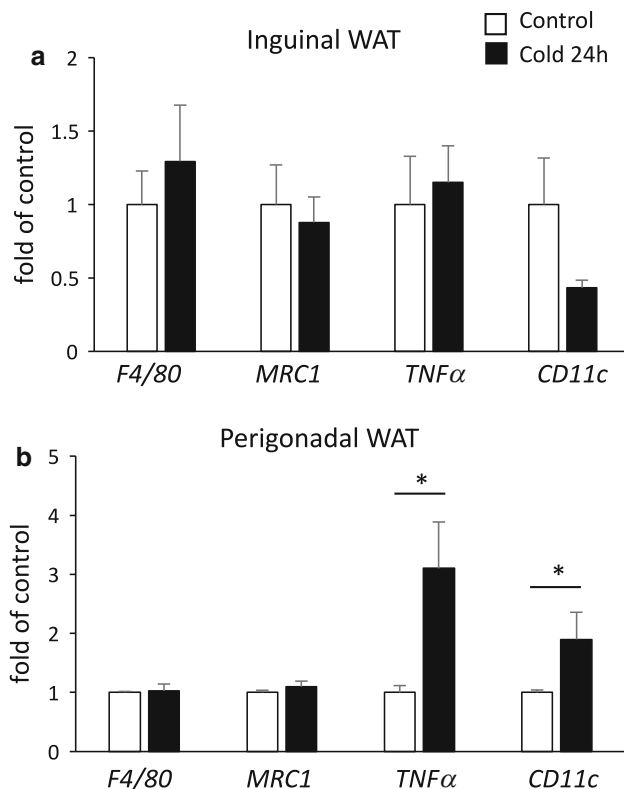
Fig. 3 Effect of cold exposure on macrophage-related gene expression in inguinal and perigonadal WAT. C57BL/6J mice were exposed to cold temperature (10 °C) for 24 h. Expression of F4/80, mannose receptor C-type 1 (Mrc1), tumor necrosis factor-α (TNF-α) and CD11c in inguinal (**a**) and perigonadal (**b**) WAT was measured by quantitative real-time PCR. Data were normalized to β-actin expression and expressed as relative to the value in the control group. Values are expressed as mean ± SE for 4 mice. *$p < 0.05$

M1 macrophages, but it showed no increase in response to cold exposure in both WAT depots. Unfortunately, clodronate injection failed to remove the macrophages in inguinal WAT due to unknown reasons in this study. Thus, we could not clarify whether M2 macrophages are involved in the browning of inguinal WAT. Also, cold exposure failed to increase the number of M2 macrophages, which is inconsistent with the previous reports [17, 18], and we could not detect the expression of tyrosine hydroxylase, an enzyme that produces NE, in SV fraction after cold exposure (Machida et al., unpublished result). In addition, it has been very recently reported that M2 macrophages do not synthesize catecholamine [24]. Thus, it is unlikely that M2 macrophages are involved in the depot-specific browning of WAT.

Our study suggested that cold exposure activates M1 macrophages and suppresses UCP1 expression in perigonadal WAT. However, even after macrophage removal, the cold-induced increase in UCP1 expression was as low as 3.3-fold, which is much lower than that in inguinal WAT (20.7-fold), suggesting that other mechanisms are also involved. It has been reported that physiological stimuli, such as fasting, activate sympathetic nerves innervating each WAT depot differently, however, cold exposure increases NE turnover similarly in inguinal and perigonadal WAT [25]. Moreover, depot-dependent difference in WAT browning is also observed after chronic injection of β3-AR agonist, although the expression of β3-AR is reported to be higher in perigonadal WAT than in inguinal WAT [13]. Consequently, the depot-dependent WAT browning may possibly be attributed to cell intrinsic characteristics. It was demonstrated that β3-AR stimulation induces the differentiation of PDGFRα-expressing progenitors existing in WAT into beige adipocytes [20]. We recently reported that the browning of inguinal WAT by adrenergic stimulation was attenuated in aged mice and is related to the reduction in the number of PDGFRα-expressing progenitor cells [26]. In this study, we observed that the number of PDGFRα-expressing cells tended to be higher in inguinal WAT than in perigonadal WAT. Thus, it is likely that the difference in the existence of progenitors also contributes to the depot-specific differences.

In summary, our study demonstrates that cold stimulation activates M1 macrophages specifically in perigonadal WAT, and the removal of macrophages enhances cold-induced UCP1 expression. M1 macrophages may be partially involved in the phenotype of perigonadal WAT that hardly undergoes browning.

between the two WAT depots. Although the number of macrophages did not change after cold exposure, mRNA expression of TNF-α and CD11c significantly increased, suggesting that M1 macrophages were activated and that these changes were specific to perigonadal WAT. In addition, the removal of macrophages by clodronate injection enhanced cold-induced UCP1 expression in perigonadal WAT. Thus, we concluded that M1 macrophages play a significant role in the suppression of browning in perigonadal WAT.
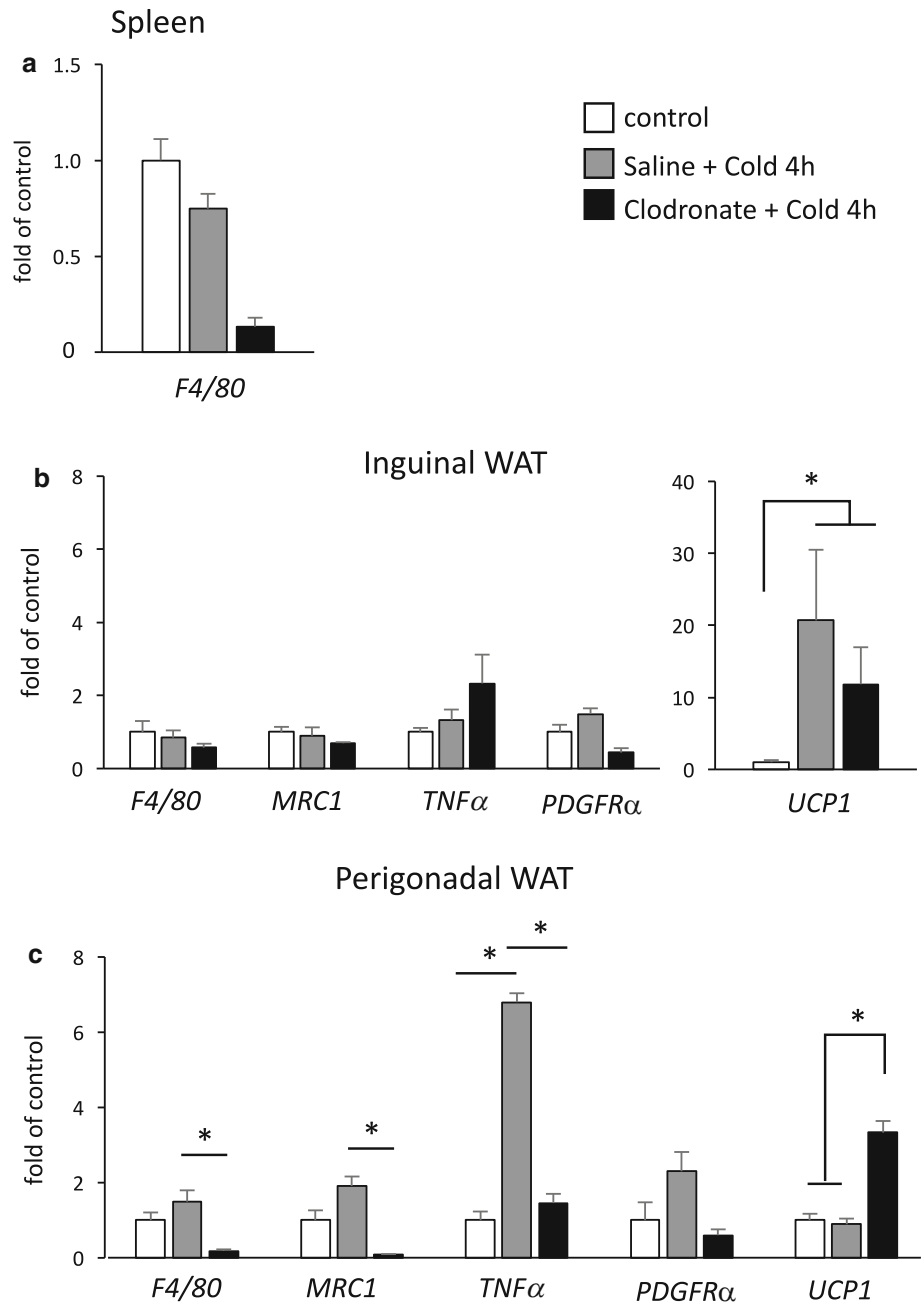
On the other hand, several reports have suggested that anti-inflammatory resident M2 macrophages are involved in the induction of beige adipocytes by synthesizing NE in response to cold stimulation [17, 18]. We found that the number of M2 macrophages was much higher than that of

Fig. 4 Effect of macrophage removal by clodronate injection on cold exposure-induced UCP1 expression in adipose tissue. C57BL/6J mice were intraperitoneally injected with clodronate- or saline-containing liposomes (65 mg/kg). Twenty hours later, mice were exposed to cold temperature (10 °C) for 4 h. Expression of F4/80, Mrc1, TNF-α, PDGFRα, and UCP1 in the spleen (**a**), inguinal (**b**), and perigonadal WAT (**c**) was measured by quantitative real-time PCR. Data were normalized to β-actin expression and expressed as relative to the value in the control group. Values are expressed as mean ± SE for 4 mice. *$p < 0.05$

Compliance with ethical standards

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