A self-sustained loop of inflammation-driven inhibition of beige adipogenesis in obesity

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In obesity, inflammation of white adipose tissue (AT) is associated with diminished generation of beige adipocytes (‘beige adipogenesis’), a thermogenic and energy-dissipating function mediated by beige adipocytes that express the uncoupling protein UCP1. Here we delineated an inflammation-driven inhibitory mechanism of beige adipogenesis in obesity that required direct adhesive interactions between macrophages and adipocytes mediated by the integrin α4 and its counter-receptor VCAM-1, respectively; expression of the latter was upregulated in obesity. This adhesive interaction reciprocally and concomitantly modulated inflammatory activation of macrophages and downregulation of UCP1 expression dependent on the kinase Erk in adipocytes. Genetic or pharmacological inactivation of the integrin α4 in mice resulted in elevated expression of UCP1 and beige adipogenesis of subcutaneous AT in obesity. Our findings, established in both mouse systems and human systems, reveal a self-sustained cycle of inflammation-driven impairment of beige adipogenesis in obesity.

The accumulation of macrophages in obese adipose tissue (AT) and their polarization into classically activated (inflammatory) M1 cells are critically involved in the inflammation of AT and metabolic dysregulation in obesity1–2. In contrast to the extensively studied chemokine-driven recruitment of monocytes and macrophages to AT3–5, little information exists about mechanisms that contribute to the retention of macrophages in AT6. The close proximity of inflammatory macrophages to adipocytes in obese AT, especially in regions designated ‘crown-like structures’ (CLSs)7, suggests that direct adhesive macrophage–adipocyte interactions might constitute a macrophage-retention mechanism in AT. Direct adhesive interactions between immune cells and other cell types are mediated largely by leukocyte integrins7. However, whether integrin-dependent adhesion mediates the retention of macrophages in obese AT and contributes to inflammation-related metabolic dysregulation in obesity has not yet been investigated.

Beyond serving as energy-storage organ, white AT has a homeostatic role in energy dissipation. The energy-dispersing function of AT is mediated by upregulation of the expression of the uncoupling protein UCP1, which uncouples oxidative phosphorylation from ATP synthesis and thereby induces thermogenic activity in white AT6–10. This function is attributed largely to a subtype of ‘beige’ adipocytes. These cells have inducible expression of UCP1, principally in response to exposure to cold, and thus share functional properties with brown adipocytes, such as thermogenic ability9,11–13. As beige fat also exists in humans13, beige adipogenesis represents a potentially important therapeutic target in obesity13. Beige adipogenesis can be derived not only from the generation of beige adipocytes from committed progenitor cells but also from the transdifferentiation of white adipocytes into beige cells9. Notably, cells of type 2 immunity stimulate beige adipogenesis in lean AT14.

Notably, the expression of UCP1 and the energy-dissipating activity of white AT are diminished in adiposity15–17, although the mechanistic underpinnings involved have remained obscure. Exacerbated inflammation, including a shift from an M2 (anti-inflammatory) macrophage state toward a predominantly pro-inflammatory M1 macrophage state is a hallmark of obese white AT1–2. However, little evidence exists indicating that inflammation can impede thermogenic beige adipogenesis in obesity18. Hypothetically, inflammatory macrophages in obese AT might secrete factors that interfere with beige adipogenesis. Alternatively (or additionally, given the proximity of macrophages and adipocytes in obese AT), adhesive interactions between these two cell types could conceivably contribute to the inhibition of beige adipogenesis. That hypothesis was confirmed in our study.

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here, which demonstrated that the interaction between \( \alpha_4 \) integrin on M1-polarized inflammatory macrophages and its counter-receptor VCAM-1 (vascular cell adhesion molecule-1) on adipocytes in obese AT initiated a self-sustained inflammatory cycle that downregulated the expression of UCP1 in adipocytes in a manner dependent on the kinase Erk. Conversely, genetic or pharmacological blockage of \( \alpha_4 \) integrin led to enhanced beige adipogenesis and prevented the metabolic dysregulation of obese AT.

**RESULTS**

**Macrophage–adipocyte adhesive interactions in obese AT**

To study macrophage–adipocyte interactions in obese AT, we focused on integrin adhesion receptors involved in leukocyte trafficking and cell–cell adhesion\(^{19} \). In this context, the integrins \( \alpha_4 \beta_1 \) and \( \alpha_5 \beta_2 \) are major participants that mediate the adhesive functions of monocytes and macrophages by recognizing their counter-receptors, VCAM-1 and ICAM-1, respectively, on target cells\(^{7,20} \). We performed adoptive-transfer experiments to identify the integrin receptors involved in macrophage accumulation in obese AT. To bypass the embryonic lethality of the deletion of \( \alpha_4 \) integrin (encoded by \( \text{Ig} \gamma_4 \)), we studied mice with inducible \( \text{Ig} \gamma_4 \) deficiency\(^{21} \), generated by breeding of mice withloxP-flanked (‘floxed’) \( \text{Ig} \gamma_4 \) alleles (\( \text{Ig} \gamma_4^{f/f} \)) with mice that have transgenic expression of Cre recombinase driven by the interferon-inducible gene \( \text{Mx}1 \) (\( \text{Mx}1^{-} \)-Cre), in which Cre activity is induced by the synthetic RNA duplex poly(I:C)\(^{21} \) (\( \text{Mx}1^{-} \)-Cre\( \text{Ig} \gamma_4^{f/f} \)-; called ‘Cre\( \text{Ig} \gamma_4^{f/f} \)- here). Cre activity is induced in several tissues in \( \text{Mx}1^{-} \)-Cre mice; however, the main recombinase activity of Cre is present in hematopoietic system and liver\(^{22,23} \). \( \text{Ig} \gamma_4 \) is expressed predominantly in hematopoietic cells\(^{21,24,25} \), whereas \( \text{Ig} \gamma_4 \) expression in isolated liver parenchymal cells was very low, relative to that in hematopoietic cells (data not shown). After administration of poly(I:C) to \( \text{Cre}^{\text{Ig} \gamma_4^{f/f}} \) mice, robust reduction in the expression of \( \alpha_4 \) integrin was observed in monocytes isolated from the bone marrow of these mice (Supplementary Fig. 1a), consistent with a published report on \( \text{Mx}1^{-} \)-Cre\( \text{Ig} \gamma_4^{f/f} \) mice\(^{21} \).

![Figure 1 The \( \alpha_4 \) integrin–VCAM-1 interaction mediates direct macrophage–adipocyte interactions. (a–c) Quantification of \( \text{Cre}^{\alpha_4^{f/f}} \) and \( \text{Cre}^{\alpha_4^{f/f}} \) macrophages (MΦ) (a) or T cells (c) in the inguinal SAT or VAT of obese wild-type mice given simultaneous adoptive transfer of a 1:1 mixture of PKH67-labeled \( \text{Cre}^{\alpha_4^{f/f}} \) cells and PKH67-labeled \( \text{Cre}^{\alpha_4^{f/f}} \) cells (monocytes or splenic T cells), and quantification (by flow cytometry) of \( \text{Cre}^{\alpha_4^{f/f}} \) and \( \text{Cre}^{\alpha_4^{f/f}} \) monocytes-macrophages in the draining lymph nodes of the SAT (SAT dLN) and those of the VAT (VAT dLN) (b); all results are presented relative to those of \( \text{Cre}^{\alpha_4^{f/f}} \) cells, set as 100%. (d) \( \text{Vcam}1 \) mRNA expression in the VAT and SAT of lean wild-type mice fed a normal-fat diet (ND; \( n = 8 \)) or obese mice fed an HFD (\( n = 10 \)) (left), in mature adipocytes from the VAT and SAT of lean mice fed a normal diet (\( n = 6 \)) or obese mice fed an HFD (\( n = 6 \)) (middle), and in CD31\(^{+} \) endothelial cells from SAT of lean mice fed a normal diet (\( n = 5 \)) or obese mice fed an HFD (\( n = 6 \)) mice (far right); results were normalized to those of 18S rRNA and are presented relative to those of mice fed a normal-fat diet, set as 1. (e) Expression of \( \text{Vcam}1 \) mRNA in CD45\(^{−} \)CD31\(^{+} \) endothelial cells and adipocytes (horizontal axis) from the SAT of obese mice; results are presented relative to those of endothelial cells, set as 1. (f) Flow cytometry (left) analyzing the surface expression of VCAM-1 on primary mouse adipocytes left untreated (UT) or treated with TNF or palmitate (Palm) (key), and summary of results, presented as median fluorescence intensity (MFI) (right). Iso, isotype-matched control antibody. (g) Adhesion of bone-marrow mononuclear cells to 3T3-L1 adipocites pretreated with TNF or palmitate (horizontal axis), assessed in the presence of \( \text{ICAM}1^{-} \)- or VCAM-1-blocking antibodies or the respective isotype-matched control antibodies (\( \text{ICAM}1^{-} \)-Iso or VCAM-1-Iso) (key); results are presented as the frequency of adherent cells. (h) Adhesion of bone-marrow mononuclear cells from \( \text{Cre}^{\alpha_4^{f/f}} \) or \( \text{Cre}^{\alpha_4^{f/f}} \) mice to 3T3-L1 adipocytes pretreated with TNF or palmitate (presented as in g). *(P < 0.05 (Mann-Whitney U-test (a,c,e,f) or Student’s t-test (b,g,h)). Data are representative of two experiments with \( n = 5 \) (a) or \( n = 4 \) (c) recipient mice (a,c); mean ± s.e.m.), are from one experiment with \( n = 5 \) recipient mice (b, mean ± s.e.m.), are from one experiment (f, mean ± s.e.m.), or are from one experiment (e, mean ± s.e.m.), or are from one experiment (d, mean ± s.e.m.), or are from one experiment with \( n = 6 \) mice (f, mean ± s.e.m.), or are from one experiment with \( n = 6 \) separate cell isolations (f, mean ± s.e.m.), or are from one experiment representative of three experiments with similar results (g, mean ± s.e.m. of \( n = 3 \) treatments per group), or are pooled from three experiments with separate cell isolations from \( n = 6 \) \( \text{Cre}^{\alpha_4^{f/f}} \) mice and \( n = 8 \) \( \text{Cre}^{\alpha_4^{f/f}} \) mice (h, mean ± s.e.m.).

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For adoptive transfer, we isolated CD11b+ monocytes from the bone marrow of Creα4-integrin−/− mice and labeled them with the green fluorochrome PKH67, and also isolated monocytes from their Igα4-sufficient (Creα4+/+) control littersmates and labeled these with the red fluorochrome PKH26, then injected the cells simultaneously, at a ratio of 1:1 in a competitive fashion, into wild-type mice with diet-induced obesity. We also implemented a similar experimental setup up with monocytes from mice deficient or sufficient in α4 integrin. 7 d thereafter, we analyzed the accumulation and retention of the cells administered in AT; the majority of adoptively transferred monocytes that had accumulated in obese AT had acquired macrophage markers (Fig. 1a). The accumulation of macrophages in both obese visceral AT (VAT) and inguinal subcutaneous AT (SAT) was dependent on the expression of α4 integrin (Fig. 1a) but not that of α2 integrin (data not shown). The diminished accumulation of Igα4-deficient macrophages relative to that of Igα4-sufficient macrophages in the SAT and VAT of obese wild-type mice was accompanied by a significantly greater number of Igα4-deficient monocytes-macrophages than Igα4-sufficient monocytes-macrophages in the draining lymph nodes of both SAT and VAT (Fig. 1b). These findings suggested that α4 integrin-deficient macrophages had egressed from SAT and VAT and hence had less retention therein. In contrast,

\begin{figure}
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\caption{Deficiency in α4 integrin ameliorates the accumulation of macrophages in obese AT and alleviates obesity-associated insulin resistance. (a) Body weight of Creα4−/− mice (n = 14) and Creα4+/+ mice (n = 15) (key) fed an HFD. (b) Micro-computed-tomography analysis of the lean and fat mass (horizontal axis) of Creα4−/− mice (n = 6) and Creα4+/+ mice (n = 6) at the end of the HFD-feeding period as in a. (c) Insulin-tolerance test of obese Creα4−/− mice (n = 14) and Creα4+/+ mice (n = 15). (d) Results from c, presented as area under the curve (AUC) relative to that of control (Creα4−/−) mice, set as 100%. (e,f) Confocal microscopy of SAT from obese Creα4−/− or Creα4+/+ mice (left margin), stained for macrophages (F4/80; green), adipocytes (caveolin-1; red) and nuclei (DNA-binding dye DAPI; blue), showing CLSs in SAT (e) and non-CLS-associated macrophages in SAT (f). Scale bars, 100 μm (e) or 20 μm (f). (g,h) Flow cytometry of M1-like pro-inflammatory macrophages (M1MΦ) (defined as F4/80+CD11b+CD206−iNOS−) and M2-like anti-inflammatory macrophages (M2MΦ) (defined as F4/80+CD11b+CD206+iNOS+) in the SVF isolated from the SAT of obese Creα4−/− mice (n = 7) and Creα4+/+ mice (n = 4) (g) or the VAT of obese Creα4−/− mice (n = 7) and Creα4+/+ mice (n = 5) (h); results (cells per gram of tissue) are presented relative to those of Creα4−/− mice, set as 100%. (i,j) TNF expression by macrophages in the SAT of obese Creα4−/− mice (n = 7) and Creα4+/+ mice (n = 4) (i) or the VAT of obese Creα4−/− mice (n = 7) and Creα4+/+ mice (n = 5) (j), analyzed by flow cytometry; results (frequency of TNF+ macrophages, defined as CD45+CD11b+F4/80+ cells) are represented relative to those of Creα4−/− mice, set as 100%. (k) Quantification (by flow cytometry) of α4 integrin in M1-like or M2-like macrophages (defined as in g,h) in the SVF prepared from the SAT and VAT of obese wild-type mice (n = 4). *P < 0.05 (Student's t-test (a,d,h) or Mann-Whitney U-test (g,j,k)). Data are pooled from five experiments (a,c,d, mean ± s.e.m. (a,c) or mean + s.e.m. (d)) or two experiments (b; mean ± s.e.m.), are representative of one experiment with three to four mice per genotype (e,f) or two experiments (g-j; mean ± s.e.m.), or are from one experiment (k; mean ± s.e.m.).
\end{figure}
Figure 3 Deficiency in α4 integrin promotes the beige adipogenesis of white AT in obesity. (a) Energy expenditure (EE) of obese Creα4f/f mice (n = 4) and Cre−/− mice (n = 5), assessed for 3 d with a metabolic-cage monitoring system and presented as kcal/h (left), and average energy expenditure of such mice in light and dark periods (right). (b) qPCR analysis of genes encoding thermogenesis-related molecules (Ucp1, Cidea, CoxBb, Cox7a1) (which encodes cytochrome c oxidase subunit 7A1) and Acsl1 (which encodes acyl-CoA synthetase long-chain family member 1)) in the SAT of obese Creα4f/f mice (n = 8) and Cre−/− mice (n = 10–11); results were normalized to those of 18S rRNA and are presented relative to those of obese Creα4f/f mice, set as 1. (c) Core temperature of Creα4f/f mice (n = 4) and Cre−/− mice (n = 6) challenged for 12 h with a temperature of 4 °C. (e) Immunoblot analysis (cropped blots) of UCP1 and actin in the SAT of mice as in d (n = 2 per genotype; one per lane), and densitometry of UCP1 in an immunoblot as at left, for SAT from Creα4f/f mice (n = 4) and Cre−/− mice (n = 5), with results normalized to those of actin and presented relative to those of Cre−/− mice, set as 1 (right). (f) Microscopy showing the staining of UCP1 in SAT from mice as in d. Scale bars, 100 μm. (g) Expression of Vcam1 mRNA in mature white and brown adipocytes isolated from the SAT and BAT of obese wild-type C57BL/6 mice (n = 6) after tissue digestion; results were normalized to those of 18S rRNA and are presented relative to those of SAT, set as 1.

Figure 4 The adhesion of macrophages to adipocytes in obesity can be modulated by integrins. (a) Administration of anti-VCAM-1 antibody (2 mg/kg) to C57BL/6 mice (n = 5) and Cre−/− mice (n = 5) once per day for 4 d by intraperitoneal injection of PBS or anti-VCAM-1 antibody (2 mg/kg) was performed. Mice were then infused with LPS (2 mg/kg) subcutaneously and administrated a high-fat diet for 7 d. After 7 d of feeding, HFD-fed mice were administered an LPS (2 mg/kg) subcutaneously, and the number of CLSs and non–CLS-associated macrophages in the peritoneal cavity, as well as their insulin sensitivity under normal-fat diet conditions (data not shown).

The accumulation of T lymphocytes in obese SAT or VAT was independent of their expression of α4 integrin (Fig. 1c).

To further elucidate the role of α4 integrin in the retention of macrophages in obese AT, we analyzed the expression of VCAM-1, the main counter-receptor of the integrin α4β1 (ref. 27). Constitutive expression of Vcam1 was observed in lean SAT and VAT (Fig. 1d). Vcam1 expression was massively upregulated in both SAT and VAT when mice developed diet-induced obesity (DIO) after being fed a high-fat diet (HFD) (Fig. 1d). The upregulation of Vcam1 in AT was attributable predominantly to its increased expression in adipocytes rather than increased expression in endothelial cells (Fig. 1d). The expression of Vcam1 by adipocytes in obese SAT was considerably higher than its expression in endothelium (Fig. 1e). VCAM-1 was detectable in both 3T3-L1 adipocytes and primary adipocytes, and its expression was upregulated after treatment with tumor-necrosis factor (TNF) or palmitate, which are both hallmark factors of the obese AT environment2–4 (Fig. 1f and data not shown). We therefore hypothesized that the interaction between α4 integrin and VCAM-1 might mediate the adhesion of macrophages to adipocytes in obese AT. Indeed, the adhesion of bone-marrow–derived mononuclear cells to adipocytes in vitro was blocked by antibody to VCAM-1 but not by antibody to the adhesion molecule ICAM-1 (Fig. 1g). Consistent with that, α4-integrin-deficient mononuclear cells displayed less adhesion to adipocytes than that of α4-integrin-sufficient cells (Fig. 1h). Thus, macrophage–adipocyte adhesion was dependent on the α4 integrin–VCAM-1 interaction, and α4 integrin mediated the retention of macrophages in obese AT.

Igα4 deficiency diminishes metabolic dysfunction in DIO

We then determined whether the α4 integrin-mediated adhesive interaction between macrophages and adipocytes in obese AT contributed to DIO-related metabolic dysregulation. Mice lacking α4 integrin (Creα4f/f) and their α4-integrin–sufficient (Creα4f/f) control littermates were fed an HFD. By flow cytometry, we observed robust deletion of α4 integrin in circulating monocytes and macrophages from the AT of Creα4f/f mice (Supplementary Fig. 1b,c). Obese Creα4f/f and Creα4f/f mice displayed no difference in terms of body-weight gain, fat mass or weight of liver, SAT or VAT (Fig. 2a,b and Supplementary Fig. 2a). Similarly, Creα4f/f mice fed an HFD displayed no significant difference in the size of adipocytes in VAT or SAT compared with that of Creα4f/f mice (Supplementary Fig. 2b). However, Creα4f/f mice with DIO displayed improved insulin sensitivity and lower glucose concentrations (although they had unaltered concentrations of triglyceride or cholesterol) compared with that of Creα4f/f mice (Fig. 2c,d and Supplementary Fig. 2c). No difference between Creα4f/f mice and Creα4f/f mice was observed in terms of their insulin sensitivity under normal-fat diet conditions (data not shown).

To study the accumulation and retention of macrophages in the AT of obese Creα4f/f and Creα4f/f mice, we performed immunofluorescence staining (using caveolin-1 and F4/80 to stain adipocytes28 and macrophages, respectively), followed by confocal microscopy of obese SAT from Creα4f/f and Creα4f/f mice. We found that the abundance of CLSs as well as that of non–CLS-associated macrophages in proximity to adipocytes was reduced in obese AT as a result of Igα4 deletion (Fig. 2e,f). Those findings were independently verified by immunohistochemical analysis of F4/80-labeled macrophages and histological quantification, which demonstrated a significant reduction in the number of CLSs and non–CLS-associated macrophages in contact with adipocytes in obese SAT after loss of expression of α4 integrin (Supplementary Fig. 3a,b).
To further substantiate our hypothesis that α4 integrin mediated the macrophage–adipocyte interactions in obese AT, we quantified the area of contact of macrophages and adipocytes, as a measure of macrophage–adipocyte adhesion. This analysis revealed that macrophages had less contact with adipocytes after deletion of Itga4 (Supplementary Fig. 3c). Moreover, three-dimensional reconstruction of macrophage–adipocyte interactions verified the diminished contact of macrophages with and spreading on adipocytes in Cre−α4inh mice relative to that in their Cre+α4inh littermates (Supplementary Fig. 4a–d). Those effects were probably independent of netrin-1 (encoded by Ntn1), which contributes to the retention of macrophages in obese AT, since deletion of Iga4 did not affect Ntn1 expression in obese SAT or VAT (Supplementary Fig. 5a,b).

To characterize the inflammatory milieu of the SAT and VAT of obese Cre+α4inh and Cre−α4inh mice, we studied the abundance of pro-inflammatory M1-like macrophages and of M2-like macrophages, defined by flow cytometry as F4/80+CD11b+CD11c−CD206− and of M2-like macrophages as F4/80+CD11b+CD11c−CD206+ (data not shown); CD11c has been used as a marker of inflammatory AT macrophages.29 Furthermore, the lower abundance of M1 macrophages in both the VAT and SAT of Cre+α4inh mice than in that of Cre−α4inh mice was confirmed by the lower number of TNF-expressing macrophages (Fig. 2f). In wild-type mice, M1-like macrophages from obese SAT and VAT displayed higher expression of α4 integrin than that of M2-like macrophages (Fig. 2k). These data suggested that α4 integrin mediated direct adhesion of macrophages to VCAM-1-expressing adipocytes and thus the retention of inflammatory macrophages within obese AT, AT inflammation and insulin resistance.

Enhanced beige adipogenesis of Cre+α4inh mice with DIO

To understand the improved metabolic phenotype of α4-integrin-deficient mice, we performed metabolic-cage analysis. Cre+α4inh mice with DIO displayed greater energy expenditure than that of their Cre−α4inh littermates (Fig. 3a), although no difference was detected in their respiratory-exchange ratio (data not shown). The absence of a difference between Cre+α4inh and Cre+α4inh mice in body weight...
Figure 5 UCP1 is essential for the beneficial effects of the pharmacological inhibition of α4 integrin on beige adipogenesis. (a) Expression of mRNA from genes encoding thermogenesis-related molecules (horizontal axis) in the SAT of C57BL/6 mice (n = 6 per group) in which DIO was established, followed by treatment with the α4 inhibitor or vehicle (key) and HFD feeding for 6 more weeks; results were normalized to those of 18S rRNA and are presented relative to that of vehicle-treated mice, set as 1. (b) Immunoblot analysis (cropped blots) of UCP1 and α4 integrin in the SAT of mice as in a (n = 3 per group; one per lane) (left), and densitometry of UCP1 in an immunoblot as at left (n = 6 mice per group), normalized to actin and presented relative to the results of vehicle-treated mice, set as 1 (right). (c, d) Insulin-tolerance tests of wild-type C57BL/6 mice in which DIO was established (with mice fed an HFD for 4 weeks), followed by implantation of an osmotic pump that delivered the α4 inhibitor or vehicle (key), followed by HFD feeding for another 4 weeks at 22 °C (n = 6 mice per group; c) or 30 °C (thermoneutrality) (n = 8 mice per group; d). (e) Expression of mRNA from genes encoding thermogenesis-related molecules in the SAT of mice as in a, d(n = 6 mice per group at 22 °C; n = 8 mice per group at 30 °C), analyzed at the end of the feeding period; results were normalized to those of 18S rRNA and are presented relative to those of vehicle-treated mice at 22 °C, set as 1. Prdm16 encodes PR (PRD1-BF1-RIZ1 homologous) domain containing 16. (f) Flow cytometry of M1-like macrophages (defined as in Fig. 2g, h) in the SVF of the SAT and VAT from mice as in d (n = 6 mice (vehicle) or n = 7 mice (α4 inhibitor), SAT; n = 5 mice (vehicle) or n = 7 mice (α4 inhibitor), VAT) maintained under conditions of thermoneutrality (isolation at the end of the experiment); results (cells per gram tissue) are presented relative to those of vehicle-treated mice, set as 100%. *P < 0.05 (Mann-Whitney U-test). Data are representative of two experiments (a, b; mean ± s.e.m. (a) or mean + s.e.m. (b)) or from one experiment (c-f; mean ± s.e.m.).

During DIO, despite the greater energy expenditure of Creα4<sup>−/−</sup> mice, could be attributed to the greater food intake of these mice (Supplementary Fig. 6a).

To elucidate the finding of greater energy expenditure in obese Creα4<sup>−/−</sup> mice, we assessed the expression of genes encoding thermogenic products in white AT or brown AT (BAT). The expression of both mRNA and protein from the gene encoding the key thermogenic protein UCP1 (UCP1) and of mRNA from genes encoding products related to brown-fat identity (Cidea, cell death-inducing DNA fragmentation factor-like effector A) and Ccox8b (cytochrome c oxidase subunit 8β)) was upregulated in the SAT of Creα4<sup>−/−</sup> mice relative to that in the SAT of Creα4<sup>−/−</sup> littermates (Fig. 3b, c). Consistent with that, Creα4<sup>−/−</sup> mice challenged with exposure to a cold temperature (4 °C) sustained a higher core temperature, accompanied by enhanced expression of UCP1, compared with that of their Creα4<sup>−/−</sup> littermates (Fig. 3d, e). Immunohistochemical analysis of UCP1 revealed a greater presence of beige-adipogenesis areas in the SAT of Creα4<sup>−/−</sup> mice exposed to the cold than in that of their Creα4<sup>−/−</sup> counterparts similarly exposed to the cold (Fig. 3f). Although deletion of Igα4 enhanced beige adipogenesis in the SAT of obese mice, it had no effect on the expression of genes encoding thermogenic products in the VAT, or on the expression of such genes and the abundance of UCP1 protein, as assessed by immunohistochemistry, in the BAT (Supplementary Fig. 6b, c). To address that difference, we compared Vcan1 expression in SAT with that in BAT and found it was much higher in the adipocytes of obese SAT than in those of obese BAT (Fig. 3g). That finding provided an explanation for the finding that deletion of Igα4 increased thermogenic beige adipogenesis in obese SAT without affecting the expression of genes encoding thermogenic products in BAT. Therefore, the greater energy-dissipating activity of obese Creα4<sup>−/−</sup> mice was derived from a more thermogenic SAT.

Pharmacological inhibition of α4 integrin in obesity

As enhanced beige adipogenesis of white AT is linked to improved metabolic control, approaches to stimulate white-fat beiging have emerged as an attractive anti-obesity therapeutic strategy<sup>30</sup>. We therefore assessed the therapeutic potential of pharmacological inhibition of α4 integrin in wild-type mice given an inhibitor of α4 integrin that ‘preferentially’ blocks α4β1 or vehicle control, via a mini-osmotic pump, for 6 weeks after establishment of DIO. Mice with DIO that received the α4 inhibitor exhibited improved insulin sensitivity and lower serum concentrations of insulin, glucose and cholesterol compared with that of mice treated with the vehicle (Fig. 4a–c). Analysis of obese SAT by confocal microscopy revealed that inhibition of α4 integrin reduced the abundance of macrophages in contact with adipocytes (Fig. 4d). Histological quantification demonstrated that the number of both CLSs and of non–CLS-associated macrophages in proximity to adipocytes in the SAT was significantly decreased by blockade of α4 integrin (Fig. 4e). Moreover, flow-cytometry analysis revealed that the abundance of M1-like macrophages (defined as F4/80<sup>+</sup>Cd11b<sup>+</sup>iNOS<sup>−/−</sup>CD206<sup>−/−</sup>) was significantly lower in the SAT of obese mice that received the α4 inhibitor than in that of mice treated with the vehicle, whereas the number of M2-like macrophages in obese SAT remained unaffected by blockade of α4 integrin (Fig. 4f). Furthermore, inhibition of α4 integrin in mice with DIO led to enhanced beige adipogenesis of the SAT, as indicated by elevated expression of Ucp1 mRNA and UCP1 protein in the SAT (Fig. 5a, b) but not in the VAT (data not shown), relative to that in mice treated with the vehicle control.
We also tested the α₄ inhibitor in ob/ob mice, a model of genetically induced obesity. The ob/ob mice treated with the α₄ inhibitor displayed improved insulin sensitivity, accompanied by enhanced thermogenesis, higher expression of UCP1 protein and ameliorated accumulation of inflammatory macrophages in the SAT, relative to that of ob/ob mice treated with the vehicle (Supplementary Fig. 7a–d). Together our findings suggested that functional inactivation of macrophage interactions in obese AT mediated by α₄ integrin ameliorated obesity-related metabolic dysregulation via improved beige adipogenesis.

To substantiate the mechanism of action of the pharmacological inhibition of α₄β₁, we treated mice with DIO with the α₄ inhibitor or vehicle, then exposed them to conditions of thermoneutrality (30 °C), wherein UCP1 is functionally incompetent. In contrast to the improved insulin sensitivity and enhanced expression of Ucp1 as a result of treatment with the α₄ inhibitor in mice kept at 22 °C, relative to that of their counterparts treated with vehicle at 22 °C, these differences were largely abolished at thermoneutrality (Fig. 5c–e). At thermoneutrality, slightly (but not significantly) higher expression of Ucp1 was observed in mice with DIO treated with the α₄ inhibitor than in their counterparts treated with vehicle (Fig. 5e). The abundance of M1-macrophages was lower in both the SAT and VAT of mice treated with the α₄ inhibitor than in their counterparts treated with vehicle (Fig. 5f). Hence, the α₄ inhibitor failed to improve insulin sensitivity or beige adipogenesis under thermoneutral conditions despite reducing the accumulation of macrophages, which suggested crucial involvement of UCP1 in the mechanism whereby pharmacological inhibition of α₄ promoted the beige adipogenesis of white AT and prevented obesity-related metabolic dysregulation.

**Macrophage–adipocyte adhesion inhibits UCP1 expression**

Studies suggest that alternatively activated M2 macrophages promote beige adipogenesis in lean AT in a paracrine fashion. However, whether M1-like inflammatory macrophages can conversely mediate the proactive inhibition of beige adipogenesis has not been demonstrated.
thus far, although it was suggested by our findings. Consistent with published findings,16,18,31 the expression of UCP1 was lower, at level of Ucp1 mRNA and UCP1 protein, in obese SAT than in lean SAT (data not shown), which indicated that the predominance of inflammatory macrophages in obese SAT correlated with the inhibition of beige adipogenesis. To conclusively demonstrate that concept and to delineate its mechanistic underpinnings, we performed macrophage–primary adipocyte co-cultures. The adipocytes used were generated from pre-adipocytes isolated from the stromal vascular fraction (SVF) of wild-type mouse SAT that maintain their ability to express beige-fat-related genes, including Ucp1 (ref. 8). White and beige adipocytes have bidirectional interconversion properties,9,13,32 probably because they originate from PDGFRα+ bipotent precursors.33 We added norepinephrine and triiodothyronine (T3) to primary adipocytes before the adipocyte–macrophage co-cultures performed here, to drive a beige-fat-like profile and activation of Ucp1 expression14 (Supplementary Fig. 8). For M1-like inflammatory macrophages, we cultured bone-marrow-derived macrophages (BMDMs) with the cytokine GM-CSF and stimulated them with lipopolysaccharide (LPS), or stimulated macrophages derived from obese SAT with LPS plus interferon-γ (IFN-γ)35,36. When adipocytes were cultured in direct contact (in the same well) with pro-inflammatory M1-like BMDMs, the Ucp1 expression in adipocytes stimulated with norepinephrine and T3 was significantly lower than that in adipocytes stimulated similarly but cultured in the absence of M1-like macrophages (Fig. 6a). Similar results were obtained for adipocytes cultured in direct contact with pro-inflammatory macrophages derived from obese SAT and stimulated with LPS plus IFN-γ (Fig. 6b). Consistent with that, deletion of Irg4 (in obese Cre+α4i4i mice) was associated with higher expression of Ucp1 in the SAT than in that of their obese Cre−α4i4i littermates (Fig. 3b). Among fat pads with thermogenic potential (SAT and BAT), adipocytes in SAT had higher thermogenic potential (SAT and BAT), adipocytes in SAT had higher
together with differentiated adipocytes. M2-like macrophages promoted Ucp1 expression by adipocytes when cultured with adipocytes in both direct-contact mode and non-contact mode (Transwell setup). Under both co-culture conditions, inhibition of α4 integrin did not affect the Ucp1 expression in adipocytes triggered by M2-like macrophages (Fig. 6c,d).

Studies have highlighted the opposing roles of the signaling pathways of the mitogen-activated protein kinases p38 (ref. 37) and Erk38,39 in beige adipogenesis and the regulation of UCP1, as they mediate stimulatory effects and inhibitory effects, respectively. We found that direct culture of inflammatory macrophages together with primary adipocytes resulted in lower abundance of phosphorylated p38 and greater abundance of phosphorylated Erk in adipocytes, relative to their abundance in adipocytes cultured alone (Fig. 6c,f); this provided a mechanistic basis for the inhibitory effect of inflammatory macrophages on the expression of Ucp1 by adipocytes. The reduction in the expression of Ucp1 by the presence of M1-like macrophages was reversed when adipocytes were pre-treated with an inhibitor of Erk (Fig. 6g). Next, we sought to determine whether the α4-integrin-dependent inhibitory effect of inflammatory macrophages on beige adipogenesis was driven by regulation of the pathways of phosphorylated p38 and phosphorylated Erk. In support of that proposal, the abundance of phosphorylated p38 was increased, whereas that of phosphorylated Erk was decreased, when the direct contact between adipocytes and inflammatory macrophages was blocked by the α4 inhibitor (Fig. 6h,i).

In addition to the ability of white adipocytes to induce UCP1 expression8,10,40, progenitors of adipocytes can also differentiate into beige adipocytes33,41. We therefore assessed whether direct contact with pro-inflammatory macrophages would also affect Ucp1 expression in the progenitors of adipocytes. First, we found that PDGFRα−Sca-1− adipocyte progenitors from the SAT of obese mice displayed significantly higher expression of Vcam1 than that of their counterparts from that of lean mice (Fig. 6j). Moreover, TNF significantly increased Vcam1 expression in adipocyte progenitors (Fig. 6k).

In addition, when adipocyte progenitors were cultured in the presence of pro-inflammatory M1-like macrophages (in a direct cell–cell contact assay), their expression of Ucp1 was significantly decreased relative to that of adipocyte progenitors cultured alone, in a manner dependent on the αβ1–VCAM-1 interaction (Fig. 6l).

We next assessed whether the direct αβ1–VCAM-1 interaction between macrophages and adipocytes would also affect the inflammatory activation of macrophages and thereby lead to a self-sustained cycle of persistent inflammation of AT. Culture of macrophages on plates pre-coated with VCAM-1 enhanced their expression of TNF (Fig. 7a,b), which is a cardinal marker of M1 macrophage polarization23,35,42. The VCAM-1-dependent upregulation of TNF in macrophages was abolished in the presence of the α4 inhibitor (Fig. 7a) and in α4-integrin-deficient macrophages (Fig. 7b). Consistent with that, inhibition of α4 integrin or α4 deficiency in macrophages prevented the enhanced TNF expression in macrophages that resulted from direct cell–cell contact with adipocytes (Fig. 7c,d). Thus, the α4-integrin-dependent crosstalk of macrophages with adipocytes was bidirectional and promoted inflammatory activation of the former and blocked beige adipogenesis of the latter.

We then studied whether inflammatory macrophages could further contribute to the inhibition of beige adipogenesis by upregulating VCAM-1 expression in the adipocyte compartment. Since TNF enhanced VCAM-1 expression in adipocytes and TNF expression itself was upregulated by the direct macrophage–adipocyte contact, we explored whether macrophage-derived TNF could stimulate VCAM-1 expression in adipocytes. We therefore performed non-contact (Transwell) macrophage–adipocyte co-cultures. We observed upregulation of VCAM-1 expression in adipocytes cultured together with SAT-derived M1-like inflammatory macrophages (Fig. 7e). This paracrine-induced increase in the expression of VCAM-1 in adipocytes via inflammatory macrophages was inhibited by antibody to TNF (Fig. 7e). In contrast, the expression of VCAM-1 in adipocytes was not affected by culture (in a Transwell setup) with SAT-derived M2-like macrophages in either the presence of the α4 inhibitor or its absence (Fig. 7f).

Thus, the α4-integrin–dependent adhesive macrophage–adipocyte interaction promoted a self-sustained inflammatory loop in obese AT that involved inflammatory activation of macrophages and resulted in enhanced TNF production that further upregulated VCAM-1 expression in adipocytes. The direct α4-integrin–dependent macrophage–adipocyte interaction also inhibited UCP1 expression, which might contribute to diminished beige adipogenesis in obesity.

**Macrophage–adipocyte interactions in the human system**

We then determined whether the adhesive inflammatory macrophage–adipocyte interaction dependent on α4 integrin–VCAM-1 also operated in the human system. At first, we observed an inverse correlation between the expression of UCP1 and that of VCAM1 in SAT samples obtained from patients undergoing abdominal surgery (Fig. 7g). We also found that the expression of VCAM1 increased in human adipocytes (differentiated from pre-adipocytes) after stimulation with TNF or palmitate (Fig. 7h). Moreover, the adhesion of human M1-like pro-inflammatory macrophages to adipocytes was partially inhibited by the α4 inhibitor (Fig. 7i). Culture of inflammatory macrophages together with primary adipocytes (with direct cell–cell contact) reduced the expression of UCP1 in the latter in a manner dependent on adhesion mediated by αβ1–VCAM-1 (Fig. 7j).

These data suggested that direct macrophage–adipocyte interactions might downregulate UCP1 expression in human SAT as well.

**DISCUSSION**

Beige adipogenesis linked with expression of the thermogenic protein UCP1 is a homeostatic function of the promotion of energy expenditure by lean AT13. In human and mouse obesity, beige adipogenesis becomes dysfunctional, and this phenomenon is associated with enhanced inflammation of AT15,17,18,33,44,45. Here we established a self-sustained loop that linked inflammation of AT and retention of inflammatory macrophages with impaired beige adipogenesis in obesity. Specifically, α4-integrin-mediated adhesion of inflammatory macrophages to VCAM-1, the expression of which was augmented in adipocytes (and their progenitors) in obesity, inhibited UCP1 expression in an Erk-dependent way.

The enhanced abundance of inflammatory macrophages in obese AT might depend on the function of integrins such as αβ1 on increased expression of monocyte-attracting chemokines1,10,27,29 or on local population expansion of macrophages44. It is conceivable that the mechanism for the α4-integrin-mediated retention of macrophages identified here might also facilitate the population expansion of macrophages in AT44. We found that TNF, the expression of which is elevated in obese AT,33,48, upregulated VCAM-1 expression in adipocytes and their progenitors and thereby promoted direct adhesive interactions of those cells with macrophages, dependent on α4 integrin–VCAM-1. That interaction promoted the retention of macrophages in obese AT and drove further pro-inflammatory M1-like polarization, including increased TNF production; it thereby further enhanced the expression of VCAM-1 by adipocytes and sustained the direct...
High incidence of metabolically active brown adipose tissue

Blockade of α4 integrin-mediated interaction of inflammatory macrophages with adipocytes downregulated signaling via p38 and upregulated signaling via Erk in the latter cells, which led to downregulation of Ucp1 expression, in accordance with published reports. Furthermore, Erk signaling in adipocytes promotes insulin resistance and diabetogenic actions by regulating the function of the nuclear hormone receptor PPARγ. Therefore, the mechanism mediated by α4 integrin–VCAM-1 delineated here might regulate further detrimental actions of Erk signaling in adipocytes.

Our findings are consistent with those of a published study that used knock-in mice expressing mutant α4 (α4(Y991A)) to demonstrate that inhibition of α4 integrin signaling ameliorates DIO-related metabolic dysfunction. However, our work has additionally introduced the new concept that α4 integrin-dependent direct adhesion of inflammatory macrophages to adipocytes inhibited UCP1 expression and has revealed a previously unknown self-sustained cycle that links inflammation of AT with impaired beige adipogenesis in obesity.

Analysis of VCAM-1 expression is usually confined predominantly to the vascular endothelium and hematopoietic cells. We found that enhanced VCAM-1 expression in obese AT was derived predominantly from adipocytes. Moreover, the most prominent VCAM-1 expression by adipocytes was observed in the SAT (rather than the VAT or the BAT), which might explain the finding that genetic or pharmacological inhibition of α4 integrin affected mainly UCP1 expression and beige adipogenesis in this particular fat pad. The α4-integrin-dependent adhesive interaction delineated might regulate beige adipogenesis in the human system, a proposal supported by the inverse correlation between the expression of VCAM-1 and that of UCP1 in human SAT. Notably, although multiple papers have described adhesive leukocyte–endothelium interactions that mediate the recruitment of inflammatory cells, little knowledge exists on mechanisms for the adhesion of macrophages with tissue-resident cells, or the functional consequences thereof for the local tissue environment. In this context, a new pathogenic principle arising from our work involves direct adhesive interactions between inflammatory cells and parenchymal cells. This previously unknown disease-promoting mechanism might underlie several other chronic inflammatory disorders and might be therapeutically important, as it is amenable to intervention. The mechanism presented here might be of therapeutic relevance, since we have pinpointed α4 integrin as a potential target for intervention in obesity. Pharmacological inhibition of α4 integrin promoted systemic insulin sensitivity by stimulating beige adipogenesis; such a therapeutic strategy has not been yet harnessed by current anti-diabetic drugs and might merit further detailed investigation in preclinical settings.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.-J.C. designed and performed experiments, analyzed and interpreted data and wrote the paper; A.C. performed experiments, analyzed and interpreted data and wrote the paper; M.E., R.G.-M., V.L.A., I.M., N.M., J.G., J.-H.L. performed experiments and analyzed data; T.P. participated in experimental design and discussion; T.P. provided mice withloxP-flanked Il6rs; M.B. performed research, analyzed and interpreted data; G.H. participated in experimental design and edited the paper; and T.C. designed the study and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Animal studies. Mx1-cre<sup>Itgal<sub>flx/</sub>flx</sup> mice (Cre<sup>α<sub>4</sub>β<sub>1</sub></sup>) were previously described<sup>13</sup>. To induce excision of α<sub>4</sub>β<sub>1</sub> integrin, Cre recombinase was induced in male Cre<sup>α<sub>4</sub>β<sub>1</sub></sup> mice by three intraperitoneal injections of poly(I:C) (Invivogen) at 2-day intervals<sup>13,15</sup>. Littermate male Mx1-cre<sup>α<sub>4</sub>β<sub>1</sub></sup> mice (Cre<sup>α<sub>4</sub>β<sub>1</sub></sup>) that also received identical treatments with poly(I:C) served as controls. The mice were fed a normal-fat diet (D12450B, 10% of kcal from fat, Research Diets) or a high-fat diet (HFD; D12492, 60% of kcal from fat, Research Diets) for up to 20 weeks starting at 2 weeks after Cre recombinase induction. Deletion of α<sub>4</sub>β<sub>1</sub> integrin was confirmed by examining α<sub>4</sub>β<sub>1</sub> integrin expression by flow cytometry (antibody to CD49d (anti-CD49d), PS/2, AbD Serotec) in leukocytes isolated from blood upon Ficol gradient purification (GE Healthcare). Mice with Itgal deletion (α<sub>4</sub>β<sub>1</sub> integrin KO) were previously described<sup>12</sup>. B6.V-<sup>Pkhi</sup>β<sup>2</sup>JR (ob/ob) mice were from Janvier Labs. Animal experiments were approved by the Landesdirektion Sachsen, Germany.

In the α<sub>4</sub>β<sub>1</sub> integrin inhibitor experiments, male C57BL/6 mice (Charles River) were fed an HFD for at least 6 weeks before subcutaneous implantation of an Alzet osmotic pump (Alzet, model 2006) filled with vehicle (PBS) or α<sub>4</sub>β<sub>1</sub> inhibitor (α<sub>4</sub>-inh.; 69.125 µM in a 200 µl total volume in the pump) that allowed continuous release for 6 weeks. Thereafter, mice were fed a HFD for another 6 weeks. The ob/ob mice were given implantation of an Alzet osmotic pump including PBS or α<sub>4</sub>β<sub>1</sub> inhibitor at 6 weeks of age. The α<sub>4</sub>β<sub>1</sub> integrin inhibitor ELND002, which has been previously used in clinical trials (NCT01318421 and NCT01144551), ‘preferentially’ blocks α<sub>4</sub>β<sub>1</sub> integrin (rather than α<sub>β</sub>integrin, and efficiently inhibits the α<sub>4</sub>β<sub>1</sub>-VCAM-1 interaction<sup>31</sup>; it is described in detail in patent US 8,269,009 B2 and was provided by Elan Pharmaceuticals and Biogen Idec.

In thermoneutrality experiments, male C57BL/6 mice were fed an HFD for a total of 8 weeks. After 4 weeks of feeding, the mice were given implantation of an Alzet osmotic pump that delivered α<sub>4</sub>-inh. inhibitor or vehicle. After pump implantation, each group of mice (α<sub>4</sub>-inh. or PBS) was divided into two sub- groups and the feeding was continued for another 4 weeks under ambient (22 °C) or thermoneutrality (30 °C) conditions. In cold-exposure experiments, obese mice were challenged with a temperature of 4 °C with free access to water and food for 12 h. Thereafter, tissues were harvested and snap frozen in liquid nitrogen for RNA and protein analysis or fixed in 10% formalin for histology.

An estimate of at least four to six mice per group was used in experiments. No blinding was performed. In the osmotic pump experiments, groups were weight matched and randomized to the two different treatments. Pre-established exclusion criteria included death of a mouse or requirement of euthanasia of a mouse following animal-protocol-defined endpoints (for example, signs of systemic illness or other forms of distress) before completion of the experiment. According to these predefined criteria, exclusion was necessary in one experiment involving overall one mouse. One mouse from the 30 °C group (involving the assays in Fig. 5d.e) was excluded according to these pre-established criteria.

Monocyte and T cell trafficking assays. Mouse bone marrow monocytes or splenic T cells were isolated from Cre<sup>α<sub>4</sub>β<sub>1</sub></sup>M (Cre<sup>α<sub>4</sub>β<sub>1</sub></sup>) mice through a mouse monocyte enrichment kit (EasySep) or a Pan T cell isolation kit II (Miltenyi Biotec). Cell purity was confirmed by flow cytometry; monocytes were defined as CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>−</sup> (CD11b<sup>+</sup>Ly6G<sup>−</sup> macrophages) or T cells were defined as CD45<sup>+</sup>CD11b<sup>−</sup>Ly6G<sup>−</sup> (T cells). Monocytes or T cells were labelled with PKH26red and PKH67green, respectively, by using PKH26- or PKH67-fluorescent cell linker kits (Sigma-Aldrich). Isolated monocytes or T cells were washed with PBS and resuspended in 1 ml of PKH26 or PKH67 (4 µM in diluent C per 2 × 10<sup>7</sup> cells) which was then incubated at 37 °C for 20 min. The staining was halted by adding 2 ml FBS, and cells were washed in medium supplemented with 10% FBS. A combination of equal numbers of PKH26- and PKH67-labelled cells (2.9 × 10<sup>6</sup> cells) was simultaneously injected into DIO C57BL/6 male mice (fed an HFD for up to 20 weeks). Seven days thereafter, the labeled cells, located in the inguinal subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT) or draining lymph nodes (inguinal lymph nodes for SAT; mesenteric lymph nodes for VAT), were analyzed by flow cytometry; in some cases, analysis also included additional staining for identification of macrophages (anti-F4/80 (BM8 from eBioscience) and anti-CD11b (identified above)) and T cells (anti-CD3 (identified above)).

Metabolic phenotyping. Blood glucose, cholesterol and triglycerides were measured in blood samples of mice fasted overnight, with a glucose meter device (Accu-Chek, Roche) and the Accutrend Plus system (Roche). Fasting insulin concentrations were measured with an ELISA Kit (Crystal Chem). For insulin-tolerance tests, mice were fasted for 6 h and then given intraperitoneal injection of insulin (1–1.5 U/kg, Huminsulin, Lilly). Glucose concentrations were measured at specific time points up to 120 min after insulin injection.

For metabolic cage analysis<sup>35</sup>, mice fed an HFD for 8 weeks were individually housed in metabolic chambers (PhenoMaster, TSE Systems) with free access to food and water, maintaining a 12 h–12 h dark–light cycle. Mice were acclimatized in metabolic chambers for 24 h before initiation of data collection. The volume of oxygen consumption (VO<sub>2</sub>) and carbon dioxide production (VCO<sub>2</sub>) were determined every 20 min for a period of 3 d. Respiratory exchange ratio (RER) was defined as VCO<sub>2</sub>/VO<sub>2</sub>, and energy expenditure (EE) was determined by using the formula 3.941 × VO<sub>2</sub> + 1.106 × VCO<sub>2</sub> food intake was also determined. Statistics were performed using analysis of covariance. Determination of lean and fat mass was performed by using computed tomography (CT, Skyscan 1178; Bruker).<sup>36</sup>

3T3-L1 cell culture. Mouse 3T3-L1 pre-adipocytes (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin-streptomycin and 10% heat-inactivated FCS (FBS) (Gibco-BRL). Cells tested negative for mycoplasma. For differentiation into adipocytes, medium was replaced with complete DMEM containing insulin (1 µg/ml, Sigma-Aldrich), 3-isobutyl-1-methylxantine (0.5 mM, Sigma-Aldrich) and dexamethasone (0.25 µM, Sigma-Aldrich) starting 2 d after cells reached confluence (day 0). On day 3, the medium was replaced every other day with complete DMEM containing insulin (1 µg/ml). Fully differentiated 3T3-L1 adipocytes (days 8–10) were used for assays. In some experiments, 3T3-L1 adipocytes were stimulated with mouse TNF (20 ng/ml, R&D) or palmitate-BSA (250 µM, Sigma-Aldrich) before further analysis.

Primary pre-adipocyte isolation and adipocyte differentiation. Mouse primary adipocyte differentiation was performed as described with modifications<sup>37</sup>. For isolation of stromal vascular (SV) cells, SAT of lean male C57BL/6 mice was dissected, minced, digested for 60 min at 37 °C in PBS containing 0.5% BSA (Sigma-Aldrich), collagenase (2 mg/ml, Gibco) and 10 mM CaCl<sub>2</sub>, filtered through a 40-µm cell strainer and centrifuged at 600g for 5 min. SV cells were resuspended in DMEM/F12 medium supplemented with 10 mM FBS, Glutamax and penicillin-streptomycin and were filtered through a 40-µm cell strainer. Afterward, the SV cells underwent CD45 negative selection to remove hematopoietic cells using MACS magnetic beads (Miltenyi) and were plated into six-well plates. After 90 min, SV cells were washed twice with PBS, to remove non-adherent cells, and then were grown to 70–80% confluence in complete DMEM/F12. For the differentiation of SV cells into adipocytes, SV cells were seeded into 24-well plates at a density of 5 × 10<sup>4</sup> cells per well in 1 ml complete DMEM/F12. When the cells reached confluence (day 0), cell medium was replaced with medium containing insulin (1 µg/ml, Sigma-Aldrich), 3-isobutyl-1-methylxantine (0.5 mM, Sigma-Aldrich), dexamethasone (0.25 µM, Sigma-Aldrich) and T3 (1 nM, Sigma-Aldrich). After 48 h, medium was exchanged (every 2 d) with complete DMEM/F12 containing insulin (1 µg/ml) and T3 (1 nM) and pioglitazone (1 µM). Differentiated adipocytes (days 8–10) were used for assays. In some experiments, adipocytes were stimulated with mouse TNF (10 ng/ml) or palmitate-BSA (250 µM) before analysis.

Co-culture of macrophages with primary adipocytes or adipocyte progenitors. Primary bone-marrow-derived macrophages (BMDMs) were prepared by flushing of the bone marrow of C57BL/6 mice<sup>26,28</sup>. After lysis of red blood cell (RBC), cells were grown in RPMI-1640 medium supplemented with 10% FBS,
Glutamax, penicillin-streptomycin and recombinant mouse GM-CSF (10 ng/ml, PeproTech) for 7 d. GM-CSF-cultured BMDMs were stimulated with LPS (100 ng/ml, Invivogen) for 12 h to induce an inflammatory M1-like phenotype. BMDMs were harvested by using a non-enzymatic cell-dissociation solution (Sigma-Aldrich) and were counted before co-culture with adipocytes.

Primary pre-adipocytes isolated from the SVF of the SAT undergoing differentiation (described above) were treated on day 8 or 9 of their differentiation with T3 (10 nM) and norepinephrine (1 μM; Sigma Aldrich) for 3 h, to induce a thermogenic response, and then washed before co-culture with BMDMs. For direct primary adipocyte–BMDM co-cultures, in which the cells were in contact with each other, adipocytes were cultured without or with BMDMs (5 × 10^4) for 1 h. Experiments were performed in the absence or presence of the αt inhibitor (ELND002; 1 nM). In other experiments, adipocytes were pre-treated with an Erk inhibitor (UO126, 10 μM, Sigma Aldrich) for 30 min and were washed with medium before the addition of M1-like polarized BMDMs. For adipocyte mRNA-expression analysis, cells were trypsinized, washed with PBS and separated from macrophages by CD45 negative selection using MACS magnetic beads (Milteny). Ucp1 mRNA expression was analyzed by qPCR; 18S rRNA was used for normalization.

For the detection of phosphorylated Erk1/2 and p38 in adipocytes after co-culture with macrophages for 30 min, cells were harvested by trypsinization, washed with PBS and incubated with mouse Fc block and APC-conjugated rat antibody to mouse CD11b (M1/70) (both from BD) for 15 min on ice. After fixation with 4% paraformaldehyde for 10 min, cells were permeabilized by the 90% methanol, treated with 10% goat serum (Sigma Aldrich) and incubated with either rabbit antibody to mouse mitogen-activated protein kinase p44/42 phosphorylated at Thr180 and Tyr182 (0.25 μg/100 μl; Cell Signaling; catalog # 9101) or rabbit antibody to mouse p38 phosphorylated at Thr180 and Tyr182 (0.25 μg/100 μl; Cell Signaling; catalog # 8691) for 1 h at 22 °C. After washing, cells were incubated with goat anti-rabbit IgG (H+L) Alexa Fluor 488 (0.1 μg/100 μl; Invitrogen; catalog # A-11034) for 1 h at 22 °C. Median fluorescence intensity (MFI) was determined by flow cytometry (FACSCanto II, BD) after gating of adipocytes (defined as CD11b+ cells).

In other experiments, co-cultures of adipocytes were performed with SAT-derived macrophages. For isolation of primary macrophages from SAT, SV cells were isolated from lean mice or obese C57BL/6 mice. Isolated SV cells were cultured with anti-F4/80 Microbeads (Milteny Biotec), and macrophages were selected as F4/80+ cells by MACS columns. The isolated macrophages were cultured in complete RPMI-1640 medium with M-CSF (10 ng/ml, eBioscience) for 24 h. To obtain M1-like polarized macrophages, macrophages derived from obese SAT were additionally treated with LPS (100 ng/ml, Invivogen) and IFN-γ (20 ng/ml, BD) for 12 h, while for obtaining M2-like polarized macrophages, macrophages derived from lean SAT were treated with IL-4 (20 ng/ml, PeproTech) for 12 h.

In direct primary adipocyte–SAT-derived macrophage co-cultures, adipocytes pre-treated with T3 and norepinephrine were co-cultured without or with SAT-derived M1-like polarized macrophages (2 × 10^4 cells) for 1 h, as described above for adipocyte–BMDM co-cultures. In other experiments, adipocytes were co-cultured without or with SAT-derived M2-like polarized macrophages (2 × 10^4 cells) for 6 h. Experiments were performed in the absence or presence of the αt inhibitor (1 nM). The cells were trypsinized, and Ucp1 mRNA expression in adipocytes was analyzed by qPCR after their separation as CD45- cells by MACS magnetic beads (Miltenyi Biotec).

The indirect primary adipocyte–primary SAT-derived macrophage co-cultures, in which the cells were not in contact, were performed with 0.4-μm pore size Transwell inserts (Corning). T3- and norepinephrine-pre-treated primary adipocytes (lower compartment) were co-cultured with 6 h without or with SAT-derived M2-like polarized macrophages (2 × 10^4 cells) that were in the Transwell inserts, in the absence or presence of the αt inhibitor. At the end of the experiment, Transwell inserts were removed, adipocytes were washed, and Ucp1 mRNA expression was analyzed by qPCR.

In other experiments, M1- or M2-like polarized SAT-derived macrophages were seeded on 0.4-μm pore size Transwell inserts (0.4-μm pore size) at a density of 2 × 10^4 cells and co-cultured with primary adipocytes. The experiments with M2-like macrophages were performed in the absence or presence of a TNF-blocking antibody (1 μg/ml, R&D; catalog # AF-410-NA). After incubation for 24 h, adipocytes were trypsinized and stained with an anti-VCAM-1 (clone 429, BD); VCAM-1 expression was analyzed by flow cytometry.

For adipocyte–progenitor-cell isolation, SV cells from SAT from lean C57BL/6 mice were washed with PBS containing 0.5% BSA (Sigma Aldrich), incubated with Fc block (BD) and stained with anti-CD31 (MEC 13.3 from BD), anti-CD45 (30-F11 from BioLegend), anti-Scal (D7 from BD) and anti-PDGFRα (APAS from BD) for 20 min at 4 °C. Adipocyte progenitors were sorted as CD31+CD45−Scal+PDGFRα+ cells in a FACSaria II cell sorter (BD). The cells were seeded into culture dishes until they reached 70–80% confluence and then were transferred to 24-well plates (5 × 10^4 cells per well) before co-culture with BMDMs. Specifically, after 3 h of pre-treatment of adipocyte progenitors with T3 and norepinephrine, direct co-cultures of adipocyte progenitors with M1-like polarized BMDMs were performed for 1 h in the absence or presence of the αt inhibitor. Thereafter, Ucp1 mRNA abundance in adipocyte progenitors was determined by qPCR, after exclusion of BMDMs as described above. In other experiments, after isolation of SV cells and adipocyte-progenitor-cell sorting, the latter were seeded for 24 h in 48-well plates (2 × 10^4 cells per well) and then treated overnight with mouse recombinant TNF (20 ng/ml, R&D systems), and mRNA expression of Vcam1 was analyzed by qPCR.

Isolation of mature adipocytes, adipocyte progenitors and endothelial cells from SAT. SAT, VAT and BAT from lean or obese C57BL/6 mice were digested with collagenase and filtered. After centrifugation, the floating cells representing the mature adipocytes were collected and washed three times with PBS, and RNA isolation was performed with Trizol. The SV cells in the pellet were washed with PBS containing 0.5% BSA and then were incubated with Fc block (BD) and stained with anti-CD31 (MEC 13.3 from BD), anti-CD45 (30-F11 from BioLegend), anti-Scal (D7 from BD) and anti-PDGFRα (APAS from BD) for 20 min at 4 °C. After being washed, CD31+CD45−Scal+PDGFRα+ adipocyte progenitors or CD45+CD31+ endothelial cells were sorted in a FACSaria II cell sorter (BD), and RNA from each individual population was isolated by Trizol.

Stromal vascular fraction (SVF) isolation and flow cytometry. SVF was prepared as reported55,59,60. In brief, inguinal SAT or VAT was isolated from euthanized mice, then was minced and digested for 60 min in DMEM containing collagenase type I (2 mg/ml per gram of tissue; Invitrogen) at 37 °C. Cell suspensions were then filtered through a 40-μm cell strainer and the SVF was collected as a pellet after centrifugation at 500g for 5 min. After RBC lysis, cells were stained with anti-CD45 (30-F11, BD), anti-F4/80 (clone BM8, BioLegend), anti-CD11b (M1/70, BD), anti-CD14 (HCD14, BD), anti-CD206 (c06802, BioLegend; MR5D3, Acris), anti-TNF (MP6-XT22, BioLegend), anti-iNOS (CXNFT, eBioscience) and anti-CD49d (clone PS/2, Abbvie, or clone 9C10 (MF4.8, BioLegend). Cells were analyzed in a FACSCanto II flow cytometer (BD).

Histological analysis. For immunohistochemistry, AT was fixed overnight in 10% formalin solution, embedded in paraffin and cut into 5-μm sections. For adipocyte size determination, images of H&E-stained SAT or VAT sections were obtained and the diameter of ~200 adipocytes per slide was measured with the Axiovision Rel. 4.8 software (Carl Zeiss MicroImaging). The diameters of both height and width were measured from each cell. For UCPI or F4/80 immunohistochemistry, sections were de-paraffinized and incubated with pronase (Sigma Aldrich) or citrate buffer, respectively. Sections were then incubated overnight with anti-UCPI (Abcam; catalog # ab10983) or anti-F4/80 (Novus Biologicals; catalog # NB600-404). The Vectastain ABC kit (Vector Laboratories) was used for UCPI or F4/80 detection. Images were obtained by a computerized microscope (Zeiss) or an Axioscan.Z1 slide scanner (Zeiss). Quantification of CLSs was performed in whole-section F4/80-stained mosaic images by counting of CLSs per mm^2 of tissue in six sections per sample. Determination of the number of non–CLS-associated macrophages was performed by counting of F4/80+ cells per 100 adipocytes from ten random low-magnification CLS-free fields per sample. The Zen Imaging Software (Zeiss) was used in both types of quantification.

For immunofluorescence detection of macrophages (F4/80 staining) and adipocytes ( caveolin-1 staining)28 in SAT, whole mounts were prepared.
The tissues were extracted and fixed with parafomaldehyde 4% (Thermo Fisher Scientific) for 2 h at 4 °C. Thereafter, the tissue was cut into small cubic pieces (each side approximately 2 mm). The samples were thoroughly washed with PBS and blocked-permeabilized in serum-free protein block (Dako) with 1% Saponin (Sigma Aldrich) for 2 h at 22 °C. The primary antibody incubation was performed in Antibody diluent (Dako) supplemented with 0.5% saponin overnight at 4 °C. A 1:100 anti-mouse F4/80 (1:100, CI:A3-1; Abcam, ab6640) and a rabbit anti-mouse ced vein-1 (1:100, Abcam, ab192869) were used. After samples were washed, incubated with fluorescence-labeled secondary antibodies (Alexa Fluor 488 donkey anti-rat IgG from Jackson Immunoresearch, 712-546-153; and Alexa Fluor 555 donkey anti-rabbit IgG from Thermo Scientific, A-31572) and counterstained with DAPI (1:2000, Sigma Aldrich), images were acquired with a Zeiss LSM 510 confocal microscope (Zeiss). For the three-dimensional reconstruction of macrophage- adipocyte interactions in the SAT, the Arivis Vision 4D software was used.

For quantification of the contact area between macrophages and adipocytes in SAT, images were acquired with an Olympus IX83 microscope equipped with a Yokogawa CSU-X1 spinning disc confocal scanner (Olympus). Quantification was performed with the Fiji software43 and its update SCF-MPI-CBG (http://sites.imagej.net/SCF-MPI-CBG) by analysis of ten arbitrarily selected cells per mouse. Annotation of the contact surface between macrophages (F4/80+ cells; green) and adipocytes (caveolin-1+ positive cells; red) was done by manual drawing of lines along the surface plane in the Fiji software. The surface area was then calculated by a custom tool (SCF-MPI-CBG) that interpolated the surface area from the drawn lines.

Analysis of human VCAM1 and UCP1 mRNA expression in adipose tissue. Paired samples of subcutaneous whole AT were obtained from 169 subjects (113 women and 56 men). The age ranged from 19 years to 88 years and the BMI ranged from 18.8 kg/m² to 73.2 kg/m². Phenotypic characterization of the study participants has been reported42. All AT samples were collected during open or laparoscopic abdominal surgery as described42. The study was approved by the Ethics Committee of the University of Leipzig (approval no: 159-12-21052012 and 017-12-23012012) and was performed in accordance to the declaration of Helsinki. All subjects gave written informed consent before taking part in this study.

AT was immediately frozen in liquid nitrogen and stored at ~80 °C. RNA was extracted from AT with an RNAeasy Lipid tissue Mini Kit (Qiagen). Quantity and integrity of RNA was monitored with NanoVue plus Spectrophotometer (GE Healthcare). 1 µg total RNA from subcutaneous AT was reverse-transcribed with standard reagents (Life Technologies). cDNA was then analyzed by TaqMan probe-based quantitative real-time polymerase chain reaction (qPCR) using the QuantStudio 6 Flex Real-Time PCR System (Life Technologies). Human VCAM1 and UCP1 expression were measured by quantitative real-time RT-PCR using the following probes: human VCAM1 (Hs01003370_m1, ThermoFisher Scientific) and UCP1 (Hs00222453_m1, ThermoFisher Scientific). Fluorescence emissions were monitored after each cycle. Human VCAM1 and UCP1 mRNA expression were calculated relative to the expression of mRNA encoding hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1) (Hs01003267_m1, ThermoFisher Scientific). For correlation analysis (as presented in Fig. 7g), only samples with detectable UCP1 expression were used, as UCP1 expression was detectable only in a subset of the AT samples.

Cell adhesion assays. Fully differentiated 3T3-L1 adipocytes in 96-well plates were stimulated with mouse recombinant TNF (20 ng/ml) or Palmitate-BSA (250 µM) for 16 h. After being washed with serum-free medium, cells were incubated with mouse Fc block (2.5 µg/ml, BD) for 10 min, and then with anti-ICAM-1 (20 µg/ml, Y1N1/7.4, BioLegend) or anti-VCAM-1 (20 µg/ml, 429, e Bioscience) or isotype-matched control IgG (20 µg/ml, rat IgG2b, x isotype control, RTRK4530, BioLegend; and rat IgG2a, x isotype control, eBR2a, e Bioscience) for 30 min, followed by addition of bone-marrow mono-nuclear cells (BMMS). BMMS were isolated from C57BL/6 mice or from Cre+α/βCre+α/β mice (2 weeks after induction of Cre recombination with poly-L:C; poly-L:C was administered to both Cre+ mice and Cre+ mice) by Histopaque 1077 (Sigma Aldrich). BMMS were labeled with BCECF AM (Invitrogen) for 15 min at 37 °C, washed twice with RPMI with 0.1% BSA and then BMMS (1 × 10⁴ to 2 × 10⁴) were added onto 3T3-L1 adipocytes in RPMI with 0.1% BSA. Cells were allowed to adhere to adipocytes for 30 min at 37 °C and then the plate was washed twice. Fluorescence intensity before and after the washings was determined by using a microplate reader (BioTek) and the percentage of adherent cells was calculated as fluorescence intensity of adherent cells/fluorescence intensity of input cells × 100.

Macrophage-activation studies. Peritoneal macrophages were harvested after mouse euthanasia by flushing of the peritoneal cavities of C57BL/6 mice, or those of Cre+α/β and Cre+α/β, mice, with 5 ml buffer (PBS+0.5% BSA). Then, the cells were incubated with Fc block (BD) and stained with anti-CD45 (30-F11, BD), anti-CD3 (145-2C11, e Bioscience), anti-CD11b (MI/70, BioLegend) and anti-F4/80 (BM8, e Bioscience) for 20 min at 4 °C, and macrophages were sorted as CD45+CD3+CD11b+F4/80+ cells in a FACSaria II cell sorter (BD). Thereafter, the isolated macrophages were seeded on 48-well plates that were pre-coated with VCAM-1–Fc (10 µg/ml, R&D) or control IgG (10 µg/ml, R&D) or onto plates with differentiated 3T3-L1 adipocytes in the absence or presence of the α4 inhibitor (ELNID002; 1 nM) for 6 h or 12 h. In some experiments, a second α4 inhibitor was used (BIO5192 from APEX-BIO), which provided similar results (data not shown). After incubation, cells were harvested, stained for CD45, CD11b and F4/80 or only CD11b and F4/80 (antibodies identified above) and were subsequently fixed-permeabilized with Foxp3 staining kit (e Bioscience) and stained with anti-TNF (M6P-XT22, BioLegend). The expression of TNF was analyzed in a FACS Canto II flow cytometer by FACSDiva 6.1.3 software (BD).

Adipocyte-macrophage interactions in the human system. Human subcutaneous pre-adipocytes (Lanza) were maintained and differentiated to adipocytes according to the manufacturer’s instructions. At day 10 after differentiation was started, cells were treated with human TNF (10 ng/ml, R&D) or palmitate (250 µM) for 12 h and VCAM1 expression was analyzed by qPCR. For cell-adhesion assays, peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers (after informed consent was obtained; the procedure was approved by the Ethics Committee of the Technische Universität Dresden) by density-gradient centrifugation (Histopaque 1077, Sigma), and monocytess were separated by the human monocyte isolation kit II (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated monocytess were seeded in 12-well plates (5 × 10⁴ cells per well) and were grown in RPMI-1640 medium supplemented with 10% FBS, Glutamax, penicillin-streptomycin and recombinant human M-CSF (20 ng/ml, R&D) for 7 d. The cells were then treated with LPS (100 ng/ml, Invivogen) and human IFN-γ (20 ng/ml, BioLegend) for 12 h, so they would acquire an inflammatory M1-like macrophage phenotype44. After trypsinization and washing with PBS, the cells were incubated with B BecF AM for 15 min at 37 °C and were washed twice with RPM1 including 0.1% BSA, then 1 × 10⁴ cells were added onto differentiated human adipocytes in 96-well plates and were pre-treated overnight with TNF (10 ng/ml, R&D). The human inflammatory macrophages were allowed to adhere to adipocytes for 15 min at 37 °C in the absence or presence of the α4 inhibitor (1 nM) and then the plate was washed twice. Fluorescence intensity before and after the washings was determined with a microplate reader (BioTek), and the percentage of adherent cells was calculated as fluorescence intensity of adherent cells/fluorescence intensity of input cells × 100. In other experiments, adipocytes in 24-well plates were differentiated for 10 d and were subsequently co-cultured with macrophages that were pre-treated with LPS and human IFN-γ for 12 h, as described above. Before co-culture, the human adipocytes were pre-treated with T3 (10 nM) and norepinephrine (1 µM) for 3 h to induce a thermogenic response, then were washed and co-cultured with or without M1-like macrophages (5 × 10⁴ cells) in the same well for 1 h. The experiments were performed in the absence or presence of the α4 inhibitor. For detection of UCP1 mRNA in adipocytes after co-culture, cells were harvested by trypsinization and washed with PBS. The adipocytes were collected as CD45+ cells by human CD45 negative selection using MACS magnetic beads (Miltenyi). After isolation of RNA, cDNA synthesis and qPCR were performed as described below (Real time PCR).

Immunoblot analysis. Fat tissues were snap-frozen in liquid nitrogen and were homogenized with the T10 basic Ultra-Turrax Homogenizer (IKA) in...
The data that support the findings of this study—human primers 66, ∆White and brown fat tissues were snap-frozen in liquid nitrogen. The samples were homogenized by Precellys 24 beads in Trizol (Invitrogen) and total RNA was isolated. In addition, RNA from cell cultures or from cells isolated from fat tissues was also extracted by Trizol. RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed with SsoFast EvaGreen Supermix (Bio-Rad) and gene-specific primers (mouse primers, Supplementary Table 1; human primers (Fig. 7h.j), Supplementary Table 2) in a CFX384 Real time PCR detection system (Bio-Rad). Relative mRNA expression was calculated according to the ΔΔCt method upon normalization to 18S rRNA or ACTB.

Real-time PCR. White and brown fat tissues were snap-frozen in liquid nitrogen. The samples were homogenized by Precellys 24 beads in Trizol (Invitrogen) and total RNA was isolated. In addition, RNA from cell cultures or from cells isolated from fat tissues was also extracted by Trizol. RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed with SsoFast EvaGreen Supermix (Bio-Rad) and gene-specific primers (mouse primers, Supplementary Table 1; human primers (Fig. 7h.j), Supplementary Table 2) in a CFX384 Real time PCR detection system (Bio-Rad). Relative mRNA expression was calculated according to the ΔΔCt method upon normalization to 18S rRNA or ACTB.

Statistical analysis. Statistical tests used are indicated in figure legends. For statistical comparisons, data were analyzed by a Student’s t-test or a Mann-Whitney U test, as appropriate. A one-tailed Pearson correlation analysis was performed for the association between VCAM1 expression and UCP1 expression in human AT samples. For metabolic cage analysis, a one-way analysis of covariance was conducted in conjunction with a Bonferroni post-hoc test. The body weight was used as the covariate. Graph Pad Prism 6 or the IBM SPSS statistics 22 were used. Significance was set at P < 0.05.

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