Alternatively activated macrophages do not synthesize catecholamines or contribute to adipose tissue adaptive thermogenesis

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Adaptive thermogenesis is the process of heat generation in response to cold stimulation. It is under the control of the sympathetic nervous system, whose chief effector is the catecholamine norepinephrine (NE). NE enhances thermogenesis through β3-adrenergic receptors to activate brown adipose tissue and by ‘browning’ white adipose tissue. Recent studies have reported that alternative activation of macrophages in response to interleukin (IL)-4 stimulation induces the expression of tyrosine hydroxylase (TH), a key enzyme in the catecholamine synthesis pathway, and that this activation provides an alternative source of locally produced catecholamines during the thermogenic process. Here we report that the deletion of Th in hematopoietic cells of adult mice neither alters energy expenditure upon cold exposure nor reduces browning in inguinal adipose tissue. Bone marrow-derived macrophages did not release NE in response to stimulation with IL-4, and conditioned media from IL-4-stimulated macrophages failed to induce expression of thermogenic genes, such as uncoupling protein 1 (Ucp1), in adipocytes cultured with the conditioned media. Furthermore, chronic treatment with IL-4 failed to increase energy expenditure in wild-type, Ucp1−/− and interleukin-4 receptor-α double-negative (Il4ra−/−) mice. In agreement with these findings, adipose-tissue-resident macrophages did not express TH. Thus, we conclude that alternatively activated macrophages do not synthesize relevant amounts of catecholamines, and hence, are not likely to have a direct role in adipocyte metabolism or adaptive thermogenesis.

Thermogenesis is crucial for the survival of endothermic mammals and birds because it enables the organism to maintain a stable body temperature even in a cold environment1,2. Hypothermia has been a major cause of mortality for these animals throughout their evolutionary history. Consequently, the need for efficient thermogenesis represents an evolutionary pressure that has shaped the biology of all endothermic homeotherms3. Thermogenesis also plays a central part in energy homeostasis, and pharmacological stimulation of energy expenditure is considered to be a valuable strategy for combatting obesity and its comorbidities4. A detailed understanding of the physiological and molecular underpinnings that regulate adaptive thermogenesis is therefore of utmost importance. It is well established that catecholamines are a key driver of thermogenesis, which they induce by stimulating uncoupled respiration in brown adipose tissue (BAT) and beige, or ‘brite’ (beige/brite), white adipose tissue (WAT). Notably, catecholamines are also key for the induction of lipolysis in WAT that prompts the release of fatty acids, the principal substrates for BAT thermogenesis5. In the periphery, catecholamines have classically been thought to originate from either sympathetic postganglionic neurons or, otherwise, the adrenal medulla. Challenging this conventional

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view, recent reports have proposed a major paradigm shift by suggesting that alternatively activated macrophages are another important source of catecholamines, and that they consequently represent a previously overlooked mechanism behind the regulation of thermogenesis. In such a model, cold exposure enhances alternative activation of macrophages, which thereby induces TH enzyme, which in turn results in the production and secretion of NE to enhance nonshivering thermogenesis in BAT, lipolysis in WAT and browning of inguinal WAT (iWAT). Most of the data on which this concept rests are derived from *in vivo* experiments that analyzed mice with a lifelong deficiency of key ‘M2 signaling nodes’, i.e., mice systemically deficient in Il4/13, Il4ra or Stat6, and mice with myeloid-cell-specific deletion of Il4ra and Th. Notably, all of these germline-knockout (KO) models share the caveat that their metabolic phenotypes can be compromised through developmental processes and/or through altered sympathetic regulation because all of these genes are also expressed in the central nervous system (CNS). Accordingly, the aim of the present studies was to assess the role of macrophages in mice with adult-onset peripheral deletion of Th, as well as to further evaluate the role of alternatively activated macrophages in the regulation of white, brown and beige/brite adipocyte function *in vitro* and *in vivo*. Collectively, we show here, using a combination of *in vivo* and *in vitro* approaches, that alternatively activated macrophages do not synthesize sufficient amounts of catecholamines and are thus unlikely to have a direct role in adipocyte metabolism or adaptive thermogenesis.

**RESULTS**

**Peripheral catecholamines control thermogenesis**

Lifelong deletion of Th results in early embryonic lethality, possibly owing to the important role of catecholamines in the CNS during development. To study the role of peripheral catecholamines in thermoregulation, we generated mice, herein called THΔper mice, in which Th can be inducibly deleted in all peripheral tissues of adult mice, including the sympathetic nervous system (SNS) and hematological cells, but not the CNS. This was accomplished by crossing a strain of mice in which Cre expression is under the control of the Rosa26 locus that is induced upon tamoxifen administration with a mouse harboring floxed alleles of Th, and owing to poor Cre expression in the CNS, the deletion of the Th allele does not occur in the CNS (Fig. 1a). Ablation of the TH protein in peripheral tissues (BAT, spleen, liver and epididymal white adipose tissue (eWAT)) after tamoxifen administration in adult mice was confirmed by western blot analysis (Fig. 1b), and this resulted in marked depletion of NE levels in all peripheral tissues analyzed, as compared to wild-type (WT) controls (Fig. 1c). In line with the prediction that THΔper mice have reduced sympathetic activity relative to WT controls, the animals exhibited impaired thermoregulation when exposed to 4 °C (Fig. 1d), which is consistent with a key role of catecholamines in thermogenesis. We also used tissue extracts from these mice to validate several commercially available antibodies against TH, several of which had a strong, nonspecific band of similar molecular size as TH, which raises the concern that, without appropriate controls, it might be difficult to ascertain the specificity of any of these antibodies in applications such as western blot (Supplementary Fig. 1).

To probe whether catecholamines produced by alternatively activated macrophages play an appreciable part in thermoregulation, we generated bone marrow–chimeric mice, in which Th is specifically deleted from hematopoietic cells (including macrophages) in an inducible manner. We first transplanted bone marrow from WT and noninduced THΔper mice into irradiated WT recipient mice.

**Figure 1** Selective deletion of Th in peripheral but not CNS tissues results in peripheral catecholamine depletion and impaired thermoregulation. (a) Schematic of the inducible peripheral Th knockout (THΔper) mouse model. (b) Representative western blot images of indicated tissues from WT or THΔper mice. Uncropped western blot images are shown in Supplementary Figure 11. For WT and THΔper mice, n = 2 and n = 3 samples (brainstem), n = 7 and n = 5 (BAT), n = 4 and n = 6 (spleen), n = 4 and n = 4 (liver) and n = 1 and n = 3 (eWAT). Gapdh of eWAT was chosen as a representative image for the loading control; comparable Gapdh loading for other tissues is shown in Supplementary Figure 11. (c) The level of norepinephrine in peripheral tissues of WT (n = 3–5) and THΔper (n = 3 or 4) mice; each dot represents one animal. (d) Body temperature of WT (n = 7) and THΔper mice (n = 7) during a cold-tolerance test at 4 °C. Data represent mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 according to a two-sided Student’s t-test (c) and two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc comparison of the individual time points (d).

Fluorescence-activated cell sorting (FACS) analysis conducted on peripheral blood ~8 weeks later revealed a >90% reconstitution with lymphocytes and granulocytes of WT or THΔper donor origin in the majority of chimeras (Fig. 2a). Subsequent tamoxifen treatment of the chimeras resulted in Th ablation from hematopoietic cells (including macrophages) in THΔper, but not WT, chimeras without affecting body weight (Fig. 2b). Notably, energy expenditure did not differ between THΔper chimeric mice and their WT controls at room temperature (21 °C) and after exposure to cold at successively lower temperatures of 15 °C, 10 °C and 6 °C (Fig. 2c,d). Furthermore, locomotor activity (Fig. 2e), substrate utilization—as assessed by the respiratory exchange ratio (RER) (Fig. 2f)—and core body temperature (Fig. 2g) were not different between the mouse types. In keeping with the observation that energy expenditure and core body temperature were not changed after Th deletion in hematologic cells, THΔper chimera also exhibited a cold-induced increase in iWAT Ucp1 mRNA levels comparable to that seen in WT controls (Fig. 2h).

**No role of macrophages in browning of white adipocytes**

To evaluate a possible role of macrophages in adipocyte metabolism *in vitro*, we isolated preadipocytes from iWAT, depleted the macrophages by magnetic-bead-mediated sorting of CD11b-positive cells and cultured and differentiated them *in vitro* (henceforth described as 'primary cells'). We confirmed successful macrophage depletion by the lack of the integrin subunit alpha M (*Itgam*, also known as *Cd11b*) and the adhesion G-protein-coupled receptor E1 (*Adgre1*) mRNA levels...
during adipocyte differentiation (Supplementary Fig. 2a,b) and absence of M1 and M2 markers (Tnf, Arg1, Mgl2, Mrc1 and Il10) in fully differentiated iWAT primary cells (Supplementary Fig. 2c,d). Lipid accumulation, as assessed by Oil Red O staining, was not affected by the absence of macrophages (Supplementary Fig. 2e,f), which indicates that macrophages are dispensable for adipocyte differentiation. Consistent with this notion, the expression profiles of markers indicative of adipocyte differentiation (Fasn, Adipoq, Fabp4 and Pparg) were not affected by the absence of macrophages (Supplementary Fig. 2g–j). Similarly, we observed no changes for gene programs indicative of fatty acid synthesis, fatty acid transport, cytokine signaling, lipoprotein metabolism, carbohydrate metabolism, lipogenesis or lipolysis (Supplementary Fig. 2k–p). Furthermore, we observed no differences in the expression profiles of genes related to mitochondrial electron transport (Cycs and Cox4i1) (Supplementary Fig. 3a,b) or of key thermogenic genes (Ucp1, Pparγc1a (also known as Pgc-1α) and Prdm16) during the differentiation of iWAT primary cells (Supplementary Fig. 3c–e).

We next asked whether the depletion of macrophages impaired the ability of isoproterenol to stimulate the expression of Ucp1 or Pgc-1α in differentiated iWAT primary cells and found no difference between the WT cells and those devoid of macrophages (Supplementary Fig. 3f,g). Consistent with the observation that isoproterenol-induced stimulation of Ucp1 and Pgc-1α is not affected by the absence of macrophages, we observed no changes in basal respiration, ATP production, maximal respiration or nonmitochondrial respiration, as assessed by the oxygen consumption rate (OCR), following treatment with isoproterenol, oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) or rotenone/antimycin A/2-deoxyglucose (Supplementary Fig. 3h–j).

To test whether macrophages first need to undergo polarization to stimulate thermogenesis, we treated bone marrow–derived macrophages (BMDMs) with various doses of IL-4 (5 ng/ml, 10 ng/ml or 20 ng/ml) and then used the conditioned media (CM) to stimulate differentiated iWAT primary cells. IL-4 treatment robustly enhanced expression of the M2 markers Arg1, Mrc1 and Mgl2 in the BMDMs, as compared to vehicle treatment (Fig. 3a–c). However, despite robust polarization, the CM failed to stimulate the expression of Ucp1 or Pgc-1α in the differentiated iWAT primary cells, indicating that the M2-polarized BMDMs fail to sufficiently secrete NE into media (Fig. 3d,e). In summary, these data suggest that macrophages are neither required for the differentiation of iWAT primary adipocytes, nor likely to play a part in browning or β-adrenergic-receptor-mediated stimulation of thermogenesis in these cells.

M2 macrophages do not affect thermogenesis in BAT primary cells

To evaluate the thermogenic effect of M2-activated macrophages in BAT, we used CM from IL-4-stimulated BMDMs (IL-4-CM) to stimulate differentiated BAT primary cells. Despite robust M2 polarization of the BMDMs (Fig. 3a–c), we observed no effect of the CM on the expression of Ucp1 and Pgc-1α (Fig. 3f,g), which is congruent with the findings obtained in iWAT primary cells (Fig. 3d,e). IL-4-CM also failed to activate hormone-sensitive lipase (HSL) at all IL-4 doses tested (Fig. 3h,i) and independently of the duration of IL-4 treatment (15 min, 1 h, 3 h or 6 h) (Supplementary Fig. 4). Notably, we confirmed the failure of M2 macrophages to induce thermogenic gene programs in iWAT and BAT primary cells in several independent experiments, which were carried out in primary cells obtained from...
Chronic IL-4 treatment has no effect on energy metabolism

We next assessed whether chronic IL-4 treatment in WT and Il4ra−/− mice exposed to various declining environmental temperatures (30 °C, 20 °C, 10 °C or 5 °C) alters energy expenditure. Daily intraperitoneal administration of IL-4 (50 µg/kg) for 12 d did not alter body weight (Fig. 4a) or energy expenditure in WT or Il4ra−/− mice at any temperature tested (Fig. 4b,c). Notably, despite robust induction of M2 polarization in the BAT (Fig. 4d–f) after IL-4 treatment—confirming that the IL-4 was fully functional—such treatment did not increase the expression of Ucp1 or Pgc-1α in BAT relative to controls (Fig. 4g,h). Mice lacking Il4ra show no elevated expression of Arg1, Mrc1 or Mgl2 after IL-4 treatment (Fig. 4d–f), which thus confirms their inability to activate M2-type macrophages through IL-4Ra-mediated signaling. The genotype of Il4ra−/− mice was confirmed by blunted gene expression of Il4ra in both vehicle-treated and IL-4-treated Il4ra−/− mice (Fig. 4i).

Consistent with our in vitro data demonstrating no effect of IL-4 on the phosphorylation of HSL (p-HSL) in iWAT and BAT primary cells (Fig. 3h,i and Supplementary Fig. 4), we found no difference in p-HSL in mice chronically treated with IL-4 relative to controls (Fig. 4j–n). Supporting these data, HPLC analysis revealed no difference in the levels of catecholamines, including NE or their metabolites, in cold-exposed mice treated chronically with IL-4 (Fig. 4o).

We next evaluated the metabolic effect of chronic IL-4 treatment in C57BL/6 WT and Ucp1−/− mice housed at thermoneutrality (30 °C) to assess a possible role of UCP1 in driving the reported effects of IL-4 on energy homeostasis. We administered IL-4 (50 µg/kg) daily for 8 d, and after the final injection, we recorded energy expenditure at 30 °C and during a gradual decrease of the ambient temperature to 10 °C. Chronic treatment with IL-4 had no effect on body weight (Fig. 5a) or body composition (Fig. 5b,c), as compared to vehicle controls. Measurement of resting metabolic rate revealed no differential effect caused by IL-4 at any tested temperature, and we observed no difference between WT and Ucp1−/− mice (Fig. 5d,e).
sub-thermoneutral conditions, also revealed no effect of IL-4 in thermal conductance in either WT or Ucp1<sup>−/−</sup> mice (Fig. 5f). IL-4 treatment induced Arg1 expression in the BAT of cold-exposed mice lacking Ucp1 relative to control, which again confirms successful M2 polarization by IL-4 (Fig. 5g). We further assessed BAT Ucp1 mRNA and protein levels to confirm the genotype of the Ucp1<sup>−/−</sup> and WT mice (Fig. 5h,i). Notably, the amounts of UCP1 protein in BAT remained unaffected by IL-4 treatment of WT mice (Fig. 5i). Taken together, these data strongly imply that alternatively activated macrophages do not directly regulate thermogenesis in vitro or in vivo.

**Macrophages do not express Th or synthesize catecholamines**

To assess the ability of macrophages to synthesize catecholamines, we studied Th expression in a reporter mouse expressing tdTomato under control of the Th promoter (Th<sup>cre</sup>:r26-tdTomato). FACS analysis readily revealed tdTomato-labeled CD11b<sup>+</sup> CD14<sup>−</sup> F4/80<sup>−</sup> cells, which represent macrophages, in Pan-r26-tdTomato control mice, which served as positive controls (Fig. 6a). However, we detected no tdTomato signal in BAT macrophages of Th<sup>cre</sup>:r26-tdTomato reporter mice maintained at room temperature or after exposure of mice to 4 °C for 8 h (Fig. 6a). This is consistent with the observation that a peripheral-hematopoietic-cell-specific Th deletion does not affect energy expenditure (Fig. 2c,d). Furthermore, we also detected no double-positive cells in histological analysis following TH stain -ing of BAT sections of mice expressing Cre under the direction of the Cx3cr1<sup>cre</sup>-stop-GFP mutant mice crossed with Rosa26-stop-GFP:Th<sup>cre</sup>:r26-YFP mice (Fig. 6b) or following two-photon microscopy of Cx3cr1-GFP:Th<sup>cre</sup>:r26-tdTomato double-reporter mice (Fig. 6c). Finally, and corroborating the inability of macrophages to produce TH, RNA sequencing of macrophage populations isolated from various tissues, including BAT, revealed no Th transcripts in any of the tested macrophage populations (Fig. 6d). Assessment of Th expression in iWAT similarly revealed no mRNA expression of Th (Supplementary Fig. 7). Besides, we detected no colocalization of TH and Mac-2 signal in the iWAT or BAT of WT mice kept at either room temperature or cold exposure (5 °C) (Supplementary Fig. 8a,b). Of note, in BAT, we were able to detect only a very low number of Mac-2-positive macrophages (Supplementary Fig. 8b,c).

We further evaluated a previously proposed role of adiponectin in M2 polarization and thermogenesis in subcutaneous iWAT<sup>3</sup>. We found that protein concentrations of adiponectin are lower, rather than higher, in cold-acclimated mice (exposed to 4 °C for 4–5 weeks) relative to mice maintained at thermoneutrality, whereas adiponectin (Adipoq) mRNA levels remained unchanged (Supplementary Fig. 9).
The exposure of WT mice to 4 °C for 6 h led to higher mRNA levels of Ucp1 and Pgc-1α (Supplementary Fig. 10a,b), but notably lower expression of markers indicative of M1 and M2 polarization (Supplementary Fig. 10c,d), which suggests that macrophage density is reduced during cold-induced sympathetic stimulation of iWAT.

HPLC analysis of catecholamines revealed higher absolute levels of dopamine, 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid in BAT of cold-exposed mice relative to mice held at room temperature (22 °C) (Supplementary Fig. 10c-h). BAT levels of NE and epinephrine are reduced in the cold-exposed mice relative...
to room temperature controls, which likely reflects enhanced NE turnover during cold stimulation (Supplementary Fig. 10i,j).

In summary, these data demonstrate that peripheral, hematopoietic-cell-specific Th deficiency does not affect energy expenditure, and that macrophages lack the capacity to produce sufficient amounts of catecholamines to promote thermogenic effects in iWAT or BAT.

**DISCUSSION**

Thermogenesis is crucial for the survival of homeotherms and plays a key part in energy homeostasis. There is solid evidence indicating that thermogenesis is regulated via the sympathetic nervous system through the release of NE, and that consequent activation of β-adrenergic receptors induces lipolysis and drives BAT thermogenesis\(^6,7\). Given the therapeutic potential of targeting thermogenesis to improve metabolic diseases, it is of high biological and clinical relevance to determine whether there are alternative mechanisms that regulate WAT lipolysis and BAT thermogenesis. Hence, it is important to examine whether catecholamines are produced by sources other than the SNS and/or the adrenal gland, because that could lead to the identification of targetable pathways for the development of novel therapeutics. Recent studies have suggested that M2 macrophages represent one such alternative source of catecholamines. Specifically, it was reported that M2 macrophages synthesize NE \textit{de novo}, and in a paracrine fashion, locally activate β-adrenergic-receptor signaling in nearby WAT and BAT adipocytes to induce lipolysis and BAT thermogenesis\(^6,7\). Furthermore, meteorin-like, a circulating hormone secreted from adipose tissue upon cold exposure, has recently been proposed to potentially promote nonshivering thermogenesis through enhanced recruitment of M2-type macrophages\(^12\). Similarly, it has been suggested that adiponectin induces browning of subcutaneous WAT by triggering M2-macrophage proliferation upon cold stimulation\(^9\). Recently, another immune cell type, type-2 innate lymphoid cells (ILC2s), has been reported to elicit the activation of alternative macrophages through IL-4-receptor signaling and to have a role in the regulation of beige/brite fat biogenesis\(^13\). Moreover, caloric restriction has recently been shown to promote browning of WAT through type-2 cytokine signaling\(^9\), although caloric restriction did not affect the levels of ILC2s in those studies. Of note, it has been shown that IL-33-elicited ILC2s promote ‘beiging’ independently of eosinophils or the IL-4Ra signaling pathway\(^15\).

If alternatively activated macrophages are indeed an important source of catecholamines through \textit{de novo} synthesis of catecholamines to induce WAT lipolysis, subcutaneous WAT browning and BAT thermogenesis, then one would predict that the elimination of catecholamine production in hematopoietic cells should impair all three processes. To test this hypothesis, we generated a mouse model that allows for inducible deletion of Th in all peripheral tissues, including the bone marrow, and we performed a bone marrow transplant on WT mice. Upon acceptance of the BM transplant more than 90% of macrophages were donor-derived. Notably, irradiation before the bone marrow transplantation also ablates tissue macrophages\(^16,17\). After we induced the Th deletion, specifically in hematopoietic cells, we did not observe any alteration in thermogenesis, energy expenditure or browning of subcutaneous WAT in TH\textsuperscript{−/−} per chimeric mice and their WT controls. We also studied the direct effects of alternatively activated macrophages on the thermogenic program of adipocytes \textit{in vitro} and observed no relevant effect of M2 macrophages on white and brown adipocyte function.

Our studies also investigated a role of alternatively activated macrophages in cold-triggered brown-fat function and thermogenesis in multiple \textit{in vivo} and \textit{in vitro} systems. Collectively, these studies indicate that IL-4-mediated polarization to M2 macrophages does not affect energy expenditure or thermogenesis in the iWAT and BAT of WT, Il4ra\textsuperscript{−/−} or Ucp1\textsuperscript{−/−} mice exposed to different ambient temperatures. Furthermore, these studies failed to detect TH expression in CX3CR1-positive mononuclear phagocytes, as revealed by flow cytometry and RNA-sequencing analysis, even after cold exposure. Absolute levels of NE, as well as of other intermediates or products of catecholamine synthesis, remained unchanged in IL-4-activated M2 macrophages or in supernatant from IL-4-stimulated BMDMs. In keeping with the lack of TH expression and NE production in macrophages, the inducible deletion of Th in bone marrow chimeras failed to demonstrate any impairment in energy metabolism, even upon cold exposure. Given that previous studies have argued for a role of M2 macrophages in thermogenesis and browning of iWAT\(^6,7,9\), the source for the discrepant results remains elusive. For instance, the divergent findings could arise from differences in the composition of the gut microbiome in the mice used here relative to those in the original reports, or they could be due to different animal-housing conditions between institutions. Such seemingly insignificant factors have been shown to influence metabolic phenotypes in mouse colonies from different facilities\(^18\). However, given that we used several mouse models and different background strains and performed these studies at several institutions on multiple continents, this scenario is unlikely. Although previous studies have reported that macrophages release NE into the culture media \textit{in vitro}\(^19\), it is important to note that the serum used in cell culture contains NE. It is also of interest that non-neuronal cells, such as osteoblasts, express the norepinephrine transporter (NET), exhibit specific NE-uptake activity through NET and can catabolize, but not generate, NE\(^20\). Hence, macrophages, similarly to neurons, might be able to take up and release catecholamines, a process that might be regulated by IL-4 signaling.

In summary, the studies presented herein from six independent laboratories repeatedly and consistently demonstrate that alternatively activated macrophages do not relevantly affect adipocyte metabolism and adaptive thermogenesis by catecholamine production. Instead, we reconfirm the essential and pivotal role of sympathetic activation in the regulation of adipose tissue lipolysis and thermogenesis.

**METHODS**

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
K.F. co-conceptualized the project, designed and performed in vitro, in vivo and ex vivo experiments, analyzed and interpreted data and wrote the manuscript. B.F. and C.C. helped to design experiments and interpreted data. H.H.R., K.J., D.J.O., V.v.d.H., A.C.S. and D.H. performed, analyzed and interpreted the inducible THAper mouse, validation of antibodies and BM chimera data. A.V.K. and N.P. performed, analyzed and interpreted in vivo experiments in Ucp1−/− mice. J.N. and K.-W.S. performed, analyzed and interpreted catecholamine data. Y.W. and S.J. performed, analyzed and interpreted tyrosine hydroxylase staining. F.B. generated the Il4ra−/− mouse. S.D., E.G., S.K., M.J., D.M., G.C., S.H., J.N. and B.C. helped to design experiments and interpreted data. S.C.W. helped with the interpretation of data and drafting the manuscript. M.H.T. co-conceptualized the project and interpreted all data. T.D.M. and C.B. co-conceptualized the project, interpreted all data, supervised the studies and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

General experimental approaches for *in vivo* experiments. For *in vivo* studies, group sizes of seven or eight mice were preferentially used, which was determined from previous experiments as optimal for *in vivo* evaluation. Smaller group sizes were used in the studies involving genetically or chemically modified animals, in case there were not sufficient numbers available to reach the preferred group size of seven or eight.

For measurements of IL-4 induction of energy expenditure, group-size estimations were based upon a power calculation to minimally yield an 80% chance of detecting a 20% difference in energy expenditure between the treatment groups, and under the assumption of an alpha level of 0.05 and a s.d. of 13% in both groups.

For *in vivo* studies, mice were randomized into the treatment groups on the basis of body weight and body composition (fat and lean tissue mass). For *in vivo* experiments, all mice except the ones used in the Ucp1−/− study were on a C57BL/6J background. Ucp1−/− and WT mice were on a C57BL/6J background. All mice were maintained under specific-pathogen-free (SPF) conditions. Experiments were performed nonblinded. We screened for statistically significant outliers using the maximum normal residual (Grubb’s) test, but no outliers were detected for *in vivo* experiments.

Mice. All animal procedures were approved by either the Mount Sinai School of Medicine, Institutional Animal Care and Use Committee (IACUC) protocols, CCHMC IACUC, the Weizmann Institute Animal Care Committee, the Animal Ethics Committee of the North Stockholm region or the regional animal-welfare committee of the state of Bavaria. We generated inducible peripheral Th knockout mice by crossing ThFoxFox mice (kindly provided by R. Palmer, University of Washington) with tamoxifen-inducible Rosa26CreERT2+ mice (Taconic #4666, Hudson, NY). The resultant ThFoxFox,Rosa26CreERT2−/− (ThAper mice) and the ThFoxFox,Rosa26CreERT2+/− (WT littermates) expressed normal levels of Th protein during development and before tamoxifen induction of the KO. Deletion of the Th gene was achieved by tamoxifen administration and was limited to the periphery, owing to low expression of Cre recombinase in the CNS22. Here we used male 28-week-old WT or ThAper mice (n = 4 each genotype).

To generate mice with inducible Th deficiency (ThAper chimera) restricted to hematopoietic cells, we injected 4 × 109 Tcell−/− depleted BM cells from noninduced ThAper (CD45.2+) (n = 2 males and n = 3 females, age of 14 weeks) or WT (CD45.1+) (n = 2 males and n = 3 females, age of 4–8 weeks) mice intravenously (i.v.) into lethally irradiated (2 × 100 rad) CD45.1+ congenic (B6.SJL-129pFcRn-Tg[CD2F1]; #000174, Jackson Laboratory, USA) (n = 2 males and n = 3 females, age of 4–8 weeks) or WT (CD45.2+) (n = 2 males and n = 3 females, age of 19–22 weeks at injection) mice, as described23. We assayed reconstitution of the immune system –8 weeks later by differentiating donor-derived (CD45.2+) and host-derived cell populations in peripheral blood, according to CD45.1/CD45.2 expression patterns, and we used mice with a donor reconstitution of >90% for all subsequent experiments.

We used the following mouse strains to generate reporter mice: Cx3cr1GFP+ (mice (JAX stock 005582 B6.129P+Cx3cr1tm1Litt/I, Cx3cr1Cre(JAX stock 025524 B6.129P(Cg)-Cx3cr1tm1.1(cre))FJ); td Tomato reporter mice (JAX stock 007908 B6.129S6-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/f+); and ThCre+ female (male 8-week-old mice).

Analysis of mouse metabolic phenotypes. We analyzed body composition (fat and lean mass) using a magnetic-resonance whole-body composition analyzer (EchoMRI, Houston, TX) and was plotted versus the ambient temperature (‘Scholander plots’)26. Using total energy expenditure calculated during the last h at each temperature by means of a modified Weir equation yielded similar results27. The slopes of the Scholander plots at and below 26–27 °C were calculated individually for each mouse, using the best linear fit. Insulation was calculated as the inverse of the slope. Two rounds of the experiment yielded similar results.

Western blot. For the THAper and chimera studies, we homogenized the tissues in 20-mM MOPS containing 2-mM EGTA, 5-mM EDTA, 30-mM sodium fluoride, 40-mM β-glycerophosphate, 10-mM sodium pyrophosphate, 2-mM sodium orthovanadate, 0.5% NP-40 and complete protease-inhibitor cocktail (Roche,的规定)， and centrifuged at 13,000g for 20 min at −4 °C. The supernatant was then collected, and protein concentration was measured using a bicinchoninic acid (BCA) protein quantification kit (Thermo Scientific, Waltham, MA). Protein extracts were separated on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA) and blotted onto Immobilon FL PVDF membranes (Millipore, Billerica, MA). Membranes were blocked at room temperature for 1 h with Odyssey LI-COR blocking buffer (LI-COR, Lincoln, NE) diluted 1:1 in Tris-buffered saline (TBS). Membranes were then incubated with primary antibodies (diluted 1:1,000 in a 1:1 blocking buffer/TBS-T solution) overnight at 4 °C. Primary antibodies against tyrosine hydroxylase (EMD Millipore, Billerica MA-MAB318, 1:1,000 dilution) and Gapdh (Santa Cruz Biotechnology, Dallas, TX-32233, 1:2,000 dilution) were used. Membranes were washed consecutively three times for 5 min each in TBS-T (0.1%). Blots were incubated with Dylight 680-conjugated goat anti-rabbit IgG and Dylight 800-conjugated goat anti-mouse IgG (Thermo Scientific, Waltham, MA) for 1 h at room temperature in blocking buffer containing 0.1% TBS-T and 0.1% SDS. Blots were washed three more times in TBS-T followed by a final wash in TBS, and the bands were then scanned with the LI-COR Odyssey (LI-COR, Lincoln, NE) and quantified with Odyssey 3.0 software on the basis of a direct fluorescence measurement. Other fat tissues were lysed in ice-cold RIPA buffer (Sigma-Aldrich, Munich, Germany) containing 1-mM PMSF (Carl Roth), 10-nM calcium A (Cell signaling) and protease/phosphatase inhibitor (Thermo Fisher Scientific, Erlangen, Germany) using a polytron. Lysates were chilled on ice for 20 min and centrifuged for 15 min, 10,000g at 4 °C. The supernatant was collected and protein concentration was determined by the method of Lowry et al.28. Proteins were separated on Criterion gel (Bio-Rad, Munich, Germany) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA) overnight at 4 °C, and HRP-coupled secondary antibody (Santa Cruz Biotechnology, Dallas, TX) was used to detect protein signal through the LI-COR imaging system. Antibodies were purchased from Cell Signaling (Phospho-HS1 [Ser660] #4126, HSL #4107, each diluted 1:2,000), Santa Cruz (Gadph G-9, #350062, diluted 1:10,000) and Abcam (Ucp1, #23841, diluted 1:2,000).

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Flow cytometry. We performed FACS analysis of BM chimera using CD45.1 and CD45.2 antibodies (BioLegend). Acquisition was performed with a BD Biosciences FACSCalibur and analysis with FlowJo software. For FACS analysis of BAT of the CX3CR1-GFP:Thy1.2-ZR-26-Tomato mice, tissue was collected, dissected by scissors and then incubated for 30 min with DMEM medium (Beit Haemek, Israel) containing 1 mg/ml collagenase-2 (Sigma-Aldrich), 2% BSA (Sigma-Aldrich) and 12.5-mM HEPES buffer (Beit Haemek, Israel). The resulting cell suspension was filtered through a 70-µm mesh, and erythrocytes were removed by ACK lysis. After cell suspension, cells were incubated in FACS buffer (PBS with 1% BSA, 2 mM EDTA and 0.05% sodium azide) in the presence of staining antibody. Antibodies used for staining were CD45 (clone 30F11, BioLegend), CD14 (clone Sa2-8, BioLegend) and F4/80 (clone BM8, Serotec).

Preparation of RNA and gene-expression analysis. Total RNA was prepared using RNase Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Gene expression of cell samples was profiled with quantitative PCR-based (qPCR-based) techniques using SYBR green or TaqMan Single Probes (Thermo Fisher Scientific, Erlangen, Germany). The relative expression of the selected genes was measured using the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Erlangen, Germany). The relative expression levels of each gene were normalized to the housekeeping gene peptidylprolyl isomerase B (Ppib), hypoxanthine phosphoribosyltransferase (Hprt) or TATA-box binding protein (Tbp). RNA expression data were quantified according to the ΔΔCt method, as described. Sequences of all primers are listed in Supplementary Table 1. TaqMan Low Density Array (Thermo Fisher Scientific, Erlangen, Germany) was performed according to instructions.

Isolation and culture of BMDMs. Bone marrow from the hind legs of 6-week-old C57BL/6 or BALB/c mice was obtained, erythrocytes were lysed with ACK buffer (151 mM NH4Cl, 10 mM KHCO3, 0.2 mM EDTA in H2O), and the bone marrow was purified using Ficoll (GE Healthcare, Munich, Germany). Monocyte differentiation into macrophages was achieved by differentiation with DMEM medium containing 30% L929 supernatant, 20% FBS and 1% penicillin/streptomycin for 5 d. BMDM polarization toward the M2 phenotype was accomplished by treatment with IL-4 (5, 10, 20 ng/ml, Peprotech, Rocky Hill, NJ) for 24 h. BMDMs and primary adipocytes were not tested for mycoplasma infection; however, Raw264.7 and L929 cells were negatively tested for mycoplasma infection.

Isolation of primary adipocytes. iWAT was obtained from 6–8-week-old male C57BL/6 or BALB/c mice. Fat pads were minced and digested for 40 min at 37°C (1 mg/ml Collagenase IV (Thermo Fisher Scientific, Erlangen, Germany), 3 U/ml Dispase II (Sigma-Aldrich, Munich, Germany); 0.01-mM CaCl2 in PBS). The cell suspension was filtered, centrifuged and resuspended in growth medium (DMEM/F12 1:1 plus Glutamax (Thermo Fisher Scientific, Erlangen, Germany)). The relative expression levels of each gene were normalized to the housekeeping gene peptidylprolyl isomerase B (Ppib), hypoxanthine phosphoribosyltransferase (Hprt) or TATA-box binding protein (Tbp). RNA expression data were quantified according to the ΔΔCt method, as described. Sequences of all primers are listed in Supplementary Table 1. TaqMan Low Density Array (Thermo Fisher Scientific, Erlangen, Germany) was performed according to instructions.

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Depletion of macrophages from primary iWAT. Macrophages were separated from iWAT primary cells by magnetic-immunofluorescence isolation using anti-CD11b antibodies conjugated to magnetic beads (MACS Cell Separation System; Miltenyi Biotec, Bergisch Gladbach, Germany). After iWAT isolation, CD11b-positive cells were separated using positive-selection columns (LD columns; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. For the validation of cell separation, cell eluents were taken before and after depletion of CD11b-positive cells, as well as from the retained cell fraction, bound to the conjugated beads. Successful depletion of macrophages was confirmed by flow cytometric and qPCR analysis.

Oil Red O staining and microscopy. Cells were fixed with 4% PFA at differentiation days 0, 3 or 6. Adipocytes were stained with Oil Red O (Sigma-Aldrich, Munich, Germany) for 10 min and immediately washed with H2O. Phase-contrast microscopy was performed before and after Oil Red O staining with a Keyence BZ-9000 microscope. For lipid quantification, Oil Red O was retrieved from the cells by 100% isopropanol and absorbance was measured at 500 nm. DAPI signal was measured to correct for cell number.

Bioenergetic analysis. Primary iWAT cells were isolated, macrophage-depleted and differentiated for 5 d on a collagen-coated XF96 well plate. On the day of the experiment (day 5), cells were washed with DMEM XF Assay medium (Seahorse Bioscience, Santa Clara, CA), supplemented with 25 mM glucose, 10 mM pyruvate and 0.3% fatty-acid-free BSA, and incubated in 180 µl of XF Assay medium in a non-CO2 incubator at 37 °C for 1 h. All port compounds were dissolved in pure DMEM XF Assay medium without supplements, and ten-fold higher concentrated compounds were loaded into the ports of a XF Assay Cartridge. Oxygen-consumption rate (OCR) was measured using an extracellular flux analyzer (XF96, Seahorse Bioscience, Santa Clara, CA). Basal OCR was recorded for 21 min, followed by measurement of OCR after injection of isoproterenol (1 µM, 35 min), oligomycin (2 µg/ml, 21 min), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1 µM, 21 min), rotenone (2.5 µM)/antimycin (2.5 µM)/2-deoxyglucose (10 mM) (28 min). For normalization, the cell plate was subsequently stained with DAPI and Nile red, and the fluorescence signal was detected to correct for cell number and differentiation.

Adipocyte treatment with conditioned media from activated macrophages. Conditioned media from 24-h polarized BMDMs or Raw264.7 cells (Sigma-Aldrich, Munich, Germany) were collected and supplemented to 6-d differentiated primary adipocytes for 6 h. Raw264.7 cells have been tested and found negative for mycoplasma contamination.

Immunofluorescence. iWAT and interscapular BAT were dissected from room temperature (23 °C) or 4-h cold-exposed (5 °C) C57BL/6J mice and subsequently fixed in 4% PFA. Fat tissues were embedded in paraffin and cut in 5-µm sections in a vibratome. After deparaffinization, sections were subjected to antigen retrieval by boiling for 20 min in citrate buffer (10 mM sodium citrate in H2O, pH = 6) in a microwave, then left to cool at room temperature for 20 min. After washing in TBS, sections were blocked in SUMI (0.25% gelatine + 0.5% TritonX in TBS) for 1 h, then incubated overnight at 4 °C with a cocktail containing primary antibodies against TH (ab152, abcam, 1:500) and Mac-2 (CL8942AP, CedarLane Labs, 1:500) diluted in SUMI. After washing in TBS, sections were incubated with a cocktail of secondary antibodies (Alexa 488 Goat anti-rabbit, R37116+ Alexa 568 Goat anti-rat, A-11077) diluted in SUMI for 1 h at room temperature. After washing in TBS, sections were incubated with 0.1% Sudan Black B (Sigma-Aldrich, Munich, Germany) in 70% EtOH for 20 min to quench autofluorescence, and then washed with TBS + 0.02% Tween20. Sections were then counterstained for 3 min with DAPI (Thermo Fisher Scientific, 62248, 1:3,000), washed in TBS, then mounted using Elvanol mounting medium. Image stacks (5-µm thick) were collected through the z axis at an interval of 1 µm using a Leica SP5 scanning confocal microscope equipped with a 20× and 40× objective, and final images obtained by maximum intensity projection of the z-stack. Number of Mac-2-positive cells was quantified using ImageJ, for each sample (n = 3 or 4), and the average number of cells was calculated from at least three different 20× fields from at least two different sections.

Statistics. Statistical analyses were performed by using statistical tools implemented in GraphPad Prism (version 6). Statistical analyses were performed on data distributed in a normal pattern using a regular one-way or two-way analysis of variance (ANOVA) with Bonferroni post hoc multiple-comparison analysis to determine statistical significance between treatment groups. Differences with P values less than 0.05 were considered to be significant. Group-size estimations were based on a power calculation to minimally yield an 80% chance of detecting a significant difference in energy expenditure or body weight of P < 0.05 between the treatment groups. All results are given as mean ± s.e.m. Data on gene expression were screened for singular, statistically significant outliers using the maximum normed residual (Grubb’s) test implemented in GraphPad prism.

Data availability. RNA-sequencing data have been deposited in the Gene Expression Omnibus and accession codes are available, as described21.

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