Thermoneutrality but Not UCP1 Deficiency Suppresses Monocyte Mobilization Into Blood

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Rationale: Ambient temperature is a risk factor for cardiovascular disease. Cold weather increases cardiovascular events, but paradoxically, cold exposure is metabolically protective because of UCP1 (uncoupling protein 1)-dependent thermogenesis.

Objective: We sought to determine the differential effects of ambient environmental temperature challenge and UCP1 activation in relation to cardiovascular disease progression.

Methods and Results: Using mouse models of atherosclerosis housed at 3 different ambient temperatures, we observed that cold temperature enhanced, whereas thermoneutral housing temperature inhibited atherosclerotic plaque growth, as did deficiency in UCP1. However, whereas UCP1 deficiency promoted poor glucose tolerance, thermoneutral housing enhanced glucose tolerance, and this effect held even in the context of UCP1 deficiency. In conditions of thermoneutrality, but not UCP1 deficiency, circulating monocyte counts were reduced, likely accounting for fewer monocytes entering plaques. Reductions in circulating blood monocytes were also found in a large human cohort in correlation with environmental temperature. By contrast, reduced plaque growth in mice lacking UCP1 was linked to lower cholesterol. Through application of a positron emission tomographic tracer to track CCR2+ cell localization and intravital 2-photon imaging of bone marrow, we associated thermoneutrality with an increased monocyte retention in bone marrow. Pharmacological activation of β3-adrenergic receptors applied to mice housed at thermoneutrality induced UCP1 in beige fat pads but failed to promote monocyte egress from the marrow.

Conclusions: Warm ambient temperature is, like UCP1 deficiency, atheroprotective, but the mechanisms of action differ. Thermoneutrality associates with reduced monocyte egress from the bone marrow in a UCP1-dependent manner in mice and likewise may suppress blood monocyte counts in man. (Circ Res. 2017;121:662-676. DOI: 10.1161/CIRCRESAHA.117.311519.)

Key Words: atherosclerosis ■ bone marrow ■ housing ■ macrophages ■ thermogenesis

Atherosclerosis remains one of the most common diseases in the United States, despite impressive gains in recent decades at reducing its prevalence. Atherosclerotic plaques are characterized by an accumulation of macrophages, lipids, smooth muscle cells, and fibroblasts, and, in advanced stages, necrotic debris within the arterial wall. The initial injury of the intimal monolayer of endothelial cells triggers the secretion of proinflammatory mediators that activate the innate and adaptive immune system and facilitate progression of the pathology.1,2 Monocyte recruitment from the blood circulation plays a crucial role in giving rise to plaque macrophages that drive plaque progression,3,4 and such recruitment along with macrophage proliferation and survival in the atherosclerotic plaque regulates overall macrophage plaque burden.5–9

Given that monocytes are recruited into plaques from blood, it is perhaps not surprising that blood monocyte counts.
Novelty and Significance

What Is Known?

- The incidence of cardiovascular events increases in colder temperatures.
- Elevated levels of circulating blood monocytes are a risk factor for atherosclerosis.

What New Information Does This Article Contribute?

- Warm environment protects from atherosclerosis progression in mice.
- Monocyte egress from bone marrow is reduced when ambient temperature is warmer.
- UCP1 (uncoupling protein 1) deficiency protects from atherosclerosis but does not regulate monocyte levels.

Environmental temperature has been linked to risk of cardiovascular disease, with elevated risk occurring in colder ambient temperatures. However, the effects of ambient temperature on the immune system in the context of atherosclerosis are not well known. We identify that circulating blood monocyte counts, which are also a known risk factor for atherosclerosis, are inversely correlated with environmental temperature. This association was observed in mice and in human samples collected throughout different times of the year. Animals with reduced circulating monocytes were protected from atherosclerosis. Further, deletion of UCP1, a gene associated with the activation of brown fat and the thermogenic program, was also protective to atherosclerotic disease. However, the protective effect of Ucp1 deletion was independent of monocyte levels. In addition to decreased monocyte counts and lower atherosclerosis, warmer temperatures promoted glucose tolerance and a longer lifespan. Finally, we illustrate that UCP1 activation in peripheral brown fat pads does not interfere with the protective effects of warm ambient temperature, raising the possibility that the combination therapies to combat metabolic disease and atherosclerosis might be devised.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<td>UCP1</td>
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seem to impact disease progression. That is, monocytosis and neutrophilia are well-established risk factors for disease evolution in humans, and data from preclinical mouse models demonstrated that blood monocyte levels correlate with the rate of cell recruitment to the plaque. One mechanism by which monocyte counts in the circulation are regulated is through sympathetic nerve activation in the bone marrow that regulates release of newly formed monocytes, explaining a link between stress and cardiovascular events.

Sympathetic nerve activation is also well known to regulate adipose depot status, with particular impact on thermogenesis within brown and beige fat because of expression of UCP1 (uncoupling protein 1) that generates heat by uncoupling electron transport from ATP generation in the electron transport chain. Induction of UCP1 expression and activity in brown and beige fat is often discussed as a desirable end point to combat obesity and insulin resistance. However, for this approach to be viable, unwanted effects must be avoided. Yet, some reports indicate that cold temperature, a powerful stimulus for brown and beige fat and UCP1 activation, enhances atherosclerosis. However, other studies suggest that thermoneutral housing at the warm temperature of 30°C—the temperature at which energy expenditure is not needed to maintain body temperature—enhances atherosclerosis. The impact of housing temperature on blood monocyte counts has scarcely been discussed, despite reported connections of sympathetic activation to both beige fat activation and monocytosis, as discussed above.

From an epidemiological perspective, cold ambient temperature is associated with cardiovascular events and may impact blood pressure, triacylglycerides, and plasma cholesterol. However, overall, how ambient temperature affects cardiovascular disease remains unclear and whether the link is simply associative and indirect is unknown. Given the momentum behind the concept that cold exposure may be beneficial because of brown and beige fat activation and the potential health effects of such an approach, we here examined the impact of various housing temperatures on experimental atherosclerosis and addressed whether ambient temperature regulates monocyte counts in the blood. The data arising from this work indicate that cold exposure potentiates atherosclerosis. We show that thermoneutral housing protects against atherosclerosis development through, at least in part, regulation of monocyte recruitment from the bone marrow. We distinguish this mechanism from the action of UCP1 in regulating disease burden and glucose homeostasis.

Methods

Mice

Mice were housed in specific pathogen-free animal facilities, maintained by Washington University School of Medicine. Six-week-old B6 (C57Bl/6J, 000664) and Ldlr−/− (B6.129S7-Ldlrtm1Her/J, 002207) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Apoe−/− (B6.129P2-Apoetm1Gbr/J, 002052), Cxcl12tm1Deb (C57Bl/6J, 002258), Cx3cr1tm1LiuJ, (C57Bl/6J, 008451), and Ucp1−/− (B6.129-Ucp1tm1/J, 003124) mice were purchased from Jackson Laboratories, then maintained at Washington University School of Medicine. High-fat diet (Teklad TD.88137) containing 21% milk fat and 0.15% cholesterol was used for atherosclerosis studies. For cold challenge and thermoneutral housing, littermates were born at 22°C and transferred to rooms maintained continuously at 4°C or 30°C when indicated. The 30°C room was a standard room within the animal facility barrier in the same hallway as the 22°C room. By contrast, 4°C housing occurred in a cold room outside the animal facility barrier that was adapted for mouse housing and maintained with monitored standard air flow. Mice were allowed to equilibrate at new housing conditions for 7 days before the start of experiment or diet treatments. All facilities, inside or outside the barrier, were maintained on identical 12-hour light cycles. All experimental procedures were approved by the Washington University School of Medicine Animal Studies Committee.

Human Blood Analysis

Human studies were reviewed and approved by the Institutional Review Board of the Washington University in St. Louis/Barnes-Jewish Hospital (main adult teaching hospital for Washington
University School of Medicine) under protocol number 201703135. These human studies were a retrospective analysis of existing data in the electronic medical record, and the Institutional Review Board protocol included an approved informed consent waiver. Peripheral blood complete blood counts with automated differentials were extracted from the electronic medical record system for outpatients tested at Barnes-Jewish Hospital (or regional affiliate clinics and hospitals) with blood draws during the second week of each month beginning March 2016 and ending February 2017. Monthly means±SEM absolute cell counts were determined for monocytes, lymphocytes, neutrophils, eosinophils, and basophils and correlated against mean ambient temperature recorded at the St. Louis Lambert International Airport weather station during the same time periods as the blood draws. Complete blood counts data included the age and sex of the patient, but not other factors, such as ethnicity/race or disease status.

These studies were designed to have 85% power to detect a 10% difference in monocyte counts between 2 separate 3-month periods, assuming an overall mean monocyte count of 0.5×10^3 cells/μL, equal SDs of 1.0×10^3 cells/μL, equal monthly mean temperatures. Student tests were performed to test whether monthly mean cell counts correlated with ambient temperature recorded at the St. Louis Lambert International Airport weather station during the same time periods as the blood draws. Complete blood counts data included the age and sex of the patient, but not other factors, such as ethnicity/race or disease status.

Counting of CXCCL12 DesRed and CX3CR1 Green Fluorescent Protein Green Voxels

To count the voxels from the red and green channels, videos were initially dye separated using Leica software. In Imaris, 5 time frames from each video were randomly selected with the random number generator provided by www.random.org. Using Fiji, the green and red channels of each time frame were then saved separately as tiff image stacks. Images were collected every 30 s, with a 25 μm step size for each sample.

Glucose Tolerance

Mice were fasted for 12 hours, then injected with 10 μL/g (20% Glucose) intraperitoneally. Blood glucose was measured (Glucocard, Vital) from the tail vein at 0, 15, 30, 45, 60, 90, and 120 minutes after glucose administration. Graphical representations were generated using GraphPad Prism 7, and the area under the curve for each animal was calculated.

Monocyte Recruitment

To label classical Ly6C+ monocytes, mice were first injected intravenously with a single 200 μL dose of clodronate-loaded liposomes to deplete total circulating monocytes. Animals were then rested for 3 days and injected intravenously with 200 μL of sterile fluoresbrite green (YG [yellow-green]) 1 μm latex beads (Polysciences, Inc), diluted 1:4 from stock solution. Ly6C+ monocyte bead labeling was confirmed by flow cytometry of the blood and recruitment of Ly6C+ monocytes into plaques were assayed 2 days after bead transfer.

Body Temperature

Animal body temperature was measured by rectal probe thermometer (Extech Instruments, EasyView 10).

Plaque Analysis

Hearts were collected and immediately fixed in 4% paraformaldehyde containing 30% sucrose. Hearts were then embedded in optimal cutting temperature medium, and cryosections (10 μm) were prepared through the aortic sinus. Samples were stained with oil red O to determine lipid deposition or immunostained with anti-CD68 to quantify macrophage content. Sections were blocked with 5% donkey serum in the presence of 0.1% Tx-100. Macrophages were identified in aortic sinus by CD68 (Bio-Rad) staining followed by secondary staining with antirat Cy3-conjugated Ab (Jackson Laboratories). CD68-, oil red O, and plaque area were measured using ImageJ analysis software. For en face analysis, aorta was fixed with 4% paraformaldehyde, adipose tissue was carefully removed, and the arteries were pinned onto wax dishes. Aortae were pretreated with 100% propylene glycol (siga P4347) for 15 minutes, then incubated with oil red O (siga 01516) for 3 hours at room temperature. Aortae were washed with 85% propylene glycol, then washed multiple times in phosphate-buffered saline. Aortae were imaged on a Zeiss dissecting microscope, and plaque area measurements were made using ImageJ analysis software.

Positron Emission Tomographic Imaging

For positron emission tomographic (PET) imaging, 0 to 60 minutes dynamic PET/computed tomography (CT) scan was performed after injection of ⁶⁸Cu-DOTA-ECL1i (3.7 MBq in 100 μL saline) via tail vein with Inveon PET/CT system (Siemens, Malvern, PA). The PET

Flow Cytometry

Blood samples were collected from the superficial temporal vein. Then red blood cells were lysed (Pharmlyse, BD), and fluorescence-activated cell sorting staining was performed. Bone marrow was extracted from the femurs and tibias. In brief, the bones were flushed with phosphate-buffered saline 1× and then tissue was disrupted using a 21-gauge needle. Red blood cells were lysed, and the samples were counted (Cellometer X4; Nexcelom Biosciences) and processed for staining. Samples were incubated in 2.4 μg/mL goat antirat IgG (Jackson Immunoresearch) and then stained on ice with specific antibodies: CD11b APC-Cy7 conjugated (M1/70, BD Biosciences), CD11c APC conjugated (AFS98, eBioscience), Ly6C PerCP-Cy5.5 conjugated (HK1.4, Biogen, Inc), Ly6G FITC conjugated (1A8, BD Biosciences), CD43 PE conjugated (1B1, Biogen), CCR2 PE conjugated (475301, R&D systems), CD45 Pacific Blue conjugated (30-F11, Biogen), Lin staining (CD3 PE-cy7 conjugated [145-2C11, Biogen], CD19 PE-cy7 conjugated [eBio1D3, eBioscience], B220 PE-cy7 conjugated [RA3-6B2, eBioscience], Ter-119 PE-cy7 conjugated [TC15-12Fl2.2, BioLegend], CD150 PE-cy7 conjugated [RA3-6B2, eBioscience], CD48 PE-cy7 conjugated [RA3-6B2, eBioscience], CD19 PE-cy7 conjugated [eBio1D3, eBioscience], B220 PE-cy7 conjugated [RA3-6B2, eBioscience], Ter-119 PE-cy7 conjugated [TC15-12Fl2.2, Biogen], CD150 PE conjugated (M1/70, BD Biosciences), CD68+ oil red O, and plaque area were measured using ImageJ analysis software. For en face analysis, aorta was fixed with 4% paraformaldehyde, adipose tissue was carefully removed, and the arteries were pinned onto wax dishes. Aortae were pretreated with 100% propylene glycol (siga P4347) for 15 minutes, then incubated with oil red O (siga 01516) for 3 hours at room temperature. Aortae were washed with 85% propylene glycol, then washed multiple times in phosphate-buffered saline. Aortae were imaged on a Zeiss dissecting microscope, and plaque area measurements were made using ImageJ analysis software.
images were reconstructed with the maximum a posteriori algorithm and analyzed by Inveon Research Workplace. The organ uptake was calculated as percent injected dose per gram of tissue in 3-dimensional regions of interest without the correction for partial volume effect.\textsuperscript{31}

**Bio Distribution of PET Tracers**

The CCR2-specific PET tracer of \(^{64}\text{Cu}-\text{DOTA-ECL1i}\) and CXC4-targeted PET tracer of \(^{64}\text{Cu}-\text{AMD3100}\) were synthesized after our previous reports.\textsuperscript{31–34} For biodistribution studies, about 370 KBq of \(^{64}\text{Cu}-\text{DOTA-ECL1i}\) or \(^{64}\text{Cu}-\text{AMD3100}\) in 100 μL saline (APP Pharmaceuticals, Schaumburg, IL) was injected into the mice via tail vein (n=4 per group). The mice were anesthetized with inhaled isoflurane during injection and reanesthetized before euthanasia by cervical dislocation at 1 hour post-injection (n=4 per group). Organs of interest were collected, weighed, and counted in a Beckman 8000 gamma counter (Beckman, Fullerton, CA). Standards were prepared and measured along with the samples to calculate the percentage of the injected dose per gram of tissue.

\[\text{\beta-3-Agonist Treatment}\]

CL-316,243 (Sigma C9576), a \(\beta\)-specific adrenoceptor agonist, or saline was administered twice daily (1 mg kg\(^{-1}\) d\(^{-1}\)) by intraperitoneal injection for 4 weeks as previously described.\textsuperscript{30,36}

**Quantitative Polymerase Chain Reaction**

Tissues were totally disrupted using homogenizer probe in Trizol LS (ThermoFisher Scientific, 10296010). Total RNA was extracted with chloroform followed by additional purification on Qiagen RNeasy mini columns (Product 74104) following the manufacturer’s instructions. RNA quantity was assessed on Nanodrop spectrophotometer, and cDNA was prepared from 1 μg RNA using the SuperScript III reverse transcriptase kit (ThermoFisher Scientific, 8080044). The following primers were designed using Universal Probe Library (Roche) and used for amplification in triplicate assays with annealing temperature of 60°C: Hprt, forward: GCTATAAAATTCTTGTGACCTGCTG; reverse: AATTACCTTTATGGTCCCTGTGATCGT, and Ucp1, forward: GGCCCTCTAGACTGACATCCA; reverse: TAAGGCCGGC TGAGATCTTGT. For quantitative polymerase chain reaction amplification, Takara SYBR mix (Takara, SYBR Premix Ex Taq II, RR820A) was used and the assays were performed on Eppendorf cycler. For normalization between samples quantitative polymerase chain reaction amplification of Hprt was performed and analyzed. ΔCt values obtained from the raw Ct values obtained from Hprt mRNA and from the Ct values obtained for the other analyzed genes.

**Food Intake Protocol**

For food intake studies, mice were single housed for 3 days. Every day at the same hour, the remaining food in the cage was weighed and recorded. Mice had unlimited access to food and water during the study, independently of the housing environmental temperature.

**Plasma Analysis**

Mouse plasma was collected from whole blood with 5 mmol/L EDTA to limit clotting. Plasma was either used immediately or stored at –80°C until use. Total cholesterol was determined using Dako Cholesterol-E kit (product 439-17501) following the manufacturer’s protocol. Plasma corticosterone was determined by ELISA following the manufacturer’s protocol (Abcam ab108821). Plasma triacylglyceride content (Infinity Triglyceride Reagent, Fisher Scientific) and nonesterified free fatty acid content (NEFA Reagents, Wako Chemicals) were analyzed by the Diabetes Model Phenotyping Core in the Diabetes Research Center at Washington University following the manufacturer’s protocols. Plasma lipoprotein content was determined by mini-high-performance liquid chromatography as previously described.\textsuperscript{39} In short, a LaChrom Elite HPLC system (Hitachi High Technologies) consisting of an L-2200 Autosampler with Peltier cooling, an L-2420 UV-Vis Detector, and 2 L-2100 SMASH pumps was used to administer \(\pm 15\) mg of cholesterol from thawed plasma. Output was analyzed with ChromPerfect Spirit Chromatography Data System, version 5.5.

**Statistical Analysis**

The statistical significance of differences in mean values was analyzed by unpaired 2-tailed Student t test, using Prism software. \(P\) values <0.05 were considered statistically significant. Error bars show the SEM.

**Results**

**Effects of Thermoneutrality on Progression of Atherosclerosis**

To determine how environmental housing temperature influenced atherosclerosis progression, we housed male or female cohorts of \(\text{Ldlr}^{−/−}\) mice fed a cholesterol-rich diet in rooms at different ambient temperatures: 4°C, 22°C, or 30°C. In males and females, food intake greatly varied between temperature groups, with cold-challenged mice consuming \(\approx 6\) g of food per day, mice housed at room temperature \(\approx 3\) g/d, and 30°C housed animals consuming only \(\approx 2\) g/d (Figure 1A). Core body temperature was also remarkably distinct between mice housed at different ambient temperature, positively correlating with food consumption (Figure 1A). Mice housed at 4°C had the highest core temperature, whereas mice living at 30°C had the lowest body temperature (Figure 1A). Cold challenge activated thermogenesis programs in brown adipose sites, and mice living at 4°C actively induced \(\text{Ucp1}\) mRNA compared with mice housed at 22°C (Figure 1B). \(\text{Ucp1}\) expression was below the level of detection when mice were housed at 30°C. Atherosclerotic plaque burden in \(\text{Ldlr}^{−/−}\) and \(\text{Apoe}^{−/−}\) mice fed a cholesterol-rich high-fat diet varied with changes in ambient temperature, with plaque burden the greatest in mice housed at 4°C and the least in those housed at 30°C, as the latter group had smaller plaques compared with the 22°C controls at both 8- and 12-week high-fat diet (HFD) time points (Figure 1C and 1D; Online Figure IA and IB), indicating that thermoneutral ambient temperature prevents the progression of the disease. Whereas the aortic sinus is a major site of atherosclerosis in mouse models, in humans it is not a dominant location for disease. Therefore, we performed en face plaque analysis of the aortic arch in mice fed HFD for an 8-, 12-, or 28-week duration (Figure 1E; Online Figure ID and IE). All anatomic locations and time points exhibited reduced atherosclerosis progression in animals housed in thermoneutral conditions compared with 22°C. This was independent of changes in relative body weight after HFD treatment in \(\text{Ldlr}^{−/−}\) mice, males and females combined (Online Figure IE). Plasma triacylglycerides and nonesterified fatty acids were markedly elevated with increasing ambient temperature in the plasma of fasted \(\text{Ldlr}^{−/−}\) mice after 8 weeks of HFD challenge (Figure 1F and 1G), which is consistent with a previous studying reporting elevated triacylglycerides but reduced atherosclerosis during 30°C housing compared with 4°C.\textsuperscript{21} Total plasma cholesterol concentration was unchanged between temperature groups in \(\text{Ldlr}^{−/−}\) or \(\text{Apoe}^{−/−}\) mice (Figure 1F; Online Figure IF), suggesting that it was not a major influence on the changes in disease progression. Finally, the stress hormone corticosterone was increased significantly in response to 4°C housing but was similar between mice housed at 22°C versus 30°C (Figure 1G). It seemed possible that the increased disease at 4°C housing was at least partially linked to this stress response,\textsuperscript{38} but it was not immediately obvious what accounted for differential disease
burden at 22°C versus 30°C. We thus next focused on identifying underlying differences that existed between cohorts housed at 22°C and 30°C.

Analysis of plasma lipoprotein composition did not uncover any significant differences in the patterns of high-density lipoprotein, low-density lipoprotein, or very-low-density lipoprotein over time after the initiation of HFD (Figure 1H). Thus, it was unlikely that altered cholesterol metabolism accounted for the decreased plaque area in animals housed at 30°C. We conclude that thermoneutrality reduces atherosclerosis progression and does so while simultaneously reducing energy consumption and energy demand for body temperature, suggestive of a lowered whole-body metabolism.

Ambient Temperature Regulates Monocyte Recruitment to Plaque

To test whether changes in plaque composition were also correlated with reduced macrophage content, we stained for CD68+ area in the aortic sinus (Figure 2A). Total macrophage (CD68+) area was consistent with total plaque measurements Figure 1. Ambient temperature regulates atherosclerosis progression. Male (top) and female (bottom) Ldlr−/− mice were housed at the indicated ambient temperature and fed high-fat diet (HFD) for 4 wk, then assayed for (A) food intake (left), rectal temperature (right), and (B) UCP1 (uncoupling protein 1) mRNA expression in brown adipose pads. C, Atherosclerotic plaque area in the aortic sinus of Ldlr−/− mice was measured after 8 wk HFD treatment at the indicated environmental temperature. D, Representative oil red O staining to highlight regions of lipid deposition. E, Representative en face plaque staining of aortic arches and quantification after 8 wk HFD. Triacylglycerides (F), nonesterified free fatty acids (G), and total cholesterol levels (H) were measured in the plasma from Ldlr−/− mice after 8 wk HFD at the indicated temperatures. I, Corticosterone levels in the plasma of Ldlr−/− mice fed HFD for 8 wk. J, Kinetic analysis of plasma lipoproteins high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) at 0, 4, and 8 wk of HFD feeding at indicated temperatures. All experiments are representative experiments with 5 to 10 animals per group, repeated in 2 or 3 independent experiments. Statistical analysis was performed by unpaired Student t test, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
and reduced in \textit{Ldlr}^{−/−} mice at thermoneutral housing after 8 and 12 weeks of HFD (Figure 2A and 2B; Online Figure IG).

Reductions in plaque macrophages could be because of a variety of possibilities, including reduced recruitment of monocytes under thermoneutral conditions.

We thus examined whether monocyte recruitment into plaques was reduced in animals housed at 30°C. We used a technique that we previously developed to quantify monocyte recruitment into plaque involving pulse labeling of monocytes with latex particles, in which the entry of latex particle+ monocytes into plaque requires recruitment in a manner that is pertussis toxin sensitive and reliant on CCR2. \textit{Ldlr}^{−/−} mice housed at 22°C or 30°C and fed HFD for 8 weeks were treated with clodronate-loaded liposomes to synchronize blood monocytes and deplete nonclassical Ly6C− monocytes, then 3 days later given beads intravenously to assess Ly6C+ monocyte recruitment into plaques. When we examined plaque sections for the appearance of latex bead+ monocytes (Figure 2C), bead accumulation at 30°C was only about 1/3 of that observed in sections from mice housed at 22°C (Figure 2D). Total leukocytes in blood and bone marrow were similar between the groups of mice housed at different temperatures after the labeling protocol (Figure 2E). However, Ly6C+ monocytes were dramatically reduced in the blood by approximately half and increased in the bone marrow (Figure 2F). Collectively, the data indicate that ambient temperature attenuates the recruitment of monocytes to the plaque and further suggests that the tendency of monocytes to emerge from the bone marrow in atherogenic mice is reduced when the mice are housed at 30°C. This potential retention in the bone marrow could promote a reduction in circulating monocytes and fewer monocytes being recruited to atherosclerotic plaques to promote plaque progression. We next addressed the possibility that monocytes in the circulation were reduced at higher ambient temperature, without the use of monocyte ablation with clodronate-loaded liposomes.

**Thermoneutral Ambient Temperature Leads to Decreased Number of Circulating Monocytes, Their Accumulation in Bone Marrow, and Reduced Recruitment to Atherosclerotic Plaques**

Although total leukocytes were unchanged in the blood (Figure 3A), even in the absence of previous monocyte ablation, Ly6C\textsuperscript{hi} monocytes (CD115\textsuperscript{+} CD11b\textsuperscript{+} Ly6C\textsuperscript{hi} CD43\textsuperscript{−} Ly6G\textsuperscript{−}), gated as shown in Online Figure II, were reduced in \textit{Ldlr}^{−/−} mice on chow or after 6 weeks of HFD (Figure 3B). The counts of Ly6C\textsuperscript{−} monocytes (CD115\textsuperscript{+} CD11b\textsuperscript{+} Ly6C\textsuperscript{−} CD43\textsuperscript{+} Ly6G\textsuperscript{−}) also showed a shift in cell numbers in the blood (Figure 3B and 3C). Next, we performed BrdU labeling and chase experiments to track monocyte appearance into blood from the bone marrow. Animals housed at 22°C or 30°C and fed an HFD...
for 4 to 8 weeks received a single bolus of BrdU intraperitoneally, then were analyzed at 24 hours for blood monocyte labeling over this period of time. Representative intracellular BrdU flow cytometric staining of Ly6C+ monocytes is shown in Figure 3D. In addition to the total cell numbers of Ly6C+ monocytes being reduced (Figure 3B and 3C), both Ldlr−/− and Apoe−/− mouse strains on HFD showed a modest, but significant reduction in the percentage of BrdU+ monocytes that appeared in the blood after BrdU injection when housed at 30°C versus 22°C (Figure 3E).

These data prompted us to consider the possibility that monocytes accumulated in the bone marrow and were relatively less prone to enter the blood circulation. To quantify net accumulation of monocytes in the bone marrow of Ldlr−/− mice, we took advantage of a PET tracer of 64Cu-DOTA-ECL1i that specifically binds to the monocyte-selective chemokine receptor CCR232 that in turn mediates monocyte egress from bone marrow.39 One advantage of the PET tracer is that most body organs can be examined simultaneously, curbing any potential selection bias with respect to the evaluation of sites where tracer might accumulate. When atherosclerotic plaque-bearing Ldlr−/− mice housed at 30°C, we used another PET tracer, 64Cu-AMD3100, designed to detect CXCR4,40 a chemokine receptor established to promote bone marrow retention.41 Biodistribution of the tracer was measured across tissues and indicated only a differential accumulation of CXCR4+ cells in the bone marrow of Ldlr−/− housed at 30°C, and no other tissue (Figure 4D), supporting the concept that 30°C housing prevents egress from the marrow.

Impact of Thermoneutrality in Wild-Type C57BL/6 Mice in the Absence of Hypercholesterolemia

We asked next whether the retention of monocytes in bone marrow was restricted to conditions of hypercholesterolemia that occurred in atherogenic Apoe−/− and Ldlr−/− mouse strains. When we housed normocholesterolemic C57BL/6 wild-type mice at 22°C and 30°C, Ly6C+ monocytes were modestly but nonetheless significantly reduced in the blood of mice housed at 30°C (Figure 5A) compared with the major shifts observed in hypercholesteremic animals. Reduced monocyte egress from the marrow was also evident (Figure 5B) when using the same 24-hour BrdU labeling approach described in Figure 3.

Using an intravital imaging approach, we were also able to image monocytes in the bone marrow of normocholesterolemic mice in the dual reporter Cx3cr1GFP/Cxcl12DsRed mouse (Figure 5C). Monocytes in this mouse are green fluorescent protein (GFP) expressing,42 and consistent with the concept that thermoneutral housing promotes monocyte retention in the bone marrow, we detected augmented GFP expression in the bone marrow compartment, defined as Cxcl12DsRed+ area, in mice housed at 30°C (Figure 5D; Online Movie I). By flow cytometry, there were similar total cell numbers but an increased number of CX3CR1GFP+expressing cells in the bone marrow of animals
housed at 30°C (Figure 5E). However, the number of stem cells, identified as Lin− Sca1+ CD117(c-Kit)−, then separated into unique precursors by CD150 and CD48 expression cells29 (gating strategy in Online Figure IVA), was unaffected (Online Figure IVB and IVC). Importantly, we observed equivalent expression levels of CCR2 protein on the surface of the bone marrow...
monocytes, suggesting that regulation of the CCR2 receptor is not the mechanism by which monocytes are being retained in the marrow (Figure 5F, gating strategy Online Figure IVD). We conclude that monocyte retention in the bone marrow occurs when mice are housed at 30°C regardless of the presence or absence of hypercholesterolemia.

Because circulating monocyte levels are associated with vascular inflammation and reduced levels are associated with reduced risk to vascular disease, we hypothesized that a thermoneutral environment may be beneficial to overall systemic health. Mice housed at 30°C since 6 weeks of age survived in a lifespan study a significantly longer time compared with littermate controls housed at 22°C environments (Online Figure V). The average lifespan of control animals was ≈758 days, which is consistent with the previous reports, whereas almost all mice housed at thermoneutral conditions lived past 1000 days. This is a small cohort of mice; however, it suggests that modest changes in overall metabolism and inflammatory mediators may have major impact on the lifespan of the mouse.

**Environmental Temperature Influences Circulating Monocytes in Humans**

We sought to determine whether environmental temperature correlated with circulating monocyte counts in human patients. We obtained complete blood counts data with automated differentials from outpatient samples over the course of a full year, with ≈1200 samples per month, and a total of 15,516 samples derived from the St. Louis, Missouri region (38°37′37.21″N). First, we compared the average monocyte counts per month (Figure 6A) and found that monocyte counts indeed changed in a seasonal manner. These seasonal changes were independent of patient age or gender (Online Figure VIA and VIB). Strikingly, monocyte counts in peripheral blood were 7.4% higher in winter months compared with summer months (Online Figure VIC). Mean monthly high, average, and low environmental temperature for the region where the hospital was located confirmed that winter months had the lowest average daily temperature and summer had the highest average daily temperature (Figure 6B). Linear regression analysis indicated an inverse correlation between average human peripheral blood monocyte counts and the mean environmental temperature (Figure 6C). We were initially concerned that the elevation in monocyte counts over the winter months might link to an increase in incidence of infection. However, all other circulating cell types, including neutrophils, eosinophils, basophils, and lymphocytes, were not correlated with environmental temperature (Figure 6D), and neutrophils or lymphocytes would have been expected to be most sensitive to bacterial or viral infection, respectively. Thus, a common thread for both mouse and human data is that monocyte counts are selectively altered in relation to ambient temperature. Although routine examination of these outpatients did not typically involve analysis of vitamin D metabolites, which
have been argued to vary seasonally in some ethnic groups above the 37th parallel, it is unlikely that vitamin D metabolite variation accounted for these findings because in persons >50, active vitamin D does not vary seasonably in the pattern consistent with pattern of monocyte counts in our data or consistent with vitamin D metabolite shifts in younger people. We conclude that, remarkably, considering the immense amount of time that humans live in controlled environments (22°C indoor temperature), outdoor ambient temperature may influence monocyte counts in peripheral blood of humans.

Thermoneutrality and UCP1 Signaling Have Unique Downstream Pathways

We next hypothesized that brown fat activation at 22°C housing might lead to augmented monocyte egress from the marrow, leading to increased atherosclerotic disease. We thus generated \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice. When housed at 22°C and fed a cholesterol-enriched diet, plaque burden in \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice was reduced compared with \( Ldlr^{-/-} \) mice and resembled that of \( Ldlr^{-/-} \) mice housed at 30°C. These data indicate that loss of UCP1, either through genetic or environmental manipulation, was atheroprotective (Figure 7A). This was not because of major shifts in animal weight (Figure 7B). In addition, plaque burden was not further reduced in \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice housed at 30°C compared with the same strain housed at 22°C after 12 or 20 weeks HFD feeding (Figure 7C; Online Figure VIIA and VIIB), supporting the concept that UCP1 expression in \( Ldlr^{-/-} \) mice housed at 22°C may account for the increased plaque burden observed over \( Ldlr^{-/-} \) mice housed at 30°C.

Changes in thermogenesis are known to affect metabolic responses in animals and humans. Therefore, we next examined glucose tolerance in \( Ldlr^{-/-} \) and \( Apoe^{-/-} \) mice fed a cholesterol-enriched diet housed at 22°C and 30°C ambient temperatures. Mice housed at the elevated ambient temperature showed improved glucose sensitivity compared with those kept at 22°C (Figure 7D). However, consistent with the previous reports with \( Ucp1^{-/-} \) mice, \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice demonstrated impaired glucose tolerance over UCP1-sufficient \( Ldlr^{-/-} \) mice at 22°C housing (Figure 7E) despite having reduced atherosclerosis outcomes. Strikingly, when we compared glucose tolerance of \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice at 22°C versus 30°C housing, the improved glucose sensitivity observed at 30°C persisted (Figure 7F). Collectively, these data implicate UCP1 in driving plaque growth in a manner independent of glucose metabolism, even as they also reveal that 30°C housing provides a means to elevated glucose sensitivity that is independent of UCP1.

Nonetheless, because our studies in \( Ucp1^{-/-} \times Ldlr^{-/-} \) mice mirrored the reduction in plaque burden observed in \( Ldlr^{-/-} \) mice housed at 30°C, we hypothesized that UCP1 activity might be directly implicated in the regulation of monocyte output from the bone marrow. We studied C57BL/6 mice and \( Ucp1^{-/-} \) mice in the absence of atherogenesis to address this question. In strong contrast to expectations for overlapping mechanisms, UCP1 deficiency did not regulate circulating monocyte numbers (Figure 8A). In addition, when housed at thermoneutral environment, UCP1-deficient mice did not show changes in circulating monocyte levels, whereas
wild-type (WT) mice examined side by side with Ucp1−/− mice did (Online Figure VIIC and VIID). We next addressed biodistribution of 64Cu-DOTA-ECL1i tracer in Ucp1−/− mice (Figure 8B). We found a significant decrease in tracer uptake in the brown fat of Ucp1−/− mice compared with WT, which was consistent with animals housed at 30°C. White fat tracer uptake was also elevated in the absence of UCP1 (Figure 8B).

Unlike the effect of 30°C housing on accumulation of 64Cu-DOTA-ECL1i in the bone marrow, Ucp1−/− mice did not display increased tracer in the bone marrow (Figure 8B).

Examining Ucp1−/− Ldlr−/− mice fed HFD for 8 weeks at 22°C, we observed no change in blood monocyte numbers compared with Ldlr−/− mice, albeit a trend toward augmentation of circulating monocytes in Ucp1−/− mice (Figure 8C). This was accompanied by no major shifts in monocyte populations in blood or bone marrow of Ucp1−/− Ldlr−/− mice housed at 22°C and 30°C (Online Figure VIID and VIIIE). We repeated 64Cu-DOTA-ECL1i biodistribution analysis in Ucp1−/− Ldlr−/− mice on HFD to test whether hypercholesterolemia might exacerbate the monocytosis associated with thermoneutral ambient temperature. Although we observed reduced tracer uptake in the aorta of the Ucp1−/− Ldlr−/− mice, consistent with reduced plaque, we did not find significant changes in other tissues, including adipose tissue compartments (Figure 8D). Finally, we asked whether Ucp1−/− Ldlr−/− mice had changes in their lipoprotein profiles that might explain the changes in atherosclerosis progression via a mechanism distinct from control of monocyte numbers in the blood. Indeed, while food intake was the same in the 2 groups, Ucp1−/− Ldlr−/− mice had reduced plasma cholesterol levels (Online Figure VIIIA and VIIIB). In particular, Ucp1−/− Ldlr−/− mice on HFD for 8 weeks showed dramatically reduced very-low-density lipoprotein and intermediate-low-density lipoprotein levels compared with controls (Online Figure VIIIC and VIIID), consistent with previous reports for the loss of Ucp1 expression,21 but distinct from mice housed at 30°C. Likely, this drop in plasma cholesterol largely accounts for the reduced atherosclerotic disease found in Ucp1−/− Ldlr−/− mice. Together, these studies indicate that the impact of housing temperature on monocyte egress from the bone marrow is not simply a product of UCP1 activation and possibly is unlinked to the activation of beige/brown fat altogether.

β3-Adrenergic Receptors Activation in Beige Fat Does Not Promote Monocyte Egress From the Bone Marrow

Next, we considered whether retention of monocytes in the bone marrow was linked with the induction of beige/brown fat. Sympathetic activation through β3-adrenergic signaling supports UCP1 expression in brown and beige fat at lower temperatures. Thus, we treated Ldlr−/− mice fed...
cholesterol-enriched HFD for 4 weeks, then began injections with \( \beta_3 \)-adrenergic receptor agonist to assess whether such stimulation would reverse the suppression in circulating monocytes observed at 30°C. Following previously described protocols\(^3\), treatment with the \( \beta_3 \)-adrenergic receptor–specific agonist CL-316,243 showed little effects on circulating monocyte numbers in \( \text{Ldlr}^{-/-} \) mice housed at 30°C on HFD (Online Figure IXA). After 4 weeks of continuous treatment, plaque area was assessed, but the activation of the \( \beta_3 \)-adrenergic receptor did not influence plaque area in male or female mice in this short period of plaque assessment (Online Figure IXB). However, indicating the efficacy of our treatment regimen to activate beige/brown fat, we observed the induction of UCP1 mRNA in beige fat depots (Online Figure IXC). In addition, body temperature was increased by the \( \beta_3 \)-adrenergic receptor agonist, returning body temperature to the equivalent of that documented at 22°C (Online Figure IXD). Furthermore, reductions in body weight were associated with animals receiving \( \beta_3 \)-adrenergic receptor agonist, consistent with what would be expected in animals with elevated activation of brown or beige fat (Online Figure IXE and Ixf).

**Discussion**

During the past decade, research on brown and beige adipose tissue has intensified tremendously. The discovery of thermogenic adipose tissue in adult humans, once thought to be absent after infancy, has fueled interest to promote thermogenesis in multiple metabolic disorders.\(^2\),\(^3\) Evidence that thermogenically active brown fat promotes glucose disposal\(^5\) and, arguably, consumption of lipids\(^6\) is among the points of logic behind the concept. Indeed, it has evolved that cold exposure is now often viewed as a natural approach to improved metabolism. Conversely, thermoneutrality is often considered experimentally as an approach equivalent to loss of active beige or brown fat. Here, we show that thermoneutrality and loss of UCP1 affect atherosclerosis similarly, by reducing disease burden. However, ultimately, these manipulations diverge mechanistically: thermoneutrality governs monocyte homeostasis, whereas UCP1 expression more closely associates with cholesterol homeostasis, particularly plasma low-density lipoprotein accumulation.

When we started these studies, we hypothesized that the anticipated metabolic benefits of cold exposure would extend to the protection against atherosclerosis. However, we were surprised to observe that thermoneutrality and genetic deficiency of UCP1 (studied in atherogenic strains of mice housed at 22°C) reduced atherosclerosis. Our findings are in general agreement with an earlier study by Dong et al,\(^2\) who observed, like us, that UCP1 deficiency in the context of experimental atherosclerosis is atheroprotective for reasons linked to plasma cholesterol. They also reported that thermoneutrality was atheroprotective compared with 4°C housing. However, this study did not include a 22°C group, the most commonly analyzed in the literature, making it difficult to determine whether cold challenge increased atherosclerosis or warm
temperatures were protective. We find that both conclusions are supported by our data and that experimental atherosclerosis is stepwise more severe as ambient temperature drops.

At first glance, our conclusions related to the impact of ambient temperature on atherosclerosis seem to be confounded by several studies that draw conclusions opposite to those of our study and that of Dong et al.21 First, Berbee et al.52 studied the impact of a pharmacological agonist to the β3-adrenergic receptor on atherosclerosis in apoE3 Leiden mice coexpressing cholesteryl ester transfer protein. In the presence of agonist, these mice showed lipid profiles consistent with reduced atherosclerosis and indeed plaque burden was lowered.53 The favorable changes in lipid profiles were not recapitulated in Apoe−/− and Ldlr−/− mice. The authors argued that cold challenge would protect against atherosclerosis but did not test it directly. It is important to recognize that the work of Berbee et al.52 more relates to the connections between UCP1 activity and blood lipid profiles, which may respond differently between mouse and man, with the apoE3 Leiden serving as a better model for human lipoprotein pathways than the other models. The more novel aspects of our work related to thermoneutrality effects on monocytes were not examined by Berbee et al.52 As we show that UCP1 activation and thermoneutrality diverge with respect to regulating monocyte counts, and that human monocyte counts are reduced in warmer months of the year, it becomes difficult to argue that the study of Berbee et al.52 is either consistent or inconsistent with our findings.

Second, Tian et al.22 examined the impact of thermoneutrality on atherosclerosis. In agreement with our findings and those of others,54 Tian et al.22 reported improved glucose clearance in mice housed at 30°C. However, they also reported worsened atherosclerosis along with modestly elevated cholesterol, albeit with minimal disease effects at the aortic arch and the most dramatic effects in the descending aorta.22 Aortic sinus disease, which we quantified in the present study along with aortic disease at multiple time points, was not examined by Tian et al.22 Giles et al.54 failed to find a major shift in plasma cholesterol but nonetheless reported that enhanced atherosclerosis was worsened in Apoe−/− mice fed a high-fat diet, but there was no disease change in Apoe−/− mice fed a Chow diet. They then went on to report that, surprisingly, WT C57BL/6 mice developed modest, but measurable, atherosclerosis when fed a high-fat diet and housed at thermoneutrality.54 For unclear reasons as of yet, these 2 studies appear directly at odds with the conclusions from our work and that of Dong et al.21 One of the critical details that deserves more attention than it has received is the conditions used for thermoneutral housing, for example, whether such housing was done within the barrier of the animal facility or in special containment units for controlling temperature, and whether air circulation and light cycles may have also varied.

To our knowledge, this is the first formal investigation of the impact of thermogenesis and thermoneutrality on leukocyte dynamics. It is interesting to note that the supplemental data included in the work of Tian et al.22 underscore a reduction in circulating neutrophils, and the data seem to support monocyte retention in the bone marrow as well. We documented, using 3 different techniques—flow cytometry coupled with BrdU labeling and cell counting, PET imaging, and 2-photon microscopy—that monocytes were retained in the bone marrow of mice housed at thermoneutrality, coupled with reduced circulating monocytes, regardless of diet or whether the mice were of an atherogenic strain or wild type. However, monocyte sequestration in the bone marrow was only a feature of thermoneutrality, not UCP1 deficiency. This sequestration of monocytes in the bone marrow was associated with reduced monocyte migration into plaques, potentially explaining the reduction in aortic sinus plaque that we report. That is, the monocyte pool in the bone marrow is dependent on the dynamical equilibrium between egress (relying on the chemokine receptor CCR2) and retention through the interaction of the chemokine receptor CXCR4 expressed on hematopoietic cells and its ligand CXCL12, mainly found in the stromal compartment, including CAR cells, endothelial cells, and nestin+ perivascular cells.55 Alteration of either of these interactions could modify the pool of monocytes in the bone marrow and peripheral blood. Blood levels of monocytes and neutrophils are strongly associated with atherosclerosis progression,4,10–12 and, therefore, reduced white blood cells counts provide a potential explanation accounting for the reduced plaque size at mice housed at 30°C. Although it remains uncertain the extent to which this mechanism can be applied to humans, we provide data examining over 1000 individuals per month in the St. Louis region that monocyte counts vary with outdoor temperature, in a manner that is consistent with the work in mice, including higher monocyte counts in blood during cooler weather, with effects on monocytes the most prominent among all leukocytes. We believe that this retrospective analysis in humans, which should be considered with due caution in full interpretation, suggests that a prospective study on the impact of ambient temperature on monocyte dynamics may be worthwhile in future studies.

Considering that UCP1 activity promotes glucose disposal, while not linked to the regulation of monocyte dynamics in the blood and bone marrow, we propose that the field take a revised view of how to approach simultaneously optimizing metabolic and cardiovascular health. We indeed argue that our experiment using synthetic β3-agonist suggests that it is technically feasible to simultaneously activate UCP1 in the beige fat pads and retain the atheroprotective effects of thermoneutrality, including reduced monocyte mobilization into blood, whether or not β3-adrenergic receptors would be the most logical target (doubtful) to activate beige fat in a translational setting. On the basis of the findings here, one might envision that developing tools that recapitulate the mechanisms conferring atheroprotection in response to thermoneutral ambient temperature, without specifically ablating UCP1 activity that confers metabolic protection, may provide the most benefit to a population that encounters a high incidence of both cardiovascular and metabolic diseases. In other words, we argue that it is critical in the cardiovascular research community to cease viewing cooler temperatures as a desired positive stimulus for arriving at the potential metabolic benefits of beige fat activation. By conceptually and practically unlinking UCP1 activity from the impact of ambient temperature, we propose that it might be more optimal to promote the healthful effects of warm environs—which based on our emerging survival data may...
extend to benefits beyond protection from atherosclerosis—while simultaneously supporting beige fat activation through the mechanisms distinct from ambient temperature. Additional research is needed to test this proposed concept emerging from the present study.

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Disclosures

None.

References


Thermoneutrality but Not UCP1 Deficiency Suppresses Monocyte Mobilization Into Blood

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Thermoneutrality but not UCP1 deficiency suppresses monocyte mobilization into the blood

Supplemental Material
Online Figure I. Reduced atherosclerosis at thermoneutral housing environment. A) Apoe<sup>−/−</sup> mice were housed at the indicated temperatures and assayed for atherosclerosis progression following 8 weeks of HFD. Ldlr<sup>−/−</sup> mice were housed at indicated temperatures and fed HFD for 12 weeks, then assayed for plaque development at aortic sinus (B) and aortic arch (C). D) Ldlr<sup>−/−</sup> mice fed HFD for 28 weeks were assayed by en face oil red o staining for plaque area on aortic arch and descending aorta. E) Percentage change relative to starting body weight of Ldlr<sup>−/−</sup> mice housed at different ambient temperatures while on HFD, (right panel) absolute weight numbers for males (upper) and female (lower). F) Plasma cholesterol levels were assayed in Apoe<sup>−/−</sup> mice after 8 weeks HFD. G) Ldlr<sup>−/−</sup> mice fed HFD for 12 weeks were assayed for CD68+ area in aortic sinus. All data include n=5 animals per group and are representative of two or three independent experiments each. Statistical analysis was performed by unpaired student T-test, * p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.
Online Figure II

**Online Figure II. Gating scheme for blood monocytes.** Monocytes were identified as CD45⁺, Ly6G⁻, CD115⁺, and CD11b⁺. Monocytes were then separated into subsets by expression of Ly6C and CD43.
Online Figure III. PET/CT Images of $^{64}$Cu-DOTA-ECL1i accumulation. \textit{Ldlr}^{-/-} mice housed at the indicated temperature and fed a HFD for 8 weeks were imaged by PET/CT for $^{64}$Cu-DOTA-ECL1i tracer.
Online Figure IV. Gating strategy for bone marrow precursor cells. A) Bone marrow was isolated and stained for stem cell markers Sca1, CD117(cKit), CD150, and CD48. Gating was first performed on total cells by fsc and ssc, then singlet gate and selected on lineage negative cells (CD3^− B220^− Gr1^− Ter119^−), as displayed in flow plot 1. B) Sca1^+ CD117^+ bone marrow stem cell progenitors were identified in flow plot 2 and C) subpopulations in flow plot 3 to identify stem cells were then separated into CD48^− CD150^+, CD48^+ CD150^−, CD48^− CD150^−, and CD48^+ CD150^+, with gate 3 (CD150^+ CD48^-) cells being the least differentiated cell in gating panel. D) Bone marrow monocytes were identified by CD115^+ , Ly6G^− CCR2^+ , and Ly6C^+ expression. A and D are representative gating panels, B and C are n= 5 mice per group and experiment was repeated two independent times.
Online Figure V. Thermoneutral housing increased lifespan of C57bl/6 mice. Animals were placed in the indicated environmental temperatures at 6 weeks of age, under normal chow diet and SPF conditions. Animals were tracked for survival, plotted on Kaplan-Meir Curve. Statistical analysis performed by Gehan-Breslow Wilcoxon Test, p-value reported. n=6-7 animals per group.
Online Figure VI. Human peripheral blood analysis. A) Average age of patients in relation to month in which blood was drawn (±SD). B) Gender (presented as % female) information for patients sampled across different months. C) Blood monocyte levels were separated by average cells in winter months vs. summer months. Data are averages of approximately 1200 samples per month.
Online Figure VII. UCP1-deficiency regulates atherosclerosis progression but not monocyte levels.

A) *Ucp1*<sup>−/−</sup> *Ldlr*<sup>−/−</sup> mice were housed at 22°C or 30°C and fed HFD for 12 weeks, Aortic arches were assayed for plaque deposition by oil red o staining. B) *Ucp1*<sup>−/−</sup> *Ldlr*<sup>−/−</sup> and *Ldlr*<sup>−/−</sup> mice were housed at indicated temperature and fed HFD for 20 weeks and assayed by en face aorta analysis for plaque area in aortic arch and descending aorta. C) *Ucp1*<sup>−/−</sup> or C57bl/6 mice were housed at indicated temperatures for 7 days and assayed for changes in Ly6C<sup>+</sup> and Ly6C<sup>−</sup> blood monocyte levels. D) Blood and E) bone marrow monocyte analysis of *Ucp1*<sup>−/−</sup> *Ldlr*<sup>−/−</sup> or *Ldlr*<sup>−/−</sup> mice housed at the indicated temperature and fed a HFD for 8 weeks, then assayed for monocyte levels. Data are representative experiments, n=5 mice per group, and repeated in one or two independent experiments. Statistical analysis was performed by unpaired student T-test, * p≤0.05, **p≤0.01
Online Figure VIII. *Ucp1<sup>−/−</sup> Ldlr<sup>−/−</sup> mice have reduced LDL levels following HFD compared to controls.*

A) *Ucp1<sup>−/−</sup> Ldlr<sup>−/−</sup>* and *Ldlr<sup>−/−</sup>* mice were housed at indicated temperatures and fed a HFD for 8 weeks, then assayed for food consumption.

B) Total cholesterol levels were measured at 0 and 4 weeks HFD.

C) After 8 weeks HFD, plasma from *Ucp1<sup>−/−</sup> Ldlr<sup>−/−</sup>* and *Ldlr<sup>−/−</sup>* mice was analyzed for lipoprotein profiles of total cholesterol, vLDL, LDL, and HDL levels.

D) Representative graph of cholesterol elution from *Ucp1<sup>−/−</sup> Ldlr<sup>−/−</sup>* and *Ldlr<sup>−/−</sup>* plasma samples. All data include n≥5 animals per group. Statistical analysis was performed by unpaired student T-test, *p<0.05, **p<0.01, ***p<0.001
Online Figure IX. β3-adrenoreceptor signaling during thermoneutrality modulates food intake and body temperature but not monocyte counts or atherosclerosis progression. A) Ldlr−/− mice were housed at the indicated ambient temperature and given twice-daily doses of β3-agonist CL or PBS for 4 weeks while on HFD. Animals were monitored for blood monocyte levels. B) Following 4 weeks of continuous treatment and HFD exposure, animals were assayed for plaque development in the aortic sinus, (C) Ucp1 expression D) core body temperature measured by rectal probe, and (E) body weight measurements (F, separating female and male weight data). Data are combined to include n≥8 animals per group and is representative of two independent experiments. Statistical analysis was performed by unpaired student T-test, * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Online Movie I

Online Movie I. Bone marrow imaging of CXCR1gfp/+ CXCL12dsred animals housed at different temperatures. Animals were housed at 22°C or 30°C for 4 weeks, then assayed by intravital bone marrow imaging for monocyte (gfp+) cell accumulation. Representative video shows dynamic behavior and organization within the bone marrow milieu.