Thermoneutral housing exacerbates nonalcoholic fatty liver disease in mice and allows for sex-independent disease modeling

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Nonalcoholic fatty liver disease (NAFLD), a common prelude to cirrhosis and hepatocellular carcinoma, is the most common chronic liver disease worldwide. Defining the molecular mechanisms underlying the pathogenesis of NAFLD has been hampered by a lack of animal models that closely recapitulate the severe end of the disease spectrum in humans, including bridging hepatic fibrosis. Here we demonstrate that a novel experimental model employing thermoneutral housing, as opposed to standard housing, resulted in lower stress-driven production of corticosterone, augmented mouse proinflammatory immune responses and markedly exacerbated high-fat diet (HFD)-induced NAFLD pathogenesis. Disease exacerbation at thermoneutrality was conserved across multiple mouse strains and was associated with augmented intestinal permeability, an altered microbiome and activation of inflammatory pathways that are associated with the disease in humans. Depletion of Gram-negative microbiota, hematopoietic cell deletion of Toll-like receptor 4 (TLR4) and inactivation of the IL-17 axis resulted in altered immune responsiveness and protection from thermoneutral-housing-driven NAFLD amplification. Finally, female mice, typically resistant to HFD-induced obesity and NAFLD, develop full disease characteristics at thermoneutrality. Thus, thermoneutral housing provides a sex-independent model of exacerbated NAFLD in mice and represents a novel approach for interrogation of the cellular and molecular mechanisms underlying disease pathogenesis.

NAFLD, a leading precursor of hepatocellular carcinoma (HCC) and liver transplantation1,2, encompasses a disease spectrum ranging from benign steatosis to nonalcoholic steatohepatitis (NASH) to cirrhosis3. Despite its clinical and public health importance, few effective therapies exist. Experimental and clinical evidence4 suggests a complex interplay of multiple biological processes in disease development, including obesity, dysbiosis of the intestinal microbiome5,6, heightened intestinal barrier permeability7, metabolic endotoxemia and various inflammatory processes8. Notably, HFD feeding, intestinal microbiome dysbiosis, augmented intestinal permeability, and metabolic endotoxemia and/or bacterial endotoxin (lipopolysaccharide; LPS) recognition in combination all contribute to activation of both innate and adaptive immune responses central to the pathogenesis of NAFLD9. TLR4 polymorphisms and elevated hepatic TLR4 expression have been associated with human NAFLD8,9. In addition to activating the innate immune system, TLR4 signaling also modulates multiple adaptive immune effector functions, including activation of the IL-17 axis10. Notably, IL-17 levels correlate with obesity and NAFLD progression in mouse models11, and the transition from steatosis to NASH in humans is associated with hepatic infiltration of IL-17-producing cells12. Inactivation of the IL-17 axis inhibits progression from steatosis to NASH in mouse models11. However, while existing

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mouse NAFLD models employing both genetic (leptin deficiency) and dietary (high-fat, high-carbohydrate and/or high-cholesterol diets) interventions have proven informative, a closer recapitulation of parameters relevant to human disease is still desired. Specifically, mouse NAFLD models are associated with a sex bias and limited progression to bridging hepatic fibrosis—characteristics not observed in human NAFLD. These limitations, and the overall lack of representative animal models for preclinical testing, may be a contributing factor in the paucity of therapeutic approaches for NAFLD.

The temperature at which mice are typically housed in research laboratories is associated with chronic cold stress that dramatically alters mouse physiology and immune responses. The thermoneutral zone (T_N), or the temperature of metabolic homeostasis, for Mus musculus is 30–32 °C. However, the standard temperature (T_S) range at which mice are usually housed is 20–23 °C, a range chosen primarily for human comfort. Housing mice under T_N, as opposed to T_S, conditions leads to remarkable physiological changes, including a heart rate increase of over 200 beats per minute, a 30% increase in mean arterial blood pressure, an overall increase in energy expenditure (50–60%) and sustained upregulation of catecholamine and corticosteroid production. In a variety of mouse models, alleviating cold stress through T_N housing alters immune function, including basal cytokine production, responses to bacterial and viral infection, and tumor immunity.

Further, mice housed at T_S fail to develop a fever after LPS challenge, whereas T_N housing promotes febrile responses. In the context of metabolic diseases, T_N housing is required to model obesity in nude mice, exacerbates adipose tissue inflammation and induces atherosclerosis in C57BL/6 wild-type (WT) mice. Importantly, atherosclerosis, the most common cause of mortality in patients with NAFLD, is a disease poorly modeled in WT mice.

The relevance of T_N housing to the modeling of human disease is emerging. The majority of people in developed nations, where obesity is classified as a disease, tend to spend most of their day within their thermoneutral zone via utility of climate control inside their dwellings. Further, exposure to nonthermoneutral conditions profoundly impacts both immune response and metabolic disease in humans. Specifically, exposure to sustained cold stress leads to dampened immune responsiveness to LPS challenge and improves glucose tolerance in type 2 diabetics. Thus, given its role in both metabolism and inflammation, we hypothesized that T_N housing would allow for development of an improved, exacerbated and more ‘human-like’ mouse model of NAFLD.

**RESULTS**

**T_N housing alters brown adipose tissue function and immune responsiveness**

Adaptation to cold stress involves, among other things, heightened brown adipose tissue (BAT) activity, energy expenditure and glucocorticoid release. In a mouse model of NAFLD, TN housing alters brown adipose tissue (BAT) function and immune responsiveness. In WT male C57BL/6 mice aged 6 weeks, T_S-housed mice fail to develop a fever after LPS challenge, whereas T_N-housed mice develop a marked inflammatory response. In the context of metabolic diseases, T_N housing is required to model obesity in nude mice, exacerbates adipose tissue inflammation and induces atherosclerosis in C57BL/6 wild-type (WT) mice.

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![Figure 1](image-url) Thermonutral housing relieves stress and augments inflammation. (a-f) WT male C57BL/6 mice aged 6 weeks were housed under either standard (T_S: 22 °C) or thermoneutral (T_N: 30 °C) conditions for a minimum of 3 weeks. (a) Expression of the indicated genes in BAT (n = 5 per group). (b) Serum corticosterone levels (n = 7 per group). (c) Expression of the indicated genes in the spleen (n = 3 per group). AU, arbitrary units. (d) Upregulated gene expression pathways in PBMCs (left) and genes within pathways (right), determined by RNA-seq analysis (P < 0.01, ****P < 0.0001). Student’s t-test (a-c,e,f), hypergeometric distribution with Bonferroni correction (d) or one-way ANOVA with post hoc Tukey’s test (g).
Thermoneutral housing exacerbates HFD-driven NAFLD pathogenesis. (a–o) WT male C57BL/6 mice aged 6 weeks were housed at T₅ (22 °C) or T₉ (30 °C) for 24 weeks and fed a chow diet or a HFD. (a) Weight gain. (b) Total body lean and fat mass. (c) Daily caloric intake. (d) Liver weight. (e) Hepatic triglyceride levels. In a–e, n = 8 per group. (f) Oil red O staining, percent area positive for lipid accumulation. (g) Representative liver morphology (n = 4 per group) by H&E staining at 20x and 40x magnification. Scale bars, 100 μm (left and middle) and 50 μm (right). (h) NAFLD activity score. (i) Expression of the indicated lipid mediator genes in the liver. (j) Expression of the indicated chemokine genes in the liver. (k) Percent hepatic CD11b+F4/80+ immune cells. (l) Colony-forming units (CFU) of aerobic bacteria cultured from liver homogenate. (m) Expression of the indicated fibrosis marker genes in the liver. In f–m, n = 4 per group. (n) Serum ALT levels (n = 8 per group). (o) Upregulated gene expression pathways (left) and genes within pathways (right), determined by RNA–seq analysis of livers from HFD-fed mice (n = 2 per group). (p) Predictability of human NAFLD based on hepatic gene expression.

Production of peripheral blood mononuclear cells (PBMCs) revealed that housing at T₅, in comparison to T₉, resulted in greater levels of gene expression in pathways known to negatively regulate immune responses, including pathways responsible for decreased inflammatory responses, increased susceptibility to infection and decreased acute inflammation (Fig. 1d and Supplementary Table 1a).

We next examined the functional relevance of the altered immune gene expression observed with differences in housing temperature for proinflammatory cytokine production. In vivo analysis revealed that T₅ in comparison to T₉ housed exacerbating systemic tumor necrosis factor (TNF) and IL-6 levels at baseline and after LPS challenge (Fig. 1e). These effects persisted ex vivo, as LPS stimulation of splenocytes and bone-marrow–derived dendritic cells (BMDCs) from T₉–housed, as compared to T₅–housed, mice resulted in heightened TNF and IL-6 production (Fig. 1f). Changes observed at T₉, in comparison to T₅, were found to be independent of alterations
in cellularity and composition of bone marrow, PBMCs, spleen and thymus (Supplementary Fig. 1a–d). These findings indicate that TN housing reverses the inhibition of immune responsiveness seen under standard housing conditions.

The anti-inflammatory effects of glucocorticoids and corticosterone are well established. We thus examined the relevance of the corticosterone axis to immune responsiveness at TN. Administration of corticosterone to TS-housed mice was sufficient to reverse the exacerbated proinflammatory cytokine production associated with TN, in comparison to TS housing (Fig. 1g), suggesting that the corticosterone axis is functional at TN and plays a role in the regulation of inflammatory vigor. Conserved effects were observed in humans following stimulation of human PBMCs with LPS in the presence of GR and β2AR agonists (data not shown). In sum, these results suggest that ambient temperature profoundly alters BAT function, glucocorticoid production and host innate immune responses in mice C57BL/6 mice and are congruent with reports indicating that TN housing promotes a more human-like immune response in mice.

TN housing exacerbates HFD-driven NAFLD pathogenesis

As the immune system plays a central role in the pathogenesis of obesity-associated sequelae, we hypothesized that TN, in comparison to TS, housing would exacerbate such effects in male WT C57BL/6 mice. TN housing in combination with HFD feeding initially accelerated weight gain in comparison to TS-housed HFD-fed mice; however, prolonged dietary exposure nullified differences in weight gain and body lean or fat mass (Fig. 2a,b). Similar weight gain occurred despite lower food intake at TN (Fig. 2c), likely owing to lower energy expenditure at TN (refs. 16,17). However, despite similar body weights and adiposity, at the time of harvest, TN-housed mice displayed heightened visceral adipose tissue immune cell infiltration and adipose tissue macrophage activation in comparison to TS-housed, HFD-fed mice (Supplementary Fig. 2a–c)—in agreement with a previous report.

Given the role of obesity and adipose tissue inflammation in glucose dysmetabolism, we next evaluated the impact of TN housing on glucose homeostasis. TN, in comparison to TS, housing exacerbated glucose intolerance only when differences in body weight existed (Supplementary Fig. 2d). These differences in glucose tolerance were diminished once body weights were normalized (Supplementary Fig. 2e). Further, with similar weight gains, TS- and TN-housed mice displayed similar insulin sensitivities (Supplementary Fig. 2f), which correlated with similar islet sizes and hepatic levels of AKT phosphorylation (data not shown). These data agree with previous reports describing the role of TN housing in the modulation of glucose metabolism and insulin resistance in obesity.

We next examined the impact of TN housing on NAFLD development and progression. Despite having similar serum triglyceride levels and serum and hepatic cholesterol levels (Supplementary Fig. 2g–i), obese mice housed at TN, in comparison to TS, had exacerbated liver

**Figure 3** Thermoneutral housing is associated with augmented intestinal permeability and dysbiosis of the microbiome. (a–c) WT male C57BL/6 mice aged 6 weeks were housed at TS (22 °C) or TN (30 °C) for 24 weeks and fed a chow diet or a HFD. (a) Flow of FITC-labeled dextran through the intestine (n = 4 per group). (b) Phylum-level differences between TS- and TN-housed mice after 24 weeks of HFD (n = 8 per group). (c, d) Relative phylum abundance (c) and principal-coordinate analysis (d) in mice housed at TS (n = 8 per group) or TN (n = 8 per group) and either healthy, lean humans (n = 16 per group) or patients with NASH (n = 22 per group) described previously. (e–h) WT male C57BL/6 mice aged 6 weeks were housed at TS (22 °C) or TN (30 °C) and fed a HFD. After 8 weeks of HFD, mice were mock treated (Ctrl) or treated with antibiotics (Tx) supplied in drinking water in addition to HFD exposure for an additional 16 weeks. (e) Flow of FITC-labeled dextran through the intestine of control chow- and HFD-fed mice at 22 °C and HFD-fed mice at 30 °C (n = 4 per group) or patients with NASH (n = 22 per group) described previously. (f) Representative liver histology (n = 4 per group) by H&E staining. Scale bars, 50 μm. (g) Inflammation score per NAFLD activity score criteria (n = 4 per group). (h) Serum ALT levels (n = 8 per group). For bar graphs, data represent mean ± s.e.m. For box plots, the midline represents the mean, boxes represent the interquartile range and whiskers show the full range of values. In a, a representative of two individual experiments is shown; in b–h, a single experiment was performed.

*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with post hoc Tukey’s test (a,e,g,h) or Mann–Whitney test (c).
weight and hepatic steatosis, as quantified by hepatic triglyceride levels, oil red O staining and NAFLD activity score (Fig. 2d–h). Histological analysis, used to determine the NAFLD activity score, suggested that hepatocytes from T N-housed, in comparison to T S-housed, obese mice exhibited elevated steatosis and hepatocyte ballooning, characterized by cellular swelling, rarefaction of the hepatocytic cytoplasm and clumped strands of intermediate filaments (Fig. 2g). These findings also correlated with reduced gene expression of key lipid mediators known to have decreased expression during NASH (Fig. 2i) 37,38.

We next examined the progression to and severity of NASH under T N housing. Modest changes in the expression of genes related to lipid handling, chemokine production and fibrosis were observed in mice fed a chow diet (Supplementary Fig. 3a–c). In the context of HFD feeding, however, T S-housed mice exhibited robust exacerbation of hepatic chemokine expression (Fig. 2j), macrophage infiltration of the liver (Fig. 2k) and bacterial translocation to the liver (Fig. 2l), in comparison to T S-housed mice. Further, T N-housed, in comparison to T S-housed, obese mice also displayed elevated expression of genes associated with induction of hepatic fibrosis (Fig. 2m) and a threefold induction in hepatocellular damage, as measured by serum alanine transaminase (ALT) levels (Fig. 2n). However, despite these robust changes, the induction of overt bridging hepatic fibrosis was not observed in WT C57BL/6 mice (Supplementary Fig. 3d).

To begin to examine the effect of T N versus T S housing on hepatic gene expression in the presence or absence of dietary modulation, we performed an unbiased approach using RNA-seq analysis of liver tissue. Although T N, in comparison to T S, housing altered hepatic gene expression in chow-fed mice, a HFD exacerbated differential gene expression (Supplementary Fig. 3e). In chow-fed mice, analysis of differential gene expression revealed alterations in liver metabolism, including elevated expression of gene pathways associated with lipid metabolism and fatty acid oxidation (Supplementary Fig. 3ef and Supplementary Table 1b). In contrast, analysis of differential gene expression in HFD-fed mice showed heightened expression of genes and gene pathways associated with collagen formation, apoptosis and HCC induction (Fig. 2o and Supplementary Table 1c). Further, comparison of the hepatic gene expression changes induced by T N versus T S housing in HFD-fed mice to known gene expression changes induced by NASH in humans 39 revealed that T N housing resulted in similar changes in the expression of genes associated with a variety of NAFLD-related pathways (e.g., inflammatory response, response to reactive oxygen species and leukocyte activation; Supplementary Fig. 3g).

Next, we determined whether changes in HFD-induced hepatic gene expression at T N or T S were more likely to predict the gene expression patterns induced by human NASH 39. Use of support vector machine analysis 40 determined that the gene expression differences induced by a HFD at T N allowed for improved prediction of the gene expression patterns of human NASH in comparison to T S housing and a HFD (Fig. 2p). These findings verify markedly exacerbated HFD-driven NAFLD pathogenesis and hepatocellular damage at T N and suggest an improved model for human disease.

Although most commonly used for obesity and NAFLD modeling, the C57BL/6 mouse strain is highly resistant to the induction of hepatic fibrosis. Thus, we next determined whether the lack of overt hepatic fibrosis observed in C57BL/6 mice was strain specific. Notably, the AKR mouse strain develops robust obesity and NAFLD when fed a HFD 41. When housed at T N, as opposed to T S, and fed a HFD, AKR mice gained more weight but maintained similar visceral and subcutaneous adiposity (Supplementary Fig. 4a,b). T N-housed, obese AKR mice exhibited greater liver weight, hepatic steatosis and NAFLD activity scores than T S-housed AKR controls (Supplementary Fig. 4c–f). The lower NAFLD activity scores observed in AKR mice as compared to C57BL/6 mice may be mouse strain dependent and/or due to shortened HFD exposure. The NAFLD activity score in AKR mice was associated with greater lobular inflammation and steatosis at T N, but limited hepatocyte ballooning was observed in both groups at this time. Augmented lobular inflammation correlated with higher hepatic chemokine gene expression, macrophage infiltration and hepatocellular damage (Supplementary Fig. 4g–i) in T N-housed, as compared to T S-housed, AKR mice. Notably, unlike in C57BL/6 mice, HFD feeding under conditions of T N but not T S housing was sufficient to induce fibrosis in AKR mice, as quantified by hepatic gene expression and Trichrome staining (Supplementary Fig. 4j,k). Together, these data indicate that the effects of T N housing on NAFLD pathogenesis are conserved across different mouse strains and suggest that the use of obese AKR mice at T N may provide a novel and accelerated model for mechanistic interrogation of NAFLD-induced hepatic fibrosis.

**Figure 4** Thermoneutral-housing-driven modulation of hematopoietic TLR4 signaling regulates NAFLD progression. (a) WT male C57BL/6 mice aged 6 weeks were housed under T S (22 °C) or T N (30 °C) conditions for 2 weeks before initiation of 8 weeks of HFD. Upregulated gene expression pathways (left) and genes within pathways (right) are shown, as determined by RNA-seq analysis (n = 2 per group). (b–d) Tlr4fl/fl; Vav1-Cre and control Tlr4fl/fl; Vav1-Cre mice on a C57BL/6 background were housed at T S (22 °C) or T N (30 °C) for 24 weeks and fed a chow diet or a HFD. (b) Representative liver histology by H&E staining at 40x magnification of Tlr4fl/fl; Vav1-Cre mice housed at 22 °C (n = 7 per group) and 30 °C (n = 5 per group) as compared to Tlr4fl/fl; Vav1-Cre mice housed at 22 °C (n = 4 per group) and 30 °C (n = 4 per group). Scale bars, 50 μm. (c) NAFLD activity score as determined by histology. (d) Serum ALT levels of Tlr4fl/fl; Vav1-Cre mice housed at 22 °C (n = 9 per group) and 30 °C (n = 8 per group) as compared to Tlr4fl/fl; Vav1-Cre mice housed at 22 °C (n = 7 per group) and 30 °C (n = 5 per group). Blue denotes T S (22 °C); red denotes T N (30 °C). For box plots, the midline represents the mean, boxes represent the interquartile range and whiskers show the full range of values. In a, a single experiment is shown; in c and d, a representative of two independent experiments is shown. *P < 0.05, **P < 0.01, ***P < 0.001, hypergeometric distribution with Bonferroni correction (a) or one-way ANOVA with post hoc Tukey’s test (c,d).
ALT levels. For box plots, the midline represents the mean, boxes represent the interquartile range and whiskers show the full range of values. In per group) mice, by H&E staining at 20× and 40× magnification. Scale bars, 100 μm (top) and 50 μm (bottom). (e) NALFD activity score. (f) Serum ALT levels. For box plots, the midline represents the mean, boxes represent the interquartile range and whiskers show the full range of values. In a, a single experiment is shown; in b–f, a representative of three individual experiments is shown. *P < 0.05, **P < 0.01, hypergeometric distribution with Bonferroni correction (a), Student’s t-test (b,c) or one-way ANOVA with post hoc Tukey’s test (e,f).

Figure 5 Thermonutral housing is associated with pathogenic upregulation of the IL-17 axis. (a) WT male C57BL/6 mice aged 6 weeks were housed under TS (22 °C) or T0 (30 °C) conditions for 2 weeks before initiation of 8 weeks of HFD feeding. The upregulated gene expression pathway (top) and genes within the pathway (bottom) are shown, as determined by RNA-seq analysis (n = 6 per group). (b,c) WT male C57BL/6 mice aged 6 weeks were housed at TS (22 °C; n = 3 per group) or T0 (30 °C; n = 4 per group) for 24 weeks and fed a HFD. Percentage of TCR-β*CD4*IL-17A* hepatic infiltrating immune cells. (c) Percentage of TCR-β*CD4*IL-17A*TNF* hepatic infiltrating immune cells. (d–f) WT (n = 5 per group), Il17ra−/− (n = 4 per group) and Il17ra−/− (n = 6 per group) male mice aged 6 weeks, all on the C57BL/6 background, were housed under T0 (30 °C) conditions for 2 weeks before initiation of 24 weeks of HFD feeding. (d) Representative liver histology of WT (n = 5 per group), Il17ra−/− (n = 4 per group) and Il17ra−/− (n = 6 per group) mice, by H&E staining at 20x and 40x magnification. Scale bars, 100 μm (top) and 50 μm (bottom). (e) NALFD activity score. (f) Serum ALT levels. For box plots, the midline represents the mean, boxes represent the interquartile range and whiskers show the full range of values. In a, a single experiment is shown; in b–f, a representative of three individual experiments is shown. *P < 0.05, **P < 0.01, hypergeometric distribution with Bonferroni correction (a), Student’s t-test (b,c) or one-way ANOVA with post hoc Tukey’s test (e,f).

The adrenal glands are a central production site for circulating corticosterone and catecholamines. Immune responsiveness under cold stress is, in part, modulated by corticosterone and catecholamine levels. Adrenalectomized male C57BL/6 mice fed a HFD exhibited similar weight gains and visceral and subcutaneous adiposity at the two housing temperatures (Supplementary Fig. 5a–d). Unlike C57BL/6 mice with intact adrenal glands, adrenalectomized C57BL/6 mice housed at TS and T0 exhibited similar liver weights, hepatic steatosis, lipid mediators and chemokine gene expression, macrophage infiltration, expression of genes associated with induction of fibrosis and hepatocellular damage (Supplementary Fig. 5e–l). Notably, the lack of adrenal glands in mice with TS housing in combination with a HFD exacerbated hepatocellular damage as compared to nonadrenalectomized mice at TS and resulted in levels of damage similar to those observed in TS-housed mice (Supplementary Fig. 5m). These data suggest that mediators produced by the adrenal glands play an important role in suppressing NAFLD progression during cold stress.

Intestinal permeability and microbiome in TN-driven NAFLD

Augmented intestinal permeability7 and dysbiosis of the intestinal microbiome6 contribute to human and mouse NAFLD progression4–6. Although histological analysis of the small intestine did not reveal differences due to housing temperature or diet in immune cell infiltration (Supplementary Fig. 6a), TS, as compared to TS, housing exacerbated paracellular intestinal permeability, as evidenced by translocation of FITC-dextran across the epithelium, and lowered transepithelial resistance on both a chow diet and a HFD (Fig. 3a and Supplementary Fig. 6b). Further, TS, as compared to TS, housing changed the intestinal microbiome in mice in as little as 2 weeks, before the mice were placed on a HFD (Supplementary Fig. 6c,d). Extended exposure (12 weeks) of randomly separated WT C57BL/6 mice to different ambient temperatures or a HFD further exacerbated these changes, with obvious differences observed in every phylum analyzed after 24 weeks of exposure (Fig. 3b and Supplementary Fig. 6e–k). Notably, TS housing enriched the representation of Bacteroidetes, whereas TS housing preferentially enriched for Firmicutes. Linear discriminant analysis effect size (LEfSe) analysis, which was employed to analyze data with specificity to the genus level, revealed congruent differences beyond the phyl level (Supplementary Fig. 7).

Although the microbiome plays a role in NAFLD in both humans and mice, the two species display an inherently different microbial composition. Nevertheless, we examined whether the mouse microbiome at TS correlated more closely with microbiomes reported in humans with NASH6. Comparison of 16S rRNA sequences demonstrated that TS housing of mice on a HFD led to greater similarity of the gut microbiome to that observed in people with NASH6. Notably, this was seen in both phylum-level alterations (Fig. 3e) and principal-component analysis (Fig. 3d), where an upward shift (PC2) corresponded with a more NASH-like metagenome.

TS housing in combination with stress from a HFD promoted expansion of Gram-negative Bacteriodetes (Fig. 3b). Antibiotic-mediated depletion of the Gram-negative microbiome in HFD-fed mice housed at TS and TS did not alter total body weight gain, visceral and subcutaneous adiposity, hepatic weight or hepatic triglyceride accumulation, although TS-housed, antibiotic-treated mice displayed elevated glucose intolerance (Supplementary Fig. 8a–g). However, antibiotic treatment obviated the greater intestinal permeability observed in HFD-fed mice housed at TS, as compared to mice housed at TS (Fig. 3e), and lowered the NALFD activity inflammation score (Fig. 3f,g) and protected against hepatocellular damage (Fig. 3h) only at TS. Of note, these protective effects in NAFLD were specific to obese TS-housed mice and were not observed in obese, antibiotic-treated TS-housed mice (Fig. 3f–h and Supplementary Fig. 8g). These data indicate that changes in intestinal microbiome composition are associated with TN-driven amplification of NAFLD.

Hematopoietic TLR4 and IL-17 axis in TN-driven NAFLD

LPS from Gram-negative bacteria activates TLR4. Our data indicate that TLR4 responsiveness is elevated at thermoneutrality (Fig. 1e,f). Additionally, TLR4 polymorphisms and hepatic TLR4 expression have been correlated with the progression of human NAFLD9. Whether TN-driven modulation of innate immune responses is maintained on a HFD has not been examined. RNA–seq analysis of PBMCs from WT C57BL/6 mice challenged with a HFD revealed greater levels of gene expression in pathways associated with cytokine production and TLR responsiveness (Fig. 4a and Supplementary Table 1d). To determine the functional relevance of differential TLR4 expression in immune
cells, \( Tlr4^{-/-} \) mice were used. Congruent with a previous report\(^{42}\), Vav1-Cre-driven hematopoietic cell deletion of \( Tlr4 \) in \( T_{30°C} \) housed mice did not hinder the progression of HFD-induced NAFLD (Fig. 4b–d). However, under \( T_{30°C} \) housing, such deletion was sufficient to protect from HFD-driven increases in weight gain, visceral and subcutaneous adiposity, glucose intolerance, liver weight, histologically identifiable steatosis, hepatocyte ballooning, and lobular inflammation and hepatic cellular damage (Fig. 4b–d and Supplementary Fig. 9). These data indicate that TLR4 signaling is fundamentally modulated by ambient temperature in the context of HFD feeding and that removing suppression of TLR4 signaling through \( T_{30°C} \) housing can contribute to NAFLD pathogenesis.

Induction of IL-6, IL-1β and IL-23 production is associated with T helper type 17 (Th17) cell polarization and IL-17 axis activation\(^{10}\). Hepatic IL-6 expression is elevated in human NAFLD, and serum concentrations of IL-1β are higher in individuals with metabolic syndrome\(^{33,44}\). RNA–seq analysis of PBMCs from WT C57BL/6 mice provided initial suggestions that \( T_{30°C} \) as compared to \( T_{22°C} \) housing coupled with a HFD resulted in greater expression of genes related to IL-17 production (Fig. 5a and Supplementary Table 1d). We next examined
whether T\textsubscript{N} housing altered TLR4-signaling-driven induction of mediators associated with activation of the IL-17 axis. LPS stimulation of BMDCs from T\textsubscript{N}-housed, as compared to T\textsubscript{S}-housed, obese mice resulted in greater IL-6 and IL-1\beta production and greater IL23a gene expression (Supplementary Fig. 10a–c). Elevated production of these mediators correlated with exacerbated hepatic infiltration of CD4\textsuperscript{+} T cells capable of both single IL-17 and dual IL-17 and TNF production in T\textsubscript{N}-housed, obese mice (Fig. 5b,c). Notably, dual IL-17- and TNF-producing CD4\textsuperscript{+} T cells exacerbate pathogenesis in mouse experimental autoimmune encephalomyelitis models\textsuperscript{45} and have been associated with higher severity of Crohn's disease in humans\textsuperscript{45,46}. Hence, we examined the contribution of IL-17 axis activation to T\textsubscript{N}-driven NAFLD pathogenesis. Despite similar weight gains, visceral and subcutaneous adiposity, hepatic steatosis and NAFLD activity scores (Fig. 5d,e and Supplementary Fig. 10d–h), T\textsubscript{N}-housed, obese IL-17 axis–deficient mice (Il17r\textsuperscript{−/−} and Il17a\textsuperscript{−/−}) were protected from glucose intolerance, exacerbated liver weight and hepatocellular damage, as compared to T\textsubscript{N}-housed WT controls (Fig. 5f and Supplementary Fig. 10i,j). These findings suggest that the IL-17 axis is a relevant factor in the regulation of NAFLD pathogenesis and that, unlike TLR4 signaling, a role for the IL-17 axis in NAFLD is conserved across housing temperatures.

Serum amyloid A (SAA), an acute-phase protein largely produced in the liver, is known to both activate TLR4 and promote T\textsubscript{H}17 cell differentiation via antigen-presenting cells\textsuperscript{47}. T\textsubscript{N}-housed, obese mice exhibited greater hepatic SAA1 and SAA2 expression in comparison to T\textsubscript{S}-housed mice (Supplementary Fig. 11a), whereas deletion of Il17ra, Il17a or hematopoietic Tbr4 was associated with lower hepatic expression of SAA1 (Supplementary Fig. 11b,c). These data suggest that T\textsubscript{N}-mediated modulation of SAA production might represent a link between activation of the TLR4 and IL-17 axes.

T\textsubscript{N} housing allows for disease modeling in female mice

Human NAFLD prevalence is similar in men and women\textsuperscript{48}. However, protection from severe HFD-induced obesity and NAFLD in C57BL/6 female mice precludes the interrogation of disease pathogenesis in a sex-independent manner. Hence, we asked whether T\textsubscript{N} housing would allow for the induction of obesity and NAFLD in female C57BL/6 mice. As expected, T\textsubscript{S}-housed female mice fed a HFD, as compared to chow, displayed mild weight gain over time (Fig. 6a). However, T\textsubscript{N}-housed female mice fed a HFD, as compared to chow, exhibited robust obesity and total body adiposity (Fig. 6a), with these increased to the levels observed in T\textsubscript{N}-housed male mice (Fig. 2). Further, in agreement with data from male mice, under conditions of robust weight gain, obese, T\textsubscript{N}-housed female mice displayed exacerbated glucose intolerance (Supplementary Fig. 12a).

Similarly, T\textsubscript{N}-housed, obese female mice exhibited greater liver weight, hepatic steatosis, hepatic chemokine expression\textsuperscript{3}, macrophage infiltration and hepatic infiltration of both IL-17-producing and dual IL-17- and TNF-producing T\textsubscript{H}17 cells, as compared to T\textsubscript{S}-housed, HFD-fed mice (Fig. 6c–i). Further, as in WT male C57BL/6 mice, these physiological changes in the liver of obese T\textsubscript{N}-housed, as compared to T\textsubscript{S}-housed, female mice correlated with greater expression of fibrosis-associated genes (Actn2, Col1a1 and Col2a1) and a greater degree of hepatocellular damage (Fig. 6j,k) but did not induce overt bridging hepatic fibrosis (Supplementary Fig. 12b). Notably, T\textsubscript{N}-housed, as compared to T\textsubscript{S}-housed, obese WT female mice exhibited greater Saa1 expression (Supplementary Fig. 12c). Thus, T\textsubscript{N} housing allows for development of robust obesity and NAFLD in WT female C57BL/6 mice and, notably, removes the sex bias typically associated with experimental modeling of mouse obesity and NAFLD. In sum, these findings demonstrate that T\textsubscript{N}-housed-driven NAFLD pathogenesis represents a novel disease model associated with altered corticosterone levels, immune responses, metabolism, intestinal barrier integrity and intestinal microbiome (Fig. 6l).

DISCUSSION

NAFLD research often involves the use of mouse models housed at a suboptimal, cold-stress-inducing ambient temperature (typically 22 °C) that fails to comprehensively recapitulate human disease. In the case of NAFLD, current models in mice include a fundamental sex bias, unlike in humans; an inability to study the interplay between atherosclerosis and NAFLD; limited induction of pathways associated with the development of hepatic fibrosis and HCC; and altered immune responsiveness as compared to humans with this condition. Although previous reports have demonstrated that housing mice at a thermoneutral temperature (30 °C) affects the pathogenesis of multiple infectious and metabolic complications, whether T\textsubscript{N} housing allows for an improved mouse NAFLD model has not previously been interrogated.

Here we demonstrate, in accordance with previous reports, that the cold stress associated with standard mouse housing procedures inhibits immune responses\textsuperscript{14}. Housing mice at T\textsubscript{N} upregulated immune responsiveness both in vivo and ex vivo, and this upregulation was suppressed by exogenous administration of corticosterone. Further, T\textsubscript{N} housing exacerbates cellular responsiveness to inflammatory ligands, without alteration of the cell type composition or cell numbers. Additional studies, however, are required to functionally examine the pathways central to altered immune cell responsiveness at T\textsubscript{N}. As such changes are sustained ex vivo, T\textsubscript{N}-driven modulation of cellular metabolism and epigenetics are likely to play a role and warrant further investigation.

Robust exacerbation of NAFLD pathogenesis in the context of T\textsubscript{N} housing and HFD-induced stress correlated with heightened hepatic steatosis, hepatic immune cell infiltration, elevated expression of genes associated with hepatic fibrosis and HCC, and hepatocellular damage. Therefore, T\textsubscript{N}-driven amplification of disease-propagating processes may allow for in-depth interrogation and improved definition of the mechanisms central to NAFLD pathogenesis, including critical sites of inflammation, cell type(s) and immune mediators, using a genetically unbiased approach. However, despite robust disease exacerbation, hepatic fibrosis was not observed in male or female WT C57BL/6 mice. Thus, additional examination of hepatic fibrosis development in C57BL/6/mice, using T\textsubscript{N} housing in combination with dietary challenges known to promote fibrosis (for example, methionine-choline-deficient diets, high-fat plus high-cholesterol diets and high-fat plus high-fructose diets)\textsuperscript{49}, might offer a clear advantage over existing experimental NAFLD models for evaluating novel therapeutics. Notably, the induction of measureable hepatic fibrosis by a HFD at T\textsubscript{N} is possible, as demonstrated in AKR mice. Employing these mice in future studies may provide a novel model for mechanistic interrogation of NAFLD-induced hepatic fibrosis. Lastly, T\textsubscript{N} housing appears to hold relevance to human disease and improve upon existing NAFLD models, even in C57BL/6 mice, as hepatic gene expression in this model provides improved prediction of human NASH.

Interactions between the microbiome and the immune system are thought to play a key role in NAFLD. Augmented intestinal permeability also correlates with NASH severity\textsuperscript{2}. Notably, T\textsubscript{N} housing and a HFD resulted in heightened intestinal permeability and intestinal microbiome dysbiosis, which mirrors the obesity- and NASH-driven...
Intestinal microbiome changes are certainly one of the many factors in the causal nexus that allow pronounced expression of the NAFLD phenotype in mice housed at thermoneutrality. However, given the consistency in the impact of TN housing on the modulation of immune responses and metabolism among multiple research institutions, it is unlikely that a unique ‘microbiome mix’, specific to a single institution, is driving observed differences independently of temperature effects. Notably, our data evoke several interesting questions and areas of future study: what are the bidirectional interactions between altered microbiome and altered immune responsiveness under TN housing? Does TN housing alter the immune response in gnotobiotic mice? Can protection from HFD-driven obesity in germ-free mice be reversed using TN housing? Are levels of specific microbial byproducts or microbial species altered at TN (refs. 11, 51)?

Our data suggest that TLR4 signaling, presumably via Gram-negative microbiome dysbiosis, plays a role in TN-driven NAFLD pathogenesis. Additionally, we demonstrate that this pathogenic role for Vav1-driven deletion of TLR4 expression is important only when mice are housed at TN. Of note, Vav1-Cre can be activated in different cells, and the necessary hematopoietic and/or endothelial cell type should be evaluated. Also, whether the role of TLR4 is dependent on LPS or induction of various endogenous TLR4 ligands (e.g., high-mobility-group box 1 protein (HMGB1), fibronectin, fibrinogen and resistin) that have been associated with NAFLD progression is unknown.

Our findings also highlight the functional effector relevance of the IL-17 axis in TN-housing-driven NAFLD. The cellular and molecular mechanisms underlying the IL-17-mediated effects in NAFLD are not well defined. IL-17-driven induction of chemokines (e.g., chemokine (C–X–C motif) ligand 1 (CXCL1) and C–C motif chemokine ligand 2 (CCL2)) is associated with both macrophage and neutrophil hepatic infiltration and activation. Further, it has been demonstrated that IL-17 plays an important role in liver fibrosis. Notably, hepatic expression of both Cxcl1 and Ccl2 and markers of fibrosis is elevated in livers from TN-housed, HFD-fed mice. Thus, definition of the critical IL-17 axis-expressing cell type(s) within the liver (e.g., hepatocytes, stellate cells, Kupffer cells, etc.) deserves examination. Similarly, definition of the critical IL-17-producing cell type(s) requires additional characterization. Notably, while CD4+ T cells are primary IL-17 producers, a variety of other cells, including CD8+ T cells and innate lymphoid cells, have been shown to produce IL-17 within the liver.

Corticosterone levels are higher in mice housed at TN. Our findings demonstrated that exogenous administration of corticosterone to TN-housed mice is sufficient to prevent augmented immune responsiveness. In contrast, adrenalectomy removes the inhibition of NAFLD pathogenesis associated with TN, as compared to TN, housing. These data suggest that specific signaling mediators released via the adrenal glands may play a suppressive role in NAFLD pathogenesis. Of note, chemical inhibition of β-adrenergic receptor signaling exacerbates NAFLD progression in mice. However, β-adrenergic receptors also have a homeostatic role in intestinal, liver and immune cells. Thus, future studies employing the use of β2,AR, β3,AR and/or GR cell-type-specific knockout are required to better define contribution of these pathways to NAFLD pathogenesis.

Human obesity and NAFLD are equally represented in males and females. The ability of TN housing to promote the development of robust obesity and NAFLD in female mice further supports the relevance of TN housing to human physiology and allows for the realization of novel pathways associated with protection from HFD-driven obesity in female mice. Notably, TN housing may allow for improved modeling of the contribution of maternal obesity to disease pathogenesis over generations of offspring. In addition, atherosclerosis, the most common cause of mortality in patients with NAFLD, is another metabolic disease that lacks an appropriate mouse model as current models are similarly sex restrained. Housing WT mice at TN in combination with a ‘Western’ diet allows for atherosclerosis induction in male C57BL/6 mice and, hence, might provide for interrogation of the pathways critical for the interplay of these two diseases, as well as the removal of sex bias in modeling atherosclerosis.

Thus, housing temperature is an overlooked and very important variable in the modeling of human disease. Close attention to this variable has promise for the discovery of novel mechanisms underlying disease pathogenesis and for improved modeling of the noncommunicable metabolic diseases that are causing an increasing burden of morbidity and mortality worldwide.

METHODS

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

ACKNOWLEDGMENTS

This work was supported in part by NIH R01DK099222 and R01DK099222-S1 (to S.D.), the CCHMC Pediatric Diabetes and Obesity Center initiative (to S.D.), R01DK033201 (to C.R.K.), K23-HD00880 (to S.S.), NIH T32AI118697 (associated with D.A.G.), NIEHS Grant P03 ES006096 University of Cincinnati Center for Environmental Genomics (associated with D.A.G.), PHS Grant P30 DK078392 Pathology of the Digestive Disease Research Core Center at CCHMC (associated with S.D.) and German Research Foundation IRTG 1911 (projects A6 and B8 to C.S. and J.R.). We would also like to acknowledge C. Chougnet (CCHMC) for providing access to human PBMC samples and C. Woods (CCHMC) for technical assistance.

AUTHOR CONTRIBUTIONS

D.A.G., M.E.M.-E., T.E.S., G.S., M.C., D.W., R.M., C.C.C., J.M.L., J.K., S.S., A.S. and D.R. participated in data generation. D.A.G., M.E.M.-E., S.G., D.R., R.K., J.B.A., S.K.S., R.S., K.A.S., D.B.H., J.R., S.P.H. and S.D. participated in data analysis and interpretation. S.S., K.S., Y.L., C.R.K., J.B.A., C.S. and C.L.K. provided materials and technical support and participated in critical review of the manuscript. D.A.G., S.S., C.R.K., J.R., C.S. and S.D. obtained the funding. D.A.G. and S.D. participated in the conception and design of the study, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. With the exception of AKR mice (Jackson), all male and female mice used were on a C57BL/6 background. WT, Thr630 (ref. 60), I317a−/− (Amgen)11 and I317a+/− (ref. 61) mice were bred at Cincinnati Children’s Hospital Medical Center (CCHMC) in a specific-pathogen-free (spf) facility maintained at 22 °C with free access to autoclaved chow (LAB Diet #5010; calories provided by carbohydrates (58%), fat (13%) and protein (29%)) and water. Adrenalectomized mice aged 6 weeks on a C57BL/6 background were obtained from Jackson. For experimental purposes, mice were randomly allocated to groups and housed in separate spf units contained within the same animal barrier but maintained at either 22 °C or 30–33 °C. All care was provided in accordance with the Guide for the Care and Use of Laboratory Animals. All studies were approved by the CCHMC IACUC. Sample size was determined by employing power size calculations based on the results of preliminary data. Blinding was only used for histological scoring of samples.

Corticosterone levels. Serum corticosterone levels were detected by ELISA according to the manufacturer’s instructions (Arbor Assays).

qRT–PCR. Tissue samples were homogenized in TRIzol (Invitrogen), and RNA was then extracted, reverse transcribed to cDNA (Verso cDNA Synthesis Kit, Thermo Scientific) and subjected to qPCR analysis (Light Cycler 480 II, Roche); all steps were performed according to the manufacturer’s instructions as previously described23. The following primer pairs were used: Nr3c1 Forward (For) CCATAATGGCAGACCCAGC Reverse (Rev) AGGCCTGCTACCTCCCAGAAG; Ppara Forward (For) CTAGGATGCATGACAGCTAGCTG; Rev ATTTTGGCAACTTCTGGTGC; Ucp1 For TCAGCTGTCCTAAGCAACAC Rev GTACAAACCGTTGGCAGTG; Ucp2 For TCTGGTACTCCCTCCAAAGGA Rev GTGAGACCTCCTAAGAAGCATT; Sreb1fc For CTGCTTACCACCCGAGATAG Rev GATGGTGACCTCAGGACACAG; Ppara For CATGGGGAGAGAGGACAGA Rev AGTTCGGGAAC

Cytokine production. Cytokines were detected employing biotinylated capture antibodies, detection antibodies and recombinant protein mouse standards. For in vivo cytokine capture assays were employed as previously described13,64,65. Briefly, cytokines were detected using IVCCA employing biotinylated capture antibodies (IL-6 (MPS-32C11), TNF (TN3-19)) detection antibodies and recombinant protein mouse standards, all from eBioscience. Biotinylated capture antibodies were injected via the tail vein or intraperitoneally, and terminal serum collection was performed 24 h later. For in vitro production, ELISAs were employed as previously described11,64. For mice, cytokine levels of TNF and IL-6 were determined according to the manufacturer’s protocol (BD OptEIA).

Cell culture and in vitro cytokine production. Mouse BMDCs were differentiated as previously described15. Mouse splenocytes and BMDCs were stimulated with ultrapure LPS (100 ng/ml; Invivogen) for 4 h or with 1 µM norepinephrine or dexamethasone as noted.

Cytokine levels were determined through two-way ANOVA with an FDR-corrected P-value cutoff of 0.05 and a fold change requirement of >2. For pathway analysis, the database at http://toppgene.cchmc.org/ was employed, which amasses ontological data from over 30 individual repositories63.

We performed candidate gene prioritization on differentially regulated genes between T55 and T56 animals on a HFD. Using the Topgene Suite Candidate Prioritization tool, we ranked genes based on their functional similarity to NASH-related genes (extracted from the NASH-profiler; healthy obese versus NASH genes identified by an FDR-corrected P value of < 0.05 (two-way ANOVA) and fold change >2). The top 20 ranked genes were submitted to ToppCluster and Cytoscape for ontological assessment and visualization (data depicted in Supplementary Fig. 3g).

Further, using a human NASH cohort, we assessed the ability of genes differentially regulated under HFD conditions as compared to chow in T55 and T56 housing conditions to discriminate between healthy obese controls and individuals with NASH. Using support vector machines (SVMs), we quantified the discriminative capabilities of the following gene sets: (1) genes differentially regulated between HFD and chow diets in T55; (2) genes differentially regulated between HFD and chow diets in T56; and (3) differentially regulated genes between healthy obese controls and NASH identified through the NASH-profiler67. Model accuracy was compared between the three gene sets (data depicted in Fig. 2p).

Obesity and the NAFLD model. At 6 weeks of age, mice were randomly separated into T55 or T56 housing facilities. After 2 weeks of acclimation, mice were fed either an irrigated HFD (Research Diets D12492; 60% of calories from fat) or a chow diet. All food was replaced weekly to avoid contamination. All mice were fasted overnight before glucose metabolism testing, insulin tolerance testing or terminal harvest (completed from 7–10 a.m.). Glucose and insulin tolerance tests were done as previously described11. Briefly, following an overnight fast, glucose tolerance levels were determined by injecting mice with 10 µl of a 10% dextrose solution per gram of bodyweight, and glucose levels were quantitated kinetically at the times shown. For insulin resistance testing, mice were given 10 µl of a 0.15 U/ml solution of insulin (Novolin) per gram of bodyweight. Hepatic triacylglyceride deposition and serum alanine transaminase levels were quantified as previously described11. NAFLD activity score (NAS) was determined from H&E staining by a certified pathologist according to standard practice36. Total body fat and lean and water mass were determined by nuclear magnetic resonance (Whole Body Composition Analyzer, Echo MRI)67.

Histology. Oil red O staining was performed on 5-µm flash-frozen tissue sections. Masson’s Trichrome and H&E staining were performed on paraffin-embedded tissue blocks. Fibrosis quantification employed the use of an Aperio AT2 Slide Scanner, ImageScope (v. 12.3.1.5011), Color Deconvolution (v9) and Positive Pixel Count (v9, all Leica). Color deconvolution was used to identify the value of the positive stain. This value (0.622712) was subsequently identified in the slides using positive pixel count and percentage positive area, which took into account the total number of pixels identified, was quantified.
Flow cytometry. Single-cell suspensions were derived from hepatic homogenate. Staining with directly conjugated monoclonal antibodies or isotype controls. Staining for cytokine expression was completed after 4 h of stimulation with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Promega), 1 µg/ml ionomycin (Calbiochem) and 10 µg/ml brefeldin A (Gold Bio). Data collection and analysis were done as previously described. Briefly, cells were stained with Live/Dead stain (Zombie UV Dye, Biolegend) and with directly conjugated monoclonal antibodies CD45-AF700 (104), CD11b-PE (M1/70), F4/80-APC-e780 (BM8), Ly6C-Perp (HK1.2), Gr1-FITC (RB6-8C5), NK1.1-PECy7 (PK136), TCR-β-PE (H57-597), CD4-APC-e780 (GK1.5), CD8-e450 (53-67), TNF-BV650 (MP6-XT22), IL-17A-Perp (17B7) and IL-17F-PE (18F10) (all antibodies from eBioscience). Flow cytometry data were then collected using an LSR Fortessa (BD) flow cytometer and analyzed using FlowJo X software (vX0.7).

Bacterial translocation. Liver tissue was homogenized in enriched thioglycollate medium and subsequently cultured on TSA II/5% sheep blood plates (BD Bioscience).

Intestinal permeability. 1 cm of freshly isolated jejenum was mounted in a U2500 dual Ussing chamber (Warner Instruments). Transepithelial resistance and FITC-dextran flux were determined as previously described.

Microbiome analysis. Bacterial DNA was isolated from fecal material when mice arrived at the facility (−2 weeks), before they were placed on a certain diet (0 weeks), and at 12 and 24 weeks after either diet was introduced. Partial 16S rRNA gene sequences were amplified, targeting the hypervariable regions v1/v2, using primers 27F (AGAGTTTGATCCTGCGTCA) and 338r (TGCTGCTCCCTCCGTTAGGAT). Equimolar amounts of all samples were subjected to sequencing using a MiSeq sequencer from Illumina. Data were then processed using mothur software to determine phylotypes and operational taxonomic units (OTUs) and subjected to statistical analysis. LEfSe analysis was performed using the online tool at https://huttenhower.sph.harvard.edu/galaxy/ (ref. 70). For comparison to the human microbiome, individual reads were assigned to taxonomies using the QIIME package. Similarly, raw human 16S sequencing data published by Zhu et al. were downloaded from http://metagenomics.anl.gov/mgmain.html?mgpage=project&project=mgp1195 and analyzed using the same QIIME assignment method and analytic pipeline as were used for the mouse 16S read data. Taxonomic data were first analyzed using Mother software to determine significantly different OTUs. Similarly, GraphPad Prism (ver 6.07) was used to plot relative species abundance and compare groups. The Mann–Whitney test was applied to the data to determine the statistical significance of observed differences. Between-class principal-component analysis was performed using the ade4 package (ver 5.12) in R; this software is available upon request from the corresponding author. Mouse microbiome raw 16S rDNA data can be accessed at http://metagenomics.anl.gov/mgmain.html?mgpage=project&project=mgp183119.

Microbiome manipulation. For antibiotic-mediated depletion of Gram-negative bacteria, a cocktail of neomycin and polymyxin B sulfate (0.5 and 0.125 mg/ml, respectively) was added to the drinking water after 8 weeks of HFD feeding.

Statistical analysis. Sample sizes were determined based on preliminary data, which with respect to obesity and NAFLD modeling included weight gain, hepatic triglyceride deposition, immune cell infiltration and hepatocellular damage. Statistical tests were employed for all data sets with similar variance. The test used was dependent on both the number of groups being compared and whether the data were normally distributed. For normally distributed data, Student’s t-test was used when the comparison was two groups, while one-way ANOVA was employed for three or more groups, with Tukey’s post hoc test to assess differences between specific groups. For non-parametric data sets, the Mann–Whitney test was employed. Statistical analysis was completed using Prism 5a (GraphPad Software, Inc.). All values are represented as means ± s.e.m. No animals were excluded from analysis. Studies were not blinded unless otherwise noted above.

Data availability. Raw data can be accessed at the Gene Expression Omnibus (GEO) under accession GSE80976.
Erratum: Thermoneutral housing exacerbates nonalcoholic fatty liver disease in mice and allows for sex-independent disease modeling

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Nat. Med.; doi:10.1038/nm.4346; corrected online 21 June 2017

In the version of this article initially published online, a grant supporting the authors’ work was omitted from the Acknowledgments section. The grant “NIH T32AI118697 (associated with D.A.G.)” has now been added. The error has been corrected in the print, PDF and HTML versions of this article.