Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice

K Stemmer, P Kotzbeck, F Zani, M Bauer, C Neff, T D Müller, P T Pfluger, R J Seeley, S Divanovic

Cite this article as: K Stemmer, P Kotzbeck, F Zani, M Bauer, C Neff, T D Müller, P T Pfluger, R J Seeley, S Divanovic, Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice, *International Journal of Obesity* accepted article preview 28 October 2014; doi: 10.1038/ijo.2014.187.

Received 29 April 2014; accepted 14 October 2014; Accepted article preview online 28 October 2014

Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice

[1,2*]Kerstin Stemmer, [1]Petra Kotzbeck, [1]Fabio Zani, [1]Michaela Bauer, [1]Christina Neff, [3]Timo D. Müller, [4]Paul T. Pfluger, [2,5]Randy J. Seeley, [6]Senad Divanovic

[1] Division of Metabolism and Cancer, Institute for Diabetes and Obesity, Helmholtz Centre Munich, Neuherberg, Germany

[2]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, and the University of Cincinnati College of Medicine, Cincinnati, OH, USA

[3] Division of Molecular Pharmacology, Institute for Diabetes and Obesity, Helmholtz Centre Munich, Neuherberg, Germany

[4]Division of Nutrition Biology, Institute for Diabetes and Obesity, Helmholtz Centre Munich, Neuherberg, Germany

[5]Department of Surgery, University of Michigan, Ann Arbor, MI, USA.

[6]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, and the University of Cincinnati College of Medicine, Cincinnati, OH, USA

[*] Corresponding Author

Address all correspondence and requests for reprints to:

Kerstin Stemmer, Institute for Diabetes and Obesity, Helmholtz Centre Munich, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany.

Email: kerstin.stemmer@helmholtz-muenchen.de

Phone: +49-89-3187-2109, Fax: +49-89-3187-2182

Running Head: Diet induced obesity in nude mice

Conflict of interest: The authors declare no conflict of interest.

ABSTRACT

Objectives: Obesity-related cancers represent public health burdens of the first order. Nevertheless, suitable mouse models to unravel molecular mechanisms linking obesity to human cancer are still not available. One translational model is the immunocompromised Foxn1 (winged-helix/forkead transcription factor) nude mouse transplanted with human tumor xenografts. However, most xenograft studies are conducted in nude mice on an in-bred BALB/c background that entails protection from diet-induced obesity. To overcome such resistance to obesity and its sequelae, we here propose the dual strategy of utilizing Foxn1 nude mice on a C57BL/6 background and housing them at their thermoneutral zone.

Methods: C57BL/6 nude and corresponding wild type mice, housed at 23°C or 33°C, were subjected to either low fat diet or high fat diet. Energy expenditure, locomotor activity, body core temperature, respiratory quotient as well as food and water intake were analyzed using indirect calorimetry. Immune function at different housing temperatures was assessed by using an *in vivo* cytokine capture assay.

Results: Our data clearly demonstrate that conventional housing protects C57BL/6 nude mice from high fat diet (HFD)-induced obesity, potentially via increased energy expenditure. In contrast, HFD-fed C57BL/6 nude mice housed at thermoneutral conditions develop adiposity, increased hepatic triglyceride accumulation, adipose tissue inflammation, and glucose intolerance. Moreover, increased circulating levels of lipopolysaccharide (LPS)-driven cytokines suggest a greatly enhanced immune response in C57BL/6 nude mice housed at thermoneutrality.

Conclusion: Our data reveals mild cold stress as a major modulator for energy and body weight homeostasis as well as immune function in C57BL/6 nude mice. Adjusting housing temperatures to the thermoneutral zone may ultimately be key to

successfully study growth and progression of human tumors in a diet-induced obese environment.

Keywords: Nude mice, metabolic phenotyping, obesity, cold stress, inflammation

INTRODUCTION

Obesity is one of the most prevalent chronic disorders worldwide and the second most preventable cause of death in developed countries [1]. It is characterized by lipid accumulation in both adipose and non-adipose tissues and accompanied by a variety of comorbidities such as insulin resistance and cardiovascular disease [2]. Recent epidemiological studies have linked obesity with an increased risk of developing numerous types of cancers, including esophageal, pancreatic, colon, breast, endometrial, kidney and liver [3-5]. Studies in rodents have suggested that elevated circulating levels of obesity-related hormones and pro-inflammatory cytokines (e.g. insulin, insulin growth factor-1 (IGF-1), leptin, interleukin-6 (IL-6) and tumor necrosis factor (TNF)) play a major role in cancer promotion and propagation [5, 6]. However, the exact molecular mechanisms of action still remain elusive.

A major challenge is the choice of the appropriate research model - a model suitable for studying the underlying mechanisms of both, cancer and obesity. In cancer research, numerous murine models, of genetically engineered mice lacking or overexpressing cancer-associated genes, have been developed to study tumor-initiation, -promotion, -progression and response to cancer therapies. However, despite providing useful tools to study the role of specific genes on tumor development, such genetic murine models do not fully represent the genetic and epigenetic complexity of a human tumor [7]. Of note, human xenograft models, where human tumor cells or biopsies are either transplanted under the skin (subcutaneous models) or into the organ of tumor origin (orthotopic models) of immunocompromised mice, provide attractive tools for translational cancer research. The most widely used murine model for human tumor xenografts utilizes nude mice carrying a single spontaneous mutation in the Foxn1 (winged-helix/forkead

transcription factor) gene that leads to an abnormal thymus morphology, a lack of thymus-derived T cells, and as such represent an immunocompromised state [8]. In fact, xenografts of human tumors into nude mice represent a commonly used model to interrogate the impact of various environmental cues on human tumors, or to evaluate the clinically efficacy of anti cancer agents [9-11].

Human tumor xenografts in the nude mouse model may also allow for analysis on the impact of diet and obesity on human tumor growth and metastasis. However, nude mice used in cancer-related studies are typically bred on an obesity-resistant BALB/c background [12, 13]. In contrast, obesity and diabetes research are mostly conducted in mice on a C57BL/6 background, which facilitates diet-induced obesity and obesity-associated comorbidities including glucose intolerance and hepatic steatosis [13-15]. Although, nude mice have been backcrossed to a pure C57BL/6 background their susceptibility to diet-induced obesity and comorbid conditions has not been studied in detail.

This study was designed to provide the first full metabolic characterization of C57BL/6 nude mice. We further aimed to assess their propensity for diet-induced obesity and its sequelae. Overall, our data address the question whether C57BL/6 nude mice can serve as a suitable translational model to study the link between obesity and cancer. For the first time, we show that nude mice on a C57BL/6 background are prone to development of high fat diet (HFD) induced obesity and glucose intolerance. However, we also show that such propensity is dependent on housing C57BL/6 nude mice at thermoneutral temperatures. At conventional, ambient housing temperatures, nude mice display increased energy expenditure and full protection from high fat diet (HFD) induced obesity, compared to wild type (WT) controls. Notably, thermoneutral housing significantly enhanced lipopolysaccharide

(LPS) driven pro- and anti-inflammtory cytokine production in C57BL/6 nude mice. Together, our data indicate that conventional housing-driven cold stress has broad effects on development of obesity and its sequelae, as well as the immune system, in C57BL/6 nude mice.

MATERIALS AND METHODS

Mice

Male nude mice on a C57BL/6 background (B6.Cg-Foxn1nu/J) and corresponding C57BL/6 wild type mice were purchased from The Jackson Laboratory (Bar Harbour, ME) at 6 weeks of age. Mice were housed in high-efficiency particulate-filtered laminar flow hoods at the Cincinnati Children's Hospital Medical Center (CCHMC), or at the animal facility of the Metabolic Diseases Institute of the University of Cincinnati, with free access to irradiated food and autoclaved water. Four independent experimental groups were used for the studies. In a first set of experiments 10 weeks old nude mice and WT controls (N=5) were ad libitum fed with either a low fat diet (LFD), 10% kcal from fat (D12450B, Research Diets Inc., New Brunswick, NJ), or a high-fat diet (HFD), 60% kcal from fat (D12492, Research Diets Inc., New Brunswick, NJ) for 35 days to compare the level of diet induced obesity in both strains. Body weights were measured weekly. Body composition was analyzed prior to, two and four weeks after feeding LFD or HFD using a whole-body composition analyzer (EchoMedical Systems, Houston, TX).

With the second experimental cohort (N = 8), we aimed to compare the effects of temperature on metabolic performance in nude and corresponding wild type mice. 10 weeks old mice were ad libitum fed with HFD. Metabolic performance (indirect calorimetry, locomoter activity, body core temperature, respiratory quotient (RQ) and caloric intake during light and dark phases) was analyzed as described below. The third cohort of C57BL/6 nude mice (N=8) were kept at an increasing temperature gradient ranging from 30°C to 33°C and were ad libitum fed with either LFD or HFD for 12 weeks. Body weights and food intake were measured weekly. Intraperitoneal (ip.) glucose tolerance tests (GTT) were performed one week before sacrifice. In a last

set of experiments we investigated the ability of LFD fed C57BL/6 nude mice (N = 8) housed either at 23°C or 33°C to respond to an inflammatory stimulus (lipopolysaccharide [LPS] challenge; see below). Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under animal study proposals approved by the CCHMC or by the University of Cincinnati Institutional Animal Care and Use Committee.

Intraperitoneal glucose tolerance test:

Mice (N = 6) were fasted for six hours, starting one hour after the initiation of the light phase. Baseline glucose levels (0 min) were measured in duplicate from tail blood using hand-held glucometers. Mice then received a 2.0 mg / kg bolus of dextrose (Phoenix Pharmaceutical, St. Joseph, MO) via intraperitoneal (ip.) injection and glucose concentration were measured after 15, 30, 45, 60 and 120 min.

Combined indirect calorimetry:

A 32-cage combined indirect calorimetry system (PhenoMaster, TSE Systems GmbH) was used to assess energy expenditure, locomotor activity, body core temperature, respiratory quotient as well as food and water intake. Mice were allowed to acclimatize to the air-tight cages for 16 h. Subsequently, volumes of oxygen consumption ($\Delta vol\%O_2$) and volumes of CO_2 production ($\Delta Vol\%CO_2$) were measured every 10 min for a total of six light and six dark phases (144 h) to determine the respiratory quotient ($RQ = VCO_2/VO_2$) and energy expenditure ($EE = VO_2 \times [3.815 + (1.232 \times (VCO_2/VO_2))] \times 4.1868$) [16]. Home-cage locomotor activity was determined by a multidimensional infrared light beam system. Stationary locomotor activity was defined as consecutive infrared light beam breaks of one single light

beam, and ambulatory movement as consecutive breaks of two different light beams. Scales integrated into the sealed cage environment continuously measured cumulative food intake. Mean body core temperature was monitored via intraperitoneal E-mitter telemetry devices (E-mitters; Mini Mitter, Bend, OR) which had been implanted one week previously, using isoflurane anesthesia and buprenorphine hydrochloride analgesia (Buprenex®, single dose of 0.05 mg/kg bodyweight). The mice were kept at a constant temperature of 23°C for 72 hours, housing temperatures were then increased to 33°C until the end of the experiment.

Sacrifice and tissue collection:

Mice were sacrificed by CO_2 anesthesia followed by heart puncture and blood collection. White and brown adipose tissue samples were excised, weighed and frozen on dry ice for subsequent sample processing.

Liver triglycerides quantification:

Lipids were isolated from 50 mg of frozen livers of HFD- and LFD-fed nude mice using a chloroform/methanol (2:1) extraction procedure as described previously [17, 18]. After evaporation of the organic solvent, total tryglyceride contents were determined by an enzymatic method (Infinity™ Triglycerides Reagent, Thermo Fisher Scientific, VA), according to the manufacturer's instructions.

Gene expression analyses

RNA from epididymal white adipose tissue was isolated using the RNeasy Lipid Tissue mini kit (Qiagen, Valencia, CA) and transcribed into cDNA by using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), according to the

manufacturer's instructions. Quantitative real-time PCR reactions were performed in a ViiA™ 7 System (Applied Biosystems, Foster City, CA) by using murine specific TaqMan probe sets for leptin (Lep, Mm00434759_m1) and Chemokine (C-C motif) ligand 2 (CCL2, Mm00441242_m1). Relative expression levels were normalized to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1, Mm01545399_m1); expression changes were evaluated using the delta-delta Ct (ΔΔCt) method.

In vivo cytokine capture assay:

Systemic TNF, IL-6, IL-17A, IFN-γ and IL-10 levels were detected using the *in vivo* cytokine capture assay (IVCCA), an assay that integrates *in vivo* cytokine production over the 24 hr period of challenge, as described before [19, 20]. Briefly, systemic systemic cytokine levels were quantified employing biotinylated capture antibodies, detection antibodies and recombinant protein murine standards. Biotinylated capture mouse antibodies (TNF, clone TN3-19; IL-6, clone MP5-32C11; IL-17A, clone eBio17B7; IFN-γ, clone R4-6A3; and IL-10, clone JES5-16E3; all eBioscience, San Diego, CA) were injected via tail vein 3 hours prior to TLR4-specific LPS challenge (25 mg/ mouse, ultrapure LPS, E. coli 0111:B4; InvivoGen, San Diego, CA) and terminal serum collection was performed 24 hours later. Detection antibodies (TNF, clone G281-2626; IL-6, clone MP5-20F3; IL-17A, clone eBio17CK15A5; IFN-γ, clone AN-18; and IL-10, clone JES5-2A5; all eBioscience) along with appropriate standards were used for cytokine level quantification.

Statistics:

All data are expressed as mean ± standard error of mean (SEM). Body weight, food intake, metabolic performance and glucose excursions following the intraperitoneal glucose tolerance test were analyzed via two-way analysis of variance (ANOVA) (variables: treatment and time) with a Bonferroni multiple comparisons test. Energy expenditure was analyzed by analysis of covariance (ANCOVA) using body weight as covariate. Statistical differences between surgical groups of all other measurements were analyzed using one-way ANOVA followed by a Bonferroni post hoc test. Analyses were done using GraphPad Prism 6.0 software. A p-value < 0.05 was considered significant.

RESULTS

Nude mice on a C57BL/6 background are protected from diet-induced obesity when exposed to mild cold stress.

Thirty-five days of HFD feeding at ambient temperature (23°C) resulted in a significantly higher body weight gain in C57BL/6 WT mice compared to LFD-fed control mice (Figure 1A - B). In contrast, C57BL/6 nude mice were protected from HFD-induced obesity as they gained similar body weight compared with LFD-fed C57BL/6 nude mice (Figure 1C - D). Further, HFD-fed WT mice, but not nude mice showed a significant increase in body fat compared to the LFD-fed controls (Figure 1E). Notably, lean mass, initially lower in nude mice, was not affected by diet in both strains (Figure 1F).

Increased energy expenditure in nude mice on a C57BL/6 background is restricted to normal housing temperatures.

We next aimed to delineate whether the protection from diet-induced weight gain in nude mice at conventional housing conditions of 23°C was the result of a decreased caloric intake, an increased energy dissipation due to an increased locomotor activity or thermogenesis, or both.

Indirect calorimetry, followed by analysis of covariance with body weight as covariate, (Figure 2A, B) revealed higher energy expenditure in HFD-fed nude mice compared to the WT controls when housed at 23°C (ANCOVA, $F = 6.093$, $p = 0.028$). Such changes in energy expenditure were observed during both, light (ANCOVA, $F = 8.267$, $p = 0.013$) and dark phases (ANCOVA, $F = 4.645$, $p = 0.05$) (Figure 2B). Notably, the difference in energy expenditure between WT and nude mice was lost after housing mice at a thermoneutral temperature of, 33°C (ANCOVA,

F = 0.625, p = 0.443) and was not influenced by either the light phase (ANCOVA, F = 0.685, p = 0.423) or the dark phase (ANCOVA, F = 0.342, p = 0.568) (Figure 2B). We corroborated our finding of enhanced energy expenditure at a standard housing temperature of 23°C in an inversed experimental setup, using an additional cohort of nude mice (Suppl. Material and Methods and Suppl. Fig. 1A). Specifically, after an initial acclimatization period of at least 2 weeks at thermoneutrality, we conducted indirect calorimetry measurements in LFD-fed nude mice and four differentially aged groups of C57BL/6J WT mice. We neither observed differences in energy expenditure (Suppl. Fig. 1A), food intake (Suppl. Fig. 1B) nor respiratory quotients (Suppl. Fig. 1C) in mice fed LFD at 33°C, nor later in the same mice fed HFD at 33°C. However, at mild cold exposure of 23°C, a significant increase in energy expenditure (ANCOVA F=5.58, p=0.019) even after correction for the covariates body weight (F = 0.268, p = 0.619), fat mass (F = 0.454, p = 0.52), lean mass (F = 0.116, p = 0.742), body length (F = 0.962, p = 0.355) and age (F = 1.217, p = 0.302) was observed. Notably, such enhancement of energy expenditure was also apparent in linear regression plots of energy expenditure against lean mass, body weight and body length (Suppl. Fig. 2).

Differences in energy expenditure were independent of any changes in locomotor activity, as both WT and nude mice exhibited similar activity at both temperatures (Figure 2C). Thermoneutral housing reduced calorie intake in both genotypes to a similar degree (Figure 2D). WT and nude mice consumed 2.8 ± 0.41 g and 2.3 ± 0.57 g of HFD at 23°C (ANCOVA, F = 1.829, p = 0.206, body weight as covariate), and 1.9 ± 0.33 g and 1.9 ± 0.34 g of HFD at 33°C (ANCOVA, F = 0.733, p = 0.503, body weight as covariate), respectively. Similarly, no difference in food intake was observed following normalization to the respective body weights (Figure 2E), to

correct for the significantly lower body weight of nude vs. WT mice (Figure 2F). The respiratory quotient (RQ) after HFD feeding was 0.7 in both strains and at both temperatures (Figure 2G), suggesting that both nude and WT mice mainly used fat as source of energy. Continuous body core temperature measurements at both housing temperatures demonstrate that both strains are able to effectively maintain homeothermia (Figure 2H). However, nude mice exhibited significant increase in the percent of thermogenic brown adipose tissue compared to WT mice (Figure 2I). Together these data indicate that nude mice have to expend more energy to keep their body core temperature constant—something that correlates also with protection from weight gain.

Housing at thermoneutrality promotes diet-induced obesity in nude mice on a C57BL/6 background.

The comparable energy expenditure at thermoneutrality between nude and WT mice suggests that exposure of C57BL/6 nude mice to HFD should lead to weight gain when housed at higher temperatures. To test this, nude mice were fed either LFD or HFD and housed at an increasing temperature gradient (30°C or 33°C). Body weights were similar in HFD and LFD mice during a feeding period of 30 days at 30°C (Figure 3A). After increasing the temperature to 33°C, HFD fed nude mice showed a significant increase in total body weight compared to the LFD fed controls (Figure 3A). Such increase in body weight directly correlated with an increase in visceral white adipose tissue consisting of epididymal and perirenal fat pads and subcutaneous inguinal fat pad (Figure 3B). Increased obesity in nude mice was also characterized by a higher leptin expression and adipose tissue inflammation, as defined by increased CCL2 expression (Figure 3C). The obese phenotype in HFD-fed nude mice was

further accompanied by a small but significant decrease in their insulin tolerance (Figure 3D-E). Further, HFD fed nude mice exhibited higher liver weights (Figure 3F), which was accompanied by a slight, however not yet significant increase of hepatic triglycerides (Figure 3G), indicative of a development of an early stage of non-alcoholic fatty liver disease (NAFLD) [19]. Food intake was similar at both temperatures (23°C and 33°C), with HFD feeding leading to a significant increased in the cumulative caloric intake (Figure 3H). However, the higher caloric intake only translated into an obese phenotype at thermoneutrality.

The housing temperature affects systemic cytokine production in nude mice on a C57BL/6 background.

Previous studies have shown that housing temperature can lead to broad (patho-) physiological changes in mice— something associated with significant modulation of immune responses [21]. To elucidate possible effects of the housing temperature on the immune response in nude mice, LFD fed nude mice were kept at housing temperatures of 23 °C and 33°C for 12 weeks, before they were challenged with TLR4-specific LPS. Systemic cytokine production in nude mice was significantly higher, when housed at 33°C than at 23°C. Specifically, nude mice displayed higher production of pro-inflammatory cytokines including, tumor necrosis factor α (TNF), IL-6, IL-17A and interferon (IFN)-γ and (Figure 4 A-D) as well as anti-inflammatory cytokine, IL-10 (Figure 4E).

DISCUSSION

The discovery of the Foxn1 nude mouse in 1962 and the subsequent development of human subcutaneous and orthotopic tumor xenograft models represented major breakthroughs in cancer research. Until today, nude mice remain powerful and clinically relevant animal models that allow for efficient screening for anti-cancer therapeutics [9-11]. We here aimed to assess whether nude mice on a C57BL/6 background, could also be a valuable model to evaluate a causal relationship between diet-induced obesity and cancer. Foxn1 nude mice not only lack mature T cells and are thus unable to mount most $CD4^+$ or $CD8^+$ T cell-dependent immune responses, including graft rejection. The mutation further leads to an abnormal hair follicle morphology, and thus to a nude phenotype. Previous findings have demonstrated that even fur carrying mice constantly loose heat at normal housing temperatures of 20-23°C [22], which requires a continuous compensatory heat production in order to maintain euthermia [23]. Indeed, hair loss and reduced insulation in Foxn1 nude mice has been associated with higher energy dissipation compared to WT mice [24]. We here corroborate and expand those findings by showing that C57BL/6 nude mice, in contrast to WT C57BL/6 controls, demonstrate a significant increased energy expenditure and protection from diet-induced obesity when kept at normal housing temperatures of 23°C. The increased energy expenditure was independent from several co-variants such as body weight, fat mass, lean mass, body length and age. Accordingly, our data suggest that the different energy homeostasis depicted in C57BL/6 nude mice at 23°C is a specific consequence of the Foxn1 mutation, and not an artifact of normalization methods or differential body morphometry.

The increase in energy expenditure in nude mice kept at 23°C was not the result of a higher locomotor activity, which was similar in both strains. Stable body core

temperatures in nude and WT mice suggest that nude mice have a higher energy demand for thermogenesis at regular housing temperatures.

Importantly, at temperatures in the thermoneutral zone (33°C), nude mice and WT controls significantly decreased their energy expenditure to a similar level. Consequently, nude mice housed at thermoneutrality were prone to the effects of HFD exposure and displayed a clear obese phenotype as well as glucose intolerance. A trend towards increased liver triglycerides further points towards an early stage development of hepatic steatosis. Overall, our data clearly show that housing temperature is a major denominator for metabolic homeostasis in nude C57BL/6 mice. Housing mice at thermoneutral conditions may in fact reflect the human situations, which usually live at thermoneutral conditions.

The thermoneutral zone of a fur-bearing mouse is about 30°C. Our nude mice were still protected from diet induced obesity at this temperature. Only a temperature increase to 33°C resulted in a significant weight gain due to HFD feeding, indicating that the thermoneutral zone in nude mice is even increased. Housing mice at 23°C represents a constant mild cold stress, which may affect numerous physiological and metabolic processes [25, 26]. Together, our data suggests that future studies - utilizing nude C57BL/6 mice and focused on the interplay between obesity and cancer - should be conducted at thermoneutrality.

Cold exposure normally leads to an increased food intake to compensate for the thermogenic loss of calories [25, 27]. Nude mice, which suffer from cold stress even at regular housing temperatures, should hence display increased food intake at room temperature, compared to WT mice. However, even when food intake was normalized to the significantly lower body weights of nude mice, nude mice were not hyperphagic compared to WT controls. Such lack of compensatory food intake during

mild cold stress may provide an additional explanation as to why nude mice but not WT mice are resistant to HFD-induced obesity at 23°C but not at 33°C.

Molecular roles for Foxn1 in food intake control have not been reported to date. Our data nevertheless point towards a central - potentially hypothalamic[28] - role of Foxn1 in controlling ingestive behavior upon cold stimulation. Recent data showed higher neurotrophin and noradrenaline concentrations and increased density of noradrenergic fibers in hypothalami of BALB/c nude mice housed at regular housing temperatures [29]. Activation of noradrenergic circuitry and enhanced hypothalamic-pituitary-adrenal (HPA) axis were previously linked with chronic cold stress in rats [30]. Accordingly, nude mice housed at mild cold stress of 23°C may fail to compensate for the enhanced energy loss due a chronic activation of the HPA axis, and a subsequent dysregulation of physiological eating patterns. Future studies should elaborate on this potential link between cold stress-induced hyperactivation of the HPA axis and nude mouse metabolism, and clarify whether Foxn1 plays a functional role in feeding circuitry in the hypothalamus.

Recent findings demonstrated that antitumor immunity is significantly increased in murine allograft tumor models of a BALB/c and a C57BL/6 background when kept at housing temperatures of 30°C compared to 22°C [31]. In contrast, increased housing temperature did not affect immunity against xenograft tumors in nude mice [31]. In our study, C57BL/6 nude mice displayed HFD-induced weight gain only after raising housing temperatures to 33°C. At 30°C, our C57BL/6 nude mouse model remained resistant to detrimental effects of HFD exposure, indicating the essential role of correctly assessing the thermoneutral zone for a specific animal model. Thermoneutral housing further augmented microbial ligand-driven activation of immune cell-driven cytokine production in our C57BL/6 nude mouse model.

Specifically, C57BL/6 nude mice housed at 33°C displayed significantly higher production of pro- and anti-inflammatory cytokines after an acute LPS challenge, compared to LPS-challenged nude mice housed at 33°C. These data suggest that cold stress induces suppression of the LPS-driven cytokine production and that such effects are independent of T cell response. Notably, LPS, a cell wall component of gram-negative bacteria, is found in the blood stream of mice with acute or chronic bacterial infection, but has also been implicated in other inflammation related diseases such as obesity and cancer. HFD feeding was shown to increase intestinal permeability and circulating LPS levels [19]. The resulting increase in circulating LPS has been hypothesized to contribute to the development of body adiposity and insulin resistance [32, 33]. On the other hand, pro-inflammatory LPS, – depending on the tumor type - can lead to a tumor promoting [34] or suppressing [35, 36] effects. Importantly, cold stress induced repression of immune cell responsiveness to LPS may therefore result in an underestimations of LPS effects on tumorigenesis and obesity.

Mice have been described as relatively insensitive towards bacterial endotoxins compared to humans [37, 38]. Our findings suggest that such reduction in LPS sensitivity may not solely be explained by a species-specific difference, but that cold stress-mediated impairment of myeloid cell activation may play a major role. Indeed, and in contrast to the *in vivo* situation, monocytes isolated from blood of mice and humans exhibit similar *in vitro* responsiveness to LPS [37, 38].

In summary, we demonstrate that thermoneutrality is a prerequisite to study metabolism in the cancer Foxn1 nude mouse model even when kept on the obesity-prone C57BL/6 background. In conventional housing, C57BL/6 nude mice, compared to C57BL/6 WT controls, are protected from diet-induced obesity and obesity-associated comorbidities due to increased energy expenditure and impaired feeding

behavior. At thermoneutrality however, C57BL/6 nude mice are prone to the detrimental effects of HFD exposure, making them a superior xenograft model to dissect potential mechanisms for obesity-induced carcinogenesis. Further augmentation of immune function in C57BL/6 nude mice housed at thermoneutrality may lead to novel discoveries on the role of immune system in obesity and cancer. In fact, future studies should directly and comprehensively elaborate on this potential impact of housing temperature on murine immune function, and determine whether immune responsiveness in other mutant mouse models should also be examined at thermoneutrality.

Supplementary Information:

Supplementary information available at the International Journal of Obesity's website.

GRANTS:

This work was supported by the German Research Foundation (STE 1466/4-1) (to K.S.) and in part by a NIH R21-HL113907 and R01-DK099222 (to S.D.).

REFERENCES

1. WHO. Obesity and Overweight, Fact sheet N°311. 2008.
2. Unger RH, Clark GO, Scherer PE, Orci L. Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta* 2010; **1801**(3)**:** 209-14.
3. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**(17)**:** 1625-38.
4. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *Lancet Oncol* 2002; **3**(9)**:** 565-74.
5. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004; **4**(8)**:** 579-91.
6. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer* 2011; **11**(12)**:** 886-95.
7. Richmond A, Su Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech* 2008; **1**(2-3)**:** 78-82.
8. Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 1966; **8**(3)**:** 295-309.
9. Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer* 2004; **40**(6)**:** 827-36.
10. Kerbel RS. Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved. *Cancer Biol Ther* 2003; **2**(4 Suppl 1)**:** S134-9.
11. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* 2004; **40**(6)**:** 852-7.

12. Montgomery MK, Hallahan NL, Brown SH, Liu M, Mitchell TW, Cooney GJ *et al.* Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. *Diabetologia* 2013; **56**(5)**:** 1129-39.

13. Waller-Evans H, Hue C, Fearnside J, Rothwell AR, Lockstone HE, Calderari S *et al.* Nutrigenomics of high fat diet induced obesity in mice suggests relationships between susceptibility to fatty liver disease and the proteasome. *PLoS One* 2013; **8**(12)**:** e82825.

14. Tschop M, Heiman ML. Overview of rodent models for obesity research. *Curr Protoc Neurosci* 2002; **Chapter 9:** Unit 9 10.

15. Kahle M, Horsch M, Fridrich B, Seelig A, Schultheiss J, Leonhardt J *et al.* Phenotypic comparison of common mouse strains developing high-fat diet-induced hepatosteatosis. *Mol Metab* 2013; **2**(4)**:** 435-46.

16. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 1949; **109**(1-2)**:** 1-9.

17. Frayn KN, Maycock PF. Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability. *J Lipid Res* 1980; **21**(1)**:** 139-44.

18. Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW. Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* 1991; **40**(2)**:** 280-9.

19. Harley IT, Stankiewicz TE, Giles DA, Softic S, Flick LM, Cappelletti M *et al.* IL-17 signaling accelerates the progression of nonalcoholic fatty liver disease in mice. *Hepatology* 2013.

20. Finkelman F, Morris S, Orekhova T, Sehy D. The in vivo cytokine capture assay for measurement of cytokine production in the mouse. *Curr Protoc Immunol* 2003; **Chapter 6:** Unit 6 28.

21. Karp CL. Unstressing intemperate models: how cold stress undermines mouse modeling. *J Exp Med* 2012; **209**(6)**:** 1069-74.

22. Dawson NJ. The surface-area-body-weight relationship in mice. *Aust J Biol Sci* 1967; **20**(3)**:** 687-90.

23. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* 2011; **214**(Pt 2)**:** 242-53.

24. David JM, Chatziioannou AF, Taschereau R, Wang H, Stout DB. The hidden cost of housing practices: using noninvasive imaging to quantify the metabolic demands of chronic cold stress of laboratory mice. *Comp Med* 2013; **63**(5)**:** 386-91.

25. Cannon B, Nedergaard J. Thermogenesis challenges the adipostat hypothesis for body-weight control. *Proc Nutr Soc* 2009; **68**(4)**:** 401-7.

26. Speakman JR, Keijer J. Not so hot: Optimal housing temperatures for mice to mimic the thermal environment of humans. *Mol Metab* 2012; **2**(1)**:** 5-9.

27. Ravussin Y, Xiao C, Gavrilova O, Reitman ML. Effect of intermittent cold exposure on brown fat activation, obesity, and energy homeostasis in mice. *PLoS One* 2014; **9**(1)**:** e85876.

28. Woods SC, Schwartz MW, Baskin DG, Seeley RJ. Food intake and the regulation of body weight. *Annu Rev Psychol* 2000; **51:** 255-77.

29. Jouda J, Wildmann J, Schafer M, Roggero E, Besedovsky HO, del Rey A. T cells affect central and peripheral noradrenergic mechanisms and

neurotrophin concentration in the spleen and hypothalamus. *Ann N Y Acad Sci* 2012; **1261:** 18-25.

30. Pardon MC, Ma S, Morilak DA. Chronic cold stress sensitizes brain noradrenergic reactivity and noradrenergic facilitation of the HPA stress response in Wistar Kyoto rats. *Brain Res* 2003; **971**(1)**:** 55-65.

31. Kokolus KM, Capitano ML, Lee CT, Eng JW, Waight JD, Hylander BL *et al.* Baseline tumor growth and immune control in laboratory mice are significantly influenced by subthermoneutral housing temperature. *Proc Natl Acad Sci U S A* 2013; **110**(50)**:** 20176-81.

32. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D *et al.* Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007; **56**(7)**:** 1761-72.

33. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008; **57**(6)**:** 1470-81.

34. Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C *et al.* Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. *Int J Cancer* 2002; **101**(5)**:** 415-22.

35. Chicoine MR, Zahner M, Won EK, Kalra RR, Kitamura T, Perry A *et al.* The in vivo antitumoral effects of lipopolysaccharide against glioblastoma multiforme are mediated in part by Toll-like receptor 4. *Neurosurgery* 2007; **60**(2)**:** 372-80; discussion 381.

36. Andreani V, Gatti G, Simonella L, Rivero V, Maccioni M. Activation of Toll-like receptor 4 on tumor cells in vitro inhibits subsequent tumor growth in vivo. *Cancer Res* 2007; **67**(21)**:** 10519-27.

37. Munford RS. Murine responses to endotoxin: another dirty little secret? *J Infect Dis* 2010; **201**(2)**:** 175-7.

38. Warren HS, Fitting C, Hoff E, Adib-Conquy M, Beasley-Topliffe L, Tesini B *et al.* Resilience to bacterial infection: difference between species could be due to proteins in serum. *J Infect Dis* 2010; **201**(2)**:** 223-32.

FIGURE CAPTIONS

Figure 1: Lack of diet-induced obesity in nude mice kept at room temperature.

Comparative body weight data in C57BL/6 WT mice (A and B) and nude littermates (C and D), kept on 10 % LFD or a 60 % HFD, respectively. Total body weights in gram (A and C). Body weight gain in percent (B and D). Comparative fat mass (E) and lean mass (F) measurements in C57BL/6 WT and nude mice, at weeks 0, 2 and 4 of LFD or HFD feeding. N = 5; mean ± SEM; 2-Way-ANOVA with Bonferroni correction, *P < 0.05; **P < 0.01.

Figure 2: Conventional housing results in an increased energy expenditure in nude mice.

(A) Significantly increased energy expenditure in nude vs. WT mice at a housing temperature of 23 °C ($p < 0.05$) but not at 33 °C. (B) Average energy expenditure of the whole dark or light phase shows that the significant increase in energy expenditure was restricted to the light phase. ($p < 0.01$). (C) Similar locomotor activity in nude and WT mice at both housing temperatures (D). Food intake was unchanged in both groups regardless of housing temperatures normalization and (E) following normalization to the significantly different body weights (F). (G) Similar respiratory quotient (RQ) after HFD feeding in both strains irrespective of housing temperatures. (H) Similar mean body core temperature during light and dark phases at housing temperatures of 23°C (left panel) and 33°C (right panel). (I) Higher percentage of brown adipose tissue mass (normalized to their total body weights) in nude vs. WT mice. N = 8; mean ± SEM; ANCOVA (A- G), Student's t- test (F - I), *P < 0.05; **P < 0.01, ***P < 0.001.

Figure 3: Diet induced obesity in nude mice is determined by the housing temperature.

(A) Body weights of LFD and HFD fed nude mice at two different housing temperatures (30°C and 33°C). Similar body weight gain during 30 days of 30°C exposure, while HFD feeding resulted in a significant body weight gain at 33°C. (B) Increased wet tissue mass of epididymal (E), retroperitoneal (RP) and subcutaneous (SC) fat in diet induced obese nude mice, housed at 33°C. (C) Increased leptin and CCL2 expression in the epididymal fat mass of obese nude mice, normalized to the housekeeping gene HPRT1. (D) Blood glucose levels before (time 0) and after an ip glucose tolerance test indicate a significantly higher glucose excursions in obese vs. lean mice. (E) Area under the curve (AUC) for plasma glucose across the 2 hours glucose tolerance test showed a significant increase in HFD fed nude mice compared to the LFD fed controls. (F) Significantly increased liver wet tissue mass in HFD fed nude mice. (G) Increased liver triglycerides in HFD fed nude mice. (H) Higher cumulative caloric intake in HFD- vs. LFD fed nude mice. N = 6 - 8; mean + SEM; 2-Way-Anova (A; D; H), Student's T-Test (B; E-G). *P < 0.05; **P < 0.01, ***P < 0.001.

Figure 4: Thermoneutral housing exacerbates LPS-driven cytokine production in nude mice.

Significantly increased circulating levels of (A) tumor necrosis factor α (TNF), (B) IL-6 ($p < 0.001$), (C) IL-17A, (D) interferon (IFN)-γ, and (E) IL-10 in nude mice housed at 33°C compared to 23°C. (A-E) N = 5 - 7; Student's t-test; *P < 0.05; **P < 0.01; ***P < 0.001.