Original article

MOLECULAR METABOLISM

Differential colonization with segmented filamentous bacteria and *Lactobacillus murinus* do not drive divergent development of diet-induced obesity in C57BL/6 mice

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ABSTRACT

Alterations in the gut microbiota have been proposed to modify the development and maintenance of obesity and its sequelae. Definition of underlying mechanisms has lagged, although the ability of commensal gut microbes to drive pathways involved in inflammation and metabolism has generated compelling, testable hypotheses. We studied C57BL/6 mice from two vendors that differ in their obesogenic response and in their colonization by specific members of the gut microbiota having well-described roles in regulating gut immune responses. We confirmed the presence of robust differences in weight gain in mice from these different vendors during high fat diet stress. However, neither specific, highly divergent members of the gut microbiota (*Lactobacillus murinus,* segmented filamentous bacteria) nor the horizontally transmissible gut microbiota were found to be responsible. Constitutive differences in locomotor activity were observed, however. These data underscore the importance of selecting appropriate controls in this widely used model of human obesity.

Keywords Microbiome; Obesity; Inflammation; Metabolism; Nicotinamide nucleotide transhydrogenase

1. INTRODUCTION

Obesity has become a full-fledged pandemic [1]. Increases in obesity prevalence are expected to drive attendant increases in morbidity and mortality, as obesity is associated with diverse pernicious sequelae, including insulin resistance, type 2 diabetes, hypertension, metabolic syndrome, atherosclerotic coronary artery disease, non-alcoholic fatty liver disease and various cancers [2–5]. In light of the increasing clinical and public health burden and the limitations of current therapies, novel therapeutic approaches to obesity are clearly needed [6,7]. For this, clearer definition of the cellular and molecular mechanisms causing obesity, and the relative contribution of such mechanisms to the recent rapid increase in obesity prevalence will likely be important.

The intestinal microbiota has been proposed to be a contributory factor in the development and maintenance of obesity [8]. The observations that gnotobiotic mice are resistant to diet-induced obesity (DIO) and that transfer of gut bacteria from obese to lean mice can transfer obesity phenotypes led to studies employing high-throughput genome sequencing technologies that have reported differences in the gut microbiomes and/or metagenomes of obese and non-obese humans [9–15]. If the gut microbiome does play a causal role in obesity, there is promise for approaches (pre-, pro- and anti-biotic) aimed at modulating the microbiota as novel therapeutic modalities for obesity and its sequelae. The field remains controversial, however (reviewed in [8]). Specifically, efforts to replicate the initial description of phylum-level shifts in the obese human microbiome have only been marginally successful [8]; it is presently unclear whether this is due to true lack of association or lack of power to detect associations of the expected effect size. Additionally, the protection of gnotobiotic mice from DIO, may depend on mouse strain and obesogenic diet [8]. More generally, efforts to rigorously define the mechanisms through which putatively obesogenic microbes might drive obesity phenotypes have been hampered by a failure to identify specific causal microbes or microbial properties [8,10,16–21].

Following on the relatively recent understanding that obesity is associated with a state of chronic, low-grade inflammation [22,23], the innate immune response to gut microbes has been implicated in the development of obesity and its pernicious sequelae [16–18,24]. In turn,

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the immune system's apparent causal contributions to the development of obesity appear to be mediated by alterations in the constitution of the intestinal microbiota. In particular, deletion of genes encoding pattern-recognition receptors that signal the presence of microbe-associated molecular patterns, such as *Tlr5* and *Pycard*, has been shown to select for microbiota that can transfer obesity to other mice [16,17]. Given the relationship between the intestinal immune system and the gut microbiota [25,26], we hypothesized that members of the gut microbiota capable of robust stimulatory effects on the intestinal immune system might also causally contribute to the development and maintenance of obesity. This hypothesis was bolstered by the observation that colonization with one such microbe, segmented filamentous bacteria (SFB), specifically induced the intestinal expression of genes with dual roles in both immunity and metabolism, such as *Retnlb*, *Saa1* and *Il17a* [25], and altered extraintestinal (patho)physiology in diverse disease models [27–29].

We thus defined the contribution of the microbiota to obesity in mice known to differ in both the development of DIO [25,30–33] and in colonization with particular species known to alter immune responses [25,34,35]. Specifically, C57BL/6 mice from two commercial vendors, Jackson Labs and Taconic Farms, are known to vary in their colonization with SFB and *L. murinus*, as well as in their body weight gain in response to high fat diet (HFD) challenge. We confirmed previously reported differences between these strains in their response to HFD challenge. However, our data indicate that these differences are not due to SFB or *L. murinus,* or, indeed, to the entire gut microbiota—as assessed by a variety of experimental designs, including cross-colonization via co-housing, oral gavage of whole gut microbiota, and oral gavage with specific members of the gut microbiota. While our data demonstrate that the gut microbiota is not responsible for the divergent response to HFD challenge in these mice, we observed constitutively increased locomotor activity in the (leaner) C57BL/6 mice from Jackson Labs. Moreover, in the course of these studies, a number of novel experimental techniques were developed to interrogate the microbiota and their role in modulating host (patho) physiology.

2. MATERIALS AND METHODS

2.1. Mice

Wild type C57BL/6J (Nnt^{B6J}) and C57BL/6NJ (Nnt^{WT}) mice were obtained from Jackson labs. Wild type C57BL/6NTac (Nnt^{WT}) mice were obtained from Taconic Farms. Gnotobiotic C57BL/6 germ-free mice were obtained from the National Gnotobiotic Rodent Resource Center at UNC-Chapel Hill. Except where otherwise indicated, the mice from Taconic farms were of the "Murine Pathogen Free™" (MPF) health status. All mice were housed in a specific pathogen-free animal facility and handled in high-efficiency particulate-filtered laminar flow hoods with ad libitum access to food and water at CCHMC, except for the experiments in the PhenoMaster/LabMaster cages from TSE Systems, which were performed at the metabolic diseases institute at the University of Cincinnati. For all experiments, animal care was performed according to the procedures outlined in the Guide for Care and Use of Laboratory Animals. All animal studies were carried out with the approval of the Institutional Animal Care and Use Committees at which they were performed: the Cincinnati Children's Hospital Medical Center IACUC (Protocol #1D05047) and the University of Cincinnati College of Medicine IACUC (Protocol #06-08-07-01).

2.2. Fecal and intestinal DNA extraction, 16S rDNA qPCR, cDNA synthesis, qPCR

DNA extraction was carried out according to a modified version of a published protocol [36]. Briefly, fecal pellets or approximately 1 cm sections of terminal ileum were placed in lysis buffer and homogenized with a TissueLyser (Qiagen) set at 30 Hz for 5 min. For fecal pellets, 0.1 mm zirconia–silica beads (BioSpec, Bartlesville, OK) were used to homogenize bacterial cells. For homogenization of terminal ilea, such beads were used in combination with 1/8'' diameter stainless steel beads (McMaster-Carr). Following extraction and resuspension in DEPC-treated H_2O, DNA quantification was performed with a NanoDrop ND-1000. 50 ng of DNA was used as the amplification template in a Light Cycler 480 II using Sybr Green I Master mix (both from Roche). The following primers for the 16S rDNA of SFB, *Lactobacillus murinus*, all *Lactobacillus* species or all Eubacterial (EUB) species were used: SFB 5′ GACGCTGAGGCATGAGAGCAT, 3′ GACGGCACGGATTGTTATTCA; *L. murinus* 5′ GCAATGATGCGTAGCCGAAC, 3′ GCACTTTCTTCTCTAACAACAGGG; All *Lactobacillus sp.* 5′ AGCAGTAGGGAATCTTCCA, 3′ CACCGCTACACATGGAG; EUB 5′ ACTCCTACGGGAGGCAGCAGT, 3′ ATTACCGCGGCTGCTGGC [37,38]. Phylum and species levels were then normalized to the total amount of Eubacterial DNA or, where indicated, total amount of *Lactobacillus* DNA. Notably, while the primers used to detect *L. murinus* were designed to detect the ASF361 isolate of *L. murinus* present in altered Schaedler flora, they have also been predicted to bind the 16S sequence from the closely related *Lactobacillus* species, *L. animalis* [37].

For RNA isolation, tissue samples were homogenized as above in TRIzol (Invitrogen) with 1/8'' diameter stainless steel beads (McMaster-Carr) to disrupt the tissue. RNA was extracted according to manufacturer instructions. Equal amounts of RNA were used as templates to synthesize cDNA with oligo-dT, random hexamers and Superscript II reverse transcriptase (Invitrogen). The cDNA was then RNase-digested, diluted 1:7 in DEPC-treated water and subjected to qPCR analysis using Light Cycler 480 II (Roche). Sybr Green I Master mix (Roche) and the following primer pairs [25,39] were used: *Gapdh* 5′ CCTCGTCCCGTAGACAAAATG, 3′ TCTCCACTTTGCCACTGCAA; *Saa1* 5′ CATTTGTTCACGAGGCTTTCC, 3′ GTTTTTCCAGTTAGCTTCCTTCATGT; *Reg3g* 5′ CCTTCC TCTTCCTCAGGCAAT, 3′ TAATTCTCTCTCCACTTCAGAAATCCT; IL-17A 5′ ACTACCTCAACCGTTCCACG, 3′ AGAATTCATGTGGTGGTCCAG.

2.3. Obesity models

Except where otherwise indicated, 6–7 week-old male mice were placed on a high-fat diet (HFD; 60% of kcal from fat, carbohydrate 20% kcal, protein 20% kcal; Research Diets #D12492i), a chow diet (Chow; fat 13.5% kcal, carbohydrate 59% kcal, protein 27.5% kcal; Lab Diet #5010), a semi-purified low fat diet (LFD; fat 10% kcal, carbohydrate 70%kcal, protein 20% kcal; Research Diet #D12450Bi), or autoclavable chow from Jackson Labs present in the shipping container, in which mice were received (Jax Chow; fat 16% kcal, carbohydrate 61.7%kcal, protein 22.2%kcal; Lab Diet#5K67). Animals were weighed weekly. Fresh food was provided on a weekly basis and food consumption was quantified weekly.

Quantification of fasting glucose was carried out as follows. Mice were fasted overnight (12 h) and the glucose level in a single drop of blood was quantified using the FreeStyle flash blood glucose monitoring system (Abbot Diabetes Care Inc.). Insulin tolerance testing was carried out as follows. Mice were fasted during the day (8 h) and challenged via intraperitoneal injection (10 μL/g of body weight) with 1.5 U/kg Novolin R insulin (Novo Nordisk Inc.). Immediately following injection, and kinetically thereafter, one drop of blood was collected and analyzed for glucose levels as above. Glucose tolerance testing was carried out as

