Original Article

MOLECULAR METABOLISM

Host–microbiota interaction induces bi-phasic inflammation and glucose intolerance in mice

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ABSTRACT

Objective: Gut microbiota modulates adiposity and glucose metabolism in humans and mice. Here we investigated how colonization of germ-free (GF) mice affects kinetics of adiposity and glucose metabolism.

Methods: Adiposity and glucose metabolism were evaluated at different time points in ex-GF and antibiotic treated mice after colonization with gut microbiota from a conventionally raised (CONV-R) mouse. Mouse physiology, microbiome configuration, serum cytokine levels, and gene expression for inflammatory markers were performed in different tissues.

Results: Colonization resulted in a bi-phasic glucose impairment: the first phase occurring within 3 days of colonization (*early phase*) and the second 14–28 days after colonization (*delayed phase*). The *early phase* co-occurred with an inflammatory response and was independent of adiposity, while the *delayed phase* was mostly ascribed to adipose tissue expansion and inflammation. Importantly, re-colonization of antibiotic treated mice displays only the *delayed phase* of glucose impairment and adiposity, suggesting that the *early phase* may be unique to colonization of the immature GF mice gut.

Conclusions: Our results provide new insights on host–microbiota interaction during colonization of GF mice and the resulting effects on adiposity and glucose metabolism in a time resolved fashion.

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Keywords Microbiota; Germ-free; Antibiotic; Colonization; Adiposity; Glucose metabolism

1. INTRODUCTION

Bacterial colonization of the mammalian gut starts at birth [1]. It is a complex process that undergoes several phases of maturation that involves several factors and results in the development of the immune system [1–3]. The gut microbiota has emerged as an important factor regulating host physiology and metabolism, in particular glucose metabolism and adiposity [4]. Several studies in humans support the central role of gut microbiota on adiposity and glucose metabolism, showing an altered microbiota composition in subjects with obesity and type 2 diabetes [5–7]. However, the underlying mechanisms are just starting to be unraveled. The microbial cell membrane component lipopolysaccharide (LPS) induces low-level inflammation and influences adiposity and glucose tolerance in both mice and humans [8–10], providing a putative mechanism for the role of microbial-derived compounds in the development of the metabolic syndrome. Thus, modulation of gut microbiota and of microbially-produced compounds in mice and humans has been suggested to improve glucose metabolism, indicating a possible causative role of bacteria in the regulation of the host metabolic status [11–14].

Germ free (GF) mice can be used to study the effect of microbiota–host interaction on metabolism [15]. GF mice have better glucose tolerance and lower adiposity than conventionally raised (CONV-R) mice that are colonized with a normal microbiota at birth [16]. Colonization of GF mice at adulthood with a normal microbiota results in impairment of glucose metabolism and fat deposition [10,16,17]. In agreement with these results, ablation of the gut microbiota in CONV-R mice reduces adiposity and improves glucose metabolism [18,19]. Colonization of GF mice allows studying the kinetics of host–microbiota interaction and provides unique opportunities to investigate the key events following colonization [15,20]. This model has been used to study the development of the immune system [20–22] and have shown that gut microbiota colonization is a rapid and dynamic process, characterized by the appearance of a low-diversity microbiota around 2 days post-colonization, followed by the succession within 2–3 weeks of a more complex community resembling the initial inoculum [20,21,23,24]. Several studies have provided mechanistic evidence for how the development of a gut microbiota can result into the establishment of an immune homeostasis promoting the maturation of the immune system in the host [20,22]. Besides the effects on the immune

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system, the development of a gut microbiota also influences the regulation of host metabolic genes in the intestine, thus indicating a possible link between gut microbiota, the immune system, and metabolism [23].

Although it is known that colonization of GF mice affects glucose metabolism and adiposity, there is limited knowledge about how the development of microbiota and its interaction with the host affects adiposity and glucose metabolism in a time-resolved fashion [10]. Here we perform a time-resolved study of how microbial colonization of GF mice affects adiposity and glucose metabolism.

2. MATERIALS AND METHODS

2.1. Mice experiments

GF male Swiss Webster mice, 10–12 weeks old, were maintained in flexible film isolators under a strict 12 h light cycle. GF status was verified regularly by anaerobic culturing in addition to PCR for bacterial 16S rRNA gene. All mouse experiments were performed on autoclaved chow diet (Labdiet) ad libitum.

Colonization and re-colonization experiments were performed in 4 h fasted mice. The cecum content of an age-, strain-, and sex-matched mouse was dissolved in 5 ml of buffer (PBS, 0.2 g/L Na_2S and 0.5 g/L cysteine as reducing agents). 200 μl of solution were gavage in 4 h fasted GF/antibiotic treated mice. For antibiotic treatment, mice were treated with a combination of 4 different antibiotics in the drinking water (1 g/L ampicillin, 1 g/L metronidazole, 0.5 g/L vancomycin and 0.5 g/L neomycin, Sigma, St Louis, MO) for 1 week. Antibiotic solution was changed every 48 h and kept in light protected bottles. Epididymal white adipose tissue, liver, small intestine, and serum were harvested 4 h after fasting.

2.2. MRI, insulin and cytokines measurement

MRI, insulin, and glucose tolerance tests and measurements of insulin levels were performed as earlier described [10]. Briefly, insulin and glucose tolerance tests were performed by injecting insulin (0.75 U/kg body weight) or glucose (2 g/kg body weight), respectively, intraperitoneally after a 4 h fast. Tail blood samples were collected at 0, 15, 30, 60, 90, and 120 min and blood glucose levels were determined using a glucose meter (Accu Check Aviva, Roche). Insulin and cytokines serum levels were measured with kits from Meso Scale (Gaithersburg, Maryland, USA), and Crystal Chem (Downers Grove, Illinois, USA), respectively, according to the manufacturers' protocols.

2.3. Immunohistochemistry of EWAT and liver

Paraffin-embedded epididymal EWAT and liver sections (7 μm) were processed as previously described [10]. Briefly, slides were deparaffinized and processed for antigen retrieval with a 2100 Retriever using 13 DIVA solution and Hot Rinse (HistoLab Products AB, Gothenburg, Sweden). Endogenous peroxidase activity was quenched with 30% H_2O_2 for 30 min. Blocking in 5% rabbit serum, 1% bovine serum albumin, and 0.1% Triton X-100 at room temperature for 30 min. EWAT macrophages were stained with MAC-2/galectin-3 antibody (diluted 1:500 in blocking buffer overnight at 4 °C), and then detected with a biotinylated anti-rat (10 mg/ml) antibody. Immune complexes were detected by Vulcan Red reagent (Vector Laboratories, USA), according to the manufacturer's instructions. Crown-like structures were counted in 15–40 mm^2 of histological sections per tissue. For the liver staining, F4/80 antibody was diluted 1:500 and incubated in blocking buffer 2 h at room temperature and then detected with a HRP anti-rat antibody diluted 1:100. Immune complexes were detected by Vectastain ABC kit reagents (Vectorlab, USA) according to the manufacturer's instructions counterstained with hematoxylin and quantitated by densitometric analysis using Biopix iQ software (version 2.1.3; Biopix, Sweden). List of antibodies is reported in online Supplementary Table 3.

2.4. Flow cytometry analysis

Adipose tissue was minced thoroughly and suspended in digestion solution (PBS, 2% bovine serum albumin, 20 mg collagenase type II, Sigma Aldrich, St Louis, Missouri, USA). Tissue digestion was performed at 37 °C using a shaker at 120 rpm for 40 min. The fat layer was removed, and cells were passed thought a 70 μm cell strainer. Cells were centrifuged (500 g, 4 °C, 5 min), washed in PBS selection buffer (PBS, 2 mM EDTA, and 2% BSA), and subsequently treated with ACK solution (NH_4Cl 150 mM, $KHCO_3$ 10 mM, and Na_2EDTA 0.1 mM) for 7 min to remove red blood cells. Cells were washed and suspended in PBS selection buffer with BD Fc Block for 5 min, followed by incubation with antibody cocktails (CD11b/CD11c/Gr1 or CD3/CD4/CD8, respectively, see Supplementary Table 3 for details). After washing, cells were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, Michigan, USA). Data was processed using FlowJo 10 analysis software (FlowJo LLC, Ashland, Oregon, USA). A similar protocol was used for spleen and blood samples without the collagen digestion steps.

2.5. LPS measurement

Blood was collected from the mouse portal vein using a pyrogen free syringe/needle, and plasma was immediately isolated and frozen in liquid nitrogen. LPS concentration was measured using Endosafe-MCS (Charles River, Lyon, France) based on the limulus amoebocyte lysate (LAL) kinetic chromogenic methodology that measures color intensity directly related to the endotoxin concentration in a sample. Plasma was diluted with endotoxin-free buffer to minimize interferences in the reaction (inhibition or enhancement) and heated for 15 min at 70 °C. Each sample was diluted with endotoxin-free LAL reagent water (Charles River) and treated in duplicate, and two spikes for each sample were included in the determination [25].

2.6. Quantitative RT-PCR

Quantitative RT-PCR RNA was isolated using RNeasy kit with on-column DNase treatment (Qiagen, Hilden, Germany). cDNA templates were synthesized from total RNAs using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. qRT-PCR assays were performed in 10 μl reactions containing 8 μl SYBR Green Master Mix buffer (Thermo Scientific, Waltham, Massachusetts, USA), and 2 μl of 900 nM gene-specific primers (300 nM primer concentrations were used to assess L32 transcripts). Gene expression data were normalized to the ribosomal protein L32. Primer sequences are reported in online Supplementary Table 4.

2.7. Liver lipid analysis

Snap frozen liver tissues were homogenized in 1.5 ml Chloroform/methanol (2:1 v/v). Lipids were measured using Infinity Triglycerides kit (Thermo Fischer Scientific).

2.8. Extraction of fecal genomic DNA and profiling of the fecal microbiota

Fresh fecal pellets for extraction of fecal genomic DNA were collected from 5 mice after 1, 3, 7, 14, and 28 days of colonization. Total fecal genomic DNA was extracted from one fecal pellet using a repeated bead beating method based on a protocol previously described [26].

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