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Long term effect of gut microbiota transfer on diabetes development

Jian Peng [a], Sukanya Narasimhan [b], Julian R. Marchesi [c,d], Andrew Benson [e], F. Susan Wong [f], Li Wen [a,*]

[a] Section of Endocrinology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA
[b] Section of Infectious Disease, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA
[c] Cardiff School of Biosciences, Main Building, Museum Avenue, Cardiff University, Cardiff, UK
[d] Centre for Digestive and Gut Health, Imperial College London, London, UK
[e] Department of Food Science and Technology, University of Nebraska, Lincoln, NE, USA
[f] Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, Cardiff, UK

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ABSTRACT

The composition of the gut microbiome represents a very important environmental factor that influences the development of type 1 diabetes (T1D). We have previously shown that MyD88-deficient non-obese diabetic (MyD88−/−NOD) mice, that were protected from T1D development, had a different composition of gut microbiota compared to wild type NOD mice. The aim of our study was to investigate whether this protection could be transferred. We demonstrate that transfer of gut microbiota from diabetes-protected MyD88-deficient NOD mice, reduced insulitis and significantly delayed the onset of diabetes. Gut bacteria from MyD88-deficient mice, administered over a 3-week period, starting at 4 weeks of age, stably altered the family composition of the gut microbiome, with principally *Lachnospiraceae* and *Clostridiaceae* increased and *Lactobacillaceae* decreased. The transferred mice had a higher concentration of IgA and TGFβ in the lumen that was accompanied by an increase in $CD8^{+}CD103^{+}$ and CD8αβ T cells in the lamina propria of the large intestine. These data indicate not only that gut bacterial composition can be altered after the neonatal/weaning period, but that the composition of the microbiome affects the mucosal immune system and can delay the development of autoimmune diabetes. This result has important implications for the development of probiotic treatment for T1D.

1. Introduction

Development of type 1 diabetes (T1D) requires a genetic predisposition that interacts with environmental factors [1]. The exact nature of these environmental factors has not been clearly understood, although infection has long been thought to play a role [2]. Recent evidence suggests that gut bacteria play a role in Non-Obese Diabetic (NOD) mouse and the BioBreeding (BB) rat models of T1D and this role is also true for humans [3].

The incidence of T1D has increased over the last 40 years, in common with allergic diseases [4–6]. To account for these changes in incidence and prevalence, the "Hygiene hypothesis" or a refinement of this, the "Old Friends hypothesis" has been suggested [5,7]. This postulates that a reduction in exposure to microorganisms in the environment can lead to a failure of immunoregulation [8–10]. These "Old Friends" could either be non-pathogenic organisms, as in *saprophytic mycobacteria* [11] or *lactobacilli* [12,13], or parasitic infections, such as with helminths [14–16] that are more common in developing countries. The idea is that these organisms influence the maturation of dendritic cells, stimulating regulatory T cells and reducing pathogenic effector cells [10]. In addition to the possible effect of increasing tolerance and/or bystander suppression, there may also be other mechanisms of importance.

It is interesting that the BB rat, the main rat model of T1D, was originally derived in germ-free (GF) conditions [17]. It was later reported that the BB rat has an abnormal intestinal barrier [18]. There are numerous studies, in both humans and animal models of human diseases, which strongly support the role of gut microbiota as an important factor in balancing health and disease. Development of inflammatory bowel disease (IBD) is influenced by gut microbiota as most, if not all, of the experimental IBD animal models are disease free if they are housed in GF conditions. There is an increasing public interest in probiotic compounds as an alternative medicine. Probiotics are cultures of beneficial bacteria from the healthy gut microbiota that improve the balance of the

* Corresponding author. TAC-S141, Mail Box 208020, 300, Cedar Street, New Haven, CT 06520, USA. Tel.: +1 203 785 7186; fax: +1 203 737 5558.
E-mail address: li.wen@yale.edu (L. Wen).

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intestinal milieu by modifying the gut microbiota and suppressing inflammatory responses caused by the host immune cells in response to harmful microbes in the intestine. Recent studies have shown that oral probiotic administration prevents diabetes development in NOD mice [19]. This suggests that normal commensal microbes and their balance in the gut are extremely important for maintenance of health. In this study, we investigated the effect of gut microbiota transfer on diabetes development in NOD mice and our results suggested that transient gut microbiota transfer at a young age could have long-lasting effects on diabetes development in adulthood in the NOD mouse model of human T1D.

2. Materials and methods

2.1. Mice

NOD/LtJ mice purchased from the Jackson Laboratory were used for studying diabetes development. NOD/Caj mice were originally obtained from the Jackson Laboratory (NOD/LtJ) and have been maintained at Yale University for over 25 years. MyD88−/−NOD mice were generated as described previously [20] and have been maintained at Yale University for over 7 years. MyD88−/−B6 mice were kindly provided by Dr. Akira [21] and have been maintained at Yale University for over 10 years. B6g7 breeders were kindly provided by Drs. Mathis and Benoist (Harvard University) and have been bred at Yale University for over 10 years. MyD88−/−B6g7 mice were generated by breeding B6g7 with MyD88−/−B6 mice. C57BL/6J (B6) mice were originally obtained from the Jackson Laboratory and have been maintained at Yale University for over 5 years. All mice used in this study were kept in the same room, in specific pathogen-free conditions, in a 12-h dark/light cycle and housed in individually-ventilated filter cages with autoclaved food at the Yale University Animal Facility. The use of the animals in this study was approved by the Yale University Institutional Animal Care and Use Committee.

2.2. Antibodies and reagents

All fluorochrome-conjugated monoclonal antibodies (mAbs) were purchased from Biolegend Inc. All the reagents for detection of mouse immunoglobulins were purchased from Southern Biotech Inc. The reagents for detection of TGFβ were from R&D Systems. The reagents for isolation of bacterial DNA and pyrosequencing were from Qiagen and Roche, respectively.

2.3. Gut microbiota transfer

Fresh feces (10 fecal pellets ~150 mg) were collected from each of the following female donor mice ($n = 3–4$/strain): MyD88−/−NOD, MyD88−/−B6, MyD88−/−B6g7 and wild type B6 mice (all at 12–15 wks of age) and resuspended in 250 ml of sterile water, containing approximately 6×10^5/ml bacteria. The treated water was given to wild type female NOD/LtJ mice for 3 weeks (at ~4 wk of age, $n = 15$/group) and the freshly treated water was changed twice a week.

2.4. Bacterial DNA isolation

Total bacterial DNA was extracted from 0.25 g fecal sample using the repeated bead beating method described by Favier [22] with modifications. Briefly, 250 mg of fresh mouse fecal samples from colon were first loosened by vortex in TE buffer before Proteinase K (200 μg/ml) digestion. Repeated bead beating was done in 50% PCI solution (phenol/chloroform/isoamyl alcohol: 25/24/1) and spun after bead beating. DNA was precipitated and washed sequentially using isopropanol and 70% (v/v) alcohol, respectively.

2.5. 16S rRNA gene sequencing

The V2 region of the 16S rRNA gene was amplified from each DNA sample using a composite broadly conserved bacterial forward primer (5′-CATGCTGCCTCCCGTAGGAGT-3′) and bar-coded broad-range bacterial reverse primer (5′-TCAGAGTTTGATCCTGGCTCAG-3′) as described by Vaishnava et al. [23]. The PCR products were purified with a Qiagen gel extraction kit (Qiagen, CA). After quantification of DNA concentration by NanoDrop, each sample was diluted to a concentration of 1×10^9 molecules/μl in TE buffer and pooled. 20 μl of the pooled sample was used for pyrosequencing with GS Junior Titanium Series 454 sequencing system according to the manufacturer's instructions (Roche 454, Life Sciences Corp., Branford, CT, USA).

2.6. Microbiota classification

The sequencing data were analyzed with QIIME software [24] package (version 1.6) to assign operational taxonomic units (OTUs). After quality filtering based on the characteristics of each sequence, any low quality or ambiguous reads will be removed. Taxonomy assignment was performed at various levels using representative sequences of each OTU. Beta-diversity was calculated to compare differences between microbial communities and the data was shown as Principal Coordinate Analysis (PCoA) [24].

2.7. Gut lumen IgA and TGFβ measurement

Intestine (small and large) was harvested from the mice and flushed with 10 ml of sterile PBS. The total material was centrifuged for 5 min at 2000 rpm. The supernatant was collected and IgA (Southern Biotech) or TGFβ (eBioscience) was measured by ELISA. The results were expressed as the total IgA or TGFβ content, which was calculated by concentration per milliliter × 10 (ml).

2.8. Lamina propria lymphocyte isolation

Mouse intestine was divided into small intestine (SI) and colon. Luminal contents were washed off with sterile PBS. After removing Peyer's patches (PP), the intestine was cut longitudinally into 0.5 cm lengths. Mucus in the gut segments was washed off by gently shaking the tube. The gut segments were then transferred to a new 50 ml tube into pre-warmed HBSS and 1 mM EDTA and shaken for 20 min at 250 rpm at 37 °C, followed by further vigorous shaking for 30 s. The sample was then filtered through nylon mesh. The remaining gut tissue was further cut into small pieces and digested with 1 mg/ml collagenase D and 500 U DNAse1 in RPMI medium and incubated for 1 h at 250 rpm, 37 °C. Lamina propria (LP) lymphocytes were isolated using 40% (w/v) Percoll.

2.9. Intracellular staining

Foxp3 staining was performed using a Foxp3 staining kit (eBioscience) following the manufacturer's instructions. For intracellular cytokine staining, 10^6 cells were cultured for 5 h in the presence of 50 ng/ml PMA (Sigma), 500 ng/ml of ionomycin (Sigma) and 1 μl/ml of Golgi plug (BD Bioscience). After staining of surface markers, cells were fixed in IC fixation buffer (eBioscience) for 20 min at room temperature. After 2 washes with permeabilization buffer (eBioscience), cells were stained with anti-cytokine antibodies.