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Non-obese type 2 diabetes patients present intestinal B cell dysregulations associated with hyperactive intestinal Tfh cells

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ABSTRACT

Most current studies of type 2 diabetes (T2D) focus on obesity in the pathogenesis of the disease. However, many individuals develop T2D at non-obese body mass index (BMI) level. It is yet unclear whether certain etiological mechanisms discovered in these obese models can apply to non-obese T2D patients. In the present study, we focused on one aspect that was potentially involved in T2D development, the intestinal inflammation, and examined the difference between non-obese T2D patients and BMI-matched healthy controls. We found that non-obese T2D patients presented significantly higher levels of fecal IgG than BMI-matched controls. Compared to active Crohn's disease patients, both T2D and healthy controls presented lower levels of fecal IgG. In the mucosal biopsies, the B cells and plasmablasts from T2D patients presented a slight but significant increase in the frequencies of cells with surface IgG expression compared to those from healthy individuals. The potential mechanism resulting in increased IgG expression was then examined. The $CD4^+CXCR5^+$ T cells (Tfh) from non-obese T2D patients were highly enriched in IFN-γ-producing cells and depleted in IL-4- and IL-17-producing cells. Presence of mucosal $CD4^+CXCR5^+$ T cells significantly increased IgG production from mucosal samples. Interestingly, when stimulated with *E. coli*, a common intestinal microbe, the $CD4^+CXCR5^+$ T cells from T2D patients presented significantly higher IFN-γ expression than $CD4^+CXCR5^+$ T cells from BMI-matched controls Together, these results demonstrated that non-obese T2D patients presented a low-grade inflammation in the intestinal tract, possibly supported by bacteria-responding $CD4^+CXCR5^+$ T cells.

1. Introduction

The prevalence type 2 diabetes (T2D) is increasing worldwide (Mayer-Davis et al., 2017). T2D develops from a variety of potential causes, including predisposing genetic factors and induced defects such as muscle insulin resistance, defective insulin secretion, defective incretin hormone secretion, altered adipokine metabolism, and elevated liver glucose production (McCarthy, 2010). A low-grade inflammation with elevated IL-1β, IL-6, TNF production contributes to the maintenance of insulin resistance. Obesity caused by sedentary lifestyle and energy-rich diet is considered a major precipitating factor in the development of T2D. However, many T2D patients do not present obesity and the etiology of T2D in these non-obese individuals is yet incompletely understood (Vaag and Lund, 2007).

In recent years, the importance of intestinal microorganisms in maintaining the metabolic and immunological homeostasis in the human body is increasingly recognized (Upadhyaya and Banerjee, 2015). Gut commensals are directly involved in the digestion of food and extraction and processing of the nutrients (Tremaroli and Bäckhed, 2012). They are also potentially immunogenic and microbial translocation across the intestinal epithelium, and can induce a systemic inflammation that alters not only the immune status but also the metabolic processes (Brenchley and Douek, 2008; Le Chatelier et al., 2013; Shu et al., 2012). An increasing number of studies have identified an association between T2D development and alterations in the composition of the intestinal microbiota (Hartstra et al., 2015). Both mice and humans show decreased *Bacteriodetes*-to-*Firmicutes* ratio when the study subjects present obesity (Ley et al., 2005; Ley et al., 2006). Fecal

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microbiota transplantation has been investigated in a number of inflammatory and metabolic diseases, such as chronic intestinal infection, inflammatory bowel diseases, and cardiovascular diseases, and has demonstrated partial or full efficacy (van Nood et al., 2013; Smits et al., 2013). A double-blind randomized trial in which feces from lean donors was infused into insulin-resistant male recipients has demonstrated that recipients present significantly improved insulin sensitivity along with significantly higher microbial diversity (Vrieze et al., 2012). Current explanation for the efficacy of microbiota transplantation is that non-pathogenic commensals from lean individuals altered nutrient uptake in the recipient and dampened inflammation (Wen and Duffy, 2017).

Previous investigations on the interplay between intestinal commensals, host immune system, and T2D development are focused on obese T2D patients and obese murine models. It is yet unclear whether intestinal inflammation contributes to T2D development in non-obese individuals. In healthy individuals without intestinal inflammation, IgA is predominantly secreted by intestinal B cells/plasmablasts to contain the microbiomes, while IgG is rarely present. During an inflammation or when active inflammatory bowel diseases are present, the higher affinity IgG can be upregulated (Macpherson et al., 1996; Macpherson et al., 2008). We used this antibody signature to investigate the intestinal inflammation in non-obese T2D patients and BMI-matched healthy controls.

2. Methods

2.1. Study participants

Study participants were recruited after providing written informed consent and under a protocol approved by Changhai Hospital institutional review board. All non-obese T2D patients presented newly diagnosed and untreated type 2 diabetes mellitus with a fasting plasma glucose level of ≥ 7 mmol/l. In addition, all T2D patients and BMI-matched controls were nonsmoking non-obese (BMI < 30) adults (age ≥ 18 years). This study was double blinded. Characteristics of the healthy controls and T2D patients are presented in Table 1.

2.2. Fecal sample processing

Fecal sample was obtained from 24 T2D patients, 14 BMI-matched healthy controls, and 11 active Crohn disease (CD) patients and was stored at -80°C until use. Before extraction, the fecal material was placed in 0.01% sodium azide and 1% protease inhibitor cocktail (Sigma) in PBS at 100 mg/mL. The feces-containing tube was then vortexed at 4° C for 10 − 15 min until reaching a complete dissolution by visual checking. The tubes were then centrifuged at 16,000*g* for 10 min at 4 °C. The upper supernatant was aspirated for ELISA. The Human IgG ELISA Kit and Human IgA ELISA Kit (Abcam) was used following the manufacturer's protocols.

2.3. Mucosal biopsy processing

Mucosal biopsy was obtained at the colon 25–30 cm from the anal verge. Immediately following isolation, the biopsy was sterilized in RPMI 1640 supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, and 2 mM L-glutamine (Gibco) for 30 min at 4 °C, during which time the sample was transferred to sterile biosafety cabinets. The sample was then washed thoroughly, and digested by two enzymatic digestion steps with collagenase type 2 (Sigma), each for 45 min at 37 °C. The digestion product was filtered across a 70-μm sterile cell strainer (Corning) and used immediately in experiments. All mucosal samples were used fresh. A total of 4 out of 14 healthy controls, and 10 out of 24 T2D patients have donated mucosal samples. Due to the fact that mucosal samples had limited availability, not all samples were used in every experiment. Analyses of B cell Ig subtype composition and Tfh cell intracellular cytokine secretion were performed on all samples. 6 out of the 10 T2D mucosal sample donors provided two sequential untreated biopsies, and their samples were used in depletion of $CD4^+CXCR5^+$ T cells and neutralization of IFN-γ experiments. Samples from all 4 healthy mucosal samples and 7 out of 10 T2D mucosal samples were used in *E. coli* stimulation experiments.

Table 1
Characteristics of the study subjects.

	Healthy (n = 14)	T2D (n = 24)	P
Age (y)	48 (30–60)	47 (34–58)	> 0.05
A1c (%)	NA	8.5 (6.6–12.9)	> 0.05
BMI (kg/m^2)	23.8 (21.1–26.5)	24.1 (22.0–26.7)	> 0.05
Glucose (ml/dl)	NA	178 (136–270)	> 0.05
Sex (M/F)	10/4	18/6	> 0.05
Ethnicity (% Chinese)	100	100	> 0.05

Values are presented as median (range). NA: not applica

2.4. Flow cytometry

To measure surface Ig expression by intestinal B cells, mucosal cells were labeled with LIVE/DEAD Fixable Violet (Invitrogen) and anti-human CD19, CD20, IgG, and IgA antibodies (BioLegend) immediately following isolation. To measure $CD4^+CXCR5^+$ T cell cytokine secretion, mucosal cells were stimulated with ImmunoCult Human CD3/CD28 T cell Activator (StemCell) for 5 h with 5 μg/mL brefeldin A and 5 μg/mL monensin (BD). Cells were then labeled with Fixable Violet and anti-human CD3, CD4, and CXCR5 antibodies (BioLegend). After removal of excess antibodies, cells were incubated in CytoFix/CytoPerm (BD) for permeabilization, and labeled with anti-human IFN-γ, IL-4 and IL-17 antibodies (BioLegend). Samples were acquired using the FACSCanto (BD) system.

2.5. $CD4^+CXCR5^+$ T cell depletion and bacterial stimulation

Mucosal cells were first with anti-human CD4 and CXCR5 antibodies, and were then processed in a FACSAria (BD) system for $CD4^+CXCR5^+$ depletion or mock sorting. 2 μg SEB (Sigma) was then added per 10^5 mucosal cells for 48 h. Supernatant IgG was examined by ELISA. Human extracellular IFN-γ was inhibited using rabbit-anti-human IFN-γ polyclonal IgG (ab25101; Abcam) at 5 μg/mL. Matching concentrations of Rabbit polyclonal IgG (ab171870; Abcam) were used as isotype control. *E. coli* (ATCC 9637) was added to mucosal cells at MOI of 20:1 for 24 h at 37° C, and 5 μg/mL brefeldin A and 5 μg/mL monensin was added for the last 5 h.

2.6. Statistical tests

Kruskal-Wallis test, 2-way ANOVA followed by Tukey's multiple comparison test, Mann-Whitney test, and Wilcoxon matched-pairs test were applied accordingly, as specified in the Figure Legend. Prism software was used to calculate the p values, of which < 0.05 was considered significant.

3. Results

3.1. Type 2 diabetes (T2D) patients presented higher IgG inflammation than BMI-matched controls

Non-obese T2D patients (N = 24) and BMI-matched healthy controls (N = 14) were recruited. The immunoglobulin (Ig) composition in stool samples was analyzed. The total IgG level in stool was significantly higher in T2D patients than in healthy controls (Fig. 1). An upregulation of intestinal IgG secretion is generally associated with the presence of gut mucosal inflammation. For comparison, we also examined stool Ig from patients with active Crohn's disease (CD; N = 11),