Presence of Segmented Filamentous Bacteria in Human Children and Its Potential Role in the Modulation of Human Gut Immunity

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Segmented filamentous bacteria (SFB) are commensal organisms that grow by anchoring a specialized holdfast structure to the intestinal walls of a variety of animals. Interaction of SFB with Peyer’s patches in mice promotes the post-natal maturation of the immune system. We previously reported that the colonization of SFB in humans mainly occurs by 36 months of age, and is difficult to be detected afterward. In this study, we measured the level of SFB in intestinal fluids of human children. SFB were found via qPCR to represent a small fraction of the whole SFB-positive microbiota ($10^5$ SFB in $10^{11}$ total bacteria). Bacteria with filamentous segmented morphology were observed in intestinal fluids via fluorescent in situ hybridization, and from gut biopsies via scanning electron microscopy. SFB-specific DNA and peptide fragments were also identified via multiple displacement amplification PCR and mass spectrometry. There was an overall positive correlation between the presence of SFB and the titer of total secretory immunoglobulin A (sIgA), which is more apparent in intestinal fluids of the age group of 8–36 months. Afterward there was a decline of SFB in numbers correlated with a reduction of total sIgA. RT-qPCR analysis of the terminal ileal biopsies revealed that the expression of Th17 pathway genes were induced in SFB-positive samples, while the markers of T and B cell receptor signaling pathways were also upregulated. Collectively, these data suggest that SFB is a rare member of microbiota, and may play an important role in the development of human gut immunity.

Keywords: SFB, immunity, S Ig A, Th17 cells, ileum microbiota
INTRODUCTION

Recently, segmented filamentous bacteria (SFB), or Candidatus Savagella (Thompson et al., 2012), have been implicated in the modulation of the host immune system (Ivanov and Littman, 2010). Due to the intimate association with the intestinal epithelium, SFB show the rare ability to induce post-natal maturation of virtually all immune system components. In particular, SFB stimulate differentiation of Th17 cell lineage and T-cell responses in mice (Ivanov et al., 2009). Also, enhanced production of secretory immunoglobulin A (sIgA) was present in SFB-colonized mice (Schnupf et al., 2013). SFB’s ability to induce gut maturation suggests a role in autoimmune diseases such as encephalitis and arthritis. Recent work indicated that co-culture with host cells is essential for cultivation of mouse SFB in vitro (Talham et al., 1999), but elucidation of SFB function still relies on this mono-associated mouse model.

Segmented filamentous bacteria have been documented to colonize the guts of various vertebrate animals, including fish, pigs, chicken, mice, and rats (Klaasen et al., 1992), and also display host specificity (Tannock et al., 1984). We previously reported that SFB exist in human feces in an age-dependent manner (Yin et al., 2013). Jonsson (2013) using PCR, also detected SFB in human ileostomy samples and Caselli’s group observed morphological SFB-like Gram-positive bacteria in histological slides of ileo-cecal valves from ulcerative colitis patients (Caselli et al., 2013). Given these pieces of evidence, humans may harbor SFB; however, direct evidence of human SFB is lacking and no other SFB genes, apart from the 16S rRNA gene, have been reported in human specimens. Furthermore, bioinformatic searches of human SFB genes in the Human Microbiome Project (HMP) database and other human metagenomic databases have been negative.

Here, we report that human SFB are present in both the Chinese and US populations. The qPCR and metagenomics analyses of the luminal fluids of children revealed the levels of SFB were extremely low in gut microbiota. Similar to the effect from mouse SFB studies, an enhanced human immune response was observed in the SFB-positive individuals by comparing total sIgA production in the terminal ilea. In addition to the Th17 pathway genes, we found SFB colonization of human terminal ileum is associated with the activation of T and B cell receptor (BCR) signaling pathways.

RESULTS

The Concentrations of SFB Are Within a Narrow Range, and Higher in Children Between 21 and 36 Months of Age Relative to Other Age Groups

To investigate the SFB prevalence in American children, we collected a total of 54 fecal samples from 5 day to 15-year-old children. DNA was extracted from the fecal materials and the presence and distribution of SFB was detected via PCR as previously reported (Yin et al., 2013). Children younger than 3 years old showed 68.29% positive identification of SFB in a total of 41 samples (Table 1). When age increased to 6 years old, the percentage of positive samples decreased to 50%. However, SFB could be detected in children up to 15 years of age. Additionally, from December 2012 to November 2014, 144 individuals in Hangzhou, China (80 males, 64 females; 2 months to 178 months-of-age) were enrolled in our SFB study (Supplementary Table S1). Luminal fluids from the junction between the cecum and ascending colon were collected via a standard endoscopic procedure. Biopsy specimens from the terminal ilea were also obtained in 54 patients with no apparent clinical symptoms for histopathological examination, scanning electron microscopy (SEM), and/or gene expression profiling (Supplementary Table S1). All samples were first screened for SFB. Sample selection was random within group, and all the human subjects were outpatients free from the inflammation such as IBD or Crohn’s disease but with abdominal pain when admitted.

The luminal fluids were used for the extraction of the microbiota DNA. These DNA samples were tested for the quantification of total bacteria and SFB via qPCR (Supplementary Table S3). We estimated the detection limit of the PCR and qPCR methods was $10^4$ CFU/ml of SFB in an average microbiota of $10^{11}$ CFU/ml of total bacteria (Figure 1). Therefore, the samples below this threshold were considered as SFB-negative. In all SFB-positive samples, the level of SFB was quantified to be between $10^4$ and $10^6$ CFU/ml, but a higher range of concentrations ($10^3$–$10^6$ CFU/ml) were found in the age group between 21 and 36 months (Figure 1). As the ages increased to 36 months, the concentration dropped to $10^4$–$10^5$ CFU/ml or below the threshold for detection.

Identification of Bacterial Structure Similar to SFB With Attachment to the Human Intestinal Walls

To examine the possible interaction of SFB-like structures with intestine, we used two methods, SEM of tissue biopsies and FISH of intestinal fluids using SFB-specific probe. SEM of samples collected from the terminal ilea showed a filamentous shape with variable lengths with a distinct structure that formed septa between two daughter cells (Figures 2a,b). However, we did not observe typical holdfast segments. Previously, SFB were identified by their unusual morphology in the terminal ilea of mouse, rats, chicken, fish, and pigs with filamentous shapes (Klaasen et al., 1993a). Our SEM results of SFB morphology are in agreement with previous observations.

| Table 1: Distribution of SFB in fecal samples of US children. |
|-----------------|-----------------|-----------------|-----------------|
| Age (months)    | Sample (n)      | SFB positive (n) | SFB* (%)        |
| 0–36            | 41              | 28              | 68.29           |
| 37–72           | 4               | 2               | 50              |
| 73–108          | 2               | 0               | 0               |
| 109–181         | 6               | 2               | 33.33           |
| Total           | 54              | 34              | 64              |
*Detected by PCR with SFB specific primers 779F/1008R.
Segmented filamentous bacteria morphology in the luminal fluids was also analyzed via FISH (Supplementary Table S2). The SFB probe specificity was previously confirmed (Yin et al., 2013). Bacteria that hybridized positively with the SFB probe had varied morphology; for example, both long typical filamentous shape with segments (Figures 2c–f) and short rods (Figures 2g,h) were observed in the gut luminal fluids of SFB-positive individuals. Both structures were found in healthy individuals as indicated in Supplementary Table S1.

Identification of Human SFB-Specific DNA and Protein Fragments in Human Intestinal Fluids

Despite the availability of mouse and rat SFB genomes, the only sequence of human SFB in NCBI database is a partial sequence of the 16S rRNA gene. In order to detect the human SFB-specific DNA, we developed a simple bioinformatics approach. Briefly, all SFB conserved genes were identified by sequence homology of SFB-rat-Yit genes to all other 10 SFB genomes in database. Genes that had some homology to any available genomes of the Clostridium spp. were subtracted. Among the 441 SFB conserved genes, 19 top ranking genes were selected for PCR analysis based on their conservation and primers were subsequently designed using Primer3 software (Table 2). The DNA was subjected to the enrichment via multiple displacement amplification (MDA). Using this enriched DNA as template, we performed PCR with the primers as listed in Supplementary Table S2. As shown in Table 2, PCR products generated by SFB33013 and R49716 belonged to two non-16S genes of Candidatus Savagella by comparison to rat and mouse SFB genomes, which encode the homologs of the penicillin-binding protein 2 and the flagellar basal-body rod protein FlgG, respectively. We next used two SFB-positive ileal fluids to test whether the SFB-specific protein fragments can be detected. As shown in Table 3, a total of 18 SFB-specific protein fragments were identified by mass spectrometry.

SFB Is Correlated With Total sIgA Concentration in Luminal Fluids of Human Terminal Ilea

Segmented filamentous bacteria have recently been identified as an important member of commensal bacteria that can manipulate the host immune system, particularly by inducing strong and broad IgA responses (Schupp et al., 2015). Monoassociation of germ-free mice with SFB colonization increased not only the production of SFB-specific IgA, but also induced non-specific total IgA production equivalent to that of the conventional mice (Klaasen et al., 1993b). Thus, we measured sIgA with ELISA in the luminal fluids (n = 47). Samples were divided into SFB-positive and -negative groups based on PCR performed on age-matched specimens (Supplementary Table S1). We observed higher titers of sIgA in SFB-positive samples (118.60 ± 6.65 ± 32.23 μg/ml, median ± SEM n = 23, median age: 33.81-month-old) vs. SFB-negative samples (69.21 ± 3.87 ± 23.91 μg/ml median ± SEM, n = 24 median age: 34.45-month-old); (p < 0.0001) (Figure 3A). The relationship between the level of sIgA to the ages of these children were analyzed, and a negative trend was noted in Figure 3B. It seems that the majority of elevated levels of sIgA was clustered to the group before the age of 36 months. Surprisingly, we also noticed that IgA decreases with age in the gut independently of whether there is or not SFB, which is particularly obvious after 36 months of age.

SFB Numbers Are Correlated With the Expression of Th17 Pathway Genes in Human Terminal Ilea

Previously, SFB colonization in the small intestine of mouse can induce the differentiation of naïve T cells into Th17 cells (Gabriels-Routhiau et al., 2009; Ivanov et al., 2009). The cytokines and transcriptional factor that promote such differentiation include IL-23, IL-17, TGF-B, and ROR-γ (Betelli et al., 2006; Ivanov et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). To identify the effects of SFB colonization on the expression of human Th17 pathway genes, we extracted mRNA from a total of 22 human biopsies (SFB positive samples n = 11 vs. SFB negative samples n = 11) without any clinical inflammation and gross pathology. The qRT-PCR was performed to assay the gene expression of Th17-related pathway markers. The results in Figure 4 demonstrates that IFN-γ, IL-10, IL-17, foxp3, Tgf-β, rtg-γ, and TNF-α expression was significantly enhanced in SFB-positive samples relative to SFB-negative samples, indicating the expression of Th17 pathway genes likely were affected in SFB-positive human ileal samples. We also measured expression serum amyloid A (SAA) and galactoside 2-alpha-l-fucosyltransferase 2 (Fut2), because both are epithelial genes and SAA production is regulated by host IL-17 (Anthony et al., 2013; Conti and Gaffen, 2015). Figure 4B shows that SAA and Fut2 expression was significantly different between SFB-positive and SFB-negative specimens. Collectively, these data show that SFB colonization in human ileum likely affects...
FIGURE 2 | SEM and FISH micrographs of human ileal biopsies. The specimens were processed for the observation of microbiota by SEM and FISH as described in the Section “Materials and Methods.” Samples processed for SEM were labeled in the SEM column in Supplementary Table S1. Terminal ilea of patient 4 (a,b) observed under SEM with magnification of 2,300 and 470, respectively. SFB-like structures as visualized with DAPI staining (c,e) and hybridization with SFB-specific probe (d,f) of patient 4 and 20 (long filamentous shape) observed under epi-fluorescent microscope with magnification of 1,000; SFB by DAPI staining (g) and hybridization with SFB-specific probe (h) of patient 22 (short rod shape).

differentiation and maturation of multiple CD4+ T helper subsets.

SFB Colonization in Human Terminal Ilea Is Associated With Activation of T and B Cell Receptor Signaling Pathways

To investigate SFB–host interactions and the host response to SFB, we compared the expression profiles in terminal ileal mucosa from three SFB-positive and three SFB-negative age-matched individuals. All these tissue biopsy specimens were collected from the location where clinical inflammation and gross pathology are absent but have abdominal pain. They micro-array slides used for hybridization contain roughly 42,545 GenBank accessions (GSE68425). When we performed two group comparison (SFB positive samples \( n = 3 \) vs. SFB negative samples \( n = 3 \)) using the standard pipeline Agilent Genespring GX, only six out of 42,545 genes entitled satisfying corrected \( p \)-value cut-off 0.05 (Supplementary Table S3). These six samples are from age closely matched, healthy individuals (Supplementary Table S1, the chips column). This suggests that there are significant variations in the gene expression profiles within group. However, when comparing person to person, metallothionein (MT)-Heavy Metal, T cell receptor (TCR) and BCR signaling emerged as significant pathways in most of comparisons (Supplementary Table S4), suggesting the samples of the human terminal ileal
The presence of SFB in the human gastrointestinal tract is debatable, although Jonsson (2013) and Yin et al. (2013) showed
SFB can be detected in the intestinal microflora. However, several genome-wide attempts to identify the SFB sequences have failed so far despite a large number of human intestinal metagenomes being interrogated (Prakash et al., 2011; Sczesnak et al., 2011). One of the main reasons is due to the very low amount of SFB in the microbiota. Lagier et al. (2015) reported that in a complex ecosystem that consists of $10^{12}$ bacteria, current metagenomic studies are unable to detect bacteria at the level of $10^5$ bacteria. Indeed, we didn’t identify SFB-like sequences from 44.28G metagenomics sequences of the microbiota. The amount of SFB, as an immune-modulator of the small intestine in mice, is tightly regulated. There is a balanced SFB-containing microbiota in the gut of normal mice. The overgrowth of SFB in mice has been linked with autoimmune diseases (Van Praet et al., 2015). Overgrowth has also been shown through the disruption of the immune pathways such as the knockout of IL-17 receptor or the suppression of the IL-17A and neutrophil in the gut (Kumar et al., 2016). These observations are consistent with current observation. Interestingly, in humans, the peak of SFB abundance coincides with a period of 21–36 months of age, which is thought
to be important for the post-natal development of the immunity (Flannigan et al., 2016). However, in our study, we didn’t observe the typical holdfast morphology through SEM. Transmission electron microscope (TEM) may provide much better resolution for visualization of this specialized structure connecting to the host epithelium.

The percentage of SFB positivity in American children before 36 months of age was 68.3 % (Table 1), which is higher than 46.1% from the same age group of Chinese children (Yin et al., 2013). This may be due to difference in where these samples are collected. The samples of our previous study came from a daycare setting while the samples of the current study are all from outpatients. Additionally, we identified two American children with SFB colonization whose ages are up to 15 years old. But due to the small sample size, this number may not truly reflect the actual percentage of SFB colonization in this age group. We can also not rule it out whether these two patients may have underlying intestinal diseases. However, in our previous survey, we noted the SFB colonization can occur in young adult group (Yin et al., 2013).

We and others previously used 16S-derived primers to detect human SFB. Since bacteria normally have multiple copies of rRNA genes in the genome, the advantage of using 16S primers is they facilitate detection of the low-concentration target such as SFB. However, the problem is the 16S rRNA genes are highly conserved. It is not clear how specific these primers are for SFB. To circumvent these problems, we identified two specific genes for human SFB (Table 2). These genes showed almost identical sequences to mouse SFB homologs. Together with the proteomic data from the luminal fluids of human ilea, we conclude that human SFB exist in the intestinal mucosa. To identify these non-16S rRNA genes, we used an MDA procedure to enrich human SFB. Since bacteria normally have multiple copies of the enzyme to make sIgA has a selective overgrowth of SFB in gut microbiota (Jiang et al., 2001). However, it has been reported that sIgA have a stimulatory effect on the presence of SFB (Klaasen et al., 1993b), which supports our observation about the association of an elevated level of sIgA with a high number of SFB during the neonatal development. Pathway associated with B-cell receptor activation were up-regulated in the SFB-positive samples relative to the SFB-negative samples, suggesting that SFB colonization is involved not only in T cell, but also B-cell development.

The induction of T and B cells by SFB colonization is a hallmark for previous SFB studies, particularly for the induction of Th17 cell proliferation in mice (Ivanov et al., 2009) SFB adhesion to the intestinal epithelial cells is considered to be integral for the induction of Th17 activation (Atarashi et al., 2015). In the current study, we observed attachment of SFB-like filamentous bacteria through SEM of biopsies to the surface of distal ileal epithelial cells. However, our FISH results also showed that SFB can exhibit a rod-like structure in the luminal fluids without attachment to the epithelium. This rod shape of SFB has an apparent relationship to pathology. Additionally, we documented markers suggestive of an increased T and B cell response when human ileal samples were colonized with SFB. A range of genes, including TNFa, IL-10, CXCL10, INFγ, CD3E, Foxp3, GABBR2, and FCAMR shown to be up-regulated in mice by the colonization of SFB, were also up-regulated in the current study with human intestinal SFB-positive ileal biopsies, suggesting that human SFB plays a similar role in the immune stimulation (Ivanov et al., 2009). Meanwhile, it is interesting to note that the expression level of IL-17 production is significantly elevated in SFB-positive ileal biopsies that are in line with the mouse data, implying a similar pattern of immune response development shared by mouse and human in response to SFB colonization.

In conclusion, SFB are present in human intestinal fluids as a low fraction of the whole microbiota. Identification of SFB-specific DNA and peptide fragments in human ileal fluids provides additional evidence that human gut harbors SFB. Previous published data indicate that SFB triggers the post-natal maturation of the intestinal immune system in mice (Ivanov et al., 2009; Schnupf et al., 2013; Atarashi et al., 2015). A similar trend in the total sIgA and Th17 pathway marker expression in SFB-positive individuals suggests that the immune stimulation pathways by the colonization of SFB may be conserved between mice and human. It is also possible that the altered levels of immunological markers as observed in this study are due to other bacteria other than SFB since the composition of two microbiotas with/without SFB is different. Since SFB is only a minority member of the human gut microbiota, its role in the...
activation of inflammatory pathways in the human gut needs to be further investigated.

MATERIALS AND METHODS

Colonoscopy and Sample Collection

Both studies were approved by the Committee of Ethics at the Children’s Hospital, Zhejiang University School of Medicine, and Marshall University Joan C. Edwards School of Medicine. All patients were informed ahead of time of all possible outcomes and adverse events following such a procedure and their samples would be used in this study in a de-identified manner. After bowel preparation, patients were given 10% chloral hydrate (0.5 ml/kg, po) and sedation with midazolam (0.1–0.2 mg/kg, iv) 30 min before the colonoscopy. Luminal fluids at the cecum and ascending colon were collected and several specimens from terminal ilea were obtained when the colonoscope was extended to the distal ileum for routine histopathological examination. Tissue specimens were immediately frozen in liquid nitrogen and stored at −80°C for further analysis. The sample information was listed in Supplementary Table S1.

Sample Selection

Endoscopic biopsy samples weighted around 10–15 mg per piece, with 2–3 pieces acquired from each patient, and combined weight of these species generally less than 50 mg per sample. First, we performed PCR screening for SFB in all 144 samples. To confirm the presence of SFB by SEM and FISH, we tested 36 SFB positive samples based on the availability of sample materials.

For CHIPS: Since SFB were detected in 6–36 months more frequently, we choose three SFB positive and three negative samples randomly from these children. For measurement of sIgA: 23 samples chosen by random from 36 SFB positive samples, and 24 samples randomly chosen from SFB negative samples. Again, this was done based on the amount of sample materials available.

mRNA: 36 SFB positive samples, and 11 samples all randomly chosen from SFB negative samples.

QPCR and non-16S gene: 24 randomly chosen from 36 SFB positive samples.

Meta sequence: Seven samples randomly chosen from 36 SFB positive samples, and four randomly chosen from SFB negative samples.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to view 0.2 mm × 1 mm samples from the terminal ilea. Biopsies were fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer, and processed as described in the literature (Liao et al., 2012). Biopsies of terminal ilea obtained from endoscopy were immediately fixed in 4% glutaraldehyde-cacodylate buffer, dehydrated and processed as described in the literature (Gaboriau-Routhiau et al., 2009; Liao et al., 2012). Samples were observed under an H-9500 Hitachi SEM microscope at 20 kV after coating samples with gold-palladium film.

FISH Procedure

Luminal fluids were centrifuged at 12,000 × g and pellets were fixed in 4% paraformaldehyde solution as previously described (Liao et al., 2012). Samples were stored in 50% ethanol at −20°C and fixed bacteria were spotted onto glass slides and allowed to air-dry for 1 h. An aliquot of 20 µl hybridization solution (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS, pH 7.2) containing 5 ng SFB-specific oligonucleotide probe (Supplementary Table S2) was spotted onto the air dried cells and incubated overnight at 50°C (Zarda et al., 1991). After slides were immersed in washing solution (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS, pH7.2) at 48°C for 15 min, slides were briefly rinsed with sterile water, air-dried, and then stained with 4',6-diamidino-2-phenylindole (DAPI) for 15 min. After washing with 80% ethanol and sterilized water, air-dried bacteria cells were observed and photographed using an Olympus epi-fluorescent microscope CX-51 (Olympus Corporation, Japan).

DNA Extraction and PCR Amplification With SFB Specific Primers

Bacterial genomic DNA from luminal fluid was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer’s instructions (Qiagen, German). DNA was quantified using a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, United States), and its integrity and size was confirmed by agar gel electrophoresis (1.0%). Oligonucleotides targeted to the SFB 16S rRNA gene region (779F/1008R, Supplementary Table S2) were used as PCR primers. Standard PCR was performed as described in the literature (Yin et al., 2013). Amplification products were separated using TAE agarose gel electrophoresis (1.0%) and bands were visualized under UV light.

Quantification of SFB in Luminal Fluids by qPCR

Quantification of bacterial DNA was performed using an ABI PRISM 7500 Real-Time PCR Detection System (Applied Biosystems) according to the manufacturer’s instructions. A 20 µl amplification reaction was performed with 10 µl Thunderbird SYBR quantitative PCR (qPCR) Mix (Toyobo Co., Ltd., Osaka, Japan), 0.04 µl 50 × ROX reference dye, 0.5 mM of each primer, 1 µl DNA template (20 ng/µl), and distilled water. Amplifications were performed as follows: one cycle at 95°C for 1 min, 40 cycles at 95°C for 15 s, at appropriate annealing temperatures for 35 s and 72°C for 35 s. Fluorescence was measured after the extension phase of each cycle. Melt curve analyses were performed by slowly heating the PCR mixtures from 55 to 95°C. These served as end point assays and were used to confirm PCR specificity. Primers for total number of bacteria and SFB were selected for quantification and the sequences are presented in Supplementary Table S2. The quantitative measurement of unknown samples was achieved using standard curves made from known concentrations of plasmid DNA containing the respective amplicons for each set of primers (Fite et al., 2004).
DNA extraction, Library Construction, and Sequencing

The luminal fluid sample DNA libraries were constructed using Illumina TruSeq DNA Sample Prep Kit according to the manufacturer's instruction. Illumina TruSeq PE Cluster and SBS Kit were used to perform cluster generation, template hybridization, isothermal amplification, linearization, blocking and denaturation and hybridization of the sequencing primers. Paired-end sequencing 2× 100bp was performed to sequence all libraries. The base-calling pipeline (Casava 1.8.2 with parameters—use-bases-mask y100n, I6n, Y100n, – mismatches 1,–adapter-sequence) was used to process the raw fluorescent images and all sequences (Qin et al., 2015).

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure total sIgA concentrations in the luminal fluid, approximately 500 µl samples were centrifuged for 20 min at 1,000 × g at 4°C. Collect the supernatant and carry out the assay immediately. The 96 plates were washed in PBS and blocked in PBS with 1% BSA. Samples were diluted 1/10, and a twofold serial dilution was made. Samples were incubated at room temperature for 2 h. Total sIgA was quantified by ELISA using a human secretory IgA ELISA detection Kit (Elabscience, Wuhan, China). The measurement was taken according to the manufacturer's instructions.

RNA Extraction and Gene Expression Analysis

Biopsy specimens of terminal ilea were lysed in Trizol (Life Technologies, Carlsbad, CA, United States) according to the manufacturer’s instructions and total RNA was extracted and purified by using RNeasy mini Kit (Qiagen, GmbH, Germany). RNA samples of each group were then used to generate fluorescent-labeled cRNA targets for the Agilent Whole Human Genome Oligo Microarray (4 × 44K). Labeled cRNA targets were then hybridized with the slides and slides were scanned on an Agilent Microarray Scanner (Agilent technologies, Santa Clara, CA, United States). Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, United States). Raw data were normalized using a quantile algorithm (GeneSpring Software 11.0, Agilent technologies, Santa Clara, CA, United States) and subsequently adjusted for multiple testing by the Benjamini and Hochberg false discovery method (*p < 0.05*). Microarray experiments were performed using an Agilent Technologies Inc.

Bioinformatics Analyses for Microarray Assay

Ratios were calculated between SFB-positive and SFB-negative patients. Genes with at least a twofold change were selected for further analysis and selected genes were grouped in functional categories using the standard pipeline Agilent Genespring GX and processes were selected based on *P*-values smaller than 0.05.

Quantitation of Gene Expression With Real-Time PCR

RNA was purified from intestinal tissues and RT-qPCR was performed with either the TaqMan gene expression assay with TaqMan Universal PCR master mix (Applied Biosystems) or human-specific primers with SYBR-Green PCR master mix (Applied Biosystems). The cd3e, cxcl10, IFNγ, IL-10, IL-17, TNFα, foxp3, Fut2 and SAA primer sequences are listed in Supplementary Table S2. cDNA samples were assayed in duplicate and gene expression for each sample was normalized relative to actin with a 2−ΔCt calculation.

Bacterial Protein Digestion and SFB Peptide Identification

Ileal wash fluids from two individuals were used for bacterial protein extraction. After gel electrophoresis, five protein bands with molecular weight ranging from 40 to 50 kDa were excised for LC-MS analysis. In-gel digestion, peptide extraction and desalting were performed as described previously (Shevchenko et al., 1996; Rappsilber et al., 2007). The eluted peptides were dried and dissolved with sample loading buffer (1% ACN and 1% formic acid in water) for MS analysis. The peptide identification was performed on a Q Exactive Exact mass spectrometers (Thermo Fisher Scientific, San Jose, CA, United States) equipped with a Ultimate 3000 ultra-performance liquid chromatography (UPLC) system (Thermo Fisher Scientific, San Jose, CA, United States). The raw peptide data were submitted to Sequest HT server using Proteome Discoverer software (Thermo Fisher Scientific, San Jose, CA, United States). Local database were builded by downloaded mouse SFB proteins only were considered as SFB unique peptide.

Data and Materials Availability

All mRNA sequences used were deposited in the National Center for Biotechnology information Gene Expression Omnibus with accession number GSE68425. The raw Illumina read data of all samples has been deposited in the NCBI Sequence Read Archive under accession numbers SRP069299.

Statistics

Significance was scored with an unpaired Student’s two-tailed *t*-test unless otherwise noted. *P*-values are presented in figures and tables. To compare the difference of sIgA level between

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1http://www.uniprot.org/
2https://blast.ncbi.nlm.nih.gov/Blast.cgi
SFB-positive and SFB-negative groups, non-linear fit curves were introduced into the data using GraphPad Prism 6.

**AUTHOR CONTRIBUTIONS**

BC, HC, YY, XS, and JL performed the experiments. LC, KP, WG, HZ, YE, and LJ collected samples. FX, JQ, JS, and LL conducted data analysis. HY, MJ, XW, and CX contributed to the design of experiments and composition of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01403/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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