INTRODUCTION

Studies have shown that gut microbiota plays an important role in the maintenance of immune function in the body. However, the abuse of antibiotics disrupts the normal balance of gut microbiota, causing an imbalance of pro-inflammatory and anti-inflammatory factors in gut-associated lymphoid tissues, which reduces both innate and adaptive immune responses in the body and induces the development of pulmonary diseases such as asthma.

As important immune recognition mechanisms in the body, Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) play key roles in the recognition of invading microorganisms, activation of innate immune system, and exertion of anti-viral activities. Our previous study demonstrated that antibiotic-induced dysbacteriosis in mice inhibited expression levels of mRNA and protein in TLR7 signaling pathway, causing increasing levels of T cell associated factors, IL-4 and IL-10, and decreasing levels of IFN-γ and IL-17. After treatment with probiotics, the IL-4 and IL-10 levels decreased significantly, while the IFN-γ and IL-17 levels increased. Administration of probiotics increased the expression levels of genes and proteins in TLR7 signaling pathway, indicating that the balance of gut microbiota has an important function in the maintenance of the recognition mechanism of TLR7 signaling.

This study used different antibiotics to establish different animal models of dysbacteriosis. On this basis, mice were infected with influenza virus type A H1N1, and the expression levels of TLR7 and RLRs immune recognition pathways were analyzed after dysbacteriosis. The results provide evidence to elucidate the importance of the balance of gut microbiota in the maintenance of immune recognition in the body.
with influenza viruses to establish an animal model with combined dysbacteriosis and influenza virus infection. The influences of gut microbiota on the immune recognition by TLR7/RLRs in the body after viral infection in the lung were analyzed in order to investigate the mechanisms for maintenance of immune recognition in the body by the balance of gut microbiota.

MATERIALS AND METHODS

Experiment grouping and model establishment

Seventy-two SPF BALB/c mice (Guangdong Medical Laboratory Animal Center) were randomly divided into 4 groups of 12, denoted as control group (C), virus infection model group (V), dysbacteriosis model group (D), and dysbacteriosis and virus infection group (D+V). The control group received no treatment. 20%LD50 FM1 virus (A/FM1/1/47, provided by the Department of Immunology and Microbiology at the Medical College of Jinan University) was used to establish the virus-infected mouse model in the group V by intranasal infection for 4 days beginning at the 14th day of the experiment. The intestinal dysbacteriosis mouse models were established by oral administration of three different antibiotics for 14 days in the group D and group D+V. The doses used for this experiment were 0.45 g/kg body weight of metronidazole and 0.30 g/kg body weight of cefradine and neomycin. The dysbacteriosis mice model were administered for 14 days, as reported in our previous work [1]. Furthermore, mice in group D+V were also infected with FM1 virus following the methods described earlier. All experiments were performed in accordance with the Guidelines for Animal Experiments Committee of Jinan University.

Bacterial colony plate counts

Ten-fold serial dilutions of a 1 g sample from the cecum were performed on day 18 and then plated onto Pfizer Selective Enterococcus Agar, Violet Red Bile Agar-MUG, MRS Agar, and BBL Agar (Huankai Microbiological Detection Company, Guangzhou), respectively, for the cultivation of Enterococcus, Bacillus coli, lactic acid Bacillus, and Bifidobacteria. Anaerobic agar was used for the anaerobic bacteria plate count. The Enterococcus, Bacillus coli, and lactic acid Bacillus plates were incubated at 37°C for 24 h under aerobic conditions, whereas the anaerobic bacteria and bifidobacteria plates were incubated at 37°C for 48 h under anaerobic conditions (AN0035, AnaeroGen sachet, Oxoid AnaeroGen System, Oxoid). Plate counting was then conducted, and the growth of each bacterial type was quantified by log CFU/g.

Semiquantitative real-time polymerase chain reaction assays and western blot

Total RNA was isolated from lung tissue using Beyozol reagent (Beyotime Biotechnology, Haimen), precipitated, and then reverse transcribed with a Reverse Transcription Kit (Tiangen Biotech Co. Ltd, Beijing). The GAPDH was used as an internal control. All primers used in this experiment were synthesized by Generay Biotech Co., Ltd., Shanghai. All proteins were quantified by way of western blotting as described in our previous work [2]. The TLR7 and RLR pathways’ antibodies were from CST Company (Boston, USA) and had a dilution of 1:1000, the GAPDH was from TransGen Biotech Beijing, and the secondary horseradish peroxidase–labeled antibody (1:10,000) was from Proteintech Group, Chicago, IL. Finally, enhanced chemiluminescence from Beyotime Biotechnology (Haimen) was used to identify the immunoreactive bands.

Statistical analysis

Data analysis was performed in SPSS for Windows (version 19). The significance of differences between groups was evaluated using one-way ANOVA, and the differences were considered statistically significant at P< 0.05. All values are represented as the means ± SEM.

RESULTS

Bacterial colony plate counts

The treatments with three different antibiotics could reduce the number of dominant microbiota as reduced the counts of Lactobacillus, Bifidobacteria, Enterobacteria, and Enterococcus (P<0.05). Viral infection of the lungs also reduced the numbers of Enterobacteria, Enterococcus, Bifidobacteria, Lactobacillus, and total anaerobic bacteria in group V. Compared to the group D, the numbers of Enterococcus, Lactobacillus, and Bifidobacteria after combined dysbacteriosis and viral infection exhibited a continuous decreasing trend; however, only the reductions of Lactobacillus and Bifidobacteria were statistically significant (P<0.05, Figure 1).

mRNA and protein level of TLR7 and RLR signal pathways

The results indicated that after viral infection, the expression of mRNA and protein of the TLR7 and RLR signaling pathways associated with TLR7, MyD88, RIG-I, NF-κB, and MAVS exhibited a significant increasing trend with statistical significance (P<0.01). Compared to the control group, the group D exhibited down-regulation of the recognition mechanism, with TLR7, MyD88, RIG-I, and NF-κB displaying significant decreasing trends (P<0.05). MAVS 25 kDa protein also exhibited a decreasing trend (P<0.01), but 75 kDa underwent an increasing trend. Compared to group V, the dysbacteriosis combined with viral infection exhibited...
a decrease in the expression of mRNA and protein in the TLR7- and RLR-associated signaling pathways, which suggesting that dysbacteriosis could inhibit the activation of TLR7 and RLR signaling and cause a decrease in the ability to recognize pathogens. Compared to the simple dysbacteriosis model in group D, viral infection could cause a slight increase in the expression of genes associated with the TLR7 and RLR immune recognition signaling pathways in mice after dysbacteriosis; however, this trend was not significant. The expression of proteins in the TLR7 and RLR signaling pathways all showed decreasing trends, of which the trends of TLR7, MyD88, and NF-κB expression were significant (P<0.01, Figures 2-4).

Figure 1. Bacterial colony plate counts in each group from the mice cecum plated onto different selective agar. The growth of each bacterial type was quantified by log CFU/g. According to student t test, P<0.05 for a vs b.

Figure 2. Real-time RT-PCR bar graphs showing alterations of relative mRNA expression of the genes from TLR7 and RLRs signal pathway. Data were normalized to GAPDH control. Data shown are the mean of three independent experiments each performed in triplicate (mean ± SE). P<0.05 for a vs b, a vs c and b vs d in each graph by ANOVA.

Figure 3. Relative protein levels of TLR7, MyD88, RIG-1, NF-κB and MAVS were determined in the lung tissue. Protein values were determined for relative to GAPDH protein levels as a loading control. Data are presented as mean values ± SE from at least three independent experiments. P<0.05 for a vs b, a vs c, b vs d, and c vs d in each graph by ANOVA.
**DISCUSSION**

Gut microbiota are associated with the regulation of innate immunity in the body and pathogen-related pattern recognition receptors. Their dynamic balance plays important roles in the maintenance of mutualism between immune function in the body and gut microbiota [3]. Dysbacteriosis of the gut microbiota will cause a reduction in immune responses in mice, damage to the gastrointestinal mucosal barrier, dysfunction of antibody synthesis or secretion, and development of diseases in the respiratory system. Abnormal intestinal immune function usually causes an overgrowth of gut bacteria, bacterial translocation, and induction or aggravation of multiple organ failure. In this study, mice were treated with antibiotics to successfully establish an animal model of dysbacteriosis. Viral infection was then conducted to investigate the influences of dysbacteriosis on the TLR7/RLR immune recognition mechanisms in the body.

The results revealed that viral infection caused changes in the gut microbiota and reductions in the numbers of Enterobacteria, Enterococcus, Bifidobacteria, anaerobic bacteria, and Lactobacillus, which indicated that changes in the gut microbiota caused by viral infection might induce inflammatory responses in the systems related to the intestinal mucosa.

As major recognition receptors targeting pathogenic microorganisms, TLRs and RLRs could induce DC maturation and promote immune cells to release inflammatory factors to fight an invasion of pathogenic microorganisms. Toll-like receptors (TLRs) recognized PAMPs and up-regulated the expression of associated factors such as MyD88, IRAK (IL-1R-associated kinase), and TRAF6 (TNF-receptor-associated factor) through the MyD88 dependent TLR/IL-1R signaling pathway, thus inducing the activation of NF-kB [4] and release of TNF, IL-6, proIL-1β and pro IL-18, IL-12p35, and type I interferon [5,6]. The RLR family contains three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and LGP2 (laboratory of genetics and physiology-2). RIG-I recognizes the 5’-ppp ssRNA and short dsRNA of viral RNA, and MDA5 recognizes long-chain dsRNA [7]. After being recognized, viruses further interacted with MAVS (mitochondria anti-viral signaling protein, IPS-1) to cause IRF3/7 activation, type I interferon production, and NF-kB activation. TLRs and RLRs have been reported to regulate CD4+ T cell differentiation through the DC p38 MAPK and IRF signaling pathways [8].

After viral infection, the expression levels of genes and proteins in the TLR7 and RLR signaling pathways significantly increased, indicating that viral infection could induce the activation of the immune recognition receptor pathway in the body to effectively clear a viral invasion. Dysbacteriosis of the gut microbiota induced down-regulation of the TLR and RLR signaling pathways, causing the release of inflammatory factors such as TNF and IL-6 induced by NF-κB as well as the release of IL-12p35 and type I interferon induced by IRF activation, thus inhibiting the production of IL-6 and IL-10. IL-6 and IL-10 were closely associated with Th2 and Treg differentiation, indicating that down-regulation of the TLR and RLR signaling pathways induced by dysbacteriosis could inhibit Th2 and Treg activation and induce the enhancement of pro-inflammatory responses and excess immune responses in the body. The expression of RIG-I exhibited an increasing trend after viral infection, which was conductive to virus clearance from the body. Dysbacteriosis of the gut microbiota caused down-regulation of the RLR signaling pathway, which was not conducive to virus clearance, indicating that dysbacteriosis of the gut microbiota affected the progression of pulmonary diseases. MAVS is a mitochondrial antiviral-signaling protein that is present in T cells, monocytes and dendritic cells. MAVS activation induces the activation of the downstream NF-κB and IRF-3 signaling pathways to regulate the levels of inflammatory factors and the expression level of IFN-γ. This study showed that MAVS protein expression exhibited an increasing trend after viral infection, indicating that viral infection could activate MAVS expression in the body to exert its anti-virus activity. The change in MAVS after dysbacteriosis was not consistent with that of RIG-I. The combination of the aforementioned antibody array data and flow cytometry data showed that the influences of the three antibiotics utilized in the study varied. In addition, MAVS activation was associated with RIG-I or MDA5; however, MDA5 was not analyzed in this study. It was possible that MDA5 also exerted its regulatory mechanism during the process of dysbacteriosis.
This study speculated that dysbacteriosis aggravated inflammatory exudates in the lung tissues of mice after infection with FM1 viruses, thereby the dysbacteriosis of gut microbiota could down-regulate TLR7 and RLR immune recognition mechanisms in the lungs and induce an abnormal innate immune mechanism in the body. Therefore, immune response functions could not be exerted, reducing the ability to clear viruses from the lungs. And the normal gut microbiota has important significance in the maintenance of immune function and recognition mechanisms in the body.

AUTHORS’ CONTRIBUTION

YB, CJ, DL and CX conceived and designed the experiments. YB, DL, SB, WX, ZC, DC and WS performed the experiments. YB, CJ, ZL, SM, and CX analyzed the data. YB, CJ, DL, JZ and CX contributed reagents, materials and analysis tools. YB, DC, CJ and CX wrote the manuscript.

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