Probiotics Alleviates Intestinal Immune Dysfunction in A Mouse Model of Irritable Bowel Syndrome

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Abstract Background and Aims: Irritable bowel syndrome (IBS) constitutes many functional gastrointestinal disorders; the pathogenesis is unclear. Probiotics can alleviate IBS symptoms in clinic. This study aims to investigate whether administration of *Clostridum butyricum* prevents the visceral hypersensitivity and intestinal immune dysfunction induced by trinitrobenzene sulfonicacid (TNBS) in an IBS mouse model. Methods: Mice were treated with TNBS together with or without gavage-feeding *Clostridum butyricum*. On day 24, the perception of pain sensation was assessed by the colorectal distension (CRD). Macrophages and memory T cells in the lamina propria were analyzed by flow cytometry. Serum levels of T helper 2 cytokines were determined by enzyme-linked immunobsorbent assay. Results: Mice treated with TNBS showed a low-grade of inflammation in the colon mucosa. A significant increase in scores of CRD and decrease in pain threshold were recorded; the frequency of macrophage and memory T cells were significantly increased in the lamina propria mononuclear cells of the colon in the TNBS-group and a Th2 cytokine profile was detected in the serum of the mice, which was restored to the levels of naïve control group after administration of *Clostridum butyricum*. Conclusions: Twenty-four days after rectal Instillation of TNBS can induce IBS-like symptoms and immune deregulation in mice. Administration of *Clostridum butyricum* can alleviate visceral pain perception and regulate the immune dysfunction in this IBS model.
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1.Introduction

Irritable bowel syndrome (IBS) is defined as a functional disorder of the gastrointestinal (GI) tract, characterized by abdominal pain, a disturbance in bowel habit and bloating (Longstreth GF et al, 2006). The prevalence of IBS is between 5 and 20% (Hillilä MT et al, 2004 and Mearin F et al, 2001). It significantly affects the quality of life and causes excessively to use health care resources (Talley NJ, 2008). IBS is a chronic disorder; patients experience relapsing and remitting symptoms (Ford AC et al, 2008); it has emerged as a general health problem worldwide.

The etiology of IBS remains obscure although the proposed mechanisms are numerous. IBS aggregates in families (Kalantar JS et al, 2003); whether this is due to genetic factors, shares upbringing, or both is unclear. Disturbances in GI motility are thought to play a role in IBS because of studies demonstrating abnormalities in transit time in the stomach, small intestine, and colon (Stanghellini V et al, 2002). Abdominal pain in IBS is thought to be caused by a combination of visceral hypersensitivity and abnormal central pain processing. Compared with controls, IBS sufferers exhibit lower thresholds to pain induced by balloon distension of the GI tract, as well as greater spatial extents of brain activity, specifically in regions associated with pain modulation and emotional arousal, during such stimulation in functional magnetic resonance imaging studies (Tillisch K et al, 2011).

Various animal models have been established for the investigation of IBS in humans, however, there is still lack of methodologic evaluation of the animal model. Trinitrobenzene sulfonic acid (TNBS), which is the most commonly used agent for IBS animal models. In this model, physiologic and reflex responses (e.g., GI transit time, frequency and consistency of stool, visceromotor reflex), or brain responses (functional brain imaging) and so on (Qin HY et al, 2011) were adopted to evaluate the IBS-like symptoms (Holschneider DP et al, 2011). In this study, we used a comprehensive assessment to measure the IBS mice model, including physiologic and reflex responses (e.g., gastro intestinal [GI] transit time, frequency and consistency of stool, visceromotor

reflex),which could have more aspects similar to IBS patients and identification of IBS subgroups (IBS diarrhea pre-dominant or constipation predominant).

Another potential explanation put forward for the pathophysiology of IBS is immunomodulation of both the brain and motor system of the gut by a low-grade inflammatory process. This concept was first proposed 20 years ago, and in latter years it has been supported by numerous studies that demonstrate a higher prevalence of symptoms compatible with IBS in individuals with prior exposure to acute enteric infection, compared with those without such exposure (Marshall JK et al, 2010). A few studies have demonstrated increased levels of pro-inflammatory cytokines (Mearin F et al, 2009),and evidence is growing to support the notion that IBS might be a post-inflammatory and stress-correlated condition (De Giorgio R et al, 2008) and chronic gut inflammatory processes are thought to play a role in its pathogenesis (Bashashati M et al, 2012).

The intestinal microbiota plays essential role in nutrient absorption and metabolism, immune stimulation, satiety and pain. An altered composition of intestinal microbiota has been reported in IBS patients(Kassinen A et al, 2007). And its modification by probiotic diet reduces visceral hypersensitivity in experimental models of abdominal pain by modulating neural functions (McKernan DPet al, 2010). *Clostridum butyricum* (*C. butyricum*) is a strictly anaerobic endospore-forming Gram-positive butyric acid producing bacillus subsisting by means of fermentation using an intracellularly accumulated amylopectin-like polyglucan (granulose) as a substrate(Seki H et al, 2003 and Urbanska AM et al, 2010).

In the present study we have made an attempt to identify mechanisms involved in beneficial effects exerted by *Clostridum butyricum* ntervention in a model of visceral hypersensitivity induced in mice by TNBS colon perfusion by definitizing the potential relationship between the mucosal immune dyfunction and visceral sensitivity. Our results indicates that rectal instillation of TNBS was a stable useful IBS animal model for studying IBS, causes both allodynia and hyperalgesia and influences mucosal immune system function, which is known to mediates inflammation and pain. Our results also demonstrate that *Clostridum butyricum* intervention was effective in both reverting TNBS-induced visceral hypersensitivity and resetting the complex mucosal immune system dysfunction involved in inflammation and pain in the intestine.

2.Materials and methods

Reagents Trinitrobenzene sulfonic acid (TNBS), 2-butyl alcohol-tribromoethyl alcohol, dithiothreitol,

collagenase D, DNase I and Percoll were purchased from Sigma Aldrich (Shanghai, China). Dispase II were purchased from Roche Applied Science (Shanghai, China). Fluorescence-labeled anti-mouse CD11c-FITC,F4/80-PE cy5.5, CD44APC and CD62L-PE were purchased from BD Biosciences (Shanghai, China). ELISA kits for IL-4, IL-10,IL-13 were purchased from R&D Systems (Shanghai, China). The probiotics *C. butyricum* (CGMCC0313-1) and *nonpathogenic Escherichia coli standard stains* were gifts from Dr. Xun He (Shandong Kexing Bioproducts Co. Ltd., China).

Establishment of IBS mouse model The animal experimental procedures were approved by the Animal Welfare Committee at The Chinese academy of sciences institute of biophysics (NO:syxk2011-211) and the Animal Care Committee at Zhengzhou University. Male C57BL/6 mice, 6–8 weeks old, were purchased from Beijing Experimental Animal Center. The TNBS-IBS model were induced by intrarectally introducing a singular dose of 0.2 ml trinitrobenzene sulfonic acid (TNBS) (2.5 mg/mouse in 30% EtOH) via a polyethylene catheter inserted about 4 cm from the anus under a light general anesthetization. Control mice were received same volume of 30% EtOH. The IBS mice were treated with either of the two probiotics (*C. butyricum* and *nonpathogenic E. coli*) during the frist week. The inflammation of the colonic mucosa was evaluated by histology. Rectal sensitivity was assessed by a barostat study using an intermittent pressure-controlled distension protocol.

Abdominal withdrawal reflex (AWR) recording Visceral sensitivity was assessed by behavioral responses to colorectal distention (CRD), which was measured by a semiquantitative score of AWR and the threshold intensity of CRD, which elicits an expression of contraction in the abdominal wall musculature (Al-Chaer E et al, 2000). CRD was performed as described previously (Jones RC 3rd et al, 2007). AWR and its thresholds were recorded during phasic balloon inflation to 15, 30, 45, 60, and 80 mm Hg respectively. The AWR scores was recorded as described previously (Mearin Fet al, 2009). The stimulus intensity that evokes a visually identifiable contraction of the abdominal wall was recorded as the threshold intensity of CRD. During the measurements, mice were given CRD for 20 s every 4 min. To achieve an accurate measure, each pressure was repeated five times.

Histopatholgical evaluation of the colonic tissues Immediately following the somatic and visceral pain testing, all mice were euthanized by peritoneal injecting of sodium pentobarbital (120mg/kg). Following euthanasia, a piece of the descending colon was removed and processed for histopathology. The tissue was fixed in formalin and processed using

standard procedures for H & E staining. The severity of the lesions in the colon mucosa were graded using a system previously described (Mearin Fet al, 2009 and Zhou Q et al, 2008). The grades of colitis included: mild (+1) infiltration of a limited number of neutrophils in the lamina propria with minimal interstitial edema; moderate (+2) infiltration of a moderate number of neutrophils in the lamina propria with moderate interstitial edema; severe (+3) diffuse infiltration of neutrophils in the lamina propria with severe interstitial edema (Mearin Fet al, 2009 and Zhou Q et al, 2008).

Isolation of lamina propria mononuclear cells (LPMCs) The colon segments were excised immediately after sacrifice; the colon was opened longitudinally and cut into 0.3 cm in size, incubated in $Ca2^+Mg2^+$-free Hank's Buffered Salt Solution(HBSS), supplemented with 5% FBS, ethylenediaminetetraacetic acid (EDTA) (5 mM), penicillin (100 unites/mL)/streptomycin (100 mg/mL), and dithiothreitol (1 mM) at 37°C for 20 min. The cell suspension was filtered through a 70-µm cell strainer and then digested in RPMI 1640 supplemented with 1.75 mg/mL collagenase D, 5mg of DNase I, and 0.3 g of dispase II for 1 h at 37°C. The LPMCs were isolated on a 40%–80% Percoll gradient centrifuged for 20 min at 1000×g (Weigmann B et al, 2007).

Flow cytometry Cells were blocked with 1% BSA for 30 min, and then stained with fluorescence-labeled antibodies (0.5-1 µg/ml) for 1 h at room temperature. After washing with PBS, the cells were analyzed by a flow cytometer (FACSCanto II, BD

Bioscience, Beijing, China). The data were analyzed by software FlowJo (Tree Star, Ashland, OR).

Enzym-linked immunobsorbent assay (ELISA) The blood was collected at the sacrifice. The serum was isolated and analyzed by ELISA using purchased reagent kits following the manufacturer's instruction.

Data analysis Abdominal withdrawal reflex scores at each pressure of CRD among the three groups were compared using the Kruskal–Wallis one-way ANOVA on ranks, if the result was significant ($P < 0.05$), a Wilcoxon rank sum test with a Bonferroni correction at 0.05/3 was used to correct for multiple comparisons. Other data were expressed as mean±standard error (SEM), and one-way anova was performed among three groups, followed by least significant difference (LSD) multiple range analysis. A value of $P < 0.05$ was considered significant. Statistical analyses were performed with Graphpad Prism 5.0 software.

3.Results

Evaluation of physiological parameters in mouse model of IBS. Compared with the control group, mice of TNBS group showed that the body weight reduced significantly in the frist week (Fig.1a); defecation time extended markedly (Fig.1b) and the defecation frequency was significantly decreased (Fig.1c), fecal water content were also reduced obviously (Fig.1d) (all $P<0.05$). These data indicated that after the rectal instillation of TNBS, the symptoms of the mice are somewhat similar to IBS of human, such as the subtype of constipation-predominant IBS.
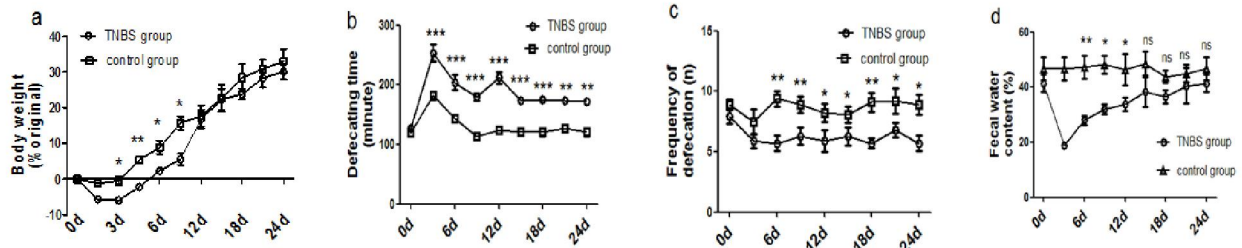

Figure 1. Physiological Parameter Changes in An IBS Mouse Model. From day 0 to day 24, the body weight (a), defecating time period (b), frequency of defecation (c) and the fecal water content (d) were recorded. The data are presented as *mean ± SEM*. Each group was consisted of 6 mice. *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001, compared with the control group.

Assessment of intestinal inflammation. All mice treated with TNBS had a low-grade mucosal inflammation in colon characterized by infiltration of a moderate number of mononuclear cells in the lamina propria and moderate interstitial edema on day 6 in the colon mucosa. The saline treated mouse colon appeared

normal. Twenty-four days after TNBS treatment, the colon mucosa appeared normal indicating recovering. As compared with *E coli*-treated group, the IBS mice treated with *Clostridum butyricum* showed less inflammatory signs in the colon mucosa (Fig. 2).
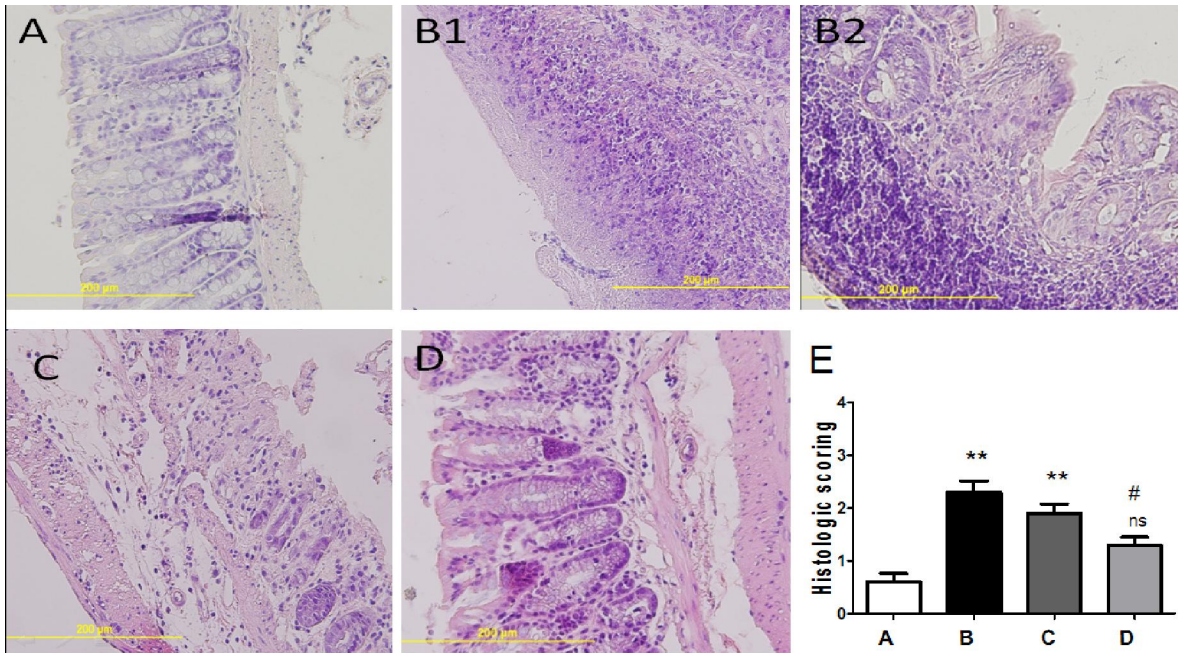
Figure 2. Anatomical Pathological Changes of The Colon Mucosa in IBS Mouse Model. The colon segments were processed for H&E staining. (A) Naïve mice. B1–B2, mice were treated with TNBS (B1: on day 6; B2: on day 24). C–D, mice were treated with TNBS and *E coli* (C), or *C. butyricum* (D). Image magnification: ×200. E, the bars indicate the histologic scores (averaged from 20 fields per mouse; *mean ± SEM*. ns $P > 0.05$ **$P < 0.01$ ***$P < 0.001$, compared with group A. # $P < 0.05$, compared with group C). Each group had 6~10 mice. Samples from individual mice were processed separately.

Visceral Pain Test. As tested with the colonic Distension, the TNBS group showed a significant increase of AWR scores (2.60±0.13, 3.33±0.19, 3.73±0.15) at intensities of 20, 40, 60 mm Hg respectively of CRD (Fig. 3a) as compared with control group (1.33±0.13, 2.33±0.13, 2.93±0.12). A significant decrease in pain threshold (day 0 = 40.87±1.15; day 24 = 21.79±0.94; P<0.001) was also observed (Fig. 3b).

Treatment with *Clostridum butyricum* restored the intestinal pain threshold to normal levels (day 0 = 36.05±1.31; day 24 = 37.58±1.12) while those treated with *E coli* did not show apparent improvement (day 0 = 36.06±1.31; day 24 = 31.80±0.69; P<0.01) (Fig. 3a, 3c).
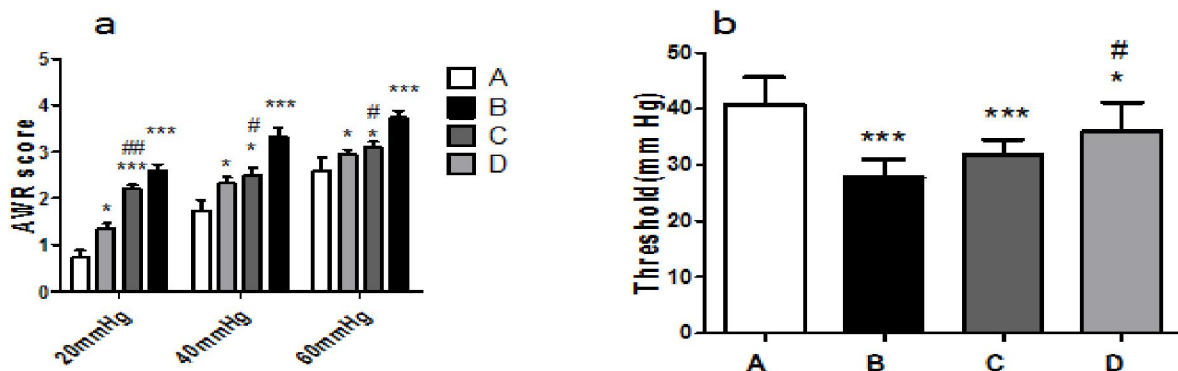

Figure 3. Abdominal Withdrawal Reflex (AWR). (a) the bars indicate the AWR scores that were recorded in the colorectal distention (CRD) test on day 24 after TNBS treatment. (b) the bars indicate the thresholds (the intensities of CRD to evoke abdominal contraction) of the AWR in CRD tests. The data are presented as *mean ± SEM*; *$P < 0.05$ **$P < 0.01$; ***$P < 0.001$; compared with group A. # $P < 0.05$; ## $P < 0.01$; ns $P > 0.05$, compared with group C. A: Naïve control group; B: TNBS group; C: *E coli* group; D: *C. butyricum* group. Each group consists of 6~10 mice.

Macrophages and memory T cells in the colon mucosa. Macrophages (CD11c+ and F4/80+) have the ability to stimulate other immune cells such as memory T cells (CD44+/CD62L+). As shown by flow cytometry data, macrophages of the colon mucosa in TNBS group were significantly more than naïve control mice (83.2±2.42% vs 27.7±1.05%, in LPMCs); treatment with *Clostridum butyricum* significantly reduced the frequency of macrophages (39.2±1.71%) (Fig. 4a). The frequency of memory T cells in LPMCs was higher in TNBS group than naïve controls (60.1±3.32% vs 18.2±1.95%), which was markedly down regulated after treatment with *Clostridum butyricum* (34.4±1.38%). Treatment with *E coli* also

suppressed the frequency of memory T cells (45.2±0.86%) (Fig. 4b). The results suggestes that treatment with *Clostridum butyricum* can regulate the immunity of the intestinal mucosa in an IBS-like environment.

Cytokines level in the serum. The sera were collected from mice at sacrifice 24 days after treatment with TNBS. As shown by ELISA data, the serum levels of IL -4, IL -13 and IL- 10 were increased in TNBS-treated mice as compared with naïve mice, which indicates a Th2 response in the mice. The Th2 cytokine levels were less in TNBS/*E coli*-treated mice and further lower in mice treated with TNBS/ *Clostridum butyricum* (Fig. 5).
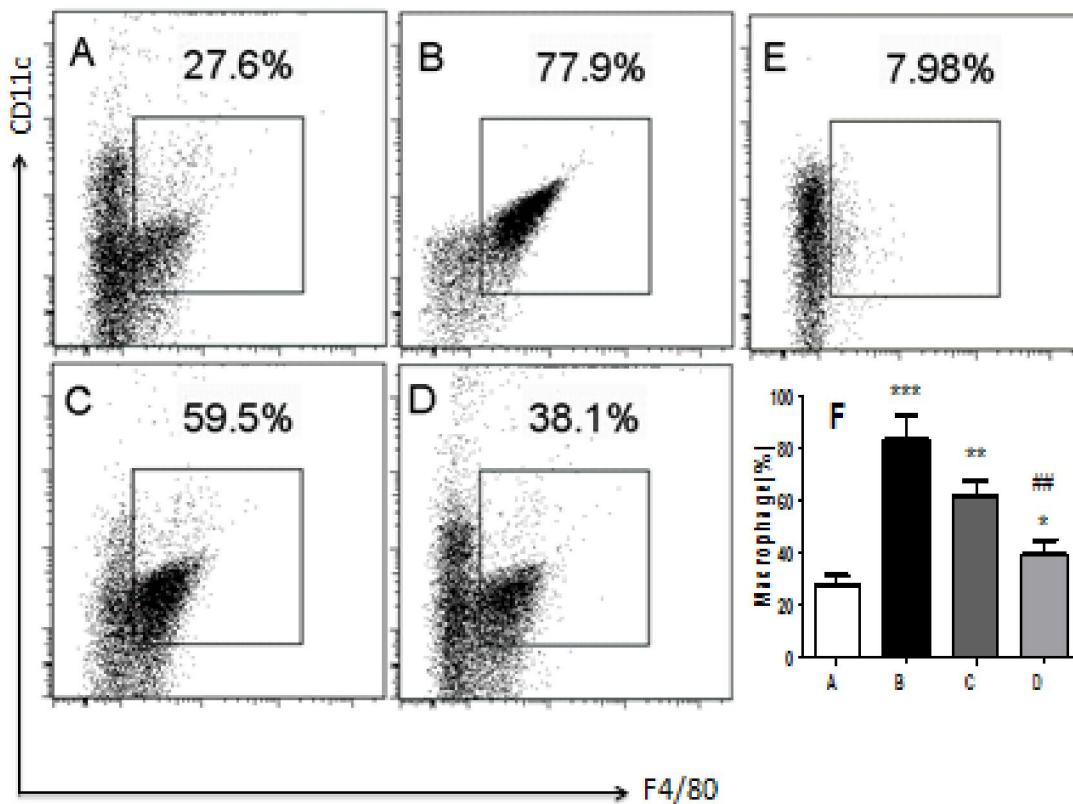

Figure 4. (a) Macrophages in Colonic Lamina Propria. Isolated colon lamina propria mononuclear cells (LPMCs) were analyzed by flow cytometry. A-D, the dot plots indicate the frequency of macrophages (CD11c+ F4/80+). E, the bars indicate the summarized data of the dot plots (mean ± SEM); *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$, compared with group A. # $P < 0.05$ ## $P < 0.01$, compared with group C). A: Naive control group; B: TNBS group; C: *E coli* group; D: *C. butyricum* group; E: Isotype IgG control. Each group consists of 6~10 mice.
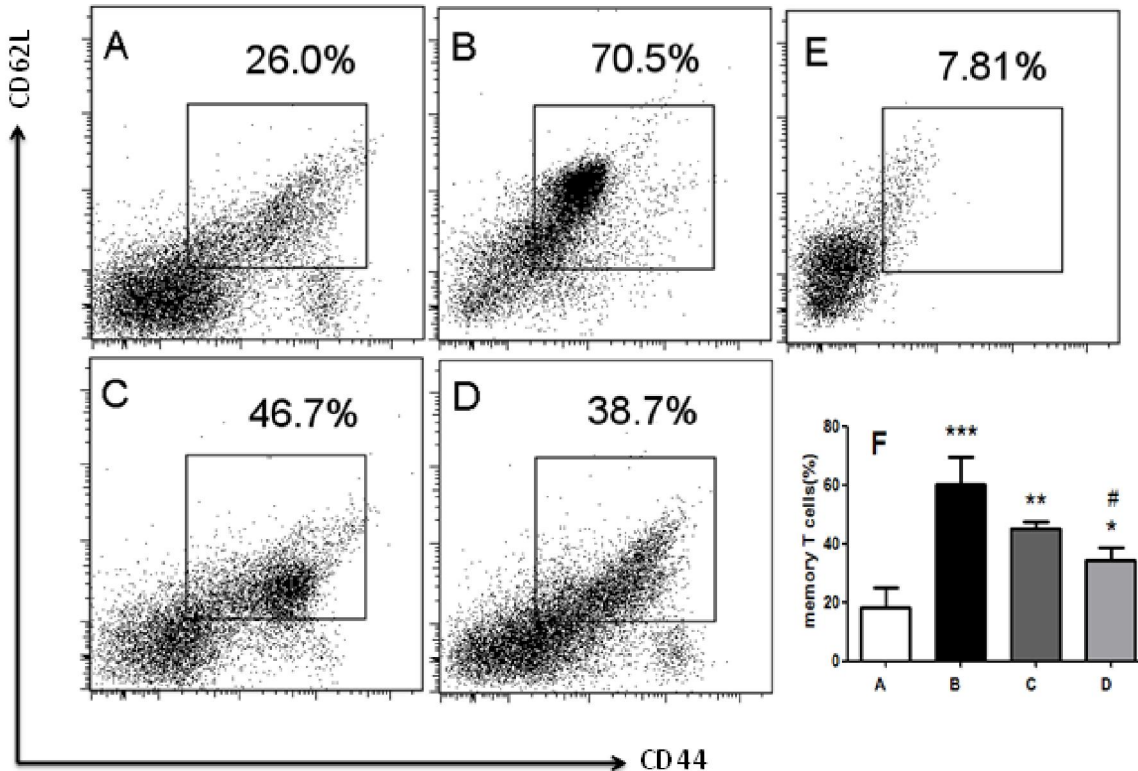
Figure 4.(b) Memory T cells in The Colonic Lamina Propria. Colon LPMCs were analyzed by flow cytometry. A - D, the dot plots indicate the frequency of memory T cell (CD44+ CD62L+) in LPMCs. E, the bars indicate the summarized data of the dot plots (the labels on X axis are the same as dot plots). The data are presented as *mean ± SEM*; *P < 0.05 **P < 0.01 ***P < 0.001, compared with group A. # *P* < 0.05 ## *P* < 0.01, compared with group C. A: Naive control group; B: TNBS group; C: *E coli* group; D: *C. butyricum* group; E: Isotype IgG control. Each group consists of 6~10 mice.
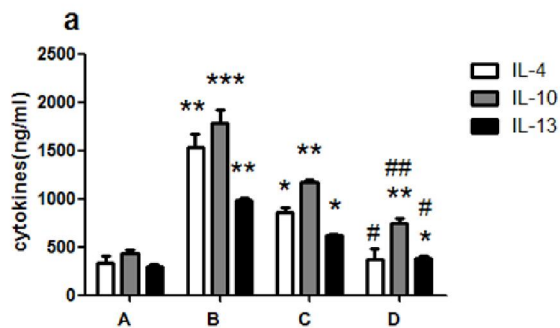

Figure 5. Levels of The Cytokines in The Serum. The serum was analyzed by ELISA. The bars indicate the levels of IL-4, IL-10 and IL-13 (*mean ± SEM*); *P < 0.05 **P < 0.01 ***P < 0.001, compared with group A. # *P* < 0.05; ## *P* < 0.01, ns *P*>0.05, compared with group C. A: Naive control group; B: TNBS group; C: *E coli* group; D: *C. butyricum* group. Each group consists of 6~10 mice.

4.Discussion

IBS is a disorder characterized by chronic abdominal pain and discomfort associated with alterations in bowel habits in the absence of a demonstrable pathology (Gaboriau-Routhiau V et al, 2009). The important role of the mucosal immune system and the potential relationship between the mucosal immune dyfunction and visceral sensitivity was still unclear (Buret AG et al, 2013). Our data show there is at least partially an low-grade mucosal inflammation in the colon, perhaps the local inflammation continually abnormal activating of the intestinal immune system, and then the disease is refractory to be improved. In this IBS model several physiological parameters are changed obviously that are similar to IBS in human, such as intestinal peristalsis changes, continuous visceral hypersensitivity and mucosal immune system dysfunction.

In IBS, the alterations in bowel habits are likely related to dysregulation of intestinal nervous system and mucosal immune system dysfunction, whereas symptoms of abdominal pain and discomfort are thought to involve additional changes in the perception of visceral events, in the form of hyperalgesia or allodynia. Our data are in line with those previous

studies by showing the symptoms of hyperalgesia and markedly changes of defecation and fecal components in mice treated with TNBS, which are similar to the IBS symptoms in human. New treatments of probiotics modulate intestinal immune system present with favorable outcomes in clinical trials (Grundmann O et al, 2010). Our data provide further insight to the mechanism of probiotic treatment by showing that probiotics can restore the immune deregulation in the intestine in an IBS mouse model.

IBS patients display an increased frequency of peripheral CD4+ and CD8+ T cells (Ohman L et al, 2005),and had increased numbers of CD3+, CD4+, CD8+ T cells and mast cells in the intestinal mucosa, and augmented frequency of lamina propria CD8+ T cells in the ascending colon (Cremon C et al, 2009 and Varol C et al, 2010 and Braak B et al, 2012). Our data showed that macrophages and memory T cells were increased in the colon of the IBS mice. Together with the data of elevation of Th2 cytokine levels in the serum, the data implicate that the TNBS-induced inflammation results a sustaining immune deregulation in the intestine.

Th2 cells can be activated by dendritic cells (DC) via presenting antigen information. After activation, Th2 cells release Th2 cytokines Including IL-4, IL-5, IL-13, etc. The normal Th2 response is one of the body's immune functions to defense microbial organism invasion and eliminating other foreign antigens. But skewed Th2 responses cause body injury and induces inflammation, such as induce allergic disorders and ulcerative colitis (Barkhordari E et al, 2010). Our data show that after inflammation, high levels of Th2 cytokines were detected in the colon in parallel to the IBS-like symptoms in the mice. After treatment with probiotics, the levels of Th2 cytokines were suppressed, the IBS-like symptoms, were also suppressed in the mice. The results suggest a potential link between the enhancement of Th2 cytokines and the IBS-like symptoms in the colon; the underlying mechanism needs to be further investigated.

The frequency of macrophage was increased in the colon of mice treated with TNBS. Macrophaes are one of the antigen presenting cells in the body. The results implicate there may be more activities of antigen presentation going on in the intestinal mucosa. The frequency of memory T cells was also increased in the colon after TNBS-induced hypersensitivity, which can be an indicator of the mucosal immune activation. Further work needs to be carried out to elucidate the subtypes of the memory T cells.

Visceral hyperagesia is one of the major symptoms of IBS. In the present study we observed that 24 days after TNBS-treatment, mice exhibit visceral hyperalgesia and allodynia in response to CRD; the results indicate that TNBS can induce long-standing hypersensitivity of the enteric nervous system that results in an increase in the pain perception. These changes were attenuated by administering mice with a probiotic *Clostridum butyricum.* The effects of *Clostridum butyricum* on TNBS-induced hypersensitivity is associated with the changes of the modification of the intestinal immune homeostasis as shown by the present data; the results implicate that probiotics may restore the immune function in GI tract after suffering inflammation.

In conclusion, this report illustrates that after inflammation, an immune deregulation may sustain in the intestinal mucosa, which contribute to the development of IBS-like symptoms, which can be prevented or restored by administration with probiotics, *Clostridum butyricum*.

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