Enterobacter Ludwigii Protects Experimental Colitis Through CD103+DC and Treg Cells

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Research

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Abstract

**Background:** Intestinal bacteria are closely related with inflammatory bowel disease (IBD), and regulatory cell-mediated immune tolerance is important to inhibit IBD. Commensal intestinal bacteria play key roles in regulating immune tolerance cell, however, bacterial strains directly involved in this regulation remain to be identified.

**Results:** In the present study, metronidazole, among nine antibiotics, was found to have the best effect on protecting mice against DSS-induced colitis. *Enterobacter ludwigi*, abundant in mouse feces after metronidazole treatment, was identified to decrease mice susceptibility to DSS-induced colitis with or without the presence of complex intestinal bacteria. *E. ludwigi* gavage increased CD103+DCs and Foxp3+Treg cells in intestinal microenvironment, and effects of *E. ludwigi* on diminishing colitis were lost in DC or Treg depletion mice. CD103+DCs isolated from *E. ludwigi*-treated mice showed enhanced ability to promote the Treg differentiation from naive T cells. DCs, directly stimulated by live *E. ludwigi* strain or its culture supernatant, had increased immune tolerance ability for Treg differentiation in vitro.

**Conclusions:** Overall, our findings identify a facultative anaerobe bacterial strain *E. ludwigi*, which directly enhances CD103+DC and Treg-mediated immune tolerance, resulting in protecting mice against DSS-induced colitis.

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), spreads globally with an incidence rate of approximately 0.3% [1]. Pathogenesis of IBD involving genetic factors and environmental triggers such as microbiome, remains to be elucidated [2].

Gut microbiota alteration is closely associated with IBD occurrence [3], and gut bacterial diversity is reduced in feces and colonic mucosa of IBD patients compared with that of patients in remission [4, 5]. Several kinds of bacteria including *Bifidobacterium species*, *Lactobacillus species*, *Clostridium* species, *Bacteroides* species, *Faecalibacterium prausnitzii*, *Roseburia* species, *Suterella* species, *Akkermansia muciniphila*, and *Eubacterium hallii*, most of which are obligate anaerobic bacteria, have been reported to play roles in reducing inflammation during IBD [6, 7]. Aerobes or facultative anaerobes are always hypothesized to contribute to IBD pathogenesis.

Antibiotics decrease the number of luminal bacteria and change the composition of intestinal flora [8]. Rifaximin is effective at inducing CD remission, which increases beneficial bacteria such as *Bifidobacteria* and *F. prausnitzii* [9]. The combination of metronidazole and ciprofloxacin improved symptoms of perianal diseases [10], meanwhile metronidazole and ornidazole successfully prevented clinical postoperative recurrence of Crohn's disease [11, 12]. The antibiotic combinations, such as metronidazole/tobramycin, metronidazole/amoxicillin/tetracycline, and metronidazole/tobramycin/vancomycin/rifaximin, show benefit of remission induction in moderate UC.
However, as antibiotics have side effects on patients and impact on intestinal flora, use of antibiotics is limited in IBD patients [15].

Inflammatory response during IBD is caused by induced Th1/Th17 effector cells and impaired regulatory cells including regulatory T cells (Treg), B cells (Breg), M2 macrophages, dendritic cells (CD103+DCs) and innate lymphoid cells (ILCs) [6, 16, 17]. Regulatory cell-mediated immune tolerance to foreign antigens and autologous proteins in the colon is important to inhibit IBD [18]. Tregs play a key role in immunological tolerance through secretion of inhibitory cytokines, and Tregs could disrupt metabolic processes in T effector lymphocytes (Teffs), induce apoptosis of Teffs, trigger cytotoxic activity against Teffs, neutralize DC function, and produce amphiregulin to repair tissue [19]. CD103+DC promotes Treg differentiation and inhibits Th1/Th17 immune response in mesenteric lymph nodes (MLN) by producing TGF-β, retinoic acid, indoleamine-2,3-dioxygenase (IDO), AhR ligands and carbonic anhydrate I epitope peptide [6, 20]. Only a few bacteria have been identified to directly induce immune tolerance cells. Due to the presence of a large number of intestinal bacteria, strains involved in immune tolerance and their mechanism remain to be clarified.

In the present study, a facultative anaerobe Enterobacter ludwigii isolated from metronidazole-treated mouse feces, was found to enhance the ability of CD103+DC to differentiate Treg cells, which increased the CD103+DC/Treg-depended tolerance response to reduce mice susceptibility to DSS-induced colitis.

Results

Mice susceptibility to DSS-induced colitis is influenced by different kinds of antibiotics

To study the role of intestinal bacteria in inflammatory bowel disease, we used a mouse model of DSS-induced acute colitis combined with antibiotic treatment. Male C57BL/6J mice were pretreated with different antibiotics for 7 days (ampicillin, chloramphenicol, ciprofloxacin, clindamycin, metronidazole, neomycin, polymycin, tetracycline, vancomycin, a broad-spectrum antibiotic cocktail composed of ampicillin, vancomycin, neomycin and metronidazole (ABX), and no antibiotic treatment as the control), and subsequently exposed to the same antibiotic plus 3% DSS for another 7 days (Fig. S1A, additional file 1). The colitis severity was evaluated through body weight loss, disease activity index (DAI) scores, colon lengths and histopathological scores. ABX treatment, which is widely used to clear normal intestinal bacteria, greatly reduced mice susceptibility to DSS treatment as reported previously [21] (Fig. S2A to D, additional file 2). Among the antibiotics used, metronidazole, vancomycin and clindamycin were observed to decrease mice susceptibility to DSS-induced colitis significantly, with less body weight loss, lower DAI scores, longer colon lengths and lower histopathological scores compared with the other antibiotic treatment and the control group (Fig. S1B to D, additional file 1 and 2). Metronidazole treatment, compared with vancomycin or clindamycin treatment, had the best effect in reducing colitis (Supplementary Fig. 2I). In addition, other antibiotic treatment was not found to significantly attenuate the severity of DSS-induced colitis (Fig. S1B to E, Fig. S2A to H, additional file 1 and 2).
Taken together, these data indicate that different kinds of antibiotics play different roles in mice susceptibility to DSS-induced acute colitis, and metronidazole treatment greatly attenuated DSS-induced colitis. As different antibiotic treatment could shape different intestinal bacteria composition, we hypothesized that metronidazole-induced intestinal bacteria may play roles in protecting mice against experimental colitis.

**Metronidazole treatment increases CD103^+DCs and Foxp3^+Treg cells in intestinal microenvironment**

CD103^+DCs are reported to induce Treg cell differentiation, resulting in intestinal homeostasis [22]. Therefore, we examined the effect of metronidazole treatment on CD103^+ DCs and Treg cells in mesenteric lymph nodes (MLN) and colonic lamina propria (cLP) using flow cytometry. In MLN, the percentage of CD103^+DCs in total DCs was significantly increased in the metronidazole group compared with that in the control and neomycin group (Fig. 1A), and a trend toward higher percentage of CD103^+DCs in total DCs was observed for the metronidazole group without statistical significance in cLP (Fig. 1B). The percentage of CD25^+Foxp3^+ Tregs in CD4^+ T cells in the metronidazole group was increased compared with that in the control and neomycin group in MLN, and compared with that in the control group in cLP (Fig. 1C to D). In addition, we examined the IFN-g^+ or IL-17A^+CD4^+ cells in cLP, which are reported to enhance intestinal inflammation and can be regulated by Treg cells [23, 24]. The percentage of either IFN-g^+ or IL-17A^+CD4^+ in total CD4^+ cells was decreased in the metronidazole group compared with that in the neomycin group in cLP, and a trend toward lower percentage without statistical significance was observed for the metronidazole group compared with the neomycin group in MLN (Fig. S3A to D, additional file 3). These results suggest that metronidazole treatment induces CD103^+DCs and Foxp3^+Treg cells in intestinal microenvironment, which is correlated with reduced experimental colitis in mice.

**Analysis of bacteria remodeled by metronidazole treatment**

To characterize the potential bacterial population related with the reduced susceptibility to DSS-induced acute colitis in mice treated with metronidazole, we performed 16S rRNA gene sequencing of bacterial DNA collected from mice feces on the zero and the fifth day during DSS treatment, and bacterial DNA collected from colon mucosal tissues on the seventh day, in the mouse model of DSS-induced acute colitis combined with antibiotic treatment described above (Fig. S1A, additional file 1). The 44 samples generated a total of 1888804 high-quality 16S rRNA sequences, and the OTUs were obtained based on an equal sequencing depth (27528 reads per sample) to be used for further analysis. The bacteria of mice treated with metronidazole had decreased alpha diversity (based on Shannon's index) compared with those of mice in the neomycin or control group (Fig. 2A). As lower bacterial alpha diversity is related with increased intestinal inflammation [25], it is suggested that bacterial alpha diversity is not involved in different mice susceptibility to DSS-induced colitis in this mouse model. The beta diversity of bacteria based on Bray-Curtis revealed that bacteria in mice treated with metronidazole were different from those in mice of the neomycin or control group, and similar bacteria in feces and colon mucosal tissues were
detected for the metronidazole group (Fig. 2B), which is corresponding to the analysis of bacterial composition at the genus level (Fig. 2C). Obviously decreased obligate anaerobes and increased facultative anaerobes were observed in all the samples from the mice treated with metronidazole compared with those from mice of the other groups (Fig. 2D), and no obvious difference of gram-positive or gram-negative bacteria were observed for all the samples from mice in the three groups (Fig. S4A, additional file 4). The top 50 dominant genera in all samples were selected to construct a heatmap, and 11 genera including *Allobaculum, Akkermansia, Bifidobacterium*, unclassified genera in *Enterobacteriaceae, Enterococcus, Klebsiella, Lactobacillus, Parabacteroides, Parasutterella, Pasteurella* and *Turicibacter* were found increased in the metronidazole group compared with the neomycin or control group, and 5 genera including unclassified genera in *Enterobacteriaceae, Enterococcus, Klebsiella, Lactobacillus*, and *Pasteurella* are belong to facultative anaerobes (Fig. 2E). Abundance of the 11 genera in mice of the three groups was analyzed, and 8 genera including *Akkermansia, Bifidobacterium*, unclassified genera in *Enterobacteriaceae, Enterococcus, Klebsiella, Lactobacillus, Parabacteroides*, and *Parasutterella* were found increased significantly in the metronidazole group compared with the neomycin or control group, and 4 genera including unclassified genera in *Enterobacteriaceae, Enterococcus, Klebsiella*, and *Lactobacillus* belong to facultative anaerobes (Fig. 2F). We focused on the facultative anaerobes induced by metronidazole treatment in the following study.

**Enterobacter ludwigii protects mice against DSS-induced acute colitis**

Six bacterial strains including *Enterobacter ludwigii, Enterococcus gallinarum, Enterococcus lactis, Klebsiella huaxiensis, Klebsiella michiganensis*, and *Lactobacillus johnsonii*, which belong to the genera including *Enterobacteriaceae, Enterococcus, Klebsiella*, and *Lactobacillus*, were isolated from fecal samples of mice treated with metronidazole and their effects on DSS-induced acute colitis in mice were examined. Male C57BL/6J mice were given $10^9$ CFU of each strain via oral gavage for 7 days, and were subsequently given the same strain plus 3% DSS for another 7 days (water gavage instead of bacteria was used in the control group) (Fig. S4B, additional file 4). Among the six strains, *E. ludwigii* gavage showed lower DAI scores, longer colon lengths and lower histopathological scores in mice treated with DSS compared with the control group, and *L. johnsonii* showed lower histopathological scores (Fig. S4C to F, additional file 4). These results suggest that *E. ludwigii* plays a role in protecting mice against DSS-induced acute colitis.

We also check whether *E. ludwigii* mixed with other isolated bacterial strains could enhance its effect on DSS-induced acute colitis. Male C57BL/6J mice were given strain mixture (the percentage of each strain was same, and a total of $10^9$ CFU was used) for 14 days, and 3% DSS were added in drinking water from the seventh day (Fig. S5A, additional file 5). However, no obvious protection effect of the mixture of different strains with *E. ludwigii* was found compared with the control group (Fig. 5B to E, additional file 5), indicating that a large number of other bacterial strains in the mixture attenuate the effect of *E. ludwigii*. 
In addition, male C57BL/6J mice were given $10^9$ CFU of *E. ludwigii* and 3% DSS at the same time for 8 days (Fig. S5F, additional file 5), and mice in the *E. ludwigii* group had less body weight loss, lower DAI scores, longer colons and lower histological scores compared with the control and *E. gallinarum* group (Fig. S5G to J, additional file 5), indicating that *E. ludwigii* could reduce the severity of DSS-induced colitis without pretreatment.

In order to examine the major effect of *E. ludwigii* on DSS-induced acute colitis, mice were pretreated with ABX for five days, and were given $10^9$ CFU of *E. ludwigii*, *E. gallinarum*, or *L. johnsonii* via oral gavage one day after ABX treatment for 5 days, and were subsequently given the same strain in addition with 3% DSS and LPS challenge (8 mg/kg body weight, LPS is used to stimulate colitis in the absence of commensal bacteria [26]) for 7 days (Fig. 3A). *E. ludwigii* gavage showed less weight loss compared with the *E. gallinarum* and *L. johnsonii* group (Fig. 3B). Notably, *E. ludwigii* gavage showed a significantly lower DAI score compared with the *E. gallinarum*, *L. johnsonii*, and the control group, beginning at the fifth day of DSS challenge and lasting until the mice were euthanized (Fig. 3C). In addition, mice with *E. ludwigii* gavage had longer colon lengths and lower histopathological scores with preserved crypt structures and less inflammatory cells infiltration, compared with the other three groups (Fig. 3D to E). We noticed that mice with *L. johnsonii* gavage had moderately higher histopathological scores compared with the control group (Fig. 3E), although *L. johnsonii* gavage showed lower histopathological scores in mice without ABX treatment (Fig. S4F, additional file 4), which suggests that *L. johnsonii* decreases DSS-induced histopathological scores in mice depending on other bacteria.

Taken together, these results indicate that *E. ludwigii* abundance induced by metronidazole treatment could protect mice against DSS-induced acute colitis with or without the presence of other intestinal bacteria.

**E. ludwigii** increases CD103^+DCs and Foxp3^+Tregs in intestinal microenvironment

As metronidazole treatment induces CD103^+DCs and Foxp3^+Treg cells in intestinal microenvironment, we examined the effect of *E. ludwigii* on CD103^+DCs and Foxp3^+Treg cells using the same mouse model for evaluating single bacterial strain (Fig. 3A). In MLN, the percentage of CD103^+DCs in total DCs cells was significantly increased in the *E. ludwigii* group compared with that in the *L. johnsonii* and control group (Fig. 4A). In cLP, the percentage of CD103^+DCs in total DCs cells was obviously increased in the *E. ludwigii* group compared with that in the *E. gallinarum* and *L. johnsonii* group (Fig. 4B). A trend toward higher percentage of CD103^+DCs without statistical significance was also observed for the *E. ludwigii* group compared with the *E. gallinarum* group in MLN (Fig. 4A), and compared with the control group in cLP (Fig. 4B). In both MLN and cLP, the percentage of CD25^+Foxp3^+ Treg cells in CD4^+ T cells was detected to be higher for the *E. ludwigii* group than that for the control and *E. gallinarum* group (Fig. 4C to D). The percentage of CD25^+Foxp3^+ Treg cells was higher for the *E. ludwigii* group compared with that for the *L. johnsonii* group in MLN (Fig. 4C), and a higher trend without statistical significance was observed for the *E. ludwigii* group compared with that for the *L. johnsonii* group in cLP (Fig. 4D). In immunofluorescence
assays, the proportion of CD103+DCs in total DCs cells and Foxp3+ Treg cell number were increased in the *E. ludwigii* group compared with that in the control group in cLP (Fig. 5A to B).

To verify that the effect of *E. ludwigii* on relieving colitis depends on CD103+DCs and Tregs, diphtheria toxin (DT)-induced DC depletion mice (CD11c-GFP-DTR) and Treg depletion mice (Foxp3-GFP-DTR) (Fig. S6, Fig. S8, additional file 6 and 8) were used to examine the role of *E. ludwigii* (Fig. S7, Fig. S9, additional file 7 and 9). *E. ludwigii* had no effect on DSS-induced acute colitis compared with the control and *L. johnsonii* group, when DCs or Treg were depleted (Fig. S7, Fig. S9, additional file 7 and 9). These results indicate that *E. ludwigii* protects mice against DSS-induced acute colitis through CD103+DC and Foxp3+Treg cells.

We also examined the effect of *E. ludwigii* on CD103+DCs and Foxp3+Tregs using the mice that were pretreated with ABX and subsequently given 10⁹ CFU of single strain for 5 days without DSS and LPS challenge (Fig. S10A, additional file 10). We found a significant increase in CD103+DC frequency in MLN and cLP of the *E. ludwigii* group compared with the control and *E. gallinarum* group (Fig. S10B to C, additional file 10), and Foxp3+Treg frequency in cLP was also increased in the *E. ludwigii* group compared with the control and *E. gallinarum* group (Fig. S10D to E, additional file 10). These results suggest that *E. ludwigii* contributes to maintenance of tolerant immune cells even in intestinal homeostasis.

*E. ludwigii* gavage induces CD103+DCs’ ability to promote Foxp3+Treg differentiation

To examine the function of CD103+DCs and Foxp3+Tregs induced by *E. ludwigii* in vivo, CD103+DCs and Foxp3+Tregs were purified from MLNs of mice in the *E. ludwigii*, *E. gallinarum*, *L. johnsonii* and control group in the above mouse model (Fig. 3A) using fluorescence-activated cell sorting and were analyzed using quantitative reverse transcription PCR (qRT-PCR). The *Tgfb1, Tgfb2, Aldh1a2* and *Pdl1* mRNA level of CD103+DCs, which are usually used to evaluate CD103+DCs’ ability in tolerogenic responses [27-29], were increased in the *E. ludwigii* group compared with those in the control, *L. johnsonii* or *E. gallinarum* group (Fig. 5C). The *Tgfb1,Tgfb2* and *Il-10* mRNA level of Foxp3+Tregs, which are used to evaluate Treg-mediated T cell suppression ability [30], were increased in the *E. ludwigii* group compared with that in the control, *L. johnsonii* or *E. gallinarum* group (Fig. 5D). To further validate that *E. ludwigii*-induced CD103+DCs play a role in Foxp3+Treg differentiation, CD103+DCs purified from MLNs of mice in the *E. ludwigii*, *E. gallinarum*, *L. johnsonii* or control group were co-cultured with naive CD4+T cells from spleens of untreated Foxp3-GFP-DTR mice. CD103+DCs from the *E. ludwigii* group enhanced conversion of naive CD4+T cells into Foxp3+Tregs, compared with those from the other three groups (Fig. 5E). These results indicate that *E. ludwigii* gavage enhances the ability of CD103+DCs in immune tolerance to drive Foxp3+Treg differentiation, resulting in decreased susceptibility of mice to DSS-induced acute colitis.

*E. ludwigii* directly enhances DCs’ ability to promote Foxp3+Treg differentiation
To examine if *E. ludwigii* had direct effect on DC, we pulsed DCs isolated from C57BL/6J mice MLN with *E. ludwigii* or *E. gallinarum* for 2 hours (MOI = 10). The Tgfβ1, Tgfβ2, Aldh1a2 and Pd1l mRNA levels of DCs, were increased in the *E. ludwigii*-challenged group compared with that in the control or *E. gallinarum*-challenged group (Fig. 6A). It was shown that *E. ludwigii* culture supernatant, but not heat-inactivated bacteria, could increase Tgfβ1, Tgfβ2, Aldh1a2 and Pd1l mRNA levels of DCs (Fig. 6B to C). We further assessed the regulatory action of *E. ludwigii*-stimulated DC by coculturing with naive CD4^+^CD62L^+^CD44^-^CD25^-^ T cells for 72h, and the phenotype of polarized T cells was then analyzed by flow cytometry and immunofluorescence. *E. ludwigii* culture supernatant-stimulated DCs, compared with the control and *E. gallinarum* group, obviously promoted the differentiation of Treg cells from naive T cells (Fig. 6D to E). Therefore, we conclude that *E. ludwigii* interacts with DCs directly to increase DCs’ immune tolerance ability, which drive Foxp3^+^Treg differentiation.

**Discussion**

The intestinal flora in balance is mainly composed of obligate anaerobes, due to a low level of oxygen tension in the distal part of the gastrointestinal tract [31]. Gut microbiota dysbiosis is usually characterized by expansion of aerobes or facultative anaerobes belonging to Proteobacteria and Fusobacteria phylum, and by reduction of obligate anaerobes belonging to Firmicutes phylum [31, 32]. Therefore, aerobes or facultative anaerobes are usually hypothesized to contribute to IBD pathogenesis. In the present study, we found that a facultative anaerobic bacterial strain isolated from metronidazole-treated mouse feces, played a role in reducing mice susceptibility to DSS-induced colitis through enhancing CD103^+^DC-mediated immune tolerance.

Metronidazole, as a nitroimidazole preventing nucleic acid synthesis, limits anaerobic bacteria, protozoans and microaerophiles[33]. Metronidazole is medicative in active Crohn’s colitis patients and preventive of post operative recurrence of CD in combination with other antibiotics, but has less benefit for ulcerative colitis [34]. Metronidazole is also effective in treating *Clostridium difficile*-associated colitis [35]. However, prolonged high doses of metronidazole have side effects, such as gastrointestinal disturbance, peripheral neuropathy and encephalopathy, which limited its widespread use in clinical practice [36]. In the acute DSS mouse colitis model, metronidazole treatment could prevent colonic inflammation, but has no therapeutic effect on established colitis [37]. Metronidazole in combination with ciprofloxacin treatment resulted in expansion of *Enterococcaceae* and *Lactobacillaceae* in mice [33]. In our study, we found *Enterobacteriaceae* was also abundant after metronidazole treatment, and a bacterial strain *E. ludwigii* was identified to promote immune tolerance through increasing CD103^+^DCs’ ability to drive Treg differentiation independent of other intestinal flora.

The interactions between commensal bacteria and immune cells are very important to maintain intestinal homeostasis. However, only a few bacteria have been reported to directly interact with intestinal immune cells or epithelia cells to enhance immune tolerance. Segmented filamentous bacteria (SFB) strongly activates non-inflammatory homeostatic Th17 responses in the small intestine [38, 39]. *B. fragilis* could
directly induce IL-10-producing Treg differentiation in the gut by activating TLR2 [40, 41]. A 15 kDa protein isolated from *F. prausnitzii* acts on intestinal epithelial cells to inhibit NF-κB pathway to prevent colitis [42]. In the present research, we proved that *E. ludwigii* directly promoted DCs’ tolerance function to enhance Treg differentiation.

*E. ludwigii* is a motile rod-shaped gram-negative bacterium, and some *E. ludwigii* isolates with antibiotic resistance genes have been reported to be associated with surgical site infections or nosocomial bloodstream infections [43, 44]. Therefore, *E. ludwigii* strains should be carefully considered for direct application. Our data demonstrate that *E. ludwigii* culture supematant has effect on DC, providing the possibility that the secreted components or metabolites of *E. ludwigii* play direct roles in immune tolerance, which may lead to novel strategies to treat IBD.

**Conclusions**

*E. ludwigii*, abundant in metronidazole-treated mouse feces, decreases mice susceptibility to DSS-induced colitis with or without the presence of complex intestinal bacteria, and has both preventive and therapeutic benefit for DSS-induced colitis. *E. ludwigii* gavage increases CD103+DCs and Foxp3+Tregs in intestinal microenvironment with or without DSS stimulation, and effects of *E. ludwigii* on diminishing colitis depend on DCs and Tregs. *E. ludwigii* gavage induces CD103+DCs’ ability to promote Foxp3+Treg differentiation, and direct interactions between *E. ludwigii* and DCs enhances DCs’ immune tolerance ability.

**Methods**

**Animals**

Wild type male C57BL/6J mice, aged 6–8 weeks, were purchased from Academy of Military Medical Science (Beijing, China). CD11c-GFP-DTR mice were a gift from Professor Xuetao Cao of Nankai University. Foxp3-GFP-DTR mice were a gift from Professor Xiaoming Feng of Institute of Hematology and Hospital of Blood Disease, Chinese Academy of Medical Sciences. All mice were bred and maintained under specific pathogen free condition and a standard chow diet ad libitum in the animal facility at Tianjin Medical University. Animal procedures and protocols were performed with 6 to 8 weeks male mice and were approved by Animal Care and Use Committee, Tianjin Medical University.

**Antibiotic treatment**

Mice were provided with the following antibiotics (SangonBiotech, Shanghai, China) in drinking water individually or in combination: ampicillin (A610028-0025, 1 g/L), chloramphenicol (A100230-0010, 500 mg/L), ciprofloxacin (A600310-0025, 500 mg/L), clindamycin (A600312-0025, 500 mg/L), metronidazole (A600633-0025, 1 g/L), neomycin (A610366-0025, 1 g/L), polymycin (A610318-0001, 1 g/L), tetracycline (A600504-0025, 500 mg/L), vancomycin (A600983-0001, 500 mg/L). Antibiotics were pretreated for 7 days and then given with DSS until the end of the experiment. In addition, we used a combination of
ampicillin, vancomycin, neomycin and metronidazole (ABX) to clear most of intestinal bacteria [45]. Antibiotic-containing water was renewed every 3 to 4 days to maintain efficacy.

DSS-induced colitis mouse model

To study antibiotic effect on mice susceptibility to DSS-induced colitis, male C57BL/6J mice were pretreated with antibiotics for seven days, and exposed with the same antibiotics in addition with 3% DSS (0216011090, M.W. = 36,000–50,000 Da; MP Biomedicals, Solon, OH, USA) in drinking water for another seven days [46]. Feces were collected on the zero and fifth day, and tissues were collected on the seventh day when mice were euthanized.

To test the effect of individual bacterial strain on DSS-induced colitis, the strain was inoculated into 10 ml of sterile BHI broth (E. gallinarum, K. huaxiensis, E. ludwigii, E. lactis and K. michiganensis) or MRS broth (L. johnsonii) containing 2.5% L-cysteine, grown in anaerobic penicillin bottles at 37 °C for 24 h. Cultured bacteria were pelleted by centrifugation (5000 × g for 5 min at 4 °C) and resuspended in PBS to obtain a density of 5 × 10^9 CFU/ml. Each male C57BL/6J mouse was gavaged with 200 μl liquid bacterial strains (10^9 CFU) once per day for fourteen days, and 3% DSS was added to the drinking water of the mice from the seventh day to the fourteenth day during the bacteria administration.

To check the effect of bacterial strain mixture on DSS-induced acute colitis, individual strain was cultured separately in anaerobic penicillin bottles containing BHI broth at 37 °C for 24h and mixed together in the same proportion before gavage. Each male C57BL/6J mice were pretreated with 10^9 CFU strain mixture for seven days, and exposed with the same strain mixture plus 3% DSS in drinking water for another seven days.

To evaluate the therapeutic effect of E. ludwigii on experimental colitis without pretreatment, male C57BL/6J mice were treated with 3% DSS in drinking water for 7 days and gavaged with single bacterial strain once a day during the same period.

To study the major effect of individual bacterial strain on DSS-induced acute colitis, male C57BL/6J mice were pretreated with ABX for 5 days and water for 1 day, then mice were gavaged with 10^9 CFU bacterial strains once per day for twelve days. 3% DSS was added to the drinking water of mice, and mice were gavaged with LPS (8 mg/kg body weight/day, L2880, Sigma-Aldrich, St. Louis, MO, USA) simultaneously from the fifth day to the twelfth day during the bacteria administration.

Body weight loss was calculated relative to starting weight before giving DSS of the same mouse. DAI is the sum of stool consistence score, stool bleeding score and body weight loss score. Stool consistency was scored as follows: 0, normal; 1, mild soft stools; 2, very soft stools; 3, watery stools. Stool bleeding was scored as follows: 0, normal; 1, brown color; 2, reddish color; 3, bloody stool. Body weight loss was scored as follows: 0 (<2%), 1 (2-5%), 2 (5-10%), 3 (10-15%), 4 (≥15%) [47]. Body weight loss and DAI were scored every day during the DSS treatment. A distal part of colon was fixed in 4% paraformaldehyde and embedded into paraffin. Slides (5μm) were stained with hematoxylin and eosin, then images acquired
with a microscope (BX46, Olympus, Tokyo, Japan) were used to assess histopathological scores. Seven parameters were used to score histopathology: (A) extent of inflammation (score of 0 to 4), (B) extent of crypt damage (score of 0 to 4), (C) infiltration of neutrophils and lymphocytes (score of 0 to 3), (D) submucosal edema (score of 0 to 3), (E) loss of goblet cells (score of 0 to 3), (F) reactive epithelial hyperplasia (score of 0 to 3), (G) crypt abscesses (score of 0 to 2) [46, 47].

DNA extraction and 16S rRNA gene sequencing

Microbial genomic DNA was extracted from each sample using the Stool DNA Kit (D4015-02, Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols. Total DNA was eluted in 50 μl of Elution buffer. The amount of DNA was determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA was evaluated using 1.5% (wt/vol) agarose gel electrophoresis. All DNA samples were stored at -80 °C until sequencing.

16S rRNA gene sequencing was performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The primers 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene by thermocycler PCR system (GeneAmp 9700, ABI, USA). The process of PCR program was as follows: 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The 20μl PCR reaction system contained 4 μL 5 × FastPfu Buffer, 2 μL dNTPs (2.5 mM), 0.8 μL V3 primer (5 μM), 0.8 μL V4 primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA and PCR reactions were performed in triplicate. The PCR products were separately extracted from a 2% agarose gel and further purified through the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Then using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol quantified the purified products. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA).

Processing of sequencing data

Illumina sequencing generated raw fastq files, and the sequence reads were then merged, trimmed, filtered, aligned, and clustered by operational taxonomic unit (OTU) using UPARSE (version 7.1 http://drive5.com/uparse/). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%. OTU sequences were clustered, and those with > 97% similarity were binned into the same OTU. Alpha diversity metrics at the OTU level based on Shannon’s index [48] were mainly calculated by MOTHUR program (https://www.mothur.org/wiki/Main_Page). Beta diversity at the OTU level based on Bray-Curtis [49], were qualitatively examined by MOTHUR program and visualized in Principal Coordinate Analysis (PCoA) using the vegan R packages [50]. Bacterial taxonomic distributions of each group communities at the genus level were visualized using the vegan R packages. A heatmap with a base-10 logarithmic transformation of the absolute abundance at the genus level was generated through R package vegan. Analyses of alpha diversity, beta diversity, bacterial taxonomic distributions, and generation of the heatmap were performed at Majorbio I-Sanger Cloud platform.
(https://cloud.majorbio.com). 16S rRNA gene sequencing data have been submitted to NCBI Sequence Read Archive (SRA) with the accession number of PRJNA657382.

**Isolation of bacterial strains**

Fresh feces pellets collected from mice with metronidazole treatment were homogenized in 1 ml sterile phosphate buffered saline containing 2.5% L-cysteine and serially diluted with PBS, seeded onto multiple agar plates (hopebiol, Qingdao, China), including Man Rogosa Sharpe agar (MRS) and Brain Heart Infusion agar (BHI). After incubated under aerobic conditions or anaerobic conditions at 37 °C for 48 h to 72 h, individual colonies with distinct morphologies were picked up and the selected colonies were stream plated in the same media for another 2 or 3 days for further purification and identification.

Bacterial genomic DNA was extracted using Bacterial Genomic DNA Extraction Kit (DP302-02, TIANGEN, Beijing, China). To identify the isolated strains, 16S rRNA gene sequence was amplified by PCR using 16S rRNA gene-specific primer pairs: 27F (5'–AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'–GGTTACCTTGTTACGACTT-3'), and sequenced in Sangon Biotech (Shanghai, China). The resulting sequences were compared to sequences in EzBioCloud 16S rRNA databases (www.ezbiocloud.net).

**Preparation of lymphocytes**

Collected colons were opened longitudinally after excising fat tissues and washed with PBS to remove luminal feces, followed by shaking in PBS containing 2 mM dithiothreitol (DTT, D1070, Solarbio, Beijing, China) for 10 min once and PBS containing 5 mM EDTA for 10 min thrice at 37 °C to remove epithelial cells. Lamina propria tissues were sliced into small pieces and digested with PBS containing 2% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mg/ml collagenase IV (C5138, Sigma-Aldrich) and 100 ng/ml DNaseI for 30 min at 37 °C in a shaking water bath. The digested cell tissues were then filtered through a 70 μm nylon cell strainer (352350, BD Biosciences, San Jose, CA, USA) to obtain single cell suspensions and resuspended in 40% percoll to perform percoll gradient separation for obtaining lamina propria lymphocytes. Spleens and mesenteric lymph nodes were ground directly into a single cell suspension mechanically, in which spleen cells were subjected to lyse erythrocytes on ice for 15 min.

**Flow cytometry**

For cell surface staining, single cell suspensions were incubated on ice at 37 °C for 30 min with following antibodies: anti-CD45 conjugated to PE-Cy7 (25-0451-82, eBioscience, San Diego, CA, USA), anti-CD11c conjugated to APC (117309, Biolegend, San Diego, CA, USA), anti-MHCII conjugated to PE (107607, Biolegend), anti-CD103 conjugated to PerCP/Cy5.5 (46-1031-82, eBioscience), anti-CD4 conjugated to APC-Cy7 (100414, Biolegend), anti-CD3 conjugated to FITC (100204, Biolegend), anti-CD4 conjugated to PE-Cy7 (25-0041-81, eBioscience), anti-CD3 conjugated to APC-Cy7 (05112-87-100, eBioscience), anti-CD25 conjugated to PE (102008, Biolegend), anti-CD44 conjugated to PE-Cy7 (103029, Biolegend), anti-CD62L conjugated to APC (104411, Biolegend).
For Treg cell analysis, after staining of cell surface with CD3, CD4 and CD25 antibodies, lymphocyte suspensions were fixed, permeabilized using Foxp3/transcription factor staining buffer sets (00-5523-00, eBioscience) according to the manufacturer's instructions and stained with anti-Foxp3-APC (320014, Biolegend). For analysis of Th1 and Th17 cells, isolated tissue lymphocytes were stimulated for 5 h with cell activation cocktail with Brefeldin A (423303, Biolegend). After incubation for 5 h, cells were washed in PBS and stained with dead cells using Zombie NIR\textsuperscript{TM} Fixable Viability Kit (423105, Biolegend), anti-CD3 conjugated to PE (100205, Biolegend) and anti-CD4 conjugated to FITC (100405, Biolegend). Stained cells were fixed in fixation buffer (420801, Biolegend), permeabilized with intracellular staining permeabilization wash buffer (421002, Biolegend), and stained with anti-IL-17A conjugated to PE-Cy7 (25-7177-80, eBioscience) and anti-IFN-γ conjugated to PerCP/Cy5.5 (505821, Biolegend).

Stained cells were then analyzed on a FACS Canto II Flow Cytometer (BD Biosciences) using the FlowJo software (FlowJo, Ashland, OR, USA) or performed flow sorting to obtain purified cells on FACS Aria\textsuperscript{TM} Cell Sorter (BD Biosciences).

**Identification of knockout mice**

Genomic DNA was extracted from mouse tail, and the mouse genotype was identified by PCR with specific primers (Table S1, additional file 11). To investigate the role of DC and Treg deletion in *E. ludwigii*-mediated protection, CD11c-GFP-DTR transgenic mice were injected intraperitoneally (i.p.) with 200 µl PBS containing 100 ng of Diphtheria toxin (4 ng DT/g body weight, D0564-1MG, DTx, Sigma-Aldrich) to rapidly deplete CD11c\textsuperscript{+}DC, and transgenic Foxp3-DTR-GFP mice were given intraperitoneal injections of 1 µg DTx (50 ng DT/g body weight) to deplete Treg. DTx was injected on one day prior to bacteria gavage, and subsequent doses were given every other day to maintain DC and Treg depletion throughout the whole phase of bacteria gavage. When the mice were sacrificed, the spleens, MLNs, and colons were removed and analyzed using flow cytometry to verify the elimination efficiency of DC and Treg cells (FACS Canto II Flow Cytometer, BD Biosciences; the FlowJo software, FlowJo).

**Tissue immunofluorescence analysis**

Small segment colons were embedded in OCT compound with liquid nitrogen and cut into 5-µm sections followed by fixing with cold acetone for 10 min. After blocked with 5% BSA for 1 h, sections were incubated with CD103 antibody (1:200, ab224202, Abcam, Cambridge, MA, USA) in blocking buffer overnight at 4 °C. After that, slides were incubated with Alexa Fluor 594-labeled second antibody (1:200, SA00006-8, Proteintech, Chicago, IL, USA) for 1 h and counterstained with DAPI to stain nuclei. Images were acquired using a confocal fluorescence microscope (Leica TCS-SP8, Leica Microsystems, Germany).

**RNA extraction and qRT-PCR**

Sorted DCs (CD45\textsuperscript{+}MHCI\textsuperscript{+}CD11c\textsuperscript{+}) from MLNs of C57BL/6J mice were seeded into a 96 well plate, then incubated with indicated live bacteria (MOI = 10, 2h), heat-killed bacteria (MOI = 10, 24h) or bacterial culture supernatant (bacterial culture supernatant: cell culture medium = 1:1, 24h). RNA of stimulated
DCs, flow sorted CD103+DCs (CD45+MHCII+CD11c+CD103+ from C57BL/6J mice), or flow sorted Foxp3+Tregs (CD3+CD4+CD25+Foxp3+ from Foxp3-GFP-DTR mice) was extracted using Micro RNA extraction kit (74104,QIAGEN, Duesseldorf, Germany) and converted to cDNA using HiFiScript cDNA Synthesis Kit (CW2569M, CWBio, Beijing, China) according to the manufacturer’s protocol. The PCR reactions were performed with Ultra SYBR Mixture (CW0957M, CWBio) on a 7900 Fast Real-Time PCR System (Roche, Basel, Switzerland). Transcript levels of the indicated genes were normalized to endogenous control β-actin for each individual sample using the primers (Table S1, additional file 11) and quantified using the comparative critical threshold cycle $2^{-\Delta\Delta Ct}$ method.

**T cell and DC co-cultures**

Sorted DCs were incubated with heat-killed *Escherichia coli* K12 (MOI = 10) and indicated bacterial culture supernatant for 24 h followed by washing. $1 \times 10^5$ flow purified Naive T cells (CD4+CD62L+CD25-CD44-, from spleens of Foxp3-GFP-DTR mice) were cultured with $1 \times 10^4$ stimulated DCs or $2 \times 10^4$ sorted CD103+ DCs (CD45+MHCII+CD11c+CD103+) from MLNs of C57BL/6J mice, in RPMI 1640 containing 1 μg/ml of immobilized anti-CD3 (100339, Biolegend), 100 U/ml penicillin, 100 U/ml streptomycin, 5 ng/ml recombinant mouse IL-2 (575402, Biolegend) and 5 ng/ml recombinant mouse TGF-β1 (7666-MB-005/CF, R & D Systems, Minneapolis, MN, USA) in 96-well plates. After 72 hours at 37 °C in 5% CO₂, cells were stained with anti-CD4 conjugated to PE-Cy7 and anti-CD25 conjugated to PE, and Foxp3 expression were detected by GFP through flow cytometry on a FACS Canto II Flow Cytometer (BD Biosciences) using the FlowJo software (FlowJo).

Stimulated DC and Naive T cell were co-cultured on a Lab-Tek chambered cover glass for 72 h, then fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 10 min and incubated with DAPI for 10 min. Cells were imaged using a confocal fluorescence microscope (Leica TCS-SP8, Leica Microsystems).

**Statistical analysis**

Data were presented as the mean ± SEM. The statistical significance of the differences between three or more groups was tested using one-way ANOVA or two-way ANOVA. All operations are performed in commercially available software (Graphpad Prism 6, San Diego, CA) and values of P < 0.05 were considered statistically significant.

**Declarations**

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Additional information

Supplementary information is available for this paper.

Author contributions

Q.W. designed the study, Q.L., X.S., K.Y., J.L., C.M., J.Y., S.W. and Z.F. performed the majority of experiments. Q.W., Z.Y., Q.L., X.S., and Z.Z analyzed the data and wrote the paper. All authors discussed the data, and reviewed the manuscript.

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Availability of data and materials

16S rRNA gene sequencing data in this study have been submitted to NCBI Sequence Read Archive (SRA) with the accession number of PRJNA657382.

Ethics approval and consent to participate

Animal procedures and protocols were approved by Animal Care and Use Committee, Tianjin Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

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References


Figures
Figure 1

Metronidazole treatment increases CD103+DCs and Foxp3+Tregs in MLN and cLP. MLN and cLP of mice at the seventh day after DSS treatment in the control, metronidazole, and neomycin groups were collected, and lymphocytes were isolated, stained and analyzed by flow cytometry. (A-B) Representative flow dot plots gated on CD45+MHCII+CD11c+ cells and the percentage of CD103+DCs in total DCs in MLN (A) and cLP (B) of mice in each group. (C-D) Representative flow dot plots gated on CD3+CD4+ cells
and the percentage of CD25+Foxp3+Tregs in CD4+ T cells in MLN (C) and cLP (D) of mice in each group. n = 9 in each group. Data are the mean ± SEM, one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 2

Analysis of bacteria remodeled by metronidazole treatment. Bacterial DNA collected from mice feces on the zeroth and the fifth day during DSS treatment, and bacterial DNA collected from colon mucosal tissues on the seventh day were used to perform 16S rRNA gene sequencing. (A) Alpha diversity
(Shannon index) of bacteria in the metronidazole, neomycin and control group. (B) Beta diversity (PCoA based on bray-curtis) of bacteria in the metronidazole, neomycin and control group. (C) The mean relative abundance at the genus level of bacteria in the metronidazole, neomycin and control group. The bars list all the genera that are more than 1% of the total genera. (D) Percentages of the obligate anaerobic bacteria and facultative anaerobic bacteria in the metronidazole, neomycin and control group. (E) Heatmap depiction of the mean relative abundance of bacteria at the genus level (belonging to the top 50 dominant genera in all samples) in the metronidazole, neomycin and control group. The genus colored in red belongs to the facultative anaerobic bacteria. (F) Comparison of the 11 genera (abundant in the metronidazole group based on analysis in (E)) in the metronidazole, neomycin and control group. The genus colored in red belongs to the facultative anaerobic bacteria. n = 5 for samples of the metronidazole and control group, and fecal samples of the neomycin group; n = 4 for colon mucosal tissue samples of the neomycin group. Data are the mean ± SEM, Wilcoxon rank-sum test (A), one-way ANOVA (D to F). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
Figure 3

E. ludwigii protects mice against DSS-induced acute colitis independent of other bacterial strains. (A) Schematic diagram for the mouse model of DSS-induced acute colitis combined with ABX treatment and single bacterial colonization. Male C57BL/6J mice were gavaged daily with 10^9 CFU of E. ludwigii, E. gallinarum, L. johnsonii or sterile water one day after five days of ABX treatment. After five days of single bacterial strain administration, mice were given 3% DSS and LPS (8 mg/kg body weight) simultaneously.
to induce colitis in addition with single bacterial strain administration for another 7 days, and mice were euthanized. (B-C) Body weight (B) and disease activity index (C) were examined every day during the course of DSS treatment. (D-E) Colon length (D) and histopathological scores by H&E staining (E) in each group. n = 9 (B to D) and n = 8 (E) in each group. Data are the mean ± SEM, two-way ANOVA (B to C) or one-way ANOVA (D to E). *p < 0.05; **p < 0.01; ***p < 0.001.
E. ludwigii increases CD103+DCs and Foxp3+Tregs in MLN and cLP. MLN and cLP of mice from the E. ludwigii, E. gallinarum, L. johnsonii and control group at the seventh day after DSS treatment (in the model of Fig. 3A) were collected, and lymphocytes were isolated, stained and analyzed by flow cytometry. (A-B) Representative flow dot plots gated on CD45+MHCII+CD11c+ cells and the percentage of CD103+DCs in total DC cells in MLN (A) and cLP (B). (C-D) Representative flow dot plots gated on CD3+CD4+ cells and the percentage of CD25+Foxp3+ Tregs in CD4+ T cells in MLN (C) and cLP (D). n = 12 in each group. Data are the mean ± SEM, one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 5

E. ludwigii gavage enhances the ability of CD103+DC to promote Foxp3+Treg differentiation. (A) Immunofluorescence analysis of CD103+DCs in mouse colons of the E. ludwigii, E. gallinarum, L. johnsonii and control group at the seventh day after DSS treatment (in the model of Fig. 3A). n = 3 in each group. Scale bar, 40 μm. Blue, nucleus; Green, CD11c; red, CD103. The white arrow indicates CD11c+CD103+DC. (B) Immunofluorescence analysis of Foxp3+Treg cells in mouse colons of the E. ludwigii, E. gallinarum, L. johnsonii and control group. n = 4 in each group. Scale bar, 40 μm. Blue, nucleus; Green, Foxp3. (C-D) CD103+DCs and Foxp3+Tregs were purified from MLNs of mice in the E. ludwigii, E. gallinarum, L. johnsonii and control group by FACS sorting. mRNA levels of Tgfb1, Tgfb2, Aldh1a2 and Pdl1 for CD103+DCs (C), and mRNA levels of Tgfb1, Tgfb2, Il-10 for Foxp3+Tregs (D) were analyzed using qRT-PCR. n = 4 in each group. (E) Naive T cells (purified from spleen of Foxp3-GFP-DTR mice) were cultured with CD103+DC (purified from MLN of mice in the E. ludwigii, E. gallinarum, L. johnsonii or control group) for 72 hours, and the percentage of CD25+Foxp3+ Treg in CD4+T cells was analyzed by flow cytometry. n = 3 in each group. Data are the mean ± SEM, one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.
Figure 6

E. ludwigi directly increases the capacity of DC to promote Treg differentiation. DCs were purified from MLN of C57BL/6J mice and stimulated with live bacteria (MOI = 10, 2 h), HK bacteria (MOI = 10, 24 h) and bacteria culture supernatant (1:1, 24 h). mRNA levels of Tgfb1, Tgfb2, Aldh1a2 and Pdl1 was evaluated by qRT-PCR of DCs incubated with the control, live E. ludwigi and E. gallinarum (A), heat-killed E. ludwigi and E. gallinarum (B), and E. ludwigi and E. gallinarum culture supernatant (C). n = 4 in each
group. (D-E) Sorted DCs were stimulated with heat-killed K12 (MOI = 10) plus control (BHI), E. ludwigii and E. gallinarum culture supernatant. After 24 h, DCs were cocultured with naïve T cells (CD4+CD62L+CD25-CD44-) at a ratio of 1:5 for 72 h. (D) Representative flow dot plots and the percentage of CD25+Foxp3+Treg in CD4+T cells after co-culture was assessed by flow cytometry. n = 4 in each group. (E) Representative immunofluorescence picture and analysis of Foxp3+ cells after co-culture. n = 3 in each group. Scale bar, 10 μm. Blue, nucleus; Green, Foxp3. Data are the mean ± SEM, one-way ANOVA. *p < 0.05; **p < 0.01.

**Supplementary Files**

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- Supplementaryinformation.docx
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