Original Article
BTLA associates with increased Foxp3 expression in CD4+ T cells in dextran sulfate sodium-induced colitis

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Abstract: Ulcerative colitis (UC) is an inflammatory bowel disease, and its pathogenesis involves a variety of genetic, environmental, and immunological factors such as T helper cells and their secreted cytokines. B and T lymphocyte attenuator (BTLA) is an immunoregulatory receptor that has a strong suppressive effect on T-cell function. However the role of BTLA in UC remains poorly understood. Here we demonstrated that the frequency of BTLA-expressing CD3+ T cells, especially CD4+ T cells, increased in blood and mucosa in mice with DSS-induced colitis. The frequency of Foxp3-expressing cells in BTLA+ CD4+ T cell from lamina propria mononuclear cells (LPMCs) was much higher in DSS-treated mice than that in controls. Similarly, the proportion of IL-17+ cells in BTLA+ CD4+ T cells from LPMCs in DSS-treated mice is much higher than that in controls, while no perceptible difference for the proportion of IFN-γ+ cells in BTLA+ CD4+ T cells was noted between DSS-treated mice and controls. Treatment of mesalazine, an anti-ulcerative colitis drug, down-regulated Foxp3 and IL-17 expression in BTLA positive T cells along with attenuated severity for colitis. Our findings indicate that BTLA may be involved in the control of inflammatory responses through increasing Foxp3 expression, rather than attenuating IL-17 production, in DSS-induced colitis.

Keywords: Colitis, dextran sulfate sodium, B and T lymphocyte attenuator, Foxp3, IL-17, IFN-γ

Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic gastrointestinal disorders characterized by chronic intestinal inflammation. Although it is widely accepted that genetic, environmental, and immunological factors are involved [1, 2], the exact mechanisms underlying IBD pathoetiologies are yet to be addressed, T cells and their cytokines are the main effectors in the induction and perpetuation of intestinal inflammation [3]. It was not until a few years ago that Th1 and Th2 paradigm was used to differentiate the underlying immunological conditions of CD and UC [4, 5]. More recently, a third subset of T helper cells, Th17 cells, has been described contributing to IBD pathogenesis [6, 7]. Th17 cells produce IL-17, IL-22, and

IL-23. IL-17 levels both in mucosa and serum were increased in active IBD patients [8, 9]. Unlike effector T cells, Foxp3+ Treg cells (regulatory T cells) play a nonredundant role in immune homeostasis by preventing pathological inflammatory responses to environmental and self-antigens [10, 11]. Loss-of-function mutations in the *Foxp3* gene lead to impaired Treg function and the development of a severe multi-organ inflammatory disease [12]; illustrating the importance of Treg cells in the control of intestinal immune function. Mouse models of intestinal inflammation have also pinpointed a key role for Treg cells in intestinal homeostasis [13].

Several models of experimental colitis have been reported to demonstrate various pathophysiological aspects of human IBD [1]. Dextran

sulfate sodium (DSS)-induced colitis is a well-established animal model of mucosal inflammation for the study of IBD pathogenesis [24, 25]. DSS-induced colitis is known as a UC model, and many studies have described UC as a Th2 disease [26, 27]. However, there were also several studies suggested that DSS colitis is dependent on Th1- or Th17-mediated inflammation [28-31]. Thus, the roles of T helper cells in DSS-induced colitis are unclear.

B and T lymphocyte attenuator (BTLA), a recently discovered inhibitory receptor belonging to the CD28 family, is expressed by most lymphocytes and shares structural and functional similarities with cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death 1 (PD1) [14]. BTLA interacts with the herpesvirus entry mediator (HVEM; TNFRSF14), a TNFR super family member found on T, B, natural killer (NK), dendritic (DC) and myeloid cells [15]. The function of BTLA was described in several models of inflammatory disorders and autoimmune diseases [16-18], suggesting that BTLA is crucial for dampening overreactive immune responses. BTLA-deficient mice exhibit enhanced specific antibody responses and sensitivity to EAE [14], rapid rejection of partially MHC-mismatched cardiac allograft [16], and acceleration of experimental colitis [17]. It has been shown that the deficiency of BTLA also disturbed self-tolerance, resulting in the development of an autoimmune hepatitis like disease and lymphocytic infiltration in multiple organs [18]. More recent studies further demonstrated emerging evidence suggesting that BTLA could also initiate pro-survival signals for activated or effector T cells relevant to the generation of immune memory other than its regulatory role in diverse immune responses [19, 20-23]. Therefore, BTLA expression on T cells might correlate with T-cell dysfunction and disease pathogenic events. However, the impact of BTLA on the modulation of T-cell responses during the course of UC is yet to be fully addressed. We thus in the present study employed an animal model with DSS-induced colitis to dissect the role of BTLA in T cells and their implication in the pathogenesis of ulcerative colitis.

Materials and methods

Mice

C57BL/6 mice (6- to 8 wk-old) were purchased from the Experimental Animal Center of Guangdong Province (Guangzhou, China). The mice were housed in the Experimental Animal Center at Guangdong Medical College for at least 1 wk before inclusion in experiments. All of the studies were approved by Committees on Use and Care of Animals at the Guangdong Medical College.

Collection of human colon tissues

Human colon tissues were collected from patients with UC via endoscopic colon biopsy. All procedures were approved by the Human Institutional Review Board of the Guangdong Medical College. Informed consent was obtained in writing and a copy was inserted in the medical records of the patients.

Antibodies and reagents

Anti-mouse CD4-FITC (GK1.5), CD8a-PE (53-6.7), CD272 (BTLA)-APC (8F4), mouse/rat IL-17A-PE (eBio17B7), IFN-gamma- PerCP-Cy5.5 (XMG1.2), Foxp3 (clone FJK-16s), and PE-Cy5.5 Foxp3 (FJK-16s) were purchased from eBioscience Inc. (San Diego, CA). Anti-mouse CD3e-PE-Cy7 (17A2) was obtained from BioLegend (San Diego, CA). Dextran sulfate sodium (DSS) was derived from MP Biomedicals (Shanghai, China). Fecal occult blood kits were originated from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

DSS-induced colitis and mesalazine treatment

Experimental colitis was induced by administration of DSS (36-50kDa) for 7 days. Briefly, mice in DSS-treated group were orally administered 5.0% DSS in drinking water, while mice provided with acidified water were served as controls. A portion of DSS-induced animals were orally treated with mesalazine (MSLZ, 0.0026/0.02 Kg/day) for 1 wk. The activity, coat color changes, stool, color and the presence of hemorrhage were monitored on daily basis.

Clinical evaluation of DSS-induced colitis

Body weight loss during the course for induction of colitis was normalized by the body weight on day 1. Stool consistency was checked for diarrhea and feces were screened for blood detection using an aforementioned fecal occult blood kit. The disease activity index (DAI) was as following [35]: (a) weight loss (0 point = none, 1 point = 1-5% weight loss, 2 points =
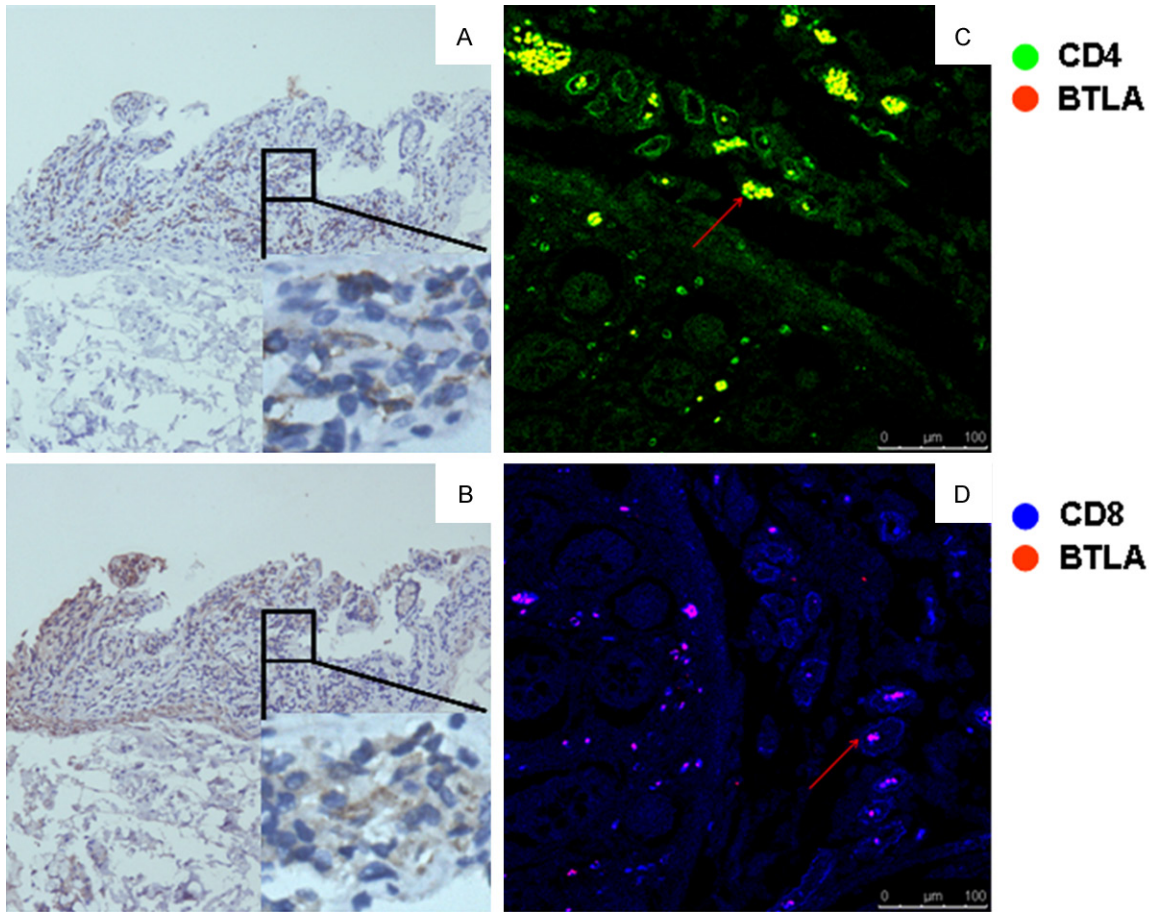
Figure 1. BTLA expression in colon tissue from the patient with UC. Colon tissues from UC patients were stained with enzyme- or fluorescence-labeled antibodies, and then observed under a light microscopy or Laser Scanning Confocal Microscopy. Same area in colon tissue were stained with anti-human CD3 (A) or anti-human BTLA (B) respectively, immunohistochemically. (C) and (D) show co-staining of BTLA (red) with CD4 (green) or CD8 (blue).

5-10% weight loss, 3 points = more than 10% weight loss), (b) stool consistency (0 point = normal, 1 point = soft stools, 2 points = very soft stools, 3 points = watery stools), and (c) the date of presence for blood in stool (0 point = no blood in stool, 1 point = day 7, 2 points = day 6 or day 5, 3 points = within day 4). The DAI was calculated as the sum of weight loss, stool consistency, and day of bleeding, with the total DAI score ranging from 0 (unaffected) to 9 (severe colitis).

Histopathological analysis and immunohistochemistry

Paraffin-embedded sections were stained with hematoxylin and eosin. The degree of colonic inflammation was analyzed according to the scoring system described previously [36]. For serial and double immunohistochemical stain-ing, frozen sections were incubated with indicated primary antibodies overnight at room temperature, followed by 30 min of incubation with appropriate secondary antibodies. Slides were then counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and analyzed using a Zeiss confocal microscope (Carl Zeiss Microimaging, Oberkochen, Germany) as instructed.

Mononuclear cell isolation

Gut LPMCs were isolated as described below. Intestinal tissue was washed extensively with RPMI 1640 medium and all the patches were removed with a scissor, followed by a longitudinal incision, and then cut into 1 mm^2 pieces. Processed tissue debris was incubated for 30 min at 37°C in calcium-and magnesium-free Hanks's balanced salt solution containing 5% FCS and 1 mM EDTA. After washed, tissue
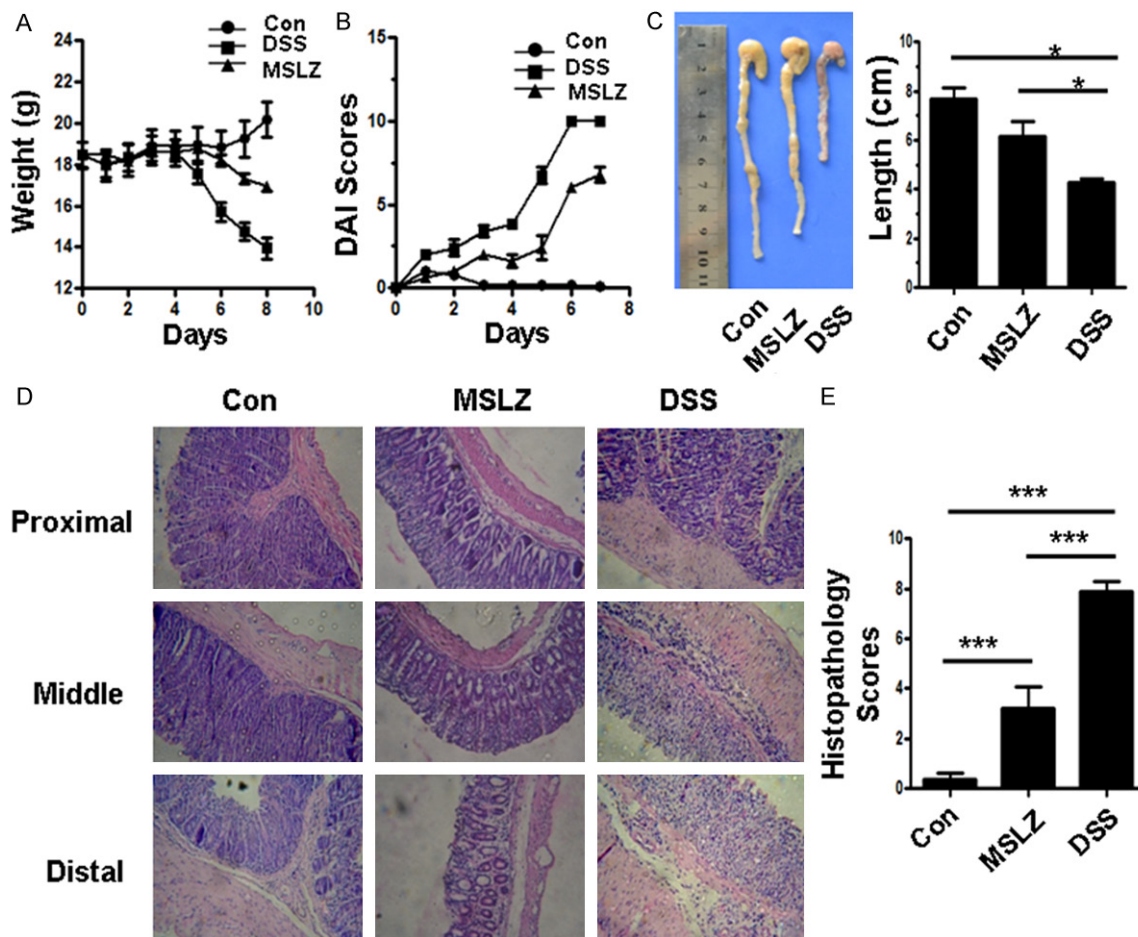
Figure 2. DAI scores and histopathology scores of mice. (A) and (B) show body weight changes and DAI scores, respectively. (C) shows the colon length of each group. (D) shows representative images for histopathological changes in each group, (E) is statistical analysis of histopathological changes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

debris were incubated for 1 h at 37°C in 30 ml complete RPMI-5 (RPMI1640 + 5% FCS) containing 1 mg/ml collagenase (GIBICO). A 20 cc syringe with a 16 g feeding needle was next employed to, aspirate tissue pieces in and out of syringe (moderately slowly) 10 times to disrupt the tissue and release remaining cells. The LPMCs preparations were washed through a nylon mesh screen into fresh 15 ml tubes. The cells were resuspended in RPMI-5 after extensive washed and then applied to Ficoll solution gently, followed by centrifugation at 3000 rpm for 20 min. LPMCs were collected from the white interface and subjected to washed with RPMI-5. The number and viability of isolated LPMCs were assessed by Trypan blue staining.

Flow cytometric analysis

The isolated cells were stimulated with PMA for 4 h and BFA was added into the culture after 2

h of stimulation. For cytofluorometric analysis, the cells were stained for 15 min at room temperature using the saturating concentrations of antibodies against CD3, CD4, CD8, and CD272 (BTLA), respectively. After washed, the cells were incubated for 15 min at room temperature in Fix/PERM A (MuLTISCIENCES Biotech Co. Ltd), followed by 20 min incubation in Fix/PERM B (MuLTISCIENCES Biotech Co. Ltd), and stained for 20 min with following antibodies: IL-17A mAb, IFN-γ mAb, and Foxp3 mAb. After washed, cells were analyzed on a FACS 440 flow cytometer (BD Biosciences, Mountain View, CA).

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 software. Data were shown as mean ± SD and were analyzed by the Mann Whitney test or the Wilcoxon matched-pairs signed-rank test.
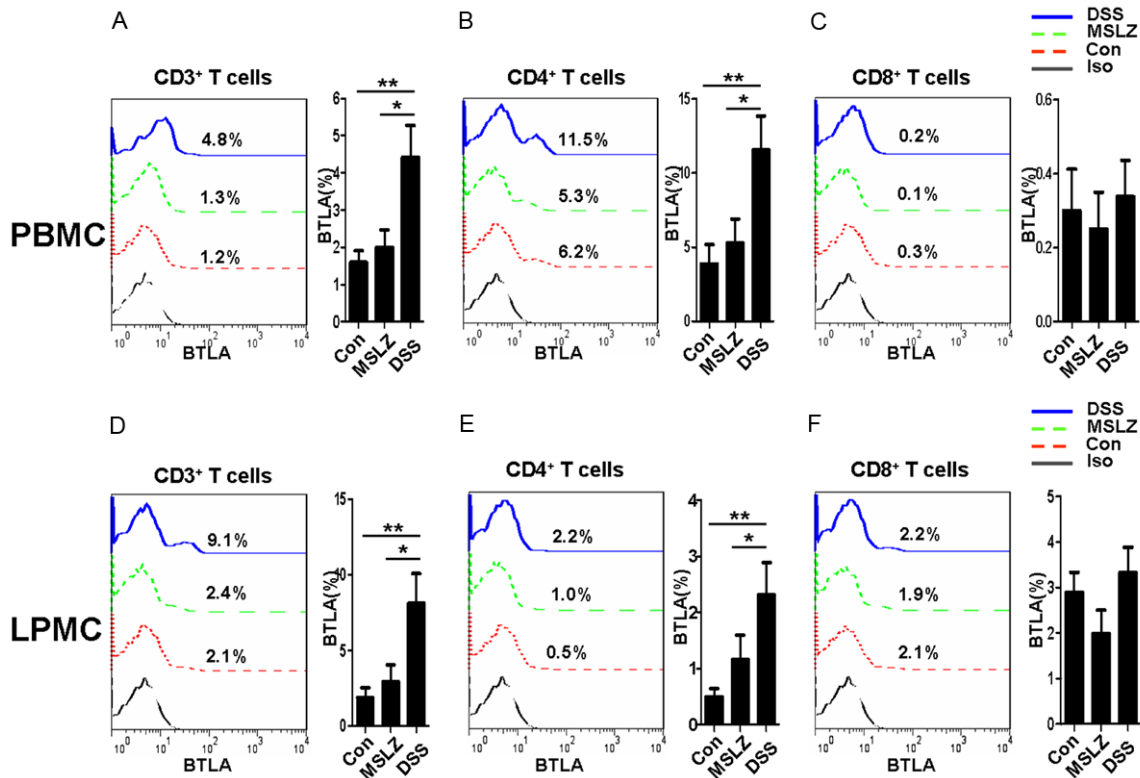
Figure 3. BTLA expression in T cell subsets. Mononuclear cells were isolated from peripheral blood and colon tissues. LPMCs and PBMC were then stained with anti-CD3, anti-CD4, anti-CD8, and anti-BTLA, and analyzed by flow cytometry. Left column, representative flow cytometric graph; Right column, statistical analysis. (A) and (D) show BTLA expressed in CD3$^+$ T cell from PBMCs (A) or LPMCs (D); (B) and (E) show BTLA expressed in CD3$^+$CD4$^+$ T cell subset from PBMCs (B) or LPMCs (E); (C) and (F) show BTLA expressed in CD3$^+$CD8$^+$ T cell subset from PBMCs (B) or LPMCs (E). *P < 0.05; **P < 0.01; ***P < 0.001.

An unpaired Student t test (two-tailed) was used to assess differences between two groups when the data met the assumptions of a t test, depending on the experiment. A P value < 0.05 was considered with statistical significance.

Results

Increased expression of BTLA in CD4$^+$ T cells in DSS-induced colitis

To assess the role of BTLA in pathogenesis of ulcerative colitis, colon biopsies were obtained in patients with UC, and then subjected to immunohistochemical analysis of BTLA expression. Interestingly, high levels of BTLA were detected in colon mucosal area of all examined patients with active UC (**Figure 1A**, **1B**). Con-immunostaining further revealed that BTLA was detected on both infiltrated CD4$^+$ and CD4$^-$ T cells (**Figure 1C**, **1D**).

To confirm these results, we next induced colitis by feeding mice with DSS water. During the course of colitis development, body weight and stool occult blood (OB) were tested daily, colon

tissues and peripheral blood were harvested at day 8 of induction. After 4 days of DSS treatment, mice displayed a progressive disease severity as manifested by the acceleration of weight loss, presence of diarrhea, reduction of mobility, stool OB, and piloerection of the fur. Notably, DSS-treated mice showed a reduction of ~15% of initial starting weight (**Figure 2A**) and increased DAI scores (**Figure 2B**), which were attenuated by MSLZ treatment. Consistently, DSS-treated mice showed severe intestinal inflammation as evidenced by the shorter length of colon (**Figure 2C**), H & E staining revealed prominent epithelial cell hyperplasia, absence of goblet cells, and a massive infiltration of mononuclear cells (**Figure 2D**). Histological analysis further demonstrated significant pathological scores in DSS-induced mice (**Figure 2E**).

BTLA expression in peripheral blood T cells or in colon infiltrated T cells was then analyzed by flow cytometry. A 2-fold increase for BTLA expression was noted both in peripheral blood CD3$^+$ T cells (**Figure 3A**) and colon infiltrated
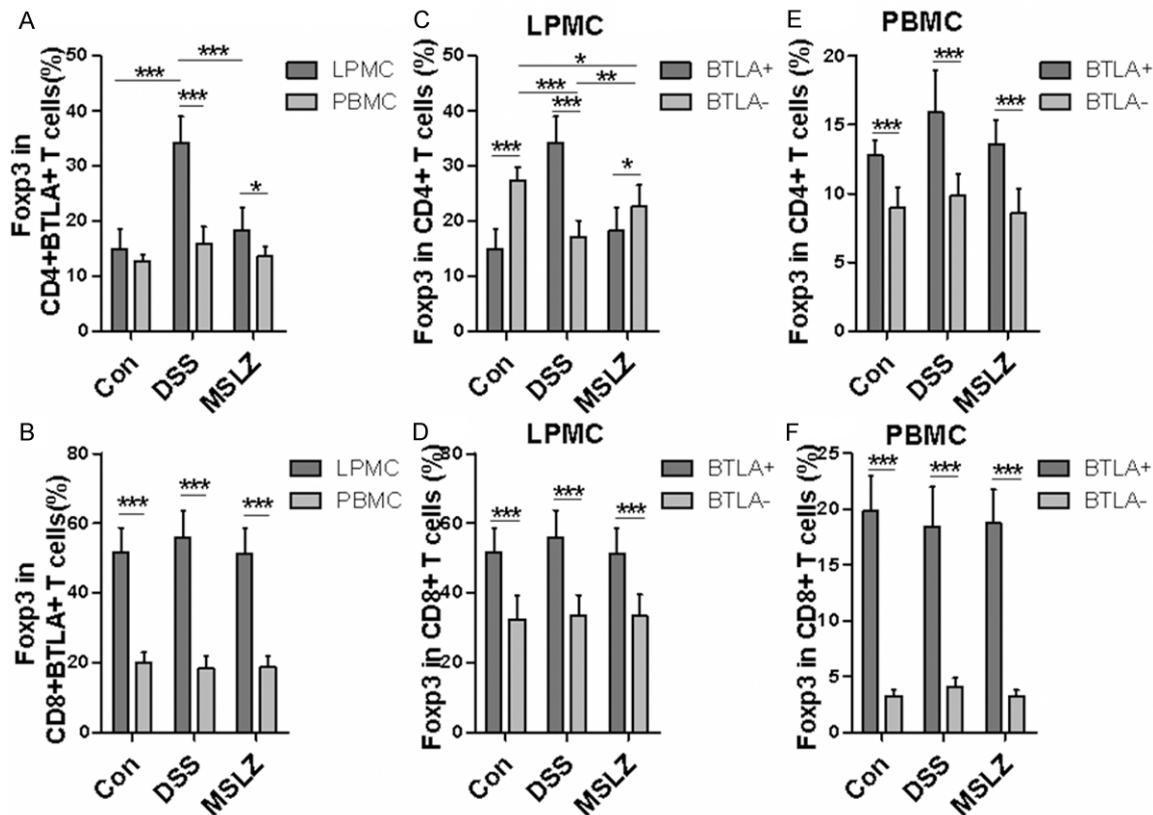
Figure 4. Foxp3 expression in BTLA-expressing T cells. LPMCs and PBMCs were stained with surface marker and intracellular Foxp3 as indicated, followed by flow cytometric analysis. (A and B) show Foxp3 expression in CD4^{+} BTLA+ and CD8^{+} BTLA+ T cells. (C and D) are comparative analyses of Foxp3 expression in BTLA+ and BTLA- T cells from LPMCs. (E and F) Foxp3 expression in BTLA+ and BTLA- T cells from PBMC. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

CD3^{+} T cells (**Figure 3D**). Furthermore, a significant increase for the number of BTLA+ CD4^{+} T cells was characterized both in PBMCs (**Figure 3B**) and LPMCs (**Figure 3E**), while perceptible difference for BTLA+ CD8^{+} T cells was detected between DSS-induced mice and controls (**Figure 3C** and **3F**). Laser Scanning Confocal Microscopy also showed an abundant expression of BTLA in DSS-induced mice (Figure S1). Together these data demonstrate that during the course of colitis development, T cells, predominantly CD4^{+} T cells, express high levels of BTLA, which may contribute to UC progression.

BTLA associates Foxp3 expression in colon tissue

As Tregs are crucial in maintaining intestinal mucosal homeostasis and take part in UC progression, we thus next sought to determine the impact of BTLA on Foxp3 expression. Interestingly, about 35% of CD4^{+} BTLA+ T cells from LPMCs expressed Foxp3 in DSS-treated mice, which were much higher than those in control mice. In addition, Foxp3+ cells in CD4^{+}

BTLA+ LPMCs was higher than that in PBMCs (**Figure 4A**). Surprisingly, over 50% of CD8^{+} BTLA+ T cells in LPMCs from DSS-treated mice expressed Foxp3, which were two times higher than those in PBMCs. However, no significant difference in terms of the frequency for Foxp3+ CD8^{+} BTLA+ cells was observed between DSS-treated and control mice (**Figure 3B**). The expression of Foxp3 in BTLA+ and in BTLA- T cells was also analyzed. In LPMCs of DSS-treated mice, higher frequency of Foxp3+ cells was found in CD4^{+} BTLA+ T cells compared to CD4^{+} BTLA- counterparts (**Figure 4C**). In control mice, however, there was a lower frequency of Foxp3+ cells in CD4^{+} BTLA+ T cells compared to that in CD4^{+} BTLA- T cells (**Figure 4C**). In contrast, the number of Foxp3+ cells in CD8^{+} BTLA+ T cells was much higher than that in CD8^{+} BTLA- T cells in LPMCs from all groups of mice examined (**Figure 4D**). Similarly, much higher number of Foxp3+ cells was noted in both CD4^{+} (**Figure 4E**) and CD8^{+} (**Figure 4F**) in PBMCs of all group of mice examined.
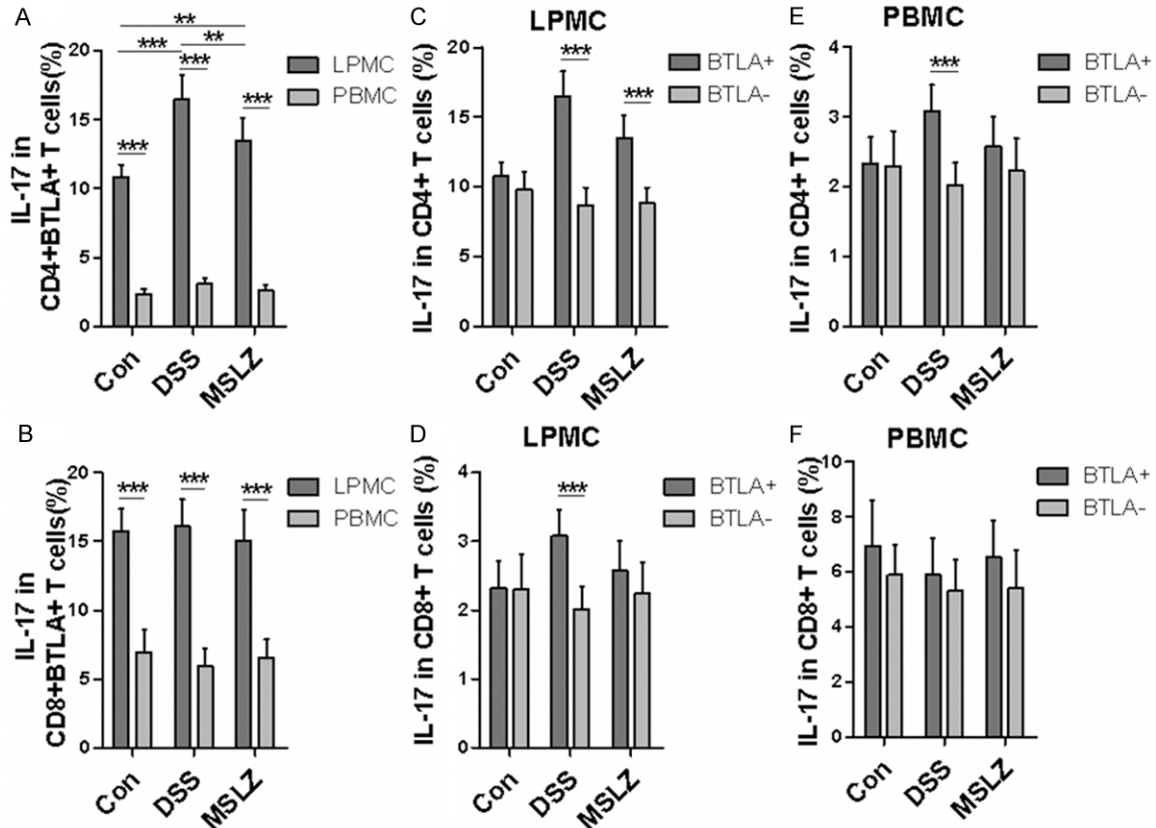
Figure 5. Intracellular cytokine staining of IL-17 in BTLA+ T cells. LPMCs and PBMCs were stained with surface marker and intracellular IL-17. A and B. Show IL-17 production in CD4+ BTLA+ and CD8+ BTLA+ T cells. C and D. IL-17 production in BTLA+ and BTLA- T cells from LPMCs. E and F. IL-17 production in BTLA+ and BTLA- T cells from PBMC. *P < 0.05; **P < 0.01; ***P < 0.001.

Collectively, these data suggest that BTLA expression is likely associated with induction of Foxp3 expression in the setting of DSS-induced colitis.

BTLA expression does not attenuate IL-17 production

Because IL-17 and IFN-γ are important inflammatory cytokines implicated in UC pathoetiology, we further explored the effect of BTLA on IL-17 and IFN-γ expression. LPMCs manifested higher frequencies for IL-17- and IFN-γ-producing cells both in CD4+ BTLA+ (**Figure 5A**) and CD4- BTLA+ T cells (**Figure 5B**) as compared with that of PBMCs. Importantly, DSS-treatment induced much more IL-17+ cells in CD4+ BTLA+ T cells from LPMCs as compared to controls (**Figure 5A**). Unexpectedly, DSS-treatment failed to show perceptible effect on IL-17 production in BTLA- T cells, and similarly, no discernable difference was noted in terms of IFN-γ+ cells between BTLA+ and BTLA- T

cells (**Figures 5C**, **5E**, **6A**, **6C** and **6E**). In DSS-treated mice, higher proportion of IL-17+ cells was observed in BTLA+ T cells compared to BTLA- counterparts. In contrast, higher proportion of IFN-γ+ cells was found in BTLA- T cells than in BTLA+ counterpart in PBMCs (**Figure 6E**). Collectively, these results demonstrate that BTLA expression may not impact the production of IL-17+ cells in the setting of DSS-induced colitis.

Mesalazine treatment inhibits BTLA expression along with attenuated production of Foxp3+ and IL-17+ T cells

Mesalazine (MSLZ) is an anti-inflammatory drug commonly used in the clinical setting for treatment of inflammatory bowel disease, especially UC. It is a bowel-specific aminosalicylate drug that acts locally in the gut, there by having few systemic side effects. We finally sought to investigate whether MSLZ attenuation of colitis symptom involves regulation of
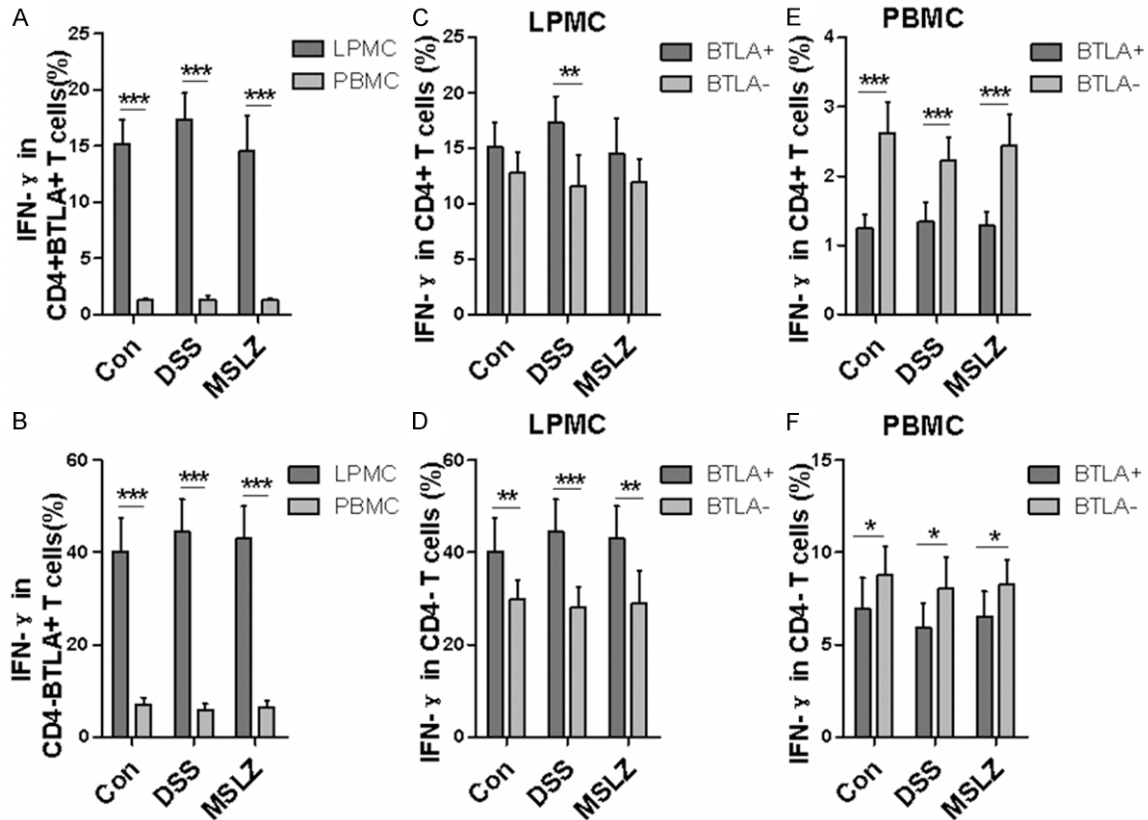
Figure 6. Intracellular cytokine stain of IFN-γ in BTLA+ T cells. LPMCs and PBMCs were stained with surface marker and intracellular IFN-γ. A and B. Show IFN-γ production in CD4+ BTLA+ and CD8+ BTLA+ T cells. C and D. IFN-γ production in BTLA+ and BTLA- T cells from LPMCs. E and F. IFN-γ production in BTLA+ and BTLA- T cells from PBMC. *P < 0.05; **P < 0.01; ***P < 0.001.

BTLA expression connected to the production of Foxp3+ or IL-17+ T cells. For this purpose, we conducted flow cytometry analysis of BTLA, Foxp3, and IL-17 expression in mice with MSLZ treatment. Indeed, MSLZ treatment significantly down-regulated BTLA expression in CD4+ T cells both in LPMCs and PBMCs (**Figure 3A**, **3B**, **3D**, **3E**). In consistent with this observation, MSLZ treatment significantly decreased the proportion of Foxp3+ and IL-17+ cells in CD4+ BTLA+ T cells, but without detectable effect on the production of IFN-γ+ cells (**Figure 5A**). Collectively, those results suggest that MSLZ could modulate suppressive immune response and inflammatory response simultaneously.

Discussion

In the present study, we demonstrate an increase of BTLA expression on CD3+ T cells (especially CD4+ subpopulation) from both LPMCs and PBMCs in DSS-induced colitis. Importantly, increased BTLA in CD4+ T cell is associated with the expression of Foxp3, a master regulator for Tregs, suggesting that BTLA might be important for suppressive function of Tregs. Particularly, we found lower IFN-γ production in BTLA+ T cells suggesting BTLA expression may suppress the production of IFN-γ. However, IL-17 production in CD4+ BTLA+ T cells was higher in LPMCs from DSS-treated mice than that in controls, indicating IL-17 production induced by DSS may not be attenuated by BTLA. These findings uncovered a previously unknown mechanism by which BTLA regulates T-cell immune responses in ulcerative colitis.

Foxp3 is an immune regulator found in Tregs and is important for the development and suppressive function of Tregs [33]. Loss-of-function mutations in the *Foxp3* gene lead to severe multigrain inflammatory diseases [12]. Studies have shown that Foxp3+ Treg increased in inflamed mucosa of Crohn's disease (CD) and ulcerative colitis (UC), despite the fact that they

were decreased in PBMCs [34]. It has been shown that Foxp3+ Tregs express BTLA and BTLA signal displayed suppressive function on effector cells [35]. However, the relationship between BTLA and Foxp3 remains to be fully elucidated. Our study suggested that DSS treatment induces an increase of Foxp3+ Tregs in BTLA+ CD4$^+$ T cells from LPMCs, while no difference was observed in BTLA+ CD4$^+$ T cells from PBMCs. These results indicate that BTLA associates with Foxp3 expression in CD4$^+$ T cells and it is likely that BTLA signal may enhance the suppressive function of Treg in DSS-induced colitis. Indeed, lower number of IFN-γ+ effector T cells was observed in BTLA4+ T cells compared with that of BTLA- counterparts.

Extensive studies have suggested a critical role for IL-17 in chronic inflammatory disease. It induces neutrophil migration and expansion in inflammatory sites. It also promotes dendritic cell maturation and secretion of inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8, GM-CSF, iNOS, PGE2, and metalloproteases [36, 37]. BTLA has been reported to be able to negatively regulate IL-17 and TNF-α production in CD27(-) γδ T cells [38]. Interestingly, our current data showed that BTLA-expressing CD3$^+$ CD4$^+$ T cells and CD3$^+$ CD8$^+$ T cells produce much more IL-17 than that in BTLA negative CD3$^+$ CD4$^+$ T cells and CD3$^+$ CD8$^+$ T cells after DSS-treatment. Furthermore, BTLA-expressing CD4$^+$ T cells produced much more IL-17 in LPMCs from DSS-treated mice than that from control mice, indicating that DSS-induced BTLA expression failed to attenuate IL-17 production in DSS-induced colitis.

Mesalazine, also known as 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug commonly used to treat inflammatory bowel disease, especially to UC by acting locally in the gut. As a derivative of salicylic acid, mesalazine is also thought to be an antioxidant that traps free radicals. Previous study has shown that TNBS induced high-level expression of Foxp3 in mucosa in a rat ileitis model, while 5-ASA administration could down-regulate IL-17 expression without affecting Foxp3 expression [39]. Interestingly, our study demonstrated that Mesalazine treatment down-regulates not only IL-17 but also Foxp3 in BTLA-expressing CD4$^+$ T cells. This discrepancy is probably because we detected different cell population. We detected the expression of Foxp3 in BTLA+ T cells, while

Requena et al detected Foxp3 in whole ileum samples in their reports [39]. Further studies are required to investigate the effect of mesalazine on Foxp3 expression and Treg suppressive function.

In summary, we demonstrated a previously unappreciated role for BTLA in T cells in UC. BTLA associates with increased Foxp3 expression and might be important for the suppressive function of Tregs in DSS-induced colitis. However, BTLA is not sufficient to attenuate IL-17 production in DSS-induced colitis. Mesalazine treatment decrease Foxp3 and IL-17 expression simultaneously. Our study indicates that BTLA may be involved in the control of inflammatory response in UC.

Disclosure of conflict of interest

None.

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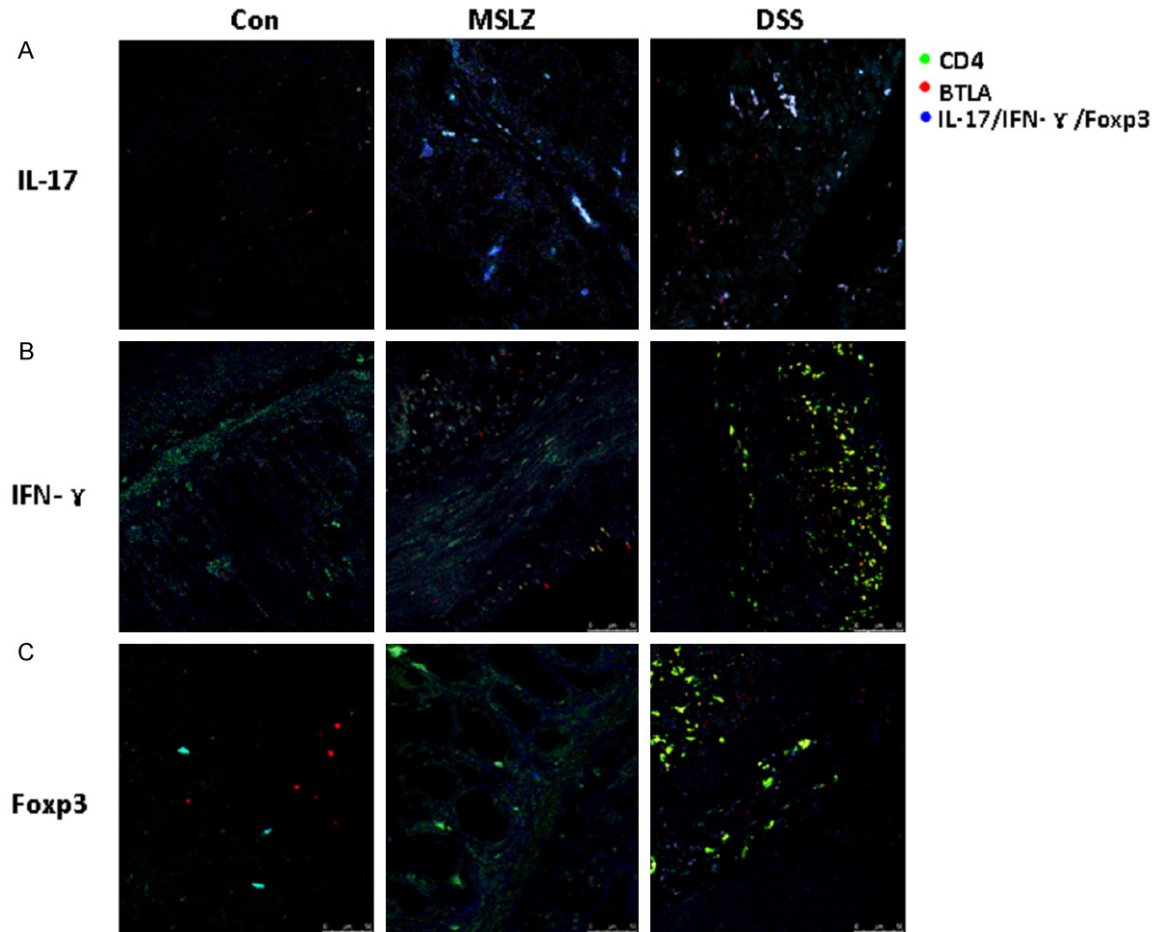
Figure S1. Foxp3, IL-17 and IFN-γ expression in BTLA+ T cells in colon tissue from DSS-induced colitis. To determine co-expression of BTLA with IL-17 ,IFN-γ and Foxp3 in colon tissues from DSS-induced colitis, paraffin sections were stained with fluorescent labeled anti-mouse CD4 ,anti-mouse BTLA, and anti-mouse IL-17 (A), anti-mouse IFN-γ (B) or anti-mouse Foxp3 (C).