**Name of journal: World Journal of Gastroenterology**

**ESPS Manuscript NO: 25188**

**Manuscript Type: ORIGINAL ARTICLE**

***Basic Study***

**Curcumin improved regulatory T cells in gut-associated lymphoid tissue in colitis mice**

Zhao HM *et al*. Curcumin improved regulatory T cells in colitis mice

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**Author contributions:** Zhao HM is primary author of the manuscript, and designed, performed the research; Zhao HM, Huang XY and Lu AP contributed to analysis of data, as well as writing and reviewing the final manuscript; Xu R, Huang MF, Yue HY, Wang X and Zou Y performed the research; and Liu DY was involved in project conception/design and data analysis, as well as writing and reviewing the final manuscript.

**Supported by** the Project of National Natural Science Foundation of China, No. 81260595 and No.81460679; and funded by Chinese Scholarship Council and Jiangxi Province as visiting scholar (CSC: 201408360106, 201408360110), and the Project of Jiangxi University of Traditional Chinese Medicine, NO. JZYC15S13.

**Institutional review board statement:** All routine colonic biopsy specimens and gut-associated lymphoid tissue samples from the mice were taken after ethical permission was obtained for participation in the study.

**Institutional animal care and use committee statement:** The experimental protocols (IACUC protocol number: JZ2015-016) were approved by Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine.

**Conflict-of-interest statement:** All authors have declared that there is no conflict of interests.

**Data sharing statement:** No additional unpublished data are available

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**Received:** February 27, 2016

**Peer-review started:** February 28, 2016

**First decision:** March 21, 2016

**Revised:** March 29, 2016

**Accepted:** April 15, 2016

**Article in press:**

**Published online:**

**Abstract**

**Aim**: To explore probable pathway of Curcumin (Cur) regulated function of Treg cells by observing expressions of costimulatory molecules of dendritic cells (DCs).

**MethodS**: Experimental colitis was induced by administering the solution of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)/ethanol. Forty male C57BL/6 mice were randomly divided into 4 groups: Normal, TNBS +Cur, TNBS + Mesalazine (Mes) and Normal groups. And the mice in the TNBS + Cur and TNBS + Mes were treated with Cur and Mes, respectively, while those in the TNBS group were treated with physiological saline for seven days. After treatment, the curative effect of Cur was evaluated by colonic weight, colonic length, weight index of colon, and histological observation and score. The levels of CD4+CD25+Foxp3+T cells (Treg cells) ,and costimulatory molecules of DCs were measured by flow cytometry. Also, related cytokines were analyzed by enzyme-linked immunosorbent assay.

**Results:** Cur alleviated inflammatory injury of colonic mucosa, decreased colonic weigh and histological score and restore colonic length to treat experimental colitis in the present study. While the number of Treg cells was increased while the secretion of TNF-α, IL-2, IL-6, IL-12 p40, IL-17 and IL-21, and the expressions of costimulatory molecules (CD205, CD54 [ICAM-1], TLR4, CD252 [OX40 L], CD256[RANK] and CD254[RANK L]) of DCs were notably inhibited in colitis mice treated with Cur.

**Conclusion:** Cur potentially modulated activation of DCs to enhance the suppressive functions of Treg cells, and restore damaged colonic mucosa of IBD.

**Key words:** Curcumin; Regulatory T cells; Dendritic cells; Costimulatory molecules

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**Core tip:**the low-level of regulatory T (Treg) cells play an important role in pathogenic process of inflammatory bowel disease. In our and other previous studies, curcumin (Cur) can effectively attenuate inflammation in human and animal with colitis. However it is unclear whether that Cur can improve the level of Treg cells, and which pathway Cur can regulate the Treg cells. In the present study, we had shown that Cur potentially modulated activation of DCs to enhance the suppressive functions of Treg cells, and restore damaged colonic mucosa of IBD.

Zhao HM, Xu R, Huang XY, Cheng SM, Huang MF, Yue HY, Wang X, Zou Y, Lu AP, Liu DY. Curcumin improved regulatory T cells in gut-associated lymphoid tissue in colitis mice. *World J Gastroenterol*2016; In press

**Introduction**

Since 1995, regulatory T cells (Treg) as a particular lineage of CD4+T cell play a central role in the effective control of self-tolerance and maintenance of immune homeostasis[1,2]. Insufficient quantity or dysfunction of Treg invariably leads to inflammatory diseases or autoimmunity or lymphoproliferative syndrome including inflammatory bowel disease (IBD), rheumatoid arthritis and systemic lupus erythematosus in human and animal[3-6]. many previous studies had demonstrated that the suppressive function of Treg cell limits convincingly favorable host effector responses and restrains inflammatory responses in diverse anatomical locations as mucosal barriers against chronic inflammations and tumors[7-10]. Distinct Treg subsets coexist in the intestinal mucosa and mesenteric lymph nodes. Some studies have demonstrated that the suppressive function of Treg cells ispartly implemented *via* modulation of dendritic cells (DCs)[11-13]. Usually, the transfer of Treg cells leads to the development of colitis *via* accumulation of the T-cell and DC[14]. However,adequate Treg or transfusion of Treg cells maintains mucosal tolerance to prevent and cure experimental colitis by directly inhibiting the expression of costimulatory molecules (as CD40, CD154, CD134 and CD134L) of DCs or migration of DCs to the MLN, or by reducing DCs activation[15,16]. These findings indicate that the interactions of DCs and Treg cells are closely related to the pathogenesis of IBD, and probably become an action target for medicine treated IBD.

Curcumin (Cur), as a major active componentisolated from the rhizome of Curcuma longa (turmeric), has been used widely as herbal remedies to treat cardiovascular disease, diabetes mellitus and IBD for several centuries in China[17,18]. Cur is known for its low toxicity and a wide range of reported pharmacological effects, which includeantioxidant, anti-inflammatory, antiplatelet, hypoglycemic, cholesterol lowering, anti-bacterial, wound-healing and anti-fungal effects[19-21]. Several studies have demonstrated that cur can effectively attenuate inflammation in human and animal with colitis[19-21]. Although anti-inflammatory actions of Cur-treated IBD maybe associated with the inhibition of nuclear factor κB (NF-κB) pathway such as p38 mitogen-activated protein kinases (MAPK) activity and reduction of pro-inflammatory cytokine response[22,23], its exact mechanisms remain unclear.In the present study, we attempt to explored pathway of Cur regulated function of Treg cells by observing expressions of costimulatory molecules of DCs in animal colitis.

**Materials and methods**

***Experimental animals***

Male C57BL/6 mice, 9-12-wk-old, were purchased from the Animal Center of Peking University Health Science Center (The animal certificate number was SCXK 2012-0001).They were housed at 23 ± 2℃ with a humidity of 50% ± 5% in a 12 h light/dark cycle and provided with standard diet and water ad libitum. Mice wererandomly assignedinto4 groups: the Normal groups (Normal), the TNBS group (TNBS), the TNBS + Cur group (TNBS + Cur), and the TNBS + Mesalazine (Mes) group (TNBS + Mes). Each group contained ten mice. The experimental protocols (JZ2015-016) were approved by Institutional Animal Care and Use Committee (IACUC) of Jiangxi University of Traditional Chinese Medicine (TCM).

***Induction of colitis***

Colitis was induced by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) (TNBS, batch number: p2297, Sigma, United States) as described by previous studies[24,25]. Except for mice in the Normal group, others mice were lightly anesthetized with pentobarbital sodium (40 mg/kg) *via* intraperitoneal injection before 100 mg/kg TNBS dissolved in 0.3 mL 50% ethanol was instilled into approximately 2 cm colon to theanus. The same volume of physiological saline, as control, was served in the Normal group.

***Treatment protocols***

Twenty-four hours after colitis was induced, the mice in the TNBS + Cur and TNBS + Mes groups were respectively administered with curcumin (200 mg/kg) (purity > 95% by HPLC, batch number: GR-133-140421, GANGRUN Biotechnology, Nanjing, China) andMesalazine (Mes, 300 mg/kg) (Mes, batch number: 130407, Sunflower Pharma Jiamusi, China) intragastrically for 7 d. The mice in the Normal and TNBS groups received equivalent volume of physiological saline every day until the end of the experiment. All the mice were sacrificed on the 8th day.

***Histological evaluation***

The whole colon (*n =* 10) was separated rapidly, measured its length, cleared its contents, and weighted colonic weigh, and then divided into two parts. The weight index of colon is equal to colonic weight/body weight × 100 %. The colonic segments were fixed in 4% polyformaldehyde solution, subsequently made into pathological section stained with hematoxylin and eosin (HE). According to the previous study reported by Nicole and his colleagues[26], the criterion of the histological damage score (*n =* 10) was implemented to evaluate colonic injury. Histological score was composed with inflammatory cell infiltration and tissue damage. Scores for infiltration are as follows: 0: no infiltration; 1: increased number of inflammatory cells in the lamina propria; 2: inflammatory cells extending into the submucosa; and 3: transmural inflammatory cell infiltration. The scores of tissue damage are as follows: 0: no mucosal damage; 1: discrete epithelial lesions; 2: erosions or focal ulcerations; and 3: severe mucosal damage with extensive ulceration extending into the bowel wall.

***Isolation of lymphocyte from gut-associated lymphoid tissue***

Gut-associated lymphoid tissue (GALT) was separated and collected from the whole small intestine to the terminal rectum. GALT was triturated in 3% fetal calf serum (FCS)/PBS solution on ice cake, and filtrated *via* 300 section stainless steel cell cribble. The cell suspensions were centrifuged at 1500 rpm/min at 4 ℃ for 5 min and suspended in 1 × 106-1 ×107/mL in 3% FCS/PBS solution.

***Assay of CD4+CD25+Foxp3+ T cells by flow cytometry***

The cell suspensions (𝑛 = 8) were incubated for 30 min with V450 -anti-mouse CD4+Ab (0.125 μg/100 μL, BD bioscience) and PerCP/Cy5.5 anti-mouse CD25+Ab (0.25 μg/100 μL,Biolegend) at 37 ℃ in the dark. Cells were centrifuged at 1500 rpm / min and 4 ℃ for 5 min, fixed in Fix/Perm Buffer (eBioscience, San Diego, CA) for at least 1 h at 37 ℃, and then incubated with APC -anti-mouse Foxp3+Ab (0.5 μg/100 μL; eBioscience) for 30 min at 37 ℃ in the dark. Cells labeled with PE rat IgG2a were used as the isotype negative control. Fluorescence-activated cell sorting analysis was performed on a FACSCalibur (BD Biosciences).

***Measurement of costimulatory molecules by flow cytometry***

Data acquisition was performed using a flow cytometer (FACSCalibur, BD-Pharmingen, San Diego, CA, United States) by collecting aminimum of 10000 events per sample. The frequency of positive cells was analyzed using the program Cell Quest in two regions. The lymphocyte region was determined using granularity (SSC) and size (FSC) plot. DCs were identified as a MHC+lineage+ [CD205+, CD54 (ICAM-1)+, TLR4+, CD252 (OX40 L)+, CD256 (RANK)+ and CD254 (RANK L)+] population, and within this gate the CD11c+ population was assessed. The following mAbs were used: APC/Cy7 anti-mouse CD11c, PerCP/Cy5.5 Anti-mouse CD205, FITC Anti-mouse CD54, PerCP-Cy™5.5 Anti-mouse I-A/I-E (MHC-II), PE-Cyanine7 Anti-mouseTLR4, APC Anti-mouseCD252, PEAnti-mouseCD256 and PE Rat Anti-mouseCD254 (eBioscience, San Diego, CA). Limits for the quadrant markers were always set based on negative populations and isotype controls.

***Enzyme-linked immunosorbent assay***

The colonic mucosa was separated from all mice and prepared into tissue homogenate. After centrifugation at 5000 rpm/min for 10 min, the supernatant was collected and used for testing the level of cytokines by enzyme-linked immunosorbent assay (ELISA). The levels of TNF-α, IL-2, IL-6, IL-12 p40, IL-17 and IL-21 (𝑛 = 8) were determined using commercial ELISA kits according to the manufacturer’s instructions (eBioscience, San Diego, CA).

***Statistical analysis***

Data are expressed as mean ± SE. The statistical significance was evaluated by one-way analysis of variance (ANOVA), and performed by Prism 4.0 (GraphPad Software, La Jolla, CA). P-values less than 0.05 were considered statistically significant.

**Results**

***Curcumin effectively alleviated inflammatory injury of colonic mucosa in colitis mice***

Administration of TNBS led to a severe illness that their pathological characters were including damaged colonic mucosa, increased colonic weight and so on. As shown in the Figure 1, compared with mice in the TNBS group, the colonic weight (Figure 1A) and weight index of colon (Figure 1C) were decreased in the TNBS + Cur, and TNBS + Mes groups, while the colonic length (Figure 1B) was lengthened. Pathological observation found that mucosal architecture was damaged, the colon wall was thicken, ulcers formatted, extensive inflammatory cell were infiltrated in the colonic mucosa from colitis mice, while its histological score was increased (Figure 1D-2 and E). Synchronously, the extent of damaged colonic mucosa was alleviated in the TNBS + Cur and TNBS + Mes groups (Figure 1D-3 and D-4) followed with decreased histological scores(Figure 1E). The results hinted Cur alleviated effectively inflammatory injury of colonic mucosa.

***Curcumin improved level of Treg cells in GALT***

As shown in figure 2, the total number of CD4+T cells in mice GALT decreased in the Normal, TNBS+ Cur, and TNBS + Mes groups compared with the TNBS group. However, the numbers of CD4+CD25+Foxp3 + T cells (Treg cells), which is a marker of Treg cells, increased in the three groups compared with the TNBS group.

***Curcumin inhibited secretion of related cytokines in colonic mucosa in mice colitis***

Significant increases in the secretions of TNF-α, IL-2, IL-6, IL-12 p40, IL-17, and IL-21, as assessed by ELISA in figure 3, were observed in the TNBS group compared to the Normal group. Notably, the increased expressions of TNF-α, IL-2, IL-6, IL-12 p40, IL-17, and IL-21 were remarkably reduced in the TNBS + Cur group after treatment with Cur compared to the TNBS group.

***Curcumin reduced expressions of costimulatory molecules in GALT***

Figure 3 show expressions of CD205 (Figure 4A), CD54 (ICAM-1) (Figure 4B), TLR4 (Figure 4C), CD252 (OX40 L) (Figure 4D), CD256 (RANK) (Figure 4E) and CD254 (RANK L) (Figure 4F) in GALT. The expressions of these costimulatory molecules were increasedin the TNBS group compared with the Normal group. Interestingly, after 7 d treatment the increased levels of costimulatory molecules were coincidently inhibited or downgraded in the TNBS+ Cur and TNBS + Mes groups compared with the TNBS group.

**Discussion**

Many studies had indicated that IBD is usually characterized as inflammatory injury of colonic mucosa with disorder immune responses in inflamed mucosa, with the dominance of IL-17-producing cells and deficiency of Treg cells[27-29]. In the present study, the result had shown Cur repaired colonic structure, decreased colonic weigh and histological injury score, recovered colonic length, and indicated Cur effectively restored damaged colonic mucosa in mice with TNBS-induced colitis. It is gratifying that the over-expression of IL-17 and decreased Treg cells in the development of TNBS-induced colitis in our experiments were in agreement with these previous studies. After treatment with Cur, the level of IL-17 decreased, and Treg cells increased, indicating that the protective effect of Cur against TNBS-induced colitis is closely associated with decreased IL-17 expression and recovered Treg cells level. Also, we found that the productions of related cytokines (as TNF-α, IL-2, IL-6, IL-12 p40 and IL-21) and expressions of costimulatory molecules were suppressed synchronously through treatment with Cur. It is known these inhibitory cytokines were secreted by DCs. In summary, the effect of Cur treated experimental colitis is closely with the suppressive function of Treg and activation of DCs.

Treg cells, also known as CD4+CD25+ Foxp3+T cells, are involved in the maintenance of peripheral tolerance and control of the immune response by initiating suppressive effects on activated immune cells[30,31]. Treg-mediated suppression can be regulated primarily by the four broad categories of mechanisms including suppression by inhibitory cytokine (IL-10, IL-35 and TGF-β), cytolysis, metabolic disruption, or via modulation of dendritic cell (DC) maturation or function[11-13]. One of Treg-mediated suppression is realized by anti-inflammatory cytokines (IL-10, IL-35) and TGF-β, which are in balance with pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, and IL-21. These pro-inflammatory cytokines are inhibited by anti-inflammatory cytokines, and, as a result, promotes Th17 cells to secrete IL-17 and eventually induce inflammatory damage. IL-17 is overwhelmingly produced by Th17 cells[12,32], and is expressed extensively in the mucosa and serum of IBD patients, and the TNBS-induced colitis mice[33]. This suggests that the balance of Treg cells and Th17 cells was possibly broken in the GALT in mucosa from mice with colitis.

Foxp3, a forkhead/winged helix transcription factor, as a specific marker can maintain generation, function, and stabilization of Treg cells[12]. Hence, the level of Treg cells is usually evaluated by the expression of Foxp3 in the cell nucleus of Treg cells. High expression of Foxp3 is essential for effector cytokines of Th1, Th2, and Th17 lineages[34,35]. For example, IL-2 can maintain the stable Foxp3 expression in Treg *via* STAT5 phosphorylation[36,37]. Moreover, IL-6 may cause Treg cells to transfer into Th17 cells and secrete abundant IL-17 and IL-21. IL-21 combined with IL-23 to induce signal transducer and activator of transcription-3 (STAT3) expression and height retinoid-related orphan receptor-γt (ROR-γt) activation, and finally improve secretion of IL-17[38-40]. In our study, down-regulation of Treg cells led to an imbalance of Treg and Th17 cells, and also an imbalance of Th1 and Th 2 cells and thus produced powerful expressions of pro-inflammatory cytokines that resulted in inflammatory damages in colonic mucosa.

As the uppermost part, dendritic cells (DCs) can modulate Treg-mediated suppressive function via expression of costimulatory molecules[41,42]. DCs are critical for regulation of intestinal immunity and mucosal immune tolerance to commensal microorganisms which is one of the pivotal inflammatory etiology induced IBD[41]. Maturation and migration of DC in peripheric lymph nodes are “danger” signals that induce inflammatory injuries in the intestinal mucosa[42,43]. The mature DCs with an expression of costimulatory molecules can regulate the balance between T helper (Th) 1 and Th 2, and balance of Treg and Th17 cells. Misra and his colleagues had demonstrated that lower level expression of costimulatory molecules of DCs can induce immature and limited antigen presentation of DCs to stimulate ineffectively T cell responses and then induce an increase in the numbers of Tregcells[44]. Certainly, Treg cells can secrete inhibitory cytokines to repress activation of MHC-Ⅱ and costimulatory molecules of DCs via negative feedback to suppress the antigen presentation of DCs[45,46]. Furthermore, DCs can induce the differentiation of Th 17 cell to regulate balance of Treg and Th 17 cells by secreting IL-6, or IL-12 p40 which produces more pro-inflammatory cytokines (as TNF-α,IL-6,IL-15 and so on) that mediate inflammatory injury[47,48]. Meanwhile, costimulatory molecules of DCs, which include TNF/TNF receptor protein families (CD40/CD40L, OX40/OX40L, TNFR/TNF and so on) and immune globulin superfamily (ICAM-1/LAF-1, CD28/CTLA4/B7, *etc.*), participate in the polarization of Th1 and Th2 cell responses. As an example, Receptor activator of NF-κB (RANK)/receptor activator of NF-κB ligand (RANKL) signal is an important costimulatory molecule that activate DCs and prolongs its lifespan. This is achieved by activating Bcl-XL gene and cooperating with other costimulatory molecules (ICAM-1, TLR4, OX40 L, *etc.*) and some cytokines (IL-6 and IL-12) to activate NF-κB to secrete pro-inflammatory cytokines (TNF-α, IL-6, IL-17, and IL-21)[49]. The ICAM-1/LFA-1 signal and B7-1 Molecule (B7/CD28 signal) can activate DC to induce Th 1 cell response via secretion of IL-12. However, B7-2 Molecule and OX40/OX40L signal promote the polarization of Th 2 cells. These costimulatory molecules of DCs were highly expressed in human and animal colitis[50,51]. Thus, DCs are closely related to the development of IBD, suppression of Treg cells and balance of Treg/Th17 cells. In the present study, Cur noticeably inhibited the expression of costimulatory molecules of DCs that ineffectively stimulated T cell response to increase the suppression or number of Treg cells, and maintain Treg-mediated suppression. Also, Cur down-regulated secretion of cytokines including IL-6 and IL-12 p40 by inhibiting DCs to prevent Treg cells transfer into Th 17 cell and inhibit productions of pro-inflammatory cytokines (TNF-α, IL-2, and IL-6) and decrease the destructive effects of IL-17 and IL-21. The mechanisms by which Cur maintains Treg-mediated suppression were only partly discovered in the present study. Therefore, further study is needed to determine whether cytolysis of Treg cells and metabolic disruption of Treg cells are also mechanisms through which Cur maintains Treg-mediated suppression.

In conclusion, Cur potentially modulated activation of DCs to enhance the suppressive functions of Treg cells and restore damaged colonic mucosa of IBD.

**COMMENTS**

***Background***

Regulatory T cells (Treg) play a crucial role in the maintenance of self tolerance and the prevention of inflammatory bowel disease. Treg-mediated suppression can be implemented primarily by the four broad categories of mechanisms including suppression by inhibitory cytokine, cytolysis, metabolic disruption, or via modulation of dendritic cells (DCs) maturation or function. In these four facts, the activation of DCs is thought as the main role to maintain the suppression of Treg cells.

***Research frontiers***

The mature DCs with an expression of costimulatory molecules can regulate the balance between balance of Treg and Th17 cells. The previous had demonstrated that higher level expression of costimulatory molecules of DCs can lead to a decreased numbers of Treg cells to induce IBD.

***Innovations and breakthroughs***

The present study is firstly shown that Cur potentially modulated activation of DCs to enhance the suppressive functions of Treg cells and restore damaged colonic mucosa of IBD.

***Applications***

It is known that Cur effectively treated experimental colitis by many pathway including the inhibition of nuclear factor κB (NF-κB) pathway and reduction of pro-inflammatory cytokine response. However the level and pathway are ambiguous that Cur can or not regulate Treg cell. In the present study, our results had hinted that Cur can height the suppressive function of Treg cells via inhibiting the activation of DCs. The results are favorable to explore the mechanism of Cur treated chronic colitis.

***Terminologs***

These costimulatory molecules of DCs, which include TNF/TNF receptor protein families (CD40/CD40L, OX40/OX40L, TNFR/TNF and so on) and immune globulin superfamily (ICAM-1/LAF-1, CD28/CTLA4/B7, *etc.*), are marker of activation of DCs, and decreased to inhibit the suppressive function of Treg cells in many previous documents .

***Peer-review***

This is a well-designed and well-presented study for examining the anti-inflammatory potential of curcumine in TNBS colitis of mice. The authors found that curcumine modulates the action of dendritic cells to enhance suppressive Treg functions, leading to an accelerated mucosal healing.

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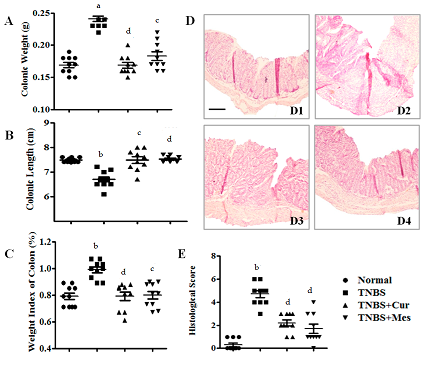
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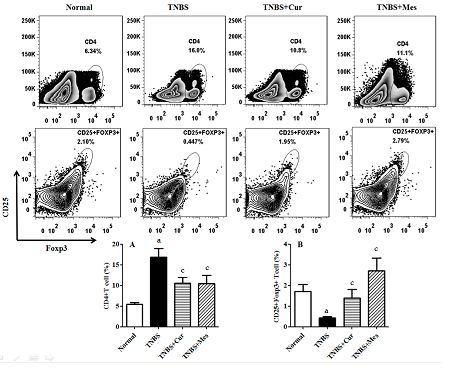
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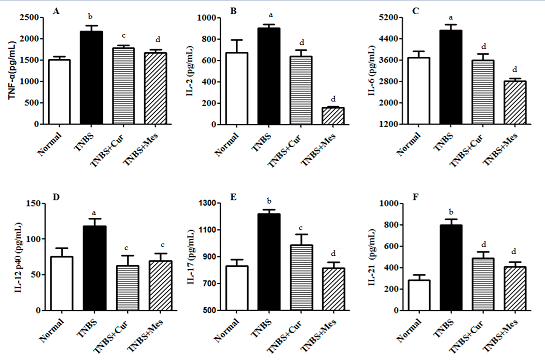
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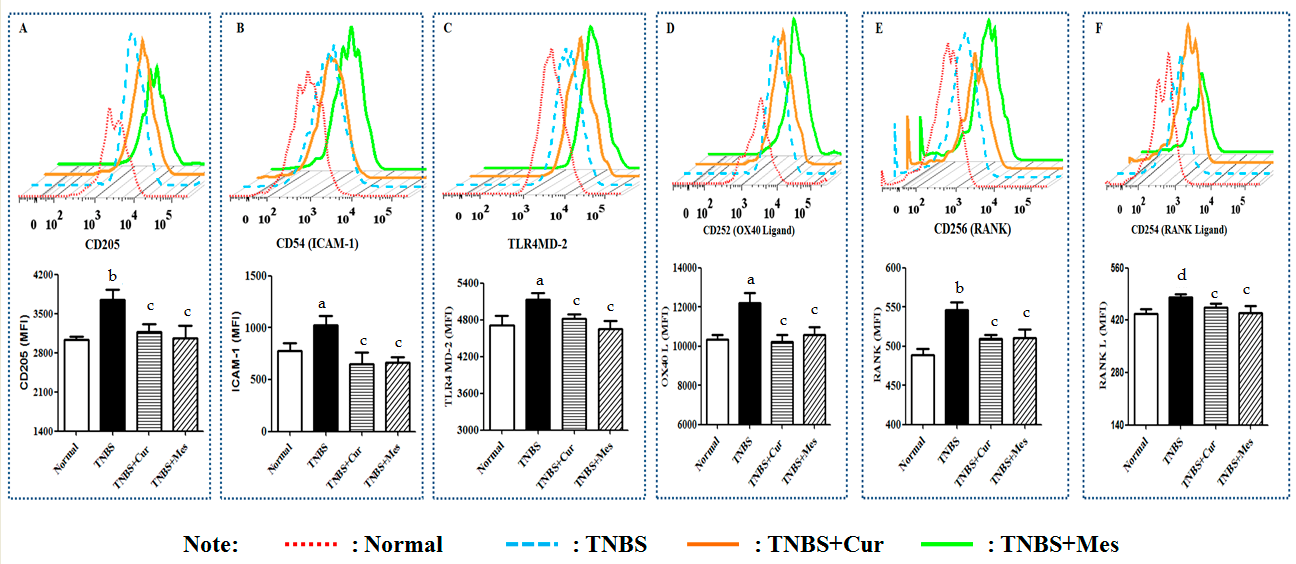
**Figure 1 Histological evaluation.** A: Colonic weight; B: Colonic length; C: Weight index of colon; D: Typical histological images stained by HE, The D1, D2, D3 and D4 respectively represent the Normal, TNBS, TNBS + Cur and TNBS + Mes animal groups. Bar = 200 μm; E: Histological scores. Data were presented as mean ± SE (*n =* 10). a*p* < 0.05 and b*p* < 0.01 *vs* Normal group; c*p* < 0.05 and d*p* < 0.01 *vs* TNBS group.



**Figure** **2 Typical Graphs and levels of CD4+ and CD25+Foxp3+ T cells.** Lymphocytes were isolated from GALT in Normal mice or mice with TNBS-induced colitis, and analyzed by flow cytometry. A: Typical graphs and levels (MFI) of CD4+ T cells; B: Typical graphs and levels (MFI) of CD25+Foxp3+ T cells. Data are expressed as mean ± SE (*n =* 8). a*p* < 0.05 *vs* Normal group; c*p* < 0.05 *vs* TNBS group.



**Figure 3 Concentrations of cytokines in colonic mucosa.** The tissue supernatant was prepared from the colonic mucosa in all mice. The level of cytokines was analyzed by ELISA. A: Concentration of TNF-α in colonic mucosa from different groups; B: Concentration of IL-2 in colonic mucosa from different groups; C: Concentration of IL-6 in colonic mucosa from different groups; D: Concentration of IL-12 p40 in colonic mucosa from different groups; E: Concentration of IL-17 in colonic mucosa from different groups; F: Concentration of IL-21 in colonic mucosa from different groups. Data are expressed as mean ± SE (*n =* 8). a*p* < 0.05 and b*p* < 0.01 *vs* Normal group; c*p* < 0.05 and d*p* < 0.01 *vs* TNBS group.



**Figure 4 Typical curveand levels of costimulatory molecules in dendritic cells.** Lymphocytes were isolated from GALT in Normal mice or mice with TNBS-induced colitis, and analyzed by flow cytometry. A: Typical pseudocolor and level (MFI) of CD205; B: Typical pseudocolor and level (MFI) of CD54 (ICAM-1); C: Typical pseudocolor and level (MFI) of TLR4; D: Typical pseudocolor and level (MFI) of CD252 (OX40 L); E: Typical pseudocolor and level (MFI) of CD256 (RNAK); F: Typical pseudocolor and level (MFI) of CD254 (RNAK L). Data are expressed as mean ± SE (*n =* 8). a*p* < 0.05 and b*p* < 0.01 *vs* Normal group; c*p* < 0.05 and d*p* < 0.01 *vs* TNBS group.