

Original Article

Immuno-modulatory mechanism of T cell immunoglobulin mucin-1 antibody in experimental colitis induced by dextran sulfate sodium

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Abstract: To evaluate the treatment effect of T cell immunoglobulin mucin-1 (TIM-1) antibody in dextran sulfate sodium (DSS)-induced colitis. 3.5% DSS was given orally for 5 days to induce acute colitis. Isotype IgG or TIM-1 antibody was given to mice 1 day before DSS intake and every 3 days thereafter. TIM-1 antibody improved the hematochezia and weight loss of DSS colitis. TIM-1 antibody treated mice showed decreased inflammatory cell infiltration and lymphocytes trafficking to the colon. CD4⁺/CD8⁺ lymphocytes ratio in spleen was down-regulated, while CD4⁺Foxp3⁺ and B220⁺TIM-1⁺ cell proportions in CLP were further increased. Moreover, CLP lymphocytes secreted less TNF- α , IL-12, IL-2, IFN- γ , IL-17, but more IL-10 after TIM-1 antibody treatment. As a conclusion, TIM-1 antibody showed immuno-modulatory effect in DSS-induced colitis, thus inhibited the inflammatory reaction in the acute phase of colitis. TIM-1 molecule may serve as a potential target for inflammatory bowel disease treatment.

Keywords: Inflammatory bowel disease, dextran sulfate sodium, colitis, T cell immunoglobulin mucin-1, biotherapy

Introduction

Inflammatory bowel disease (IBD), consisting of Crohn's disease and ulcerative colitis (UC), is characterized by relapsing and remitting condition of abdominal pain and diarrhea. In IBD patients, the improper activation of mucosal immune system by luminal flora is facilitated by the defects of the mucosal epithelial barrier [1]. In vitro culture of lymphocytes isolated from colonic lamina propria (CLP) in UC patients showed increased production level of T_H2 type cytokine IL-5, while in Crohn's disease patients increased secretion of T_H1 type cytokine IFN- γ was found [2]. Based on the imbalance of T_H1/T_H2 cytokines, several monoclonal antibodies have been developed researchers. TNF- α antibody has been widely used, but it is not effective in some patients [3]. IFN- γ antibody and IL-6R antibody showed some beneficial effects in early trials, but their definite functions still need to be verified [4, 5].

The induction of colitis by dextran sulfate sodium (DSS) was mediated by its toxicity towards

mucosal epithelial cells and the tight junction complex [6, 7]. The broken down of intestinal epithelial barrier is followed by the dissemination of luminal flora and antigens into the underlying tissue. The innate immune system is activated firstly. The local inflammation is characterized by hyperemia, edema, hemorrhage and infiltration of inflammatory cells. It was shown that in the acute phase of DSS colitis, the numbers of pre-B cells in bone marrow and pre-T cells in thymus decreased, while the numbers of granulocytes and monocytes in bone marrow increased [8]. Studies also showed that in the acute phase of DSS colitis, the expression levels of IL-1 β , IL-6, IL-12, IL-17, TNF- α , IFN- γ and G-CSF in colon tissue were increased [9, 10].

Tim gene mediates the secretion of T_H2 type cytokines and airway hyper responsiveness in mice [11]. TIM-1 protein provides co-stimulating signals for T cell activation, and promoted the transcription of IL-4 [12]. TIM-1 can also interact with macrophages, up-regulating the expression of co-stimulating signal molecules and

promoting the secretion of TNF- α and IL-6 [13]. Animal experiments showed that TIM-1 antibodies had broad regulatory functions in immune system and were effective in the treatment of hyper-sensitivity disease, transplant rejection, and ischemia-reperfusion injury models [14-16]. TIM-1 antibody also blocked the activation of T cells by dendritic cells (DCs) and provided therapeutic effects in colonic allergy model [17]. However, the effect of TIM-1 antibody hasn't been evaluated in DSS colitis model.

Materials and methods

Animals

Female C57BL/6 mice were purchased from HFK Bioscience (Beijing, China). They were used for the study at 6-8 weeks of age (body weight: 21.6 ± 1.6 g). The mice were reared under specific pathogen-free conditions at 23°C with a 12-hour light/dark cycle and were given food and tap water ad libitum. The mice were acclimatized for 2 weeks before entering the study. All animal husbandry and experimental procedures were approved by the Beijing Hospital ethics committee.

Induction of colitis

Mice receiving only water were studied as normal control. To induce acute colitis, 3.5% DSS (molecular weight of 36,000-50,000; MP Biomedicals, LLC, Illkirch, France) was added to drinking water for 5 days. On day 6, DSS was withdrawn and the colitis progressed to chronicity. Mice were sacrificed on day 5 or day 19 (14 days after stopping DSS) to study the acute and chronic phase of DSS colitis, respectively.

Antibody delivery

Rat monoclonal anti-TIM-1 antibody (RMT1-10; Bio X Cell, West Lebanon, NH, USA), and its isotype control, Rat IgG2a (2A3; Bio X Cell) were used for colitis treatment. Colitis mice were randomly divided into RMT1-10 treatment group and IgG group, each group containing 20 mice. 200 μ l PBS containing 100 μ g of antibodies were intraperitoneally injected on the day before DSS intake and every third day thereafter. Ten mice were killed on day 5, and the other 10 mice were killed on day 19.

Removal of organs and isolation of lymphocytes

Mice were sacrificed by isoflurane overdose. Peripheral blood (PB) was collected by cardiac puncture. The spleen and mesenteric lymph nodes (MLNs) were aseptically removed. MLNs were taken along the ileocecal artery. Single-cell suspensions were prepared using a standard mechanical disruption procedure. The entire colon was then removed and gently cleared of feces. To obtain cell suspensions from the colonic lamina propria (CLP), the tissues were digested using 0.5 mg/ml of type VIII collagenase (C2139; Sigma-Aldrich, St. Louis, MO, USA). To extract the lymphocytes in the PB and the CLP, cell suspensions were then centrifuged over Histopaque-1077 (Sigma-Aldrich).

Histological evaluation

Three portions (proximal, middle and distal) of the dissected colons were fixed in 4% formaldehyde, embedded by paraffin, sectioned at 5 mm, and stained with hematoxylin and eosin. Histopathologic alterations were assessed by a scoring system built up by Corazza et al [18]. Briefly, mucin depletion, epithelial erosion, crypt abscesses, cellular infiltration in the CLP, hyperemia and mucosal thickness were evaluated and quantified in this system. Each mouse acquired a histological score ranging from 3 to 45.

Flow cytometric analysis

Immunofluorescent analysis was performed using FACSCalibur (BD Biosciences, San Jose, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8 α , anti-CD3 ϵ , and phycoerythrin (PE)/FITC-conjugated anti-CD45R/B220 were purchased from eBioscience (San Diego, CA, USA). PE-conjugated RMT1-10 was purchased from Biolegend (San Diego, CA, USA). For measurement of apoptosis, Annexin V PE (eBioscience) was also used. Cells were incubated with anti-Fc receptor mAb (BD Biosciences Pharmingen, San Jose, CA, USA) before flow cytometric analysis to prevent non-specific staining. Cell Quest software (BD Biosciences) was used for analyzing results.

Costimulation of lymphocytes and measurement of cytokines and proliferation

CLP and spleen lymphocytes (10^6 cells/well) were cultured in 300 μ l 10% fetal calf se-

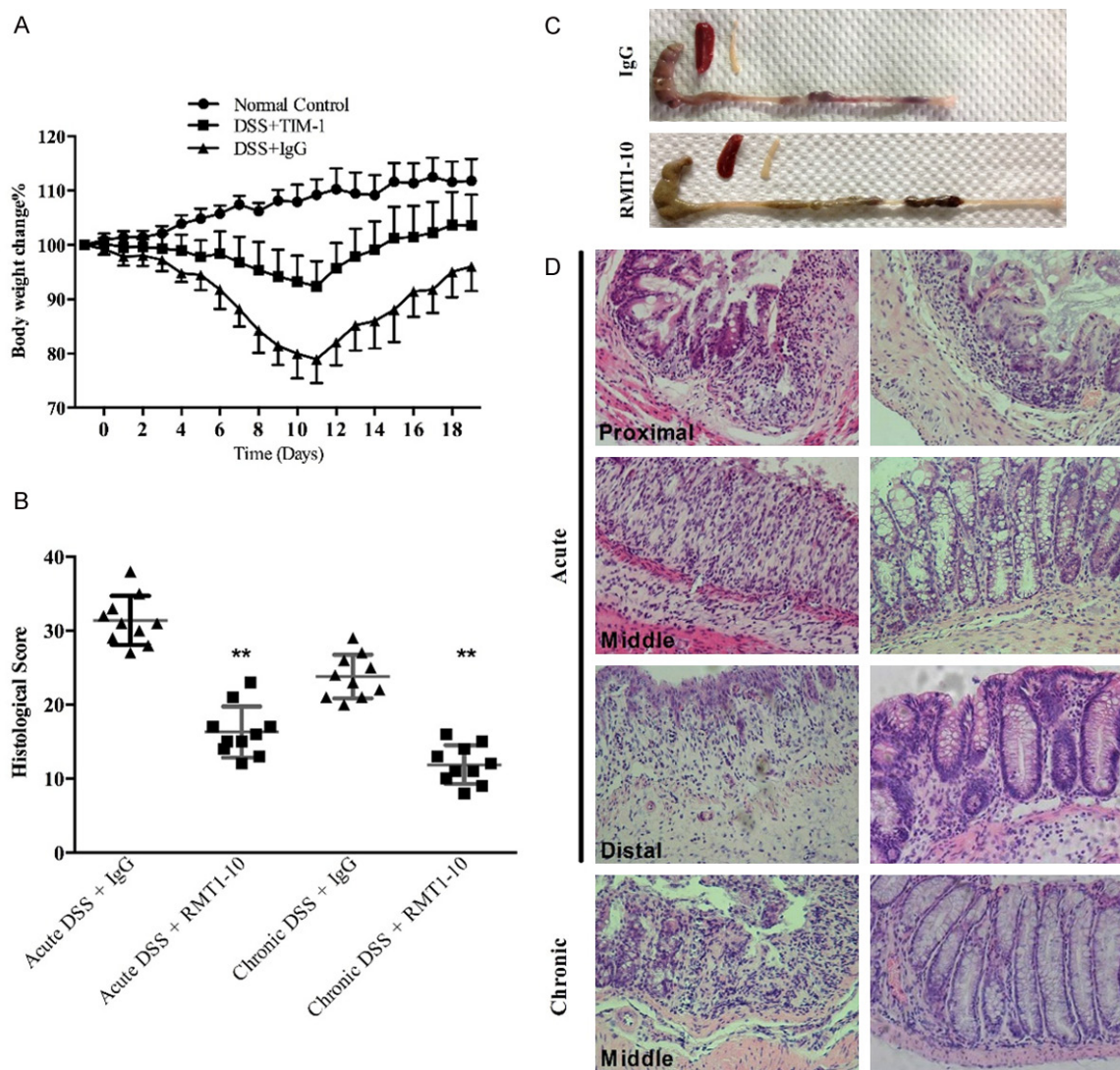


Figure 1. RMT1-10 treatment improved DSS colitis *in vivo* and histologically. **A.** Body weight changes in normal control mice, IgG-treated DSS colitis control mice and RMT1-10-treated DSS colitis mice. **B.** Histological scores for colon tissues from IgG- and RMT1-10-treated mice. $**P < 0.01$. **C.** Representative gross specimens from IgG- and RMT1-10-treated acute colitis mice. **D.** Representative histopathology slices from different portions of colon in IgG- and RMT1-10-treated colitis mice ($\times 20$, HE staining). $n = 10$ mice per group. DSS, dextran sulfate sodium.

rum-containing RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and stimulated by pre-coated anti-CD3 ϵ (BD Biosciences Pharmingen) and 1 μ g/ml soluble anti-CD28 (BD Biosciences Pharmingen) for 48 h in 48-well Costar plates. Supernatants were assayed for TNF- α , IL-2, IL-4, IL-10, IL-12, IL-17 and IFN- γ using solid phase sandwich enzyme-linked immunosorbent assay kits (R&D Systems). Optical densities were read by a microplate reader (model-680; Bio-Rad, Hercules, CA, USA) at 450 nm. For measurement of proliferation, repeated cultures were pulsed with 1 μ Ci [3 H] Thymidine (GE Healthcare, London, UK) in the last 16 h,

and the radioactivity was tested by the Wallac Liquid Scintillation Counter (PerkinElmer, Waltham, MA, US).

Statistical analysis

Data were presented as mean \pm SD. Analyses were carried out using GraphPad Prism 5.01 (San Diego, CA, USA). Differences between two groups were evaluated using Student's *t* test; data from three or more groups were evaluated with one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. $P < 0.05$ was considered statistically significant.

Table 1. Impact of RMT1-10 treatment on the length of colon, the weight of spleen and of MLNs

Group	Colon length (cm)	Spleen weight (mg)	Weight of MLNs (mg)
Normal control	11.5 ± 0.7	82.0 ± 10.5	22.6 ± 3.3
IgG-treated acute DSS colitis	8.0 ± 0.7**	79.5 ± 8.9	20.6 ± 3.4
RMT1-10-treated acute DSS colitis	10.1 ± 1.1**	93.3 ± 13.6	22.9 ± 6.0
IgG-treated chronic DSS colitis	8.2 ± 0.6**	103.3 ± 8.6*	20.1 ± 2.1
RMT1-10-treated chronic DSS colitis	11.2 ± 0.8**	105.6 ± 9.1	25.4 ± 3.1

Values are shown as mean ± SD. *n* = 10 mice per group. IgG-treated colitis mice were compared with normal mice, while RMT1-10-treated colitis mice were compared with IgG-treated colitis controls. *, *P* < 0.05 and **, *P* < 0.01. Significances were determined by Bonferroni's multiple comparison tests following one-way ANOVA. DSS, dextran sulfate sodium.

Results

RMT1-10 improved the DSS colitis in vivo and histologically

Mice with acute DSS colitis treated with RMT1-10 loss less body weight compared with the IgG-treated colitis control mice (**Figure 1A**). By day 4, the difference in body weight changes between RMT1-10- and IgG-treated groups became significant ($-1.01 \pm 2.90\%$ vs. $-5.17 \pm 1.64\%$, respectively, *P* < 0.01). Both two groups recovered from rectal bleeding and regained their body weight after DSS withdrawal. However, RMT1-10-treated mice recovered from rectal bleeding (11.4 ± 1.4 d vs. 16.0 ± 1.8 d, *P* < 0.01) and diarrhea (16.4 ± 2.6 d vs. > 19 d) more quickly.

RMT1-10 improved DSS colitis histologically

Grossly, acute DSS colitis is characterized by mucosa bleeding and decreased length of colon. Mucosal bleeding was markedly reduced by RMT1-10 treatment (**Figure 1C**), and colon length was increased in both the acute (*P* < 0.01) and the chronic (*P* < 0.05) phases (**Table 1**). Spleen weight in chronic colitis mice was increased, while the weight of MLNs was unchanged after DSS intake. RMT1-10 treatment showed no effect on the weight of spleen and MLNs (**Table 1**). Microscopically, RMT1-10-treated C57BL/6 mice showed relatively intact colonic mucosal epithelium and reduced inflammatory infiltration into the lamina propria in the acute phase and displayed less infiltration of lymphocytes, more regular mucosal epithelium and thinner bowel wall in the chronic phase compared with IgG-treated mice

(**Figure 1D**). The effect of RMT1-10 treatment was significant both in the acute and chronic phases measured by histological score (*P* < 0.01, **Figure 1B**).

Effect of RMT1-10 treatment on lymphocyte numbers

Compared to normal mice, in the acute phase of DSS colitis treated by IgG, the numbers of lymphocytes in spleen and MLNs decreased (both

P < 0.01), while the numbers of lymphocytes in CLP and PB increased (both *P* < 0.05). However, RMT1-10 treatment increased the numbers of splenocytes and MLN lymphocytes (both *P* < 0.01, **Figure 2A**), while decreased the numbers of CLP (*P* < 0.01) and PB (*P* < 0.05) lymphocytes in the acute phase of colitis. In the chronic phase, the number of CLP lymphocytes also increased. Again, RMT1-10 treatment decreased these CLP lymphocytes (**Figure 2B**).

Effect of RMT1-10 treatment on lymphocytes subsets

Compared to normal mice, the ratios of CD4⁺/CD8⁺ T lymphocytes in spleen and MLNs were increased, and the proportions of CD4⁺Foxp3⁺ T lymphocytes and B220⁺ cells in MLNs and CLP were increased in IgG-treated acute colitis (data not shown). However, RMT1-10 treatment decreased the ratios of CD4⁺/CD8⁺ cells in spleen and MLNs (both *P* < 0.01), and increased the proportions of CD4⁺Foxp3⁺ T lymphocytes in MLNs (*P* < 0.05) and CLP (*P* < 0.01). We found that the proportions of B220⁺ cells were unchanged after RMT1-10 treatment (**Figure 2C-E**).

RMT1-10 induced TIM-1 expression on B cells

By flow cytometry, we found that TIM-1 was mainly expressed on CD4⁺ and B220⁺ cells. In the acute phase of colitis treated by IgG, the expression of TIM-1 on CD4⁺ cells was unchanged; however, that on B220⁺ cells increased significantly. Moreover, the expression of TIM-1 on B220⁺ cells was further increased by RMT1-10 treatment (**Figure 3A and 3B**).

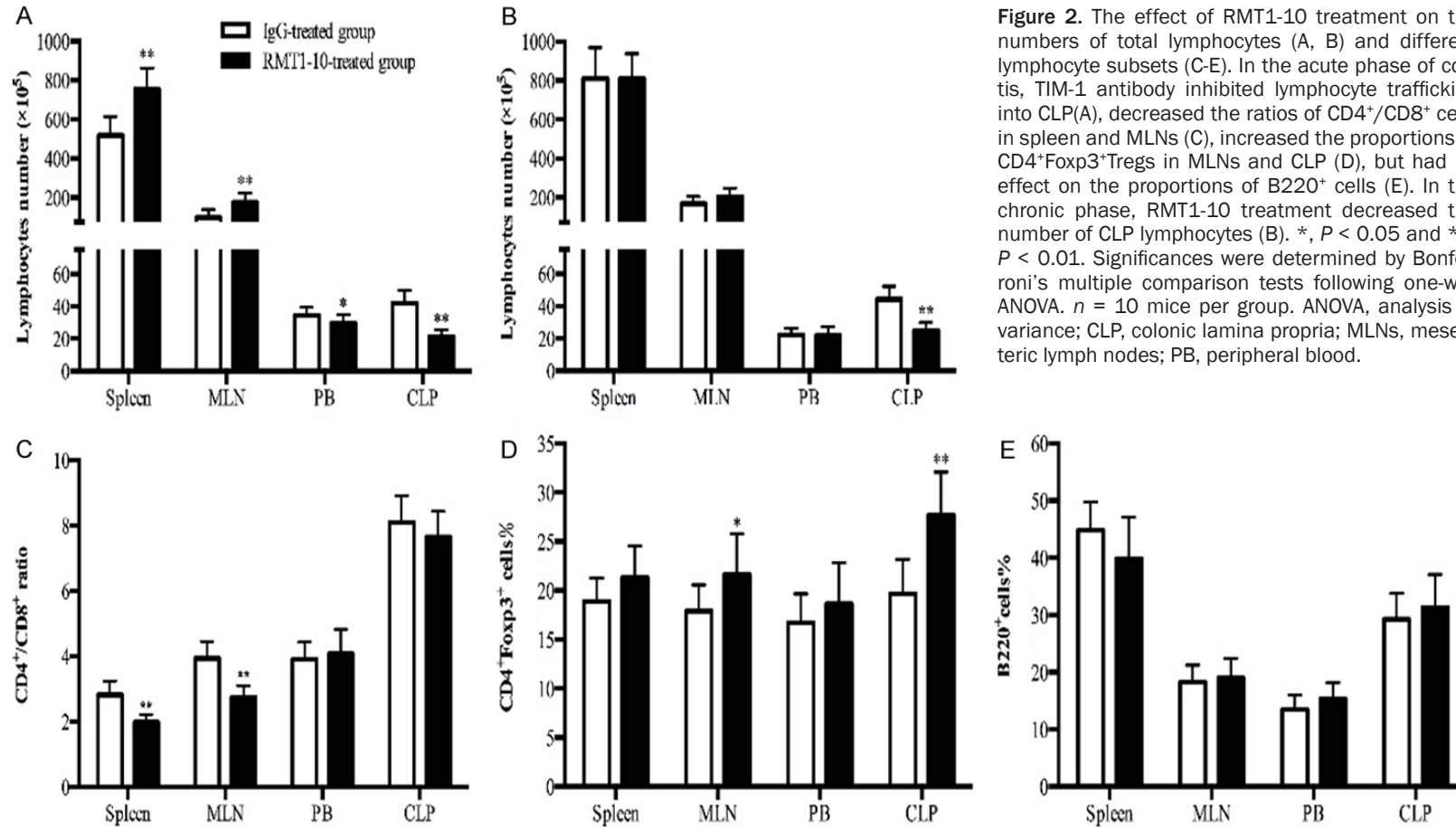


Figure 2. The effect of RMT1-10 treatment on the numbers of total lymphocytes (A, B) and different lymphocyte subsets (C-E). In the acute phase of colitis, TIM-1 antibody inhibited lymphocyte trafficking into CLP(A), decreased the ratios of CD4⁺/CD8⁺ cells in spleen and MLNs (C), increased the proportions of CD4⁺Foxp3⁺Tregs in MLNs and CLP (D), but had no effect on the proportions of B220⁺ cells (E). In the chronic phase, RMT1-10 treatment decreased the number of CLP lymphocytes (B). *, $P < 0.05$ and **, $P < 0.01$. Significances were determined by Bonferroni's multiple comparison tests following one-way ANOVA. $n = 10$ mice per group. ANOVA, analysis of variance; CLP, colonic lamina propria; MLNs, mesenteric lymph nodes; PB, peripheral blood.

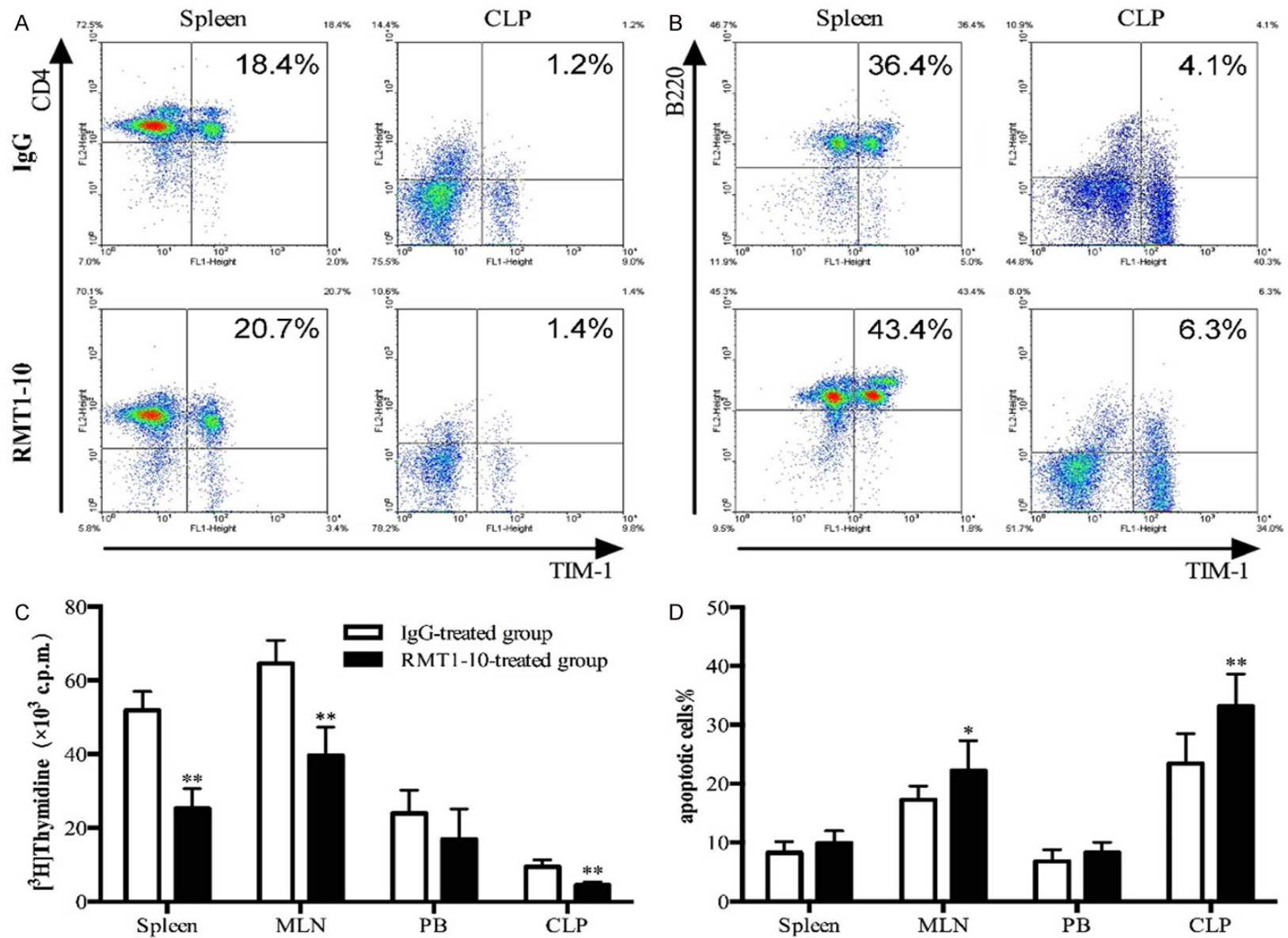


Figure 3. The effect of RMT1-10 treatment on the expression of TIM-1 and the proliferation and apoptosis of lymphocytes. (A, B) Representative flow cytometry plots showing TIM-1 expression on CD4⁺ and B220⁺ cells in the acute phase of colitis. TIM-1 expression on B220⁺ cells was induced by RMT1-10 treatment in acute coli-

tis. RMT1-10 treatment inhibited the proliferation (C) and induced the apoptosis (D) of lymphocytes. The proliferation was measured by pulsing with [^3H] thymidine, while the apoptosis was measured by Annexin V staining. *, $P < 0.05$ and **, $P < 0.01$. Significances were determined by Bonferroni's multiple comparison tests following one-way ANOVA. $n = 10$ mice per group. c.p.m., counts per minute.

RMT1-10 treatment inhibited the proliferation and induced the apoptosis of lymphocytes

In the acute phase of colitis, IgG-treated mice showed enhanced proliferation of lymphocytes in spleen, MLNs, and CLP. Compared to IgG-treated group, RMT1-10-treated mice showed declined proliferation of these lymphocytes (all $P < 0.01$, **Figure 3C**). We also found that the proportions of apoptotic CD3 $^+$ T cells in MLNs and CLP were decreased in IgG-treated acute colitis. However, by RMT1-10 treatment, the apoptosis of CD3 $^+$ T cells in MLNs and CLP was induced (**Figure 3D**).

RMT1-10 treatment enhanced the production of IL-10 by lymphocytes

Following in vitro stimulation, the productions of TNF- α , IL-12, IL-2, IFN- γ , IL-17 and IL-10 by spleen and CLP lymphocytes were increased in IgG-treated acute colitis mice. Compared to IgG-treated mice, splenocytes and CLP lymphocytes from RMT1-10-treated acute colitis group showed less productions of TNF- α , IL-12, IL-2, IFN- γ and IL-17 and more secretion of IL-10 (**Figure 4**). However, we didn't find any change in the production of IL-4 in colitis mice (data not shown).

Discussion

In the acute phase of colitis, the numbers of lymphocytes in spleen and MLNs decreased, while those in PB and CLP increased. Lymphocytes trafficking seems to play an important role in the pathology of colitis. In our previous study, we showed that sphingosine-1-phosphate antagonist KRP-203 improved colitis in IL-10 $^{-/-}$ mice by inhibiting lymphocytes trafficking [19]. As shown by Angiari et al, TIM-1 also participates in the regulation of lymphocytes trafficking by binding with P-selectin [20]. In our current study, TIM-1 antibody increased the numbers of lymphocytes in spleen and MLNs, while decreased those in PB and CLP. Concomitantly, T $_{\text{H}}$ 1- and T $_{\text{H}}$ 17-type cytokines produced by CLP lymphocytes decreased. These results suggest inhibiting lymphocytes trafficking may be one of the mechanisms used by TIM-1 antibody to treat colitis.

Early studies showed the change in the number of B cells in mice colon is associated with the concentration of DSS used to induce colitis [21]. We used 3.5% DSS to induce colitis, and found that B cell numbers in MLNs and CLP increased significantly in the acute phase. Besides its classical role as the antibody producer, B cells were also shown to be an immune regulator in various inflammatory processes, including colitis [22]. Latest studies suggest TIM-1 expressed on B cells to be a surface marker for regulatory B cells (Bregs) [23]. Compared to TIM-1 $^{-}$ cells, TIM-1 $^{+}$ B cells secreted more IL-10. In our current work, TIM-1 antibody induced the expression of TIM-1 on B cells, but had no effect on the numbers of B cells. It is possible that TIM-1 improved colitis by enhancing the regulatory function of Bregs.

Regulatory T cells (Tregs) are characterized by the expression of CD4 $^{+}$ Foxp3 $^{+}$. They secreted TGF- β and IL-10, and inhibited the function of antigen presenting cells (APCs) by interacting with cytotoxic T lymphocyte associated antigen-4 (CTLA-4). Tregs have an immuno-modulatory effect in mice colitis [24]. In an allo-transplantation model, TIM-1 antibody increased the proportion and enhanced the regulatory function of Tregs [15]. However, when TIM-1 antibody was administered in B cell depleted mice, the proportions of IL-10-producing Tregs decreased, with a shorter survival time of the transplant [23]. These studies suggest B cells may be essential for the function of Tregs. In our current work, CD4 $^{+}$ Foxp3 $^{+}$ cell numbers increased in colitis mice, and was further induced by TIM-1 antibody. However, it is unclear whether the induction of Tregs is dependent on B cells.

DSS-induced damage to epithelial barrier facilitates the dissemination of bowel antigens and bacteria. They activate macrophages and lead to the secretion of TNF- α , IL-6 and G-CSF. Then, T cells are activated by APCs. T $_{\text{H}}$ 1 cells are induced by IL-12 and produce IFN- γ . T $_{\text{H}}$ 17 cells secrete IL-17, they are able to induce G-CSF and are associated with the infiltration of neutrophils in the inflammatory site. We showed here that productions of both the T $_{\text{H}}$ 1 (TNF- α , IL-12, IL-2, and IFN- γ)- and T $_{\text{H}}$ 17-type cytokines were

The immuno-modulatory mechanism of TIM-1 antibody in experimental colitis

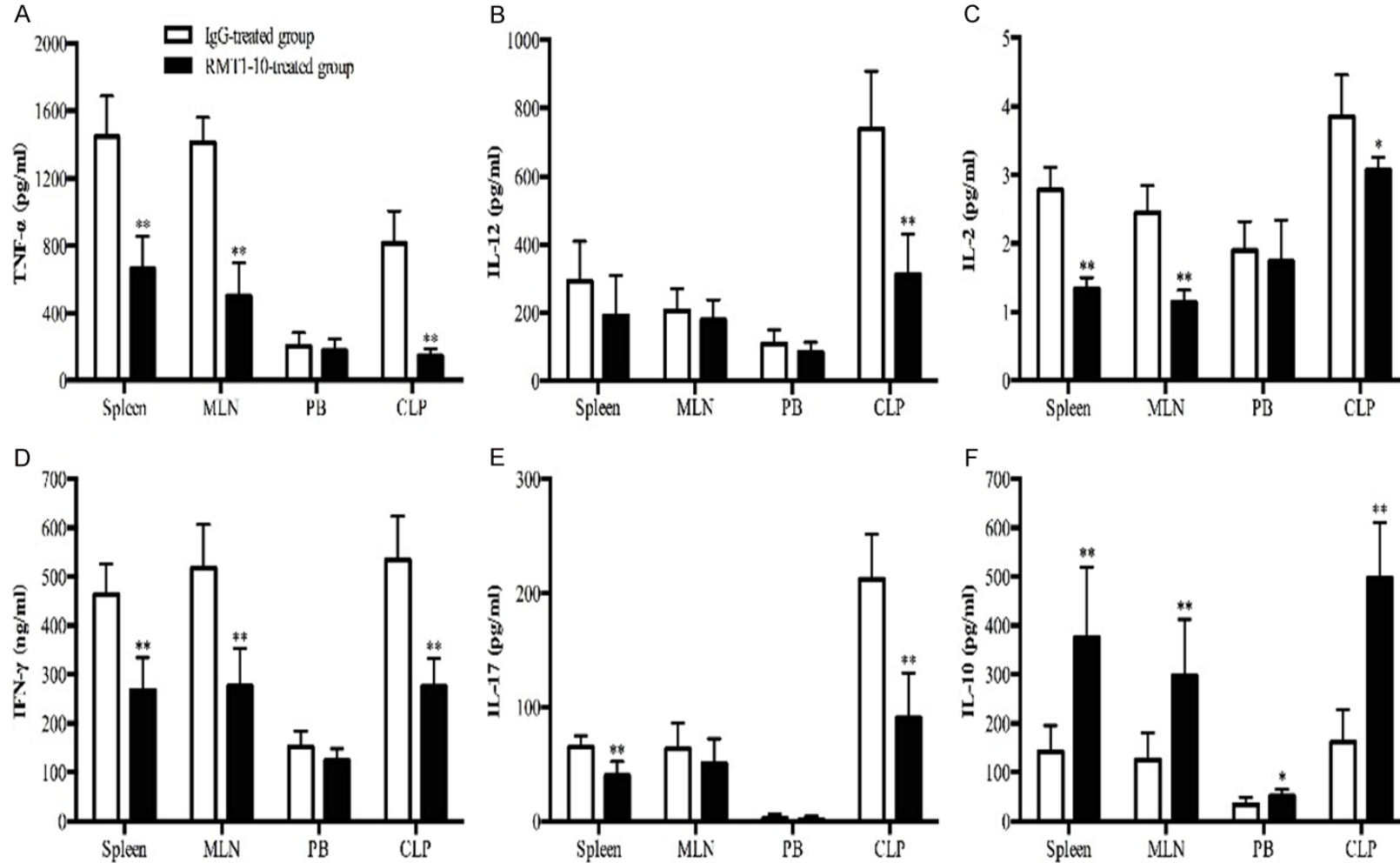


Figure 4. Productions of TNF-α (A), IL-12 (B), IL-2 (C), IFN-γ (D), IL-17 (E) and IL-10 (F) by cultured lymphocytes from acute colitis mice. *, $P < 0.05$ and **, $P < 0.01$. Significances were determined by Bonferroni's multiple comparison tests following one-way ANOVA. $n = 10$ mice per group.

declined by TIM-1 antibody. As was evaluated by pathology examination, the infiltration of neutrophils to colon was also abated. IL-10 is secreted by macrophages and Tregs. They inhibited the secretion of IL-1, TNF and IL-12 by DCs and macrophages and inhibited T_H1-type immune response. In both human and mice, IL-10 deficiencies lead to colitis [19]. Therefore, IL-10 plays important roles in gut immune modulation. In the acute phase of colitis, we found the production of IL-10 increased significantly, paralleled with the increased number of Tregs. In TIM-1 antibody treated group, the production of IL-10 was further increased.

We also found enhanced proliferation ability and decreased apoptosis of lymphocytes in the acute phase of colitis. TIM-1 antibody down-regulated lymphocytes proliferation and increased the number of apoptotic T lymphocytes. The proliferation of lymphocytes is mainly mediated by antigen-stimulating signals. A previous study showed that macrophages activated by DSS itself can stimulate the proliferation of T lymphocytes. Recent studies suggest the interaction of TIM-1 and TIM-4, expressed on APCs, provides co-stimulatory signals for T cells [25]. TIM-1 antibody may block the interaction of TIM-1 and TIM-4, and inhibited the activation of T cells, thus inhibiting proliferation and inducing apoptosis.

In conclusion, our findings indicated TIM-1 antibody is effective in the treatment of DSS-induced colitis. It inhibited lymphocytes trafficking, up-regulated the proportions of regulatory lymphocytes and modulated the cytokine profiles. However, further investigation is required to elucidate the role of TIM-1 in IBD and test the effect of TIM-1 antibody in IBD treatment.

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Disclosure of conflict of interest

None.

Authors' contribution

J.S. and J.W. designed research; X.N., Q.A., Y.L. and W.S. performed research; Y.L. contributed

new reagents/analytic tools; W.S. analyzed data; and K.Y., W.S. and Q.A. wrote the paper.

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