International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2023.222.229

Research Article
Metformin Alleviates Dextran Sulfate Sodium-Induced Colitis in a Mouse Model by Increasing IgA Secretion in Intestinal B cells

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Abstract

Background and Objective: The B cells involved in the alleviation of ulcerative colitis (UC) potentially through antibody and cytokine production. The hypoglycemic drug metformin (MET) is known to have anti-inflammatory and antioxidative effects in UC, however, its relation to B cell regulation in UC has not yet been clarified. Therefore, the current study aims to explore the mechanism by which MET improves UC in a mouse model. **Materials and Methods:** Twenty-one male C57BL/6 mice were randomly grouped into the control group, sodium dextran sulfate (DSS) and sodium dextran sulfate+metformin (DSS+MET) groups. The mRNA or protein expression level of IL-10, IL-35 and IgA in the colon was detected by RT-qPCR or ELISA and analyzed by flow cytometry. Anti-cd40, TGF-β, IL-5, IL-6 and retinoic acid were added to the cultured spleen $CD19^+$ B cells *in vitro* to obtain IgA-producing B cells. The mRNA level of vdj-Cα was detected after B cells were co-cultured with different concentrations of MET. **Results:** The MET significantly improved the 14 days survival rate of DSS-induced mice, from 0 (DSS group) to 57.1%. The MET significantly reduced the DSS-induced colitis-associated weight loss, disease activity index score increase and colon constriction. The MET treatment effectively increased the production of IL-10, IL-35 and IgA in the intestinal tissue ($p<0.05$) and promoted the number of $CD138^+IgA^+$ cells in the colon epithelia and lamina propria of DSS-treated mice. The MET increased the mRNA level of vdj-Cα in the B cells *in vitro*, suggesting that MET alleviates UC by increasing IgA production by intestinal B cells. **Conclusion:** The current study provides insights that could facilitate the clinical treatment of UC through B cell regulation and anti-inflammatory cytokine and IgA production.

Key words: Metformin, ulcerative colitis, immunoglobulin A, anti-inflammatory cytokines, B cells/lymphocytes

Citation: Xinjian, D., Z. Zhe, Y. Le, Z. Jin and L. Sha, 2023. Metformin alleviates dextran sulfate sodium-induced colitis in a mouse model by increasing IgA secretion in intestinal B cells. Int. J. Pharmacol., 19: 222-229.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ulcerative colitis (UC) is a subtype Inflammatory Bowel Disease (IBD) of unknown etiology but is commonly characterized by bloody diarrhea, a damaged epithelial barrier, immune response disorder and equilibrium in the intestinal immune system[1,2]. Many researchers consider UC to be an autoimmune condition[3]. Over the last two decades, B cells have been recognized as therapeutic targets for autoimmune diseases, indicating that B cells may have an indispensable role in UC. Studies have found that removing $CD20^+$ B cells can lead to various disorders, as well as exacerbate or result in a spontaneous attack of clinical colitis[4,5].

The B cells play important roles in antibody production (e.g., immunoglobulins A, G and M), antigen presentation and cytokine production, all of which contribute to immune responses[6]. The IgA is the most abundant antibody secreted into the gastrointestinal tract and provides initial immune protection[7]. A major function of IgA is to exclude pathogenic microbes by restricting their access to the epithelial surfaces[8]. The IL-10 inhibits pro-inflammatory, effector innate and adaptive immune cells and helps maintain mucosal homeostasis[9]. The $B220^+$ B cells and $CD138^+$ plasma cells have been observed to secrete both IL-10 and IL-35 to inhibit inflammation[10]. Intestinal IL-35-producing B cells play an important role in inflammatory bowel disease and have been found in patients with Crohn's disease. Furthermore, suppression of Th1 and Th17 responses which were mediated by Breg was restored by the addition of IL-35in patients with UC[11].

Metformin (MET) is a first-line hypoglycemic drug to treat type 2 diabetes. The MET has both anti-inflammatory and antioxidative effects, which improve intestinal inflammation[11]. Studies have shown that MET can reduce inflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, or upregulate IL-10 in animals with colitis[12,13]. However, it remains unclear whether MET causes B cells to exert protective effects via anti-inflammatory effects in patients with UC.

In this experimental study, the pharmacological effect of MET on the antibody and anti-inflammatory cytokine production by the B cells in mice with dextran sodium sulfate (DSS)-induced colitis was investigated.

MATERIALS AND METHODS

Study area: The experiments were conducted between February, 2022 and January, 2023 at the National Beijing Center for Drug Safety Evaluation and Research, Beijing Institute of Pharmacology and Toxicology in Beijing, China.

Animal model: Twenty one male C57BL/6 mice (age, 6-8 weeks and weight, 22-24 g) were provided by SPF Biotechnology (Beijing, China). All animal studies were performed at the Beijing Institute of Pharmacology and Toxicology. The animals were housed under standard conditions (specific pathogen-free, 45±5% humidity, 25°C and day/night cycle). The mice were randomly grouped into control, DSS (Shanghai Yuanye Bio-Technology Co., Ltd.) or DSS+MET (Sigma-Aldrich, St Louis, MO) group. Mice were administered MET (400 mg/kg/day) via intragastric gavage for seven days. Subsequently, mice were administered MET combined with DSS. Mice in the DSS+MET group received MET (400 mg/kg/day) dissolved in filter-sterilized drinking water daily from day 1 to 14 and the control and DSS groups received only sterilized drinking water in the same volume. The DSS was administered in drinking water at 3% in the DSS and DSS+MET groups from the eighth to the fourteenth day.

Ethical consideration: This study was approved by the Animal Research Ethics Committee of the Beijing Institute of Pharmacology and Toxicology (Approval No. IACUC-DWZX-2021-622) in accordance with the guide for the care and use of laboratory animals of the institute.

Disease activity index (DAI) and histopathological symptoms: Body weight, rectal bleeding and stool consistency were observed daily and a DAI score was generated. Each parameter was graded from 0 to 4. Body weight loss was scored as follows: 0, <5, 2, 6-10, 4 and >10%. Fecal blood was rated as follows: Negative hemoccult test: 0, Visible bleeding and blood clotting on the anus: 2 and Gross bleeding: 4[14]. A stool consistency score of 0 was considered to indicate well-formed pellets, a score of 2 indicated loose stools and a score of 4 indicated diarrhea. After sacrificing the mice, the colon length was measured. The colon tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of about 5 μm in thickness were prepared and stained with hematoxylin-eosin. The scores were assessed following the protocol described by Zhou *et al.*[15].

Gut cell isolation: The Lamina Propria Dissociation Kit (Miltenyi Biotec, Germany) was used to isolate intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from mice. We used 800 mg of colon tissue for each digestion. After cleaning with Hank's buffered salt solution (HBSS, containing 1.26 mM $CaCl_2$, 0.407 mM $MgSO_4$, 0.493 mM $MgCl_2$, 0.441 mM KH_2PO_4, 5.33 mM KCl, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.338 mM Na_2HPO_4 and 5 mM glucose) the fat tissue and

Peyer's patches of the colon were removed. The intestine was longitudinally cut into pieces approximately 0.3-0.5 cm in length. The tissue pieces were incubated into predigestion solution (1×HBSS buffer containing 10 mM HEPES without Ca^{2+} and Mg^{2+}, 5 mM ethylenediaminetetraacetic acid, 5% fetal bovine serum and 1 mM dithiothreitol) in a tube using the MACS (magnetic-activated cell sorting) mix Tube Rotator (Miltenyi Biotec, Cologne, Germany) and then applied to a 100 µm MACS Smart Strainer (Miltenyi Biotec, Cologne, Germany) to collect the flow through containing IELs. The entire dissociation process included three iterations of IEL separation and all the obtained flow throughs were mixed. For the isolation of LPLs, the colon tissue was transferred into a gentle MACS C Tube containing the enzyme mix. It was then placed to the gentle MACS Dissociator (Bergisch Gladbach, Germany) to run the dissociation program. After that, a short spin-up was performed to collect sediment at the bottom of the detached tube. Subsequently, PB buffer (PBS containing 0.5% FBS) was added and the cell suspension was used to the 100 µm MACS SmartStrainer. The SmartStrainer was then washed with PB buffer. The cell suspension was centrifuged and the supernatant was aspirated to obtain the LPLs. As few cells were collected from the colon lamina propria, IELs and LPLs were mixed for the flow cytometry analysis.

Flow cytometry: Highly purified $CD45^+$ cells were isolated using the EasySep™ Mouse CD45 Positive Selection Kit (StemCell Technologies, Canada). The anti-mouse antibodies IL-10-Pe/Cyanine7, CD19-PerCP/Cyanine5.5 and CD138-APC were purchased from BioLegend (San Diego, CA) and IgA-FITC, B220-BV421, Fixable Viability Stain 510 and Purified Rat Anti-Mouse CD16/CD32 (Mouse Fc Block) were purchased from BD Pharmingen™ (Franklin Lakes, NJ). The P35-FITC was purchased from Thermo Fisher Scientific (Waltham, MA) and EBI3-APC from R&D Systems (Emeryville, CA). The Leukocyte Activation Cocktail with BD GolgiPlug™ (San Diego, CA) was used for intracellular accumulations of IgA, IL-10, P35 and EBI3 according to the manufacturer's recommended protocol. Cells were acquired on a BD LSR flow cytometer (BD Biosciences, Oxford, UK) and the data were analyzed using FlowJo v10.0 software.

Cell culture: The spleens of male C57BL/6 mice were ground on the SmartStrainer (Miltenyi Biotec) using the piston handle of a syringe to obtain a single cell suspension. The $CD19^+$ B cells were obtained using CD19 MicroBeads (Miltenyi Biotec) following the producer's manual. The isolated cells were cultured in RPMI 1640 medium containing 10% FBS. Lipopolysaccharide (3 µg mL^{-1}) was used to stimulate IL-10 and IL-35 secretion. An anti-CD40 group containing anti-CD40 (20 µg mL^{-1}, TGF-β (1 ng mL^{-1}), IL-5 (5 ng mL^{-1}), IL-6 (5 ng mL^{-1}) and retinoic acid (RA, 10 nM) was used to induce IgA secretion.

Real-time PCR (RT-qPCR): Total RNA from colon samples was isolated with the RNeasy Mini Kit (QIAGEN, Germany) according to standard protocol. First-strand cDNA synthesis was performed using QuantScript RT Kit (Tiangen Biotech, Beijing, China). Real-time-qPCR was performed using BioRad CFX96 with SuperReal PreMix Plus (Tiangen Biotech).

Three set primers were used to detect the expression of IgA, IL-10 and β-actin, namely, IL-10-F: 5 -AGCCGGGAAGACAA TAACTG-3 , IL-10-R: 5 -CATTTCCGATAAGGCTTGG-3, VDJ-Cα-F: 5-CATCTGAGGACTCTGCNGTCTAT-3, VDJ-Cα-R: 5-GAGCTG GTGGGAGTGTCAGTG-3, β-actin-F: 5-GTCCCTGACCCTCCC AAAAG-3 and β-actin-R: 5-GCTGCCTCAACACCTCAACCC-3. The β-actin was kept as the normalization control to calculate relative mRNA expression levels using the $2^{-\Delta\Delta CT}$ method.

Enzyme-Linked Immunosorbent Assay (ELISA): The intestinal concentrations of IL-10, IL-35 and IgA were detected by ELISA using IL-10 (Boster Biotech, Wuhan, China), IL-35 (Cloud-Clone Corp., Wuhan, China) and IgA (Thermo Fisher Scientific, USA) kits, respectively.

Statistical analysis: Data are presented as the Mean±SEM. Statistical analyses were performed using the Gehan-Breslow-Wilcoxon test, for survival comparison and the Student's t-test, for comparing means between two groups, using GraphPad Prism version 8.0 (San Diego). A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

MET alleviated DSS-induced colitis in mice: All mice in the DSS group died on the 10th day, but the survival rate of DSS+MET treated mice was 57.1% (Fig. 1a). The MET attenuated colitis in DSS-treated mice, as indicated by less weight loss, reduced DSS-mediated severe DAI scores and less shortening of the colon length (Fig. 1b-d). The histopathological status of observed colon samples showed that MET alleviated the injuries caused by DSS owing to the decreased infiltration of immune cells and epithelial cell death (Fig. 1e). These results suggest that the clinical symptoms of mice with DSS-induced colitis can be relieved by MET.

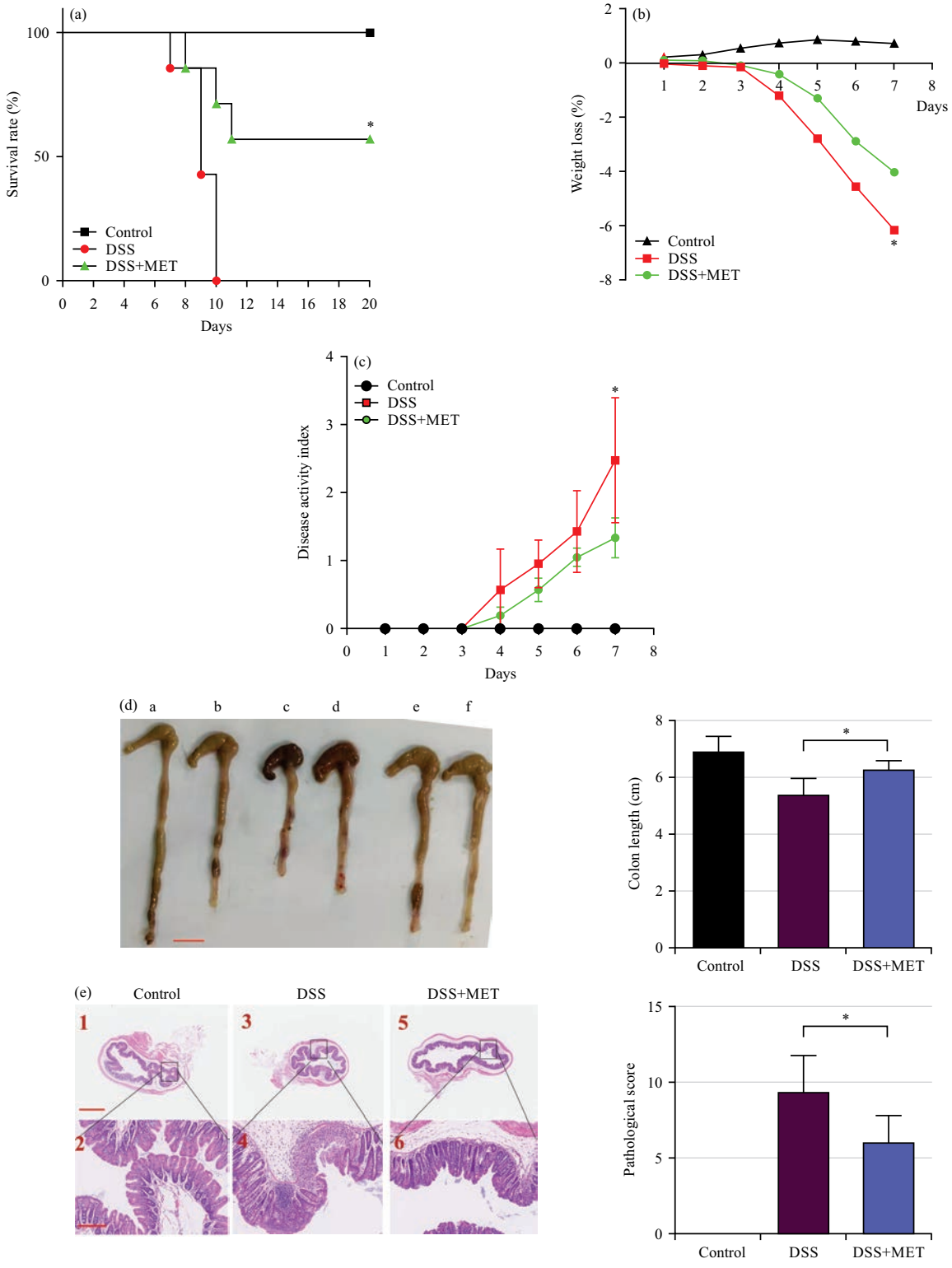

Fig. 1(a-d): MET alleviated DSS-induced colitis in mice, (a) Metformin (MET) improves the survival rate of DSS treated mice, (b) MET reduces the weight loss ratio of mice with UC, (c) MET improves the disease activity index (DAI) of mice with UC, (d) MET improved the DSS reduced colon length of mice with UC and (e) MET improves the symptoms of colonic strictures induced by dextran sulfate sodium (DSS)

Scale bar: 1 cm (1, 2: Control group, 3, 4: DSS group and 5, 6: DSS+MET group, *p<0.05), Scale bar of 1, 3 and 5: 1 mm and Scale bar of 2, 4 and 6: 200 μm

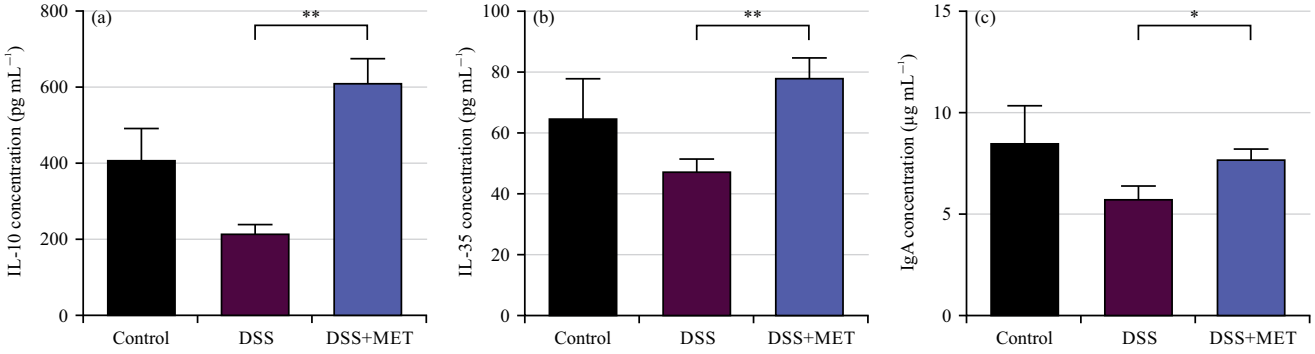

Fig. 2(a-c): Comparison on the levels of (a) IL-10, (b) IL-35 and (c) IgA in the colon tissue of control, colitis and MET treated colitis mice

n = 5 and *,**Significant difference

***In vivo* effect of MET on IL-10, IL-35 and IgA:** The concentrations of IL-10, IL-35 and IgA decreased after DSS treatment, whereas, MET significantly increased the levels of IL-10, IL-35 and IgA in the detected tissues (Fig. 2). These results suggested that MET promotes the production of anti-inflammatory cytokines and IgA in UC mice.

Effect of MET on the proportion of IL-10$^+$ and IgA$^+$ B cells: We further detected the percentage of IL-10$^+$ B cells in the DSS and DSS$^+$MET groups. No statistically significant differences were observed in the proportion of total IL-10$^+$, B220$^+$IL-10$^+$ or CD138$^+$IL-10$^+$ cells in the mixture of IELs and LPLs between the control and DSS groups (Fig. 3a) or between the DSS and DSS+MET groups (Fig. 3a-b). These data demonstrated that IL-10$^+$ B cells may have a limited effect on MET-mediated alleviation of DSS-induced UC and that IL-10 may be secreted by other cells in the colon tissue. The proportion of CD45$^+$CD138$^+$IgA$^+$ cells was lower in the mixture of IELs and LPLs in the DSS group than that in the vehicle-treated mice. However, MET treatment elevated the proportion of CD45$^+$CD138$^+$IgA$^+$ cells in the DSS-treated group (Fig. 3c). These data suggest that MET alleviates colitis by increasing the proportion of IgA-expressing CD138$^+$ B cells.

MET increases the mRNA expression level of vdj-Cα in B cells *in vitro*: Mature B cells that secrete IgA express the vdj-Cα gene, reflecting the differentiation and development level of B cells[16]. After adding stimulators to mouse CD19$^+$ B cells, the expression level of vdj-Cα mRNA increased, indicating that the induction condition was effective (Fig. 4). The MET (0.2 mM, 1 mM) significantly promoted the expression of vdj-Cα dose dependently, implying that MET promoted the secretion of IgA by B cells.

Mizoguchi *et al.*[17] found that B cells in intestinal-associated immune organs regulate intestinal inflammation by secreting IL-10 during spontaneous intestinal inflammation. Although the MET enhanced the production of IL-10 in the intestine of the DSS treated mice in this study, no significant difference was found in the proportion of B220$^+$IL-10$^+$ or CD138$^+$IL-10$^+$ cells between DSS-treated and MET+DSS-treated mice, suggesting that MET ameliorated the clinical symptoms of colitis in mice by some mechanism other than regulating B cells to secret IL-10. The lower expression capacity of IL-35 in B cells was previously reported in the peripheral blood of patients with Crohn's disease[18]. Previously, Wang *et al.*[19] found that after intraperitoneal injection of IL-35, the numbers of regulatory T cells and IL-35$^+$ regulatory B cells increased, thus decreasing the savageness of experimental autoimmune uveitis[19]. In this study, the expression of IL-35 in the intestine increased after MET treatment, however, no difference was found in the proportion of B220$^+$ cells producing IL-35 in each group, indicating that MET did not improve UC by inducing B cells to produce IL-35.

The MET also affects the gut microbiota and has a positive effect on IBD[20]. Caruso *et al.*[21] found that pathogenic intestinal microbiota prevents intestinal inflammation by stimulating plasma cells in intestinal-associated lymphoid tissues to produce IgA. In this study, MET increased IL-10, IL-35, IgA expressions and the percentage of CD138$^+$IgA$^+$ B cells in the intestinal tissues. Xu *et al.*[22] found that endometrial regenerative cells attenuated DSS-induced UC and increased the production of regulatory B cells and IL-10 level. However, the percentages of B220$^+$IL-10$^+$, B220$^+$IL-35$^+$ and CD138$^+$IL-10$^+$ cells, were not affected by MET, indicating that the MET attenuated IL-10 and IL-35 and were not secreted by B cells in

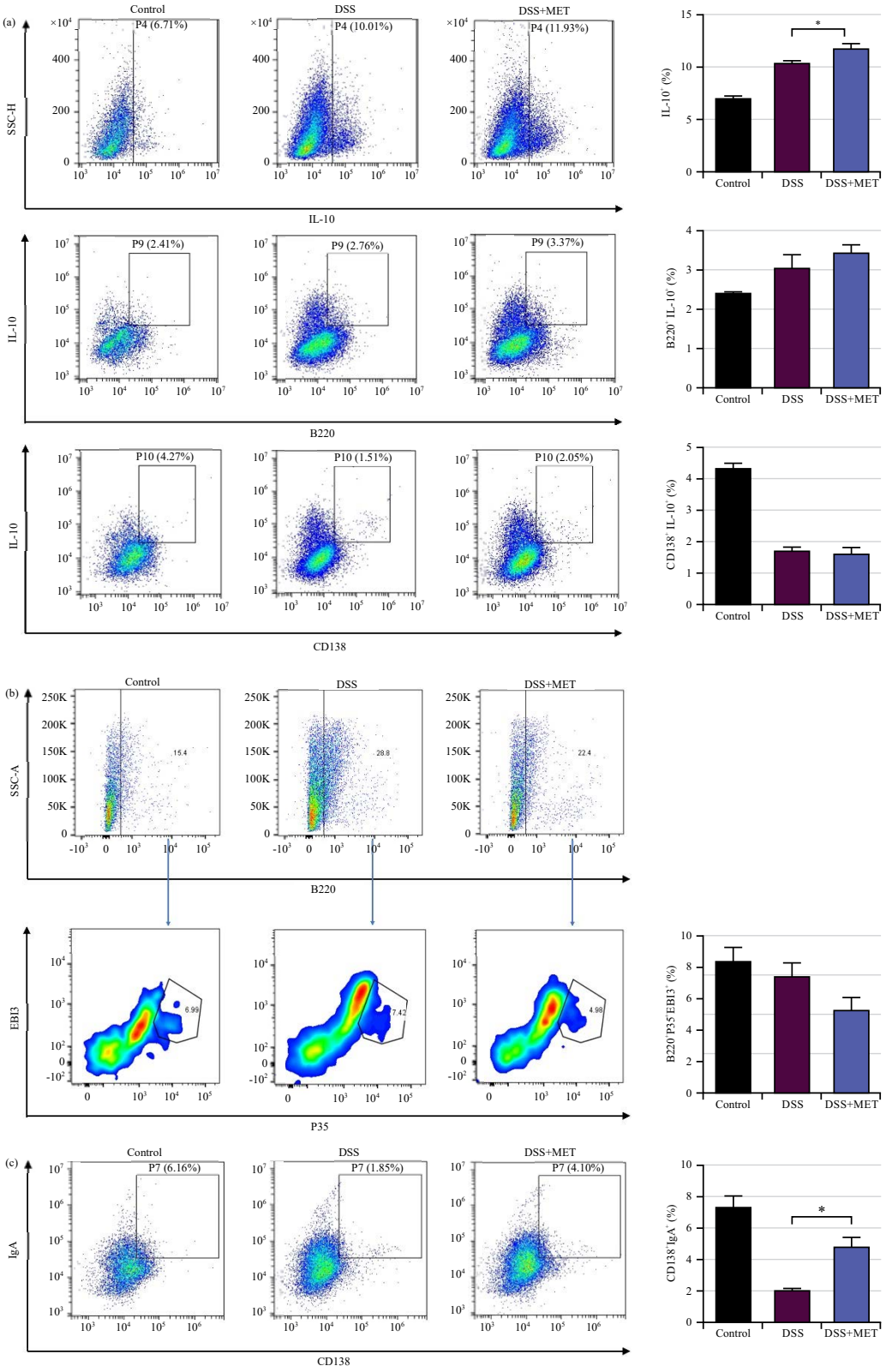

Fig. 3(a-c): Effect of MET on the proportions of (a) IL-10$^+$, B220$^+$IL-10$^+$, CD138$^+$IL-10$^+$ cells, (b) B220$^+$p35$^+$ebi3$^+$ cells and (c) CD138$^+$IgA$^+$ cells in the colon tissue of the mice

n = 5 and *,**Significant difference

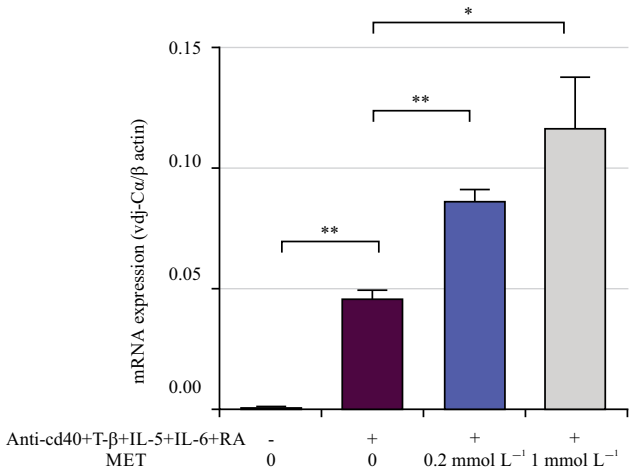

Fig. 4: Effect of MET on vdj-Cα mRNA expression in cultured CD19$^+$ B cells
n = 3, *p<0.05 and **p<0.01

the present study. Therefore, further experiments are required to clarify the source of MET-attenuated IL-10 and IL-35. Our result makes a significant contribution to the literature and provides a scientific basis for the potential application of MET in the prevention and treatment of UC. However, more investigation is necessary to completely understand the mechanism of how MET induces the intestinal IL-10 and IL-35 expression in this UC model.

CONCLUSION

The therapeutic role of MET on DSS-induced colitis in mice by regulating the B cells that produce IgA in intestinal tissues was demonstrated. The results indicated that some small molecules can directly stimulate intestinal B cells to secrete IgA and thereby improve UC. This study may provide important clues for the discovery of novel small-molecule drugs for UC treatment.

SIGNIFICANCE STATEMENT

The B cells are involved in the production of immunoglobulins, which provide immune protection and anti-inflammatory cytokines. Metformin was found to increase the level of the antibody IgA and cytokines IL-10 and IL-35, improve ulcerative colitis, improve the symptoms of colitis by regulating the production of IgA-secreting B cells in mice and IL-10 and IL-35 were produced by some other mechanism. We believe that our study makes a significant contribution to the literature because it is the first to describe the mechanism by which metformin acts on the IgA to alleviate ulcerative colitis.

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