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Estrogen receptor β activation ameliorates DSS-induced chronic colitis by inhibiting inflammation and promoting Treg differentiation

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ABSTRACT

Estrogen receptor (ER) β activation has anti-inflammatory activity. However, its effect on the development of inflammatory bowel disease (IBD) and the underlying mechanism have not been clarified. This study aimed to assess the clinical value of $ER\beta^+CD4^+$ T cells in IBD patients and examine the anti-inflammatory role of ERβ activation in dextran sulfate sodium (DSS)-induced chronic colitis in mice. We investigated the effects of ERB041 (an ERβ-specific agonist) on inflammatory cytokines and pro-inflammatory T-cell and regulatory T-cell (Treg) responses in murine colitis. We tested the role of ERβ activation on Treg differentiation and its activity to suppress T-cell proliferation in vitro. We found that reduced frequency of circulating $ER\beta^+CD4^+$ T cells in IBD patients was negatively correlated with inflammation and disease severity. ERβ and FoxP3 expression co-localized in the intestinal tissues of IBD patients. Treatment with ERB041 significantly mitigated colitis-induced weight loss, inflammation, and disease severity. It also restored the $ER\beta^+CD4^+$ T cell population in the spleen and colon lamina propria of these mice. ERB041 treatment inhibited $CD4^+CD25^-$ and $CD8^+$ T cell infiltration and restored Tregs and activated T-cell immunoreceptor with Ig and ITIM domains $(TIGIT)^+$ Tregs in the colon lamina propria. In vitro, we found that ERβ activation enhanced Treg differentiation, immunosuppression, and TGF-β1/Smad signaling in $CD4^+$ T cells. Our data suggest that $ER\beta^+CD4^+$ T cells represent a potential biomarker for evaluating IBD disease severity, and ERβ activation may be valuable for the treatment of IBD by enhancing the Treg response.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two main forms of inflammatory bowel disease (IBD) characterized by an aberrant mucosal immune response to intestinal microbiota resulting from environmental triggers or genetic predisposition [1–3]. Previous studies demonstrated that pro-inflammatory $CD4^+$ effector Th1, Th17, and Th2 cells could migrate into the inflammatory lesions in the intestinal organs to secrete inflammatory cytokines (e.g., IFNγ, TNFα, IL-2, IL-6, IL-17A), which activate macrophages and NK cells, leading to inflammatory damage to the intestinal organs and IBD development [4,5]. In contrast, $CD4^+FoxP3^+$ regulatory T cells (Tregs) are potent inhibitors, which can secrete anti-inflammatory cytokines (e.g., IL-10, TGF-β1) to downregulate autoimmune inflammation, maintain self-tolerance, and promote tissue repair [6]. Therefore, an imbalance between effector T cell (Teff) and Treg responses is crucial for the development and progression of IBD [1,7]. Therefore, modulation of this imbalance represents a potential new treatment strategy for autoimmune IBD [8]. However, the mechanisms underlying the regulation of these aberrant $CD4^+$ T-cell responses and the imbalance between pro-inflammatory and Treg responses in IBD have not been defined.

Estrogen receptor (ER) β, a member of the nuclear receptor family, is a critical regulator of inflammation, immune responses, cancer, neurodegeneration, and metabolism [9–11]. Previous studies showed that ERβ has potent anti-inflammatory activity, and treatment with an ERβ agonist ameliorates experimental autoimmune encephalomyelitis, adjuvant-induced arthritis, and HLA-B27 transgenic rat colitis [12–15]. In the intestinal tract, ERβ is the predominant ER subtype in both men and women [16–18]. There is ample expression of ERβ in healthy intestinal mucosa, but its expression is markedly downregulated in active UC and CD [19,20]. Furthermore, Saleiro et al. reported that ERβ deficiency is associated with increased severity of inducible colitis in mice

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[21]. Conversely, ERβ-specific agonists can inhibit autoimmune inflammation in rodents [14,15]. Taken together, these data suggest that activation of ERβ may be crucial for the inhibition of colitis. Christaki et al. demonstrated that ERβ agonism could increase survival in experimental sepsis and reduce the transcription of multiple pro-inflammatory proteins [22]. In addition, the ERβ-selective agonists repressed inflammatory gene transcription in peripheral blood mononuclear cells (PBMCs) by recruiting the coactivator steroid receptor coactivator-2, highlighting its anti-inflammatory properties in immune cells [23]. Moreover, an ERβ-selective agonist had a therapeutic effect against multiple sclerosis by suppressing activated microglia and blocking NF-κB activation and iNOS expression in both microglia and T cells that were invading the spinal cord [12].

Little is known about how ERβ activation inhibits the development of colitis. ERβ is highly expressed in the intestinal epithelium, and a previous study suggested a potential role for this receptor in the maintenance of epithelial permeability in the colon of mice [19]. In addition, ERβ deficiency is associated with an increase in epithelial cell proliferation, decrease in cellular adhesion molecules, disrupted tight-junction formation, and abnormal colonic architecture [24,25]. The protective effects of this epithelial barrier mediated by ERβ may be one of its anti-inflammatory mechanisms to relieve colitis after exposure to an ERβ agonist. However, whether ERβ has a role in regulating intestinal immune responses during the development of colitis has not been thoroughly investigated.

Emerging evidence has implicated $CD4^+$ T cells in the pathogenesis of IBD [1,8]. Tregs represent a subset of $CD4^+$ T cells, and studies suggest that Treg defects can play distinct causative roles in IBD [7,26]. The expression of ERβ in $CD4^+$ T cells and its association with IBD severity and whether ERβ activation can modulate pro-inflammatory and Treg responses during the development of colitis are unclear. In the present study, we demonstrated that circulating $ER\beta^+CD4^+$ T cells were significantly decreased in patients with active IBD. This reduction was negatively associated with the development and severity of this disease. Treatment with the ERβ-specific agonist ERB041 significantly mitigated DSS-induced colitis by inhibiting inflammatory cytokines and pro-inflammatory T cell responses and promoting Treg differentiation and suppression of T-cell proliferation. This study provides novel immunological explanations as to why ERβ activation ameliorates colitis, which may aid in the design of new therapies for IBD.

2. Materials and methods

2.1. Subjects

All experimental protocols in this study were performed in accordance with the guidelines approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Written informed consent was obtained from each subject.

A total of 30 patients with UC, 31 with CD, and 29 healthy controls (HCs) were recruited by the Department of Gastroenterology at Wuhan Union Hospital, China. Individual patients with IBD were diagnosed according to clinical, endoscopic, and histological criteria. Individuals with infectious colitis or chronic systemic diseases were excluded. Clinical disease activity was assessed by the Mayo Clinic scoring index [27] or the Crohn's Disease Activity Index (CDAI) clinic score [28]. HCs had normal endoscopy and histology results. Mayo scores of ≤ 2 and > 2 were defined as remission (R) and active disease (A), respectively. Similarly, CDAI scores of ≤ 150 and > 150 were defined as R and A, respectively. The demographic and clinical characteristics of the subjects are shown in Table 1.

2.2. Sample preparation

Peripheral blood samples from individual subjects were prepared for flow cytometry. Two intestinal biopsy samples were collected from the inflamed mucosal sites of patients with IBD and non-inflamed tissues of HCs during endoscopic examination. One sample was formalin-fixed and paraffin-embedded for histological and immunofluorescence analysis. The second sample was immediately stored in Trizol at −80 °C for gene expression analysis.

Table 1
Clinical characteristics of patients and health controls.

Characteristics	UC	CD	HCs
Patients (n)	30	31	29
Age (years)	41.17 ± 2.24	28.65 ± 1.80	39.31 ± 2.09
Sex (male/female)	21/9	21/10	17/12
Disease duration (months)	36.73 ± 8.25	27.81 ± 5.14	–
Active/Remission (n)	22/8	11/20	–
Enteric resection (n)	0	15	0
Mayo score	6.87 ± 0.66	–	–
CDAI score	–	103.30 ± 12.36	–
CRP (mg/L)	24.08 ± 6.68**	17.95 ± 3.82***	1.27 ± 0.28
ESR (mm/h)	24.67 ± 4.32***	18.45 ± 3.08**	6.47 ± 0.78
Albumin (g/L)	38.59 ± 1.24**	40.33 ± 0.86**	43.27 ± 0.68
BMI	19.89 ± 0.51**	19.73 ± 0.50**	21.87 ± 0.44

(a) Data are shown as mean ± SEM, **P < 0.01, ***P < 0.001 vs. the Health Controls.
(b) Abbreviations: UC: Ulcerative colitis; CD: Crohn's disease; HCs: Health Controls; CDAI: Crohn's Disease Activity Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; BMI: Body Mass Index.

2.3. Mice and disease model

The care and use of mice were performed in accordance with institutional guidelines with approval from the Institutional Animal Care and Use Committee at the Animal Care Unit.

Male C57BL/6 mice (8 to 10 weeks; 22 to 25 g) were obtained from HFK Bioscience (Beijing, China). The mice were housed in a specific pathogen-free facility at the Experimental Animal Center (Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China). The mice were randomized into control, colitis, and colitis + ERB041 groups (n = 10 per group). Mice were given free access to food and water. The mice in the colitis and colitis + ERB041 groups were provided with water containing 2% (wt/vol) DSS (MP Biomedicals, IIIkirch, France) for three cycles (5 days on 2% DSS and 4 days on normal water). Beginning on day 7 post-induction, mice were injected s.c. with vehicle (the control and colitis groups) or 5 mg/kg body weight of the ERβ-specific agonist ERB041 (1 mM in ethanol, Tocris Biotech, Minneapolis, USA) daily for up to 30 days post-induction.

2.4. Evaluation of chronic colitis

The severity of DSS-induced chronic colitis in individual mice was evaluated by monitoring the body weight, stool consistency, rectal bleeding, and anal prolapse daily throughout the study period in a blinded manner. The disease activity index (DAI) was assessed by combining the scores of weight loss, stool consistency, and bleeding [29]. At the end of the experiment, all mice were euthanized, and their colons dissected and photographed. The colons were fixed in 10% buffered formalin overnight and embedded in paraffin. The 5-μm tissue sections were stained with hematoxylin and eosin (H&E) and then examined by light microscopy.

2.5. Chemicals

ERB041 and the ERβ antagonist PHTPP were purchased from Tocris, USA. Both compounds were dissolved in ethanol at a concentration of

1 mM.

2.6. Isolation of cells

Mouse splenic mononuclear cells (MNCs), mesenteric lymph node (MLN) cells, colonic intraepithelial lymphocytes (IEL), and lamina propria mononuclear cells (LPMC) were isolated as previously described [30]. The splenic MNCs and MLN cells were isolated after lysis of red blood cells using red blood cell lysis buffer (BD Biosciences, USA). After being washed, the cells were used for following experiments.

To isolate IEL and LPMC, the colons were flushed with cold PBS and cut longitudinally into small pieces, followed by washing with 1 mM DTT in Hank's balanced salt solution (HBSS) at 37 °C with gently shaking for 20 min. To remove the epithelium, the pieces were incubated in 1.3 mM EDTA in HBSS at 37 °C for 30 min. The IELs were enriched by 40%/70% Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation. To isolate LPMCs, the remaining colon pieces were digested with 0.1 mg/ml collagenase D (Roche Applied Science, Basel, Switzerland) in serum-free Iscove's modified Dulbecco's medium at 37 °C for 1 h and then filtered through a cell strainer followed by centrifugation. The LPMCs were enriched by 40%/70% Percoll gradient centrifugation. The cells at the interface were harvested for further experiments.

2.7. Flow cytometry

Human whole blood samples were stained with anti-CD4-FITC and anti-CD25-APC antibodies (BD PharMingen, USA) for 30 min at 4 °C. The cells were fixed, permeabilized, and intracellularly stained with anti-ERβ-PE (Novus Biologicals, USA) and anti-FoxP3-PE CF594 (BD PharMingen) for 60 min at 4 °C. The stained cells were characterized by flow cytometry (BD LSR Fortessa X-20). Peripheral lymphocytes were first gated according to their SSC and FSC. The cells were gated for CD4+, and the $CD25^+FoxP3^+$ Tregs were analyzed. $ER\beta^+CD4^+$ T cells in the total $CD4^+$ T cells were analyzed.

Mouse MNCs were stained with anti-CD4-FITC, anti-CD25-APC, anti- FoxP3-BV421, anti-TIGIT- percpcy5.5, anti-CD39-PE-Cy7 (BD PharMingen, USA), and anti-ERβ-PE (Novus Biologicals, USA). Isotype IgGs were used as controls. The data were analyzed using FlowJo software.

2.8. Quantitative real-time PCR

Total RNA was extracted from individual biopsy specimens and mouse colon using TRIzol reagent (Takara, Shiga, Japan). cDNA was reversely transcribed from 1 μg of RNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Relative gene expression levels were determined by quantitative RT-PCR (qRT-PCR) on a Roche Light Cycler 480 System using SYBR Green Real-Time PCR Master Mix (Toyobo) and specific primers. The data were normalized to the β-actin (human samples) or GAPDH (mouse samples) control and analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Supplemental Table 1.

2.9. Cell culture

$CD3^+CD4^+CD44^-CD62L^+$ naïve T cells, $CD4^+CD25^+$ Tregs, $CD4^+CD25^-$ Teffs and $CD8^+$ T cells were isolated from the spleens and MLNs of unmanipulated C57BL/6 mice by MACS (Miltenyi Biotec, Germany). Preparations with a purity of 93% were used for flow cytometry. The purified naïve T cells were pretreated with 100 nM ERB041 or 1000 nM PHTPP in 96-well round-bottom plates to induce $CD4^+CD25^+FoxP3^+$ Tregs in vitro as previously described [31]. Briefly, the purified naïve T cells were stimulated with 12 μg/ml immobilized anti-mouse CD3ε, 2 μg/ml anti-mouse CD28 (BD PharMingen, USA), 10 ng/ml recombinant mouse IL-2, and 5 ng/ml recombinant mouse transforming growth factor (TGF) β1 (R&D Systems, USA) for 5 days in a 5% CO_2 incubator at 37 °C. To analyze phosphorylated Smad2/3, naïve T cells were purified and cultured as described for the induction of Treg differentiation in the presence or absence of TGF-β1 (R&D Systems, USA), ERB041 and/or PHTPP (Tocris, USA). On day 3, the cells were treated with or without 10 ng/ml TGF-β1, 100 nM ERB041, or 1000 nM PHTPP for 60 min in a 5% CO_2 incubator at 37 °C. The cells were then fixed, permeabilized, and stained with anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425)-PE mAb and analyzed by flow cytometry.

T cell proliferation was measured by CFSE dilution-based flow cytometry. Briefly, purified $CD4^+CD25^-$ and $CD8^+$ T cells were labeled with 1 μM CFSE (Invitrogen) for 15 min at 37 °C. The CFSE-labeled $CD4^+CD25^-$ or $CD8^+$ T cells alone or co-cultured with Tregs (a ratio of Treg to CD4/CD8 of 1:4) were stimulated with anti-CD3/CD28 beads for 72 h in the presence or absence of ERB041, or PHTPP and analyzed by flow cytometry.

2.10. Immunofluorescence

To evaluate the intestinal expression of ERβ and the presence of Tregs, the human mucosal sections (4 μm) were stained with rabbit-anti-FoxP3 and mouse-anti-ERβ antibodies (both 1:400 dilution, R&D Systems, USA) and then FITC-anti-rabbit and PE-anti-mouse IgGs, respectively. The staining was analyzed using laser confocal microscopy.

$CD3^+CD4^+CD44^-CD62L^+$ naïve T cells were purified by MACS and stained by immunofluorescence to confirm ERβ expression. Fresh cell suspensions were loaded on polylysine-coated coverslips for 20 min. After washing, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 at room temperature for 20 min. After washing, the cells were blocked with 3% normal donkey sera and stained with rabbit-anti ERβ (1:500 dilution, Abcam, UK) overnight at 4 °C. The bound antibodies were detected with FITC-donkey anti-rabbit antibody (1:400 dilution, Jackson ImmunoResearch, USA) and counterstained with DAPI (Sigma, USA). The cells were analyzed by confocal laser microscopy.

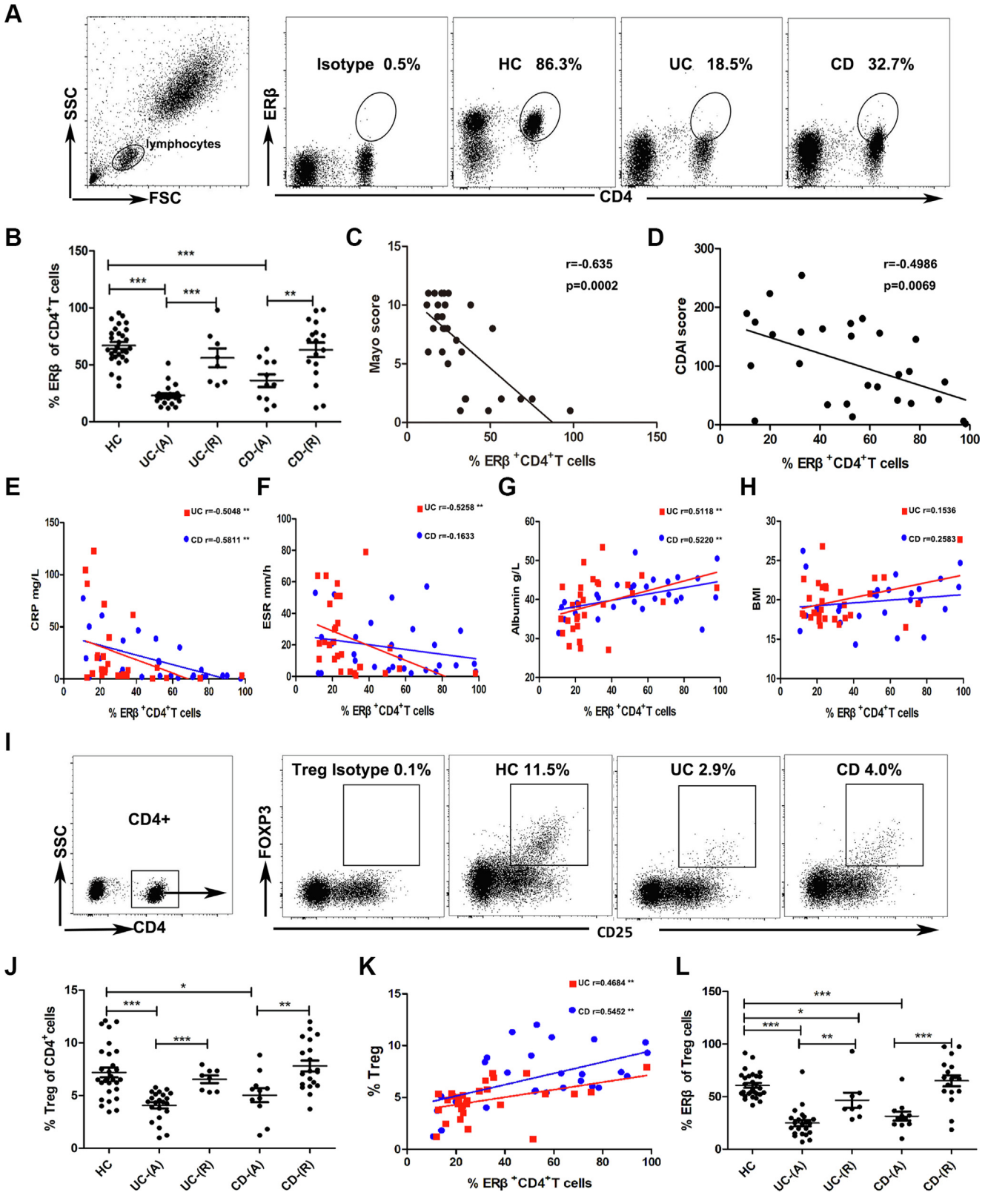
2.11. Statistical analysis

Statistical analyses were performed using SPSS version 17.0 and GraphPad Prism version 5.0. Patient data are presented as medians and 25th to 75th interquartile ranges and analyzed by the Mann-Whitney *U* test. Other normally distributed data are expressed as the mean ± SEM. The difference between two groups was analyzed by the unpaired Student *t*-test. Differences among three or more groups were analyzed by one-way analysis of variance (ANOVA). The potential correlation between variants was analyzed by the Spearman correlation coefficient. A two-tailed *P*-value of < 0.05 was considered statistically significant. The asterisks indicate *P < 0.05, **P < 0.01, ***p < 0.001.

3. Results

3.1. ERβ expression in circulating $CD4^+$ T cells is significantly associated with the severity of IBD in human patients

To understand the regulatory role of ERβ, IBD patients and HCs were recruited. As expected, IBD patients displayed significantly higher levels of serum C-reactive protein (CRP) and blood erythrocyte sedimentation rate (ESR), but lower albumin and body mass index (BMI), relative to the HCs (Table 1). The percentage of peripheral $ER\beta^+CD4^+$ T cells in patients with active disease was significantly lower than in the HCs (all P < 0.001; Fig. 1A and B). Furthermore, the percentage of $ER\beta^+CD4^+$ T cells in patients with active CD or UC was significantly lower than in patients in remission (P < 0.001 for UC, P < 0.01 for



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CD; Fig. 1A and B). More importantly, regression analysis indicated that the percentage of circulating ERβ$^+$CD4$^+$ T cells was inversely correlated with the Mayo and CDAI scores in patients with UC ($r = -0.635$, $P = 0.0002$) and CD ($r = -0.4986$, $P = 0.0069$), respectively (Fig. 1C and D). Further analyses revealed that the percentage of circulating ERβ$^+$CD4$^+$ T cells was negatively correlated with the serum CRP levels in patients with UC ($r = -0.5048$, $P = 0.0072$) or CD ($r = -0.5811$, $P = 0.0015$, Fig. 1E) and blood ESR in UC patients ($r = -0.5258$, $P = 0.0041$ for UC; $r = -0.1633$, $P = 0.4063$ for CD; Fig. 1F). In contrast, the percentage of peripheral ERβ$^+$CD4$^+$ T cells was positively

Fig. 1. ERβ expression in circulating $CD4^+$ T cells is significantly associated with the severity of IBD in human patients. The percentage of $ER\beta^+CD4^+$ T cells in the total $CD4^+$ T cells was analyzed by flow cytometry (A) and the percentages (B) of circulating $ER\beta^+CD4^+$ T cells were calculated in the HCs and IBD patients. (C-D) Correlation analysis between the percentage of circulating $ER\beta^+CD4^+$ T cells and disease activity assessed by the Mayo or CDAI score. (E-H) Correlation analysis between the percentage of circulating $ER\beta^+CD4^+$ T cells and serum C-reactive protein (CRP) levels, blood erythrocyte sedimentation rate (ESR), serum albumin levels, and body mass index (BMI). Red, patients with UC; blue, patients with CD. (I) Treg analysis in different groups by flow cytometry. Illustrators as Tregs represent the percentage of peripheral $CD4^+CD25^+FoxP3^+$ Tregs in the $CD4^+$ T cells. (J) Percentages of peripheral Tregs in the HCs and IBD patients. (K) Correlation analysis between the percentages of peripheral Tregs and $ER\beta^+CD4^+$ T cells. Red, patients with UC; blue, patients with CD. (L) Percentage of circulating $ER\beta^+$ Tregs in HCs and IBD patients were analyzed by flow cytometry. (A) as active stage, (R) as remission stage. Data are presented as representative images or expressed as the mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlated with serum albumin levels ($r = 0.5118$, $P = 0.0045$ for UC; $r = 0.5220$, $P = 0.0044$ for CD, Fig. 1G), but not with BMI values ($r = 0.1536$, $P = 0.4176$ for UC; $r = 0.2583$, $P = 0.1844$; Fig. 1H) in this population. Hence, ERβ expression in circulating $CD4^+$ T cells was decreased in active IBD patients and negatively associated with IBD progression.

Tregs are important regulators of autoimmune inflammation [32]. Therefore, we analyzed the frequency of circulating $CD4^+CD25^+FoxP3^+$ Tregs in the different groups of subjects by flow cytometry. We found that the percentage of circulating Tregs in patients with active UC or CD was significantly lower than in patients in remission or the HCs (all $P < 0.05$; Fig. 1I and J). Furthermore, the percentage of circulating Tregs was positively correlated with the percentage of peripheral $ER\beta^+CD4^+$ T cells in patients with UC ($r = 0.4684$, $P = 0.009$) or CD ($r = 0.5452$, $P = 0.0027$, Fig. 1K). In addition, the percentage of $ER\beta^+$ Tregs in patients with active UC or CD was significantly lower than in patients in remission or the HCs (all $P < 0.05$; Fig. 1L). Thus, impaired circulating Tregs were positively associated with $ER\beta^+CD4^+$ T cells, which could contribute to IBD progression in humans.

3.2. Impaired ERβ expression in the intestine of IBD patients

We next analyzed ERβ expression in the intestinal biopsy samples from the different groups of subjects by immunofluorescence. While high ERβ expression levels were detected in the HC samples, lower levels were detected in the intestinal biopsies from both UC and CD patients (Fig. 2A). To assess whether the ERβ activation could modulate Treg responses, we analyzed the histological localization of ERβ and FoxP3. Interestingly, there were merged ERβ and FoxP3 fluorescent nuclear signals in the lamina propria of HCs and IBD patients (Fig. 2A).

In addition, quantitative RT-PCR analysis indicated that the relative *ESR2* expression levels in the intestinal biopsies from UC and CD patients were significantly lower than that in the HC samples ($P < 0.01$ or $P < 0.001$, respectively). However, there was no significant difference in the relative *ESR2* expression levels between UC and CD patients (Fig. 2B). Together, these data indicated that there was impaired ERβ expression in the intestines of patients with UC and CD.

3.3. ERB041 ameliorates DSS-induced chronic colitis and inflammation in vivo

Activation of ERβ can inhibit inflammation [11,15]. Therefore, we tested whether treatment with the ERβ agonist ERB041 could modulate inflammation in a DSS-induced mouse model of chronic colitis. We found that while the colitis group displayed a gradual reduction in body weights, treatment with ERB041 significantly mitigated the colitis-induced weight loss compared to the colitis group. The body weights in the ERB041-treated mice remained slightly lower than the control mice (Fig. 3A). Compared to the colitis group, treatment with ERB041 significantly decreased the DAI scores and mitigated the reduction in colon length and the pathological changes (e.g., architectural derangements, epithelial necrosis, diffuse lymphocytic infiltration) in the colon of mice induced by DSS (Fig. 3B-D). Furthermore, treatment with ERB041 significantly eliminated the upregulation of IL-1β, IFNγ, IL-17A, IL-18, and TNFα and the downregulation of IL-10 and TGF-β1 mRNA transcripts observed in the intestines of mice with colitis (Fig. 3E and F).

Given that impaired ERβ expression was associated with a lower frequency of Tregs during UC and CD pathogenesis, we questioned whether activation of ERβ could mitigate the impaired ERβ expression and Treg response in a mouse model of chronic colitis. Following the induction of chronic colitis by DSS, we found that the frequency of splenic, IEL, and LPMC, but not MLN, $ER\beta^+CD4^+$ T cells in the colitis group were significantly lower than that of the control and colitis + ERB041 groups (Fig. 3 G and H). There was no significant difference in the percentage of $ER\beta^+CD4^+$ T cells between the control and colitis + ERB041 groups. A similar pattern of *ESR2* mRNA transcripts was detected in the intestines of mice from the three groups (Fig. 3I). Taken together, these data revealed that activation of ERβ by its agonist ERB041 dramatically ameliorated colitis and the related inflammation by restoring the colitis-impaired $ER\beta^+CD4^+$ T cell population, especially in the colon lamina propria and spleen of mice with chronic colitis.

3.4. ERB041 treatment inhibits $CD4^+CD25^-$ and $CD8^+$ T cell infiltration and restores the Treg quantity in the colon mucosal lamina propria

UC is characterized by multiple types of inflammatory infiltrates, especially gut-specific T cell accumulation in the lamina propria [4]. To further elucidate the anti-inflammatory mechanisms of the ERβ agonist, we characterized the frequency of different subsets of T cells in the different treatment groups by flow cytometry. Compared to the control mice, significantly lower frequency of splenic and MLN $CD4^+$ and $CD8^+$ cells, $CD4^+CD25^-$ effector T cells, Tregs and IEL Tregs were detected in the colitis group (Fig. 4A-D), suggesting that $CD4^+$, $CD8^+$, and $CD4^+CD25^-$ T cells may infiltrate the lamina propria of the colonic mucosa during the process of chronic colitis in mice, similar to human UC. In contrast, there was a significantly increased frequency of $CD4^+$ and $CD8^+$T cells, but decreased Tregs in the colonic lamina propria of the colitis group. Treatment with ERB041 eliminated the colitis-mediated increase in the frequency of $CD4^+$, $CD8^+$, and $CD4^+CD25^-$ T cell and restored the frequency of Treg in the colon mucosal lamina propria of mice.

In addition, treatment with ERB041 significantly increased FoxP3 expression in the colon compared to the colitis group (Fig. 4E). Consistent with the observations in IBD patients, the percentage of Tregs was positively correlated with that of the $ER\beta^+CD4^+$ T cells in the colon mucosal lamina propria of mice ($r = 0.7684$, $P = 0.0001$; Fig. 4F). Collectively, these data demonstrated that ERB041 treatment modulated the imbalance between pathogenic T-cell and Treg responses in the colon mucosal lamina propria of mice with colitis.

3.5. ERB041 increases the frequency of activated Tregs in the colon mucosal lamina propria of mice

A recent study showed that the co-inhibitory molecule TIGIT is a distinct marker for activated Tregs [33]. Extracellular adenosine triphosphate (ATP) exhibits multiple pro-inflammatory effects, and the degradation of extracellular ATP by CD39 is another immunosuppression mechanism of Tregs [34,35]. Thus, $CD39^+$ Tregs have potent

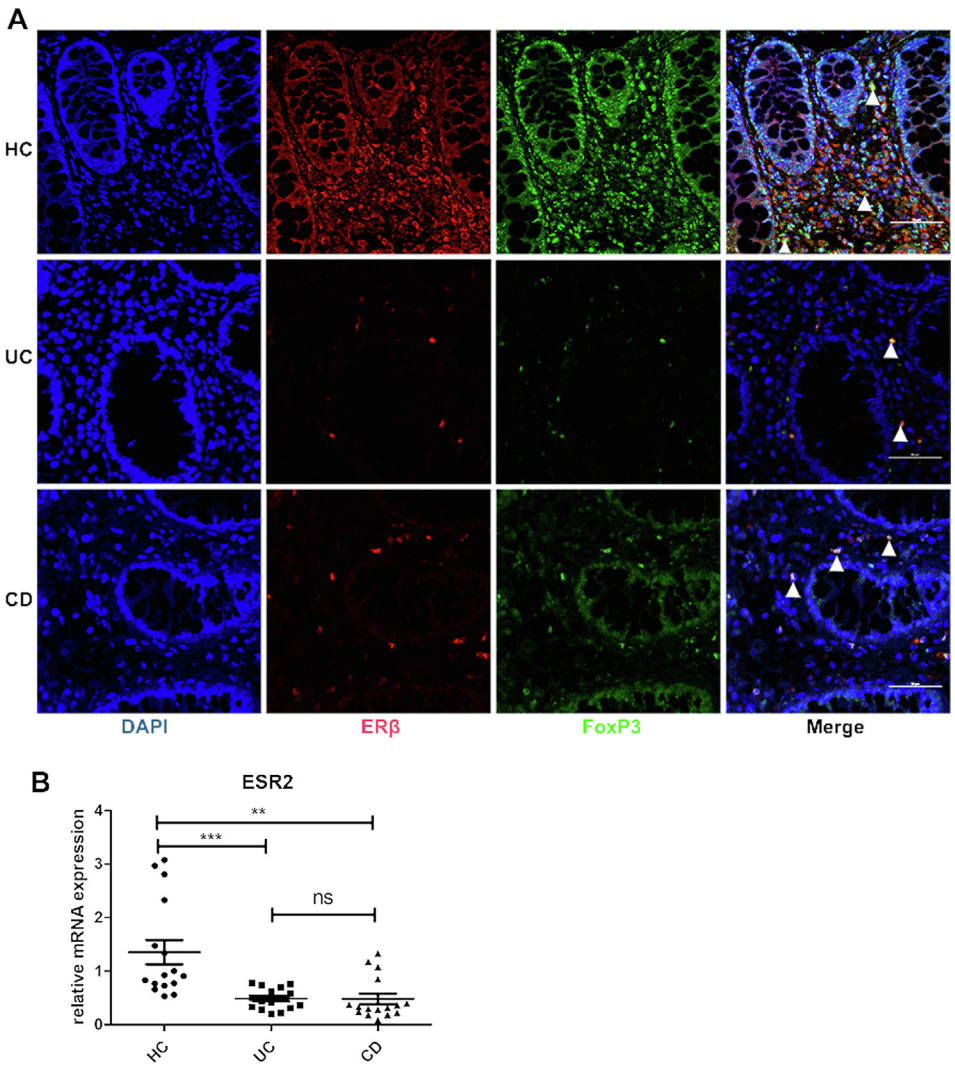

Fig. 2. Impaired ERβ expression in the intestine of IBD patients. (A) Immunofluorescence staining of ERβ (red) and FoxP3 (green) in the intestinal tissues visualized by confocal microscopy. Cell nuclei were stained with DAPI (blue). Arrows represent co-expression of ERβ and FoxP3 (scale bar, 50 μm, original magnification × 600). (B) Relative *ESR2* mRNA expression levels in the mucosal specimens from patients with IBD or HCs determined by qRT-PCR using β-actin as the control gene. Data are presented as medians and 25th to 75th interquartile ranges. **P < 0.01, ***P < 0.001; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitory activity by producing immunosuppressive adenosine [36]. To further understand the regulatory effect of ERB041, we examined the frequency of TIGIT$^+$ or CD39$^+$ Tregs in the different organs of the different groups of mice by flow cytometry. Compared to the control mice, there were significantly lower frequencies of splenic, MLN, IEL, and LPMC TIGIT$^+$ Tregs and LPMC CD39$^+$ Tregs in the colitis group (Fig. 5A and B). Treatment with ERB041 significantly increased the percentages of MLN, IEL, and LPMC TIGIT$^+$ Tregs, but did not significantly change in the frequency of LPMC CD39$^+$ Tregs in the colitis mice. Hence, treatment with ERB041 increases the frequency of activated Tregs, but it has no regulatory effect on the CD39$^+$ Tregs in this model.

3.6. ERβ activation enhances Treg differentiation and increases its activity to suppress T-cell proliferation in vitro

Because our results demonstrated that ERB041 restored the intestinal Treg in vivo, we tested whether ERβ activation promoted Treg differentiation and its immunosuppressive activity in vitro. MLN CD3$^+$CD4$^+$CD44$^-$CD62L$^+$ naïve T cells were purified by MACS (Fig. 6A), and ERβ was expressed by nearly all naïve T cells (Fig. 6B), further demonstrating ERβ expression in CD4$^+$ T cells. We found that stimulation with anti-CD3, anti-CD28, and IL-2 did not effectively induce FoxP3$^+$ Tregs (Fig. 6C and D, group 1). Consistent with previous studies [37], we found that TGF-β1 could effectively induce the differentiation of naïve T cells into CD4$^+$CD25$^+$ Tregs (Fig. 6C and D, group 2), which was partially attenuated by the ERβ antagonist PHTPP (group 4) in our experimental system. Interestingly, stimulation with ERB041 also significantly increased the frequency of FoxP3$^+$ Tregs (group 3) and enhanced TGF-β1-mediated Treg differentiation (group 6); both effects were abrogated by PHTPP (groups 5 and 7, respectively). Collectively, these data indicated that activation of ERβ enhanced Treg differentiation in vitro.

A previous study demonstrated that TGF-β1 could induce the differentiation of suppressive Tregs through the activation of Smad signaling [37]. Furthermore, dysregulated TGF-β signaling may have a role in the pathogenesis of IBD [38]. Recently, an ERβ/TGF-β1/Smad3 signaling pathway was reported in renal cell carcinoma and the colon crypt base [39,40]. Thus, we tested the hypothesis that enhanced Treg differentiation mediated by ERβ activation may occur through its signaling cross-talk with the TGF-β1/Smad pathway in CD4$^+$ T cells. We found that treatment with TGF-β1 significantly increased Treg differentiation and Smad2/3 phosphorylation levels in T cells (Fig. 6D and E). Moreover, ERβ activation by ERB041 enhanced Smad signaling by increasing Smad2/3 phosphorylation, which was dramatically mitigated by PHTPP (Fig. 6E). Treatment with ERB041 also enhanced TGF-β1 mediated Smad2/3 phosphorylation. Our data demonstrated that ERβ activation enhanced Treg differentiation by promoting the cross-talk between ERβ-related signaling and TGF-β1/Smad signals in CD4$^+$ T cells.

We further tested the effects of ERβ activation on the immunosuppressive activity of Tregs using an in vitro suppression assay.

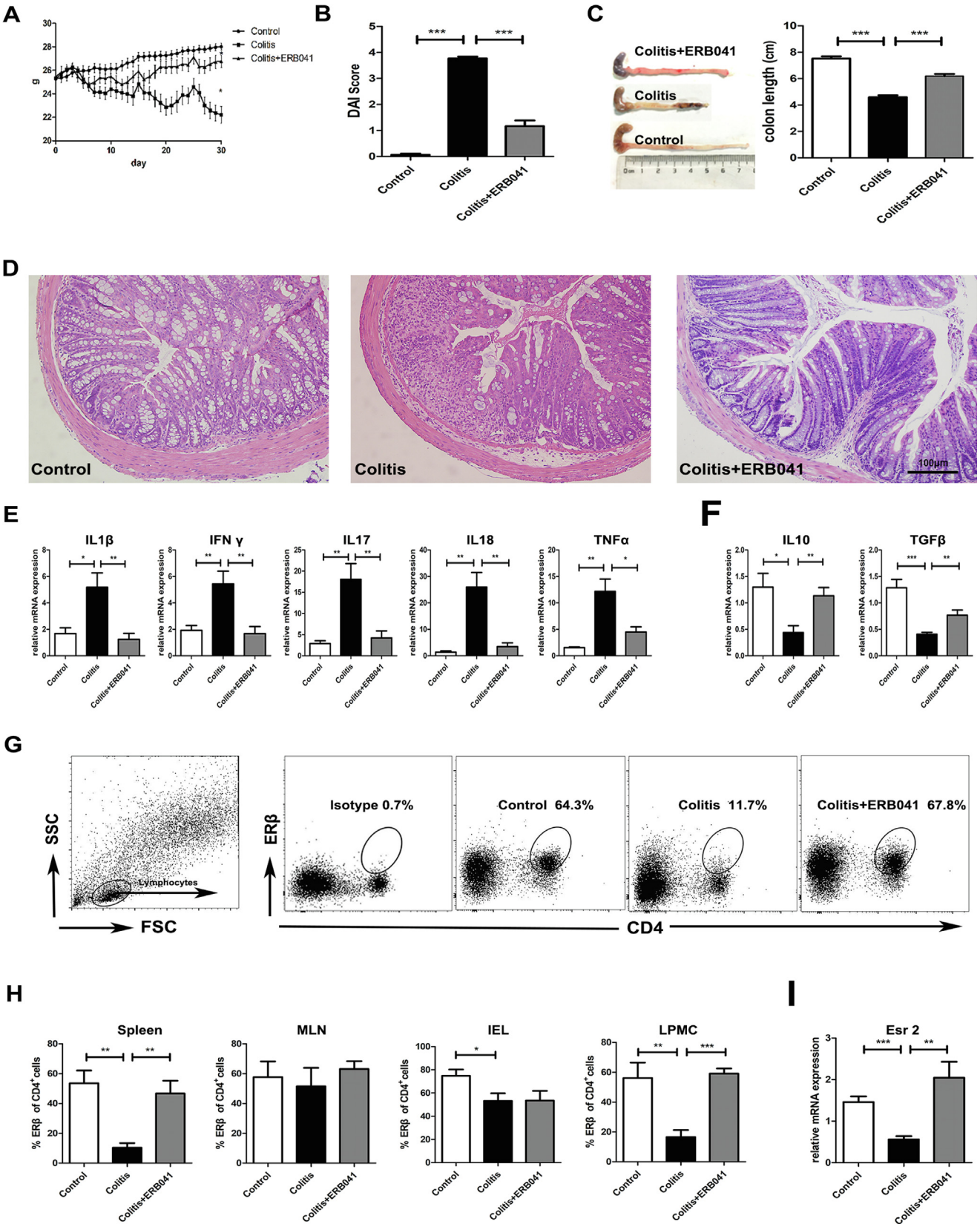

Fig. 3. ERβ agonist ERB041 ameliorates DSS-induced chronic colitis and inflammation in vivo. (A-C) Body weights (g) (A), DAI scores (B), and colon length (cm) (C) for individual groups of mice (n = 10 per group). (D) Pathological changes in the colons of mice (H&E staining, scale bar, 50 μm, magnification × 200). (E, F) Relative expression levels of proinflammatory mediators (E) and anti-inflammatory cytokines (F) determined by qRT-PCR using GAPDH as the control gene. Mouse splenic mononuclear cells (MNCs), mesenteric lymph node (MLN) cells, colonic intraepithelial lymphocytes (IEL), and lamina propria mononuclear cells (LPMC) were isolated from individual groups of mice, and the percentage of ERβ$^+$CD4$^+$ T cells was analyzed by flow cytometry (G) and the percentages (H) of ERβ$^+$CD4$^+$ T cells in the spleen, MLN, IEL and LPMC of different groups of mice. (I) Relative *Esr2* expression levels in different groups of mice determined by qRT-PCR using GAPDH as the control gene (n = 5 per group). Data are presented as representative images or expressed as the mean ± SEM for three separate experiments, *P < 0.05, **P < 0.01, ***P < 0.001.

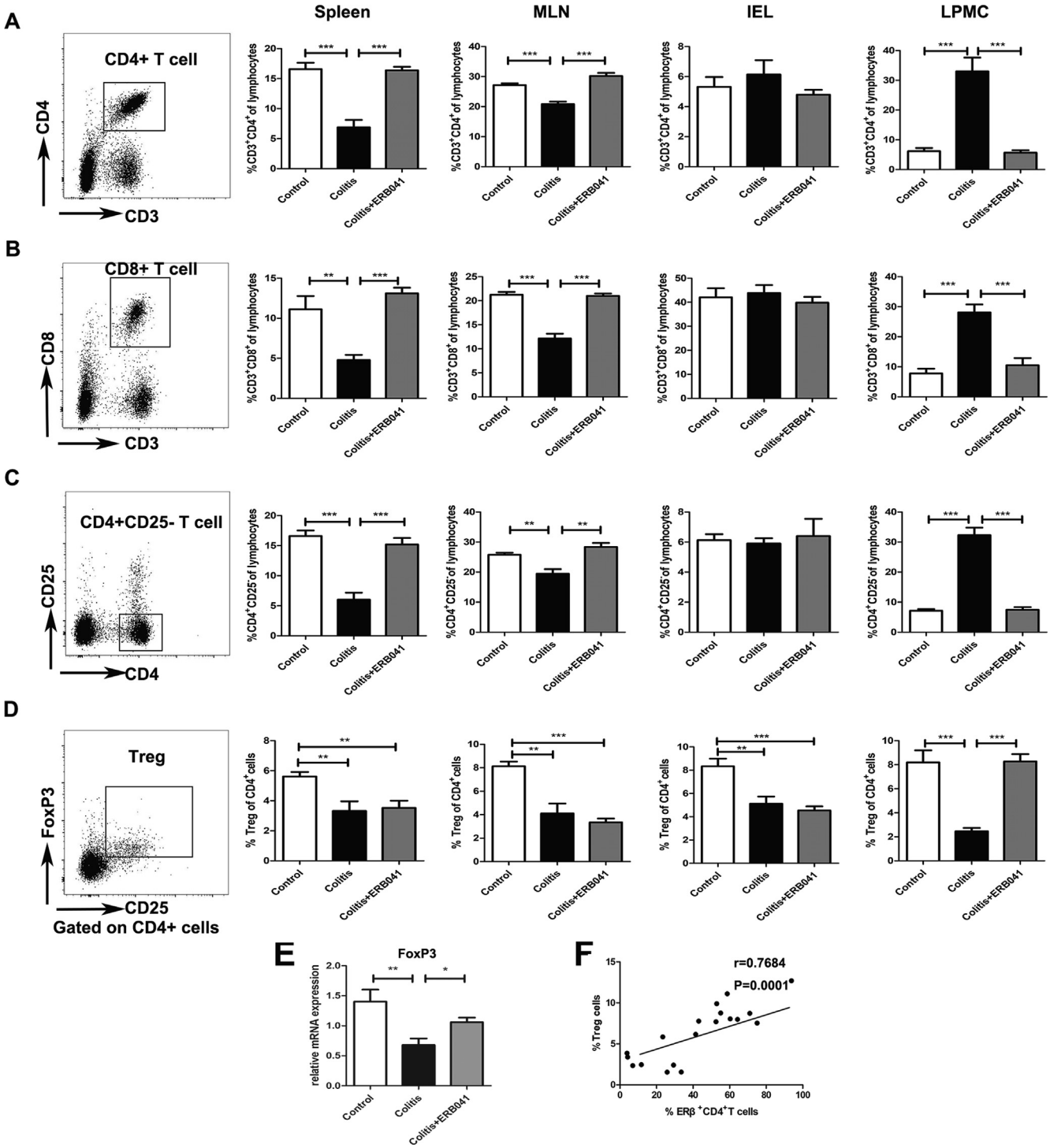

Fig. 4. ERB041 treatment inhibits $CD4^+CD25^-$ and $CD8^+$ T cell infiltration and restores the Treg quantity in the colon mucosal lamina propria. The percentages of $CD3^+CD4^+$ T cells (A), $CD3^+CD8^+$ T cells (B), and $CD4^+CD25^-$ T cells (C) in the spleen, MLN, IEL, and LPMC from individual groups of mice were analyzed by flow cytometry. (D) Flow cytometry analysis of the $CD4^+CD25^+FoxP3^+$ Treg in individual groups of mice. MNCs were gated in $CD4^+$ and the percentages of $CD25^+FoxP3^+$ Tregs were analyzed in the spleen, MLN, IEL, and LPMC from individual groups. (E) Relative *FoxP3* expression levels in the colons of individual groups of mice were determined by qRT-PCR using GAPDH as the control gene. (F) Correlation analysis between the frequency of Tregs and $ER\beta^+CD4^+$ T cells in the colon mucosa lamina propria of mice (n = 5 per group). Data are presented as representative images or expressed as the mean ± SEM for three separate experiments, *P < 0.05, **P < 0.01, ***P < 0.001.

$CD4^+CD25^+$ Tregs, $CD4^+CD25^-$ effector T cells and $CD8^+$ T cells were enriched from the spleen and MLN by MACS. The quality of the purified cells was > 94% (Fig. 6F). For the suppression assay, $CD4^+CD25^-$ T cells and $CD8^+$ T cells were cultured alone or co-cultured with Tregs followed by stimulation. We found that treatment with either ERB041 or PHTPP did not affect the proliferation of $CD4^+$ and $CD8^+$ T cells in the absence of Tregs (Fig. 6G-H). However, co-culture of the $CD4^+$ or $CD8^+$ T cells with Tregs inhibited their proliferation. Treatment with ERB041, but not PHTPP, further decreased the proliferation of these T cells (all P < 0.01). These data indicated that ERβ

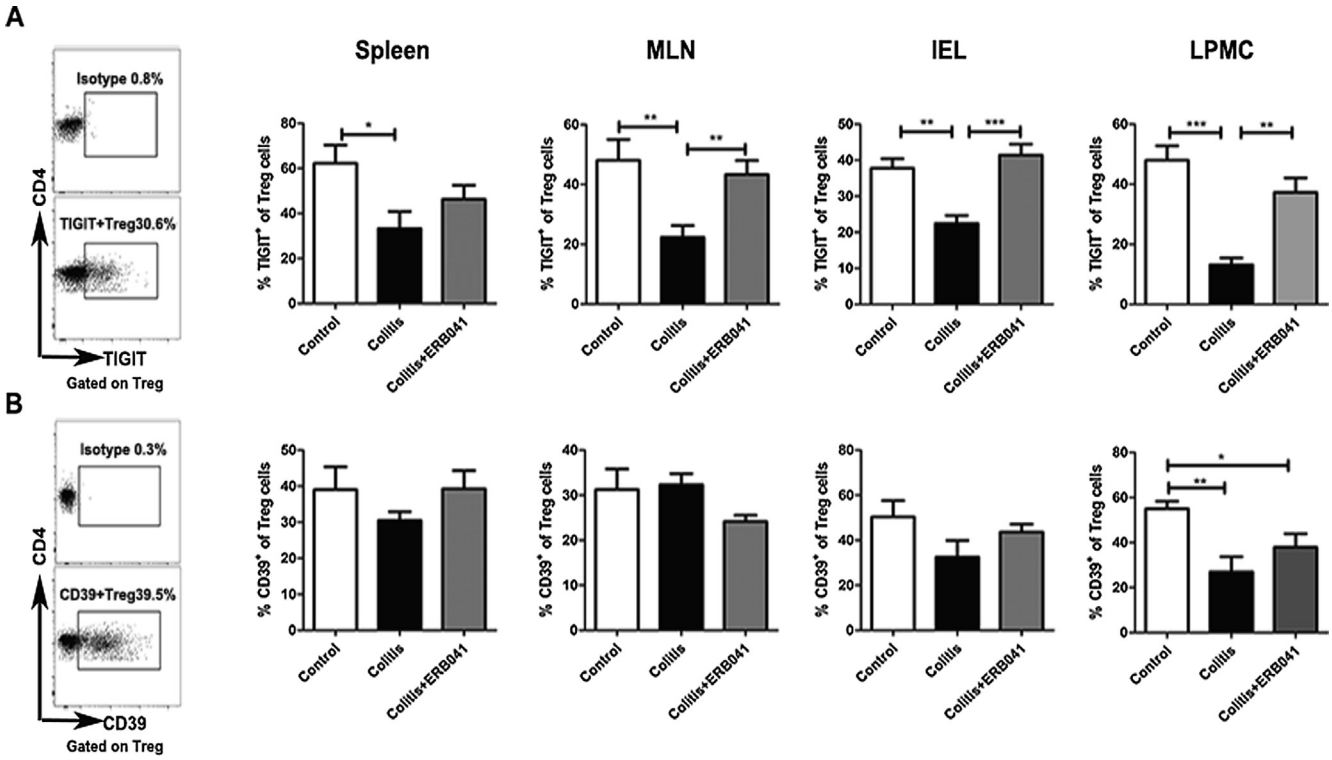

Fig. 5. ERB041 increases the frequency of activated Tregs in the colon mucosal lamina propria of mice. Representative flow cytometry analysis and the percentages of TIGIT$^+$ Tregs (A) and CD39$^+$ Tregs (B) in the spleen, MLN, IEL, and LPMC from individual groups of mice were calculated (n = 5 per group). CD4$^+$FoxP3$^+$ Tregs were gated in MNCs. TIGIT and CD39 expression on Tregs were analyzed by flow cytometry. Data are presented as representative images or expressed as the mean ± SEM for three separate experiments, *P < 0.05, **P < 0.01, ***P < 0.001.

activation enhanced the suppressive activity of Tregs against T effector cell proliferation in vitro.

4. Discussion

Reduced ERβ expression has been reported in colon biopsies from IBD patients [20]. Similarly, Maselli et al. [41] noted that intracellular ERβ expression was significantly lower in T cells from patients with systemic lupus erythematosus and was negatively correlated with disease activity. However, ERβ expression in CD4$^+$ T cells and its specific modulatory effects on pro-inflammatory and Treg responses in IBD remain poorly understood. In the current study, we demonstrated that the reduced frequency of ERβ$^+$CD4$^+$ T cells in UC and CD patients were inversely correlated with disease activity and severity, suggesting that quantifying these cells in patients may be valuable for evaluating the severity of IBD in the clinic.

Tregs play a key role in the maintenance of immune homeostasis and the prevention of IBD [7,42,43]. They suppress inflammation through immunoregulation (consumption or production of TGF-β1, IL-10, IL-35, and surface-bound enzymes, such as CD39 and CD73) or direct cytotoxic effects on antigen present cells (APCs) or Teffs by perforins and granzymes [7,44]. Immune suppression mediated by Tregs is crucial to the control of excessive inflammation [45,46], and Tregs efficiently ameliorate chronic colitis [47]. In addition, traditional IBD therapies, including aminosalicylates, glucocorticoids, and infliximab, can increase intestinal Tregs [7,48]. Our data indicated that ERβ and FoxP3 have a co-localized foundation. We next demonstrated that ERβ regulate Treg function during the progression of DSS-induced chronic colitis.

In this study, we found that activation of ERβ by its specific agonist ERB041 significantly mitigated the colitis-related pathological changes in the mouse colon and inhibited inflammation. Inhibition of pro-inflammatory T-cell infiltration and the production of inflammatory cytokines by ERB041 may be responsible for the inhibition of inducible colitis, which is consistent with previous reports [14,15,49]. Interestingly, ERB041 treatment significantly increased the relative expression levels of the anti-inflammatory IL-10 and TGFβ1 cytokine genes in the mouse colon. ERB041 treatment also restored the colitis-decreased ERβ$^+$CD4$^+$ T cell and Treg, as well as, FoxP3 expression in these mice. These data suggested that ERB041 treatment could modulate the imbalance between pathogenic and regulatory T cell responses in the colon of mice affected by colitis. In addition, ERB041 treatment significantly restored the frequency of TIGIT$^+$ Treg, implicating the activation of ERβ in the enhanced Treg activation observed in vivo. How did ERβ activation restore the intestinal Treg in mice? Firstly, natural FoxP3$^+$ Tregs are produced in the thymus. However, naïve T cells can also differentiate into FoxP3$^+$ Tregs (induced Tregs) in the periphery, especially in the intestinal mucosa of mice [50]. Induced Tregs can develop from naïve CD4$^+$ T cells in vitro upon antigen stimulation in the presence of an appropriate combination of cytokines, including TGF-β1 and IL-2 [51]. Interestingly, we found that ERB041 treatment promoted Treg differentiation and enhanced the growth factor-mediated differentiation of naïve T cells to Tregs in vitro, which were abrogated and dramatically mitigated by an ERβ-specific antagonist PHTPP. TGF-β1 can induce Treg differentiation by activating Smad signaling [37], and we also observed that ERβ activation increased Smad2/3 phosphorylation in CD4$^+$ T cells. These data suggest that ERβ activation may promote Treg differentiation by increasing the TGF-β1/Smad signaling in this model. Secondly, ERB041 eliminated the colitis-induced CD4$^+$CD25$^-$ and CD8$^+$ T cell infiltration in the lamina propria in vivo. We found that ERβ activation enhanced the suppressive activity of Tregs against the proliferation of CD4$^+$ and CD8$^+$ T cells in vitro. Thus, the activation of ERβ may initiate downstream signaling cross-talk with the TGF-β1-related Smad signaling pathway to promote FoxP3 expression and functional Treg development, leading to the inhibition of autoimmune inflammation. Therefore, our findings suggest a novel

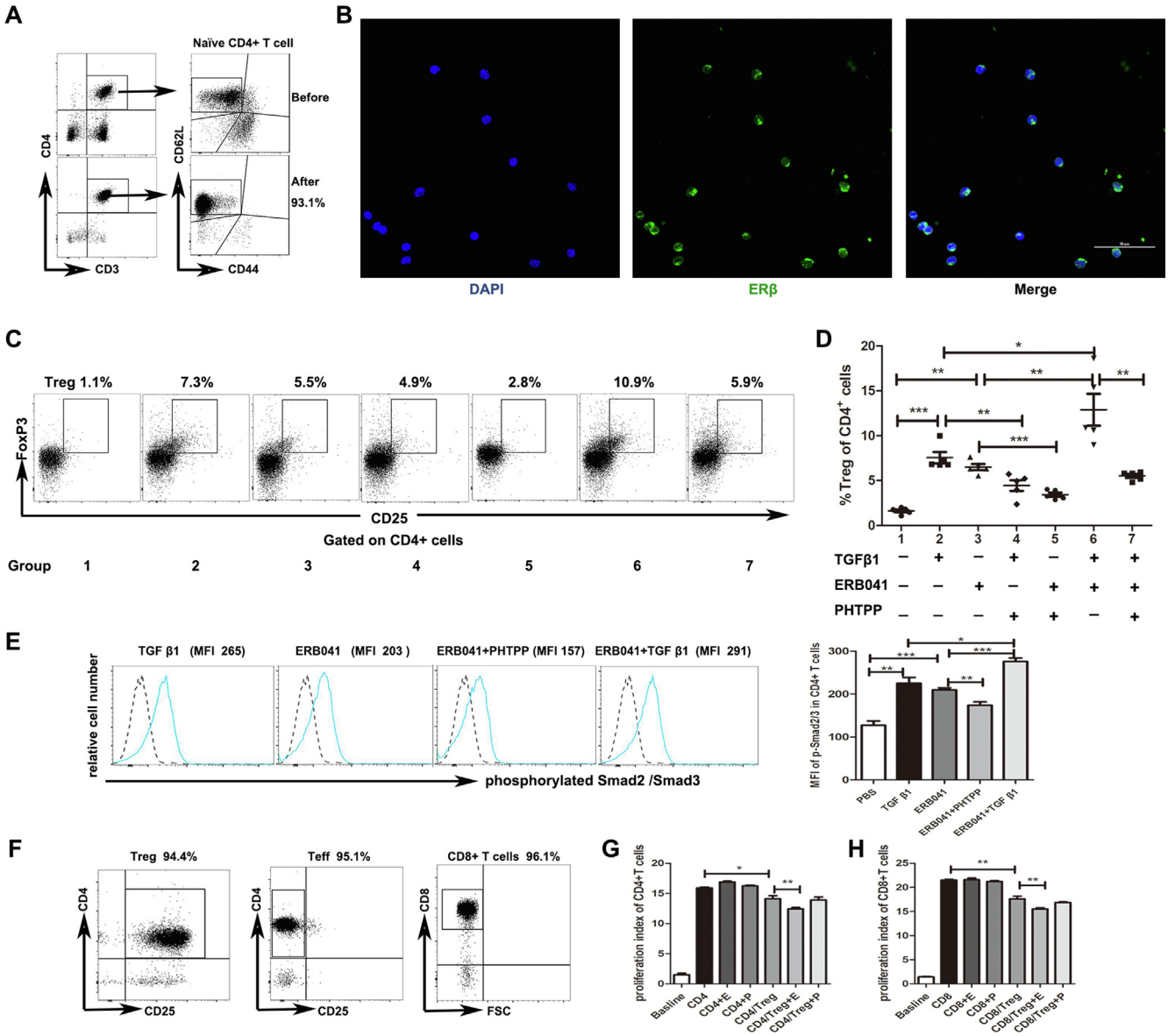

Fig. 6. ERβ activation enhances Treg differentiation and increases its activity to suppress T-cell proliferation in vitro. (A) The purity of $CD3^+CD4^+CD44^-CD62L^+$ naïve T cells before (top) and after (bottom) isolation. (B) ERβ expression (green) in naïve T cells by confocal microscopy. Cell nuclei were stained with DAPI (blue). Scale bar, 50 μm; original magnification × 600. Representative flow cytometric dot plots in different groups (C) and the percentage of Tregs (D) are presented. The cells were gated on CD4 + T cells and the percentage of $CD25^+FoxP3^+$ Tregs was analyzed. (E) Representative flow cytometry analysis of phosphorylated Smad2 (pS465/pS467)/Smad3 (pS423/pS425) levels in naïve $CD4^+$ T cells (left) treated with PBS (dashed line), 10 ng/mL TGF-β1, 100 nM ERB041 or 1000 nM PHTPP (solid line). Mean fluorescence intensity (MFI) of the Smad2/3 phosphorylation in naïve $CD4^+$ T cells are shown for each group (right). (F) The purity of $CD4^+CD25^+$ Tregs (left), $CD4^+CD25^-$ Teff (middle), and $CD8^+$ T (right) cells are presented. (G, H) Treg immunosuppression assay. $CD4^+CD25^-$ Teff and $CD8^+$ T cells were labeled with CSFE. The CSFE-labeled T cells were cultured alone or co-cultured with Tregs in the presence or absence of ERB041 (E) or PHTPP (P) for 72 h. The proliferation index for $CD4^+CD25^-$ Teff (G), and $CD8^+$ T (H) cells are shown. Data are presented as representative images or expressed as the mean ± SEM for three separate experiments, *P < 0.05, **P < 0.01, ***P < 0.001.

immunological explanation for why treatment with ERβ agonists can inhibit autoimmune inflammation in multiple types of autoimmune disease.

We recognize that our study has some limitations, such as its relatively small sample size and the lack of precise information about the $ERβ^+FoxP3^+$ Tregs in the patient intestinal biopsy samples due to the limited size of the specimens. In addition, understanding the function of the $ERβ^+FoxP3^+$ Tregs and the molecular mechanisms underlying the cross-talk between ERβ-related signaling and the cytokine-mediated Jak/Stat and Smad signaling pathways in different subsets of T cells in the context of colitis requires further study. We are currently interested in investigating whether ERβ activation can directly regulate the immunosuppressive actions of Tregs, such as the modulation of APC activity, secretion of anti-inflammatory cytokines, and the induction of Teff apoptosis [52–54]. Additional studies on larger patient populations are needed to understand the relationship between ERβ activation and the regulation of inflammation and the balance between autoreactive T-cell and Treg responses during the development and progression of IBD. Moreover, because the action of ERB041 is systemic and many kinds of cells and tissues express ERβ, future research needs to include other cell types to fully understand the therapeutic effects of ERβ activation against IBD.

In conclusion, our study demonstrated that reduced frequency of circulating $ERβ^+CD4^+$ T cells was inversely correlated with disease

severity in UC and CD patients. Accordingly, it may be a valuable biomarker for evaluating the severity of IBD. Activation of ERβ is protective against DSS-induced chronic colitis, and this effect may be achieved, at least in part, by inhibiting the production of inflammatory cytokines, suppressing pathogenic T cell responses, and enhancing the Treg response. Our data suggest that ERβ may be a novel therapeutic target for IBD.

Declaration of Competing Interest

All authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105971.

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