**Roseburia intestinalis** stimulates TLR5-dependent intestinal immunity against Crohn’s disease

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**Summary**

**Background** Crohn’s disease (CD) is a chronic inflammatory disorder characterized by intestinal immune dysfunction. Multiple factors, including gut dysbiosis, are involved in the pathogenesis of CD. However, the effect of commensal bacteria on controlling the inflammatory response in individuals with CD remains unclear.

**Methods** We detected Toll-like receptor 2 (TLR2), TLR4, and TLR5 expression in *Roseburia intestinalis* (*R. intestinalis*)-treated mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. Then, we quantified the signs of colonic inflammation, the proportion of regulatory T cells (Tregs) and the expression of thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-β in TLR-5-deficient (*Tlr5*−/−) mice, bone marrow chimera mice (generated using wild-type (WT) and *Tlr5*−/− mice), and anti-TSLP/anti-TGFβ-treated C57BL/6 mice with colitis induced by TNBS. In vitro, we used the lipopolysaccharide (LPS)-stimulated human intestinal epithelial cell line Caco-2 as an inflammatory colon cell model treated with or without the TLR5-siRNA intervention in the presence of *R. intestinalis* and incubated human monocyte-derived dendritic cells (DCs) with the supernatant of Caco-2 cells. Then, we cocultured human CD4+ T cells with the aforementioned DCs to determine the differentiation of Tregs. Additionally, samples from patients with CD were collected to analyse the correlation between TLR5/TSLP/TGF-β expression and the percentage of *R. intestinalis*.

**Findings** Here, we show that *R. intestinalis* inhibits the development of CD by increasing the differentiation of anti-inflammatory Tregs. Mechanistically, *R. intestinalis* stimulates TSLP production in intestinal epithelial cells (IECs) through TLR5 but not TLR2 or TLR4. TSLP produced by IECs specifically induces the secretion of interleukin-10 (IL-10) and TGF-β from DCs, which is necessary for subsequent Treg differentiation. Consequently, the depletion of TLR5 (using *Tlr5*−/− mice) or inhibition of TSLP (using anti-TSLP neutralizing antibodies) attenuates the protective effect of *R. intestinalis* on experimental colitis in mice. Importantly, the expression of TSLP in patients with CD is positively correlated with the level of *R. intestinalis*.

**Interpretation** These findings reveal the previously unknown mechanism of *R. intestinalis*-mediated intestinal immune regulation, which may provide the basis for new therapeutic strategies for CD.

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**Keywords:** Crohn’s disease; *R. intestinalis*; Toll-like receptor 5; Tregs; TSLP

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**Introduction**

Inflammatory bowel disease (IBD), which primarily includes ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic and recurrent intestinal inflammatory disease. The increased incidence of IBD is related to urbanization and a shift towards a Westernized diet. Although the pathogenesis of IBD is not completely...
Commensal bacteria are essential for controlling the inflammatory response in patients with CD. We previously showed that the abundance of *R. intestinalis* decreased significantly in patients with CD compared with healthy volunteers. *R. intestinalis* inhibits the development of experimental colitis by promoting Treg differentiation.

**Added value of this study**

Our data add novel evidence that *R. intestinalis*-mediated intestinal immune regulation resists CD. Specifically, we show that *R. intestinalis*-mediated alleviation of colitis inflammation depends on TLR5. TLR5 expression on IECs activates DCs and promotes DC-induced Treg cell differentiation. The cytokine TSLP secreted by IECs suppresses colitis pathogenesis.

**Implications of all the available evidence**

In this paper, we identified that stimulation of the TLR5-dependent intestinal immune response by *R. intestinalis* contributes to DC-induced Treg differentiation. These findings further improve our understanding of the amelioration of colitis by *R. intestinalis* in individuals with CD, which may provide profound insights into host-microbe interactions and novel treatment strategies for CD.

**Methods**

**Human samples and ethical statement**

The collection of human samples was approved by the local Ethics Committee and the Review Board of Third Xiangya Hospital of Central South University (Changsha, China) (No. 2016-S094). CD was diagnosed through the combined analysis of clinical manifestations and pathological, endoscopic and imaging examinations by experienced clinical doctors according to published guidelines. All volunteers provided informed consent for the collection of peripheral blood, faeces, and paraffin-embedded colon sections. The basic information of all participants, including age, sex and disease location, is shown in Table S1.

**Animals and ethical statement**

Six-week-old male C57BL/6 (B6 WT) mice were acquired from SJA Laboratory Animal Co., Ltd. (Hunan, China) and raised under specific pathogen-free (SPF) conditions. Breeder pairs of *Tlr5<sup>−/−</sup>* mice were donated by the Collaborative Innovation Center of Model Animals at Wuhan University (Wuhan, China) and housed under SPF conditions at SJA Laboratory Animal Co., Ltd. The *Tlr5<sup>−/−</sup>* mice were maintained under SPF conditions and their care and handling were in accordance with the ARRIVE guidelines and the guidelines of the National Animal Research Facility of China. All animal experiments were conducted under a protocol approved by the local Ethics Committee of the SJA Laboratory Animal Co., Ltd. (Hunan, China) and conformed to all guidelines of the Chinese Government.
mice were on a C57BL/6 genetic background. Approval of all processes for animal experiments was provided by the Ethical Committee of Medical Research of SJA Laboratory Animal Company (No. 20170047) and Central South University (No. 2018-S092). All animal experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.16

Bacterial strains

A bacterial strain (Roseburia intestinalis DSMZ-14610 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)) was cultured with lytic anaerobic medium (BD Diagnostics, 510794) as previously described.10 For in vivo experiments, R. intestinalis was washed three times and resuspended in saline, and mice were administered an R. intestinalis suspension (at 1 × 10^9 CFUs/100 μl). For in vitro studies, R. intestinalis was washed three times and resuspended (at 1 × 10^9 CFUs/ml) in RPMI-1640 (HyClone, SH3025501).10

Experimental colitis

For the induction of colitis, mice were treated with 150 μl of presensitization solution (5% 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma-Aldrich, P2297) mixed with a mixture of acetone/olive oil [4:1 v/v]) or with the acetone/olive oil mixture alone through the shaved dorsal skin (day 1).10 Then, mice were anaesthetized with isoflurane before the intrarectal administration of 100 μl of the TNBS solution [5% TNBS and 50% ethanol (1:1 v/v)] or 50% ethanol (days 8 and 10).17 The control group was administered normal saline (100 μl), while the R. The 1 group was intragastrically administered the bacterial suspension (100 μl) (day 12 to day 14). Mice were euthanized on day 15 to collect tissue samples.

In vivo neutralization of TSLP was performed as described previously with certain modifications; briefly, C57BL/6J mice were intraperitoneally injected with 500 μg of TSLP-neutralizing antibody (R&D Systems, MAB335-500) every three days, a total of two injections, starting 1 h before TNBS administration.8,10,13

For in vivo neutralization of TGFβ, C57BL/6J mice received intraperitoneal injections of the TGFβ neutralizing antibody (R&D Systems, MAB1835-500) every three days, a total of two injections, starting 1 h before TNBS administration.5,9,19

Bone marrow transplantation (BMT)

Six-week-old male mice were randomly grouped into donors or recipients. Recipient mice were treated with 2 mg/ml neomycin in water 1 week prior to irradiation. All recipient mice received irradiation with 2 doses of 5 Gy (gamma rays) at 4-h intervals (dose rate 1.0 Gy/min). Irradiation was performed by a technician from the Radiology Department of Central South University Third Xiangya Hospital. Within 24 h after irradiation, bone marrow (BM) cells from the femur, pelvis and tibia from the hind leg of donors were prepared, and the recipient mice were transplanted with 5,000,000 live BM cells through the lateral tail vein. The chimeric mice were maintained on water supplemented with neomycin for 2 weeks. After antibiotic treatment, the chimeric mice were standardized by exposure to the microbiota of non-BMT mice; namely, WT or Tlr5−/− mice were cohoused in the same cage with non-BMT WT or Tlr5−/− mice. Peripheral blood was analysed using flow cytometry to examine the reconstitution efficiency at least 6 weeks after BMT. Experimental colitis was induced in all reconstituted mice after 6 weeks of recovery from transplantation.

The reconstituted mice were administered the TNBS presensitization solution through the cutaneous route, treated with the TNBS solution twice via the rectum, and R. intestinalis treatment was administered by gavage for 3 days. The mice were monitored for 14 days after the first skin exposure to TNBS. Peripheral blood and colon tissues were collected for histological and expression analyses.21,22

Disease activity index (DAI)

Loss of body weight, characteristics of faeces, and the occurrence of haematochezia were observed during the modelling period. The severity of the induced colitis was assessed by calculating the DAI (Table S2), as described previously.12

Histology

Haematoxylin and eosin (H&E) staining was performed using standard procedures. For H&E detection, washed colon tissues were immersed in a 4% formaldehyde solution, embedded in paraffin and sliced, followed by H&E staining. The sections were evaluated independently by two pathologists who were blinded to the groups of experiments using published criteria14 (Table S4). For IHC detection, a series of consecutive sections was soaked in xylene and ethanol for deparaffinization and hydration, respectively, and then treated with sodium citrate buffer. Next, the sections were incubated with the following primary antibodies: rabbit polyclonal anti-TLR5 (1:200, Proteintech, 19810-1-AP, RRID: AB_2909813), rabbit polyclonal anti-TSLP (1:250, Abcam, ab188766, RRID: AB_2894705), rabbit polyclonal anti-IL-10 (1:200, Proteintech, 20850-1-AP, RRID: AB_10699876), rabbit polyclonal anti-TLR5 (1:200, Proteintech, 19810-1-AP, RRID: AB_2909813). The sections were incubated with the secondary antibody (Mai New Biotechnology Development Company, KIT 9710) according to the manufacturer’s instructions. The sections were evaluated by two independent pathologists who were blinded to the
experimental intervention under a light microscope (DP72; Olympus Corporation), with at least 5 high-powered fields at ×200 or ×400 counted per sample.

**Enzyme-linked immunosorbent assay (ELISA)**

Samples, including peripheral blood serum, tissue homogenates or cultured cell supernatants, were collected for quantitative ELISAs of TSLP (R&D Systems, DTSLP0 for human, MTLPOO for mouse), TGFβ (R&D Systems, SB100B) and IL-10 levels (eBioscience, 88-7106 for human, 88-7105 for mouse) according to the manufacturer’s instructions.

**Reverse transcription polymerase chain reaction (RT–PCR)**

RNA was isolated from purified cells or colon tissues using TRIzol (Invitrogen, 15596026) according to the manufacturer’s protocol. Reverse transcription was performed using a cDNA synthesis kit (Thermo Fisher Scientific, K1652) according to the manufacturer’s instructions. RT–PCR amplification using SYBR Green qPCR master mix (Vazyme, Q311-02) was performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The primer sequences are described in Table S3.

**Quantitative real-time polymerase chain reaction (qPCR) of R. intestinalis**

DNA was extracted from human faecal samples using a genomic DNA extraction kit (Tsingke, TSP201). The qPCR analysis of *R. intestinalis* 16S rRNA was performed as described previously.12 Detection was performed using SYBR Green PCR master mix (Vazyme, Q311-02) and a real-time PCR system (Bio-Rad Laboratories). The copy number was calculated from 10-fold serial dilutions compared with the standard. The primer sequences are shown in Table S3.

**Flow cytometry**

Collected cells were incubated with a Zombie UV viability staining kit (BioLegend, 423101) to separate live cells, followed by an incubation with the following human or mouse conjugated antibodies: CD4 (FITC conjugate; BioLegend, 100406, RRID: AB_312691), CD25 (APC conjugate; BioLegend, 101910, RRID: AB_2280288), FOXP3 (PE conjugate; BioLegend, 126404, RRID: AB_1089117), CD45 (APC/Cy7 conjugate; BioLegend, 109823, RRID: AB_310788), CD3 (PE conjugate; BioLegend, 100205, RRID: AB_312662), CD12 (FITC conjugate; BioLegend, 101205, RRID: AB_312788), and CD19 (PE conjugate; BioLegend, 152408, RRID: AB_2629817). Experiments were performed using a FACS Aria II flow cytometer (BD Biosciences). Data were analysed using FlowJo 7.0 FACS software (FlowJo).

**Western blotting**

Proteins were extracted from colon tissues or cells using lysis buffer containing complete protease inhibitor. A BCA assay kit (Thermo Fisher Scientific, 23227) was used to determine the protein concentration. Thirty micrograms of processed sample (1 μg/ml) were subjected to SDS–PAGE and immunoblotting. The primary antibodies used for immunoblot analyses were rabbit monoclonal anti-TLR2 (1:500, Abcam, ab209217, RRID: AB_2814691), rabbit monoclonal anti-TLR4 (1:500, Cell Signaling Technology, 14318, RRID: AB_2798460), and rabbit polyclonal anti-TLR5 (1:500, Thermo Fisher Scientific, PAI-4139, RRID: AB_2205279). Subsequently, a goat anti-rabbit-HRP antibody (1:5000, Proteintech, SA00001-2, RRID: AB_2722564) was used as the corresponding secondary antibody, and then protein levels were quantified using an enhanced chemiluminescence reaction (Bio-Rad Laboratories).

**In vitro model of inflammation**

Caco-2 cells (ATCC HTB-37, RRID: CVCL_0025) were cultivated with RPMI-1640 (HyClone, SH3025501) containing 10% FBS (Biological Industries, 04-001-1ACS) with 5% CO2 at 37°C. For the induction of the in vitro model of inflammation, Caco-2 cells were stimulated with lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich, L2630) for 24 h. Then, the LPS-induced Caco-2 cells were treated with *R. intestinalis* for 24 h. The supernatant of the Caco-2 cells was centrifuged, isolated, and stored at −80°C to measure the cytokine levels using ELISAs or used to stimulate DCs in subsequent cell-based experiments.

**Primary cell culture**

For the isolation and cultivation of DCs, human peripheral blood mononuclear cells (PBMCs) were extracted from healthy participants and seeded. After 2 h, the...
suspended cells were carefully removed by washing, and recombinant human granulocyte-macrophages (GM-CSF) (1,000 U/ml, PeproTech, 300-03) and recombinant human interleukin 4 (IL-4) (500 U/ml, PeproTech, 200-04) were added to the adherent cells. On the 6th day of induction culture, recombinant human tumour necrosis factor (10 ng/ml, PeproTech, 300-01A), IL-1β (10 ng/ml, PeproTech, 200-01B), IL-6 (1000 U/ml, PeproTech, 200-06) and prostaglandin E2 (1 µg/ml, PeproTech, 3632464) were used to induce the maturation of DCs. On days 7–8 of induction culture, mature DCs were harvested. Flow cytometry was performed to detect the expression of CD80 (FITC conjugate; BioLegend, 305205, RRID: AB_314501), CD83 (PE conjugate; BioLegend, 305307, RRID: AB_314515) and CD86 (APC conjugate; BioLegend, 374207, RRID: AB_2721448).

Human PBMCs were separated, and a Naive CD4+ T Cell Isolation Kit (Miltenyi Biotec, 130-091-894) was used to sort and culture the initial T cells.

The DCs described above were cocultured with initial T cells at a 1:1 ratio for 48 h. The proportion of Tregs used to sort and culture the initial T cells. Cell Isolation Kit (Miltenyi Biotec, 130-091-894) was

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**SiRNA transfection**

Caco-2 cells were suspended and seeded in a six-well plate at a density of 5 × 10^5 cells/well. After 12–24 h of culture, a mixture of transfection reagent and TLR5-siRNA was prepared. The whole experimental process was performed strictly in accordance with the instructions provided with the Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) transfection reagent. Cells were transfected with Silencer Select siRNA against TLR5 (SANTA CRUZ, siRNA ID—sc-40262) or a Silencer Select Negative siRNA.

**Treatment with recombinant TSLP and TGFβ proteins in vitro**

Human colon cells were treated with 100 ng/ml recombinant TSLP (R&D Systems, 1398-TS) or 1 ng/ml recombinant TGFβ (R&D Systems, 240-B) for 24 h to inhibit the expression of TSLP and TGFβ, and the experiments were subsequently performed.

**Fluorescence in situ hybridization (FISH)**

For the FISH analysis, FAM (488)-tagged 5'-TCA-GACTTGGCYYACCGC-3' was applied to detect the presence of R. intestinalis, and the procedure was conducted as previously described. Briefly, serial paraffin sections were treated with xylene and ethanol solutions for deparaffinization and rehydration, respectively. The sections were treated with freshly prepared 4% paraformaldehyde and lysozyme in Tris–HCl (pH 6.5). Subsequently, sections were hybridized with hybridization buffer (0.9 mol NaCl and 20 mmol Tris–HCl, 0.02% SDS and formamide), followed by FISH probes (30 mg/l). Then, each section was washed with washing buffer (0.9 mol NaCl and 20 mmol Tris–HCl). Finally, sections were mounted with DAPI and air dried. All FISH sections were observed using a fluorescence microscope at ×100 and ×200 magnification and were analysed with CaseViewer image processing software (JAVS).

**Intestinal permeability detection**

Fluorescein-labelled FITC-dextran (Sigma, 46944) was administered by gavage according to the body weight of the mice (400 µg/g). The mice were sacrificed 4 h later to collect blood plasma and detect plasma levels of FITC-dextran.

**Mice and AAV delivery**

Six-week-old male Villin-cre mice (purchased from Cyagen Biosciences, Co01243) were used. AAV9 targeting Cre recombinase TLR5-Flox-AAV (AAV9-RFP-DIO-CMV-GFP-shRNA, 8.81E13 v.g./ml) and Control AAV (AAV9-RFP-DIO-CMV-GFP-shRNA, 6.06 E13 v. g./ml) was ordered from Venegen (Jinan, China).

Before TNBS induction, 1 × 10^12 particles of TLR5-Flox-AAV or Control-AAV in 0.15 ml of saline were delivered to Villin-cre mice by enema once and then supplemented with 1 × 10^11 AAV in 0.2 ml of saline by tail vein injection two days later to downregulate TLR5 expression in IECs. Then, the mice were subjected to the TNBS intervention 1 week after the first AAV administration.

**Ethical approval and consent to participate**

This study was approved by the Ethics Committee and the Review Board of Third Xiangya Hospital of Central South University, and informed consent was obtained from all participants. All animal studies adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Statistical analysis**

Significant differences were assessed using GraphPad Prism 8.0 software (GraphPad Software). The Kruskal-Wallis test with Dunn’s multiple comparisons test were applied to compare single variables among three or more groups. The Mann–Whitney U test was used to compare single variables between two groups. Spearman’s correlation analysis was performed to evaluate the correlations of two variables. Other details are shown in the figure legends. Data are presented as individual points and the means ± standard errors of the means. P < 0.05 was regarded as statistically significant.

**Role of funders**

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Results

TLR5 is required for R. intestinalis-induced Treg differentiation

TLRs, particularly TLR2, TLR4 and TLR5, are essential for regulating the interaction between the intestinal microbiota and intestinal mucosal immunity.30,31 Therefore, we explored the expression of TLR2, TLR4, and TLR5 in the colon tissues of mice treated with TNBS and R. intestinalis. Administration of R. intestinalis prevented the TNBS-induced downregulation of TLR5. In contrast, the expression of TLR2 and TLR4 was not increased by R. intestinalis. Based on these results, TLR5 is involved in mediating the effects of R. intestinalis (Figure 1a-b). Previously, we showed that R. intestinalis increases the proportion of Tregs in the colon and inhibits colitis in WT mice.10 We investigated whether TLR5 plays a key role in R. intestinalis-induced Treg differentiation by treating Tlr5+/− mice with TNBS to induce colitis and then administered R. intestinalis. We identified the absolute absence of TLR5 in Tlr5−/− mice (Figure S1a-d) and normal gut barrier function (Figure S1e-f) compared with WT mice before the overall experiment, and no signs of major organ injury were observed in Tlr5−/− (control) mice (Figure S1g-k). Compared to Tlr5+/− (TNBS) mice, Tlr5−/− (TNBS + R. I) mice showed indistinguishable body weight changes and DAI scores, which indicated epithelial damage and leukocyte infiltration, suggesting that TLR5 is required for the R. intestinalis-induced inhibition of colon inflammation (Figure 1c-f). Consistently, the Tlr5−/− (TNBS + R. l) mice showed no increase in the differentiation of Tregs or the colonization of R. intestinalis compared with Tlr5+/− (TNBS) mice (Figures 1g-h and S1 i-m).

Thus, R. intestinalis-induced Treg differentiation requires TLR5 expression.

IECs expressing TLR5 play a major role in R. intestinalis-mediated inhibition of inflammation in vivo

TLR5 is widely expressed in nonimmune cells (e.g., IECs) and immune cells (e.g., DCs and macrophages).15 We generated chimeric mice to determine which cell component expressing TLR5 is required for the R. intestinalis-mediated inhibition of inflammation in vivo. We lethally irradiated WT and Tlr5−/− mice that were treated with the antibiotic neomycin for 1 week before irradiation. The use of neomycin before and after BMT prevented most bacterial contamination, as previously reported.20,21 Then, we transferred bone marrow (BM) from the WT and Tlr5−/− donors to generate WT mice containing immune cells deficient in TLR5 (Tlr5−/− → WT) and control mice (WT → WT). We also generated Tlr5−/− mice expressing TLR5 only in immune cells (WT→ Tlr5−/− → WT) and control mice (Tlr5−/−→ Tlr5−/−) (Figure 2a). After BMT, the mice received antibiotics for another 2 weeks and were then exposed to the SPF microbiome environment for 4 weeks to avoid antibiotic-induced dysbiosis. Accordingly, we analysed the peripheral blood cells of the chimeric mice using flow cytometry to confirm effective immune reconstitution20 (Figure S2a-b). Flow cytometry combined with RT–PCR analysis were used to identify the absence of TLR5 expression in the colon of WT→ Tlr5−/− mice and the presence of TLR5 expression in the colon of Tlr5−/−→ WT mice (Figure S2c-d). Subsequently, the chimeric mice were sequentially treated with TNBS and R. intestinalis (Figure 2a).

The effects of R. intestinalis on TNBS-induced colitis in chimeric mice were investigated. The WT→WT (TNBS + R. l), WT→Tlr5−/− (TNBS + R. l) and Tlr5−/−→WT (TNBS + R. l) mice showed an increased body weight and decreased DAI scores compared with WT→WT (TNBS), WT→Tlr5−/− (TNBS) or Tlr5−/−→WT (TNBS) mice, while Tlr5−/−→Tlr5−/− (TNBS + R. l) mice did not show changes in body weight or DAI compared with Tlr5−/−→ Tlr5−/− (TNBS) mice. The WT→WT (TNBS + R. l) mice showed decreased DAI scores and similar body weight changes compared with the WT→Tlr5−/− (TNBS + R. l) mice, and showed similar changes in both body weight and DAI scores compared with Tlr5−/−→WT (TNBS + R. l) mice. The WT→Tlr5−/− (TNBS + R. l) and Tlr5−/−→WT (TNBS + R. l) mice both displayed significantly greater body weight and lower DAI scores than the Tlr5−/−→Tlr5−/− (TNBS + R. l) mice (Figure 2b-c). These results indicate that nonimmune cells (IECs) expressing TLR5 play a major role in reducing the changes in body weight and DAI score induced by R. intestinalis and that immune cells may be involved in the process. The histological analysis of colon sections showed no or minimal signs of colitis and colon damage in the WT→WT (TNBS + R. l) and Tlr5−/−→WT (TNBS + R. l) mice compared with WT→WT (TNBS) or Tlr5−/−→WT (TNBS) mice, respectively, whereas WT→Tlr5−/− (TNBS + R. l) and Tlr5−/−→Tlr5−/− (TNBS + R. l) mice showed no obvious relief of mucosal injury, crypt damage or inflammatory cell infiltration and exhibited decreased histopathological scores compared with the WT→Tlr5−/− (TNBS) mice or Tlr5−/−→Tlr5−/− (TNBS) mice (Figure 2d-e). Therefore, pathological colonic inflammation was only relieved in the WT→WT (TNBS + R. l) and Tlr5−/−→WT (TNBS + R. l) mice, which revealed the indispensable role of IECs expressing TLR5 in inhibiting colitis. The colon shortening (Figure S2e) and the colonization of R. intestinalis (Figure S2f-g) corresponded to the severity of colon inflammation. Consistent with the histological findings, the proportion of Tregs showed an obvious increase in the WT→WT (TNBS + R. l) and Tlr5−/−→WT (TNBS + R. l) mice compared with WT→WT (TNBS) or Tlr5−/−→WT (TNBS) mice, respectively, while WT→Tlr5−/− (TNBS + R. l) or Tlr5−/−→Tlr5−/− (TNBS + R. l) mice did not show a higher proportion of Tregs than the WT→Tlr5−/− (TNBS) or Tlr5−/−→Tlr5−/− (TNBS + R. l) mice (Figure 2f-g). Taken together, immune...
**Figure 1.** *R. intestinalis*-induced differentiation of Tregs requires TLR5 expression in vivo.
TLR5 is required for \textit{R. intestinalis}-induced expression of TSLP and TGFβ

We hypothesized that TLR5 signalling in IECs stimulates the production of cytokines, thereby inhibiting intestinal inflammation and driving \textit{R. intestinalis}-mediated and DC-induced Treg differentiation. Studies have shown that TSLP secreted by IECs activates DCs, thereby promoting the differentiation of Tregs and the subsequent immune tolerance of the intestinal mucosa.\textsuperscript{14-35} Furthermore, IECs stimulated with the microbiota or its components also express TGFβ and IL-6 through TLR activation.\textsuperscript{36,37} Therefore, we measured the expression of cytokines in LPS-induced Caco-2 cells after \textit{R. intestinalis} treatment. TSLP and TGFβ were upregulated, and IL-6 levels were not obviously changed compared to that of the controls (Figure S4a).

Next, we used specific siRNAs to suppress the expression of TLR5 in Caco-2 cells in vitro (Figure S4b), and the results showed that \textit{R. intestinalis} required TLR5 to induce the secretion of TSLP and TGFβ in Caco-2 cells (Figure 3a). We analysed the expression or concentration of TSLP and TGFβ in colon tissues (Figures 3b-c and S3a-d) and plasma (Figure 3d-e) from the chimeric mice in vivo. IL-10, an anti-inflammatory cytokine, was also detected, and its levels showed a trend similar to that of TGFβ and TSLP levels (Figs. S5c-h). Similar to the in vitro tests, these in vivo studies confirmed that the upregulation of TGFβ and TSLP expression was mainly induced by IECs (nonhaematopoietic cells) expressing TLR5.

IEC-mediated DC activation is required for \textit{R. intestinalis}-induced Treg differentiation in vitro

In the intrinsic lamina propria, various types of myeloid and lymphoid cells shape an integrated intestinal immune response through cytokine production or cell–cell contact. Intestinal mucosal DCs, which produce immune factors, promote the development and differentiation of Tregs.\textsuperscript{38} The signal from IECs activates DCs, which produce Tregs in mesenteric lymph nodes.\textsuperscript{39} Researchers have not clearly determined whether either or both IECs and DCs are involved in \textit{R. intestinalis}-induced Treg differentiation.

We determined whether IEC and DC activation were necessary for the regulation of \textit{R. intestinalis}-induced Treg differentiation using the LPS-stimulated human intestinal epithelial cell line Caco-2 as an inflammatory cell model.\textsuperscript{10,25} In the presence or absence of \textit{R. intestinalis}, the supernatant from Caco-2 cells treated with LPS was used to stimulate human monocyte-derived DCs, and naive CD4+ T cells were incubated with DCs treated with the supernatant as described above to examine the differentiation of Tregs (Figure 4a). Caco-2 cells treated with LPS alone and \textit{R. intestinalis} were used as controls. The supernatant of Caco-2 cells treated with LPS inhibited DC-induced Treg differentiation, while the supernatant of Caco-2 cells treated with LPS plus \textit{R. intestinalis} significantly reversed the reduction in DC-induced Treg differentiation. Notably, \textit{R. intestinalis} treatment without IECs or without DCs failed to increase the differentiation of Tregs (Figure 4b-c). Moreover, \textit{R. intestinalis} or LPS alone failed to upregulate DC production of IL-10 and TGFβ (Figure 4d). In contrast, the supernatant collected from Caco-2 cells treated with LPS plus \textit{R. intestinalis} significantly upregulated the expression of IL-10 and TGFβ in DCs, and the concentration of TGFβ in the DC supernatant increased more than 1000 times compared with that in the IEC.
Figure 2. TLR5-expressing IECs are required for _R. intestinalis_-mediated amelioration of colitis in TNBS-induced mice.
supernatant (Figure 4d), indicating a role for R. intestinalis in the regulation of LPS-induced immune mediator production in IECs, controlling subsequent DC activation. Therefore, these results indicate that both DC activation and subsequent DC-induced Treg differentiation require the cytokines produced by R. intestinalis-activated IECs (but not R. intestinalis alone).

We first suppressed TLR5 expression in Caco-2 cells with specific siRNAs to examine whether TLR5 has a direct role in modulating R. intestinalis-induced Treg differentiation. Indeed, R. intestinalis-upregulated expression of TLR5 in LPS-stimulated Caco-2 cells was blocked following TLR5-siRNA transfection (Figure 4e). Then, we used the supernatant of the aforementioned Caco-2 cell culture system to stimulate human monocyte-derived DCs, cocultured them with Tho cells, and detected the proportion of Tregs. We found that knockdown of TLR5 in Caco-2 cells inhibited Treg differentiation induced by R. intestinalis (Figure 4f-g). Thus, TLR5 expressed in IECs contributes to R. intestinalis-mediated Treg differentiation.

**TLSP is a key mediator of R. intestinalis-induced Treg differentiation**

We used specific siRNAs to suppress TLSP or TGFβ expression in Caco-2 cells as a method to investigate whether TLSP and TGFβ are the key factors mediating R. intestinalis-induced DC activation and subsequent Treg differentiation. Supernatant-induced release of TGFβ and IL-10 from DCs was suppressed by the knockdown of TLSP and TGFβ in IECs (Figure 5a); the percentage of DC-induced Tregs was decreased when the expression of TLSP and TGFβ on IECs was inhibited (Figure 5b-c). In contrast, this process was reversed by the addition of TLSP or TGFβ proteins (Figure 5a-c). Thus, TLSP and TGFβ produced by IECs promote the differentiation of DC-induced Tregs in response to R. intestinalis.

We used neutralizing antibodies to antagonize TLSP or TGFβ in TNBS- and R. intestinalis-treated C57BL/6 mice as a method to evaluate the roles of TLSP and/or TGFβ in modulating Treg differentiation in vivo (Figure 5d, S6a). The TNBS+ R.I mice showed minimal signs of colitis, including decreased weight loss and increased DAI score (Figure 5e-f), increased colon length (Figure 5a), reduced colonic epithelial injury and decreased pathological score (Figure 5g-h), compared with the TNBS mice. In contrast, intestinal inflammation was not completely reversed in the anti-TLSP+TNBS+R.I mice compared with the anti-TLSP alone. Consistent with the analysis of intestinal inflammation, the proportion of Tregs (Figure 5i-j) and the expression of TLSP, TGFβ, and IL-10 (Figure 6k, S7i-j) were increased in the TNBS+R.I mice but not in the anti-TLSP+TNBS+R.I mice compared with the anti-TLSP+TNBS mice. Anti-TLSP mAb treatment did not affect R. intestinalis colonization (Figure S7k-l). These findings indicate that TLSP is a key molecule contributing to R. intestinalis-mediated inflammatory inhibition in mice. Notably, although the knockdown of TGFβ or TLSP exerted a similar effect on IEC-induced Treg differentiation in vitro, the administration of the anti-TGFβ antibody failed to alter the R. intestinalis-mediated anti-inflammatory effects and Treg differentiation in the TNBS-induced animal models (Figure S6b-j). Collectively, these data indicate that TLSP, but not TGFβ, might be the key mediator of R. intestinalis-dependent inhibition of inflammation in the model of TNBS-induced colitis.

**Lower TLR5, TGFβ, TLSP and IL-10 expression in intestinal tissues from patients with CD**

We detected the levels of TLR5, TLSP, TGFβ and IL-10 in the colon of patients with CD using IHC, which were significantly reduced (Figure 6a-h). The level of
R. intestinalis in the faeces of the patients with CD was also decreased (Figure 6i). Additionally, the levels of TLR5, TSLP, TGF\(\beta\) and IL-10 were positively associated with R. intestinalis (Figure 6j-m). Thus, the expression of TLR5, TSLP, TGF\(\beta\) and IL-10 in patients with CD was downregulated, potentially due to the loss of R. intestinalis. Collectively, these results suggest that TLR5 expression in response to R. intestinalis stimulation leads to the upregulation of TSLP, TGF\(\beta\) and IL-10 and the subsequent DC-mediated anti-inflammatory responses.

**Discussion**
The gut microbiota plays an indispensable role in inducing the development of a mature intestinal immune system. The host and the intestinal microbiota form an intestinal mucosal steady state to ensure the normal function of the intestine. Immune disorders caused by dysbiosis exert extensive effects on the pathogenesis of CD. Clarifying the role of microorganisms in the pathogenesis of CD may provide new strategies for preventing and treating this disease. Previously, we reported that the abundance of R. intestinalis was substantially decreased in patients with untreated CD compared with healthy controls in China, indicating that this factor upregulates the expression of Tregs and plays a role in inhibiting colonic inflammation. R. intestinalis interacts with plant polysaccharides, inhibits glycolysis, promotes fatty acid utilization, and ameliorates atherosclerosis. R. intestinalis also improved alcoholic fatty liver in mice by reducing the expression of genes related to liver inflammation and lipid transport. However, researchers have not clearly determined how R. intestinalis mediates the expression of these genes.

**Figure 3.** R. intestinalis-mediated expression of TSLP and TGF\(\beta\) requires TLR5 signalling in IECs.

(a) Caco-2 cells transfected with the TLR5-siRNA or scrambled RNA were treated with PBS, LPS, LPS plus R. intestinalis in different amounts. ELISAs of TSLP (left panel) and TGF\(\beta\) (right panel) levels in the supernatant of Caco-2 cells. R.I\(_1\), R.I\(_2\) and R.I\(_3\): bacteria to cell ratios of 10: 1, 5: 1 and 1: 1, respectively.

(b and c) Colon TSLP (b) and TGF\(\beta\) (c) mRNA expression was detected in the reconstituted mice using RT–PCR (n=6).

(d and e) ELISAs of the blood serum concentrations of TSLP (d) and TGF\(\beta\) (e) in the reconstituted mice (n=6).

*\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), and ns, not significant. Data represent the cumulative results from two or three independent experiments.
Figure 4. *R. intestinalis* induces Treg differentiation through IEC-mediated DC activation in a TLR5-dependent manner in vitro.

(a) Schematic of the induction of Tregs. Caco-2 cells were stimulated with LPS (24 h), followed by treatment with *R. intestinalis* (24 h). Human monocyte-derived DCs (from human-derived monocytes) were incubated with Caco-2 supernatants for 48 h. Then,
R. intestinalis modulates intestinal immunity in individuals with CD. Here, we found that IECs expressing TLR5 and producing TSLP are essential for the R. intestinalis-mediated differentiation of DC-induced Tregs, which further clarified the anti-inflammatory effects and mechanism by which R. intestinalis modulates gut mucosal immunity in individuals with CD (Figure 7). Our research suggests that R. intestinalis is a potentially beneficial microorganism that exerts a protective effect on CD.

TLRs are critical regulators of intestinal mucosal immunity that recognize and interact with gut microorganisms. TLRs, including TLR2, TLR4 and TLR5, are related to bacterial recognition and represent some molecules initiating inflammation. In particular, the deletion of TLR2, TLR4 and TLR5 is related to the pathogenesis of IBD. Our results showed an increase in TLR expression induced by TNBS. Consistently, dysbiosis of the gut microbiota is caused by an inflammatory state, including TNBS, and LPS from the outer membrane of gram-negative bacteria activates TLR4 in immune and nonimmune cells. Upregulated expression of TLR4 is involved in an enhanced inflammatory response. Here, we explored the roles of TLRs in R. intestinalis-induced alleviation of inflammation, and we first showed that TLR5, but not TLR2 or TLR4, is necessary for R. intestinalis to promote Treg differentiation. Salmonella typhimurium activates NF-κB in a TLR5-dependent manner to promote IL-8 secretion and result in inflammation. TLR5 deficiency in mouse IECs induces spontaneous colonic inflammation. E. coli H18 flagellin was used to stimulate TLR5 in Caco-2 cells, and flagellin-treated Caco-2 supernatants activated DCs and led to inflammatory responses. These studies show that TLR5 activation may exert a complex regulatory effect on colonic inflammation. We found that TLR5 is required for the anti-inflammatory effects of R. intestinalis, and R. intestinalis flagellin may be the key component interacting with TLR5, which requires further confirmation. We also compared the effect of heat-killed bacteria with live bacteria. Heat-killed R. intestinalis treatment exerted a similar effect on inducing TSLP expression to R. intestinalis treatment (Figure S4c), indicating that the components of R. intestinalis, such as flagellin, may play a main role in its anti-inflammatory activity. We will use recombinant R. intestinalis flagellin to investigate this possibility in future explorations.

TLR5 is widely expressed in intestinal IECs, DCs and macrophages (Møs). These cells continuously recognize microorganism-associated molecular patterns through TLR5 to maintain mucosal immune homeostasis in the intestine. Studies have found that microorganisms such as E. coli induce IECs to produce β-defensin through TLR5 to enhance host defences and inhibit bacterial invasion. Another study reported that S. typhimurium triggers the IL23-IL22 pathway through TLR5 on DCs to enhance mucosal innate immune defences. This bacterium also induces Treg differentiation and stimulates B cells to produce IgA to restrain inflammation. The microbiota activate Møs to produce IL-1β through TLR5 and regulate intestinal mucosal immunity. Studies have reported that BMT promotes immune reconstitution and contributes to restoring intestinal mucosal immune cell populations, but obvious evidence of enterocyte regeneration has not been reported. We investigated the role of TLR5 expressed on intestinal nonimmune cells (IECs) compared with immune cells by generating bone marrow chimeric mice, including WT mice with immune cells deficient in TLR5 and TLR5−/− mice with IECs deficient in TLR5, as well as control mice, which first confirmed that TLR5-expressing IECs play a key role in the alleviation of intestinal inflammation induced by R. intestinalis; TLR5-expressing immune cells (e.g., macrophages and DCs) may also participate in R. intestinalis-induced Treg differentiation and inhibition of inflammation. This finding indicates that a sophisticated cell communication network regulates the effect of the gut microbiota on the gut mucosal immune reaction and inflammation, and the presence of preponderant immunoregulatory cells has a dominant function in this network.

Extensively washed DCs were cocultured with human naive CD4+ T cells (Th0) for 48 h to induce Treg differentiation. Human-derived monocytes and Th0 cells were collected from peripheral blood samples from healthy participants. FCM, Flow Cytometry.

(b) Caco-2 cells were treated with PBS, LPS, R. intestinalis, and LPS plus R. intestinalis. DCs were stimulated with the supernatant from Caco-2 cells (48 h). The DCs were washed and cocultured with CD4+ Th0 cells, and the supernatant of Caco-2 cells without DCs was used as a control. The proportion of Tregs was evaluated using flow cytometry analysis.

(c) The proportion of Tregs was analysed statistically.

(d) The levels of IL-10 and TGFβ in the supernatant of the DCs mentioned above in (b) were detected using ELISAs.
(e) Caco-2 cells were transfected with the TLR5-siRNA or scrambled RNA. Transfected Caco-2 cells were treated with different concentrations of PBS, LPS, and LPS plus R. intestinalis. Western blot analysis of TLR5 levels in Caco-2 cells. The quantification of the TLR5 protein levels in Western blots using ImageJ software is shown. Tubulin was used as a reference.

(f and g) DCs were treated with supernatants from the different groups of Caco-2 cells mentioned above in (e), and then washed DCs were cocultured with CD4+ Th0 cells. A flow cytometry analysis of the number of Tregs was performed (gated on CD4+ cells), and data were processed for statistical analysis.

*p<0.05, ***p<0.001, and ns, not significant. Data represent the cumulative results from three independent experiments.
**Figure 5.** *R. intestinalis* promotes DC-induced differentiation of Tregs to inhibit inflammation through a mechanism requiring TSLP expression in IECs.

(a) Caco-2 cells transfected with the TSLP-siRNA or TGFβ siRNA were stimulated with LPS+R.I in the presence or absence of the recombinant TSLP/TGFβ proteins. The supernatant of the Caco-2 cells was used to induce human monocyte-derived DCs. TGFβ and IL-10 levels in DC culture supernatants were measured using ELISAs.

(b and c) DCs were treated with the supernatants described above in (a) and then extensively washed and cocultured with human naive CD4+ T cells (Th0). The proportion of Tregs was analysed using flow cytometry (gated on CD4+ cells), and a statistical analysis was performed.

(d) Schematic of C57BL/6 WT mice treated with the anti-TSLP (neutralization of TSLP) mAb, TNBS and *R. intestinalis*.

(e and f) Changes in body weight (e) and DAI score (f) of each group (n=6). ***p<0.001, control vs. TNBS; #p<0.05, TNBS vs. TNBS+R.I; 6p<0.05, anti-TSLP+TNBS vs. anti-TSLP+TNBS+R.I; 7p<0.05, TNBS+R.I vs. anti-TSLP+TNBS+R.I.

(g and h) The colon histopathological score and representative images of colon tissues stained with H&E (n=6).

(i and j) Representative flow cytometry plots of Treg cells (gated on CD4+ cells) in colon tissues and statistical analysis (n=6).

(k) ELISAs of TSLP, TGFβ and IL-10 levels in colon tissue homogenates (n=6).

*p<0.05, **p<0.01, ***p<0.001, and ns, non-significant. Data represent the cumulative results from two or three independent experiments.
Figure 6. TLR5, TSLP, TGFβ and IL-10 levels in the intestine are positively related to R. intestinalis in patients with CD.

(a-d) Representative images of IHC detection of colonic TLR5, TSLP, TGFβ and IL-10 expression in healthy controls (ctrl) or patients with CD.

(e-h) Each section stained using IHC was evaluated, and five images from each slide were randomly selected and measured using ImageJ. The mean densities of TLR5, TSLP, TGFβ and IL-10 were statistically analysed (ctrl: n=22; patients: n=24).

(i) Comparative analysis of R. intestinalis in the faeces of the healthy controls or the patients with CD.

(j-m) The correlations between the levels of TLR5, TSLP, TGFβ and IL-10 with R. intestinalis in controls and patients with CD were analysed (Spearman’s correlation analysis with two-tailed p values).

**p<0.01 and ***p<0.001. Data represent the cumulative results from two or three independent experiments.
IECs are the intermediary bridges connecting intestinal bacteria and gut homeostasis and diseases. Segmented filamentous bacteria act on IECs to trigger the release of serum amyloid A, which stimulates DCs to secrete IL-1β and IL-23 and enhance Th17-induced mucosal defences. The gut microbiota may act on IEC-intrinsic IKKa to promote the secretion of TSLP and regulate ILC3 responses to maintain immune homeostasis in the intestinal mucosa. In this study, the regulation of IEC-derived cytokines by R. intestinalis and the subsequent regulation of immune cells in vitro were investigated, and the results showed that R. intestinalis promotes the production of TSLP and TGFβ in IECs. TSLP and TGFβ do not directly regulate the differentiation of Th0 cells to Tregs and may indirectly induce this process by amplifying signals or increasing the secretion of other cytokines by intermediate cells. DCs exert an instrumental effect on modulating the immune response of the intestinal lamina propria. The supernatant of IECs promoted the differentiation of tolerogenic DCs, and tolerogenic DCs upregulated the expression of CD103, CD80, CD83 and CD86 and promoted the differentiation of Tregs (data not shown), consistent with previous research. The addition of DCs to the system of R. intestinalis-treated IECs induced Th0 cells to differentiate into Tregs, which was related to the secretion of IL-10 by DCs and the amplification of TGFβ signalling. We found that Tho differentiation into Tregs was mediated by TGF-β in a concentration-dependent manner. IEC-derived TSLP plays an indispensable role in inducing DC-dependent Treg differentiation. Additionally, in our experiment, TGF-β and IL-10 expression in the colon of mice treated with anti-TSLP, were also altered. We surmised that TSLP may also induce IL-10 production and TGFβ expression in DCs, which requires further experimental confirmation. Decreased TSLP expression may be related to the development of Th1 cell-associated CD. Thus, we concluded that R. intestinalis-induced TSLP production in IECs may contribute to inhibiting the inflammation and progression of CD.

Contributions
W.X.Y. supervised the project and revised the manuscript. S.Z.H. performed the in vitro experiments, generated the figures, and revised the manuscript. L.W.W. performed most of the in vivo experiments, completed the analysis of the results and wrote the manuscript. T.B. performed some of the in vivo experiments and analysed the results. D.M.Z. and W.S. contributed to the study design. X.M.W. cultured bacterial strains. Z. C. and M.K.J. contributed to some of the in vivo experiments and collection of samples. L.Y.J. and X.J.H.

Figure 7. A proposed model illustrating the inhibitory effect of R. intestinalis on inflammation and colitis. R. intestinalis increases TSLP expression in IECs expressing TLR5 and induces IL-10 production and TGFβ expression in DCs. These cytokines then promote the differentiation of Tregs to inhibit colonic inflammation. TLR5, Toll-like receptor 5; IECs, intestinal epithelial cells; DCs, dendritic cells; Treg, regulatory T cell.
collected some of the samples. W.X., N.K. T.T. and M.X. R. collected the data and contributed to the data analysis. S.Z.H. and L.W.W. assessed and verified the data. W.X.Y. were responsible for the decision to submit the manuscript.

Data sharing statement
All data that support the findings of this study are available within this paper and its Supplementary Information files or from the corresponding author upon reasonable request.

Declaration of interests
The authors have declared that no competing interests exist.

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Supplementary materials
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