Research Article

Oxytocin signalling in dendritic cells regulates immune tolerance in the intestine and alleviates DSS-induced colitis

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Background: Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) that is associated with immune dysfunction. Recent studies have indicated that the neurosecretory hormone oxytocin (OXT) has been proven to alleviate experimental colitis.

Methods: We investigated the role of OXT/OXT receptor (OXTR) signalling in dendritic cells (DCs) using mice with specific OXTR deletion in CD11c+ cells (OXTRfloxflox × CD11c-cre mice) and a dextran sulfate sodium (DSS)-induced colitis model.

Results: The level of OXT was abnormal in the serum or colon tissue of DSS-induced colitis mice or the plasma of UC patients. Both bone marrow-derived DCs (BMDCs) and lamina propria DCs (LPDCs) express OXTR. Knocking out OXTR in DCs exacerbated DSS-induced acute and chronic colitis in mice. In contrast, the injection of OXT-pretreated DCs significantly ameliorated colitis. Mechanistically, OXT prevented DC maturation through the phosphatidylinositol 4,5-bisphosphate 3-kinase (Pi3K)/AKT pathway and promoted phagocytosis, adhesion and cytokine modulation in DCs. Furthermore, OXT pre-treated DCs prevent CD4+ T cells differentiation to T helper 1 (Th1) and Th17.

Conclusions: Our results suggest that OXT-induced tolerogenic DCs efficiently protect against experimental colitis via Pi3K/AKT pathway. Our work provides evidence that the nervous system participates in the immune regulation of colitis by modulating DCs. Our findings suggest that generating ex vivo DCs pretreated with OXT opens new therapeutic perspectives for the treatment of UC in humans.

Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) with symptoms including diarrhoea, abdominal pain, weight loss and rectal bleeding [1]. This disease has a high incidence rate in Western countries, exceeding 0.3% [2], and has increased rapidly in newly industrialised countries over the past 10 years [3]. Although the aetiology of UC remains unknown, the disease is generally considered to be associated with genetic predispositions, immunological responses, intestinal flora, environmental factors and some psychological problems [4–6]. Dysregulated immune responses in the intestinal mucosa lead to the overproduction of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-17, interferon (IFN)-γ and tumor necrosis factor-α (TNF-α). Dendritic cells (DCs), the major type of antigen-presenting cells, contribute to both innate and adaptive immune responses. The activated DCs together with pro-inflammatory...
cytokines prime T cell differentiation into the T helper 1 (Th1) or Th17 [7–11] and perpetuate the inflammatory responses in the colon, eventually leading to tissue damage and the development of colitis [12].

In the last few years, researchers displayed the communication between neuro and immunity in mucosa [13,14]. Oxytocin (OXT), a neuropeptide synthesised in the brain, plays an essential role in mammalian labour, lactation, maternal bonding, and social affiliation [15]. Recent studies including ours, have proven that OXT is also synthesised and released by enteric neurons [16–18]. Previous findings have demonstrated that OXT alleviates experimental colitis [19] and participates in the anti-inflammatory response [20]. Here, we reveal for the first time that the OXT/OXT receptor (OXTR) signalling pathway mediated immune tolerance in DCs that participates in protection against colitis.

Materials and methods

Mice

C57BL/6 mice, 8–10 weeks old, were purchased from Shandong University. CD11c-cre mice were purchased from the Model Animal Research Centre of Nanjing University, and OXTRfl/fl (OXTRΔDC) mice were from Jackson Laboratories. Transgenic mice were cross-fertilised to generate OXTRΔDC/ΔDC mice. The cre negative littermates (OXTRfl/fl) were used as control groups in the experiments. The OXTRfl/fl and OXTRΔDC male and female mice were used between 8 and 12 weeks. The experimental mice were raised under pathogen-free conditions in animal care facilities. Mice were killed by cervical dislocation and were anaesthetised by intraperitoneal injection of sodium pentobarbital (100 mg/kg) if needed. All animal experiments were carried out in the Laboratory Animal Centre of Shandong University and conducted by the guidelines of the Animal Care and Use Committee of Shandong University (LL-201602017).

Human colonic tissue and plasma samples

The UC tissue samples were obtained from patients undergoing UC surgery at Qilu Hospital of Shandong University. The samples were categorised as discard tissue. The plasma samples were taken from 24 patients with UC or 20 healthy persons. Characteristics of UC patients and healthy controls are shown in Table 1. The detail information of the UC patients is shown in Supplementary Table S1. Each participant was informed consent with an approved protocol (LL201601005). The use of human tissue and plasma was approved by the Medical Institutional Ethics Committee of Shandong University from China.

Cell preparation

Bone marrow-derived DCs (BMDCs) were induced from bone marrow (BM) cells as described by Zhao et al. [21]. Femurs and tibias of C57BL/6 mice (6–8 weeks) were removed and purified from surrounding muscle tissue. The BM cells flushed with PBS. After lysing red blood cell, BM cells (2 × 10⁶/ml) were resuspended and cultured in RPMI 1640 with 10% FBS, 10 ng/ml rmGM-CSF (recombinant Murine Granulocyte-macrophage Colony Stimulating Factor, Biolegend, San Diego, CA, U.S.A.) and 5 ng/ml rmIL-4 (recombinant Murine interleukin-4, Biolegend, San Diego, CA, U.S.A.) and cultured in tissue culture dishes. On days 3 and 5 culture, two-thirds of the medium was replaced with the medium containing rmGM-CSF and rmIL-4. At day 7, non-adherent cells were collected and analysed for the percentage of CD11c positive cells by flow cytometry (>90%). To assess the function and mechanism, BMDCs were cultured with OXT (10⁻⁸ M, Abcam, Cambridge, UK) or normal saline (CISEN, China) and then stimulated with lipopolysaccharides (LPSs, 100 ng/ml, Solabio, China) to mature the DCs. In the experiments for investigating the phosphatidylinositol 4,5-bisphosphate 3-kinase (Pi3K)/AKT pathway in DCs treated with OXT, DCs were incubated with AKT inhibitor MK2206 (10⁻⁷ M, Selleck, Shanghai, China), Pi3K inhibitor AS605240 (2 × 10⁻⁷ M, Selleck, Shanghai, China), Wortmannin (2 × 10⁻⁷ M, Selleck, Shanghai, China), or Ly294002 (10⁻⁷ M, Selleck, Shanghai, China), or mammalian target of rapamycin (mTOR) inhibitor Rapamycin (2 × 10⁻⁷ M, HY-10219, MCE, Monmouth Junction, NJ, U.S.A.). Colon lamina propria mononuclear cells (LPMCs) were isolated by using a modified technique described previously [22].

<table>
<thead>
<tr>
<th>UC (n=24)</th>
<th>HC (n=20)</th>
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<tr>
<td><strong>Age, median (range)</strong></td>
<td>52.5 (25–73)</td>
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<td><strong>Gender (F/M)</strong></td>
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Table 1 Characteristics of UC patients and HCs
Lamina propria DCs (LPDCs) were isolated from LPMCs by using CD11c+ magnetic-activated cell sorting (MACS, MojoSort Mouse FCD11c Nanobeads, 480077, Biolegend, San Diego, CA, U.S.A.) following the manufacturer’s instruction.

CD4+ T cells were purified from mesenteric lymph node (MLN) cells in dextran sulfate sodium (DSS)-treated mice by MojoSort Mouse CD4 Naive T Cell Negative Isolation kit (480039, Biolegend, San Diego, CA, U.S.A.) following the manufacturer’s instructions.

**Coculturing CD4+ T cells with DCs**

Protein extracts were acquired by homogenisation of DSS-induced mice colonic segments (50 mg tissue/ml) as described [22]. BMDCs were pulsed with the protein extraction (50 μg/ml) for 8 h, then cultured with OXT and LPS for 24 h. After washing with PBS, the BMDCs (10^5) were cocultured with CD4+ T cells (10^6) for 4 days in the existence of anti-CD3 (20 μg/ml, 05112-25-500, BioGems, Westlake Village, CA, U.S.A.), anti-CD28 (20 μg/ml, 10312-25-500, BioGems, Westlake Village, CA, U.S.A.), IL2 (10 ng/ml, NBP2-35111-5 μg, NOVUS, Abingdon, U.K.) and transforming growth factor-β (TGFβ; 10 ng/ml, 100-21, Peprotech, Cranbury, NJ, U.S.A.). After that, the samples were analysed by flow cytometry.

**DC phagocytosis test**

FITC-dextran particles (40000 Da, 1 mg/ml, FD40, Sigma–Aldrich, Germany) were loaded to BMDCs for 1 h at 37°C and fluorescence microplate (Molecule Devices, San Jose, CA, U.S.A.) was used to determine the extent of phagocytosis.

**Bacteria adhesion bacterial strain**

*Salmonella typhimurium* and *Lactobacillus crispatus* provided by the laboratory at Shandong Normal University or Shandong university was used in the present study. *S. typhimurium* was incubated in nutrient broth (NB) at 37°C for 24h with shaking and *L. crispatus* was statically cultured in De Man, Rogosa, Sharpe (MRS) at 37°C for 12 h. The bacteria were harvested by centrifugation at 7000 × g for 3 min, washed with PBS for three times and added to DCs.

**Adhesive capability of pathogens to DCs**

*S. typhimurium* and *L. crispatus* (10^8) were respectively labelled with FITC (0.5 mg/ml), incubated for 1 h at room temperature and washed three times with PBS. The FITC-labelled bacteria (bacteria:cell with 1000:1) were added to BMDCs. They were incubated for 45 min at 37°C and washed with PBS. The adhesion of BMDCs with bacteria was observed by fluorescence microscopy. The adhesion capability was determined by fluorescence microplate with an absorption wavelength of 495 nm and an emission wavelength of 521 nm.

**DSS-induced colitis**

DSS (36–50 kDa, MP Biomedicals, Illkirch, France) was dissolved in sterile drinking water. For acute colitis, the mice were induced with different concentrations of DSS in drinking water and killed. BMDCs (10^6 cells/mouse/day) were injected via the tail vein at days 4 and 6 in DC therapy experiment [22].

Chronic colitis was induced by three cycles of a 5-day treatment with 3% DSS, 1.5% DSS and 2% DSS, respectively and followed with a 7-day recovery on normal water drink.

The entire colon was quickly excised and washed by pre-cooled PBS. The proximal colons were used for protein extraction and the middle part was kept in RNA fixer (Aidlab, Beijing, China) for RNA isolation. The distal parts were fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained by H&E (Hematoxylin-eosin staining). [23]

The severity of colitis was determined as the disease activity index (DAI) and inflammatory cell infiltration in the lamina propria score as described previously [24].

**Western blotting**

Colon sections and cells were lysed by RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitor cocktail (Bimake, Houston, TX, U.S.A.) and phosphatase inhibitor cocktail (Bimake, Houston, TX, U.S.A.). Protein extracts were separated by SDS/PAGE and detection was performed referring to the standard protocol. The blot was incubated with antibodies as described in Supplementary Table S2.
Immunofluorescence
BMDCs or paraffin sections were stained by American hamster anti-CD11c-FITC antibody (1:300, Biolegend, San Diego, CA, U.S.A.) and rabbit anti-OXTR antibody (1:300, Abcam, Cambridge, U.K.) at 4°C overnight. Secondary antibodies were performed with Alexa 568 conjugated donkey-anti-rabbit antibody for an hour and DAPI (4',6-diamidino-2-phenylindole) stained for 5 min.

Flow cytometry
Cells were blocked by anti-CD16/CD32 (Biolegend, San Diego, CA, U.S.A.) for 10 min. For surface staining, cells were stained with fluorobently labelled anti-mouse antibodies diluted in PBS at an indicated concentration (Supplementary Table S3) for 30 min at 4°C, before extensive wash. For intracellular staining of cytokines and transcription factors, cells were incubated in complete RPMI 1640 medium with cell activation cocktail (Biolegend, San Diego, CA, U.S.A.) and Brefeldin A (Biolegend, San Diego, CA, U.S.A.) for 4 h in the CO2 incubator. Cells were fixed and permeabilised according to the manufacturer's instructions (eBioscience™ Foxp3/Transcription Factor Staining Buffer Set, Thermo Fisher Scientific van Allen Way Carlsbad, CA, U.S.A.). Cells were then immuno-stained following the same protocol described above. The volumetric acquisition was performed on a GALLIOUS or a CytoflexS (Beckman Coulter, Inc.250 S.Kraemer Boulevard Brea, CA, U.S.A.). Cell analysis was done using FlowJo software (Version 10; Tree Star).

ELISA
ELISA kits were used to measure cell-cultured supernatant anti-IL12p70 (Dayou, Beijing, China) and IL6 (Dayou Beijing, China), mouse serum or colon protein extracts OXT (OmnimAbs, ALHAMBRA, CA, U.S.A.), or human plasma OXT (CUSABIO, Wuhan, China) according to the manufacturer's instructions.

RNA detection
RNA was isolated from RNA fixer fixed tissue using an RNAeasy mini kit (R0027, Beyotime, Shanghai, China). Isolated RNA was reverse-transcribed using QuantiTect Rev. Transcription Kit (R223-01, Vazyme, Nanjing, China) and real-time PCR was performed on 3.8 μl cDNA product (diluted five times) using SYBR Green qPCR Mix (Q711-02, Vazyme, Nanjing, China) on Bioer Lightcycler or Bio-Rad CFX Connect Real-Time System. The primers were described in Supplementary Table S4 and synthesised by the Beijing Genomics Institute (Beijing, China).

Stool sample collection and RNA extraction
Stool samples freshly collected from OXTR<sup>ADC</sup> or OXTR<sup>Bfl</sup> mice were immediately frozen at −80°C, and transported to the laboratory with drikold pack. Bacterial RNA was extracted and sequencing at Novogene Bioinformatics Technology Co., Ltd.

Statistical analysis
Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences in groups was assayed by one-way ANOVA or <i>t</i> test using GraphPad Prism 8 (GraphPad Software). All the differences were considered significant at <i>P</i> < 0.05.

Results
Abnormal OXT signalling in patients with UC and mice with DSS-induced colitis
The neuro–immune communication between the enteric nervous system (ENS) and immune effector cells such as DCs and macrophages has been proven to play important roles in gastrointestinal (GI) physiology, host immunity and GI pathologies, which are most likely mediated by neuropeptides and non-peptidergic neurotransmitters [25,26]. Our results revealed that the level of the neuropeptide OXT was elevated in the serum or colon tissues of mice with DSS-induced colitis (Figure 1A). Consistent with this finding, UC patients exhibited high levels of plasma OXT (Figure 1B). Next, we analysed the expression of OXTR in BMDCs and LPDCs by Western blotting and immunofluorescence. As shown in Figure 1C,D, both BMDCs and LPDCs from mice expressed OXTR. Similarly, LPDC from human colon expressed OXTR (Figure 1C). Interestingly, LPDCs from mice with DSS-induced colitis exhibited significantly lower expression of OXTR than those from control mice (Figure 1E).
Deletion of OXTR in DCs exacerbated DSS-induced acute colitis

We generated mice with DC-specific deletion OXTR (OXTR<sup>ΔDC</sup> mice) to study the function of OXTR-expressing DCs in experimental colitis. As shown in Figure 2A, OXTR expression was notably reduced in OXTR<sup>ΔDC</sup> DCs. OXTR<sup>ΔDC</sup> mice exhibited normal colonic goblet cells, villus lengths, crypt depth (Supplementary Figure S1) and submicroscopic structures (Supplementary Figure S2) compared with those of OXTR<sup>fl/fl</sup> mice. Moreover, there was no significant difference in weight between the mice in the two control groups (Figure 2B). DSS-induced colitis was more severe in OXTR-deficient mice. Compared with OXTR<sup>fl/fl</sup> mice, OXTR<sup>ΔDC</sup> mice that were administered 2% DSS for 7 days exhibited significantly more weight loss (Figure 2B), reduced colon lengths (Figure 2C) and more severe disease as measured by DAI scores (Figure 2D). Histologically, epithelial damage, focal crypt injury and inflammatory cell invasion were more severe in OXTR<sup>ΔDC</sup> mice than OXTR<sup>fl/fl</sup> mice (Figure 2E–G).

Dynamic changes in DC subsets, the proportion of Th cells and cytokine release in the colon lamina propria of OXTR<sup>ΔDC</sup> acute colitis mice

The LPDCs can be classified by CD11b and CD103 [27,28]. The gating strategy is shown in Supplementary Figure S3. Compared with OXTR<sup>fl/fl</sup> mice, OXTR<sup>ΔDC</sup> mice exhibited a much higher ratio of the CD11b<sup>+</sup>CD103<sup>+</sup> DC subpopulation (Figure 3A). The frequencies of Th1 and Th17 cells in the colon were high in OXTR<sup>ΔDC</sup> mice compared with WT mice (Figure 3B). Consistent with this finding, the expression of T-bet and Rorγt but not Foxp3 or Gata3 was markedly increased in the colon tissue of OXTR<sup>ΔDC</sup> mice compared with OXTR<sup>fl/fl</sup> mice (Figure 3C). IL23 is thought to regulate the proliferation and survival of Th17 cells, while TGFβ and IL6 promote the differentiation of Th17 cells [29]. Therefore, we evaluated the expression of various pro-inflammatory and anti-inflammatory cytokines in the mouse colon. The mRNA levels of the pro-inflammatory cytokines Il23 and Il6 were higher and the anti-inflammatory cytokine Tgfβ was lower in OXTR<sup>ΔDC</sup> mice than in OXTR<sup>fl/fl</sup> mice (Figure 3D).

OXTR<sup>ΔDC</sup> exacerbated DSS-induced chronic colitis

To confirm the role of OXTR-expressing DCs in colitis, DSS-induced chronic colitis was used. As shown in Figure 4A,
To induce acute colitis, OXTR<sup>ΔDC</sup> mice and the wildtype OXTR<sup>fl/fl</sup> littermates were received 2% DSS dissolved in water. (A) Western blot analysed the expression of OXTR in LPDCs from OXTR<sup>fl/fl</sup> and OXTR<sup>ΔDC</sup> mice. (B) Relative weight changes of OXTR<sup>fl/fl</sup> and OXTR<sup>ΔDC</sup> mice during the experiment period with or without DSS. (C) Representative photograph of the colon from OXTR<sup>fl/fl</sup> and OXTR<sup>ΔDC</sup> mice, and colon lengths were measured after the mice were killed. (D) The DAI was scored on the final day. (E) Histological score of the colon segment. (F,G) H&E and AB-PAS stained sections from the distal colon. The experiment was repeated twice. Data are the means ± SEM (n=6); compared by unpaired t test, *P<0.05, **P<0.01, ***P<0.001 vs OXTR<sup>fl/fl</sup> DSS mice. Scale bar = 100 μm.

Figure 2. OXTR<sup>ΔDC</sup> promoted the acute colitis in mice

To further investigate the role of the OXT system in DCs, a DC transfer experiment was established. BMDCs were incubated with normal saline (NS, Control) or LPS pretreated with OXT (DC<sub>OXT</sub>) or without OXT (DC<sub>control</sub>). In the DC therapy experiment, mice were injected with the three kinds of DCs (10<sup>6</sup>/mouse/day) on days 4 and 6 after 2.5% DSS administration. DC<sub>OXT</sub> treatment but not DC<sub>control</sub> treatment protected against weight loss (Figure 5A), reduced
Figure 3. OXT promoted colon mucosal homeostasis in acute colitis via activating OXTR
(A) Expression of CD11b and CD103 by live single CD45^+CD11c^+MHCII^+CD64^- DCs (left) from the LPMCs and relative frequencies of DC subsets (right). (B) Representative flow plots of IFN\(\gamma\), IL17A or Foxp3 gated on CD4^+ T cells from the LPMCs (left) and the percentage of Th1 cells, Th17 cells and Treg cells in CD4^+ T cells from LPMCs (right). Data are combined from more than five independent experiments. (C) Relative expression of t-bet, rorc, foxp3 and gata3 was measured by qRT-PCR. (D) Relative expression of tgf\(\beta\), il6 and il23a was measured by qPCR. All the experiments were repeated twice. Data are the means \(\pm\) SEM (\(n\geq 4\)), compared by unpaired t test, *\(P<0.05\), **\(P<0.01\) vs OXTR\(^{fl/fl}\) DSS mice.

Colon lengths (Figure 5B,C), high DAI scores (Figure 5D) and histological damage in the colon compared with those of the control group (Figure 5E,F).
Figure 4. OXTR<sup>ΔDC</sup> aggravated DSS-induced chronic colitis

(A) The experimental schedule for the treatment of DSS-induced chronic colitis. (B) Relative weight changes during the experiment period. (C) The colon lengths were measured after the mice were killed. (D) The DAI scored on the final day. (E) Histological score of the colon. Data are the means ± SEM (n=5), compared by unpaired t test, *P<0.05, **P<0.01, ***P<0.001 vs OXTR<sup>fl/fl</sup> DSS mice. (F) Representative H&E stained sections from the distal colon. (G) Expression of t-bet, gata3, foxp3, rorγt in the colon from DSS-induced chronic colitis mice was measured by qPCR. (H) qPCR analysed the level of aldh1a2, tgfβ, il23a and il6 in the colon from DSS-induced chronic colitis mice. The experiment was repeated twice. Data are the means ± SEM (n≥3), compared by unpaired t test, *P<0.05, **P<0.01, ***P<0.001 vs OXTR<sup>fl/fl</sup> DSS mice. Scale bar = 100 μm.

OXT prevented DC maturation, pro-inflammatory cytokine release, phagocytosis and adhesion to Lactobacillus in vitro

Based on the effects of OXTR<sup>ΔDC</sup> and DC<sub>OXT</sub> treatment on DSS-induced colitis, we investigated the phenotype and function of OXT-treated DCs in vitro. Flow cytometric analysis showed that OXT suppressed the expression of CD80, CD86 and MHC II in LPS-stimulated BMDCs (Figure 6A). Tnfo, Il12a, Il23a and Il6 expression levels were increased in LPS-stimulated OXTR<sup>fl/fl</sup> BMDCs. Pretreatment with OXT inhibited the production of pro-inflammatory cytokines (Figure 6B,C). However, OXT did not affect the expression of Tnfo, Il12a, Il23a or Il6 in OXTR<sup>ΔDC</sup> BMDCs. In addition, OXT enhanced DC phagocytosis, as shown by FITC-dextran uptake, promoted the adhesion of FITC-labelled Lactobacillus to DCs, and prevented the adhesion of Salmonella (Figure 6D,E).
Figure 5. DC(OXT) injection significantly ameliorated DSS-induced colitis

Colitis was induced by 2.5% DSS. BMDCs stimulated with LPS in the presence of OXT (DCOXT) or the absence of OXT (DCcontrol). Mice were treated with vein injection with normal saline (Control), DCcontrol or DCOXT (10⁶ cells/mouse) at days 4 and 6. (A) Relative weight changes of mice during the experiment period. (B) Representative photograph of the colon from each group. (C) Colon lengths were measured after the mice were killed. (D) The DAI score on the final day. (E) Histological score of the colon was shown. (F) Representative H&E stained sections from the distal colon. The experiment was repeated twice. Data are the means ± SEM (n=7), and were analysed by ordinary one-way ANOVA with Tukey’s multiple comparisons test. *P < 0.05, ***P < 0.001 vs control mice, #P < 0.05, ###P < 0.001 vs DC (control) mice. Scale bar = 100 μm.

Activation of OXTR in LPS-stimulated DCs inhibited CD4⁺ T cells differentiate into Th1 and Th17

Because the most important function of DCs is to present antigens and prime T cells, we cocultured BMDCs with CD4⁺ T cells sorted from the MLNs of DSS-induced colitis mice [22]. OXT-pretreated BMDCs from OXTR⁺/⁺ mice but not OXTRΔDC mice markedly reduced the differentiation of Th1 and Th17 cells (Figure 7A,B).

OXT prevented DC maturation via the PI3K/AKT pathway

Next, we explored the potential mechanisms responsible for OXT-induced DC tolerance. The phosphorylation of AKT (RAC serine/threonine-protein kinase) was increased in OXT-treated BMDCs (Figure 8A). The phosphorylation of AKT and mTOR could be blocked by the AKT inhibitor MK2206, the PI3K inhibitor AS605240, wortmannin, Ly294002, and the mTOR inhibitor rapamycin (Figure 8B). Nuclear factor κ B (NFκB) is a core transcription factor involved in the inflammatory response that can be activated by LPS in DCs [30]. The NFκB inhibitor IκB was phosphorylated and degraded in LPS-stimulated DCs, which was impaired by OXT (Figure 8C). Notably, either MK2206 or wortmannin prevented the suppressive effect of OXT after LPS treatment (Figure 8C). Furthermore, preincubation with MK2206, AS605240, wortmannin and rapamycin reversed the effect of OXT on the expression of CD86 and MHC II (Figure 8D).
Figure 6. OXT regulated DCs function

(A) Flow cytometry was performed to analyse the expression of CD80, CD86 and MHCII on the BMDC (n=4) stimulated by LPS (100 ng/ml) for 12 h in the presence or absence of OXT (10^{-8} M). Data are compared by paired t test, *P<0.05 vs NS-treated DCs. (B, C) Relative expressions of Tnfα, Il12a, Il23a and Il6 were analysed by qPCR and the concentrations of IL12p70 and IL6 were detected by ELISA from OXTR^{fl/fl} DCs or OXTR^ΔDC DCs. Data are the means ± SEM (n≥3) and were compared by ordinary one-way ANOVA with Tukey’s multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 vs NS-treated BMDCs. N.D., not detected. (D) BMDC phagocytosis of FITC-Dextran was analysed by fluorescence microplate. BMDCs were incubated with different doses of OXT in the absence (left) or presence of LPS (right) for 24 h, then loaded with 1 mg/ml FITC-Dextran for 1 h and determined by fluorescence microplate. Data are the means ± SEM (n=3), compared by unpaired t test, *P<0.05 vs NS-treated DCs (left), *P<0.05 vs LPS-treated DCs (right). (E) FITC-labelled Lactobacillus or Salmonella adhered to DC was detected by fluorescence microplate. Data are the means ± SEM (n=3), compared by unpaired t test, *P<0.05, **P<0.01, ***P<0.001 vs NS-treated DCs. All the experiments were repeated at least twice.

Discussion

In the present study, we revealed that OXTR was expressed in BMDCs and LPDCs. OXTR-expressing DCs play a role in maintaining immune homeostasis in the colon during colitis. The deletion of OXTR in DCs exacerbated DSS-induced colitis. In contrast, DC_{OXT} injection significantly alleviated experimental colitis. Mechanistically, OXT suppressed DC maturation through the PI3K/AKT/mTOR pathway, thus inhibiting Th1 and Th17 cell differentiation.

Gershon et al. found that OXT and OXTR not only in many brain regions, but also in a variety of peripheral tissues not only in intestinal epithelium and ENS but also in kidney, pancreas, testis and heart [31], suggest that OXT/OXTR signalling may also be of relevance to immune inflammatory diseases in those organs, as well as the GI tract. Moreover, work in the early 1980s established the action of OXT on thymocytes and, via an insulin-like signalling mechanism, on rat adipocytes [32] and insulin-like action on rat adipocytes [33]. Previous studies have shown that OXT inhibits...
Figure 7. Activation of OXTR in LPS-stimulated DC inhibited CD4+ T cells differentiate into Th1 and Th17

CD4+ T cells were isolated from MLN in colitis mice and co-cultured with BMDCs from OXTR\textsuperscript{fl/fl} or OXTR\textsuperscript{ΔDC} mice activated by LPS with or without OXT for 4 days. Representative flow plots of IFN\textgamma, IL17A and Foxp3 in CD4+ T cells (left) and the percentages of IFN\textgamma, IL17A and Foxp3 in CD4+ T cells (right) (A, B). All the experiments were repeated at least twice. Data are the means ± SEM (n=3), compared by unpaired t test, *P<0.05 vs LPS-stimulated BMDCs.

LPS-induced inflammation in microglial cells [34] and acute lung injury [35]. Klein et al. found that colostrum OXT attenuated stress in newborn villi and increased inactive p-eIF2a, p-PKR and IκB but reduced p-IκB, BiP and LC3A [36]. We also found that mice with tamoxifen-induced global OXTR knockout (OXTR\textsuperscript{fl/fl}; UBC-cre-ert2 mice) exhibited serious colitis compared with WT mice (Supplementary Figure S4). Intraperitoneal injection of OXT protected against DSS-induced colitis [19]. Multiple studies have characterised the endogenous OXT signalling system in the ENS [16–20]. The concentration of OXT in colon tissue was higher than that in serum (Figure 1A), indicating that OXT in colon tissue may be mainly secreted by the ENS. Therefore, we propose that OXT released by enteric neurons
may regulate LPDC function and that communication between DCs and neurons in the intestine may be involved in the development of colitis. Anxiety and stress significantly activate the OXT system [37]. Colitis patients always experience anxiety [38] and stress [39]. Stress and anxiety may lead to elevated OXT levels observed in the serum and colon tissue of DSS mice (Figure 1A) or in the plasma of UC patients (Figure 1B). Furthermore, long-term exposure to OXT results in the desensitisation and down-regulation of OXTR [40] (Figure 1E), which increases anxiety-related behaviour in mice [41]. Therefore, we hypothesised that injecting OXT-pretreated DCs is better than injecting OXT for UC therapy. The OXT/OXTR signalling system plays an important role in the neuro–immune axis in the gut.

Intestine is one of our most important immune organs and contains large numbers of innate and adaptive immune cells. CD103⁺CD11b⁺DCs are unique to the intestinal mucosa [42] and play an essential role in regulating Th17 cell differentiation. The depletion of CD103⁺CD11b⁺ DCs reduced the frequency of Th17 cells [43]. In contrast, TLR agonist-activated CD103⁺CD11b⁺ DCs induce the differentiation of Th17 cells [44]. Our in vitro and in vivo experiments suggested that OXT inhibited the differentiation of Th17 cells by modulating intestinal CD103⁺CD11b⁺ DCs. Moreover, OXTR-knockout DCs promoted IL23 and IL6 expression, which could facilitate CD4⁺ T cell differentiation into Th17 cells. Fujino et al. [45] reported that IBD patients had increased numbers of Th17 cells compared with healthy controls and that active patients had up-regulated levels of Th17 cells compared with inactive patients. Additionally, activated host Th17 cells promoted intestinal microbe adhesion to epithelial cells [46]. Moreover, OXTRΔDC mice exhibited a lower ratio of Firmicutes/Bacteroidetes and a higher percentage of Proteobacteria (Supplementary Figure S5) compared with WT mice, indicating disturbed microbial flora in OXTRΔDC mice. Notably, our present study revealed that OXT promoted the adhesion of BMDCs to the probiotic Lactobacillus. Lactobacillus regulates
immature DC and T cell functions, protecting the intestine from colitis [47,48]. Further, the results of the DC therapy experiments were consistent with that of conditional knock mice experiments, which confirmed that the OXT/OXTR signalling system in DCs participates in protection against the colitis (Figures 2 and 5).

Importantly, although AKT can activate NFkB, OXT itself does not affect the phosphorylation of IκB (Supplementary Figure S6)[49]. Pretreatment of DCs with PI3K, mTOR or AKT inhibitors prevented OXT-induced suppression of DC maturation after LPS treatment. PI3K is the key molecule for AKT activation. Pharmacological or genetic targeting of PI3K promotes the expression of inflammatory factors and suppresses the expression of anti-inflammatory factors, thereby exacerbating inflammation [50,51]. FOXO1, which was inhibited by AKT, promotes DC-mediated inflammation by regulating the transcription of pro-inflammatory signalling molecules and chemokine receptors [52]. AKT activates β-catenin by phosphorylating β-catenin at Ser522 [53,54]. The β-catenin signalling pathway drives fatty acid oxidation, suppressing IL6 and IL12 expression [55]. Therefore, we believe that the anti-inflammatory effect of OXT is mediated by the PI3K/AKT pathway.

Conclusion
Our results suggest that OXT-induced tolerogenic DCs efficiently protect against experimental colitis via PI3K/AKT pathway. Our work provides evidence that the nervous system participates in the immune regulation of colitis by modulating DCs. Our findings suggest that generating ex vivo DCs pretreated with OXT opens new therapeutic perspectives for the treatment of UC in humans.

Clinical perspectives
- UC is a type of IBD that is associated with immune dysfunction. Recent studies have indicated that the neurosecretory hormone OXT has been proven to alleviate experimental colitis.
- The present study showed that OXT signalling in DCs participated in protection against colitis. Mechanistically, OXT suppressed DC maturation by activating the PI3K/AKT pathway and prevented Th1 and Th17 cell differentiation.
- OXT together with DCs participates in the communication between neuro and immunity. Our findings suggest that generating ex vivo DCs pretreated with OXT opens new therapeutic perspectives for the treatment of UC in humans.

Data Availability
All data generated and analysed in the present study are included in this published article or in the data repositories listed in References.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution
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Abbreviations
BM , bone marrow; BMDC , bone marrow-derived DC; DAI , disease activity index; DAPI , 4',6-diamidino-2-phenylindole; DC , dendritic cell; DSS , dextran sulfate sodium; ENS , enteric nervous system; GI , gastrointestinal; H&E , hematoxylin-eosin staining; IBD , inflammatory bowel disease; IFN-γ , Interferon-γ; IL , interleukin; LPDC , lamina propria DC; LPS , lipopolysaccharide; MLN , mesenteric lymph node; MRS , De Man, Rogosa, Sharpe; mTOR , mammalian target of rapamycin; NB , nutrient broth; NFκB , nuclear factor-κ B; OXT , oxytocin; OXTR , oxytocin receptor; Pi3K , phosphatidylinositol 3-kinase; rmGM-CSF , recombinant murine granulocyte-macrophage colony stimulating factor; rmIL , recombinant murine interleukin; Th1 , T helper 1; TGFβ , transforming growth factor-β; TNF-α , tumor necrosis factor-α; UC , ulcerative colitis.

References