



Oxytocin suppresses epithelial cell-derived cytokines production and alleviates intestinal inflammation in food allergy

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ABSTRACT

Food allergy is a growing healthcare problem worldwide, but prophylactic options and regulatory therapies are limited. Oxytocin (OXT), conventionally acknowledged as a hormone, was recently proven to have potent anti-inflammatory and immunomodulatory activities in certain diseases. Here, we reported the novel function and its underlying mechanisms of OXT on food allergy in vivo and in vitro. We showed that the levels of OXT were elevated in ovalbumin (OVA)-allergic mice and patients with food allergy. In HT-29 cells, OXT inhibited the production of the epithelial cell-derived cytokines thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 and IL-33 by suppressing NF- κ B signaling, in which β -arrestin2 participated. These functions of OXT were abolished by oxytocin receptor (OXTR) depletion. Treating OVA-induced BALB/c mice with OXT suppressed TSLP, IL-25 and IL-33 production and attenuated systemic anaphylaxis and intestinal inflammation. OXTR^{-/-} mice showed extreme increases in TSLP, IL-25 and IL-33 levels as well as severe systemic anaphylaxis and intestinal inflammation. In conclusion, through OXTRs, OXT has a promising antiallergic effect on experimental food allergy by suppressing epithelial TSLP, IL-25 and IL-33 production via inhibiting NF- κ B signaling and upregulating β -arrestin2 expression. Our study provides a new therapeutic perspective for food allergy in humans.

1. Introduction

Food allergy is an immune-mediated adverse reaction to food protein antigens. Food allergy is a prevalent condition, with up to 6%–8% of children < 5 years of age and 2%–3% of adults having a food allergy in developed countries, and has become a growing concern in developing countries [1,2]. Gastrointestinal dysfunctions such as nausea, vomiting, diarrhea, and abdominal pain are common manifestations of food allergy [3]. Food proteins that survive gastrointestinal digestion may cross the enteric epithelium via different routes and induce allergic sensitization in susceptible individuals [4]. Increasing evidence suggests that the intestinal epithelium, as the first barrier of intestinal immunity, plays an important role in the initiation and development of food allergy. In addition to providing a passive mucosal barrier, a layer of mucus-covered cells joined by tight junctions, intestinal epithelial cells (IECs) may actively participate in food allergy by recognizing antigens and secreting proinflammatory mediators. In susceptible individuals,

exposure to environmental stimuli can lead to aberrant production of thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 and IL-33, but why only certain individuals mount allergic responses to such stimuli remains an unanswered question [5]. The epithelial cell-derived cytokines TSLP, IL-25, and IL-33 are usually characterized as “alarmins” of allergic diseases and shape the development of downstream immune responses [6]. TSLP, IL-25, and IL-33 are central regulators of allergic inflammation that initiate, propagate, and sustain aberrant type 2 responses [7]. There is growing interest in developing therapeutics for allergic diseases that target TSLP, IL-25 and IL-33, for which antibodies are under development or have entered clinical trials [7,8]. However, curing food allergy remains significantly challenge in clinical medicine.

Oxytocin (OXT), a neurohypophysial nonapeptide that is known as a hormone and to bind to oxytocin receptors (OXTRs), plays roles in parturition and lactation [9,10]. Recently, the OXT/OXTR signaling system, which participates in immune surveillance, strengthens immune defense and maintains immune homeostasis, has emerged as an

Abbreviations: IEC, intestinal epithelial cell; I κ B, inhibitor of NF- κ B; IL, interleukin; LPS, lipopolysaccharide; KO, knockout; mMCP-1, mouse mast cell protease-1; NF- κ B, nuclear factor κ B; OVA, ovalbumin; OXT, oxytocin; OXTR, oxytocin receptor; TSLP, thymic stromal lymphopoietin; WT, wild type.

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Table 1
Characteristics of patients with food allergy and healthy controls.

Participants	Age	Sex
Children (n = 25)	Median age, range (months)	Boys/Girls (n)
Patients (n = 13)	14, 1–31	8/5
Healthy controls (n = 12)	18.5, 3–38	7/5
Adults (n = 23)	Median age, range (years)	Male/Female (n)
Patients (n = 12)	33, 24–51	7/5
Healthy controls (n = 11)	30, 22–46	7/4

important component of the neuroendocrine immune network [11,12]. An increasing number of studies have indicated that OXT can act as a “natural medicine” to fulfill anti-inflammatory roles [13]. Many protective effects of OXT appear to be mediated through its receptors, OXTRs. OXTRs, which belong to G protein-coupled receptors (GPCRs), are widely distributed throughout central and peripheral organs, tissues, and cells [9]. Studies from our group [14–16] and other studies [17–20] have demonstrated that OXT is produced by enteric neurons and that OXTRs are expressed in the intestinal epithelium. However, no studies have investigated the effect of OXT on food allergy, especially in relation to the proallergic cytokines TSLP, IL-25 and IL-33, which are secreted by IECs.

In the current study, we investigated the protective properties and underlying mechanisms of OXT/OXTR signaling in food allergy. For the first time, we analyzed the OXT levels in patients with food allergy compared with healthy controls. We evaluated whether the OXT/OXTR signaling pathway influenced the production of TSLP, IL-25 and IL-33 in human intestinal epithelial cells line and in mouse models and sought to explore its related molecular mechanisms.

2. Materials and methods

2.1. Patients and controls

Human plasma samples were obtained from patients with food allergy and nonallergic healthy controls at Shandong Provincial Hospital. The exclusion criterion was the presence of major physical or gastrointestinal diseases. None of the female participants were pregnant or breastfeeding. The characteristics of the participants are shown in Table 1.

2.2. Experimental animals

BALB/c mice were obtained from the Animal Center of Shandong University. OXTR floxed (OXTR^{fl/fl}) mice were purchased from the Jackson Laboratory (stock No. 008471). Ubc-Cre transgenic mice were gifts from Dr. Liu Shangming (Shandong University). OXTR knockout (OXTR^{-/-}) mice were obtained by crossing OXTR floxed (OXTR^{fl/fl}) mice with ubc-Cre transgenic mice, and their OXTR^{fl/fl} littermates were used as wild-type (WT) controls.

All experimental mice were male and six-to-eight weeks old. All experimental mice were cage-housed and maintained under pathogen-free conditions in animal care facilities. All mice were sacrificed by cervical dislocation and were anesthetized with sodium pentobarbital (100 mg/kg) if needed. All animal experiments were carried out in the Experimental Animal Center of Shandong University.

2.3. Drug administration and experimental design of mouse models

Mice were sensitized and orally challenged with ovalbumin (OVA) as previously described [21]. On day 0 and day 14, the mice were sensitized by intraperitoneal injection with 50 µg of OVA (grade V, A-5503; Sigma-Aldrich, St. Louis, Missouri, USA) adsorbed to 1 mg of aluminum potassium sulfate adjuvant (A-7210; Sigma-Aldrich) (OVA/alum) in 100 µl of sterile saline. From day 28, the mice were deprived of food for

4 h and treated by oral gavage with 50 mg of OVA in 250 µl of sterile saline. Intragastric OVA challenge was repeated every other day. The control mice were treated identically with sterile saline.

From day 0, OXT (O6379; Sigma-Aldrich) dissolved in sterile saline was injected intraperitoneally once a day. The control mice were treated identically with sterile saline. The mice were treated with OXT or sterile saline 30 min prior to OVA administration.

The BALB/c mice were randomized into four groups (n = 6 per group): control group, OVA group, OXT group, and OVA+OXT group. The OXTR knockout mice and their littermate mice were divided into four groups (n = 6 per group): WT-control group, KO-control group, WT-OVA group, and KO-OVA group. All the experiments were repeated three times independently.

The diarrhea symptoms and fecal characteristics of all mice were monitored for up to 1 h after oral gavage. All mice were sacrificed 1 h after the last intragastric OVA challenge, and blood or jejunal samples were collected for analysis.

2.4. Assessment of diarrhea scores

Diarrhea of the mice with food allergy was scored as previously described in a blinded fashion by two independent investigators [22]: 0 = normal; 1 = soft; 2 = runny; 3 = liquid; 4 = bloody. Mice demonstrating liquid stool were recorded as positive for diarrhea.

2.5. Assessment of symptom scores

Symptom scores of the food allergy model mice were determined in a blinded fashion by two independent investigators as explained below. Allergic symptoms were assessed by using a scoring system as previously described [23,24]: 0 = no symptoms; 1 = rubbing, scratching, or both of the head, nose, or feet; 2 = puffiness around the eyes and mouth, pilar erection, reduced activity or standing still with an increased respiratory rate; 3 = wheezing, labored breathing, and cyanosis around the mouth and the tail; 4 = symptoms as those in no.3 with loss of consciousness, tremor or convulsion; 5 = death.

2.6. Hematoxylin-Eosin (HE) staining

Mouse jejunal tissues were fixed in 4% paraformaldehyde for 24 h and then rinsed, dehydrated, and embedded in paraffin. The tissues were sectioned into slices. Four- to five-micrometer-thick sections were stained with a hematoxylin-eosin staining kit (G1120, Solarbio, Beijing, China) and observed under a microscope.

2.7. Toluidine blue staining

Mouse jejunal tissues were fixed in 4% paraformaldehyde for 24 h and then rinsed, dehydrated, and embedded in paraffin. The tissues were sectioned into slices. Four- to five-micrometer-thick sections were stained with toluidine blue solution (G3670, Solarbio, Beijing, China) to distinguish mast cells.

2.8. Cell culture

HT-29 cells (Cell Bank of Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (HyClone, Utah, USA) containing 10% fetal bovine serum (Gibco, NY, USA) and 1% penicillin-streptomycin solutions (Gibco) in an incubator at 37 °C with 5% CO₂ and 95% humidity.

Lipopolysaccharide (LPS) (Sigma-Aldrich) was used to induce TSLP, IL-25 and IL-33 production. Bay11-7082 (Sigma-Aldrich) was used as a selective NF-κB inhibitor.

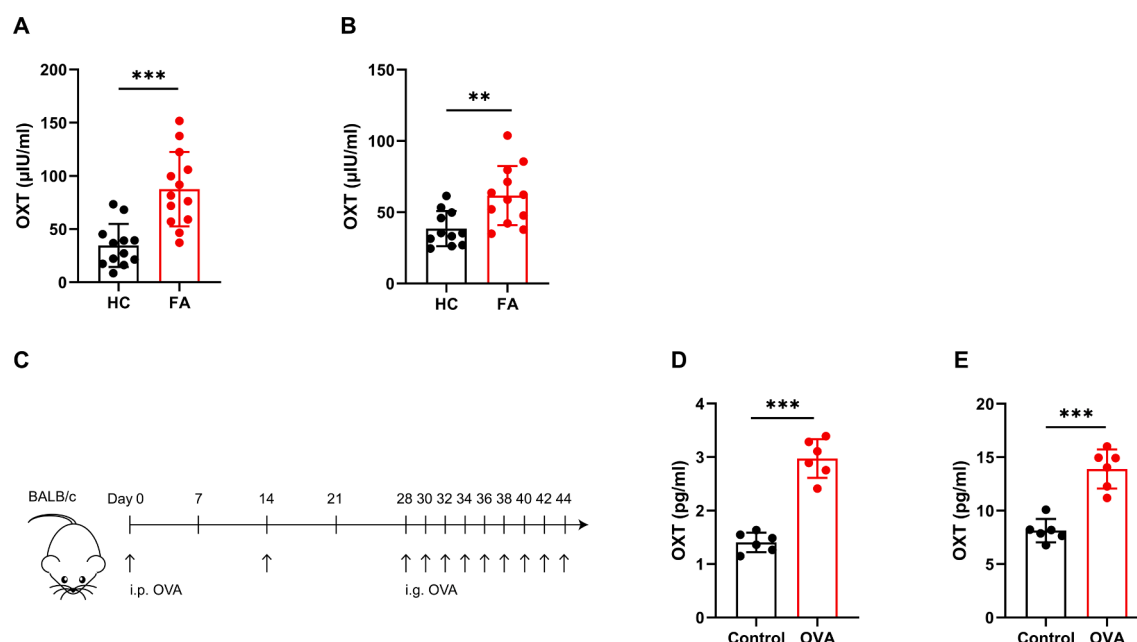


Fig. 1. The concentration of OXT in patients with food allergy and in OVA-allergic mice. (A) The concentration of OXT in plasma from 25 children was measured by ELISA. HC, healthy controls, $n = 12$. FA, patients with food allergy, $n = 13$. (B) The concentration of OXT in plasma from 23 adults was measured by ELISA. HC, healthy controls, $n = 11$. FA, patients with food allergy, $n = 12$. (C) According to the experimental protocol, BALB/c mice were sensitized to OVA/alum twice by intraperitoneal injection and challenged with OVA every other day. The concentrations of OXT in serum (D) and the jejunum (E) in each group were measured by ELISA. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.9. Small interfering RNA (siRNA)

HT-29 cells were planted in six-well plates at 40% confluence before transfection. Scrambled (Scr) siRNA, which was used as a negative control, and OXTR siRNA or β arr2 siRNA (GenePharma, Shanghai, China) were transfected into HT-29 cells using transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. The sequence of the siRNA targeting OXTR was 5'-GCGU-CAAGCUCAUCUCCAAUU-3', and the sequence of the negative control siRNA was 5'-UUCUCCGAACGUGUCACGUUU-3'. The sequence of the siRNA targeting β -arrestin2 was 5'-CCAACCUCUAUUGAAUUUGATT-3', and the sequence of the negative control siRNA was 5'-UUCUCCGAACGUGUCACGUTT-3'. The transfection efficiency was analyzed by western blotting after 72 h.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were left undisturbed at room temperature for 2 h and then centrifuged at 3000 rpm for 10 min at room temperature. Serum samples were collected for cytokine determination. Serum levels of total IgE (BioLegend, San Diego, CA, USA), OVA-specific IgE (BioLegend), mouse mast cell protease-1 (mMCP-1) (Invitrogen, Waltham, MA, USA) and OXT (CUSABIO, Wuhai, China) were quantified by ELISA kits according to the manufacturer's instructions.

Jejunal tissue samples were weighed and homogenized on ice. The homogenates were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatants were harvested for cytokine determination. The concentrations of histamine (Abcam, Cambridge, UK), IL-4 (R&D Systems, Minneapolis, MN, USA), IL-5 (R&D Systems), IL-9 (R&D Systems), IL-13 (R&D Systems) and OXT (CUSABIO) in tissue homogenates were quantified by ELISA kits according to the manufacturer's instructions.

The concentrations of TSLP (R&D Systems), IL-25 (R&D Systems) and IL-33 (R&D Systems) in mouse jejunal epithelium homogenates from which the serosa and muscularis had been gently stripped away were determined by ELISA kits according to the manufacturer's

instructions.

HT-29 cells were planted and cultured in six-well plates. After stimulation with drugs, the cells were homogenized and harvested for cytokine determination according to the manufacturer's instructions. The concentrations of TSLP (R&D Systems), IL-25 (R&D Systems) and IL-33 (R&D Systems) in HT-29 cell homogenates were determined by ELISA kits according to the manufacturer's instructions.

2.11. Western blotting

After treatment with specific drugs, fresh jejunal tissues from which the serosa and muscularis had been gently stripped away or HT-29 cells were collected in 200 μ l of RIPA-lysis buffer (Beyotime Biotechnology, Shanghai, China), homogenized, and centrifuged at 12000 rpm for 20 min at 4 °C. All protein samples were electrophoresed on a 5% to 10% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred onto 0.45-nm polyvinylidene fluoride (PVDF) membranes. After being blocked in blocking buffer (5% nonfat dry milk dissolved in Tween/Tris-buffered salt solution) for 1 h at room temperature, the PVDF membranes were immunoblotted with primary antibodies against OXTR (1:1000, Abcam, Cambridge, UK), NF- κ B (1:500, CST, Danvers, MA, USA), phospho-NF- κ B (1:500, CST), I κ B- α (1:500, CST), β -arrestin2 (1:500, CST), and β -actin (1:1000, ZSGB-Bio, Beijing, China) at 4 °C overnight. After three washes for 10 min each, the membranes were incubated with HRP-conjugated secondary antibodies (1:2000, ZSGB-Bio) for 1 h at room temperature. After three washes, chemiluminescence was detected using ECL Plus (Millipore Corporation, Billerica, MA, USA). The results were analyzed with ImageJ software.

2.12. Statistical analysis

The results between each group were analyzed by one-way analysis of variance (ANOVA) or two-tailed Student's t -test. The Kolmogorov-Smirnov test was used to determine whether the variables were

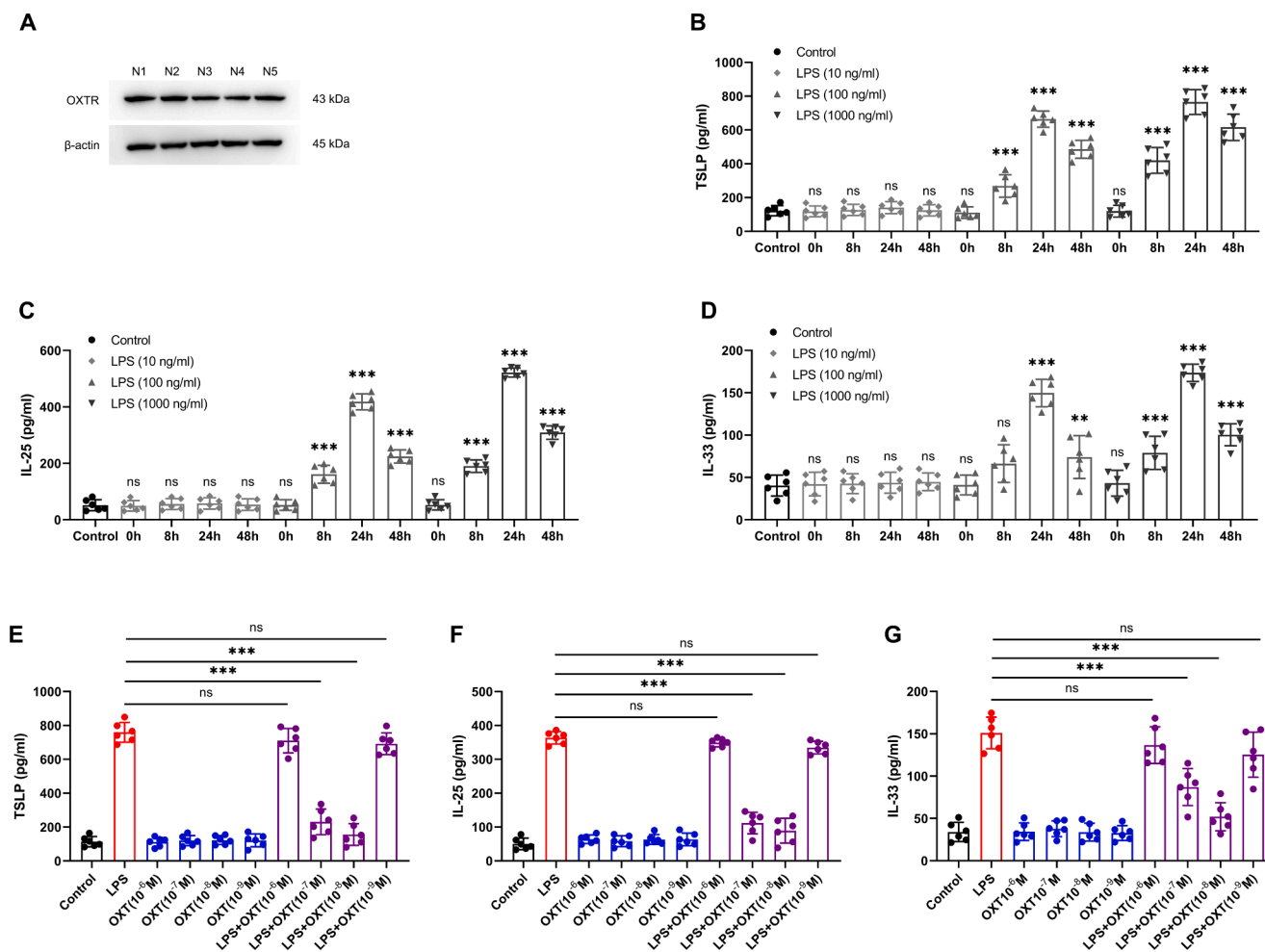


Fig. 2. OXT suppresses the production of TSLP, IL-25 and IL-33 in LPS-stimulated HT-29 cells. (A) OXTR expression in HT-29 cells was measured by western blotting. $n = 5$ in each group. HT-29 cells were stimulated with different concentrations (10 ng/ml, 100 ng/ml, or 1000 ng/ml) of LPS for different periods of time (0 h, 8 h, 24 h, 48 h). The levels of TSLP (B), IL-25 (C) and IL-33 (D) in HT-29 cell lysates from each group were measured by ELISA. $n = 6$ in each group. HT-29 cells were pretreated with different concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M) of OXT for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. The levels of TSLP (E), IL-25 (F) and IL-33 (G) in HT-29 cell lysates from each group were measured by ELISA. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

normally distributed. When variances were significantly different, the Kruskal-Wallis test or Mann-Whitney test was used. The data are expressed as the mean $\pm$ standard deviation (SD). All statistical analyses were carried out using GraphPad Prism 8 (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

2.13. Study approval

All the participants or their legal guardians were informed and consented to an approved protocol. The usage of human plasma samples was approved by the Medical Ethics Committee of Shandong University School of Basic Medicine Sciences (ECSBMSSDU2018-1-039).

All animal procedures were performed in accordance with the Medical Ethics Committee for Experimental Animals of Shandong University School of Basic Medicine Sciences (ECSBMSSDU2020-2-006).

3. Results

3.1. Abnormal OXT levels in patients with food allergy and OVA-allergic mice

Interestingly, we found that patients with food allergy exhibited higher plasma levels of OXT than the nonallergic healthy controls

(Fig. 1A-B). To further investigate this finding, we developed OVA-allergic mice as shown in Fig. 1C. The levels of OXT were elevated in the serum and intestines of OVA-allergic mice (Fig. 1D-E). OXT and OXTRs have been proven to be widely expressed in the gastrointestinal tract [17,20]. The concentration of OXT in the intestinal tissues was higher than that in the serum (Fig. 1D-E), indicating that OXT in the intestines may be mainly released from enteric neurons and may be involved in the development of intestinal inflammation of food allergy. Considering the immunologic and anti-inflammatory roles of OXT [13], we proposed that OXT might participate in protection against intestinal inflammation of food allergy.

3.2. OXT alleviates LPS-stimulated TSLP, IL-25 and IL-33 production in HT-29 cells

Our findings prompted us to study whether and how OXT participates in the regulation of allergic inflammation of the intestine. The epithelium-derived cytokines TSLP, IL-25 and IL-33, which can be triggered by endotoxin, are central regulators of allergic inflammation [7]. IECs, which are major sources of these proinflammatory cytokines, may be target cells of OXT. Therefore, we selected a human IEC line for a preliminary study. OXTRs are constitutively expressed in HT-29 cells (Fig. 2A). LPS stimulated the production of TSLP, IL-25 and IL-33 in a

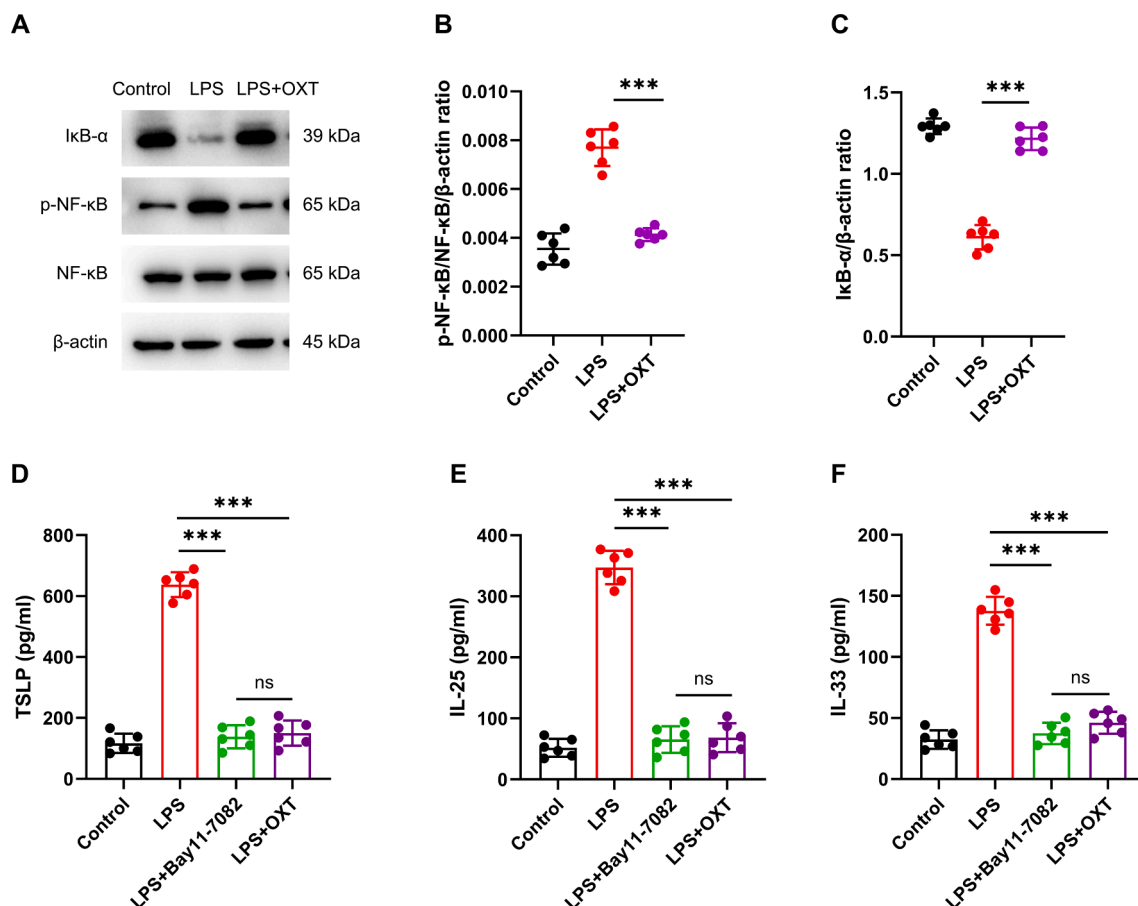


Fig. 3. OXT suppresses TSLP, IL-25 and IL-33 production in LPS-stimulated HT-29 cells by inhibiting NF-κB signaling. (A–C) HT-29 cells were pretreated with OXT (10^{-8} M) for 1 h and then stimulated with LPS (100 ng/ml). The protein expression of NF-κB signaling in HT-29 cells was measured by western blotting. The phosphorylation of NF-κB and protein levels of IκB-α were normalized to β-actin. $n = 6$ in each group. HT-29 cells were pretreated with OXT (10^{-8} M) or Bay11-7082 (5 μM) for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. The levels of TSLP (D), IL-25 (E) and IL-33 (F) in HT-29 cell lysates from each group were measured by ELISA. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

time-dependent manner and a concentration-dependent manner in HT-29 cells (Fig. 2B–D). Thus, in further studies, we stimulated cells with 100 ng/ml LPS for 24 h. HT-29 cells were pretreated with different concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M) of OXT for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. Pretreatment with 10^{-8} M or 10^{-7} M OXT had an inhibitory effect on the LPS-stimulated production of TSLP, IL-25 and IL-33; OXT alone had no influence on TSLP, IL-25 and IL-33 production (Fig. 2E–G). We chose 10^{-8} M OXT as the therapeutic concentration for further study in vitro.

3.3. In HT-29 cells, OXT inhibits TSLP, IL-25 and IL-33 production by downregulating NF-κB signaling

NF-κB is a significant transcription factor that initiates the transcription of genes associated with allergic inflammation. NF-κB binding with IκB-α is inactivated in the cytoplasm and becomes active through translocation to the nucleus after degradation of IκB-α. Western blot analysis demonstrated that pretreatment with OXT (10^{-8} M) effectively inhibited the phosphorylation of NF-κB and blocked the degradation of IκB-α in LPS (100 ng/ml)-stimulated HT-29 cells (Fig. 3A–C). To further investigate the possible mechanism by which OXT alleviates LPS-stimulated epithelial TSLP, IL-25 and IL-33 production, HT-29 cells were pretreated with OXT (10^{-8} M) or Bay11-7082 (5 μM) for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. We found that pretreatment with OXT (10^{-8} M) largely inhibited LPS-stimulated TSLP, IL-25 and IL-33 production in HT-29 cells and that the inhibitory efficiency

of OXT and Bay11-7082 was not significantly different (Fig. 3D–F). These results, although preliminary, suggest that OXT may decrease epithelial TSLP, IL-25 and IL-33 production by suppressing the activation of NF-κB signaling.

3.4. OXT suppresses TSLP, IL-25 and IL-33 production, in which β-arrestin2 participates

β-arrestin2 is a ubiquitously expressed cytosolic protein, and recent reports have shown that β-arrestin2 may modulate NF-κB signaling pathways by interacting with IκB-α [25,26]. Our study showed that pretreatment with OXT (10^{-8} M) enhanced the expression of β-arrestin2 in LPS (100 ng/ml)-stimulated HT-29 cells (Fig. 4A–B). We transfected HT-29 cells with a specific siRNA to knock down β-arrestin2 expression, and the control group was transfected with Scr siRNA (Fig. 4C–D). After transfection with βarr2 siRNA in HT-29 cells, pretreatment with OXT (10^{-8} M) had no effect on TSLP, IL-25 and IL-33 production stimulated by LPS (100 ng/ml) (Fig. 4E–G). Taken together, these results demonstrate that OXT reduces epithelial TSLP, IL-25 and IL-33 production largely by inhibiting the activation of NF-κB signaling, in which β-arrestin2 plays a key role in the connection between OXT and NF-κB signaling.

3.5. OXT inhibits TSLP, IL-25 and IL-33 production in vitro through OXTRs

We deleted OXTR in HT-29 cells to further investigate the

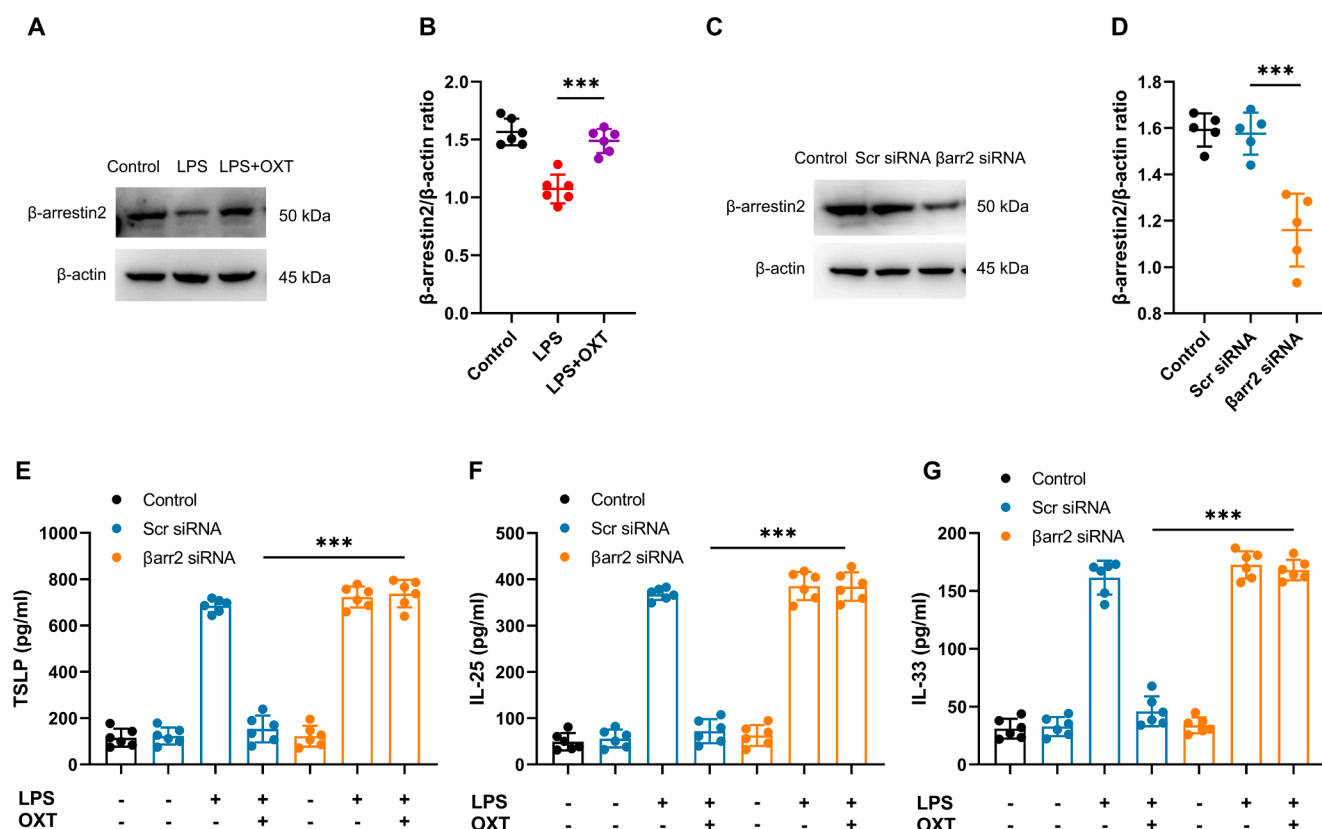


Fig. 4. OXT suppresses TSLP, IL-25 and IL-33 production in LPS-stimulated HT-29 cells by improving β -arrestin2 expression. (A-B) HT-29 cells were pre-treated with OXT (10^{-8} M) for 1 h and then stimulated with LPS (100 ng/ml). The protein expression of β -arrestin2 in HT-29 cells was measured by western blotting. The protein levels of β -arrestin2 were normalized to β -actin. $n = 6$ in each group. (C-D) In HT-29 cells, β -arrestin2 was knocked down by transfection with β arr2 siRNA for 72 h, and Scr siRNA was used as a control. The protein expression of β -arrestin2 in transfected HT-29 cells from each group was measured by western blotting. The protein levels of β -arrestin2 were normalized to β -actin. $n = 5$ or $n = 4$ in each group. After transfection, HT-29 cells were pretreated with OXT (10^{-8} M) and then stimulated with LPS (100 ng/ml). The levels of TSLP (E), IL-25 (F) and IL-33 (G) in HT-29 cell lysates from each group were measured by ELISA. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

mechanism of OXT. We reduced OXTR expression in HT-29 cells by using a specific siRNA against OXTR, and control HT-29 cells were transfected with Scr siRNA. The expression of OXTR was largely depleted in HT-29 cells transfected with OXTR siRNA compared with control cells (Fig. 5A-B). The production of TSLP, IL-25 and IL-33 stimulated by LPS (100 ng/ml) was significantly inhibited in OXT (10^{-8} M)-pretreated HT-29 cells transfected with Scr siRNA, while the anti-inflammatory role of OXT was abolished in cells transfected with OXTR siRNA (Fig. 5C-E). These results prove that OXTR is necessary for the anti-inflammatory effect of OXT, which involves a decrease in the production of epithelial TSLP, IL-25 and IL-33.

LPS (100 ng/ml)-stimulated phosphorylation of NF- κ B and degradation of I κ B- α were mainly inhibited by preincubation with OXT (10^{-8} M) in Scr siRNA transfected HT-29 cells, while OXT function was abolished in OXTR siRNA transfected cells (Fig. 5F-H). Moreover, the ability of OXT to upregulate β -arrestin2 expression was reversed in LPS-stimulated HT-29 cells by transfection with OXTR siRNA (Fig. 5I-J). After OXTR was depleted, OXT failed to suppress the activation of NF- κ B signaling stimulated by LPS in HT-29 cells, and its protective effect involving upregulation of β -arrestin2 expression was also eliminated. These results prove that OXT must rely on its receptor OXTR to suppress NF- κ B signaling and improve β -arrestin2 expression and thus inhibit epithelial cell-derived cytokines production in vitro.

3.6. OXT attenuates the development of allergic diarrhea in BALB/c mice

To explore the anti-inflammatory effects of OXT, an OVA-induced food allergy murine model was used. As shown in Fig. 6A, we

sensitized BALB/c mice to OVA/alum by intraperitoneal injection on days 0 and 14 and then repeatedly challenged them by intragastric OVA treatment every other day. BALB/c mice were treated with sterile saline or different concentrations (0.3 mg/kg, 1 mg/kg or 3 mg/kg) of OXT by intraperitoneal injection every day. We observed that 1 mg/kg OXT had a protective effect against the onset of OVA-induced allergic diarrhea, but neither 0.3 mg/kg nor 3 mg/kg OXT had a protective effect (Fig. 6B). Therefore, we identified 1 mg/kg as the therapeutic concentration of OXT for further study. We followed the protocol shown in Fig. 6A and found that BALB/c mice began to develop acute diarrhea upon the fourth OVA challenge, and all mice exhibited systemic anaphylaxis upon the ninth OVA challenge. BALB/c mice treated daily with 1 mg/kg OXT by intraperitoneal injection were more resistant than mice not treated with OXT up to the ninth OVA challenge; OXT alone had no influence on BALB/c mice (Fig. 6C). During OVA challenges, BALB/c mice treated with 1 mg/kg OXT had lower diarrhea scores and symptom scores than those not treated with OXT (Fig. 6D-E). This study indicates that OXT significantly protects BALB/c mice from the onset of OVA-induced diarrhea and moderates systemic anaphylaxis during OVA challenges.

3.7. OXT prevents TSLP, IL-25 and IL-33 production and alleviates allergic inflammation in OVA-induced BALB/c mice

BALB/c mice challenged with OVA showed a significant increase in the production of TSLP, IL-25 and IL-33 in the intestinal epithelium compared to control mice, and the production of TSLP, IL-25 and IL-33 was markedly decreased in mice treated with OXT (Fig. 7A-C). As is known, the secretion of TSLP, IL-25 and IL-33 controls the fate of

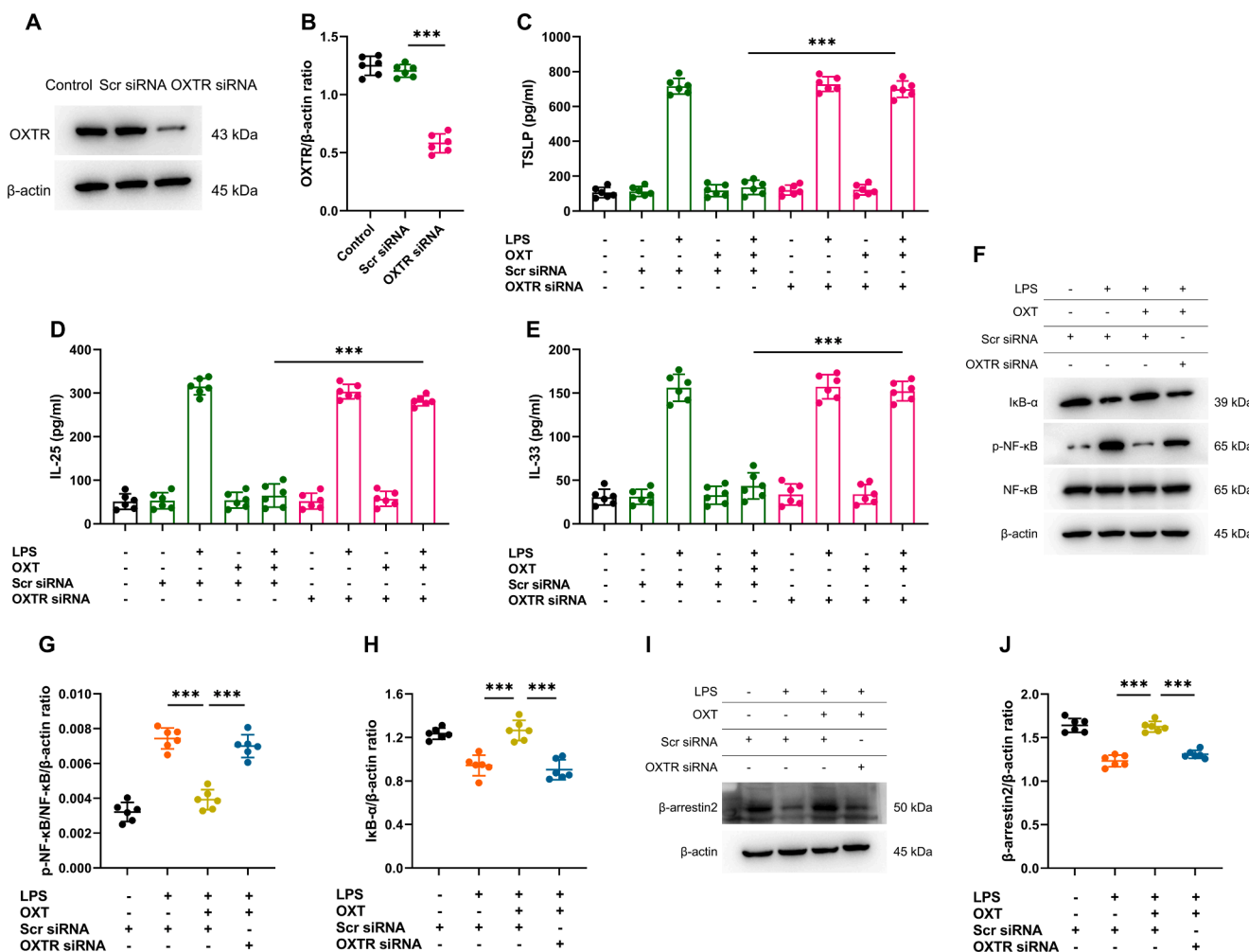


Fig. 5. OXT depresses TSLP, IL-25 and IL-33 production in LPS-stimulated HT-29 cells through OXTRs. (A–B) In HT-29 cells, OXTR was knocked down by transfection with an OXTR siRNA for 72 h, and Scr siRNA was used as a control. The protein expression of OXTR in transfected HT-29 cells from each group was measured by western blotting. The protein levels of OXTR were normalized to β-actin. $n = 6$ in each group. After transfection, HT-29 cells were pretreated with OXT (10^{-8} M) and then stimulated with LPS (100 ng/ml). The levels of TSLP (C), IL-25 (D) and IL-33 (E) in HT-29 cell lysates from each group were measured by ELISA. $n = 6$ in each group. (F–H) The protein expression of NF-κB signaling in HT-29 cells was measured by western blotting. The phosphorylation of NF-κB and protein levels of IκB-α were normalized to β-actin. $n = 6$ in each group. (I–J) The protein expression of β-arrestin2 in HT-29 cells was measured by western blotting. The protein levels of β-arrestin2 were normalized to β-actin. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

downstream allergic immune responses. Therefore, we evaluated the expression of various inflammatory mediators and cytokines on the final OVA challenge. BALB/c mice treated with OXT exhibited significantly decreased serum levels of IgE, OVA-specific IgE and mMCP-1 compared to mice not treated with OXT (Fig. 7D–F). The release of histamine and production of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 in the jejunum were notably reduced by OXT treatment (Fig. 7G–K). Histological assessment of mice treated with OXT showed milder epithelial damage, less inflammatory cell infiltration and less mast cell degranulation in comparison with mice treated without OXT (Fig. 7L–M). These results indicate that OXT depresses TSLP, IL-25 and IL-33 production and plays a potent and comprehensive anti-inflammatory role in OVA-allergic BALB/c mice.

3.8. OXT inhibits NF-κB signaling and upregulates β-arrestin2 expression in OVA-induced BALB/c mice

We evaluated the signaling pathways in the epithelium in OVA-induced BALB/c mice. OVA-allergic BALB/c mice showed intense activation of NF-κB signaling, including upregulation of the expression levels of phospho-NF-κB and downregulation of the expression levels of

IκB-α. OXT treatment significantly decreased the expression levels of phospho-NF-κB and increased the expression levels of IκB-α (Fig. 8A–C). Moreover, OXT treatment significantly enhanced the expression levels of β-arrestin2 (Fig. 8A, D). These results prove that OXT suppresses NF-κB signaling and improves β-arrestin2 expression in vivo, which is consistent with the in vitro results.

3.9. Deletion of OXTR in vivo promotes epithelial cell-derived cytokines and aggravates OVA-induced diarrhea

OXT has the highest affinity for OXTR, but it can also bind to vasopressin receptors (AVPR1A, AVPR1B, AVPR2) [27]. We successfully generated OXTR^{-/-} mice (Fig. 9A). OXTR^{-/-} mice are on a C57BL/6 background. Previous study showed that C57BL/6 mice were less sensitive to OVA sensitization and challenges than BALB/c mice [21]. As shown in Fig. 9B, OXTR^{-/-} mice exhibited acute diarrhea symptoms upon the second OVA challenge, and 100% of mice were symptomatic upon the fifth OVA challenge; however, littermates were resistant up to the sixth OVA challenge (Fig. 9C). OXTR^{-/-} mice had obviously higher diarrhea scores and symptom scores than their littermates, which showed no sign of diarrhea symptoms during OVA challenges (Fig. 9D–

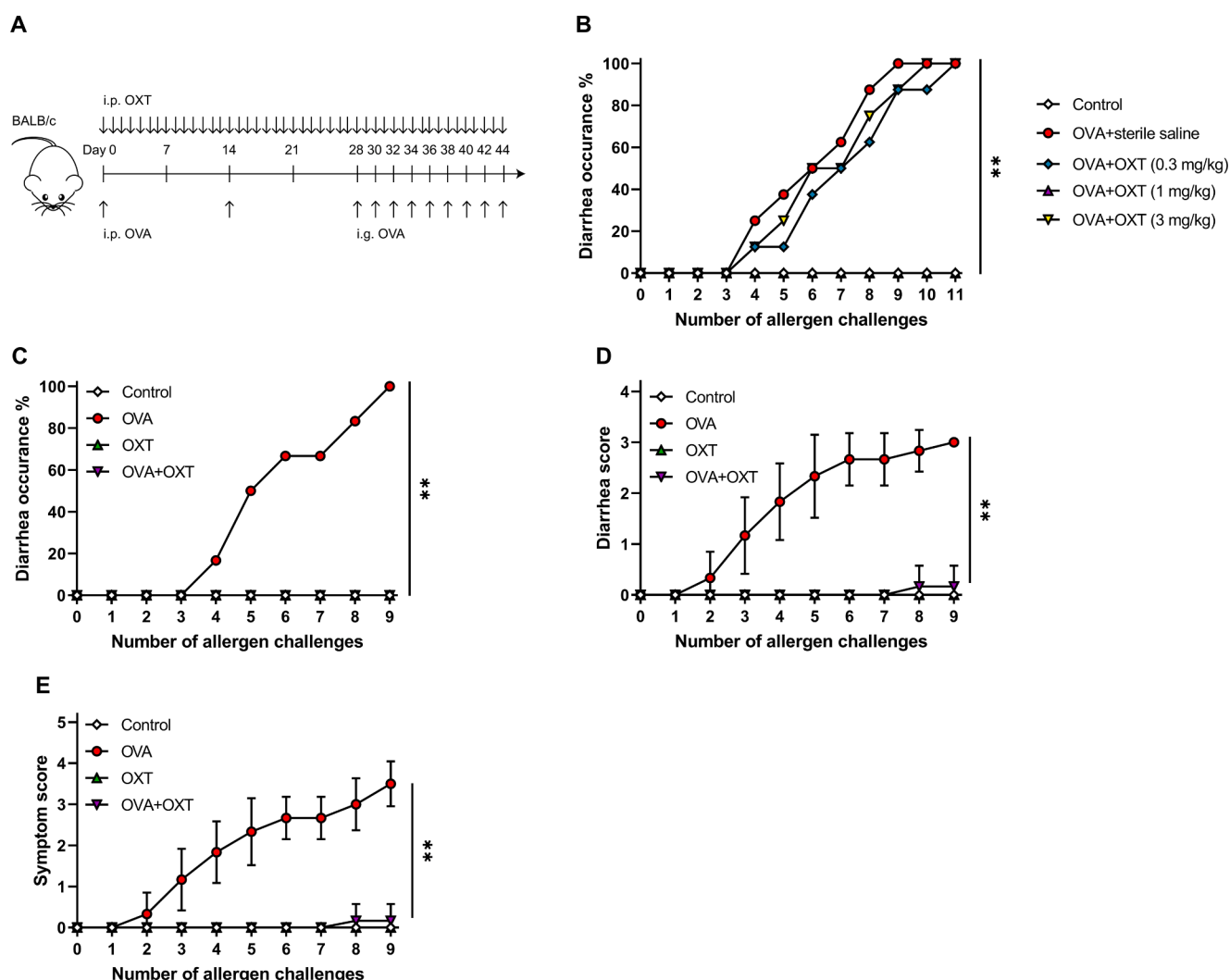


Fig. 6. OXT ameliorates acute diarrhea and systemic anaphylaxis in OVA-induced BALB/c mice. (A) According to the experimental protocol, BALB/c mice were sensitized by intraperitoneal injections of two OVA/alum and challenged intragastrically with sterile saline or OVA every other day. The mice were treated with sterile saline or OXT by intraperitoneal injection every day. (B) The mice were treated with sterile saline or different concentrations (0.3 mg/kg, 1 mg/kg, or 3 mg/kg) of OXT by intraperitoneal injection every day. Diarrhea occurrence in BALB/c mice in each group. $n = 8$ in each group. Diarrhea occurrence (C) and diarrhea scores (D) and symptom scores (E) of the different groups of BALB/c mice. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

E). The symptoms of food allergy induced by OVA in C57BL/6 mice knocking out OXTR were exacerbated, which mirrored the capacity of OXT to alleviate the food allergy.

The production of TSLP, IL-25 and IL-33 in the epithelium was significantly increased in OVA-induced OXTR^{-/-} mice compared to their littermates (Fig. 10A-C). Serum levels of IgE, OVA-specific IgE and mMCP-1 were distinctly elevated in OXTR^{-/-} mice compared to their littermates (Fig. 10D-F). The release of histamine and the levels of IL-4, IL-5, IL-9, and IL-13 in the jejunum were greatly increased in OVA-induced OXTR^{-/-} mice compared to their littermates (Fig. 10G-K). Histological assessment of OVA-induced OXTR^{-/-} mice showed more severe epithelial damage, more inflammatory cell infiltration and more mast cell degranulation than littermates (Fig. 10L-M).

In the epithelia of OVA-induced OXTR^{-/-} mice, NF- κ B signaling remained extremely activated, as manifested by sustained phosphorylation of NF- κ B and enhanced degradation of I κ B- α ; moreover, down-regulation of β -arrestin2 expression was also observed (Fig. 11A-D).

In conclusion, these results prove that OXTRs are necessary for OXT to depress TSLP, IL-25 and IL-33 production and to perform antiallergic effects in vivo.

4. Discussion

For the first time, this study demonstrates the potential of OXT in the treatment of an antiallergic medicine for food allergy by suppressing the production of the epithelial cell-derived cytokines TSLP, IL-25 and IL-33. In vivo and in vitro, OXT, through OXTR, inhibited NF- κ B signaling in IECs, in which β -arrestin2 played an important role (Fig. 12). Our study was also the first to reveal abnormal levels of OXT in food-allergic humans and food-allergic mice. Exogenous OXT served as a “natural medicine” and alleviated systemic anaphylaxis and intestinal allergic inflammation in experimental food allergy. OXTR^{-/-} mice exhibited more severe allergic inflammation induced by OVA, suggesting that OXT has endogenous antiallergic capacities in the gut.

OXT is known to be a hormone produced by the supraoptic nucleus and paraventricular nucleus of the hypothalamus, stored in the posterior pituitary and released into circulation [9]. Previous studies have revealed that OXT can serve as a “natural medicine” capable of both preventing and treating various disorders, including autism, depression, anxiety, schizophrenia, addiction, pain, metabolic and digestive disorders, diabetes, cardiovascular diseases, infectious diseases and cancer

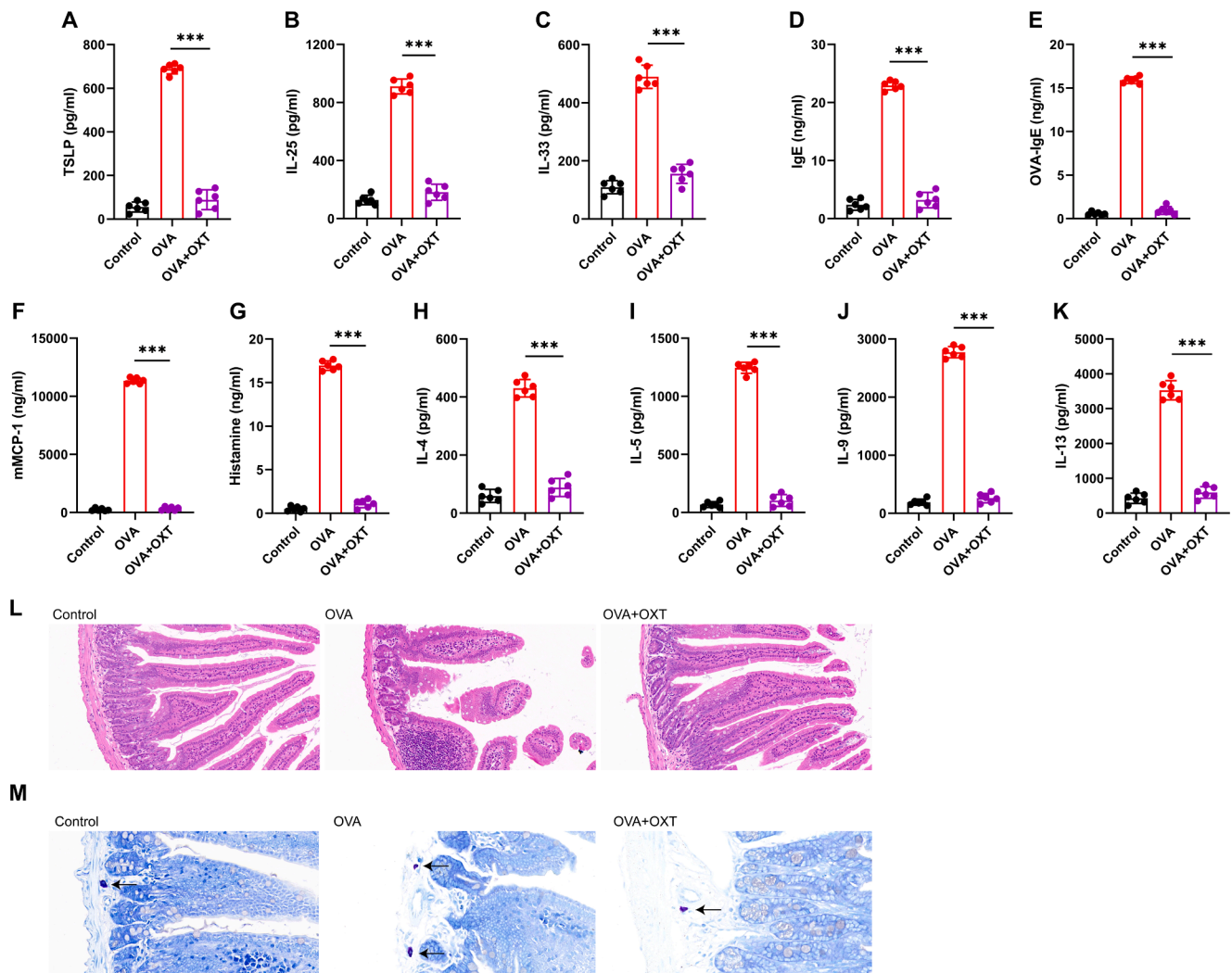


Fig. 7. OXT inhibits TSLP, IL-25 and IL-33 and alleviates allergic inflammation in OVA-induced BALB/c mice. BALB/c mice were treated with OXT during OVA administration. The levels of TSLP (A), IL-25 (B), and IL-33 (C) in the epithelium of jejunal tissues isolated from BALB/c mice were measured by ELISA. $n = 6$ in each group. The serum levels of IgE (D), OVA-specific IgE (E) and mMCP-1 (F) in each group were measured by ELISA. $n = 6$ in each group. The levels of histamine (G), IL-4 (H), IL-5 (I), IL-9 (J), and IL-13 (K) in the jejunum were measured by ELISA. $n = 6$ in each group. (L) Representative HE-stained jejunal sections. (M) Representative toluidine blue-stained jejunal sections. The black arrows indicate mast cells. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

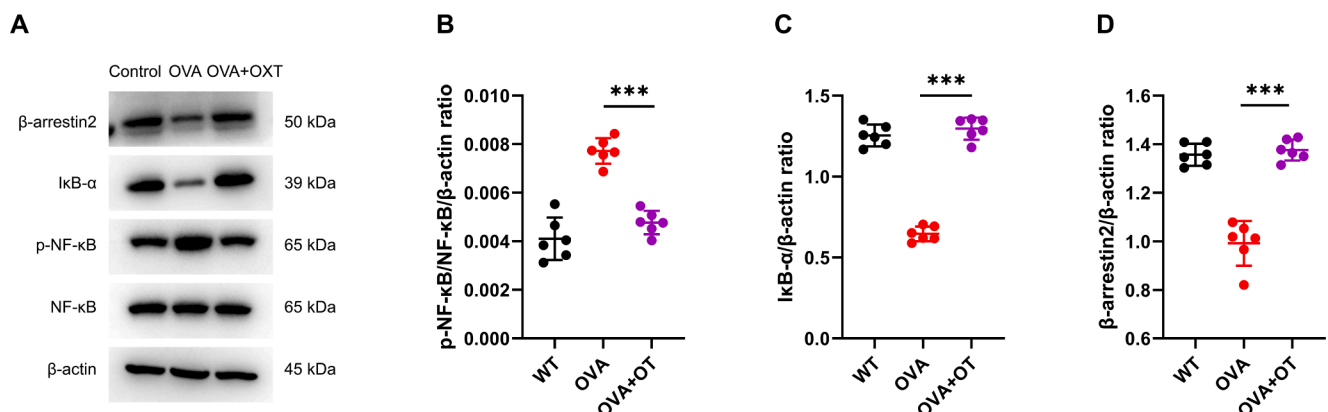


Fig. 8. OXT inhibits NF- κ B signaling and promotes β -arrestin2 expression in OVA-induced BALB/c mice. BALB/c mice were treated with OXT during OVA administration. (A-D) The protein expression of NF- κ B signaling and β -arrestin2 in the epithelium of the jejunum was measured by western blotting. The phosphorylation of NF- κ B and the protein levels of I κ B- α and β -arrestin2 were normalized to β -actin. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

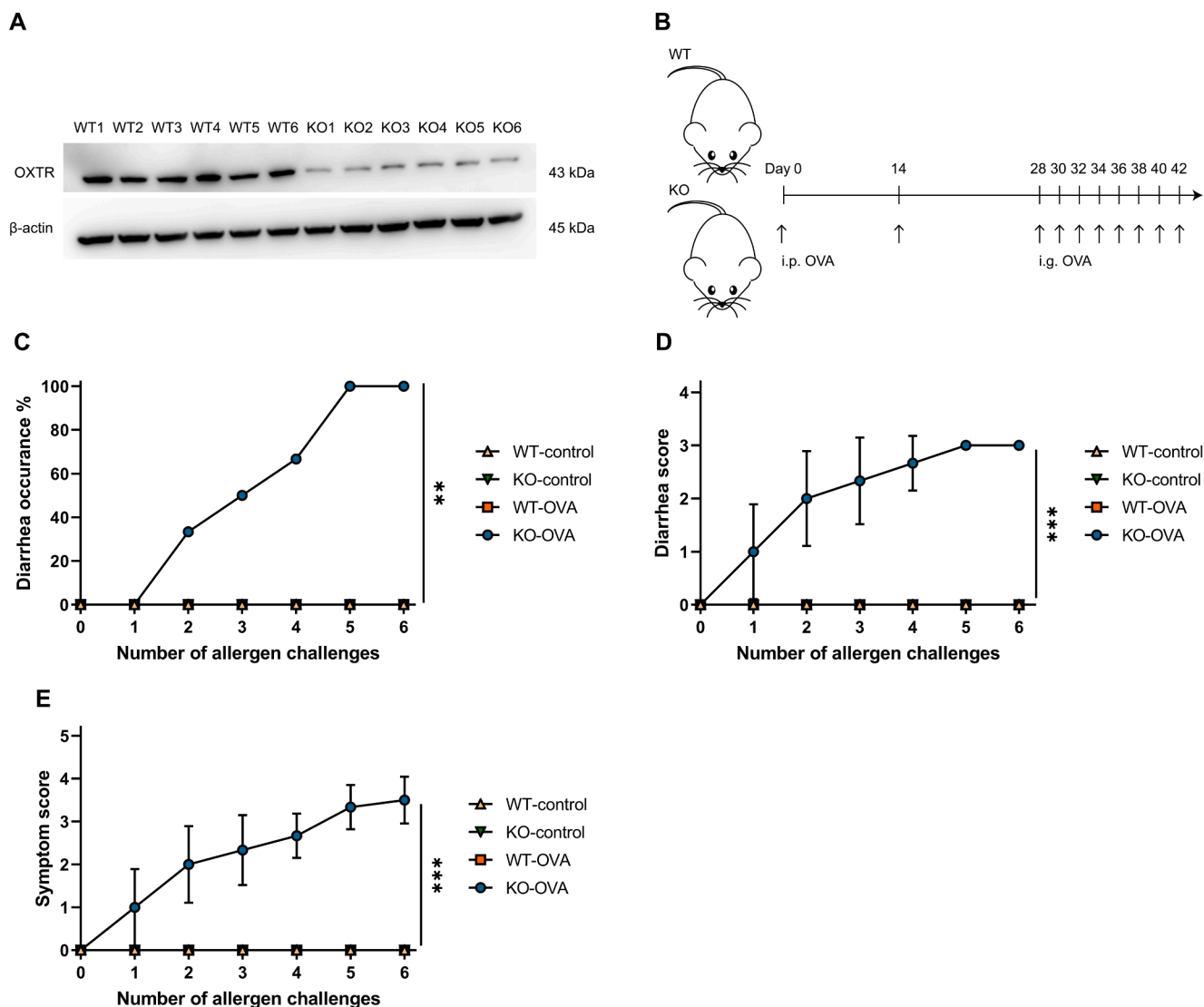


Fig. 9. OXTR^{-/-} mice develop more severe acute diarrhea and systemic anaphylaxis induced by OVA. (A) OXTR expression in OXTR^{-/-} mice and their littermates was measured by western blotting. $n = 6$ in each group. (B) According to the experimental protocol, OXTR^{-/-} mice and their littermates were sensitized to OVA/alum by two intraperitoneal injections and challenged intragastrically with sterile saline or OVA every other day. Diarrhea occurrence (C), diarrhea scores (D) and symptom scores (E) of the different groups of OXTR^{-/-} mice compared with their littermates. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[13]. Endogenous and exogenous OXT exerts its effects via OXTRs, which belong to the class I GPCR superfamily. OXT and OXTRs have been reported to be extensively distributed throughout central and peripheral tissues, including the gastrointestinal tract [20]. Recently, an increasing number of studies have shown that OXT, characterized as a crucial neuroimmunomodulator, has anti-inflammatory functions and that OXT signaling affects immunologic surveillance, defense and homeostasis [11,12]. OXT and OXTRs are constitutively expressed in the enteric nervous system and intestinal epithelium [17,19]. OXT/OXTR signaling appears to play an important protective role in gut development and function, but the underlying mechanism needs to be further investigated [19,28,29]. Our previous studies have proven that OXT may alleviate colonic inflammation in dextran sulfate sodium (DSS)-induced colitis in mice [30]. However, no studies have investigated the interaction between OXT signaling and food allergy, which is a prevalent and distressing disease in humans. In addition, it has been revealed that OXT can be present and stable in human milk [31,32]. Nevertheless, there is nearly no information about the influence of OXT ingested via breastmilk in infants. As mentioned above, we wished to elucidate the important therapeutic role of OXT in food allergy and offer new insights

into the immunological benefits of breastfeeding.

Food allergy is an immune response to a specific food and usually begins in the first two years of life [1]. Common food antigens include eggs, milk, peanuts, wheat, fish, and so on. Egg and milk allergies are generally outgrown in childhood, but allergic reactions to peanuts and fish often persist throughout life [33]. Common manifestations of food allergy include nausea, vomiting, diarrhea, abdominal pain, early satiety and food impaction. The gastrointestinal tract is the main route of exposure to food allergens. As the first line of defense against food antigens, IECs serve an essential role in allergic sensitization by maintaining immune homeostasis through intestinal barrier function and the ability to actively regulate the inflammatory reaction. Allergens (proteins with unique properties or abundant nonprotein components of allergenic sources such as endotoxin and lipids), cell damage and recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) can promote the secretion of epithelium-derived cytokines (TSLP, IL-25, and IL-33), which “set” the immune system to Th2 responses and recruit effector cells to the gastrointestinal mucosa [5,6]. It has been proven that IECs constitutively express TSLP, IL-25 and IL-33 and are regarded as the

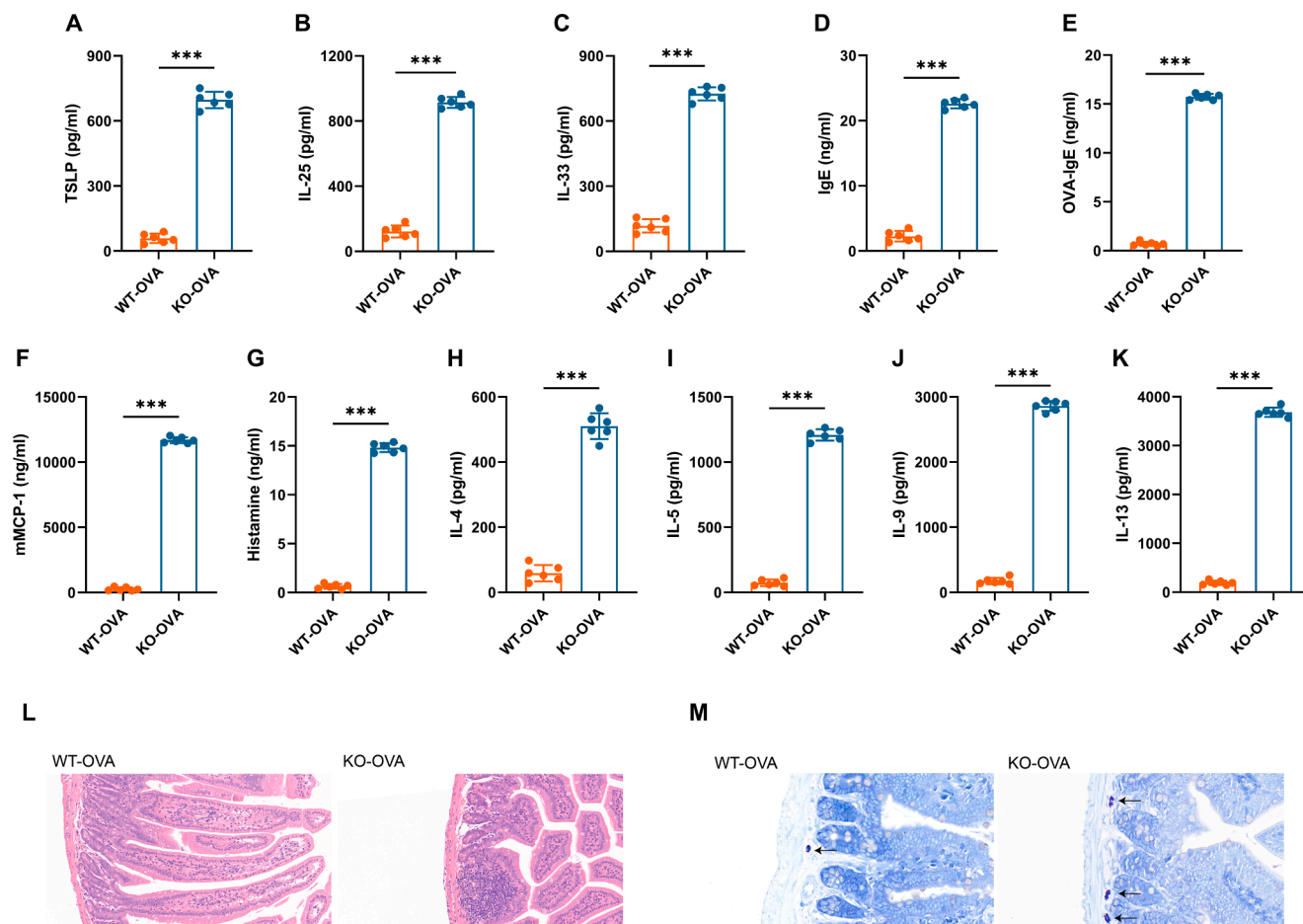


Fig. 10. OVA-induced OXTR^{-/-} mice develop aggravated epithelial cell-derived cytokines production and exacerbated allergic inflammation. Mice were administered with OVA. On the final challenge, the levels of TSLP (A), IL-25 (B) and IL-33 (C) in the epithelium of jejunal tissues isolated from OXTR^{-/-} mice and their littermates were measured by ELISA. $n = 6$ in each group. The serum levels of IgE (D), OVA-specific IgE (E) and mMCP-1 (F) in OXTR^{-/-} mice and their littermates were measured by ELISA. $n = 6$ in each group. The levels of histamine (G), IL-4 (H), IL-5 (I), IL-9 (J) and IL-13 (K) in the jejunum of OXTR^{-/-} mice and their littermates were measured by ELISA. $n = 6$ in each group. (L) Representative HE-stained jejunal sections. (M) Representative toluidine blue-stained jejunal sections. The black arrows indicate mast cells. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

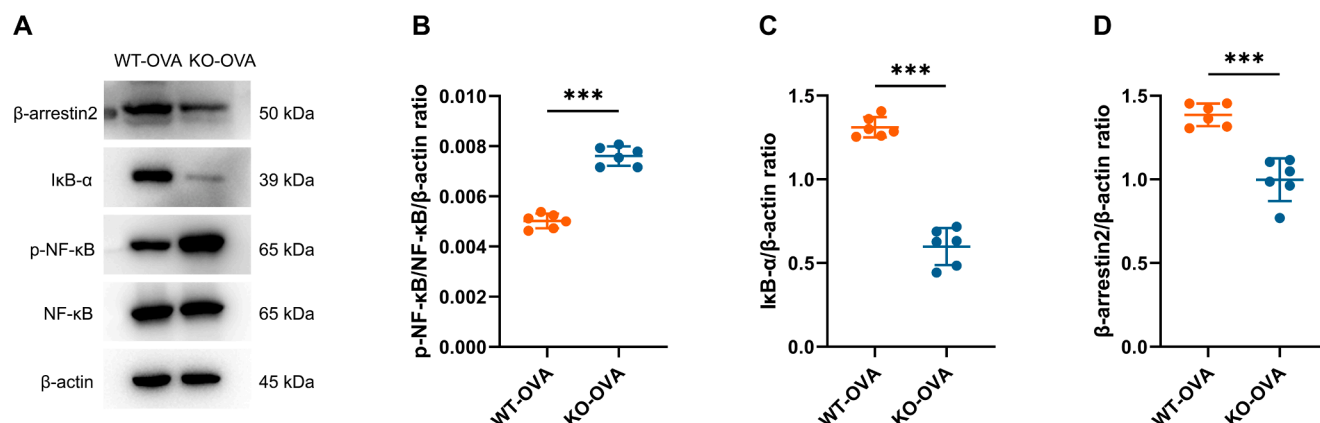


Fig. 11. OVA-induced OXTR^{-/-} mice develop promoted NF- κ B signaling and downregulated β -arrestin2 expression. Mice were administered with OVA. (A-D) The protein expression of NF- κ B signaling and β -arrestin2 in the epithelium of the jejunum isolated from OXTR^{-/-} mice and their littermates was measured by western blotting. The phosphorylation of NF- κ B and the protein levels of I κ B- α and β -arrestin2 were normalized to β -actin. $n = 6$ in each group. The results are expressed as the mean $\pm$ SD. The data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

primary source of TSLP, IL-25 and IL-33 under inflammatory conditions [34–38]. Previous studies have demonstrated that TSLP, IL-25 and IL-33 triggered by environmental stimuli are important initiators and

amplifiers of mucosal inflammation, particularly in eliciting an allergic Th2 response and suppressing inflammatory Th1 and Th17 responses [39–43]. Once oral tolerance is broken down and sensitization is

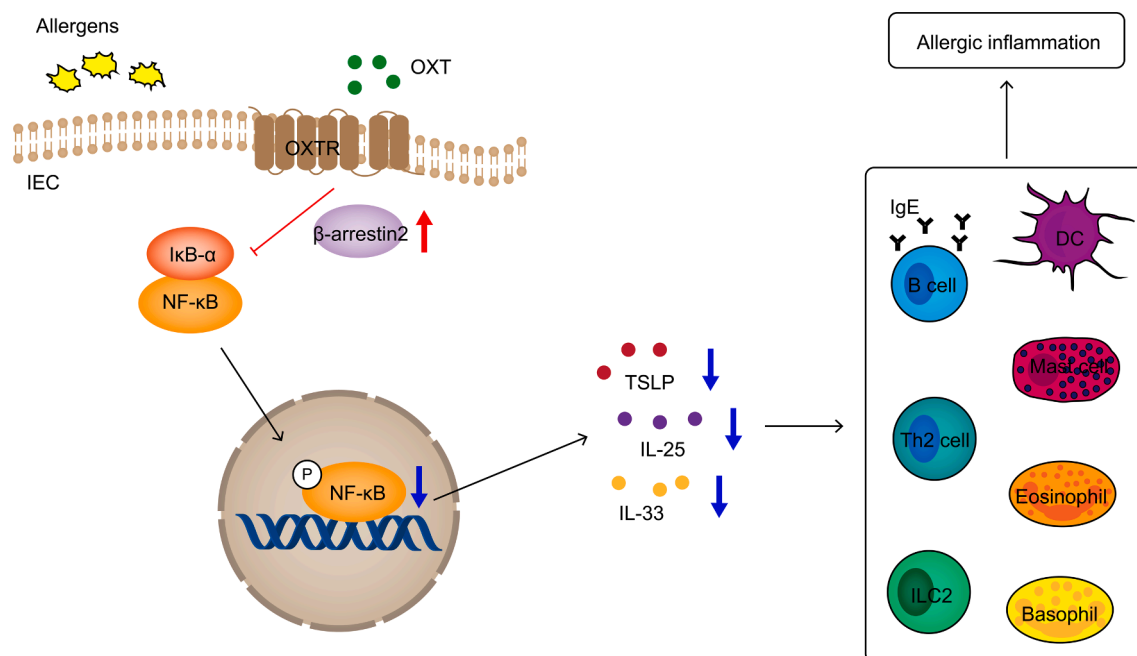


Fig. 12. Proposed mechanisms of the antiallergic effects of OXT in food allergy. OXT/OXTR signaling suppresses the production of TSLP, IL-25 and IL-33 in IECs, which are central regulators of allergic inflammation. OXT depends on OXTR to decrease the activation of NF- κ B signaling, in which β -arrestin2 participates.

established, individuals who are re-exposed to specific food allergens develop a cascade of food-specific IgE antibodies and a unique expression profile of inflammatory mediators and cytokines such as histamine, platelet-activating factor (PAF), IL-4, IL-5, IL-9 and IL-13 [44–48]. Three cytokines, i.e., TSLP, IL-25, and IL-33, have emerged as critical mediators throughout the process of allergic inflammation, including the initiation, regulation, and persistence of allergic disease, and may prove to be key targets for therapeutic intervention even when allergic responses are well established [7,49,50]. Combined neutralization of all three cytokines can effectively suppress murine food allergy [8]. While the anti-IL-25 antibody is still in the early stage of development, the anti-TSLP antibody tezepelumab and the anti-IL-33 antibody etokimab have entered clinical trials [7]. Thus, it is valuable to develop a drug that targets all three epithelial cell-derived cytokines. Here, in vitro and in murine models of food allergy, we concentrated on whether and how OXT influences the expression of epithelial cell-derived cytokines, with the expectation of providing a novel approach for attenuating food allergy in humans.

NF- κ B signaling is known as a prototypical proinflammatory signaling pathway and plays essential roles in inflammatory diseases, including gene expression and protein secretion of the epithelial cell-derived cytokines [6,51,52]. In this study, we demonstrated that NF- κ B signaling was involved in IECs to regulate the production of epithelial cell-derived cytokines and modulate the allergic response. Previous studies have confirmed that β -arrestin2, known as an adaptor protein and signal transducer, is a negative regulator of GPCR-dependent or GPCR-independent signaling pathways. Accumulating evidence has indicated that β -arrestin2 has the ability to negatively modulate the inflammatory response [53–57]. Moreover, β -arrestin2 has been reported to be a novel negative regulator of NF- κ B signaling [25,26,58,59]. β -arrestin2 may directly interact with I κ B- α to modulate NF- κ B activation [26]. Our previous study showed that β -arrestin2 plays an indispensable intermediate role between OXT signaling and NF- κ B signaling in macrophages [30]. Thus, we speculated that β -arrestin2 might contribute to the anti-inflammatory role of OXT signaling in IECs. Although the discovery in this article that β -arrestin2 participates in regulating the epithelial cell-derived cytokines production is innovative and needs to be further investigated, we preliminarily infer that the

strong anti-inflammatory role of OXT/OXTR signaling is owed to the direct interaction between β -arrestin2 and I κ B- α .

In summary, for the first time, we demonstrate that OXT/OXTR signaling has an antiallergic effect and suppresses the production of epithelial cell-derived cytokines (TSLP, IL-25 and IL-33) in vivo and in vitro. We reveal that OXT, through its receptor OXTR, inhibits TSLP, IL-25 and IL-33 by downregulating the activation of NF- κ B signaling and upregulating the expression of β -arrestin2. Moreover, we demonstrate that OXT effectively suppresses intestinal inflammation and alleviates OVA-induced allergic diarrhea. It is worth noting that patients with food allergy maintained high levels of OXT, indicating that OXT/OXTR signaling participates in the immune regulation of food allergy in humans. Although utilizing OXT as a “natural medicine” or creating safe drugs based on OXT may face various obstacles considering its unique evolutionary characteristics and complex physiologic properties, our present study paves new roads for the development of therapeutic and preventive strategies for food allergy in humans.

CRediT authorship contribution statement

Yiang Yu: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing - original draft. **Jingxin Li:** Conceptualization, Writing - review & editing. **Chuanyong Liu:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

The data supporting the findings of this study are available in the article and its supplementary material.

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