

Cold stress promotes IL-33 expression in intestinal epithelial cells to facilitate food allergy development

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ABSTRACT

Background: The causative factors and pathogenesis of food allergy (FA) is not fully understood yet. Cold stress (CS) occurs frequently in human life that influences physiological activities in the body. In this study, we aimed to investigate the chronic CS (CS) effects on promoting the expression of IL-33 in intestinal epithelial cells.

Methods: CS was carried out by placing mice at 4 °C for 1 h daily for 7 consecutive days. We developed a mouse model used to test the effects of CS on the FA development.

Results: We found that, similar to conventional FA mouse model, CS induced the core body temperature to drop markedly in mice, increased intestinal epithelial barrier permeability and facilitated FA development. CS promoted interleukin (IL)-33 expression in intestinal epithelial cells through the adrenocorticotrophic hormone (ACTH)/cortisol axis and via inducing the *Il33* promoter methylation. CS facilitated the FA development in mice, that could be blocked by depletion of IL-33 expression in intestinal epithelial cells.

Conclusions: CS induces IL-33 expression in intestinal epithelial cells to promote Th2 polarization in the intestinal tissues and facilitates FA development.

1. Introduction

Food allergy (FA) is an adverse immune response to food antigens by the intestinal immune system [1]. The FA pathogenesis is unclear. So far, we do not have any remedies to prevent FA, nor are any specific remedies available for curing FA [2]. FA symptoms vary from person to person; some have slightly abdominal discomfort while others may suffer from life-threatening anaphylactic shock [3]. If no medical intervention is carried out properly, complications may be caused by FA, such as FA-related asthma, dermatitis, inflammatory bowel disease (IBD) or autoimmune disorders [4–6]. Therefore, FA has been a negative impact on social economy and human health [7]. It is urgent to further study the FA pathogenesis and develop more effective and safe novel therapeutic remedies for FA.

Skewed T helper (Th)2 polarization is the canonical immunopathological feature of FA [8]. In the Th2 polarization status, Th2 cells over populate the local tissue to over produce Th2 cytokines, e.g.,

interleukin (IL)-5, IL-4 and IL-13 [9]. These cytokines, especially IL-4, induce plasma cells to produce antigen specific IgE [3]. IgE-sensitized mast cells can be activated by specific antigens to release allergy-related mediators to induce allergic responses [10]. Although the allergic pathological processes look like clear, the causative factors of allergic disorders remain elusive.

It is reported that IL-33 plays a critical role in the development of Th2 polarization [11]. IL-33 can be produced by several cell types, including endothelial cells, epithelial cells, fibrocytes, dendritic cells, mast cells, macrophages and osteoblasts [11]. By ligation its receptor, ST2, IL-33 can activate many inflammatory cells, including group 2 innate lymphoid cells (ILC2s), CD4⁺ T cells, B cells, mast cells, eosinophils and macrophages [11]. Yet, the underlying mechanism by which IL-33 involves the FA development needs to be further investigated.

The ambient temperature change is a natural phenomenon that occurs often in all seasons of a year. It is well-documented that cold

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stress (CS) triggers the virus-induced common cold, influenza or airway infection and affects immune function [12,13]. Previous studies showed that CS induced human intestinal barrier dysfunction [14,15]. While intestinal barrier disruption is associated with the pathogenesis of FA [16], whether chronic CS contributes to FA development remains uninvestigated. To this end, in this study, we observed the effects of CS on altering IL-33 expression by intestinal epithelial cells and facilitating FA development.

2. Materials and methods

2.1. Reagents

Antibodies of NFATc1 (7A6), occludin (E-5), ZO-1 (R40.76), IL-33 (APC), EpCAM (FITC), CD4 (Alexa Fluor® 488; GK1.5) and IL-4 (Alexa Fluor® 546; OX81), IL-5 (Alexa Fluor® 594, B-2), IL-13 (Alexa Fluor® 647, A-9,) were purchased from Santa Cruz Biotech (Santa Cruz, CA). RU486, ELISA kits of ACTH, cortisol, OVA-specific IgE, mMCP1, IL-5, IL-4 and IL-13 were provided by Dakewe Biomart (Beijing, China). TRIzol reagents, cDNA synthesis reagent kit, SYBR Green Master Mix, polyacrylamide gels, buffers for electrophoresis, protein G and chromatin immunoprecipitation kit were purchased from Invitrogen (Carlsbad, CA). Alum, KNK437, tamoxifen and FITC-dextran (40 kDa) were provided by Sigma Aldrich (St. Louis, MO).

2.2. Mice

C57BL/6 mice (6–8-week-old) were provided by the Guangzhou Experimental Animal Center (Guangzhou, China). The experimental mice were kept in an SPT (specific pathogen free) facility. The mice were allowed to access food and water freely. Mice with the *Il33* gene conditionally knocked out in epithelial cells were constructed by the Animal Institute, Agricultural University of China (Beijing, China). The development protocol is placed in the supplemental materials. The experimental protocol was approved by the Shenzhen University Animal Ethical Committee.

2.3. Treating mice with cold stress (CS)

A fridge (247 L) was purchased from Beijing Fuyi Ltc. (Beijing, China). The temperature of the fridge can be adjusted between room temperature and 4 °C. The fridge is equipped with an air circulation system and a glass door. To treat mice with CS, mice were placed in the fridge for 1 h. Before and after CS, we measured the core temperature from each mouse. Sham CS was carried out by placing mice in the same fridge for 1 h with temperature adjusted to 22 °C.

2.4. Development of an FA mouse model together with antigen and CS

Mice were treated with CS or sham stress together by gavage-feeding with OVA at 200 µg/mouse in 100 µl saline daily for 7 consecutive days. On day 8, mice were challenged by gavage-feeding 5 mg OVA/mouse in 0.1 ml saline and sacrificed 2 h later. After the challenge, the core temperature of each mouse was measured at 0, 30, 60 and 120 min. In the 2-h period after challenge, diarrhea was recorded. From each mouse, blood samples were collected; the serum samples were prepared and stored at –80 °C until use. Jejunal segments were excised. Proteins were extracted from the intestine and processed for further experiments. The mouse FA-related parameters were assessed in the routine procedures in our laboratory that can be found elsewhere [17]. Core temperatures of each mouse were recorded before and after CS using a rectal thermometer (Rectal Probe for Mice, Kent Scientific Corporation, Torrington, USA).

2.5. Development of FA mouse model with conventional procedures

Mice were subcutaneously injected with OVA (100 µg/mouse) mixed in 0.1 ml Alum on the back skin on day 0; the injection was repeated on day 6. The mice were then treated by gavage-feeding with OVA at 200 µg/mouse in 100 µl saline daily for 7 consecutive days (from day 7 to day 14). On day 15, mice were challenged by gavage-feeding 5 mg OVA/mouse in 0.1 ml saline and sacrificed 2 h later. The rest procedures were the same as the CS group.

2.6. Preparation of LPMCs (intestinal lamina propria mononuclear cells)

Intestinal tissues were cut into 2 × 2 × 2 mm pieces, that were incubated in a collagen IV solution at 1 mg/ml for half an hour. Samples were filtered through a cell strainer (70 µm diameter first, then 40 µm diameter). Single cells were processed with the Percoll gradient density centrifugation to isolate LPMCs. As assessed by Trypan blue exclusion assay, cell viability was greater than 99%.

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed with specific reagent kits according to the manufacturer's protocol to determine the levels of OVA-specific IgE and cytokines including ACTH, cortisol, IL-33, IL-4, IL-5 and IL-13 in the protein extracts of the intestinal tissues or serum. Each sample was tested in triplicate.

2.8. Permeability assay of intestinal epithelial barrier

Mice were gavage-fed with FITC-dextran (500 mg/kg, in 0.3 ml PBS, MW 40 kDa). One hour after the gavage-feeding, blood samples were collected. Serum FITC intensity was assessed with a fluorescent spectrometer (BioTek). A standard curve was created by serial dilution of FITC-dextran used to calculate FITC-dextran concentrations in the serum.

2.9. Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted with TRIzol reagents from cells of interest. The cDNA was synthesized with the RNA using a high-capacity cDNA reverse transcription Kit (Bio Rad) following the manufacturer's protocol. Samples were processed in a qPCR device (CFX96 Touch qPCR Detection System) with the SYBR Green Master Mix and the IL-33 primers (tcctgtctgctgaagtcaat aaggacgaagaaggcctgtt). The results were processed with the $2^{-\Delta\Delta C_t}$ method.

2.10. Preparation of protein extracts

Cells were lysed with a lysis buffer (10 mM KCl; 1.5 mM MgCl₂; 10 mM HEPES; 0.05% NP40; 0.5 mM DTT; 1 mM EDTA and protease inhibitor cocktail). The samples were centrifuged at 13,000 g for 10 min. Supernatant was used as the cytosolic protein extracts. The pellets were resuspended in a nuclear lysis buffer (0.2 mM EDTA; 4.6 M NaCl; 1.5 mM MgCl₂SO₄; 5 mM HEPES; 26% glycerol; 0.5 mM DTT) for 30 min. The samples were centrifuged at 13,000 g for 10 min. Supernatant was used as the nuclear protein extracts. Extract protein levels were determined by the BCA method. The experiments were performed at 4 °C.

2.11. Western blotting

Proteins (50 µg/ml) were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), that were transferred onto a polyvinylidene difluoride (PVDF) membrane. The following procedures were performed with the membrane sequentially: Blocking with 5% skim milk for 30 min; incubating with the primary antibodies

(1:500) of interest at 4 °C overnight; washing with TBST (Tris-buffered saline containing 0.05% Tween 20) 3 times; incubating with HRP-labeled secondary antibodies at ambient temperature for 2 h; washing with TBST 3 times. The immunoblots were revealed with the enhanced chemiluminescence, and then, the blots were photographed with an imaging device (UVP BioSpectrum Imaging System; Upland, CA). The integrated density of immunoblots was quantified with software ImageJ.

2.12. Chromatin immunoprecipitation (ChIP)

Cells were fixed with 1% formalin for 15 min to cross link the surrounding proteins and chromatin. Cells were lysed with a lysis buffer (10 mM KCl; 1.5 mM MgCl₂; 10 mM HEPES; 0.05% NP40; 0.5 mM DTT; 1 mM EDTA and protease inhibitor cocktail). The lysed cellular components were further treated with sonication to shear the DNA into small pieces (200–500 bp). The samples were added with protein G agarose beads and incubated for 2 h. The samples were centrifuged for 5 min at 5,000 g, the beads were discarded. Supernatant was collected and incubated with relevant antibodies or isotype IgG (negative controls) for 16 h. Samples were added with protein G agarose and incubated for 2 h to precipitated immunocomplexes. The beads were harvested by centrifugation for 5 min at 5,000 g. Beads were treated with an eluting buffer to elute protein/chromatin complexes. The samples were processed using a DNA extracting kit (Qiagen) following the manufacturer's protocol. The DNA was analyzed by qPCR following established protocols in the presence of a pair of *Il33* promoter primer (gagaaaggaccaaggagct and ctctccattgatgaccga).

2.13. Flow cytometry

In the surface staining, cells were incubated with antibodies (1:100; labeled with fluorochromes) of interest or isotype IgG (used as controls) for 30 min on ice. The cells were washed with PBS 3 times and analyzed with a flow cytometer (FACSCanto II; BD Bioscience). In the intracellular staining, cells were fixed with 1% paraformaldehyde containing 0.05% Triton X-100 for 1 h (using the latter for increasing the membrane permeability); washing with PBS 3 times, and then processed with the same procedures of the surface staining. The Data were processed with flowjo (TreeStar Inc., Asland, OR) with the data of isotype IgG staining as a gating reference.

2.14. Intestinal epithelial cell purification

Single intestinal cells were prepared first as described in the section of LPMC preparation. The epithelial cells were incubated with FITC-labeled anti-EpCAM (an epithelial cell marker) antibody for 30 min. The FITC⁺ cells were purified by cell sorting with a flow cytometer. Cell purity was more than 96% by assessing with flow cytometry.

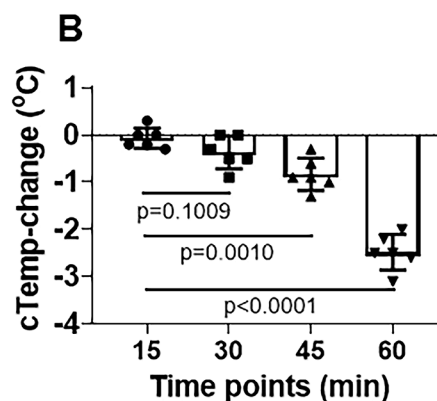
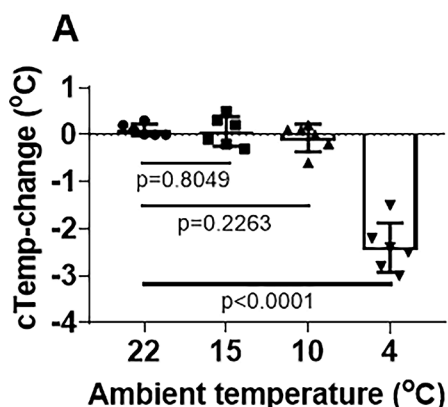


Fig. 1. Swiftly dropping ambient temperature (CS) alters core body temperature (cTemp). Mice were treated with CS at indicated temperature and indicated times. Bars show cTemp changes (mean $\pm$ SEM). Each dot presents data obtained from each mouse. Statistics: ANOVA followed by Dunnett's test. Results of ANOVA: $p < 0.0001$ in both A and B. Results of individual Dunnett's test are shown in the figures. The "22 °C" of panel A was used as the sham group.

2.15. Immune cell culture

RPMI1640 medium was used for cell culture. The medium was supplemented with fetal calf serum (10%), streptomycin (0.1 mg/ml), penicillin (100 U/ml), and glutamine (2 mM). Cell viability was assessed by Trypan blue exclusion assay, that was greater than 99%.

2.16. Methylation status of *Il33* promoter

The CpG island methylation status of *Il33* promoter was analyzed following published procedures [18]. DNA isolations from intestinal epithelial cells were carried out using a QIAGEN Blood & Tissue DNA extraction Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. The DNA samples were then analyzed with a DNA Methylation Enzyme Kit (SA Biosciences Corp; Frederick, MD) following the manufacturer's protocol together with *Il33* promoter primers (gagaaaggaccaaggagct and ctctccattgatgaccga).

2.17. Statistics

The "mean $\pm$ SEM" was used to present data in this study. The Student *t*-test was employed to determine difference between two groups. Analysis of variance (ANOVA) followed by the Bonferroni test or the Dunnett's test was employed in multiple comparisons. $P < 0.05$ was set as a significant criterion. Each sample was processed in triplicate.

3. Results

3.1. CS causes the core body temperature (cTemp) drop

A session of CS was to place mice at 22 °C, or 15 °C, or 10 °C, or 4 °C, for 1 h, that was carried out daily for 7 consecutive days. The cTemp was recorded from each mouse before and after the treatment. The results showed that 22 °C did not alter cTemp; 15 °C and 10 °C decreased cTemp variously and 4 °C evenly decreased the cTemp at 2–3 °C. In separate experiments, mice were treated with 4 °C for different time period, including 15 min, or 30 min, or 45 min or 60 min. The results showed that treating mice with 4 °C for 15 min or 30 min or 45 min could alter cTemp but not as stable as that altered by 4 °C for 1 h (Fig. 1). Thus, we set "treating mice with 4 °C for 1 h" as one session of CS.

3.2. CS increases cortisol levels in the intestinal tissues

Cortisol is one of the stress hormones [19]. CS is a kind of stress, that may alter the release of stress hormones, such as cortisol. Thus, after CS, we collected blood samples, serum samples were isolated and excised jejunal tissues. Proteins were prepared with the sampled tissues.

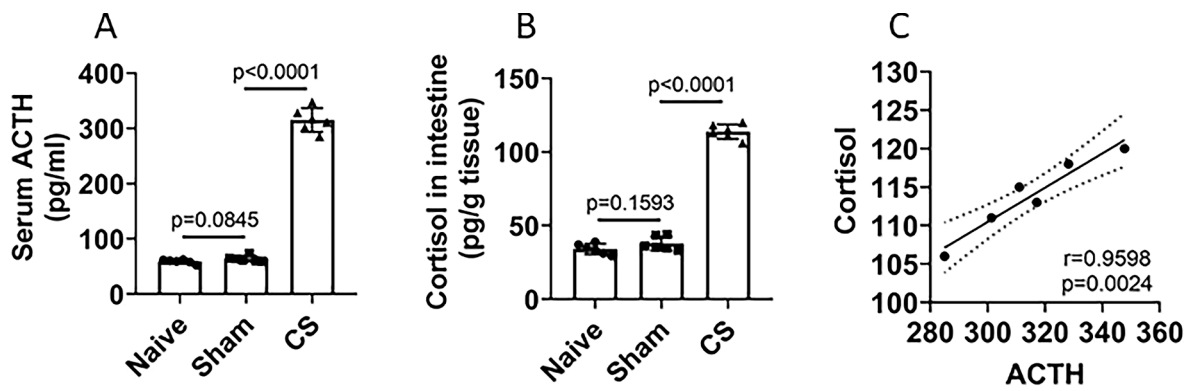


Fig. 2. CS alters mouse serum ACTH and intestinal cortisol. A, after CS, mice were sacrificed. Blood samples were collected from each mouse. The serum was analyzed by ELISA (in triplicate). Bars show serum ACTH levels in mice. B, gut tissues were excised. Proteins were extracted and processed by ELISA (in triplicate). Bars show cortisol levels in the proteins. C, a positive correlation between cortisol levels and serum ACTH in the gut tissues. Statistics: ANOVA followed by the Dunnett's test (A, B) and Pearson correlation assay (C).

Serum samples and intestinal tissues were analyzed by ELISA and Western blotting to assess serum ACTH levels and cortisol levels in the intestinal tissues. We found that markedly increased serum ACTH levels and cortisol levels in the intestinal tissues after the CS exposure (Fig. 2A-B). The serum ACTH levels and intestinal cortisol levels showed a positive correlation (Fig. 2C).

3.3. CS induces intestinal epithelial cells to express IL-33

Jejunal tissues were collected from mice 48 h after CS. Proteins were extracted from the intestinal tissues and analyzed by Western blotting. We observed that CS significantly increased IL-33 levels in the intestine (Fig. 3A-B); the IL-33 levels of the CS group were positively correlated with cortisol levels in the intestine (Fig. 3C). Single cells were isolated with the intestinal samples and analyzed by flow cytometry. The results showed that CS significantly increased the IL-33 expression in intestinal cells (Fig. 3D-E); IL-33⁺ cell counts were positively correlated with cortisol levels in the intestinal tissues (Fig. 3F). Furthermore, we found that more than 80% IL-33⁺ cells of the CS group were also EpCAM positive (Fig. 3G), indicating that most IL-33⁺ cells in the CS group are intestinal epithelial cells. The results demonstrate that CS induces intestinal epithelial cells to express IL-33.

3.4. CS induces NFATc1 expression to promote IL-33 expression in intestinal epithelial cells

In line with previous reports that NFATc1 was involved in regulating IL-33 expression [20], we also found the NFATc1 protein levels were significantly increased by CS in intestinal epithelial cells, that was abolished by pretreatment with the cortisol receptor antagonist, RU486 (Fig. 4A-C). We also found that CS increased the levels of NFATc1 at the *Il33* promoter, such an effect was also blocked by pretreatment with RU486 (Fig. 4D). CS also markedly up regulated the IL-33 expression in gut epithelial cells (Fig. 4E-G). The NFATc1 levels in the *Il33* promoter were positively correlated with the IL-33 expression in intestinal epithelial cells (H). In addition, we also found that CS induced hypomethylation status in the *Il33* promoter in intestinal epithelial cells (Fig. 4I).

3.5. Intestinal epithelial cell-derived IL-33 mediates chronic CS to facilitate Th2 polarization development in the intestine

As IL-33 plays a canonical role in Th2 polarization development [21], we assessed the Th2 response in the intestinal tissues of mice treated with CS for 7 consecutive days (this is the so called chronic CS) and mice treated with the conventional FA procedures. We found that CS induced marked Th2 polarization in the intestine, including high

Th2 cell frequency and high levels of Th2 cytokines in the intestinal tissues (Fig. 5A-C). Such an effect was abolished in mice with IL-33-deficient epithelial cells (Fig. 5D-F). The FA response in the CS group was similar to that in mice treated with conventional FA procedures (Fig. 5). The results demonstrate that chronic CS can induce the Th2 polarization in the intestine, in which intestinal epithelial cell-derived IL-33 plays a critical role.

3.6. IL-33 derived from intestinal epithelial cells mediates CS-facilitated FA development

WT mice and KO mice (intestinal epithelial cells in KO mice were IL-33 deficient) were treated with CS daily for one week with gavage-feeding with ovalbumin as a specific antigen. FA responses were assessed. We observed that after treating with CS, WT mice showed FA clinical responses, including core temperature drop and diarrhea (Fig. 6A-B), serum mouse mast cell protease-1 (mMCP1) and specific IgE (Fig. 6C-D) and increase in IL-33 expression in gut epithelial cells (Fig. 6E) and increase in intestinal epithelial barrier permeability (Fig. 6F), which was not detected in KO mice. Th2 cells and Th2 cytokines were also markedly increased in the gut tissues of mice treated with CS + OVA (not shown). The results demonstrate that CS can facilitate FA development, in which gut epithelial cell-derived IL-33 plays a critical role.

4. Discussion

The present study identified a novel factor in the induction of Th2 polarization in the intestine that facilitated FA development. By treating mice with CS, the core body temperature was dropped markedly and increased Th2 response in the intestine; the increase in IL-33 expression in intestinal epithelial cells was observed in CS-treated mice. These phenomena were positively correlated with the increase in cortisol in the intestinal tissues, indicating that cortisol mediates the effects of CS on IL-33 expression in intestinal epithelial cells and induces Th2 polarization as well as FA-like responses in the intestinal tissues. It is noteworthy that the FA response in the CS group was similar to those treated with the conventional FA procedures.

CS is a natural phenomenon that often occurs in human daily life. It is a common sense that CS is an important facilitating factor for virus-induced common cold and influenza [12,13]. The present data show that CS induced the core temperature drop in mice, this could be a natural response to ambient temperature changes to conserve energy as suggested by Reitman ML [22], while we found that in addition to the core temperature alteration, CS also impaired the epithelial barrier function, which is an important factor in the disruption of homeostasis in the body. Santos et al reported that, upon ice-cold water-stimulating

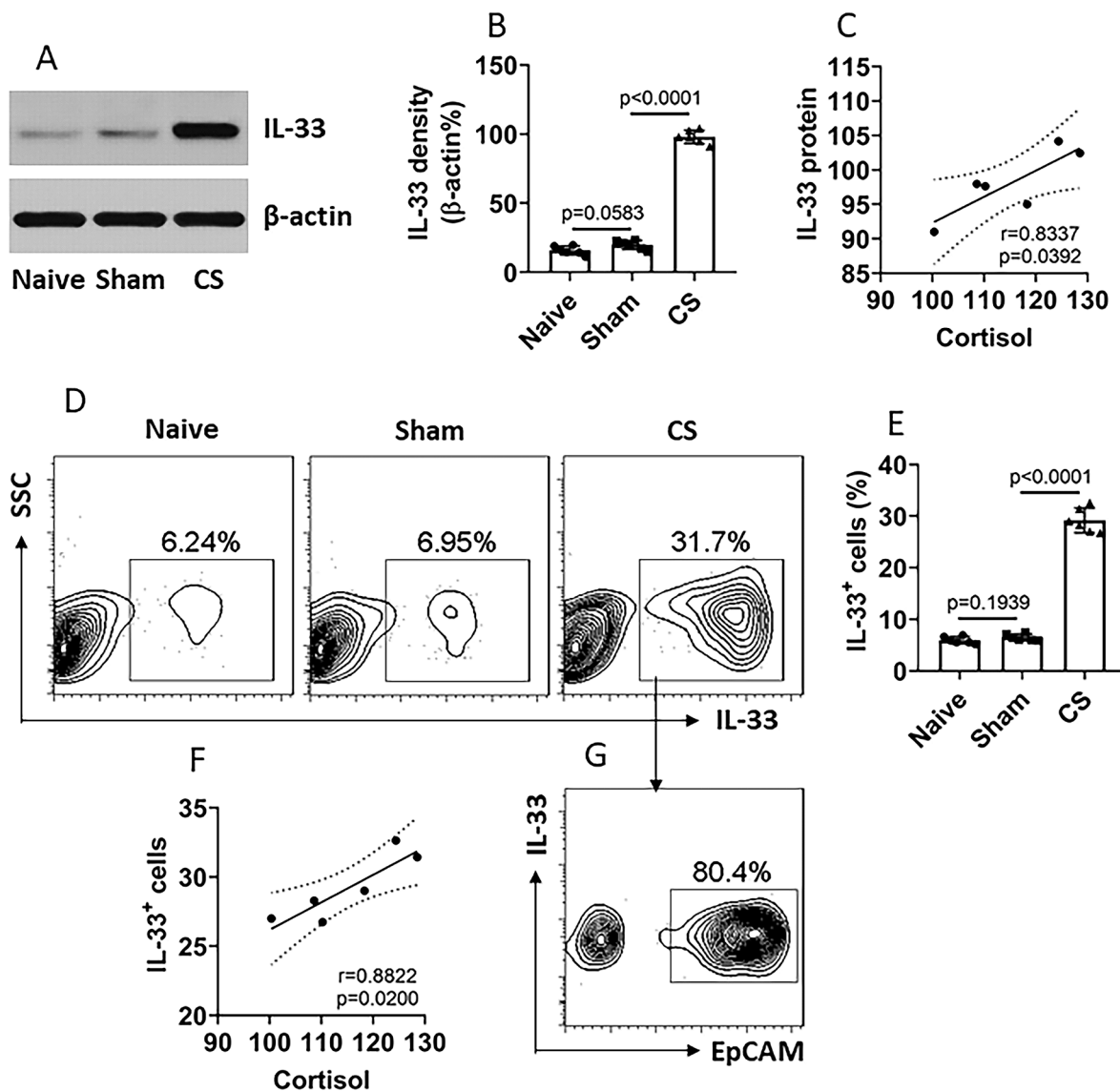


Fig. 3. CS increases IL-33 expression in the intestinal epithelial cells. After treating with CS for 1 h, mice were sacrificed; the jejunal tissues were excised from each mouse; proteins were prepared with the samples and analyzed by ELISA and Western blotting. A, immunoblots show IL-33 in the intestine. B, bars show the immunoblot image density. C, scatter dot plots show positive correlation between cortisol levels (data in Fig. 2) and IL-33 levels. D, gated flow cytometry plots show IL-33⁺ cells in mixed intestinal cells. E, bars show summarized IL-33⁺ cells from 6 samples per group. F, positive correlation between CS mouse intestinal IL-33⁺ cells and intestinal cortisol levels. G, gated plots show more than 80% IL-33⁺ cells in panel D (CS group) are also EpCAM positive. Each group consists of 6 mice. Statistics: ANOVA + the Dunnett's test.

human feet, significant increase in intestinal barrier permeability was observed [14,15]. Samak et al also observed this phenomenon that CS increased mouse intestinal epithelial barrier permeability [23]. Others found that passively cooled the human body skin delayed neural activities in somatosensory processing and the cognitive function in human [24]. CS causes tachycardia in mice was also reported [25]. Thus, it is the consensus that CS does alter the physiological activities in the body. The present data specify that CS also can alter gut epithelial cell activities through the ACTH/cortisol axis; this is consistent with previous studies; Zong et al found that cortisol could compromise gut epithelial barrier function through altering the tight junction associated protein expression [26].

It is reported that the cold ambient temperature is associated with human epigenetic modifications [27]. Allergic subjects show epigenetic changes; such as a number of epigenetic changes can be found in

allergic skin disorders [28]; food allergy subjects show the epigenetic dysregulation in the T cell receptor complex in the early stages of signal transduction [29]; influence of environmental factors in modification of epigenetic systems of patients with allergic diseases has attracted attention in recent years [30]. Our data suggest that CS is one of the environmental factors involves altering the epigenetic system in the body. The data show that upon CS, *Il33* promoter hypomethylation and increase in IL-33 expression were detected in mouse gut epithelial cells. Although the epithelial cells express IL-33 involving in the pathogenesis of allergic diseases was found in previous work [31], the causative factors of the IL-33 expression increased in epithelial cells remain to be elucidated. Our data provide novel information to this research slut that CS can increase IL-33 expression in gut epithelial cells by modifying its epigenetic aspects.

It is well-documented that IL-33 is a primary cytokine in the Th2

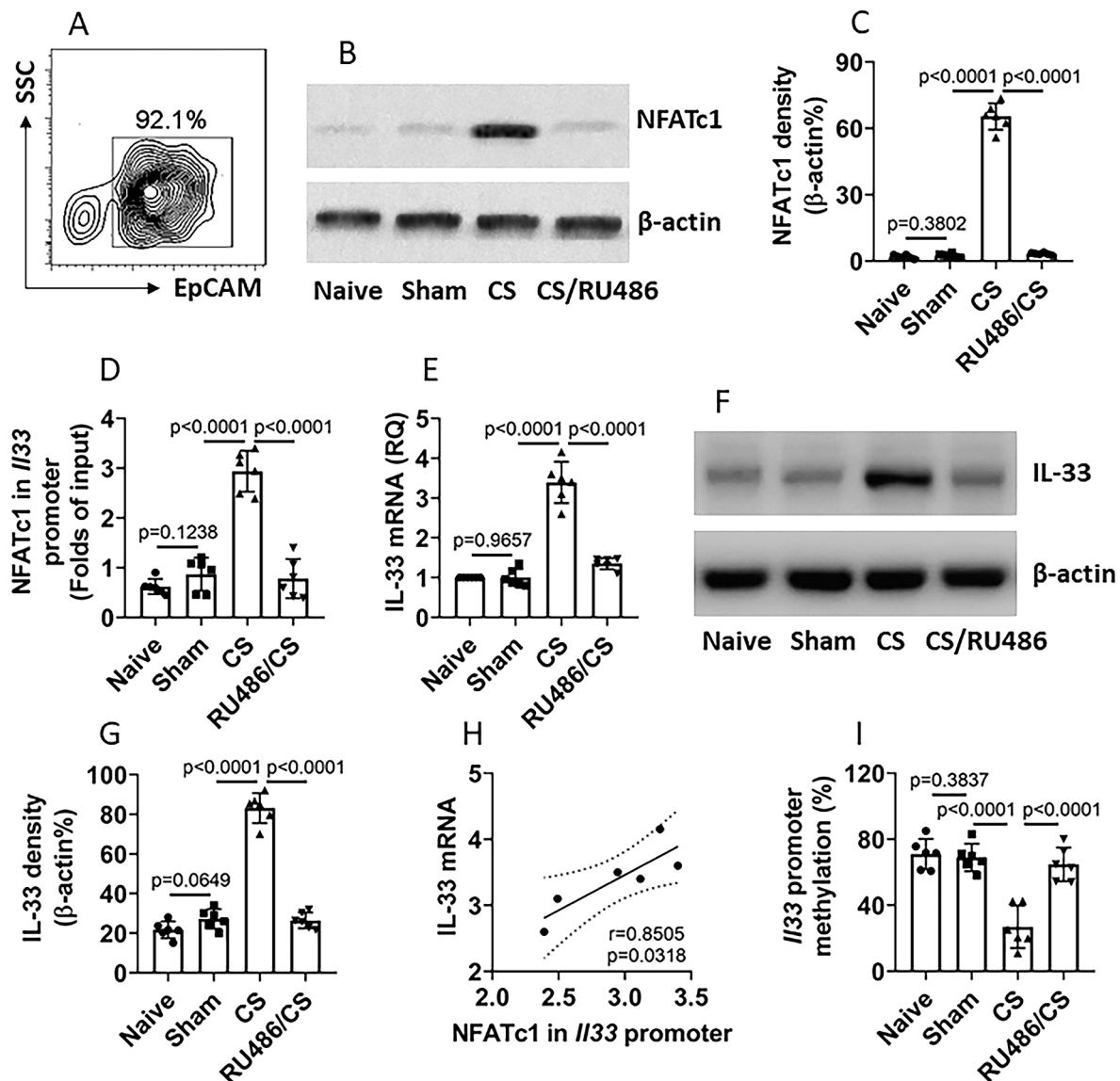


Fig. 4. NFATc1 mediates CS-increased IL-33 expression in intestinal epithelial cells. Mice were treated with CS; one of the groups of mice were pretreated with RU486 (a cortisol receptor antagonist). Jejunal segments were excised from each mouse after CS. Epithelial cells were purified from the gut tissues by flow cytometry cell sorting with the EpCAM as a cell marker. A, the gated flow cytometry plots indicate the cell purity of isolated gut epithelial cells. B-C, intestinal epithelial cell proteins were analyzed by Western blotting. Immunoblots indicate NFATc1 proteins in intestinal protein extracts. Bars show the immunoblots image integrated density. D, nuclear proteins of gut epithelial cells were assessed by ChIP. Bars show NFATc1 levels in the *Il33* promoter of intestinal epithelial cells. E-F, proteins and RNA were isolated from intestinal epithelial cells and assessed by Western blotting and RT-qPCR. Bars show *IL-33* mRNA (E) and immunoblots show *IL-33* proteins (F) in intestinal epithelial cells. G, bars show immunoblot integrated density of panel F. H, the dot plots show a positive correlation between NFATc1 in the *Il33* promoter and *IL-33* mRNA levels in intestinal epithelial cells. I, bars show *Il33* promoter methylation status in intestinal epithelial cells. Six mice in each group. Statistics: ANOVA + the Dunnett's test. RQ: Relative quantification. Each sample was analyzed in triplicate.

polarization initiation in the body [11]. Th2 polarization plays a critical role in the FA pathogenesis. The Th2 polarization causative factors remain to be further investigated. Our data provide mechanistic evidence that CS can increase the IL-33 expression by intestinal epithelial cells; IL-33 in turn initiates Th2 polarization in the intestine. After a relevant long time of CS exposure, or chronic CS, FA response was observed in mice. In this animal study, we observed epithelial cell-derived IL-33 played a critical role in FA development. Although IL-33 can be produced by several cell types, including endothelial cells, epithelial cells, fibrocytes, dendritic cells, mast cells, macrophages and osteoblasts [11], depletion of IL-33 expression in epithelial cells abolished

the CS-induced FA, indicating that the IL-33 from epithelial cells is the major source contributing to the CS-induced FA.

Our data indicate that chronic CS facilitates the FA development through inducing intestinal epithelial cells to produce IL-33. Others may ask a question whether people live in cold areas is more susceptible to suffering from FA. We have not found such information available in current literature; this needs to be further investigated.

In summary, our data show that CS can induce Th2 polarization in the intestine by promoting intestinal epithelial cells to produce IL-33. Chronic CS can facilitate the FA development.

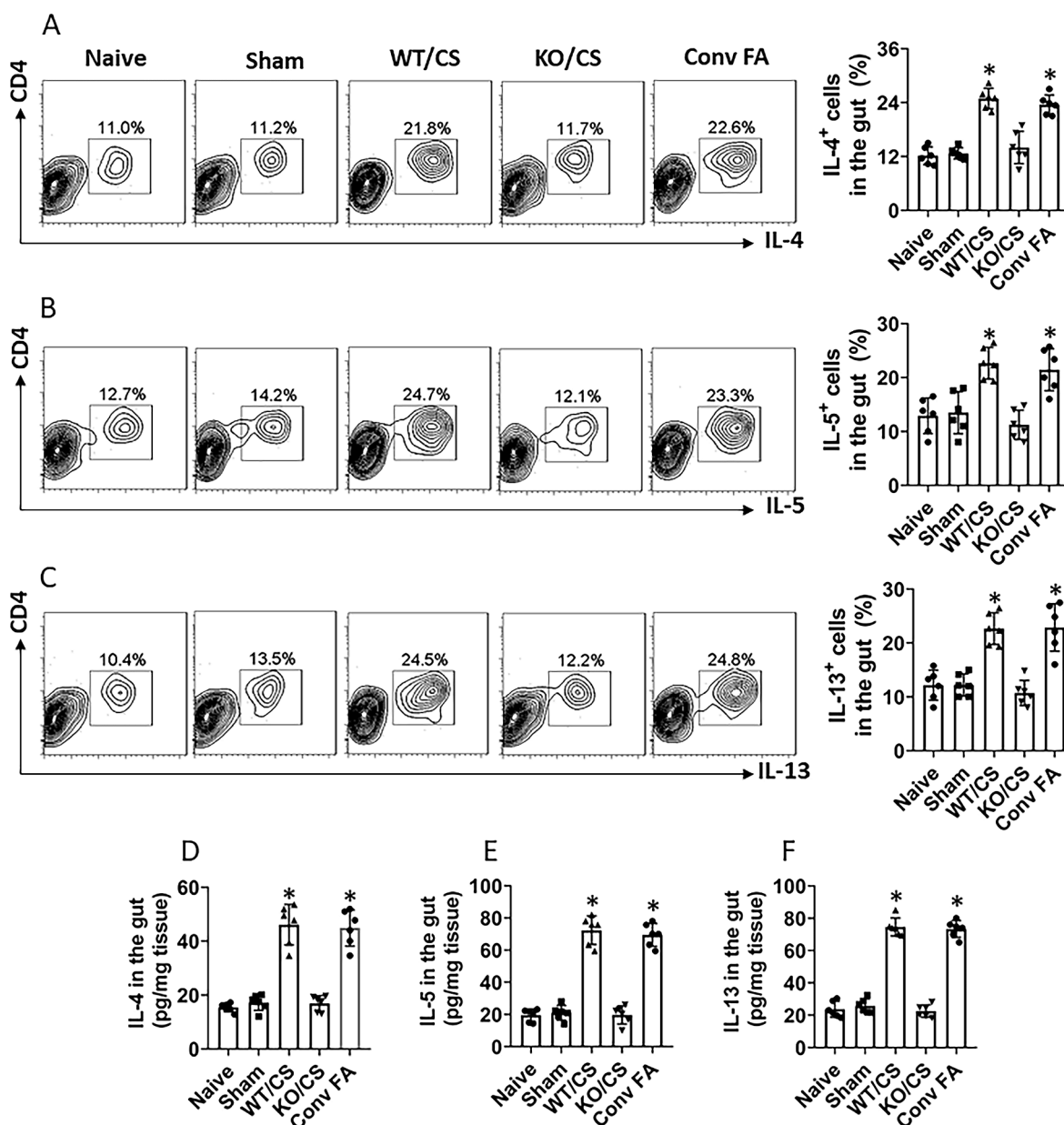


Fig. 5. Intestinal epithelial cell-derived IL-33 mediates CS-induced Th2 polarization in the gut. Wild type (WT) mice and KO mice (intestinal epithelial cells in KO mice were IL-33 deficient) were treated with CS daily for 7 days. A group of mice were treated with the conventional FA procedures (Conv). Jejunal tissues were collected from each mouse. A-C, LPMCs were separated from the gut tissues and assessed by flow cytometry. The gated plots indicate the Th2 cell counts in LPMCs. The bars indicate the summarized Th2 cell counts of 6 mice per group. D-F, the proteins were prepared with the gut tissues and assessed by ELISA. The bars indicate the Th2 cytokine amounts in the protein extracts. Each sample was tested in triplicate. Statistics: ANOVA followed by the Dunnett's test.

CRediT authorship contribution statement

Jiang-Qi Liu: Data curation, Investigation, Paper review. **Tian-Yong Hu:** Data curation, Investigation, Paper review. **Kai-Yuan Diao:** Data curation, Investigation, Paper review. **Dian Yu:** Data curation, Investigation, Paper review. **Yan-Nan Song:** Data curation, Investigation, Paper review. **Li-Hua Mo:** Data curation, Investigation, Paper review. **Gui Yang:** Data curation, Investigation, Paper review. **Zhi-Qiang Liu:** Investigation, Study management, Paper review. **Zhi-Gang Liu:** Investigation, Supervision, Paper review. **Ping-Chang Yang:** Conceptualization, Project design, Original paper preparation, Supervision.

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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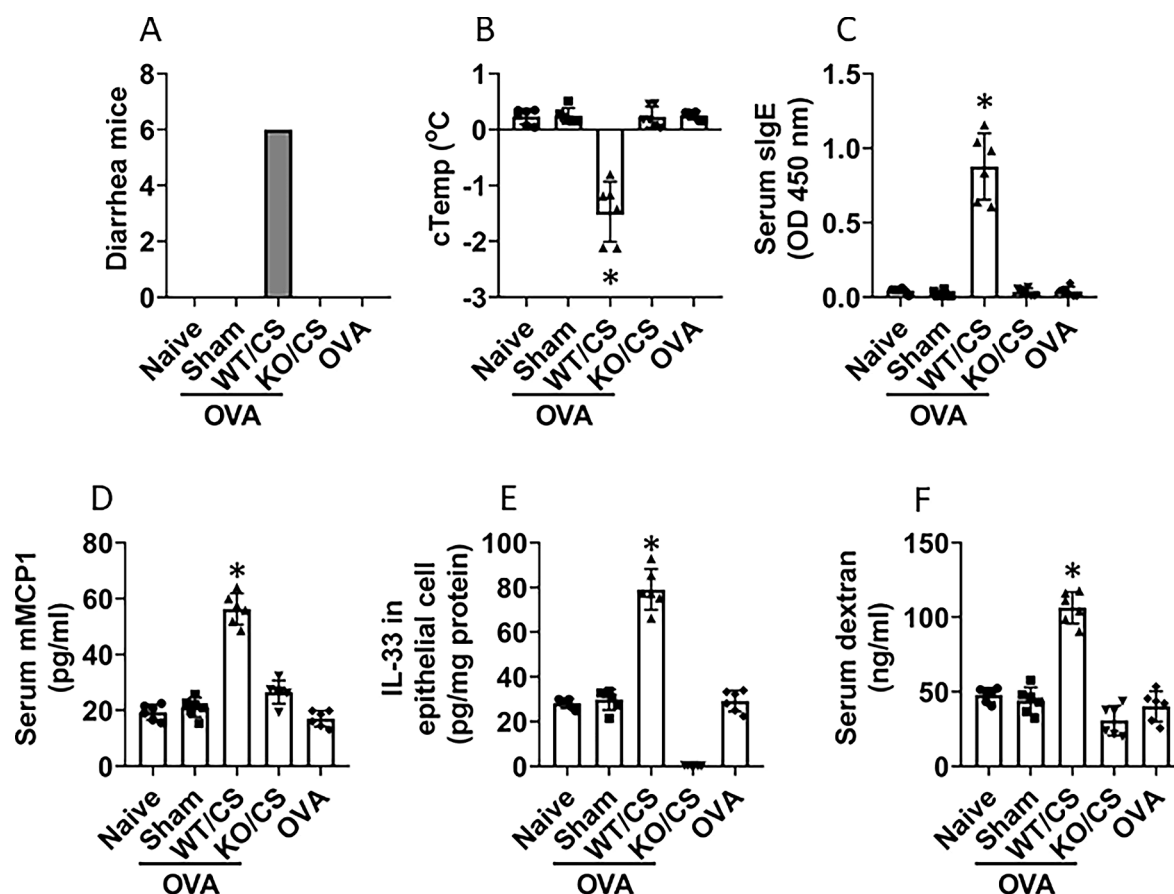


Fig. 6. CS facilitates FA development. WT mice and epithelial cell IL-33 KO mice were treated with CS with or without addition of OVA gavage daily for 1 week. On day 8, mice were challenged with a large dose of OVA (gavage with 0.3 ml OVA solution, 50 mg/ml). Bar graphs show FA responses, including FA clinical symptoms [diarrhea (A) and core temperature (cTemp) drop (B)], serum specific IgE (sIgE, C) and mouse mast cell protease-1 (mMCP1, D), IL-33 in gut epithelial cell proteins (E) and gut epithelial barrier permeability (reflexed by serum FITC-dextran levels, F). Each sample was tested in triplicate. Statistics: ANOVA + the Dunnett's test.

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Author contributions

JQL, DY, TYH, ZQL, YNS, LHM and GY performed experiments, analyzed data and reviewed the manuscript. PCY, ZQL and ZGL organized the study and supervised experiments. PCY designed the project and wrote the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cyto.2020.155295>.

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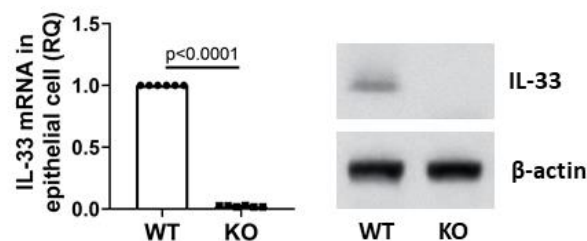
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Supplemental Materials

Generation of epithelial cell IL-33-knockout (KO) mice

The *Il33* gene in epithelial cells were specifically knocked out in C57BL/6 mice through genetic engineering approach. To avoid affecting the role of IL-33 in other cells, the *Il33* gene was specifically depleted from epithelial cells with EpCAM as the specific marker. According to published procedures (J Clin Invest 1996; 98: 600-3; Elife 2014; 3: e01949), mice with loxP-flanked *Il33* gene (flox) were developed. In the first step, a gene targeting vector containing 3 loxP sites was constructed, in which two of them flanking the neomycin resistance gene. The genomic locus was modified between vector and the *Il33* gene in embryo stem (ES) cells by homologous recombination. The loxP-flanked *Il33* gene was targeted by EpCAM-Cre expression in ES cells (EpCAM is specifically expressed in epithelial cells). Using the modified ES cells, a loxP-*Il33* containing mouse line was generated. Then, the mouse strain harboring two loxP sites in the *Il33* gene was crossed with another strain expressing EpCAM-Cre recombinase. Thus, only in cells expressing EpCAM-Cre the *Il33* gene becomes inactivated but remained active in other cells of the body. To keep the *Il33* gene active until the experimental period, the Cre gene was modified to be functional only in the ribosomes, but could not penetrate the nuclear membrane. Thus, the loxP-*Il33* gene was remained intact in the mice. One week before the experiments, the mice were treated with tamoxifen citrate salt (Sigma Aldrich, St. Louis, MO; 0.3 g/kg in 0.2 ml 10% EtoH in corn oil) through gavage-feeding daily for 5 days. Control mice received oral gavage of 0.2 ml 10% EtoH in oil. Tamoxifen increases the permeability of the nuclear membrane to allow the EpCAM-Cre penetrate the nuclei to cleave the flox-*Il33* gene. As assessed by RT-qPCR and Western blotting, all EpCAM⁺ epithelial cells in the intestine did not express IL-3 (see below).



Assessment of expression of IL-33 in the mouse intestine. Intestinal segments were excised from mice with depletion of *Il33* in epithelial cells. The samples were analyzed by RT-qPCR and Western blotting. Bar graph show mRNA levels and immunoblots show protein levels of IL-33 in the samples. The data of bars are presented as mean $\pm$ SEM. Statistics: Student *t* test. The data represent 6 independent experiments.