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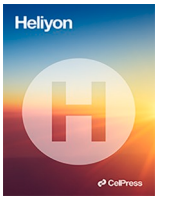

TRPM2 exacerbates airway inflammation by regulating oxidized-CaMKII in allergic asthma

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ABSTRACT

Background: Airway epithelial cells play important roles in allergic asthma. Transient receptor potential melastatin-related 2 (TRPM2) and oxidized Ca^{2+}/calmodulin-dependent protein kinase II (ox-CaMKII) participate in the airway inflammation. This study aimed to analyze the effects of TRPM2 on ox-CaMKII in the airway epithelial cells during allergic asthma.

Methods: BEAS-2B cells were treated with different dose of IL-13 (0, 5, 10, 20 ng/mL) for 24 h to analyze the changes of TRPM2 and ox-CaMKII protein. Cells expressing different level of TRPM2 were obtained by transfection of TRPM2 siRNA or TRPM2-short cDNA. The transfected cells were treated with 10 ng/mL of IL-13 to analyze the effects of TRPM2 on the ox-CaMKII. A CaMKII inhibitor KN-93 was used to confirm the effects of TRPM2 on levels of ox-CaMKII, *p*-MEK and *p*-ERK in the IL-13-treated BEAS-2B cells. Wild-type (WT) mice and TRPM2-knockout ($TRPM2^{-/-}$) mice were induced by ovalbumin (OVA) to compare the differences of inflammation, levels of ox-CaMKII, *p*-MEK and *p*-ERK in airways.

Results: Cell viability was clearly decreased by the 20 ng/mL of IL-13. The levels of TRPM2 and ox-CaMKII protein in cells were increased with increasing doses of IL-13. Transfection of TRPM2 siRNA or TRPM2-short cDNA respectively decreased or increased the levels of ox-CaMKII in the IL-13-stimulated cells. The results of KN-93 treatment were similar to the results of TRPM2 siRNA transfection, that the levels of ox-CaMKII, *p*-MEK and *p*-ERK were significantly decreased in the IL-13-treated cells. Compared with the OVA-induced WT mice, levels of inflammation, ox-CaMKII, *p*-MEK and *p*-ERK in the airways were significantly weakened in the OVA-induced $TRPM2^{-/-}$ mice.

Conclusions: TRPM2 plays a vital role in regulating ox-CaMKII in airway epithelial cells during allergic asthma.

1. Introduction

Allergic asthma is characterized by airway hyperresponsiveness (AHR), T-helper type 2 (Th2) airway inflammation and eosinophil infiltration [1]. Airway epithelial cells can initiate Th2 immune response by secreting interleukin (IL)-25 and IL-33 in the allergic asthma [2]. Production of reactive oxygen species (ROS) in the airway epithelial cells also participates in the airway inflammation [3].

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However, the molecular mechanisms of ROS-mediated inflammation in the airway epithelial cells are still unclear during the allergic asthma.

In asthma, Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII), a serine/threonine-specific protein kinase family, is associated with inflammation in the airway epithelial cells [3,4]. The levels of oxidized-CaMKII (ox-CaMKII) increase in the airway epithelial cells and contribute to the airway inflammation and airway remodeling. The relevant mechanisms of CaMKII in the asthma include the CaMKII-mediated MEK/ERK pathway [5], ROS and autophagy [3]. Transient receptor potential melastatin-related 2 (TRPM2) acts as an important molecular mechanism conferring susceptibility to ROS-induced cell death [6–8]. Furthermore, the TRPM2 can be activated by ROS generation in the inflamed airway epithelial cells [9]. However, the effects of TRPM2 on the ox-CaMKII-mediated inflammation are unclear during allergic asthma.

There are different opinions regarding the roles of TRPM2 in the asthma. Sumoza-Toledo et al. [10] found that ablation of TRPM2 channels did not alter airway inflammation and did not affect cytokine levels in the ovalbumin (OVA)-induced severe allergic asthma. However, wang et al. [11] showed TRPM2 participated in the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammation in mice with $PM_{2.5}$-induced acute lung injury. Importantly, Li et al. [12] reported that TRPM2 ubiquitination contributed to macrophage inflammation in asthma. These findings promote us to further study the roles and mechanisms of TRPM2 in the airway inflammation of allergic asthma.

In this study, BEAS-2B cells were administrated with different doses of IL-13 to analyze the changes of TRPM2 and ox-CaMKII. Cells expressing different levels of TRPM2 were obtained by transfection of TRPM2 siRNA or TRPM2-short cDNA to analyze the effects of TRPM2 on ox-CaMKII in the IL-13-induced BEAS-2B cells. Furthermore, wild-type (WT) mice and TRPM2-knockout ($TRPM2^{-/-}$) mice were induced by OVA to compare the differences of inflammation and levels of ox-CaMKII in the airways.

2. Materials and methods

2.1. Cell culture

Bronchial epithelial cells (BEAS-2B, Procell, Wuhan, China) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 1 % penicillin and streptomycin (P/S) at 37 °C with 5 % CO_2. In order to prevent changes of cell viability, the same cell line was used in this study. At approximately 80 % confluency, cells (2×10^5 per well) were used for experiments.

2.2. Treatment with different doses of IL-13

According to a reported study [13], the cells were treated with different doses (0, 5, 10, 20 ng/mL) of IL-13 ($^{\#}$SRP3274, Sigma–Aldrich) for 24 h. The IL-13 was dissolved in 1 % bovine serum albumin (BSA), and 1 % BSA was used as a vehicle control. The cell viability, cell apoptosis, and levels of TRPM2 and ox-CaMKII protein were measured.

2.3. Cell transfection

Human TRPM2 siRNAs (TRPM2 $siRNA^{\#1}$ and TRPM2 $siRNA^{\#2}$) and scrambled control siRNA were obtained from RiboBio (Guangzhou, China). The cells were transfected with 40 nM TRPM2 siRNA or control siRNA for 6 h at 37 °C with 5 % CO_2 using 2 μL of Lipofectamine 2000 reagent diluted in serum and antibiotic-free DMEM [14]. After transfection, the cells were cultured for 24 h.

Cells transfected by TRPM2-full length are susceptible to apoptosis [15]. Therefore, cells were transfected with a short splice variant of TRPM2-short cDNA (TRPM2-s cDNA). The TRPM2-s cDNA was inserted into pTracer-CMV (Invitrogen). In short, cells were transfected with 1 μg/mL of TRPM2-s cDNA or vector control using Lipofectamine Plus for 6 h, and then cultured for 24 h.

The sequences of TRPM2 siRNAs and TRPM2-s cDNA in this study were showed in Table 1.

2.4. Cellular experiments

The program of cellular experiments was showed in Fig. 1A.

First, cells expressing different level of TRPM2 were treated with 10 ng/mL of IL-13 for 24 h to analyze the effects of TRPM2 on ox-CaMKII expression. Control cells were treated with 1 % BSA. The cell viability, levels of TRPM2 and ox-CaMKII protein were measured.

Second, a CaMKII inhibitor KN-93 (Sigma–Aldrich, Shanghai) was used to confirm the effects of TRPM2 on ox-CaMKII in the IL-13-

Table 1
The sequences of TRPM2 siRNAs and TRPM2-s cDNA in this study.

Name	Sequence
TRPM2 $siRNA^{\#1}$	Forward: GCCCAAGATCATCATTGTGAA
TRPM2 $siRNA^{\#2}$	Forward: GACCTTCTCATTTGGGCCATT
Control siRNA	Forward: UGGUUUACAUGUCGACUAA
TRPM2-s cDNA	Forward: CGGCGGCGGTACCGAATGGAGCCCTCAGCCCTGAGGAAAGCTGGC
	Reverse: CCGCCGCTCGAGTCAGTAGTGAGCCCCGAACTCAGCGGCTGC

treated cells. In detail, cells were divided into five groups: control group, 1 % BSA group, TRPM2-siRNA$^{\#2}$ group, KN-93 group, and TRPM2-s cDNA + KN-93 group. Except the control group, cells in the other groups were treated with 10 ng/mL of IL-13 for 24 h. In the KN-93 group or TRPM2-s cDNA + KN-93 group, cells were treated with 10 μM KN-93 [14]. The levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK (Tyr204) were observed in each group.

2.5. Cell viability

Cell viability was measured using a CCK-8 kit (Beyotime, Shanghai, China). In short, the cells were cultured with 10 μL of CCK-8 solution for 4 h, and then the absorbance of each well was measured at 450 nm.

2.6. Cell apoptosis

Cell apoptosis was measured using an Annexin V-FITC apoptosis kit (Beyotime, Shanghai, China). In short, the centrifuged cells were stained with 5 μL of Annexin V and 10 μL of propidium iodide (PI) for 15 min in the dark. The results were detected by flow cytometry within 60 min. Overall percentage of cell death (%) = Quadrant 1 (Q1)-UR (apoptotic) + Q1-LR (necrotic) [16].

2.7. Western blotting

According to reported methods [17], the prepared membranes were obtained through collection, separation and transmembrane, and then incubated with rabbit primary antibodies for overnight at 4 °C. Antibodies included anti-TRPM2 (1:500, Thermo Fisher Scientific), anti-ox-CaMKII (1:500, 07–1387, EMD Millipore), anti-*p*-ERK(Tyr204) (1:500, AF1014, Affinity), anti-*p*-MEK (Thr292) (1:500, AF3383, Affinity) and anti-GAPDH (1:1000, AF7021, Affinity). After washed with TBS-0.01 % Tween 20 for 3 times, the membranes were incubated with the secondary antibody (1:3000, S0001, Affinity) for 2 h at 25 °C. An enhanced chemiluminescence

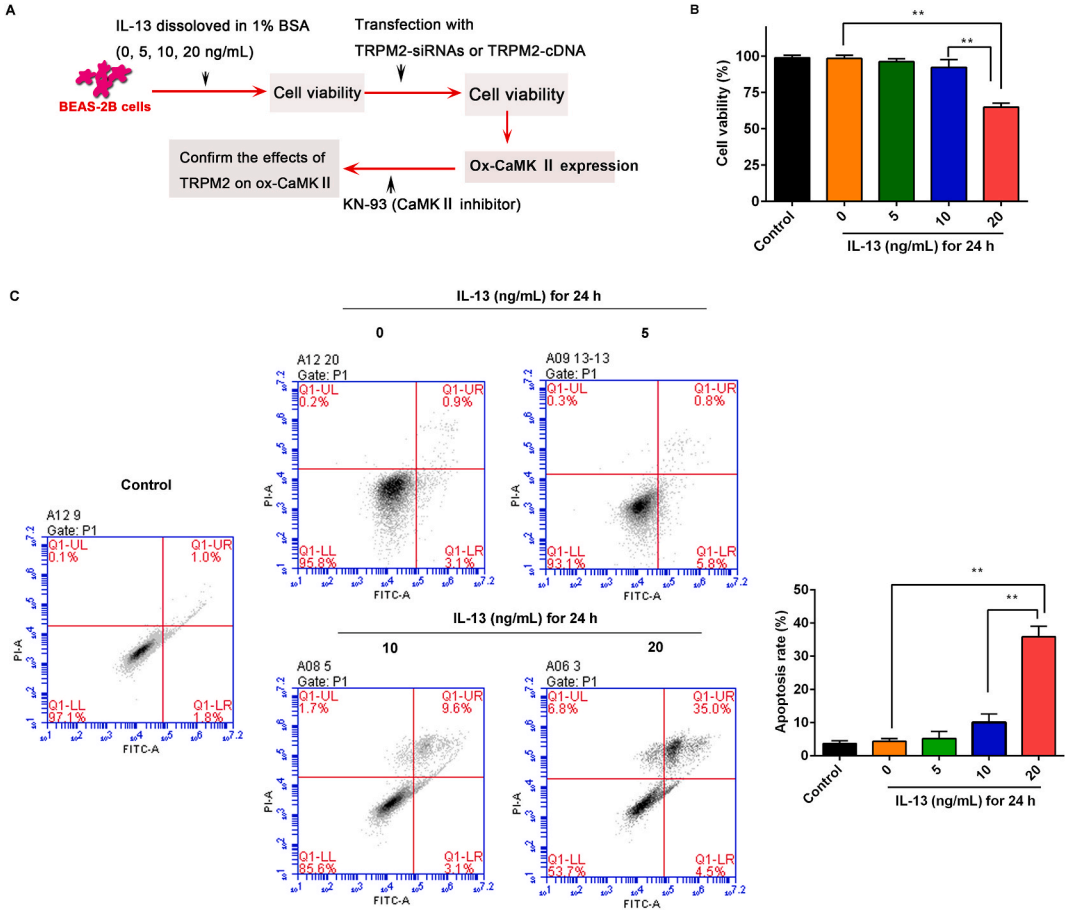

Fig. 1. Effects of different doses of IL-13 on cell viability and cell apoptosis in BEAS-2B cells. The IL-13 was dissolved in 1 % bovine serum albumin (BSA). (A) The program of cellular experiments. (B) Cell viability was measured by a CCK-8 kit. (C) Cell apoptosis was measured by an Annexin V-FITC/PI apoptosis kit. Apoptosis rate (%) = Q1-UR + Q1-LR. **p < 0.01.

reagent (D085075, Bio-Rad, China) was used to visualize the bands.

2.8. Immunofluorescence

Cells were fixed with 4 % paraformaldehyde for 30 min and blocked with TBS-T containing 5 % BSA for 60 min. After washing, the cells were incubated with rabbit primary antibodies, anti-TRPM2 (1:200, Thermo Fisher Scientific) and anti-ox-CaMKII (1:200, 07–1387, EMD Millipore), overnight at 4 °C without light. After washing, the cells were incubated with Cy3-labeled goat anti-rabbit IgG (H + L) antibody (1:200, S0008, Affinity) for 60 min at 25 °C without light. Nuclei of cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI).

3. Animals

Ten wild-type C57BL/6J male mice (6–8 weeks old) and ten TRPM2$^{-/-}$ C57BL/6J male mice (C57BL/6J-Trpm2^{em1cyagen}, Cyagen, 6–8 weeks old) were used in this study. The exon 2–4 region of TRPM2 was knocked out. The TRPM2$^{-/-}$ mice were backcrossed with C57BL/6J mice for seven to ten generations to eliminate any background effects on the phenotypes. The detailed information of TRPM2$^{-/-}$ mice was provided in the Supplemental information. All animals were housed under specific pathogen free conditions for at least 5 days before experiments.

3.1. Animal model

The program of mice model was showed in Fig. 8A.

According to reported methods [18], the mice were intraperitoneally sensitized with 20 μg of OVA (Macklin) plus 1 mg of Alum in a total of 200 μL saline on Day 1 and 8, then inhaled aerosolized 5 % (V/V) OVA in PBS on Day 15, 16 and 17 for 30 min in a whole-body exposure chamber. The control mice were intraperitoneally treated with 20 μL of PBS in 200 μL saline on Day 1 and 8, and then inhaled aerosolized 5 % PBS on Day 15, 16 and 17 for 30 min. For the aerosolized inhalation, OVA or PBS was treated by a high-frequency nebulizer (AER-AL, TOW-INT TECH, Shanghai, China).

3.2. Airway mechanics

Twenty-four hours after the last challenge with OVA, AHR of mice was evaluated [19]. The anaesthetized mice were mechanically ventilated with a Sci-Req FlexiVent machine with 150 breaths/min and 2 cmH_2O positive end-expiratory. Different concentrations of nebulized methacholine (0, 12.5, 25, 50, 100 mg/mL) were administrated to measure the airway resistance of mice.

3.3. Levels of leukocyte counts and cytokines

Forty-eight hours after the last challenge with OVA, total numbers of cells in the bronchoalveolar lavage (BAL) fluids (BALFs) were counted, and the numbers of eosinophils, neutrophils and lymphocytes were obtained using Wright stain solution.

The levels of IL-4, IL-5 and IL-13 in the supernatants of BALFs were respectively analyzed by enzyme-linked immunosorbent (ELISA) kits. All ELISA kits were purchased from Beyotime (China).

3.4. Pathological observation

The lung tissues were observed using hematoxylin-eosin (HE) staining and periodic acid-Schiff (PAS) staining [20]. For the HE staining, sections (3 μm) were stained with HE solution (Solarbio, China). For the PAS staining, sections (3 μm) were oxidized for 6 min, and stained with PAS staining solution (Solarbio) for 15 min.

3.5. Immunohistochemistry

As reported methods [20], sections (3 μm) were incubated with incubated with ox-CaMKII primary antibodies (1:100, 07–1387, EMD Millipore) overnight at 4 °C. After washing, the sections were incubated with the secondary antibody (1:500, S0001, Affinity) for 120 min at 25 °C.

3.6. Statistical analysis

The results of western blotting, immunofluorescence and immunohistochemistry were analyzed using ImageJ software (National Institutes of Health, USA). Data significance was analyzed by SPSS 19.0 software (IBM, Chicago, IL, USA), and the results were showed as mean ± standard deviation. One-way analysis of variance following the LSD test was used to compare the significance among groups. A value of $p < 0.05$ is defined as statistical significance.

4. Results

Levels of TRPM2 and ox-CaMKII protein were upregulated with increasing dose of IL-13 in BEAS-2B cells.

BEAS-2B cells were treated with different doses of IL-13 (0, 5, 10, 20 ng/mL), and the cell viability (Fig. 1B) and apoptosis rate (Fig. 1C) were observed. The cell viability was significantly declined and the apoptosis rate was significantly increased after 20 ng/mL of IL-13 treatment ($p < 0.01$). Meanwhile, the levels of TRPM2 and ox-CaMKII protein were measured in BEAS-2B cells (Fig. 2A), and their levels were upregulated with increasing dose of IL-13 (Fig. 2B and C). The levels of TRPM2 and ox-CaMKII protein were significantly increased after the 10 ng/mL or 20 ng/mL of IL-13 treatment by comparation with the control treatment ($p < 0.01$). On the basis of cell viability and apoptosis rate, 10 ng/mL of IL-13 was used for following experiments.

4.1. Effects of TRPM2 on ox-CaMKII in the IL-13-induced BEAS-2B cells

TRPM2 silence in BEAS-2B cells was obtained by transfection of TRPM2 siRNA$^{\#1}$ or TRPM2 siRNA$^{\#2}$. No significance in cell viability was found after different treatment (Fig. 3A). The levels of TRPM2 and ox-CaMKII protein were obviously upregulated in the IL-13-treated cells compared with the control cells (Fig. 3B, $p < 0.05$). Compared with the siRNA-control transfection, transfection of TRPM2 siRNA$^{\#1}$ or TRPM2 siRNA$^{\#2}$ was obviously declined the levels of TRPM2 and ox-CaMKII protein in the IL-13-treated cells ($p < 0.01$). Additionally, the levels of TRPM2 and ox-CaMKII in cells were measured by immunofluorescence (Fig. 4A). The mean gray value of TRPM2 and ox-CaMKII was significantly increased after the IL-13 treatment, and the mean gray value was suppressed by the transfection of TRPM2 siRNA$^{\#1}$ or TRPM2 siRNA$^{\#2}$ in the IL-13-treated cells (Fig. 4B, $p < 0.05$). No significance was found between the TRPM2 siRNA$^{\#1}$ and TRPM2 siRNA$^{\#2}$ ($p > 0.05$), and the TRPM2 siRNA$^{\#2}$ was used for the following experiments.

TRPM2 overexpression in BEAS-2B cells was obtained by transfection of TRPM2-s cDNA. No significance in cell viability was found after different treatment (Fig. 5A). By comparation with the control cells, the levels of TRPM2 and ox-CaMKII protein were clearly upregulated after the IL-13 treatment ($p < 0.01$). In the IL-13-treated cells, transfection of TRPM2-s cDNA obviously increased the levels of TRPM2 and ox-CaMKII protein (Fig. 5B, $p < 0.01$). The levels of TRPM2 and ox-CaMKII in cells were also measured by immunofluorescence (Fig. 6A). The mean gray of TRPM2 and ox-CaMKII was clearly increased after the IL-13 treatment contrasted to the control cells (Fig. 6B, $p < 0.01$). Transfection of TRPM2-s cDNA also significantly increased the mean gray of TRPM2 and ox-CaMKII in the IL-13-treated cells (Fig. 6B, $p < 0.01$).

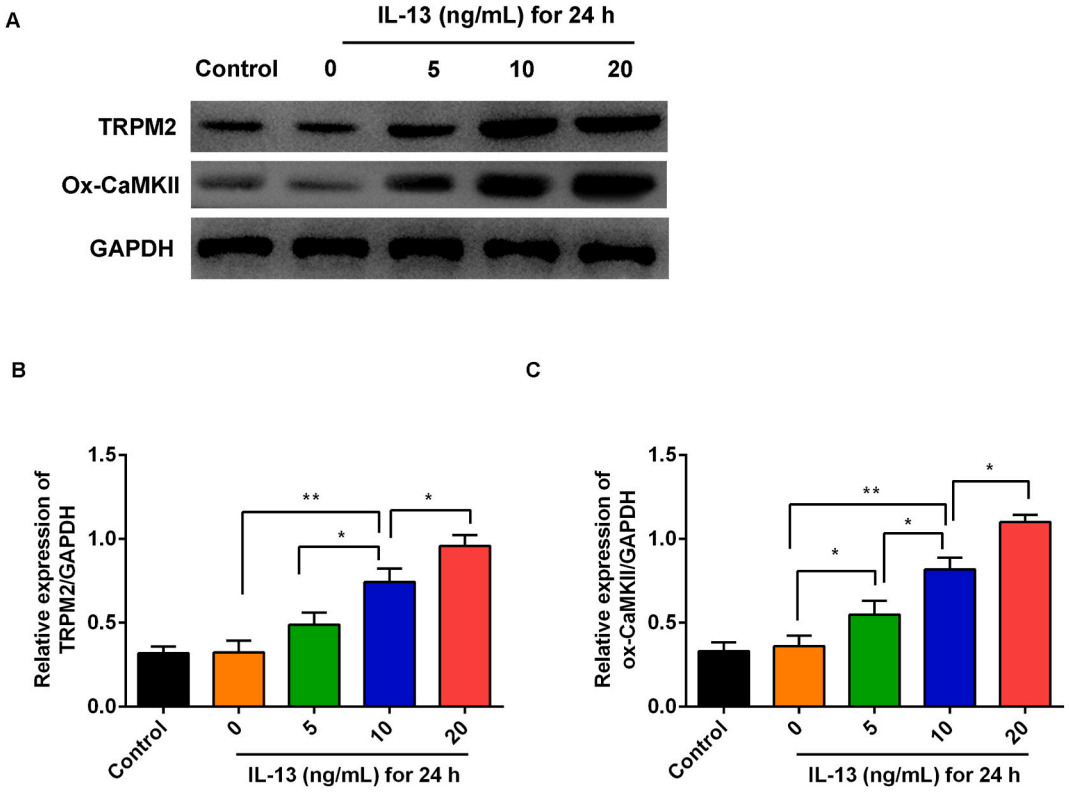

Fig. 2. Effects of different doses of IL-13 on levels of TRPM2 and ox-CaMKII protein in BEAS-2B cells. (A) Levels of TRPM2 and ox-CaMKII protein were measured by western blotting. The full length of TPRM2, ox-CaMKII and GAPDH blots was provided in the Supplemental file. (B) Relative expression of TRPM2 protein. (C) Relative expression of ox-CaMKII protein. The relative expression of protein was normalized by GAPDH. *$p < 0.05$, **$p < 0.01$.

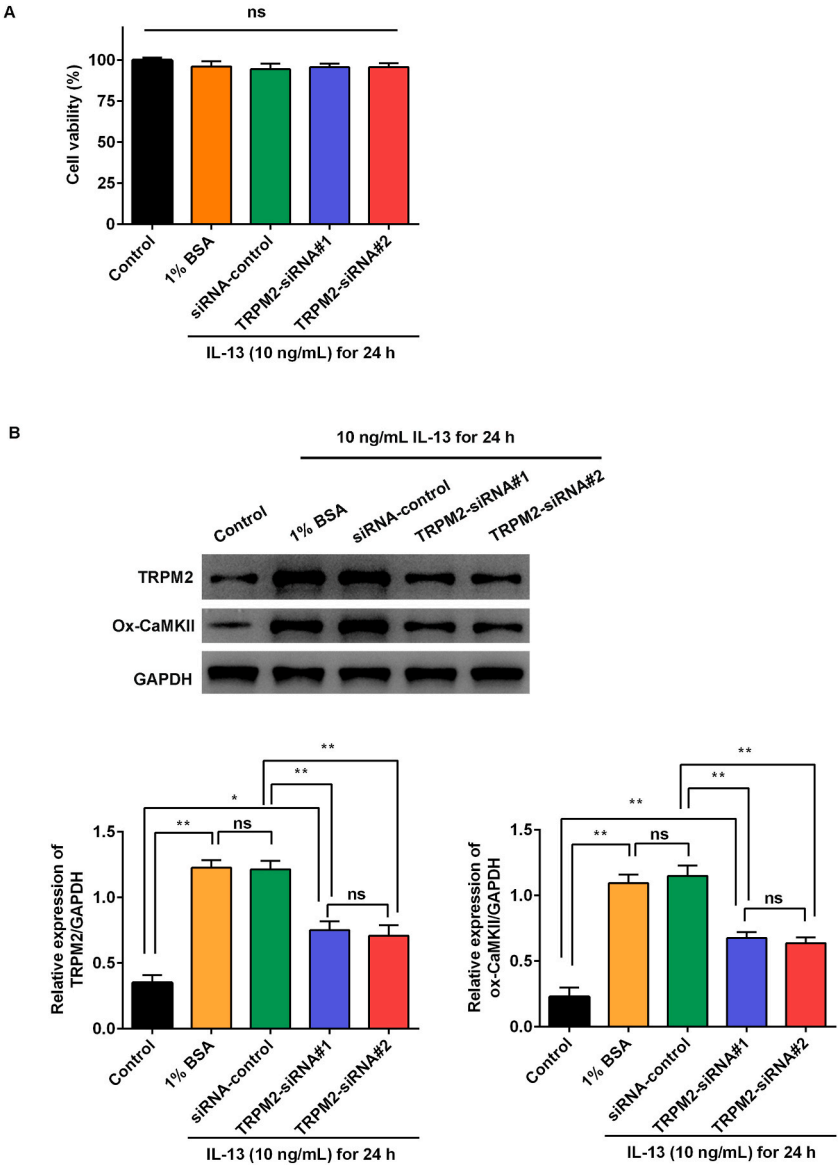

Fig. 3. TRPM2 silence downregulated the levels of ox-CaMKII protein in the IL-13-treated BEAS-2B cells. (A) Cell viability. (B) Levels of TRPM2 and ox-CaMKII protein. The full length of TPRM2, ox-CaMKII and GAPDH blots was provided in the Supplemental file. ns: no significance, *$p < 0.05$, **$p < 0.01$.

4.1.1. TRPM2 affected MEK/ERK pathway by regulating ox-CaMKII in the IL-13-induced BEAS-2B cells

A CaMKII inhibitor KN-93 (10 μM) was used to confirm the regulation of TRPM2 on ox-CaMKII in the IL-13-induced cells. The levels of ox-CaMKII were measured by immunofluorescence (Fig. 7A), and the mean gray value of ox-CaMKII was significantly suppressed by the TRPM2 siRNA$^{\#2}$ transfection or KN-93 treatment in the IL-13-treated cells (Fig. 7B, $p < 0.01$). However, the TRPM2-s cDNA transfection significantly weakened the effects of KN-93 on the level of ox-CaMKII ($p < 0.01$). The levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK(Tyr204) protein were measured by western blotting (Fig. 7C). The levels of these proteins were clearly upregulated after the IL-13 treatment compared with the control group (Fig. 7D–F, $p < 0.01$). Meanwhile, the levels of these proteins were significantly suppressed by the TRPM2-siRNA$^{\#2}$ transfection or KN-93 treatment ($p < 0.01$), and the effects of KN-93 were blocked by the TRPM2-s cDNA transfection ($p < 0.05$).

4.2. TRPM2 knockout improved airway inflammation in the OVA-induced mice

The WT mice and TRPM2$^{-/-}$ mice were induced by OVA treatment (Fig. 8A). In the two genotypes, a significant increase in the airway resistance was observed in the OVA-induced mice by comparation with the PBS-treated mice (Fig. 8B, $p < 0.01$). The level of

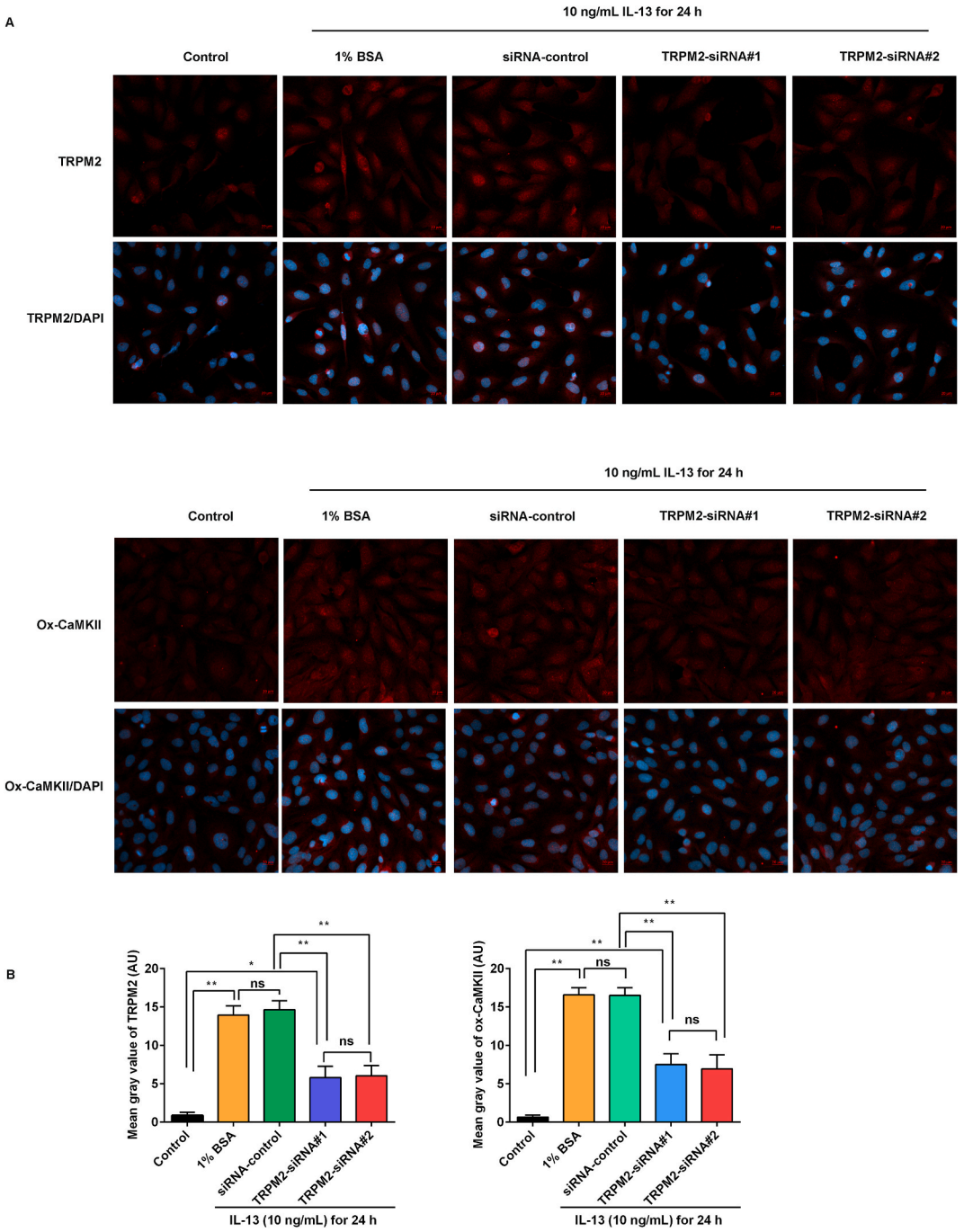

Fig. 4. TRPM2 silence downregulated the levels of ox-CaMKII in the IL-13-treated BEAS-2B cells. (A) Levels of TRPM2 and ox-CaMKII were measured by immunofluorescence, scale = 20 μm. (B) Mean gray value of TRPM2 and ox-CaMKII was analyzed using ImageJ software. *$p < 0.05$, **$p < 0.01$, ns: no significance.

airway resistance in the OVA-induced TRPM2$^{-/-}$ mice was clearly lower than that in the OVA-induced WT mice ($p < 0.05$).

The airways of the two genotypes were observed using the HE (Fig. 8C) and PAS (Fig. 8D). In the two genotypes, OVA challenge induced inflammatory infiltration (black arrows, Fig. 8C) and goblet cell metaplasia (black arrows, Fig. 8D) in the airways. Importantly, the severity of inflammatory infiltration and goblet cell metaplasia was clearly improved in the OVA-induced TRPM2$^{-/-}$ mice compared with the OVA-induced WT mice.

In the two genotypes, the numbers of eosinophils, lymphocytes and neutrophils in BALFs were notably increased after the OVA treatment (Fig. 9A, $p < 0.05$). The numbers of above factors were significantly declined in the OVA-induced TRPM2$^{-/-}$ mice compared

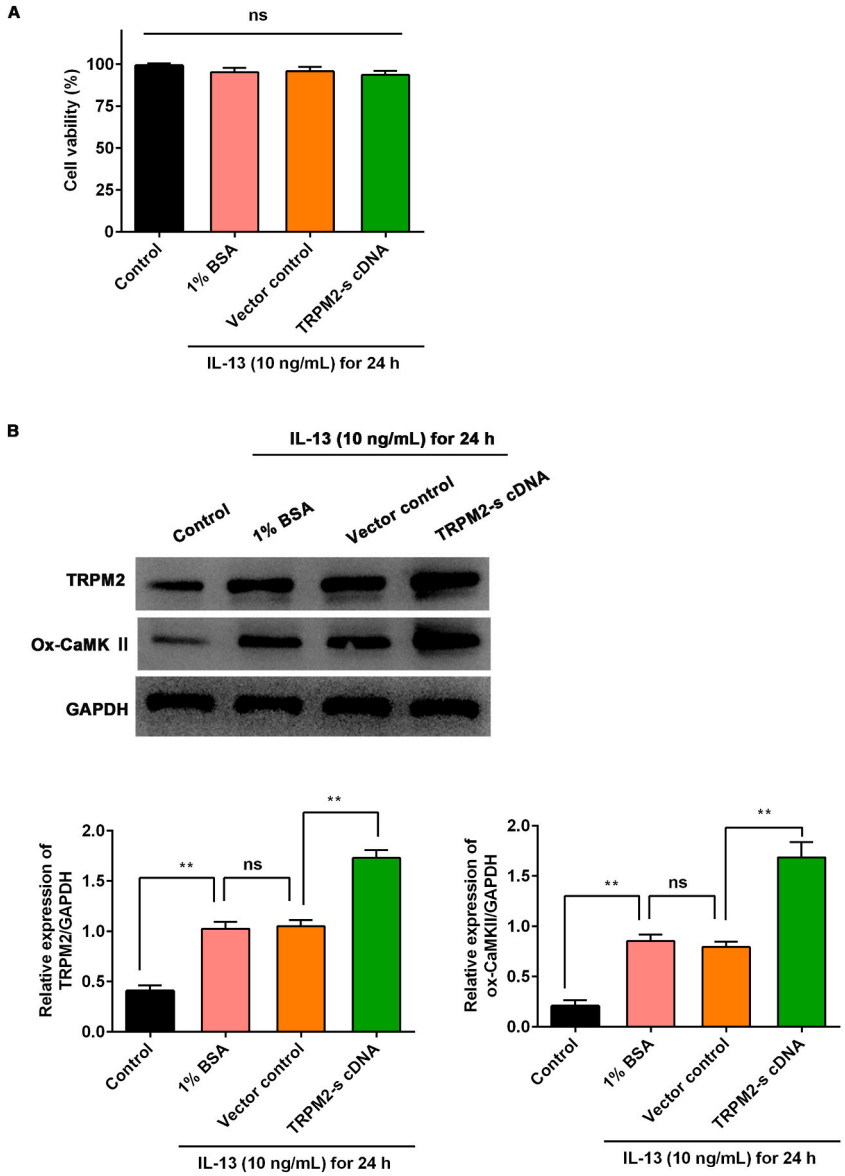

Fig. 5. TRPM2 overexpression upregulated the levels of ox-CaMKII protein in the IL-13-treated BEAS-2B cells. (A) Cell viability. (B) Levels of TRPM2 and ox-CaMKII protein. The full length of TPRM2, ox-CaMKII and GAPDH blots was provided in the Supplemental file. ns: no significance, $^{**}p < 0.01$.

with the OVA-stimulated WT mice ($p < 0.01$). The levels of IL-4, IL-5 and IL-13 in BALFs were significantly increased after the OVA treatment in the two genotypes (Fig. 9B, $p < 0.01$), and these inflammatory factors were markedly declined in the OVA-challenged TRPM2$^{-/-}$ mice contrasted to the OVA-challenged WT mice ($p < 0.01$).

4.3. *TRPM2 knockout suppressed ox-CaMKII expression and MEK/ERK pathway in the OVA-induced mice*

The levels of ox-CaMKII in the airways were observed by immunohistochemistry (Fig. 10A). The levels of ox-CaMKII were significantly increased after OVA treatment in the two genotypes ($p < 0.05$). Compared with the OVA-induced WT mice, the levels of ox-CaMKII were significantly decreased in the OVA-induced TRPM2$^{-/-}$ mice ($p < 0.01$). The levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK(Tyr204) protein in the airways were observed by western blotting (Fig. 10B). The levels of these proteins were significantly increased after the OVA treatment in the two genotypes ($p < 0.05$). Additionally, the levels of these proteins were significantly declined in the OVA-induced TRPM2$^{-/-}$ mice contrasted to the OVA-induced WT mice ($p < 0.01$).

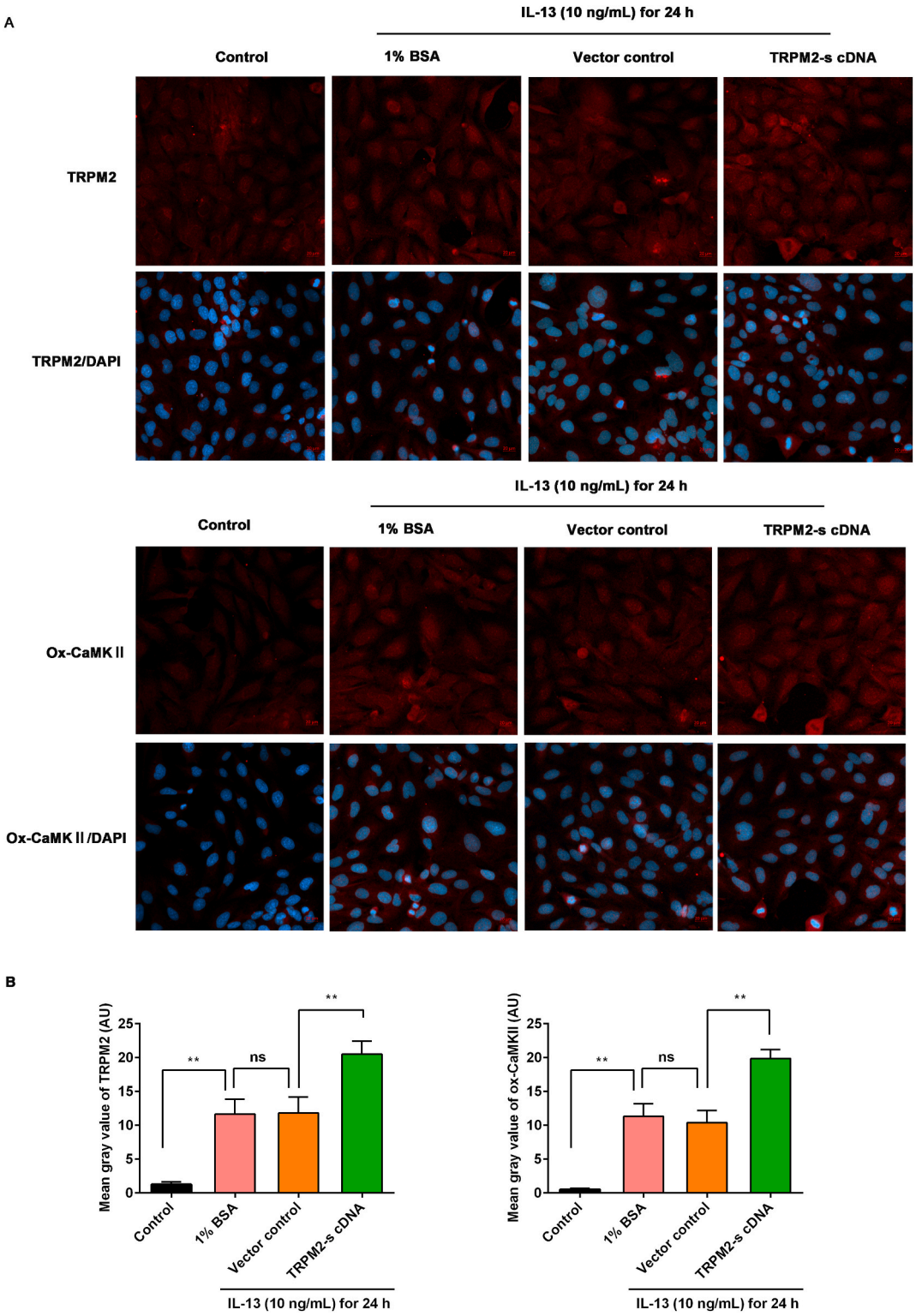

Fig. 6. TRPM2 overexpression upregulated the levels of ox-CaMKII in the IL-13-treated BEAS-2B cells. (A) Levels of TRPM2 and ox-CaMKII, scale = 20 μm. (B) Mean gray value of TRPM2 and ox-CaMKII. **p < 0.01, ns: no significance.

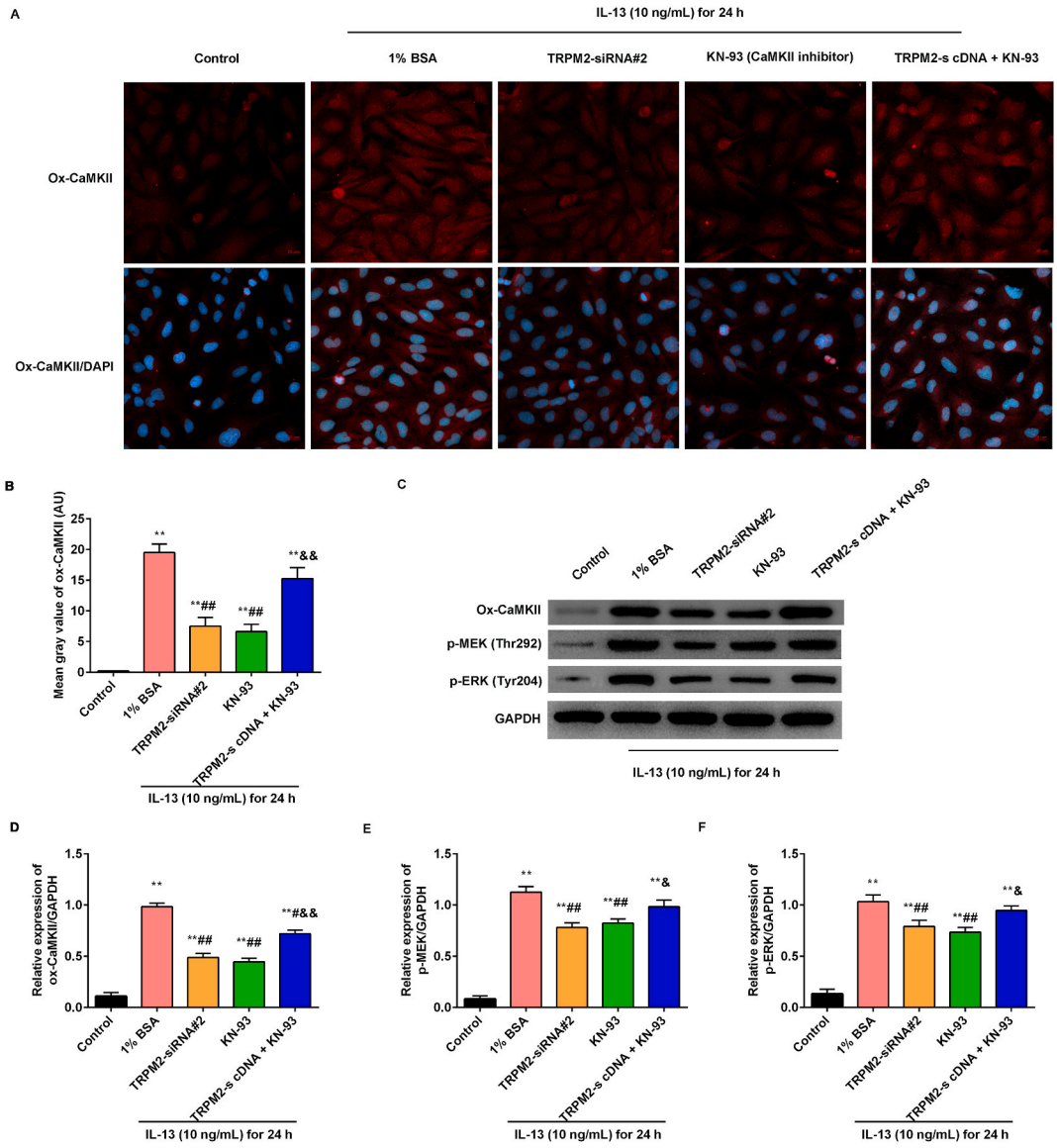

Fig. 7. TRPM2 regulated the ox-CaMKII in the IL-13-treated BEAS-2B cells. Cells were treated with 10 μM KN-93 (a CaMKII inhibitor). (A) Levels of ox-CaMKII in cells, scale = 20 μm. (B) Mean gray value of ox-CaMKII. (C) Levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK(Tyr204) protein in cells. The full length of ox-CaMKII, *p*-MEK (Thr292), *p*-ERK(Tyr204) and GAPDH blots was provided in the Supplemental file. (D) Relative expression of ox-CaMKII protein. (E) Relative expression of *p*-MEK protein. (F) Relative expression of *p*-ERK protein. vs. the control group, $^{**}p < 0.01$. vs. the 1 % BSA group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. vs. the KN-93 group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$.

5. Discussion

Accumulating evidences show that inhibition of ox-CaMKII can improve AHR and airway inflammation in cockroach-challenged or OVA-challenged allergic asthma [3,5,21,22]. In these studies, researchers found that the mechanisms of ox-CaMKII in allergic asthma included mitochondrial ROS production and autophagy in human bronchial epithelial cells [3] and human aortic endothelial cells [21]. As a pivotal regulator of Ca^{2+} signaling, the TRPM2 channel is activated by intracellular ADP-ribose (ADPR) and by several ROS messengers [9]. In PM_{10}-induced BEAS-2B cells, intracellular Ca^{2+} signaling and ROS signaling were suppressed by TRPM2 inhibitors [9]. In this study, the regulation of TRPM2 on ox-CaMKII in allergic asthma was firstly observed.

In the airway epithelial cells, the contribution of IL-13 to redox balance has been highlighted [23]. In this study, the BEAS-2B cells were treated with different doses of IL-3, and the cell viability was obviously affected by 20 ng/mL of IL-13. Therefore, 10 ng/mL of IL-13 was used to observe the effects of TRPM2 on ox-CaMKII in cells. Here, we found that silencing or overexpressing TRPM2 separately suppressed or promoted the levels of ox-CaMKII in the IL-13-stimulated BEAS-2B cells. Furthermore, a CaMKII inhibitor KN-93, which can suppress the activation of CaMKII [24], was used to confirm the effects of TRPM2 on ox-CaMKII in the IL-13-treated

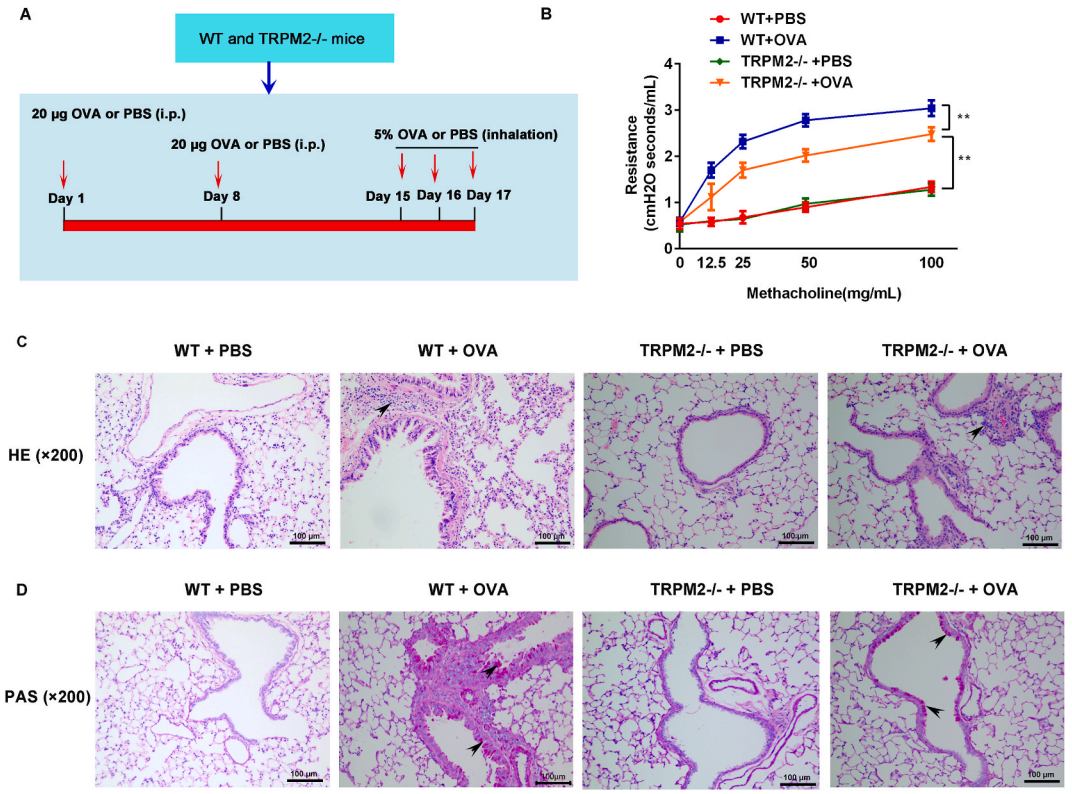

Fig. 8. Effects of TRPM2 on airway inflammation in the OVA-induced mice. (A) The program of mice model. (B) Airway hyperreactivity. **p < 0.01. (C) Inflammatory infiltration (black arrows) was observed by HE staining, scale = 100 μm. (D) Global cell metaplasia (black arrows) was observed by PAS staining, scale = 100 μm.

cells. It showed that the effects of overexpressing TRPM2 on the levels of ox-CaMKII were significantly suppressed by the KN-93 treatment in the IL-13-treated cells. Furthermore, this study showed that TRPM2 inhibition suppressed the levels of *p*-MEK (Thr292) and *p*-ERK(Tyr204) protein in the IL-13-treated cells, suggesting that TRPM2 regulates MEK/ERK pathway by regulation of ox-CaMKII in the IL-13-treated BEAS-2B cells.

Mitogen-activated protein kinase (MAPK) pathway is activated by three-tiered sequential phosphorylation of MAPK kinase kinase (MKKK), MAPK kinase (MEK) and MAPK [25]. Extracellular signal regulated protein kinase (ERK) is one of distinct MAPK, and the MEK/ERK pathway is involved in modulating cellular processes [26,27]. In asthma, inflammatory response of various cell types in the lung and bronchus was activated by the MEK/ERK pathway, such as lung lymphocytes, airway smooth muscle cells and bronchial epithelial cells [28,29]. In an OVA-induced asthma model, the CaMKII/ERK pathway was excessively activated, and then aggravated airway inflammation and contributed to airway remodeling [5]. In this study, TRPM2 inhibition not only suppressed the level of ox-CaMKII but also suppressed the MEK/ERK pathway in the IL-13-treated cells. In the OVA-induced animal models, the TRPM2 knockout also suppressed the levels of ox-CaMKII, *p*-MEK and *p*-ERK in the airway tissues. These results indicate that TRPM2 affects the MEK/ERK pathway by regulating the ox-CaMKII in allergic asthma.

However, Sumoza-Toledo et al. [10] found that the TRPM2 channel was not required for acute airway inflammation in OVA-induced severe allergic asthma. This contradictory result may be related to the severity of the asthma model. In a previous report, severe asthma in TRPM2$^{-/-}$ mice was established by 50 μg of OVA sensitization and 100 μg of OVA challenge. In our study, asthma mice were established by 20 μg of OVA sensitization and 5 % OVA inhalation challenge. The mechanisms of allergic asthma are complex, and the airway inflammatory response in severe allergic asthma may be achieved through other signals. In addition, the conditional TRPM2 knockout mice may be more suitable animals, because the phenotype may be caused by the TRPM2 deficiency in immune cells or airway epithelial cells or other cells type or the cooperation of various cell types. In the next study, the conditional TRPM2 knockout mice will be used to confirm our findings, and to study other mechanisms of TRPM2 in the allergic asthma.

6. Conclusions

This study shows that TRPM2 plays a vital role in regulation of ox-CaMKII in airway epithelial cells during asthma, providing a possible mechanism of TRPM2 on promoting airway inflammation in allergic asthma.

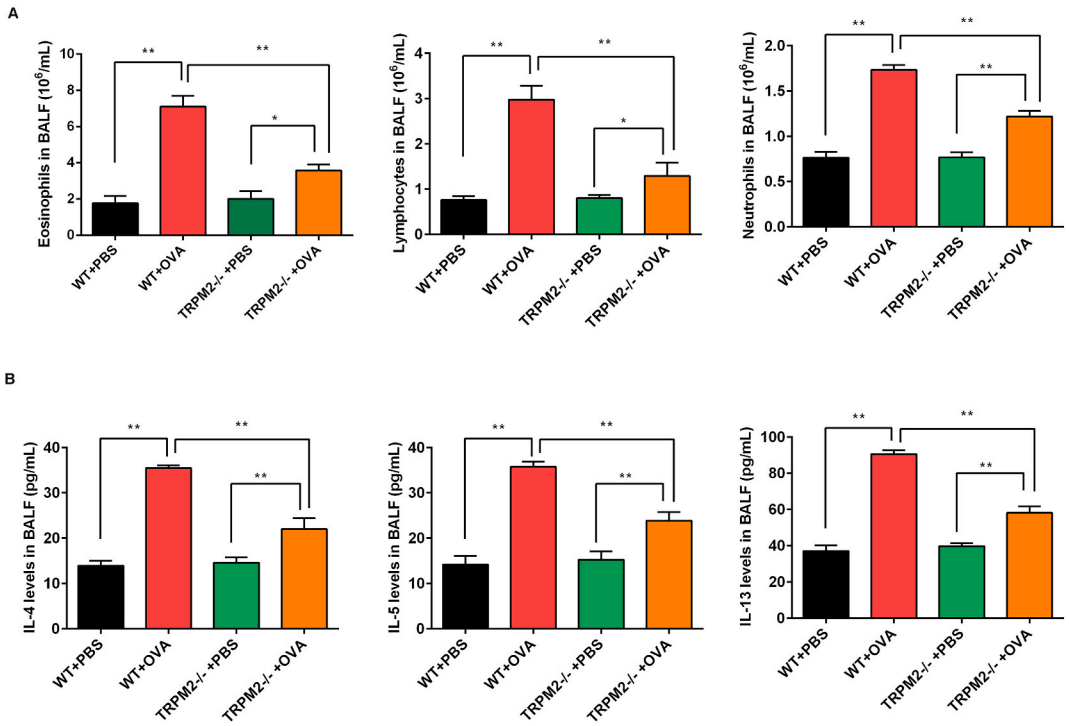

Fig. 9. TRPM2 knockout suppressed airway inflammation in the OVA-induced mice. (A) Numbers of eosinophils, neutrophils and lymphocytes in bronchoalveolar lavage fluids (BALFs). (B) Levels of IL-4, IL-5 and IL-13 in the BALFs. *$p < 0.05$, **$p < 0.01$.

Ethics statement

In this study, all experimental protocols were approved by the Ethics Committee of Yantai Yuhuangding Hospital (No. 2023-343). All methods were carried out in accordance with the ARRIVE guidelines for the reporting of animal experiments.

Consent for publish

Not application.

Data availability statement

All data generated in this study can be made available on request to the corresponding author.

Funding

Not application.

CRediT authorship contribution statement

Xueping Liu: Writing – original draft, Project administration, Investigation, Data curation, Conceptualization. **Lingyan Zhao:** Writing – review & editing, Project administration, Investigation, Data curation, Conceptualization. **Rui Wang:** Writing – review & editing, Validation, Software, Resources, Formal analysis. **Zhaoying Tang:** Writing – review & editing, Validation, Software, Resources, Formal analysis.

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.

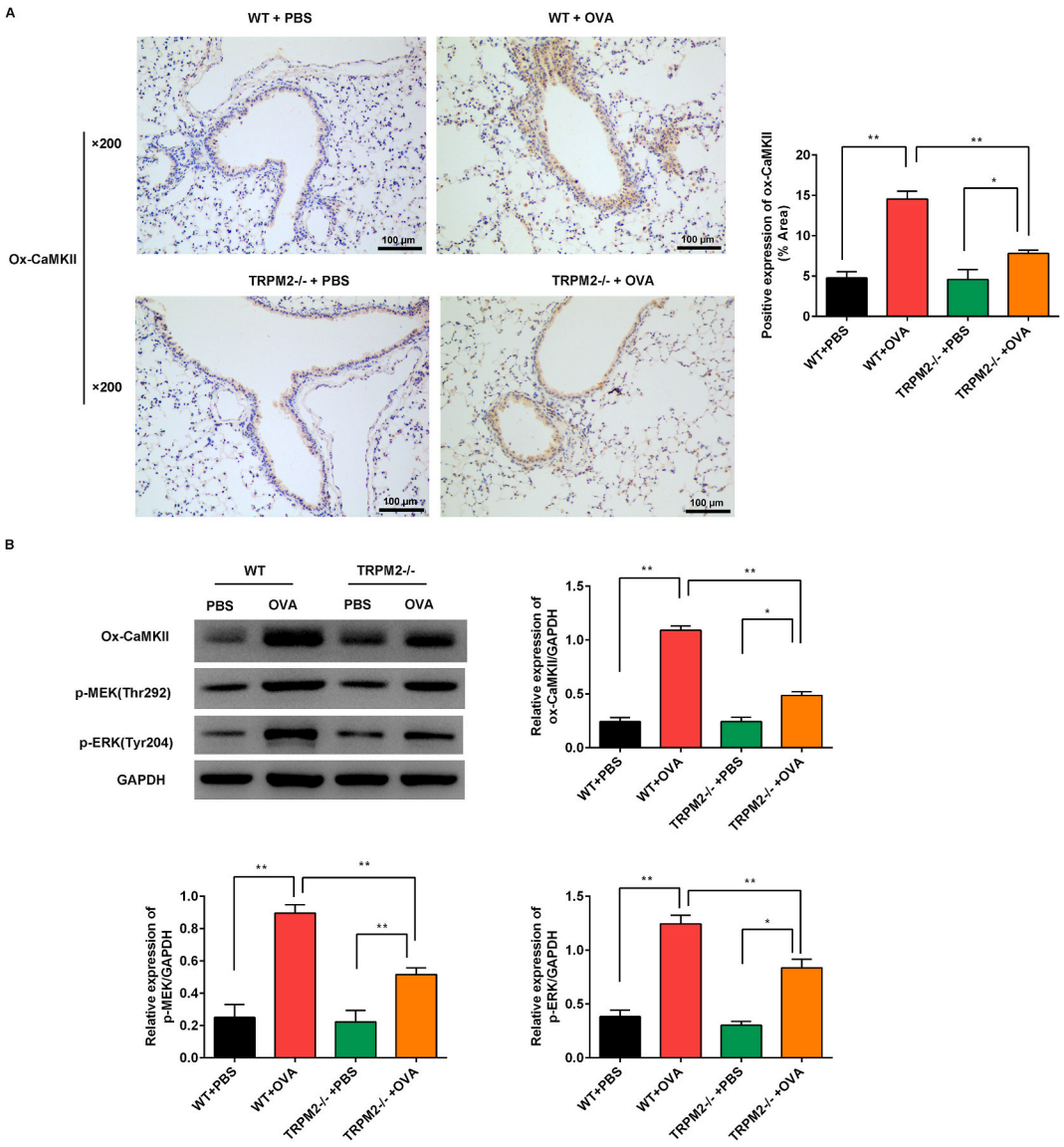

Fig. 10. TRPM2 knockout suppressed the levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK(Tyr204) in the OVA-induced mice. (A) The level of ox-CaMKII was observed by immunohistochemistry, scale = 100 μm. (B) Levels of ox-CaMKII, *p*-MEK (Thr292) and *p*-ERK(Tyr204) protein were measured by western blotting. The full length of ox-CaMKII, *p*-MEK (Thr292), *p*-ERK(Tyr204) and GAPDH blots was provided in the Supplemental file. *p < 0.05, **p < 0.01.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23634.

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