House Dust Mite Induces IL-33 Release From Human Nasal Epithelial Cells via P2Y Purinergic Signals and ERK/P38 MAPK Pathways.

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Research

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Abstract

Background

Allergic rhinitis (AR) is an inflammatory disease of the nasal mucosa, which is triggered by stimulations of environmental allergens such as house dust mite (HDM). Th2-type proinflammatory factor interleukin 33 (IL-33) plays an important role in the pathogenesis of AR, but it remains unknown how IL-33 products in human nasal epithelial cells (HNECs) mediated by HDM.

Methods

We investigated the effect of HDM allergens by analyzing the accumulation of Ca^{2+} levels and IL-33 release in HNECs. Involvements of Adenosine triphosphate (ATP)-dependent activation of P2Y-PLC-IP3 pathways, downstream of Ca^{2+} signaling and P38/ERK pathways were studied, using the P2Y-PLC-IP3 pathways agonists, the calcium chelators, and P38/ERK pathways inhibitors.

Results

Der p induced expression of IL-33 mRNA and protein in HNECs via ERK/P38 pathways. Average 69.4% of co-localization of quinacrine and Lyso-tracker fluorescent puncta revealed that ATP was mainly stored in the lysosomes of nasal epithelial cells. After stimulation with Der p, ATP released from lysosomes by P2Y-PLC-IP3-Ca^{2+}pathways. The ATP assay of HNECs culture supernatants implied an acute accumulation of extracellular ATP immediately after the Der p stimulation. Using P2Y-PLC-IP3 signaling inhibitors, we found that the Der p-induced IL-33 release was dependent on ATP-P2Y-PLC-IP3 signaling, followed by abolishing the ERK/P38 pathways.

Conclusion

Der p induced an acute accumulation of extracellular ATP which activated PY2-PLC-IP3 pathways to induce Ca^{2+} releasing from endoplasmic reticulum (ER), and intracellular Ca^{2+} induced ATP release from lysosomes from HNECs. ATP activated PY2-PLC-IP3 pathways followed by transactivation of ERK/P38 pathways which induced the expression of IL-33 mRNA and protein.

1. Introduction

Allergic rhinitis (AR) is an inflammatory disease of the nasal mucosa characterized by nasal itching, sneezing, runny nose and nasal congestion, which exists worldwide but is still not fully understood[1] .The incidence and prevalence of AR is pretty high, affecting more than 10%-40% of the global population, and continue to grow rapidly in recent years[2]. The widespread prevalence of AR in the world has caused a considerable economic burden on the patients and society. According to a telephone survey of 47,216 people conducted by Professor Zhang Luo's team in 2011, the incidence of AR in the Chinese population is about 17.6%, which demonstrates a significant increase compared to the results in

2005[3]. AR can be divided into seasonal AR and perennial AR[4],and inhaled air allergens (pollen, mold, and house dust mites) are also the main causes of AR. Among them, house dust mite (HDM) is the main allergen of perennial AR and contributes to the development of allergic respiratory diseases[5]. For about 10% to 20% of the world's population is allergic to HDM [6, 7]. The group 1 and group 2 molecules are the main allergens in HDM, namely D. pteronyssinus (Der p 1 and Der p 2) and D. farinae (Der f 1 and Der f 2) [8]. HDM stimulates airway smooth muscle cells through Toll-like receptors (TLRs) and NOD-like receptors (NLRs) signaling pathways to trigger MyD88 signaling pathway, which further activates NF-κB and MAPK pathways, thereby inducing cells to release interleukin 6 (IL-6) and interleukin 33(IL-33)[9].

IL-33 is a nuclear-related cytokine of the IL-1 family and an effective inducer of type II allergic reactions[10]. IL-33 binds to its orphan receptor ST2, thereby inducing the release of Th2-type inflammatory factors[11]. The IL-33/ST2 signaling pathway can not only promote the accumulation of eosinophils, basophils, mast cells and other inflammatory cells in the nasal mucosa, but also promote the release of inflammatory cytokines, thereby mediating allergic diseases[12]. Studies have shown that the higher expression level of IL-33 in the serum of AR patients is, the higher expression levels of IL-33 and ST2 in nasal mucosal epithelial cells are, which indicates that IL-33/ST2 may play an important role in AR[13].

Adenosine triphosphate (ATP) acts as an "alarm factor" when the immune system is attacked[14]. Studies have shown that ATP levels significantly increase in the alveolar lavage fluid of asthmatic patients and antigen-stimulated sensitized mice, and neutralized ATP can inhibit Th2 airway inflammation[15]. Kuozaki also found that the exposure to inhaled allergens induces the rapid release of ATP, while neutralizing the effect of ATP or blocking the P2 purinergic pathway could significantly inhibit the release of IL-33[16]. In primary cultured bronchial epithelial cells, HDM promotes the release of intracellular endoplasmic reticulum Ca^{2+} by activating ATP-dependent P2 purine receptors, thereby inducing cells to secrete CCL20[17]. Our teams' previous studies have confirmed that ATP induces the release of Ca^{2+} from the endoplasmic reticulum (ER) calcium store of the marginal cells through the P2Y2-PLC-IP3 signaling pathway, and ATP releases from lysosomes through exocytosis[18]. In keratinocytes, when stimulated by HDM, the cells quickly release a large amount of ATP, which rapidly induces the release of Ca2 + from the endoplasmic reticulum via activating extracellular P2Y purinergic receptor signaling, and enables activated keratinocytes to release IL-33[19]. HDM has also been confirmed to induce keratinocytes to release IL-1β through the ERK and P38 MAPK pathways, thereby inducing allergic dermatitis[20]. In vascular smooth muscle cells, the release of IL-33 induced by virus stimulation can be inhibited by Suramin, a broad-spectrum inhibitor of purine signaling[20]. Phospholipase C (PLC) inhibitor U73122 attenuates the release of IL-6 and IL-8 from human adipocytes by inhibiting the ERK MAPK pathway[21].In the present study, whether HDM would induce ATP release and regulate IL-33 released, which might contribute to the treatment of AR .

2. Methods

2.1 Cell Culture

Human nasal epithelial cell lines (BNBIO, BNCC340481, China) were grown in medium containing DMEM (GIBCO, 11965-092, Australia) mixed with 10% fetal bovine serum (GIBCO, 10099-141, Australia) and 100 U/ml penicillin (GIBCO, Australia), in an incubator (Thermo Scientific HERA CELL 150i CO2 incubator) with 5% CO2 at 37 °C.

2.2 Real-time PCR

Human nasal epithelial cells were first treated with 25μg/ml HDM extracts from Dermatophagoides pteronyssinus (Der p, XPB82DD3A2.5, Greer Laboratories, Lenoir, NC, USA) for 0,3,6,12,24 hours. After pre-treated with 5μM SB203580(Selleck, S1076, USA), 500nM trametinib (Selleck, S2673, USA) and 1μM SCH772984(Selleck, S7101, USA) for 30 mins, cells were then treated with Der p (25μg/ml) for 12 hours. After pre-incubation with P2Y-PLC-IP3 inhibitors 30μM Suramin (TOPSCIENCE, T2160, China), 2μM U73122 (TOPSCIENCE, T6243, China),100μM 2-APB (TOPSCIENCE, T4963, China) and 2μM Xestospongin C (APExBIO, B6668,USA), Ca^{2+} chelators 2mM EGTA (TOPSCIENCE, T13673, China), 30μM BAPTA-AM (TOPSCIENCE, T6245, China), an inhibitor of SERCA 2μM TG (TOPSCIENCE, TQ0302, China) and 4U/ml apyrase (Sigma Aldrich, A6535, USA) for 30 mins, cells were then treated with Der p (25μg/ml) for 12 hours. Total RNA was isolated from the cells using the trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. RNA pellets were dissolved in diethylpyrocarbonate-treated water (Sangon Biotech, B300592, China). The yield of RNA was quantified by spectroscopy at 260 nm. Complementary DNA (cDNA) was synthesized using 5× Prime Script RT Master Mix (TAKARA, R036A, Japan). Then, IL-33 and β-actin cDNA was amplified using an Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in conjunction with the 5× SYBR green Master Mix reaction kit. Primers were as follows: IL-33: forward 5′-GCCTGTCAACAGCAGTCTACTG-3′, reverse 5′-TGTGCTTAGAGAAGCAAGATACTC-3′. β-actin,5'-GCCGATCCACACGGAGTACTT-3' for the forward primer and 5'-TTGCCGACAGGATGCAGAA-3' for the reverse primer. The average transcript levels of genes were normalized to β-actin. The comparative threshold cycle (2-ΔΔCT) method was used to quantify the mRNA of the target genes. All samples were performed in three independent experiments. The relative amplification efficiencies of the primers were tested and found to be similar.

2.3 Western blot

The cultured human nasal epithelial cells controls were seeded in a six-well plate. Cells were first treated with 25μg/ml Der p for 0,7.5,15,30,60 mins and 0,3,6,12,24 hours. After pre-treated with 5μM SB203580, 500nM trametinib and 1μM SCH772984 for 30 mins, cells were then treated with Der p (25μg/ml) for 15 mins and 6 hours. Cells were also incubation with P2Y-PLC-IP3 inhibitors, Ca^{2+} chelators, inhibitor of SERCA TG and apyrase for 30 mins, cells were then treated with Der p (25μg/ml) for 15 mins and 6 hours. The protein was extracted with 100μL of RIPA Lysis Buffer (Beyotime, P0013B, China) addition of 50x Protease and phosphatase inhibitor cocktail (Beyotime, P1045, China) per well and quantified using a NanoDrop2000 spectrophotometer. The gel was prepared using a 10% Polyacrylamide Gel Electrophoresis (PAGE) gel kit (PG112, EpiZyme, China). Each well was loaded with 5-10μL of the gel, and 5μL of pre-stained protein ladder (Thermo Scientific, 00594048, USA) was added on both sides, followed

by electrophoresis at a constant voltage of 90 V for 90 min. The suspension was sealed with 5% skimmed milk powder and then incubated with p-P38 MAPK antibody 1:1000 (Cell signaling Technology, 4511T, USA), P38 MAPK antibody 1:1000 (Cell signaling Technology, 8390T, USA), p-ERK MAPK 1:1000 (Cell signaling Technology, 4370T, USA), ERK MAPK 1:1000 (Cell signaling Technology, 4695T, USA), IL-33 1:1000 (Absin, abs137796, China) and HRP conjugated β-actin antibody 1:3000 (Abways, AB2001, USA) overnight at 4°C. The membrane was then incubated with the corresponding horseradish peroxidase-labeled secondary goat anti-rabbit IgG antibodies (Beyotime, A0208, China). Immunoreactive proteins were measured with the Enhanced Chemiluminescence (ECL) Western blot detection system (Millipore, USA).

2.4 Immunocytochemistry

Cells grew on coverslips in 24-well cluster plates for 24 hours. Human nasal epithelial cells were then fixed for 15 min with ice-cold 4% paraformaldehyde in 0.1 M PBS at room temperature.

After washing with 0.01M PBS (Beyotime, C0221A, China), the sections were pre-incubated in 10% normal donkey serum and 0.1% Triton X-100 in 0.01 M PBS for 1 h at room temperature to block nonspecific antigenic sites. The sections were incubated with polyclonal antibodies to the IL-33 1:1000 (Absin, abs137796, China) in 5% normal donkey serum and 0.1% Triton X-100 (Beyotime, ST795, China) in 0.01 M PBS overnight at 4 °C. After extensive washing with 0.01 M PBS three times, the sections were incubated with Alexa 488-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch, USA) at 37 °C for 1 h. Samples were washed three times with 0.01 M PBS, and coverslips were overlaid onto glass slides with mounting medium and observed under an inverted fluorescence microscope (Nikon, USA).

2.5 Intracellular calcium measurements

Cells were incubated with 2μM Fura-2 AM (Beyotime, S1052, China) in Hanks' Balanced Salt Solution (Beyotime, C0219, China) plus 0.01% (w/v) pluronic F-127 (Beyotime, ST501, China) and 2mM EGTA for 30 min at 37 °C. Human nasal epithelial cells were first treated with 25μg/ml Der p, and cells were then pre-incubated with 30μM suramin, 100μM 2-APB, 2μM U73122, 2mM EGTA, 30μM BAPTA-AM, 4U/mL apyrase and 2μM TG before treatment with 25μg/ml Der p. Fura-2 AM fluorescence (340/380nm excitation; 505nm emission) was determined with a multimode reader (SpectraMax M2e, Molecular Devices, USA).

2.6 ATP measurement

Cells were plated on opaque 96-well plates (Coring, 3912, USA). An ATP bioluminescence assay kit (Promega, G7571, USA) was used to measure extracellular ATP release as described as previously[18].

2.7 ELISA

Cells were seeded in a 6-well tissue culture plate and grew until 90% confluence, usually 3 days. These cells were then stimulated with 25μg/ml Der p. After pre-treated with SB203580, trametinib and

SCH772984for 30 mins, cells were then treated with Der p (25μg/ml) for 24 hours. Cells were also incubation with P2Y-PLC-IP3 inhibitors, Ca^{2+} chelators and apyrase for 30 mins, and then treated with Der p (25μg/ml) for 24 hours. Cell-free supernatants were collected and analyzed for IL-33 by ELISA (Absin, abs551019, China). Then the absorbance was measured at 450 nm with a multimode reader (SpectraMax M2e, Molecular Devices, USA).

2.8 Fluorescence imaging

Cells were incubated with 5μM quinacrine dihydrochloride (Sigma-Aldrich, Q3251, USA) for 30 min and then incubated with 75nM Lyso-tracker red (Beyotime, C1046, China) for 60 min at room temperature in the dark. The cells were then incubated with Der p (25μg/ml) for 3mins. Fluorescence images of quinacrine dihydrochloride (green) and Lyso-tracker red (red) were obtained with λexcitation = 488 nm, λexcitation = 488 nm or λexcitation = 594 nm. All fluorescence images were observed in the dark by Inverted fluorescence Microscope (Nikon, USA).

2.9 Statistical analysis

Data analysis was performed in SPSS 26.0 software (IBM SPSS Inc.) and GraphPad Prism v5.0 (GraphPad Software, Inc.); data were analyzed by one-way analysis of variance.

3. Results

3.1 Der p induced IL-33 release from human nasal epithelial cells through ERK/P38 MAPK pathways.

To investigate whether HDM allergens would induce IL-33 mRNA expression in human nasal epithelial cells, Der p (25μg/ml) was incubated with cells for 3,6,12 and 24 hours. Der p effectively induced IL-33 mRNA expression at 12 h with a nearly ten-fold peak effect (Figure.1A). Furthermore, Der p significantly induced IL-33 protein releasing from human nasal epithelial cells since 15 min, then the level of IL-33 protein reached the peak (Figure.1B-C). After the addition of Der p for 3 h, IL-33 was released to the cytoplasm which stored in the nuclei of human nasal epithelial cell (Figure.1D). Moreover, IL-33 levels were detected by ELISA in supernatant from human nasal epithelial cells exposed to Der p (25μg/ml) for 3,6,12 and 24 hours. Incubation with (25μg/ml) Der p induced a significant increase in IL-33 secretion from 6 h, which reached the peak at 24 h (Figure.1E).

To research the mechanisms of Der p induced IL-33 release, the phosphorylation of ERK and P38 were detected by Western blot. After incubation with Der p, the phosphorylation of P38 and ERK were detected as early as 7.5 min and 3 h, which reached the peak at 15 min and 6 h (Figure.1B-C). Pre-treatment of nasal epithelial cells with P38 MAPK inhibitor SB203580 (5μM), MEK inhibitor trametinib (500nM) and ERK MAPK inhibitor SCH772984 (1μM) significantly reduced the expression of IL-33 mRNA compared with Der p group (Figure.2A). Apart from that, pre-incubation with P38/ERK inhibitors also abolished the phosphorylation of P38 and ERK after the addition of Der p for 30 min and 6 h. Consistent with the results of the phosphorylation of P38 and ERK, the expression of IL-33 protein was reduced from nasal

epithelial cells (Figure.2C-D). Additionally, pretreatment of cells with P38/ERK inhibitors inhibited the Der p-induced IL-33 secretion in human nasal epithelial cells, indicating that Der p induced IL-33 mRNA expression and IL-33 protein release via ERK/P38 MAPK pathways (Figure.2B).

3.2 Der p induced ATP release from lysosomes of human nasal epithelial cells through P2Y-PLC-IP3-Ca^{2+} pathways.

To find which organelles ATP stores in the cells, immunofluorescence experiments were performed. Immunostaining of human nasal epithelial cells with quinacrine for 30 mins, which was an acknowledged marker of ATP, resulted in numerous granule-like fluorescent puncta in the cytoplasm in cultured cells (Figure.3A). Then, nasal epithelial cells loaded with ATP marker quinacrine (green) were next immune-stained with a specific marker for lysosomes (Lyso-tracker red) for 1 h (Figure.3B). Average 69.4% of co-localization of quinacrine and Lyso-tracker fluorescent puncta revealed that ATP was mainly stored in the lysosomes of nasal epithelial cells (Figure.3C,E). After stimulation with 25μg/ml Der p for 3 mins, the fluorescent puncta of quinacrine (green) decreased, while there was no change in control vesicles, thus demonstrating that Der p triggered ATP release from lysosomes (Figure.3F-G).

However, the mechanisms of Der p induced ATP release from nasal epithelial cells remain unclear. After incubation with 25μg/ml Der p, a significant increase of intracellular Ca^{2+} (F_{340}/F_{380} peak=2.029) was observed compared with control group (F_{340}/F_{380} peak=0.724, *P<0.01, Figure.4A, D). After treatment with 30μM Suramin, a broad-spectrum P2Y receptor antagonist, Ca^{2+} response (F_{340}/F_{380} peak=1.328 vs. Der p *P<0.01, Figure.4B, D) was inhibited compared with Der p treated group. Furthermore, after pre-incubation with 2μM phospholipase C (PLC) inhibitor U73122 (F_{340}/F_{380} peak=1.248, *P<0.01 vs. Der p, Figure.4B, D) or 100μM IP3 inhibitor 2-APB (F_{340}/F_{380} peak=1.343, *P<0.01 vs. Der p, Figure.4B, D) for 15 mins, Ca^{2+} responses were also significantly inhibited. These results indicated that Der p induced Ca^{2+} responses relied on P2Y-PLC-IP3 pathways. Additionally, after pre-treatment with 30μM Ca^{2+} chelator BAPTA-AM (F_{340}/F_{380} peak=0.769, Figure.4C, D, **P<0.01vs. P2Y-PLC-IP3 pathways inhibitors) and 4U/ml apyrase (F_{340}/F_{380} peak=0.773, Figure.4C, D, **P<0.01 vs. P2Y-PLC-IP3 pathways inhibitors) for 15 mins, the Ca^{2+} also significantly decreased significantly compared with P2Y-PLC-IP3 pathways inhibitors. After pre-treatment with an inhibitor of SERCA 2μM thapsigargin (TG), the concentration of Ca^{2+} was also significantly decreased compared with Der p groups and P2Y-PLC-IP3 pathways inhibitors groups (F_{340}/F_{380} peak=1.329, Figure.4C, D). To research the mechanism of Der p induced ATP release from human nasal epithelial cells, an ATP bioluminescence assay kit was used in our study. Pre-treatment with P2Y-PLC-IP3 pathways inhibitors mentioned above notably decreased the concentration of ATP compared with incubation with Der p alone, which indicated Der p induced ATP release relied on P2Y-PLC-IP3 pathways (Figure.4E). After pre-incubation with 30μM BAPTA-AM, 4U/ml apyrase and 2μM TG, the concentration of ATP was much lower than Der p induced (Figure.4F). Therefore, we could come to the conclusion that Der p induced ATP release from lysosomes of human nasal epithelial cells via P2Y-PLC-IP3- Ca^{2+} pathways.

3.3 Der p stimulated transactivation of ERK/P38 MAPK pathways through ATP/P2Y-PLC-IP3 signaling but not Ca^{2+} signaling to induce IL-33 release from human nasal epithelial cells.

After pre-incubation with 30μM Suramin and 2μM U73122 for 1 h, the expression of IL-33 mRNA significantly decreased compared with the addition of Der p alone. However, pre-treatment with 100μM 2-APB could not inhibit the expression of IL-33 mRNA (Figure.5A). These results of western blot were consistent with qPCR that Suramin and U73122 but not 2-APB inhibited IL-33 protein expression (Figure.5C-D). Importantly, in combination with Der p, 30μM Suramin and 2μM U73122 significantly and synergistically inhibited IL-33 secretion in supernatant (Figure.5B). However, Xestospongin C, a more potent selective IP3R inhibitor which targeted on IP3R1, IP3R2 and IP3R3, significantly inhibited the expression of IL-33 mRNA and IL-33 secretion in supernatant (Figure.6A-B). To sum up, ERK/P38 MAPK inhibitors and P2Y-PLC-IP3 inhibitors could both reduce the release of IL-33 from human nasal epithelial cells, but the relationship between them still unclear. To find out whether ATP/P2Y-PLC-IP3 signals regulated ERK/P38 MAPK pathways, the phosphorylation of ERK and P38 were detected after incubation with P2Y-PLC-IP3 inhibitors. Then, we confirmed the phosphorylation of ERK and P38 and IL-33 protein decreased after incubation with 30μM Suramin, 2μM U73122 and 2μM Xestospongin C but not 100μM 2-APB for 30 min and 6 h which revealed that inhibition P2Y-PLC-IP3 signaling also suppressed the ERK/P38 MAPK pathways and IL-33 expression of human nasal epithelial cells (Figure.5C-D, 6C-D). Incubation with 2mM extracellular Ca^{2+} chelator EGTA and 30μM BAPTA-AM but not 2μM TG induced a significant increase of IL-33 mRNA expression (Figure.7A). Moreover，treatment with 2mM EGTA significantly reduced the phosphorylation of P38, and the incubation with 30μM BAPTA-AM enhanced the phosphorylation of P38/ERK，while none of them had inhibitory effects on IL-33. On the contrast, pre-incubation with 30μM BAPTA-AM enhanced the phosphorylation of P38/ERK but not the expression of IL-33(Figure.7C-D). Consistent with these results of Western blot, the secretion of IL-33 in supernatant of cells showed no differences after the addition of Ca^{2+} chelators and TG, which implied Ca^{2+} pathways did not participate in Der p-induced IL-33 release (Figure.7B). Furthermore, we investigated the roles of ATP in the release process of IL-33 by human nasal epithelial cells. We exposed cells to Der p and 30μM ATP with or without 4U/ml apyrase, and RT-PCR, Western blot and ELISA were then performed. Pre-incubation with 4U/ml apyrase which hydrolyzed ATP into ADP efficiently suppressed the expression of IL-33 mRNA, the phosphorylation of P38/ERK and IL-33 expression compared with the addition of Der p alone. In addition, 30μM ATP was used to stimulate the nasal epithelial cells with or without pre-treatment with apyrase, and the results of which were consistent with Der p treatment (Figure.8A, C, D). In addition, we observed that apyrase also inhibited IL-33 secretion from cells after treatment with Der p and ATP(Figure.8B).

Taken together, we confirmed that Der p induced IL-33 release from human nasal epithelial cells relied on the transactivation of ATP/P2Y-PLC-IP3 signaling.

4. Discussion

Recently, a common hypothesis emphasizes that epithelial-derived proinflammatory cytokines, namely IL-33 and IL-25, are the key factors to the immune-pathogenesis of AR and chronic rhinosinusitis (CRS), mainly involving type 2 inflammatory response[22]. IL-33 expression is significantly higher in nasal mucosa epithelial cells of AR patients and AR rats than it in the control groups, which can induce the production of Th2 inflammatory cytokines via binding to ST2[23, 24]. HDM, as the main allergen of perennial AR, is reported to induce IL-33 release from keratinocytes[19], pulmonary epithelial cells[25] and bone marrow[26]. Our findings are consistent with those reports that HDM (Der p) induces IL-33 protein(30Kda) to be released from nuclei to cytoplasm and then to extracellular space of human nasal epithelial cells (Figure.1).HDM is reported to stimulate airway smooth muscle cells through TLRs and NLRs signaling pathways to trigger MyD88 signaling pathway, which further activates NF-κB and MAPK pathways, thereby inducing cells to release IL-33[9]. Furthermore,, HDM can also activate P38 MAPK pathway to induce the release of inflammatory cytokines in 16HBE and BEAS-2B cell lines[27]. As also found by us, pre-incubation with P38 MAPK inhibitor SB203580, MEK inhibitor trametinib and ERK MAPK inhibitor SCH772984 could largely abolish Der p-induced phosphorylation of ERK/P38 MAPK and the expression of IL-33 of human nasal epithelial cells (Figure.1-2).

However, recent studies reveal that allergen extracts including HDM, Artemisia vulgaris, Betula pendula and Altenaria alternata induce dose-dependent rapid release of ATP from HBECs, while which organelles ATP stores in and how ATP release from the cells are still unclear[28, 29]. Our previous study revealed ATP induced the release of Ca^{2+} from the ER Ca^{2+} store of the marginal cells through the P2Y2-PLC-IP3 signaling pathway, and ATP releases from lysosomes through exocytosis[18].In the present research, it demonstrated that Quinacrine-labeled ATP was stored in lysosomes of human nasal epithelial cells which had an average 69.4% of co-localization with lysosomes (Figure.3). As reported, Quinacrine-labeled ATP appeared as puncta in large vesicles of olfactory epithelial cells[30].ATP functions through ATP-gated channels (P2X receptors) and G protein-coupled P2Y receptors, including P2X3,P2X4,P2X7,P2Y2,P2Y6,and P2Y11 receptors, all of which are expressed in the human nasal epithelial cells, which might play an important role in controlling the mucus and fluid secretion[31]. ATP is reported to evoke ER Ca^{2+} release via P2Y-PLC-IP3 pathway and P2X ion channels in marginal cells, human monocytes and macrophage[18, 32]

In our study, pre-incubation with apyrase which hydrolyzed ATP into ADP efficiently suppressed HDM induced Ca^{2+} responses. These results were consistent with those found in human keratinocytes which confirmed HDM induced acute extracellular accumulation of ATP activating $P2Y_2$ receptors[19]. Then, we found HDM-induced intracellular Ca^{2+} responses were partially inhibited by P2Y-PLC-IP3 pathway inhibitors and ER inhibitor TG, thereby confirming that P2X ion channels were also involved in HMD-induced Ca^{2+} release and ER was not the only source of intracellular Ca^{2+} store. Intracellular Ca^{2+} has been concerned to be stored in the lysosomes through H^+/Ca^{2+} exchange (CAX) with the help of the vacuolar(V)-type H^+-ATPase, which aids in maintaining the luminal environment at a pH of 4.6–5.0 and controlling cellular migration[33]. However, it is pretty difficult to measure the Ca^{2+} concentration inside lysosomes, because many fluorescent probes for measuring Ca^{2+} are sensitive to pH[34]. In the present

study, pre-treatment with P2Y-PLC-IP3 pathway inhibitors and Ca^{2+} inhibitors all abolished Der p-induced ATP release from human nasal epithelial cells, thus suggesting that Der p-induced ATP release is dependent on intracellular Ca^{2+}(Figure.4). In human monocytes[35], astrocytes[36], microglia[37]and marginal cells[18], ATP triggers ATP release from lysosomes via Ca^{2+}-dependent exocytosis. Release of ATP was induced with treatment with Ca^{2+}-dependent lysosomal exocytosis agonist NH_4Cl and inhibited with inhibitor vacuolin-1[38, 39].

Circulating IL-33, which triggers or exacerbates nasal allergic inflammation through amplifying Th2 inflammatory responses, is highly detected in AR patients[40]. In our study, we identified rapid IL-33 release from human nasal epithelial cells after Der p stimulation. IL-33 is constitutively expressed in the nucleus of epithelial cells and can be released into the extracellular space after cell damage[41]. Exposure to allergens induced acute extracellular accumulation of ATP, which sustained increases in intracellular Ca^{2+} concentration and releases IL-33 of human bronchial airway epithelial cells through activation of P2Y purinergic receptors[42]. Furthermore,ATP-P2 purinergic signaling-mediated IL-33 release was also detected in corneal epithelial cells and astrocytes[43, 44]. In our study, we found that Xestospongin C which was a selective IP3R inhibitor but not 2-APB inhibited the phosphorylation of ERK/P38 MAPK and secretion of IL-33. Although 2-APB was considered as an IP3R inhibitor and often used to inhibit IP3-evoked Ca^{2+} release, but it didn't achieve effective inhibition of IP3Rs without affecting other Ca^{2+}-regulating proteins, and only show selectivity for IP3R1[45]. As reported, Xestospongin C is the most potent of a series of xestospongins isolated from Australian sponges, which can act as membrane permeable, functional inhibitors of all subtypes of IP3Rs [46].We confirmed that pretreatment with ATP-P2Y-PLC-IP3 signaling inhibitors significantly abolished the phosphorylation of ERK/P38 MAPK pathways and secretion of IL-33 protein. HDM extract-induced IL-33 expression was reported to depend on extracellular ATP/$P2Y_2$ pathway mediated by transactivation of EGFR, followed by activation of the ERK MAPK pathway in human keratinocytes[19]. Pharmacological inhibition of P2Y receptor suramin or deficiency in the $P2Y_2$ gene both abrogate IL-33 release and Th2 inflammatory responses in the Alternaria-induced airway inflammation model in naïve mice[16].Suramin, a board P2Y receptor inhibitor, was reported to suppress the activation of the puromycin-triggered P38 activation [47] and the phosphorylation of ERK in PC12 cells[48]. The PLC inhibitor U73122 abolishes trans-10, cis-12 conjugated linoleic acid-Mediated inflammatory signaling and insulin resistance in human adipocytes via ERK MAPK pathways[21].The same results were also reported that U73122 which suppressed IP3 production prevented ERK activation evoked by DNIMI[49]. Additionally, the phosphorylation of P38 was diminished using PLC inhibitor U73122 which initiated by EGFR in THP-1 and Raji cell lines[50]. IP3 is in the downstream of PLC which binds to IP3 receptor to induce Ca^{2+} release from the membrane systems of the ER[51]. Several studies revealed that 2-APB was able to partially inhibit the phosphorylation of P38/ERK MAPK pathways[52, 53]. Ca^{2+} chelators BAPTA-AM and EGTA were reported to inhibit Cd-induced Ca^{2+} elevation and block Cd activation of P38/ERK MAPK pathways in PC12, SH-SY5Y cells[54].BAPTA-AM also inhibits RANKL-induced bone marrow macrophages differentiation through MEK/ERK, P38 MAPK pathways[55]. However, several studies revealed that BAPTA-AM had no inhibitory

effects on the H_2O_2 induced phosphorylation of P38 MAPK and ERK[56, 57]. Thapsigargin(TG), an ER stress inducer, activated gene and protein expression of IL-6 and induced phosphorylation of p38 MAPK[58]. In the present study, Xestospongin C significantly inhibited the phosphorylation of ERK/P38 MAPK pathways and secretion of IL-33 protein. IP3R are intracellular Ca^{2+} channels expressed in the membranes of the ER in the most eukaryotic cells. IP3Rs are essential links between many extracellular signals which stimulate PLC and initiation of cytosolic Ca^{2+} signals triggered by IP3-evoked Ca^{2+} release from the ER[45]. However, we did not see any difference of the phosphorylation of P38/ERK after pre-treatment with Ca^{2+} chelators and TG in human nasal epithelial cells in our research which implied that Ca^{2+} signaling had no effects in Der p-induced IL-33 release. Recently, Murat Bastepe found that IP3/PKC pathway activation without involving in Ca^{2+} signaling induced FGF23 elevation was dependent on MAPK signaling in Hpy mice[59]. However, the relationship between PLC/IP3 pathway and P38/ERK MAPK signaling remains more research.

In the current study, the concentration of ATP was significantly decreased after preincubation with apyrase which implied Der p-induced IL-33 expression is dependent on acute accumulation of extracellular ATP. Moreover, the phosphorylation of P38/ERK and the secretion of IL-33 were also inhibited with the addition of apyrase. Our results were consistent with several studies which revealed that apyrase abolished the expression of IL-33 through inhibition of ERK MAPK pathways[19]. IL-6 release was reduced by apyrase via p38 MAPK-dependent pathways in small airway epithelial cells[60].

5. Conclusion

Der p induced an acute accumulation of extracellular ATP which activated P2Y-PLC-IP3 pathways to induce intracellular Ca^{2+} releasing from ER. Then, intracellular Ca^{2+} induced ATP to release from lysosomes from human nasal epithelial cells. Moreover, ATP activated P2Y-PLC-IP3 pathways followed by transactivation of ERK/P38 MAPK pathways which induced the expression of IL-33 mRNA and protein. Our research demonstrated the new mechanism controlling Der p-induced IL- 33 in human nasal epithelial cells, a new sight that might play a role in AR development or cure (Figure.9).

Declarations

Authors' contributions

JP.L conceived the study and participated in writing the manuscript.
B.L, P.Z, TT.F, performed experiments, analyzed the data and wrote the manuscript.

All authors commented on and approved the final version of the manuscript.

Acknowledgment

Not applicable

Consent for publication

All authors agree the manuscript for publication.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

References

[1] A. Greiner, Allergic rhinitis, Lancet 378(9809) (2011) 2112-2122.

[2] X.a. Wang, T.i. Ma, X.a. Wang, Y. Zhuang, D.u. Wang, Prevalence of pollen-induced allergic rhinitis with high pollen exposure in grasslands of northern China, Allergy 73(6) (2018).

[3] X.D. Wang, M. Zheng, H.F. Lou, C.S. Wang, Y. Zhang, M.Y. Bo, S.Q. Ge, N. Zhang, L. Zhang, C. Bachert, An increased prevalence of self-reported allergic rhinitis in major Chinese cities from 2005 to 2011, Allergy 71(8) (2016).

[4] Skoner, P. David, Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis, Journal of Allergy & Clinical Immunology 108(1) (2001) S2-S8.

[5] Y. Meng, C. Wang, L. Zhang, Recent developments and highlights in allergic rhinitis, Allergy (3) (2019).

[6] Q. Meng, X. Liu, P. Li, L. He, J. Xie, X. Gao, X. Wu, F. Su, Y. Liang, The influence of house dust mite sublingual immunotherapy on the TSLP-OX40L signaling pathway in patients with allergic rhinitis, International Forum of Allergy & Rhinology (2016) 862-870.

[7] J. Sunyer, D. Jarvis, J. Pekkanen, S. Chinn, C. Janson, B. Leynaert, C. Luczynska, R. Garcia-Esteban, P. Burney, J.M. Antó, Geographic variations in the effect of atopy on asthma in the European Community Respiratory Health Study, J Allergy Clin Immunol 114(5) (2004) 1033-1039.

[8] W.R. Thomas, W.A. Smith, B.J. Hales, K.L. Mills, R.M. O Brien, Characterization and Immunobiology of House Dust Mite Allergens, INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY (2002).

[9] A. Jacquet, The role of innate immunity activation in house dust mite allergy, Trends in Molecular Medicine 17(10) (2011) 604-611.

[10] W.C. Gause, T.A. Wynn, J.E. Allen, Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths, Nature Reviews Immunology 13(8) (2013) 607-14.

[11] X. Zhou, T. Wei, C.W. Cox, Y. Jiang, W.R. Roche, A.F. Walls, Mast cell chymase impairs bronchial epithelium integrity by degrading cell junction molecules of epithelial cells, Allergy (2019).

[12] IL-33/ST2 axis controls Th2/IL-31 and Th17 immune response in allergic airway diseases, Immunobiology 220(8) (2015) 954-963.

[13] Y. Haenuki, K. Matsushita, S. Futatsugi-Yumikura, K.J. Ishii, T. Kawagoe, Y. Imoto, S. Fujieda, M. Yasuda, Y. Hisa, S. Akira, A critical role of IL-33 in experimental allergic rhinitis, Journal of Allergy & Clinical Immunology 130(1) (2012) 184-194.e11.

[14] Burnstock, G., Pathophysiology and Therapeutic Potential of Purinergic Signaling, Pharmacological Reviews 58(1) (2006) 58-86.

[15] Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells, Nature Medicine (2007).

[16] Kouzaki, Hideaki, Iijima, Koji, Kobayashi, Takao, O'Grady, Scott, M., Kita, The Danger Signal, Extracellular ATP, Is a Sensor for an Airborne Allergen and Triggers IL-33 Release and Innate Th2-Type Responses, Journal of Immunology (2011).

[17] S. Post, M.C. Nawijn, M.R. Jonker, N. Kliphuis, d.B. Van, M., A.J.M. Van Oosterhout, I.H. Heijink, House dust mite-induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production, Allergy 68(9) (2013) 1117-1125.

[18] B. Liu, W. Cao, J. Li, J. Liu, Lysosomal exocytosis of ATP is coupled to P2Y 2 receptor in marginal cells in the stria vascular in neonatal rats, Cell Calcium 76 (2018) 62-71.

[19] X. Dai, M. Tohyama, M. Murakami, K. Shiraishi, K. Sayama, House dust mite allergens induce interleukin 33 (IL-33) synthesis and release from keratinocytes via ATP-mediated extracellular signaling, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1866(5) (2020) 165719.

[20] R. Utsunomiya, X. Dai, M. Murakami, H. Okazaki, T. Tsuda, H. Mori, K. Shiraishi, M. Tohyama, K. Sayama, Heparinoid suppresses Der p-induced IL-1β production by inhibiting ERK and p38 MAPK pathways in keratinocytes, Experimental Dermatology (2018).

[21] W. Shen, K. Martinez, C.C. Chuang, M. Mcintosh, The phospholipase C inhibitor U73122 attenuates trans-10, cis-12 conjugated linoleic acid-mediated inflammatory signaling and insulin resistance in human adipocytes, Journal of Nutrition 143(5) (2013) 584.

[22] H. Hong, S. Liao, F. Chen, Q. Yang, D.Y. Wang, Role of IL-25, IL-33, and TSLP in triggering united airway diseases toward type 2 inflammation, Allergy (2020).

[23] R. Huang, W. Mao, G. Wang, J. Ding, Z. Sun, Synergistic relationship between TSLP and IL/ST2 signaling pathways in allergic rhinitis and the effects of hypoxia, 2020.

[24] R., Kamekura, T., Kojima, K., Takano, M., Go, N., Sawada, The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis, Clinical & Experimental Allergy 42(2) (2011) 218-228.

[25] Z. Sun, N. Ji, Q. Ma, R. Zhu, M. Zhang, Epithelial-Mesenchymal Transition in Asthma Airway Remodeling Is Regulated by the IL-33/CD146 Axis, Frontiers in Immunology 11 (2020) 1598.

[26] E. Boberg, K. Johansson, C. Malmhll, J. Weidner, M. Rdinger, House Dust Mite Induces Bone Marrow IL-33-Responsive ILC2s and TH Cells, International Journal of Molecular Sciences 21(11) (2020) 3751.

[27] C.K. Wong, M. Li, C.B. Wang, W.K. Ip, Y.P. Tian, C. Lam, House dust mite allergen Der p 1 elevates the release of inflammatory cytokines and expression of adhesion molecules in co-culture of human eosinophils and bronchial epithelial cells, International Immunology (8) (2006) 1327-1335.

[28] H. Kouzaki, K. Iijima, T. Kobayashi, S.M. O'Grady, H. Kita, The Danger Signal, Extracellular ATP, is a Sensor for Airborne Allergens and Triggers IL-33 Release and Innate Th2-type Responses, Journal of Allergy & Clinical Immunology (2011).

[29] S. Ramu, M. Menzel, L. Bjermer, C. Andersson, H. Akbarshahi, L. Uller, Allergens produce serine proteases-dependent distinct release of metabolite DAMPs in human bronchial epithelial cells, Clinical & Experimental Allergy (2017).

[30] Sébastien, Hayoz, Cuihong, Jia, CC, Hegg, Mechanisms of constitutive and ATP-evoked ATP release in neonatal mouse olfactory epithelium, Bmc Neuroscience (2012).

[31] Jae, Young, Choi, Wan, Namkung, Ji-Hyun, Shin, Joo-Heon, Yoon, Uridine-5'-triphosphate and Adenosine Triphosphate γS Induce Mucin Secretion Via Ca2+-dependent Pathways in Human Nasal Epithelial Cells, Acta Oto Laryngologica (2016).

[32] J.A. Layhadi, S.J. Fountain, P2X4 Receptor-Dependent Ca2+ Influx in Model Human Monocytes and Macrophages, International Journal of Molecular Sciences 18(11) (2017) 2261.

[33] M. Melchionda, J.K. Pittman, R. Mayor, S. Patel, Ca2+/H+ exchange by acidic organelles regulates cell migration in vivo, Journal of Cell Biology 212(7) (2016) 803-813.

[34] F.A. Lattanzio, D.K. Bartschat, The effect of pH on rate constants, ion selectivity and thermodynamic properties of fluorescent calcium and magnesium indicators, Biochemical and Biophysical Research Communications 177(1) (1991) 184-191.

[35] V. Sivaramakrishnan, S. Bidula, H. Campwala, D. Katikaneni, S.J. Fountain, Constitutive lysosome exocytosis releases ATP and engages P2Y receptors in human monocytes, Journal of Cell Science (2012).

[36] Z. Zhang, G. Chen, W. Zhou, A. Song, T. Xu, Q. Luo, W. Wang, X.S. Gu, S. Duan, Regulated ATP release from astrocytes through lysosome exocytosis, Nature Cell Biology 9(8) (2007) 945-953.

[37] Y. Dou, H.J. Wu, H.Q. Li, Q. Song, S. Duan, Microglial migration mediated by ATP-induced ATP release from lysosomes, Cell Research 22(6) (2012) 1022-1033.

[38] J.J.A.B. C, Y.H.S. A, H.K.B. C, S.J.L. A, H.K.B. C, Possible ATP release through lysosomal exocytosis from primary sensory neurons, Biochemical and Biophysical Research Communications 430(2) (2013) 488-493.

[39] J. Cerny, Y. Fe Ng, A. Yu, K. Miyake, B. Borgonovo, J. Klumperman, J. Meldolesi, P.L. Mcneil, T. Kirchhausen, The small chemical vacuolin-1 inhibits Ca2+-dependent lysosomal exocytosis but not cell resealing, EMBO reports 6(9) (2005) 883–888.

[40] H. Hong, S. Liao, F. Chen, Q. Yang, D. Wang, Role of IL-25, IL-33, and TSLP in triggering united airway diseases toward type 2 inflammation, Allergy (2020).

[41] Lefran?ais, Cayrol, Mechanisms of IL-33 processing and secretion: Differences and similarities between IL-1 family members, European cytokine network (2012).

[42] Kouzaki, Hideaki, Iijima, Koji, Kobayashi, Takao, O'Grady, M. Scott, Kita, Hirohito, The Danger Signal, Extracellular ATP, Is a Sensor for an Airborne Allergen and Triggers IL-33 Release and Innate Th2-Type Responses, Journal of Immunology (2011).

[43] C. Hudson, G.P. Christophi, R.C. Gruber, J.R. Wilmore, D.A. Lawrence, P.T. Massa, Induction of IL-33 expression and activity in central nervous system glia, Journal of Leukocyte Biology 84(3) (2008).

[44] L. Zhang, R. Lu, G. Zhao, S.C. Pflugfelder, D.Q. Li, TLR-mediated Induction of Pro-allergic Cytokine IL-33 in Ocular Mucosal Epithelium, other 43(9) (2011).

[45] H. Saleem, S.C. Tovey, T.F. Molinski, C.W. Taylor, Interactions of antagonists with subtypes of inositol 1,4,5-trisphosphate (IP3) receptor, British Journal of Pharmacology 171(13) (2014) 3298-3312.

[46] A. Milsom, Xestospongin C, Xestospongin C2011.

[47] H. Piao, Y. Chi, X. Zhang, Z. Zhang, K. Gao, M. Niimi, M. Kamiyama, J. Zhang, M. Takeda, J. Yao, Suramin inhibits antibody binding to cell surface antigens and disrupts complement-mediated mesangial cell lysis, Journal of Pharmacological Sciences 132(4) (2016) 224-234.

[48] Y. Liu, K.Z. Guyton, M. Gorospe, Q. Xu, N.J. Holbrook, Differential activation of ERK, JNK/SAPK and P38/CSBP/RK map kinase family members during the cellular response to arsenite, Free Radical Biology & Medicine 21(6) (1996) 771.

[49] M. Tomizawa, J.E. Casida, Desnitro-imidacloprid Activates the Extracellular Signal-Regulated Kinase Cascade via the Nicotinic Receptor and Intracellular Calcium Mobilization in N1E-115 Cells, Toxicology & Applied Pharmacology 184(3) (2002) 180-186.

[50] L. Saryeddine, K. Zibara, N. Kassem, B. Badran, N. El-Zein, EGF-Induced VEGF Exerts a PI3K-Dependent Positive Feedback on ERK and AKT through VEGFR2 in Hematological In Vitro Models, Plos One 11(11) (2016) e0165876.

[51] M.J. Berridge, P. Lipp, M. Bootman, The versatility and universality of calcium signalling, Nat Rev Mol Cell Biol (2000).

[52] Y. Li, H. Jiang, C. Ruan, J. Zhong, P. Gao, D. Zhu, W. Niu, S. Guo, The interaction of transient receptor potential melastatin 7 with macrophages promotes vascular adventitial remodeling in transverse aortic constriction rats, Hypertension Research Official Journal of the Japanese Society of Hypertension 37(1) (2014) 35-42.

[53] Z. Zeng, T. Leng, X. Feng, H. Sun, I. Koichi, L. Zhu, Z.G. Xiong, A. Ken, Silencing TRPM7 in Mouse Cortical Astrocytes Impairs Cell Proliferation and Migration via ERK and JNK Signaling Pathways, PLOS ONE 10(3) (2015) e0119912.

[54] B. Xu, S. Chen, Y. Luo, Z. Chen, L. Liu, H. Zhou, W. Chen, T. Shen, X. Han, L. Chen, Calcium Signaling Is Involved in Cadmium-Induced Neuronal Apoptosis via Induction of Reactive Oxygen Species and Activation of MAPK/mTOR Network, Plos One 6(4) (2011) e19052.

[55] S. Zhou, X. Yuan, Q. Liu, X. Zhang, X. Pan, L. Zang, L. Xu, BAPTA-AM, an intracellular calcium chelator, inhibits RANKL-induced bone marrow macrophages differentiation through MEK/ERK, p38 MAPK and Akt, but not JNK pathways, Cytokine 52(3) (2010) 210-214.

[56] Q.S. Ji, G. Carpenter, Role of basal calcium in the EGF activation of MAP kinases, Oncogene 19(14) (2000) 1853.

[57] K. Niwa, O. Inanami, T. Ohta, S. Ito, T. Karino, M. Kuwabara, p38 MAPK and Ca2+ contribute to hydrogen peroxide-induced increase of permeability in vascular endothelial cells but ERK does not, Free Radical Research Communications 35(5) (2001) 519-527.

[58] O. Krupkova, A. Sadowska, T. Kameda, W. Hitzl, K. Wuertz-Kozak, p38 MAPK Facilitates Crosstalk Between Endoplasmic Reticulum Stress and IL-6 Release in the Intervertebral Disc, Frontiers in Immunology 9 (2018) 1706.

[59] Q. He, L.T. Shumate, J. Matthias, C. Aydin, M. Bastepe, A G protein–coupled, IP3/protein kinase C pathway controlling the synthesis of phosphaturic hormone FGF23, JCI Insight 4(17) (2019) e125007.

[60] Douillet, D. Christelle, R. Iii, P. William, Milano, M. Peter, Boucher, C. Richard, Rich, B. Preston, Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent

pathways, American Journal of Physiology: Lung Cellular & Molecular Physiology (2006).

Figures

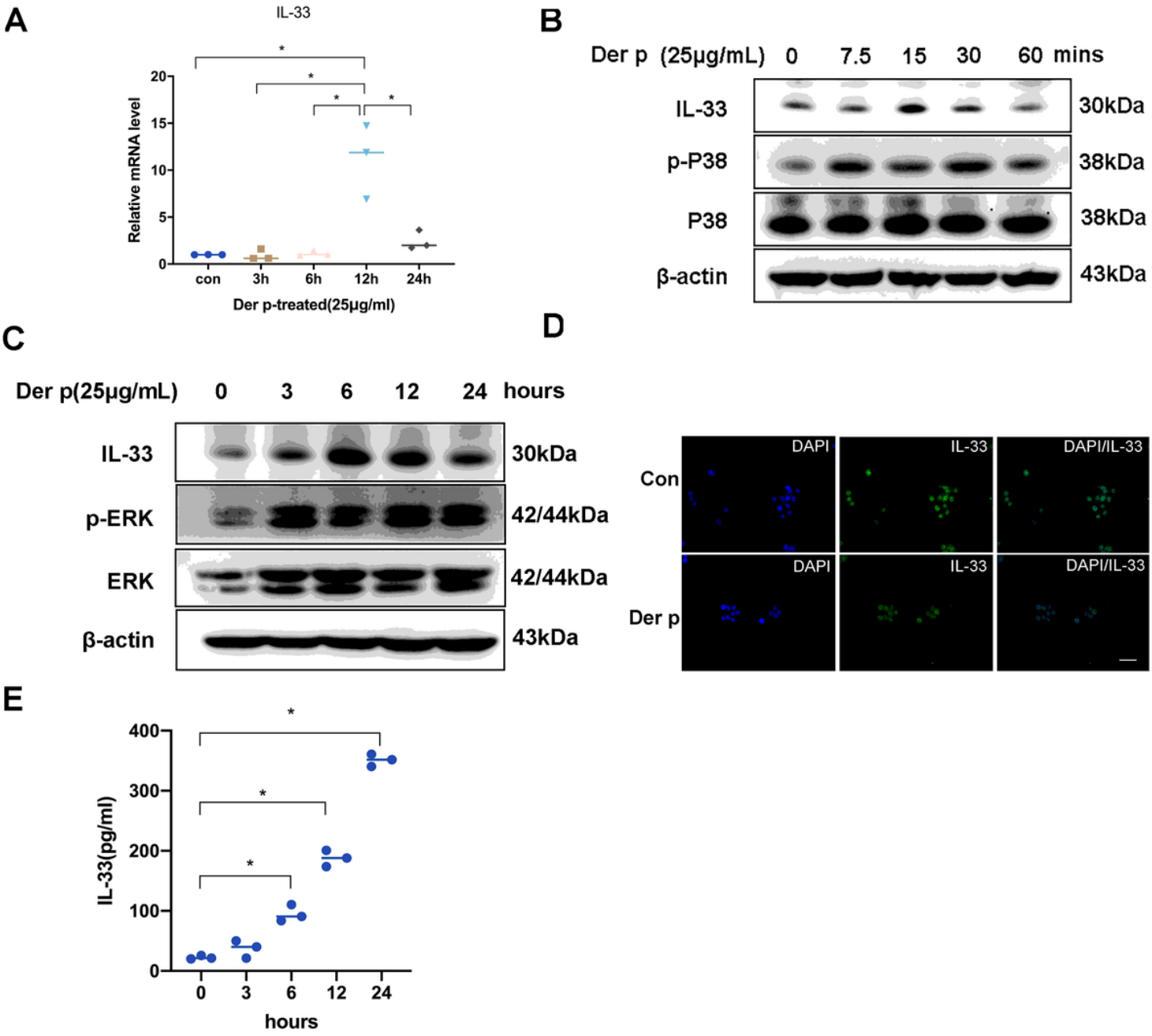

Figure 1

Der p induced IL-33 expression in human nasal epithelial cells. (A) Der p (25μg/ml) induced IL-33 mRNA expression at 12 h by using real-time PCR. (B-C) IL-33 protein was analyzed by Western blotting of cell extracts. Der p significantly induced IL-33 protein release from human nasal epithelial cells since 15 min, then reach the peak at 6 h and 12 h. The phosphorylation of P38 and ERK were detected as early as 7.5 min and 3 h, which reached the peak at 15 min and 6 h.(D) IL-33 released to the cytoplasm from the

nuclei of human nasal epithelial cell after the addition of Der p for 3 h. (E) IL-33 levels increased from 6 h and reached the peak at 24 h by ELISA in supernatant from human nasal epithelial cells after exposing to Der p for 3,6,12 and 24 hours. Relative mRNA levels are expressed as means ± SDs (n = 3), and ELISA levels are expressed as means ± SDs (n = 3). (* $p < 0.05$).

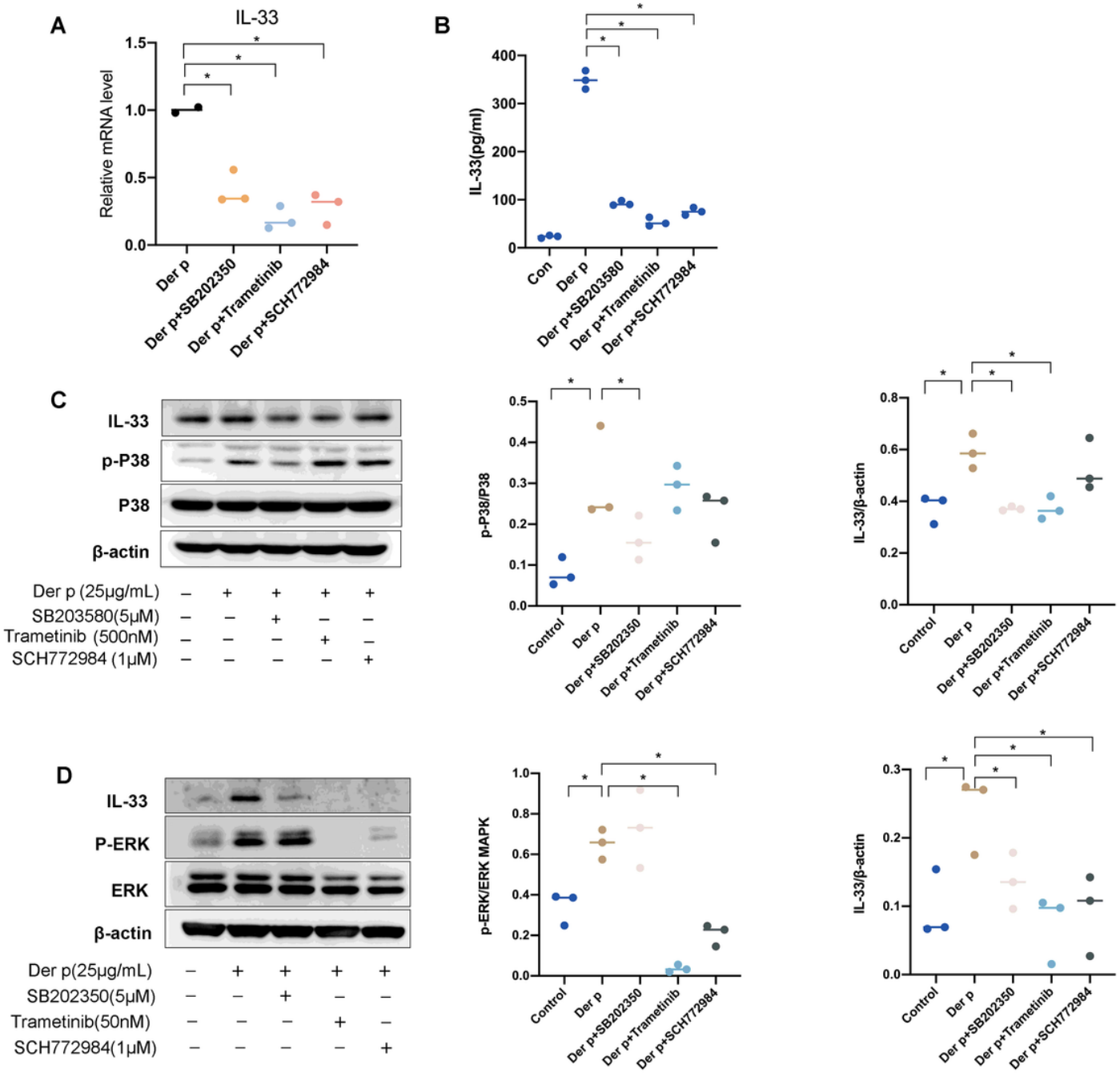

Figure 2

Der p induced IL-33 expression in human nasal epithelial cells via P38/ERK MAPK pathways. (A) Der p-induced expression of IL-33 mRNA was significantly inhibited with P38 MAPK inhibitor SB203580 (5μM), MEK inhibitor trametinib (500nM) and ERK MAPK inhibitor SCH772984 (1μM). (B) IL-33 levels were decreased at 24 h by ELISA in supernatant from human nasal epithelial cells after pre-treatment with SB203580 (5μM), trametinib (500nM) and SCH772984 (1μM). (C) IL-33 protein and the phosphorylation

of P38 were significantly abolished after incubation with SB203580, trametinib and SCH772984 from human nasal epithelial cells. (D) IL-33 protein and the phosphorylation of ERK were significantly abolished after incubation with SB203580, trametinib and SCH772984 from human nasal epithelial cells. Relative mRNA levels are expressed as means ± SDs (n = 3), ELISA levels are expressed as means ± SDs (n = 3), Western blot levels are expressed as means ± SDs (n = 3) (* $p < 0.05$).

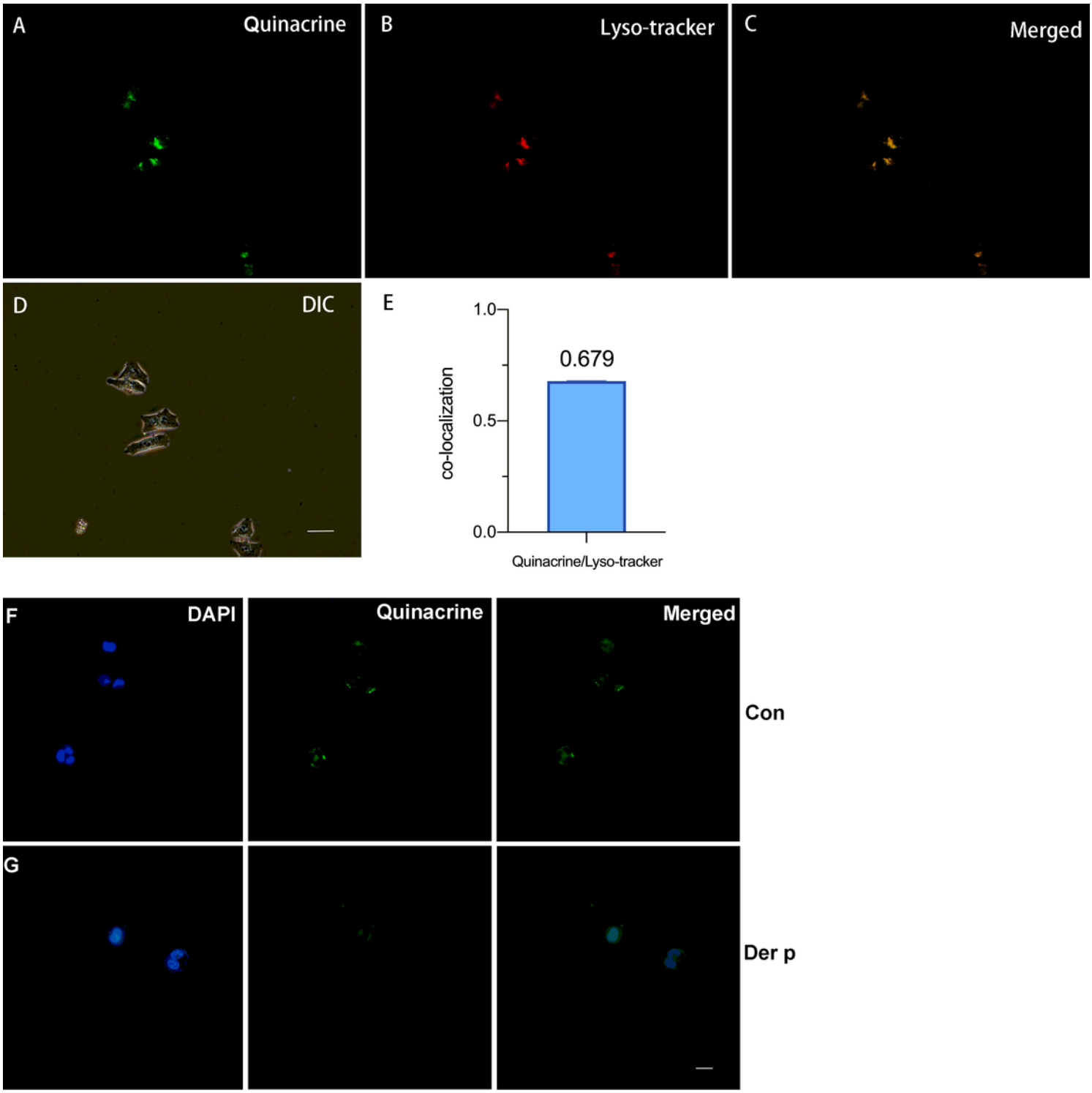

Figure 3

Der p induced ATP release from lysosomes from human nasal epithelial cells. (A) Fluorescence image of human nasal epithelial cells labelled with quinacrine. (B) Cells stained with Lyso-tracker in the cytoplasm. (C) Merged image of quinacrine and Lyso-tracker. (D) Cells in DIC. (E) Summary of the co-localization of quinacrine and Lyso-tracker of human nasal epithelial cells. (F)Left: Immunolabelling of cells labelled with quinacrine. Middle: cells stained with DPAI. Right: Merged image of quinacrine and DAPI; (G) Left: Immunolabelling of cells labelled with quinacrine which incubation with Der p. Middle: Cells stained with DAPI. Right: Merged image of quinacrine and DAPI; scale bar: 50μm. The number above column refers to mean co-localization coefficient. 3 images obtained from 3 independent experiments were calculated for co-localization analysis in each group.

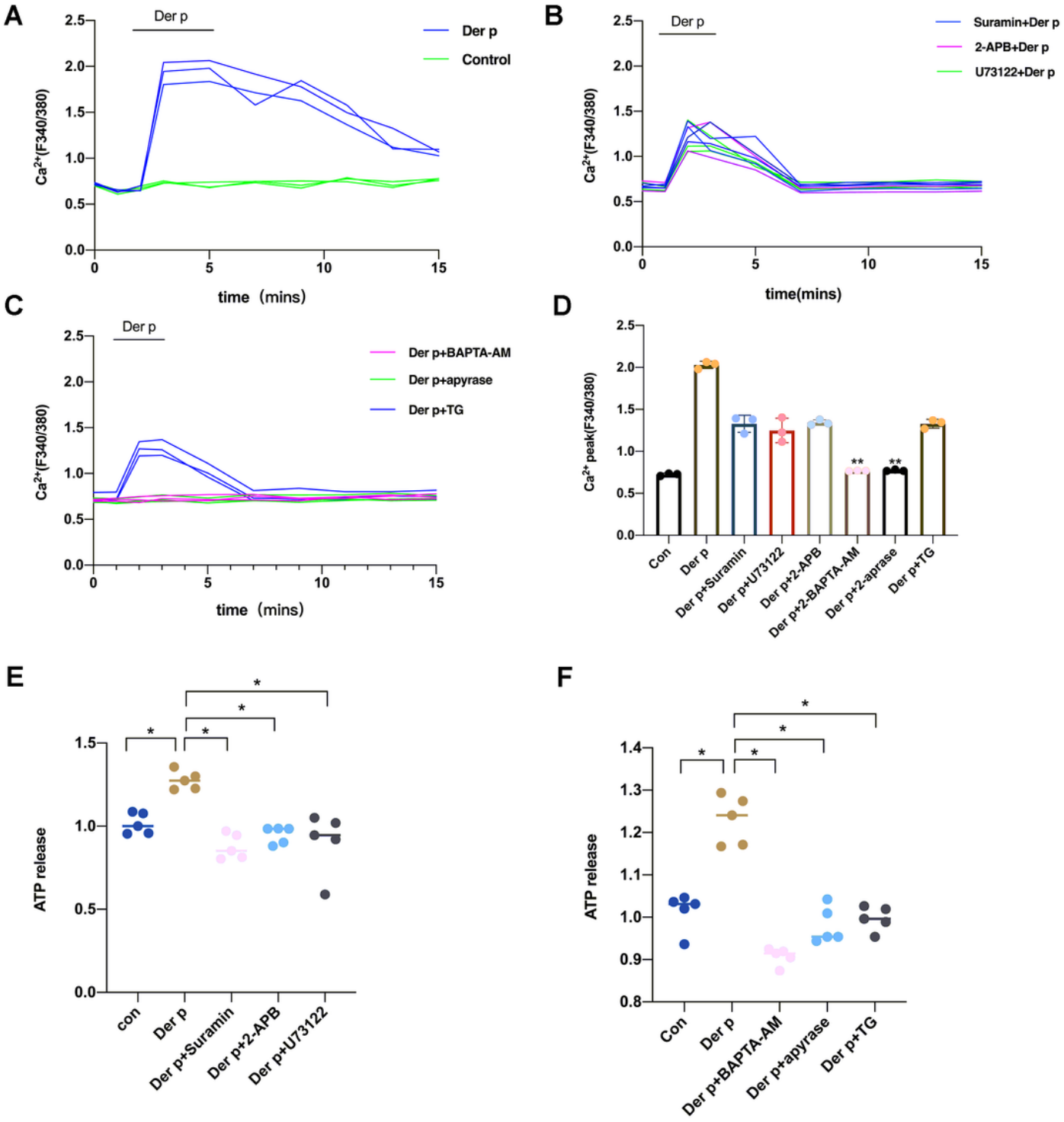

Figure 4

Der p induced ATP release from human nasal epithelial cells via P2Y-PLC-IP3-Ca2+. (A) Ca2+ signals were evoked by (25μg/ml) Der p. (B) Der p-evoked Ca2+ responses were inhibited by pre-incubation with 30μM suramin, 2μM U73122 and 100μM 2-APB. (C) Der p-evoked Ca2+ responses were inhibited by pre-incubation with 4U/mL apyrase, 2μM TG and 30μM BAPTA-AM. (D) Average peak Ca2+ responses (Fmax/F0) after treatment with 0.1% DMSO and antagonists above (n =3, *P < 0.01, **P < 0.01 compared

with P2Y-PLC-IP3 pathway antagonists). (E) Treatment with different reagents (30μM suramin, 2μM U73122 and 100μM 2-APB) resulted in ATP release from cells (n = 9, *P < 0.01 compared with Der p groups). (F) Treatment with different reagents (4U/mL apyrase, 2μM TG and 30μM BAPTA-AM) resulted in ATP release from cells (n = 9, *P < 0.01 compared with Der p groups). Error bars indicate SD; data were analyzed by one-way analysis of variance.

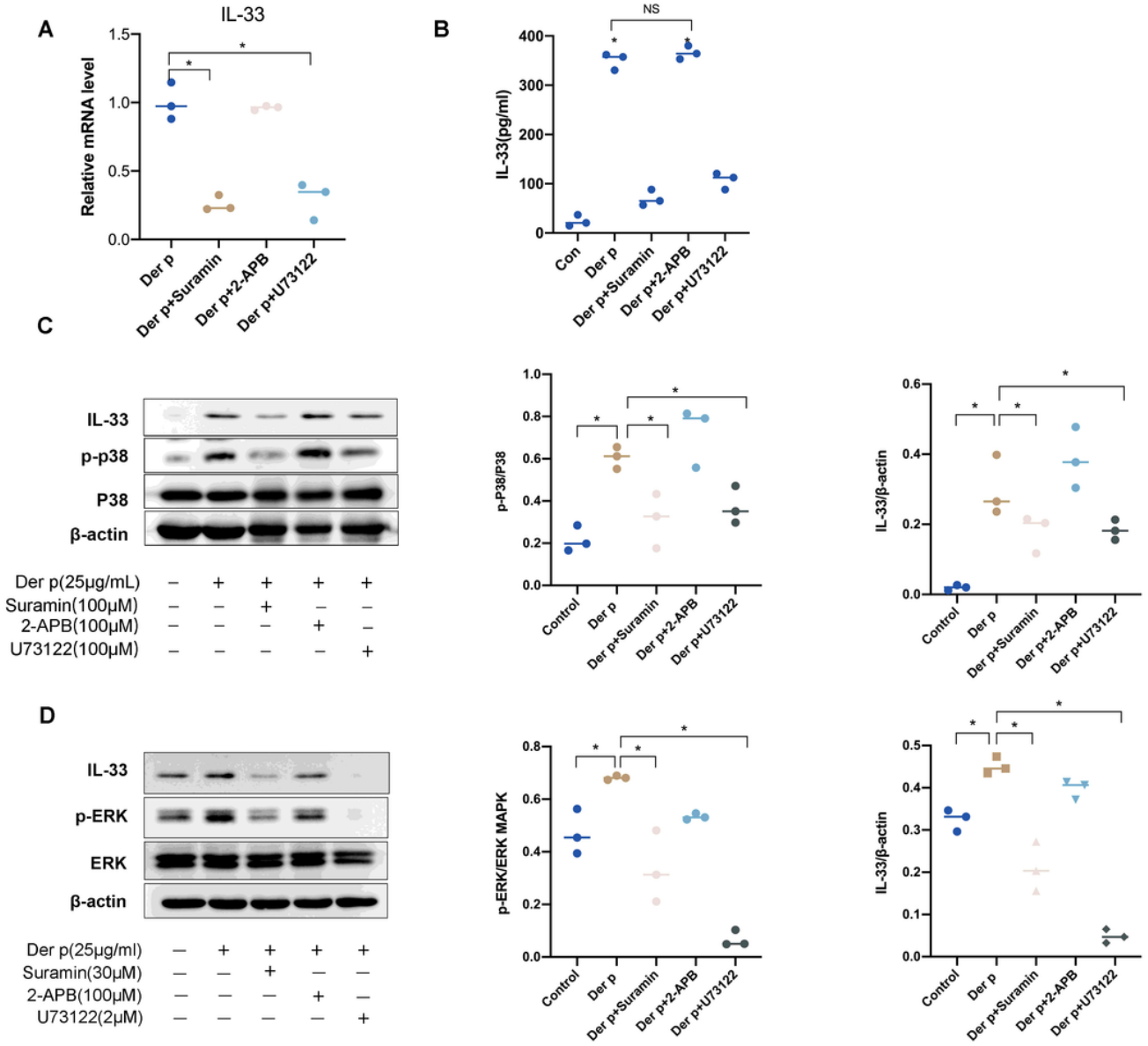

Figure 5

Der p induced IL-33 expression via P2Y-PLC signaling. (A) Der p-induced expression of IL-33 mRNA was significantly inhibited with 30μM suramin, 2μM U73122 and 100μM 2-APB. (B) IL-33 levels were decreased at 24 h by ELISA in supernatant from human nasal epithelial cells after pre-treatment with 30μM suramin, 2μM U73122 and 100μM 2-APB. (C) IL-33 protein and the phosphorylation of P38 were significantly abolished after incubation with 30μM suramin, 2μM U73122 and 100μM 2-APB from human nasal epithelial cells. (D) IL-33 protein and the phosphorylation of ERK were significantly abolished after incubation with 30μM suramin, 2μM U73122 and 100μM 2-APB from human nasal epithelial cells.

Relative mRNA levels are expressed as means ± SDs (n = 3), ELISA levels are expressed as means ± SDs (n = 3), Western blot levels are expressed as means ± SDs (n = 3) (* $p < 0.01$). Error bars indicate SD; data were analyzed by one-way analysis of variance.

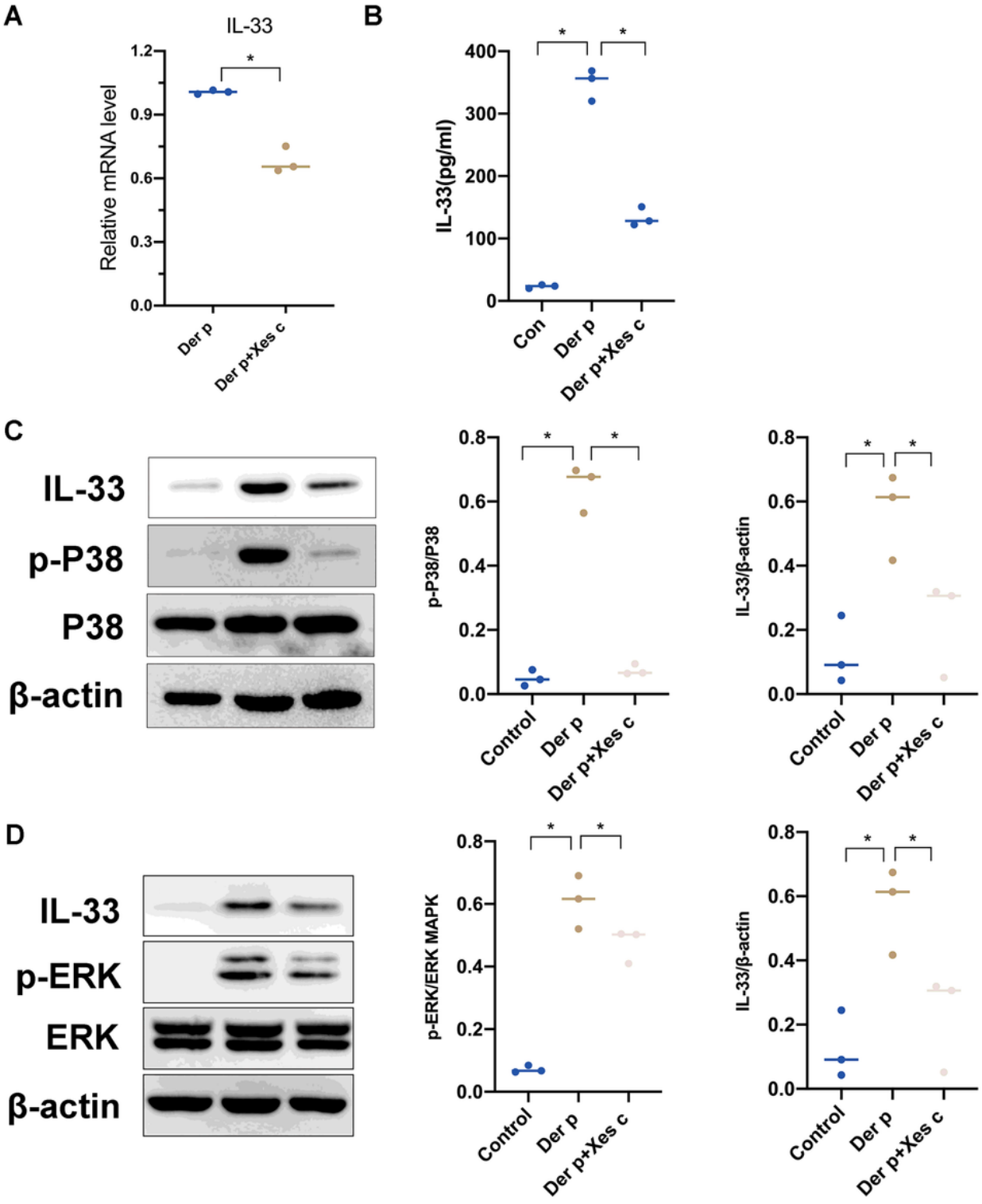

Figure 6

Der p induced IL-33 expression via P2Y-PLC-IP3 signaling. (A) Der p-induced expression of IL-33 mRNA was significantly inhibited with 2μM Xestospongin C. (B) IL-33 levels were decreased at 24 h by ELISA in

supernatant from human nasal epithelial cells after pre-treatment with 2μM Xestospongin C. (C) IL-33 protein and the phosphorylation of P38 were significantly abolished after incubation with 2μM Xestospongin C from human nasal epithelial cells. (D) IL-33 protein and the phosphorylation of ERK were significantly abolished after incubation with 2μM Xestospongin C from human nasal epithelial cells. Relative mRNA levels are expressed as means ± SDs (n = 3), ELISA levels are expressed as means ± SDs (n = 3), Western blot levels are expressed as means ± SDs (n = 3) (* $p < 0.01$). Error bars indicate SD; data were analyzed by one-way analysis of variance.

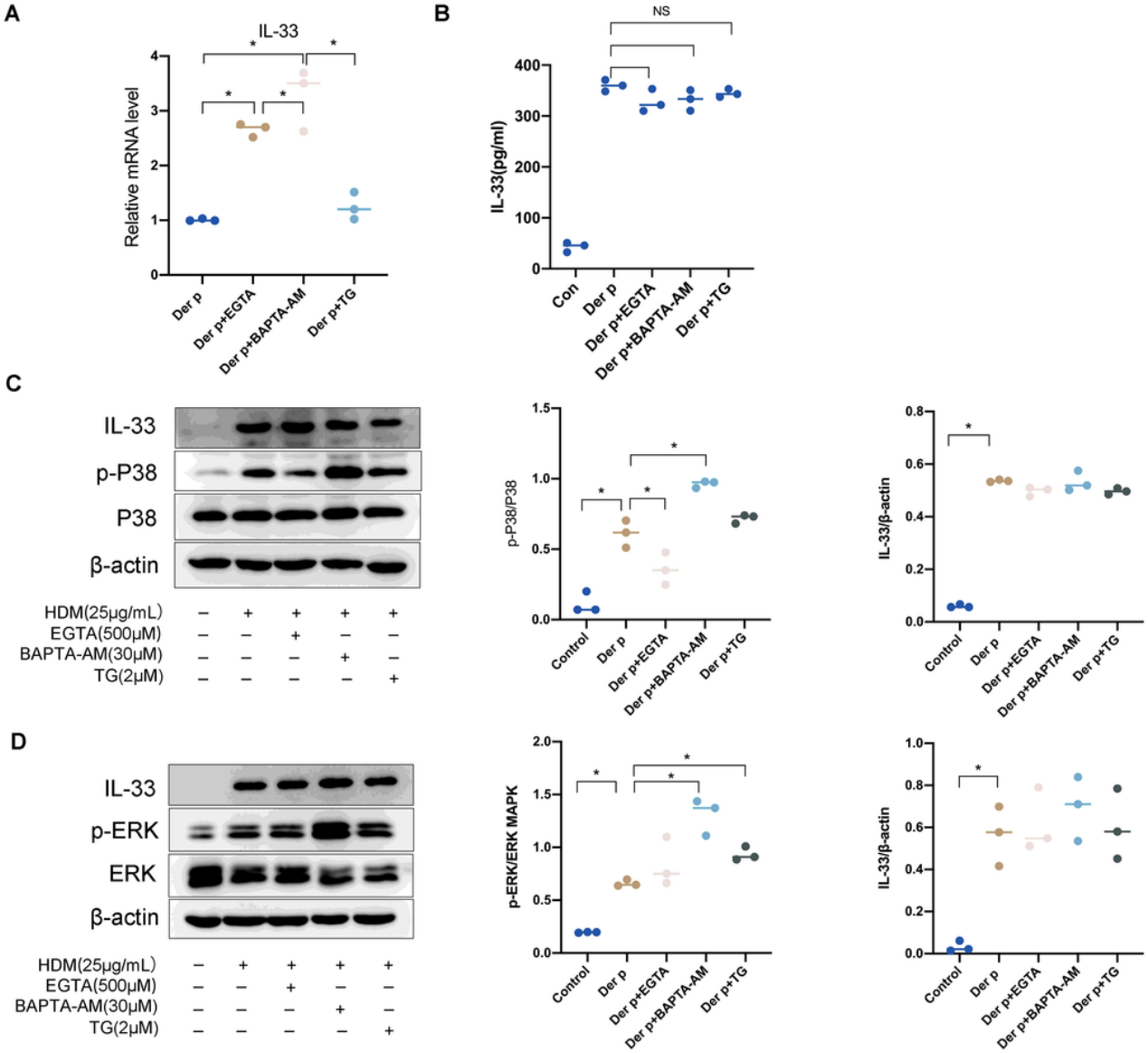

Figure 7

Der p induced IL-33 expression via extracellular ATP-mediated signal pathways. (A) Der p-induced expression of IL-33 mRNA was significantly increased with 2mM EGTA and 30μM BAPTA-AM. (B) IL-33 levels were decreased at 24 h by ELISA in supernatant from human nasal epithelial cells after pre-treatment with 2mM EGTA and 30μM BAPTA-AM and 2μM TG. (C) The phosphorylation of P38 was decreased after pre-treatment with 2mM EGTA and increased after pre-treatment with 30μM BAPTA-AM. IL-33 protein was not affected by pre-incubation with 2mM EGTA, 30μM BAPTA-AM and 2μM TG. (D) The

phosphorylation of ERK was increased after pre-treatment with 30μM BAPTA-AM and 2μM TG, and IL-33 protein was not affected by pre-incubation with 2mM EGTA, 30μM BAPTA-AM and 2μM TG. Relative mRNA levels are expressed as means ± SDs (n = 3), ELISA levels are expressed as means ± SDs (n = 3), Western blot levels are expressed as means ± SDs (n = 3) (* p < 0.01). Error bars indicate SD; data were analyzed by one-way analysis of variance.

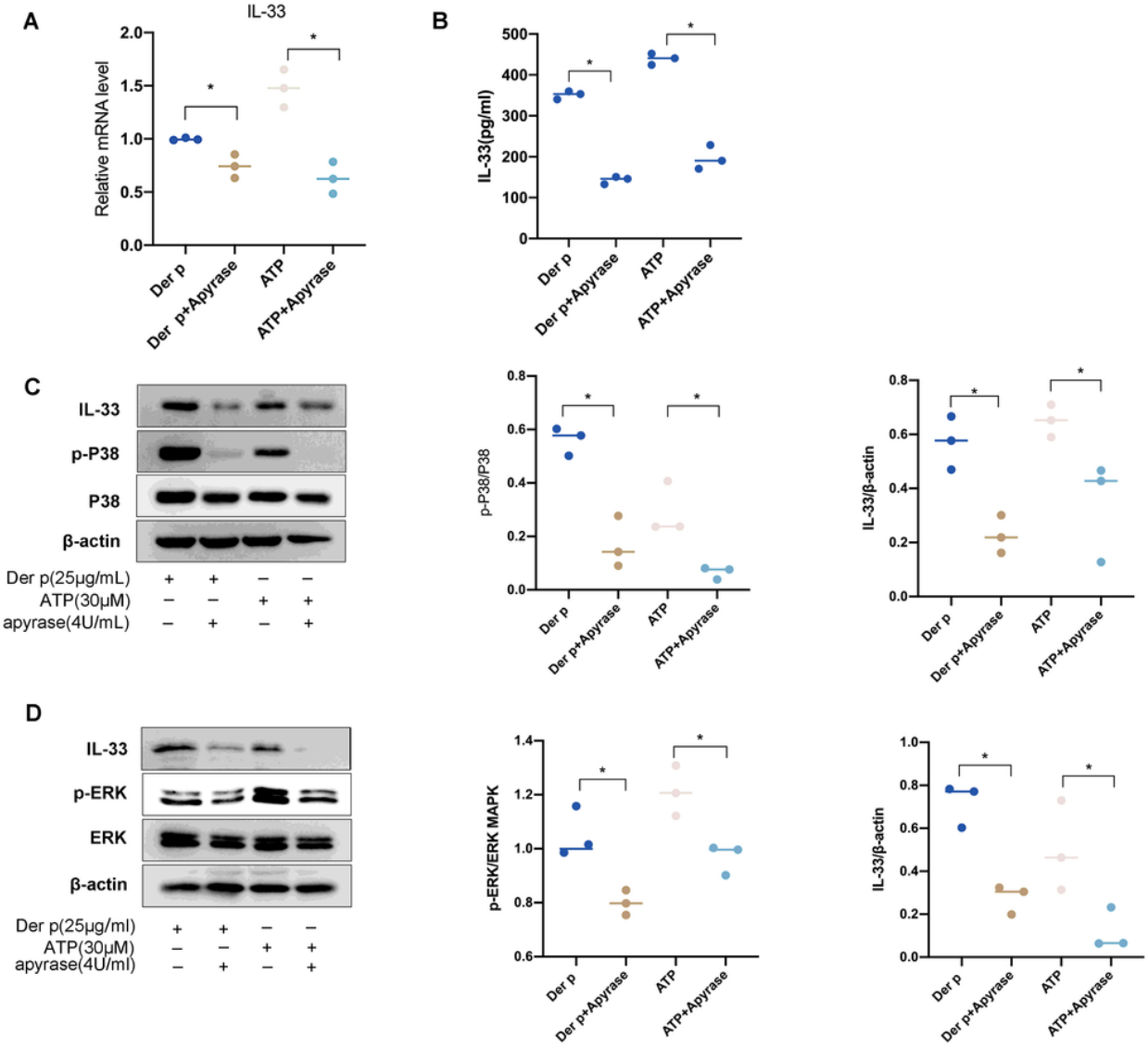

Figure 8

Der p induced IL-33 expression relied on extracellular ATP. (A)Der p-induced expression of IL-33 mRNA was significantly increased with Der p and 30μM ATP with or without 4U/mL apyrase. (B) IL-33 levels were decreased at 24 h in supernatant from human nasal epithelial cells after pre-incubation with 4U/mL apyrase after the addition of Der p and ATP. (C)IL-33 protein and the phosphorylation of P38 were significantly abolished after pre-incubation with 4U/mL apyrase after the addition of Der p and ATP from human nasal epithelial cells. (D) IL-33 protein and the phosphorylation of ERK were significantly inhibited after pre-incubation with 4U/mL apyrase after the addition of Der p and ATP from human nasal epithelial cells. Relative mRNA levels are expressed as means ± SDs (n = 3), ELISA levels are expressed as means ±

SDs (n = 3), Western blot levels are expressed as means ± SDs (n = 3) (* p < 0.01). Error bars indicate SD; data were analyzed by one-way analysis of variance.

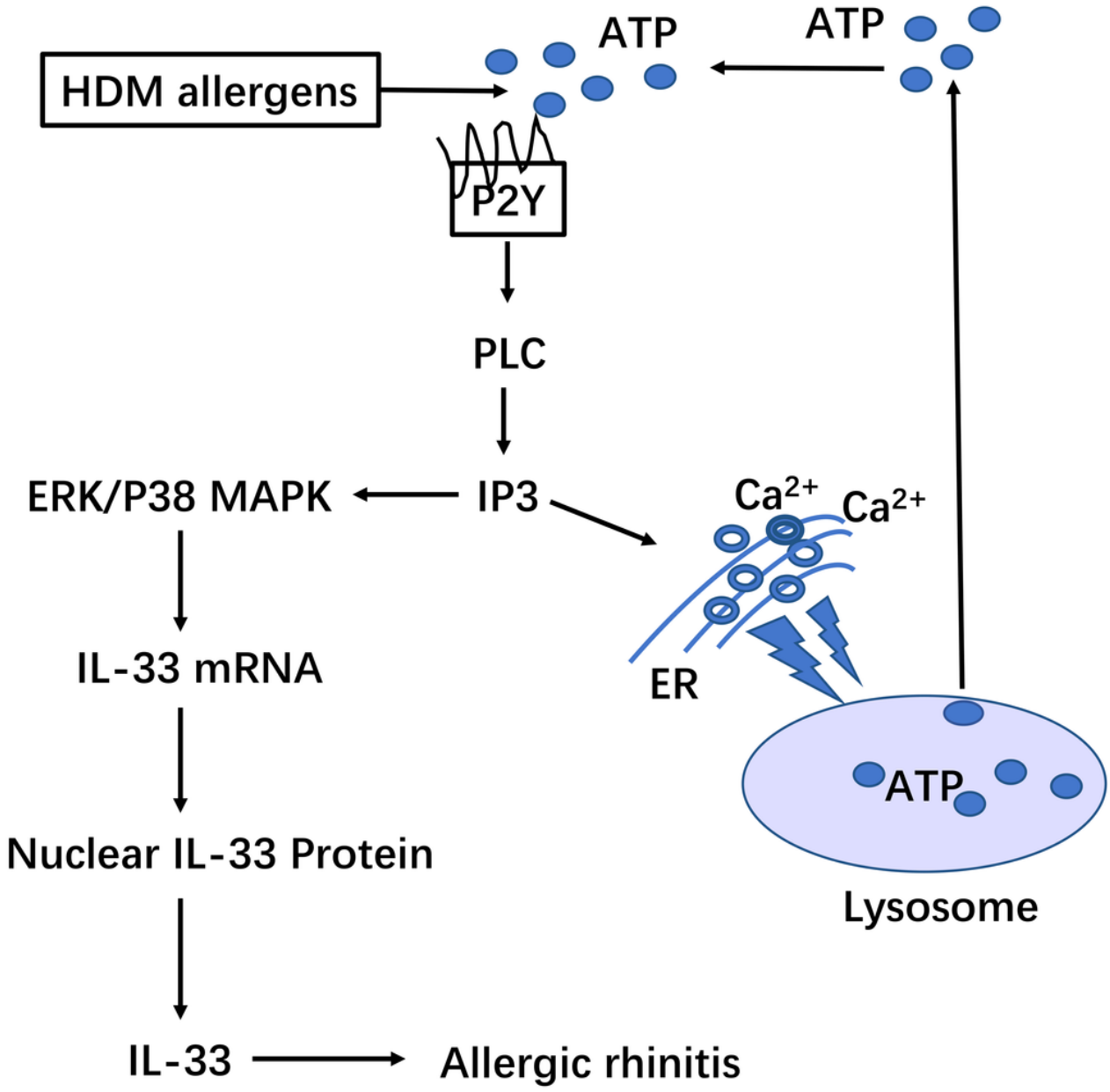

Figure 9

Summary of the molecular mechanisms involved in Der p-induced IL-33 release in human nasal epithelial cells. Der p induced an acute accumulation of extracellular ATP which activated P2Y-PLC-IP3 pathways to induce intracellular Ca2+ releasing from ER. Then, intracellular Ca2+ induced ATP release from lysosomes from human nasal epithelial cells. Moreover, ATP activated P2Y-PLC-IP3 pathways followed by transactivation of ERK/P38 MAPK pathways which induced the expression of IL-33 mRNA and protein.