

# Resveratrol attenuates IL-33-induced mast cell inflammation associated with inhibition of NF- $\kappa$ B activation and the P38 signaling pathway

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**Abstract.** Resveratrol (RSV), a natural polyphenol found in grapes and other herbal plants, has been reported to possess anti-inflammatory, anti-oxidative and anti-proliferative activities. The aim of the present study was to investigate the effect of RSV on interleukin (IL)-33-induced inflammatory responses in mast cells and identify the underlying molecular mechanisms. Rat basophilic leukemia (RBL-2H3) cells were stimulated with IL-33 in the presence or absence of RSV. MTT, ELISA, reverse transcription-quantitative PCR and western blot analyses were then performed in order to assess cytotoxicity, inflammatory cytokine production, suppression of tumorigenicity 2 receptor expression, protein expression involved in mitogen-activated protein kinase (MAPK) and nuclear factor (NF)- $\kappa$ B signaling, respectively. Finally, rats were used to determine the biological effect of RSV *in vivo*. The results revealed that RSV inhibited cell viability and increased cytotoxicity in a dose-dependent manner. Medium concentration of RSV (10  $\mu$ M) treatment attenuated inflammatory cytokine production, such as IL-6, IL-13, tumor necrosis factor- $\alpha$  and monocyte chemoattractant protein-1, and curbed IL-33-induced enhancement of immunoglobulin E-mediated responses in RBL-2H3 cells, which were associated with the suppression of NF- $\kappa$ B-mediated transcription and inhibition of P38 phosphorylation in response to IL-33 stimulation, but not extracellular signal regulated kinase or JNK. Notably, RSV application also decreased the levels of inflammatory cytokines in rats induced by IL-33 injection, which was

similar to the anti-inflammatory effect *in vitro*. The data from the present study demonstrated that RSV played a regulatory role in antagonizing the effects of IL-33 on mast cells both *in vitro* and *in vivo*, suggesting that it has therapeutic potential in IL-33-mediated inflammatory diseases that are associated with mast cells.

## Introduction

According to functional difference, the cytokine system can be divided into two distinct phenotypes: Proinflammatory cytokines and anti-inflammatory cytokines. Generally, proinflammatory cytokines include interleukin (IL)-1 $\beta$ , IL-2, interferon (IFN)- $\gamma$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6, IL-9, IL-12, IL-18, IL-17 and IL-23, which are produced predominantly by Th1, Th9, Th17 cells and M1 macrophages. Anti-inflammatory cytokines include IL-4, IL-10, IL-13 and transforming growth factor (TGF)- $\beta$ , which are represented by Th2, Th3 cells and M2 macrophages. Type 1 (IL-2, IFN- $\gamma$  and TNF- $\alpha$ ) cytokines have been identified to play a role in organ-specific autoimmune diseases, such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis and autoimmune hepatitis (1). As an alarm molecule in the IL-1 family (2), IL-33 has attracted increasing attention in recent years due to its immunoregulatory role in inducing type 2 immune responses. Higher expression of IL-33 and soluble spliced variant of suppression of tumorigenicity 2 (sST2) was identified in the sera and endobronchial biopsies of asthmatic patients, as well as in mouse models of asthma induced by ovalbumin through increased Th2 cytokine production, such as IL-4, IL-5 and IL-13 (3,4). In addition, IL-33 promoted eosinophil infiltration and pathogenic Th2 immune responses, leading to chronic experimental ileitis (5). In dextran sulfate sodium-induced experimental colitis, IL-33 played a protective role via goblet cell induction and also exhibited proinflammatory properties as a Th2 cytokine (6). In addition, male-specific IL-33 expression regulates sex-dimorphic experimental autoimmune encephalomyelitis susceptibility as attenuators of the pathogenic Th1/Th17 response (7).

Including endothelial cells, fibroblasts, basophils, dendritic cells, macrophages and mast cells can produce IL-33 in response to local or systemic balance disorders, such as cell

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damage, stress, inflammation or microbial invasion (8). IL-33 has also been reported to promote mast cell maturation, activation and survival (9). Through binding to the IL-1R/Toll-like receptor (TLR) superfamily member ST2 receptor, IL-33 stimulates target cells and induces subsequent activation of NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways via identical signaling events to those observed for IL-1 $\beta$ , resulting in the production of cytokines and chemokines (10). Although it is a weak inducer of mast cell degranulation, IL-33 can augment the amplitude of cell degeneration in response to cross-linking stimulation triggered by antigens and immunoglobulin E (IgE) receptors (11). In addition, IL-33 is also a nuclear factor that is abundantly expressed in high endothelial venules from lymphoid organs that is associated with chromatin, exhibiting transcriptional regulatory properties (12).

Resveratrol (RSV), a natural polyphenol found in grapes and other herbal plants, has been reported to be beneficial in allergic diseases (13), fibrogenetic disorders (14) and immunoinflammatory pathologies characterized by upregulated type 2 cytokine production, including some forms of inflammatory bowel disease, ileitis and systemic lupus erythematosus (8). A previous study demonstrated that RSV inhibited the release of IgE-associated mediators from bone marrow-derived mouse mast cells *in vitro*, such as histamine, TNF- $\alpha$ , leukotrienes and prostaglandin D2 (15). Another study reported that RSV suppressed IgE-mediated basophilic mast cell degranulation *in vitro*, including  $\beta$ -hexosaminidase and histamine. In addition, RSV decreased IgE-mediated passive cutaneous anaphylaxis and alleviated allergic inflammation, such as monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 (16). A recent study indicated that RSV-curcumin hybrids exhibited anti-inflammatory efficacy as potential therapeutic agents for inflammatory lung diseases, decreasing lipopolysaccharide (LPS)-induced TNF- $\alpha$ , IL-6, IL-12 and IL-33 mRNA expression (17). Although these studies suggest that RSV can inhibit the mast cell activation involved in inflammatory responses, no direct evidence has yet demonstrated the effect of RSV on IL-33-induced inflammatory responses in mast cells and the detailed underlying molecular mechanisms should be elucidated.

In the present study, the effect and underlying molecular mechanisms of RSV on IL-33-induced mast cell inflammation were investigated. The data revealed that RSV decreased IL-33-stimulated inflammatory cytokine production *in vivo* and *in vitro* and also inhibited IL-33-induced enhancement of IgE-mediated responses in mast cells, at least partly, contributing to inhibition of NF- $\kappa$ B activation and P38 signaling. These findings indicate the potential application of RSV as an immunoregulator in allergic disorders associated with mast cell inflammation.

## Materials and methods

**Cell culture.** Rat basophilic leukemia (RBL)-2H3 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. The cells were grown in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml) and 10% heat-inactivated fetal bovine serum (Hyclone; GE

Healthcare Life Sciences) at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Toluidine Blue Staining.** RBL-2H3 cells (1 $\times$ 10<sup>5</sup>) were washed three times with PBS and subsequently fixed with 4% paraformaldehyde at 4°C. After 24 h, the slides were washed with distilled water for 5 min and stained using 1% toluidine blue solution for 2 h at room temperature. Subsequently, excess solution was removed and three fields of view were observed under a light microscope (magnification,  $\times$ 200).

**RSV treatment.** RSV (Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) as a stock solution. Various concentrations of RSV (0-100  $\mu$ M) were then used as a working solution in the cell culture for the indicated time with or without IL-33 (50 ng/ml; BioLegend, Inc.) administration.

**Cell viability assay.** RBL-2H3 cells (4 $\times$ 10<sup>4</sup>) were seeded in 96-well plates and treated with RSV at the indicated concentrations (0-100  $\mu$ M) and times (0-72 h). Cell viability was assessed using an MTT assay (Sigma-Aldrich; Merck KGaA). Briefly, MTT (5 mg/ml) was added to the plates and incubated at 37°C for 4 h. DMSO was used to dissolve the formazan crystals. The absorbance was measured at 590 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Unless stated otherwise, 4 $\times$ 10<sup>5</sup> RBL-2H3 cells were used for the subsequent experiments.

**Cytokine measurement by ELISA.** Rat IL-6 (cat. no. BGK20607), IL-13 (cat. no. BGK42203), TNF- $\alpha$  (cat. no. BGK16599) and MCP-1 (cat. no. 900-M59) ELISA kits were purchased from PeproTech, Inc. Cytokine measurement was performed according to the manufacturer's protocol.

**IgE-mediated mast cell activation.** RBL-2H3 cells were first sensitized to IgE (500 ng/ml) (Sigma-Aldrich; Merck KGaA) overnight at 37°C prior to antigen (Ag) stimulation in the presence or absence of RSV (10  $\mu$ M). The cells were then stimulated with 500 ng/ml DNP-HSA (Sigma-Aldrich; Merck KGaA) for 6 h at 37°C. Where indicated, IL-33 (50 ng/ml) was added at the same time as the Ag. IL-33 stimulation (for 6 h at 37°C) with or without RSV treatment (24 h prior to IL-33 application) was used as the control. Cytokine production in the resultant supernatant was assayed using an ELISA.

**Reverse transcription-quantitative PCR (RT-qPCR).** RBL-2H3 cells were harvested and total RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RNA concentration (406 ng/ $\mu$ l) was calculated and 1  $\mu$ g RNA was reverse transcribed (42°C for 10 min and 80°C for 10 min) into complementary DNA (cDNA) using a Reverse Transcription kit (Takara Bio Inc.), according to the manufacturer's protocol. cDNA was used for qPCR with SYBR Green Supermix kit (Bio-Rad Laboratories), according to the manufacturer's protocol. The primers used in the present study included: ST2 forward, 5'-CGCCTGTTCAGTGTTTA-3' and reverse, 5'-TGGTTCCGTTCTCCGTGT-3';  $\beta$ -actin forward, 5'-GGAGATTACTGCCCTGGCTCCTAGC-3' and reverse,

5'-GGCCGGACTCATCGTACTCTGCTT-3', which were synthesized by Sangon Biotech, Co., Ltd. The amplification conditions for the PCR consisted of an initial incubation at 50°C for 2 min and denaturation for 10 min at 95°C; followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 60°C for 1 min; final extension at 72°C for 5 min; and storage at 4°C. All melting curve analyses were performed between 50-95°C. mRNA expression was quantified using the  $2^{-\Delta\Delta C_q}$  method and normalized to the internal reference gene  $\beta$ -actin (18).

**Western blot analysis.** RBL-2H3 cells were collected and lysed with RIPA lysis buffer containing 1 mM inhibitor PMSF (Wuhan Boster Biological Technology, Ltd.). The cytoplasmic and nuclear proteins were extracted via their subcellular structure with a cytoplasm protein and nucleoprotein extraction kit (grant no. AR0106; Wuhan Boster Biological Technology, Ltd.), according to the manufacturer's protocol. Protein concentration was determined using the bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Samples (20  $\mu$ g per lane) were separated via SDS-PAGE (10% gel) and transferred onto PVDF membranes (EMD Millipore), which were then blocked with 5% non-fat powdered milk for 2 h at room temperature. Membranes were incubated overnight at 4°C with primary rabbit anti-rat antibodies against: IkB $\alpha$  (cat. no. 4812; 1:1,000), phosphorylated (p)-IkB $\alpha$  (cat. no. 2859; 1:1,000), NF- $\kappa$ B (p65; cat. no. 8242; 1:1,000), p-ERK (cat. no. 4370; 1:2,000), ERK (cat. no. 4695; 1:1,000), p-JNK (cat. no. 4668; 1:1,000), JNK (cat. no. 9252; 1:1,000), p-P38 (cat. no. 4511; 1:1,000), P38 (cat. no. 8690; 1:1,000),  $\beta$ -actin (cat. no. 4970; 1:1,000) and Lamin B1 (cat. no. 13435; 1:1,000; all purchased from Cell Signaling Technology, Inc.). On the next day, the membranes were washed with TBST and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. Peroxidase-labeled protein bands were detected using the Immobilon Western Chemiluminescent HRP substrate (EMD Millipore) and the protein intensity was analyzed using ImageJ software (version 1.52; National Institutes of Health) with  $\beta$ -actin and Lamin B1 as the loading controls.

**Signal transduction inhibitors assessment.** BAY 11-7082 (NF- $\kappa$ B inhibitor; 5  $\mu$ M) was obtained from Santa Cruz Biotechnology (cat. no. sc-200615). PD98059 (ERK inhibitor; 10  $\mu$ M; cat. no. 9900), SP600125 (JNK inhibitor; 10  $\mu$ M; cat. no. 8177) and SB203580 (P38 inhibitor; 10  $\mu$ M; cat. no. 5633), were purchased from Cell Signaling Technology, Inc. Signal transduction inhibitors were added to the culture medium 2 h at 37°C prior to activation with IL-33 (50 ng/ml). After 6 h of stimulation at 37°C, supernatants were collected for ELISA.

**In vivo evaluation.** Male 6-week-old Sprague-Dawley rats (200-220 g; n=32) were purchased from the Hubei Research Center of Laboratory Animals (no. 42000600024858) and housed in an air-conditioned room (22 $\pm$ 1°C; 12-h light/dark cycles) with free access to food and water. The care and use of the animals in the present study were approved by the Animal Care and Use Committee of Hubei University of Chinese Medicine (no. SYXK2017-0067). Rats were treated with RSV

(5 mg/kg) or PBS (5 mg/kg) via intraperitoneal injection for 7 days consecutively (n=8 per group). On the eighth day, IL-33 (5  $\mu$ g) was administered into rats via intraperitoneal injection to induce inflammatory cytokine production. After 6 h, rats were euthanized and blood samples were collected for cytokine detection via ELISA.

**Statistical analysis.** Results were presented as the mean  $\pm$  standard deviation and data were analyzed using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. Comparisons between two groups were performed using an unpaired Student's t-test.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of RSV on cell viability of RBL-2H3 cells.** RBL-2H3 cells were used in the present study to mimic mast cells *in vitro*. Fig. 1A and B present the morphological features of RBL-2H3 cells in the culture medium and toluidine blue staining at resting state, respectively. First, the present study investigated the cytotoxic effects of RSV in RBL-2H3 cells. As presented in Fig. 1A, RSV inhibited cell viability in a dose-dependent manner ( $\geq 50$   $\mu$ M). Furthermore, the cells were treated with 1, 10 or 50  $\mu$ M RSV for up to 72 h and cell viability was determined at each 24 h interval. Although a low concentration of RSV (1  $\mu$ M) had no effect on cell viability, a high concentration of RSV (50  $\mu$ M) resulted in significant cytotoxicity following the extension of cell culture time ( $P<0.05$ ; Fig. 1B). Based on these findings, 10  $\mu$ M RSV was used for further experiments.

**RSV attenuates IL-33-induced inflammatory cytokine production.** RBL-2H3 cells were pretreated with RSV for 24 h and the cells were then stimulated with IL-33 (50 ng/ml) for 6 h. Subsequently, inflammatory cytokine production, such as IL-6, IL-13, TNF- $\alpha$  and MCP-1, were detected by ELISA. IL-33 stimulation significantly increased the release of inflammatory cytokines ( $P<0.001$ ). In contrast, RSV treatment significantly attenuated IL-33-induced inflammatory cytokine production in RBL-2H3 cells ( $P<0.001$ ; Fig. 2).

**RSV restrains IL-33 and IgE-regulated synergistic effect in RBL-2H3 cells.** Due to the synergy of IL-33 combined with IgE on the cytokine production in mast cells, the present study further investigated the effect of RSV on IL-33-induced enhancement of IgE-mediated responses in RBL-2H3 cells. As presented in Fig. 3, an overt additive effect was observed in the presence of IL-33 alongside Ag stimulation, resulting in amplification of cytokine production. RSV restrained cytokine secretion with a similar trend under all conditions, decreasing IL-33 and IgE-regulated synergistic responses in RBL-2H3 cells.

**RSV inhibits IL-33-mediated P38 MAPK signaling and NF- $\kappa$ B activation.** In order to investigate the potential molecular mechanisms involved in the suppressive effects of RSV on IL-33-induced inflammation in mast cells, the

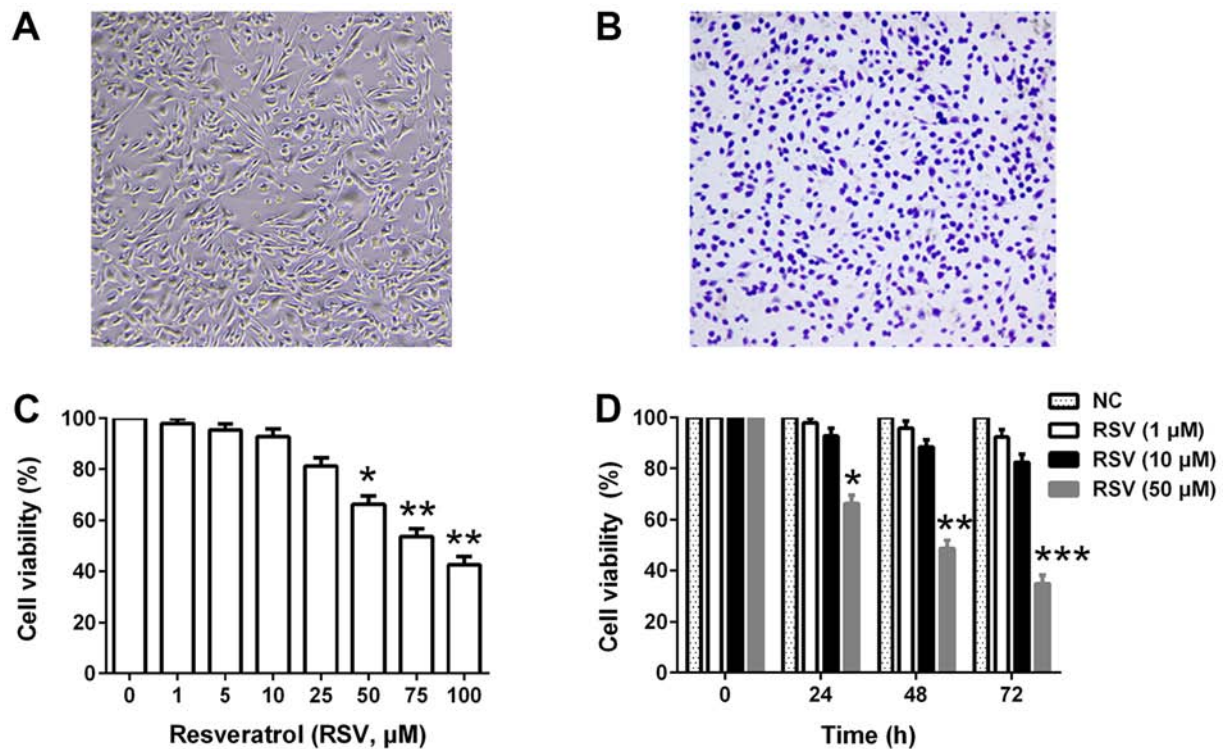


Figure 1. Effects of RSV on cell viability of RBL-2H3 cells. The morphological features of RBL-2H3 cells in (A) culture medium and (B) toluidine blue staining at resting state are presented (magnification, x200). (C) RBL-2H3 cells ( $4 \times 10^4$ ) were treated with various concentrations of RSV (0-100  $\mu\text{M}$ ) for 24 h at 37°C. Cell viability was then assessed using an MTT assay. (D) RBL-2H3 cells ( $4 \times 10^4$ ) were incubated with different concentrations of RSV (1, 10 and 50  $\mu\text{M}$ ) for 24, 48 and 72 h at 37°C. Following incubation, cell viability was analyzed using an MTT assay. Cells with culture medium alone were used as the negative control. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. 0  $\mu\text{M}$  RSV group. RSV, resveratrol; RBL, rat basophilic leukemia; NC, negative control.

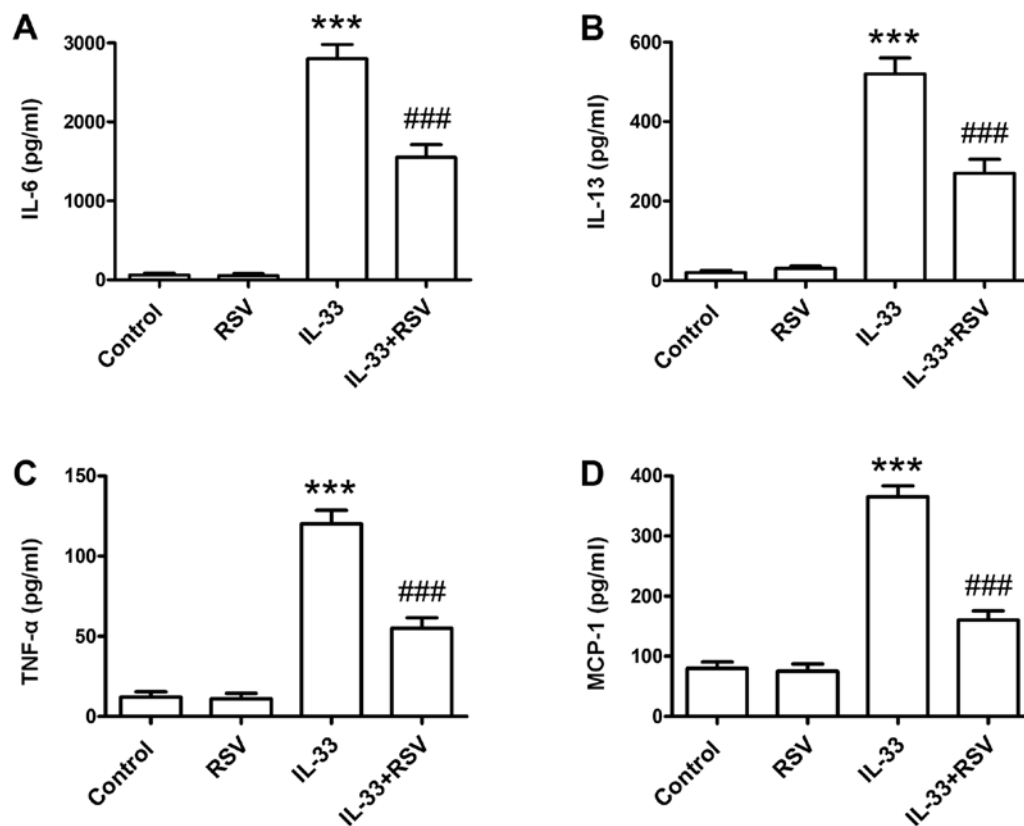


Figure 2. Effects of RSV on cytokine production by IL-33-stimulated RBL-2H3 cells. RBL-2H3 cells ( $4 \times 10^5$ ) were treated with RSV (10  $\mu\text{M}$ ) for 24 h at 37°C. Cells were then stimulated with IL-33 (50 ng/ml) for 6 h at 37°C. Supernatants were collected and the production of proinflammatory cytokines, including (A) IL-6, (B) IL-13, (C) TNF- $\alpha$  and (D) MCP-1, was detected using an ELISA. \*\*\* $P < 0.001$  vs. Control. ### $P < 0.001$  vs. IL-33 only stimulated group. RSV, resveratrol; RBL, rat basophilic leukemia; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein.

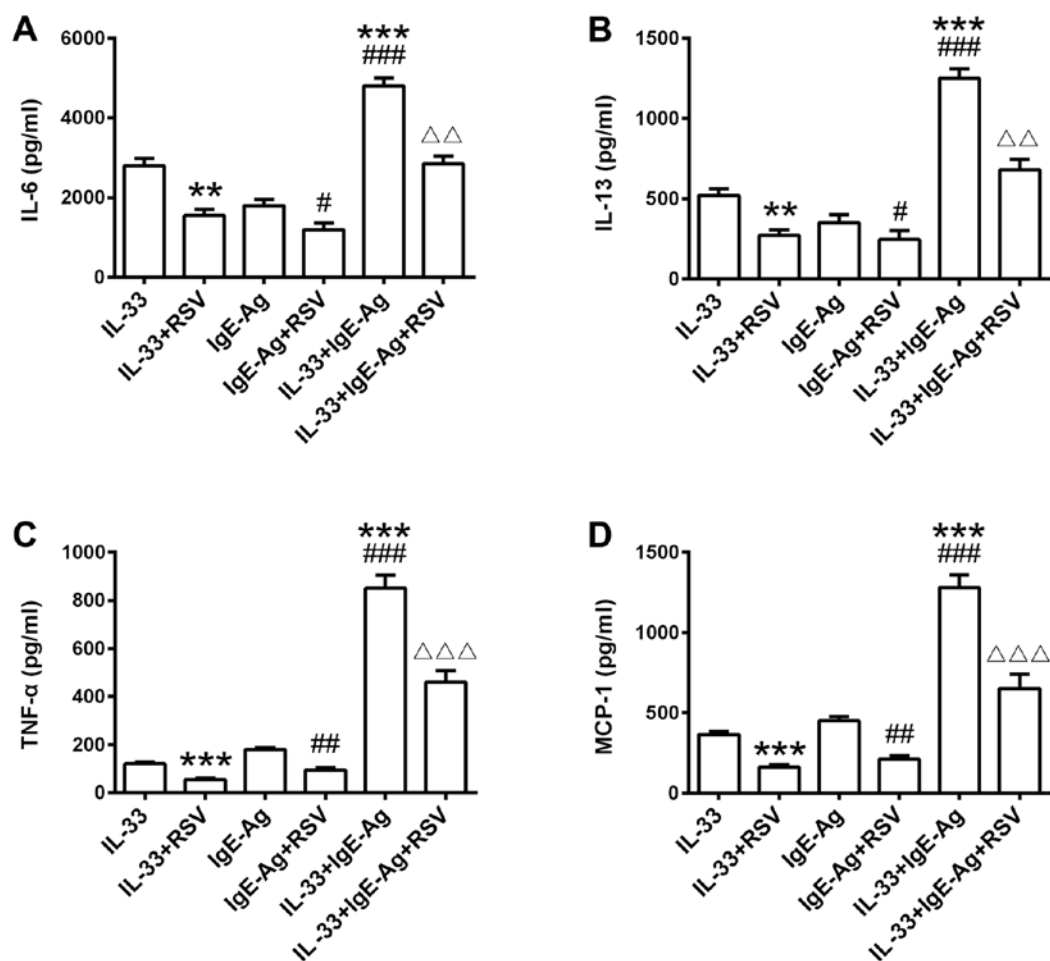


Figure 3. Effects of RSV on IgE-induced cytokine secretion. RBL-2H3 cells were sensitized with IgE (500  $\mu$ g/ml) overnight at 37°C with RSV treatment. Cells were then stimulated with 500 ng/ml DNP-HSA, IL-33, or both together for 6 h at 37°C. (A) IL-6, (B) IL-13, (C) TNF- $\alpha$  and (D) MCP-1 secretion was measured by ELISA. \*\*P<0.01 and \*\*\*P<0.001 vs. IL-33 only stimulated group. #P<0.05, ##P<0.01 and ###P<0.001 vs. IgE-Ag group. <sup>△△</sup>P<0.01 and <sup>△△△</sup>P<0.001 vs. IL-33 + IgE-Ag group. RSV, resveratrol; RBL, rat basophilic leukemia; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor; MCP-1, monocyte chemoattractant protein; Ag, antigen; Ig, immunoglobulin.

present study assessed ST2 receptor expression promoted by IL-33 activity. RSV had no distinct effect on ST2 expression compared with the other three groups (Fig. 4). The effects of RSV on IL-33-mediated MAPK signaling and NF- $\kappa$ B activation were then investigated. RSV significantly inhibited both NF- $\kappa$ B-mediated transcription and P38 phosphorylation in response to IL-33 stimulation (P<0.001; Fig. 5). Furthermore, the application of signal transduction suppressor, including NF- $\kappa$ B and P38 inhibitor (Fig. 6C and D), significantly reversed IL-33-induced TNF- $\alpha$  release (P<0.01). This was not true for the ERK and JNK inhibitors, which demonstrated no marked effect on TNF- $\alpha$  production (Fig. 6A and B).

**RSV suppresses IL-33-induced inflammation in rats.** Finally, the present study assessed the effect of RSV on IL-33-induced inflammation *in vivo* (n=8 rats per group). Similar to the *in vivo* results, IL-33 injection significantly increased production of circulating inflammatory cytokines, such as IL-6, IL-13, TNF- $\alpha$  and MCP-1 (P<0.001; Fig. 7). However, RSV-treated rats exhibited lower levels of IL-6, IL-13, TNF- $\alpha$  and MCP-1 compared with the rats that received PBS injection alone (Fig. 7), suggesting RSV is a potent inhibitor of IL-33-mediated inflammation.

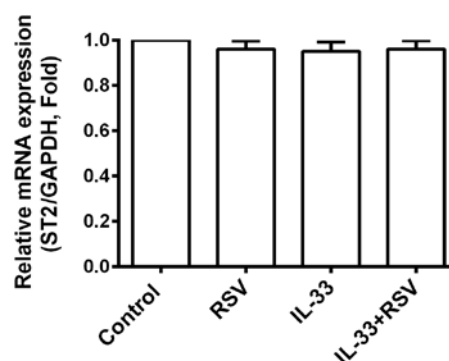


Figure 4. Effects of RSV on ST2 receptor expression. Rat basophilic leukemia-2H3 cells were treated with RSV. After 24 h at 37°C, the cells were stimulated with IL-33 for 6 h at 37°C. ST2 receptor expression at the mRNA level was assessed by reverse transcription-quantitative PCR and statistically analyzed. RSV, resveratrol; ST2, suppression of tumorigenicity 2; IL, interleukin.

## Discussion

To the best of our knowledge, the data obtained in the present study demonstrates for the first time that RSV can effectively

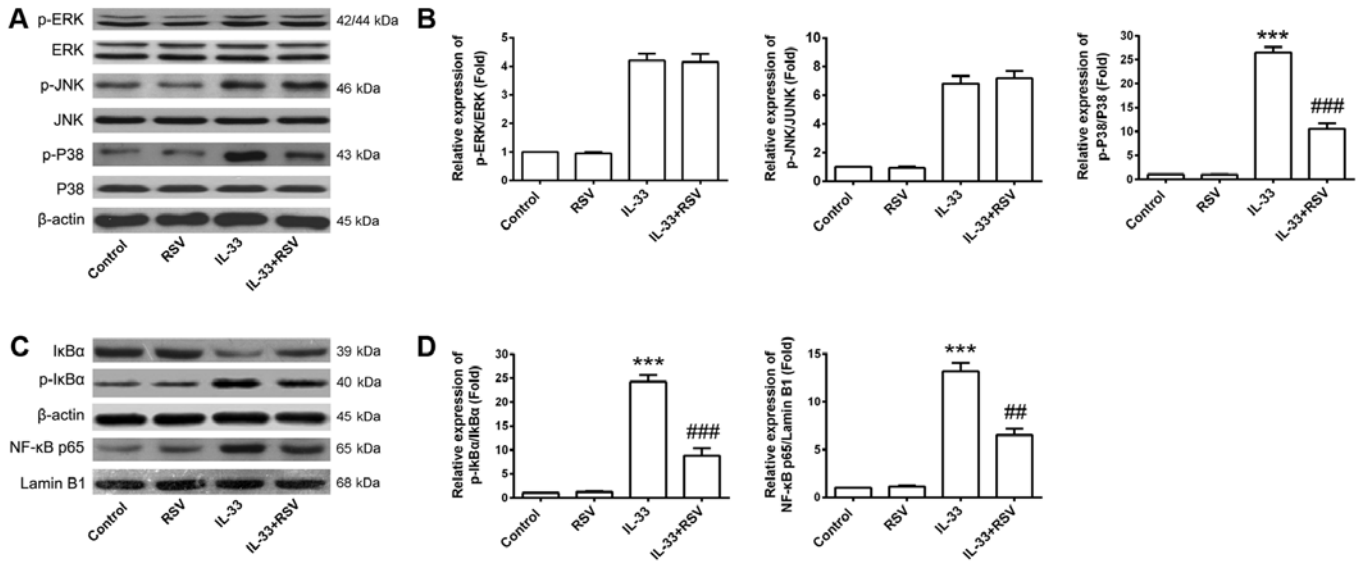


Figure 5. Effects of RSV on IL-33-mediated mitogen associated protein kinase signaling and transcription factor activation. RBL-2H3 cells were treated with RSV and stimulated with IL-33. (A) Lysates were collected for western blot analysis. The expression of cytosolic (B) ERK, p-ERK, JNK, p-JNK, P38 and p-P38 proteins were detected and statistically analyzed. The expression of cytosolic IκBα and nuclear NF-κB (p65) proteins were (C) detected and (D) statistically analyzed. β-actin and Lamin B1 were used to confirm equal sample loading. The representative results are presented. \*\*\*P<0.001 vs. Control. \*\*P<0.01 and ###P<0.001 vs. IL-33 only stimulated group. RSV, resveratrol; IL, interleukin; p, phosphorylated; NF, nuclear factor.

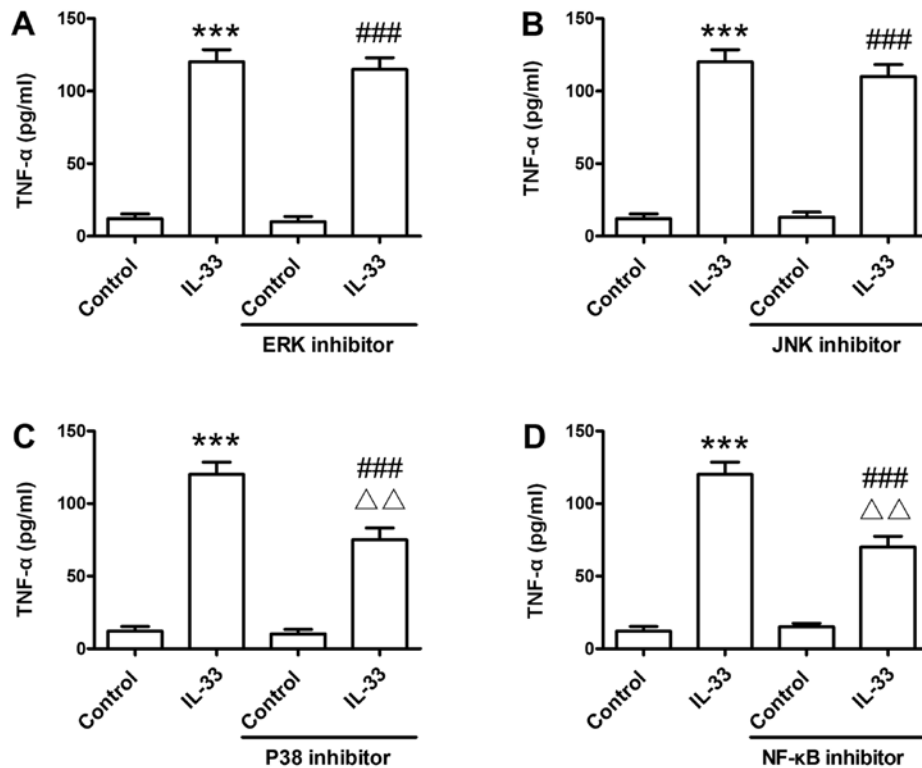


Figure 6. Effects of signal transduction inhibitors on IL-33-induced inflammation in RBL-2H3 cells. RBL-2H3 cells were pretreated with different signal transduction inhibitors for 2 h, including (A) PD98059 (ERK inhibitor; 10 μM), (B) SP600125 (JNK inhibitor; 10 μM), (C) SB203580 (P38 inhibitor; 10 μM) and (D) BAY 11-7085 (NF-κB inhibitor; 2 μM). Cells were then treated with IL-33 for 6 h at 37°C and the supernatants were collected for the ELISA. \*\*\*P<0.001 vs. Control. ###P<0.001 vs. Control with inhibitor. ΔΔP<0.01 vs. IL-33 only stimulated group. RBL, rat basophilic leukemia; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemoattractant protein; NF, nuclear factor.

attenuate IL-33-induced inflammatory responses in mast cells *in vitro* and inflammatory cytokine production *in vivo*. The potential beneficial effects of RSV offer an alternative and promising treatment strategy for allergic inflammation associated with mast cells.

Mast cells are attracting increasing attention due to their roles in regulating a broad spectrum of immune responses, particularly allergic and hypersensitivity responses (19). RBL-2H3 cells are a basophilic leukemia cell line with high affinity IgE receptors and can be activated to secrete histamine

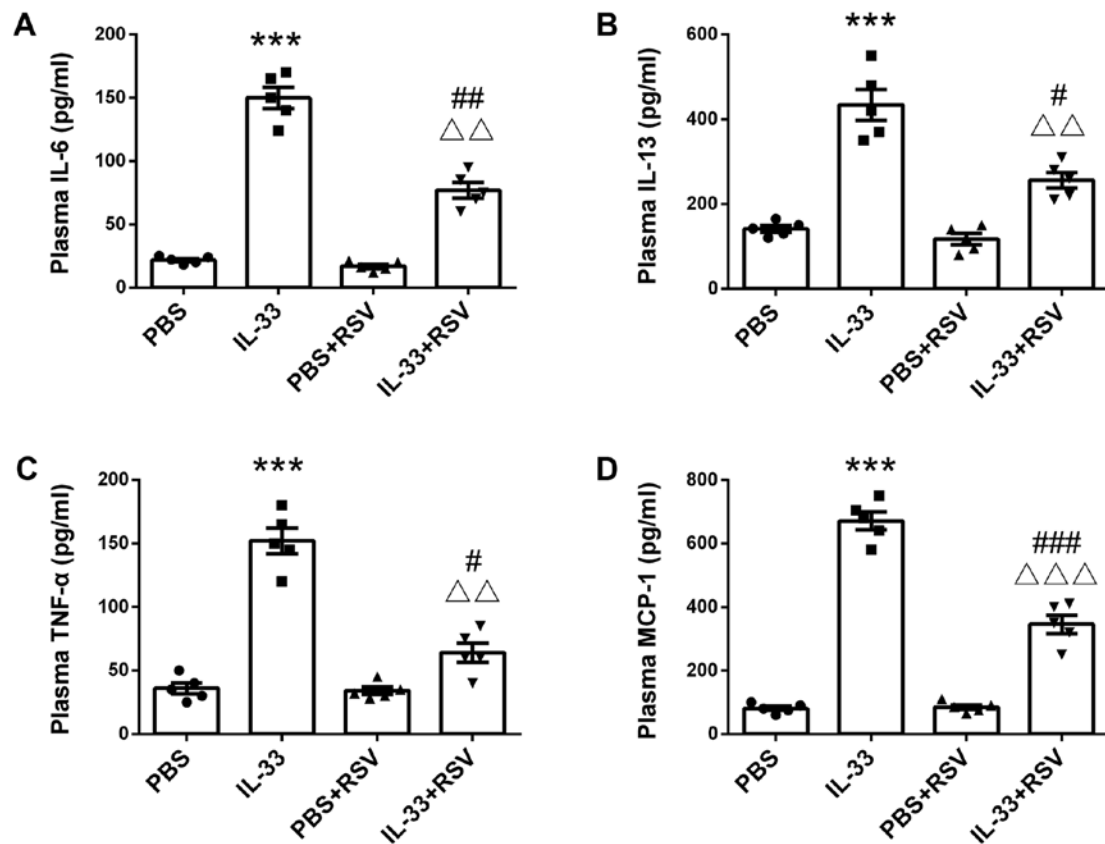


Figure 7. Effects of RSV on IL-33-mediated inflammation *in vivo*. Rats were injected intraperitoneally with either RSV (5 mg/kg) or PBS (5 mg/kg) for 7 days consecutively. On the eighth day, 5  $\mu$ g IL-33 was administered via intraperitoneal injection. After 6 h, rats were euthanized and blood samples were collected for cytokine detection via ELISA, including (A) IL-6, (B) IL-13, (C) TNF- $\alpha$  and (D) MCP-1. Each of the symbols (including dots, squares, up and down-facing triangles) in the different groups represent a rat (n=5 per group). \*\*\*P<0.001 vs. PBS. #P<0.05, ##P<0.01 and ###P<0.001 vs. PBS + RSV group.  $\Delta$ P<0.01 and  $\Delta\Delta$ P<0.01 vs. single IL-33-injected group. RSV, resveratrol; RBL, rat basophilic leukemia; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemoattractant protein.

and other mediators. As a result of this, RBL-2H3 cells have been used extensively to mimic the activation and characteristics of mast cells involved in allergic disease *in vitro*. Activation of mast cells results in the release of a diverse panel of inflammatory mediators, including histamine, leukotriene and potent inflammatory cytokines (e.g., IL-6 and TNF- $\alpha$ ), depending on the type and strength of the stimuli (20). The most characterized pathway associated with mast cell activation is Ag-mediated crosslinking of IgE and Fc $\epsilon$ RI (21). However, a previous study reported that mast cell-produced IL-33 could regulate IgE-dependent inflammation in marrow-derived mast cells via the IL-33/ST2 axis, indicating the potency of IL-33 on IgE-mediated mast cell activation (22). Another study demonstrated that mast cells and the IL-33/ST2 axis were involved in pulmonary and cardiovascular diseases, which was associated with the decrease of neutrophil infiltration and IL-6 expression at the mRNA level (23). 3,4-dihydroxybenzohydroxamic acid suppressed IL-33-induced cytokine production in primary mouse mast cells, such as IL-6, IL-13, TNF and MIP-1 $\alpha$  (24). These studies suggest that inflammatory cytokines induced by IL-33 participate in the immune responses associated with mast cell activation. In line with these findings, moderate concentrations of RSV treatment significantly inhibited the production of inflammatory cytokines induced by IL-33 or the synergistic effect of IL-33 and IgE in the present study, including IL-6, IL-13, TNF- $\alpha$  and MCP-1. Notably, the suppressive effects of

RSV on IL-33-stimulated inflammatory cytokine production was also confirmed in rats in the present study.

The bioactivity of IL-33 is controlled by the regulation of IL-33 binding to the receptor, IL-1R/TLR superfamily member ST2. On the one hand, IL-33 combines with the ST2 receptor and results in subsequent signaling cascades, contributing to allergic diseases, such as asthma and atopic dermatitis (25). On the other hand, sST2 directly binds to IL-33 and curbs the biological activation of IL-33 as a decoy receptor produced by mast cells and Th2 cells in asthmatic patients (26). Cytokines that are produced due to activation of the IL-33-ST2 axis, such as IL-5, IL-6, IL-8, IL-13, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , chemokines and prostaglandins, participate in inflammatory responses (27). In addition, ST2 was also important for the development of peripheral airway hyper-responsiveness and inflammation in a house dust mite mouse model of asthma (28). The levels of the inflammatory cytokines IL-1 $\beta$ , IL-5, IL-13, IL-33, granulocyte-macrophage-colony stimulating factor, thymic stromal lymphopoietin and mast cell protease MCP-1 were decreased in house dust mite-treated ST2(-/-) mice compared with wild-type controls (29). In the present study, RSV exhibited little effect on ST2 receptor expression, although it inhibited the synergistic effect of IL-33 and IgE in cytokine production. The results also promoted further investigations into the molecular mechanisms underlying IL-33-mediated signaling in mast cells accompanying RSV administration.

As is already established, MAPK signaling pathways are responsible for and involved in a variety of cellular activities, such as cell proliferation, differentiation, apoptosis and autophagy. There are three major MAPK pathways that have been identified thus far, including ERK, JNK and P38 MAPK (30). Accumulating evidence demonstrates the anti-inflammatory properties of RSV as an immunoregulator (31-33). Due to its potential as a new drug candidate for the treatment of inflammation, the anti-inflammatory properties of RSV have always been of interest. In LPS-treated RAW264.7 murine macrophages, RSV inhibited the activation of MAPK and NF- $\kappa$ B signaling, decreasing proinflammatory cytokine levels, such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (34). Using an LPS-mediated acute inflammation rat model, RSV treatment decreased the production of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and cyclooxygenase-2, which is associated with inhibition of the TLR4/NF- $\kappa$ B p65/MAPKs signaling cascade (35). These results indicate that MAPK and NF- $\kappa$ B signaling is involved in the biological effects of RSV. It is well known that NF- $\kappa$ B is a protein complex that plays a central role in the transcription of DNA, cytokine production and cell survival. In the classical pathway, following the degradation of I $\kappa$ B, the NF- $\kappa$ B complex is then free to enter the nucleus and induce target gene expression (36). In the present study, RSV restrained IL-33-mediated P38 MAPK signaling and NF- $\kappa$ B activation, at least in part, contributing to the suppression of inflammatory responses in mast cells. Other molecular mechanisms have also been proposed that attempt to explain the inhibitory effects of RSV on inflammatory and allergic diseases, including the activation of AMP-activated protein kinase, Fc $\epsilon$ RI-MAPK or JAK1-signal transducer and activator of transcription 3 signaling, as well as the inhibition of phosphoinositide dependent kinase 1 kinase, phosphoinositide 3 kinase-protein kinase B or TGF- $\beta$ 1/mothers against decapentaplegic homolog 9 pathways (37-41). As the present study did not assess the effects of RSV on all the key proteins involved in intracellular signaling pathways, additional studies are required in order to elucidate the details.

In conclusion, the results from the present study demonstrate that RSV administration both *in vitro* and *in vivo* effectively attenuated IL-33-induced inflammatory cytokine production associated with mast cell inflammation, which were mediated by the inhibition of NF- $\kappa$ B activation and P38 signaling. These results at least partially provide a theoretical basis for RSV as a potential therapeutic agent against the development of allergic diseases.

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## Availability of data and materials

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

## Authors' contributions

GZ conceived the study. YDX and GZ designed the study. QL, YDX and XHG performed the experiments (including cell culture, ELISA analysis and western blotting), analyzed the data and wrote the manuscript. LX contributed to the animal breeding and treatment. XHG and GZ revised the manuscript.

## Ethics approval and consent to participate

Animal care and use were performed according to the guidelines of the Animal Care and Use Committee of Hubei University of Chinese Medicine (No. SYXK2012-0067).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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