



Anti-allergic and anti-inflammatory effects of resveratrol via inhibiting TXNIP-oxidative stress pathway in a mouse model of allergic rhinitis

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ABSTRACT

Background: Allergic rhinitis (AR) is a type I hypersensitivity mediated by IgE in the nose. Thioredoxin-interacting protein (TXNIP) plays a pivotal role in the process of producing reactive oxygen species (ROS). Resveratrol is a TXNIP inhibitor. Nonetheless, its role and mechanism in AR are still undetermined. The present study aimed to explore the effect and mechanism of resveratrol on an ovalbumin (OVA) induced mouse model of AR.

Methods: AR murine model was established using OVA and administrated intranasally with resveratrol or *N*-acetylcysteine (NAC). Hematoxylin and eosin (HE) stain was used for evaluating eosinophils. Immunohistochemistry (IHC) staining and real-time PCR were employed to evaluate immunolabeling and mRNA expression of TXNIP in nasal mucosas of mice. Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in nasal tissue homogenates were measured using MDA and SOD Assay Kit. Concentrations of OVA-specific IgE and histamines in serum, and OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-33 and TNF- α in nasal lavage fluid (NLF) were assayed by ELISA. *In vitro* studies, western blotting, real-time PCR, ELISA, ROS detecting dye DCFH-DA, MDA, and SOD Assay Kit were performed to evaluate the effects and mechanisms of OVA, resveratrol or NAC on spleen mononuclear cells.

Results: We found significant alternations of sneezing, nasal rubbing, inflammatory cytokines, eosinophil numbers, TXNIP, MDA, and SOD levels in resveratrol or NAC treated mice compared with untreated AR mice. In cultured spleen mononuclear cells, TXNIP, MDA, SOD, ROS and inflammatory cytokines levels were altered by OVA but reversed by resveratrol or NAC.

Conclusions: Resveratrol could effectively alleviate murine AR by inhibiting TXNIP-oxidative stress pathway.

Keywords: Allergic rhinitis, TXNIP, Oxidative stress, Ovalbumin, Reactive oxygen species, Resveratrol

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INTRODUCTION

Allergic rhinitis (AR) is a type I hypersensitivity mediated by IgE in the nose characterised by sneezing, rhinorrhea, and nasal congestion, which increases patients' economic burden and negatively affects their quality of life.¹⁻³ Accumulating evidence has shown that AR is a Type 2 T helper (Th2) immune response-mediated nasal allergic and inflammatory disease triggered by allergens in the air,^{4,5} and antihistamines and corticosteroids are effective in the treatment of AR.⁶ Whereas, the precise pathogenesis of AR remains undetermined, and some AR patients remain insensitive to antihistamines and corticosteroids.^{7,8} Thus, a novel drug with better effect on AR is urgently needed to be explored.

Considerable data indicate that excess reactive oxygen species (ROS) accumulation and oxidative damage play crucial roles in the process of allergic and inflammatory response.^{9,10} Thioredoxin-interacting protein (TXNIP) is a multi-functional protein mediating redox homeostasis by increasing ROS production and inducing oxidative stress.¹¹

Resveratrol (trans-3,5,4'-trihydroxystilbene), an inhibitor of TXNIP,¹² is a stilbenoid belonging to the polyphenols, and the sources of resveratrol in food comprise grapes, berries, and peanuts.^{13,14} Resveratrol can exert antioxidant, anti-inflammatory, antiproliferative, and angioregulatory effects in a variety of diseases.¹⁵ Resveratrol could inhibit ROS production and reduce oxidative stress, resulting in the alleviation of inflammatory damage and apoptosis induced by ischemia/reperfusion injury in the brains of rats.¹⁶

The present study aimed to explore the effect of resveratrol in an ovalbumin (OVA) induced mouse model of AR. First, AR murine model was established using OVA and administrated intranasally with resveratrol or *N*-acetylcysteine (NAC, an ROS scavenger). Immunohistochemistry (IHC) staining and real-time PCR were employed to evaluate immunolabeling, and mRNA levels of TXNIP in nasal mucosas of mice. Concentrations of OVA-specific IgE and histamines in serum, and OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-

33, and TNF- α in nasal lavage fluid (NLF) were assayed by enzyme-linked immunosorbent assay (ELISA). Second, *in vitro* studies, western blotting, flow cytometry, real-time PCR, and ELISA were performed to evaluate the effects and mechanisms of OVA and resveratrol on spleen mononuclear cells. We found significant alternations of sneezing, nasal rubbing, inflammatory cytokines, TXNIP, MDA, and SOD levels in resveratrol or NAC treated mice compared with untreated AR mice. In cultured spleen mononuclear cells, significant alternations of TXNIP, MDA, SOD, ROS, and inflammatory cytokines levels were induced by OVA but reversed by resveratrol or NAC.

MATERIALS AND METHODS

Animals

A total of 50 female BALB/c mice (6-8 weeks of age) were included and kept in a specific-pathogen free facility. The mice were divided into 5 groups (n = 10 per group): normal control group, untreated AR group, 200 μ g resveratrol treated AR group, 400 μ g resveratrol treated AR group, and 100 μ g NAC treated AR group.

AR mouse model and treatment

The murine AR model was induced by ovalbumin (OVA) as previously described.^{17,18} A schematic diagram of AR mouse model establishment and resveratrol or NAC treatment protocol is depicted in Fig. 1. First, to establish AR murine model, mice were sensitized with 0.2 mL suspension including 0.5 mg/mL ovalbumin (OVA, Sigma-Aldrich) and 20 mg/mL aluminum hydroxide (Sinopharm Chemical Reagent Co) by intraperitoneal injection on day 1, 8, and 15, respectively. Furthermore, normal control mice were intraperitoneally administered with 0.2 mL normal saline on day 1, 8, and 15, respectively. On days 22-29, the mice in all AR groups were challenged daily with 20 μ L OVA (40 mg/mL) by intranasal instillation; then, normal control mice and untreated AR mice were administrated with 30 μ L vehicle of 0.5% carboxymethyl cellulose solution. In addition, on days 22-29, resveratrol treated AR mice were instilled intranasally with resveratrol (200 μ g or 400 μ g, Sigma-Aldrich) dissolved in 30 μ L 0.5% carboxymethyl cellulose

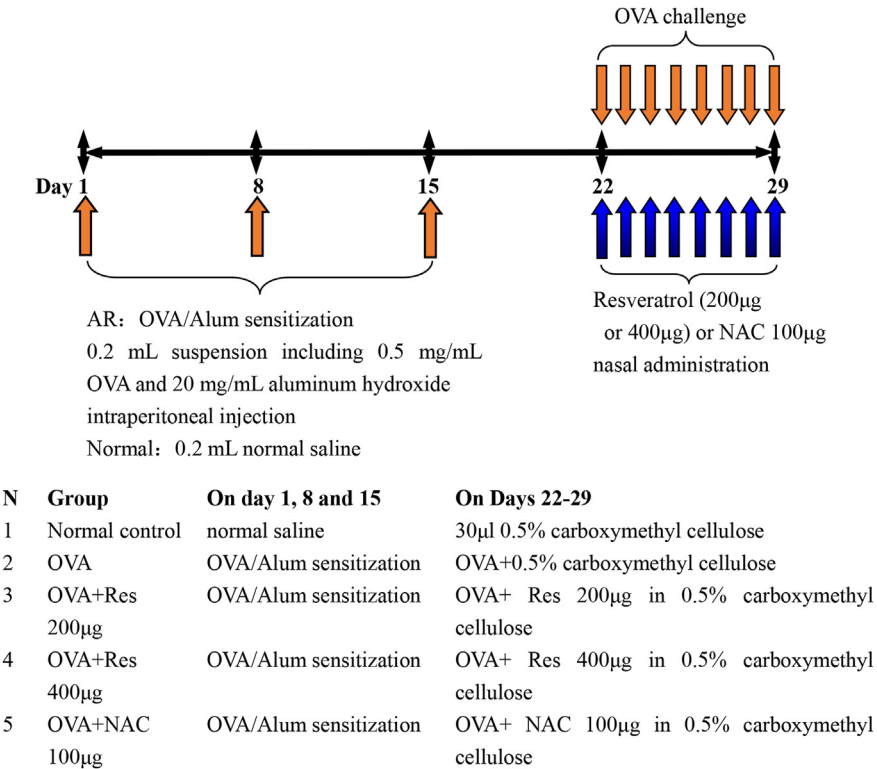


Fig. 1 Schematic diagram demonstrating the process of AR mouse model establishment and resveratrol treatment

solution on days 22-29. Moreover, during this period, NAC treated AR mice were instilled intranasally with NAC (100 µg) dissolved in 30 µl 0.5% carboxymethyl cellulose solution.

Mice nasal symptoms evaluation and sample preparation

Mice nasal symptoms including frequency of sneezes and nasal rubbing were evaluated for 10 min on the 29th day, immediately following the final OVA provocation. After 24 h, the mice were killed, and the blood and NLF samples were collected for ELISA. Nasal mucosas were collected and assayed by IHC staining and real-time PCR methods. More detailed protocols can be found in the Online Supplementary Material.

ELISA assay in the serum and NLF

Serum OVA-specific IgE and histamines levels and concentrations of OVA-specific IgE, PGD2, LTC4, ECP, IL-4, IL-5, IL-6, IL-33 and TNF-α in NLF were assayed with specific ELISA kits. Each sample was analyzed in triplicate. More detailed protocols can be found in the Online Supplementary Material.

HE and IHC staining

Nasal mucosas of mice were paraffin-embedded, serially sectioned (4 µm). Following deparaffinization and rehydration, HE staining was used for evaluating eosinophils counts and IHC staining was employed to evaluate TXNIP immunolabeling. More detailed protocols can be found in the Online Supplementary Material.

Quantitative real-time reverse transcriptions PCR

Quantitative real-time PCR was employed to assess TXNIP expression in nasal mucosas of mice. Total RNA in the collected tissues was extracted utilizing RNeasy commercial kit (Qiagen, Chatsworth, CA, USA). More detailed protocols can be found in the Online Supplementary Material.

Measurement of MDA, and SOD in nasal tissue homogenates

MDA level and SOD activity in nasal tissue homogenates were detected using MDA Assay Kit and SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturer's protocol.

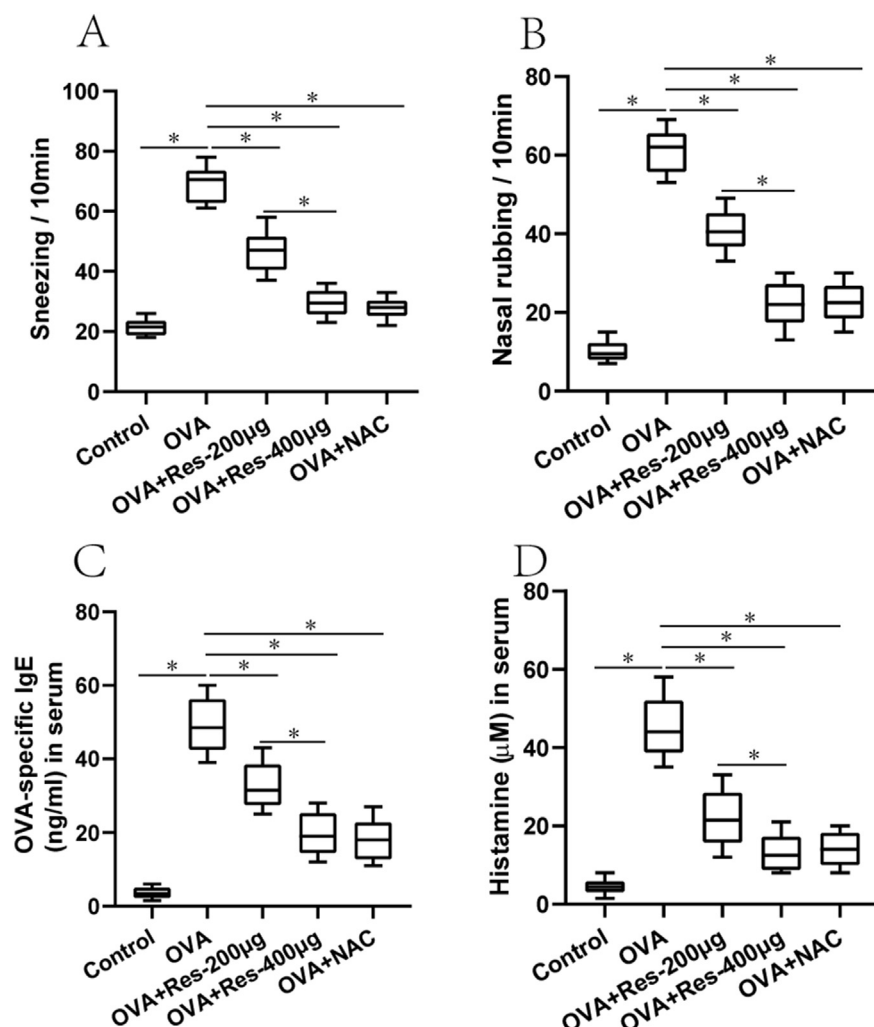


Fig. 2 (A–D) Sneezes, nasal rubbing and OVA-specific IgE and histamines levels in the serum from normal control group ($n = 10$), untreated AR group (OVA, $n = 10$), 200 µg resveratrol treated AR group (OVA + Res-200µg, $n = 10$), 400 µg resveratrol treated AR group (OVA + Res-400µg, $n = 10$) and NAC treated AR group (OVA + NAC-100µg, $n = 10$). $P < 0.05$. One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Each experiment was repeated three times at short time intervals

Additional information can be found in this article's Online Supplementary Material.

Spleen mononuclear cells culture and stimulation

Spleen mononuclear cells were isolated from 6 untreated AR mice and cultured as previously described.¹⁹ Briefly, spleen mononuclear cells were stimulated with control PBS, OVA (10 µg/mL) alone or with resveratrol (10 µM or 20 µM) or NAC (10 µM) for 24 h. Following stimulation, the cells and supernatants were collected for western blotting, real-time PCR, flow cytometry, and

ELISA. More detailed protocols can be found in the Online Supporting Information.

Western blotting

TXNIP protein levels in the spleen mononuclear cells were assayed with western blotting methods. Additional information can be found in this article's Online Supplementary Material.

Quantitative real-time reverse transcriptions PCR

TXNIP mRNA levels in the spleen mononuclear cells were assayed with quantitative real-time PCR

methods. Additional information can be found in this article's Online Supplementary Material.

Measurement of MDA, and SOD in spleen mononuclear cells

Following exposure, MDA level and SOD activity in spleen mononuclear cells were detected using MDA Assay Kit and SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute), respectively, according to the manufacturer's protocol. Additional

information can be found in this article's Online Supplementary Material.

Intracellular reactive oxygen species (ROS) assay

Following stimulation, intracellular ROS in spleen mononuclear cells was assayed using DCFH-DA (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. Additional information can be found in this article's Online Supplementary Material.

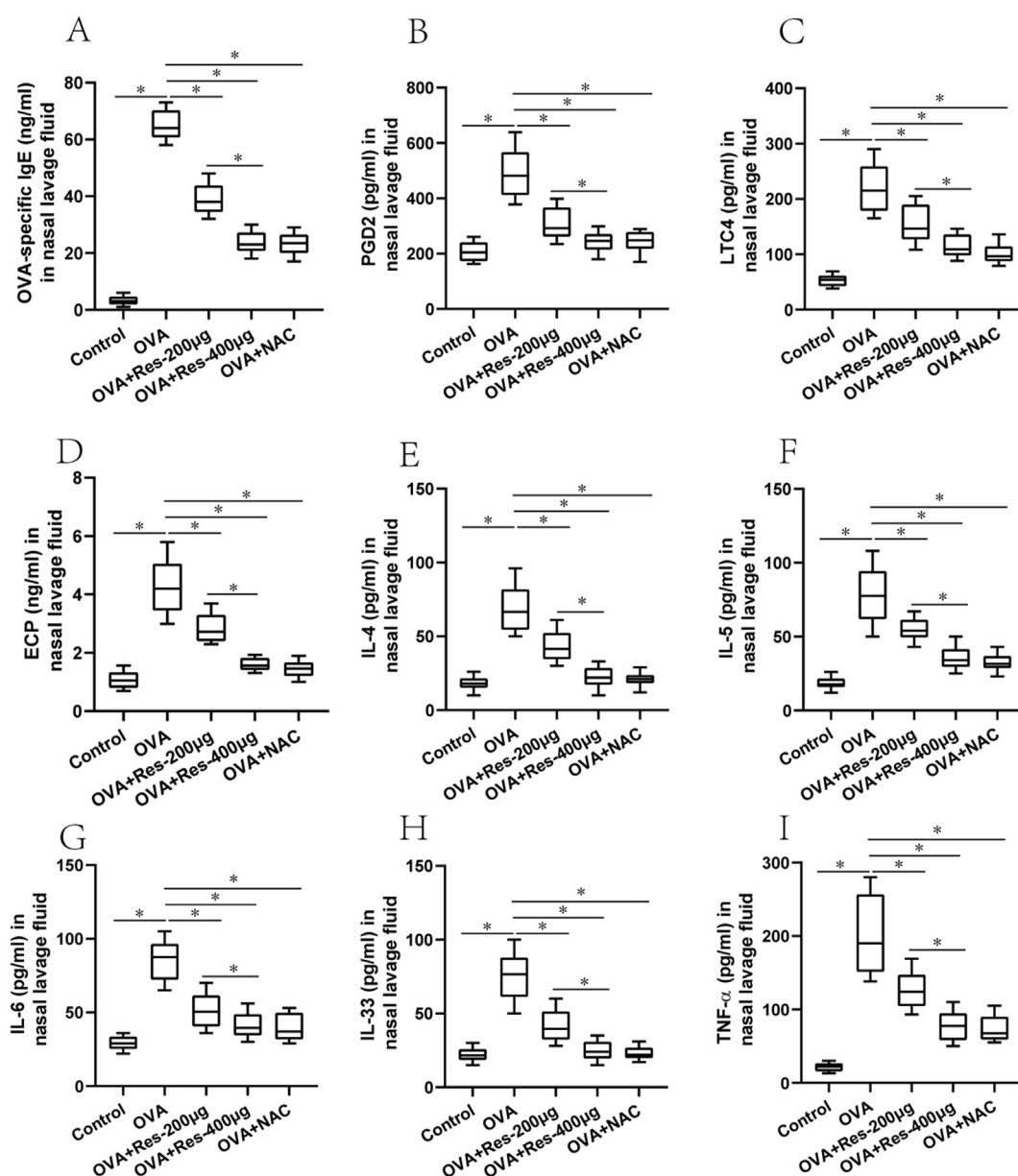


Fig. 3 (A-I) OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-33 and TNF- α levels in the NLF from normal control group (n = 10), untreated AR group (OVA, n = 10), 200 μ g resveratrol treated AR group (OVA + Res-200 μ g, n = 10), 400 μ g resveratrol treated AR group (OVA + Res-400 μ g, n = 10) and NAC treated AR group (OVA + NAC-100 μ g, n = 10). $P < 0.05$. One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Each experiment was repeated three times at short time intervals

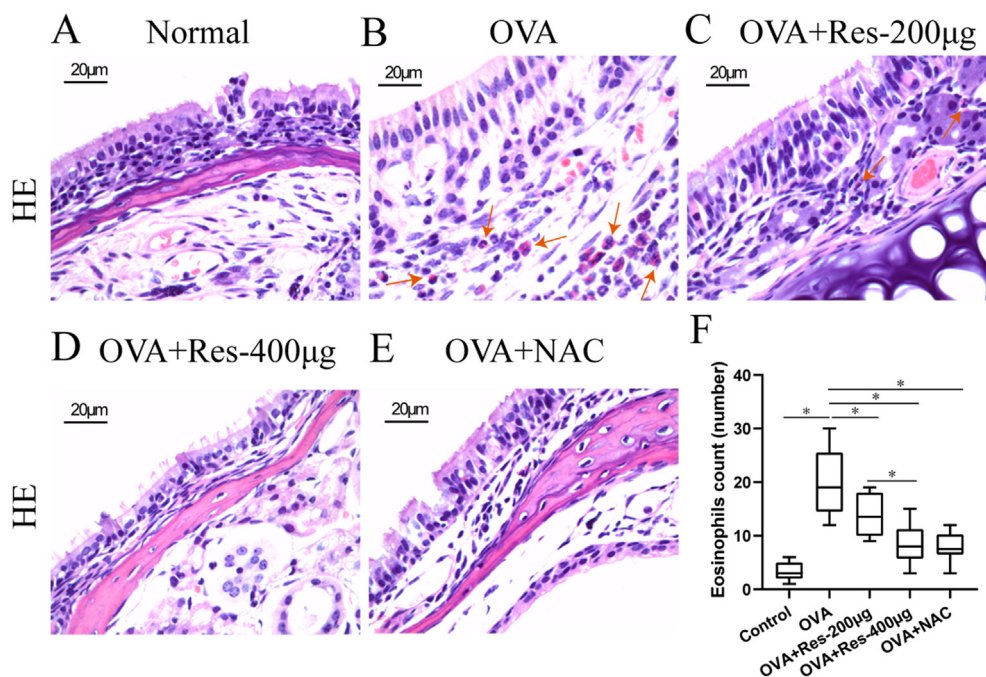


Fig. 4 (A-F) HE staining for eosinophils in nasal tissues from normal control group ($n = 10$), untreated AR group (OVA, $n = 10$), 200 μg resveratrol treated AR group (OVA + Res-200 μg , $n = 10$), 400 μg resveratrol treated AR group (OVA + Res-400 μg , $n = 10$) and NAC treated AR group (OVA + NAC-100 μg , $n = 10$). Red arrows indicate eosinophils. Scale bar = 20 μm . * $P < 0.05$. One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Each experiment was repeated three times at short time intervals.

ELISA assay in the supernatants

Briefly, following treatment, the supernatants were collected for assaying IL-4, IL-5, IL-6, IL-33, and TNF- α protein levels using ELISA methods. Additional information can be found in this article's Online Supplementary Material.

Statistical analysis

Values were expressed as mean $\pm$ standard error of mean (SEM). A P value of less than 0.05 was considered statistically significant. One-way ANOVA with Bonferroni *post hoc* test was employed for intergroup comparison. Correlations were assessed by Spearman's test. Statistical analyses were performed with SPSS Software (Version 22.0, Chicago, IL, USA) and GraphPad Prism 7 software (GraphPad Software, San Diego, Calif, USA).

RESULTS

Effects of resveratrol on nasal symptoms in AR mice

Notably, significant upregulation of frequencies of sneezes and nasal rubbing were found in the

untreated AR group compared with the normal control group (Fig. 2A and B), and these effects were attenuated in the resveratrol (200 μg or 400 μg) treated AR group and NAC treated AR group compared with the untreated AR group (Fig. 2A and B). In addition, the resveratrol (400 μg) treated AR group exhibited significant downregulation of frequencies of sneezes and nasal rubbing in comparison to the resveratrol treated AR (200 μg) group (Fig. 2A and B).

Effects of resveratrol on OVA-specific IgE and cytokines levels in the serum and NLF

Consistent with above findings, OVA-specific IgE and histamines levels in the serum, and OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-33, and TNF- α levels in the NLF from the untreated AR group were significantly elevated compared with the normal control group (Fig. 2C and D, and Fig. 3A-I). Furthermore, OVA-specific IgE and cytokine levels in the serum and NLF were diminished in the resveratrol (200 μg or 400 μg) treated AR group and NAC treated AR group compared with the untreated AR group (Fig. 2C and D, and Fig. 3A-I). In addition, these effects were more

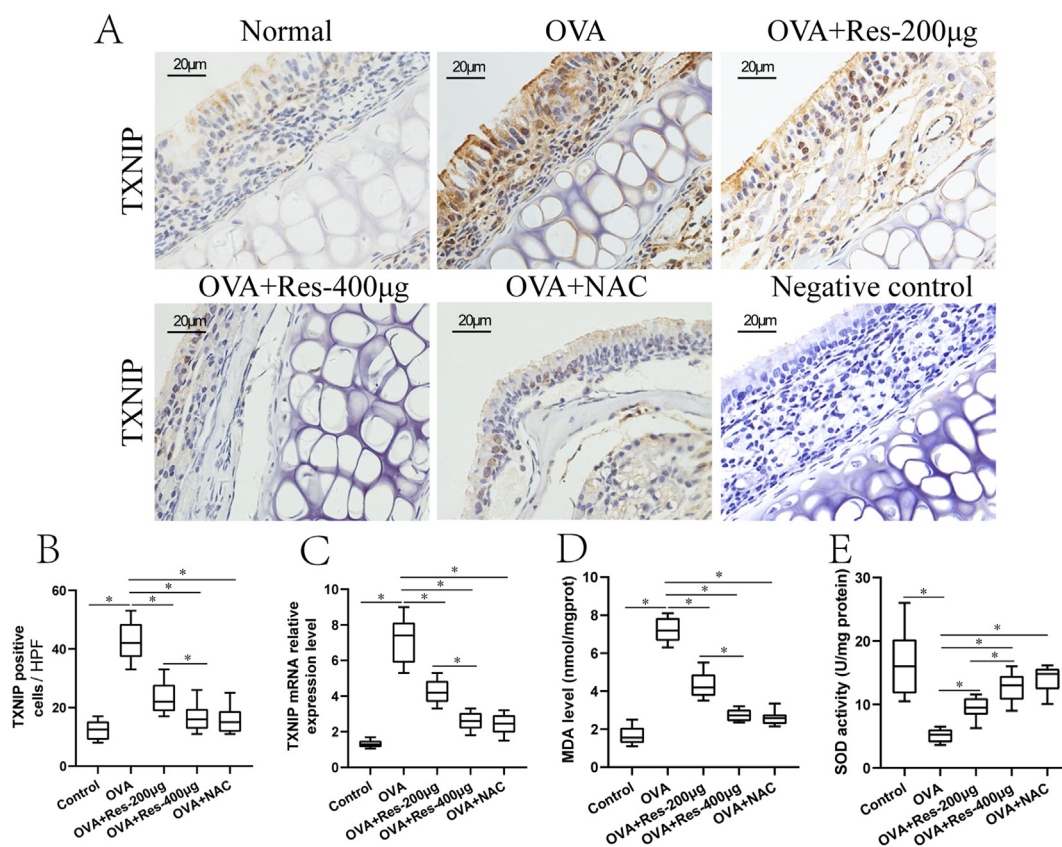


Fig. 5 (A-E) Immunolabeling and mRNA expression of TXNIP, MDA levels and SOD activities in nasal tissues from normal control group (n = 10), untreated AR group (OVA, n = 10), 200 µg resveratrol treated AR group (OVA + Res-200µg, n = 10), 400 µg resveratrol treated AR group (OVA + Res-400µg, n = 10) and NAC treated AR group (OVA + NAC-100µg, n = 10). Scale bar = 20 µm. *P < 0.05. One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Each experiment was repeated three times at short time intervals.

obvious in the resveratrol (400 µg) treated AR group in comparison to the resveratrol (200 µg) treated AR group (Fig. 2C and D, and Fig. 3A-I).

Effects of resveratrol on eosinophil numbers in nasal tissues from AR mice

Notably, eosinophil numbers were significantly elevated in the untreated AR group compared with the normal control group and resveratrol (200 µg or 400 µg) or NAC treatment significantly diminished the effects in comparison to the untreated AR group (Fig. 4A-F). Of note, these effects were more obvious in resveratrol (400 µg) treated AR group in comparison to the resveratrol (200 µg) treated AR group (Fig. 4A-F).

Effects of resveratrol on TXNIP immunolabeling and mRNA in nasal tissues from AR mice

Notably, TXNIP positive cells were mainly epithelial cells and inflammatory cells in the nasal

mucosa (Fig. 5A). TXNIP positive cells were significantly elevated in the untreated AR group compared with the normal control group and resveratrol (200 µg or 400 µg) or NAC treatment significantly diminished the effects in comparison to the untreated AR group (Fig. 5A and B). Of note, these effects were more obvious in the resveratrol (400 µg) treated AR group in comparison to the resveratrol (200 µg) treated AR group (Fig. 5A and B).

As indicated in Fig. 5C, TXNIP mRNA levels were significantly increased in nasal tissues from the untreated AR group compared with the normal control group and TXNIP mRNA levels were diminished in the resveratrol (200 µg or 400 µg) or NAC treated group compared with the untreated AR group. Of note, these effects were more obvious in the resveratrol (400 µg) treated AR group in comparison to the resveratrol (200 µg) treated AR group (Fig. 5C).

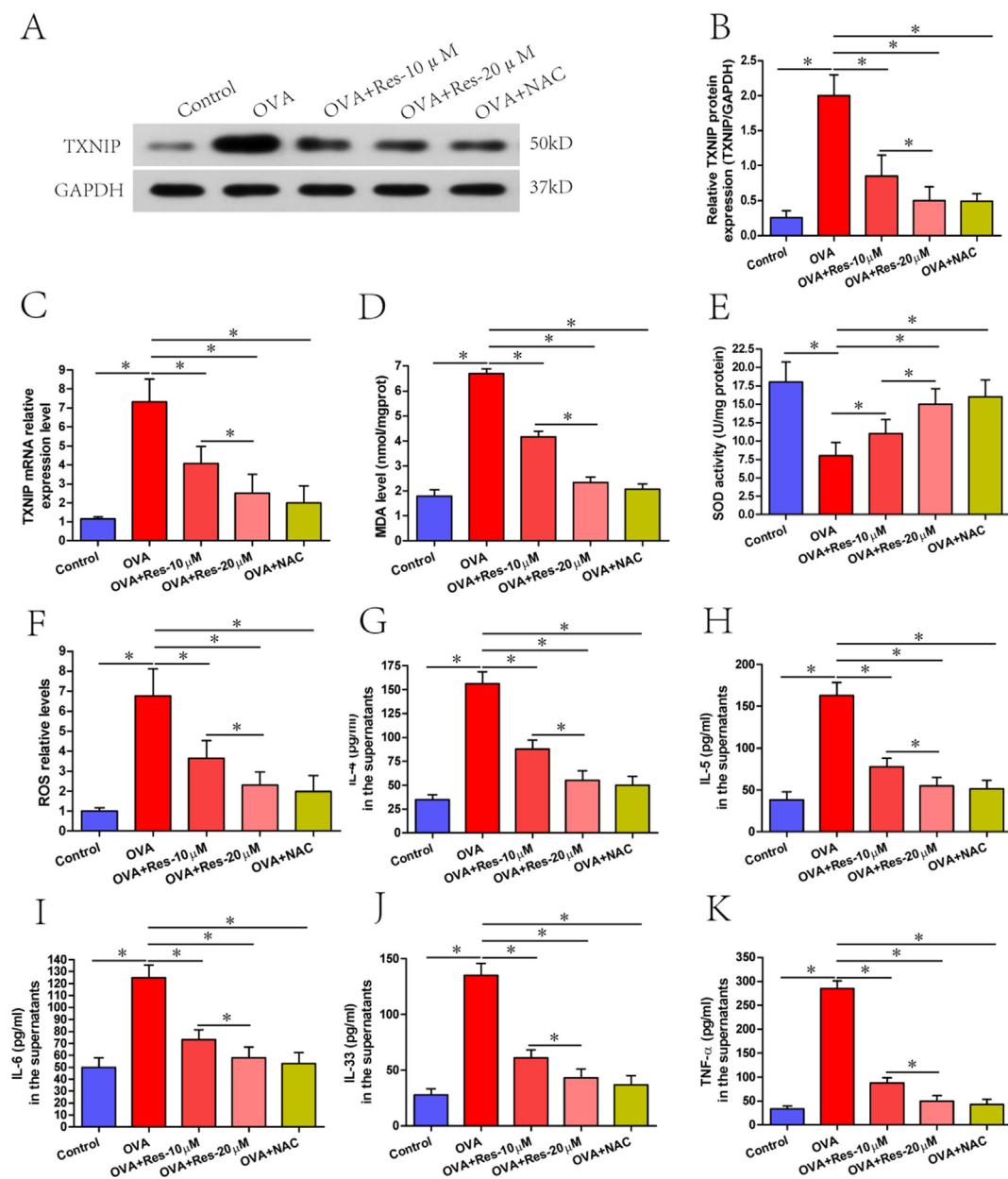


Fig. 6 (A-K) TXNIP protein and mRNA levels, MDA levels, SOD activities, ROS levels and inflammatory cytokines levels in five groups of spleen mononuclear cells classified as control cells, OVA treated cells, OVA + resveratrol (10 μ M) treated cells, OVA + resveratrol (20 μ M) treated cells and NAC treated cells. $P < 0.05$. One-way ANOVA with Bonferroni post hoc test was employed for intergroup comparison. Each experiment was repeated six times at short time intervals

Effects of resveratrol on MDA level and SOD activities in nasal tissue homogenates from AR mice

Significantly increased MDA levels and decreased SOD activities were found in nasal tissue homogenates from the untreated AR group compared with the normal control group, and these alternations were reversed in the resveratrol

(200 μ g or 400 μ g) or NAC treated group compared with the untreated AR group (Fig. 5D and E). In addition, these effects were more obvious in the resveratrol (400 μ g) treated AR group in comparison to the resveratrol (200 μ g) treated AR group (Fig. 5D and E).

Interestingly, MDA levels were positively correlated with TXNIP protein levels (Spearman's test,

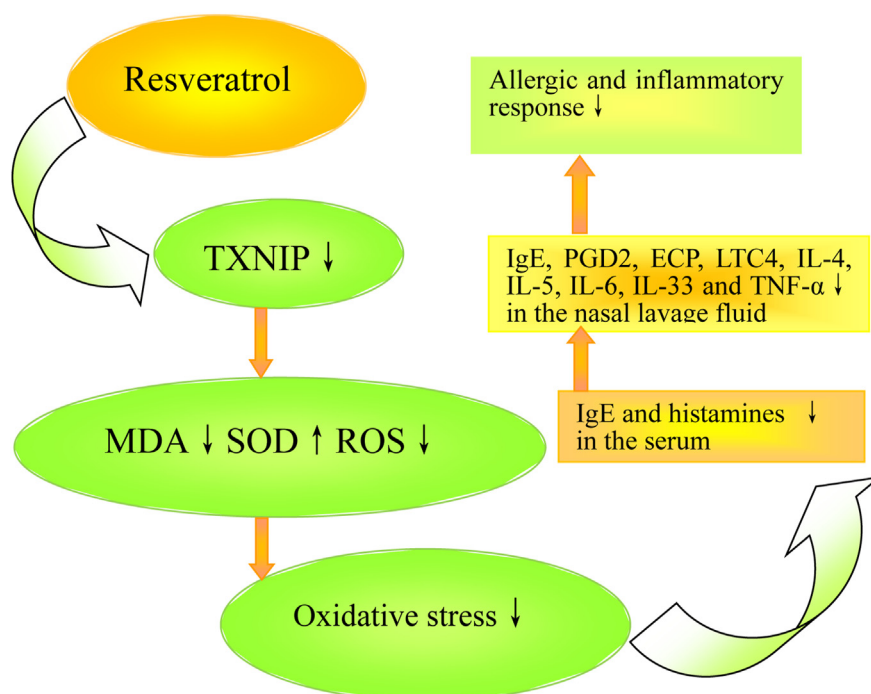


Fig. 7 Schematic diagram displaying a hypothetical chain of events in which resveratrol treatment could attenuate the allergic and inflammatory response via inhibiting TXNIP-oxidative stress signaling pathway in a murine AR model

$r = 0.689$, $P < 0.001$), whereas, SOD activities were negatively correlated with TXNIP protein levels (Spearman's test, $r = -0.730$, $P < 0.001$).

TXNIP protein and mRNA levels in spleen mononuclear cells

Notably, stronger bands and upregulated mRNA levels of TXNIP were found in OVA treated cells in comparison to control cells, and these effects were attenuated in resveratrol (10 μM or 20 μM) or NAC treated cells (Fig. 6A-C). Of note, these effects were more obvious in resveratrol (20 μM) treated cells in comparison to resveratrol (10 μM) treated cells (Fig. 6A-C).

MDA, SOD and ROS levels in spleen mononuclear cells and inflammatory cytokines levels in the supernatants

Significantly, increased levels of MDA and ROS, and decreased SOD activities in spleen mononuclear cells, were found in OVA treated cells in comparison to control cells, and these effects were attenuated in resveratrol (10 μM or 20 μM) or NAC

treated cells (Fig. 6D-F). In addition, inflammatory cytokines including IL-4, IL-5, IL-6, IL-33 and TNF- α levels in the supernatants were significantly upregulated following OVA treatment in comparison to control cells, and these effects were attenuated following resveratrol (10 μM or 20 μM) or NAC treatment (Fig. 6G-K). Furthermore, these effects were more obvious in resveratrol (20 μM) treated cells in comparison to resveratrol (10 μM) treated cells (Fig. 6D-K).

DISCUSSION

Considerable data indicate that TXNIP is essential for regulating redox homeostasis.²⁰ Activation of TXNIP could enhance ROS generation and trigger oxidative stress, leading to inflammatory response exacerbation,²⁰ and these cell activities could be inhibited by TXNIP inhibitor resveratrol.¹³

Accumulating evidence indicates that antioxidant supplementation with resveratrol is capable of attenuating asthma symptoms in patients with asthma,²¹ and resveratrol could reduce asthma-

induced airway inflammation and remodeling in a mouse or rat model of asthma.^{22,23} In addition, intranasal resveratrol is effective for improving nasal symptoms in adults and children with AR.^{24,25} Nonetheless, its precise role and mechanism in AR are still undetermined.

In the present study, frequencies of sneezes and nasal rubbing, OVA-specific IgE and histamines levels in the serum, and OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-33 and TNF- α levels in the NLF were significantly upregulated in untreated AR group compared with normal control group, and these effects were attenuated by resveratrol or NAC treated AR group compared with untreated AR group. These results indicate that AR murine model is successfully established, and resveratrol treatment could ameliorate nasal symptoms and attenuate allergic and inflammatory response in AR mice.

In the nasal tissue samples of mice, significant alternations of eosinophil numbers, TXNIP positive cells and mRNA levels, MDA levels, and SOD activities were found in the untreated AR group compared with the normal control group and resveratrol or NAC treatment significantly diminished the effects in comparison to the untreated AR group. Furthermore, TXNIP level was positively correlated with MDA level, and negatively correlated with SOD activities respectively, indicating that resveratrol treatment could decrease TXNIP and oxidative stress levels in nasal tissue samples from AR mice, which are consistent with a previous report indicating that resveratrol could ameliorate obesity-associated allergic airway inflammation in mice by elevating SOD levels and reducing ROS production in lung tissues of obese mice.²⁶

In the *in vitro* studies, TXNIP protein and mRNA levels, MDA, SOD, ROS, and inflammatory cytokines levels were significantly altered following OVA treatment compared with control cells, and these effects were reversed by resveratrol or NAC. Consistent with the above findings, resveratrol treatment could alter TXNIP, MDA, SOD, ROS, and inflammatory cytokine levels in spleen mononuclear cells. These results indicate that resveratrol could suppress TXNIP and SOD activities, and then reduce MDA and ROS generation, resulting in the downregulation of inflammatory mediators in the spleen mononuclear cells, which are consistent

with previous reports that resveratrol could exert antioxidant and anti-inflammatory effects by inhibiting TXNIP and oxidative stress in a variety of cells.²⁷⁻²⁹

As depicted in Fig. 7, resveratrol could suppress TXNIP and increase SOD activity, leads to reduction of MDA and ROS levels; thus, oxidative stress are reduced, and then OVA-specific IgE and histamine levels in the serum, and OVA-specific IgE, PGD₂, LTC₄, ECP, IL-4, IL-5, IL-6, IL-33, and TNF- α levels in the NLF are attenuated, resulting in the reduction of allergic and inflammatory responses.

Some limitations of the present study should be interpreted. First, with respect to the studies on guandose-dependent effects of resveratrol on AR murine model, only 2 doses were used, and studies with more doses are warranted to further clarify this finding. Second, in splenocytes culture and stimulation experiment, we only isolated splenocytes from untreated AR mice, and splenocytes should be isolated from different mice groups and splenocyte response should be compared among these groups in the future. In conclusion, resveratrol could effectively alleviate murine AR by inhibiting TXNIP-oxidative stress pathway. Resveratrol may be a promising therapeutic strategy for AR.

Abbreviations

AR: Allergic rhinitis; TXNIP: Thioredoxin-interacting protein; ROS: Reactive oxygen species; OVA: Ovalbumin; NAC: N-acetylcysteine; IHC: Immunohistochemistry; ELISA: Enzyme-linked immunosorbent assay; MDA: Malondialdehyde; SOD: Superoxide dismutase; NLF: Nasal lavage fluid; Th2: Type 2T helper

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Ethics statement

All animal procedures and experimental protocols were approved by the Animal Ethics Committee of Shanghai Sixth People's Hospital.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional information files. Any dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

All authors contributed to design this study, read and approved the final version of manuscript. HL designed the study. WTZ, RT and HL carried out the experiments, analyzed data, prepared figures, prepared the manuscript and edited the manuscript. RT and GYB carried out the conduction of animal experiments and edited the manuscript. MXL contributed to the nose histopathology, captured the images and prepared the manuscript. WTZ, RT and HL contributed to edit the manuscript.

Consent for publication

All authors agreed to publication of the work.

Declaration of competing interest

The authors declare that they have no relevant conflicts of interest.

Acknowledgements

Not applicable.

Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.waojou.2020.100473>.

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Supplementary Material

Anti-allergic and anti-inflammatory effects of resveratrol via inhibiting TXNIP-oxidative stress pathway in a mouse model of allergic rhinitis

Materials and methods

Animals

A total of 50 female BALB/c mice (6–8 weeks of age, Shanghai Experimental Animal Center, Shanghai, China) were included and kept in a specific-pathogen free facility. The mice were divided into five groups (n=10 per group): normal control group, untreated AR group, 200µg resveratrol treated AR group, 400µg resveratrol treated AR group and NAC treated AR group.

AR mouse model and resveratrol treatment

The murine AR model was induced by ovalbumin (OVA) as previously described.^{1,2} First, to establish AR murine model, mice were sensitized with 0.2 mL suspension including 0.5 mg/mL ovalbumin (OVA, Sigma-Aldrich) and 20 mg/mL aluminum hydroxide (Sinopharm Chemical Reagent Co) by intraperitoneal injection on day 1, 8, and 15, respectively. Furthermore, normal control mice were intraperitoneally administered with 0.2 mL normal saline on day 1, 8, and 15, respectively. Then, on days 22 to 29, the mice in AR groups were challenged daily with 20µl OVA (40

mg/ml) by intranasal instillation, normal control mice and untreated AR mice were administrated with 30µl vehicle of 0.5% carboxymethyl cellulose solution. In addition, on days 22 to 29, resveratrol treated AR mice were instilled intranasally with resveratrol (200µg or 400µg, Sigma-Aldrich) dissolved in 30µl 0.5% carboxymethyl cellulose solution on days 22 to 29. Moreover, during this period, NAC treated AR mice were instilled intranasally with NAC (100µg) dissolved in 30µl 0.5% carboxymethyl cellulose solution.

Mice nasal symptoms evaluation and sample preparation

Mice nasal symptoms including frequency of sneezes and nasal rubbing were evaluated for 10 minutes on the 29th day, immediately following the final OVA provocation. After 24 hours, the mice were killed and the blood samples were harvested by cardiac puncture and were centrifuged to obtain serum for enzyme-linked immunosorbent assay (ELISA). Tracheas of the mice were partially resected and a 22-gauge catheter was inserted via the tracheal opening to the nasopharynx in order to perfuse the nasal passages from choana to nostril with 1 ml PBS. Nasal lavage fluid (NLF) was collected from the nares and centrifuged (3,500 rpm, 10 minutes, 4°C), and the supernatants were collected and stored at -80°C for ELISA. Nasal mucosas were collected and assayed using immunohistochemistry (IHC) staining and real-time PCR methods.

ELISA assay in the serum and NLF

OVA-specific immunoglobulin E (IgE) and histamines levels in the serum, and PGD2 in the NLF were assayed using specific ELISA kit (BlueGene Biotech, Shanghai, China) and OVA-specific IgE, LTC4, ECP, IL-4, IL-5, IL-6, IL-33 and TNF- α in the NLF were assayed with specific ELISA kits (BlueGene Biotech). Each sample was analyzed in triplicate.

HE and IHC staining

Nasal mucosas of mice were paraffin-embedded, serially sectioned (4 μ m). Following deparaffinization and rehydration, HE staining was used for evaluating eosinophils counts and IHC staining was employed to evaluate histopathological characteristics and TXNIP immunolabeling. IHC was performed using a streptavidin biotin complex (SABC) kit (Boster Biological Technology, Wuhan, China). Briefly, the sections were then retrieved in citrate buffer for 15 min at 98°C and then 3% hydrogen peroxide was added to block endogenous peroxidase for 20 min. Nonspecific binding was blocked using 5% bovine serum albumin. Sections were incubated with primary antibodies (rabbit anti-mouse TXNIP, 1:100 dilution, Abcam) overnight at 4°C. The sections stained by species and subtype-matched antibodies instead of the primary antibodies were chosen as negative controls. Following 20 min incubation with a horseradish peroxidase (HRP) labeled secondary anti-rabbit antibody, 3,3'-diaminobenzidine (DAB) was applied for visualizing immunoreactivity. Positively stained cells in ten different areas were counted under high power and quantified as cells per high-powered field (HPF, $\times 400$). All slides images were reviewed by two

independent investigators who were blinded to the study.

Quantitative real-time reverse transcriptions PCR

Total RNA in the collected tissues or cells was extracted utilizing RNeasy commercial kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. The purity and integrity were assessed by measuring absorbance ratios at 260/280nm (1.8~2.0 was considered eligible) and agarose gel electrophoresis, respectively. Two microgram of total RNA was reverse-transcribed to cDNA as a template for real-time PCR utilizing the PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa Biotechnology, Dalian, China) with specific primers (Table E1). Incubation conditions of PCR were as follows: 95°C/30 sec, 40 cycles of 95°C/10 sec, annealing at 60°C/60 sec and extension at 72°C/20 sec. Each sample was analyzed in triplicate. β -actin was used as an internal control gene. Relative mRNA levels were expressed as the relative fold change and calculated using the $2^{-(\Delta\Delta CT)}$ method as previously described.³ A normal control sample was designated as the calibrator.

Measurement of MDA, and SOD in nasal tissue homogenates

Nasal tissue samples were weighed and homogenized in 1 mL 0.9% sodium chloride solution on ice, and then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatants were collected for assaying MDA level and SOD activity using MDA Assay Kit and SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing,

China) respectively according to the manufacturer's protocol. Protein concentration was quantified using bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, St Louis, MO, USA).

Spleen mononuclear cells culture and stimulation

Spleen mononuclear cells were isolated from six untreated AR mice and cultured as previously described.⁴ Briefly, spleen mononuclear cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco), and stimulated with control PBS, OVA (10µg/ml) alone or with resveratrol (10µM or 20µM) or NAC (10µM) for 24h. Following stimulation, the cells and supernatants were collected for western blotting, real-time PCR, flow cytometry and ELISA.

Western blotting

The cells were homogenized and lysed in RIPA buffer (Pierce). Then, the samples were then centrifuged at 14000 rpm at 4°C for 15 min and the supernatants containing total protein were harvested. After quantification of protein concentration using bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich), the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Invitrogen, Carlsbad, CA, USA) and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation for 1h at room temperature in 5% skimmed milk to block excess protein binding sites, the PVDF membranes were incubated with primary rabbit anti mouse antibodies

(TXNIP, 1:1000 dilution, Abcam) at 4°C overnight. Following TBST washing, a secondary goat anti-rabbit IgG-HRP antibody was used (Abcam) for a 1h incubation. Finally, Enhanced Chemiluminescence (ECL) kit (Pierce) was used to visualize immunoblotting. We used GAPDH as an internal control. Densitometric analyses were conducted utilizing Gel-Pro Analyzer 4.0 software (Media Cybernetics).

Measurement of MDA, and SOD in spleen mononuclear cells

Following exposure, culture medium was aspirated and spleen mononuclear cells were gently rinsed with PBS twice and lysed with cell lysis buffer. Then, the lysate was centrifuged at 10,000 g and 4 °C for 10 min to remove debris. The supernatants were collected for assaying MDA level and SOD activity using MDA Assay Kit and SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute) respectively according to the manufacturer's protocol. Protein concentration was quantified using bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich).

ROS assay in spleen mononuclear cells

Spleen mononuclear cells were centrifuged at 12,000 rpm for five min, and resuspended in complete RPMI medium. Then, cells were stained with DCFH-DA dye (10 μ M) in the dark at 37 °C for 30 min. Following PBS washing for three times, the cells were collected and resuspended in 500 μ l PBS and then analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The ROS mean fluorescence intensity (MFI) was calculated by Cell Quest software. Intracellular ROS levels were assessed by the relative ratio of MFI of targeted cell sample versus that of

a control cell sample whose relative MFI was set at one.

ELISA assay in the supernatants

Following treatment, the supernatants were collected for assaying IL-4, IL-5, IL-6, IL-33 and TNF- α protein levels using specific ELISA kits (BlueGene Biotech).

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TABLE E1. Primers used for quantitative real-time PCR analysis

Primer	Sequence	Expected product size (bp)
TXNIP	(F) 5'-CCTGTGAAGGGTTGTGGTTG-3' (R) 5'-CTGGACAGGAAATGGGGTCT-3'	107
β -actin	(F) 5'-CGCCACCAGTTCGCCATGGA-3' (R) 5'-TACAGCCCGGGGAGCATCGT-3'	105