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SIRT1 attenuates murine allergic rhinitis by downregulated HMGB 1/TLR4 pathway

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Short Title: SIRT1, a therapeutic agent of allergic rhinitis

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Abstract

Conventional allergic rhinitis (AR) treatments have limitations due to the lack of safety and complete cure strategy. We evaluated the effects of Silent information regulator 1 (SIRT1), a multifunctional molecule involved in a variety of inflammatory pathways, on murine AR model. Ovalbumin (OVA)-induced murine model was constructed, and recombinant SIRT1 was administered into the nostril continuously. The expression of SIRT1 was measured at mRNA and protein levels, and the allergic symptoms were evaluated. Protein levels of OVA-specific IgE, leukotriene C4 (LTC4), eosinophil cation protein (ECP), Prostaglandin D2 (PGD2), as well as different inflammatory cytokine mediators in the serum and nasal lavage fluid (NLF) were assessed by ELISA. The effects of SIRT1 on human primary nasal epithelial cells challenged with Tumor necrosis factor (TNF)- α were also evaluated by investigation the HMGB1/TLR4 signaling pathway. Administration of SIRT1 significantly alleviated OVA-induced AR symptoms with lower numbers of sneezing and nasal rubbing events, decreased levels of OVA-specific IgE, LTC4, ECP, PGD2, less inflammatory cells, and downregulated levels of Th2 type cytokines. SIRT1 also reduced the genes of HMGB1/TLR4 signaling pathway in the murine model and cultured human nasal epithelial cells. Expression of SIRT1 is impaired in OVA-induced AR model. The administration of SIRT1 alleviates the allergic symptoms of mice, regulates the production of pro-inflammatory mediators predominantly produced by Th2 cells in AR, and attenuates expressions of proteins relevant to HMGB1/TLR4 signaling pathway. All the results showed that SIRT1 is promising as a therapeutic agent of AR.

Keywords: Allergic Rhinitis, SIRT1, sirtuin, HMGB1, Th2 type cytokines

Introduction

Allergic rhinitis (AR) is one of the most common chronic illnesses worldwide, and its prevalence has increased rapidly over the last few decades [1]. AR is allergic inflammation in the upper airway associated with the mediators released by several types of the hyper-responsive immune cells including antigen presenting cells (APCs), B cells, mast cells, and eosinophils etc. [2]. Currently the most common accepted therapy for AR consists of disease control through the use of anti-inflammatory agents, glucocorticoid-based drugs, anti-histamines, and anti-leukotrienes agents. Despite the alleviation of symptoms in the majority of the patients by these available drugs, they do not cure the underlying disease in long-term run. Further, these conventional AR treatments have limitations because of the side effects or low compliance with allergen avoidance [3]. Therefore, the discovery and development of novel therapeutic agents that target the origin of the pathogenesis of AR remain pivotal.

Silent information regulator 1 (SIRT1) belongs to the mammalian sirtuin family, which is composed of seven NAD⁺-dependent protein deacetylases (SIRT1-7). It was previously revealed that SIRT members have conserved sequences, expressed widely in bacteria, mammalian and humans [4]. The functional roles of sirtuins have been implicated in a variety of biological processes including cell cycle, apoptosis, stress response, neuronal protection, metabolism, tumorigenesis, and transcription of rRNA genes [5]. Notably, SIRT1 deacetylates histones and other non-histone proteins, and is a multifunctional molecule involved in different pathways, such as cell differentiation, cell apoptosis, cell aging, tumor suppression and asthmatic inflammation [4, 6]. The inhibition of SIRT1 distorts oxidative energy metabolism and stimulates the NF- κ B-induced inflammatory responses, while SIRT1 activity raises apoptosis in response to TNF- α by inhibiting the transactivation of the RelA/p65 protein [7-9]. SIRT1 is also essential in the termination of the inflammatory response [9, 10]. Myeloid deletion of SIRT1 in the mice challenged with a high-fat diet

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demonstrated high levels of activated macrophages in liver and adipose tissue [11]. A previous study showed that overexpression of SIRT6 led to the hypoacetylation of GATA-3, preventing cellular differentiation toward Th2 [12]. SIRT1 also represses the activity of the nuclear receptor peroxisome proliferator-activated receptor- γ in dendritic cells, thus promoting the differentiation toward a pro-Th2 phenotype [5]. SIRT1 as an important regulator of macrophage inflammatory responses in insulin resistance using primary and cell line of macrophages [13]. These findings indicate the potential functional role of SIRT1 in therapy against AR. Therefore, in this study we aim to investigate protective effects of the administration of SIRT1 in OVA-induced murine AR model, and to explore the possible underlying action mechanisms.

Materials and Methods

OVA-induced allergic rhinitis (AR) and intranasal SIRT1 treatment

The AR murine model was constructed as previously described with slight modification [14]. Specifically, female BALB/c mice (6 weeks old) were housed in an isolated room with controlled temperature and at a 12/12 (light/dark) schedule and were allowed ad libitum access to sterile food and water in a specific pathogen-free facility. All the animals were handled in accordance with the protocol approved by the Care and Use of Laboratory Animals committee. The mice were randomly assigned into three experimental groups (n = 10 each): AR group (AR): mice were given intraperitoneal injection of 100 μ g of OVA and 2 mg of aluminum hydroxide [Al(OH)₃] dissolved in 100 μ l saline on days 0, 7 and 14, then challenged daily with an OVA solution at 40 mg/ml (20 μ l/mouse) into the nostrils on days 21-28; Control group (Ctrl): Al(OH)₃ solution was injected, then challenged with saline on days 21-28; AR plus SIRT1 group (SIRT1): mice were injected intraperitoneally with OVA and Al(OH)₃ on days 0, 7 and 14, then administered SIRT1 (0.1 mg/ml, 30 μ l/mouse, Abcam) into the nostril 30 min before OVA challenge daily on days 21-28.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Nasal mucosa was removed and total RNA was extracted using an RNeasy mini kit (Qiagen, CA, USA). RNA was reverse-transcribed into cDNA using PrimeScript First Strand cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's protocol, and the complementary DNA was then subjected to qPCR on the MiniOpticon™ Real-Time PCR System (Biorad, CA, USA). The primers used in the experiments were as follows: *sirt1*: forward, ctgttgaccgatggactct and reverse, gccacagcgtcatatcatcc; β -actin: Forward, gtgggaatgggtcagaagga and reverse, tcatttttcacggttggcc were the same as described previously [15]. The amplification protocol was performed as follows: 1 cycle at 98°C for 1 min, followed by 40 cycles at 98°C for 10 sec, 56°C for 20 sec, and 72°C for 30 sec. The relative quantification of *SIRT1* gene expression compared with β -actin was analyzed.

Western blot analysis

Total protein was extracted from the cells in mice nasal mucosa with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific, Shanghai, China). Totally 20 μ g of proteins were separated by a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked by 5% non-fat milk for 2 h at room temperature and incubated with the primary antibodies for overnight at 4 °C. After washing the membrane for 5 times with 1xPBST, the secondary antibodies were added and incubated for 1 h at room temperature. The membrane was developed by enhanced luminol-based chemiluminescence and the results were photographed using the UVP BioSpectrum Imaging system (BioSpectrum, CA, USA).

Assessment of allergic symptoms and NLF sample preparation

Within 20 min of the final nasal challenge with OVA on day 28, the early phase responses including sneezing frequencies and number of nasal rubbing events of all the mice were counted and recorded for a total of 10 min in a blind way. Under anesthesia, blood and nasal lavage fluid (NLF) samples were collected from mice 2 h after the final challenge, followed by centrifugation at 3,000 rpm for 10 minutes. The plasma and NLF supernatant were stored at -80 °C for subsequent measurements. Moreover, the skull was sagittally bisected, with septal and turbinate mucosa removed, the nasal mucosa tissues were stored at -80 °C for further western blot analysis.

Enzyme-linked immunosorbent assays (ELISAs)

Protein levels of OVA-specific IgE, leukotriene C4 (LTC4), eosinophil cation protein (ECP), and Prostaglandin D2 (PGD2) in the plasma and NLF samples were measured by the corresponding ELISA kits (Blue Gene, Shanghai, China). Briefly, plasma and NLF samples were serially diluted and added into 96-well plates coated with purified primary polyclonal antibody, followed by the wash and incubation steps described in the kits. The reactions were developed with tetramethylbenzidine and terminated with H₂SO₄. The absorbance at 450 nm was measured by a Spectramax microplate reader (Molecular Device, CA, USA).

Inflammation cells counting

The cells in the NLF were resuspended with 1 ml 100 mM PBS with 1% bovine serum albumin (BSA), and the total cell number of leukocytes was counted using a hemocytometer (Mindray3000, Shenzhen, China). The numbers of eosinophils, neutrophils and lymphocytes were determined by cell counting with Wright's-Giemsa staining.

Human nasal epithelial (HNE) cells culture

Primary nasal epithelial cells were collected from the mid-part of the inferior turbinate of 2 healthy volunteers with interdental brushes. Both subjects signed consents to participate in the study granted by Research Ethical Committee of Qingdao Municipal Hospital. Cell populations were expanded through two passages with airway epithelial growth medium (Promocell, Heidelberg, Germany) containing antibiotics and seeded into sterile plastic collagen-coated flasks at a density approximately 150,000 cells/cm². All air-liquid interface culture (ALI) were carried for 21 days before exposure. Mucus production was observed by day 10 of ALI and excess mucus was rinsed with saline every two days. HNE cells were pretreated with SIRT1 (500 ng/ml) for 24 h, then stimulated with TNF- α (10 ng/ml, R&D Systems) for 30 min, and expressions of proteins of HMGB1/TLR4 signaling pathway were detected by western blotting as described earlier.

Statistical analysis

All values in the study are presented as mean $\pm$ standard deviation (SD) of a minimum of three independent experiments. Statistically, the values were analyzed by one-way analysis of variance, followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

SIRT1 was downregulated in the nasal mucosa of AR mice

The mouse model of AR was induced by OVA injection. Upon the establishment of the AR mice model, we first analyzed expression levels of SIRT1 in the nasal mucosa of the established AR mice. As shown in Figure 1A, the level of SIRT1 mRNA in AR mice is

significantly lower than the one in the control mice ($P < 0.01$). Consistently, western blotting results showed that the protein level of SIRT1 in AR mice decreased to approximately 20% of the level in the normal control group (Figure 1B and C, $P < 0.01$). These findings indicated the potential modulation of SIRT1 on AR.

SIRT1 alleviated symptoms of AR in mice

To examine the regulation of SIRT1 in AR, SIRT1 was administered into the nostril before OVA challenge daily for one week, and the numbers of sneezes and nasal rubbing were recorded after the last OVA challenge. As presented in Figure 2, the AR group showed significant increase in sneezing numbers compared to the normal control group without OVA stimulation. The average sneeze count of SIRT1 treated AR mice was significantly smaller than that of AR mice ($P < 0.01$; Figure 2A). Similarly, the number of nasal rubbings in AR mice showed increase compared to the normal mice, and SIRT1 treated AR mice demonstrated significantly lower nasal rubbings events than AR mice ($P < 0.01$; Figure 2B). Of note, it was found that SIRT1 reduced infiltration of inflammatory cells into nasal cavity (supplementary Figure S1), which could promote the initial irritation and sneeze reflex of mice. These results clearly indicated that the intranasal administration of additional SIRT1 alleviated allergic symptoms in mice.

SIRT1 decreased levels of allergic inflammatory cytokines and OVA-specific IgE in the of AR mice

To further explore the underline mechanisms of the attenuation of SIRT1 in AR, we evaluated the levels of allergic inflammatory cytokines and immunoglobulins including OVA-specific IgE, LTC₄, ECP, and PGD₂ in the NLF and serum of the mice. The levels of OVA-IgE and LTC₄ of AR mice were both elevated compared to those of the normal group. In contrast, the NLF levels of OVA-IgE and LTC₄ of the SIRT1 group were significantly reduced compared

to those of AR mice ($P < 0.01$, Figures 3A, 3B, 3D; $P < 0.05$, 3E). Additionally, the ECP level in serum and the PGD2 level in NLF showed similar trends of changes in the normal control group, AR group, and SIRT1 group. Specially, both of these two factors elevated in AR mice compared to those in normal control, and were reduced by SIRT1 administration significantly ($P < 0.01$, Figures 3C, 3F). These results showed that additional SIRT1 in the nasal mucosa inhibited both local and systemic allergic responses.

SIRT1 decreased inflammatory cell numbers in the NLF of AR mice

As shown in Figure 4, the total leukocytes number, as well as the numbers of eosinophils, lymphocytes, and neutrophils in the NLF of AR mice was significantly greater than that of normal control mice (Figure 4A-D). The total and differential cell numbers of all inflammatory cells in the NLF of SIRT1 treated AR mice were all decreased compared to AR mice ($p < 0.01$).

SIRT1 impacted inflammatory cytokines secretion in the serum of AR mice

The levels of several pro-inflammatory cytokines and anti-inflammatory cytokines including IL-4, IL-13, IL-5, IL-10, IL-6, IL-17, TNF- α , IFN- γ and IL-2 in the serum from BALB/c mice were assessed by a two-site sandwich ELISA. As shown in Figure 5A-I, the cytokine levels of IL-4, IL-13, IL-5, IL-10, IL-6, IL-17, TNF- α were increased in AR mice compared to normal control mice, and the introduction of exogenous SIRT1 significantly decreased the levels of these cytokines in comparison with AR mice ($P < 0.01$). In contrast, the levels of IFN- γ and IL-2 were lower in the AR mice than those in the normal control mice, and the additional SIRT1 inversed the levels of these two factors with statistical significance ($P < 0.05$). Interestingly, both of IL-33 and IL-35 were elevated in AR group, but downregulated in SIRT1 group (supplementary Figure S2).

SIRT1 downregulated the genes of HMGB1/TLR4 signaling pathway

It has been suggested that SIRT1 plays important roles in regulating HMGB1 acetylation [16], we investigated changes in these proteins to determine whether SIRT1 treatment alleviated the HMGB1-regulated inflammation in the AR mice. Western blotting results showed that administration of SIRT1 decreased the expression of HMGB1 in the nasal mucosa of AR mice significantly ($P < 0.01$, Figure 6 A-B). In addition, cultured human nasal epithelial cells were stimulated with pro-inflammatory cytokine TNF- α , the results showed that TNF- α induced an increase levels of HMGB1, TLR4, and Myd88 in HNE cells, and additional SIRT1 decreased the levels of these proteins in HNE cells significantly ($P < 0.01$ for HMGB1 and Myd88; $P < 0.05$ for TLR4, Figure 6C-D). Furthermore, we detected the expression level of p65, one of the important components of NF- κ B signaling, our data showed that the TNF- α stimulation induced the p65 level normalized to Lamin B, and addition of SIRT1 inversed the p65 level significantly ($P < 0.01$, Figure 6C-E).

Discussion

Cumulative studies have demonstrated that allergic inflammation is characterized by a T helper 2 (Th2) cell-driven immune response. It has been reported that inhibition of sirtuins damaged adaptive Th2 responses and the subsequent allergic inflammation [5]. In the present study, we used the OVA-induced AR mouse model to investigate the therapeutic effects of SIRT1, a member of sirtuin family. Our data showed that the expression of SIRT1 protein was significantly downregulated in the established AR mice compared with normal control. Intriguingly, the expression of SIRT1 mRNA transcripts quantified by qRT-PCR did not show changes between groups as significantly as protein level, indicating that the reduction of SIRT1 is not only due to reduced gene transcription. This result is strikingly consistent

with previous cohort study which showed that SIRT1 protein expression in peripheral blood mononuclear cells was significantly reduced in patients with severe asthma in comparison with that of healthy subjects, whereas the change of SIRT1 mRNA expression was not that statistically significant [12].

To further investigate the potential role of SIRT1, exogenous SIRT1 was administered in the AR mice, and the results showed the allergic symptoms marked by sneezing and nasal rubbing were significantly alleviated with the additional administration of SIRT1. During the pathogenesis of AR, an allergen triggers a Th2-predominant immune response that produce antigen-specific IgE to a certain extent, which binds to mast cells. Exposure to the same allergen activates these IgE-binding mast cells which release inflammatory mediators, such as histamine and leukotriene C4 (LTC4), promoting the initial irritation and sneeze reflex of mice [17]. We found SIRT1 decreased the levels of OVA-specific IgE and LTC4 in serum and NLF, suggesting the alleviation of local and systematic allergic inflammation. Moreover, SIRT1 downregulated the levels of eosinophil cationic protein (ECP), which is the most widely used marker to monitor diseases involving eosinophils [18], and prostaglandin D2 (PGD2), which plays an important role in interactions between different immune cells such as mast cells, Th2 cells, eosinophils, and dendritic cells [19]. Consistently, SIRT1 reduced the numbers of the lymphocytes, eosinophils and neutrophils in the NLF.

Moreover, compared with the AR induced mice, the SIRT1 treatment groups showed a significant reduction in serum levels of IL-4, IL-13, IL-5, IL-10, IL-6, IL-17, and TNF- α , which are main mediators produced by Th2 cells ($p < 0.01$) and less significant increase of the levels of IFN- γ and IL-2 ($p < 0.05$), which are mainly produced by Th1 cells. IL-4 plays a pivotal role in the allergic response, including induction of isotype switching to IgE, expression of vascular cell adhesion molecule-1, promotion of eosinophil migration across the endothelium, mucus secretion, and differentiation of Th2 lymphocytes. IL-5 is essential

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cytokine for the differentiation and maturation of eosinophils [20], while IL-13 is the most potent inducer of goblet cell differentiation and mucus production [21]. In addition, IL-17 is related to airway hyperreactivity and mucus hypersecretion in the upper airway, including in AR [22]. Interestingly, IL-10 is usually considered as anti-inflammatory factor. Different from our initial expectation, we saw the elevation of IL-10 in the AR model. AR is characterized by a Th2 cell-driven immune response, and we speculated the observed IL-10 was predominantly released by the differentiated Th2 under AR condition. In summary, we conclude that SIRT1 has a therapeutic potential for the alleviation of AR in mice indicated by the downregulation of pro-inflammatory cytokines predominantly produced by Th2 cells, which play a central role in induction of allergic symptomatology.

It has been reported that the concentrations of HMGB1 were significantly higher in nasal secretions from patients with AR compared with the control subjects [23]. HMGB1 has recently been shown to be a mediator in different chronic inflammatory disorders such as acute-on-chronic liver failure [24]. HMGB1 triggers downstream signaling via activating a wide range of receptors, among which TLR4 signaling is thought to be the key for its cytokine-inducing activities [25, 26]. Binding of additional adaptor proteins with TLR is essential for triggering intracellular signaling cascades, while the myeloid differentiation factor 88 (MyD88) is one of the identified adaptor proteins identified [27]. A previous study has also reported that the acetylation on HMGB1 is highly relevant with the deacetylase activity of SIRT1, indicating that SIRT1 interacts with HMGB1 via deacetylation, thus preventing the release of HMGB1 [28]. In this study, the results showed that SIRT1 decreased the HMGB1 expression in the AR mice significantly. Further, we applied an *in vitro* inflammatory mucosal model by stimulating human nasal epithelial cultures with TNF- α to stimulate the production and secretion of HMGB1, and SIRT1 inhibited the expression of HMGB1, TLR4 and adaptor protein Myd88 induced by TNF- α in the cell culture system. In

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addition, SIRT1 has been shown to inhibit NF- κ B transcriptional activity through the deacetylation of the p65 subunit, leading to the reduced inflammatory cytokine production [29]. Our results consistently showed the increase of p65 in AR mice, and the attenuation of SIRT1 on the p65 level in AR mice, indicating the successful establishment of the cell model as well as the protection role of SIRT1.

Taken together, the expression of SIRT1 is impaired in OVA-induced AR model. The administration of SIRT1 alleviates the allergic symptoms of mice and regulates the production of pro-inflammatory mediators in AR. Current study showed that SIRT1 was effective in the alleviation of allergic symptoms of AR mice, and is promising to be explored as potential therapeutic agent for AR. Future studies will be performed to clarify the safety of SIRT1 in animals by investigating the cells viability and apoptosis, as well as the metabolisms of this protein *in vivo*. Further, the change of the deacetylase activity of the SIRT will be evaluated by assessing the acetylation of GATA-3, a Th2 transcription factor that is increased in the airways of asthmatic patients [12]. The detailed molecular mechanisms of SIRT1 on AR therapy will be helpful to evaluate whether it is a new strategy for clinical management of AR, broadening the effective targets selections towards the long-term symptomatic therapy of AR.

Conclusion

Expression of SIRT1 is impaired in OVA-induced AR model. The administration of SIRT1 alleviates the allergic symptoms of mice, regulates the production of pro-inflammatory mediators predominantly produced by Th2 cells in AR, and attenuates expressions of proteins which are relevant to HMGB1/TLR4 signaling pathway. All the results showed that SIRT1 is promising as a therapeutic agent of AR.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

None.

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Figure legends

Figure 1. SIRT1 was decreased in the nasal mucosa of AR mice. (A) Relative SIRT1 mRNA levels were examined by RT-PCR and normalized to β -actin control. (B-C) SIRT1 protein expressions were analyzed by western blotting. Beta-actin was employed as a loading control. AR: AR group were given intraperitoneal injection of OVA and aluminium hydroxide [Al(OH)₃]f periodically, then challenged daily with an OVA solution; Ctrl: normal control group, saline plus Al(OH)₃ without OVA was injected, then challenged with saline. Each value represents the mean $\pm$ SD of 10 mice in each group. **P<0.01 significant difference with Ctrl group.

Figure 2. SIRT1 attenuates allergic symptoms in AR mice.. (A) Number of sneezing. (B) Number of nasal rubbing events. Each value represents the mean $\pm$ SD of 10 mice in each group. **P<0.01 significant difference with AR group.

Figure 3. SIRT1 decreased OVA-specific IgE, LTC₄ and ECP in the serum (A-C), and OVA-specific IgE, LTC₄ and PGD₂ in the NLF (D-F) of AR mice. Each value represents the mean $\pm$ SD of 10 mice in each group. *P<0.05 and **P<0.01 significant difference with AR group.

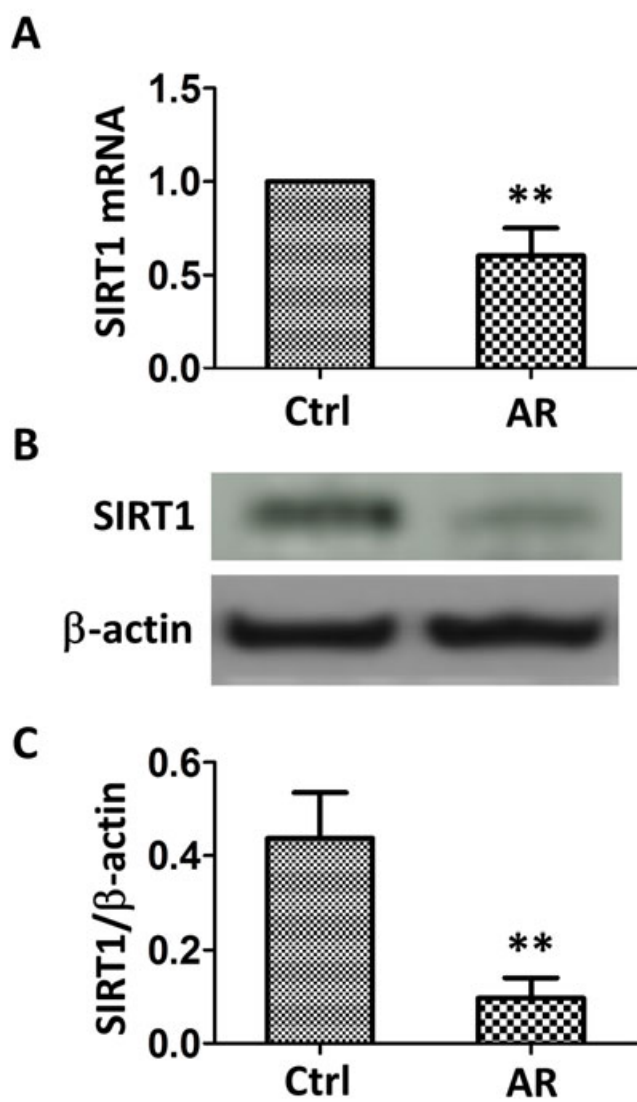
Figure 4. SIRT1 decreased the number of leukocytes (A), eosinophils (B), lymphocytes (C) and neutrophils (D) in the NLF of AR mice. Each value represents the mean $\pm$ SD of 10 mice in each group. **P<0.01 significant difference with AR group.

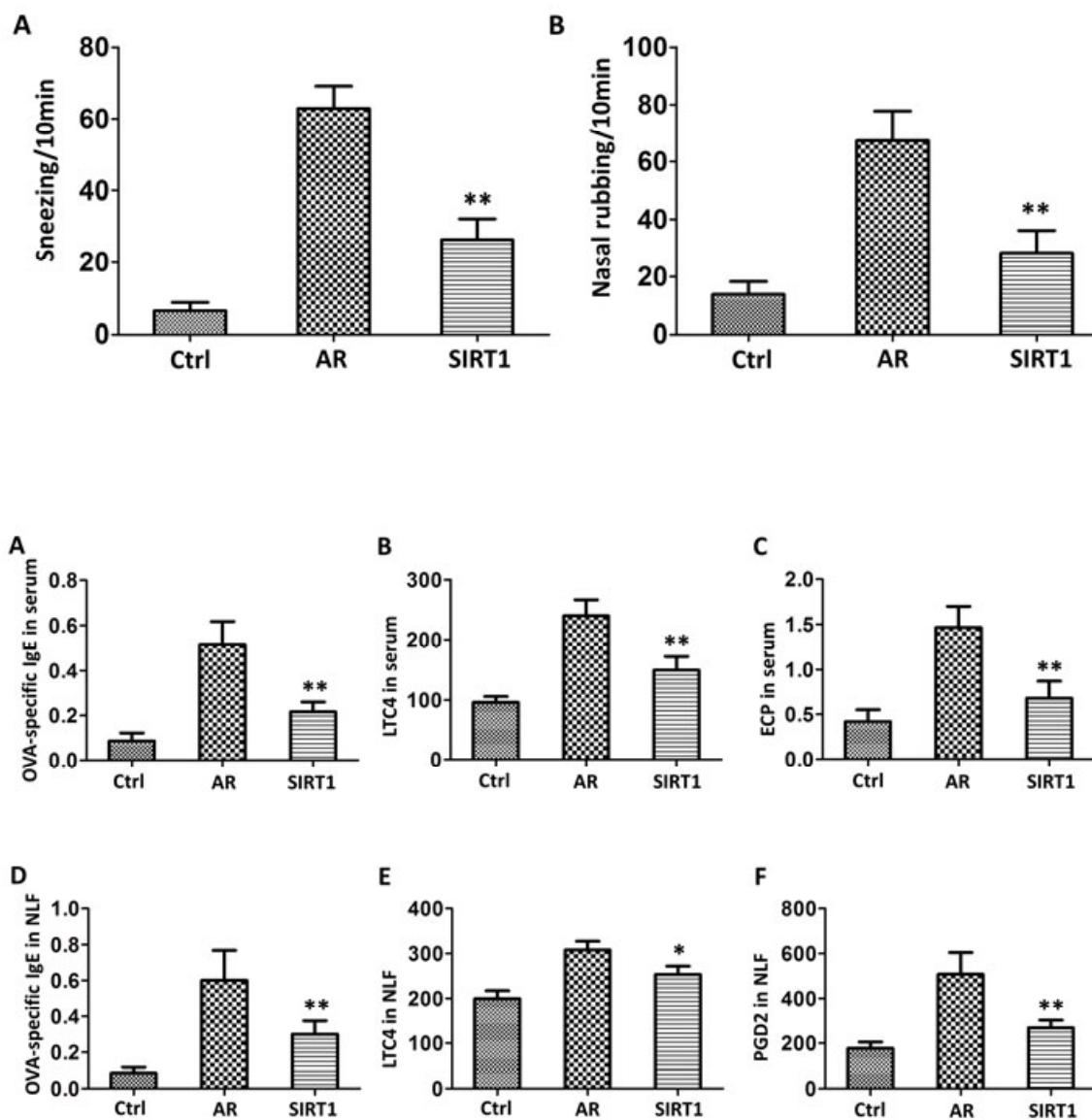
Figure 5. SIRT1 impacted inflammatory cytokines secretion in the serum of AR mice. (A) IL-4 secretion; (B) IL-13 secretion; (C) IL-5 secretion; (D) IL-10 secretion; (E) IL-6 secretion; (F) IL-17 secretion; (G) TNF- α secretion; (H) IFN- γ secretion; (I) IL-2 secretion. Each value

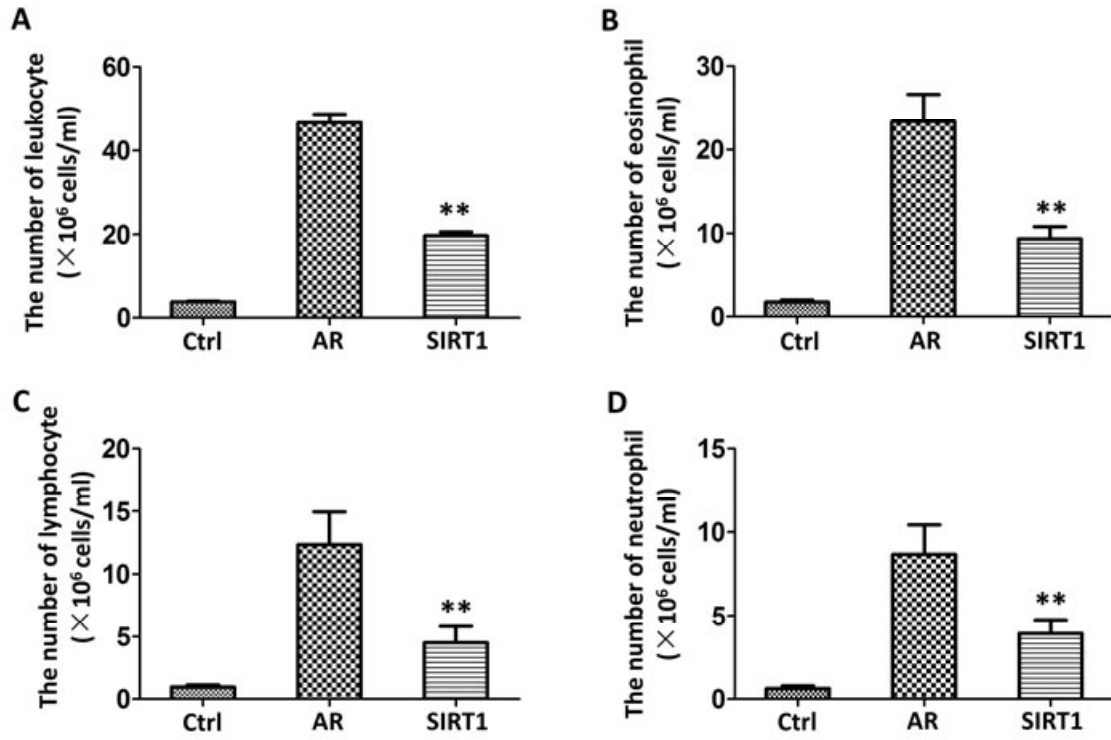
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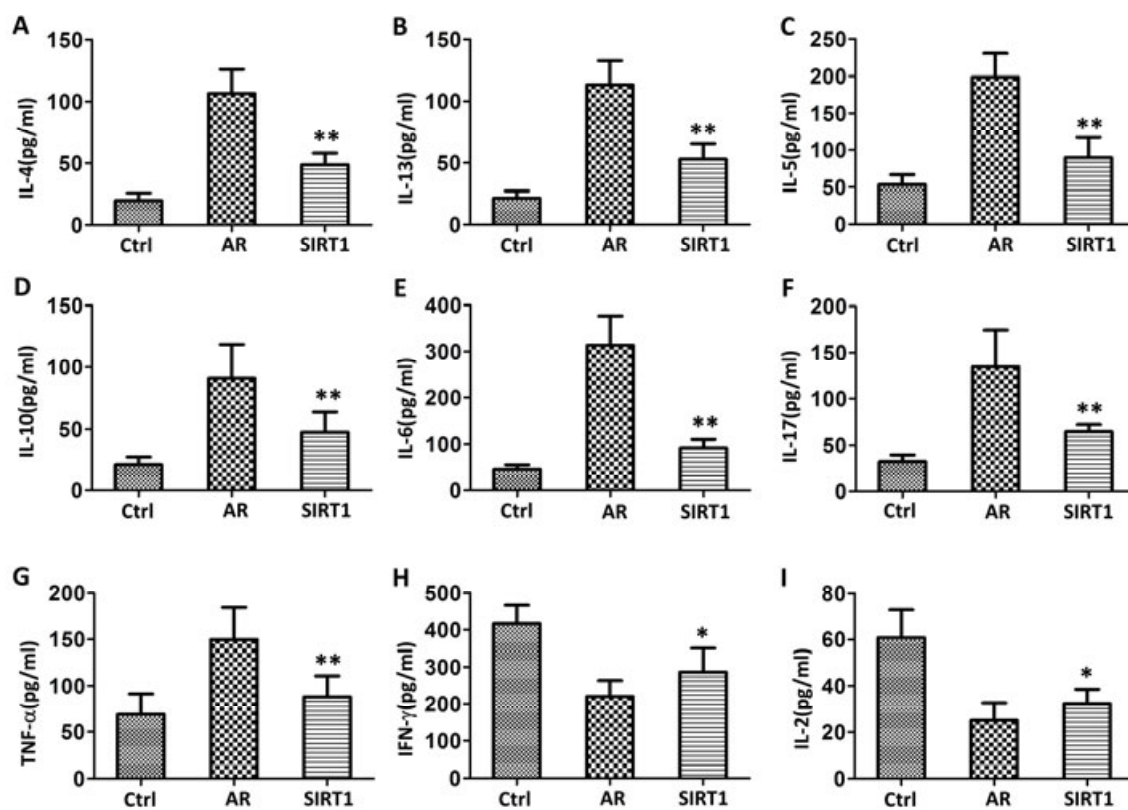
represents the mean $\pm$ SD of 10 mice in each group. *P<0.05 and **P<0.01 significant difference with AR group.

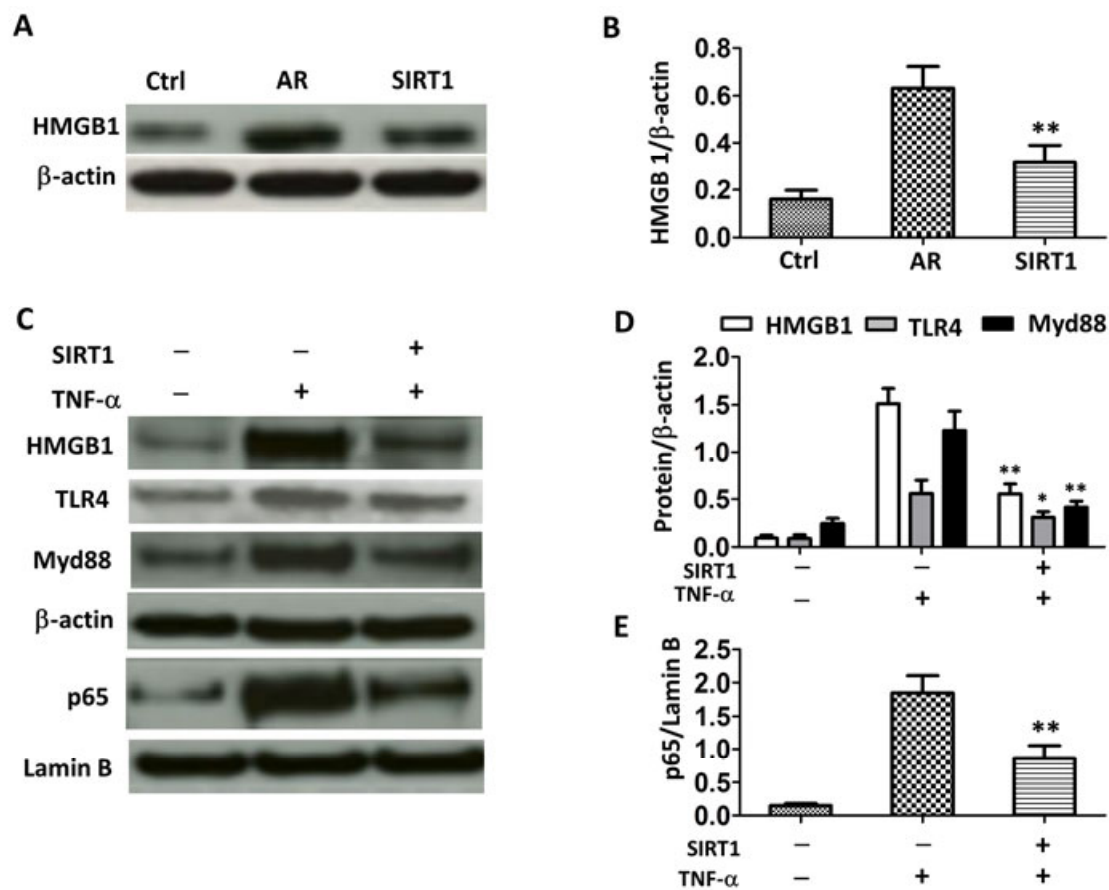
Figure 6. SIRT1 downregulated the genes of HMGB1/TLR4 signaling pathway. (A-B) Additional SIRT1 decreased HMGB1 in the nasal mucosa of AR mice. Each value represents the mean $\pm$ SD of 10 mice in each group. **P<0.01 significant difference with AR group. (C-E) Additional SIRT1 reduced the level of genes in HMGB1/TLR4 signaling pathway in human nasal epithelial cells. Results are expressed as the mean $\pm$ SD. *P<0.05 and **P<0.01 significant difference with the samples that stimulated with TNF- α but untreated with SIRT1.



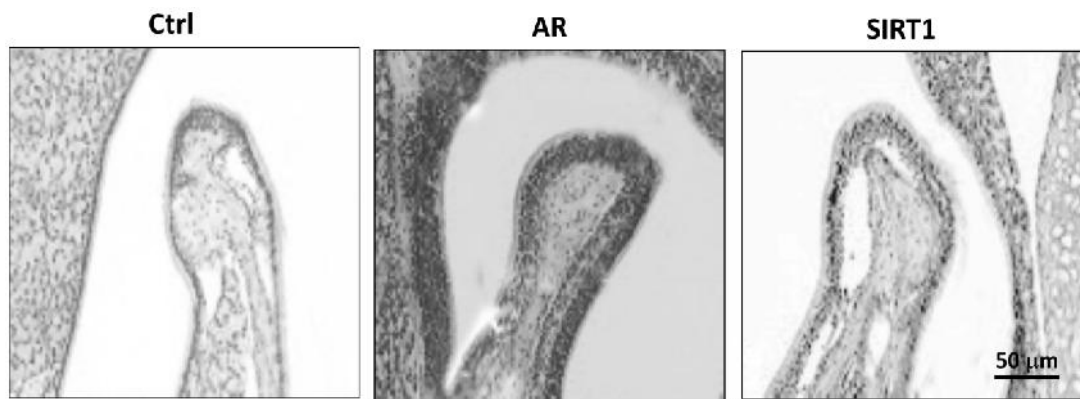




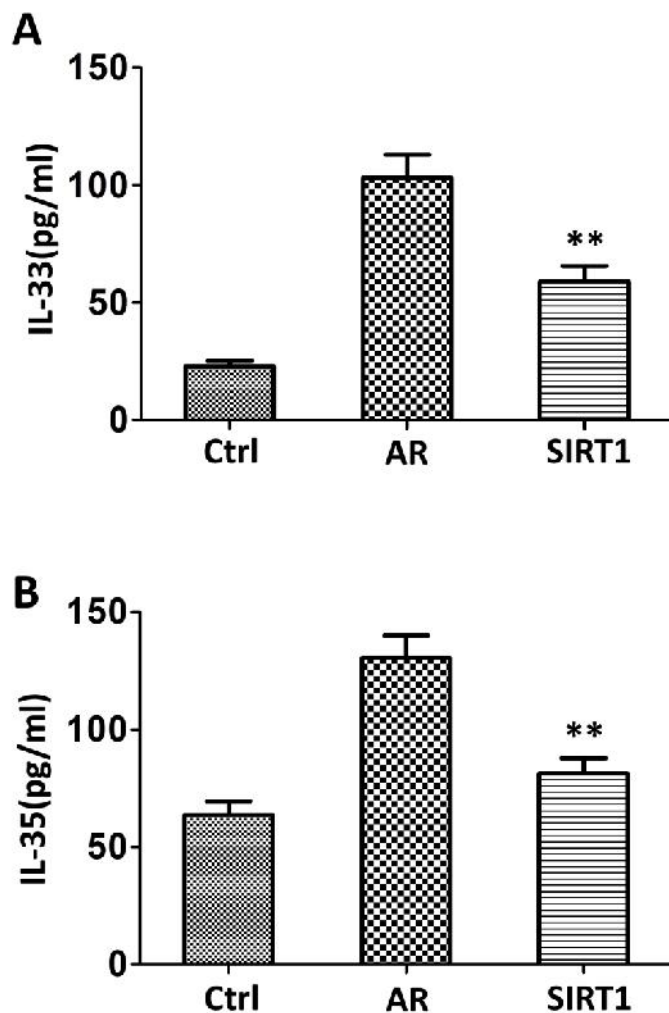




Supplementary Materials



Supplementary Figure S1. SIRT1 reduced infiltration of inflammatory cells into nasal cavity. The nasal mucosa sections were stained with hematoxylin and eosin. Sections were evaluated using microscope at an original magnification of 400x.



Supplementary figure S2. SIRT1 impacted inflammatory cytokines secretion in the serum of AR mice. (A) IL-33 secretion; (B) IL-35 secretion. Each value represents the mean $\pm$ SD of 10 mice in each group. **P<0.01 significant difference with AR group.