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TLR4 antagonist ameliorates combined allergic rhinitis and asthma syndrome (CARAS) by reducing inflammatory monocytes infiltration in mice model

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ABSTRACT

The present study aims to investigate the effects of toll-like receptor 4 (TLR4) antagonist in an ovalbumin (OVA)-induced mouse model of combined allergic rhinitis and asthma syndrome (CARAS). An OVA-induced mouse model of CARAS was established and TLR4 antagonist, TAK-242, was administrated intranasally or intraperitoneally. The number of sneezing and nasal rubbing was counted. The frequency of different cell types in the bronchoalveolar lavage fluid (BALF) and nasal lavage fluid (NLF) was analyzed using flow cytometry. Expressions of protein in nasal mucosa and lungs were determined using western blotting. Levels of interleukin (IL)-4, IL-5, and IL-13 were determined using Enzyme-linked Immunosorbent Assay (ELISA). Histological scores were applied for the assessment of lung injury. Treatment of TAK-242 downregulated CCL2 expression and reduced monocyte infiltration in nasal mucosa and lung tissues. Additionally, treatment of TAK-242 ameliorated upper airway symptoms including the sneezing and nasal rubbing by the regulation of cytokines including IL-4, IL-5, and IL-13. Furthermore, treatment of TAK-242 ameliorated lower airway symptoms including decreasing the frequency of $CD45^+SiglecF^+CD11b^+CD11c^-$ eosinophils in BALF and $IL13^+$ Th2 cells in the lungs. In conclusion, treatment of TAK-242 ameliorated CARAS-related lung injury by inhibiting lymphocyte infiltration, reducing monocytes infiltration, as well as regulating the frequency of eosinophils and Th2 cells.

1. Introduction

Allergic rhinitis and asthma often coexist. Up to 40% of patients with asthma were affected by allergic rhinitis, and as many as 80% of patients with allergic rhinitis were experienced by asthma [1]. The terminology combined allergic rhinitis and asthma syndrome (CARAS) has been introduced to describe the patients who have two manifestations of the same disease [2]. Allergic rhinitis is mediated by an inflammatory response which is characterized by the infiltration of a series of cell types including T cells, mast cells, basophils, and eosinophils. These cells release cytokines, chemokines, and inflammatory mediators, which further trigger a systematic inflammatory response [1,3]. Similarly, asthma is a chronic inflammatory disease and airway inflammation is a key feature of asthma. The recruitment of T cells, eosinophils, basophils, and mast cells contributes to the development of an asthmatic response [4]. Additionally, the immunologic and epidemiologic studies also demonstrate the link between allergic rhinitis and asthma [1].

As a type of pattern recognition receptor, toll-like receptors (TLRs) are able to recognize exogenous microbial products and endogenous molecules released upon cell damage [5]. Activation of TLRs results in the activation of cellular signaling pathways and the release of inflammatory cytokines and chemokines. TLR4 is one of the important sensing receptors in the immune system and expressed on many types of cells including T cells, cardiac myocytes, macrophages, and endothelial cells. TLR4 can be triggered by a series of ligands including lipopolysaccharide, tenascin-C, hyaluronic acid, morphine-3-glucuronide [6]. Two major pathways are initiated called TLR4-myeloid differentiation primary response gene 88 (MyD88) pathway and TLR4-TIR domain-containing adapter-inducing interferon-β (TRIF) pathway. In addition, intracellular endogenous ligands including heat shock proteins and high-mobility group box 1 (HMGB1) are also able to activateTLR4.

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HMGB1 was identified as an inflammatory cytokine as well as a ligand for TLR4. Fan and colleagues have demonstrated that HMGB1-TLR4 axis mediates NAD(P)H oxidase activation induced by hemorrhagic shock plus resuscitation [7]. Another study performed by Ding and colleagues have reported that HMGB1-TLR4 axis triggers cardiomyocyte apoptosis during myocardial I/R injury by accelerating of the productions of inflammatory cytokines [8]. More recently, Yuan and colleagues have reported that HMGB1-TLR4 axis plays an important role in the development of allergic rhinitis and the down-regulation of HMGB1-TLR4 is a promising therapeutic strategy to attenuate allergic rhinitis [9]. However, the roles of HMGB1-TLR4 in the development of CARAS are still unknown. Therefore, the present study was designed to explore the effects of HMGB1-TLR4 axis activation in an ovalbumin (OVA)-induced mouse model of CARAS. Besides, the underlying mechanisms of HMGB1-TLR4 in the development of CARAS were investigated. More importantly, for the first time, the present study found that TLR4 antagonist attenuates symptoms of lower- and upper airway the mice model of CARAS by downregulation of CCL2 expression and reduction of monocyte infiltration in nasal mucosa and lung tissues.

2. Materials and methods

2.1. Reagents and antibodies

Phosphate-buffered saline (PBS) was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Ovalbumin (OVA) and TAK-242, an antagonist for TLR4, were purchased from Sigma Aldrich (St. Louis, MO, USA). FACS antibodies including CD3, CD4, CD11b, CD11c, CD45, Ly6C, SiglecF, and IL13 were purchased from BioLegend (San Diego, CA, USA). Polyclonal antibodies against HMGB1, TLR4, Myd88, p56, CCL2, and β-actin were purchased from Abcam (Cambridge, MA, USA).

2.2. Animals and protocols

Female BALB/c mice (weighing 22 ± 2.5 g, 6–8 week) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The animals were housed in a 12-hour light-dark cycle and fed under experimental conditions with a temperature of 22–24 °C and humidity of 50 ± 5%. The animal experiments performed in this study were approved by Qingdao Municipal Hospital.

Construction of OVA-induced mice model of CARAS was according to a previously described method [10]. As shown in Fig. 1A, the mice were sensitized on days 0 and 7 *via* intraperitoneal injection of OVA emulsified in $AL(OH)_3$ (OVA/alum). After two weeks, the mice were challenged by instilling a 10 μL droplet of OVA (1 mg/mL) into each nostril using a micropipette. The mice were challenged for 3 successive days on 3 consecutive weeks. 24 h after the final intranasal application, mice were challenged with 5 mL of 2% OVA using an aerosol exposure technique for 30 min on 5 consecutive days. The mice with the same age were used as the control group.

As shown in Fig. 3A, in the TLR4 antagonist TAK-242 treatment group, mice models of CARAS were established and TLR4 antagonist TAK-242 was treated 1 h after the nasal challenge. CARAS mice were administrated with 5 μg TAK-242 (dissolved in 10 μL 1% dimethyl sulfoxide, DMSO) intranasally or 50 μg TAK-242 (dissolved in 100 μL 1% DMSO) intraperitoneally. 24 h post aerosol challenge, mice were sacrificed and analyzed.

2.3. Western blotting

The animals were harvested and nasal mucosa and lung tissues were collected. The protein extraction was according to the previously reported methods [11,12]. Briefly, tissues were homogenized in Radioimmunoprecipitation assay (RIPA) buffer supplied with protease inhibitors. Next, the lysate was centrifuged at 13000*g* to remove insoluble material. The supernatant was collected and bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, Waltham, MA, United States) was used to qualify protein concentrations.

Equal amounts of protein from cell lysates were separated by 10% sodium dodecyl sulfate (SDS) gel. Next, the SDS gel was then transferred to a polyvinylidene fluoride membrane and 5% non-fat milk was used to block the membrane. After the membrane was probed with antibodies against HMGB1, TLR4, Myd88, p65, CCL2, and β-actin overnight at 4 °C, the membrane was then incubated with appropriated secondary antibodies. ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA) was used to examine chemiluminescence. The expressions of proteins of interest were normalized to the internal control β-actin.

2.4. Flow cytometry analysis

After the mice were sacrificed, the lung tissue was collected. Cell suspensions of lung tissues were prepared according to the previously reported methods [13,14]. Nasal lavage fluid (NFL), and bronchoalveolar lavage fluid (BALF) were collected. The fluids were centrifuged and the cells were resuspended. Cell suspensions were stained with the indicated antibodies and analyzed on flow cytometry (BD Biosciences, CA, United States) using FlowJo (Ashland, OR, United States).

2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Cytokines including interleukin (IL)-4, IL-5, and IL-13 in the NLF were measured using an ELISA kit (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions.

2.6. Histopathological examination

After the mice were sacrificed, the lung tissue was collected. The tissues were fixed and embedded into paraffin. A slicer was used to cut lung sections in 4-μm slices. Next, the tissue sections were stained with hematoxylin and eosin (H&E) and observed under an optical microscope. Histopathologic scoring was performed to assess the lung injury.

2.7. Statistical analysis

SPSS (SPSS, Chicago, IL, USA) was used to statistical analysis. All Data were expressed as mean value ± SD. To evaluate the significance, one-way analysis of variance with multiple comparisons and Student-Newman-Keuls (SNK) test were performed. A P-value < 0.05 was considered as a statistical significance.

3. Results

3.1. Establishment of an OVA-induced mice model of CARAS

An OVA-induced mice model of CARAS was established as shown in Fig. 1A. We observed that the numbers of sneezing and nasal rubbing at every 10 mins in the OVA-induced group were significantly increased when compared with those in the control group (Fig. 1B and C, $P < 0.001$). Additionally, we did not observe eosinophils in the presence of lung tissues in the control group. However, lung histology of OVA-induced group showed the abundance of eosinophils (Fig. 1D). Flow cytometry analysis demonstrated $CD45^+SiglecF^+CD11b^+CD11c^-$ cells, identified as eosinophils, were dramatically increased in the BALF of OVA-induced mice in comparison to the control group (Fig. 1E, $P < 0.001$). These results supported that an OVA-induced mice model of CARAS was successfully established.

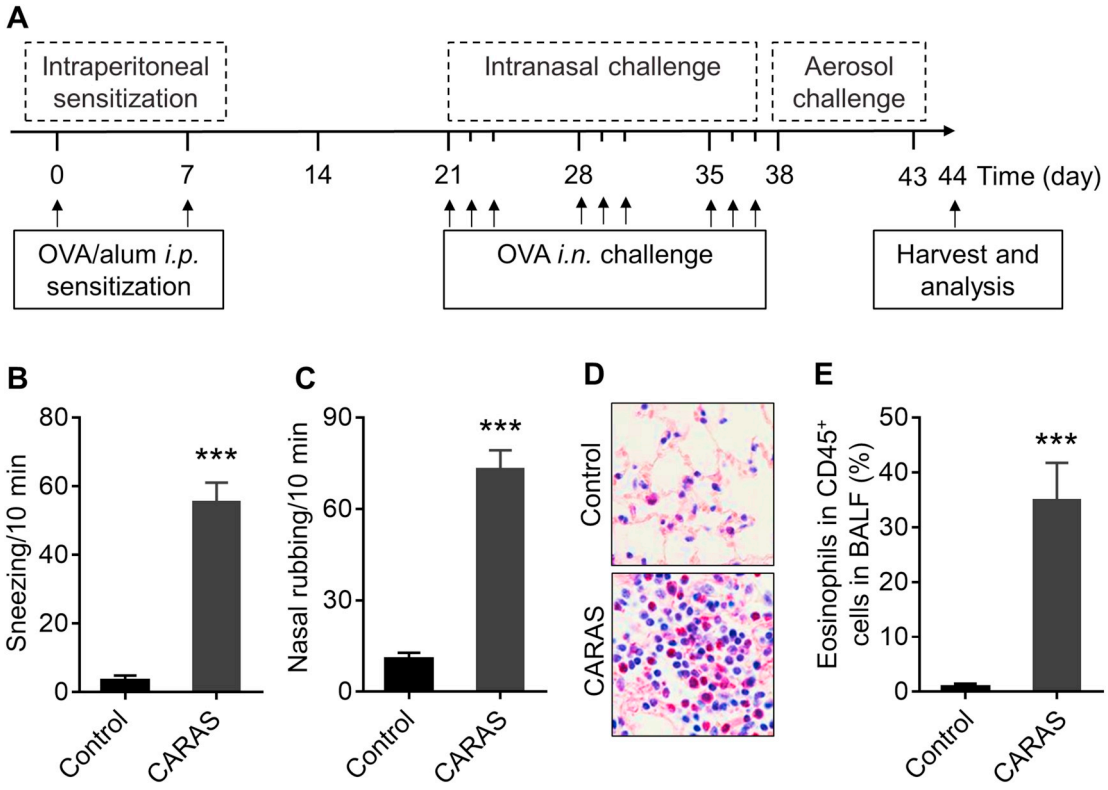

Fig. 1. Establishment of ovalbumin (OVA)-induced mice model of combined allergic rhinitis and asthma syndrome (CARAS). (A) To induce mice model of CARAS, the BALB/c mice were sensitized on days 0 and 7 *via* intraperitoneal injection of OVA emulsified in $AL(OH)_3$ (OVA/alum). Two weeks later, the mice were challenged by instilling a 10 μL droplet of OVA (1 mg/mL) into each nostril using a micropipette. The mice were challenged for 3 successive days on 3 consecutive weeks. 24 h after the final intranasal application, mice were challenged with 5 mL of 2% OVA using an aerosol exposure technique for 30 min on 5 consecutive days. The number of sneezing (B) and nasal rubbing (C) were counted on day 44. (D) Representative photomicrographs of H&*E*-stained lung sections, showing the abundance of eosinophils. (E) Flow cytometry analysis of eosinophils in $CD45^+$ cells in bronchoalveolar lavage fluid (BALF) of control mice and OVA-induced CARAS mice. Eosinophils were identified as $CD45^+SiglecF^+CD11b^+CD11c^-$ cells. Data represent means ± SD (n = 8). ***$p < 0.001$.

3.2. HMGB1/TLR4 signaling pathway was activated and CCL2 was upregulated in nasal mucosa and lungs in mice model of CARAS

We then examined the activation of the HMGB1/TLR4 signaling pathway in the nasal mucosa and lungs. The expressions of HMGB1, TLR4, Myd88, and p56 were determined using Western blotting. We found that the expressions of HMGB1, TLR4, Myd88, and p56 in the nasal mucosa were significantly increased in mice model of CARAS when compared with that in the control group (Fig. 2A). Similarly, the expressions of HMGB1, TLR4, Myd88, and p56 in the lungs were also significantly increased in the lungs (Fig. 2B). These results demonstrated that HMGB1/TLR4 signaling pathway was activated in the mice model of CARAS.

To investigate whether the recruitment of monocytes was affected due to CARAS, we examined the expressions of a chemokine (C—C motif) ligand 2 (CCL2) in nasal mucosa and lungs. CCL2 is also called monocyte chemoattractant protein 1. One of the most important functions of CCL2 is to recruit monocytes. In this study, we found that the expressions of CCL2 were significantly increased in the mice model of CARAS (Fig. 2C and D).

Taken together, these results suggested that HMGB1/TLR4 signaling pathway was activated and CCL2 was upregulated in nasal mucosa and lungs in mice model of CARAS.

3.3. Treatment of TAK-242 downregulated CCL2 expression and reduced monocyte infiltration in nasal mucosa and lung tissues in mice model of CARAS

We observed that HMGB1/TLR4 signaling pathway was activated in nasal mucosa and lungs in mice model of CARAS. We then investigated the effects of TAK-242, a TLR4 antagonist, on the recruitment of monocytes and basophils.

As shown in Fig. 3A, TLR4 antagonist TAK-242 was treated 1 h after the nasal challenge. First, the expressions of CCL2 in nasal mucosa and lungs were examined. The results demonstrated that treatment of TAK-242 significantly downregulated CCL2 expressions in nasal mucosa and lungs in mice model of CARAS (Fig. 3B and C). Next, we analyzed the populations of monocytes in NFL and BALF. Interestingly, we found the populations of $Ly6C^{hi}$ monocyte were significantly decreased in OVA-induced CSRAS mice treated with TAK-242 in comparison to the vehicle-treated group (Fig. 3D and E). These results supported that treatment of TAK-242 significantly downregulated CCL2 expression and reduced monocyte recruitment in nasal mucosa and lung tissues in mice model of CARAS.

3.4. Treatment of TAK-242 attenuated symptoms of the upper airway in the mice model of CARAS

The symptoms of the upper airway in the mice model of CARAS treated with TAK-242 or vehicle were observed. The numbers of sneezing and nasal rubbing events were counted 24 h after aerosol challenge. We observed that the numbers of sneezing and nasal rubbing events were significantly increased in the OVA-induced CARAS mice. Interestingly, treatment of TAK-242 dramatically decreased the numbers of sneezing and nasal rubbing events when compared with that in the vehicle-treated CARAS mice (Fig. 4A and B, $P < 0.01$).

We observed the TAK-242 treatment attenuated the inflammatory cells infiltration including eosinophils and monocytes. Accumulation of

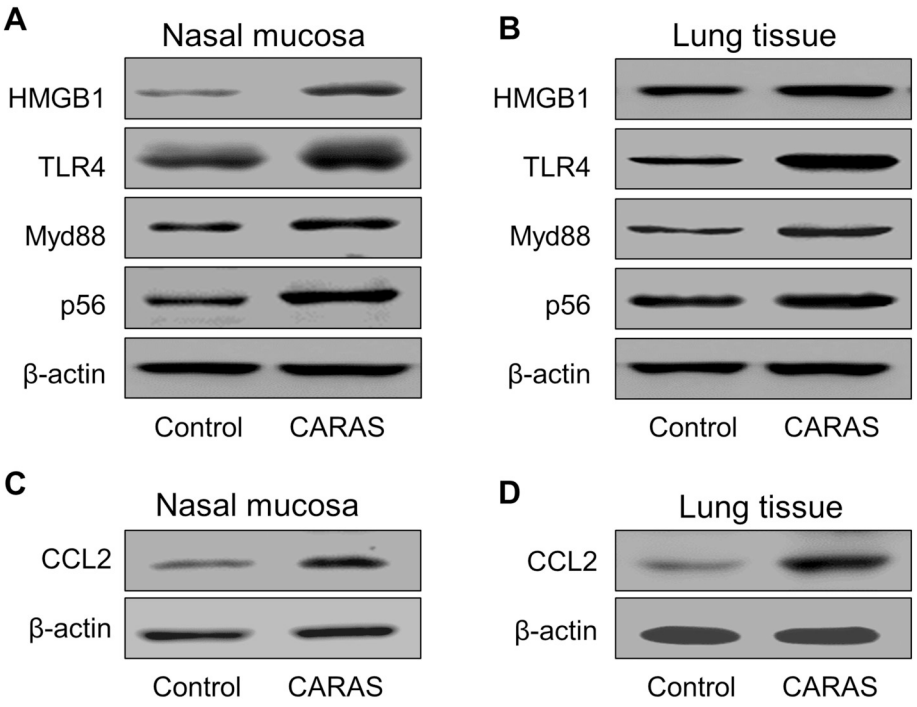

Fig. 2. HMGB1/TLR4 signaling pathway was activated and CCL2 was upregulated in nasal mucosa and lungs in mice model of CARAS. Protein expressions of the HMGB1/TLR4 signaling pathway (including HMGB1, TLR4, Myd88, and p56) in the nasal mucosa (A) and lung tissues (B) isolated from control mice and OVA-induced CSRAS mice. Expression of CCL2 in the nasal mucosa (C) and lung tissues (D) isolated from control mice and OVA-induced CSRAS mice. β-actin was used as a control protein.

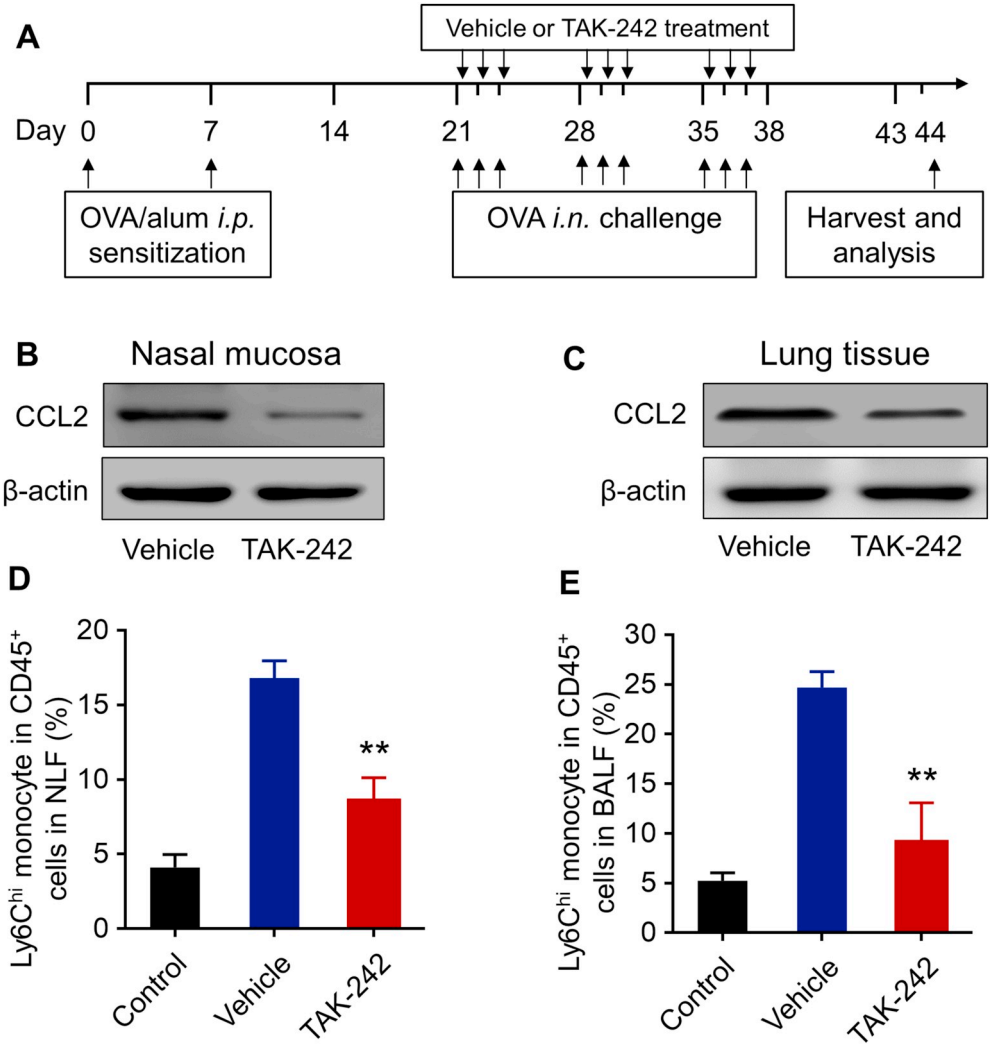

Fig. 3. Treatment of TLR4 antagonist TAK-242 downregulates CCL2 expression and reduces monocyte infiltration in nasal mucosa and lung tissues in mice model of CARAS. (A) Mice models of CARAS were established and TLR4 antagonist TAK-242 was treated 1 h after the nasal challenge. CARAS mice were administrated with 5 μg TAK-242 (dissolved in 10 μL 1% DMSO) intranasally or 50 μg TAK-242 (dissolved in 100 μL 1% DMSO) intraperitoneally. 24 h post aerosol challenge, mice were sacrificed and analyzed. The expressions of CCL2 in the nasal mucosa (B) and lung tissues (C) isolated from control mice and OVA-induced CSRAS mice treated with vehicle or TAK-242. (D) and (E) $Ly6C^{hi}$ monocyte in $CD45^+$ cells in nasal lavage fluid (NFL) and bronchoalveolar lavage fluid (BALF) of OVA-induced CSRAS mice treated with vehicle or TAK-242. Data represent means ± SD (n = 8). $^{**}p < 0.005$, *vs* vehicle-treated CARAS mice.

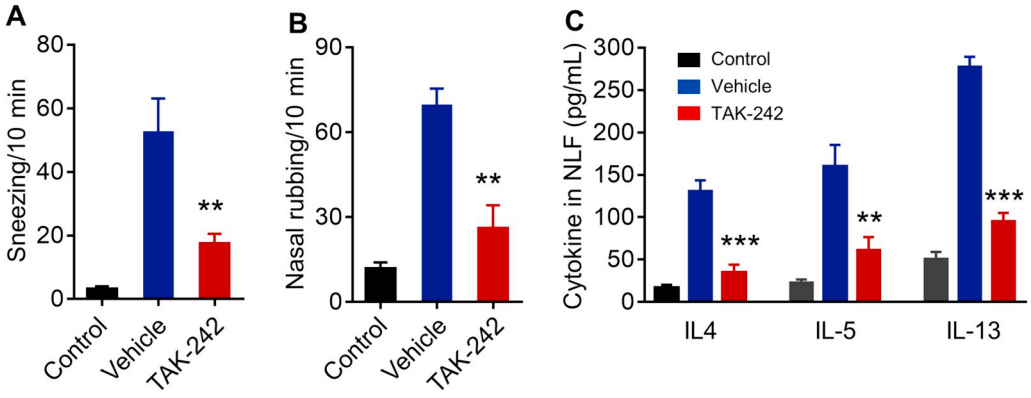

Fig. 4. TLR4 antagonist TAK-242 attenuates symptoms of the upper airway in the mice model of CARAS. OVA-induced CARAS mice were treated TAK-242, the number of sneezing (A) and nasal rubbing events (B) were counted 24 h post aerosol challenge. (C) Concentrations of cytokines (including IL4, IL5, and IL13) in NFL were examined by ELISA. Data represent means ± SD (n = 8). $^{**}p < 0.005$, $^{***}p < 0.001$ *vs* vehicle-treated CARAS mice.

inflammatory cells resulted in the release of inflammatory mediators and cytokines. Next, we analyzed the levels of inflammatory cytokines in NFL. We found that the levels of cytokines including IL-4, IL-5, and IL-13 were significantly increased in the OVA-induced CARAS mice. Treatment of TAK-242 dramatically decreased the expressions of IL-4, IL-5, and IL-13, respectively, when compared with those in the vehicle-treated group (Fig. 4C, $P < 0.001$, $P < 0.01$, $P < 0.001$).

3.5. Treatment of TAK-242 attenuated symptoms of the lower airway in the mice model of CARAS

We further evaluated the effects of TAK-242 on the symptoms of the upper airway in the mice model of CARAS. First, the populations of eosinophils in BALF were evaluated. Flow cytometry analysis demonstrated that the populations of eosinophils were significantly decreased in BALF of CARAS mice treated with TAK-242 when compared with that of CARAS mice treated with vehicle (Fig. 5A and B, $P < 0.01$).

Treatment of TAK-242 dramatically decreased the expressions of IL-4, IL-5, and IL-13. It is known that IL-4, IL-5, and IL-13 are markers for the Th2 cells. Therefore, we further analyzed the frequency of $IL13^+$ Th2 cells in total $CD3^+CD4^+$ T cells from the lungs. Interestingly, we found that treatment of TAK-242 significantly decreased the frequency of $IL13^+$ Th2 cells when compared with those in the vehicle-treated group (Fig. 5C and D, $P < 0.01$).

Histopathological examination was performed and histological scoring was used to assess the lung injury. We observed that lung injury was significantly attenuated in the CARAS mice treated with TAK-242 when compared with that in the CARAS mice treated with vehicle (Fig. 5E, $P < 0.01$). Taken together, these results demonstrated that treatment of TAK-242 attenuated symptoms of the lower airway in the mice model of CARAS.

4. Discussion

Both allergic rhinitis and asthma are mediated by similar inflammatory mechanisms [1,15]. CARAS has been introduced to describe the patients with two manifestations of the same disease [1,16]. HMGB1-TLR4 axis has been identified to play an important role in the inflammatory response. In 2018, Yuan and colleagues, for the first time, identified the roles of HMGB1-TLR4 axis in the development of allergic rhinitis and inhibition of HMGB1-TLR4 axis is a promising therapeutic strategy to attenuate allergic rhinitis [9]. However, the roles of HMGB1-TLR4 axis in the development of CARAS are still poorly understood. Therefore, in the present study, we constructed an OVA-induced mice model of CARAS and determined the activations of HMGB1-TLR4 signal pathway in both the nasal mucosa and lungs. Interestingly, we found that the expressions of HMGB1, TLR4, Myd88, and p65 were significantly increased in the OVA-induced mice model of CARAS. The MyD88-dependent pathway is one of the essential pathways after TLR4 activation [17]. It is important for p65 nuclear translocation and the following transcriptions of cytokines (including IL-1, IL-6, and tumor necrosis factor (TNF)-α), chemokines, and inflammatory mediators, which trigger an inflammatory cascade [18,19].

Apart from the activations of HMGB1-TLR4 signal pathway, we also found that the expressions of CCL2 the nasal mucosa and lungs were significantly increased. CCL2, as the first discovered human chemokine, has been identified to recruit monocytes and natural killer cells to the inflammatory sites [20,21]. Interestingly, we found that the numbers of $Ly6C^{hi}$ monocyte were also significantly increased in NFL and BALF. These data suggested that the activations of HMGB1-TLR4 signal pathway also accompanied with an increase of CCL2 expression and recruitment of monocytes in the development of CARAS.

To examine the therapeutic potential of HMGB1-TLR4 signal pathway, a TLR4 antagonist, TAK-242, was used in this study to treat OVA-induced CARAS mice. Surprisingly, we found that treatment of TAK-242 significantly ameliorated symptoms of the upper airway including the sneezing and nasal rubbing in CARAS mice. To our knowledge, Th2 cells are important for the host defense mechanism and closely associated with allergic rhinitis and asthma [22]. For instance, Th2 cells secret IL-4, IL-5, and IL-13, which mediate eosinophil differentiation, expansion, and recruitment in response to allergic rhinitis [22,23]. We, therefore, determined cytokines including IL-4, IL-5, and IL-13 in NLF, to explore the potential effects of TAK-242 on Th2 cells. Interestingly, we found that levels of IL-4, IL-5, and IL-13 were significantly decreased in CARAS mice treated with TAK-242. These results suggested that TAK-242 treatment ameliorated symptoms of the upper airway in part by the regulation of Th2 cells.

Th2 cells mediate the initiation of the allergic response, then eosinophils are attracted to the inflammation site from the circulation [23,24]. Eosinophils have been identified to play a critical role in the symptoms of asthma and allergies including participating in allergic airway remodeling and regulation of airway inflammatory response [25,26]. First, we determined the effects of TAK-242 on the symptoms of the lower airway by analyzing numbers of eosinophils in BALF. The results showed a significant decrease of eosinophils in BALF of the CARAS mice treated with TAK-242. In addition, we found a dramatical decrease of Th2 cell related cytokines in NLF of CARAS mice treated with TAK-242. Therefore, we further analyzed the frequency of $IL13^+$ Th2 cells in total $CD3^+CD4^+$ T cells from the lungs. The results showed treatment of TAK-242 significantly decreased the frequency of $IL13^+$ Th2 cells. Third, to evaluate the effects of TAK-242 on the histopathology of the lung, histological scores were used to assess lung injury. We found that treatment of TAK-242 significantly inhibited lymphocyte infiltration in the lung. Taken together, these results suggested that TAK-242 treatment ameliorated symptoms of the lower airway in part by the regulation of frequency of eosinophils and Th2 cells.

In the present study, for the first time, we demonstrated that HMGB1/TLR4 signaling pathway was activated and CCL2 was upregulated in nasal mucosa and lungs in the mice model of CARAS. We identified that TAK-242, an antagonist of TLR4, downregulated CCL2

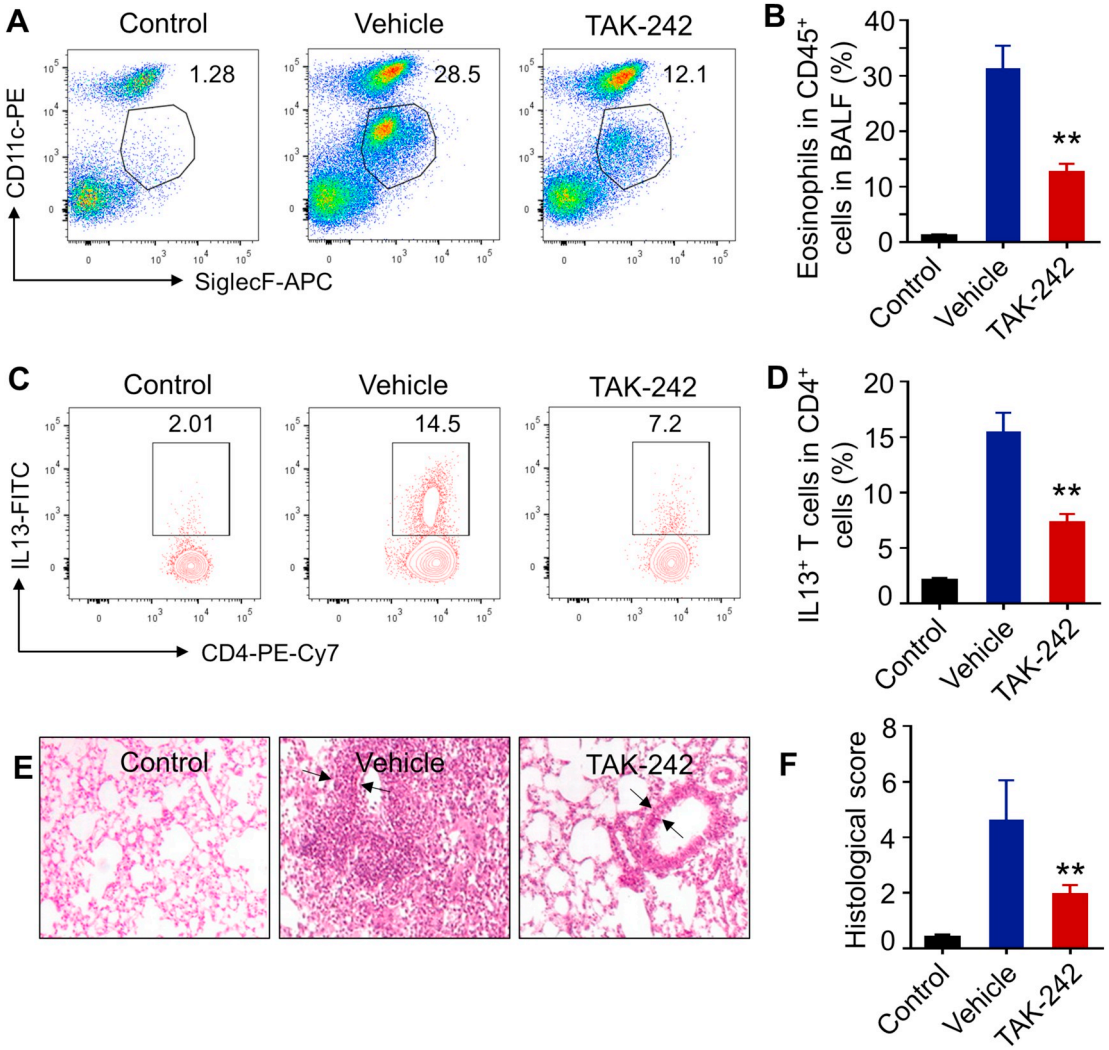

Fig. 5. TLR4 antagonist TAK-242 attenuates symptoms of the lower airway in the mice model of CARAS. (A–B) Flow cytometry analysis of the abundance of eosinophils in bronchoalveolar lavage fluid (BALF) from control mice and CARAS mice treated with TAK-242 or vehicle. Eosinophils were identified as $CD45^+SiglecF^+CD11b^+CD11c^-$ cells. (C–D) Flow cytometry analysis of the frequency of $IL13^+$ Th2 cells in total $CD3^+CD4^+$ T cells from lungs isolated from control mice and CARAS mice treated with TAK-242 or vehicle. (E) Histologic sections of lungs from each group. Pictures showed representative samples of each group. (F) Histological scores for assessment of lung injury in each group. Data represent means ± SD (n = 8). $^{**}p < 0.005$ *vs* vehicle-treated CARAS mice.

expression and reduced monocyte infiltration in nasal mucosa and lung tissues in mice model of CARAS. Treatment of TAK-242 ameliorated symptoms of the upper airway including reducing the numbers of sneezing and nasal rubbing events and decreasing the levels of Th2 cell cytokines in NFL. Moreover, treatment of TAK-242 ameliorated symptoms of the lower airway including decreasing the populations of eosinophils in BALF and Th2 cells in the lungs. These results suggested HMGB1/TLR4 axis might serve as a potential target for CARAS therapy.

5. Conclusion

In summary, the present study identified that HMGB1-TLR4 signaling pathway was activated in the development of CARAS. In addition, the activation of HMGB1-TLR4 signaling pathway was associated with an increase of CCL2 expression and recruitment of monocytes in the development of CARAS. Furthermore, we found TAK-242, an antagonist of TLR4, downregulated CCL2 expression and reduced monocyte infiltration in nasal mucosa and lung tissues in mice model of CARAS. Notably, TAK-242 treatment ameliorated symptoms of the upper- and lower airway in mice model of CARAS.

List of abbreviations

TLR4	toll-like receptor 4
OVA	ovalbumin
CARAS	combined allergic rhinitis and asthma syndrome
BALF	bronchoalveolar lavage fluid
NLF	nasal lavage fluid
IL	interleukin
ELISA	Enzyme-linked Immunosorbent Assay
PBS	phosphate-buffered saline
DMSO	dimethyl sulfoxide
SDS	sodium dodecyl sulfate

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None.

Declaration of Competing Interest

The authors declare that there is no conflict of interests.

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