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Melatonin biosynthesis restored by CpG oligodeoxynucleotides attenuates allergic airway inflammation via regulating NLRP3 inflammasome

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ABSTRACT

Aims: Both CpG oligodeoxynucleotide (CpG-ODN) and melatonin have been reported to induce Th1 response and contribute to allergic asthma resistance. Here, we aimed to reveal how they confer such effect as well as whether they crosstalk with each other.

Main methods: Six-week-old Female C57BL/6 mice were challenged by OVA to induce allergic airway inflammation, and were treated with CpG-ODN, CpG-ODN plus Luzindole or melatonin respectively. Bronchoalveolar lavage fluid (BALF) cellularity was classified and counted by Wright's-Giemsa staining. HE and PAS staining were used to analyze airway inflammation. The levels of IL-4, IL-5, IL-13,GM-CSF and IFN-γ, as well as IL-1β and IL-18 were analyzed by ELISA. Protein expressions of ASMT, AANAT, NLRP3, IL-1β and caspase-1 in lung tissue were detected by Western blotting, expression of ASMT and AANAT were further observed by immunohistochemistry.

Key findings: We found that CpG-ODN considerably suppressed OVA-induced airway leukocytes infiltration, goblet cell hyperplasia and Th2 cytokines production. Furthermore, the resolution effect of CpG-ODN on OVA-induced allergic airway inflammation occurred in parallel with decreased-activation of NLRP3 inflammasome and increased biosynthesis of melatonin. Blocking the effect of endogenous melatonin by Luzindole abolished the suppressive effect of CpG-ODN on OVA-induced airway inflammation and activation of NLRP3 inflammasome, suggesting such effect was mediated by endogenous melatonin. Moreover, exogenous melatonin pronouncedly ameliorated airway inflammation and decreased the activation of NLRP3 inflammasome.

Significance: These results proven that CpG-ODN protects against allergic airway inflammation via suppressing the activation of NLRP3 inflammasome, and such effect may be resulted from the restored-production of melatonin.

1. Introduction

During exacerbation, persistent or non-resolving airway inflammation precedes bronchospasm, therefore asthma is now increasingly acknowledged as an inflammatory disease [1]. Cytokines especially Th2 cytokines releasing from T cells have been increasingly reported to play a critical role in the inflammation of asthma, and the homeostasis between Th1 and Th2 cells activity is thought to be important in controlling disease symptoms [2].

Bacterial DNA, which contains unmethylated CpG dinucleotides, has been reported to induce the secretion of Th1 cytokines. This process is generally considered to suppress Th2 responses [2–5]. Therefore, bacterial infection during childhood has been considered to protect against asthma, and this may be due to the induced Th1 response by bacterial DNA [6]. Synthetic CpG oligodeoxynucleotide (CpG-ODN) which can activate immune responses mimicking the effect of bacterial DNA [7] has been already proven to suppress airway inflammation and hyper-responsiveness in the established asthma model [8–11], and even

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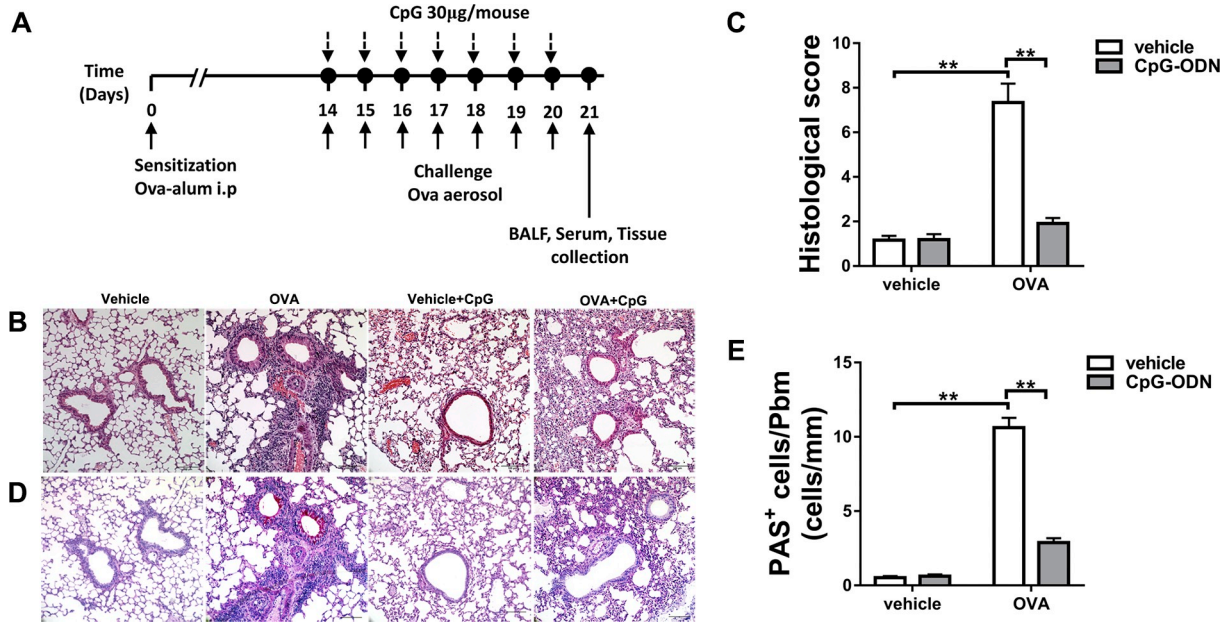

Fig. 1. CpG-ODN treatment prevents peribronchial inflammation and goblet cell hyperplasia
(A) Experimental protocol of establishing murine allergic airway diseases model and the treatment of CpG-ODN. (B) Lung sections from vehicle and OVA-challenged mice treated with or without CpG-ODN were stained with H&E to analyze cell infiltration. (C) The extent of peribronchial inflammation was scored. (D) Lung sections from vehicle and OVA-challenged mice treated with or without CpG-ODN were stained with periodic acid-Schiff (PAS) to evaluate mucus production and goblet cell hyperplasia. (E) PAS^+ cells in the airway were counted and quantified. Data were expressed as mean ± SEM of 6 mice per group, $^{**}p < .01$.

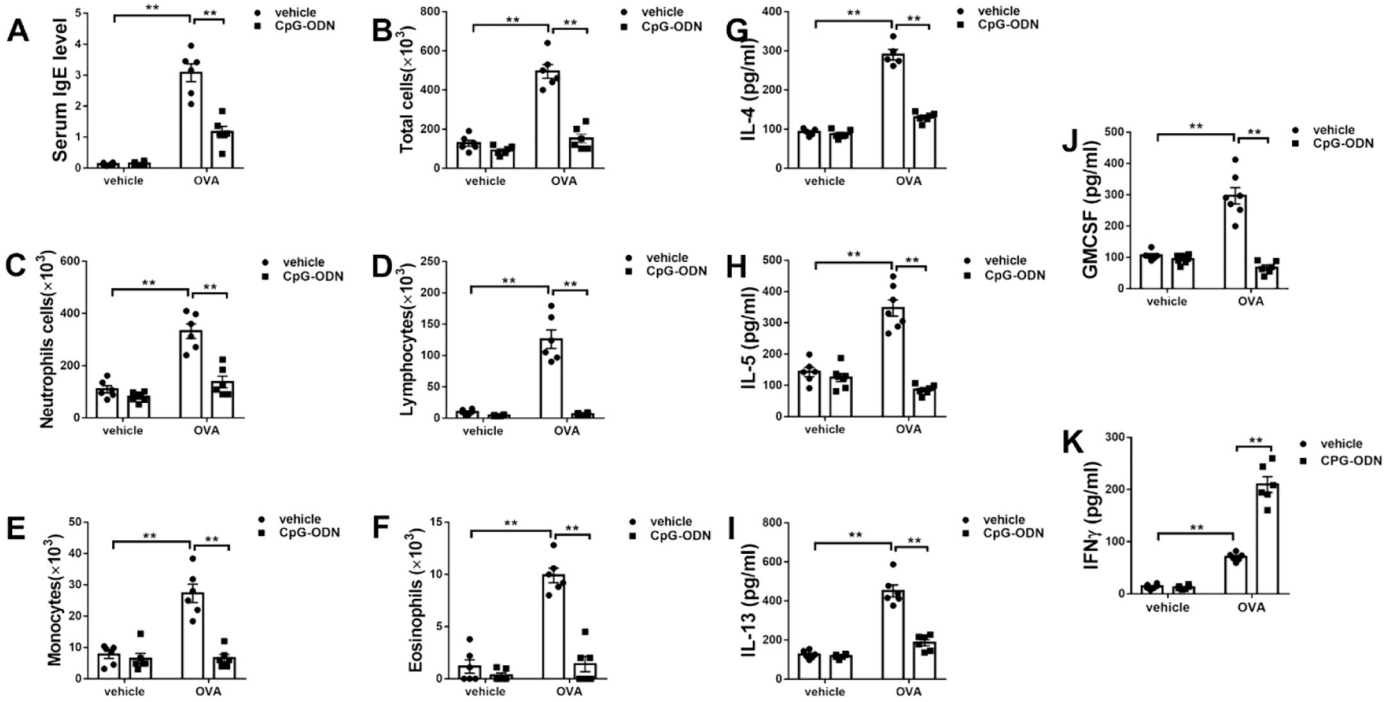

Fig. 2. CpG-ODN inhibits serum IgE, BALF inflammatory cells infiltration and regulates Th1/Th2 cytokines production
(A) The level of OVA-specific IgE in serum. (B–F) BALF cellularity from vehicle and OVA-challenged mice treated with or without CpG-ODN. (G-J) Productions of Th2 associated cytokines such as IL-4, IL-13, IL-5 and GM-CSF in BALF. (K) The level of Th1 cytokine IFN-γ. Data were expressed as mean ± SEM of 6 mice per group, $^{**}p < .01$.

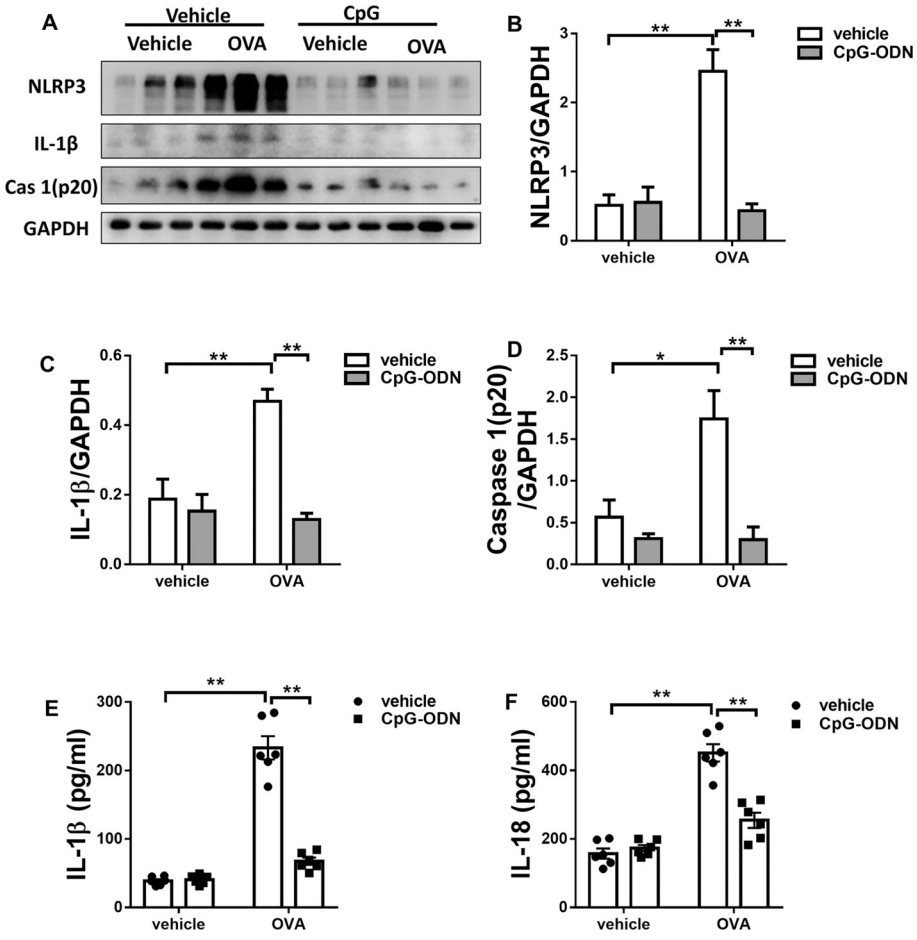

Fig. 3. CpG-ODN suppresses OVA-induced NLRP3 inflammasome activation

(A) The expression of NLRP3, mature IL-1β and caspase 1(p20) in the lung tissues was analyzed by western blot. (B-D) The relative levels of NLRP3, mature IL-1β and caspase 1(p20) in the lung tissues. (E, F) Concentration of IL-1β and IL-18 in BALF was analyzed by ELISA. Data were expressed as mean ± SEM of 6 mice per group. * $p < .05$, ** $p < .01$.

in human clinical trials [12–14]. Consequently, CpG-ODN has been studied as a potent preventive as well as a therapeutic immune modulator in allergic inflammatory diseases. However, how CpG-ODN exerts these immunomodulatory effects remains elusive.

Accumulating evidence revealed that hyper-activation of NLR pyrindomain containing 3 (NLRP3) inflammasome contributes to allergic airway inflammation [15–17]. The protein levels of NLRP3, caspase-1 and IL-1β protein in sputum or alveolar lavage fluid (BALF) were elevated in asthmatic patients [15,18,19], and is closely related to the degree of neutrophilic inflammation, steroid resistance in asthmatic patients [20]. In OVA-induced murine asthma model, NLRP3 inflammasome is activated [21]. In NLRP3-deficient mice, OVA-induced airway inflammation is suppressed, Th2-type response is reduced [16]. Additionally, inhibitors of caspase-1, anti-IL-1β antibody and NLRP3 inhibitor MCC950 can inhibit allergic airway inflammation and steroid resistance in murine asthma model, while IL-1β can induce steroid resistance again [20]. More interestingly, NLRP3 itself is an important transcriptional regulator of Th2 cell differentiation [22]. Therefore, we hypothesize that the resolution effect of CpG-ODN on allergic airway inflammation may be related to its regulation of the activation of NLRP3 inflammasome.

Moreover, it has been reported that the TLR/NF-κB pathway can induce the synthesis of pro-IL-1β and pro-IL-18 and the assembly of NLRP3 inflammasome, leading to the activation of caspase-1 which subsequently hydrolyzes pro-IL-1β and pro-IL-18 to mature and active IL-1β and IL-18 [23,24]. Interestingly, experiments show that melatonin attenuates the transcriptional activity of NF-κB and inhibit the activation of NF-κB-dependent activation of NLRP3 inflammasome [25,26], suggesting NLRP3 inflammasome is a new target of melatonin.

As we known, melatonin not only regulates biological rhythm, but also has immunomodulatory, anti-inflammatory and anti-oxidant functions [27]. Melatonin has been shown to activate Th1 response [28] and suppress Th2 immune response [29], thus conferring it be a powerful potential therapeutic drug for asthma. To date, many studies have reported that administration of exogenous melatonin significantly inhibits allergic airway inflammation [30,31]. Also, endogenous melatonin, which is mainly converted from serotonin (5-HT) by aralkylamine *N*-acetyltransferase (AANAT) and Acetylserotonin O-Methyltransferase (ASMT), has been reported to be correlated with the severity of asthma in asthmatic patients [32] and in animal model [33].

In the present study, we aimed to investigate whether CpG-ODN ameliorates allergic airway inflammation via regulating the activation

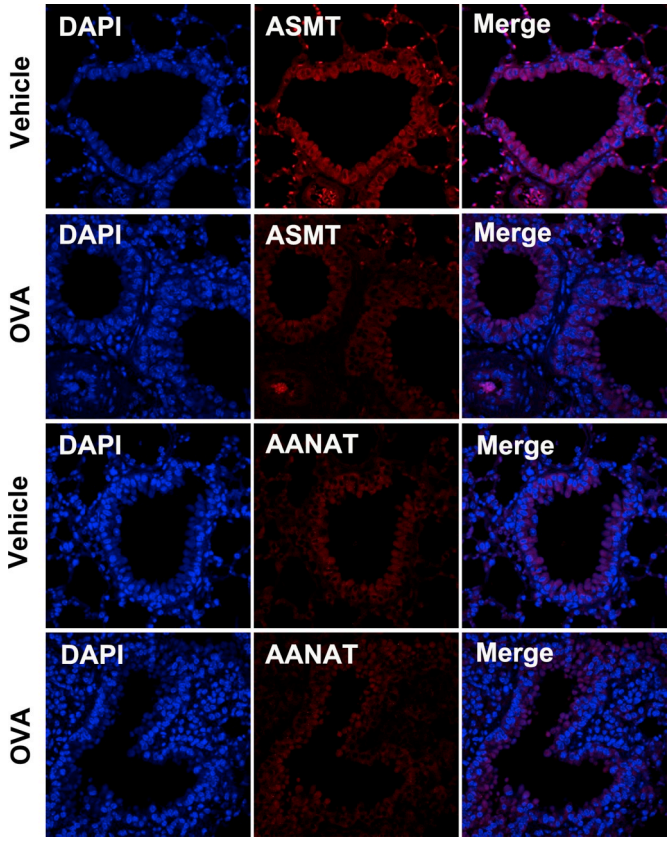

Fig. 4. The expression of ASMT and AANAT in lung tissues
By using immunofluorescence, the expression of ASMT and AANAT in the lung tissues was detected by confocal microscopy (magnification ×40). Nuclei were stained by DAPI.

of NLRP3 inflammasome, and further reveal whether such effect of CpG-ODN is due to the restored melatonin level in allergic airway diseases.

2. Materials and methods

2.1. Animals

Six-week old, female C57BL/6 mice, were obtained from Shanghai SLAC Laboratory Animal Center. Mice were housed under specific pathogen-free conditions on a 12 h light-dark cycle. All protocols were reviewed and approved by the Animal Care and Use Committee of Anhui Medical University.

2.2. Oligodeoxynucleotides

The CpG ODN 1668 (5'-TCC ATG ACG TTC CTG ATG CT-3′) was synthesized and supplied by Sangon Biotech (Shanghai, China), CpG ODN (30 μg/mice) was administrated by i.p. injection.

2.3. Murine model of allergic airway inflammation and treatments

Mice in the allergic airway inflammation model received intraperitoneally injection of OVA (10 μg, Sigma, St. Louis, MO, USA) precipitated with alum (1 mg) on day 0 for sensitization, followed by challenge with nebulized 1% OVA for consecutive seven days. Control mice received saline-sensitization and inhalation of nebulized saline solution. Some mice received 30 μg of CpG oligonucleoides per mice or 10 mg/kg melatonin (Sigma) by intraperitoneal injection one hour before OVA challenge. Luzindole (30 mg/kg, Sigma) was given intraperitoneally for consecutive seven days during OVA challenge. Mice were sacrificed 24 h after the last challenge.

2.4. Bronchoalveolar lavage fluid (BALF) collected from left lung

After the mice being killed, the trachea was cannulated and the right principal bronchus was ligatured. The left lung was lavaged with 0.3 ml of ice-cold phosphate-buffered saline (PBS) for five times. The lavages were processed for total and differential cell counts by using a haemocytometer or Wright-Gimsa stain as previously [34,35], respectively. The supernatants were saved for Enzyme-linked immunosorbent assay (ELISA).

2.5. Histopathology

After BALF collection, part of the right lung was fixed in 4% paraformaldehyde. Lung tissues were then embedded in paraffin, and 5-μm-thick sections were cut and stained with hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) to determine the cellular infiltration and global cell hyperplasia, respectively. The score of cellular infiltration or global cell hyperplasia were assessed as previously reported [36,37].

2.6. Immunofluorescence and immunohistochemistry and confocal microscopy

Paraffin lung tissues were cut into 5-μm-thick sections. Rinsing the sections in PBS (phosphate saline buffer, 0.01 M, pH = 7.4) three times for 5 min each. For antigen retrieval, incubating sections in 0.05 M (Citrate buffer Ph = 6.0) for 20 min at 100 °C. After rinsing, blocking sections with 5% donkey serum for 30 min at 37 °C. Next, incubating sections with primary antibodies diluted in PBS for 1 h at 37 °C, then overnight at 4 °C, the primary antibodies were anti-AANAT (1:200, Abcam, Boston, MA, USA), anti-ASMT (1:100, Abcam) and anti-CD45 (1:50, Santa cruz, CA, USA). Next day, for immunofluorescence, sections were incubated with the secondary antibody donkey anti-rabbit (Alexa Fluor® 594, Abcam) for 1 h at 37 °C, then washed with PBS 3 times. After incubation the sections with DAPI (Sigma) for 5 min at room temperature, mounting the sections and coverslip using 80% glycerin. For immunohistochemistry, sections were incubated with the HRP-conjugated secondary antibody (Beijing Golden Bridge Biotechnology, Beijing, China) for 30 min. Diaminobenzidine (DAB) was used for visualization.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The productions of IL-5, GM-CSF, IFN-γ in the BALF and OVA-specific IgE in the serum were measured by ELISA kits (Cusabio, Wuhan, China). The levels of IL-4, IL-13, IL-1β and IL-18 in BALF, and the level

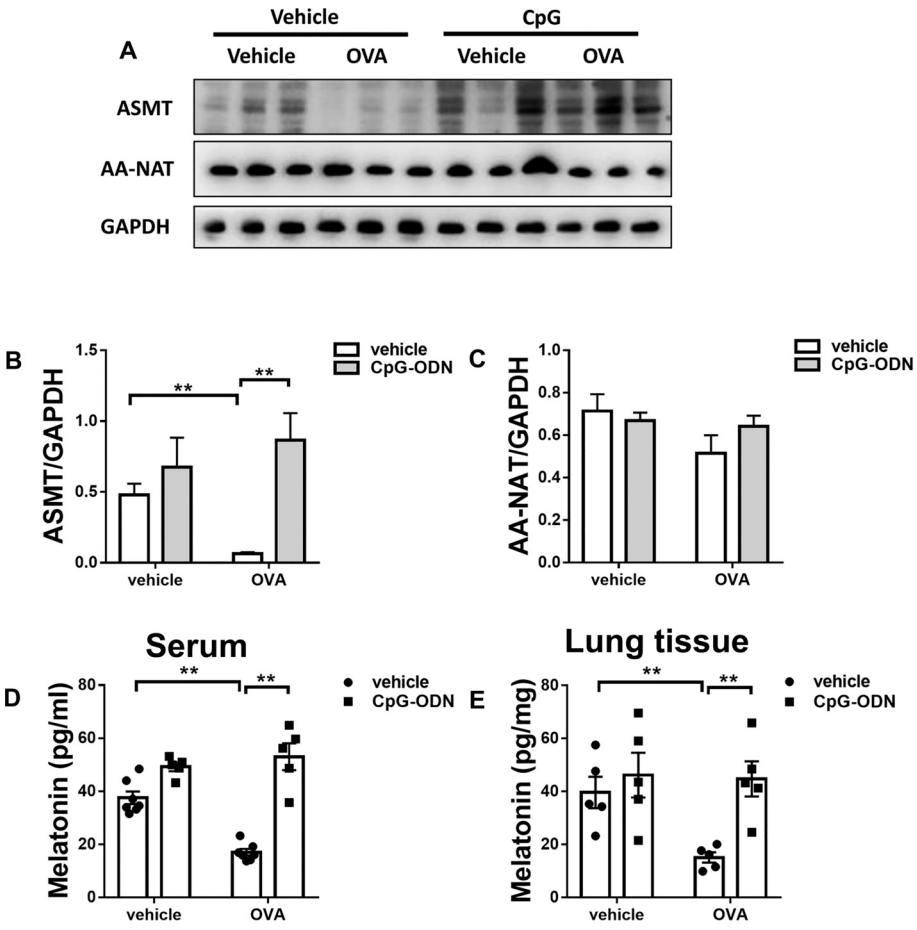

Fig. 5. CpG-ODN restores melatonin biosynthesis in OVA-challenged mice
(A) The expression of ASMT and AA-NAT in lung tissues was analyzed by western blot. (B-D) The relative protein levels of ASMT and AA-NAT. (E, F) Concentration of melatonin in serum and lung homogenates was analyzed by ELISA. Data were expressed as mean ± SEM of 6 mice per group. ** $p < .01$.

of melatonin in serum and lung tissue were assessed by ELISA kits (Cloud-Clone Crop, Wuhan, China) according to the protocols from the manufacturer.

2.8. Western blotting analysis

Lung tissues were homogenized in ice-cold RIPA lysis buffer using a homogenizer as previously described [33]. Proteins were separated by 12% SDS-PAGE and then transferred to PVDF membrane. The blots were tested with antibodies as below: anti-NLRP3 (Adipogen, San Diego, CA, USA), anti-IL-1β (R&D Systems, Minneapolis, MN, USA), anti-Caspase-1 (p20) (Adipogen, San Diego, CA, USA), anti-AANAT (Abcam), anti-ASMT (Abcam) and anti-GAPDH (KANGCHEN Biotech, Shanghai, China) for 1 h at 37 ˚C, followed by at 4 ˚C overnight. Blots were washed thrice with TBST and probed with HRP-conjugated secondary antibodies, which were revealed using the ECL detection kit (Pierce). The densitometry value of the bands was assessed by ImageJ software (NIH, Bethesda, MD, USA).

2.9. Statistics

Results are showed as mean ± SEM. A one-way analysis of variance followed by Bonferroni multiple comparison test was used to determine the differences among the groups (SPSS 19.0).

3. Results

3.1. CpG-ODN treatment prevents peribronchial inflammation and goblet cell hyperplasia

To study the effect of CpG-ODN on OVA-induced allergic mice, 30 μg of CpG-ODN was intraperitoneally administrated before OVA challenge for consecutive seven days (Fig. 1A). Tissue sections demonstrated that marked peribronchial inflammatory cells infiltration in OVA-challenged mice as compared to mice of control group, which was almost completely abrogated in CpG-ODN-treated mice (Fig. 1B, C, Supplemental Fig. 1).Additionally, PAS-stained histological analysis of lung tissue showed that goblet cell hyperplasia and mucus production was enhanced in mice after OVA challenge, while administration of CpG-ODN remarkably reduced airway PAS^+ cells (Fig. 1D, E).

3.2. CpG-ODN inhibits serum IgE, BALF inflammatory cells infiltration and regulates Th1/Th2 cytokines production

To further confirm the effect of CpG-ODN on resolution of allergic inflammation in mice, serum IgE levels, infiltration of inflammatory

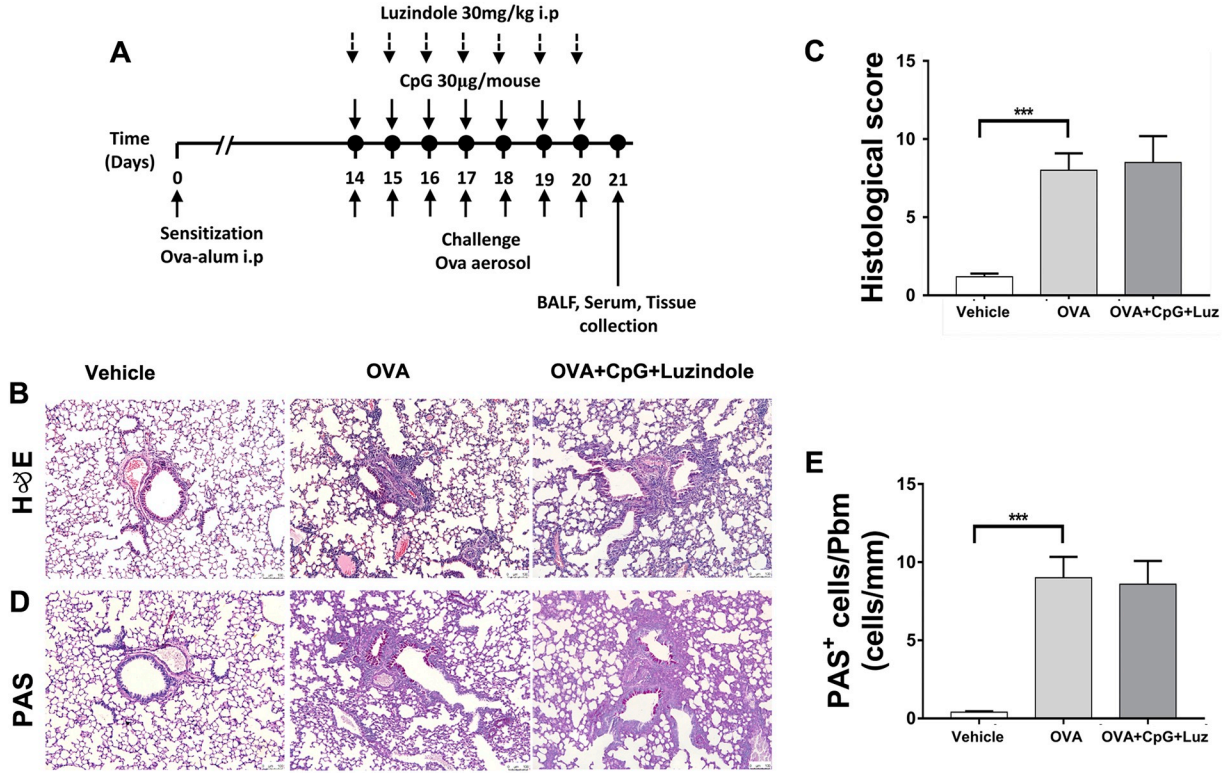

Fig. 6. Inhibiting the effect of endogenous melatonin by luzindole abolished the effect of CpG-ODN on OVA-induced airway inflammation
(A) Experimental protocol of treatment of CpG-ODN and luzindole during establishing murine allergic airway diseases model. (B) Lung sections were stained with H& E to analyze cell infiltration. (C) The extent of peribronchial inflammation was scored. (D) Lung sections were stained with periodic acid-Schiff (PAS) to evaluate mucus production and goblet cell hyperplasia. (E) PAS^+ cells in the airway were counted and quantified. Data were expressed as mean ± SEM of at least 6 mice per group, $^{***}p < .01$.

cells and production of Th2 cytokine in BALF were analyzed. Serum IgE level from the mice in the OVA-challenged group was significantly increased compared to that of saline-challenged control group, while administration of CpG-ODN markedly reduced IgE production (Fig. 2A). Also, repeated OVA challenge with CpG-ODN pretreatment dramatically reduced the number of total cells, neutrophils, lymphocytes, monocytes, and eosinophils in the BALF (Fig. 2B–F). In addition, the levels of Th2 cytokines such as IL-4, IL-13, IL-5 and GM-CSF in the BALF were effectively reduced by CpG-ODN treatment (Fig. 2G–J), while the level of Th1 cytokine IFN-γ was further increased by CpG-ODN (Fig. 2K). Taken together, these data implied that CpG-ODN treatment strongly ameliorated OVA-induced allergic airway inflammation.

3.3. CpG-ODN suppresses OVA-induced NLRP3 inflammasome activation

However, the mechanism of ameliorated allergic inflammation related to CpG-ODN treatment is still elusive. Since NLRP3 inflammasome has been demonstrated to play crucial role in allergic airway inflammation [38,39], we hypothesized CpG-ODN may prevent allergic airway inflammation via suppressing the activation of NLRP3 inflammasome. In the present study, we found that protein levels of NLRP3, IL-1β (p17) and Caspase 1(p20) were low in the vehicle group and increased greatly after OVA challenge (Fig. 3A, B, C, D). Of note, the OVA-induced activation of NLRP3 inflammasome was markedly suppressed by CpG-ODN treatment (Fig. 3A, B, C, D). Moreover, NLRP3 inflammasome-associated cytokine IL-1β and IL-18 were notably inhibited by CpG-ODN. These results suggested the role of CpG-ODN in regulating the activation of NLRP3 inflammasome in allergic airway inflammation (Fig. 3E, F).

3.4. CpG-ODN restores melatonin biosynthesis in OVA-challenged mice

Meanwhile, we found the protein level of ASMT but not AANAT was significantly decreased in OVA-challenged mice as compared to that of vehicle mice by using immunofluorescence (Fig. 4) and western blotting (Fig. 5A, B, C), however, CpG-ODN treatment substantially restored such reduction (Fig. 5A, B, C). Similarly, CpG-ODN significantly restored the level of melatonin in serum and homogenate lung tissue in OVA-challenged mice (Fig. 5D, E).

3.5. Inhibiting the effect of endogenous melatonin by Luzindole abolished the effect of CpG-ODN on OVA-induced airway inflammation

To further confirm melatonin which restored by CpG-ODN could inhibit allergic airway inflammation, Luzindole, an antagonist of melatonin receptor was applied to block the effect of endogenous melatonin as shown in Fig. 6A. The results demonstrated that after Luzindole treatment, the suppressive effect of CpG-ODN on OVA-induced

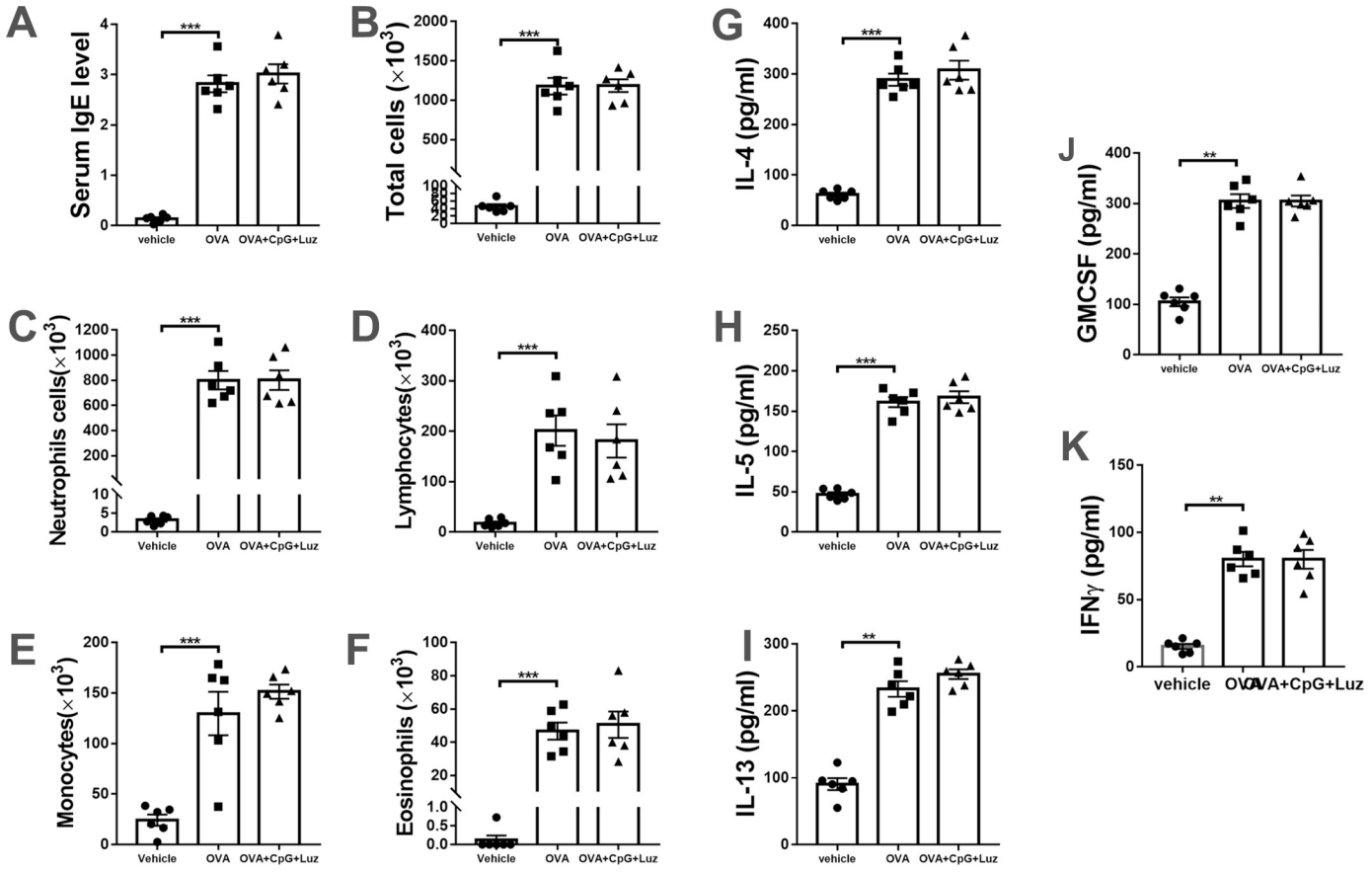

Fig. 7. Luzindole abrogated the effect of CpG-ODN on OVA-induced serum IgE, BALF inflammatory cells infiltration and Th1/Th2 cytokines production
(A) The level of OVA-specific IgE in serum. (B—F) BALF cellularity counts. (G-J) Concentration of Th2 associated cytokines such as IL-4, IL-13, IL-5 and GM-CSF in BALF. (K) The level of Th1 cytokine IFN-γ. Data were expressed as mean ± SEM of at least 6 mice per group, $^{***}p < .01$.

peribronchial inflammatory cells infiltration (Fig. 6B, C, Supplemental Fig. 1), goblet cell hyperplasia and mucus production were not detected (Fig. 6D, E).

3.6. Luzindole abrogated the effect of CpG-ODN on OVA-induced serum IgE, BALF inflammatory cells infiltration and Th1/Th2 cytokines production

Moreover, when simultaneously treated with Luzindole, CpG-ODN had no effect on the level of IgE in serum (Fig. 7A), the number of total cells, neutrophils, lymphocytes, monocytes and eosinophils in BALF (Fig. 7B–F). Similarly, the effect of CpG-ODN on the levels of Th2-cell related cytokines such as IL-4, IL-13, IL-5 and GM-CSF (Fig. 7G–J) as well as Th1-cell related cytokine IFN-γ (Fig. 7K) was not found in Luzindole pretreated mice.

3.7. Luzindole dampened the effect of CpG-ODN on OVA-induced activation of NLRP3 inflammasome

Additionally, the protein expressions of NLRP3, mature IL-1β and caspase 1(p20) (Fig. 8A–D), as well as the productions of IL-1β and IL-18 (Fig. 8E, F) were not significant different in OVA-challenged mice with or without CpG-ODN plus Luzindole treatment. These data revealed that when blocking the effect of endogenous melatonin, the suppressive effect on airway inflammation and NLRP3 inflammasome activity conferred by CpG-ODN was abolished, suggesting such effect of CpG-ODN may be due to the restored level of melatonin.

3.8. Exogenous melatonin ameliorates OVA-induced airway inflammation

Finally, to directly demonstrate the effect of melatonin on allergic airway inflammation and NLRP3 inflammasome activity, exogenous melatonin was applied as shown in Fig. 9A. Melatonin pretreatment markedly alleviated OVA-induced recruitment of leukocytes to peribronchial (Fig. 9B, C, Supplemental Fig. 1). Similarly, increased goblet cell hyperplasia and mucus production in OVA-challenged mice were also abolished after melatonin pretreatment (Fig. 9D, E).

3.9. Exogenous melatonin suppresses serum IgE, BALF inflammatory cells infiltration and regulates Th1/Th2 cytokines production

Furthermore, the level of OVA-specific IgE in serum (Fig. 10A), the number of total cells, neutrophils, lymphocytes, monocytes and eosinophils in BALF (Fig. 10B–F), and the levels of Th2-cell related cytokines such as IL-4, IL-13, IL-5 and GM-CSF (Fig. 10G–J) were greatly

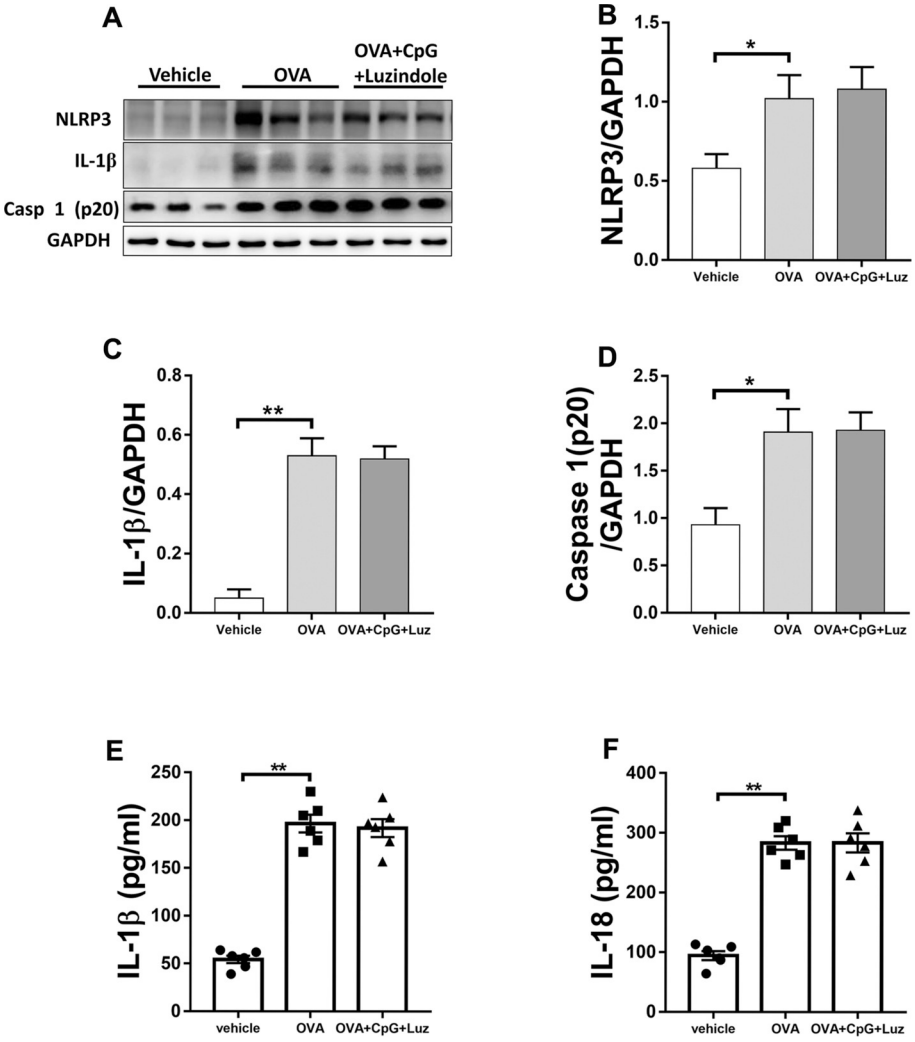

Fig. 8. Luzindole dampened the effect of CpG-ODN on OVA-induced activation of NLRP3 inflammasome
(A) The expression of NLRP3, mature IL-1β and caspase 1(p20) in the lung tissues was analyzed by western blot. (B-D) The relative levels of NLRP3, mature IL-1β and caspase 1(p20) in the lung tissues. (E, F) Concentration of IL-1β and IL-18 in BALF was analyzed by ELISA. Data were expressed as mean ± SEM of at least 6 mice per group. $^{**}p < .01$, $^{***}p < .001$.

reduced when melatonin treatment in OVA-challenged mice. However, the OVA-induced production of Th1 cytokine IFN-γ was further increased when application of melatonin (Fig. 10K).

3.10. Exogenous melatonin inhibits NLRP3 inflammasome activation

Moreover, administration of melatonin significantly suppressed the level of NLRP3, mature IL-1β and caspase-1(p20) in lung homogenate of OVA-challenged mice (Fig. 11A–D). The levels of IL-1β and IL-18 in BALF were consistent with the results in lung homogenate, i.e. melatonin decreased the level of IL-1β and IL-18 during allergic airway inflammation (Fig. 11E, F). Basing on these data, exogenous administration of melatonin has protective effects on allergic airway inflammation via inhibiting the activation of NLRP3 inflammation, and such effect was comparable with that of CpG-ODN.

4. Discussion

In the present study, we demonstrated that OVA challenge exacerbated lung inflammation in mice. However, pretreated with CpG-ODN, such airway inflammation including the migration of inflammatory cells into the lungs, the number of inflammatory cells in BALF, the level of OVA-specific IgE, and productions of Th2 cytokines such as IL-4, IL-13, IL-5 and GM-CSF was markedly suppressed in OVA-challenged mice. Conversely, the OVA-induced production of Th1 cytokine IFN-γ was further upregulated when application of CpG-ODN. IL-4, IL-5, IL-13 and GM-CSF, which are Th2 inducing factors have been extensively investigated for the development of therapeutic drugs for asthma [40]. These data highlight the preventive effect of CpG-ODN in allergic airway diseases. However, the mechanism underlying such effect is elusive in the literature.

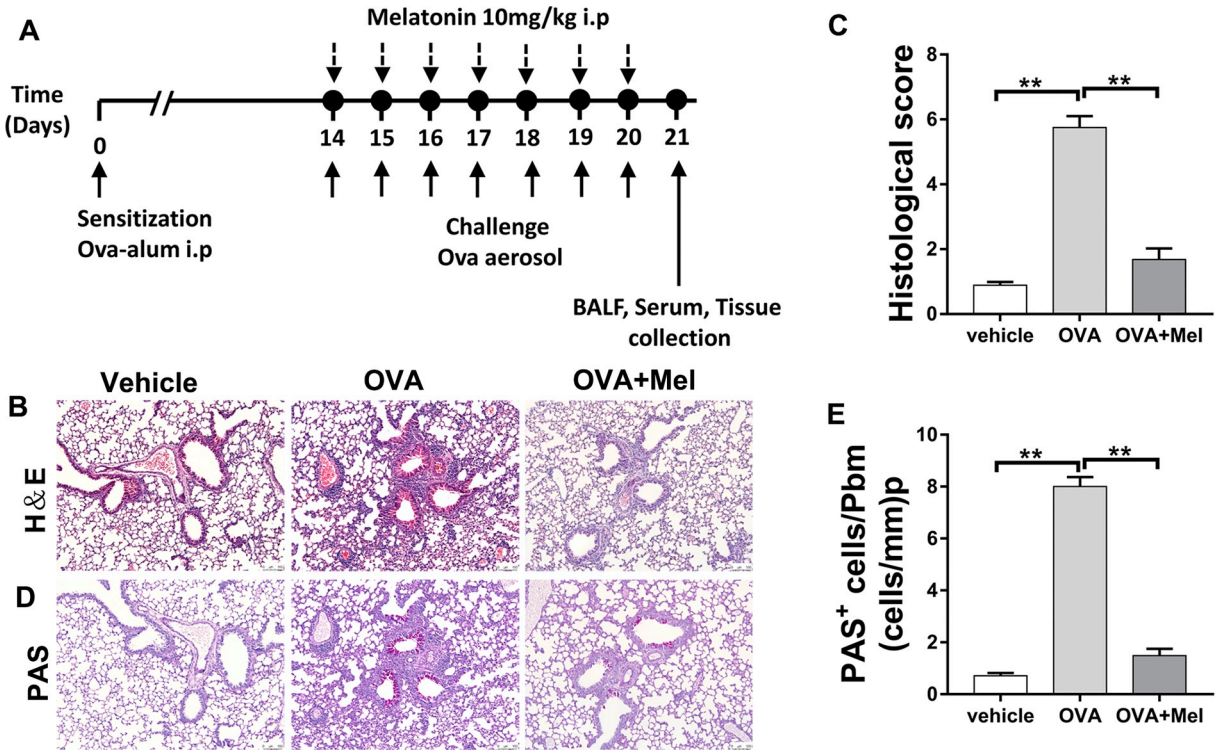

Fig. 9. Exogenous melatonin ameliorates OVA-induced airway inflammation
(A) Experimental protocol of melatonin treatment during establishing murine allergic airway diseases model. (B) Lung sections were stained with H&E to analyze cell infiltration. (C) The extent of peribronchial inflammation was scored. (D) Lung sections were stained with periodic acid-Schiff (PAS) to evaluate mucus production and goblet cell hyperplasia. (E) PAS^+ cells in the airway were counted and quantified. Data were expressed as mean ± SEM of 6 mice per group, $^{**}p < .01$.

Recently, the contribution of NLRP3 inflammasome in the development of allergic airway inflammation has been attracting more and more attention. NLRP3 inflammasome is composed of NLR protein NLRP3, ASC and caspase-1 [41,42]. When activated, NLRP3 inflammasome is assembled and promotes active caspase-1 to convert pro-IL-1β and pro-IL-18 into their bioactive form IL-1β and IL-18 [43]. Notably, elevated level of IL-1β in BALF [44], serum [45] and sputum [46] has been found from asthmatic patients. And IL-18 enhances secretion of IL-4 and IL-13, thereby induces bias of adaptive immunity to Th2 responses [47]. However, in the absence of NLRP3, mice were resistant to allergen-induced airway inflammation [16,48]. In agreement with these findings, our present study showed that the activation of NLRP3 inflammasome was induced after OVA challenge, including elevated protein expression of NLRP3, caspase-1 and IL-1β as well as increased secretion of NLRP3-associated cytokines IL-1β and IL-18. However, pretreatment of CpG-ODN remarkably suppressed OVA-induced activation of NLRP3 inflammasome. Our results link the protective effect of CpG-ODN in allergic airway inflammation to its suppressive effect on the activation of NLRP3 inflammasome.

Interestingly, NLRP3 inflammasome is recently reported to be a new target for melatonin [25,49], which is a strong immune-modulatory and anti-inflammatory agent [27]. Here, our study showed that the protein level of melatonin synthetic enzyme ASMT, as well as the level of melatonin in serum and lung homogenate was significantly decreased in OVA-challenged mice as compared to control mice. Similar to our results presented here, the level of melatonin in saliva of asthmatic patients [32] as well as in serum and BALF of asthmatic animal [33] is lower. Moreover, the melatonin level is lowest in patients with moderate to severe asthma as compared to that of mild asthma [32], suggesting that the level of melatonin is correlated with the severity of asthma. Of note was that, the present study found the protein expression of ASMT and the level of melatonin in OVA-challenged mice pretreated with CpG-ODN were considerably restored, which were even similar to that of control mice. Although it has been reported respectively that either CpG-ODN or melatonin significantly suppresses allergic airway inflammation [4,31], our study was the first to reveal that CpG-ODN can regulate the level of endogenous melatonin in allergic airway diseases, thus linked these two potential therapeutic agent for allergic airway diseases together.

Although previous study has suggested that restored endogenous melatonin is beneficial in prevention of allergic airway diseases such as asthma [33], the mechanism underlying such effect is not fully understood. Luzindole, a melatonin receptor antagonist has been reported to diminish the protective effect of melatonin in human mesenchymal stem cells [50] and SH-SY5Y cell line [51]. Therefore, in this study, Luzindole was used to determine whether endogenous melatonin

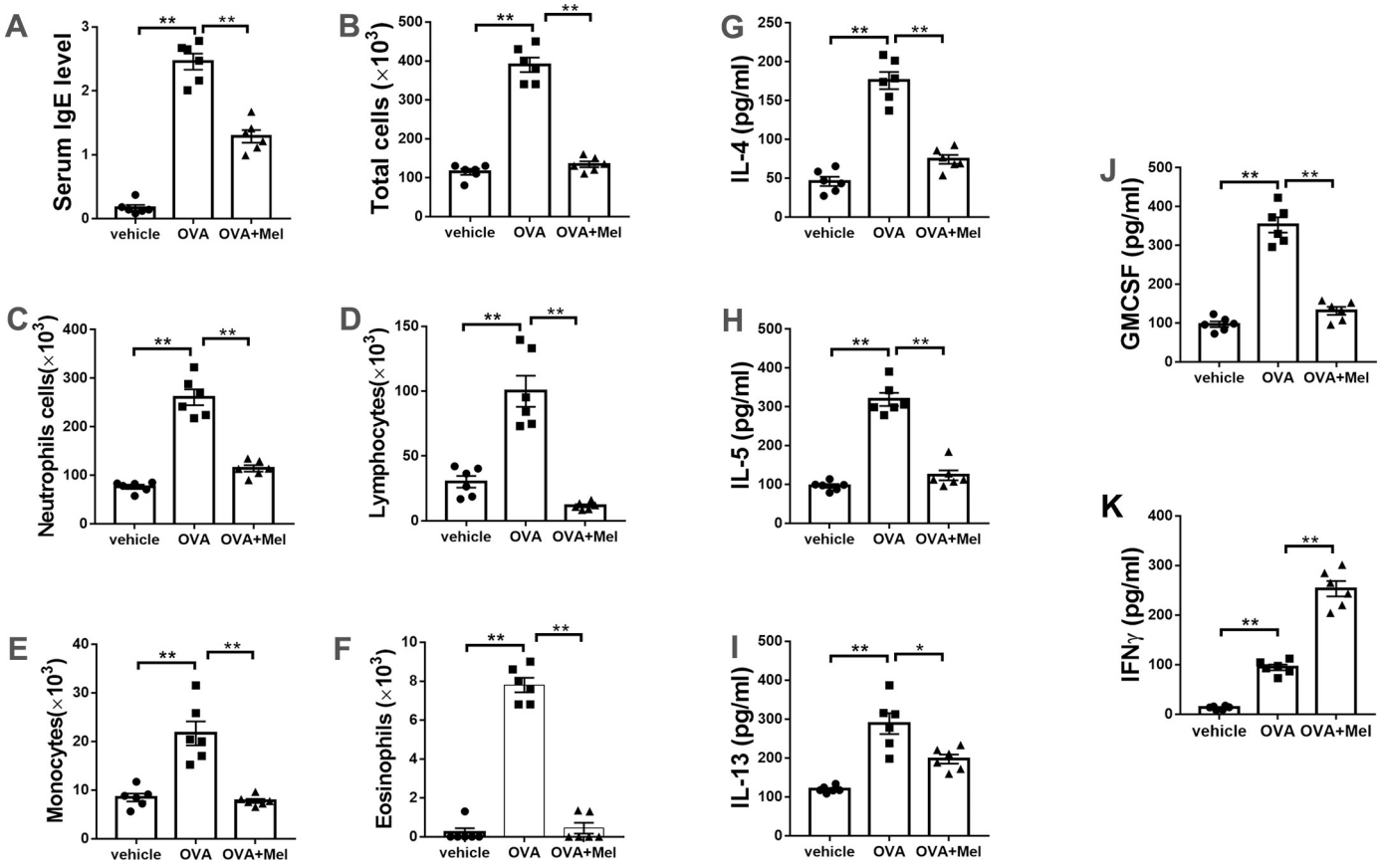

Fig. 10. Exogenous melatonin suppresses serum IgE, BALF inflammatory cells infiltration and regulates Th1/Th2 cytokines production
(A) The level of OVA-specific IgE in serum. (B—F) The number of total cell, neutrophils, lymphocytes, monocytes and eosinophils in BALF. (G-J) Productions of Th2 associated cytokines such as IL-4, IL-13, IL-5 and GM-CSF in BALF. (K) The level of Th2 cytokine IFN-γ. Data were expressed as mean ± SEM of 6 mice per group, $^{*}p < .05$, $^{**}p < .01$.

biosynthesis restored by CpG-ODN suppressed the activation of NLRP3 inflammasome in allergic airway disease. We found that Luzindole abolished the effect of CpG-ODN on airway leukocytes infiltration, goblet cell hyperplasia and Th1/Th2 cytokines production, as well as the activation of NLRP3 inflammasome. These results suggested that CpG-ODN attenuate allergic airway inflammation and suppress the activation of NLRP3 inflammasome via endogenous melatonin.

To obtain direct evidence that melatonin suppresses allergic airway inflammation via inhibiting the activation of NLRP3 inflammasome, exogenous melatonin was used. In agreement with recent report that exogenous melatonin attenuates airway inflammation in rats with COPD via suppressing the activation of NLRP3 inflammasome [52], our present study demonstrated that exogenous melatonin administration significantly alleviates allergic airway inflammation and concomitantly inhibited the activation of NLRP3 inflammasome.

In conclusion, our findings demonstrated that CpG-ODN exerted a protective effect against allergic airway inflammation. And this protective effect of CpG-ODN was attributed to the restored melatonin which inhibited the activation of NLRP3 inflammasome in the lung tissues. Both CpG-ODN and melatonin are reported to inhibit allergic airway inflammation and activate Th1 response [1,28], our study showed firstly that the resolution effect of CpG-ODN on allergic airway inflammation maybe mediated by melatonin. Melatonin is a natural hormone which secretes by our body, making it a more suitable and safety target for allergic airway diseases, thus our study reinforced the application of melatonin for allergic airway diseases such as asthma.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117067.

Declaration of competing interest

The authors have no conflict of interest to declare.

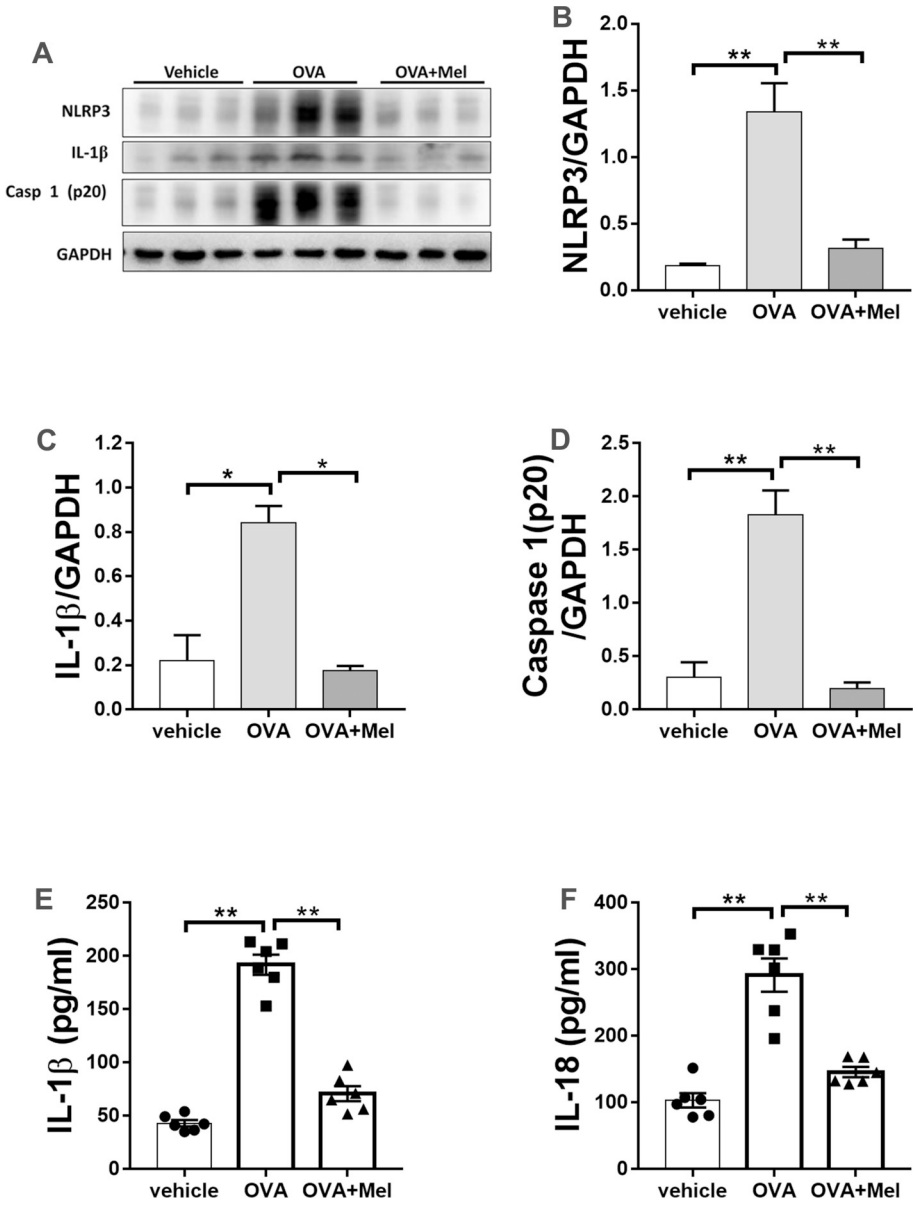

Fig. 11. Exogenous melatonin inhibits NLRP3 inflammasome activation
(A) The protein expression of NLRP3, mature IL-1β and caspase 1(p20) in the lung homogenates was analyzed by western blot. (B-D) The relative levels of NLRP3, mature IL-1β and caspase 1(p20) in the lung tissues. (E, F) Concentration of IL-1β and IL-18 in BALF was analyzed by ELISA. Data were expressed as mean ± SEM of 6 mice per group. $^{*}p < .05$, $^{**}p < .01$.

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Author contributions

W.H.M designed the experiments, analyzed the data and wrote the manuscript. X.Q.M and Z.C.C. established the murine model of allergic airway disease, performed the bronchoalveolar lavage cell counts, western blotting, immunohistochemistry and confocal. X.J performed ELISA assays. F.X.Y revised the manuscript. F.G.H revised the manuscript, helped to design and to coordinate the experiment.

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