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Anti-inflammatory activity of SintMed65, an *N*-acylhydrazone derivative, in a mouse model of allergic airway inflammation

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ABSTRACT

Asthma is a chronic, complex and heterogeneous inflammatory illness, characterized by obstruction of the lower airways. About 334 million people worldwide suffer from asthma, and these estimates, as well as the severity of the disease, have increased in the last decades. Glucocorticoids are currently the most widely used drugs in the treatment and control of asthma symptoms, but their prolonged use can cause serious adverse effects. *N*-acylhydrazone derivatives have been tested in pre-clinical studies in models of inflammatory diseases. Here we tested SintMed65 (*N'*-[(1E)-3-(4-nitrophenylhydrazono)]-(2E)-propan-2-ylidene-3,5-dinitrobenzohydrazide), a compound belonging to a novel class of immunosuppressive drugs, in a mouse model of allergic airway inflammation. BALB/c mice were sensitized previously and challenged with ovalbumin for five consecutive days and SintMed65 treatment was performed orally 1 h prior to challenge with ovalbumin. Administration of SintMed65, as well as the reference drug dexamethasone, reduced cellularity and the number of eosinophils in the bronchoalveolar fluid (BALF). SintMed65 also reduced the production of Th2 cytokines IL-4, IL-5 and IL-13 in the BALF, and IL-4, IL-10 and CCL8 gene expression in lung, compared to vehicle-treated mice. Importantly, a reduction in the number of leukocytes and in the mucus production in lungs of SintMed65-treated mice was found, compared to the vehicle-treated group. In contrast, IgE production was not significantly altered after treatment with SintMed65. Our results demonstrate that compound SintMed65 possesses anti-inflammatory characteristics, suggesting its therapeutic potential for the treatment of allergic diseases.

1. Introduction

Asthma is a common chronic disease that affects about 334 million people of all ages in all parts of the world [1]. Although some countries have reported a decline in asthma-related hospitalizations and deaths, many patients show signs of disease exacerbation, with an increase in symptoms by almost 30% in the last 20 years [2]. Asthma is a complex disease, characterized by airway edema, remodeling and hyperresponsiveness [3], associated with the accumulation and activation of inflammatory cells such as type 2 T-helper (Th2) lymphocytes, eosinophils and mast cells within the bronchial mucosa [4]. Cytokines, such as the interleukins (IL)-4, IL-5, IL-9 and IL-13 produced by allergen-sensitized Th2 $CD4^+$ T cells, are involved in airway eosinophilia, chronic inflammation and pathological changes characteristic of atopic asthma. Allergen-specific IgE production is implicated in the activation of mast cells and perpetuation of the inflammation [5–7].

The challenge in asthma treatment is to control symptoms and reduce future risks of exacerbation [8]. Currently, the most recommended treatment for asthma is the use of corticosteroids [9]. However, long-term exposure to oral corticosteroids may cause several side effects, including hypertension, gastrointestinal bleeding ulcers, depression and sepsis, among others [10]. Although progress has been made in the understanding asthma, the development of alternative treatments is relatively slow and new effective therapies that control severe asthma are still needed [11].

Hydrazones constitute an important class of biologically active drugs which has attracted the attention of medicinal chemists due to their wide range of pharmacological properties, such as antioxidants,

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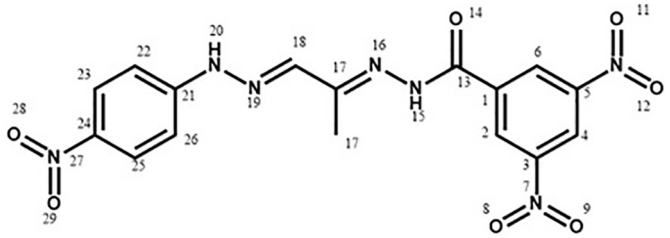

Fig. 1. Chemical structure of SintMed65 (*N′*-[(1E)-3-(4-nitrophenylhydrazono)]-(2E)-propan-2-ylidene-3,5-dinitrobenzohydrazide).

anti-inflammatories, anticonvulsants, analgesics and antimicrobials [12–14]. One such new molecule is the compound SintMed65 (Fig. 1), a derivative from an *N*-acylhydrazone moiety, considered a privileged structure shared by many compounds with diverse pharmacological activities [15]. Recently, several studies with bioactive *N*-acylhydrazone (NAH) derivatives demonstrated antitumor [16], analgesic, anti-inflammatory, antiplatelet [12,17] and antithrombotic [18] activities. In this paper, we hypothesized that SintMed65 compound ameliorates inflammation in a murine model of allergic airway inflammation induced by chicken egg ovalbumin.

2. Materials and methods

2.1. Animals

Male BALB/c mice, 4–6 weeks old, were housed under controlled temperature (22 ± 2 °C) and humidity (60 ± 5%) conditions with a 12-hour light/dark cycle. Food and water were available *ad libitum*. Animals were handled according to the National Institutes of Health (NIH) guidelines for animal experimentation. Experimental protocols were approved by the Ethics Committee for the Use of Animals in Research IGM-FIOCRUZ/BA (license number 005/2015).

2.2. Drugs and reagents

SintMed65 was synthesized and characterized as previously described [19]. The solutions were prepared right before use and were not stored for later use. For a more homogeneous dilution, the compound was dissolved in a mixture of 30% sorbitol and 10% tween 80 in saline. Chicken egg ovalbumin (OVA), grade V, 98% pure, sorbitol and dexamethasone were obtained from Sigma Chemical Company, (St Louis, MO, USA) and aluminum hydroxide (alum; AlumImject) was purchase from Pierce (Rockford, IL, USA). TRIZOL® was purchased from Thermo Fisher Scientific, Waltham, MA, USA). High-Capacity cDNA Reverse Transcription Kit, Taqman® Universal Master Mix, Taqman® Gene expression assay and all genes used were purchase from Taqman® (Thermo Fisher Scientific).

2.3. Model of allergic airway inflammation and treatment

Allergic airway inflammation was induced as previously described [20]. Briefly, groups of seven mice received systemic immunization by subcutaneous injection of 10 μg of OVA adsorbed in 2 mg/mL alum followed by a booster injection at day 14. A nasal challenge was performed starting at day 28, by inhalational exposure to an aerosolized solution of 1% OVA for 15 min/day, for five consecutive days. One hour before each aerosol delivery, mice were treated orally with SintMed65 (5, 20 or 80 mg/Kg), dexamethasone (25 mg/kg) or vehicle (sorbitol/tween 80 in saline). A group of animals (naive group), which were not injected with OVA for induction of airway inflammation and received no treatment, was also included in experimental design.

2.4. Collection of blood and bronchoalveolar lavage fluid (BALF)

Forty-eight hours after the last inhalation exposure, mice were euthanized with a lethal dose of 5% ketamine (Vetanarcol®; Konig, Avellaneda, Argentina) and 2% xylazine (Sedomin®; Konig) by intraperitoneal injection. Blood was attained *via* the brachial plexus for collection of samples used to estimate the IgE production. BALF was collected twice by intratracheal instillation of 1 mL of PBS each time. The first lavage fluid was centrifuged, and aliquots of the supernatant were kept at −80 °C until use for cytokine measurements. The second lavage fluid was centrifuged and the two cell pellets were resuspended in a PBS final volume of 1 mL. The number of total leukocytes in BALF was estimated in a Neubauer chamber. Differential counts were obtained using hematoxylin and eosin (H&E) stained cells in cytospin preparations. Slides were analyzed under light microscopy (Olympus, Tokyo, Japan) at ×60 magnification by an observer blinded to the specimen identities. Differential counts were completed on 400 cells per slide using standard morphological criteria.

2.5. Histopathological and morphometric analyses

The tracheas of the mice were cannulated for perfusion of the lungs with 4% buffered formalin. The right lobe of the lungs from each animal was removed, fixed in 4% buffered formalin for 24 h and then processed for histological analysis. H&E stained sections of the lungs were used for quantification of inflammatory cells by optical microscopy. For each lung, 25 fields (40 ×) per section were analyzed and the data were used to calculate the mean number of cells per mm^2. The presence of mucus was analyzed in periodic acid-Schiff (PAS)-stained sections. All images were digitalized using a color digital video camera (CoolSnapcf) adapted to a BX41 microscope (Olympus, Tokyo, Japan) calibrated with a reference measurement slide and were analyzed using Image Pro image program (version 6.1; Media Cybernetics, San Diego, CA, USA).

2.6. Measurement of IgE and cytokine production

After collection, blood samples were centrifuged at 1200*g*, 10 min, 4 °C and the serum supernatant transferred to microcentrifuge tube and stored at −20 °C until subsequent analysis. The IgE antibody levels to OVA were used as a marker of allergic sensitization and measured using enzyme-linked immunosorbent assay (ELISA). Briefly, microplate wells were sensitized with 100 μg/mL of OVA for 2 h, followed by blocking the reaction with PBS containing 10% fetal bovine serum (FBS; GIBCO) for additional 2 h. Dilutions of the test samples were incubated overnight with in PBS containing 5% FBS. Detection of bound ovalbumin-specific IgE was performed by sequential biotinylated anti-IgE antibody incubation. After incubation with streptavidin-peroxidase conjugate, the reaction was developed using 3,3′,5,5′ tetramethylbenzidine (TMB; Sigma Chemical Company) peroxidase substrate and read at 450 nm. Concentrations of interleukin (IL)-4, IL-5 and IL-13 in the BALF were also determined by ELISA using specific antibody kits (R&D System, Minnesota, MN, USA), according to the manufacturer's instructions.

2.7. Real-time polymerase chain reaction (RT-PCR)

The RNA extraction was performed using TRIZOL®, following the manufacturer's recommendations. The tissue fragment was homogenized in TissueLyserII (Qiagen). After extraction, the purity and concentration of the RNA was checked by the NanoDrop ™ 1000 spectrophotometry (Thermo Fisher Scientific). For reverse transcription, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used. The real-time PCR reactions were conducted on the ABI7500 thermal cycler (Applied Biosystems ™) under the conditions recommended by the manufacturer. Each 10 μL reaction contains: 30 ng cDNA, 2× Taqman® Universal Master Mix (Applied Biosystems ™) and 20× Taqman® Gene expression assay. We evaluated the genes

for: IL-4 (Mm00445259_m1), IFNγ (Mm00801778_m1), TGFβ1 (Mm00441724_m1), CCL8 (Mm01297183_m1), IL-10 (Mm00439616_m1), CCL2 (Mm00441242_m1), CCL7 (Mm00443113_m1), CCL17 (Mm00516136_m1), CCL22 (Mm00436439_m1), CXCL12 (Mm00445552_m1). For normalization of the samples two reference genes were used: HPRT (Mm00496968_m1) and ACTB (Mm00607939_s1). The 2-ΔΔCt method was used for comparing relative changes in gene expression [21].

2.8. Statistical analysis

Results were expressed as mean ± SEM of 7 mice per group. Statistical comparisons between groups were performed by one-way analysis of variance and Newman-Keuls multiple comparison tests using Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when the values of P were < 0.05.

3. Results

3.1. SintMed65 suppresses OVA-induced inflammatory cell infiltration in asthmatic mice but not IgE production

To evaluate the effects of SintMed65 on allergic airway inflammation, we treated AAI mice with different doses of SintMed65 by gavage, for five consecutive days 1 h before OVA aerosol challenge. BALF was collected 48 h after the last OVA aerosol challenge, and then total and differential cell counts were performed. OVA-inhalation significantly increased total cell and eosinophil counts in asthmatic mice compared to the naive controls. Treatment with SintMed65 at doses of 5, 20 and 80 mg/kg significantly reduced the number of total inflammatory cells (Fig. 2a) and eosinophils (Fig. 2b) in a dose-dependent manner, compared to the vehicle-treated group. The inhibition promoted by SintMed65 at 80 mg/kg in total cell counts was similar to that found with dexamethasone, a gold standard glucocorticoid. A statistically significant reduction was also observed in lymphocyte counts (Fig. 2c). No differences were observed in neutrophils cell counts in BALF of SintMed65 or dexamethasone treated mice compared to the vehicle-treated group (Fig. 2d). Finally, both SintMed65 and dexamethasone increased the macrophage cell counts in BALF (Fig. 2e).

To evaluate whether SintMed65 affects the levels of OVA-specific IgE antibodies, we assessed the serum IgE levels by ELISA. Vehicle-treated mice had high levels of serum anti-ovalbumin IgE antibodies compared to naive mice (Fig. 2f). While a statistically significant reduction in OVA-specific IgE antibodies was observed in mice treated with the standard drug dexamethasone, SintMed65-treated mice had no reduction in OVA-specific IgE antibodies compared to vehicle-treated mice.

Histopathological evaluations of H&E-stained lung sections showed that OVA challenge induced an intense infiltrate of inflammatory cells containing lymphocytes, macrophages and eosinophils, when compared to the naive group (Fig. 3a and b). Dexamethasone-treated and SintMed65-treated lung section had a reduced number of inflammatory cells compared to the vehicle group (Fig. 3c–e).

3.2. SintMed65 inhibited OVA-induced airway mucus production

The presence of mucus in the lungs of the animals was analyzed in lung sections stained with PAS. In the lungs of the naive mice, rare PAS^+ cells were seen in the respiratory epithelium, indicating low mucus production compared to vehicle-treated group, in which a marked presence of mucus was found (Fig. 4a–b and e). Treatment with the highest dose of SintMed65 (80 mg/Kg) reduced the presence of mucus, although it was less potent than dexamethasone (Fig. 4c–d and e).

3.3. SintMed65 modulates OVA-induced cytokine expression

To investigate whether SintMed65 could modify an OVA-specific Th2 response, Th2 cytokine levels in the BALF and gene expression of cytokines and chemokines in the lung were determined by ELISA and qRT-PCR, respectively. OVA inhalation in sensitized mice (vehicle group) caused a marked increase in IL-4, IL-5 and IL-13, as compared with naive group (Fig. 5a–c), indicating an induction of a Th2 profile. Treatment with SintMed65 potently decreased the levels of IL-4 and IL-13 (similar to dexamethasone), and caused a partial reduction of IL-5, in the BALF compared to vehicle-treated group (Fig. 5a–c). Furthermore, the chemokine/cytokine transcripts were evaluated in the lungs of vehicle and SintMed65 (80 mg/Kg). The expression of IFN-γ, TGF-β and CCL17 genes were upregulated after SintMed65 treatment, while IL-4, IL-10 and CCL8 gene expression were downregulated (Fig. 5d). Collectively, these results show that SintMed65 suppressed the Th2 immune response in OVA-induced murine AAI model.

4. Discussion

Inflammatory process in the lung of asthmatic individuals occurs in response to allergens, and is a highly orchestrated process, leading to an infiltrate of inflammatory cells, especially eosinophils [22]. These events need to occur in a self-limiting way so as not to generate large expenditures for metabolic energy, host tissue damage, and organ failure [23,24].

Treatment with the SintMed65 compound led to a significant improvement in the inflammatory response, controlling the Th2 profile cytokine levels. The compound was also able to decrease significantly the total number of cells and the eosinophils in BALF. These findings indicate that this hydrazone inhibited the recruitment of eosinophils and lymphocytes into the airway, as well as the presence of mucus in lung tissue. We can relate this reduction of mucus with the low levels of Th2 cytokines found in the treated mice, since IL-13 stimulates the production of mucus, in addition to promote a significant remodeling of the airways in asthma, favoring goblet cell hyperplasia [25]. Additionally, SintMed65 also reduced the production of IL-4, a cytokine which plays a key role in mucus production through the recruitment of Th2 cells into the lungs and the induction of inflammation [22].

These data are consistent with other studies that used compounds with anti-inflammatory activity in a murine model of inflammation of the lower airways [20,26,27,28]. Here we found that SintMed65 also attenuated the inflammation, especially reducing the eosinophilic infiltrate. Eosinophilia is a significant pathological feature of allergic diseases, possibly contributing to airway damage through the release of mediators and cytokines. Infiltration of eosinophils in the airways is linked to the production of IL-5, which is important for eosinophil proliferation and activation [22]. IL-5 is also essential for the migration of eosinophils from the bone marrow into the peripheral blood, acting in the differentiation and terminal proliferation of eosinophil precursors, as well as the activation of mature eosinophils [29]. Significant evidence supports the theory that the reduction of eosinophil infiltrates is also related to decreased levels of IL-4 and IL-13, which are involved in the recruitment of eosinophils to the airway, inducing the synthesis of eotaxin and positive endothelial adhesion to regulating molecules [25,30]. Our study corroborates this idea, since the marked reduction of eosinophil counts in BALF after oral SintMed65 treatment was accompanied by a reduction in the levels of IL-4, IL-5 and IL-13 cytokines.

The compound 2-(4-{2-[(phenylthio) acetyl]-carbonohydrazonoyl}-phenoxy) acetamide, also belonging to the hydrazone class, was shown to regulate IgE, IL-1B, IL-5, IL-13, COX-2 and reduce eosinophilic infiltrate in OVA allergic rhinitis model, which corrobores the data observed in the present study [31]. The effect of hydrazones on cells of the immune system has been reported previously. In an experimental autoimmune encephalomyelitis model, the tetravalent guanylhydrazone CNI-1493 was able to modulate dendritic cells and macrophages [32].

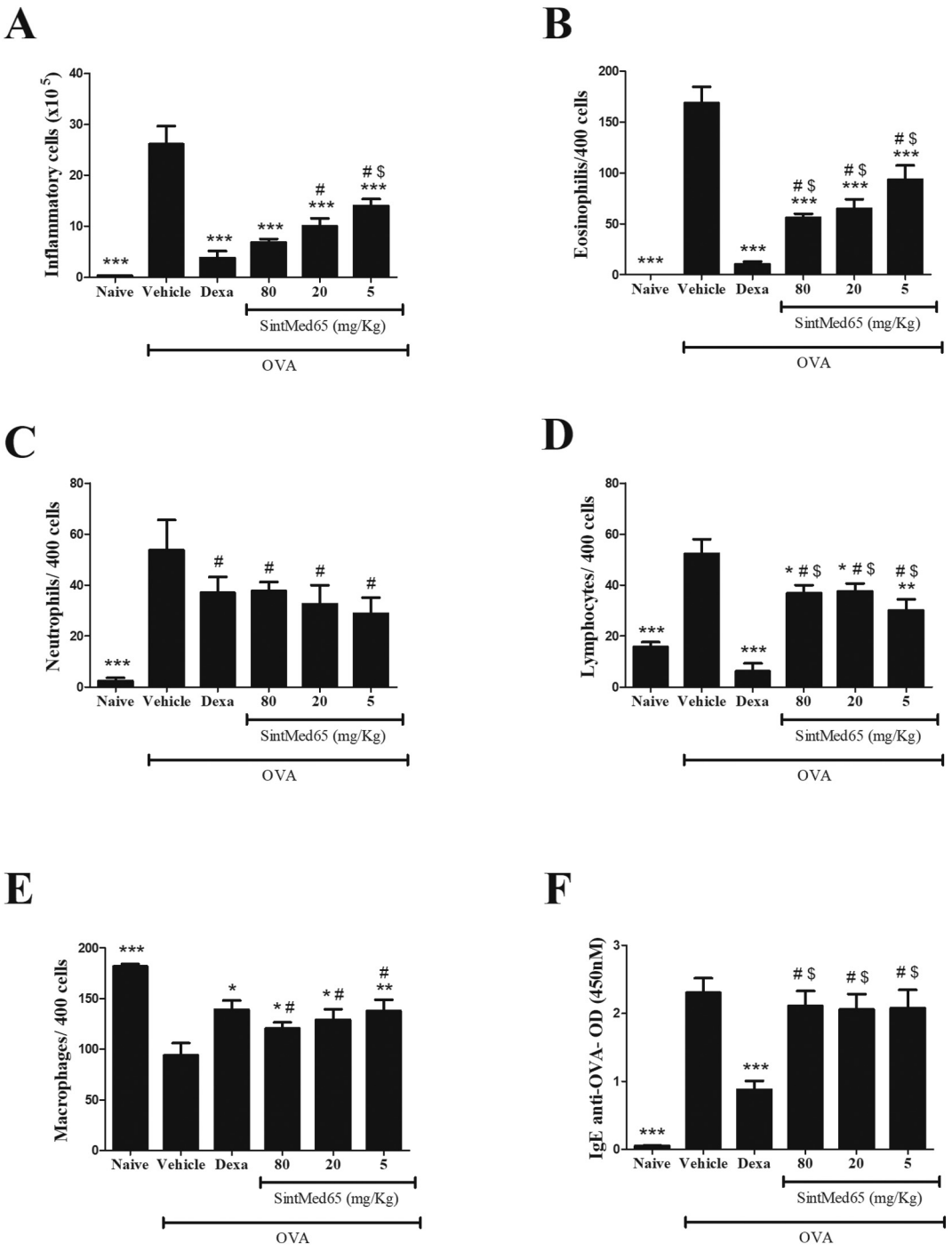

Fig. 2. Cellularity in BALF and IgE antibodies production from naive or ovalbumin-challenged mice treated with vehicle, dexamethasone (Dexa; 25 mg/kg) or SintMed65 (80, 20, and 5 mg/kg), was evaluated. Total cell count (A), eosinophils (B), lymphocytes (C), neutrophils (D) and macrophages (E) in BALF samples obtained from mice submitted to different treatments. The levels of ovalbumin-specific IgE antibodies were determined in the sera of individual mice (F). The data are expressed as mean ± S.E.M. of 7 mice per group. $p < 0.001$ compared to vehicle-treated mice; $p < 0.01$ compared to vehicle-treated mice; $p < 0.05$ compared to vehicle-treated mice; #$p < 0.05$ compared to naive group; \$$p < 0.05$ compared to dexamethasone-treated mice.

Additionally, a previous work from our group has shown that SintMed65 has anti-inflammatory effects on macrophages and lymphocytes [19]. In the context of airway inflammation, the effect of other hydrazone compounds was demonstrated in eosinophils and mast cells [31] and eosinophilic progenitors [33]. To the best of our knowledge, our work was the first to evaluate the effect of a hydrazone (SintMed65) in an allergy model. Further studies, however, are needed to better elucidate the mechanism of action of this drug on allergic airway inflammation, aiming to reinforce its potential for asthma treatment.

We did not observe a reduction of ovalbumin-specific IgE antibody production in animals treated with SintMed65. In contrast, dexamethasone caused a considerable decrease in IgE levels. IgE production is associated with allergic reactions, playing an essential role in type I hypersensitivity, including allergic asthma. The presence of allergic disease or parasitic infection may cause an increase in specific IgE. This specific IgE antibody binds to its receptors in basophils and mast cells, leading to the release of different inflammatory mediators that result in the symptoms of infection and asthma [34,35]. The fact

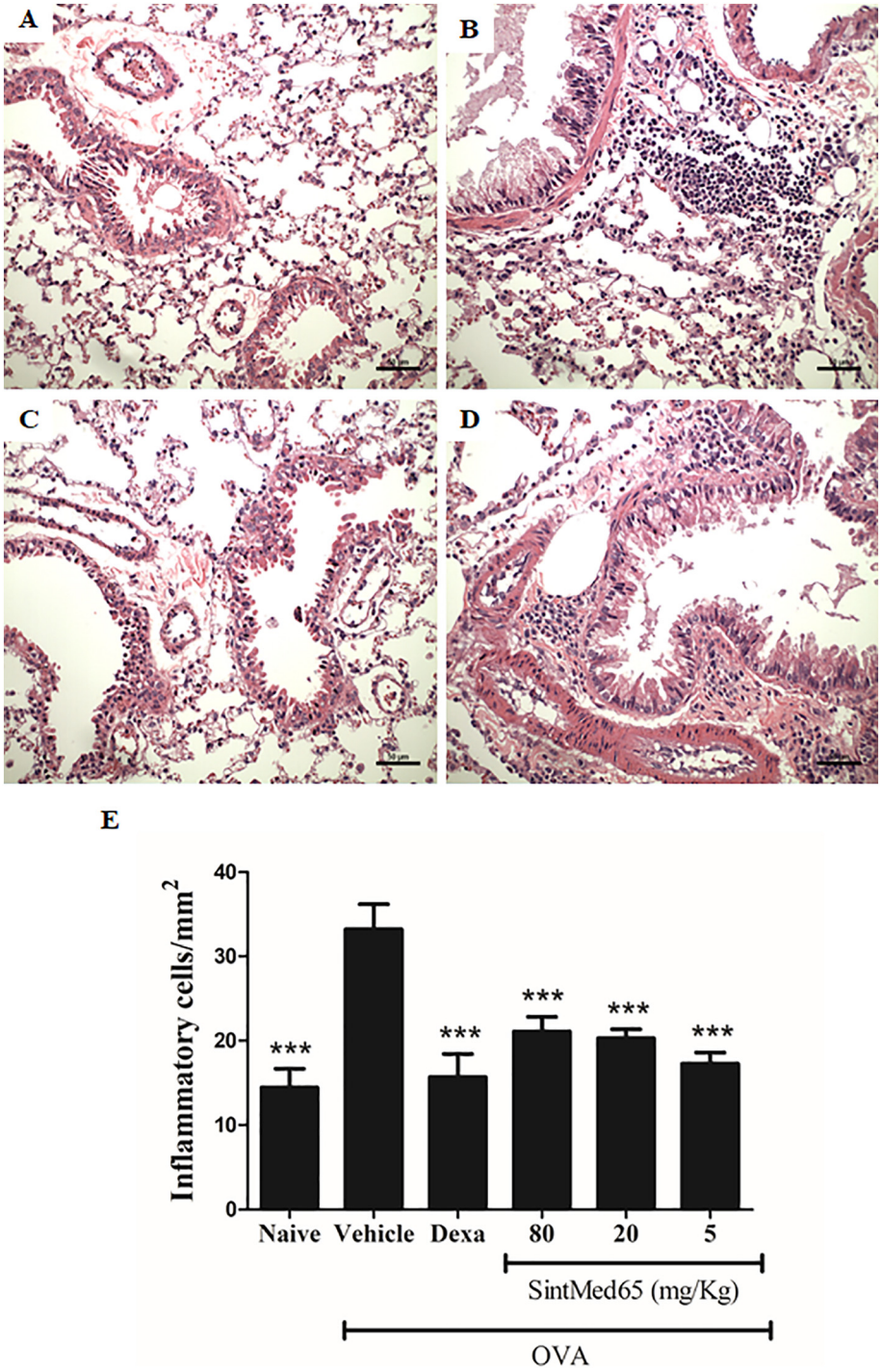

Fig. 3. Histopathology of lungs from naive or ovalbumin-challenged mice treated with vehicle, dexamethasone (Dexa; 25 mg/kg) or SintMed65 (80, 20, and 5 mg/kg). Lung sections of naive mice (A) and asthmatic mice treated with vehicle (B), dexamethasone (C), or SintMed65 80 mg/Kg (D) stained with H&E. The number of inflammatory cells was determined by morphometrical analysis (E). The data are expressed as mean ± S.E.M. of 7 mice per group. $p < 0.001$ compared to vehicle-treated mice.

that SintMed65 and dexamethasone had different effects on IgE levels suggests that these two compounds act through different molecular mechanisms. Further studies are needed to show the molecular mechanisms induced by SintMed65 responsible for the protective effects of this compound in the AAI model.

Manise et al. (2013) evaluated the cytokine profile in asthmatic and non-asthmatic individuals, and linking high levels of IgE to high levels of IL-5, IL-6 e TNF-α, showing that IL-5 together with IL-6 can promote IgE synthesis and increase IL-4-dependent IgE synthesis [36] in conditions such as allergic asthma, allergic rhinitis and sinusitis [37]. In the context of allergic inflammation induced by OVA, we observed that SintMed65 compound has anti-inflammatory effects, minimizing the production of cytokines and mucus, as well as inflammation in lung tissue.

A limitation of the present study was a non-achievement of Th1 cytokine measurements, to evaluate the relation between Th1/Th2 response. We believe that the analysis used in the present study, focused mainly on Th2 cell activity does not significantly alter the final

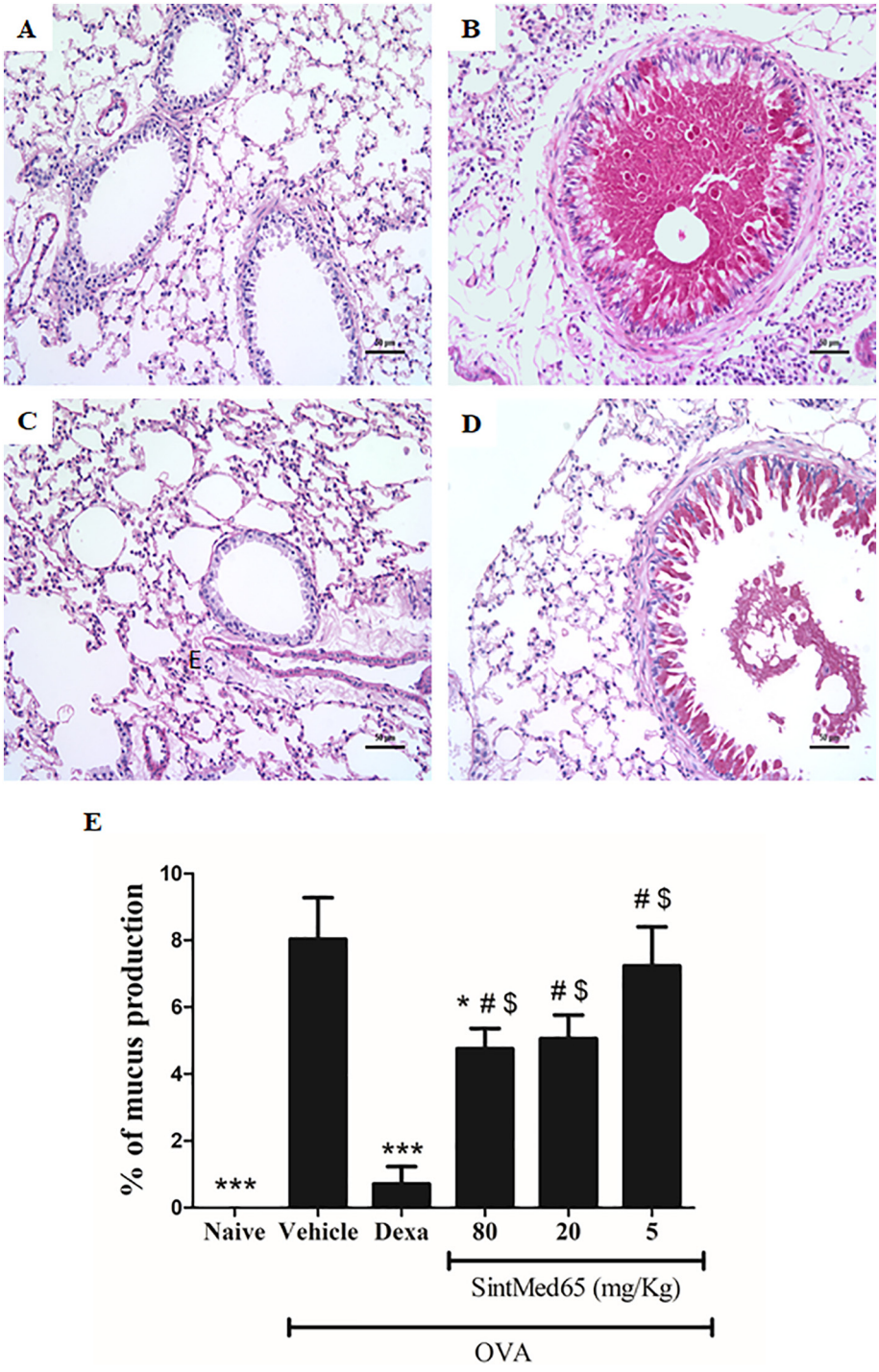

Fig. 4. Analysis of mucus production on PAS-stained lung sections. Representative areas are illustrated. Naive (A) and asthmatic mice treated with vehicle (B), dexamethasone (C), or SintMed65 80 mg/Kg (D). The area of PAS staining was estimated by morphometric analysis (E). The data are expressed as mean ± S.E.M. of 7 mice per group. $p < 0.001$ compared to vehicle-treated mice; $p < 0.05$ compared to vehicle-treated mice; #$p < 0.05$ compared to naive group; \$$p < 0.05$ compared to dexamethasone-treated mice.

interpretation of the results. However, the authors understand that further studies need to be carried out, with a better characterization of the cytokines involved in the inflammatory response, for better understanding our model.

5. Conclusion

Our results demonstrated that the SintMed65 compound attenuates the airway inflammation in a mouse model, reducing the migration of inflammatory cells induced by OVA, as well as the number of eosinophils in BALF. This indicates a potential to control allergic inflammation by inhibiting cells and production of Th2 cytokines, such as IL-4, IL-5 and IL-13, a reduction of inflammatory infiltrate and mucus in lung tissue. Our findings suggest that SintMed65 compound has a therapeutic potential for a future drug in the treatment of patients with allergic airway inflammation conditions.

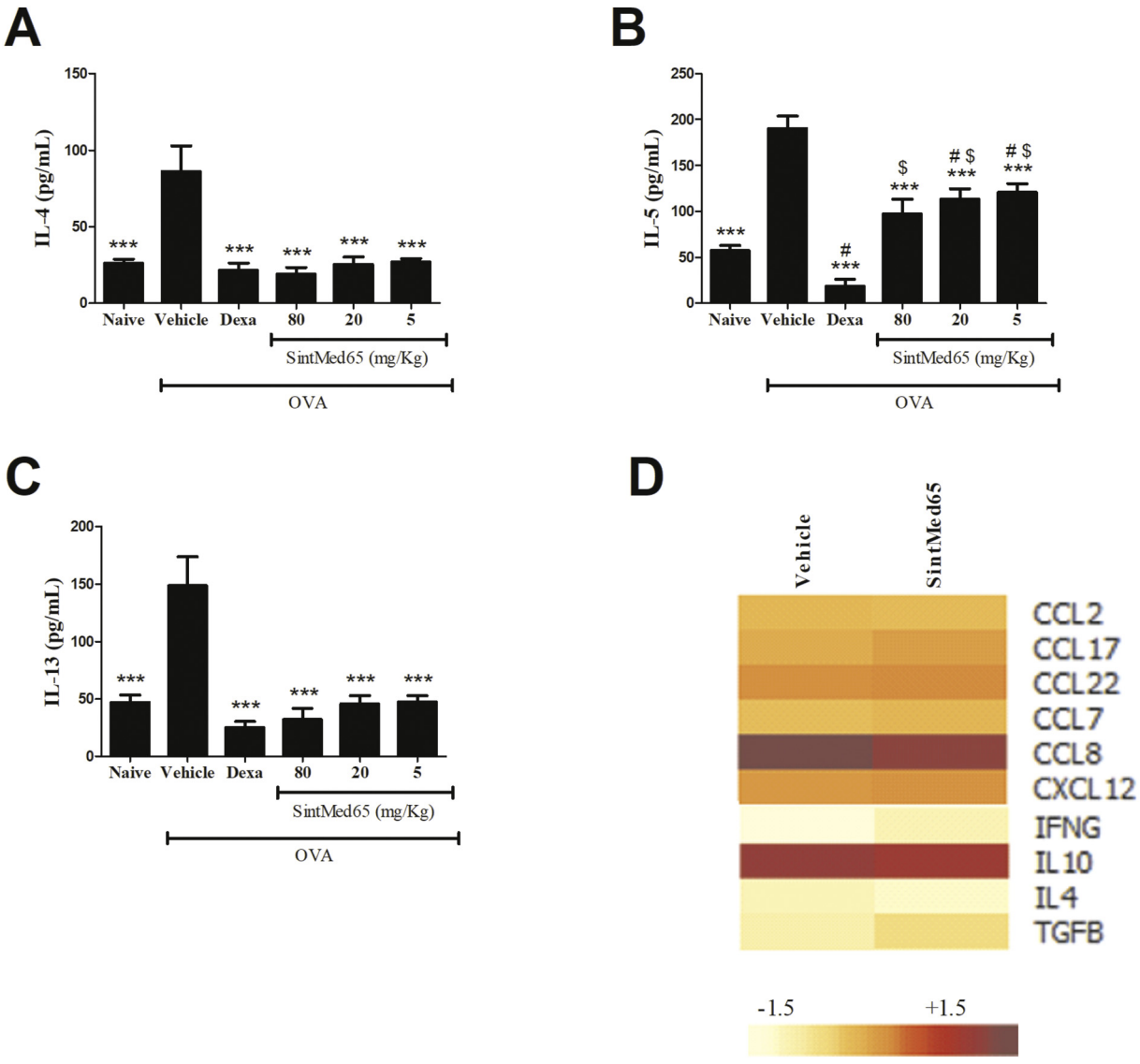

Fig. 5. Th2 cytokine and chemokine production in SintMed65-treated mice. Cytokine levels in mice from each experimental group were determined by ELISA. In the BALF it was analyzed (A) IL-4, (B) IL-5 and (C) IL-13. (D) Heatmap of chemokine/cytokine gene expression of vehicle and SintMed65 (80 mg/Kg) treated OVA-immunized mice. Color gradient bar representing lower expression in beige and higher expression in dark red. The data are representative of the expression levels of pooled samples (n = 7, per group). The data are expressed as mean ± S.E.M. of 7 mice per group. $p < 0.001$ compared to vehicle-treated mice; #$p < 0.05$ compared to naive group; $$p < 0.05$ compared to dexamethasone-treated mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

All authors have no conflict of interest to disclose.

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