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The effects of neutralizing anti-murine interleukin-17A monoclonal antibody on ozone-induced inflammation and glucocorticoids insensitivity in a murine model of asthma

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ABSTRACT

Background and objective: Exposure to ozone contributed to the worsening of inflammation and glucocorticoids insensitivity in OVA-challenged asthma. Interleukin-17A participates centrally in stages of the inflammatory response and glucocorticoids insensitivity. In this study, the effect of neutralizing anti-murine interleukin-17A monoclonal antibody (IL-17A mAb) on inflammation and glucocorticoids insensitivity in ozone-exposed and ovalbumin (OVA)-challenged mice was investigated.

Methods: Mice were sensitized and challenged with OVA and then exposed to ozone. Dexamethasone (Dex) and IL-17A mAb were administrated in corresponding periods.

Results: Compared with OVA-challenged mice, combination administration of ozone exposure and OVA challenge increased the recruitment of inflammatory cells in bronchoalveolar lavage fluid, enhanced the inflammation scores and levels of inflammatory cytokines and IL-17A mRNA, and caused the activation of p38 MAPK together with down regulation of glucocorticoids recepters (GR) in lung tissue. Monotherapy of IL-17A mAb partially attenuated lung inflammation in OVA-challenged and ozone-exposed mice, while the combination treatment of Dex and IL-17A mAb effectively reduced lung inflammation, inactivated p38 MAPK and up regulated GR in lung tissue.

Conclusions: Ozone exposure worsened OVA-challenged airway inflammation, activation of p38 MAPK and down regulation of GR in OVA-sensitized and -challenged mice, which was effectively counteracted by IL-17A mAb, and combination treatment of IL-17A mAb and Dex shows profound efficacy in inhibiting airway inflammation and improving glucocorticoids insensitivity synergistically.

1. Introduction

Allergic asthma is a global epidemic and a public health problem, and it currently affects approximately 20% of the world's population [1–3]. Air pollutants are known to exacerbate the symptoms of asthma, and may be involved in the initiation of this disease. Epidemiological investigations have demonstrated that the number of asthma outpatients is being adversely affected by air pollutants, such as ozone (O_3) [4]. The previous study also suggested that air pollutants are positively associated with the increasing prevalence of children's respiratory symptoms including asthma symptoms in China [5]. Air pollutants, particularly particulates, have also been associated with an increase in the number of emergency visits [6] and hospitalizations for acute asthma attacks [7]. Ozone (O_3) is an important constituent of environmental pollution and can induce airway hyperresponsiveness (AHR) and lung neutrophilia in animals and humans [8,9]. Recent evidence also has suggested that additional O_3 exposure promotes the pathogenesis of allergen-induced asthma [10]. Ovalbumin (OVA)

Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; GR, glucocorticoid receptors; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; OVA, ovalubumin; PBS, phosphate-buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; TNF-α, tumour necrosis factor-α

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challenge and O_3 exposure increased airway eosinophilia in C57/BL6 mice [11] and caused higher AHR and higher levels of IL-4 in asthma model [12]. O_3 exposure has also been related to asthma-related hospital visits [13]. Using a chronic allergic asthma model, we found that repeated O_3 exposures reduced the response to systemically administrated dexamethasone (Dex) of the experimental animals [14]. Furthermore, a human study reported that transient ozone exposure exhibited glucocorticoids insensitivity in stable asthmatic patients, compared with those air-exposed patients [15]. In regard to the above detailed corticosteroid insensitivity of asthma therapy, and high risk of adverse effects of glucocorticoids including diabetogenesis, osteoporosis, muscle wasting, skin thinning and weight gain [16,17], more effective therapeutic approaches are needed to explore.

As a proinflammatory cytokine, Interleukin (IL)-17A, is closely related to many cytokines and other inflammatory mediators, such as TNF-α, IL-8. It is now clear that IL-17A can specifically and selectively recruit neutrophils into the airways and enhance airway smooth muscle contraction and proliferation, and epithelial permeability of the airways to allergen [18]. In mice with OVA inhalation, inhibition of IL-17A activity with anti-IL-17A antibodies (IL-17AmAb) remarkably reduced the increased numbers of inflammatory cells of airways [19]. Our previous work also demonstrated that IL-17A mAb suppressed chronic ozone-induced airway inflammation by reducing the inflammatory cytokines, such as IL-6, IL-8 and regulating p38MAPK and NF-κB signaling pathways [20]. However, whether and how IL-17A mAb inhibits ozone-exposed and OVA-sensitized and -challenged asthma inflammation and affects glucocorticoids insensitivity is unknown. Therefore, the aim of the present study was to evaluate the effects of IL-17A mAb on asthma airway inflammation caused by repeated OVA challenge and O_3 exposure.

2. Materials and methods

2.1. Mice

Forty-eight 8-week-old male C57/BL6 mice (SLAC Laboratory Animal Co. Ltd, Shanghai, China) were housed under specific pathogen-free conditions maintained in a controlled temperature (19 ~ 23℃) and humidity (55% ± 10%), facility with a strict 12-h light–dark cycle and had free access to conventional laboratory feed and water. All experimental procedures involving animals were approved by the laboratory animal ethics committee of Shanghai General Hospital.

2.2. Study design

OVA challenge and O_3 exposure as described was carried out in six groups (eight mice per group) as outlined in Table 1. Detailed description is seen in *Suplementary Information* in the online supporting information. In addition, we examined the effects of IL-17A mAb, Dex, combination treatment with Dex and IL-17A mAb on OVA-sensitized and -challenged mice with O_3 exposure.

2.3. Bronchoalveolar lavage fluid (BALF)

Collection of BALF were obtained as described previously [21]. In brief, the lungs were lavaged using a cannula inserted in the trachea and the lungs were instilled with 0.6 ml PBS after exsanguination. Cytospin slides were prepared by Wright-Giemsa staining. Total cell and eosinophil counts were counted under the microscope in a blinded method.

2.4. IgE and cytokines in BALF and serum

Before mice were sacrificed, blood was obtained from the right ventricle, then centrifuged at 3000 r/min for 10 min at 4℃. The serum was stored at-80℃ for further determination. All the cytokines, including IL-5, IL-13, IL-8 and IL-17A in both supernants of BALF and serum and TNF-α, IFN–γ in BALF and total IgE in serum were determined by ELISA (R&D systems, Abingdon, UK) following the manufacturer's instructions. The limit of detection was 0.2 ng/ml. Data were expressed as ELISA units, which was the product of the dilution and the optical density.

2.5. Histology

After BAL, the right lung lobe was fixed in 4% formaldehyde and later embedded in paraffin. Lungs were then dissected and placed in fresh paraformaldehyde for 48 h. Routine histological techniques were used to paraffin-embed the tissue, and 3 μm sections of lung tissue were stained with hematoxylin–eosin (HE) for detection of inflammation scores [22,23]. Inflammation scores can be found in *Suplementary Information* in the online supporting information.

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

RT-PCR analysis was used to detect changes in IL-17A mRNA expression in lung tissue. Detailed information is seen in *Suplementary Information* in the online supporting information.

2.7. Western blotting

Lung tissues were homogenized and qualified by bicinchoninic acid assay analysis. Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane and then incubated with primary antibodies against phospho-p38 MAPK, total p38MAPK, GR, phosphor-GR (Cell Signaling Technology, Beverly, CA) for blot detection.

2.8. Statistical analysis

Data are presented as mean ± SEM for each experimental group. The statistical analysis and graphics were performed using GraphPad PRISM, version 6.0 (GraphPad Software, San Diego, CA). One-way ANOVA was carried out for comparison between individual groups.

Table 1
Study Design.

Groups (Name)	On days 0 and 7	From day 14, every 2 days for 6 weeks		
	Sensitization by i.p. injection	administration 30 min before challenge	Challenge by aerosol	Exposure 1 h after challenge
Control	Saline	None	Saline	Air
OVA	OVA	PBS	OVA	Air
OVA + O_3	OVA	PBS	OVA	O_3
OVA + O_3 + Dex	OVA	Dex(2 mg/kg)	OVA	O_3
OVA + O_3 + IL-17A mAb	OVA	IL-17A mAb(50 μg/ml)	OVA	O_3
OVA + O_3 + Dex + IL-17A mAb	OVA	Dex(2 mg/kg) + IL-17A mAb(50 μg/ml)	OVA	O_3

Dex, dexamethazone; i.p., intraperitoneal; O_3, ozone; OVA, ovalbumin; PBS, phosphate buffer saline.

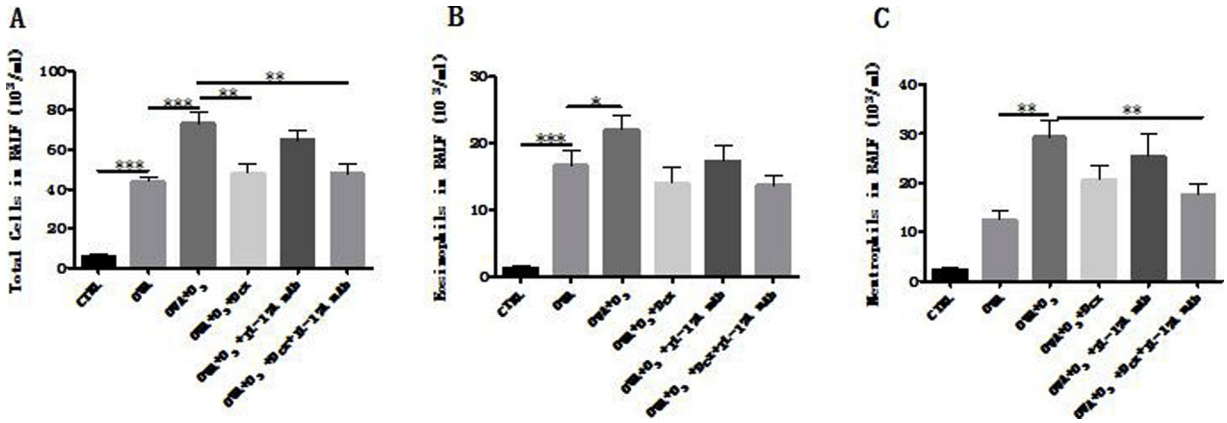

Fig. 1. Mean numbers of total cells (A), eosinophils (EOS) (B) and neutrophils (NEU) (C) in BALF for the six experimental groups. Data are expressed as means ± SEM for n = 8 for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Dex, dexamethasone; O_3, ozone; OVA, ovalbumin.

$P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of IL-17A mAb on BALF cells

BALF was collected 24 h after the last administration of all the animals. OVA sensitization and challenge provoked a significant increase in total cell counts in BALF, including increased eosinophils compared with control mice ($P < 0.001$ and $P < 0.001$, respectively, Fig. 1A, B), but except neutrophils (Fig. 1C). Exposure to O_3 induced further significant increases in total cell counts, as reflected by neutrophils and eosinophils, compared with the numbers of those after only OVA-challenged controls ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively, Fig. 1A, B, C). In OVA-challenged and O_3-exposed mice, dexamethasone (Dex) treatment significantly decreased total cell counts, but did not affect neutrophils or eosinophils, and the concurrent treatment with Dex and IL-17A mAb significantly reduced total cell counts, and the number of neutrophils, while the monotherapy of IL-17A mAb did not affect the numbers of all the cell counts, neutrophils or eosinophils.

3.2. Effects of IL-17A mAb on IgE and cytokines in BALF and serum

OVA challenge caused an increase in IL-5, IL-13, IL-8, IL-17A, and TNF-αin BALF, IL-13, IL-17A, and IgE in serum, compared the control mice (Fig. 2). But O_3 exposure further enhanced only part of cytokines, such as IL-17A in BALF and serum, TNF-α in BALF (Fig. 2). In OVA-challenged and O_3-exposed mice, IL-17A mAb treatment decreased the levels of IL-5, IL-13, IL-8, IL-17A in BALF and IL-17A in serum (Fig. 2). Dex treatment reduced part of cytokines, including IL-5, IL-13, IL-17A, and TNF-αin BALF and IL-13, IL-17A and IgE in serum, but combination treatment attenuated all the parameters except IL-8 in serum (Fig. 2), suggesting that Dex and IL-17A mAb showed synergistic effects in regulating the above parameters.

Furthermore, we also measured the levels of IFN-γ, one of the Th1-driven cytokines. OVA challenge caused an decrease in the levels of IFN-γ, O_3 exposure further reduced the levels of IFN-γin OVA-challenged mice (Fig.2). IL-17A mAb treatment and combination treatment increased the levels of IFN-γin OVA-challenged mice. We also found that combination treatment significantly enhanced IFN-γlevels in BALF compared with Dex treatment only.

3.3. Effects of IL-17A mAb on lung histology

OVA challenge caused an increase in inflammation scores with peribronchial and perivascular inflammatory cell infiltrates in lung sections compared with control mice ($P < 0.01$, Fig. 3B), and OVA challenge combined with O_3 exposure caused no significant increase in inflammation scores compared with OVA challenge alone. The treatment with IL-17A mAb significantly reduced inflammation scores in OVA-challenged and O3-exposed mice ($P < 0.001$, Fig. 3B). The treatment with both Dex and IL-17A mAb significantly reduced inflammation scores in OVA-challenged and O3-exposed mice compared with no treatment or Dex alone ($P < 0.001$, $P < 0.05$, respectively, Fig. 3B). Representative photomicrographs in Fig. 3A also showed different inflammatory scores occurring in different administration mice.

3.4. Effects of IL-17A mAb on IL-17A mRNA and protein levels in lung tissue

Both OVA challenge and combination of OVA challenge and O_3 exposure increased IL-17A mRNA expression, compared with the control mice ($P < 0.001$ and $P < 0.001$, respectively, Fig. 4A), but there was no significant difference between OVA challenge mice and combination of OVA challenge and O_3 exposure mice in the expression of IL-17A mRNA. In OVA-challenged and O_3-exposed mice, mice with IL-17A mAb or combination treatment showed a lower level of IL-17A mRNA expression ($P < 0.01$ and $P < 0.001$, respectively, Fig. 4A), and combination treatment significantly reduced the expression of IL-17A mRNA ($P < 0.01$, Fig. 4A).

OVA challenge alone significantly up-regulated p38 MAPK phosphorylation compared with control mice ($P < 0.001$, Fig. 4B). The p38 MAPK phosphorylation was further increased in OVA-challenged and O_3-exposed mice, compared with only OVA-challenged mice ($P < 0.05$, Fig. 4B). In OVA-challenged and O_3-exposed mice, mice with IL-17A mAb or Dex or combination treatment showed a lower level of p38 MAPK phosphorylation, ($P < 0.05$, $P < 0.05$ and $P < 0.05$, respectively, Fig. 4B), but there was no significant difference among all the treatment groups.

OVA challenge alone did not down-regulate the expression of GR compared with control mice (Fig. 4C). While O_3 exposure and OVA challenge down-regulated the expression of GR compared with no administration or OVA challenge alone ($P < 0.001$, $P < 0.05$, respectively, Fig. 4C). IL-17A mAb treatment alone and combination of Dex and IL-17A mAb inhibited this down-regulation of GR in OVA-challenged and O3-exposed mice ($P < 0.01$, $P < 0.001$, respectively, Fig. 4C). But Dex treatment alone did not prevent down-regulation of GR in OVA-challenged and O3-exposed mice (Fig. 4C).

4. Disscussion

We demonstrate that O_3 exposure of OVA-challenged mice leads to enhanced recruitment of inflammatory cells in BALF and increases

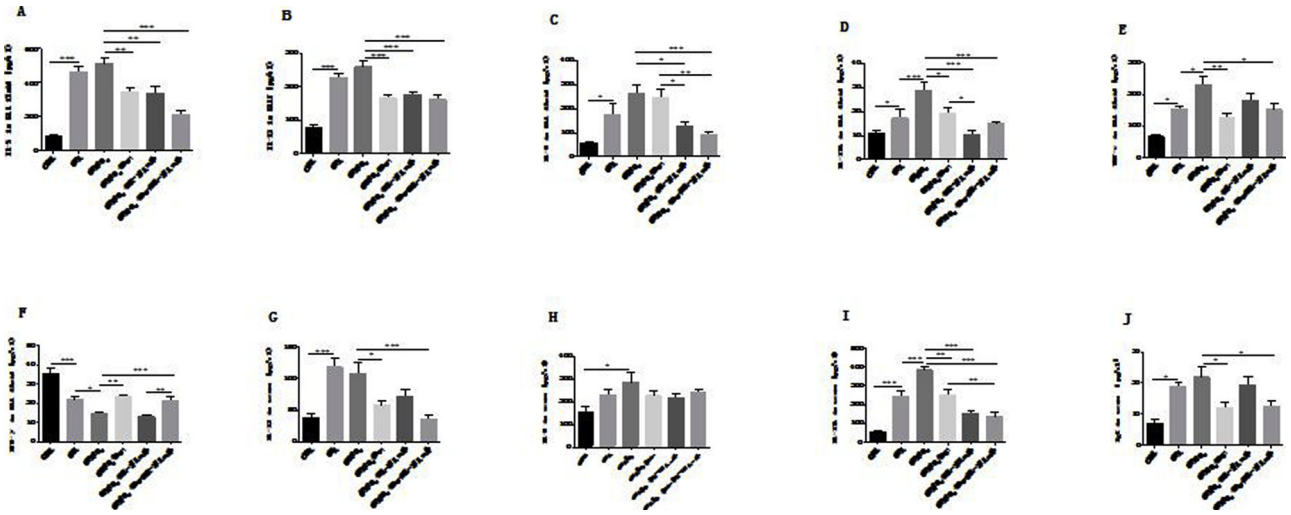

Fig. 2. Cytokine levels in BALF and serum. Mean Levels of IL-5 (A), IL-13 (B), IL-8 (C), IL-17A (D), TNF-α (E) and IFN-γ(F) in BALF, IL-13 (G), IL-8 (H), IL-17A (I) and IgE (J) in serum of the six groups. Data are expressed as means ± SEM for n = 8 for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Dex, dexamethasone; O_3, ozone; OVA, ovalbumin.

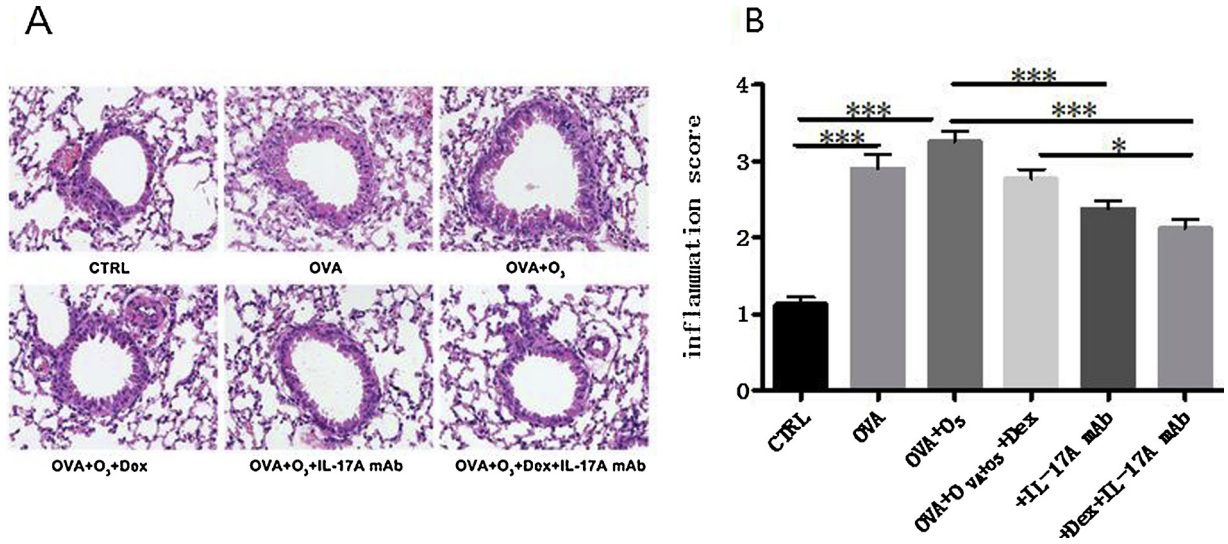

Fig. 3. Representative photomicrographs of haematoxylin and eosin (H&E) staining of lung sections from the corresponding groups (original magnification × 400) (A). Mean values of inflammation score in the airways measured from H&E-stained sections (B). Data are expressed as means ± SEM for n = 8 for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Dex, dexamethasone; O_3, ozone; OVA, ovalbumin.

inflammation scores. It also causes an increase in levels of inflammatory cytokines such as IL-5, IL-13 and IL-8, activation of p38 MAPK and down regulation of GR. Treatment with IL-17A mAb only partially attenuates lung inflammation, while combination treatment with corticosteroid and IL-17A mAb effectively reduces lung inflammation, inactivates p38 MAPK and up regulates GR in lung tissues synergistically. The corticosteriod on its own has a weak effect. Thus, these results highlight that IL-17A mAb could suppress OVA-challenged and -sensitized and O_3-exposed asthma inflammation and partially restored corticosteroid sensitivity via up-regulating GR expression and that the use of IL-17A mAb might be a potential therapeutic approach to severe asthma.

As a direct oxidant, O_3 can induce oxidative stress, which adversely affects human health by irritating the mucosa and harming the respiratory system [24], and has been implicated in the development of asthma.

It is increasingly being recognized that the generation of reactive oxygen species (ROS) from O_3 exposure could augment allergic airway inflammation [25,26] and could also inhibit the maturation of dendritic cells thereby interfering in Th1-promoting response pathways and then altering the balance of Th1/Th2, which is a central mechanism in asthma initiation and development [27]. In our experiment, we detected the production levels of Th1-driven cytokine IFN-γ in BALF, however, we observed that in OVA-challenged plus ozone-exposed mice, there is no significant effect of IL-17A mAb on the BALF concentration of IFN-γ. But surprisingly, combination treatment of IL-17A mAb and corticosteroid enhanced the concentrations of IFN-γ in BALF compared with the experimental mice. The results demonstrated that IL-17A mAb attenuated asthma inflammation by partly inhibiting certain inflammatory cytokines but except promoting IFN-γ.

IL-17A has also been investigated in the development of asthma. Previous studies have provided results regarding the role of IL-17A in asthma. IL-17A is known to play an important role in activating T cells [28], enhancing neutrophil accumulation, and inducing IL-6, and TNF-α production, which increase airway inflammation [29].

We found that OVA challenge caused increases in numbers of eosinophils and neutrophils, and enhancement in levels of IL-5, IL-8 and TNF-α. OVA challenge combined with O_3 exposure cause further

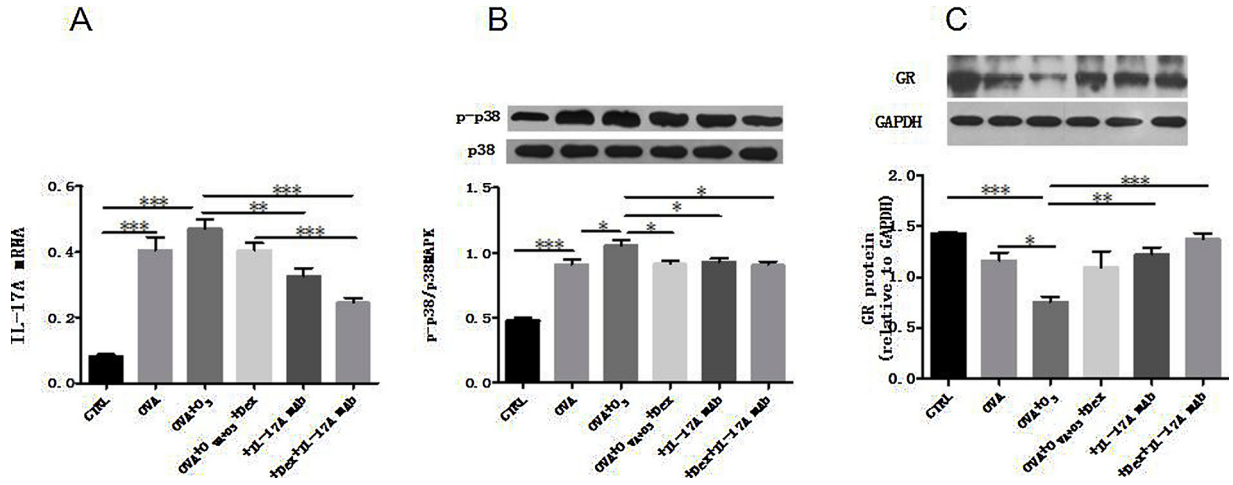

Fig. 4. Mean expression of IL-17A (A) in lung tissue measured by quantitative RT-PCR and western blot analysis of ratio of phosphorylated p38 mitogen-activated protein kinase (MAPK) to total p38 MAPK (B), phosphorylated GR to total GR (C) in lung tissue from the six groups. Each panel shows representative Western blots of phosphorylated p38 MAPK and total p38 MAPK, phosphorylated GR and total GR. Data are expressed as means ± SEM for n = 8 for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Dex, dexamethasone; O_3, ozone; OVA, ovalbumin.

increases in IL-5, IL-8 and TNF-α, and increases in IL-17A. TNF-α could increase the contractile response in airway smooth muscle, thus playing a role in AHR of asthma, and mediate the recruitment of neutrophils and eosinophils into the airways in antigen-driven airway inflammation in mice [30]. IL-5 plays an essential role in airway remodeling formation and regulates the accumulation, migration, and prolonged survival of eosinophils in the lung [31,32]. IL-13 overexpression not only induces AHR in animal models of asthma but also results in IgE production, inflammation and eosinophilia. Several previous studies have demonstrated that inhibiting IL-13 revealed significantly decreased airway inflammation, and airway responsiveness in mouse models of asthma [33]. In addition, blocking antibodies to IL-8 and related chemokines inhibits certain types of neutrophilic inflammation in experimental animals [34]. Our results suggested that IgE level was not reduced by IL-17A mAb, but was inhibited by Dex, indicating IgE production not mainly resulted from the increased IL-13 overexpression. From Fig.2, we also observed that IL-17A mAb did not regulate inflammatory cytokines except IL-17A in peripheral blood, suggesting that IL-17A mAb may partly act on airway-targeted inflammation, but not in systemic inflammation.

As a clinical feature of severe asthma, glucocorticoids insensitivity is characterized by the reduced effect of Dex in inhibiting inflammatory cytokines release in our study. Glucocorticoids insensitivity may also due to the reduced numbers and the attenuated activation of glucocorticoids receptors (GR). Besides, the involvement of p38MAPK is associated with the ozone exposure on the response to glucocorticoid in a mouse model of asthma and inhibition on the phosphorylation of p38MAPK could improve the response to glucocorticoids, and reverse the airway inflammation [35]. Moreover, IL-17A induces glucocorticoid insensitivity in human bronchial epithelial cells [36]. Increased phosphorylation of p38 MAPK has been observed in allergen or O_3-induced airway inflammation [37,38]. In addition to directly activating transcription factors and inducing phosphorylation of other kinases, recently, p38 MAPK has been shown to play a critical role in inflammatory cytokine production and that down-regulation of p38 MAPK expression attenuates asthma in mice [39–41]. p38 MAPK activation may lead to a reduction in corticosteroid actions through down-phosphorylation of GR, IL-17A mAb may up-regulate GR expression in O_3-exposed inflammation [20], and this were also confirmed in our present experiment of OVA-challenged and O_3-exposed asthma.

In current study, while corticosteroids, the cornerstone of asthma therapy, failed to inhibit airway inflammation, or block the phosphorylation of p38 MAPK or down-regulate IL-17A mRNA expression when these OVA-challenged mice were additionally exposed to O_3, indicating that O_3 exposure of OVA-challenged mice may lead to glucocorticoids insensitivity. The inflammatory effects of OVA challenge and O_3 exposure, however, were inhibited by a combination of corticosteroids and IL-17A mAb. This effect showed a synergistic effect of the combination treatment because monotherapy of Dex or IL-17A mAb on its own had partial effect on airway inflammation. Our data also indicates that IL-17A mAb may restore corticosteroid sensitivity via up-regulating GR expression, demonstrating that IL-17A mAb is a promising new candidate for the treatment of severe asthma.

Disclosures

The authors have no financial or commercial conflicts of interest to disclose in relation to publication of the manuscript.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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