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Silencing IL-23 expression by a small hairpin RNA protects against asthma in mice

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Abbreviations: Alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CIA, collagen-induced arthritis; DAB, diaminobenzidine; EAE, encephalomyelitis; HE, hematoxylin and eosin; IBD, inflammatory bowel disease; OVA, ovalbumin; RT-PCR, reverse transcription polymerase chain reaction; RNAi, RNA interference; VCAM-1, vascular cell adhesion molecule; VLA4, very late antigen-4

Abstract

To determine the impact of IL-23 knockdown by RNA interference on the development and severity of ovalbumin (OVA)-induced asthmatic inflammation, and the potential mechanisms in mice, the IL-23-specific RNAi-expressing pSRZsi-IL-23p19 plasmid was constructed and inhaled into OVA-sensitized mice before each challenge, as compared with that of control mice treated with alum or budesonide. Inhalation of the pSRZsi-IL-23p19, significantly reduced the levels of OVA-challenge induced IL-23 in the lung tissues by nearly 75%, determined by RT-PCR. In addition, knockdown of IL-23 expression dramatically reduced the numbers of eosinophils and neutrophils in BALF and mitigated inflammation in the lungs of asthmatic mice. Furthermore, knockdown of IL-23 expression significantly decreased the levels of serum IgE, IL-23, IL-17, and IL-4, but not IFNγ, and its anti-inflammatory effects were similar to or better than that of treatment with budesonide in asthmatic mice. Our data support the notion that IL-23 and associated Th17 responses contribute to the pathogenic process of bronchial asthma. Knockdown of IL-23 by RNAi effectively inhibits asthmatic inflammation, which is associated with mitigating the production of IL-17 and IL-4 in asthmatic mice.

Keywords: asthma; interleukin-23; mice; ovalbumin; RNA interference

Introduction

Bronchial asthma is a serious chronic illness with variable clinical symptoms. Because of increased environmental pollution, the incidence of bronchial asthma is increasing worldwide (Eder *et al.*, 2006). Pathogenic studies have revealed that bronchial asthma is characterized by leukocyte infiltration in the bronchial tissues, excessive mucus production, epithelial damage, basement membrane thickening, and smooth muscle hypertrophy in airway epitheial tissues (Barnes, 1989; Boushey and Fahy, 1995). Although many therpaeutic strategies have been utilized for the management of asthmatic patients, the efficacy of these therapies is limited. Hence, development of new effective and safe therapies for the treatment of human asthma will be of great significance.

Interleukin (IL)-23 is a member of the IL-12 heterodimeric cytokine family. IL-23 is composed of p19 and p40, a subunit of IL-12 (Oppmann *et al.*, 2000). IL-23 is a growth factor and inducer of pro-inflammatory Th17 cells, which secrete IL-17 (Aggarwal *et al.*, 2003). Indeed, IL-23 and Th17 cells are critical players in the development of autoimmune diseases, such as experimental allergic encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) (McKenzie *et al.*, 2006). Notably, high levels of serum IL-23p19 and IL-17 are detected in various human lung diseases, including asthma and chronic obstructive pulmonary disease (Barczyk *et al.*, 2003; McAllister *et al.*, 2005). Apparently, IL-23 is a pathogenic factor, participating in the pathogenic process of human asthma. Conceivably, we hypothesized that down-regualtion of IL-23 expre-

ssion might reduce airway inflammation and mitigate the severity of allergen-induced asthma.

Sensitization and aerosol challenge of mice with ovalbumin (OVA) has been widely used as a mouse model of bronchial asthma (Braun *et al.*, 1998; Hopfenspirger *et al.*, 2001; Nakanishi *et al.*, 2001). In the present study, we first generated a new recombinant plasmid, IL-pSRZsi-IL-23p19, that expressed the IL-23p19-specific shRNA. Subsequently, we employed a mouse OVA bronchial asthma model to test whether treatment with IL-pSRZsi-IL-23p19 for the knockdown of IL-23 expression *in vivo* could modulate OVA-induced bronchial inflammation in mice. We found that treatment with the IL-23p19-specific shRNA significantly reduced the OVA-induced pulmonary inflammation in mice. We discussed the implications of our findings.

Results

Knockdown of IL-23 expression by the IL-23-specific shRANA-expressing plasmid *in vivo*

To determine the effect of IL-23 silencing *in vivo*, we constructed a plasmid of pSRZSi-IL-23p19 that expressed the IL-23-specific shRNA. Next, we tested the efficacy of IL-23 knockdown in mouse model of bronchial asthma by sensitizing and challenging BALB/c mice with OVA (Figure 1). We found that groups of mice that had been treated with the pSRZSi-IL-23p19 plasmid in Lipofectamine 2000 at 48 h post the last treatment displayed the expression of EGFP in the lung tissues (Figure 2A). Similar patterns of EGFP expression were observed in the mice that had been treated with the plasmid at 24 or 72 h post the last treatment, respectively (data not shown). Characterization of the relative levels of IL-23 mRNA transcripts in the lung tissues of different groups of mice revealed that OVA-sensitization and challenges induced significantly higher levels of IL-23 transcription in the lungs, as compared with that in the alum-treated alone controls, ($P < 0.01$, Figures 2B and 2C), and treatment with budesonide inhibited the expression of IL-23 by nearly 65% ($P < 0.01$). Notably, treatment with the pSRZSi-IL-23p19, but not empty vector, also greatly reduced the levels of IL-23 mRNA transcripts in the lungs, and the levels of IL-23 mRNA transcripts in the lungs of mice at 48, and 72 h post the last treatment were significantly lower than that of the budesonide-treated mice ($P < 0.01$). Similar patterns of IL-23 proteins were observed in the lungs of different groups of mice (Figures 2D and E). As shown in Figure 2D, increased IL-23-immunostaining (dark brown) was found in the bronchial epithelial cells, alveolar epethelial cells, and bronchopulmonary capillary endothelial cells of the lung tissues in the OVA-challenged or empty vector-treated groups of mice. The numbers of IL-23-positive cells in the lung tissues were reduced in the budesonide-treated and IL-23 silencing mice. Given that IL-23 is a critical regulator of the development of Th17 cells (Ivanov *et al.*, 2007), we characterized the expression of IL-17 and found that the relative levels of IL-17 mRNA transcripts in the lungs of the mice treated with budesonide or with the pSRZSi-IL-23p19 were significantly reduced, particularly for the mice treated with the pSRZSi-IL-23p19 for 48 and 72 h (Figures 2B and 2C). Therefore, treatment with the pSRZSi-IL-23p19 not only silenced the expression of IL-23, but also reduced the expression of IL-17 in the mouse model of bronchial asthma. Accordingly, we therefore chose the time point of 48 hr after the final challange for the following experiments.

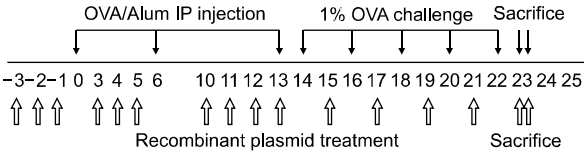

Figure 1. Schematic illustration of the experimental protocol. IP, Intraperitoneal injection.

Knockdown of IL-23 reduces the OVA-induced pulmonary inflammatory

Allergen-specific IgE and inflammatory eosinophils and neutrophils have been thought to be crucial mediators for the development of bronchial asthma. We next examined the impact of IL-23 silencing on the levels of serum OVA-specific IgE and eosinophilic and neutrophilic infiltrates in the BALF of different groups of mice. As expected, OVA-challenge induced significantly higher levels of OVA-specific IgE responses, as compared with that of alum control, while treatment with budesonide or with the pSRZSi-IL-23p19, but not with empty vector, dramatically inhibited the IgE responses (Figure 3A). Similarly, treatment with budesonide or with the pSRZSi-IL-23p19 also greatly inhibited the infiltration of inflammatory eosinophils and neutrophils in the lungs (Figures 3B and 3C). Evidentially, the numbers of eosinophils and neutrophils in the BALF from the mice that had been treated with budesonide or with the pSRZSi-IL-23p19 were significantly reduced, and knockdown of IL-23 expression had a stronger inhibition on the infiltration of neutrophils than that of treatment with

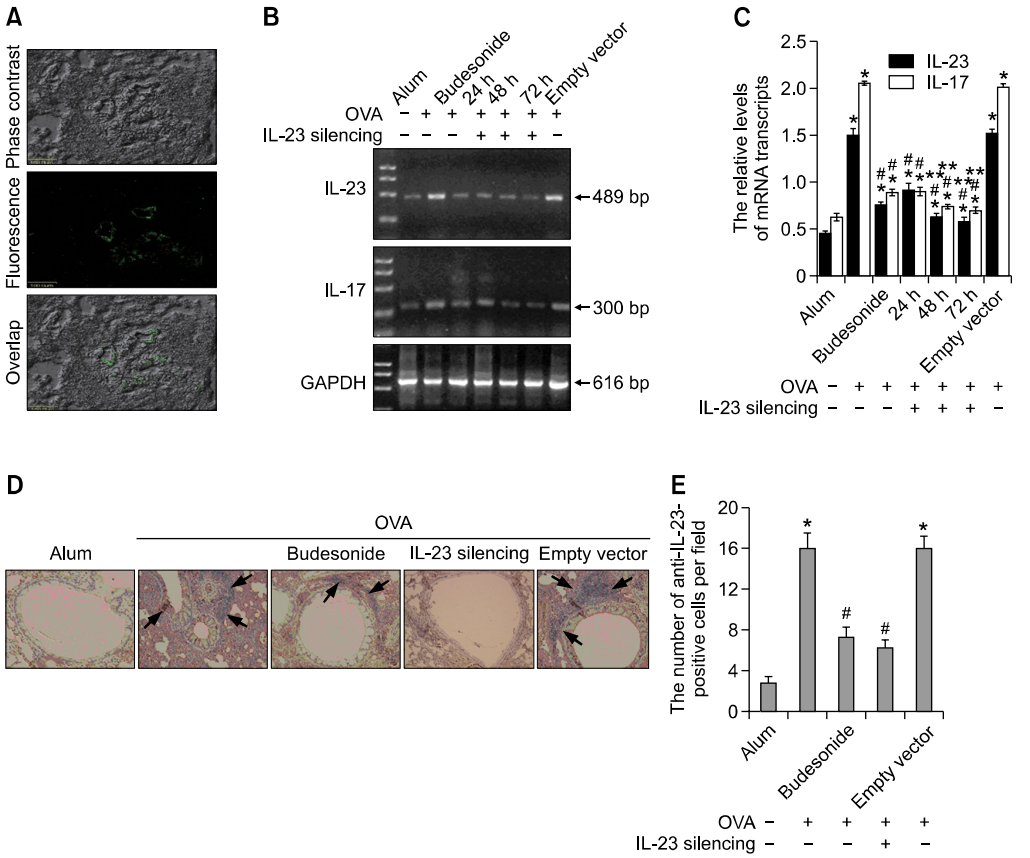

Figure 2. Knockdown of IL-23 expression. Groups of BALB/c mice were sensitized with OVA and treated with the IL-pSRZsi-IL-23p19 before sensitization and challenge, and the transfection efficiency in the lung tissues was examined under a fluorescent microscope (A). (B) The relative levels of IL-23 and IL-17 mRNA transcripts were determined by RT-PCR. Data shown are representative images of each group of mice (n = 10) from three separate experiments. M, DL2000 DNA marker. (C) Quantification of the relative levels of IL-23 and IL-17 mRNA trascripts to control GAPDH. Data are expressed as mean ± SD of individual groups of mice. (D) Charaterization of IL-23 expressing cells in the lung tissues by immunohistochemistry using an anti-IL-23 antibody. Data shown are representative images (×200) of each group. IL-23$^+$ cells were indicated by arrows. (E) Quantitative analysis of IL-23 expressing cells in the lung tissues by immunohistochemistry. Data are expressed as mean ± SD of each field in individual groups of mice. Ten fields from 3 sections of individual mice were counted. *$P < 0.01$ compared with Alum control; $^{\#}P < 0.01$ compared with OVA-challenged model group; **$P < 0.01$ compared with budesonide pespules-treated positive control.

budesonide. Characterization of inflammation in the lungs of different groups of mice indicated that OVA-challenge induced leukocyte infiltration in the bronchial tissues, excessive mucus production, epithelial damage, basement membrane thickening, and smooth muscle hypertrophy, accompanied by goblet cell proliferation and congestion (Figure 4A). Treatment with budesonide or with the pSRZSi-IL-23p19, but not with empty vector, dramatically reduced the OVA-challenge induced inflammation in the lungs (Figure 4B). Collectively, our data indicated that knockdown of IL-23 expression significantly mitigated the OVA-induced pulmonary inflammation in mice.

Effects of IL-23 silencing on the levels of serum cytokines in OVA-sensitized and -challenged mice

Antigen-specific Th2, Th17, and Th1 responses regulate the development of allergen-induced bronchial asthma. To evaluate the effect of IL-23 silencing on the development of systemic inflammation, we examined the levels of serum cytokines by ELISA assays. As shown in Figure 5, the levels of serum IL-23, IL-17, and IL-4 were elevated significantly, but the levels of IFNγ were reduced in the OVA-sensitized and challenged mice and the mice that had been treated with empty vector. In contrast, treatment with budesonide significantly reduced the levels of serum IL-23, IL-17, and IL-4, but increased the levels of serum IFNγ. Furthermore, treatment with the pSRZSi-IL-23p19 also

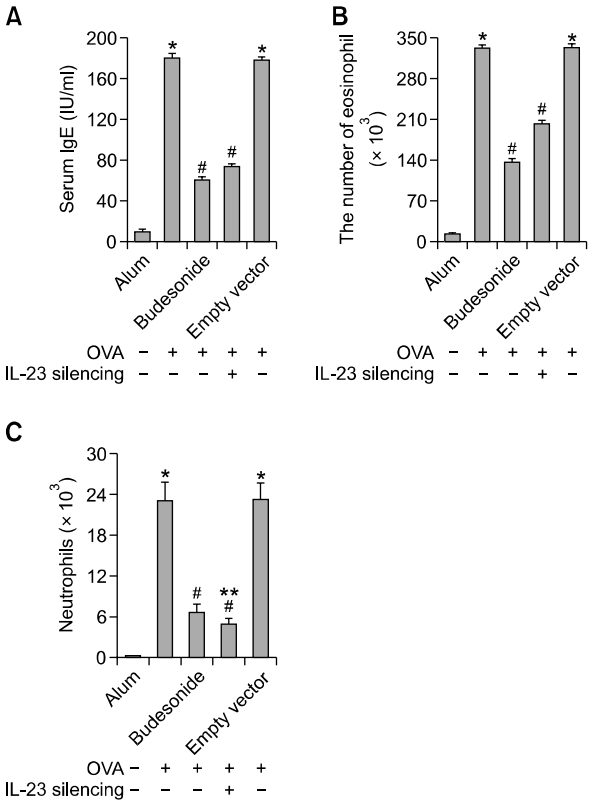

Figure 3. Knockdown of IL-23 expression mitigates the OVA-induced pulmonary inflammatory in asthmatic mice. The numbers of eosinophilic and neutrophilic infiltrates in BALF were characterized under a light microscope, and the levels of serum IgE were determined by ELISA. (A) The levels of serum IgE. (B) The number of eosinophils in BALF. (C) The number of neutrophils in BALF. Data are expressed as mean ± SD of each group (n = 10 per group). $^{*}P < 0.01$ compared with Alum control; $^{\#}P < 0.01$ compared with OVA-challenged model group; $^{**}P < 0.01$ compared with budesonide pespules-treated positive control.

significantly decreased the levels of serum IL-23, IL-17, and IL-4, but had no effect on the levels of serum IFNγ. In addition, the levels of serum IL-23 were positively correlated with that of IL-17 in these mice ($r = 0.92$, $P < 0.01$). Apparently, knockdown of IL-23 expression not only down-regulated systemic Th17 responses, but also modulated systemic Th2 responses in our experimental system, contributing to the therapeutic effect of IL-23 silencing on the OVA-induced bronchial asthma in mice.

Discussion

In the current study, we employed the OVA-based mouse model of bronchial asthma to investigate the impact of IL-23 silencing on the development of OVA-induced pulmonary and to determine potential mechanisms underlying the effect of IL-23 knockdown on inflmmatory cytokine production in mice. We found that knockdown of IL-23 expression significantly mitigated the OVA-induced pulmonary inflammation, accompanied by significantly reduced expression of IL-17 and IL-4, indicating that IL-23 contributed to the pathogenic process of allergic bronchial inflammation in mice.

IL-23 is crucial for the development of Th17 response (Aggarwal *et al.*, 2003), and IL-23 and Th17 cells have been found to be pathogenic factors in the development of autoimmune diseases (McKenzie *et al.*, 2006). Previous studies have found high levels of serum IL-23 and IL-17 in patients with asthma and chronic obstructive pulmonary disease (Barczyk *et al.*, 2003; McAllister *et al.*, 2005). In this study, we found that challenge with OVA resulted in a 3-fold increased levels of IL-23 expression in the lung tissues (Figures 2B-E). Our data are consistent with a previous report [14] and suggest that IL-23 may participate in the asthmatic process in this model. To determine the role of IL-23 in the asthmatic process, we specifically silenced the expression of IL-23 by treating the mice with the IL-23 siRNA-expressing plasmid. We found that inhalation of the plasmid significantly inhibited the IL-23 mRNA transcription in a time-dependent manner and dramatically reduced the number of IL-23 expressing cells in the lung tissues of asthmatic mice. These novel data clearly demonstrated that inhalation of a plasmid that expressed the IL-23-sepcific siRNA effectively silenced the expression of IL-23 for at least three days in the target lung tissues of asthmatic mice. We are interested in further examining the dynamics of IL-23 expression in the target tissues of asthmatic mice following treatment with the plasmid-mediated IL-23 siRNA.

Eosinophilic and neutrophilic infiltration in the bronchial tissues is a hallmark of bronchial asthma-related airway inflammation and regulated by a variety of factors, such as cytokines IL-4, IL-5, IL-13, vascular cell adhesion molecule (VCAM-1), and very late antigen-4 (VLA4) (Koo *et al.*, 2003). Neutrophilic infiltration is related to the pathogenic process of chronic obstructive pulmonary disease (COPD) (Thompson *et al.*, 1989). Indeed, neutrophils are detected in sputum from asthmatic patients, particularly for those with acute exacerbation (Fahy *et al.*, 1994). Our previous study showed massive infiltration of eosinophils in the lungs of asthmatic IL-23R transgenic mice, while significantly reduced airway inflammation in asthmatic IL-23$^{-/-}$ mice, which suggest that IL-23 may promote airway inflammation and eosinophil infiltration in the lung of asthmatic mice (Peng *et al.*, 2010). Consistent with this notion, we found a

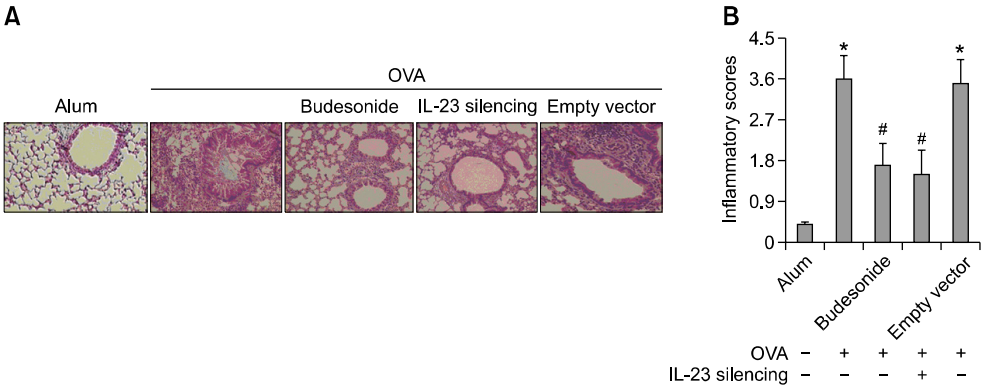

Figure 4. Histopathological analysis of inflammation in the lung of asthmatic mice. (A) HE staining. (B) Inflammatory scores. Data shown are representative images ($\times$200) or mean $\pm$ SD of individual groups of mice. Alum, Mice received alum injection without OVA and used as controls. Other groups of mice were indicated at the top of figure. *P $<$ 0.01 compared with Alum control, $^{\#}P$ $<$ 0.01 compared with OVA-challenged model group.

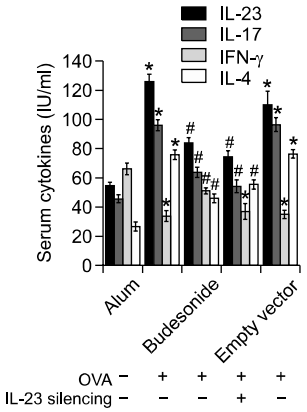

Figure 5. Knockdown of IL-23 expression alters the levels of serum cytokines in asthmatic mice. The levels of serum IL-23, IL-17, IFNγ, and IL-4 were determined by ELISA 48 h after the last OVA challenge. Data are expressed as mean $\pm$ SD of individual groups of mice. *P $<$ 0.01 compared with Alum control, $^{\#}P$ $<$ 0.01 compared with OVA-challenged model group.

dramatically increased number of eosinophilic and neutrophilic infiltrates in BALF collected from the OVA-challenged asthmatic mice. Interestingly, we found that knockdown of IL-23 significantly reduced the number of infiltrated eosinophils and neutrophils in BALF, accompanied by decreased inflammation in the lungs of asthmatic mice and inhibitory effect of IL-23 knockdown on neutrophilic infiltration was stronger than that of treatment with budesonide. Our data are similar with a previous report that treatment with anti-IL-23 antibody decreased antigen-induced eosinophil infiltration in the airway (Wakashin *et al.*, 2008). Apparently, treatment with IL-23 specific siRNA to silence IL-23 is capable of effectively inhibiting inflammation in asthmatic mice. Notably, treatment with anti-p19 only slightly reduces the numbers of neutrophilic infiltrates in the lung of asthmatic mice (Wakashin *et al.*, 2008). The different effects of inhibiting IL-23 may stem from variable experimental protocols and sample sizes. Alternatively, the significant inhibition of neutrophilic inflammation by the IL-23 siRNA is likely attributed to the inhibition of Th17 response in our model as Th17 cells are crucial for neutrophilic inflammation in asthmatic mice (Wakashin *et al.*, 2008). Given that the inhibitory effect of IL-23 silencing on the neutrophilic and eosinophilic infiltration and airway inflammation are similar to or better than that of budesonide treatment, the IL-23 silencing may provide a novel strategy for the control of bronchial asthma. We are interested in further investigating whether treatment with both could synergistically inhibit pulmonary inflammation in asthmatic model.

IL-23 is a critical regulator of the development of Th17 responses, and can enhance Th2 polarization and allergic airway inflammation (Peng *et al.*, 2010). On the other hand, IFNγ, an important Th1 cytokine, can down-regulate the development of Th17 response and antagonize the function of Th2 cells. To understand the potential mechanisms underlying the therapeutic effect of IL-23 silencing, we analyzed the levels of serum cytokines and found that high levels of serum IL-17 and IL-4 were accompanied by reduced levels of serum IFNγ in OVA-challenged asthmatic mice. In contrast, treatment with the plasmid for the IL-23-specific siRNA expression not only significantly reduced the levels of serum IL-23, but also decreased IL-17 in asthmatic mice, and the levels of serum IL-23 were positively correlated with the levels of IL-17. However, knockdown of IL-23 did not significantly modulate the levels of serum IFNγ in these mice. Notably, IL-17 has been suggested to promote inflammatory infiltration (Wakashin *et al.*, 2008). It

is possible that IL-23, through up-regulating the development of Th17 and Th2 responses and IL-17 and IL-4 production, promotes the infiltration of inflammatory cells in the lungs of asthmatic mice. Conceivably, it is likely that inhibition of IL-17 and IL-4 production contributes to the therapeutic effect of IL-23 silencing on the asthmatic inflammation in mice.

Allergen-specific IgE response is an important biomarker for allergic asthma. IgE can trigger mast cells to release asthmatic mediators, such as cytokines, histamine, cysteinyl-leukotrienes, prostaglandin D_2, and tryptase, leading to the pulmonary inflammatory (Peachell, 2005). Ghilardi and colleagues reported that IL-23-deficient mice exhibited an impaired IgE response following allergic challenge (Ghilardi *et al.*, 2004). Consistent with their observation, we found that knockdown of IL-23 expression significantly reduced the levels of serum IgE in asthmatic mice. Given that IL-4 is crucial for the differentiation of allergen-activated IgE-producing B cells and IL-23 can enhance Th2 polarization, knockdown of IL-23 expression may, through down-regulation of IL-4 expression, inhibit IgE response in asthmatic mice.

Notably, budesonide and other corticosteroids have been used for the control of asthma at clinic. We found that treatment with budesonide not only inhibited eosinophilic and neutrophilic infiltration and airway inflammation, but also significantly reduced the levels of serum IFNγ and IL-4 in asthmatic mice. More interestingly, treatment with budesonide decreased the levels of IL-23 expression in the target lung tissues and reduced the levels of serum IL-23 and IL-17 in asthmatic mice. These novel data provide new insights into understanding the pharmacological effect of corticosteroids on the control of bronchial inflammation and asthma at clinic.

In summary, our data suggest that IL-23 contributes to the pathogenic process of baronial asthma through promoting IL-17 secretion and Th2 polarization. Therefore, silencing IL-23 expression by the specific RNAi may provide an efficient therapy for bronchial asthma.

Methods

Reagents

Restriction enzymes *BamH I* and *EcoR I*, *E. coli* DH5α competent cells, DNA Ligation Kits were purchased from TaKaRa Biotechnology. LipofectamineTM 2000 was obtained from Invitrogen, USA. Monoclonal antibody against mouse IL-23 and biotinylated rabbit anti-mouse IgG antibodies were from Santa Cruz Biotechnology. The FITC-conjugated mouse anti-rabbit IgG was obtained from Beijing Boisynthesis Biotechnology. The streptavidin-perosidase and diaminobenzidine (DAB) reagents were purchased from Fuzhou Maxim Company. Murine IL-23 and IL-17 ELISA kits were purchased from SunBio, Beijing and Tianjin Genomapping Technology, respectively. The IFN-γ and IL-4 ELISA kits were provided by Jingmei BioTech, Shenzheng. The serum IgE detection kit was obtained from BioKey, USA.

Plasmid construction

Oligonucleotides coding for siRNA that targeted to mouse IL-23p19 (GenBank accession no. NM031252) were designed. The forward and reverse oligonucleotide primers were annealed and ligated into the linearized retroviral vector RNAi-Ready pSIREN-RetroQ-ZsGreen (Clontech, BD Biosciences). The recombinant pSRZSi-IL-23p19 plasmid was double-digested with *BamH I* and *EcoR I* restriction enzymes and characterized by 2% agarose gel electrophoresis. The recombinant plasmid pSRZsi-IL-23p19 was further confirmed by sequencing (Applied Biosystem). After that, the identified plasmid was transformed into *E. coli* and purified with TaKaRa MiNiBEST Plasmid Purification Kit, according to the manufacturers' instruction (TaKaRa Biotechnology).

Mice and induction of asthma

The experimental protocol was approved by the Ethic Committee of our university. Female BALB/c mice at 6-8 weeks of age, weighing 18-22 g, were obtained from the Center of Laboratory Animals, School of Basic Medical Sciences, Jilin University, and maintained at a specific pathogen free facility with a constant humidity and temperature at 12 h/12 h light/dark cycle with free access to food and water. To induce bronchial asthma, the mice were randomized and sensitized by intraperitoneal injection with 10 μg ovalbumin (OVA, grade V, Sigma) and 0.2 mg aluminum hydroxide (Alum) in 100 μl PBS on day 0, 6, and 13. Two weeks after the first immunization, the mice were challenged with aerosolized 1% OVA for 30 min every other day for 5 times over the course of 9 days. Sham groups of mice were injected intraperitoneally with 0.2 mg Alum alone in 100 μl PBS on Day 0, 3, and 6, and were challenged aerosolized 1% OVA for 30 min every other day for 5 times. Another group of mice were inhaled with 2 ml of 50% (1 mg) budesonide pespules (AstraZeneca) for 30 min at 1 h prior to 1% OVA challenges and used as the positive therapeutic controls. The experimental and empty vector groups of mice were inhaled daily with 100 μl of the mixture of 4 μg plasmid or vector DNA and 8 μl Lipofectamine 2000 in 492 μl DMEM for three consecutive days before every sensitization with OVA/Alum. The mice were treated nasally with the same amount of plasmid transfection complex one day prior to 1% OVA challenge. The experimental protocol and manipulation schedule for different groups of mice (n = 10 per group) are illustrated in Figure 1. Twenty-four hours after the final challenge, blood samples were obtained from individual mice for the determination of serum cytokines, and the mice were sacrificed. Their left lungs were collected and fixed in 10%

formalin for histological examination. The right lungs of individual mice were frozen at -80°C for the determination of the relative levels of cytokine mRNA transcripts. The transfection efficiency was determined by visualization of the green fluorescence in the crystal lung sections under a confocal microscope (Olympus, Japan).

RT-PCR

The relative levels of IL-23 and IL-17 mRNA transcripts in the lung tissues were examined by RT-PCR, using the specific primers (Supplemental data Table S1) on a PTC-100TM thermocycler (MJ. Research). Total RNA was extracted from individual lung tissues by conventional technology, reversely transcribed into cDNA and used as the templates. The PCR reactions were performed in duplicate at 94°C for 3 min, and subjected to 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s, followed by at 72°C for 5 min. The GAPDH was used as an internal control with a program of 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and 72°C for 5 min. The amplified products were characterized by 1.5% agarose gel electrophoresis and imaged using the gel imaging system (Kodak Digotal Science ID).

Immunohistological and immunohistochemistric analysis

The formalin-fixed and paraffin-embedded left lung tissues were sectioned at 5 μM and immunostained after deparaffinization and rehydration. The tissue sections were subjected to antigen retrieval and treated with endogenous peroxidase blocking solution. Subsequently, the tissue sections were blocked with goat serum and probed with anti-IL-23 monoclonal antibody or isotype control IgG (4 μg/ml) overnight at 4°C. After washing, the sections were incubated with biotinylated rabbit anti-mouse IgG at room temperature for 20 min and the bound antibodies were detected by peroxidase-conjugated streptavidin, followed by visualizing with DAB. Individual cells with yellow-brown-staining membrane and/or cytoplasm were recognized as positive immunostaining cells. A total of 10 fields were randomly selected from 5 sections of a single mouse under a microscope, and the average number of IL-23-positive cells in individual mice was calculated.

In addition, some of the lung tissue sections were stained with hematoxylin and eosin (HE) for characterizing inflammatory infiltrates. The inflammatory scores were graded using a 0-4 grade scoring system (0: no inflammation; 1: mild inflammation; 2: moderate inflammation; 3: severe inflammation; and 4: extreme inflammation), as described previously (Henderson *et al.*, 2002).

Characterization of lung morphology and leukocytes in blood, tissue, and bronchoalveolar lavage fluid (BALF)

The BALF was collected from the lungs of individual mice by washing the lungs three times with 0.8 ml of Ca^{2+}- and Mg^{2+}-free cold PBS supplemented with 0.1% BSA and 0.05 mM EDTA. After that, the BALF was centrifuged, and the contained cells were stained with Wright-Giemsa. The frequency of eosinophils in individual BALF samples was counted with a hemocytometer. The frequency of neutrophils was determined in the HE-stained sections. The numbers of eosinophils and neutrophils were expressed over the 1,000 cells in each field, and at least four fields under a phase contrast microscope were recorded for data analysis.

ELISA

Individual sera were prepared by centrifugation and the levels of serum IL-23, IL-17, IL-4, IFN-γ, and IgE were determined by ELISA using the specific kits, according to the manufacturers' instructions.

Statistical analysis

Data are presented as mean ± SD. The difference among different groups was determined by ANOVA and between the two groups was analyzed by F-test or q test. Statistical analyses were performed using SPSS 16.0 statistics software (SPSS Inc., Chicago, IL). A P value of < 0.01 was considered statistically significant.

Supplemental data

Supplemental data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-4-04.pdf.

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