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Original Article

YKL-40 siRNA downregulates the expression of eotaxin, IL-5, GM-CSF in an epithelial cell model of asthma

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Abstract: Background: YKL-40 is highly expressed in airway inflammation and remodeling, and higher YKL-40 levels are detected in patients with inflammatory diseases. YKL-40 may participate in the mechanism of inflammatory mediators that regulate eosinophil-mediated airway inflammation and contribute to the pathogenesis of asthma. In the epithelium, YKL-40 could modulate the expression of eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-5, and the latter played a important role in eosinophilic airway inflammation. Objectives: YKL-40 could be an attractive target in the design of asthma therapies. Methods: Mouse airway epithelial cells were isolated and identified by immunofluorescent staining. The primary mouse tracheal epithelial cells were cultured with OVA for 48 h and transfected with YKL-40 siRNA. The relative levels of YKL-40, IL-5, GM-CSF, and eotaxin mRNA transcripts were determined by semi-quantitative RT-PCR, and their proteins were determined by ELISA and Western blot assays, respectively. Results: The YKL-40 protein levers in cells transfected with siRNA-YKL-40 (YKL-40-si) were decreased by 70% compared with the levels in primary mouse tracheal epithelial cells transfected with siRNA-negative control (YKL-40-NC) or non-transfected epithelial cells (YKL-40-0) by Western blotting. The levels of eotaxin, GM-CSF, IL-5 mRNA and protein in cells transfected with siRNA-YKL-40 (YKL-40-si) were markedly decreased compared with those in cells transfected with siRNA-negative control (YKL-40-NC) or non-transfected epithelial cells (YKL-40-0). Conclusions: This finding could extend the prospective use of YKL-40 siRNA in asthma research and enlarge the armamentarium for treating asthma.

Keywords: Asthma, eotaxin, epithelium, GM-CSF, IL-5, YKL-40

Introduction

Asthma is an allergic inflammation disease characterized by chronic airway eosinophilia and is attributed to pulmonary infiltration of lymphocytes, particularly of the T helper 2 (Th_2) subtype, macrophages and mast cells. Currently, the pathogenesis of asthma is not fully understood, and the quality of life of patients with asthma has remained essentially unchanged over the last several decades. Controlling asthma remains a challenge in many patients.

The allergen-stimulated airway epithelium secretes various cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-5, and eotaxin, which play a crucial role in eosinophilic airway inflammation [1-3]. IL-5 plays an important role in promoting airway inflammation and is required for the development of airway eosinophilia and immunoglobulin (Ig) E production [4]. Eotaxin, a C-C chemokine, is highly expressed in asthmatic airway epithelium and is pivotal for the recruitment of eosinophils [5, 6]. Thus, it has been proposed that airway eosinophilia, together with the various inflammatory cytokines mentioned above, may contribute to eventual air hyperresponsiveness in asthma [7, 8].

Most recently, several authors have reviewed the field of chitinase-like proteins (CLPs) whose main value resides in being novel biomarkers in asthma [1, 9]. YKL-40, also known as chitinase-3-like protein 1, is a glycoprotein secreted by macrophages, chondrocytes, neutrophils, syno-

vial cells, and epithelial cells [1, 10-13]. In patients with asthma, high levels of serum YKL-40 are associated with an increased risk for the development of asthma and are positively correlated with the severity of asthma and thickness of the sub-epithelial basement membrane [14, 15]. These findings suggest that serum YKL-40 levels are a promising prognostic predictor and potential therapeutic target for asthma patients.

Therefore, this study examined the roles of YKL-40 in epithelial cells. We found that OVA enhanced YKL-40, IL-5, GM-CSF, and eotaxin expression simultaneously in epithelial cells *in vitro*. We defined the important role of YKL-40 interactions *in vitro* in the allergic context.

Several authors have concluded that additional studies using small-interfering RNA (siRNA) technology are required to investigate the potential role of chitinases in the pathogenesis of asthma [16]. Gene silencing by RNA interference is becoming a valuable technique for the experimental knockdown of gene expression in mammalian cells [17]. Therefore, we proposed silencing YKL-40 expression in epithelial cells using small interfering RNA (siRNA), and the present findings confirmed this to be the case. We investigated the expression of YKL-40, eotaxin, GM-CSF, and IL-5 in primary mouse tracheal epithelial cells, and further investigated whether inhibition of eotaxin, GM-CSF, and IL-5 activity using the small interfering (YKL-40 siRNA) approach has immunomodulatory effects in such a model. In this study, we showed that YKL-40 expression knockdown using siRNA markedly attenuated the OVA-induced expression of eotaxin, GM-CSF and IL-5 in epithelial cells, and further that YKL-40 knockdown may lead to the suppression of eosinophilic airway inflammation. These findings suggest that YKL-40 siRNA is effective at preventing the development of allergic asthma.

Materials and methods

Subjects

Female and male 5- to 8-week-old C57BL/6N mice were purchased from the Animal Experiment Center of Nantong University and housed in a conventional animal facility. All experimental protocols were approved by the Ethics Committee for Animal Studies of Nantong University, China.

Cell culture and the siRNA transfection method

Primary airway epithelial cells were cultured and identified, as described previously [18]. The identified mouse tracheal cells were cultured with filtered OVA (200 μg/mL) for 48 h and used in our experiments [19]. For siRNA transfection, these cells were washed and seeded at a concentration of 2×10^5 cells/well and were cultured for 24 h in six-well plates to 70% confluence before transfection. Briefly, Lipofectamine 2000 (Invitrogen) was used to transfect siRNA to a final concentration of 80 nM. Inhibition of gene expression by siRNA was determined after 48 h by Western blot analysis. The sequence of the YKL-40 siRNA (YKL-40-si) used in this study was as follows: 5'-CCACCCUAAUCAAGGAAAUTTAUUUCCUUGAUUAGGGUGGTT-3'. A negative control siRNA was used for negative control transfection (YKL-40-NC). The YKL-40 or negative control siRNA (Invitrogen) was dissolved separately in antibiotic- and serum-free Dulbecco's modified Eagle's medium containing nutrient mixture F-12 (DMEM/F12). The cells were incubated for 6 h, and the medium was subsequently changed to antibiotic- and serum-containing DMEM/F12 medium. After 48 h of transfection, the cells were used in our experiments.

Western blot analysis

The transfected or non-transfected epithelial cells (8×10^5/well) were washed three times, and then harvested, followed by lysis in lysis buffer. After determining the protein concentrations using a NanoPhotometer™ kit (IMPLEN, Westlake Village, CA, USA), the protein lysates (20 μg/lane) from the various groups were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). The membranes were blocked in 5% bovine serum albumin and incubated with a primary antibody against YKL-40 (1:200; Bios, Beijing, China) or β-tubulin (1:5000; Bioworld Technology, St. Louis, MO, USA) for 16 h at 4°C. After washing, bound antibodies were detected using a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000; Beyotime Biotechnology, Nantong, China) and visualized using Ultra enhanced chemiluminescence Western blotting detection

Table 1. Primer sets for reverse transcriptase-polymerase chain reaction analysis

Targets	Sequences	
	Forward (5'-3')	(5'-3')
YKL-40	CAACATCAGCAGCGACAA	CCATAAGAACGCAGGAACG
IL-5	TATGCTTTCTGTTGGCATGG	GACTGTGCCATGACTGTGC
GM-CSF	GCTCACCCATCACTGTCACC	GTCTGGTAGTAGCTGGCTGTC
Eotaxin	GCTTTCAGGGTGCATCTGTT	TTCCATCTGCTCCCTCCA
GAPDH	CCATTTGCAGTGGCAAAG	CACCCCATTTGATGTTAGTG

reagents (Beyotime Biotechnology). The relative levels of YKL-40 to β-tubulin were analyzed by densitometric scanning using the Image J software.

Enzyme-linked immunosorbent assay (ELISA)

The epithelial cells (6×10^5/well) were treated in triplicate with 200 μg/mL OVA (Sigma-Aldrich, St. Louis, MO, USA) for 48 h, and then transfected for 48 h (or not), as described previously. Levels of YKL-40, IL-5, eotaxin and GM-CSF protein secretion by the cells were measured by collecting the supernatants and subjecting them to ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Each sample was analyzed in triplicate. Protein concentrations were determined according to optical density values at 450 nm using the Bio-Rad Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. After quantification, one portion of the total RNA (3 μg) was reverse transcribed in triplicate into complementary DNA (cDNA) using the First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following a standard protocol. The relative levels of YKL-40, IL-5, eotaxin, and GM-CSF mRNA transcripts were determined by semi-quantitative PCR using Taq Master Mix (2×; OMEGA) and specific primers. The sequences of the primers are shown in **Table 1**. Amplifications were performed at 95°C for 2 min and then were subjected to 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 10 min. The PCR products were resolved in 2% agarose gels containing Gold View (Solarbio, Beijing, China). The relative levels mRNA transcripts of each target gene to that of the control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined by densitometric analysis.

Statistical analysis

Data are presented as the means ± standard errors of the mean. The differences among the groups were analyzed by one-way analysis of variance and Student's *t*-test using SPSS 18.0 (SPSS, Chicago, IL, USA) or GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The *P*-values for significance were set to 0.05 for all tests.

Results

Identification of primary murine tracheal epithelial cells

The cultured primary airway epithelial cells were stained with Pan-cytokeratins and FITC-goat anti-mouse IgG, and examined under a fluorescent microscope. Approximately 95% of the cultured cells were stained by Pan-cytokeratins, a family of intermediate filaments in the airway epithelial cell cytoskeleton (**Figure 1**). These data indicate that the majority of cultured cells had the features of epithelial cells.

Adoptive transfer of YKL-40-siRNA-treated airway epithelial cells reduces the expression of YKL-40

Further experiments were performed to determine whether YKL-40 siRNA inhibition affected eotaxin, GM-CSF and IL-5 levels *in vitro*. To examine the effect of YKL-40-specific siRNA treatment on airway inflammation, the tracheal epithelial cells were cultured with OVA (200 μg/mL) for 48 h, and then transfected with YKL-40 siRNA (YKL-40-si) or negative control siRNA (YKL-40-NC), or were left untransfected (YKL-40-0) for 48 h, as described previously. The YKL-40 protein levels in epithelial cells transfected with YKL-40-Si were decreased by 70% compared with those in epithelial cells transfected with YKL-40-NC or YKL-40-0, as determined by Western blotting (**Figure 2C**, **2D**). The YKL-40 mRNA and protein levels in epithelial cells were analyzed by RT-PCR (**Figure 2A**, **2B**)

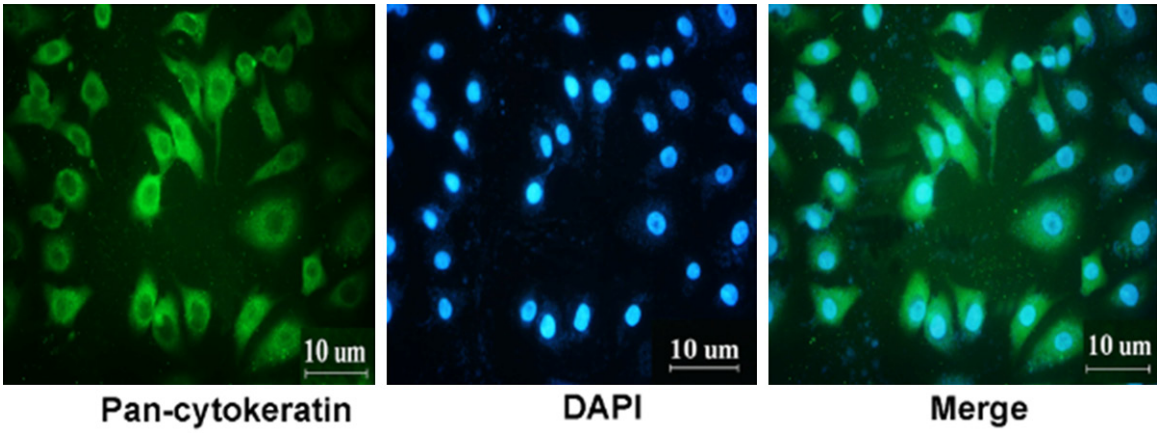

Figure 1. Immunofluorescent analysis of the isolated primary mouse airway epithelial cells. The primarily cultured airway epithelial cells from C57BL/6N mice were fixed, and incubated with mouse antibodies against Pan-cytokeratins. The antibodies were detected with FITC-labeled goat anti-mouse IgG (green) and then stained with DAPI (blue), followed by analysis using a confocal microscope. Data shown are images (magnification ×400) from three separate experiments. Scale bars indicate 10 μm.

and ELISA (**Figure 2E**), respectively. The YKL-40 siRNA, but not negative control siRNA or non-transfected cells, reduced YKL-40 expression by approximately 70% in the tracheal epithelial cell model of asthma.

Downregulation of eotaxin, GM-CSF and IL-5 by siRNA suppresses YKL-40 in the cell model of asthma

The mRNA (**Figure 3A**, **3B**) and protein (**Figure 3C-E**) levels of eotaxin, GM-CSF, and IL-5 in OVA-pulsed cells transfected with siRNA-YKL-40 (YKL-40-Si) were markedly downregulated compared with in epithelial cells transfected with siRNA-negative (YKL-40-NC) and non-transfected epithelial cells (YKL-40-0). siRNA repressed the upregulated expression of eotaxin, IL-5 and GM-CSF in OVA-pulsed epithelial cells by decreasing the activity of the YKL-40 promoter, mRNA transcription, and protein levels *in vitro.* Together, these data clearly indicate that siRNA-targeted YKL-40 in epithelial cells attenuates eosinophil airway inflammation under allergic conditions by downregulating the expression of eotaxin, IL-5, and GM-CSF.

In summary, we examined the effects of YKL-40 silencing by siRNA on the expression of OVA-induced eotaxin, IL-5, and MG-CSF in an adenocarcinoma cell line *in vitro*. We found that OVA induced the expression of YKL-40, eotaxin, IL-5, and MG-CSF in cells and the levels of these molecules were correlated positively in the cells. Furthermore, knockdown of YKL-40 significantly reduced OVA-promoted eotaxin, IL-5, and GM-CSF expression in the cells. We conclude that YKL-40 expression status correlates well with the eotaxin, GM-CSF, and IL-5 expression in the epithelium in an asthmatic context. Furthermore, YKL-40 siRNA effectively suppressed the upregulated expression of eotaxin, IL-5 and GM-CSF in OVA-stimulated epithelium. This finding could extend the prospective use of YKL-40 siRNA in asthma research and enlarge the armamentarium for treating asthma.

Discussion

The prevalence of asthma is increasing worldwide. Consequently, there is an increased need for the development of new agents for the treatment of asthma, particularly for patients who respond poorly to conventional therapy such as corticosteroids. Glucocorticoids are widely recognized as the most effective anti-inflammatory therapy currently available for asthma. However, in the past decade, it has become clear that a subset of asthma sufferers exists who are clinically unresponsive, or “resistant” or “insensitive” to glucocorticoid therapy. However, this group of patients with difficult-to-treat asthma, who are also refractory to treatment with corticosteroids, likely contributes almost 50% of the economic burden of the disease [20-22]. The molecular mechanisms leading to steroid resistance are now better understood, which has resulted in the identification of new

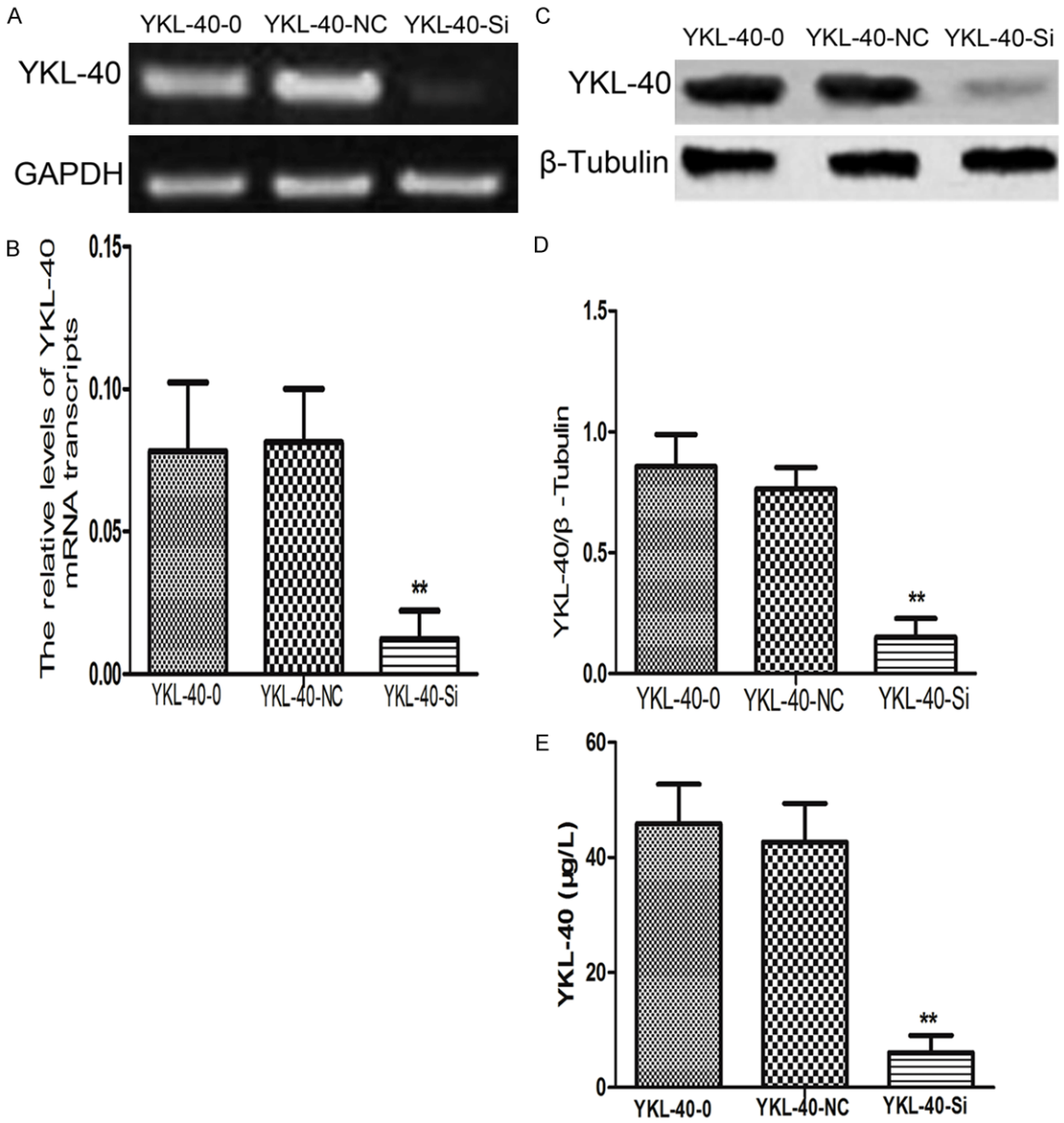

Figure 2. Small-intefering (Si) RNA (siRNA) effectively reduced expression of the YKL-40 in tracheal epithelial cells *in vitro*. A, B. The identified primary mouse tracheal epithelial cells were incubated with OVA for 48 h, then these cells were transfected with siRNA-YKL-40 (YKL-40-si), siRNA-negative control (YKL-40-NC), or non-transfected cells (YKL-40-0), and RNA from these transfected cells, the down-regulation of YKL-40 expression was measured at the RNA level by reverse transcriptase-polymerase chain reaction analysis. C, D. YKL-40 protein levels in transfected cells. The cells lysates from these transfected cells were collected to assess the protein expression of YKL-40 by Western blotting. β-Tubulin was used as a loading control. The levels of YKL-40 protein in cells transfected with siRNA-YKL-40 (YKL-40-si) were decreased by-70% compared with levels in cells transfected with siRNA-negative control (YKL-40-NC) or non-transfected epithelial cells (YKL-40-0) by western blotting. E. The concentrations of YKL-40 in the supernatants of cultured epithelial cells were determined by enzyme-linked immunosorbent assay (ELISA). Results are from three independent experiments. The data for each group are expressed as means ± SEM. Significant differences between YKL-40-si and - YKL-40NC or YKL-40-0. $^{**}P<0.01$.

targets for therapy [20, 23]. YKL-40 plays a vital role in airway inflammation and airway remodeling [14], and higher levels of YKL-40 are detected in patients with inflammatory diseases [24, 25]. A recent study has shown that YKL-40 was upregulated in the circulation and lungs of a subpopulation of patients with asthma in whom the YKL-40 levels were positively corre-

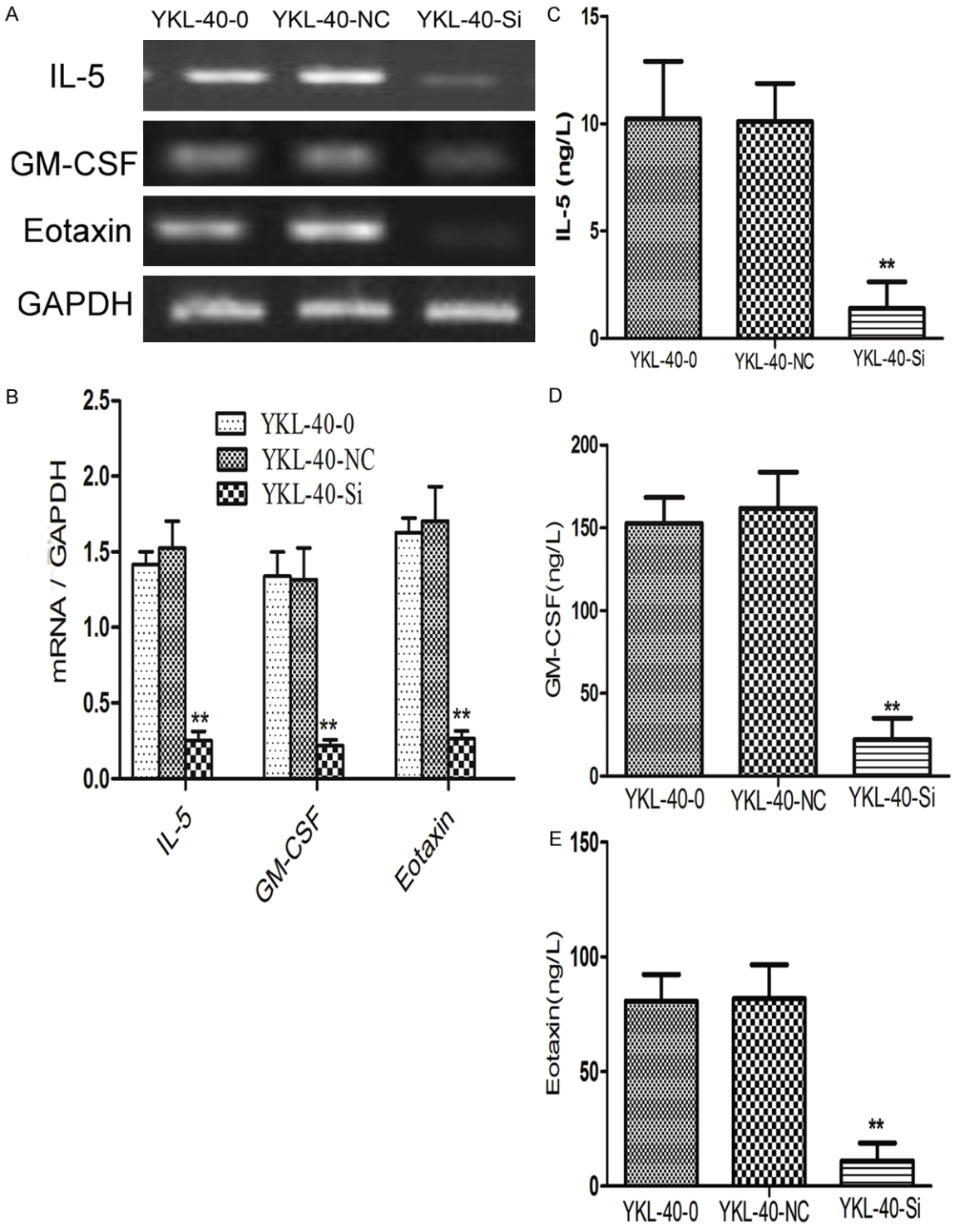

Figure 3. Administration of OVA/cells transfected with siRNA-YKL-40 reduced Eotaxin, GM-CSF, IL-5 levels *in vitro*. The OVA/cells were transfected with siRNA-YKL-40 (YKL-40-si), siRNA-negative control (YKL-40-NC), or non-transfected cells (YKL-40-0) for 48 h and RNA from these transfected cells were collected to assess the expression of Eotaxin, GM-CSF, IL-5 by RT-PCR. A, B. RT-PCR analysis of Eotaxin, GM-CSF, IL-5 levels in transfected tracheal epithelial cells. Results are from three independent experiments. The data for each group are expressed as means ±SEM. $^{**}P<0.05$, significant differences between YKL-40-si and YKL-40-NC or YKL-40-0. C-E. Eotaxin, GM-CSF, IL-5 protein levels in transfected cells. cells were unmanipulated (YKL-40-0), transfected with siRNA-negative control (YKL-40-NC), or siRNA-YKL-40 (YKL-40-Si), and supernatants from these transfected cells were collected to assess the expression of Eotaxin, GM-CSF, IL-5 by ELISA. $^{**}P<0.01$, significant differences between YKL-40-0 or YKL-40-NC and YKL-40-Si.

lated with the severity of disease and thickness of the subepithelial basement membrane and were inversely proportional to lung function [26]. Patients with higher levels of YKL-40 had more frequent rescue-inhaler use, greater oral corticosteroid use, and a greater rate of hospitalization than patients with lower levels [27].

Eosinophilic infiltration is a hallmark of allergic inflammation in mouse models of asthma and human patients [28-31] and is associated with epithelial damage [7, 32]. The airway epithelial cells secrete inflammatory mediators, such as eotaxin, GM-CSF, and IL-5, which are potent chemotactic factors for the recruitment of eosinophils through the CCR3 receptor, contributing to airway inflammation. Furthermore, these mediators also recruit inflammatory progenitor cells into the inflammatory site and promote their differentiation [33-36]. It is notable that the levels of YKL-40 are associated with the degree of eosinophil infiltration in asthmatic patients [37] and that YKL-40 stimulates the migration of IL-4$^+$ eosinophils and basophils into the inflammatory lungs [38]. In the our previous study, YKL-40 was found to be upregulated in epithelial cells pulsed with OVA compared with non-pulsed epithelial cells in a time-dependent manner, and the levels of YKL-40 were positively correlated with those of eotaxin, GM-CSF and IL-5 in primary mouse epithelial cells [19]. Given that inflammation in the airway epithelial cells is crucial for the development and progression of asthma, we examined the effect of treatment with OVA, a common allergen in mouse, on the expression of YKL-40, IL-5, GM-CSF, and eotaxin in primary mouse tracheal epithelial cells *in vitro*. The increase in gene expression was paralleled by the increase in YKL-40 protein levels. These data suggest YKL-40 to be an active player during airway inflammation-mediated epithelial damage and identify YKL-40 as a critical initiator and regulator of the development of allergen-induced allergic airway inflammation. The YKL-40 concentration is a reflection of the asthma status, and is predictive of the prognosis and therapeutic outcomes. To investigate the effects of YKL-40 interactions on epithelial cells in the context of allergen-driven responses, *in vitro* approaches followed the silencing of YKL-40 expression in epithelial cells, and the use of an epithelial cell transfer protocol. Given this role for YKL-40 in epithelial cells, we next determined whether the increase in YKL-40 gene expression and protein levels in OVA-pulsed epithelial cells was the critical event in these responses, by transfection to silence YKL-40 expression. Transfection of epithelial cells with siRNA was effective in silencing the targeted genes and facilitated assessment of the capacity of allergen-pulsed and siRNA-modified epithelial cells to alter the allergen-specific immune response. In transfected epithelial cells, we demonstrated that siRNA could target the expression of YKL-40 at the transcription and protein levels, resulting in functional consequences. These findings demonstrate for the first time that the effects of YKL-40 and eotaxin, GM-CSF, IL-5 expression through siRNA-YKL-40 silencing in epithelial cells reduced the eotaxin, GM-CSF and IL-5 levels in a model of asthma. Therefore, studies designed to analyze the roles of cytokines in human asthma should be performed to improve our understanding of the disease and evaluate YKL-40 as a target of asthma therapy. Identification of approaches or drugs that inhibit YKL-40 expression will improve the effect of conventional therapies, thereby improving the prognosis of asthma patients who have allergic disease that express YKL-40. Therefore, the downregulation of YKL-40 in epithelial cells might be a useful therapeutic approach for allergic asthma.

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Disclosure of conflict of interest

None.

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