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ORIGINAL ARTICLE

Inhibition of MicroRNA-21 by an antagomir ameliorates allergic inflammation in a mouse model of asthma

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ABSTRACT

Aim of the Study: MicroRNA-21 (miR-21) is up-regulated during allergic airway inflammation, reflecting a Th2 immune response. We investigated the effects of an miR-21 antagomir and its mechanism of action in a mouse model of acute bronchial asthma. **Materials and Methods:** BALB/c mice were sensitized and challenged with ovalbumin (OVA). The anti-miR-21 antagomir was administered by intranasal inhalation from the day of sensitization. Changes in cell counts, Th2 cytokine levels in bronchoalveolar (BAL) fluid, and airway hyper-responsiveness (AHR) were examined. Histopathological changes and expression levels of miR-21 in lung tissues were analyzed. The mechanism of action of the antagomir was investigated by counting CD4⁺/CD8⁺ T cells in splenocytes and by measuring the expression levels of transcription factors associated with T cell polarization. **Results:** MiR-21 expression was selectively down-regulated in the lung tissues of mice treated with anti-miR-21. The antagomir suppressed AHR compared with that of the OVA-challenged and scrambled RNA-treated groups. It also reduced the total cell and eosinophil counts in BAL fluid and the levels of Th2 cytokines, including *IL-4*, *IL-5*, and *IL-13*. The direct target of miR-21, *IL-12p35*, was induced in the antagomir-treated group, decreasing the CD4⁺/CD8⁺ T cell proportions in splenocytes. The levels of transcription factors involved in the Th2-signaling pathway were reduced in lung tissues on treatment with the antagomir. **Conclusions:** The miR-21 antagomir suppresses the development of allergic airway inflammation in a mouse model of acute bronchial asthma, inhibiting Th2 activation. These results suggest that this antagomir might be useful for treating bronchial asthma.

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allergy; asthma; MicroRNA-21

Introduction

Asthma is a chronic airway disease characterized by inflammation and airway remodeling, which causes lung function to decline.^[1] Allergic airway inflammation is central to the pathophysiology of disease, and is associated with the balance between the levels of Th1 and Th2 cytokines.^[2,3]

MicroRNAs (miRNAs) are short, single-stranded RNA molecules that modulate the expression levels of hundreds of proteins via post-transcriptional silencing. MicroRNAs are involved in many biological processes, including cell differentiation and development, apoptosis, inflammation, and homeostasis. They also contribute to the pathogenesis of numerous diseases, including respiratory conditions, non-small cell lung cancer, pulmonary fibrosis, and chronic inflammatory airway disease and asthma. MiR-21, miR-145, miR-146a, miR-155, and miR-221 are known

to be involved in asthma.^[4–6] Of these, miR-21 is one of the most important mediators of the disease, being overexpressed in inflamed lung tissues of animals with experimentally induced allergic asthma. MiR-21 is up-regulated not only in doxycycline-induced lung-specific interleukin (IL)-13 transgenic mice but also in the ovalbumin (OVA)- and *Aspergillus fumigatus*-induced mouse models of asthma. *MiR-21* is expressed principally in macrophages and dendritic cells, and the 3' UTR of *IL-12p35* contains a conserved *miR-21* target sequence.^[7] In humans with bronchial asthma, *miR-21* expression increases in bronchial epithelial cells to a greater extent than in controls.^[8] Moreover, non-treated patients express *miR-21* to a significantly greater extent than do patients prescribed an inhaled corticosteroid. Sawant et al.^[9] found that children with eosinophilic esophagitis, an allergic disease, had elevated levels of *miR-21*, suggestive of the existence

of a strong association between *miR-21* expression and various allergic diseases. In patients with allergic asthma, *miR-21* has been suggested to regulate immune polarization, directly targeting *IL-12p35*. This, in turn, limits the production of Th1 cytokines and increases signaling through the Th2 pathway.^[7,10,11] In an OVA-stimulated mouse model of asthma, *miR-21*-deficient animals exhibited a reduction in eosinophilic lung infiltration, increased expression of *IL-12/IFN- γ* , and a decreased level of Th2 cytokine *IL-4*.^[10]

Antagomirs (inhibitors of miRNA action) have been reported to reduce airway inflammation in models of bronchial asthma.^[6,12–14] Collison et al.^[6] showed that anti-miR-126 significantly improved acute asthma, Th2-driven airway inflammation, and airway hyper-responsiveness (AHR) in a model of acute asthma. In addition, antagomir-mediated inhibition of miR-145 reduced eosinophilic inflammation, secretion of Th2 cytokines, and AHR.^[15] However, few studies have explored any possible utility of anti-miR-21 therapy in asthma models.

We explored the effects of an miR-21 antagonist on allergic airway inflammation and related Th1/Th2 polarization in an OVA-induced model of acute bronchial asthma. We also measured changes in the expression levels of relevant transcription factors during helper T cell differentiation.

Materials and methods

Animals and experimental design

Female BALB/c mice (Dae-Han Experimental Animal Center, Daejeon, South Korea), aged 6 weeks, were used. Mice were housed in a controlled environment with a 12-hour light/dark cycle with free access to food and water, and were maintained on an OVA-free diet. All procedures of animal research were provided in accordance with the Laboratory Animals Welfare Act, the guide for the care and use of laboratory animals and the guidelines and policies for rodent experiment provided by the Institutional Animal Care and Use Committee (IACUC) in School of Medicine, The Catholic University of Korea.

Mice were randomly assigned into one of the four groups: (1) control; (2) OVA challenge; (3) OVA challenge plus intranasal inhalation of scrambled control (anti-scrambled) RNA; and (4) OVA challenge plus intranasal inhalation of anti-miR-21 antagomir.

Sensitization and antigen challenge protocol

The animals were sensitized and challenged with OVA as described previously.^[16] The mice were sensitized by subcutaneous injection of OVA (Sigma-Aldrich, St. Louis, MO, USA) adsorbed to 1 mg of aluminum hydroxide (Aldrich, Milwaukee, WI, USA) in 200 μ L of normal saline. After sensitization on days 1 and 8, mice were challenged by intranasal inhalation of OVA (20 ng/50 μ L in phosphate buffer solution (PBS)) on days 21, 23, 25, and 28 under isoflurane (Vedco, St. Joseph, MO, USA) anesthesia. The control group was treated in the same way with PBS without OVA. Mice were sacrificed 24 hours after the final OVA challenge.

Antagomirs administration

We purchased commercially available anti-miR-21 antagomir for in vivo study from Ambion[®] (Austin, TX, USA), and anti-scrambled RNA from Cosmo-genetech (Shanghai GenePharma Ltd, Shanghai, China). The antagomir sequence was as follows: 5'-*mA*mC*mG mGmCmA mAmCmA mCmCmA mGmUmC mGmAmU mGmGmG mC*mU*mG*mU*-3'-chol*; where *m* denotes 2'-O-methyl-modified phosphoramidities, *denotes phosphorothioate linkages, and *chol* denotes hydroxypropylol-linked cholesterol. We administered 50 μ g of anti-miR-21 or anti-scrambled RNA mixed in 50 μ L of sterile saline by intranasal route 30 minutes before the OVA challenge on the day of sensitization (days 1 and 8) and then once a day from the day of challenge until sacrifice (days 20, 21, 22, 23, 24, and 27).

Measurement of airway hyper-responsiveness

Airway hyper-responsiveness to metacholine (Mch; Sigma-Aldrich) was measured 24 hours after the final OVA inhalation using the flexiVent system (SCIREQ, Montreal, Quebec, Canada) as described previously.^[17] Briefly, the mice were anesthetized with an intraperitoneal injection of rompum and zoletil mixture (1:4). The trachea was exposed and cannulated, then connected to a computer-controlled small-animal ventilator and ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. Each mouse was exposed to PBS and the

increasing concentrations of Mch (6.25, 12.5, 25, 50 mg/mL) in PBS, and the peak airway response to the inhaled Mch were recorded.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage was performed immediately after the measurement of AHR. BAL fluid (BALF) was withdrawn after instillation of 1 mL of sterile PBS through trachea into the lung. The total number of cells in BAL fluid was counted using a hemacytometer. The BAL fluid was cytospun (7 minutes at 2000 rpm) onto microscope slides and stained with Diff-Quick (Sysmax, Kobe, Japan). The percentage values of macrophages, eosinophils, lymphocytes, and neutrophils in BAL fluid were obtained by counting 400 leukocytes on randomly selected areas of slide using light microscopy. Supernatants were stored at -70°C .

Real-time polymerase chain reaction

For quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of *miR-21* and *IL-12p35* gene expression, total RNA was isolated from lung homogenates using TRIzol reagentTM (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The total RNA (1 μg) was reverse-transcribed directly using a TaqMan[®] MicroRNA Reverse Transcription Kit (4366596, AB, CA, USA) according to manufacturer's protocol. The reverse-transcription reaction was incubated at 16°C for 30 minutes and at 42°C for 30 minutes, inactivated at 85°C for 5 minutes, and cooled at 4°C . Taqman probe RT-PCR was performed using a TaqMan[®] Universal Master Mix II, with or no UNG (4440040, AB) for *miR-21* (002493, AB) and *IL-12p35* (Mm00434165_m1, AB) according to manufacturer's protocol. PCRs were carried out in RT-PCR machine (CFX96 Touch; Bio-rad, CA, USA). The samples were incubated at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and then at 60°C for 60 seconds. The fluorescence emitted by the reporter dye was detected in real time, and the threshold cycle (Ct) of each sample was recorded as a measure of the amount of PCR product in the sample. The Ct is the fraction cycle number at which the fluorescence generated by the reporter dye exceeds a set level above baseline. When indicated, the target signal was normalized against the relative quantity of GAPDH and expressed as $\Delta\text{Ct} = \text{Ct}_{\text{Target}} - \text{Ct}_{\text{GAPDH}}$. The change in the target

signal relative to the total amount of genomic DNA was expressed as $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{Treatment}} - \Delta\text{Ct}_{\text{Control}}$. Relative changes were then calculated as $2^{-\Delta\Delta\text{Ct}}$.

Enzyme-linked immunosorbent assay (ELISA)

The level of *IL-4*, *IL-5*, *IL-13*, *IL-12p70*, and interferon (IFN)- γ was measured in the supernatant of BAL fluid with an ELISA kit (R&D Systems, Minneapolis, MNUSA). The protocol was according to manufacturer's instructions.

Western blotting

Separated lung tissues frozen in liquid nitrogen were disrupted using a Polytron homogenizer (Tissue TreaorTM, Biospec Products Inc, Bartlesville, OK, USA), and centrifuged. The proteins were purified from the supernatant, and the concentration was assessed using the Bradford method.^[18] The protein samples were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). After blocking with 10% skimmed milk (BD Difco, Franklin Lakes, NJ, USA), the membrane was incubated with antibodies including Signal transducer and activator of transcription (STAT)4, STAT6, phosphorylated (p)-STAT4, p-STAT6, T-bet, GATA-binding protein 3 (GATA3), and β -actin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. The membrane was washed for three times with PBS and incubated with secondary antibody for 2 hours. The target protein was detected using the ECL Western Blotting Analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and X-ray film.

Lung tissue histopathology

After BAL fluid was obtained, the lungs were inflated, fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin wax. Sections of 4- μm thickness were cut using a microtome, and stained with hematoxyline and eosin (H&E) using standard techniques for histological changes. The paraffin-embedded tissues were sectioned at 5–6 μm and stained with periodic acid-Schiff (PAS) to identify goblet cells in the epithelium. To quantify goblet cell hyperplasia, we used the method described by Padrid et al.^[19] The pathological changes were evaluated according to the modified five-point scoring system (grade 0–4) and expressed as the percentage of goblet cells in the epithelium scored

as follows: grade 0 (no goblet cells); grade 1 (<25%); grade 2 (25%–50%); grade 3 (51%–75%); and grade 4 (>75%). The mean score of goblet cell hyperplasia was calculated for each mouse.

Flow cytometric (FACS) analysis of Th1 and Th2 cells

To isolate cells from the spleen of mice, tissue was dissected and minced into small pieces with 5-mL RBC lysis buffer and centrifuged at 1500 rpm for 7 minutes at 4°C. After centrifugation, the supernatant was discarded and cell pellets were washed in PBS. Cells were resuspended with cell freezing medium (1×10^7 cells/mL) and stored in LN₂ tank.

Stained cells were sorted using MoFlo (Beckman Coulter Inc., CA, USA). The liquid volume in the FACS tube was adjusted to 50 µL, and added fluorescence-labeled CD4 and CD8 antibodies. Cells were incubated with Fc block for 5 minutes before staining 1×10^6 cells with antibodies against CD4 (0.1 µg), and CD8 (0.2 µg), which were conjugated to FITC, or peridinin chlorophyll-a protein (PerCP) (BD Biosciences, CA, USA). Sorted CD4⁺/CD8[−] T cells were seeded at 1×10^6 cells/mL and cultured in X-VIVO 15 medium (Lonza Inc., MD, USA) at 37°C in a 5% CO₂ incubator. After 24 hours, cells were activated with 50-ng/mL phorbol myristate acetate (PMA) and 750 ng/mL ionomycin in GolgiStopTM, and cultured at 37°C in 5% CO₂. After 4 hours, cells were fixed and permeabilized using cytofix/cytoperm (BD Biosciences) for 20 minutes at 4°C. The cells were washed (Perm/WashTM buffer, centrifuged at 1500 rpm for 5 minutes at 4°C) and stained with IFN-γ (PE-Cy7, 0.2 mg/mL) and IL-4 (PE, 0.2 mg/mL) or appropriate controls (corresponding IgG negative isotypes) for 30 minutes at 4°C in dark. Cells were washed twice with Perm/WashTM buffer and finally resuspended in 0.4-mL stain buffer. Samples were stored at 4°C until flow cytometric analysis. Intracellular stained lymphocyte samples were analyzed with a FACS CANTO II flow cytometry (BD Biosciences). Voltages were set with unstained control, and compensation was adjusted using single-stained cells.

Data analysis

The data were subjected to one-way ANOVA followed by Dunnett's multiple range tests using Graph-Pad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). All data were expressed as mean

values $\pm$ SEM, and $P < .05$ was considered statistically significant for all cases.

Results

MiR-21 antagomir suppresses airway inflammation and hyper-responsiveness

Figure 1 shows the total and differential counts of airway inflammatory cells in BAL fluid. Compared with control mice, OVA-sensitized mice exhibited elevated total cell and eosinophil counts. Intranasal inhalation of the antagomir, commencing at sensitization, significantly suppressed rise in both total cell ($P < .001$) and eosinophil counts ($P < .01$) compared with those of OVA- and scrambled RNA-treated mice. Otherwise, counts of macrophages and lymphocytes were not changed following OVA sensitization and antagomir inhalation. Neutrophil counts were increased with OVA challenge and decreased after antagomir treatment; however, these changes were statistically insignificant. AHR to metacholine fell in the antagomir-treated group compared with the OVA- and scrambled RNA-treated groups ($P < .001$; Figure 2). Histopathologically, the extent of peribronchial inflammatory cell infiltration was higher in OVA- and scrambled RNA-treated mice than in controls. The antagomir significantly reduced such infiltration (Figure 3). Moreover, the extent of lung goblet cell hyperplasia (revealed by PAS staining) was greater in OVA- and scrambled RNA-treated mice than in treated animals ($P < .05$).

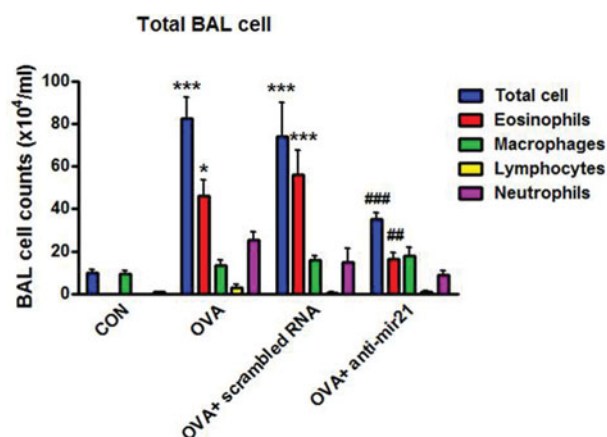


Figure 1. The effects of miRNA-21 on total and differential cell counts in BAL fluid of experimental mice. Mice were sacrificed 24 hours after the final OVA challenge, and BAL cells were isolated. Cell numbers are expressed as mean values $\pm$ SEMs ($n = 5-9$ /group). * $P < .05$ and *** $P < .01$ compared with the control group, ## $P < .01$ and ### $P < .001$ compared with OVA + scrambled RNA group.

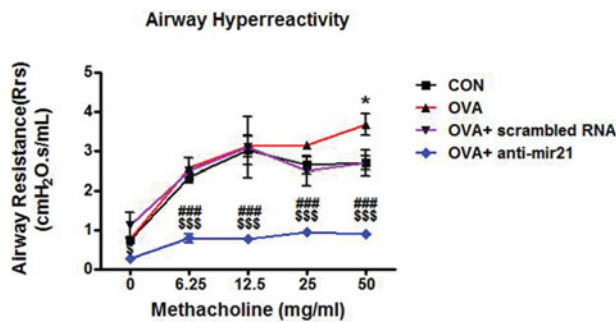


Figure 2. Effects of miRNA-21 on AHR to methacholine. AHR was measured 24 hours after the final OVA challenge using a Flexivent system, in which mice were exposed to increasing concentrations of methacholine (6.25–50 mg/mL). Values are expressed as mean values $\pm$ SEMs ($n = 5$ –9/group). * $P < .05$ compared with the control group. ### $P < .001$ compared with the OVA group, \$\$\$ $P < .001$ compared with the OVA + scrambled RNA group.

MiR-21 antagomir alters the levels of T helper cell cytokines in BAL fluid

We measured the expression levels of T helper cell cytokines in BAL fluid. The expression of Th2-associated cytokines (*IL-4*, *IL-5*, and *IL-13*) was suppressed in the antagomir-treated mice compared with OVA- and scrambled RNA-treated animals; the expression levels of the Th1 cytokines *IL-12p70* and *IFN- γ* increased in the treated group (*IL-12p70*: $P < .001$; *IFN- γ* : $P < .01$) (Figure 4). The *IFN- γ* level was slightly elevated in scrambled RNA-treated group. However, the difference had no statistical significance between the OVA and the scrambled RNA group.

MiR-21 antagomir reduces the activation of STAT6 and GATA3 in lung tissues

The efficacy of the antagomir treatment was confirmed by qRT-PCR of lung tissue. *MiR-21* expression was suppressed in the antagomir-treated mice compared with scrambled RNA-treated animals, indicating that the intranasal antagomir selectively inhibited *miR-21* transcription. Moreover, levels of the direct target of *miR-21*, *IL-12p35*, increased in lung tissues of the antagomir-treated compared with the scrambled RNA-treated mice (Figure 5A).

To explore the possible effects of the antagomir on T helper cell polarization, we measured the expression levels of relevant transcription factors. STAT4, STAT6, GATA3, and T-bet are known to play essential signaling roles during generation of Th1 and Th2 cells from naïve T cells.^[20,21] Signaling through *IL-4* receptors activates the transcription factors STAT6 and GATA3, which

favor the expression of Th2 cytokines. *IL-12* induces signaling pathways involving both STAT4 and T-bet, culminating in the expression of the genes of Th1 pathway.^[20] Western blotting of homogenized lung tissue showed that STAT6 activation (to yield p-STAT6, p-STAT6/STAT6) and expression of GATA3 decreased upon treatment with the antagomir, whereas the levels of p-STAT4/STAT4 and T-bet increased (compared with the levels in scrambled RNA-treated animals; Figure 5B).

MiR-21 antagomir attenuates the generation of CD4⁺ T cells in the spleen

The pathophysiological action of *miR-21* in bronchial asthma involves creation of an imbalance between Th1 and Th2 cell numbers. Th2 polarization plays a major role in the disease, and we thus measured the splenic populations of CD4⁺ T cells via fluorescence-activated cell sorting. The proportion of anti-CD4-stained CD4 splenocytes (CD4⁺/CD8[−] cells) (Figures 6A and B) was significantly lower in antagomir ($P < .05$) than in OVA-treated mice. The proportion of CD4⁺/CD8[−]/*IFN- γ* [−]/*IL-4*⁺ cells (Figure 6C) was similarly low.

Discussion

We found that the *miR-21* antagomir effectively suppressed bronchial hyper-responsiveness and inflammation in an OVA-induced mouse model of asthma. The intranasal antagomir selectively down-regulated *miR-21* expression and enhanced expression of *IL-12p35*. Induction of *IL-12* activated the transcription factors STAT4 and T-bet. Ultimately, the proportions of CD4⁺/CD8[−] T cells decreased in the spleens of the antagomir-treated mice, suggesting that this antagomir may be useful for treating bronchial asthma, ameliorating the Th1/Th2 imbalance.

Bronchial asthma is a chronic airway inflammatory disease characterized by infiltration of inflammatory cells, including eosinophils, basophils, mast cells, and CD4⁺ T-helper cells. Inhalation of allergens stimulates the innate immune system to secrete cytokines, promoting the Th2 response. Such Th2 cytokines induce airway changes, including hyper-responsiveness and remodeling, trigger epithelial cell proliferation accompanied by goblet cell hyperplasia, increase mucus secretion, trigger smooth muscle

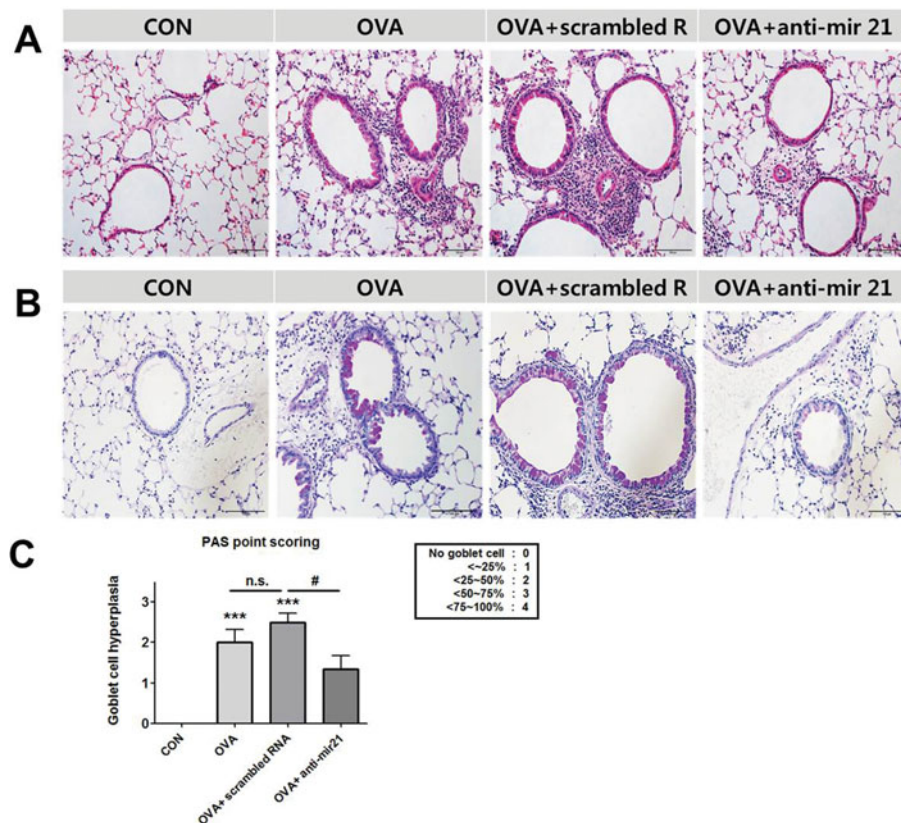


Figure 3. Effects of miRNA-21 on goblet cell hyperplasia and histological changes in OVA-induced lungs. Representative photomicrographs of (A) H&E- and (B) PAS-stained lung sections from mice of various groups are shown. (C) Quantification of PAS-stained areas by point scoring. Values are expressed as mean values $\pm$ SEMs ($n = 5-9/\text{group}$). *** $P < .001$ compared with the control group, # $P < .05$ compared with the OVA + scrambled RNA group.

hyperplasia and hypertrophy, and increase the number of sub-epithelial cells.^[22,23] Current treatments include inhaled corticosteroids and long-acting β -agonists, which are generally effective in most of the patients.^[1] However, some difficult-to-treat patients respond poorly to even optimal therapy.^[24] New targeted therapies are necessary in clinical practice. Monoclonal antibodies, including anti-IL4, anti-IL5, and anti-IL13, anti-thymic stromal lymphopoietin (TSLP), and anti-IgE have been clinically investigated in this context.^[22]

Micro-RNAs are regulators of human disease which interferes with the transcription of specific mRNAs. Most reports to date have explored the roles played by miRNAs in malignancy.^[25-28] In terms of allergic disease, recent reports have shown that certain miRNAs are involved in immunological responses to bronchial asthma, and regulate the expression of cytokines and chemokines that cause acute airway inflammation and fibrosis.^[14,29] Moreover, miRNAs might play potent roles in corticosteroid-resistant asthma,^[30] suggesting that they fine-tune the expression of genes, contributing to the development of various endotypes and phenotypes of bronchial asthma.

The effects of miRNAs on allergic inflammation have been attributed to the polarization of adaptive immune response, activation of specific T cell subtypes, regulation of eosinophil development, and modification of *IL-13*-driven epithelial responses.^[11] Liu et al.^[31] recently suggested that amelioration of smooth muscle cell proliferation by miRNAs might possibly reduce the extent of airway remodeling in asthmatic patients. Certain miRNAs control the Th1/Th2 balance, which, in turn, dictates the expression levels of pro-inflammatory cytokines associated with bronchial asthma. For example, miR-155 may affect the Th1/Th2 balance by targeting *IL-13RA*, *SOCS1*, and *SHIP1* of macrophages.^[32] MiR-375 elevates the expression of TSLP in primary bronchial epithelial cells.^[33] In addition, *IL-10* induces miR-187, which, in turn, negatively regulates the expression of *TNF- α* , *IL-6*, and *IL-12p40*.^[34] Lu et al.^[7,10] showed that miR-21 was up-regulated in animal models on allergic airway inflammation; the *IL-12/IFN- γ* pathway was involved in Th1 polarization in various models of asthma, including asthma induced by OVA, *A. fumigatus*, and lung-specific *IL-13*. Wu et al.^[35] found that miR-21

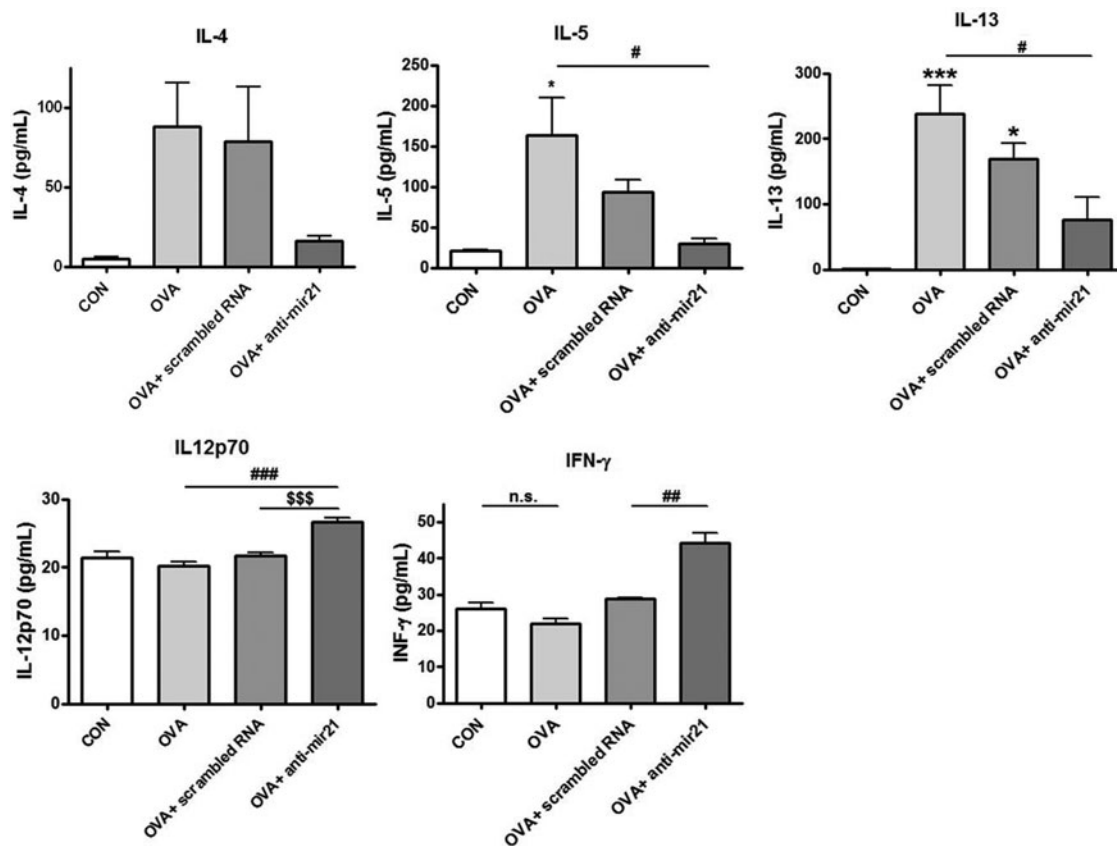


Figure 4. Effects of miRNA-21 on the expression levels of various cytokines by T helper cells in BAL fluid. The levels of Th2 cytokines (IL-4, IL-5, and IL-13) and Th1 cytokines (IL-12p70 and IFN- γ) were measured by ELISA. Values are expressed as mean values $\pm$ SEMs ($n = 5-9/\text{group}$). * $P < .05$ and *** $P < .001$ compared with the control group, # $P < .05$, ### $P < .001$ compared with the OVA group, ## $P < .01$ compared with the OVA + scrambled RNA group, \$\$\$ $P < .001$ compared with the OVA + scrambled RNA group.

expression was remarkably increased in an OVA-induced asthma model, and there was decrease in the expression of IL-12/STAT4 proteins.

In the present work, we confirmed that miR-21 expression significantly increased and both IL-12p70 and IL-12p35 were down-regulated in mice with OVA-induced asthma. Moreover, suppression of miR-21 by intranasal administration of the antagomir improved asthma symptoms, including airway inflammation and AHR, inhibited Th2 polarization of splenic CD4⁺/CD8⁻ cells, and changed the cytokine levels in BAL fluid. These findings are consistent with previous work on the association between miR-21 and asthma, and further provide the evidence that miRNA antagomirs may beneficially control the Th2 deviation triggered by IL-12.

There is only one previous report on the miR-21 antagomir.^[15] In a house dust mite (HDM) model of allergic asthma, antagomirs of miR-21 and let-7b did not protect against airway inflammation, whereas an antagomir of miR-145 inhibited allergic inflammation,

different from what we found. Several explanations are possible. First, the materials used to induce asthma and challenge the animals differed. We used OVA combined with an aluminum adjuvant; the cited report employed an HDM allergen. OVA is a toll-like receptor (TLR)4-independent antigen that activates TLR4-independent pathways to induce asthma; the HDM allergen is TLR4-dependent. Different stimulatory materials may have evoked the expression of distinct miRNAs and other target genes. Second, we gave anti-miR-21 antagonist by intranasal route during both sensitization and challenge; the cited report used the intranasal route only during the challenge period. Since the effects of anti-miR-126 differ in both chronic and acute mouse models of bronchial asthma induced by OVA challenge,^[6] and since the success of delivery and efficacy of antagomirs vary with the cell-type tested,^[36] different modes of anti-miR-21 administration used in the two studies may explain the different results obtained. We were suspicious about the systemic effect of intranasal inhalation therapy of

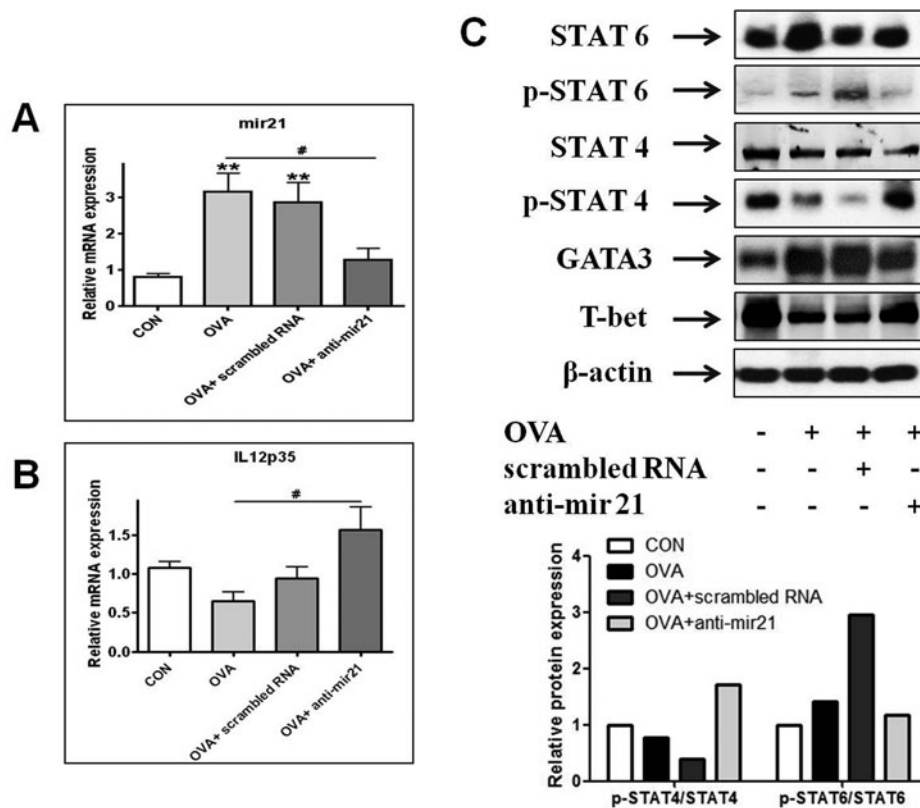


Figure 5. Effects of miRNA-21 inhibition on the expression levels of T cell signal transducer and activator of transcription (STAT) and IL-12p35 proteins in lung tissue. (A) and (B) Total RNAs were extracted from lung tissues and the levels of mRNAs encoding *miR-21* and *IL-12p35* were determined via TaqMan RT-PCR. The relative expression levels of miR-21 were determined by qRT-PCR (snoRNA served as an internal control). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a control for *IL-12p35* PCR. The comparative C_T method ($\Delta\Delta C_T$) was used to quantify gene expression levels. ** $P < .01$ compared with the control group. # $P < .05$ compared with the OVA group. (C) Expression and phosphorylation levels of STAT4 and STAT6 and the expression levels of GATA3 and T-bet were measured by Western blotting. Relative protein expressions of p-STAT4/STAT4 and p-STAT6/STAT6 were also documented.

the miR-21 antagomir. However, previously we had published the decreased serum OVA-specific IgE and IgG levels via intraperitoneal administration of anti-IL33/ST2 antibodies.^[37] Moreover, there were many reports describing the effects of intranasal inhalation^[21,38,39] and oral gavage^[40] treatment on mouse splenic T cells. These reports revealed that intranasal or intraperitoneal approach also had a systemic effect such as decreased IgE in serum and modulation of Th cell polarization in spleen. Further experiments using protocols other than OVA sensitization would help to confirm the efficacy of this antagomir. In addition, comparison of the antagomir and steroid treatments would yield valuable data.

We found that the antagomir affected the miR-21-induced expression of *IL-12*, thereby influencing the differentiation of naïve T cells; it also modulated the activation of the transcription factors STAT, GATA, and T-bet. Activation of STAT4 and STAT6 is crucial for the differentiation of Th1 and Th2 cells respectively.^[20] GATA3 is a transcription factor downstream of STAT6

that promotes Th2 cytokine expression.^[41] T-bet is a key transcription factor in Th1 cells that promotes Th1 signaling.^[42] Signaling through IL-12 receptor is associated with a polarized Th1 response; STAT4 activation triggers the expression of cytokine *IFN- γ* , inducing the synthesis of T-bet proteins. *IL-4* activates STAT6, which increases the level of GATA3 and triggers the polarized Th2 response.^[20,43]

In the OVA-induced bronchial asthma model, biological agents, including mangiferin, targeted various molecules to regulate Th1/Th2 polarization; the agent exhibited anti-asthmatic effects.^[21] However, the effects of miR-21 antagonists on the expression of transcription factors driving Th1/Th2 polarization have not been studied to date. In one study, dexamethasone lowered the expression levels of *IL-12* and STAT4.^[35] However, to the best of our knowledge, our findings that the miR-21 antagomir directly inhibits miR-21 may be the first preclinical evidence suggesting that this antagomir may be useful for treating bronchial asthma.

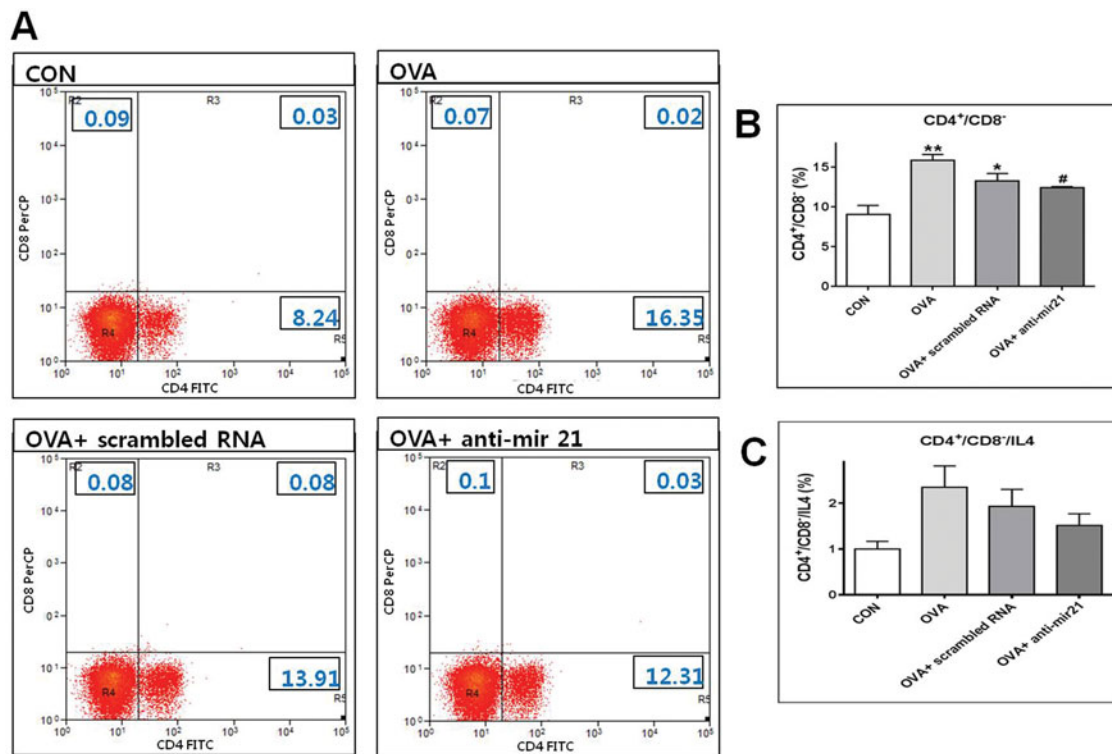


Figure 6. Effects of miR-21 on the levels of *IL-4* and *IFN-γ* expressed by splenic CD4⁺/CD8⁻ T cells of experimental mice. (A) Flow cytometric analysis of splenocytes successively stained with anti-CD4 (FITC) and anti-CD8 (PerCP). (B) A bar plot of the average proportions of CD4⁺/CD8⁻ cells. (C) A bar plot of the average proportions of CD4⁺/CD8⁻/*IFN-γ*⁻/*IL-4*⁺ cells. All data are mean values ± SEMs and are representative of three independent experiments each. **P* < .05 and ***P* < .01 compared with the control group. #*P* < .05 compared with the OVA group.

In conclusion, miR-21 was significantly up-regulated in an acute OVA-induced model of bronchial asthma, and an miR-21 antagonist effectively protected against the development of allergic airway inflammation. The MiR-21 antagonomir modulated the Th1/Th2 balance by changing the expression level of *IL-12* and activating transcription factors, including STAT, GATA, and T-bet.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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