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# Antagonism of mmu-mir-106a attenuates asthma features in allergic murine model

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**Sharma A, Kumar M, Ahmad T, Mabalirajan U, Aich J, Agrawal A, Ghosh B.** Antagonism of mmu-mir-106a attenuates asthma features in allergic murine model. *J Appl Physiol* 113: 459–464, 2012. First published June 14, 2012; doi:10.1152/jappphysiol.00001.2012.—Micro-RNAs (miRs) regulate immunological pathways in health and disease, and a number of miRs have been shown to be altered in mouse models of asthma. The secretion of interleukin-10 (IL-10), an anti-inflammatory cytokine, has been shown to be defective in many inflammatory diseases including asthma. We recently demonstrated that miR-106a inhibits IL-10 in a post-transcriptional manner. In this study, we investigated the effect of inhibition of mmu-miR106a in asthmatic condition to find its possible role as a therapeutic target. Our in vitro experiments with mouse macrophage, RAW264.7, revealed that mmu-miR-106a potentially decreased IL-10 along with increase in proinflammatory cytokine. Furthermore, administration of mmu-miR-106a to naive mice reduced IL-10 levels in lungs in a dose-dependent manner without altering lung histology. Most interestingly, knockdown of mmu-miR-106a in an established allergic airway inflammation has significantly alleviated most of the features of asthma such as airway hyperresponsiveness, airway inflammation, increased Th2 response, goblet cell metaplasia, and subepithelial fibrosis along with increase in IL-10 levels in lung. This represents the first in vivo proof of a miRNA-mediated regulation of IL-10 with a potential to reverse an established asthmatic condition.

IL-10

MICRORNAs (miRs), novel class of noncoding RNAs, regulate gene expression and fine tune many biological processes (11, 29). Recent findings demonstrated the involvement of miRs in immune regulation indicating the crucial importance of miRs in immune-mediated diseases in therapeutic aspect (4, 13). Evidently, alterations in miRNA expression have been implicated in many diseases including asthma (4, 9). In this context, we recently demonstrated that miR-106a inhibits interleukin-10 (IL-10) expression in lymphoid and myeloid cells (26). IL-10 is a well-known cytokine with prominent anti-inflammatory effects. It is produced by numerous cell types, such as cells of innate immune response like macrophage, dendritic cells, neutrophils and eosinophils, and of adaptive immune response like Th1, Th2, Th17, Treg, CD8+ T cells, and B cells, indicating its central role in many inflammatory diseases including asthma (2, 6, 21, 31). Thus inhibition of IL-10, a potent anti-inflammatory cytokine, via increased miR-106a may represent a critical regulatory mechanism that determines the balance between pro-inflammatory and anti-inflammatory responses. So we hypothesized that the allergic inflammation

may be alleviated by antagonizing mmu-miR-106a. Here, we used a murine model of asthma to determine the consequences of knockdown of mmu-miR-106a in lungs on asthma phenotype in vivo including IL-10 levels. We showed for the first time that knockdown of mmu-miR-106a increased IL-10 expression and alleviated asthmatic features.

## MATERIALS AND METHODS

This study was performed in conformity with the American Physiological Society's Guiding Principles in the Care and Use of Animals. Experimental protocols and study design were approved by the designated institutional review boards at the Institute of Genomics and Integrative Biology.

**Maintenance of cell culture and transfection.** Mouse macrophage cell line RAW 264.7 were cultured in RPMI (Sigma) containing 10% fetal bovine serum, streptomycin (50 µg/ml), and penicillin (50 IU/ml) and incubated at 37°C and in 5.0% CO<sub>2</sub>.

RAW 264.7 cells were transfected with or without 25 nM–200 nM end modified mmu-miR-106a oligonucleotides or cel-miR-67 (Dharmacon) prior to stimulation with LPS for 24 h using transfection reagent Lipofectamine 2000 (Invitrogen) as described previously (26). IL-10 and TNF-α (BD Biosciences) levels were determined in culture supernatants by ELISA.

**Animals.** Male BALB/c mice (8–10 wk old) were obtained from National Institute of Nutrition (Hyderabad, India) and acclimatized for 1 wk prior to starting the experiments. All animals were maintained as per CPCSEA guidelines, and protocols were approved by the Institutional Animal Ethics Committee.

**Grouping of mice and treatment with mmu-mir-106a oligonucleotide.** Normal mice were treated without (optiMEM media, Invitrogen) or with increasing concentrations of mmu-miR-106a (2, 4, 8 mg/Kg) or cel-miR-67 (8 mg/Kg) oligonucleotides (Dharmacon). Oligonucleotides were dissolved in water, and the working dilution was prepared in optiMEM (Invitrogen). It was delivered into mice intranasally using computer-controlled inExpose nose-only exposure system integrated with aeroneb, ultrasonic nebulizer (Scireq, Canada) for two consecutive days followed by death 24 h after second aerosol treatment.

**Development of OVA-sensitized mouse model of asthma and treatment of mice with anti-mmu-mir-106a.** Mice were sensitized and challenged as described (18) and shown in Fig. 2A. Mice were divided into four groups as indicated, each group ( $n = 6$ ) was named according to sensitization/challenge/treatment: Sham/PBS/vehicle (Veh) (normal controls) called Sham, OVA/OVA/Veh (allergic controls, OVA, chicken egg ovalbumin, Grade V, Sigma) called OVA, OVA/OVA/anti-mmu-miR-106a (allergic mice treated with 4 mg/kg anti-mmu-miR-106a) called Anti-mmu-miR-106a, and OVA/OVA/scrambled oligonucleotide (allergic mice treated with 4 mg/kg anti-Cel-miR-67) called Scrambled, respectively. The anti-mmu-miR oligonucleotides were dissolved in water, and the working dilution was prepared in optiMEM (vehicle, Invitrogen). Vehicle, anti-mmu-miR oligonucleotides, or scrambled nucleotide were administered by nebulization using InExpose on days 26 and 27 (Fig. 2A). Twenty-four hours after last challenge, mice were killed.

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**Measurement of airway hyperresponsiveness.** AHR to methacholine (MCh, Sigma) was determined in anesthetized mice using the FlexiVent system (Scireq, Canada) as described earlier (17).

**Flow cytometry in single cell suspension of lung.** To determine whether intranasally delivered oligos have been taken in and altered by IL-10 by one of its major producers, macrophages, DY547 labeled anti-mmu-miR106a, or anti-Cel-67 (Dharmacon) were delivered, and a single cell suspension was made from lungs after death and stained with CD11b, IL-10, or IL-13 (eBioscience).

**Measurements of cytokines (IL-4, IL-5, IL-10, and IL-13) in the lung and OVA specific immunoglobulins in sera and differential count in BAL fluid.** Lung homogenates were used for IL-4, IL-5, and IL-10 ELISA (BD Pharmingen, San Diego, CA) and IL-13 ELISA (R&D systems, Minneapolis, MN) as per the manufacturer's protocol. Results were expressed in picograms and normalized by protein concentrations. OVA specific IgE and IgG1 and differential cell counts in BAL fluid were estimated as described earlier (18).

**Lung histology.** Histological examination of the lungs was performed using hematoxylin and eosin (H&E) staining, periodic acid-Schiff (PAS) staining for mucus production, and Masson trichrome (MT) staining to detect subepithelial fibrosis as described (18). Inflammation scoring and morphometry were performed in H&E, PAS, MT-stained lung sections as described earlier (17).

**Isolation of whole lung RNA and real-time PCR.** The tissue was processed to isolate total RNA using Trizol (Invitrogen) according to manufacturer's protocol. To study the relative expression of mmu-miR-106a, real-time PCR was performed (Applied Biosystem) in

Light Cycler-480 (Roche) and normalized with sno202 (5). Analysis was performed using mathematical model as described earlier (23).

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Significant differences between selected two groups were estimated using unpaired Student *t*-test. Statistical significance was set at  $P \leq 0.05$ . Each experiment was performed at least three times with six mice in each group.

## RESULTS

**Expression of IL-10 is downregulated by mmu-miR-106a.** Earlier we demonstrated an inverse correlation between miR-106a and IL-10 in human cell lines (26). Interestingly, we observed that human and mouse microRNAs have two nucleotide differences (Fig. 1A), although the seed sequence was conserved. Nonetheless, we first confirmed the suppression of IL-10 production by mmu-miR-106a in RAW 264.7 cells. As shown in Fig. 1B, mmu-miR-106a downregulated the levels of IL-10 in transfected RAW 264.7 cells. However, cel-miR-67 that has no sequence homology with mouse or human microRNA did not affect IL-10 levels. As IL-10 is one of the immune-regulatory cytokine and is known to modulate TNF- $\alpha$ , we estimated the levels of TNF- $\alpha$  in LPS-induced and mmu-miR-106a- or cel-miR-67-transfected RAW 264.7 cells. As shown in Fig. 1C, there was a dose-dependent increase in

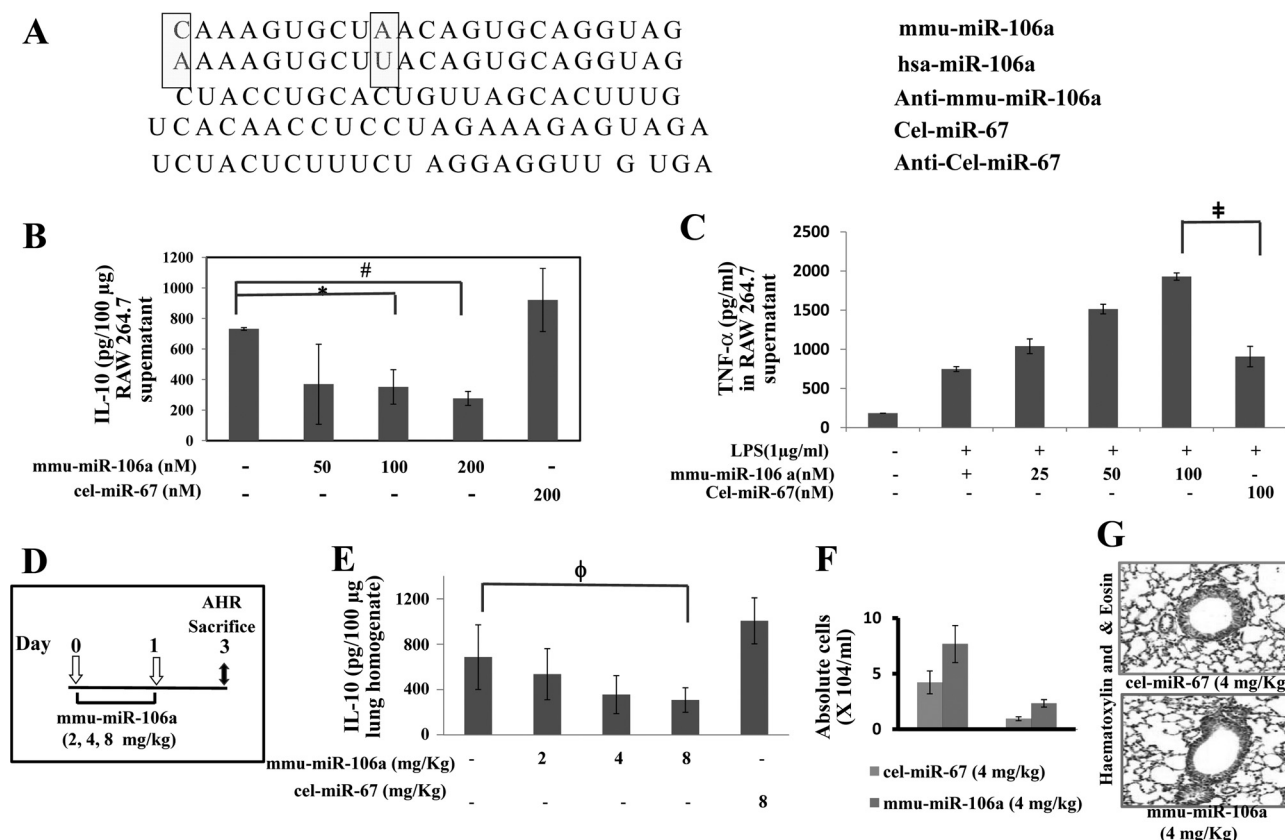


Fig. 1. Conservation of miR-106a-mediated regulation of IL-10 in mice. A: Sequence alignment of human and mice variants of miR-106a shows differences in 2 nucleotide positions just outside the seed region in the micro-RNAs and sequences of oligos used in this study. The levels of IL-10 (B) and TNF- $\alpha$  (C) from supernatants of RAW 264.7 cells transfected without or with increasing concentrations of mmu-miR-106a or cel-miR-67 are shown ( $n = 3$  each). \*, #,  $\phi P = 0.014$ , 0.003, 0.002, respectively. D: schematic representation of miR-106a mimics delivery in naive mice. E: IL-10 levels in lung homogenate of naive mice nebulized without or with mmu-miR-106a oligonucleotide or control cel-miR-67 oligonucleotide ( $n = 6$  each group). Data were means  $\pm$  SE of four independent experiments.  $\phi P = 0.04$ . The effects of mmu-miR106a and cel-miR67 on absolute BALF cell counts (F) and lung histology (G) in normal mice. Macro, macrophage; Mono, mononuclear cells such as lymphocytes, monocytes.

TNF- $\alpha$  levels with mmu-miR-106a transfection, whereas no effect was found with cel-miR-67 transfection. Furthermore, nebulization of mmu-miR-106a to naive BALB/c mice (Fig. 1D) downregulated the levels of IL-10 in lungs in a concentration-dependent manner compared with vehicle or cel-miR-67 nebulization (Fig. 1E), without altering differential cell counts or lung histology (Fig. 1, F and G). These experiments demonstrate that mmu-miR-106a could be a prominent physiological regulator of IL-10.

**Mmu-mir-106a is increased and IL-10 is reduced in allergic lung inflammation.** To further confirm the physiological effect of miR-106a, we administered anti-mmu-miR-106a or scrambled oligonucleotide to the lungs of mice with or without AAI (Fig. 2A) and estimated IL-10 levels in lungs. The levels of mmu-miR-106a were found to be significantly increased in lungs of mice with AAI, and its levels were reduced with anti-mmu-miR-106a treatment, whereas such reduction was not found with scrambled miRNA treatment (Fig. 2B) indicating the efficiency of knockdown. We then estimated the effect of anti-mmu-miR-106a or scrambled oligonucleotide on the IL-10 expression in the lungs of mice. IL-10 was found to be significantly reduced in lungs of mice with AAI, and its levels were restored with anti-mmu-miR-106a treatment compared with scrambled miRNA treated mice (Fig. 2C). Because macrophage is one of the major producers of IL-10 (2, 7), flow cytometry in single cell suspension of lung was performed to see whether macrophages have taken anti-mmu-miR106a or scrambled and its effect on IL-10 expression. CD11b-positive cells took up fluorescently labeled anti-mmu-miR106a (gating

was done with DY547) and this was associated with modest increase in IL-10 expression (Fig. 2D1), whereas there was no effect on IL-13 expression (Fig. 2D2). These results are consistent with our earlier observation where we observed a high level of mir-106a expression in macrophage compared with T cells, B cells, and epithelial cells (26). In addition, as macrophage is the dominant producer of IL-10 in vivo (2), we suggest that anti-mmu-miR106a was primarily taken up by macrophages and controls IL-10 expression in allergic mice (26).

**Anti-mmu-mir-106a ameliorates airway inflammation and reduced Th2 response in lung.** To study the effect of blocking mmu-miR-106a on airway inflammation, H&E staining of the lung sections was performed. Delivery of anti-mmu-miR-106a, but not the scrambled control oligonucleotide, significantly reduced infiltration of inflammatory cells both in perivascular (PV) and peribronchial (PB) regions (Fig. 3A1). The semi-quantitative assessment of inflammation by blind scoring confirmed that treatment with anti-mmu-miR-106a resulted in a significant reduction in inflammatory cell infiltration (Fig. 3A2).

To determine the effect of anti-mmu-miR-106a on other immune parameters of AAI; Th2 cytokines such as IL-4, IL-5, IL-13; and OVA specific immunoglobulins such as IgE and IgG1 were estimated in lung homogenates and sera, respectively. As shown in Fig. 3, anti-mmu-miR-106a significantly reduced the levels of IL-4 (Fig. 3B), IL-5 (Fig. 3C), IL-13 (Fig. 3D), and OVA specific IgE (Fig. 3E), but had no effect on OVA specific IgG1 (Fig. 3F). It is important to note that anti-mmu-miR-106a reduced IL-13 in lung homogenates,

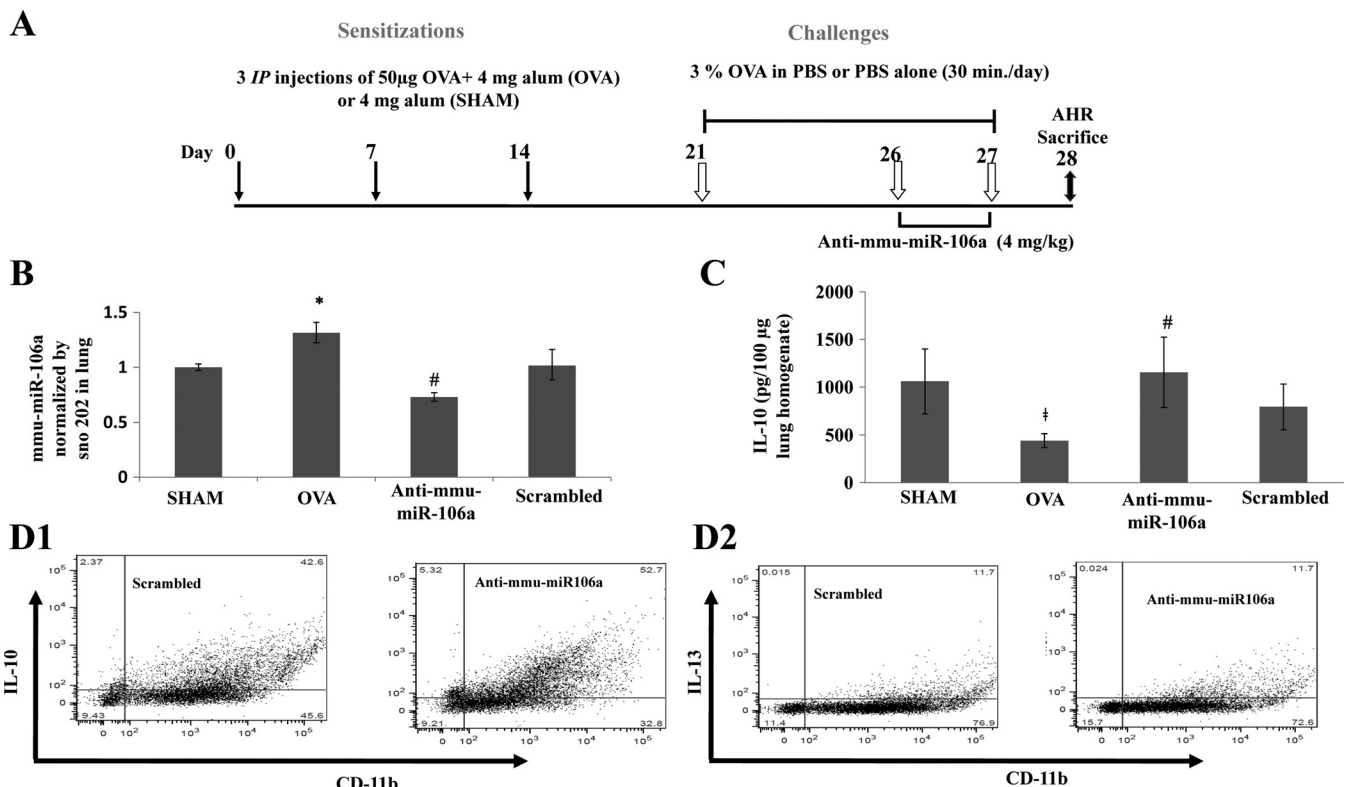


Fig. 2. Administration of anti-mmu-miR-106a blocks miR-106a and elevates IL-10 expression in mice with AAI: anti-mmu-miR-106a (A) was delivered to OVA sensitized and challenged mice as described in the METHODS. Levels of mmu-miR-106a which were normalized by sno202 (B) and IL-10 (C) in lung. \* $P = 0.04$ , 0.02 vs. Sham, respectively, and # $P < 0.05$  vs. OVA ( $n = 5$ , all measurements in triplicate). Data were means  $\pm$  SE of 4 independent experiments. (D1, D2) Flow cytometry analysis of IL-10 (D1) or IL-13 (D2) positive CD11b cells, first gated for the uptake of DY547 labeled either Scrambled or anti-mmu-miR-106a.

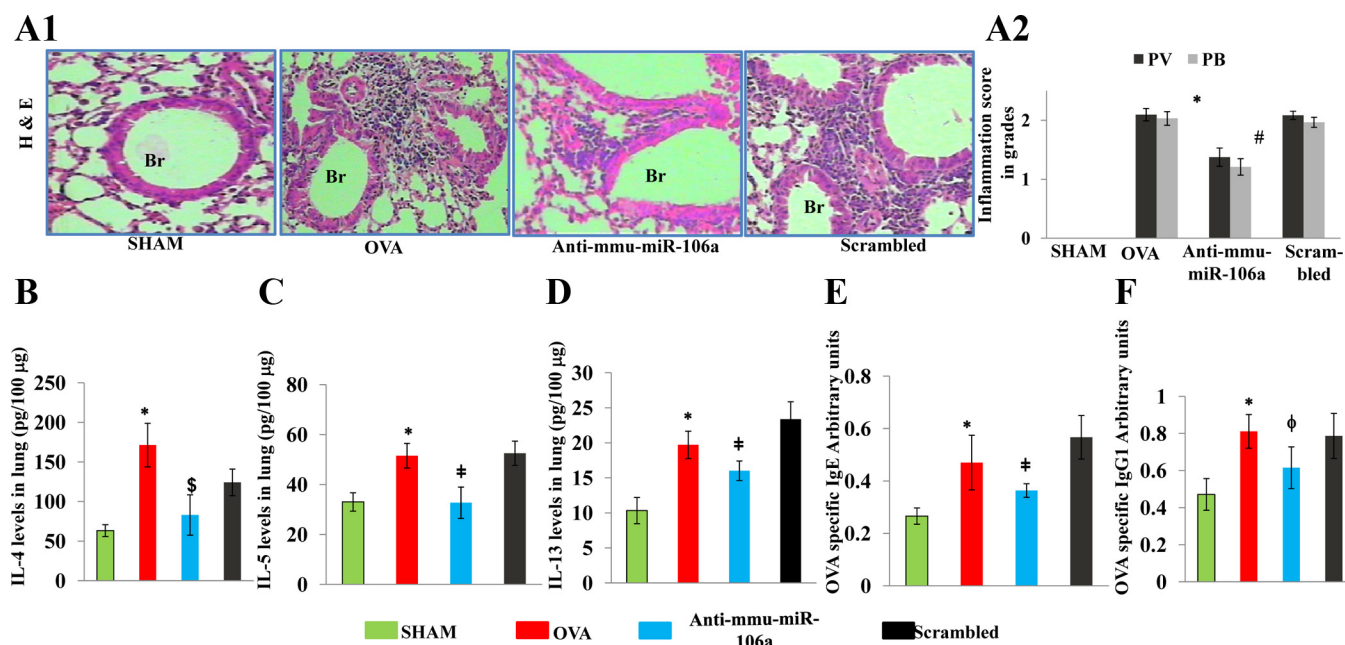


Fig. 3. Administration of Anti-mmu-miR-106a reduces airway inflammation and Th2 response. Lung sections from different groups were stained using hematoxylin and eosin (H&E; A1) and semi-quantitative assessments on airway inflammation (A2) are also shown. ELISA was performed to determine the levels of IL-4 (B), IL-5 (C), and IL-13 (D) in lung homogenates and to determine the relative levels of OVA specific IgE (E) and IgG1 (F) in sera. Results are expressed as means  $\pm$  SE of 2 independent experiments with 6 mice in each group. \* $P < 0.005$  vs. Sham, # $P < 0.05$  vs. OVA, and \$,‡,ϕ $P < 0.05$ , = 0.02, not significant vs. Scrambled, respectively. Data are means  $\pm$  SE of 4 independent experiments. Br, bronchi; PV, perivascular inflammation; and PB, peribronchial inflammation.

whereas this effect was not observed in macrophages (Fig. 2D2), indicating possible cell specific effects.

**Anti-mmu-mir-106a alleviates airway hyperresponsiveness.** To determine the effect of anti-mmu-miR-106a on airway hyperresponsiveness (AHR), airway resistance was estimated using FlexiVent invasive airway mechanic system. As shown in Fig. 4A, OVA mice showed greater dose-dependent increase in airway resistance with methacholine than normal sham control mice. Restoration of the normal airway response to methacholine was found with anti-mmu-mir-106a treatment but not with scrambled control oligonucleotide.

**Anti-mmu-mir-106a reduces goblet cell metaplasia.** During the development of allergic asthma, there is an increased production of mucus from the goblet cells, which results in obstruction in breathing (1). So we next wanted to determine the effect of anti-mmu-miR-106a on goblet cell metaplasia and airway mucin content. It was observed that mice with OVA-induced allergic asthma had marked goblet cell metaplasia and significantly increased airway mucin content (Fig. 4, B1 and B2). Anti-mmu-miR-106a treatment significantly reduced goblet cell metaplasia and airway mucin content (Fig. 4, B1 and B2), whereas scrambled oligonucleotide had no effect.

**Anti-mmu-mir-106a reduces airway fibrosis.** Because sub-epithelial fibrosis is one of the important pathological features of asthma, we sought to investigate the effect of anti-mmu-miR-106a on subepithelial fibrosis. Sensitization and challenge with OVA resulted in substantial increase in collagen deposition in mice lungs (Fig. 4, C1 and C2). Treatment with anti-mmu-miR-106a was able to partially reduce collagen deposition, whereas scrambled oligonucleotide had no effect.

## DISCUSSION

In this study, we demonstrate for the first time that inhibition of mmu-miR106a with anti-mmu-miR106a increases IL-10 and alleviate the features of allergic airway inflammation. This extends our previous finding that miR-106a targets IL-10 in vitro; showing that such mechanism may also be operational in vivo and have pathophysiological relevance in asthma though the involvement of other targets of miR106a cannot be ruled out.

It is interesting to note that despite the lack of sequence conservation between mouse and human variants of both IL-10 and miR-106a, there is conservation in the biological process of miR-106a-mediated IL-10 regulation. This strongly suggests that it is an important biological regulatory mechanism. We found that lungs from naive mice are rich in IL-10, which was reduced upon sensitization and challenge with allergen, in accordance with many existing reports (3, 10, 28). We also found negligible basal expression of mmu-miR-106a in the lungs of normal mice, which is in consonance with other reports (9). Furthermore, we found that allergic airway inflammation in mice is associated with substantially increased mmu-miR-106a and decreased IL-10. In earlier studies, knockout of mmu-miR-106a-363 cluster was observed to have no apparent change in baseline phenotype (30). This could be due to very low basal expression of mmu-miR-106a in mouse lungs, and it would be interesting to see whether these knockout mice become resistant to asthma. Our finding that mmu-miR-106a importantly regulates IL-10 is only one piece of the larger puzzle of IL-10 regulation, in which additional miRs may be relevant. Previously we reported that miR-106b (that belongs to the same family as miR-106a), may also regulate IL-10 (26).

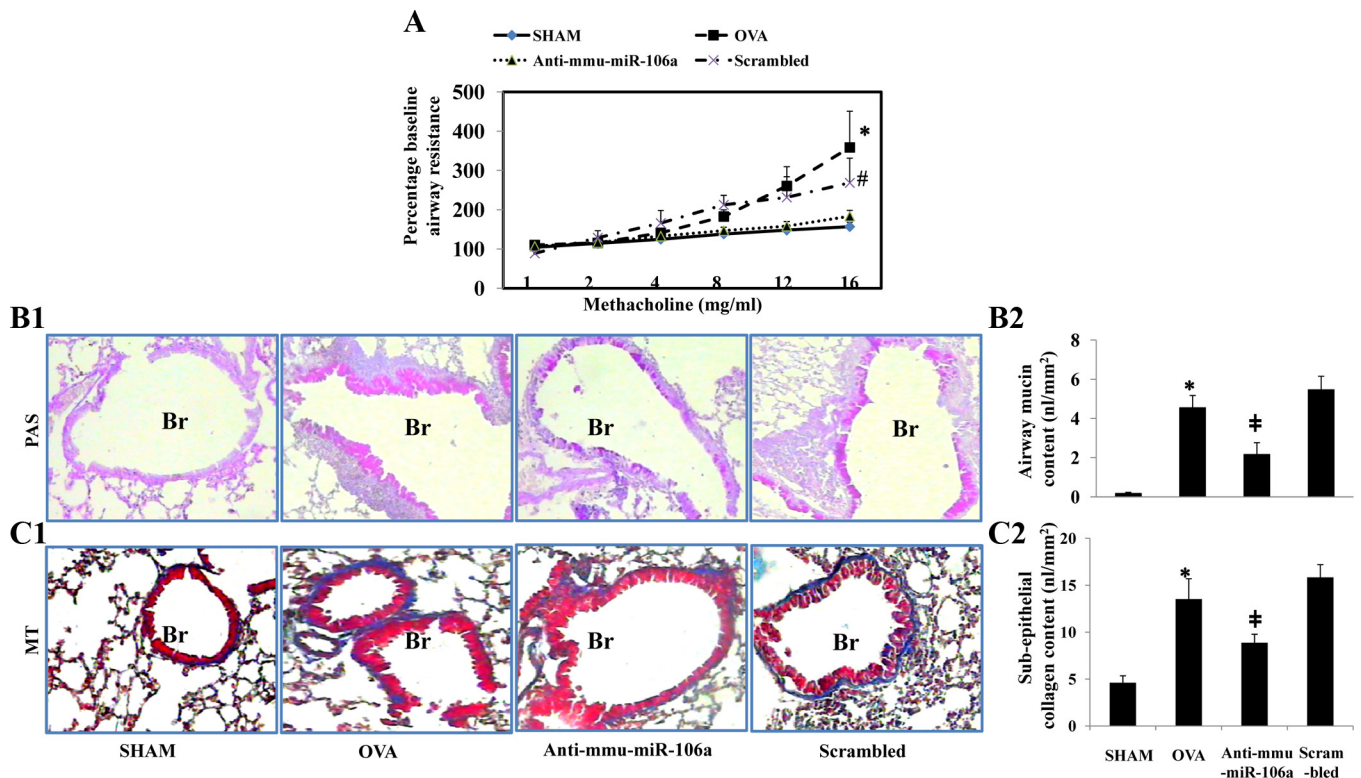


Fig. 4. Administration of anti-mmu-miR-106a reduces airway hyperresponsiveness, goblet cell metaplasia, and subepithelial fibrosis. **A**: airway resistance was estimated with increasing concentrations of methacholine. Periodic acid-Schiff (PAS; **B1**) and Masson's trichrome (MT; **C1**) stainings were performed to assess the effect of anti-mmu-miR-106a on goblet cell metaplasia and subepithelial fibrosis, respectively. Semi-quantitative assessments on airway mucin content (**B2**) and subepithelial collagen content (**C2**) are also shown. Data were means  $\pm$  SE of 4 independent experiments. \* $P < 0.005$  vs. Sham, #,  $\ddagger P = 0.003$  and  $< 0.05$  vs. OVA, respectively.

In this context, it is notable that recent results suggest that miR-106b expression is reduced in the lungs of asthmatic mice (24), opposite to the changes we find for miR-106a. Further studies incorporating cell-specific investigation of relative expression of miR-106b with respect to miR-106a in models of allergic asthma will be required to correctly understand the interplay between miR-106a, miR-106b, and IL-10. Also, our results do not rule out the possibility that the observed anti-inflammatory effects of mmu-miR-106a knock-down could be due to other targets in addition to IL-10 (12).

Whether changes in miRNA expression are associated with the pathogenesis of asthma is still unclear. Very few studies are available to explore the expression profiles of miRNA in asthmatic conditions. One human study did not find any significant asthma-associated alteration in the miRNA expression profile of airway tissue (32). In mouse studies, however, a number of miRNAs have been found to be altered (9). This discrepancy may be due to the selection of only either mild asthmatic patients or steroid-treated patients in that study and also due to the larger biological variability in human studies. The technology for miRNA profiling has also substantially improved in the intervening period (5). Interestingly, mmu-miR-106a was found to be upregulated about twofold in the short-term mouse model of asthma (9). The effect of miR-106a on IL-10 may not be exclusive because there are other miRNAs such as miR4661 (16) and miR98 (14) that are known to regulate IL-10. On the other hand, IL-10 upregulates few miRNAs such as 146a, 146b, and 125a (20) and inhibits

miR-155 (19). It is also important to note that miR-146a controls the suppressive function of Treg cells (15). Any efforts to decipher the influence of these miRNAs on the asthmatic phenotype is further complicated by the heterogeneous evidence of the role of IL-10 within asthma, with short-term anti-inflammatory effects but long-term effects being less clear. In support of the dominant anti-inflammatory effects, a significant defect in IL-10 synthesis has been demonstrated in asthmatic patients (28). IL-10 is shown to mediate resolution of inflammation by suppression of IL-5, GM-CSF, and nitric oxide. Inhaled corticosteroid and allergen specific immunotherapies increase the levels of endogenous IL-10 and alleviate allergic features (3). Also, short-term administration of recombinant IL-10 or adenovirus expressing IL-10 has been shown to reduce eosinophilic airway inflammation in mouse models (8). In contrast, variable outcomes have been found with IL-10 knockout mice (22). Interestingly, in the long-term mouse model of asthma, miR-98 was found to be downregulated and miR-146a was found to be upregulated, indicating the complicated interactions between various miRNAs and cytokines such as IL-10. These considerations limit the potential use of IL-10 knockout mice to further dissect the role of miR-106a in asthma.

In summary, we demonstrate for the first time that allergic airway inflammation in mice is associated with substantially increased mmu-miR-106a and decreased IL-10. This was confirmed by administration of anti-mmu-miR-106a by aerosol, which alleviated most of the features of asthma along with

increase in IL-10 levels. Importantly, anti-mmu-miR-106a was administered after allergic sensitization and five challenges, by which time the experimental asthma phenotype is firmly established. Furthermore, allergen exposure was continued during anti-mmu-miR-106a delivery. Thus the observed attenuation of the asthma phenotype is potentially relevant to treatment of clinical disease. Further research focusing on the usage of microRNAs in the development of future asthma therapeutics is an avenue that remains to be investigated.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: A.S., M.K., A.A., and B.G. conception and design of research; A.S., M.K., T.A., U.M., and J.A. performed experiments; A.S., M.K., T.A., U.M., J.A., A.A., and B.G. analyzed data; A.S., M.K., T.A., U.M., J.A., A.A., and B.G. interpreted results of experiments; A.S., M.K., U.M., A.A., and B.G. prepared figures; A.S., M.K., J.A., A.A., and B.G. drafted manuscript; A.S., M.K., T.A., U.M., J.A., A.A., and B.G. edited and revised manuscript; A.S., M.K., T.A., U.M., J.A., A.A., and B.G. approved final version of manuscript.

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