Antagonism of microRNA-126 suppresses the effector function of T_H2 cells and the development of allergic airways disease

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Allergic asthma is an inflammatory disease of the lung characterized by abnormal T helper-2 (T_H2) lymphocyte responses to inhaled antigens. The molecular mechanisms leading to the generation of T_H2 responses remain unclear, although toll-like receptors (TLRs) present on innate immune cells play a pivotal role in sensing molecular patterns and in programming adaptive T cell responses. Here we show that in vivo activation of TLR4 by house dust mite antigens leads to the induction of allergic disease, a process that is associated with expression of a unique subset of small, noncoding microRNAs. Selective blockade of microRNA (miR)-126 suppressed the asthmatic phenotype, resulting in diminished T_H2 responses, inflammation, airways hyperresponsiveness, eosinophil recruitment, and mucus hypersecretion. miR-126 blockade resulted in augmented expression of POU domain class 2 associating factor 1, which activates the transcription factor PU.1 that alters T_H2 cell function via negative regulation of GATA3 expression. In summary, this study presents a functional connection between miRNA expression and asthma pathogenesis, and our data suggest that targeting miRNA in the airways may lead to anti-inflammatory treatments for allergic asthma.

animal model | asthma | inflammation | microRNA | T_H2 cytokines

Asthma is a chronic inflammatory disease of the airways that has been described as an epidemic in developed countries (1, 2). The clinical condition is characterized by episodic breathlessness and wheezing, together with enhanced airways hyperresponsiveness (AHR) to a variety of stimuli that is associated with aberrant allergen-specific $CD4^+$ T helper-2 lymphocyte (T_H2 cells) responses (1, 3). These findings have lead to the paradigm that activation of T_H2 cells by inhalation of allergens plays a pivotal role in disease development and progression through the actions of their secreted cytokines. Indeed, the hallmark features of allergic asthma, such as elevated serum IgE, airway eosinophil infiltration, mucus hypersecretion and AHR have all been linked to the effector functions of the T_H2 cytokines interleukin (IL)-4, -5, -9, and -13 (3, 4). An emerging concept for a mechanism potentially causing asthma is that the innate immune system inappropriately senses allergens as foreign and dangerous and responds with a programmed adaptive T_H2 immune response (5, 6).

Toll-like receptors (TLRs) differentially sense microbial and viral bioproducts and act as sentinels for the activation of innate host defense pathways (7, 8). Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacterial, activates cells through TLR4 and the common TLR adaptor protein myeloid-differentiation-primary-response-gene-88 (MyD88) resulting in activation of transcription and proinflammatory pathways (7, 9, 10). LPS is also a prominent constituent of asthma-inducing house dust mite (HDM) allergens and can instruct the immune response to inhaled antigen to generate T_H2 responses (5). Indeed it is likely that signals derived from early activation of the innate immune system play an important role in the generation of aberrant T_H2 responses. Furthermore, clinical studies have shown a direct correlation with the levels of LPS in HDM and the severity of asthma, supporting the concept that the innate immune system plays as significant role in disease pathogenesis (11, 12).

microRNAs (miRNAs) are emerging as important regulators of gene expression in the immune system by functioning as endogenous inhibitors of translational processes (13). A single miRNA can target hundreds of messenger-(m) RNAs and thereby modulate protein output from their respective genes (14). Therefore a single or specific set of miRNAs may control discrete physiological processes by regulating the production of a few critical proteins that coordinate single or interrelated cellular events (e.g., cell proliferation) (13, 14). Very recently, TLR signaling and miRNA expression have been linked to one another, and it has been proposed that the coupling of these pathways form elemental regulatory signals of the innate immune system, which lead to activation of inflammatory pathways (15–17). Thus, TLRs and miRNAs may collaborate to promote host defense responses and the programming of the adaptive immune response (13). Although promising, the role of miRNA in the regulation of immunological processes is in its infancy, and their contribution to the pathogenesis of diseases of the immune system, of which many have chronic inflammatory components, remains unknown (13, 18). However, there are several pioneering studies, which indicate that manipulation of miRNA expression has the potential for therapeutic efficacy (19–23).

Currently there are no functional data that relate specifically to the role of miRNA in the regulation of asthma and allergen-induced T_H2 responses. However, given that miRNA expression can be controlled through activation of TLRs by microbial and viral bioproducts (15, 16), we hypothesized that TLR regulation of miRNA expression in the airways may be an early and fundamental step in the host response to allergen exposure, and that under certain conditions, expression of unique miRNAs may lead to programming of aberrant T_H2 responses.

Results

Exposure of the Airways to House Dust Mite Increases the Expression of Specific miRNAs in the Airways through TLR4- or MyD88-Dependent Mechanisms. Initially we determined if miRNAs were expressed in the airways in response to allergen exposure by employing a well characterized and clinically relevant BALB/c mouse model of HDM-induced allergic asthma (24). In this model, allergen exposure within the airways results in direct sensitization and the

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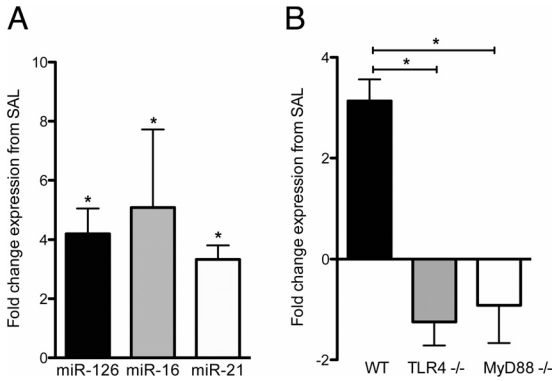


Fig. 1. HDM-induced miR-126 expression is TLR4- and MyD88-dependent. (*A*) Fold change in miRNA-126, -16, and -21 expression in the airway wall of mice challenged twice with HDM as compared to baseline (nonallergic, SAL mice). (B) Fold change in miR-126 expression in HDM allergic WT, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice as compared to baseline (SAL mice). Results are mean ± SEM (n = 3–4 mice/group). *, $P < 0.05$, HDM versus SAL. Data are representative of two independent experiments.

development of allergen-specific T_H2 cells. We modified exposure times to allow a temporal analysis of the effect of HDM on the regulation of miRNA expression in the airway wall. To achieve this, we separated the airway wall compartment from the parenchymal tissue by blunt microscopic dissection (25). Naive wild-type (WT) BALB/c mice were exposed to a single dose of HDM by directly instilling the allergen into the airways, and we then characterized the expression of miRNAs in the airway wall at 6, 12, and 24 h after challenge. At 24 h, we observed a significant increase in miRNA-16, -21 and -126 (expression increased by ≈2-fold). Ten days later, we rechallenged the airways with the single dose of HDM and observed a further increase in the expression levels of these miRNA (expression increased >2-fold). miRNAs were not upregulated by challenge with the control vehicle (saline). The increased expression of miR-16, -21 and -126 was then validated by quantitative PCR (Fig. 1*A*). The potential role miR-126 in regulating the asthma phenotype was then investigated. The rapid upregulation of miR-126 in the airway wall suggested that pathways linked to innate host defense might be activated by components such as LPS, which are found in HDM extracts. Consistent with this hypothesis miR-126 was not upregulated in the airways of TLR4- or MyD88- deficient mice after HDM sensitization and repeated challenges (Fig. 1*B*). These data are consistent with our hypothesis that specific miRNA may be expressed as an early component of the innate host response to allergens to potentially modulate gene expression and to promote T_H2-mediated allergic inflammation.

TLR4- or MyD88- Pathways Regulate miR-126 Expression and Hallmark Features of Allergic Inflammation. As the TLR4- or MyD88- pathways regulated miR-126 expression we next evaluated their role in the development of pathophysiological features of allergic disease. WT, TLR4, or MyD88 deficient ($^{-/-}$) mice were sensitized with HDM daily for 3 days, and 11 days later, the airways were rechallenged with allergen for 4 consecutive days to induce fulminant allergic inflammation and characteristic lesions (Fig. 2). The severity of disease was determined quantitatively 24 h after the last HDM exposure (day 18). Only in WT mice did exposure of the airways to HDM induce hallmark features of allergic asthma (Fig. 2). HDM challenge of WT but not TLR4$^{-/-}$ or MyD88$^{-/-}$ mice induced AHR to methacholine (Fig. 2 *A*, *B*, *C*, respectively). Suppression of AHR in allergen-challenged TLR4$^{-/-}$ or MyD88$^{-/-}$ mice was associated with a significant reduction in the number of eosinophils recruited to the airways (Fig. 2 *D–F*). In allergic MyD88$^{-/-}$ mice, mucus-producing cells (mean/SEM: 1.9/0.5 cells), IL-5 (2.64/0.32 ng/mL), IL-4 (less than 0.5 pg/mL) levels, and IgG1 serum levels (353/97 ng/mL) were also significantly reduced ($P < 0.05$) when compared to allergic WT mice (Fig. 3, Fig. 4, and below). Thus, activation of the MyD88 signaling pathway by HDM plays a critical role in the

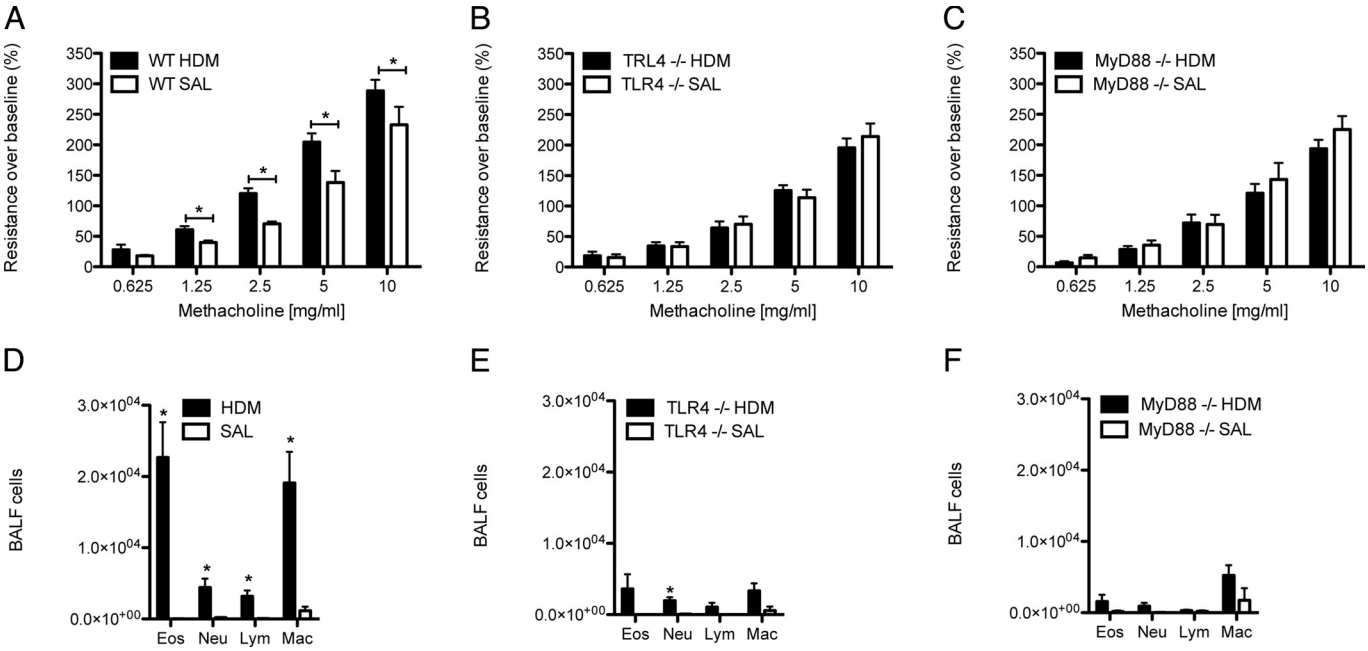


Fig. 2. TLR4/MyD88 deficiency abolishes HDM-induced AHR and reduces airway inflammation. (*A–C*) Total lung resistance is presented as a percentage change over baseline measurement (water) in response to inhaled methacholine in allergic (HDM) versus nonallergic (SAL) wild-type (WT), TLR4$^{-/-}$, and MyD88$^{-/-}$ mice. Results are mean ± SEM (n = 6–10 mice/group). *, $P < 0.05$. [*D* (WT), *E*, and *F*] Number of cells in BALF. Results are mean ± SEM (n = 3–4 mice/group). *, $P <$ 0.05. Data are representative of two independent experiments.

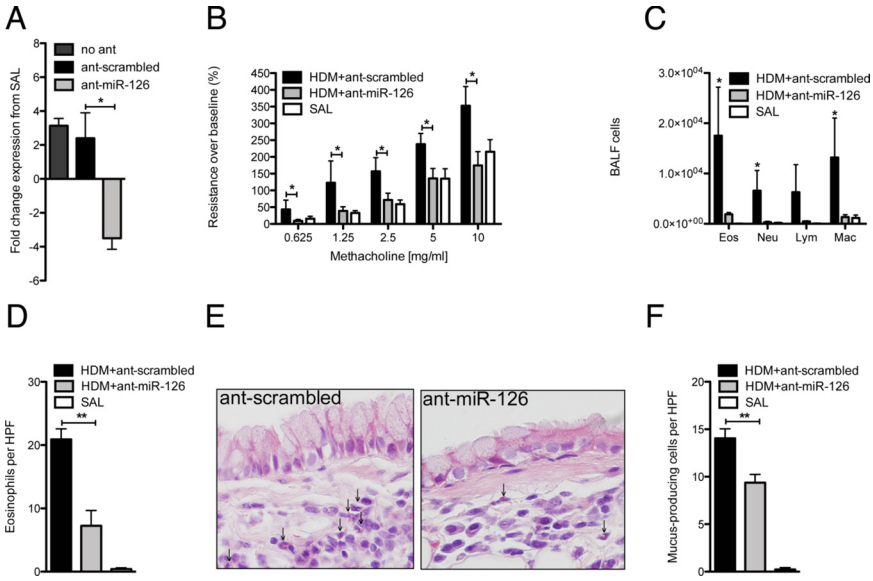


Fig. 3. Silencing miR-126 function by antagomir abolishes HDM-induced airways hyperreactivity and reduces allergic inflammation. (*A*) HDM-induced miR-126 expression after no treatment (no ant), treatment with a scrambled control sequence (ant-scrambled), or treatment with antagomir specific for the miR-126 sequence (ant-miR-126) in allergic mice. Results are mean ± SEM of the fold change from nonallergic mice treated with saline (SAL) only (baseline) (n = 3–4 mice/group). *, $P < 0.05$. (*B*) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine in HDM challenged allergic mice treated with ant-miR-126 or ant-scrambled versus nonallergic (SAL) mice. Results are mean ± SEM (n = 6–10 mice/group). *, $P < 0.05$. (*C*) Number of inflammatory cells in BALF. Results are mean ± SEM (n = 3–4 mice/group). *, $P < 0.05$. (D) Number of peribronchial eosinophils (×1000) per high-power field (HPF), (*E*) representative H&E stained histological section showing decreased eosinophil numbers in tissue (arrows), and (*F*) number of mucus-producing cells (×1000) per HPF. Results are mean ± SEM (n = 3–4 mice/group). **, $P < 0.01$. Data are representative of two independent experiments.

induction of miR-126 expression and critical features of allergic inflammation.

Inhibition of miR-126 Function Suppresses HDM-Induced AHR and Hallmark Features of Allergic Airways Inflammation. To demonstrate a regulatory role for miR-126 in the development of the phenotypic features of allergic asthma, we designed antagomirs (cholesterol-linked single-stranded antisense RNA) to block miR-126 function. HDM-sensitized WT mice were treated intranasally (i.n.) with antagomirs (ant-miR-126) or with scrambled antagomir (ant-scrambled) as a control. Antagomirs were administered 24 h before the first HDM rechallenge (10 days after HDM sensitization) and then given every 48 h. Disease severity was determined at day 18, 24 h after the last exposure to both HDM and antagomirs. HDM induced miR-126 expression was completely inhibited in the airway wall after treatment with ant-miR-126 but not with scrambled control antagomir (Fig. 3*A*). Inhibition of miR-126 function in the airway wall abolished HDM-induced AHR to methacholine (Fig. 3*B*). The number of eosinophils and neutrophils infiltrating the bronchoalveolar lavage fluid (BALF) (Fig. 3*C*) and lung tissue (Fig. 3 *D* and *E*) were significantly diminished. Histological examination of ant-miR-126-treated lungs also showed decreased mucus hypersecretion (Fig. 3*F*). Mice treated with antagomirs did not display any ill effects, identifying the potential for modulation of miRNA function in the lung as a therapeutic approach.

miR-126 Suppresses the Effector Function of Lung T_H2 Cells. We and others have previously shown that T_H2 cells and their effector cytokines IL-4, IL-5, and IL-13 play critical roles in the regulation of antibody production, AHR, mucus cell hypersecretion, and eosinophil accumulation in the lung (26–30). In HDM sensitized and challenged mice, exposure of lung to ant-miR-126 suppressed the secretion of IL-5 and IL-13 from allergen-activated peribronchial lymph (PBLN) node T_H2 cells (Fig. 4*A*), which was associated with inhibition of the crucial pathophysiological responses they induce (Fig. 3). IL-4 was also significantly reduced in mice treated with ant-miR-126 as compared to those treated with scrambled antagomir (mean/SEM: 16.1/3.5 versus 313.3/7.8 pg/mL, $P < 0.01$) although IL-4 release by PBLN cells was altogether markedly lower as compared to IL-13 and IL-5 levels. By contrast, T cell IFNγ-secretion was not altered significantly after exposure to ant-miR-126 (Fig. 4*A*). The percentage (Fig. 4 *B* and *C*) and total numbers of $CD4^+$ T cells (T_H2 cells) and myeloid dendritic cells (DCs) ($CD11b^+$ $CD11c^+$ MHC class II ^{high}) infiltrating the lung were also suppressed by ant-miR-126 treatment in HDM sensitized and challenged mice. No effect of miR-126 inhibition in the lung on serum levels of allergen-specific IgG1 and IgG2a levels as well as total IgE levels were observed (mean/SEM in ant-miR-126 treated versus untreated mice for IgG1: 593/70 versus 973/156 ng/mL, $P = 0.07$; IgG2a: below 0.4 ng/mL in all samples; total IgE: 33/9 versus 38/5 ng/mL, $P = 0.63$).

Inhibition of miR-126 Function Alters Gene Expression in the Airways of Allergic Mice. To gain insights into the downstream inflammatory pathways regulated by miR-126 we performed a gene array analysis. We compared the transcriptomes of the airway walls isolated from HDM sensitized and challenged WT mice that had been treated with ant-miR-126 or scrambled control. Interestingly, the expression of only a limited number of RNA transcripts was affected by ant-miR-126 treatment, and most of these differentially regulated genes that encode for regions in the Ig (Ig) kappa (n = 15), lambda (n = 3), or heavy (n = 11) chains. Moreover, the only non-Ig gene significantly upregulated by miR-126 inhibition in vivo was POU domain class 2 associating factor 1, also named Oct binding factor 1 (OBF.1) or B-cell Oct binding protein 1 (BOB.1) (Fig. 4*D*). Importantly, OBF.1/BOB.1 is not only a critical regulator of antibody production but also the transcriptional factor PU.1, which negatively regulates TLR4 expression and T_H2 responses by suppressing GATA3 expression

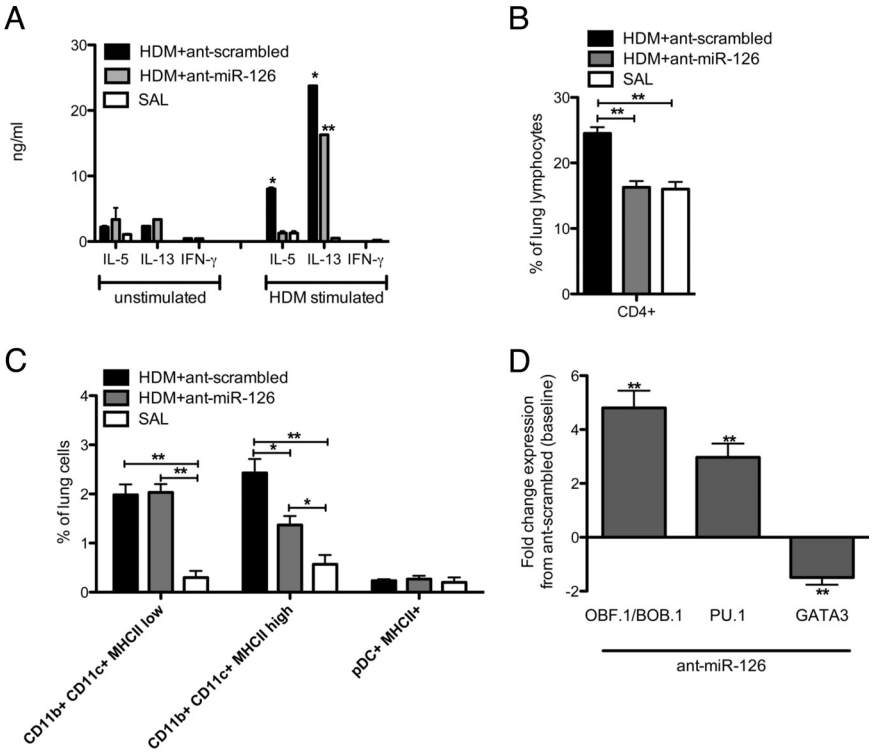


Fig. 4. Silencing miR-126 function by antagomir impairs T_H2 responses in the lung. (*A*) IL-5, IL-13, and IFN-γ release from in vitro HDM-stimulated peribronchial lymphnode cells after no treatment (SAL) or treatment with a scrambled control sequence (ant-scrambled) or treatment with antagomir specific for the miR-126 sequence (ant-miR-126) in allergic mice and in nonallergic mice (SAL). Results are mean ± SEM (n = 6–10 mice per group). *, $P < 0.05$, ant-scrambled versus SAL; **, $P < 0.01$, ant-miR-126 versus SAL. (*B*) Percentages of $CD4^+$ cells in lung homogenates and (*C*) percentages of $CD11b^+$ $CD11c^+$ MHC class II ^{high} (myeloid DCs) and pDC^+ MHC class II ^{high} cells (plasmacytoid DCs) in lung homogenates. Results are mean ± SEM of three experiments (n = 4–6 mice/group per experiment). (*D*) Fold change in OBF.1/BOB.1 and PU.1 expression in the airway wall and GATA3 expression in the parenchyma of HDM-treated allergic mice that were exposed to ant-miR-126 as compared to those mice treated with ant-scrambled (baseline). **, $P < 0.01$, ant-miR-126 versus ant-scrambled. Results are mean ± SEM (n = 4 mice/group). Data are representative of two independent experiments.

(31, 32). Indeed, quantitative analysis of PU.1 and GATA3 expression after ant-miR-126 treatment showed increased and decreased expression, respectively (Fig. 4*D*).

Discussion

The role of miRNAs in the regulation of immunological processes is beginning to emerge (13, 16–18, 21, 23, 33–36), however their contribution to inflammatory diseases such as asthma remains unknown. In this investigation, we employ a well characterized model of HDM induced allergic asthma to demonstrate that a select set of miRNAs are rapidly upregulated in the airway wall after allergen exposure. Increased expression of miRNAs and the development of HDM-induced T_H2-mediated allergic inflammation were dependent on the TLR4/MyD88 pathway. This data indicated that miRNA expression is intimately linked to the innate host defense response in the lung and could potentially contribute to the regulation of the subsequent immune response. This study also supports emerging data that miRNA expression and function are linked to innate immune responses (35, 37, 38).

Of the miRNAs upregulated by HDM exposure, we were able to demonstrate a specific role for miR-126 in the regulation of critical features of allergic disease. By exposing the airways to a specific antisense inhibitor, ant-miR-126, we abolished HDM induced expression of miR-126, which resulted in complete suppression of AHR, attenuation of mucus hypersecretion, and inhibition of eosinophil accumulation in the airways and lung tissue. AHR, eosinophil migration, and mucus production are regulated by the T_H2 cytokines IL-5 and IL-13 (26–30), and production of these cytokines were inhibited by ant-miR-126 treatment. Antagomir treatment also inhibited the recruitment of CD4 T cells (predominately T_H2 cells in this model) and T_H2 promoting myeloid DCs into the airways, which are critical for the expression of allergic airways disease (39–41). Importantly, overall suppression of the T_H2 response by ant-miR-126 occurred without evidence of a switch to a T_H1 response, as T cell IFNγ-secretion and neutrophil recruitment were not promoted by antagomir treatment. Collectively, this data demonstrates that miR-126 is a potent and specific activator of the T_H2 regulated allergic inflammatory response and that targeting miRNA expression in the respiratory epithelium may represent an important anti-inflammatory strategy to treat disease.

The way in which miRNAs function has not been fully characterized and identification of the putative transcript targets are currently based on bioinformatics analyses. Examination of databases available in the public domain (e.g., DIANA-microT, miRanda, PicTar, TargetScan) indicates that miR-126 has the potential to modulate hundreds of mRNA transcripts, some of which encode inflammatory mediators and transcriptional factors that modulate their expression. However, the biological relevance of most putative targets has not yet been demonstrated. Indeed, the physiological importance of miRNAs are being primarily ascertained by gain- and loss-of-function experiments. In the context of immune responses, studies on miRNA function suggest critical involvement in activation of transcriptional programs within feedback loops (17). In this context, we demonstrate that inhibition of miRNA-126 function results in the increased expression of two factors whose functional activity is integrated to modulate T_H2 responses and TLR4 expression. Comparisons of expression patterns of the genes in the airway

walls treated with ant-miR-126 or scrambled control showed that the only non-Ig gene upregulated was POU domain class 2 associating factor 1 or OBF.1/BOB.1. OBF.1/BOB.1 was originally described as a B-cell-specific transcriptional coactivator and enhancer of octamer-dependent transcription when recruited to DNA via protein-protein interactions with Oct1 or Oct2 (42, 43). OBF.1/BOB.1 is essential for V(D)J recombination by directly enhancing Ig kappa gene transcription, and in OBF.1/BOB.1$^{-/-}$ mice, the production of Ig isotypes is profoundly reduced (43). The increase in OBF.1/BOB.1 expression by ant-miR-126 treatment resulted in high expression of transcripts that code for Ig chains and are likely to be derived from plasma cells that infiltrate the airways wall during allergic airways inflammation (44). Recently, a critical role of OBF.1/BOB.1 in activation of the transcriptional factor PU.1 and for T_H2 cell function has been shown in vivo. T_H2 cells from OBF.1/BOB.1$^{-/-}$ mice had reduced levels of PU.1, which resulted in higher GATA3 activity and consequently increased T_H2 cytokine release and susceptibility to *Leishmania major* infection (32). Conversely, we show that increased expression of OBF.1/BOB.1 results in increased levels of PU.1 and a suppression of T_H2 cell function (cytokine production) and GATA3 expression. Notably, PU.1 is also involved in directing the transcription initiation complex to the proximal TLR4 promoter, thereby representing a major regulator of TLR4 expression (31), and is a direct target of miR-155 (36). Thus higher expression of OBF.1/BOB.1 and PU.1 observed after the loss of miR-126 function may be directly linked to the enhanced expression of Ig kappa genes and the impaired T_H2/GATA3 regulated allergic responses observed in this study. Furthermore, TLR4-induced miR-126 expression may be integrated in a negative feedback loop, whereby lower OBF.1/BOB.1 levels repress transcriptional activity at the TLR4 promoter through impaired PU.1 binding. Of note, although upregulated after miR-126 inhibition, OBF.1/BOB.1 does not contain putative binding elements for miR-126 in the 3′- or 5′-untranslated region, precluding us to further investigate a direct effect of miR-126 on OBF-1/BOB.1 translation by employing reporter assays. This indicates the complexity of miRNA function in vivo and of how miR-126 modulates gene translation to bridge TLR4 signaling and T_H2 responses to regulate hallmark features of allergic airways disease. However, the data provides important molecular insight into the anti-inflammatory potential of targeting miRNA-126 and its subsequent impact on key downstream mechanisms that modulate allergic inflammation.

By inducing loss-of-function, we describe a physiological role for miR-126 in linking the innate and adaptive immune responses that respond to HDM allergen and result in allergic disease of the lung. The relevance of our observations to human asthma remains to be determined and will require detailed spatial and temporal analyses of miRNA expression patterns in airway wall cells in response to factors that trigger asthma.

The recognition of the contribution of airway inflammation to pathogenesis of asthma has lead to the employment of broad-spectrum anti-inflammatory agents, mostly glucocorticoids, for the treatment of disease. Steroid therapies primarily focus on the management of disease and function by suppressing a range of known and unknown components of the inflammatory response. However, to the best of our knowledge, they do not target factors that initiate inflammatory cascades that perpetuate progression of disease. Our study not only highlights the potential of miRNAs as targets for anti-inflammatory treatments for allergic airways disease, but also draws attention to the importance of identifying the gene regulatory elements that are pivotal switches for the induction of immunological networks that propagate inflammatory cascades and disease.

Materials and Methods

Mice. BALB/c mice (6–14 weeks) were obtained from the Special Pathogen Free Facility of the University of Newcastle. Experiments were approved by the animal ethics committee of the University of Newcastle, Australia. Mice were housed in an approved containment facilities.

Induction of Allergic Airways Disease. We sensitized and challenged mice by exposing them i.n. to HDM extract (crude dermatophagoides pteronyssinus extract; Greer Laboratories) (50 μg daily at days 1, 2, and 3 for sensitization followed by 5 μg daily at days 14, 15, 16, and 17 for rechallenge delivered in 50 μL sterile saline). A single dose of HDM used in initial studies was 50 μg dissolved in 50 μL sterile saline. Nonsensitized mice received sterile saline only.

AHR Measurement. We assessed AHR invasively in separate groups of anesthetized mice by measurement of total lung resistance and dynamic compliance (41). Percentage increase over baseline (water) in response to nebulized methacholine was calculated.

Antagomirs. Target miRNA sequences were downloaded from miRBase, Wellcome Trust, Sanger Institute, Cambridge, UK (Sanger database; http://microrna.sanger.ac.uk/sequences/). We ordered scrambled antagomir from Dharmacon (nonspecific RNA VIII, blasted against the mouse genome) (41). The sequence of ant-miR-126 was: 5′mG.*.mC.*.mA.mU.mU.mA.mU.mU.mA.mC.mU.mC.mA.mC.mG.mG.mU.mA.*.mC.*.mG.*.mA.*. 3′-Chl, where ''m'' were 2′-OMe modified phosphoramidites, ''*'' were phosphorothioate linkages, and ''-Chl'' was hydroxyprolinol-linked cholesterol. We administered 50 μg antagomir/50 μL sterile saline i.n. at day 13 (24 h before the first out of four daily HDM rechallenges) and then every second day until mice were sacrificed at day 18.

Isolation of mRNA and miRNA. Total RNA including miRNA was isolated with mirVana miRNA Isolation kit (Ambion) from lower airway tissue (25). Briefly, the trachea and lungs were isolated, and using two pairs of forceps, the parenchyma was separated from the larger airways by blunt dissection. This facilitated effective separation of the airway wall from the parenchyma leaving several generations of airway for analyses. The preparation consists of resident airway cells, such as epithelial cells, fibroblasts, smooth muscle cells, and the basement membrane as well as infiltrating inflammatory cells.

Quantitative RT-PCR. qRT-PCRs were performed with the TaqMan Gene Expression Assays for the respective miRNA (Applied Biosystems). miRNA expression was normalized to 18S RNA.

miRNA Microarraying. Mice received HDM 50 μg intratracheally or saline-treated (SAL) and were killed at 2, 8, and 24 h post-challenge. Total RNA was extracted from bluntly dissected airways employing the Ambion mirVANA kit according to the manufacturer's protocol. miRNAs were enriched with the Ambion flashPAGE system. The Ambion 1564V1 probeset was printed on microarray epoxy slides by the Australian Genome Research Facility, Parkville, Australia. Analysis of microarray data were conducted using Genespring GX 7 software.

mRNA Microarraying. Total RNA was extracted from the airways using Ambion mirVANA kits according to the manufacturer's protocol. Whole genome Illumina microarrays (SM6V2) were conducted at the SRC Microarray Facility, Brisbane, Australia. Analysis of microarray was conducted using Genespring GX 10.

Flow Cytometry Analysis. We stained cells with anti-CD4-FITC, anti-CD11b-PerCP, anti-CD11c-PE, biotinylated anti-MHCII conjungated to Streptavidin-FITC, respective isotype controls (BD Biosciences PharMingen), and anti-mPDCA-1-PE (Miltenyi). Numbers of positive cells were quantified by flow cytometry (FACS Calibur).

Airway Morphology Studies. Lung tissue was stained, cells identified by morphological criteria, and quantitated by counting 10 HPFs in each slide (41).

Cytokine Analysis. Peribronchial lymph node cells were excised, filtered, and cultured in the presence or absence of 50 μg/mL HDM (optimal concentration) for 6 days. We determined IL-13, IL-5, and IFN-γ by ELISA (BD Biosciences PharMingen).

Statistics. The significance of differences between groups was analyzed using Student's *t*-test or Mann-Whitney test as appropriate.

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