MicroRNA-21 Is Up-Regulated in Allergic Airway Inflammation and Regulates IL-12p35 Expression

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MicroRNA-21 Is Up-Regulated in Allergic Airway Inflammation and Regulates IL-12p35 Expression[1]

Thomas X. Lu,*†‡ Ariel Munitz,* and Marc E. Rothenberg[2]*

Allergic airway inflammation is characterized by marked in situ changes in gene and protein expression, yet the role of microRNAs (miRNAs), a new family of key mRNA regulatory molecules, in this process has not yet been reported. Using a highly sensitive microarray-based approach, we identified 21 miRNAs with differential expression between doxycycline-induced lung-specific IL-13 transgenic mice (with allergic airway inflammation) and control mice. In particular, we observed overexpression of miR-21 and underexpression of miR-1 in the induced IL-13 transgenic mice compared with control mice. These findings were validated in two independent models of allergen-induced allergic airway inflammation and in IL-4 lung transgenic mice. Although IL-13-induced miR-21 expression was IL-13Rα1 dependent, allergen-induced miR-21 expression was mediated mainly independent of IL-13Rα1 and STAT6. Notably, predictive algorithms identified potential direct miR-21 targets among IL-13-regulated lung transcripts, such as IL-12p35 mRNA, which was decreased in IL-13 transgenic mice. Introduction of pre-miR-21 dose dependently inhibited cellular expression of a reporter vector harboring the 3′-untranslated region of IL-12p35. Moreover, mutating miR-21 binding sites in IL-12p35 3′-untranslated region abrogated miR-21-mediated repression. In summary, we have identified a miRNA signature in allergic airway inflammation, which includes miR-21 that modulates IL-12, a molecule germane to Th cell polarization. ***The Journal of Immunology,*** **2009, 182: 4994–5002.**

Asthma is a chronic inflammatory disease characterized by inflammation of the airways, tissue remodeling, and a decline in respiratory function (1–3). In the United States, 5–10% of the population suffer from asthma, representing a common diagnosis for pediatric hospital admission and a major cause for lost days at work and school (4). Despite intense ongoing research, the incidence of the disease continues to rise, necessitating the need for new scientific inquiry.

MicroRNAs (miRNAs)[3] are noncoding ssRNAs of 19–25 nt in length that mediate posttranscriptional silencing of target genes (5, 6). In animals, miRNAs usually bind to complementary sites in the 3′-untranslated region (UTR) of target genes and regulate target gene expression by either translational inhibition, mRNA degradation, or both (7). MiRNAs are involved in diverse biological processes, including development, stress response, cancer, and cardiac hypertrophy, implicating them in normal and pathological processes (8). However, to the best of our knowledge, studies relevant to asthma or asthma risk are still lacking, except for a recent report demonstrating that a single nucleotide polymorphism at the 3′-UTR of HLA-G, an asthma-susceptibility gene, affects the binding of three miRNAs to this gene (9). Allergic airway inflammation may be particularly sensitive to miRNA regulation because it is characterized by marked changes in gene and protein expression in the lung (10–12). For example, lung overexpression of IL-13, a key Th2 cell-derived effector cytokine in asthma pathogenesis, induces allergic airway inflammation characterized by prominent inflammatory cell accumulation, goblet cell metaplasia (mucus production), smooth muscle hyperplasia, and airway hyperresponsiveness, processes that are mediated by marked changes in gene and protein expression, including cytokines and chemokines (13).

In this study, we used both miRNA microarray- and quantitative RT-PCR (qRT-PCR)-based approaches to assess miRNA expression in murine models of allergic asthma. We define a miRNA signature consisting of 21 differentially regulated miRNAs in IL-13-induced experimental asthma. Focusing on the most highly induced miRNA, we subsequently demonstrate that miR-21 regulates murine IL-12p35, a key cytokine associated with balancing Th cell polarization.

Materials and Methods

Mice

Bitransgenic mice bearing CCSP-rtTA and $(tetO)_7$CMV-IL-13 transgenes were generated in which IL-13 was expressed in a lung-specific manner that allowed for external regulation of transgene expression (14). Transgene expression was induced by feeding bitransgenic mice doxycycline-impregnated food for 4 wk. Constitutive IL-4 lung transgenic mice under the control of the CC10 promoter were provided by F. Finkelman (Cincinnati Children's Hospital, Cincinnati, OH) (15). The IL-13Rα1 and STAT6-deficient mice were described previously (16, 17).

Experimental asthma induction

Experimental asthma was induced by injection with 100 μg of OVA and 1 mg of aluminum hydroxide as adjuvant twice, followed by two 50 μg OVA or saline intranasal challenges 3 days apart, starting a least 10 days after the second sensitization. Mice were sacrificed 18–24 h after the second challenge (10). *Aspergillus fumigatus* Ag-associated asthma was induced by challenging mice intranasally three times per week for 3 wk with 100 μg

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[3] Abbreviations used in this paper: mi, micro; HPRT, hypoxanthine phosphoribosyltransferase; LNA, locked nucleic acid; NFIB, nuclear factor IB; qRT-PCR, quantitative RT-PCR; UTR, untranslated region.

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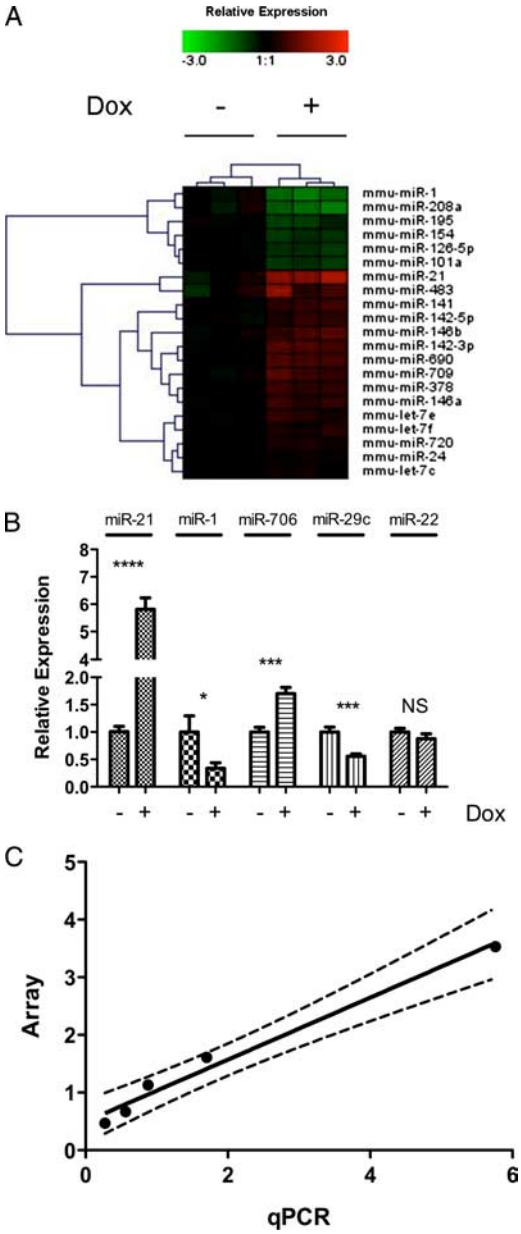

FIGURE 1. MiRNA expression profile in IL-13 transgenic mice lung. *A*, Heat map of 21 differentially expressed miRNAs following no doxycycline (−) and doxycycline (+) exposure for 28 days. Relative expression is $\log_2$ transformed. *B*, qRT-PCR validation of a selected set of miRNA probes normalized to snoRNA202. ∗, $p < 0.05$; ∗∗∗, $p < 0.001$; ∗∗∗∗, $p < 0.0001$. *C*, Correlation of miRNA microarray and qRT-PCR validation; dashed line represents 95% confidence interval. Data are represented as mean ± SEM; n = 5–7 mice per group; data representative of three experiments.

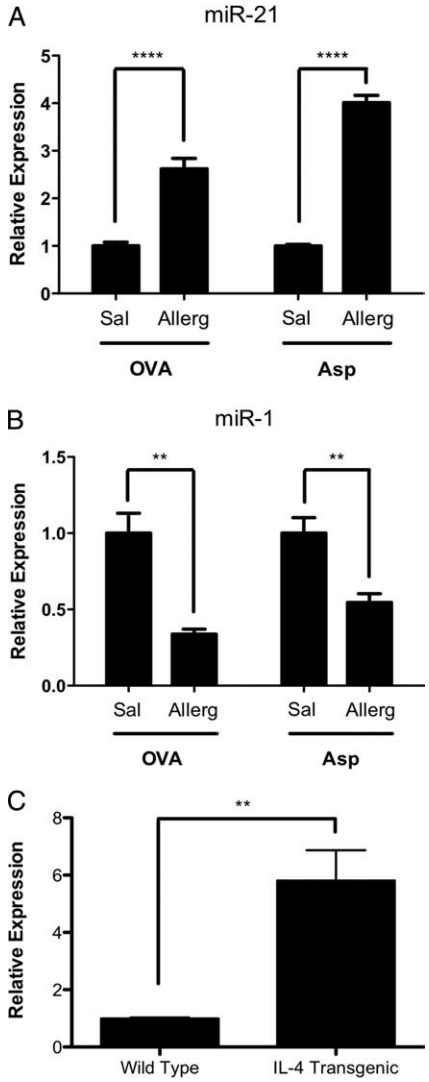

FIGURE 2. Expression of miR-21 and miR-1 in experimental asthma models. MiR-21 (*A*) and miR-1 (*B*) expression were assessed in OVA and *A. fumigatus* asthma models. *C*, MiR-21 expression was determined in IL-4 lung transgenic mice. The relative expression levels were determined by qRT-PCR normalized to snoRNA202; ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$; ∗∗∗∗, $p < 0.0001$. Data are represented as mean ± SEM; n = 3–7 mice per group; data representative of three experiments.

(50 μl) of *A. fumigatus* extract or 50 μl of saline each time. Mice were sacrificed 48 h after the last challenge (18). Experimental asthma from IL-13 bitransgenic mice was induced by feeding bitransgenic mice doxycycline-impregnated food for 4 wk, as described (19). The control group received no doxycycline. Mice were sacrificed at the end of 4 wk of IL-13 induction. Intratracheal delivery of IL-13 was performed, as previously described (20). All animals were housed under specific pathogen-free conditions in accordance with institutional guidelines. The use of animals in these experiments was approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Medical Center.

RNA extraction and microarray experiments

Total RNA was isolated from lung tissue using miRNeasy mini kit, according to manufacturer's protocol (Qiagen). RNA quality was assessed by using the Agilent 2100 bioanalyzer (Agilent Technologies), and only samples with RNA integrity number >8 were used. RNA samples from these tissues were subsequently fluorochrome labeled by using the miRCURY Hy3/Hy5 labeling kit and hybridized to Exiqon miRCURY locked nucleic acid (LNA) array (version 10.0), comprising LNA-modified probes for all mouse miRNAs in the release 10.0 of the miRBase miRNA registry (21, 22). The microarray analysis was conducted in the Genomics and Microarray Core Facility at the University of Cincinnati. Data were normalized to a common reference sample using R statistical software (R Foundation for Statistical Computing) (23). MiRNA expression data were analyzed and displayed using Genesis (version 1.7.2) (24). The microarray data have been submitted to ArrayExpress database in compliance with minimum information about microarray experiment standards (ArrayExpress accession E-MEXP-1992; www.ebi.ac.uk/arrayexpress).

Quantitative assessment of miRNA levels

Levels of miRNA expression were measured quantitatively by using TaqMan microRNA assay (Applied Biosystems), as described following manufacturer's protocol, and assayed on the Applied Biosystems 7300 real-time PCR system (25). Normalization was performed with snoRNA202 (26). Comparative real-time PCR was performed in triplicate, including

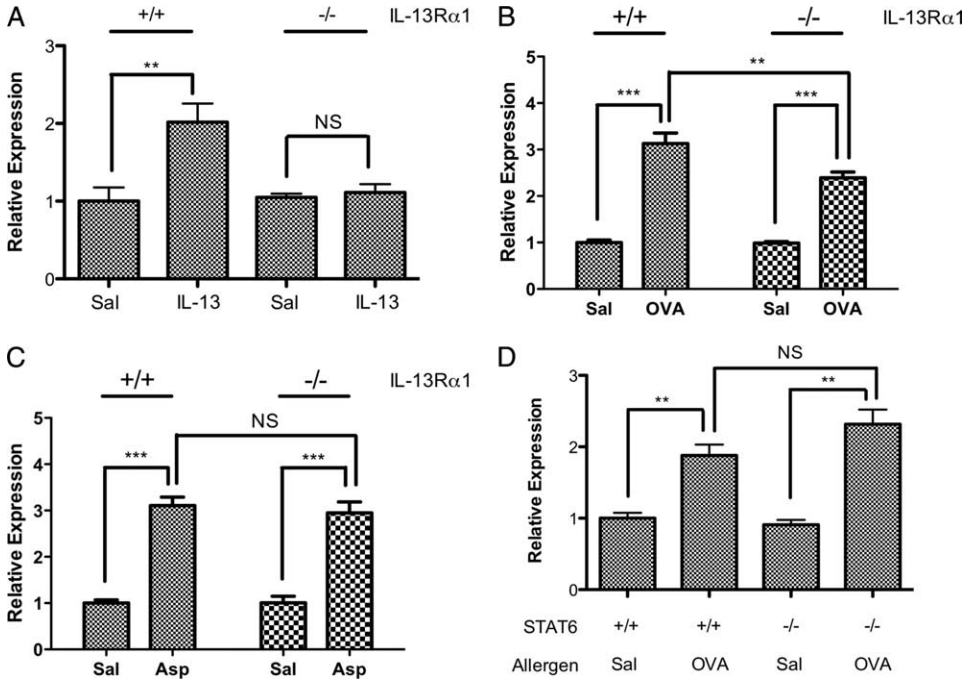

FIGURE 3. Expression of miR-21 in allergen-challenged IL-13Rα1-deficient mice. *A*, MiR-21 expression was determined in IL-13-challenged wild-type and IL-13Rα1-deficient mice. *B* and *C*, MiR-21 expression was determined in OVA (*B*) and *A. fumigatus* (Asp) models (*C*) using IL-13Rα1 (−/−) mice and wild-type (+/+) controls. *D*, MiR-21 expression was determined in OVA-challenged wild-type and STAT6-deficient mice. The relative expression levels were determined by qRT-PCR normalized to snoRNA202. **, $p < 0.01$; ***, $p < 0.001$. Data are represented as mean ± SEM; $n = 3–8$ mice per group; data representative of three experiments.

no-template controls. Relative expression was calculated using the comparative cycle threshold method, as previously described (27).

miRNA in situ hybridization

In situ hybridizations were performed in 8-μm cryosections from the lung of saline and *A. fumigatus*-challenged mice (28). Slides were stained using an automated system, the Discovery XT (Ventana Medical Systems), according to manufacturer's protocols. Slides were pretreated with 100 μl of RiboPrep (Ventana Medical Systems) for 20 min at 37°C. After rinsing the slides with reaction buffer (Ventana Medical Systems), slides were then treated with 100 μl of RiboClear (Ventana Medical Systems) for 12 min at 37°C. The slides were rinsed again and then digested with Protease 3 (Ventana Medical Systems) for 12 min at 37°C. After protease digestion, the digoxin-labeled LNA-scrambled control probe and LNA miR-21 antisense probe (Exiqon) were hybridized to the slides at 52°C for 6 h. Following posthybridization washes with 0.1× SSC buffer at 47°C, 100 μl of rabbit anti-digoxin (Sigma-Aldrich) Ab, diluted 1/2000 in Discovery Ab Diluent (Ventana Medical Systems), was applied to the slides for 30 min at room temperature. The slides were rinsed and then incubated with 100 μl of UltraMap anti-rabbit alkaline phosphatase (Ventana Medical Systems) for 16 min at room temperature. Color detection was done using a ChromoMap Blue kit (Ventana Medical Systems). Slides were counterstained with Nuclear Fast Red (Polyscientific), coverslipped, and mounted for viewing.

Cell culture

Raw264.7 (American Type Culture Collection No. TIB-71) and NIH3T3 (American Type Culture Collection No. CRL-1658) cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The murine lung epithelial cell line MLE15 and murine fetal lung mesenchyme cell line MFLM4 were provided by J. Whitsett (Cincinnati Children's Hospital, Cincinnati, OH) and were maintained in HITES medium supplemented with 4% FBS and 1% penicillin/streptomycin (29). Type II alveolar cells were isolated and cultured, as previously described (30), and were provided by T. Weaver (Cincinnati Children's Hospital, Cincinnati, OH). Bone marrow-derived macrophages were prepared by culturing cells in medium containing 30% L929 conditioned medium (31). Bone marrow-derived dendritic cells were prepared by culturing cells in medium supplemented with 40 ng/ml GM-CSF, 20 ng/ml IL-4, 10% FBS, and 1% penicillin/streptomycin (32). Neutrophils were generated by using the thioglycolate-induced peritonitis model according to previously published methods (33). After 4 h, mice were sacrificed, the peritoneal cavity was rinsed with 10 ml of PBS, and neutrophil purity was 95%, as determined by Diff-Quik staining. For LPS activation of Raw264.7 cells, the cells were stimulated with 1 μg/ml LPS (strain 055:B5; Sigma-Aldrich) for 24 h.

Target predictions

miRNA targets were predicted by using miRanda (34) and TargetScan (35) algorithms. Targets predicted by both algorithms were intersected with IL-13 down-regulated genes (19) and considered for further analysis. Nuclear factor I/B (NFIB) binding site was searched from the 1-kilobase region upstream of first exon of all IL-13-induced genes in the lung using the TraFaC program, which analyzes noncoding genomic sequences that are evolutionarily conserved between mouse and human (36).

IL-12p35 expression analysis

Total RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Samples were analyzed by TaqMan qRT-PCR for IL-12p35 transcripts and normalized to hypoxanthine phosphoribosyltransferase (HPRT)1 (primer and probe sets from Applied Biosystems; IL-12p35 assay ID, Mm00434169_m1; HPRT1 assay ID, Mm00446968_m1). Relative expression was calculated using the comparative cycle threshold method.

Luciferase reporter plasmid construction

The full-length mIL-12p35 3′-UTR was amplified with the following primers: mIL-12p35, forward, ggccactagtGAAAGGCTCAAGGCCCTCT; mIL-12p35, reverse, ggccaagcttGAACCACAAAATAAGGTATGTTTCAA, and cloned between the *Spe*I and *Hind*III sites of the multicloning region in the 3′-UTR of the firefly luciferase expression vector pMIR-report (Ambion) and designated pmIL-12p35. The pMIR-21 vector has a perfect miR-21 binding site cloned into the 3′-UTR region of the pMIR-report vector (Ambion).

Site-directed mutagenesis of miR-21 binding site in pmIL-12p35

The mutations in the miR-21 binding site of pmIL-12p35 were introduced with the QuikChange II XL site-directed mutagenesis kit (Stratagene), according to the manufacturer's protocol, and designated pmIL-12p35 Mut. The mutagenesis primers are as follows: mIL-12p35, Mut forward, 5′-GGGTGACTGAGTGTTTTCATAAACACTTTGGCACAAAAACAATTCGAATTCAGTTCTTGCTCTTCTGCTAA-3′, and mIL-12p35 Mut reverse, 5′-TTAGCAGAAGAGCAAGAACTGAATTCGAATTGTTTTTGTGCAAAGTGTTTATGAAAACACTCAGTCACCC-3′. The constructs were sequenced to prove sequence integrity.

Transfection of pre-miRNA expression plasmid and reporter plasmids

The 293T cells were cotransfected with 500 ng of firefly reporter plasmids, 25 ng of reference *Renilla* luciferase reporter plasmid pGL4.73

(Promega), and 500 ng of pMIRNA1 pre-miR-21 or 500 ng of pMIRNA1 control vector (SystemBiosciences) using Lipofectamine reagents, as per the recommended conditions (Invitrogen). Lysates were prepared at 36 h posttransfection.

Dual-luciferase reporter assays

Transfected cells were lysed in 300 μl of passive lysis buffer (Promega) for 30 min at room temperature. Firefly and *Renilla* luciferase activity were measured using the Promega Dual Luciferase Assay kit following the manufacturer's instructions and a Veritas Microplate luminometer (Turner Biosystems). All measurements were normalized for *Renilla* luciferase activity to correct for variations in transfection efficiencies and non-miR-21-specific effects of miRNA transfection on enzymatic activity.

Results

Expression profiling of miRNA in IL-13 lung transgenic mice

To identify miRNAs differentially expressed after IL-13 induction in IL-13 lung transgenic mice, miRNA expression of total lung tissue was profiled using Exiqon miRCURY LNA array (version 10.0), comprising LNA-modified probes for all mouse miRNAs available in the miRBase miRNA registry (21, 22). Of the 579 mouse miRNAs assayed, 131 miRNAs were expressed above background levels. In agreement with previous reports, several miRNAs, including miR-23b, miR-24, miR-30b, miR-451, and members of the let-7 family, were highly enriched in the mouse lung, giving strong hybridization signals on the miRNA arrays (data not shown) (37). Comparing doxycycline-induced IL-13 transgenic mice with control mice that received no doxycycline, 21 miRNAs were found to be differentially expressed at $p < 0.01$, suggesting that they were regulated directly or indirectly by IL-13 (Fig. 1*A*). Notably, miR-21 was the most up-regulated miRNA on the array, and miR-1 was the most down-regulated miRNA. To validate the results of the microarray platform, we determined the expression of a subset of miRNAs by real time RT-PCR (Fig. 1*B*). We found strong correlation between our microarray profiling and real-time RT-PCR data (Pearson correlation coefficient: 0.99, $p = 0.001$; Fig. 1*C*).

MiR-21 is induced in three separate models of experimental asthma, whereas miR-1 is repressed in the same models

We aimed to determine levels of miR-21 and miR-1 in three independent asthma models (OVA, *A. fumigatus*, and induced IL-13 bitransgenic mice) using real-time RT-PCR analysis. In the OVA model, mice were sensitized by two i.p. injections of OVA and aluminum hydroxide. The *A. fumigatus* model involves a unique mucosal sensitization route (intranasal) compared with the OVA model (10, 18, 20, 38, 39). Although the methods of experimental asthma induction are different, all three asthma models have similar phenotypes, including Th2-associated eosinophilic inflammation, mucus production, and airway hyperresponsiveness (10, 18, 19, 40, 41). First, we examined the inducible IL-13 bitransgenic system and indeed validated that doxycycline-treated IL-13 bitransgenic mice had a 4.62-fold increase in miR-21 ($p < 0.01$) and a 0.28-fold repression of miR-1 ($p < 0.05$) compared with control mice that received no doxycycline (Fig. 1*B*). Second, we examined the OVA-induced asthma model and demonstrated that OVA-challenged mice had a 2.64-fold ($p < 0.0001$) induction of miR-21 and a 0.34-fold repression of miR-1 ($p < 0.001$) compared with saline-challenged mice (Fig. 2, *A* and *B*). Third, we examined the *A. fumigatus* model of experimental asthma and demonstrated that Ag-challenged mice have a 4.01-fold increase in miR-21 level ($p < 0.0001$) and a 0.55-fold repression of miR-1 ($p < 0.01$) compared with control mice (Fig. 2, *A* and *B*). In these models, the bronchoalveolar lavage fluid eosinophil levels were $1.15 \pm 0.34 \times 10^4$, $3.39 \pm 0.76 \times 10^6$, and $4.94 \pm 0.88 \times 10^5$ cells following IL-13, OVA, and *A. fumigatus* challenge, respectively, as reported

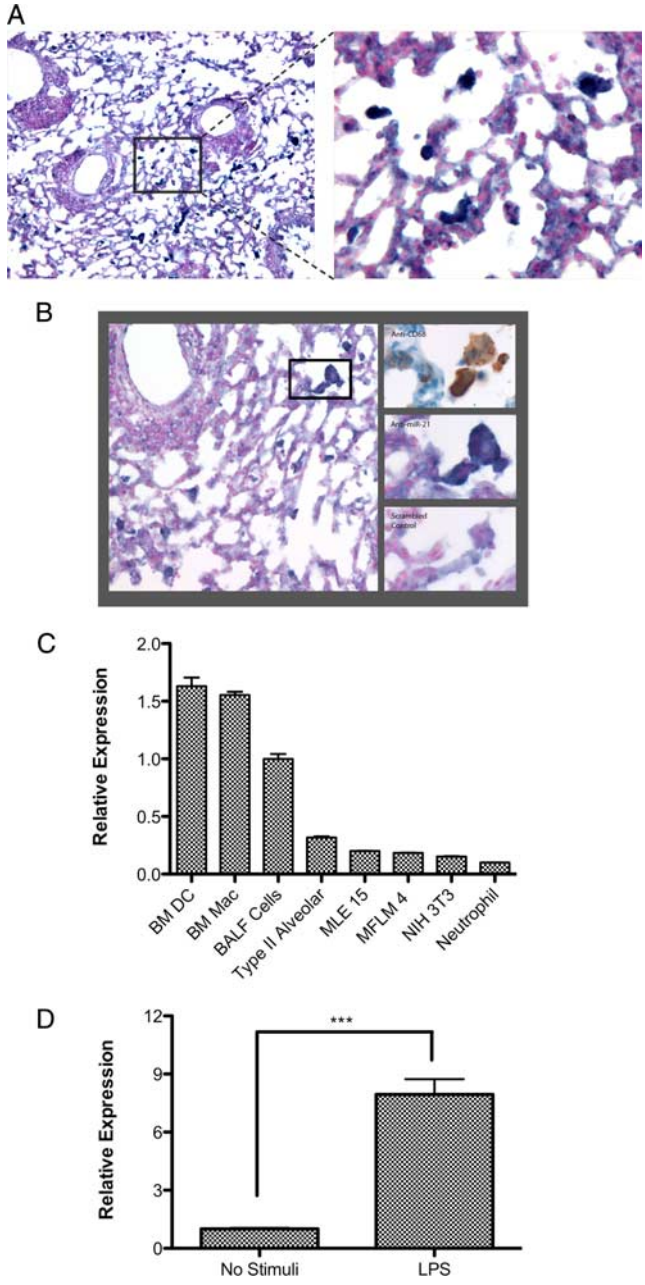

FIGURE 4. In situ hybridization of miR-21 in *A. fumigatus*-challenged wild-type mouse lung. Expression of miR-21 in (*A* and *B*) *A. fumigatus*-challenged wild-type mouse lungs was determined by LNA-based in situ hybridization. *A*, LNA anti-miR-21: *left*, ×100 field; *right*, ×400 field. *B*, *Left*, ×200 field, LNA anti-miR-21; *right*, serial sections at ×600; *right top*, LNA anti-miR-21; *right middle*, anti-CD68; *right bottom*, LNA-scrambled control probe. *C*, Relative expression of miR-21 in different cell types; BM DC, bone marrow-derived dendritic cells; BM Mac, bone marrow-derived macrophages; BALF, bronchoalveolar lavage fluid cells; type II alveolar cells, MLE15, murine lung epithelial cell line; MFLM4, murine lung mensenchyme cell line; NIH3T3, murine fibroblasts and murine neutrophils. *D*, Relative expression of miR-21 in LPS-stimulated murine macrophage cell line Raw264.7; ***, $p < 0.001$. Data are representative of three experiments.

(39). Finally, to determine whether the induction of miR-21 could be mediated by IL-4, we determined miR-21 expression level in IL-4 lung transgenic mice and found that IL-4 transgenic mice had a 5.81 ± 1.06-fold induction of miR-21 ($p < 0.01$) compared with wild-type mice (Fig. 2*C*).

Table I. *Predicted targets of miR-21 that are common between miRanda and TargetScan algorithms*

Gene Symbol	Description	Transcript ID
Arhgap24	Rho GTPase-activating protein 24	ENSMUST00000094559
Aspn	Asporin	ENSMUST00000021820
Brd1	Bromodomain containing 1	ENSMUST00000023022
Cbx4	Chromobox homolog 4	ENSMUST00000026665
Ccl1	Chemokine (C-C motif) ligand 1	ENSMUST00000021043
Cntfr	Ciliary neurotrophic factor receptor	ENSMUST00000102962
Dazl	Deleted in azoospermia-like	ENSMUST00000010736
Hnrpu	Heterogeneous nuclear ribonucleoprotein U	ENSMUST00000037748
Il12A	IL-12a	ENSMUST00000029345
Jag1	Jagged 1	ENSMUST00000028735
Kcna3	Potassium voltage-gated channel, shaker-related subfamily, member 3	ENSMUST00000052718
Krit1	KRIT1, ankyrin repeat containing	ENSMUST00000080085
Matn2	Matrilin 2	ENSMUST00000022947
Mrpl9	Mitochondrial ribosomal protein L9	ENSMUST00000029786
Mtap	Methylthioadenosine phosphorylase	ENSMUST00000058030
Nfib	NFIB	ENSMUST00000050872
Ntf3	Neurotrophin 3	ENSMUST00000050484
Pcbp1	Poly(rC)-binding protein 1	ENSMUST00000053015
Pcbp2	Poly(rC)-binding protein 2	ENSMUST00000077037
Pdcd4	Programmed cell death 4	ENSMUST00000025931
Peli1	Pellino 1	ENSMUST00000093290
Pitx2	Paired-like homeodomain transcription Factor 2	ENSMUST00000029657
Plag1	Pleiomorphic adenoma gene 1	ENSMUST00000003369
Ppara	Peroxisome proliferator-activated receptor α	ENSMUST00000057979
Psrc1	Proline/serine-rich coiled-coil 1	ENSMUST00000102630
Reck	Reversion-inducing cysteine-rich protein With kazal motifs	ENSMUST00000030198
Rnf103	Ring finger protein 103	ENSMUST00000064637
Satb1	Special AT-rich sequence-binding protein 1	ENSMUST00000024720
Ski	Sloan-Kettering viral oncogene homolog	ENSMUST00000030917
Sox2	SRY box containing gene 2	ENSMUST00000099151
Spg20	Spastic paraplegia 20, spartin (Troyer syndrome) Homolog (human)	ENSMUST00000044116
Stag2	Stromal Ag 2	ENSMUST00000069619
Tgfbi	TGF, β induced	ENSMUST00000045173
Tiam1	T cell lymphoma invasion and metastasis 1	ENSMUST00000002588
Wwp1	WW domain containing E3 ubiquitin protein Ligase 1	ENSMUST00000035982
Xkr6	X Kell blood group precursor related family Member 6 homolog	ENSMUST00000100485
Zcchc3	Zinc finger, CCHC domain containing 3	ENSMUST00000099207

MiR-21 is induced predominantly by an IL-13Rα1-independent pathway

We focused on miR-21 because this was the most markedly changed miRNA and one implicated in processes germane to asthma, including cell growth and differentiation, tissue remodeling, and myeloid cell function (42–45). Having identified miR-21 as both an IL-13- and allergen-induced gene, we were interested in determining whether the IL-13- or allergen-induced expression was IL-13Rα1 dependent, because we have recently reported that this receptor mediates some of the key cardinal features of experimental asthma (airway hyperresponsiveness and goblet cell metaplasia), but not leukocyte accumulation (39). First, we delivered IL-13 intratracheally to wild-type and IL-13Rα1 gene-deficient mice and demonstrated that IL-13-induced miR-21 was IL-13Rα1 dependent (Fig. 3*A*). We then examined both the OVA-induced model and the *A. fumigatus* model of experimental asthma using wild-type and IL-13Rα1 gene-deficient mice. Notably, this analysis revealed that both OVA and *A. fumigatus* induced miR-21 expression largely through an IL-13Rα1-independent pathway. OVA-challenged wild-type mice had a 3.13 ± 0.22-fold induction of miR-21, whereas OVA-challenged gene-targeted mice had a 2.39 ± 0.13-fold induction ($p < 0.01$ for saline vs OVA in both wild-type and gene-targeted mice). *A. fumigatus*-challenged wild-type mice had a 3.11 ± 0.10-fold induction of miR-21, whereas *A. fumigatus*-challenged gene-targeted mice had a 2.95 ± 0.23-fold induction ($p < 0.01$ for saline vs *A. fumigatus* in both wild-type and gene-targeted mice) (Fig. 3, *B* and *C*). We subsequently determined that miR-21 was induced through a STAT6-independent mechanism in OVA-challenged mice (Fig. 3*D*).

MiR-21 is expressed in cells of monocyte/macrophage lineage in the allergic lung

To determine the cell type-specific localization of miR-21, in situ hybridization was conducted on cryosections of *A. fumigatus*-challenged lungs using LNA anti-miR-21 and scrambled control probes (28, 46). MiR-21 was primarily detected in the cytoplasm of mononuclear and multinucleated myeloid cells with morphology resembling monocytes/macrophages (Fig. 4*A*). To determine whether these cells are indeed in the monocyte/macrophage lineage (both are $CD68^+$), we performed anti-CD68 staining on serial sections. Serial sectioning revealed that anti-CD68 staining was associated with miR-21 expression, at least in part (Fig. 4*B*). Staining of doxycycline-induced IL-13 bitransgenic mouse lungs

Table II. *Predicted targets of miR-21 that are common between miRanda, TargetScan, and IL-13 down-regulated genes*

Name	Transcript ID	TargetScan Prediction	miRanda Prediction
Cntfr	ENSMUST00000102962	AGUUGUAGUCAGACUAUUCGAU CCACCATCAGATT-ATAAGCTC	aGUUGUAGUCAGACUAUUCGAu cCACCATCAGATT-ATAAGCTc
Il12A	ENSMUST00000029345	AGUUGUAGUCAGAC-UAUUCGAU GAAGAGCAAGAACTGATAAGCTA	agUUGUAGUCAGACUAUUCGAU agAGCAAGA-ACTGATAAGCTA
Pitx2	ENSMUST00000029657	AGUUGUAGUCAGACUAUUCGAU TTAACATTACT---ATAAGCTT	AGUUGUAGUCAGACUAUUCGAu TTAACATTA--CT-ATAAGCTt
Plag1	ENSMUST00000003369	AGUUGUAGUCAGACUAUUCGAU CTAGCCTCTTCG-ATAAGCTT	aGUUGUAGUCAGACUAUUCGAu cTAGCCTC--TTCGATAAGCTt
Psrc1	ENSMUST00000102630	AGUUGUAGUCAGACUAUUCGAU GGTCAATCAGTT-ATAAGCTT	aguugUAGUCAGACUAUUCGAu ggtcaATCAGTT--ATAAGCTt
Reck	ENSMUST00000030198	AGUUGUAGUCAGAC--UAUUCGAU TGTTTTACAGTTTGAAATAAGCTA	aguuguaGUCAGAC--UAUUCGAU tgttttaCAGTTTGAAATAAGCTA
Satb1	ENSMUST00000024720	AGUUGUAGUCAGACUAUUCGAU TTACTATCATGCAAATAAGCTT	AGUUGUAGUCAGACUAUUCGAu TTACTATCATGCAAATAAGCTt
Sox2	ENSMUST00000099151	AGUUGUAGUCAGAC-UAUUCGAU CAAATGTCCATTGTTTATAAGCTG	agUUGUA-GUCA-GACUAUUCGAU caAATGTCCATTGTTTATAAGCTG
Spg20	ENSMUST00000044116	AGUUGUAGUCAGACUAUUCGAU GGATTTTCAGCAG-ATAAGCTA	agUUGUAGUCAGACUAUUCGAU ggATTTTCAG-CAGATAAGCTA
Wwp1	ENSMUST00000035982	AGUUGUAGUCAGAC-UAUUCGAU GCACAGATAGTGTGTATAAGCTG	aGUUGUAGUCAGAC-UAUUCGAU gCACAGATAGTGTGTATAAGCTG

showed similar results (supplemental Fig. 1).[4] We quantified the number of miR21-positive cells as a function of total airway macrophages as determined by $CD68^+$ staining in serial sections and found that 64 ± 6.8% of (mean ± SD, $n = 3$ mice) $CD68^+$ cells were miR-21 positive. In addition, we determined the expression profile of miR-21 in different cell types and found that bone marrow-derived macrophages and dendritic cells expressed relatively high levels of miR-21 compared with lung epithelial cells, fibroblasts, and neutrophils (Fig. 4*C*). The cell composition of bronchoalveolar lavage fluid cells can be found in supplemental Fig. 2.[4] We subsequently demonstrated that miR-21 is inducible in a murine macrophage cell line by LPS treatment (Fig. 4*D*).

MiR-21 targets in the allergic lung

We searched for miR-21 mRNA targets using target-prediction software miRanda and TargetScan. It has been reported that integration of miRNA target predictions from multiple algorithms substantially increases the functional correlations and decreases the false-positive rate compared with single algorithms (47, 48). Common predicted targets between miRanda and TargetScan algorithms arrived with a list of 37 predicted targets (Table I). Although some of the targets of miRNAs are modulated through translational inhibition only, the majority of targets have mRNA level changes inverse to their respective miRNA regulator (49, 50). We thus intersected the 37 predicted targets with IL-13 down-regulated genes and arrived at a list of IL-13-regulated target genes (Table II). Notably, IL-12p35 was predicted to have a strong miR-21 binding site conserved across species (Fig. 5*A*). We determined the expression level of IL-12p35 and found that it was indeed down-regulated in all three of the used asthma models (Fig. 5*B*).

[4] The online version of this article contains supplemental material.

MiR-21 targets IL-12p35

To determine whether IL-12p35 is a molecular target of miR-21, we constructed a luciferase reporter vector containing the full-length mIL-12p35 3′-UTR, as well as a positive control vector harboring a perfect miR-21 complementary sequence in the 3′-UTR region. Transfecting the miR-21 expression vector inhibited the expression of the luciferase reporter vector containing the mIL-12p35 3′-UTR as well as the expression of the positive control vector, whereas there was no effect in the luciferase vector that did not contain a miR-21 binding site (Fig. 5*C*). The reduced luciferase activity of mIL-12p35 and pMiR-21 vectors at baseline is most likely due to inhibition from both the endogenously expressed miRNAs, as well as other translational inhibition mechanisms, such as the 5′-3′ circularization disruption by endogenous translational inhibitor proteins that bind to the 3′-UTR region (51). The inhibition of mIL-12p35 reporter was dose dependent with an ED_{50} of 29.84 ± 1.24 ng (7.19 ± 0.30 nM) and a plateau reached at 250 ng (60 nM) (Fig. 5*D*). Mutation of the seed sequence of the predicted miRNA binding site abrogated this effect (Fig. 5, *E* and *F*).

Discussion

Our study has provided a comprehensive global miRNA expression profile of an IL-13-induced asthma model. Nearly 4% of 579 miRNAs assayed displayed differential expression in IL-13 transgenic mice compared with control mice (Fig. 1). This level of miRNA change is consistent with other disease states, such as breast cancer, leukemia, and myocardial infarction (52–54). Notably, each miRNA has been predicted to potentially target hundreds of genes (35, 55), indicating the potential significance of small changes in miRNAs. We found that miR-21 and miR-1 were the most induced and repressed miRNAs, respectively. We validated the induction of miR-21 and repression of miR-1 in two additional

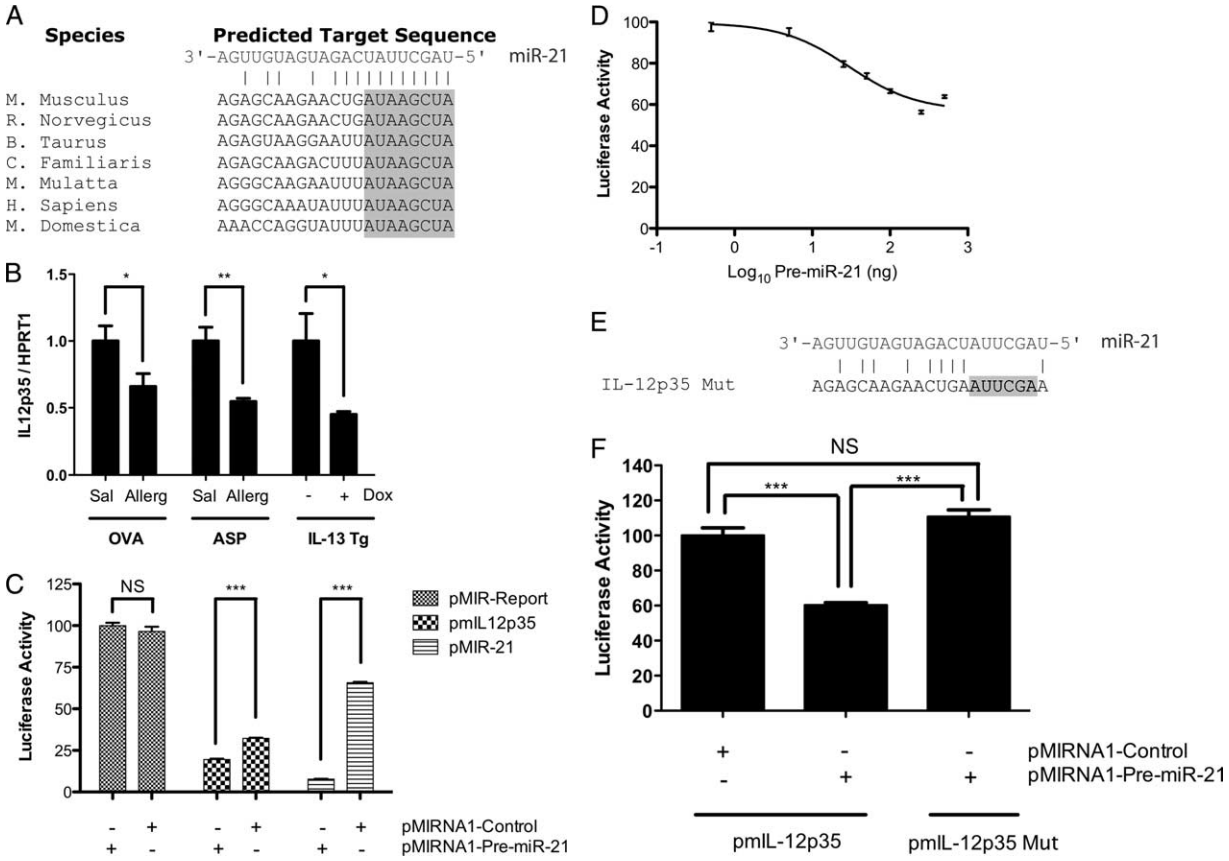

FIGURE 5. MiR-21 targets IL-12p35. *A*, Predicted highly conserved binding site for miR-21 in 3′-UTR of IL-12p35. The 8-mer seed sequence is shaded in gray. *B*, IL-12p35 expression was determined in OVA, *A. fumigatus* (ASP), and doxycycline-induced IL-13 bitransgenic models. The relative expression levels were determined by qRT-PCR normalized to HPRT1. *, $p < 0.05$; **, $p < 0.01$; $n = 5$–7 mice per group. *C*, Relative luciferase activity in 293T cells cotransfected with control firefly luciferase vector (pMIR-Report), or a firefly luciferase reporter vector containing the 3′-UTR of IL-12p35 (pmIL12p35), or a firefly luciferase vector with perfect miR-21 binding site in the 3′-UTR (pMIR-21), and either the pre-miR-21 expression vector (pMIRNA1-Pre-miR-21) or control vector (pMIRNA1-Control). Firefly luciferase activity was normalized to the *Renilla* luciferase activity, and then to the average of the control firefly luciferase reporter; ***, $p < 0.001$; $n = 4$ per group, data representative of three experiments. *D*, A dose-response study of pre-miR-21 expression vector on the luciferase activity of the luciferase vector containing the 3′-UTR of IL-12p35; $n = 4$ per group; data representative of three experiments. *E*, Mutation (shaded in gray) in the 3′-UTR of mIL-12p35. *F*, Relative luciferase activity in 293T cells cotransfected with reporter plasmid containing either the wild-type or mutant mIL-12p35 3′-UTR. Firefly luciferase activity was normalized to the *Renilla* luciferase activity, and then to the average of the wild-type mIL-12p35 firefly luciferase reporter; ***, $p < 0.001$; $n = 4$ per group; data representative of three experiments. All data are represented as mean ± SEM.

independent models of allergic airway inflammation, as well as the induction of miR-21 by chronic overexpression of the IL-4 transgene in the lung. We analyzed the receptor dependency of miR-21 induction and found that IL-13-induced expression of miR-21 was dependent on IL-13Rα1, but allergen-induced miR-21 expression was mediated largely by an IL-13Rα1-independent pathway. Although IL-13 alone completely uses IL-13Rα1 to induce experimental features of asthma, this receptor has a key, but not complete role in the development of allergen-induced experimental asthma. Notably, leukocyte recruitment to the lung occurs independent of IL-13Rα1 (39). As such, the finding that miR21 induction occurs independent of IL-13Rα1 indicates that miR21 induction most likely is derived from (or associated with) the sustained leukocyte recruitment and/or activation in IL-13Rα1-deficient mice. Consistent with this, allergen-induced miR-21 was demonstrated to occur independent of STAT6, consistent with sustained leukocyte recruitment in these mice (56). This implies that miR-21 induction may be related to the large portion of asthma signature genes that are STAT6 independent (56). The STAT6- and IL-13Rα1-independent genes that correlate with miR-21 include the C3a receptor and several chemokines (Ccl8, Ccl12, Cxcl10; data not shown) (39, 56). Notably, recent studies demonstrate that certain aspects of asthma occur through a STAT6-independent pathway (57, 58). In situ hybridization of asthmatic lung revealed that miR-21 was expressed by inflammatory leukocytes most consistent with myeloid cells and in agreement with recent studies identifying miR-21 in hematopoietic cells (59–61). Cell-type expression profile of miR-21 confirmed that macrophages and dendritic cells indeed had the highest level of expression compared with other cell types analyzed. Although the expression of miR-21 was in cells most consistent with macrophages and/or dendritic cells (both $CD68^+$), this does not rule out expression in other cells that may contain less readily detectable RNA (e.g., eosinophils). Notably, miR-21 has been shown to be inducible in a human promyelocytic cell line HL-60 after PMA treatment, which induces macrophage-like differentiation (60). We subsequently demonstrated that miR-21 is inducible in the murine macrophage cell line Raw264.7 by LPS treatment. The relationship between the LPS-induced pathway and our in vivo finding has yet to be determined. In addition, miR-21 has been reported to target the transcriptional repressor NFIB (43), providing a mechanism by which miR-21 induction could up-regulate gene expression. We performed in silico analysis of all IL-13-induced genes in the lung, and identified 130 genes with potential NFIB sites in the 1-kilobase region upstream of the first exon (supplemental Table I).[4]

Using bioinformatic approaches, we identified candidate target genes of miR-21. We further intersected this with our previous IL-13-repressed mRNA expression profiling data to identify candidate target genes that were differentially expressed, possibly because of action of these miRNAs. This analysis identified IL-12p35 as a putative target of miR-21. Transfection and reporter assays indeed identified mIL-12p35 as a target gene of miR-21, with miR-21 having the ability to reduce IL-12p35 expression via the 3′-UTR. The magnitude of miR-21-mediated repression of the mIL-12p25 reporter vector was ~40%, similar with the reported effects of miRNA-mediated mRNA repression in other systems (44, 49, 50, 62–67). These results imply that the increased levels of miR-21 in experimental asthma contribute to the observed decrease in IL-12. IL-12 is a key cytokine derived from macrophages and dendritic cells (both CD68 positive and consistent with our in situ hybridization results) involved in adaptive immune responses involving Th1 cell polarization (68, 69). The ability of miR-21 to down-regulate IL-12 indicates a novel checkpoint for regulating the level of this key immune mediator. These results suggest that strategies designed to deliver miR-21 may prime for Th2- and IL-13-associated responses; conversely, miR-21 inhibition has potential to drive Th1 polarization, promoting cellular responses (and classic adjuvant activity) (70, 71). Down-regulation of IL-12p35 could also affect IL-35 production and T regulatory cell function, leading to a proinflammatory phenotype (72). In addition to regulating IL-12 levels, miR-21 may regulate other processes germane to allergic airway inflammation. Notably, MiR-21 has been shown to regulate the level of the matrix metalloprotease inhibitor RECK (42, 73, 74), and indeed we have identified this gene to be down-regulated by IL-13 in the lung (Table II); it is interesting to speculate that this could account for some of increased matrix metalloprotease activity seen in asthma (75, 76).

In our study, we identified miR-1 as the most down-regulated gene in the IL-13 lung transgenic mice. MiR-1 is considered to be a muscle-specific miRNA (77–79), implicated in the determination of the differentiated state of muscle cells, in myogenesis and in cardiogenesis (78, 80). Down-regulation of miR-1 has been associated with cardiac and skeletal muscle hypertrophy (81, 82). Notably, disruption of just one of the two miR-1 family members, miR-1-2, has profound consequences for development and maintenance of the heart, because miR-1-1 did not compensate for loss of miR-1-2 (80). This indicates that a stable level of miR-1 is very important for normal muscle physiology. MiR-1 is down-regulated in all three of our asthma models ranging from 1.82- to 3.57-fold. It is interesting to speculate that down-regulation of miR-1 contributes to the smooth muscle hypertrophy and remodeling seen in asthma (83).

In conclusion, we report a miRNA signature of experimental asthma. We found miR-21 is the most up-regulated miRNA, and the up-regulation is largely through an IL-13Rα1-independent pathway. Using computational and tissue culture-based assays, we identify mIL-12p35 as a molecular target of miR-21. As such, miR-21 induction in experimental asthma most likely leads to a concomitant decrease in mIL-12p35, which probably contributes to polarization of Th cells toward a Th2 response. This increase in the expression of miR-21 most likely contributes to the action of IL-13 in the lung (and possibly asthma pathogenesis). Taken together, these results suggest a key role for miRNA in allergic lung inflammation.

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Disclosures

The authors have no financial conflict of interest.

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