

Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation

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Background: IL-13, a cytokine secreted by T_H2 lymphocytes and other cells, critically modulates allergic inflammation and tissue remodeling in allergic asthma. Although much is known about transcriptional regulation of IL-13, posttranscriptional regulation is poorly understood.

Objective: Because many inflammatory pathways are known to be regulated by microRNAs, permitting a rapid and fine-tuned response, the role of microRNA-mediated regulation of IL-13 was investigated using both *in vitro* and *in vivo* studies.

Methods: A combination of *in silico* approaches and *in vitro* transfections in A549 cells and primary cultured T cells was used to demonstrate the involvement of let-7 in IL-13 regulation. Furthermore, intranasal delivery of let-7 microRNA mimic in mice was performed to study its effects in allergic airway inflammatory conditions.

Results: Using a combination of bioinformatics and molecular approaches, we demonstrate that the let-7 family of microRNAs regulates IL-13 expression. Induced levels of IL-13 in cultured T cells were inversely related to let-7 levels. In an IL-13-dependent murine model of allergic airway inflammation, we observed that inflammation was associated with a reduction in most of the members of the let-7 family. Exogenous administration of let-7 mimic to lungs of mice with allergic inflammation resulted in a decrease in IL-13 levels, resolution of airway inflammation, reduction in airway hyperresponsiveness, and attenuation of mucus metaplasia and subepithelial fibrosis. **Conclusion:** Let-7 microRNAs inhibit IL-13 expression and represent a major regulatory mechanism for modulating IL-13 secretion in IL-13-producing cell types and thereby T_H2 inflammation. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Let-7 microRNA, IL-13, allergic airway inflammation, posttranscriptional regulation, primary T cells, airway hyperresponsiveness

IL-13, a cytokine secreted by T_H2 lymphocytes and other cells, promotes host defense in parasitic infection and also critically

Abbreviations used

AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
DY547: Dyomics 547
HuR: Human antigen R
OVA: Ovalbumin
PMA: Phorbol 12-myristate 13-acetate
UTR: Untranslated region

modulates allergic inflammation and tissue remodeling in diseases like allergic asthma.^{1,2} The critical nature of IL-13 in such inflammatory conditions can be understood from the fact that IL-13 alone can induce most of the features of asthma, such as epithelial cell hyperplasia, goblet cell metaplasia, deposition of various extracellular matrix proteins in subepithelial regions, and increases in airway smooth muscle cell contractility.³ Although it is difficult to attain the complete resolution of airway inflammation because of its multifactorial nature,⁴ IL-13 blockade alone was sufficient for successful amelioration of allergen-induced airway inflammation.¹ Human genetic studies have established a strong association of the *IL4/IL13* genetic cluster on human chromosome 5q31.1 and mutations in *IL4RA*, (a coreceptor for IL-4 and IL-13) with asthma and atopic diseases.⁵ Transcriptional regulation of IL-13 by various transcription factors, such as nuclear factor of activated T cells, GATA-3, and muscle-specific transcription factor 1 has been well characterized.⁶⁻⁸ Because there is a very high expression of *IL13* transcripts in T_H2 inflammation, simply switching off further transcription would be an inefficient strategy for resolution of inflammation.⁹ This is important in the context of inflammation being a double-edged sword. On one hand, IL-13 is necessary for the elimination of parasites; on the other hand, the increase in IL-13 levels is deleterious to the host. Therefore, posttranscriptional regulation of IL-13 expression might be an extremely important mechanism for fine tuning its function. The absence of IL-13 protein expression despite detectable mRNA in cultured T_H2 cells indicates the existence of translational inhibition.¹⁰ Other posttranscriptional regulatory mechanisms, such as stabilization of *IL13* mRNA by the AU rich element binding protein human antigen R (HuR), are also known.¹¹ However, a detailed account of *IL13* posttranscriptional regulation is yet to emerge.

MicroRNAs are a novel class of posttranscriptional regulators of gene expression that are usually inhibitory in nature and might be involved in the regulation of acute inflammatory processes, such as in response to LPS.¹² Recent reports indicate that microRNAs have a central role in modulating various immune responses, such as regulation of germinal center responses, B-cell maturation, thymic

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TABLE I. List of *in silico*-predicted microRNAs having potential binding sites in the IL13 3'UTR

MicroRNA	Start site	End site	Context score
<i>hsa-let-7d</i>	549	574	-0.26
<i>hsa-let-7f</i>	549	574	-0.26
<i>hsa-let-7g</i>	549	574	-0.27
<i>hsa-let-7i</i>	549	574	-0.26
<i>hsa-miR-98</i>	549	574	-0.26
<i>hsa-miR-155</i>	275	299	-0.15
<i>hsa-miR-558</i>	361	379	-0.30
<i>hsa-miR-595</i>	367	389	-0.13
<i>hsa-miR-621</i>	452	474	-0.19
<i>hsa-miR-632</i>	348	372	-0.11
<i>hsa-miR-645</i>	139	168	-0.06

T-cell selection, and sensitivity toward antigens.¹³ Also, differential expression of microRNAs in naive and effector T cells suggests the importance of microRNAs in effector cell function.¹⁴ Let-7 (lethal-7) was found as a second microRNA, first discovered in *Caenorhabditis elegans* and conserved functionally from lower invertebrates to higher mammals. Its members have been shown to be crucial in various cancerous conditions.¹⁵ Let-7i-mediated regulation of Toll-like receptor 4 expression suggests its involvement in the inflammatory response.¹²

Here we hypothesized the involvement of microRNAs in fine tuning of IL-13 at the posttranscriptional level. We demonstrated for the first time that intranasal administration of let-7 mimic reduced IL-13 levels in allergic lungs along with alleviation of asthma features, such as airway hyperresponsiveness (AHR), airway inflammation, and goblet cell metaplasia.

METHODS

In silico identification of microRNA binding sites

A database search for all human microRNAs (miRBase v9) was made against the human *IL13* 3' untranslated region (UTR) using miRanda, RNAhybrid, and TargetScan.¹⁶⁻¹⁸ A consensus approach was used, as described earlier¹⁹ and listed in Table I, to minimize false-positive hits.

Plasmids

The human *IL13* 3'UTR was amplified from genomic DNA, cloned in pMIR-REPORT vector (Ambion, Austin, Tex), and designated as *pMIR-REPORT-IL13* 3'UTR. PCR-based site-directed mutagenesis was performed as described previously to generate the microRNA target site deletion mutant.¹⁹

Cell culture and transfection

A549 cells were grown in 75-mm² culture flasks in Dulbecco modified Eagle medium (Sigma, St Louis, Mo) containing 10% FBS, streptomycin (50 µg/mL), and penicillin (50 IU/mL) at 37°C in 5% CO₂. These cells were transfected using siPORT-NeoFX (Ambion), according to the manufacturer's instructions. For reporter expression, A549 cells were transfected with 500 ng of intact *pMIR-REPORT-IL13* 3'UTR, single-mutant *pMIR-REPORT-IL13* 3'UTR, or double-mutant *pMIR-REPORT-IL13* 3'UTR with or without end-modified microRNA oligonucleotides at a final concentration of 10 nmol/L (Dharmacon, Lafayette, Colo). The reporter gene assay was performed 24 hours after transfection using the Dual luciferase assay kit (Promega, Madison, Wis). The cells were also cotransfected with 5 to 10 ng of Renilla luciferase reporter plasmid control for normalizing transfection efficiency. PBMCs were isolated from apparently normal healthy volunteers and cultured as described previously.²⁰ These cells were grown in RPMI (Sigma) containing

10% heat-inactivated FBS, streptomycin (50 µg/mL), and penicillin (50 IU/mL) at 37°C in 5% CO₂ under T cell-polarizing conditions.²⁰ Briefly, freshly isolated PBMCs were stimulated with 2 µg/mL PHA (Sigma) for 72 hours, followed by culture in the presence of IL-2 (20 ng/mL; R&D Systems, Minneapolis, Minn)—supplemented media, and all cells were used between the 10th and 15th days.

Cultured T cells were transfected in 12-well plates using siPORT-NeoFX (Ambion). One million (10⁶) cells were transfected with 50 to 200 nmol/L end-modified microRNA or 25 to 100 nmol/L let-7 family inhibitor oligonucleotides (Dharmacon), incubated for 24 hours before stimulation with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and PHA (5 µg/mL), and further incubated for 24 hours in case of let-7 mimics and 8 hours for let-7 inhibitors.

RNA extraction and RT-PCR

RNA was prepared from cells and murine lungs with Trizol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions, and stored at -70°C until further use. Quantitative RT-PCR (Kapa Biosystems, Woburn, Mass) and RT-PCR were performed using specific primers for luciferase, *IL13*, β₂-microglobulin, and glyceraldehyde-3-phosphate dehydrogenase, according to the standard protocol. For studying the relative expression of different let-7 family members, TaqMan-based real-time PCR was performed (Applied Biosystems, Foster City, Calif) with a Light Cycler-480 (Roche, Mannheim, Germany), normalized with 18S, and analyzed by using a mathematic model, as described earlier.^{21,22}

Northern blotting

RNA (40 µg) isolated from primary cultured T cells and murine lung tissue was resolved on a 15% acrylamide gel under denaturing conditions, as described previously,¹⁹ and hybridized with γP³²-labeled let-7 oligonucleotide probe at 42°C overnight in Ultrahyb-oligo hybridization buffer (Ambion). The membranes were washed twice with wash buffer containing 5× standard saline citrate and 2% SDS. They were dried and exposed in a Phosphor Imager (FLA 2000IR; Fujifilm, Tokyo, Japan) for 48 hours, and intensity was quantified with Multi Gauge software (Fujifilm).

Flow cytometric analysis

Surface staining for CD3 (Becton Dickinson, San Diego, Calif) was performed per the manufacturer's instruction. All samples were acquired with the FACSCalibur (Becton Dickinson), and data were analyzed using Cell Quest software (PRO, Version 6.0, Becton Dickinson).

Animals

Male BALB/c mice (8-10 weeks old) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for a week before the experiments. All animals were maintained as per guidelines and protocols approved by the institutional animal ethics committee.

Development of the ovalbumin-sensitized murine model of asthma and treatment of mice with let-7

Mice were sensitized and challenged as described earlier.^{23,24} Mice were divided into 4 groups as indicated, and each group (n = 6) was named according to sensitization/challenge/treatment: *sham*, sham/PBS/vehicle (normal control animals); *OVA*, ovalbumin (OVA)/OVA/vehicle (allergic control animals, OVA, chicken egg OVA, Grade V, Sigma); *let-7*, OVA/OVA/let-7 mimic (allergic mice treated with 6 mg/kg per dose of let-7 mimic); and *Cel-67*, OVA/OVA/Cel-67 (allergic mice treated with 6 mg/kg per dose of Cel-67).

MicroRNA design and delivery

Let-7 and Cel-67 oligonucleotides were designed in such a manner that the 3' terminal in both strands were chemically modified with 2'-O-methoxy substitution to impart stability (Dharmacon). The oligonucleotides were dissolved in water, and the working dilution was prepared in 1× PBS (vehicle)

and administered to mice by using the InExpose inhalation exposure system (Scireq, Montreal, Quebec, Canada) on 3 consecutive days (days 24, 25, and 26) 30 minutes before OVA challenge. The mice were killed the next day.

Measurement of AHR

AHR in the form of airway resistance was estimated in anesthetized mice by using the FlexiVent system (Scireq), which uses a computer-controlled murine ventilator and integrates with respiratory mechanics, as described previously.²⁵ Final results were expressed as airway resistance with increasing concentrations of methacholine.

Measurement of cytokine (IL-4, IL-5, IL-13, and IFN- γ) levels in culture supernatant and lung homogenate and estimation of differential count in bronchoalveolar lavage fluid

Cultured T cells were stimulated with physiological stimuli, such as α -CD3/ α -CD28 (Sigma; 1 and 0.3 μ g/mL, respectively), or with chemical stimuli, such as PMA (50 ng/mL) and PHA (5 μ g/mL), for indicated time points, and the supernatant was used to perform ELISA for IL-13 (R&D Systems). Lung homogenates were used for ELISA of IL-4, IL-5, and IFN- γ (BD PharMingen, San Diego, Calif) and IL-13 (R&D Systems) per the manufacturer's protocol. Results were expressed in picograms and normalized by protein concentrations. Additionally, IL-13 levels were also measured in bronchoalveolar lavage (BAL) fluid supernatants and sera of mice.

Differential cell counts were estimated as described earlier.²⁵ Briefly, BAL was performed with 1.5 mL of cold PBS, recovered BAL fluids were centrifuged and the cell pellets were washed 3 times, and resuspended cell suspensions were used for differential cell counting (Leishman stain), which includes macrophages, mononuclear leukocytes (eg, monocytes and lymphocytes), neutrophils, and eosinophils.

Lung histology

Formalin-fixed, paraffin-embedded lung tissue sections were examined for airway inflammation, goblet cell metaplasia, and subepithelial fibrosis with hematoxylin and eosin, Periodic acid-Schiff, and Masson trichrome stains, respectively, as described previously.^{24,25} The inflammation score was calculated by 2 independent observers who were blinded to the experiment, as described earlier with few modifications.²⁵ Briefly, grades of 0 to 4 were given; for no inflammation (grade 0), occasional cuffing with inflammatory cells (grade 1), and when most bronchi or vessels were surrounded by a thin layer (1-2 cells: grade 2), a moderate layer (3-5 cells: grade 3), and a thick layer (>5 cells deep: grade 4) of inflammatory cells. An increment of 0.5 was given if the inflammation scores were in between 2 grades, and the total inflammation score was calculated by the addition of both peribronchial and perivascular inflammation scores. Further morphometry was performed to determine the airway mucin content, as described earlier.²⁵

Statistical analysis

Data are expressed as means $\pm$ SEs. Significant differences between 2 selected groups were estimated using the unpaired Student *t* test or *post hoc* testing and the Bonferroni correction for multiple comparisons. Statistical significance was set at a *P* value of .05 or less.

RESULTS

Let-7 targets *IL13* 3' UTR

Consensus prediction using 3 independent software programs indicated potential binding of 11 microRNAs to the *IL13* 3'UTR. Five of these belonged to the let-7 family (namely miR-98, let-7d, let-7f, let-7g, and let-7i), showing a high degree of conservation in the let-7 family²⁶ and its predicted targeting of *IL13* (Table I). The

entire *IL13* 3'UTR was cloned into a luciferase reporter system and cotransfected with individual microRNA mimics (10 nmol/L) in A549 cells to experimentally validate these findings because these cells express low levels of let-7 microRNAs.^{26,27} It was observed that transfection with any of the let-7 family microRNAs (miR-98, let-7d, let-7i, let-7g, and let-7f) significantly reduced luciferase expression (Fig 1, A) by approximately 2.5-fold. However, Cel-67 (control microRNA) had no significant effect on luciferase expression. Deletion of the consensus target site (Table I and Fig 1, B) for the let-7 family microRNAs in the *IL13* 3'UTR abolished the reduction of luciferase expression by the let-7 family microRNAs ("intact" and "mutant" in Fig 1, C, and see Fig E1 in this article's Online Repository at www.jacionline.org). Incomplete restoration of luciferase expression with the single mutant indicates the possible existence of additional target sites for let-7. RNA22 software was used to predict such a site,²⁸ which was then deleted (double mutant) by means of site-directed mutagenesis as described in the Methods section. Transfection of the double mutant in the A549 cell line completely restored luciferase expression (see the last 3 bars of Fig 1, C). However, luciferase RNA levels were unchanged in both intact and deletion mutant, suggesting that translational repression might be a possible mechanism of action (Fig 1, D).

Let-7 family microRNAs regulate IL-13 secretion in primary cultured T cells

T cells were isolated from human PBMCs and cultured under T cell-polarizing conditions, as described in the Methods section, to understand the functional relevance of the let-7 family on IL-13 regulation. The purity of these cells was verified based on CD3 positivity by performing flow cytometric analysis (Fig 2, A). By using different stimulatory conditions, it was observed that the combination of PMA and PHA was able to substantially potentiate IL-13 expression (Fig 2, B). Time kinetics of IL-13 protein expression indicated a monophasic response to PMA/PHA induction. IL-13 levels remained undetectable as late as 8 hours after stimulation and then increased suddenly (Fig 2, C). Interestingly, stimulation with PMA/PHA also increased let-7 levels (by using a radiolabeled probe that does not distinguish between individual family members), which were maximal at the 4-hour time point and started decreasing by 8 hours (Fig 2, D). Thus the increase in IL-13 levels in culture supernatants was associated with a decrease in let-7 levels. In contrast, *IL13* transcript levels increased throughout the observed period, with a 100-fold increase in transcript levels seen as early as 4 hours (see Fig E2 in this article's Online Repository at www.jacionline.org). Taken together, these results suggested a possible inverse correlation between let-7 and IL-13 levels in response to PHA and PMA stimulation.

When primary cultured T cells were transfected with let-7 family members followed by stimulation with PHA/PMA for 24 hours, it was observed that each of these microRNAs except miR-98 reduced the expression of IL-13, as detected in the culture supernatant (Fig 3, A). In contrast to our *in vitro* experiment with *IL13* 3'UTR in A549 cells (Fig 1, A), miR-98 had no effect on IL-13 protein when overexpressed in cultured T cells, although this might be due to different experimental conditions, such as cell types; this needs to be further investigated. Because there was an insignificant difference between the effects of individual members on IL-13 expression, let-7i alone was used for

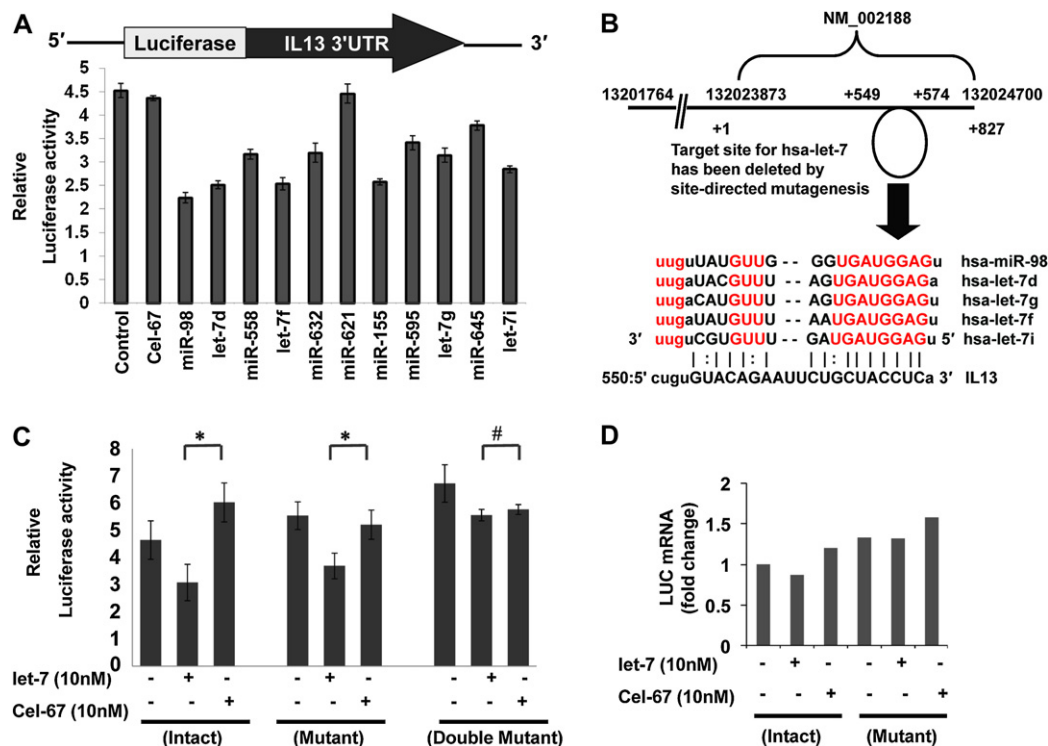


FIG 1. Let-7 microRNA regulates IL-13 expression by targeting its 3'UTR in the A549 cell line. A549 cells were cotransfected with microRNAs along with either intact *pMIR-IL13* 3'UTR vector (**A**) or its mutant (**C**) and luciferase activities (Fig 1, A and C), or quantitative RT-PCR result for luciferase (*LUC*; **D**) were determined. **B**, Let-7 binding site on the *IL13* 3'UTR. Fig 1, A and C, were plotted as means $\pm$ SEs of relative luciferase activity of 3 independent experiments. * $P < .05$. #Not significant. Fig 1, D, is a representative of 3 independent experiments.

dose-response experiments. It was observed that different doses of let-7i or the let-7 pool (equimolar mixture of let-7d, let-7f, let-7g, and let-7i) reduced PHA/PMA-stimulated IL-13 expression (Fig 3, B, and see Fig E3 in this article's Online Repository at www.jacionline.org, respectively). However, a saturation effect was observed at high doses (200 nmol/L in Fig 3, B, and 100 nmol/L in Fig E3), which might be due to system-level factors, such as cellular concentrations of the target transcripts and small RNAs loaded in RNA-induced silencing complex, which determines the kinetics of the regulation.²⁹ It is also important to note that let-7-mediated reduction of IL-13 levels occurred without apparent reduction in its mRNA expression (Fig 3, C). In addition, although transfection of cultured T cells with hairpin inhibitors of let-7d, let-7f, let-7g, and let-7i followed by PHA/PMA stimulation resulted in insignificant changes in IL-13 expression, an equimolar pool of inhibitors of the let-7 family members significantly increased IL-13 expression at 8 hours (Fig 3, D). Importantly, in experiments in which inhibitors of let-7 were used, IL-13 was detectable in culture supernatants at 8 hours (Fig 3, D) in contrast to being undetectable until 16 hours normally (Fig 2, C), confirming that high levels of endogenous let-7 (Fig 2, D) inhibit IL-13 secretion. Inefficiency of single hairpin inhibitors of let-7 compared with a pool suggested that the presence of multiple endogenous let-7 family members leads to a redundancy in the system that can only be overcome by inhibiting many of them simultaneously. Furthermore, *in vivo* experiments focused on simulation of let-7 action, which, in contrast to inhibition, can be achieved by any of the family members.

Intranasal delivery of let-7 reduces IL-13 levels in mice with allergic airway inflammation

To determine the role of let-7 microRNAs in IL-13-mediated inflammation, we used a well-established murine model of allergic airway inflammation (OVA) resembling asthma that is IL-13 dependent (Fig 4, A).³⁰ Real-time PCR analysis revealed that most of the members of the let-7 family were expressed in healthy lungs, and let-7b was the predominant form (Fig 4, B). Interestingly, levels of various members were found to be reduced in lungs of mice with allergic inflammation compared with levels seen in healthy lungs (Fig 4, B). Furthermore, Northern blot analysis showed a significant reduction in the expression of let-7 in lungs with allergic inflammation (OVA) compared with that seen in healthy control lungs (sham; Fig 4, C). We therefore administered let-7 microRNA mimics or control Cel-67 oligonucleotides in OVA-sensitized and OVA-challenged mice.³¹ Two experiments were performed to confirm that the intranasal microRNA mimic is efficiently delivered to the lung and taken up by the lung tissue. First, delivery of exogenous mature let-7 mimic to lungs through the intranasal route resulted in an 8.5-fold increase in let-7 levels above the baseline value (Fig 4, D, and see Fig E4 in this article's Online Repository at www.jacionline.org). Second, intranasal administration of Dyomics 547 (DY547)-labeled let-7 or Cel-67 oligonucleotides to OVA mice followed by staining of lung single-cell suspensions with CD3, Gr-1, and F4/80 revealed that labeled oligonucleotides were taken up by various cell types, including T cells (see Fig E5 and Table E1 in this article's Online Repository at www.jacionline.org). Uptake of let-7

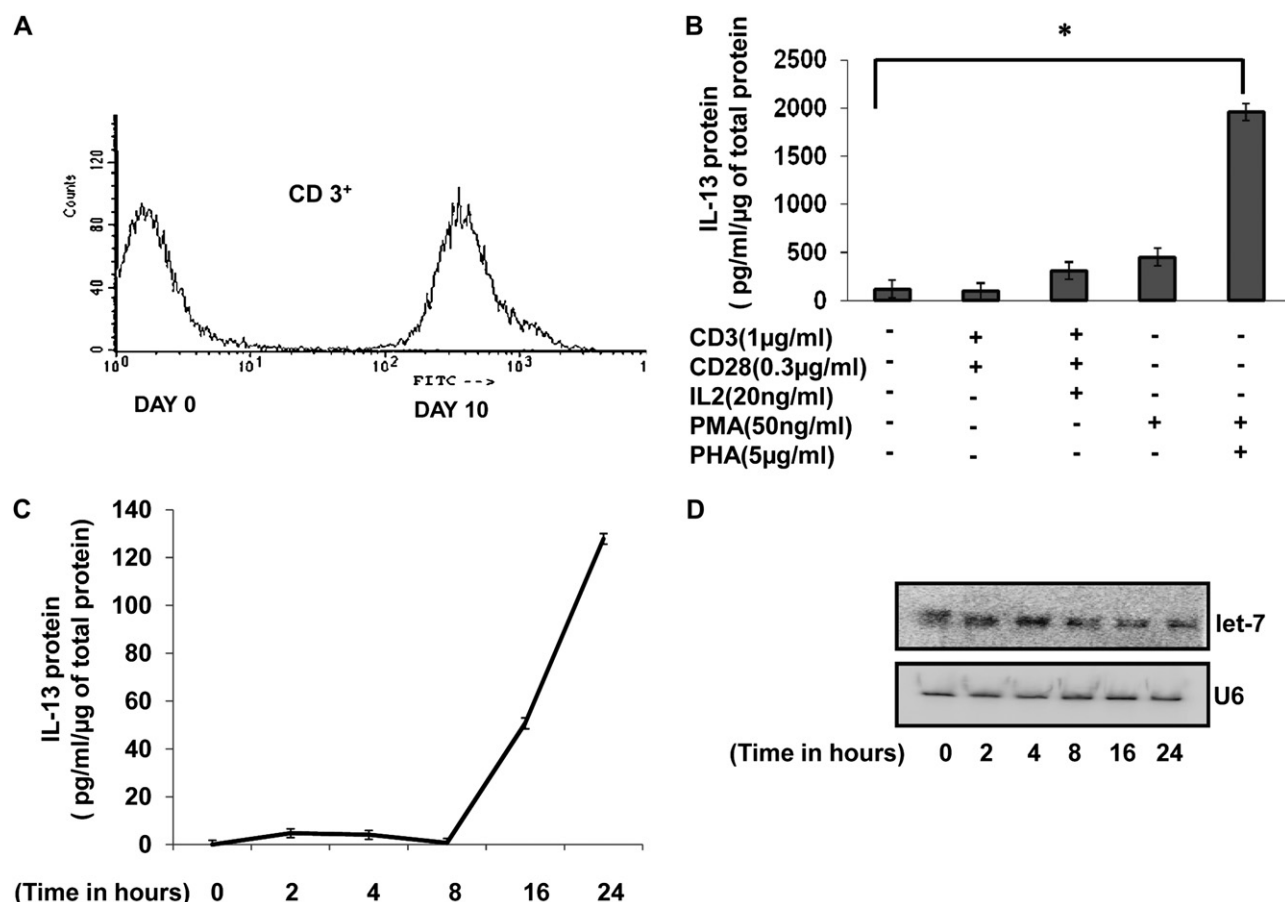


FIG 2. Let-7 inhibits IL-13 secreted by PMA/PHA-stimulated T cells. **A**, CD3 staining of primary lymphocytes. FITC, Fluorescein isothiocyanate. **B**, IL-13 levels in culture supernatants of stimulated T cells. **C** and **D**, Effect of time on IL-13 protein levels in culture supernatants of PMA/PHA-stimulated T cells (Fig 2, **C**) and let-7 RNA (Fig 2, **D**, Northern blot). Fig 2, **B** and **C**, are plotted as means $\pm$ SEs of 3 independent experiments. * $P < .0001$. Fig 2, **A** and **D**, are representative of 3 independent experiments.

mimic in the lungs of mice with allergic inflammation was associated with significant reductions in IL-13 levels in the lung homogenates, BAL fluid supernatants (Fig 4, *E1*), and sera (Fig 4, *E2*) of OVA mice compared with those seen in Cel-67 mice without having significant effects on the levels of other cytokines, such as IL-4, IL-5 (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org), and IFN- γ (see Fig E6, *B*) within the time frame of the experiment.

Intranasal delivery of let-7 mimic alleviates asthma features

To determine whether let-7-mediated reduction in IL-13 levels translated into improvement of the asthmatic condition, we determined the effects of intranasal let-7 on asthma features, such as AHR and airway inflammation, in a murine model. Administration of let-7, but not Cel-67, significantly reduced the increase in airway resistance in response to increasing concentrations of methacholine (Fig 5, *D*) and also substantially reduced the infiltration of inflammatory cells in the peribronchial and perivascular regions (Fig 5, *A*), and this reduction was further confirmed by means of inflammation scoring (Fig 5, *E*). Differential cell counts showed that treatment with the let-7 mimic significantly reduced eosinophil counts without affecting neutrophil counts, whereas no such reduction

was observed with Cel-67 (Fig 5, *F*). Because IL-13 is crucial in goblet cell metaplasia, we also determined the effect of let-7 on this feature using periodic acid-Schiff staining. As seen in Fig 5, *B*, OVA mice showed increased goblet cell metaplasia (morphometry for airway mucin content in means $\pm$ SEMs: OVA, 5.9 ± 0.2 vs sham, 1.4 ± 0.1) compared with that seen in healthy control mice. Let-7, but not Cel-67, inhibited goblet cell metaplasia (let-7, 3.9 ± 0.4 vs Cel-67, 6.6 ± 0.8). In addition, let-7, but not Cel-67, treatment reduced subepithelial fibrosis (Fig 5, *C*).

DISCUSSION

IL-13 is a key effector T_H2 cytokine. Because of its well-established role in modulating T_H2 immune responses, including asthma, efforts are being made to therapeutically modulate IL-13 function. For instance, the anti-IL-13 antibodies, such as QAX576, were under clinical trials for the treatment of IL-13-related disorders³²; the human anti-IL-13 IgG₄ mAb CAT-354 has shown a significant reduction in airway eosinophilia and airway reactivity in a murine model of airway and esophageal inflammation.³³

The aim of this study was to explore the molecular events underlying postranscriptional regulation of IL-13 in patients with asthma and related disorders. Using a combination of

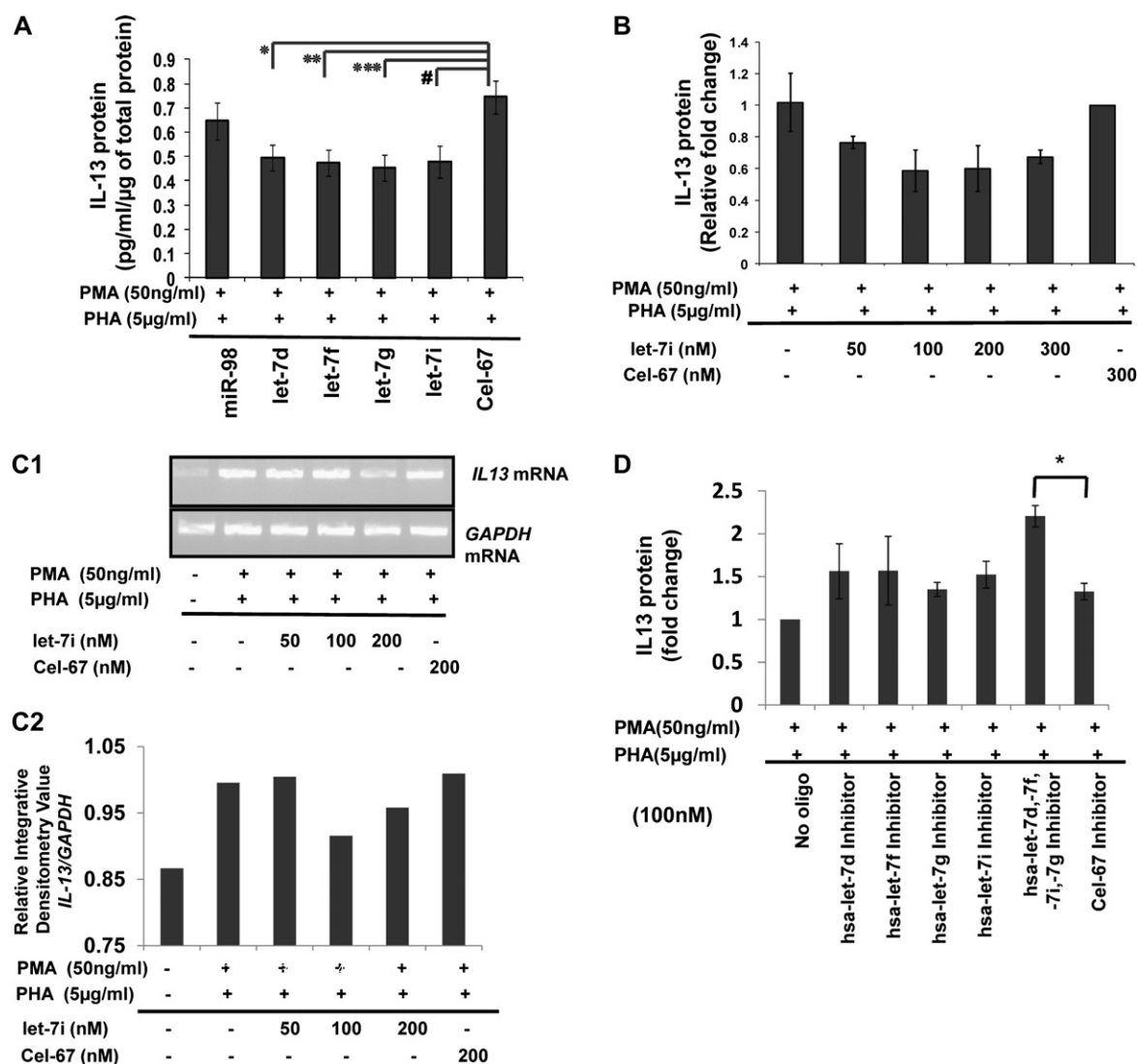


FIG 3. Let-7 modulates IL-13 levels in T cells. Protein levels (**A**, **B**, and **D**) in culture supernatants and mRNA levels (**C1** and **C2**) of IL-13 in T cells that were transfected with the let-7 family mimics or Cel-67 (Fig 3, **A**) or increasing concentrations of let-7i or Cel-67 (Fig 3, **B**, **C1**, and **C2**) or inhibitors of either let-7 members or its equimolar pool (Fig 3, **D**) followed by stimulation with PMA/PHA are shown. Fig 3, **A**, **B**, and **D**, were presented as means $\pm$ SEs of 3 independent experiments *, **, ***, # $P < .05$. Fig 3, **C1** and **C2**, are representative of 3 independent experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

in vitro and *in vivo* model systems, we demonstrate the involvement of microRNA (let-7) in the regulation of IL-13. This supplements the current understanding of transcriptional and posttranscriptional regulation of *IL13* and, most importantly, reveals the existence of a molecular process that can potentially counterbalance increased *IL13* transcription and lead to rapid resolution of the IL-13-mediated inflammation. Also, this information about the physiological regulation of a cytokine by microRNAs will help to evolve a better understanding of the interplay between complex regulatory events in disease outcome.

Let-7 microRNAs belong to a highly conserved microRNA family consisting of 12 genes encoding for 9 different microRNAs (let-7a to let-7i).¹⁵ The let-7 family members are also known as tumor suppressors because their levels are downregulated in various cancerous conditions and they also target

oncogenes, such as *RAS* and *HMGA2*.^{34,35} A high degree of sequence similarity in the seed region of the members (Fig 1, **B**) might explain the functional redundancy in IL-13 regulation (Fig 3, **D**). Such functional redundancy has also been observed in downregulation of *HMGA2* protein expression by let-7g and let-7d.³⁵ However, discrete spatial distribution of let-7 members suggests their differential regulation by multiple transcription factors under various conditions and contexts. Indeed, the let-7 microRNA family has been considered one of the airway-specific microRNAs, and let-7b was found to be highly expressed in human lungs compared with other members; our data show a similar trend in healthy murine lungs, with a general reduction seen across most of the let-7 family members during allergic airway inflammation (Fig 4, **B**). Although such a reduction was not found in asthmatic human lungs,³⁶ it should be recognized that the murine model of asthma exclusively represents an IL-13-driven

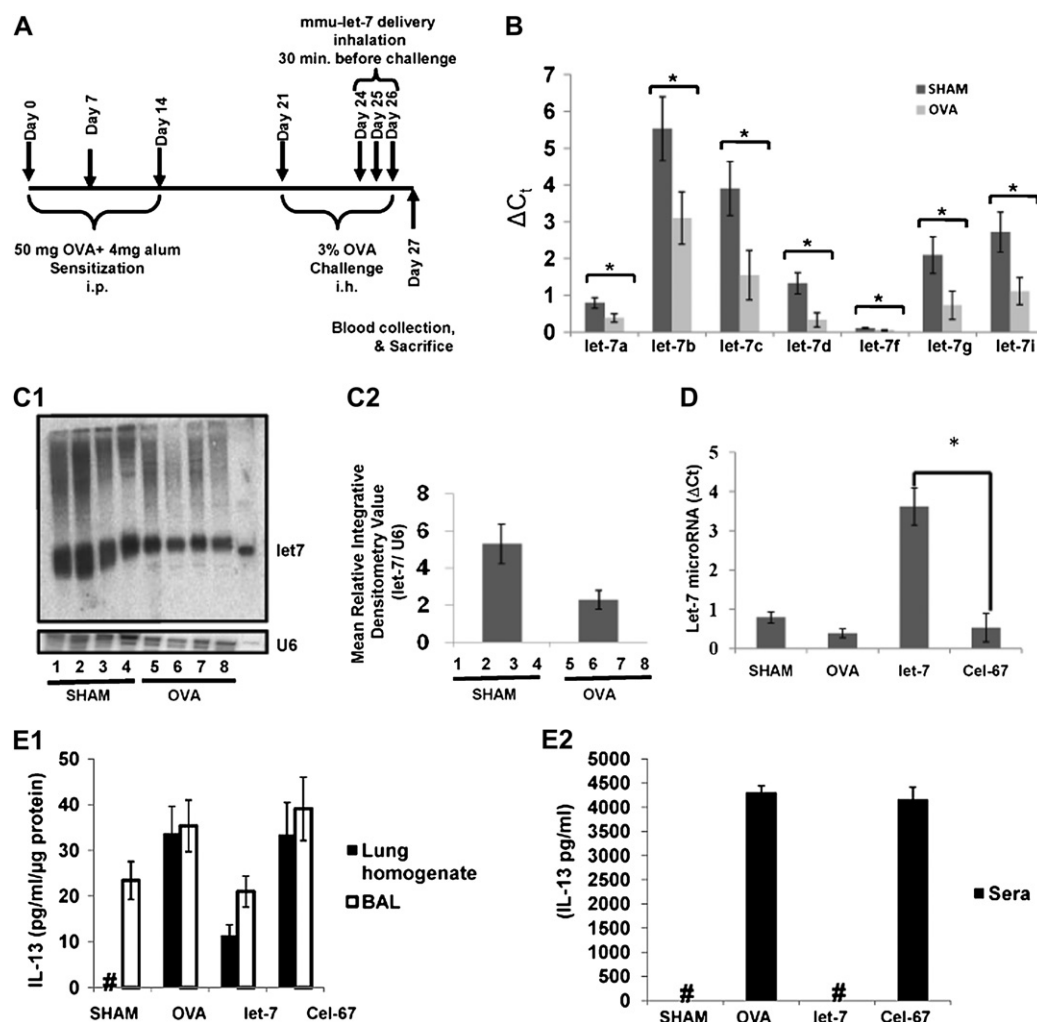


FIG 4. Exogenous let-7 reduces IL-13 levels in IL-13-dependent airway inflammation. **A**, OVA-induced asthma model. *i.p.*, Intraperitoneal; *i.h.*, inhalational. **B**, Levels of different let-7 members in lungs with respect to 18S ribosomal RNA. **C1** and **C2**, Northern blot analysis and densitometry of let-7 and U6 RNA. **D**, **E1**, and **E2**, Levels of let-7 microRNA in lungs (Fig 4, **D**) and IL-13 protein in lung homogenates, BAL fluid supernatants (Fig 4, **E1**), and sera (Fig 4, **E2**) of mice. Fig 4, **C**, is representative of 2 independent experiments. * $P < .05$. Fig 4, **E1** and **E2**, were plotted as means $\pm$ SEs of 2 independent experiments. #Undetectable.

T_H2 type of inflammatory process, whereas real asthma is far more diverse.³⁷ Within the context of the murine model, involvement of a master regulator cannot be ruled out. Most let-7 family members are posttranscriptionally regulated by Lin28, which prevents let-7 maturation by binding to its precursor and inhibits Drosha processing.^{38,39} Nuclear factor κ B, an important transcription factor during the initial phase of inflammation, is known to reduce let-7 levels by upregulating Lin28 levels. Lin28-mediated downregulation of let-7 was shown to be crucial in inflammation and cancer.⁴⁰

That let-7 microRNAs target *IL13* 3'UTR, resulting in IL-13 inhibition, is very clear from our study. In addition to the let-7 target site described, we have found evidence for an additional let-7 target site in the *IL13* 3'UTR (Fig 1, **C**). Because HuR is known to stabilize *IL-13* 3'UTR,¹¹ we ectopically overexpressed HuR and let-7i and observed that HuR had no effect on let-7i-mediated IL-13 inhibition (see Fig E7 in this article's Online Repository at www.jacionline.org). However, it would be interesting to further

explore the interplay between HuR and let-7 microRNA during inflammatory conditions.

The role of IL-13 in inflammation and asthma is well established. Exogenously delivered mature let-7 not only reduced IL-13 expression but also significantly alleviated airway inflammation, subepithelial fibrosis, and AHR. FACS analysis of inflamed lungs demonstrated that labeled let-7 oligonucleotides have been taken up by many cell types, including T cells (see Methods and Results in this article's Online Repository at www.jacionline.org, Fig E5 and Table E1), which are major IL-13 producers, and thus let-7 can exert its anti-inflammatory effects. Although these observations are in agreement with earlier reports of the effect of rIL-13 and *IL13*-specific small interfering RNA in asthma models,⁴¹ it would be interesting to identify other targets of let-7. Efforts are in progress to study the molecular regulation of the let-7 family, especially in inflammatory processes. *In silico* analysis suggests the presence of a putative Egr-1 binding site upstream of let-7i. This is interesting because IL-13 is known

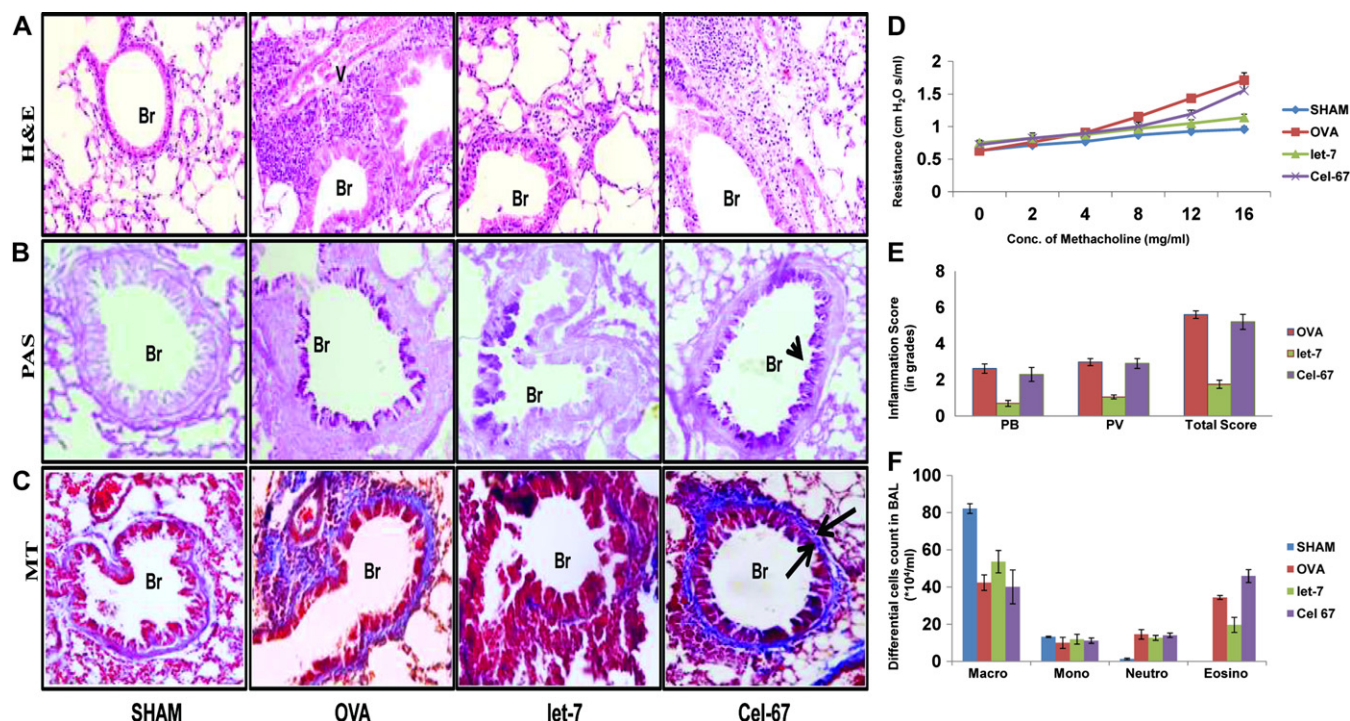


FIG 5. Exogenous let-7 attenuates airway inflammation, mucus hypersecretion, subepithelial fibrosis, and AHR in asthmatic mice. **A-C**, Lung sections stained with hematoxylin and eosin (H&E; Fig 5, A), periodic acid-Schiff (PAS; Fig 5, B), and Masson trichrome (MT; Fig 5, C) stains shown at $\times 20$ magnification. Br, Bronchus. **D**, Airway resistance with increasing concentrations of methacholine. **E** and **F**, Inflammation scores (Fig 5, E) and differential cell counts (Fig 5, F) in BAL fluid. PB, Peribronchial; PV, perivascular. Fig 5, A, B, and C, are representative of 2 independent experiments, and Fig 5, D, E, and F, were plotted as means $\pm$ SEs of 2 independent experiments.

to modulate Egr-1 during inflammation,⁴² thus suggesting a possible feedback loop.

Our results indicate the anti-inflammatory role of let-7 in a murine model of asthma. An anti-inflammatory role of let-7 has also been suggested in patients with idiopathic pulmonary fibrosis and cancerous conditions.^{40,43} However, Polikepahad et al⁴⁴ recently suggested its proinflammatory role in a similar murine model of asthma. This might relate to methodological differences. Although we used local delivery of let-7 mimics in an *in vivo* study, they used systemic delivery of let-7 inhibitors. Importantly, *in vitro* data from both studies support an anti-IL-13 anti-inflammatory role. As seen from our *in vitro* data, inhibition of let-7 family members has complex effects on IL-13, probably because of functional redundancy at the *IL13* 3'UTR and endogenous abundance of multiple let-7 members. One explanation for the apparent anti-inflammatory effects of selective let-7 inhibition reported by Polikepahad et al could be due to the Dicer negative feedback loop.^{45,46}

In summary, we report for the first time the role of let-7 microRNA in the posttranscriptional regulation of IL-13 and its anti-inflammatory role in alleviating asthmatic features in an experimental allergic asthma model in mice. This information might not only lead to a better understanding of the molecular regulation of inflammation but might also help in devising better therapeutic modalities for inflammatory disorders, including asthma.

M.K. and A.S. acknowledge the help of CSIR for their fellowships. We also thank Kamiya Mehla, Jyotirmoi Aich, Sudipta Das, Lokesh Makhija, Neha Singhal, and Himpriya Chopra for their help.

Key messages

- IL-13 is a direct target of let-7 microRNAs.
- The level of let-7 microRNAs significantly decreases in allergic airway inflammatory condition.
- Exogenous administration of a let-7 microRNA mimics alleviates asthmatic features.

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METHODS

Cell culture and transfection

A549 cells were grown in 75-mm² culture flasks in Dulbecco modified Eagle medium (Sigma) containing 10% FBS, streptomycin (50 µg/mL), and penicillin (50 IU/mL) at 37°C in 5% CO₂. Cells were transfected with siPORT-NeoFX (Ambion), according to the manufacturer's instructions. For reporter expression, A549 cells were transfected with 500 ng of either the *pMIR-REPORT-IL13* 3'UTR (intact) or the *pMIR-REPORT-IL13* 3'UTR (mutant) with or without end-modified microRNA oligonucleotides at a final concentration of 10 nmol/L (Dharmacon). The reporter gene assay was performed 24 hours after transfection with the Dual luciferase assay kit (Promega). The cells were also cotransfected with 5 to 10 ng of Renilla luciferase reporter plasmid control for normalizing transfection efficiency.

Cultured T cells were transfected in 12-well plates by using siPORT-NeoFX (Ambion). One million (10⁶) cells were transfected with 50 to 100 nmol/L end-modified microRNA of the let-7 family or Cel-67 oligonucleotides (Dharmacon) for 24 hours before stimulation with PMA/PHA (50 ng/mL and 5 µg/mL, respectively) for 24 hours.

Preparation of single-cell suspensions of lung and flow cytometry

Oligonucleotides were delivered intranasally, as described in the [Methods](#) section to analyze the uptake of fluorescent oligonucleotides, such as DY547-labeled let-7 or Cel-67 mimics (Dharmacon), by lung tissue. Lungs were harvested after death and mechanically disrupted with a 70-µm cell strainer (Becton Dickinson); RBCs were lysed and removed with ACK lysis buffer (Invitrogen) and stained with CD3 (peridinin-chlorophyll-protein), Gr-1 (peridinin-chlorophyll-protein), and F4/80 (fluorescein isothiocyanate), followed by acquisition and analysis with a FACSCalibur equipped with the Cell Quest software package (Becton Dickinson).

RNA extraction and RT-PCR

RNA was prepared from cells treated with or without a combination of PHA/PMA (5 µg/mL and 50 ng/mL) and from murine lung tissues by using Trizol reagent (Invitrogen), according to the manufacturer's instructions, and stored at -70°C until further use. Quantitative RT-PCR (Kapa Biosystems) was performed with specific primers for *IL13* and β_2 -microglobulin per the standard protocol. For studying the transfection efficiency, quantitative RT-PCR (Kapa Biosystems) of Cel-67 was performed with a Light Cycler-480 (Roche) by using stem-loop primers^{E1} and was normalized with 18S. Analysis was performed with a mathematic model, as described earlier.^{E2}

Measurement of cytokine (IL-4, IL-5, and IFN- γ) levels in lung homogenates

Lung homogenates were used for IL-4, IL-5, and IFN- γ ELISA (BD PharMingen) per the manufacturer's protocol. Results were expressed in picograms and normalized by protein concentrations.

Statistical analysis

Data are expressed as means $\pm$ SEs. Significant differences between 2 selected groups were estimated by using the unpaired Student *t* test or *post hoc* testing and the Bonferroni correction for multiple comparisons. Statistical significance was set at a *P* value of .05 or less.

RESULTS

Let-7 targets *IL13* 3'UTR

The entire *IL13* 3'UTR was cloned into a luciferase reporter system and cotransfected with individual let-7 microRNA mimics (10 nmol/L) in A549 cells because these cells express low levels of let-7 microRNAs.^{E3,E4} It was observed that transfection with any of the let-7 family microRNAs (let-7d, let-7g, and let-7f)

significantly reduced luciferase expression ([Fig E1](#)) by approximately 2.5-fold. However, Cel-67 (control microRNA) had no significant effect on luciferase expression. Deletion of the consensus target site ([Table I](#)) for the let-7 family microRNAs in the *IL13* 3'UTR has variable effect on luciferase expression, suggesting the presence of multiple binding sites for different let-7 members ([Fig E1](#)).

Time kinetics of *IL13* transcripts in stimulated T cells

Cells were stimulated with PMA (50 ng/mL) and PHA (5 µg/mL) at different time points up to 16 hours to study the kinetics of *IL13* transcripts in cultured T cells. We observed that *IL13* transcript levels increased throughout the period, with a 100-fold increase in transcript levels seen as early as 4 hours ([Fig E2](#)).

Let-7 regulates IL-13 secretion in cultured T cells

Cultured T cells were transfected with a let-7 pool at different concentrations, as described in the [Methods](#) section. Let-7 significantly downregulates IL-13 at 50 nmol/L ([Fig E3](#)), which is similar to let-7i-mediated regulation of IL-13 ([Fig 3, B](#)).

Intranasally delivered let-7 microRNA is efficiently taken up by lymphocytes

To confirm the cell types that efficiently take up intranasally delivered oligonucleotides, we delivered DY547-labeled let-7 or Cel-67 microRNA mimics in OVA mice on the last 3 consecutive days (days 25, 26, and 27). The mice were killed on day 28, and FACS analysis of single-cell suspensions of lungs revealed that there was a significant uptake of labeled oligonucleotides primarily by lymphocytes, F4/80⁺ cells, and Gr-1⁺ cells ([Fig E5](#) and [Table E1](#)).

Estimation of transfection efficiency of intranasal delivery by real-time quantitation of Cel-67 in the lungs of mice

To confirm that intranasally delivered microRNA mimic is efficiently delivered to the lung and is taken up by the lung tissue, we performed quantitative real-time PCR for Cel-67 in total lung RNA, as described in the [Methods](#) section. Cel-67 is a *C elegans* microRNA that lacks homology in mammals. We found strong expression of Cel-67 (threshold cycle, 16.5; difference in threshold cycles [normalized with 18S RNA], 1.2) in the lung tissue of OVA mice previously treated with Cel-67 for 3 consecutive days before death and thoroughly washed with PBS after BAL to prevent contamination from nontransfected Cel-67. Mice transfected with let-7 oligonucleotides showed only nonspecific PCR product, as expected ([Fig E4](#)).

Intranasal delivery of let-7 mimic did not affect IL-4, IL-5, and IFN- γ levels

Levels of T_H2 cytokines, such as IL-4 and IL-5, have been found to be increased in the lungs of OVA mice with allergic inflammation. Levels of these cytokines were measured in lung homogenates of different groups of mice to determine whether let-7 can have any effect on these cytokines, and they were found to remain unaffected, irrespective of treatment with respect to OVA ([Fig E6, A](#)). Additionally, IFN- γ levels were measured to determine any nonspecific effects of oligonucleotide

sequences and were found to remain unaltered among different groups (Fig E6, B).

Overexpression of let-7 overrides HuR mediated stabilization of *IL13* 3'UTR

Because HuR is known to stabilize the *IL13* 3'UTR, we ectopically overexpressed HuR in the A549 cell line by transfecting pTAP-HuR. We observed that cotransfection of pTAP-HuR in the presence of let-7i or Cel-67 had no effect on let-7i-mediated IL-13 inhibition (Fig E7).

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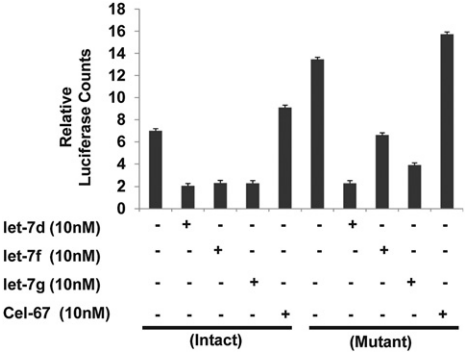


FIG E1. Deletion of the target site significantly restores luciferase expression. A549 cells were cotransfected with end-protected microRNA mimics of let-7d, let-7f, let-7g, or Cel-67 along with either *pMIR-IL13* 3' UTR (intact) or *pMIR-IL13* 3' UTR (mutant). Luciferase levels were measured 24 hours after transfection, as described in the [Methods](#) section. Data represent means $\pm$ SEs of 2 independent experiments.

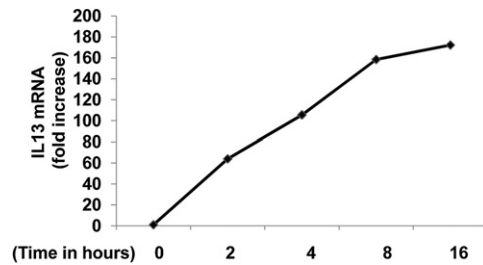


FIG E2. Time kinetics of *IL13* mRNA induction after treatment with PMA/PHA indicated a linear increase up to 16 hours. Cultured T cells were stimulated with PMA/PHA, as described in the Methods section, and RNA was prepared at different time points, as indicated in the figure, followed by real-time quantitation of *IL13* mRNA. Results were plotted as the fold increase in *IL13* mRNA levels with respect to those seen in unstimulated cells and normalized with β_2 -microglobulin expression.

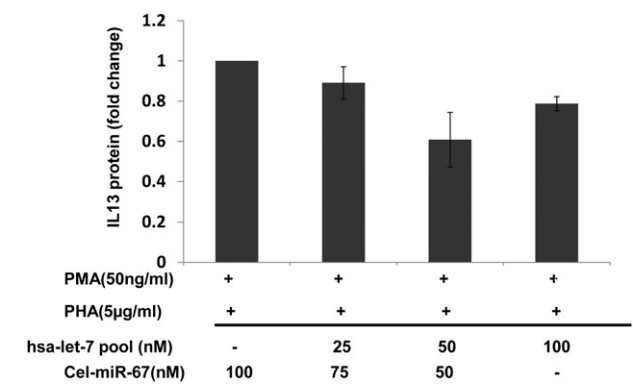


FIG E3. Ectopic expression of let-7 (pool) regulates IL-13 secretion from cultured T cells. IL-13 levels were measured in the culture supernatants of T cells that were transfected with or without increasing concentrations of an equimolar mixture of let-7 (pool) members (let-7d, let-7f, let-7g, and let-7i) or Cel-67 followed by stimulation with PMA/PHA and plotted as the relative fold change. Data represent means $\pm$ SEs of 3 independent experiments.

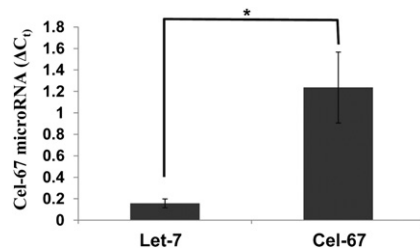


FIG E4. Increased levels of Cel-67 in lungs of mice treated with intranasal Cel-67. Cel-67 levels were measured in lungs of mice treated with intranasal let-7 and Cel-67, as described in the Methods section. Results were presented as relative levels of Cel-67 with respect to 18S. Data represents means $\pm$ SEs of 5 mice in each group.

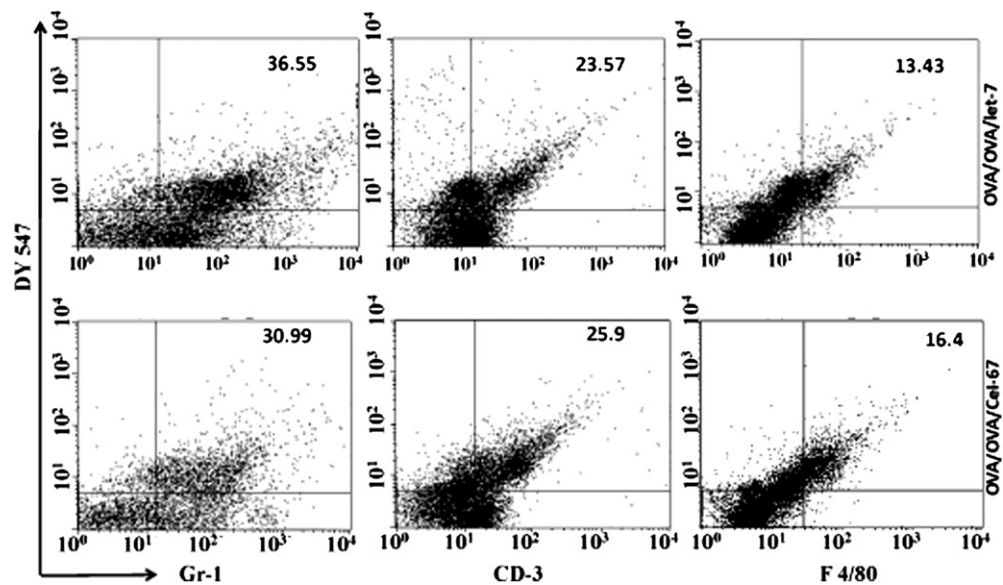


FIG E5. Intranasally delivered let-7 microRNA is efficiently taken up by lymphocytes. FACS analysis of single-cell suspension for the uptake of DY547-labeled let-7 or Cel-67 oligonucleotides in the lungs is shown. Cells were characterized by using cell-surface markers for lymphocytes (CD3⁺), granulocytes (Gr-1), and macrophage (F4/80).

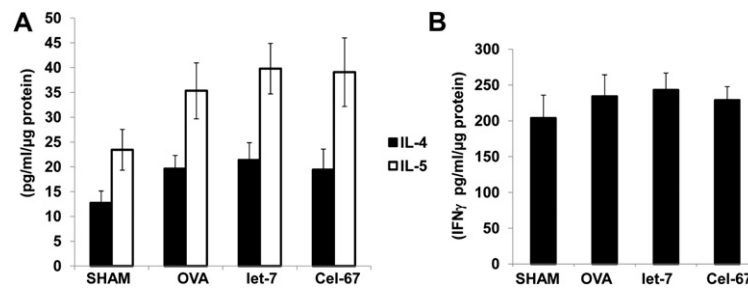


FIG E6. Ectopic expression of let-7 mimic does not affect IL-4, IL-5, and IFN- γ . T_H2 -specific cytokines, such as IL-4 and IL-5 (**A**) and the T_H1 cytokine IFN- γ (**B**) were detected in lung homogenates, as described in the Methods section, and plotted as means $\pm$ SEs of 2 independent experiments.

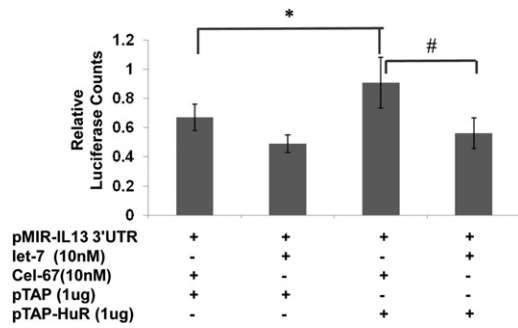


FIG E7. Overexpression of let-7i overrides HuR-mediated stabilization of the *IL13* 3'UTR. The *IL13* 3'UTR was cotransfected with let-7 and HuR overexpression of plasmid *pTAP-HuR* in A549 cells. Luciferase levels were measured 24 hours after transfection and normalized to Renilla luciferase expression. *pTAP* and Cel-67 were used as controls. Data represents means $\pm$ SEs of 4 independent experiments. * $P < .05$.

TABLE E1. Percentage of the double-stained cell population in lungs of OVA mice transfected either with let-7 (OVA/OVA/let-7) or Cel-67 (OVA/OVA/Cel-67)

	OVA/OVA/let-7, mean $\pm$ SD (n = 3)	OVA/OVA/Cel-67, mean $\pm$ SD (n = 3)
CD3/DY547	10.7 $\pm$ 2.3	17.2 $\pm$ 1.8
Gr-1/DY547	32.7 $\pm$ 3.9	19.5 $\pm$ 6.8
F4/80/DY547	11.1 $\pm$ 1.2	13.6 $\pm$ 2.3