

MicroRNA-155 is essential for T_H2-mediated allergen-induced eosinophilic inflammation in the lung

Carina Malmhäll, BSc, Sahar Alawieh, BSc, You Lu, PhD, Margareta Sjöstrand, PhD, Apostolos Bossios, MD, PhD, Maria Eldh, PhD, and Madeleine Rådinger, PhD Gothenburg, Sweden

Background: Allergic asthma is a chronic disease of the conducting airways characterized by T_H2 inflammation and tissue remodeling after exposure to inhaled allergens. Although the T_H2 profile is undisputed, the underlying molecular mechanisms leading to this abnormal T_H2 profile remain largely unclear. MicroRNAs (miRNAs) are short noncoding RNAs that are important regulators of gene expression in the immune system. However, the role of miRNAs, specifically miR-155, in the regulation of allergic airway inflammation is unexplored.

Objectives: We sought to assess the contribution of miR-155 in a mouse model of allergic airway inflammation.

Methods: To investigate a role for miR-155 in the regulation of allergic inflammation *in vivo*, we used miR-155 knockout (KO) and wild-type (WT) mice sensitized and exposed to ovalbumin. **Results:** miR-155 deficiency resulted in diminished eosinophilic inflammation and mucus hypersecretion in the lungs of allergen-sensitized and allergen-challenged mice compared with WT control animals. This was supported by a reduction in T_H2 cell numbers and airway T_H2 cytokine levels and complete abrogation of allergen-induced airway eotaxin-2/CCL24 and periostin levels in miR-155 KO mice. Intranasal instillation of eotaxin-2/CCL24 before allergen challenge partially restored airway eosinophilia in miR-155 KO mice, and adoptive transfer of CD4⁺ T cells resulted in a similar degree of airway eosinophilia in miR-155 KO and WT mice. Furthermore, the transcription factor PU.1, a negative regulator of T_H2 cytokine production, was upregulated in the airways of allergen-challenged miR-155 KO mice compared with WT mice.

Conclusions: Our data provides evidence that miR-155 contributes to the regulation of allergic airway inflammation by modulating T_H2 responses through the transcription factor PU.1. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: MicroRNA, miR-155, allergy, asthma, eosinophils, inflammation, T_H cells

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
CBA:	Cytometric Bead Array
FOXP3:	Forkhead box protein 3
KO:	Knockout
MCP:	Monocyte chemoattractant protein
MIG:	Monokine induced by IFN-γ
MIP:	Macrophage inflammatory protein
miRNA:	MicroRNA
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
PBLN:	Peribronchial lymph node
ROR:	Retinoic acid-related orphan receptor
TARC:	Thymus and activation-regulated chemokine
T-bet:	T-box transcription factor
Treg:	Regulatory T
WT:	Wild-type

Allergen-induced airway inflammation is characterized by T_H2-mediated eosinophilic inflammation in the lung. T_H2 cells play a key role in the pathogenesis of allergic asthma through their secretion of the T_H2 cytokines IL-4, IL-5, and IL-13.¹⁻⁵ Although these cytokines are hallmarks of T_H2 immunity observed in models of allergic asthma, the molecular mechanisms leading to this abnormal T_H2 response remain largely unclear.

MicroRNAs (miRNAs) are short noncoding RNAs that function as posttranscriptional modulators of gene expression by either promoting mRNA degradation or blocking protein translation.^{6,7} This results in a subsequent reduction in protein expression. A single miRNA can regulate many biological functions by suppressing hundreds of target mRNAs simultaneously. Dysregulation in expression of miRNAs is associated with human disease, including cancer and inflammatory diseases.^{8,9} There is emerging evidence that miRNAs are important regulators of gene expression in the immune system,⁷ and this is supported by recent studies demonstrating a role for miRNAs in allergic airway inflammation. miR-21 has a role in T_H2 cell polarization through negative regulation of IL-12p35 in models of allergic airway inflammation.¹⁰ Another miRNA, miR-126, links innate and adaptive immune responses in a house dust mite mouse model of allergic airway inflammation. Antagonism of miR-126 significantly suppressed effector functions of T_H2 cells and the development of eosinophilic airway inflammation.¹¹ Likewise, antagonism of miR-145 in the same model demonstrated suppressed eosinophilic airway inflammation, T_H2 cytokine production, mucus hypersecretion, and airway hyperresponsiveness.¹² The above studies highlight the contribution of miRNAs to the pathogenesis of allergic inflammation and their potential role as anti-inflammatory targets in the treatment of allergic diseases.

From the Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, University of Gothenburg.

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Corresponding author: Madeleine Rådinger, PhD, Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Box 424, SE-405 30 Göteborg, Sweden. E-mail: madeleine.radinger@gu.se.

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miR-155 is known as a multifunctional miRNA that has been implicated in carcinogenesis, as well as in innate and acquired immunity.¹³ In terms of immunity, miR-155 is upregulated in activated T cells, B cells, dendritic cells, and macrophages.⁷⁻⁹ miR-155-deficient T cells produced more IL-4 and less IFN- γ under neutral conditions *in vitro*.¹⁴ miR-155 is also important for regulatory T (Treg) cell homeostasis and survival.^{15,16} These data suggest that miR-155 is involved in T_H cell differentiation and survival. Furthermore, a role for miR-155 in animal models of disease has been demonstrated. miR-155 deficiency dampens T cell-mediated induction of colitis in an animal model of inflammatory bowel disease, and miR-155 KO mice are resistant to antigen-specific T_H17 cell responses in a model of collagen-induced arthritis.^{17,18} In terms of allergic diseases, it is interesting to note that the B-cell integration cluster/*miR-155* gene is located within a region on chromosome 21q2 associated with pollen sensitivity and asthma and atopic dermatitis susceptibility.¹⁹ Also, recent studies demonstrate miR-155 overexpression in the nasal mucosa of patients with allergic rhinitis and the skin of patients with atopic dermatitis,^{20,21} suggesting a role for this miRNA in the pathogenesis of allergic diseases.

Although miRNAs are important regulators of gene expression in the immune system, there are limited functional data on the role of these RNAs in the regulation of allergic airway inflammation. Therefore the aim of our study was to determine the role of miR-155 in allergen-induced airway inflammation. We hypothesized that miR-155 modulates allergic inflammation by influencing allergen-mediated T_H2 responses.

METHODS

Mice

Gene knockout (KO) mice lacking the *bic/miR-155* function/reporter allele (B6.Cg-*MiR155^{tm1.1Rsky}/J*) and control C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Me). Age- and sex-matched mice were used in all experiments at 6 to 10 weeks old. Mice were kept in pathogen-free conditions with food and water *ad libitum*. The *in vivo* study was approved by the Animal Ethics Committee at the University of Gothenburg, Gothenburg, Sweden (permit no. 323-2011).

Induction of allergic airway inflammation

Wild-type (WT) and miR-155 KO mice were sensitized and exposed to ovalbumin (OVA; OVA/OVA mice) to induce allergic airway inflammation. Some mice were kept naive, and some mice were sensitized to OVA but exposed to PBS (OVA/PBS mice). See the [Methods](#) section and [Fig E1](#) in this article's Online Repository at www.jacionline.org for more details on the protocol.

Flow cytometry

Viable lung cells were identified as 7-amino-actinomycin D-negative cells in a gated population based on forward- and side-scatter profiles. T cells were then identified as CD3⁺ or CD4^{hi} cells from a mononuclear cell gate, and within these gates, the subsequent T-cell subsets were identified as T_H1 cells (CD4⁺ T-box transcription factor [T-bet]⁺), T_H2 cells (CD4⁺GATA-3⁺), T_H17 cells (CD4⁺ retinoic acid-related orphan receptor [ROR] γ t⁺), and Treg cells (CD4⁺ forkhead box protein 3 [FOXP3]⁺). All flow cytometric analyses (400,000-500,000 events per sample) were performed with a BD FACS-Aria Flow Cytometer running BD FACS Diva version 6.0 Software (BD Biosciences, San Jose, Calif) and analyzed with FlowJo Software (TreeStar, Ashland, Ore). See the [Methods](#) section in this article's Online Repository for flow cytometric staining.

In vitro activation of lymphocytes

Single-cell suspension of splenocytes and peribronchial lymph node (PBLN) cells from allergen-sensitized and allergen-exposed treated WT and

miR-155 KO mice were obtained by passing exudate through cell strainers (BD Falcon; BD, Franklin Lakes, NJ). Further enrichment of untouched splenic CD4⁺ T cells was performed by using magnetic separation, according to the manufacturer's protocol (CD4⁺ T cell Isolation kit II, MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Lymph node cells and spleen CD4⁺ T cells were cultured separately *in vitro* at a density of 5×10^6 /mL in complete media (RPMI 1640 medium, 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% L-glutamine) in the presence or absence of 200 μ g/mL OVA for 6 days. Cell-free supernatants were harvested and stored at -80°C until analysis.

Chemokine and cytokine analysis

Chemokine and cytokine analysis was performed with ELISA DuoSets (R&D Systems, Minneapolis, Minn) or the BD Cytometric Bead Array (CBA; BD Biosciences). CBA data were acquired on a BD FACSARIA Flow Cytometer running BD FACSDiva version 6.0 Software and analyzed with FCAP Array Software (BD Biosciences). All analysis was carried out according to the manufacturer's protocol. See the [Methods](#) section in this article's Online Repository for more details.

Adoptive transfer of CD4⁺ T cells

Splenocytes from OVA-sensitized WT mice were used to generate OVA-specific T cells. CD4⁺ T cells were enriched by using magnetic separation, as described above, and cultured for 4 days at 37°C in the presence of OVA (200 μ g/mL). After washing and resuspension in PBS, CD4⁺ T cells (4×10^6 cells per mouse) were then transferred into naive recipient WT and miR-155 KO mice by means of intraperitoneal injection (see [Fig E1, C](#)). Twenty-four hours after cell transfer, mice were exposed to OVA (100 μ g) once daily for a total of 5 days. Lung tissue and bronchoalveolar lavage fluid (BALF) were isolated 24 hours after the final exposure, and airway eosinophilia was assessed by using differential cell counts.

miRNA and mRNA expression

LNA Primer sets for miR-155 (Exiqon A/S, Vedbaek, Denmark) were used to determine miR-155 expression in lung and spleen tissue, as detailed in the [Methods](#) section in this article's Online Repository. Mouse Custom Array CAPM11696 (SABiosciences, Qiagen, Hilden, Germany) was used for determination of target mRNAs of c-Maf, CTLA4, GATA-3, PU.1, SHIP1, SMAD1, SOCS1, STAT3, STAT6, TGF β 1, and TNFSF9 in PBLNs. Details are available in the [Methods](#) section in this article's Online Repository.

Statistical analysis

Data are expressed as means $\pm$ SEMs. Statistical analysis was performed with the unpaired Student *t* test or Mann-Whitney test, where appropriate, by using GraphPad Prism 6 Software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

RESULTS

miR-155 is required for allergen-induced eosinophilic airway inflammation

To investigate a role for miR-155 in the regulation of allergic inflammation *in vivo*, we used miR-155 KO mice sensitized and exposed to OVA. Sampling was performed 24 hours after 5 consecutive days of OVA exposure. Allergen-challenged miR-155 KO mice had reduced numbers of BALF and lung tissue cells compared with WT mice (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Next, we determined the number of inflammatory cells in the airways based on differential cell counts. As can be seen in [Fig 1, A](#), a significant reduction in the absolute numbers of eosinophils, neutrophils, lymphocytes, and

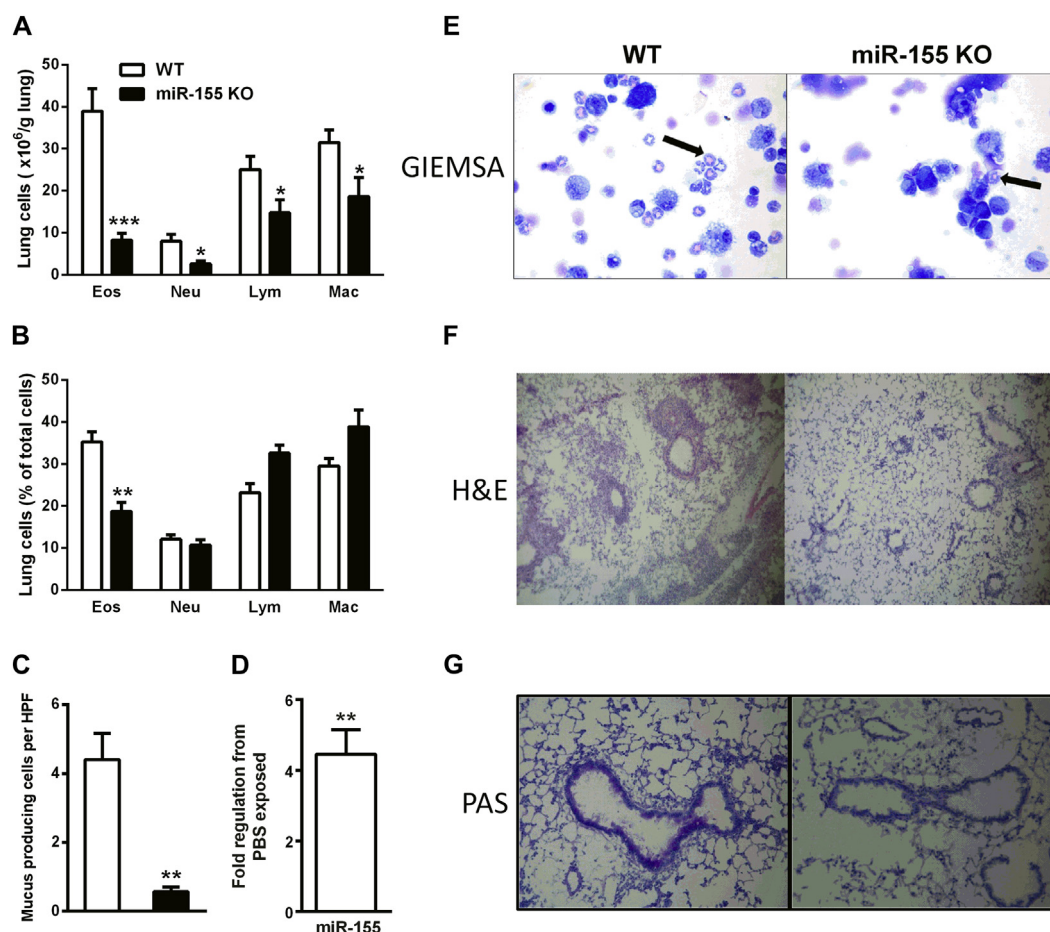


FIG 1. miR-155 deficiency results in altered inflammatory and mucus-producing cells in the lungs after allergen challenge. **A** and **B**, Total (**A**) and relative (**B**) numbers of eosinophils, neutrophils, lymphocytes, and macrophages in lungs of OVA/OVA WT and miR-155 KO mice. **C**, Number of mucus-producing cells. **D**, Fold regulation in miR-155 expression in lungs of OVA/OVA WT mice compared with OVA/PBS WT mice. **E-G**, Representative Giemsa-stained cytospin preparations from single-cell tissue preparation (**E**) and hematoxylin and eosin (**H&E**)-stained (**F**) and PAS-stained (**G**) lung sections. Means $\pm$ SEMs are shown ($n = 3-10$ per group). * $P < .05$, ** $P < .01$, and *** $P < .001$, WT versus miR-155 KO mice.

macrophages was found in the lungs of allergen-challenged miR-155 KO mice when compared with values in allergen-challenged WT mice (**Fig 1, A and E**, and see **Fig E2, C**). In contrast, when relative numbers of inflammatory cells in the lung were evaluated, a significant reduction was found only in tissue eosinophil numbers in allergen-challenged miR-155 KO mice when compared with those in WT mice (**Fig 1, B**, and see **Fig E2, E**). Furthermore, histologic examination of lungs from allergen-challenged miR-155 KO mice demonstrated a significant decrease in numbers of infiltrating eosinophils (**Fig 1, F**) and decreased mucus hypersecretion, as determined by using periodic acid-Schiff (PAS) staining, when compared with values in allergen-challenged WT mice (**Fig 1, C and G**). No mucus-positive cells were found in either WT or miR-155 KO mice that were PBS exposed. When evaluating miR-155 expression in the lungs of allergen-sensitized WT mice, we found the levels to be more than 4-fold upregulated after allergen challenge compared with those after PBS challenge (**Fig 1, D**).

Allergen-induced eosinophil levels in bone marrow, spleen, and blood were assessed to further determine the point of the inflammatory pathways at which eosinophil influx to the lung was

disrupted in miR-155 KO mice. There was no effect of miR-155 deficiency on allergen-induced bone marrow or spleen eosinophilia (**Fig 2, A and B**). In contrast, there was a slight but significant reduction in allergen-induced blood eosinophilia in miR-155 KO mice when compared with that seen in allergen-induced WT mice (**Fig 2, C**). However, when airway eosinophilia was evaluated, a markedly diminished eosinophilia was found in allergen-challenged miR-155 KO mice in both lung tissue and BALF when compared with that seen in allergen-challenged WT mice (**Fig 2, D and E**). Thus these data suggest that miR-155 has a significant role in the regulation of airway eosinophilia at the site of inflammation or possible regulation in the recruitment of eosinophils to the airways after allergen challenge.

miR-155 deficiency alters allergen-induced chemokine expression in the airways

We and others have previously shown that eotaxin-1/CCL11 and eotaxin-2/CCL24 play pivotal roles in regulating eosinophil recruitment locally in the airways in response to allergen challenge.^{22,23} Thus we measured the levels of these chemokines in BALF from

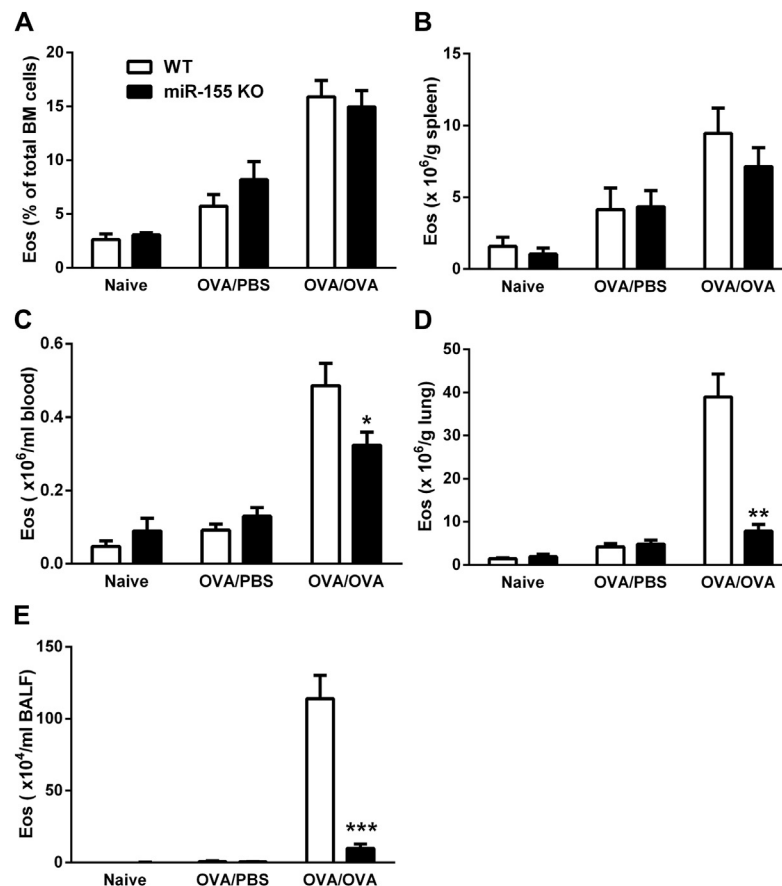


FIG 2. miR-155 deficiency abolishes allergic eosinophilic airway inflammation without affecting allergen-induced bone marrow eosinophilia. Total numbers of eosinophils in bone marrow (A), spleen tissue (B), blood (C), lung tissue (D), and BALF (E) in naive, OVA/PBS, and OVA/OVA WT mice and miR-155 KO mice. Means $\pm$ SEMs are shown ($n = 4$ -10 per group). * $P < .05$, ** $P < .01$, and *** $P < .001$, WT versus miR-155 KO mice. Eos, Eosinophils.

allergen-challenged WT and miR-155 KO mice, respectively. Eotaxin-1/CCL11 levels were decreased in the BALF of allergen-challenged miR-155 KO mice compared with that seen in allergen-challenged WT mice (Fig 3, A). Furthermore, in line with previous studies, eotaxin-2/CCL24 was the strongest induced chemokine in the BALF (Fig 3, B).²³ In the lung there was a trend toward a significant reduction in eotaxin-1/CCL11 levels (Fig 3, C), whereas eotaxin-2/CCL24, thymus and activation-regulated chemokine (TARC)/CCL17, and macrophage inflammatory protein (MIP) 1 α (Fig 3, D and E, and see Table E1 in this article's Online Repository at www.jacionline.org) levels were significantly decreased in allergen-challenged miR-155 mice compared with those in WT mice. In contrast, levels of RANTES/CCL5, monocyte chemoattractant protein (MCP) 1/CCL2, or monokine induced by IFN- γ (MIG)/CXCL9, a negative regulator of eosinophil recruitment, were not altered in allergen-exposed miR-155 KO mice compared with those seen in WT mice (Fig 3, E, and Table E1).

Effector function of lung T_H2 cells and airway periostin levels are impaired in miR-155 KO mice

It is well known that T_H2 cells and their effector cytokines IL-4, IL-5, and IL-13 play important roles in the pathophysiology of allergic inflammation. To further elucidate the role of miR-155 in regulating allergen-induced T_H2 responses in the lung, we sampled

PBLNs from allergen-challenged WT and miR-155 KO mice and restimulated these *ex vivo* with OVA for 6 days. IL-4, IL-5, and IL-13 release by PBLN cells were significantly reduced in miR-155 KO mice compared with that seen in WT mouse-derived PBLNs (Fig 4, A-C). Notably, there was a tendency toward a decrease in the release of IL-17A in miR-155 KO mouse-derived PBLN cells compared with that seen in WT mouse-derived PBLN cells (Fig 4, D). By contrast, IFN- γ release was not altered in miR-155 KO mouse-derived PBLN cells compared with that seen in WT mice (see Table E2 in this article's Online Repository at www.jacionline.org). Because IL-13 is known to have direct profibrotic and remodeling effects in patients with allergen-induced asthma and regulates eotaxin production and extracellular matrix proteins, including periostin, we measured IL-13 and periostin release in BALF from allergen-exposed WT and miR-155 KO mice. Protein levels of both IL-13 and periostin were abolished in allergen-exposed miR-155 KO mice when compared with those seen in allergen-challenged WT mice (Fig 4, E and F). Furthermore, CD4⁺ splenocytes derived from miR-155 KO showed similar allergen-induced IL-5, IL-13, and IL-17 release compared with that seen in WT control animals. However, there was a tendency toward increased IL-4 release in miR-155 KO compared with WT mouse-derived CD4⁺ splenocytes (see Table E2). Thus these findings might indicate that miR-155 is associated with T_H2-mediated leukocyte infiltration and activation locally in the

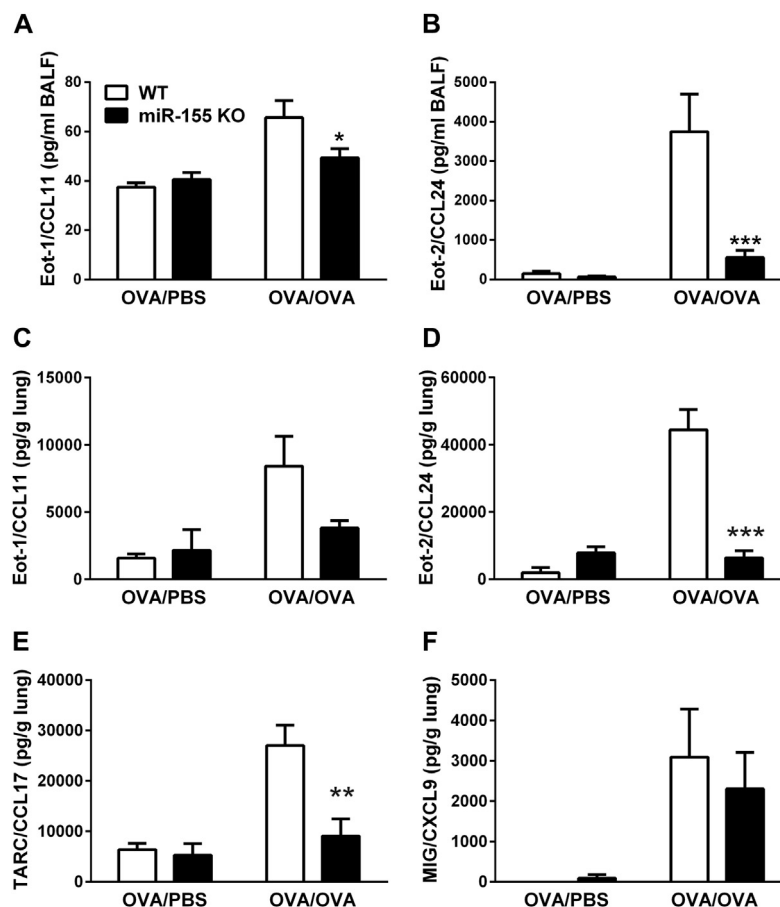


FIG 3. miR-155 deficiency reduces eotaxin-1/CCL11, eotaxin-2/CCL24, and TARC/CCL17 levels in the airways of allergen-challenged mice. **A** and **B**, Eotaxin-1/CCL11 (Fig 3, A) and eotaxin-2/CCL24 (Fig 3, B) release in BALF of OVA/PBS and OVA/OVA WT and miR-155KO mice. **C-F**, Eotaxin-1/CCL11 (Fig 3, C), eotaxin-2/CCL24 (Fig 3, D), TARC/CCL17 (Fig 3, E), and MIG/CXCL9 (Fig 3, F) in lung tissue homogenates of OVA/PBS and OVA/OVA WT and miR-155KO mice. Means $\pm$ SEMs are shown ($n = 4-6$ per group). * $P < .05$, ** $P < .01$, *** $P < .001$, WT versus miR-155 KO mice.

airways after allergen challenge. Furthermore, no upregulation of miR-155 was found in spleens after allergen exposure compared with levels seen after saline exposure in WT mice (data not shown).

miR-155 deficiency results in impaired numbers of activated CD4⁺CD25⁺ T cells and defective inflammatory T-cell development in the lung after allergen challenge

Because T cells rapidly increase miR-155 expression upon activation,²⁴ we next examined the number and phenotype of T cells from the lungs of allergen-sensitized and allergen-exposed WT and miR-155 KO mice. No significant differences were found in the relative numbers of CD4⁺ T cells between WT and miR-155 KO mice in either group tested (Fig 5, A). However, the relative numbers of CD4⁺CD25⁺ T cells were significantly altered in miR-155 KO mice, with a significant increase after PBS exposure and a significant decrease after allergen challenge (Fig 5, B). The normal function of the immune system requires the generation of different T-cell subtypes, which is dysregulated during disease.²⁵ Because miR-155 is suggested to play a role in the homeostasis of the immune system and T cells from miR-155 KO mice preferentially differentiate toward T_H2 *in vitro*,¹⁴ we next evaluated the relative numbers of T-cell subtypes in naive, PBS-exposed, and

allergen-exposed WT and miR-155 KO mice. This was achieved by assessing CD4⁺ T-cell expression of the master regulatory transcription factors T-bet, GATA-3, ROR γ t, and FOXP3 associated with the differentiation of T_H1, T_H2, T_H17, and Treg cells, respectively. In all conditions tested, there was no significant difference in the relative numbers of T_H1 cells in WT and miR-155 KO mice (Fig 5, C). Although an initial reduction in both T_H17 and Treg cell numbers was observed in naive miR-155 KO mice, these differences were lost after allergen sensitization (Fig 5, E and F). However, after allergen challenge, miR-155 deficiency resulted in impaired numbers of T_H2, T_H17, and Treg cells compared with those seen in WT mice (Fig 5, D-F). These data suggest a role for miR-155 in the development of T cells of the T_H2, T_H17, and Treg lineages, which might have important consequences in allergic inflammation.

Intranasal instillation of eotaxin-2/CCL24 before allergen challenge partially restores airway eosinophilia in miR-155 KO mice

To further investigate whether eotaxin-2/CCL24 could restore airway eosinophilia in miR-155 KO mice, we administered eotaxin-2/CCL24 intranasally to sensitized miR-155 KO mice 1 hour before allergen challenge (see Fig E1, B). A significant

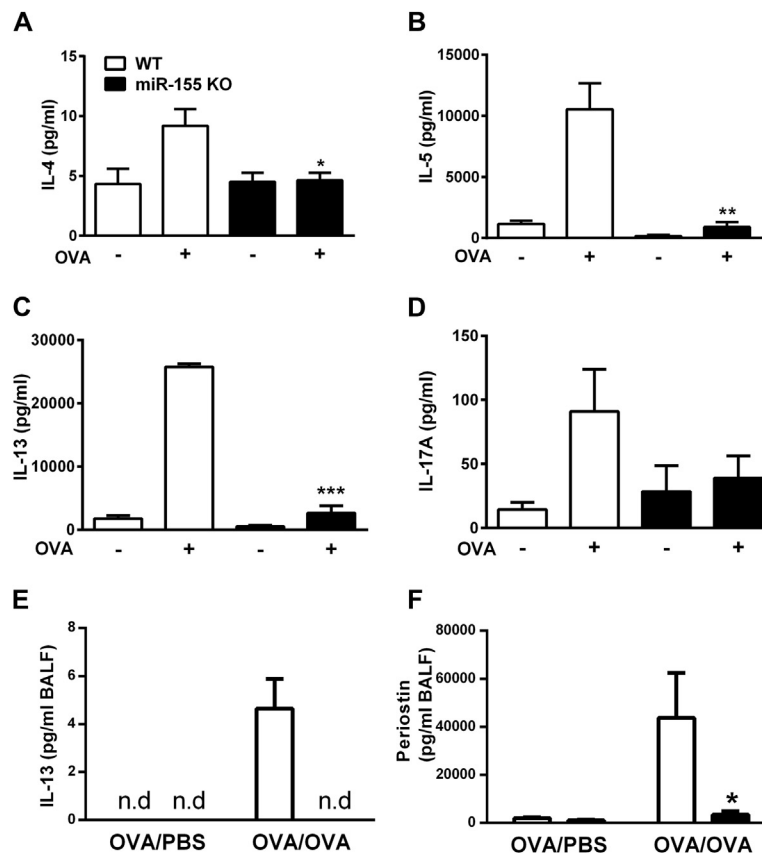


FIG 4. miR-155 deficiency impairs lung T_H2 responses. **A-D**, IL-4 (Fig 4, A), IL-5 (Fig 4, B), IL-13 (Fig 4, C), and IL-17A (Fig 4, D) release of PBLN cells from WT and miR-155 KO mice restimulated *in vitro* with OVA or saline for 6 days. **E and F**, IL-13 (Fig 4, E) and periostin (Fig 4, F) release in BALF of OVA/PBS and OVA/OVA WT and miR-155 KO mice. Means $\pm$ SEMs are shown ($n = 4-6$ per group). * $P < .05$, ** $P < .01$, and *** $P < .001$, WT versus miR-155 KO mice. n.d., Not detectable.

influx of eosinophils in BALF and lung tissue was seen after eotaxin-2/CCL24 treatment compared with that seen in miR-155 KO mice administered vehicle control (PBS), and thus eotaxin-2/CCL24 treatment partially restored both lung tissue and BALF eosinophilia in allergen-challenged miR-155 KO mice to levels observed in allergen-exposed WT mice (Fig 6, A and B).

Adoptive transfer of WT-derived $CD4^+$ T_H2 cells restores airway eosinophilia in miR-155 KO mice

OVA-specific $CD4^+$ T cells from WT mice were adoptively transferred to naive WT and miR-155 KO mice to determine whether the diminished T_H2 -dependent eosinophilia in allergen-challenged miR-155 KO mice was due to a defect in $CD4^+$ T cells. Adoptive transfer of OVA-specific $CD4^+$ T cells from WT mice resulted in comparable airway eosinophil numbers in miR-155 KO mice as in WT mice (Fig 6, C and D), arguing that the impaired allergy parameters in miR-155 KO mice are due to a defect in T_H2 cells.

miR-155 deficiency upregulates transcription factor PU.1 expression

Because miR-155 has been shown to be a multifunctional miRNA, we explored a number of mRNAs previously found to be directly or indirectly targeted by miR-155. The selected mRNAs evaluated in PBLNs from allergen-challenged WT and miR-155

KO mice were c-Maf, CTLA4, GATA-3, PU.1 (Sfp1), SHIP1 (Inpp5d), SMAD1, SOCS1, STAT3, STAT6, TGFB1, and TNFSF9. As shown in Fig 7 and Fig E3 in this article's Online Repository at www.jacionline.org, PU.1 was the only transcription factor to be significantly upregulated in miR-155 KO mice. PU.1 is a direct target of miR-155²⁶ and negatively regulates T_H2 cytokine production.²⁷ These data suggest that PU.1 might be an important transcription factor contributing to the reduced T_H2 cytokine profile in allergen-challenged miR-155 KO mice.

DISCUSSION

In this study we used a well-characterized OVA model to determine the role of miR-155 in allergen-induced airway inflammation. We clearly demonstrate that miR-155 is involved in the regulation of allergen-induced T_H2 -mediated eosinophilic inflammation in the airways. miR-155 expression was significantly increased in the lungs of WT mice after allergen exposure, and miR-155 KO mice failed to develop allergen-induced airway eosinophilia and mucus hypersecretion, both of which are dependent on T_H2 cytokines (IL-4, IL-5, and IL-13).^{5,28,29} In addition, both eotaxin-2/CCL24 and periostin levels were decreased in the airways of allergen-challenged miR-155 KO mice to levels comparable with those in saline-exposed WT mice. Intranasal administration of eotaxin-2/CCL24 before allergen challenge in miR-155 KO mice partially restored airway eosinophilia.

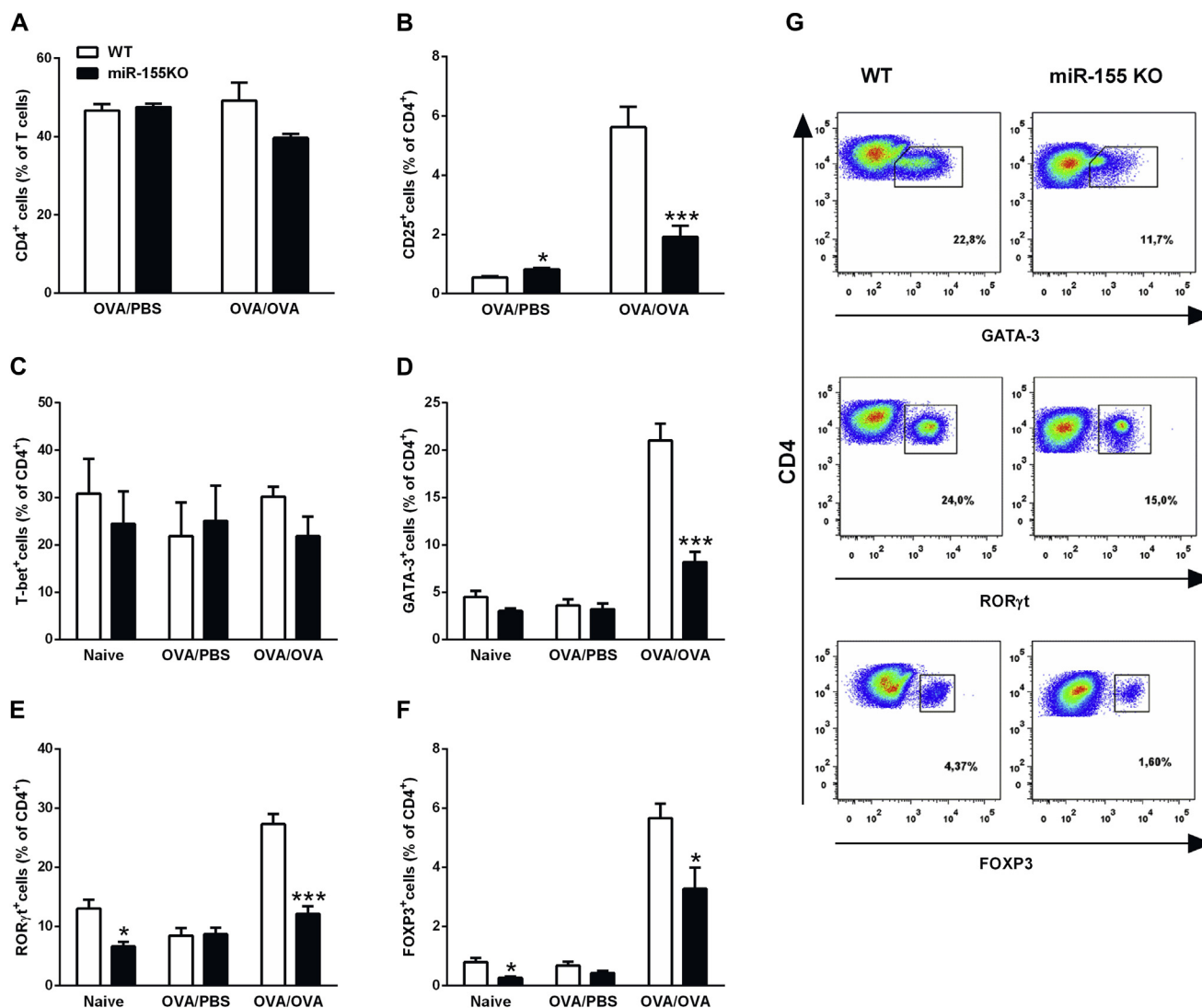


FIG 5. miR-155 deficiency reduces CD4⁺ T-cell activation and transcription factor expression in the lung *in vivo*. Fresh lung tissue was collected from naive, OVA/PBS, and OVA/OVA WT and miR-155KO mice. **A-F**, Relative number of CD4⁺ T cells (Fig 5, A) and CD4⁺CD25⁺ (Fig 5, B), T-bet⁺ (Fig 5, C), GATA-3⁺ (Fig 5, D), RORγt⁺ (Fig 5, E), and FOXP3⁺ (Fig 5, F) cells. Means ± SEMs are shown (n = 4-6 per group). **G**, Representative flow cytometry plots showing decreased transcription factor expression in OVA/OVA miR-155KO mice compared with that seen in OVA/OVA WT mice. **P* < .05 and ****P* < .001, OVA/OVA WT versus miR-155 KO mice.

Furthermore, *in vitro* studies showed that PBLN cells from miR-155 KO mice had reduced capacity to release T_H2 cytokines and that adoptive transfer of WT OVA-specific CD4⁺ T cells into naive WT and naive miR-155 KO mice restored allergen-induced airway eosinophilia in the 2 mouse strains to a similar degree.

Allergen-induced eosinophil recruitment into the airways is mainly regulated by 2 eosinophil-specific chemoattractants, namely eotaxin-1/CCL11 and eotaxin-2/CCL24.³⁰ The fact that levels of these 2 chemokines were significantly reduced in the airways of allergen-challenged miR-155 KO mice compared with WT mice and intranasal instillation of eotaxin-2/CCL24 before allergen exposure partly restored the deficient airway eosinophilia in miR-155 KO mice demonstrates the involvement of miR-155 in eosinophilic recruitment. Earlier studies have shown that eotaxin-1/CCL11 release is regulated in an IL-4- and IL-13-dependent manner and that eotaxin-1/CCL11 acts synergistically

with IL-5 to promote tissue eosinophilia.^{30,31} Likewise, cooperation between eotaxin-2, IL-5, and IL-13 for the development of allergic airway inflammation has been demonstrated.³²⁻³⁴ Furthermore, CD4 KO mice have diminished eotaxin-2/CCL24 levels in BALF compared with those seen in allergen-challenged WT mice,³⁵ suggesting that the establishment of an allergen-induced eotaxin-2/CCL24 gradient is partially dependent on CD4⁺ T_H2 cells in the airways. A significant reduction in the type 2 chemokine TARC/CCL17 in allergen-exposed miR-155 KO mice compared with WT mice also suggests an impaired activation of M2 macrophages in the airways. M2 macrophages have recently been demonstrated to release both eotaxin-2/CCL24 and TARC/CCL17 in response to T_H2 cytokines, such as IL-4 and IL-13.³⁶ Further supporting this is the observation that levels of the M1 macrophage chemokine MIG/CXCL9 were not altered in our model.

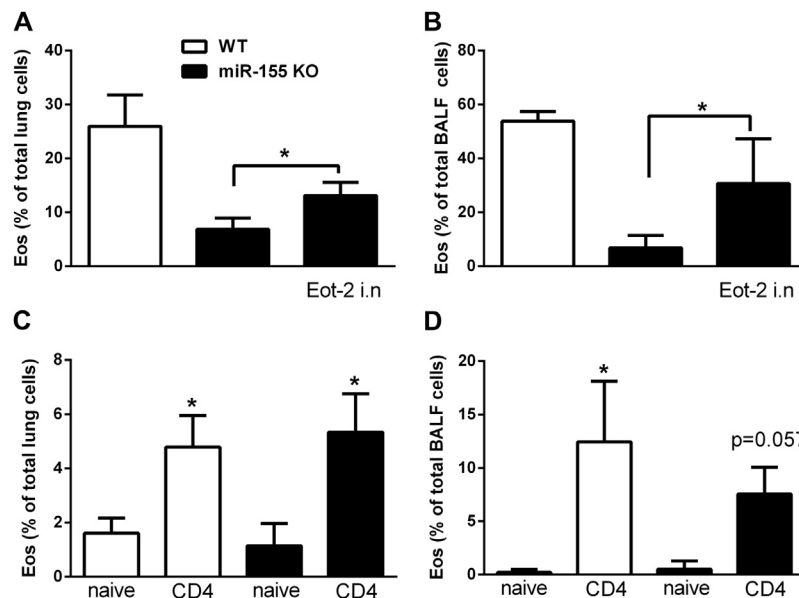


FIG 6. Eotaxin-2/CCL24 treatment and adoptive transfer of OVA-specific CD4⁺ T cells, respectively, demonstrate increased airway eosinophilia in miR-155 KO mice. **A** and **B**, Relative number of eosinophils in lung tissue (Fig 6, **A**) and BALF (Fig 6, **B**) of sensitized WT and miR-155 KO mice with or without eotaxin-2/CCL24 instillation before allergen challenge. **C** and **D**, Relative number of eosinophils in lung tissue (Fig 6, **C**) and BALF (Fig 6, **D**) of naive WT and miR-155 KO mice adoptively transferred with OVA-specific WT-derived CD4⁺ T cells before allergen challenge. Means $\pm$ SEMs are shown (n = 3–8 per group). **P* < .05.

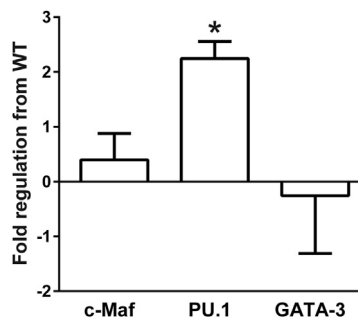


FIG 7. miR-155 deficiency increases expression of the transcription factor PU.1. Fold regulation in expression of c-Maf, PU.1, and GATA-3 in airway lymph nodes (PBLNs) of OVA/OVA miR-155 mice compared with OVA/OVA WT mice. Means $\pm$ SEMs are shown (n = 6 per group). **P* < .05.

Allergen-induced inflammation is associated with remodeling responses in the airways of asthmatic patients, and the T_H2 cytokines IL-4 and IL-13 play an important role in this process.^{37,38} These cytokines regulate production of periostin, an extracellular matrix protein, which have been shown to be increased in airway epithelial cells and to contribute to remodeling in the airways through basement membrane thickening, subepithelial fibrosis, and eosinophilic inflammation in asthma.^{39–41} It is tempting to speculate that a part of the reduced eosinophilic inflammation observed in the airways of allergen-exposed miR-155 KO mice might be due to a reduced production of periostin in these mice.

Increased expression of miR-155 in activated T cells has previously been demonstrated both in human and animal models of disease.^{14,16,21,42,43} Here we show that miR-155 deficiency resulted in impaired numbers of activated CD4⁺ T cells (ie, CD25⁺CD4⁺ cells), which was reflected by a reduced

airway T_H2 cytokine (ie, IL-4, IL-5, and IL-13) production to levels comparable with those seen in unstimulated cells. There was also a trend toward a reduction in levels of the T_H17-derived cytokine IL-17 without any effect on release of the T_H1 cytokine IFN- γ . Similarly, miR-155 KO mice had an impaired number of T_H2 and T_H17 cells in the airways compared with numbers seen in allergen-challenged WT mice, without any significant difference in airway T_H1 cell numbers. Interestingly, previous studies have demonstrated that miR-155 KO T cells are prone to T_H2 differentiation because miR-155-deficient T cells were shown to produce more IL-4, IL-5, and IL-13 and less IFN- γ .¹⁴ This phenomenon was speculated to occur due to increased expression of the IL-4-specific transcription factor c-Maf in miR-155 KO-polarized T_H2 cells. However, in our study we were not able to detect an increase in c-Maf expression in PBLN tissue from allergen-exposed miR-155 KO mice. A possible explanation for the discrepancy between this study and ours could be the source of cells used. In the study by Rodriguez et al,¹⁴ naive CD4⁺ cells were polarized *in vitro*, whereas ours were from allergen-challenged *in vivo* mice that were restimulated *ex vivo*. This suggests differential regulation of T cells by miR-155 in acquired immunity *in vivo* compared with *in vitro* stimulating conditions, supporting miRNAs as tightly regulated fine tuners of protein expression that are highly context dependent.⁴⁴ Notably, adoptive transfer of OVA-specific CD4⁺ T cells from WT mice restored airway eosinophilia in miR-155 KO mice to levels comparable with those observed in WT mice. These data suggest that the diminished allergic airway inflammation in miR-155 KO mice is due to a defect in T_H2 cell responses. Furthermore, we found an increased expression of the transcription factor PU.1, a direct target of miR-155, in PBLN tissue after allergen challenge in miR-155 KO mice compared with that seen in WT mice. PU.1

overexpression has been demonstrated to decrease T_H2 cytokine production in T_H2 cell cultures. Thus PU.1 might be an important transcription factor that, at least in part, can explain the reduced production of T_H2 cytokines in the airways of allergen-challenged miR-155 KO mice. It is interesting to note that inhibition of miR-126 was shown to modulate effector functions of T_H2 cells and the development of eosinophilic airway inflammation in a model of allergic airway inflammation, probably indirectly through PU.1 expression.¹¹ PU.1 functions partially by binding directly to GATA-3 and thus interfering with GATA-3 DNA binding.²⁷ Although we could not detect decreased expression of GATA-3 as a result of an increased PU.1 expression in miR-155 KO, we suggest that PU.1 could negatively regulate GATA-3 without affecting GATA-3 expression through decreased GATA-3 DNA binding activity. This decreased DNA binding activity would alter the T_H2 cytokine loci and thus regulate the T_H2 phenotype as observed in the allergen-challenged miR-155 KO mice.

In addition to reduced numbers of T_H2 effector cells, we also found a significant reduction in T_H17 and Treg cells in the airways of allergen-challenged miR-155 KO mice. This is in agreement with previous studies demonstrating that miR-155 modulates T_H17 and Treg cell differentiation *in vitro*.^{42,43} Moreover, miR-155 promotes autoimmune inflammation as miR-155 KO mice are resistant to experimental autoimmune encephalomyelitis through reducing T_H17 cell numbers.⁴³ With regard to Treg cells, we found a reduction in numbers of these cells in miR-155 KO mice. This is in agreement with recent studies that have also demonstrated reduced numbers of Treg cells under steady-state conditions and during inflammation.¹⁵ Thus in our present study the reduced number of Treg cells was reflected in a reduced production of IL-10 in the airways of miR-155 KO compared with WT mice (see Table E2). By contrast, no significant change in T_H1 cell numbers was demonstrated in the airways, indicating a limited involvement of T_H1 cells in local regulation of allergic airway inflammation. We have previously demonstrated similar findings in the nasal mucosa of patients with allergic rhinitis, in whom T_H1 cell numbers did not significantly change after pollen exposure.⁴⁵

In conclusion, although a considerable body of evidence suggests a role for miRNAs in inflammatory diseases, there are currently limited functional data on the role of miRNAs in the regulation of allergen-induced airway inflammation *in vivo*. Here, for the first time, we demonstrate that miR-155 is involved in the local regulation of T_H2 responses in allergen-induced eosinophilic airway inflammation. This study highlights the role of miR-155 as a potential target in patients with allergic inflammatory diseases.

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Key messages

- miR-155 plays an essential role in regulating allergen-induced airway inflammation.
- miR-155 regulates T_H2 responses locally in the airways in response to allergen challenge.

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METHODS

Induction of allergic airway inflammation

Mice were sensitized twice, on days 1 and 6, by means of intraperitoneal injection of 0.5 mL of 8 µg of chicken OVA (Sigma-Aldrich, St Louis, Mo) bound to 4 mg of aluminum hydroxide (Sigma-Aldrich) in PBS. Eight days after the second sensitization, mice were then divided into 2 groups: a control group and an OVA challenge group. Both groups were briefly anesthetized with isoflurane (Baxter, Deerfield, Ill). The OVA exposure group (OVA/OVA) received an intranasal administration of 100 µg of OVA in 25 µL of PBS on 5 consecutive days, whereas the control group (OVA/PBS) received only PBS (Fig E1, A). In some experiments miR-155 KO mice received intranasal instillation of eotaxin-2 (5 µg in 25 µL of PBS/0.1% BSA) 1 hour before OVA exposure on 5 consecutive days (Fig E1, B).

Sample collection

Blood, bronchoalveolar lavage fluid (BALF), lung tissue, spleen tissue, lymph nodes, and bone marrow were collected 24 hours after the final OVA exposure. The animals were deeply anesthetized with a mixture of xylazine (130 mg/kg; Rompun, Bayer, Leverkusen, Germany) and ketamine (670 mg/kg; Ketalar, Apoteket AB, Motala, Sweden). First, animals were killed by puncturing the right heart ventricle, and blood was collected. Second, the mice were tracheotomized, and bronchoalveolar lavage was performed by instilling 0.25 mL of PBS through the tracheal cannula, followed by gentle aspiration and a second lavage with 0.20 mL of PBS. The BALF was later separated into cell-free BALF and BALF cells. An additional 1 mL of PBS was used to wash away airway lumen inflammatory cells before the lungs were perfused and removed to harvest parenchymal inflammatory lung cells. The left lobe was filled with 1 mL of OCT compound (Tissue-Tek; Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and PBS solution containing 20% sucrose (Sigma-Aldrich) and immediately frozen in liquid nitrogen. The apical lobe was stored at -80°C , and another 3 lobes, without any connective tissue, were stored on ice in Hanks balanced salt solution (Sigma-Aldrich) before use. Third, spleen tissue and PBLNs were removed and further processed for *in vitro* assays. Finally, bone marrow was harvested by excising one femur, which was cut at the epiphysis and flushed with 2 mL of PBS, and the cells were collected in tubes through a 70-µm cell strainer (BD Falcon).

Preparation of lung single-cell suspensions

The right lung lobes were weighed and rinsed in a Petri dish before being transferred to a gentleMACS C Tube (Miltenyi Biotec) containing 5.0 mL of Hanks balanced salt solution supplemented with 10% FCS (Sigma-Aldrich), 100 µL of Collagenase D solution (final concentration, 2 mg/mL), and 20 µL of DNase I solution (final concentration, 80 U/mL). The mouse lung was dissociated with a gentle MACS Dissociator (Miltenyi Biotec), according to the manufacturer's instructions. Single lung cells were washed in PBS supplemented with 10% FCS and used for flow cytometric staining and analysis and differential cell count. The total number of cells was determined by using standard hematologic procedures.

Blood and bone marrow

One part blood was added immediately to 4 parts 2 mmol/L EDTA (Sigma-Aldrich) in PBS. Red blood cells were lysed in 5 mL of lysing solution (0.1% potassium bicarbonate and 0.83% ammonium chloride in distilled water) for 15 minutes at room temperature. White blood cells were washed and resuspended in 1% FCS in PBS. Cell counts were performed with a standard hemocytometer, and cytospin preparations for differential cell counts were prepared. Bone marrow samples were washed, and the number of cells was counted, as performed for blood.

Lung histology

The left lobe of the lung was embedded in Tissue-Tek O.C.T. Compound (Miles, Elkhart, Ind), snap-frozen, and stored at -80°C until being sliced into 5-µm sections with a cryostat Leica CM1900 UV-cryostat (Leica Microsystems Nussloch GmbH, Solms, Germany). Hematoxylin and eosin quick staining was performed by means of fixation in a solution consisting of equal

parts 37% formaldehyde and 95% ethanol for 30 seconds, and slides were rinsed in dH₂O and placed in Mayer hematoxylin (Sigma) for 1 minute. After a second rinse in dH₂O, slides were dipped in a saturated lithium carbonate solution (Merck, Darmstadt, Germany), rinsed in distilled water, and then placed in an eosin solution consisting of 100 µg of eosin (Merck Chemicals, Darmstadt, Germany), 50 mL of 95% ethanol, and 2 drops of acetic acid for 45 seconds. Excess eosin staining was removed by washing in absolute alcohol, and slides were mounted in Mountex. PAS staining was performed with a PAS kit (Sigma Aldrich), according to the manufacturer's protocol, after fixation in equal parts 4% formaldehyde solution (HistoLab Products AB, Göteborg, Sweden) and 95% ethanol for 10 minutes. The PAS-stained samples were assessed by using an Axioplan 2 microscope (Carl Zeiss Jena GmbH, Eching, Germany) at a magnification of $\times 400$ or $\times 1000$. Cells were identified based on morphologic criteria and quantified by counting 10 high-power fields in each slide.

Preparation of lung homogenates

The left lobe was used for measurement of cytokines and chemokines in lung homogenates. Briefly, each lobe was weighted and then put in a gentleMACS C tube (Miltenyi Biotec GmbH) containing 1 mL of PBS supplemented with 1 dissolved tablet of protease inhibitor (cOmplete Mini; Roche, Mannheim, Germany). Lobes were then sliced with the gentleMACS Dissociator. Tissue were further disrupted by mixing with a pipette and centrifuged at 3000 rpm for 10 minutes at room temperature. Supernatants were collected and kept at -80°C until cytokine or chemokine measurement.

Chemokine and cytokine analysis

Eotaxin-1/CCL11, eotaxin-2/CCL24, and periostin levels in cell-free BALF and eotaxin-1/CCL11, eotaxin-2/CCL24, MCP-1/CCL2, and TARC/CCL17 levels in lung were quantified by using ELISA (Mouse CCL11/Eotaxin DuoSet, Mouse CCL24/Eotaxin-2/MIPF-2, Mouse MCP-1/CCL2, Mouse TARC/CCL17 DuoSet, and Mouse Periostin/OSF-2 DuoSet; R&D Systems). The BD CBA Mouse T_H1/T_H2/T_H17 Cytokine kit (BD Biosciences) was used to measure IL-2, IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 *in vitro* cell-free supernatants. The Mouse Flex set was used to measure IL-5 and IL-13 in *in vitro* cell-free supernatants and BALF. The Mouse Flex set was used to measure MIG/CXCL9, MIP-1 α , and RANTES/CCL5 levels in lung homogenate supernatants (BD Biosciences). CBA data were acquired on a FACSAria flow cytometer running FACSDiva 6 software (BD Biosciences) and analyzed by using FCAP Array Software (BD Biosciences). All analysis was carried out according to the manufacturer's protocol.

Flow cytometry

The following antibodies were used to detect surface antigens: CD3-fluorescein (clone 145-2C11), CD4-phycoerythrin (clone H129.19), CD4-Alexa Fluor 700 (clone RM4-5), and CD25-allophycocyanin (clone PC61), all from BD PharMingen. In some samples the nucleic acid dye 7-amino-actinomycin D (BD PharMingen) was used to select viable cells. After washing in PBS supplementing with 1% FCS, cells were run immediately or subjected to further intracellular staining after fixation with Fix/Perm solution, according to the manufacturer's protocol for the Foxp3 staining buffer set (eBioscience, San Diego, Calif). Antibodies for intracellular antigens were as follows: T-bet-PerCP-Cy5.5 (clone eBio4B10), GATA-3-phycoerythrin (clone TWAJ), ROR γ t-allophycocyanin (clone AFKJS-9), FOXP3-Alexa Fluor 488, and FOXP3-phycoerythrin (clone FJK-16s), all from eBioscience.

Isolation of miRNA and mRNA

After termination of the experiment, lung, spleen, and PBLN tissue was immediately placed in RNeasy lysis buffer (Qiagen, Crawley, UK) and stored at -80°C . Tissues were then homogenized and lysed with QIAzol Lysis Reagent (Qiagen) in gentleMACS M Tubes by using a gentleMACS Dissociator (Miltenyi Biotec GmbH). Total RNA, including miRNA, was isolated with the miRNeasy Mini kit (Qiagen), and contaminating DNA was removed by means of DNase treatment of the samples with the Turbo DNA-free kit (Ambion, Life Technologies), according to the manufacturer's protocol. RNA yield and integrity were evaluated by using the

Agilent RNA 6000 Nano kit on an Agilent 2100 Bioanalyzer running 2100 Expert Software (Agilent Technologies, Waldbronn, Germany).

miR-155 expression

Reverse transcription of miRNA was performed in replicates by using 40 ng of total RNA per cDNA reaction with the Universal cDNA Synthesis kit (Exiqon A/S), according to the manufacturer's protocol. Quantitative real-time PCR was performed on a CFX-96 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) by using diluted reverse transcription product, LNA Primer sets for miR-155 and U6 snRNA, and the SYBR Green Master mix kit, as described by the manufacturer (Exiqon). miR-155 expression was normalized to U6 snRNA, and fold regulation in allergen-exposed WT mice was calculated from PBS-exposed WT mice with CFX Manager 3.0 software (Bio-Rad Laboratories).

mRNA expression

First-strand cDNA synthesis was performed by using 1.5 µg of total RNA per cDNA reaction and the RT2 First strand kit (Qiagen), according to the manufacturer's protocol. Quantitative real-time PCR was performed on a CFX-96 Real Time PCR Detection System (Bio-Rad Laboratories) by adding the cDNA and RT2 SYBR Green qPCR Master mix to a Mouse Custom Array CAPM11696, as described by the manufacturer (SABiosciences, Qiagen). The array was preset with primers for the target mRNAs of c-Maf, CTLA4, GATA-3, PU.1 (Sfp1), SHIP1 (Inpp5d), SMAD1, SOCS1, STAT3, STAT6, TGFB1, and TNFSF9 and the reference genes β_2 -microglobulin, GAPDH, and β -actin. Each target mRNA was normalized to all reference genes, and fold regulation in miR-155 KO allergen-exposed mice was calculated from WT allergen-exposed mice with CFX Manager 3.0 software (Bio-Rad Laboratories).

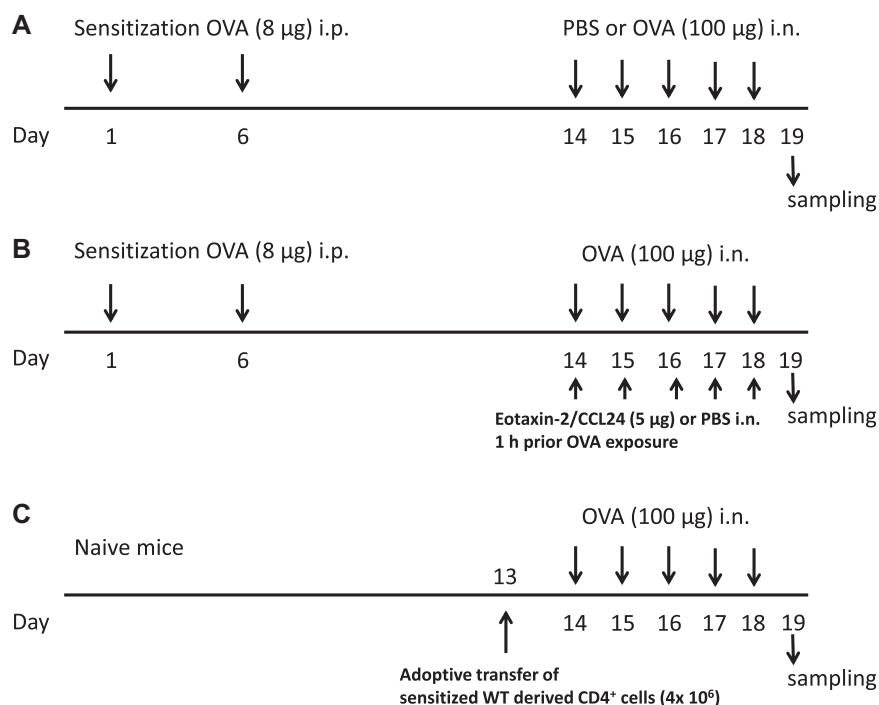


FIG E1. Mouse experimental protocols. **A**, Standard protocol for the model of allergen-induced airway inflammation. WT and miR-155 KO mice were allergen sensitized and exposed (OVA/OVA) or allergen sensitized and PBS exposed (OVA/PBS) or kept naive. **B**, Intranasal instillation of eotaxin-2/CCL24 protocol. All mice were allergen sensitized and exposed (OVA/OVA). miR-155 KO mice received eotaxin-2/CCL24 by means of intranasal instillation 1 hour before OVA exposure. Control mice received vehicle (PBS) 1 hour before OVA exposure. **C**, Adoptive transfer protocol. Naive WT and miR-155 KO mice were adoptively transferred with WT mouse-derived OVA-sensitized CD4⁺ T cells. Twenty-four hours after cell transfer, mice were exposed to OVA once daily for a total of 5 days. Naive WT and miR-155 KO mice served as controls. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

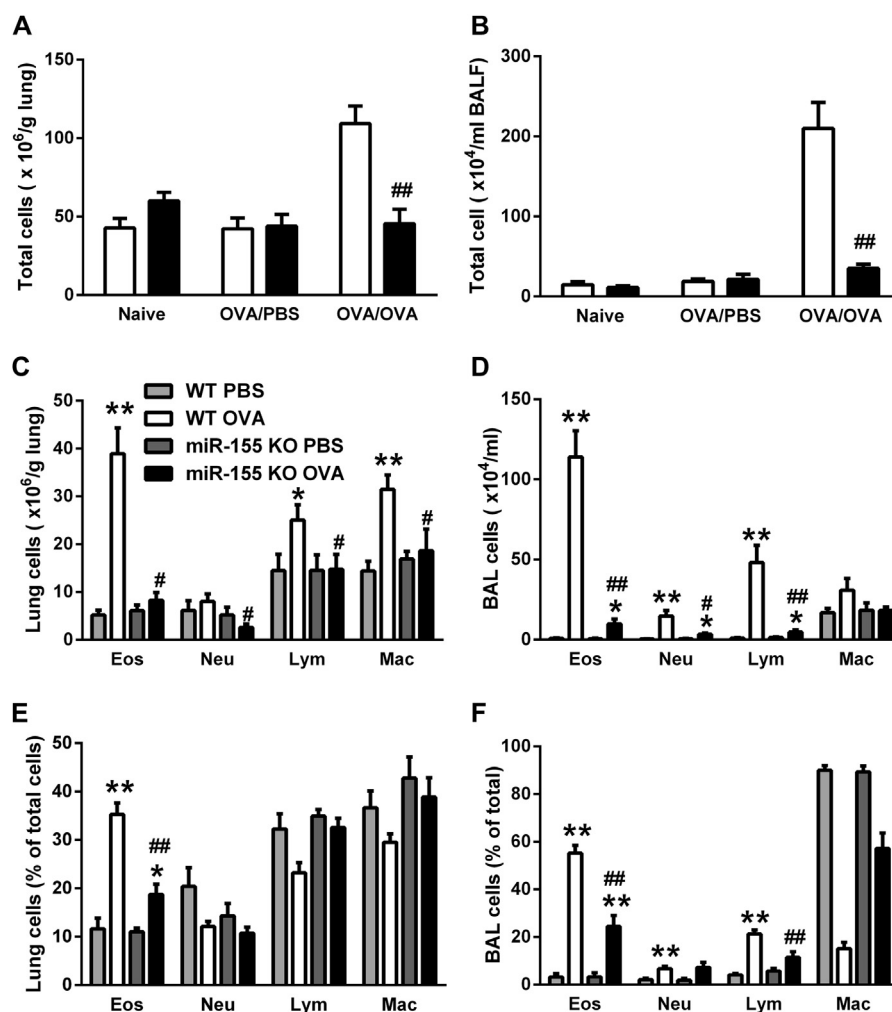


FIG E2. miR-155 deficiency reduces the total number of BALF and lung tissue cells in a model of OVA-induced allergic airway inflammation. **A** and **B**, Total number of cells in lung tissue (Fig E2, A) and BALF (Fig E2, B) in naive, allergen-sensitized, and saline-exposed (OVA/PBS) and allergen-sensitized and allergen-exposed (OVA/OVA) WT mice and miR-155 KO mice. **C-F**, Differential cell counts of lung cells (Fig E2, C and E) and BALF (Fig E2, D and F) in allergen-sensitized and saline-exposed (OVA/PBS) and allergen-sensitized and allergen-exposed (OVA/OVA) WT mice and miR-155 KO mice. Results are means $\pm$ SEMs ($n = 4-10$ mice per group). $*P < .05$ and $**P < .01$, OVA/PBS WT versus OVA/OVA WT and OVA/PBS miR-155 KO mice versus OVA/OVA miR-155 KO mice, respectively. $\#P < .05$ and $##P < .01$, OVA/OVA WT versus OVA/OVA miR-155 KO mice.

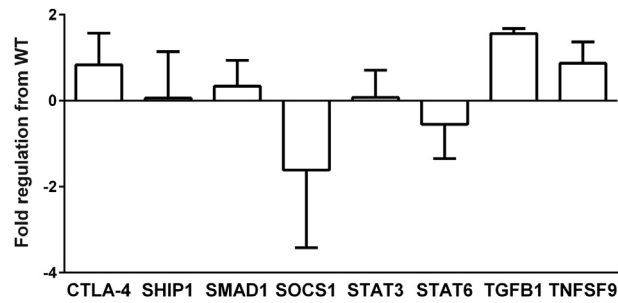


FIG E3. Fold regulation in expression of CTLA4, SHIP1, SMAD1, SOCS1, STAT3, STAT6, TGFB1, and TNFSF9 mRNA in PBLNs of OVA/OVA miR-155 mice compared with OVA/OVA WT mice. Means $\pm$ SEMs are shown (n = 6 per group).

TABLE E1. Chemokine levels in lungs

Parameter	Units	WT		miR-155 KO	
		OVA/PBS (n = 3)	OVA/OVA (n = 4)	OVA/PBS (n = 3)	OVA/OVA (n = 4)
RANTES/CCL5	pg/g lung, mean (SEM)	741.3 (108.9)	3083.8 (750.1)	1160.2 (276.4)	3470.9 (938.0)
MCP-1/CCL2	pg/g lung, mean (SEM)	2341.2 (571.9)	4396.3 (971.7)	1563.3 (904.9)	4795.2 (2372.3)
MIP-1 α /CCL3	pg/g lung, mean (SEM)	425.5 (70.4)	1267.6 (170.6)	377.6 (118.0)	539.6 (155.5)*

* $P < .05$, WT versus miR-155 KO mice.

TABLE E2. Cytokine levels in *in vitro* cell supernatants

Parameter	Units	WT		miR-155 KO	
		Medium (n = 6)	OVA (n = 6)	Medium (n = 6)	OVA (n = 6)
PBLNs					
IL-2	pg/mL, mean (SEM)	4.8 (1.7)	4.5 (0.7)	59.4 (35.0)	6.5 (2.4)
IL-6	pg/mL, mean (SEM)	1,523.2 (1,064.1)	583.2 (169.83)	1,508.6 (614.7)	2,752.1 (893.8)*
IL-10	pg/mL, mean (SEM)	474.3 (352.8)	3,266.5 (805.8)	149.4 (73.1)	451.2 (162.6)†
IFN-γ	pg/mL, mean (SEM)	194.4 (98.2)	348.3 (172.4)	109.4 (37.6)	120.3 (53.3)
TNF	pg/mL, mean (SEM)	113.5 (17.1)	283.8 (17.0)	115.8 (23.6)	140.6 (23.7)‡
Spleen					
IL-2	pg/mL, mean (SEM)	114.0 (94.8); n = 3	3.6 (0.7); n = 5	34.3 (29.3); n = 3	3.6 (0.2)
IL-4	pg/mL, mean (SEM)	6.6 (0.4); n = 3	5.9 (1.2); n = 5	8.3 (3.6); n = 3	23.2 (7.8)
IL-5	pg/mL, mean (SEM)	246.6 (131.0); n = 3	1,256.2 (179.1); n = 4	13.2 (7.1); n = 3	1,059.0 (316.0); n = 4
IL-6	pg/mL, mean (SEM)	398.8 (292.7); n = 3	287.8 (85.7); n = 5	313.5 (292.6); n = 3	1,271.6 (445.6)
IL-10	pg/mL, mean (SEM)	641.7 (566.2); n = 3	3,070.2 (524.8); n = 5	574.7 (550.4); n = 3	1,597.1 (422.4)
IL-13	pg/mL, mean (SEM)	711.4 (152.0); n = 2	11,107.5 (1,368.3); n = 3	444.2 (217.4); n = 3	11,034.6 (3,337.3); n = 3
IL-17A	pg/mL, mean (SEM)	51.1 (22.8); n = 3	119.0 (33.4); n = 5	27.1 (25.2); n = 3	111.6 (56.1)
IFN-γ	pg/mL, mean (SEM)	583.4 (515.3); n = 3	16.2 (6.0); n = 5	1,120.6 (1,028.8); n = 3	219.7 (74.7)
TNF	pg/mL, mean (SEM)	172.7 (47.8); n = 4	211.1 (41.0); n = 5	228.7 (177.7); n = 3	294.9 (42.9)

* $P < .05$, † $P < .01$, and ‡ $P < .001$, WT OVA/OVA versus miR-155 KO OVA/OVA mice.