ORIGINAL RESEARCH

Suppression of Allergic Asthma by Loss of Function of Miz1-mediated Th1 Skewing

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Abstract

Asthma is the most prevalent chronic respiratory disease worldwide. There is currently no cure, and it remains an important cause of morbidity and mortality. Here we report that lung-specific loss of function of the transcription factor Miz1 (c-Myc-interacting zinc finger protein-1) upregulates the pro–T-helper cell type 1 cytokine IL-12. Upregulation of IL-12 in turn stimulates a Th1 response, thereby counteracting T-helper cell type 2 response and preventing the allergic response in mouse models of house dust mite- and OVA (ovalbumin)-induced asthma. Using transgenic mice expressing Cre under a cell-specific promoter, we demonstrate that Miz1 acts in lung epithelial cells and dendritic cells in asthma. Chromatin immunoprecipitation coupled with high-throughput DNA sequencing or quantitative PCR reveals the binding of Miz1 on the *Il12* promoter indicating direct repression of IL-12 by Miz1. In addition, HDAC1 (histone deacetylase 1) is recruited to the *Il12* promoter in a Miz1-depdendent manner, suggesting epigenetic repression of *Il12* by Miz1. Furthermore, Miz1 is upregulated in the lungs of asthmatic mice. Our data together suggest that Miz1 is upregulated during asthma, which in turn promotes asthma pathogenesis by preventing Th1 skewing through the transcriptional repression of IL-12.

Keywords: asthma; gene regulation; Th1/Th2 immunity; Miz1; IL-12

Asthma is one of the most prevalent chronic diseases worldwide and affects around 10% of adults and an even greater proportion of children (1, 2). Although recent advances in diagnosis and management have improved the quality of life for many patients with asthma, there is no cure, and asthma remains an important cause of morbidity and mortality.

Asthma is characterized by reversible airflow obstruction, bronchial hyperresponsiveness, and airway inflammation that is predominantly characterized as type 2 immune response often accompanied by airway and peripheral eosinophilia. Both lung epithelial cells and dendritic cells (DCs) express pattern recognition receptors (PRRs) and can be activated directly by allergens. It is generally accepted that the allergic immune response is initiated by interactions of allergens, including house dust mites (HDMs), pollen, and fungal spores, with barrier epithelial cells (Ecs) through PRRs. This results in the release of the epithelial cytokines, including TSLP (thymic stromal lymphopoietin), IL-33, IL-25, and GM-CSF (granulocyte–macrophage colony–stimulating factor). TSLP mediates migration of DCs to local draining lymph nodes and their maturation via upregulation of OX40L. These mature DCs activate I CD4 T cells in lymph nodes to an IL-4–competent state. In turn, IL-4–competent T-helper (Th) cells in lymph nodes migrate to B-cell zones and differentiate into T-follicular-helper (TFH) cells or egress from the lymph nodes and enter the airway, where they mature into T-helper type 2 (Th2) cells. TFH cells in B-cell zones mediate IgE class switching in B cells, and IgE binds to mast cells and basophils, enhancing their survival, activation, and/or degranulation. Th2 cells produce large quantities of the type 2 cytokines, including IL-4, IL-5, and IL-13,

which drive a cascade of downstream events, including IgE-triggered hypersensitivity to aeroallergens, activation of airway epithelial cells, alternative macrophage activation, chemo attraction, and/or survival of effector cells (mast cells, eosinophils, and basophils), and remodeling of the epithelium and subepithelial matrix (epithelial mucus metaplasia, smooth muscle hypertrophy, increased subepithelial fibrosis, enhanced deposition of subepithelial matrix glycoproteins, etc.). Type 2 cytokines are also produced by non-Th2 cells; for example, IL-33 stimulates IL-4 release from basophils, and IL-33 and IL-25 promote IL-13 and IL-5 production from $IL25R^+$ group 2 innate lymphoid cell (ILC2) (3–7).

Human asthma is associated with airway infiltration of Th2 cells. It is generally recognized that a dominant Th2 cell activation and an inadequate Th1 cell response are responsible for the development of allergic asthma. Th1 cells are induced by IL-12 and express the transcription factor T-bet and the hallmark cytokine of Type 1 immunity, IFN-γ, which repress IL-4 and suppress eosinophil lung infiltration and airway hyperresponsiveness. Reduced expression of T-bet is observed in T cells from airways of patients with asthma compared with that from patients without asthma (8). T-bet deficiency spontaneously skews toward a Th2 phenotype and induces airway changes consistent with human asthma in mice (8, 9). Administration of IFN-γ inhibited allergic inflammation (10–12), and administration of IL-12 abolished the allergic response in an IFN-γ–dependent manner in mouse models (13), whereas blocking IL-12 during the sensitization process worsened allergic airway inflammation (14). Decreased $IFN-γ^+CD4^+$ cells and reduced IL-12 and IFN-γ concentrations have been detected in patients with allergic asthma (15, 16). All these together suggest that study of the pathway of Th1 skewing is important in understanding Th2 development in asthma and might therefore identify targets for therapy.

Miz1, also known as Zbtb17 (c-Myc-interacting zinc finger protein-1), is a transcription factor that mediates both transcriptional activation and repression. It has an amino-terminal POZ (poxvirus and zinc-finger) domain that is required for its transcriptional activity, and 13 zinc fingers at its carboxyl terminus (17, 18). Miz1 plays a critical role in regulating proliferation, differentiation, cell-cycle progression, and apoptosis through the transcriptional regulation of its target genes (17, 19–21). We previously reported that loss of function of Miz1 results in augmented and prolonged lung inflammation induced by LPS (major component of the outer membrane of the gram-negative bacteria) or enhanced innate immune response to the gram-negative bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*) (22). Furthermore, we recently reported that lung epithelial cell–specific loss of function of Miz1 causes spontaneous, age-related progressive chronic obstructive pulmonary disease (COPD)-like phenotype in mice (23). Whether Miz1 plays a role in another inflammatory lung disease, asthma, is not known. Here we report that loss of function of Miz1 skews toward a Th1 response by augmenting IL-12 expression and thereby suppresses allergic asthma, in sharp contrast to its proinflammatory role in response to LPS and *Pseudomonas* pneumonia as well as in COPD (22, 23).

Methods

Mice
Miz1(POZ)$^{fl/fl}$ mice (C57BL/6 background) have been described as we previously reported (22). *SPC-Cre* mice were kindly provided by Brigid L. Hogan (24). *CD11c-Cre* and *LysM-Cre* mice were from Jackson Laboratories. The animal care and animal experiments were performed in compliance with the institutional and U.S. National Institutes of Health regulations and were approved by the Northwestern University Animal Care and Use Committee and the University of Illinois at Chicago Animal Care Committee.

Mouse Model of HDM-induced Allergic Airway Inflammation
HDM extract (Greer Laboratories) was dissolved in saline and delivered by intranasal instillation, with the mice anesthetized with 5% isoflurane. Mice were treated with saline or HDM (50 μg in 50 μl saline) every other weekday (Monday, Wednesday, and Friday) for three 3. Twenty-four hours after the last HDM or saline exposure, mice were harvested.

Mouse Model of OVA-induced Allergic Airway Inflammation
Mice were sensitized with intraperitoneal OVA (ovalbumin) (10 μg; Sigma-Aldrich) plus alum (2 mg/ml) as adjuvant in 0.1 ml saline on Days 0 and 14, followed by challenges by exposure for 30 minutes to nebulized 1% OVA or saline alone delivered via an ultrasonic nebulizer on Days 21, 22, and 23. Twenty-four hours after the last exposure, mice were harvested.

Intratracheal Adenovirus Instillation
Ad-null, Ad-Cre, Ad-Miz1(WT), and Ad-Miz1(V89M) were from ViraQuest. Mice were infected intratracheally with 1×10^9 plaque-forming units of adenovirus per mouse.

Multiplex Analysis of Cytokines and IgE ELISA
The concentration of cytokines, including IL-4, -5, and -13, TNF, and IFN-γ in the BAL fluid were quantified by multiplex (IL-4, EPX010–20613–901; IL-5, EPX010–20610–901; IL-13, EPX010–20615–901; TNF, EPX010–20607–901; IFNγ, EPX010–20606–901; eBioscience). Concentrations of IgE in sera were determined by ELISA (88–50460; eBioscience).

Antibodies for Western Blot
Miz-1 Antibody (H-190, 1:500): sc-22837 was used until it was discontinued by the vendor, and then Miz-1 Antibody (B-10, 1:250): sc-136985 was used in replacement of Miz-1 Antibody (H-190): sc-22837. β-actin antibody (A5441, 1:20,000; Sigma-Aldrich).

Histological Analysis, Trichrome and Periodic Acid–Schiff Staining
Tissue samples were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Tissue sections were prepared at 5 μm and mounted on slides for staining with hematoxylin and eosin, Masson's trichrome stains, or periodic acid–Schiff (25). The staining was performed by the Northwestern University Mouse Histology and Phenotyping Laboratory. The images were captured with Nikon ECLIPSE E800, or with the TissueGnostics Tissue/Cell High Throughput Imaging Analysis System and TissueFAXS software (TissueGnostics) at the Northwestern University Cell Imaging Facility.

Immunohistochemistry
Immunohistochemistry staining of eosinophils from mouse lungs was performed by the Northwestern University Mouse Histology and Phenotyping Laboratory. Briefly, mouse lungs were fixed in 4% paraformaldehyde for 24 hours and

embedded in paraffin. Deparaffinized slides were incubated with eosinophil major basic protein antibody (Santa Cruz sc-33938; 1:1500) overnight at 4°, followed by detection with the biotin-streptavidin peroxidase system (Vector Lab). Nucleus was visualized concomitantly with DAPI using Aqua Mount.

Quantitative PCR, Chromatin Immunoprecipitation Assay and Sequencing

Quantitative PCR (qPCR) was performed using iQ^{TM} SYBR Green Supermix (BIO-RAD) on a CFX Connect^{TM} Real-Time PCR Dection System (BIO-RAD). mRNA expression of a specific gene was normalized to HPRT (hypoxanthine-guanine phosphoribosyltransferse). Primer sequences were listed in Table E1. For qRT-PCR, total RNA was extracted from lung tissues or isolated cells using NucleoSpin RNA kit (Macherey-Nagel). cDNA was synthesized using M-MuLV Reverse Transcriptase (NEB) according to the manufacturer's instructions. Chromatin immunoprecipitation (ChIP) assays were performed as we previously reported (22). The following antibodies were used for immunoprecipitation: anti-Miz1 (H-190 X; Santa Cruz Biotechnology), anti-HDAC1 (05–100; Millipore), and RelA/p65 (sc-372X; Santa Cruz Biotechnology). Primer sequences for ChIP-qPCR were listed in Table E1. ChIP sequencing (ChIP-seq) was performed and analyzed as we previously reported (26). Briefly, DNA samples were quantitated with Qubit, and libraries were prepared using Illumina TruSEQ. Libraries were then sequenced on an Illumina NovaSEQ6000 (100 bp, paired-end) at University of Chicago Genomics Facility. Raw sequencing data were demultiplexed using Illumina bcl2fastq, and sequencing raw reads were aligned to the mouse genome mm10 using BWA MEM. PCR duplicates were removed using Picard MarkDuplicates so that downstream results were not influenced by PCR duplication artifacts. ChIP peaks were called relative to inputs using Macs2. Normalized bedgraph tracks were produced with the –SPMR flag and converted to bigWig tracks using UCSC tool bedGraphToBigWig.

Airway Hyperresponsiveness to Metacholine Administration

Mice were tracheotomized and mechanically ventilated on a computer-controlled piston ventilator (FlexiVent; SCIREQ) with a tidal volume of 10 ml/kg at 150 breaths per minute against 2–3 cm H_2O positive end-expiratory pressure. A standard volume history was obtained with two total lung capacity operations. Two baseline assessments of respiratory system input impedance were then obtained. This was followed by an inhalation of either aerosolized methacholine or control saline for 40 seconds by directing the inspiratory flow from the ventilator through the nebulizer chamber. Each dose of methacholine aerosol was administered in a dose–response manner (10, 25, 50, and 100 mg/ml). Respiratory system input impedance was then measured every 10 seconds for 3 minutes.

Fluorescence-activated Cell Sorting and Isolation of Bone Marrow–derived DCs

The lungs were digested with Dispase (Corning) and 0.1 mg/mL DNase I, followed by red blood cells removal with 1x BD Pharm Lyse solution (BD Biosciences). Cells were then washed in MACS buffer (Miltenyi Biotech) and the number of cells was counted with a Countess automated cell counter (Invitrogen). For isolation of AT2 cells and AMs, single cell suspensions were first incubated with 0.5 μg Fc Block (BD Biosciences) for 10 minutes at 4°C, then stained with FITC conjugated anti-mouse CD45 (eBioscience), APC conjugated anti-mouse EpCAM (eBioscience), PE conjugated anti-mouse CD31 (eBioscience), PE-CF594 conjugated anti-mouse SiglecF (BD Biosciences) and efluor450 conjugated anti-mouse CD11b (eBioscience). Cell sorting were performed with a BD FACSAria SORP 5-Laser instrument (BD Immunocytometry Systems) equipped with 355, 405, 488, 561 and 640 nm excitation lasers at the Northwestern University Flow Cytometry Core Facility. Data collection and sorting were implemented with BD FACS Diva software (BD Biosciences) and data analyses were performed with FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one (FMO) controls were included for gating analyses to allocate positive staining cells from negative staining populations. Compensation was performed using single color controls prepared from BD Comp Beads (BD Biosciences). Compensation matrix was calculated by FlowJo software (Tree Star). Biexponential transformation was calibrated manually if needed. For isolation of AT2 together with other myeloid cells, after Fc blocking, cells were incubated with Biotin conjugated anti-mouse CD45 antibody for 10 minutes at room temperature. Cells were then separated using MagniSort Streptavidin Positive selection beads (eBioscience) according to the manufacturer's instructions. From the CD45 negative fraction, AT2 cells were further purified by flow sorting using FITC conjugated anti-mouse CD45 (eBioscience), APC conjugated anti-mouse EpCAM (eBioscience) and PE conjugated anti-mouse CD31 (eBioscience). From the CD45 positive fraction, the myeloid cell populations were isolated by flow sorting using FITC conjugated anti-mouse CD45 (eBioscience), PerCPCy5.5 conjugated anti-mouse MHC II (BioLegend), eFluor450 conjugated anti-mouse Ly6C (eBioscience), APC conjugated anti-mouse CD24 (eBioscience), Alexa700 conjugated anti-mouse Ly6G (BD Biosciences), APCCy7 conjugated anti-mouse CD11b (BioLegend), PE conjugated anti-mouse CD64 (BioLegend), PE-CF594 conjugated anti-mouse SiglecF (BD Biosciences) and PECy7 conjugated anti-mouse CD11c (eBioscience)(27). For isolation of bone marrow–derived DCs (BMDCs), bone marrow (BM) cells were isolated and cultured with GM-CSF.

Statistical Analysis

Data were analyzed using an unpaired Student's t test, with the assumption of normal distribution with equal variance. Sample sizes were selected on the basis of preliminary results to ensure an adequate power.

Results

Lung-Specific Loss of Function of Miz1 Results in Reduced HDM-induced Airway Hyper Reactivity in Mice

To understand the role of Miz1 in a chronic inflammatory lung disease such as asthma, we used a mouse model of asthma induced by the clinically relevant allergen, HDM (28). The POZ domain of Miz1, which is essential for its transcriptional activity (17), was deleted in the lungs by intratracheal administration of an Ad/Cre (adenovirus encoding *Cre* recombinase) into *Miz1*(POZ)$^{fl/fl}$ mice, in which the coding exons of the POZ domain of Miz1 were flanked by loxP sites (22). An empty vector (Ad/null) was used as a control. We have reported lung-specific deletion of the Miz1 POZ domain with this approach (22).

Thirty days after virus administration, mice were treated intranasally with saline or HDM every other weekday for three weeks (Figure E1 in the data supplement). Although loss of function of Miz1 augmented LPS- or *P. aeruginosa*–induced lung inflammation (22), it reduced the production of the Th2 cytokines, including IL-4, IL-5, and IL-13, in the BAL fluid in the HDM-induced asthma model (compare Ad/Cre-treated mice [*Miz1ΔPOZ*] to Ad/null-treated mice [control]) (Figure 1A). Consistently, the mRNA concentrations of these Th2 cytokines were decreased in whole-lung homogenates of *Miz1ΔPOZ* mice compared with Ad-Null control mice (Figure 1B). Accordingly, loss of the Miz1 POZ domain attenuated airway hyperresponsiveness in the HDM-challenged mice (Figure 1C). Loss of function of Miz1 also inhibited the systemic allergic response, as shown by reduced serum total IgE concentrations in HDM-treated mice (Figure 1D).

Airway epithelial–derived cytokines, including IL-25, IL-33, TSLP, and GM-CSF, which are rapidly released in exposure to allergens, activate and potentiate innate and adaptive immunity in allergic responses (29). On the other hand, Th2 cytokines can act on epithelial cells as a feedback mechanism to induce the production of TSLP, GM-CSF, IL-25, and IL-33 to perpetuate dendritic cell–driven Th2 cell development (30–33). Consistently, the mRNA concentrations of epithelial-derived cytokines, including *Il33*, *Csf2* (encoding GM-CSF), *Il25*, and *Tslp*, were also decreased in HDM-treated *Miz1ΔPOZ* mice compared with HDM-treated control mice (Figure 1E).

Lung-Specific Loss of Function of Miz1 Also Results in Suppression of OVA-induced Allergic Asthma in Mice

To further validate our observations in HDM-induced asthma model, we used another mouse model of asthma induced by OVA (34, 35). Mice were sensitized with OVA plus aluminum hydroxide (36) and challenged as indicated (Figure E2). Similar to the HDM model, loss of the Miz1 POZ domain also reduced the Th2 response in the OVA model. The observed effects included decreased production and mRNA expression of the Th2 cytokines, including IL-4, IL-5, and IL-13 (Figures 2A and 2B), attenuated airway hyperresponsiveness (Figure 2C), and decreased expression of epithelial-derived cytokines, including *Tslp*, *Il33*, *Il25*, and *Csf2*

(Figure 2D). Taken together, these data suggest that lung-specific loss of function of Miz1 results in reduced Th2 response in HDM- or OVA-induced mouse model of asthma.

Lung Epithelial Cell-Specific Loss of Function of Miz1 Results in Reduced HDM-induced Airway Hyperreactivity in Mice

To determine the cell types in the mouse lung in which Miz1 promotes the allergic response, we crossed transgenic mice expressing Cre driven by the human SPC promoter (*SPC-Cre* mice) (37) with *Miz1(POZ)^{fl/fl}* mice as we recently reported (23). As SPC is expressed early in lung epithelial progenitors, developmental expression of SPC-Cre results in Cre-mediated recombination in the airway and alveolar epithelium that persists over the lifespan of nonconditional *SPC-Cre* mice (37). Note, although SPC is an AT2 (alveolar type 2) cell marker, it is expressed in endodermal lung progenitors, which generate all distal conducting airway and alveolar cell types. In addition, SPC-driven recombination is also observed within epithelial cells of more proximal conducting airway epithelium because progeny of early lung progenitor cells contribute to these structures (38). We recently reported that the *Miz1(POZ)* was partially deleted (∼50%) in primary AT2 cells flow-sorted from heterozygous *SPC-Cre^+/Miz1(POZ)^{+/f}* littermates, and there was no deletion of *Miz1(POZ)* in alveolar macrophages (AMs) from heterozygous mutant mice (23), indicating the specificity of the *Miz1(POZ)* deletion in lung epithelial cells. We also reported that although homozygous *SPC-Cre^+/Miz1(POZ)^{f/f}* mice exhibited age-related spontaneous COPD-like phenotype starting from a young age (<4 mo), heterozygous *SPC-Cre^+/Miz1(POZ)^{+/f}* mice appeared histologically normal at a relatively young age (<6 mo) compared with control *Miz1(POZ)^{fl/fl}* mice (also *see* Figure E3A). Therefore, for all the experiments here, we used heterozygous *SPC-Cre^+/Miz1(POZ)^{+/f}* mice at a much younger age (8–12 wk). When challenged with HDM, *SPC-Cre^+/Miz1(POZ)^{+/f}* mice exhibited attenuated airway inflammation (Figure 3A), decreased eosinophil infiltration and mucus secretion in the airways (Figures 3B and 3C), reduced production of IL-5 in the

BAL fluid (Figure 3D), and reduced *Tslp* mRNA concentrations from whole-lung homogenates (Figure 3E). Together, these data suggest that Miz1 in lung epithelial cells contributes to HDM-induced allergic response.

Mice with Dendritic Cell- or Myeloid Cell-Specific Loss of Function of Miz1 Have Reduced HDM-induced Airway Hyperreactivity

DCs play an important role in determining Th1 and Th2 differentiation and also in maintaining effector Th2 response in asthma. We sought to determine whether Miz1 in DCs is important for asthma promotion. We crossed *Miz1(POZ)^{fl/fl}* mice with transgenic *CD11c-Cre* mice as we recently reported (23). CD11c-Cre induces efficient Cre-mediated recombination in DCs and AMs (39). We have reported a significant deletion of the Miz1 POZ domain in CD11C^+ DCs and AMs from the lungs of CD11c-Cre/*Miz1(POZ)^{fl/fl}* mice, and that at a younger age (<6 mo), CD11c-Cre/*Miz1(POZ)^{fl/fl}* mice were indistinguishable from the control *Miz1(POZ)^{fl/fl}* mice (also *see* Figure E3B) (23). Reduced allergic response was observed in HDM-treated CD11c-Cre/*Miz1(POZ)^{fl/fl}* mice, as shown by attenuated airway inflammation and reduced eosinophil infiltration and mucus production in the airways (Figures 3F–3H), decreased production and mRNA expression of the Th2 cytokines (Figures 3I and 3J), and decreased serum total IgE concentrations (Figure 3K). These data suggest that Miz1 in DCs and/or AMs contributes to allergic asthma. We also generated mice with myeloid cell–specific deletion of the Miz1 POZ domain [LysM-Cre/*Miz1(POZ)^{fl/fl}*]. There was a significant deletion of the Miz1 POZ domain in DCs, AMs, neutrophils, monocytes, and AT2 cells from the lungs of LysM-Cre/*Miz1(POZ)^{fl/fl}* mice (Figure E3C). At a younger age (<6 mo), LysM-Cre/*Miz1(POZ)^{fl/fl}* mice were indistinguishable from the control *Miz1(POZ)^{fl/fl}* mice, as analyzed by lung histology (Figure E3B). Similar to CD11c-Cre/*Miz1(POZ)^{fl/fl}* mice, LysM-Cre/*Miz1(POZ)^{fl/fl}* mice also showed a reduced allergic response to HDM, including attenuated airway inflammation, and reduced eosinophil infiltration and mucus production in the airways (Figures 3F–3H), as well as decreased mRNA expression of the Th2 cytokines (Figure 3L).
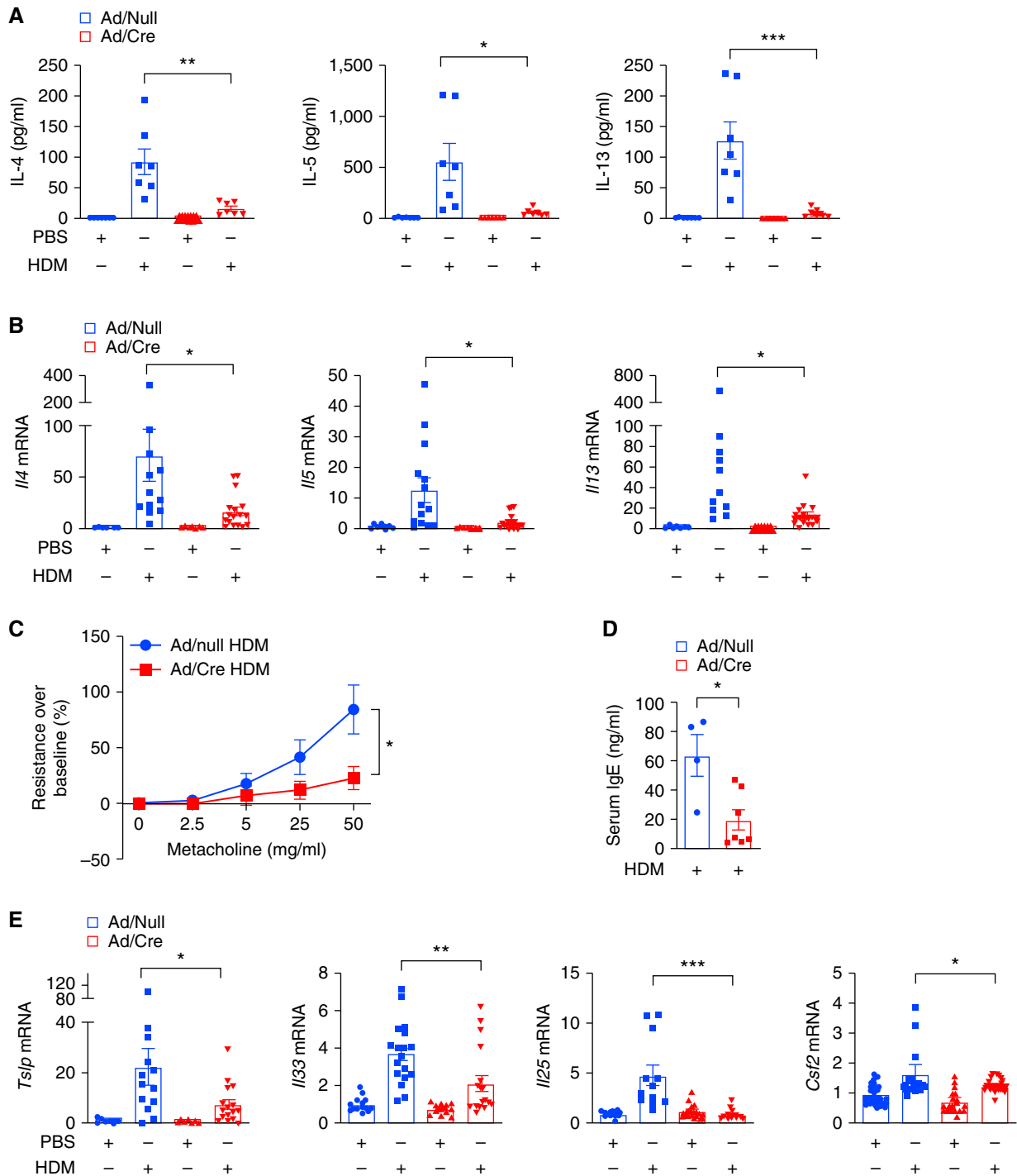
Figure 1. Lung-specific loss of function of Miz1 (c-Myc-interacting zinc finger protein-1) inhibits house dust mite (HDM)-induced allergic asthma in mice. *Miz1*(POZ)[fl/fl] mice were intratracheally instilled with Ad/Null or Ad/Cre. Thirty days later, allergic asthma was induced in mice with HDM. (*A–E*) Production of Th2 cytokines in the BAL fluid (*A*), or mRNA concentrations of cytokines in whole-lung homogenates as indicated (*B* and *E*), or airway hyperresponsiveness to aerosolized metacholine in increasing concentrations (*C*), or serum total IgE concentrations (*D*) from Ad/Null- or Ad/Cre-pretreated, HDM-challenged *Miz1*(POZ)[fl/fl] mice. *n* = 5–7 for *A*, *C*, and *D*. *n* = 5–7 with technical replicates for *B* and *E*. Values represent the mean ± SEM. Unpaired Student's *t* test was used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.
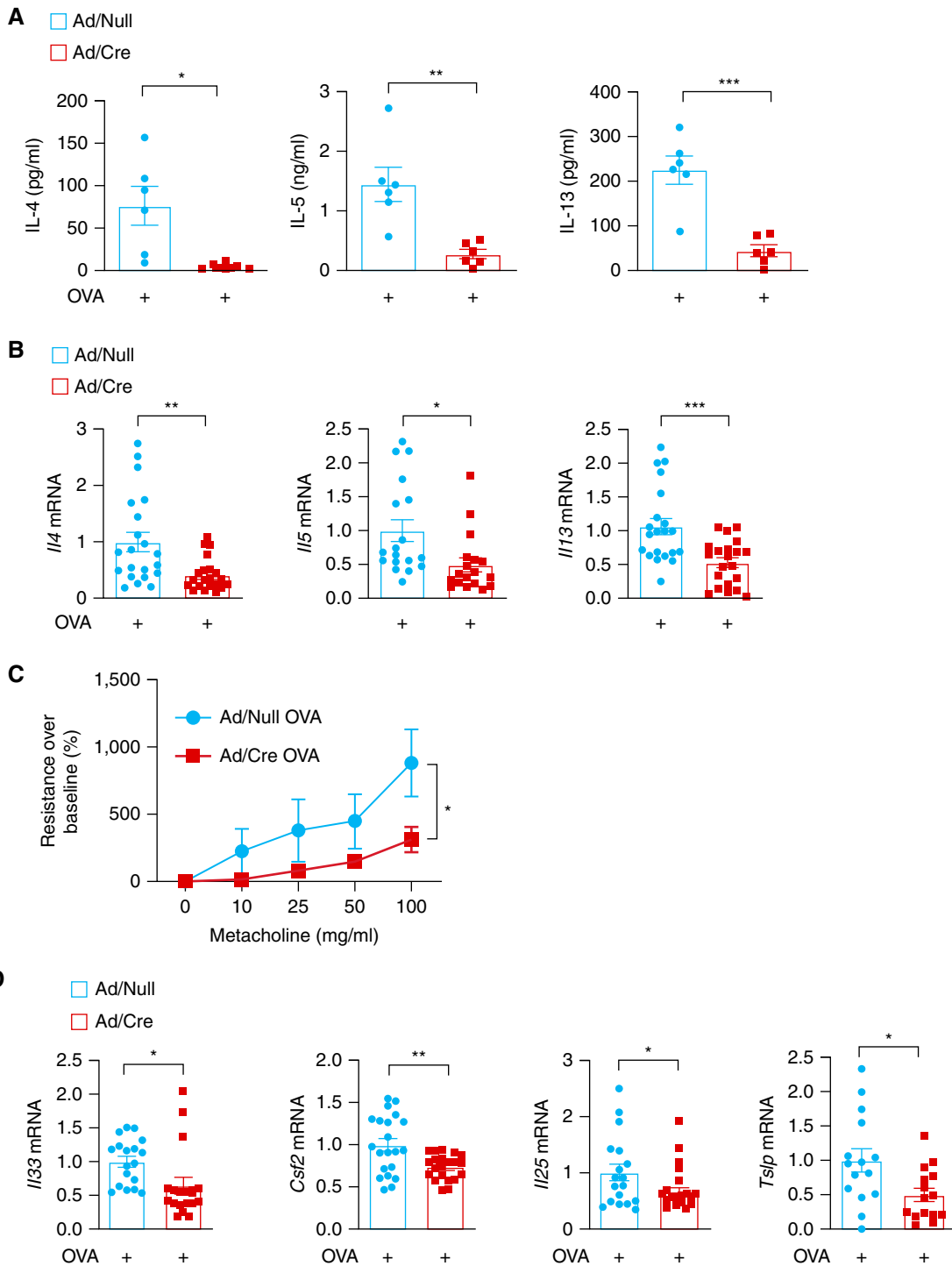
Figure 2. Lung-specific loss of function of Miz1 suppresses OVA (ovalbumin)-induced allergic asthma in mice. *Miz1*(POZ)[fl/fl] mice were intratracheally instilled with Ad/Null or Ad/Cre. Thirty days later, allergic asthma was induced in mice with OVA/alum (40). (*A–D*) Production of T-helper cell type 2 (Th2) cytokines in the BAL fluid (*A*), or mRNA concentrations of cytokines in whole-lung homogenates as indicated (*B* and *D*), or airway hyperresponsiveness to aerosolized metacholine in increasing concentrations (*C*) from Ad/Null- or Ad/Cre-pretreated, OVA-challenged *Miz1*(POZ)[fl/fl] mice. $n = 5–7$ for (*A* and *C*). $n = 5–7$ with technical replicates for (*B* and *D*). Values represent the mean ± SEM. Unpaired Student's *t* test was used. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
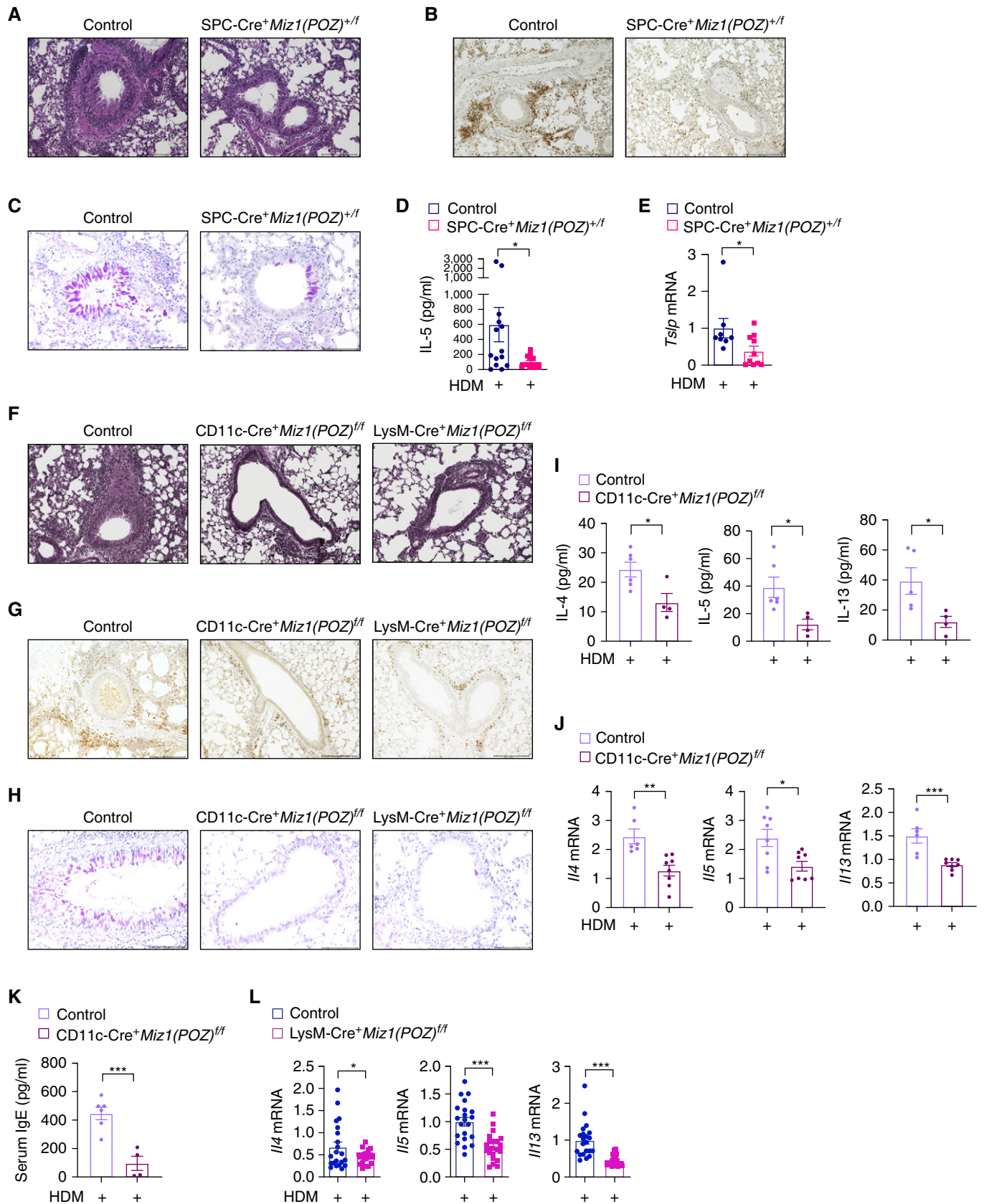
Figure 3. Miz1 in lung epithelial cells or dendritic/myeloid cells contributes to HDM-induced allergic asthma. (*A–C*) Lung histology by hematoxylin and eosin staining (*A*), eosinophil major basic protein (EMBP) staining of eosinophils (*B*), or Periodic Acid–Schiff (PAS) staining (*C*) in the airways of HDM-treated control *Miz1*(POZ)^fl/fl or heterozygous *SPC-Cre*^+/*Miz1(POZ)*^+/f mice. Eosinophils are stained brown/dark brown
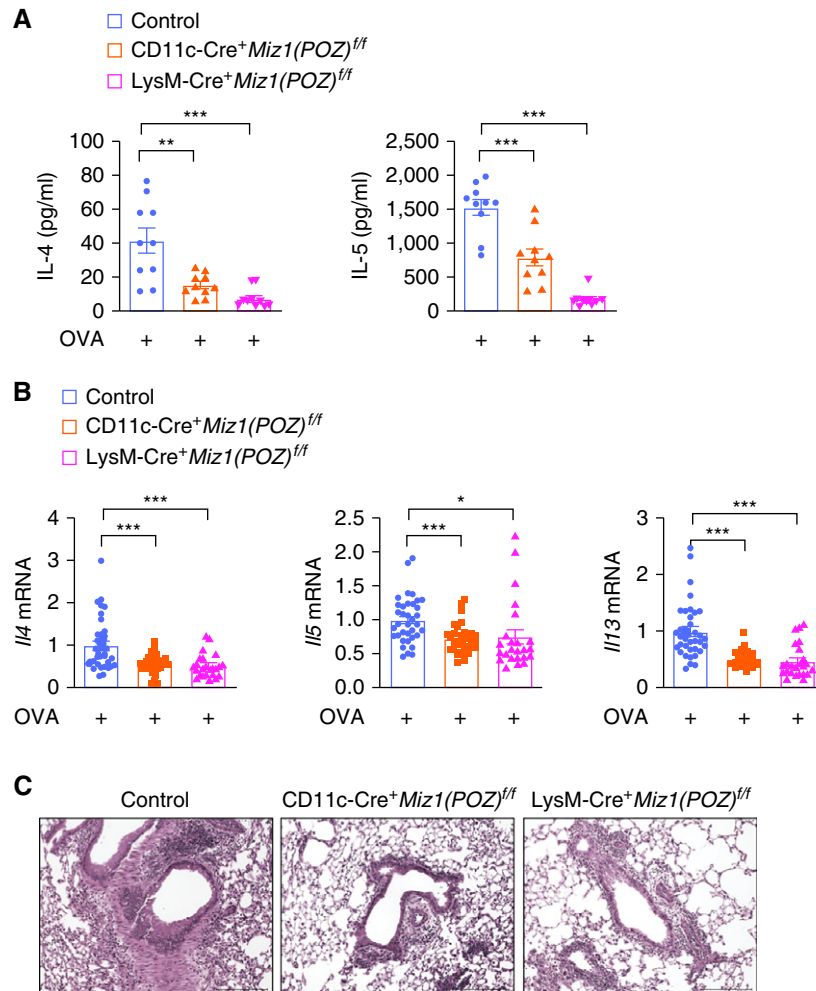
Figure 4. Miz1 in dendritic or myeloid cells contributes to OVA-induced asthma. Control *Miz1*(POZ)$^{fl/fl}$, CD11c-Cre/*Miz1*(POZ)$^{fl/fl}$, or LysM-Cre/*Miz1*(POZ)$^{fl/fl}$ mice were subjected to OVA/alum-induced allergic asthma. (*A*) Production of the Th2 cytokines in the BAL fluid of OVA-treated mice as indicated. *n* = 5 with technical replicates. (*B*) mRNA concentrations of the Th2 cytokines in whole-lung homogenates of OVA-treated mice as indicated. *n* = 5 with technical replicates. (*C*) Lung histology by hematoxylin and eosin staining in the airways of OVA-treated mice as indicated. Values represent the mean ± SEM. Unpaired Student's *t* test was used. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Miz1 in Dendritic or Myeloid Cells Also Contributes to OVA-induced Asthma

Similar to the HDM-induced asthma model, CD11c-Cre/*Miz1*(POZ)$^{fl/fl}$ or LysM-Cre/*Miz1*(POZ)$^{fl/fl}$ mice also had reduced OVA-induced allergic asthma, as shown by decreased Th2 cytokine production or mRNA expression (Figures 4A and 4B), and attenuated airway inflammation (Figure 4C). These data suggest that Miz1 in dendritic or myeloid cells contributes to the pathogenesis of asthma.

Loss of Function of Miz1 Results in Th1 Skewing

Because Th1 cells antagonize Th2 cell functions (41, 42), the decreased Th2 response by loss of the Miz1 POZ domain we observed in the mouse asthma models may be owing to an increase in the Th1 response.

Figure 3. (*Continued*). by EMBP staining. The mucin produced by goblet cells is stained a purple-magenta color by PAS staining. The nuclei are stained blue. (*D* and *E*) Production of IL-5 in the BAL fluid (*D*) and mRNA concentrations of *Tslp* in whole-lung homogenates (*E*) as indicated in HDM-treated control *Miz1*(POZ)$^{fl/fl}$ or heterozygous *SPC-Cre*$^+$/*Miz1*(POZ)$^{+/f}$ mice. *n* = 5 with technical replicates. (*F–H*) Lung histology of the airways by hematoxylin and eosin staining (*F*), EMBP staining of eosinophils (*G*), or PAS staining (*H*) in the airways of HDM-treated control *Miz1*(POZ)$^{fl/fl}$ or CD11c-Cre/*Miz1*(POZ)$^{fl/fl}$ or LysM-Cre/*Miz1*(POZ)$^{fl/fl}$ mice. Eosinophils are stained brown/dark brown by EMBP staining. The mucin produced by goblet cells is stained a purple-magenta color by PAS staining. The nuclei are stained blue. (*I–K*) Production of the Th2 cytokines in the BAL fluid (*I*), mRNA concentrations of the Th2 cytokines in whole-lung homogenates (*J*), or serum total IgE concentration (*K*) from HDM-treated control *Miz1*(POZ)$^{fl/fl}$ (*n* = 3) or CD11c-Cre/*Miz1*(POZ)$^{fl/fl}$ mice (*n* = 4). (*L*) mRNA concentrations of the Th2 cytokines from whole-lung homogenates of HDM-treated control *Miz1*(POZ)$^{fl/fl}$ or LysM-Cre/*Miz1*(POZ)$^{fl/fl}$ mice (*n* = 7 with technical replicates). Values represent the mean ± SEM. Unpaired Student's *t* test was used. *$P < 0.05$ and ***$P < 0.001$.
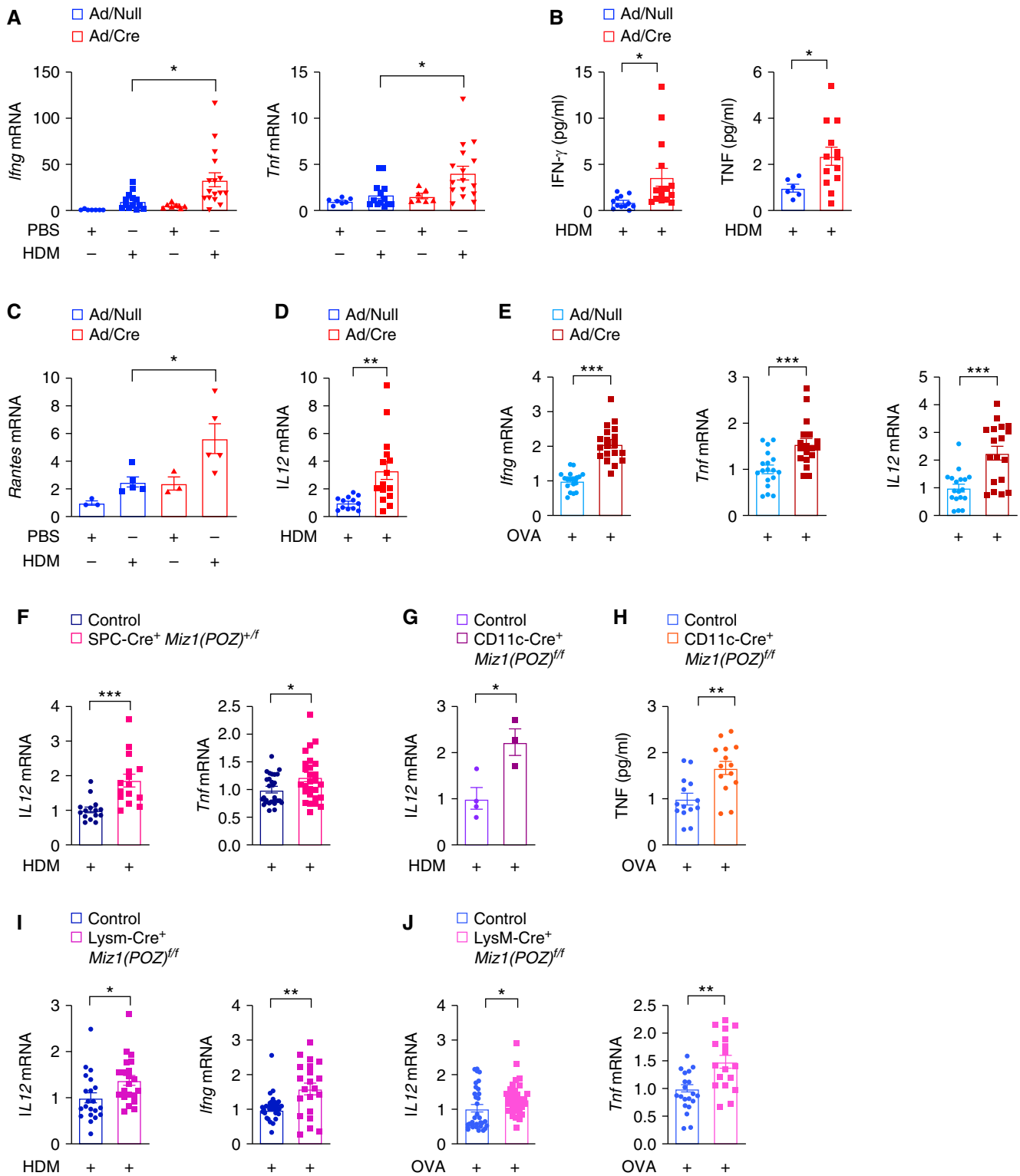
Figure 5. Loss of function of Miz1 results in Th1 skewing. Ad/null or Ad/Cre-preinfected *Miz1*(POZ)^fl/fl mice, or control *Miz1*(POZ)^fl/fl, heterozygous *SPC-Cre⁺/Miz1(POZ)*^+/f, CD11c-Cre/*Miz1*(POZ)^fl/fl, or LysM-Cre/*Miz1*(POZ)^fl/fl mice were subjected to HDM- or OVA-induced allergic asthma as indicated. (*A, C–G, I,* and *J*) mRNA concentrations of *Ifng, Tnf, Rantes,* or *Il12* in whole-lung homogenates of HDM- or OVA-treated mice as indicated. (*B* and *H*) Production of IFNγ and TNF in the BAL fluid from HDM- or OVA-treated mouse as indicated. *n* = 5–7 except in PBS groups in *C* (*n* = 3) and in *G* (3–4). Values represent the mean ± SEM. Unpaired Student's *t* test was used. *P < 0.05, **P < 0.01, and ***P < 0.001.
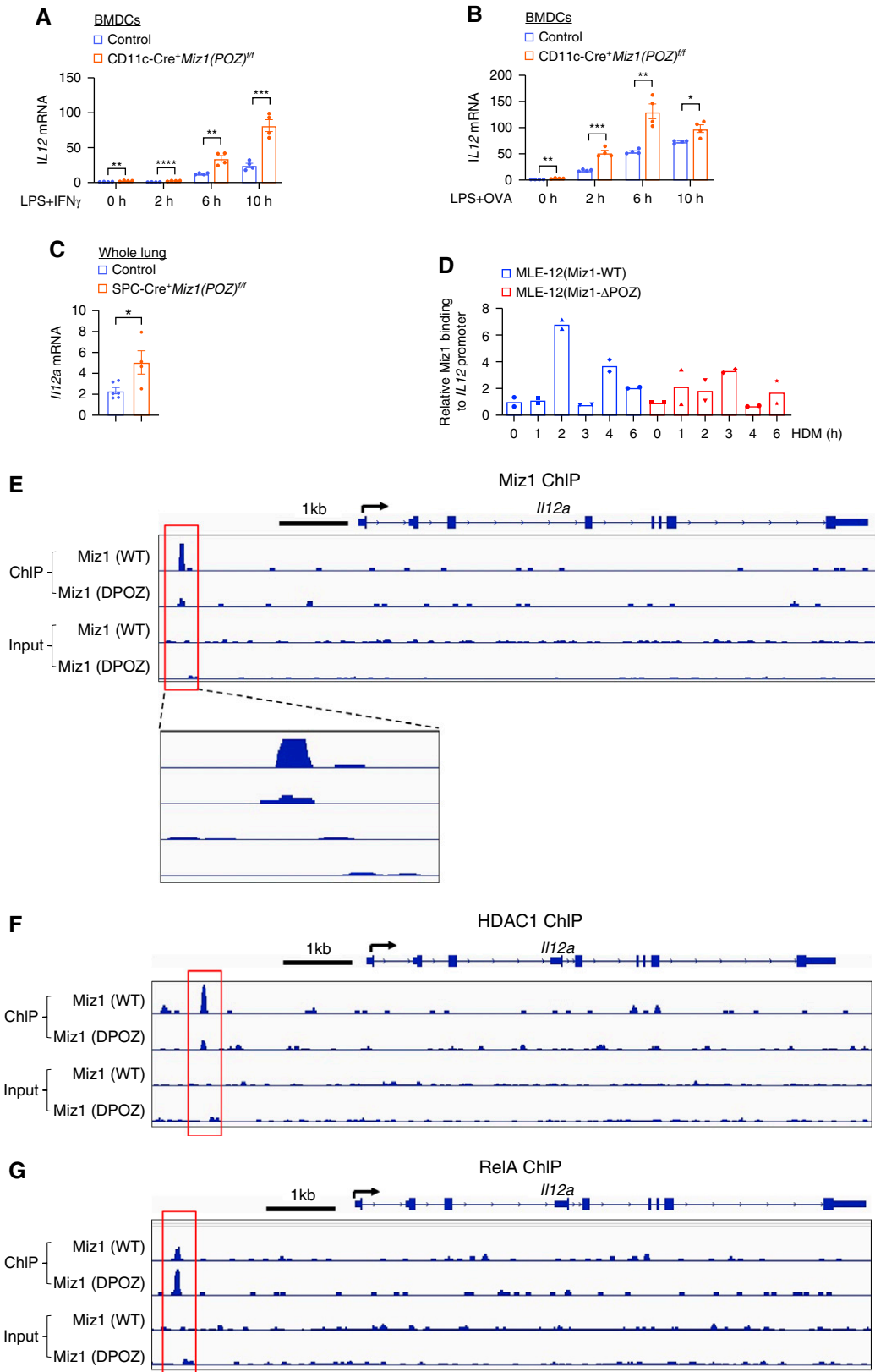
Figure 6. Miz1 binds to the promoter of il12 and represses its transcription. (*A* and *B*) mRNA concentrations of *Il12* in IFNγ- (*A*) or OVA-treated (*B*) LPS-primed bone marrow–derived dendritic cells (BMDCs) isolated from control *Miz1*(POZ)^fl/fl or CD11c-Cre/*Miz1*(POZ)^fl/fl mice. LPS, 0.1ng/ml; IFNγ, 10ng/ml; OVA, 10 μg/ml. (*C*) mRNA concentrations of *Il12* in whole-lung homogenates from control *Miz1*(POZ)^fl/fl or heterozygous *SPC-Cre*^+/*Miz1*(POZ)^+/f mice. *n* = 4–6. (*D*) Chromatin immunoprecipitation (ChIP) analyses of Miz1 binding on the *Il12* promoter in HDM-treated

Indeed, mRNA concentrations (Figure 5A) and secretion (Figure 5B) of the Th1 cytokines, including IFN-γ and TNF, were increased from the lungs of HDM-treated *Miz1*ΔPOZ mice compared with HDM-treated control mice. Consistently, the expression of *Rantes* (regulated upon activation, normal T cell expressed and presumably secreted), which is induced by the Th1 cytokines, was increased by loss of the Miz1 POZ domain in the HDM model (Figure 5C). IL-12, which is predominantly produced by activated antigen-presenting cells such as DCs and also by lung epithelial cells, is pivotal for Th1 differentiation and is among the most critical factors skewing the immune response toward a Th1 cytokine profile (high IFN-γ and low IL-4) (26, 43). We found that the mRNA concentrations of *Il12* were augmented in the lung by loss of the Miz1 POZ domain in response to HDM (Figure 5D). Similar results were obtained in the OVA model—increased *Ifng*, *Tnf*, and *Il12* expression by loss of function of Miz1 (Figure 5E). Similar to *Miz1*ΔPOZ mice, heterozygous *SPC-Cre⁺/Miz1(POZ)⁺/f*, CD11c-Cre/*Miz1*(POZ)^fl/fl, or LysM-Cre/*Miz1*(POZ)^fl/fl mice also showed increased expression of IL-12 or IFN-γ or TNF in the lung in exposure to HDM or OVA (Figures 5F–5J). These data collectively suggest that Miz1 suppresses the Th1 response, thereby promoting the Th2 response in the asthma models.

Miz1 Binds to the Promoter of *Il12* and Represses Its Transcription

Our data showed that loss of function of Miz1 resulted in a decreased Th2 response and increased expression of the Th1-skewing cytokine IL-12 in HDM- and OVA-induced asthma models. We sought to determine whether Miz1 directly represses IL-12 expression. We generated BMDCs by isolating BM cells from CD11c-Cre/*Miz1*(POZ)^fl/fl or control *Miz1*(POZ)^fl/fl mice and treated them with GM-CSF. Expression of *Il12* was augmented by loss of the Miz1 POZ domain in INF-γ– or OVA-treated, LPS-primed BMDCs (Figures 6A and 6B). Note, OVA- or HDM-treated, LPS-primed BMDCs are commonly used for *in vitro*
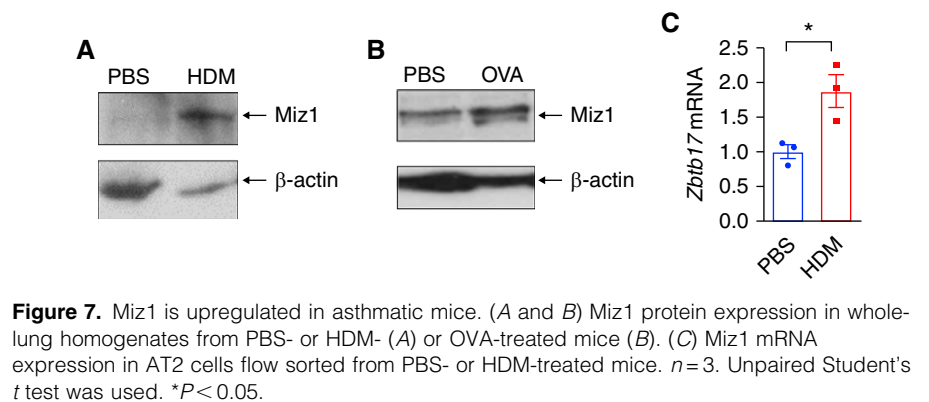
experimental model to study asthma in DCs, in which LPS is commonly used to induce DC "maturation" (44). These data suggest that Miz1 inhibits *Il12* expression in DCs.

IL-12 is also produced by lung epithelial cells (45). The lungs from *SPC-Cre⁺/Miz1(POZ)^f/f* mice showed increased *Il12* expression compared with the control *Miz1(POZ)^f/f* mice (Figure 6C). To determine whether Miz1 directly represses *Il12* transcription by binding to its promoter, we used stable murine type II–like lung epithelial cells (MLE-12–Miz1[ΔPOZ] or MLE12–Miz1[WT]), in which endogenous Miz1 was stably knocked down by specific short hairpin RNA (shRNA) and simultaneously the exogenous Miz1(ΔPOZ) mutant or wild-type Miz1, which contains silent mutations making it resistant to shRNA against endogenous Miz1, was stably expressed in amounts similar to the endogenous protein, as we previously reported (22). Chromatin immunoprecipitation assays showed that Miz1 was recruited to the *Il12* promoter in HDM-treated MLE12–Miz1(WT) cells, while the recruitment of the Miz1(ΔPOZ) mutant was reduced (Figure 6D). ChIP sequencing (ChIP-seq) also revealed Miz1 binding on the *Il12* promoter, which was almost diminished by the Miz1(ΔPOZ) mutation (Figure 6E). These data suggest that Miz1 binds to the *Il12* promoter and represses its transcription. We previously reported that Miz1 epigenetically represses gene transcription through histone deacetylation via recruiting HDAC1 (histone

deacetylase 1) (22). ChIP-seq showed recruitment of HDAC1 to the *Il12* promoter in Miz1 POZ domain–dependent manner (Figure 6F), suggesting that Miz1 epigenetically represses *Il12* through histone deacetylation. We previously also reported that Miz1 represses NF-κB–dependent gene through histone deacetylation-mediated exclusion of NF-κB binding (22). IL-12 is an NF-κB target gene (46, 47). ChIP-seq showed enhanced recruitment of RelA (also called p65), the predominant transactivating submit of the NF-κB family, on the *Il12* promoter by loss of function of Miz1 (Figure 6G). These data suggest that Miz1 recruits HDAC1 to the *Il12* promoter, resulting in histone deacetylation of the promoter and thereby excluding NF-κB binding. Altogether, our data suggest that Miz1 represses *Il12* transcription in monocyte derived DCs as well as in lung epithelial cells, thereby promoting the Th2 response in asthma.

Miz1 Is Upregulated in Asthmatic Mouse Lungs

We sought to determine whether Miz1 expression is subjected to regulation during asthma. Miz1 protein concentrations were increased in whole-lung homogenates from HDM- (Figure 7A) or OVA-treated mice (Figure 7B). Miz1 mRNA expression was also upregulated in lung epithelial cells flow-sorted from HDM-treated mice (Figure 7C). These data suggest that Miz1 is upregulated during asthma, which in turn promotes asthma pathogenesis.

Figure 7. Miz1 is upregulated in asthmatic mice. (*A* and *B*) Miz1 protein expression in whole-lung homogenates from PBS- or HDM- (*A*) or OVA-treated mice (*B*). (*C*) Miz1 mRNA expression in AT2 cells flow sorted from PBS- or HDM-treated mice. $n = 3$. Unpaired Student's *t* test was used. *$P < 0.05$.

Figure 6. (*Continued*). MLE-12/Miz1(WT) or MLE-12/Miz1(ΔPOZ) cells. (*E–G*) ChIP-seq traces of Miz1, HDAC1, or RelA proteins on the promoter of *Il12a* in MLE-12/Miz1(WT) or MLE-12/Miz1(ΔPOZ) cells. Input is shown as control. Top line indicates the *Il12a* gene (exons were shown with blue boxes and introns were shown with line). Black arrow indicates transcription start site (TSS). Red columns indicate peaks callings, which are enlarged for viewing in *E*. Values represent the mean ± SEM. Unpaired Student's *t* test was used. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Discussion

It is generally considered that type 1 immunity contributes to the eradication of intracellular pathogens, whereas type 2 immunity is predominantly responsible for the pathogenesis of allergic diseases such as asthma and is also important for protection against extracellular organisms such as parasites. Overactivation of either pathway may lead to detrimental inflammation, and therefore, the type 1/type 2 balance is important to maintain immune homeostasis. We previously reported that Miz1 suppresses lung inflammation induced by LPS (a major component of the outer membrane of the gram-negative bacteria) or the immune response to gram-negative bacterium *P. aeruginosa*. Moreover, we recently reported that lung epithelial cell–specific loss of function of Miz1 causes spontaneous, age-related progressive COPD-like phenotype in mice (23). Here we report that Miz1 inhibits type 1 immunity, resulting in promotion of type 2 immune response in allergic asthma. Our observations are in line with the prevailing paradigm regarding the Th1/Th2 counterregulation.

In this report, we provide evidence that loss of function of Miz1 skews toward a Th1 response by upregulating IL-12, thereby counteracting Th2 response and protecting against asthma. Our findings are consistent with a growing body of evidence from patients with asthma, which supports the

inverse relationship between the concentrations of IL-12 and the development of asthma (48–52). Causal studies in animal models of asthma show that the administration of IL-12 or IL-12–producing agents prevent allergen-induced Th2 response and airway eosinophilia (53–60), whereas blocking IL-12 during the sensitization process worsened allergic airway inflammation (14). Intriguingly, a recent genome-wide association study identified a SNP (rs4410871) in the Miz1 interacting protein Myc, whose transcriptional repression is mediated by Miz1 (61), as being associated with asthma (62). Most importantly, the same SNP was reported to be associated with protection against multiple sclerosis, with the risk allele (T) in asthma being the protective allele for multiple sclerosis, a Th1 disease (63), implying a crucial role for this SNP in the Th1/Th2 balance.

Our data show that Miz1 acts to shift the balance of cytokines toward a Th2 phenotype in multiple cell populations in the lung including epithelial, dendritic, and myeloid cells, indicating a general mechanism of Miz1 action in different cell types. This is consistent with our previous report that Miz1 in both epithelial cells and hematopoietic-derived cells is important to suppress LPS-induced lung inflammation. The lung epithelium was initially viewed to function only as a physical barrier in asthma; however, it is now considered to play a central role in Th2 sensitization by inducing the activation and Th2 cell–polarizing

capacity of DCs through the production of cytokines, including TSLP, GM-CSF, IL-33, and IL-25 (30). Our data that Miz1 in lung epithelial cells is as important as in DCs in asthma are in line with this notion. The Th1/Th2 paradigm has been extended to several of other T-cell subsets, including Th17 cells, Th9 cells, and Treg cells, as well as ILC2 cells, all of which may contribute to the development of asthma in subsets of patients. Future studies will investigate whether Miz1 is engaged in regulation of these subsets of cells.

In summary, our studies demonstrate that Miz1 promotes Th2-mediated allergic asthma by counteracting Th1 response. Additionally, Miz1 is upregulated in asthmatic mice. These studies directly implicate MIZ1 in asthma pathogenesis. ■

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