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Na^+/H^+ exchanger regulatory factor 1 (NHERF1) mediates the pathogenesis of airway inflammation in a murine model of house dust mite-induced asthma

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Abstract

Na^+/H^+ exchanger regulatory factor 1 (NHERF1), a class I PDZ binding protein, regulates G-protein coupled receptors (GPCR) signaling in some cell types. NHERF1 also functions as a scaffolding protein and activates non-GPCR signaling pathways, thereby contributing to the pathogenesis of various diseases. While we have previously shown that NHERF1 regulates mast cell functions, there is little information regarding the role of NHERF1 in other immune cells. How NHERF1 regulates the pathogenesis of allergic disease such as asthma also remains unknown. In the current study, we show that NHERF1 promotes allergic airway inflammation in a house dust mite extract (HDME)-induced mouse model of asthma. Specifically, HDME-specific serum IgE levels, airway leukocyte numbers and goblet cell hyperplasia were reduced in $NHERF1^{+/-}$ mice as compared to $NHERF1^{+/+}$ mice. Interestingly, the gene expression of inflammatory (interleukin (IL)-17a, IL-25 and IL-33) as well as T helper 2 (Th2) cytokines (IL-4, IL-5 and IL-13) and several chemokines that recruit eosinophils, neutrophils and lymphocytes were also decreased in the lungs of $NHERF1^{+/-}$ mice exposed to HDME. Consistent with these observations, microRNAs regulating mucus production, inflammation, Th2 effector functions and IL-13 expression were increased in the lungs of HDME-treated $NHERF1^{+/-}$ mice. Overall, our studies reveal a unique role for NHERF1 in regulating asthma pathogenesis and further elucidation of the mechanisms through which NHERF1 modulates allergic inflammation will lead to the development of new therapeutic strategies for asthma.

Keywords

Na^+/H^+ exchanger regulatory factor 1; NHERF1; house dust mite extract; asthma; airway inflammation; airway hyperresponsiveness and allergic diseases

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AUTHORSHIP CONTRIBUTIONS

A.K.K. performed experiments and analyzed the data. C.Y. and D.B. performed experiments. R.A.P. provided lung samples and edited the manuscript. R.D., and H.S. conceived the study, planned the experiments, analyzed the data and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

INTRODUCTION

Asthma represents an inflammatory airway syndrome whose global prevalence is increasing worldwide. Asthma in part is mediated by an inflammatory response amplified by leukocytes in the upper and lower airways (1). Two distinct inflammatory endotypes of corticosteroid-dependent asthma have been proposed based on the presence or absence of eosinophils in lungs and bronchoalveolar lavage fluid (BALF); type 2 (T2)-high and T2-low (also called as “non T2-high”). The T2-high endotype is characterized by the presence of eosinophilic airway inflammation, whereas the T2-low endotype is usually is accompanied by the presence of neutrophils in the sputum or paucigranulocytic (i.e., normal sputum levels of both eosinophils and neutrophils) airway inflammation(2). Additionally, a recent report demonstrated a T1-skewed signature in some severe asthma patients, marked by the production of the Th1 cytokine, IFN-γ(3). Elevated IFN-γ was associated with high airway resistance, increased inflammatory infiltrates and corticosteroid resistance. The pathogenesis of asthma in different patient endotypes is complex; however, the cardinal features of this disease in most patients include airway inflammation, mucus production and airway hyperresponsiveness (AHR).

The NHERF1 protein belongs to the NHERF family of PDZ (**P**ost-synaptic density protein 95, **D**rosophila disc large tumor suppressor and **Z**onula occludens-1) scaffold proteins(4). NHERF1 was identified as a regulator of the exchanger NHE3 and hence named Na^+/H^+ exchanger 3 (NHE3) regulatory factor 1(5). NHERF1 is a major regulator of G protein coupled receptor (GPCR) signaling(4). NHERF1 binds to GPCRs that have a class I PDZ motif S-T-X-Φ, (where “Φ” indicates hydrophobic amino acid and “X” indicates any amino acid) at their carboxyl-terminus (C-terminus). Notably, the interaction between NHERF1 and GPCRs modulate their trafficking, stability, and signaling properties. Specifically, NHERF1 interacts with class I PDZ motif in the C-terminus of GPCRs and anchors them to the membrane and restricts receptor internalization and desensitization(6). Thus, NHERF1 functions as a positive regulator of GPCR signaling. NHERF1 also indirectly regulates GPCR signaling in a receptor-independent manner by associating with downstream signaling proteins such as protein kinase A (PKA), phospholipase C (PLC)-β, and protein kinase B (PKB/Akt)(7–9). Thus, NHERF1 can mediate its effects via GPCR-dependent and -independent mechanisms to affect cellular functions.

Several reports have shown that NHERF1 regulates GPCRs in the airway epithelium and smooth muscle cells to alter their response. NHERF1 is crucial to β-2-adrenoceptor (β_2AR)-mediated activation of cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells(10, 11). In addition, NHERF1 could mediate the recycling of internalized β_2AR back to the cell membrane. Pera et al.(12) recently demonstrated that NHERF1 regulates cAMP signaling and promotes IL-6 production in human airway smooth muscle cells. NHERF1 also promotes mast cell responses via both the complement C3a receptor and IgE receptor and in its absence these cells produce lower levels of chemokines and cytokines when activated by C3a or allergen(13–15). On the contrary, NHERF1 inhibits T cell responses by facilitating C-terminal Src kinase (Csk) activation resulting in attenuation of lymphocyte-specific protein tyrosine kinase (Lck)(4, 16). NHERF1 also forms a macromolecular complex with ezrin and protein kinase A (PKA), which allows cAMP-

mediated inhibition of T-cell receptor signaling. Though these findings highlight the pivotal role of NHERF1 in regulating responses of different cell types involved in allergic airway disease, there is no study till date that has examined the role of this adapter molecule in mediating the pathophysiology of asthma.

In the current study, we investigated whether NHERF1 regulated the development of asthma using a T2-high mouse model. NHERF1$^{-/-}$ mice manifest several abnormalities such as reduced bone mineral density(17), defective kidney and colon functions(18), and hydrocephaly(19). In addition, female NHERF1$^{-/-}$ mice have high mortality rates and die very early after birth; and those that survive have multiple bone fractures and must be euthanized within 6–8 weeks of age. Therefore, we used NHERF1$^{+/-}$ mice and littermate NHERF1$^{+/+}$ (as controls) for our experiments. Exposure of wild type NHERF1$^{+/+}$ mice to an airborne allergen such as HDME elicited an inflammatory response including AHR, lung eosinophilic infiltration, goblet cell hyperplasia, high levels of serum IgE, Th2 cytokines and mucus production. While lung inflammation, mucus production, goblet cell hyperplasia and Th2 cytokines were abrogated in NHERF1$^{+/-}$ mice, AHR was unaltered suggesting that NHERF1 differentially modulates allergen-induced inflammatory responses that are uncoupled from the augmented agonist-induced bronchoconstriction in our mouse model. Mechanistically, we demonstrate that NHERF1 critically modulates gene expression of several chemokine and chemokine receptors required for migration of immune cells, alarmins such as IL-25, IL-33, the proinflammatory cytokine IL-17A and several microRNAs that have known roles in the pathogenesis of allergic asthma.

MATERIALS AND METHODS

Mice

NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice (on C57BL/6 [B6] background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred and housed under specific pathogen-free conditions. Six to eight-week-old male and female mice used for all the experiments were approved by the Institutional Animal Care and Use Committee at MSU.

Human lung samples

Lung samples from deceased subjects were obtained from either the International Institute for the Advancement of Medicine (IIAM) or National Disease Resource Interchange (NDRI) and their use was approved by Institutional Review Board at Rutgers University. All donor tissue samples were harvested anonymously and de-identified. The available information for individual donors is provided in Supplemental Table 1. Limited information on the donor tissues is available since these were obtained in a de-identified manner. All donors with asthma died of asthma and are therefore considered severe asthma patients. The participants' use of pre-mortal medication is unavailable. Participants with fatal asthma had no other co-morbidity and no pulmonary function tests data are available for the fatal asthma donors.

Mouse Model of Asthma

HDME-induced murine model of asthma (Supplemental Figure 2A) was used as described previously(20). Twenty-four hours after the final HDME challenge, mice were anesthetized

for measurement of AHR and then sacrificed for collection of blood, bronchoalveolar lavage fluid (BALF) and lung tissue for various endpoint analysis(20).

Immunohistochemistry (IHC)

IHC for NHERF1 was performed on human and mouse lung tissues using methods previously described(21). Tissue sections were the probed with the anti-NHERF-1 (ab3452, Abcam, Cambridge, UK) and anti-rabbit secondary antibodies (Vector Laboratories, California, USA) followed by DAB staining. Hematoxylin and eosin (H&E) were used as the counter stains. Staining intensity for NHERF1 (relative density) for the entire lung tissue section on the slide was evaluated in a blinded fashion using ImageJ software.

AHR

Mice were anesthetized with intraperitoneal (i.p.) injection of 100 mg/kg ketamine (Henry Schein Animal Health, Dublin, OH), 10 mg/kg xylazine (Akorn, Lake Forest, IL) and 3 mg/kg acepromazine (Henry Schein Animal Health, Dublin, OH) and tracheostomized. Airway mechanics was measured using forced oscillation technique by flexiVent, a small animal ventilator (SCIREQ®, Quebec, Canada). Parameters of AHR such as airway resistance (Rrs), elastance (Ers), Newtonian resistance (R_N), tissue elastance (H) and tissue damping (G) were assessed by a methacholine (MCh; Sigma-Aldrich, St. Louis, MO) challenge test with increasing doses of MCh (0–100 mg/ml).

Evaluation of lung inflammation and goblet cell hyperplasia

Lungs sections were prepared and stained with H&E (for lung inflammation) or Periodic acid-schiff (PAS, goblet cell hyperplasia)(20). Briefly, the lungs were infused via the trachea with 10% buffered formalin. After excision, the lungs were immersed in fresh 10% formalin overnight. Samples were then embedded in paraffin, cut into 5-μm-thick sections and stained with H&E or Periodic acid-schiff (PAS). Digital images of sections were obtained using a Nikon Eclipse 50i microscope (Nikon, Japan) equipped with a INFINITY-3 digital color camera (Lumenera Corporation, Canada), and INFINITY ANALYZE 6.5.4 software.

Sections were coded and scored in a blinded fashion.

Inflammation scoring

Slides were coded and graded in a blinded fashion using a reproducible scoring system. Multiple pictures covering the whole lung section at 4X magnification were collected and the total number of inflamed bronchioles (surrounded by infiltrated cells) in each picture was counted. A value from 0 to 4 was adjudged to each bronchiole scored. A value of 0–1 was decided when no inflammation or occasional cuffing with inflammatory cells was detected, a value of 2–4 for bronchi surrounded by a thin layer (2 to 4 cells) of inflammatory cells and a value of >4 when bronchi were surrounded by a thick layer (more than five cells) of inflammatory cells. Number of bronchioles within each category (0–1, 2–4, or >4) was then divided by the total number of inflamed bronchioles to obtain percent bronchioles within each category of inflammation. For calculating the percent severity of inflammation, the number of inflamed bronchioles that received a score of 4 or more was divided by the total number of inflamed bronchioles.

Goblet cell hyperplasia was evaluated on PAS-stained lung sections. Each lung sample was divided into 9 imaginary sections and digitally imaged at 10X magnification in an effort to consistently observe identical regions across all samples and experiments. The intensity of PAS staining was assessed using ImageJ software (NIH, Bethesda, MD) to determine PAS positive cells as well as the percent area of PAS positive cells in each section.

Blood Serum Collection

Blood was drawn from the superior mesenteric vein of the mouse and left at 4 °C overnight. Serum was collected the next day and analyzed for total IgE and total IgG1 using commercially available ELISA kits from Invitrogen (Carlsbad, CA). HDME-specific IgE and HDME-specific IgG1 levels were also analyzed from the serum samples using ELISA kits from Chondrex Inc. (Woodinville, WA).

Total and differential leukocyte count from bronchoalveolar lavage fluid (BALF)

Mice were euthanized and whole lung was lavaged with 0.5 ml of sterile PBS three times and the resultant BALF was centrifuged to separate the cellular components from the supernatants. Cell-free BALF was analyzed for cytokines including, IL-4 (BD Biosciences, San Diego, CA) and IL-13 (Invitrogen) by ELISA. Total BALF cellularity was determined using a hemocytometer, and the BALF composition was evaluated morphologically following differential staining. Briefly, 50,000 cells were cytospun onto a clean glass slide, air- dried and then stained with Giemsa Wright stain (Sigma-Aldrich, St. Louis, MO) for 3 minutes. The stained slides were washed with distilled water, dried, dipped in xylene (Avantor, Radnor Township, PA) for 2 seconds and a cover slip was placed immediately over the cells. For each sample, a total of 200 cells were counted at 40X magnification and the number of monocytes, lymphocytes, and eosinophils was enumerated.

Isolation of lung mononuclear cells

Lung mononuclear cells were isolated and used for flow cytometry(20) or *ex vivo* re-stimulation experiments. For the latter, 2×10^5 cells/well were plated in a 96-well plate and stimulated with HDME (3 μg/well). After 72 h, the supernatant was harvested and analyzed for cytokines by ELISA.

Flow cytometry

The anti-mouse antibodies used for flow cytometry are listed as follows: B220 (clone RA3-6B2), CD4 (clone RM4-5), NK 1.1 (clone PK136), CD11b (clone M1/70), and Siglec F (clone E50-2440) were from BD Biosciences. Antibodies for TCR-β (clone H57-597), CD8 (clone 53-6.7), CD11c (clone N418,), MHCII IA/IE (clone M5/114.15.2), and Ly6G (clone 1A8) were from Biolegend. Fluorochrome conjugated CD1d-tetramer (CD1d-Tet) was provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Data was collected on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC, Ashland, OR). The gating strategy used to determine cell populations is shown in Supplemental Figure 3A.

Quantitative real-time PCR

RNA from mouse lungs were reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit or the Taqman Advanced miRNA cDNA synthesis kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using Quant Studio™ 3 system (Applied Biosystems) with validated Taqman primers and Fast Advanced Master Mix. Relative gene expression data (fold change) between samples was accomplished using the $2^{-\Delta\Delta Ct}$ method. GAPDH (for gene expression) or 18S (for miRNA analysis) was used as the internal control.

Western blotting

Cells (3×10^6) isolated from the thymus, spleen and lungs of mice were lysed in RIPA (150 mM NaCl, 1.0% NP-40, 0.5% Sodium-deoxycholate, 0.10% SDS, 50 mM Tris [pH 8.0], 5 mM EDTA, 10 mM NaF, and 10 mM Na-pyrophosphate) containing protease inhibitor cocktail (Roche Applied Sciences; Mannheim, Germany). Proteins (25 μg) were separated on 10% SDS PAGE gels, immunoblotted onto nitrocellulose membranes and probed with the NHERF1 (Abcam, Cambridge, MA, 16 h) and β-Actin antibodies (Cell Signaling Technology). Bound antibodies were detected using the appropriate anti-IgG coupled to HRP (Biorad, Hercules, CA), SuperSignal West Pico and SuperSignal West Femto chemiluminescent substrates (Thermo Fisher Scientific Rockford, IL) and imaged on Amersham Imager 600 (GE Healthcare).

Statistics

All *in vivo* experiments were performed with age- and sex-matched mice and each experiment was repeated 2–4 times with 3–5 mice per cohort. Statistical significance was determined by Student's unpaired *t* test with equal standard deviation or two-way ANOVA with Sidak's multiple comparisons test (for analysis of AHR parameters) using GraphPad Prism software (GraphPad, San Diego, CA). Please see the figure legends for details regarding each experiment. Significance is shown as *$p<0.05$ and **$p<0.01$.

RESULTS

NHERF1 is expressed in the lungs of human asthmatic patients and mice exposed to HDME.

NHERF1 expression levels are altered during inflammation in various cell types and tissues. While systemic inflammation leads to upregulation of NHERF1 in macrophages(9), lower NHERF1 levels are found in the human bronchi of cystic fibrosis patients(11). Additionally, NHERF1 down regulation in the colon is associated with colitis(22). To determine if NHERF1 expression is altered during asthma, we performed qPCR and IHC staining on lung tissue sections from deceased non-asthma human volunteers and asthma patients. NHERF1 (at both the mRNA and protein level) was significantly elevated in the lungs of asthmatic individuals (Figs. 1A, 1B and Supplemental Figure 1). Quantitative analysis of NHERF1 staining in the lung tissue sections from different human donor samples revealed that there was an overall increase in NHERF1 expression in the entire tissue from asthma patients as compared to the lung sections from non-asthma human subjects (Figs. 1C). To

test if NHERF1 levels are also similarly affected in a mouse model of human asthma, we treated wild-type B6 mice with HDME on alternative days (for a total of 7 exposures, Supplemental Figure 2A) and harvested the lung tissue 24 h following the final exposure. Consistent with our results with human lung samples, NHERF1 expression was increased in the lung tissues of mice sensitized with HDME as compared to control animals that received PBS (Figs. 1D–1F).

NHERF1 regulates IgE production and lung inflammation but not AHR in HDME-induced allergic asthma.

As NHERF1 expression was elevated in the HDME-challenged mouse lung tissues, we next determined if it regulated the allergen-induced asthmatic response. We used NHERF1$^{+/-}$ mice (B6 background) for our *in vivo* experiments. These mice have been described previously(17) and we have recently reported that NHERF1 levels are reduced in mast cells cultured from the NHERF1$^{+/-}$ mice(15). Additionally, we observed a reduction in NHERF1 protein levels (by ~50%) in thymocytes, splenocytes and lung mononuclear cells from NHERF1$^{+/-}$ mice (Supplemental Figure 2B). To determine the role of NHERF1 in allergic airway inflammation, we exposed NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice to PBS and HDME as shown in Supplemental Figure 2A. AHR parameters including central airway resistance (Rrs), lung elastance (Ers), Newtonian resistance (R_N), tissue elastance (H) and tissue damping (G) were assessed in response to methacholine (Mch) challenge. Baseline values of the all these parameters were virtually identical in the NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice administered with PBS (Fig. 2A). Compared to PBS controls, HDME-exposed NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice demonstrated elevations in Rrs, Ers, R_N, H and G measurements in a dose-dependent manner over a range of Mch (6.25–100 mg/ml). While the NHERF1$^{+/-}$ mice demonstrated a slightly increased AHR responses at the higher doses of methacholine as compared to the control NHERF1$^{+/+}$ mice, the values were not significant. Thus, our data suggests that NHERF1 does not regulate lung mechanics of the airways in our mouse model.

T2-high allergic asthma is characterized by elevated levels of serum antibodies such as IgE and IgG1 and increased airway inflammation(1). We observed that serum IgE and IgG1 was significantly higher in HDME-treated NHERF1$^{+/+}$ mice as compared to PBS controls (Fig. 2B). There was no difference in either total IgG1 and HDME-specific IgG1 levels between HDME-treated NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice (Figs. 2B and 2C). In contrast, NHERF1$^{+/-}$ mice had lower total serum IgE levels following HDME exposure. Furthermore, HDME-specific IgE levels were also significantly decreased in the HDME NHERF1$^{+/-}$ mice (Fig. 2C). suggesting that NHERF1 regulates IgE levels in allergic asthma. HDME treatment also resulted in a significant increase in airway inflammation in NHERF1$^{+/+}$ mice as shown by the intense H&E staining especially near the peri-bronchial and perivascular areas of the lung tissue (Figs. 3A and 3B). However, this inflammation was attenuated in the NHERF1$^{+/-}$ mice exposed to HDME (Figs. 3A and 3B).

NHERF1 promotes infiltration of eosinophils and lymphocytes in an HDME-induced asthma model.

Airway inflammation is characterized by an influx of inflammatory cells such as monocytes, eosinophils, neutrophils and lymphocytes into the lungs. To determine whether the

decreased lung pathology observed in NHERF1$^{+/-}$ mice was due to a reduction in the recruitment of inflammatory cells to the airways, we first harvested the BALF and analyzed for total and differential inflammatory cell counts (Fig. 4A). Following exposure to HDME, an acute inflammation was seen in the BALF of NHERF1$^{+/+}$ mice. The inflammatory infiltrate consisted of eosinophils (~60%) along with lower percentages of monocytes and lymphocytes. Consistent with lung histology, NHERF1$^{+/-}$ HDME mice exhibited reduced total BALF cell counts (Fig. 4A). Specifically, eosinophil and lymphocyte counts were significantly lower in the BALF of NHERF1$^{+/-}$ HDME mice, with no change in monocyte counts (Fig. 4A). We were unable to detect any neutrophils in the BALF of mice exposed to HDME. We also characterized the immune cell populations in the lungs of these mice by flow cytometry (the gating strategy used is shown in Supplemental Figure 3A and 3B). We observed a significant reduction in T cells (both CD4 and CD8), and eosinophils in the lungs of NHERF1$^{+/-}$ HDME mice as compared to NHERF1$^{+/+}$ HDME mice (Fig. 3B). Additionally, there was a reduction in neutrophils in the lungs of NHERF1$^{+/-}$ HDME. This is surprising given that we were unable to detect neutrophils in the BALF. We are currently unsure why there are no neutrophils in the BALF, but we speculate that in our model, the kinetics of neutrophil migration and extravasation is such that it is only detected in the lungs and not in the BALF. Our data is consistent with a previously published HDME-induced mouse model of asthma(23) where the presence of neutrophils in the BALF decreased dramatically within a week after allergen administration. There was no difference observed in other immune cell populations such as B, NK, NKT, macrophages or DCs (Fig. 3B). Importantly, the homeostatic immune cell populations in the lungs of NHERF1$^{+/-}$ were identical to the cell counts in the lungs of NHERF1$^{+/+}$ animals (Supplemental Figure 3C and 3D) suggesting that NHERF1 does indeed promote infiltration of eosinophils, lymphocytes and neutrophils into the lung following HDME exposure. Furthermore, analysis of chemokines and chemokine receptors that attract neutrophils, lymphocytes and eosinophils to the lungs revealed that *Cxcl1* (keratinocytes-derived chemokine (KC)), *Cxcl2* (macrophage inflammatory protein 2-alpha, (MIP2-α)), *Cxcl5* (epithelial-derived neutrophil-activating peptide 78, (ENA-78)) (Fig. 5A), *Ccr8* (Fig. 5B), *Ccl3* (MIP-1-α), *Ccl4* (MIP-1-β), *Ccl5* (regulated on activation, normal T cell expressed and secreted, RANTES), *Ccl11* (eotaxin-1) and *Ccl24* (eotaxin-2) (Fig. 5C) were significantly reduced in NHERF1$^{+/-}$ HDME mice.

NHERF1 modulates inflammatory cytokines and alarmins in HDME-induced allergic asthma.

HDME treatment results in a T2-high asthma endotype, so we next analyzed Th2 cytokines in the lung tissues of different cohorts of mice (Fig. 6A). As expected the mRNA levels of *Il4*, *Il5* and *Il13* were elevated in the lungs of HDME-exposed NHERF1$^{+/+}$ mice as compared to PBS controls. However, the mRNA expression of these cytokines was significantly reduced in the lungs of NHERF1$^{+/-}$ HDME mice (Fig. 6A). Interestingly, though the expression of *Il10* was unaffected by HDME treatment in NHERF1$^{+/+}$ mice, it was significantly increased in the lungs of NHERF1$^{+/-}$ HDME mice suggesting that NHERF1 reciprocally regulates Th2 cytokines (IL-4, IL-5 and IL-13) and IL-10 in our mouse model.

A previous report showed that cytokines of the IL-17 family are also increased in BALF samples from patients with asthma(24). Accordingly, we observed higher levels of *Il17a* and *Il17f* in the lungs of HDME-exposed NHERF1$^{+/+}$ mice (Fig. 6B). However, only *Il17a* was reduced in the NHERF1$^{+/-}$ mice. Furthermore, epithelial cell-derived cytokines such as IL-25 and IL-33 (also known as alarmins) play a major role in the initiation of Th2-high asthma(25). We observed a significant increase in both *Il25* and *Il33* levels in HDME-treated NHERF1$^{+/+}$ mice that was reduced in NHERF1$^{+/-}$ HDME mice. (Fig. 6B). Consistent with the gene expression data, we also observed a reduction in both IL-4 and IL-13 protein levels in the BALF of NHERF1$^{+/-}$ HDME mice as compared to allergen-challenged NHERF1$^{+/+}$ mice (Fig. 6C). Additionally, this defect in IL-4 and IL-13 cytokine production was also observed when lung mononuclear cells from HDME-treated NHERF1$^{+/-}$ mice are re-exposed to the allergen *in vitro* (Fig. 6D). Taken together, our studies reveal that NHERF1 regulates asthma pathogenesis by modulating the levels of inflammatory cytokines and alarmins.

HDME-induced gene expression of polymeric mucins and goblet cell hyperplasia is reduced in NHERF1$^{+/-}$ mice.

Mucus hypersecretion and airflow obstruction occurs primarily due to an increase in numbers of goblet cells (goblet cell hyperplasia) in the lungs. Additionally, the presence of increased levels of Th2 cytokines such as IL-13 is a major contributor of goblet cell hyperplasia during asthma(26). We analyzed goblet cell hyperplasia through PAS staining of lung sections from NHERF1$^{+/+}$ and NHERF1$^{+/-}$ HDME-treated mice. Consistent with the diminished Th2 cytokine (IL-13) expression, HDME-treated NHERF1$^{+/-}$ mice exhibited reduced PAS staining (Fig. 7A). We also quantified percent area of PAS+ staining and PAS+ cell count and observed a decrease in these parameters in the HDME-challenged NHERF1$^{+/-}$ mice (Fig. 7B). The polymeric mucins, Muc5ac and Muc5b are primarily responsible for mucus production in mouse models of asthma(27). We observed that both *Muc5ac* and *Muc5b* gene transcripts were significantly upregulated in the lungs of HDME-treated mice (Fig. 7C). In agreement with the PAS histology data (Fig. 7A), mRNA levels of *Muc5ac* and *Muc5b* were decreased in HDME-treated NHERF1$^{+/-}$ mice.

NHERF1 regulates various miRNA expression in asthma.

Several miRNAs have been reported to play a protective or detrimental role in mediating allergic asthma. As shown in Fig. 8, we screened for various miRNAs that have known roles in different aspects of asthma pathogenesis. Both NHERF1$^{+/+}$ and NHERF1$^{+/-}$ PBS controls had comparable levels of most miRNAs analyzed. Furthermore, most of these miRNAs with the exception of miR-142-3p and miR-126-5p, were increased in NHERF1$^{+/+}$ HDME mice in comparison to their PBS controls. Strikingly, NHERF1$^{+/-}$ HDME-treated mice had higher expression of miRNAs that regulate mucus production(28) (miR-145-5p) (Fig. 8A), inflammation(29) (miR-155-5p and miR-221-3p) (Fig. 8B), Th2 effector functions(30) (miR-126-53p and miR-126-5p) (Fig. 8D) and IL-13 cytokine production(31) (Let-7g-5p) (Fig. 8E). However, miR-142-3p (that has been previously reported to regulate airway smooth muscle cell proliferation(32)) was unaltered in the NHERF1$^{+/-}$ HDME-treated mice (Fig. 8C). Thus, our data suggests that NHERF1 regulates airway inflammation and Th2

cytokine production at least in part via modulating miRNA expression in HDME-induced allergic asthma model.

DISCUSSION

We have previously demonstrated that NHERF1 promotes mast cell response to the C3a receptor(13) and the IgE receptor(14), both of which, play an important role in mediating allergic reactions in humans. In the current study, we uncover a novel role for NHERF1 in regulating airway inflammation in a T2-high HDME mouse model of asthma. Specifically, all characteristic features of airway inflammation such goblet cell hyperplasia, HDME-specific serum IgE levels and Th2 cytokines in the lungs were significantly attenuated in mice that had reduced levels of NHERF1. Additionally, there was a substantial reduction in lung eosinophils and lymphocytes in NHERF1$^{+/-}$ mice exposed to the allergen HDME. Interestingly, we observed that miRNAs that regulate mucus production, inflammation, Th2 effector functions, and IL-13 levels were altered in these mice suggesting that NHERF1 possibly regulates airway inflammation by modulating the levels of these miRNAs. Given that we have used NHERF1$^{+/-}$ mice for all of our experiments, our study highlights a key role for NHERF1 and provides evidence that even a partial reduction in NHERF1 levels is enough to attenuate T2-high asthma.

NHERF1 is expressed in many different cell types and it is upregulated in certain pathological conditions such as cancer(33). It is also abundantly expressed in T cells and mast cells(4), two critical cell types that have been associated with allergy in humans. However, it is currently unclear if the expression levels of NHERF1 are altered during inflammation. Previous studies showed that NHERF1 levels are increased in macrophages following systemic inflammation(9) while cystic fibrosis patients have reduced NHERF1 levels in their bronchi compared to heathy individuals(11). We observed enhanced NHERF1 staining intensity in the lung sections from human asthma patients (Fig. 1C). NHERF1 was also significantly elevated in the lungs of mice that were treated with HDME. The enhancement in NHERF1 expression could be due to multiple reasons. For e.g., it is possible that the overall increase in NHERF1 levels may be because of the influx of inflammatory cells into the lungs that express NHERF1. Alternatively, NHERF1 may be upregulated in different lung cell types such as alveolar macrophages, airway epithelial cells and smooth muscle cells following asthma induction. While it was difficult to determine if NHERF1 was upregulated in different cell types in the asthmatic lungs, we did observe a moderate enhancement of NHERF1 staining in the epithelial cells around the airways in both the human and mouse asthma samples (Fig. 1 and Supplemental Figure 1). Additionally, we also observed areas of NHERF1 staining in the inflammatory foci around the airways (Fig. 1). Our data possibly suggests that the increase in NHERF1 levels in the lungs is a result of both elevated NHERF1 expression in different cell types and increased migration of immune cells into the lungs and further experiments need to performed to test this contention.

NHERF1 has previously been shown to bind to ezrin and α-actinin via PDZ domain interactions(34, 35). Because NHERF1 tethers to these cytoskeletal proteins, it plays an important role in regulating the migratory activity of cells. Additionally, NHERF1 interacts with certain chemokine receptors such as CXCR2 and affect the migration of

neutrophils(36). We observed reduced eosinophil, neutrophil and lymphocyte recruitment in the lungs of NHERF1$^{+/-}$ mice treated with HDME. Additionally, the expression of chemokines and chemokine receptors that are responsible for the migration of these cells to lungs such as CXCL1, CXCL2, and CXCL5 (chemoattractant for neutrophils), CCR8 (chemoattractant receptor for lymphocyte), CCL3, CCL4, CCL5, CCL11 and CCL24 (chemoattractant for eosinophils and lymphocytes)(37, 38) were also decreased in the lungs of these mice. Thus, reduced levels of these chemotactic mediators may account for the decreased immune cell counts in the lungs of allergen-treated NHERF1$^{+/-}$ mice. It is also possible that the reduction in leukocyte numbers in the lungs of NHERF1$^{+/-}$ mice is actually because of altered migratory ability of these cells due to improper cytoskeletal protein composition and/or abrogation of chemokine receptor signaling. Whether these or other mechanisms contribute to the attenuated airway leukocyte infiltration in the lungs of NHERF1$^{+/-}$ mice remains to be elucidated.

An unexpected finding of the current study is that allergen-induced AHR is not altered in NHERF1$^{+/-}$ mice although the airway inflammation was substantially attenuated. While inflammation plays a critical role in mediating AHR, other mechanisms that regulate airway smooth muscle contraction and relaxation do exist(39). We used methacholine, a muscarinic receptor agonist to induce bronchoconstriction in our mouse model. The AHR response is mediated by the contraction of the airway smooth muscle cells that is dependent on both the muscarinic receptor activation as well as the physical blockade of airways due to inflammation and mucus secretion. M2 and M3 muscarinic receptors are GPCRs that are expressed in the airway smooth muscle cells(40, 41). Thus, we posit that NHERF1 directly affects the M2 and M3 muscarinic receptor activation and inhibits bronchoconstriction by airway smooth muscle cells. This response is reduced in NHERF1$^{+/-}$ mice resulting in enhanced bronchoconstriction that counterbalances the effects of decreased inflammation on AHR. Thus, overall, we see no difference in AHR between the NHERF1$^{+/-}$ and NHERF1$^{+/+}$ mice. Methacholine-induced bronchoconstriction is reversed by epinephrine-β_2AR-mediated relaxation of the airway smooth muscle cells(42). Since NHERF1 promotes β_2AR signaling, it is also plausible that the bronchorelaxation response is abrogated in the NHERF1$^{+/-}$ mice. Specific deletion of NHERF1 in murine airway smooth muscle cells will shed more light into the mechanisms by which NHERF1 regulates AHR responses independent of inflammation during asthma.

Consistent with reduced airway inflammation and leukocyte infiltration into the lungs, we observed reduced expression of Th2 cytokines such as IL-4, IL-5 and IL-13 in lungs as well as in BALF of NHERF1$^{+/-}$ mice exposed to HDME. Additionally, we also observed decreased IL-4 and IL-13 production by lung mononuclear cells obtained from NHERF1$^{+/-}$ mice following re-exposure to HDME. These data suggest that NHERF1 regulates Th2 cytokine production *in vitro* and *in vivo* and is in line with previous reports from our laboratory and others that have demonstrated that this adapter molecule regulates cytokine (IL-6) production in mast cells(13, 14) and airway smooth muscle cells(12). IL-4 and IL-13 cause immunoglobulin class switch to IgE during asthma. In agreement with this, IgE levels were reduced in the serum of NHERF1$^{+/-}$ mice. Several reports have highlighted the role of IL-13 on mucus production during asthma. Specifically, previous reports have shown that transgenic mice overexpressing IL-13 have increased mucus production(43, 44) whereas

Il13$^{-/-}$ mice have a marked reduction in mucus secretion(45). Herein, we demonstrate that gene expression of the polymeric mucins, Muc5ac and Muc5b, mucus production and goblet cell hyperplasia are significantly reduced in the lungs of allergen-treated NHERF1$^{+/-}$ mice. An interesting finding of our study is that the IL-10 levels are significantly upregulated in the lungs of NHERF1$^{+/-}$ mice exposed to HDME. Earlier studies that examined the role of IL-10 in allergic mouse models have shown that IL-10 primarily functions as a suppressive cytokine. For e.g., co-instillation of IL-10 by the intranasal route significantly inhibits Th2 cytokines and lung eosinophilia in allergic mice(46). Additionally, allergen-induced eosinophilic inflammation, IL-5 production and AHR were reduced in mice that lacked IL-10(47). IL-10 also attenuates Th2 effector functions and decreases IL-4 and IL-13 production by these cells in an allergic dermatitis model(48). Collectively, our data suggests that the increased IL-10 levels in allergen exposed NHERF1$^{+/-}$ mice may in part be responsible for the reduced Th2 cytokines and attenuated inflammation observed in these mice.

Airway epithelial cells produce alarmins such as IL-25 and IL-33 following allergen exposure. These cytokines promote Th2-associated immune responses via maturation and activation of effector Th2 cells, innate lymphoid cells and other innate immune cells such as eosinophils(25). In the current study we observed that these cytokines were significantly downregulated in HDME-exposed NHERF1$^{+/-}$ mice. Recent evidence suggests that cytokines of the IL-17 family are also increased in BALF samples from patients with asthma(24). Specifically, IL-17A levels are upregulated in these individuals and IL-17 polymorphisms is frequently associated with asthma(49). In addition, IL-17A was originally proposed to contribute to airway neutrophilia(50). Human airway epithelial cells stimulated with IL-17A produced IL-8 that is chemotactic for neutrophils(51). In agreement with these studies, we observed a significant reduction in IL-17A levels of HDME-exposed NHERF1$^{+/-}$ mice that corelated with reduced neutrophils in the lungs of these mice. Collectively our data suggests that NHERF1 modulates the effector functions of different cell types specifically the airway epithelial cells and leukocytes ultimately resulting in decreased airway inflammation.

Several miRNAs that have been implicated in the regulation of asthma pathogenesis were increased in HDME-treated NHERF1$^{+/-}$ mice. Elevated levels of miR-145-5p downregulates Muc5ac expression in an OVA-induced model of allergy(28). Both miR-155-5p and miRNA 221-3p play varied roles in asthma in many different cell types and affect airway inflammation and remodeling(29, 52). Additionally, Let-7 miRNAs can directly target IL-13 transcripts and intranasal delivery of Let-7 miRNA attenuates allergen-induced AHR in ovalbumin-treated mice(31). Thus, it is plausible that increased Let-7g-5p miRNA in HDME-treated NHERF1$^{+/-}$ mice downregulates IL-13, that in turn ameliorates goblet cell hyperplasia via regulation of Muc5ac and Muc5b production. These data suggest that NHERF1 modulates allergen-induced airway immune response via altering the expression of various miRNAs. In a recent publication(15), we showed that NHERF1 expression in the nucleus is enhanced with 15 mins following mast cell activation suggesting that it probably rapidly translocates to the nucleus. Therefore, it is quite possible that NHERF1 regulates the expression of the miRNAs directly by interacting with them and targeting them for degradation or NHERF1 mediates its effects by affecting the transcription machinery

required for miRNA production. Alternatively, NHERF1 can indirectly regulate miRNA expression via cytokines. Consistent with this notion, it has been shown previously that miRNAs can regulate and in turn be regulated differentially by pro- and anti-inflammatory cytokines(53–56). The exact mechanism by which NHERF1 regulates the expression of various miRNAs in specific immune cell types, that in turn, control HDME-induced allergic response will be the subject of our future investigation.

In summary our study provides evidence that a partial reduction in NHERF1 attenuates airway inflammation, mucus production, Th2 cytokines and serum IgE levels in a T2-high mouse model of asthma. Surprisingly, NHERF1 has little effect on allergen-induced AHR, and therefore examination of the role of NHERF1 in airway smooth muscle excitation-contraction coupling warrants further study. Nevertheless, we believe that targeting NHERF1 will modulate airway inflammation in T2-high asthma and uncover molecular mechanisms by which airway inflammation can be uncoupled from AHR. Further investigation into the role of NHERF1 in cell-specific targets using conditional deletion models will provide valuable insights to delineate the pathophysiology of asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points:

- NHERF1 promotes airway inflammation in a HDME mouse model of asthma
- NHERF1 regulates the levels of Th2 cytokines in the lungs of mice exposed to HDME

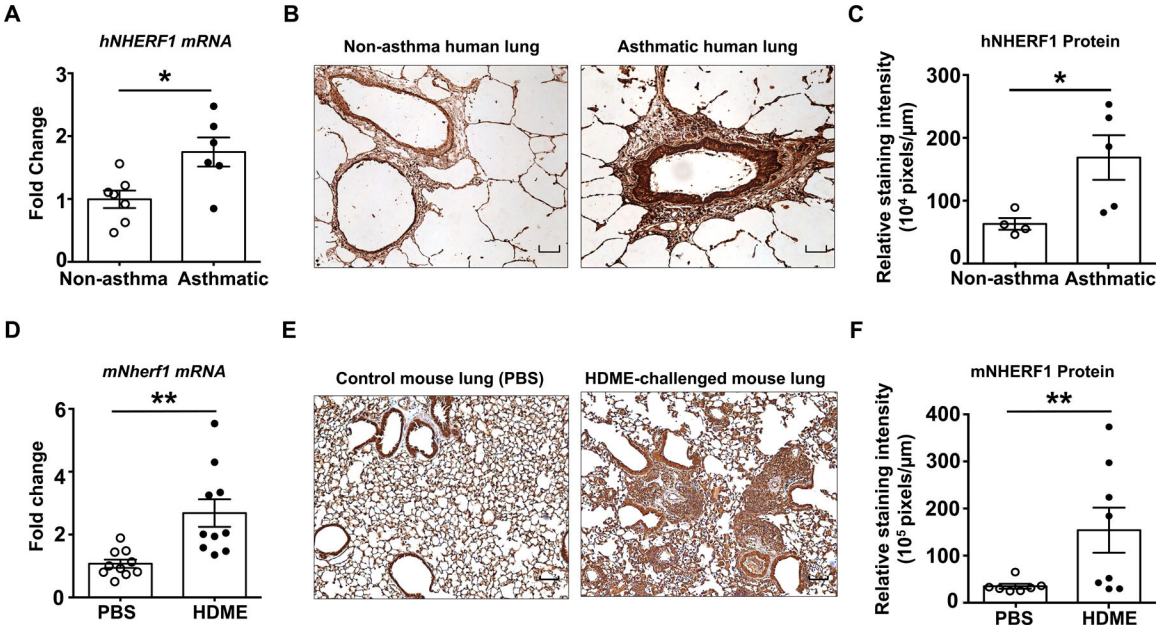

Figure 1. Increased levels of NHERF1 in the lungs of human asthmatics and HDME-challenged mice.

(A) Whole lung tissue from non-asthma donors and asthma patients were analyzed for gene expression of human (h) NHERF1. Data is shown as mean fold change ± SEM with a total of 6–7 samples per cohort. (B, C) Immunohistochemistry (IHC) was performed to determine NHERF1 levels in the lungs of non-asthma donors and asthma patients. (B) Representative images from a 4–5 donors for each group are shown. Scale bar = 100 μm. (C) Relative staining intensity of NHERF1 expression from IHC images was analyzed using Image J analysis software and is shown. (D-F) WT B6 mice were challenged intranasally (i.n.) with PBS or HDME (50 μg) on alternate days for a total of 7 injections. Twenty-four h after the last treatment mice were euthanized and the lung tissue was harvested and (D) analyzed for gene expression of mouse (m)NHERF1. Data is shown as mean fold change ± SEM from 10 samples per cohort. (E, F) IHC of mouse lung tissues were performed and (E) representative images of NHERF1 staining in the lungs of 7–8 mice from each cohort is shown. Scale bar = 200 μm. (F) Relative staining intensity of NHERF1 expression in the mouse lung samples was calculated using image J and is shown. Statistical significance was determined by using Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

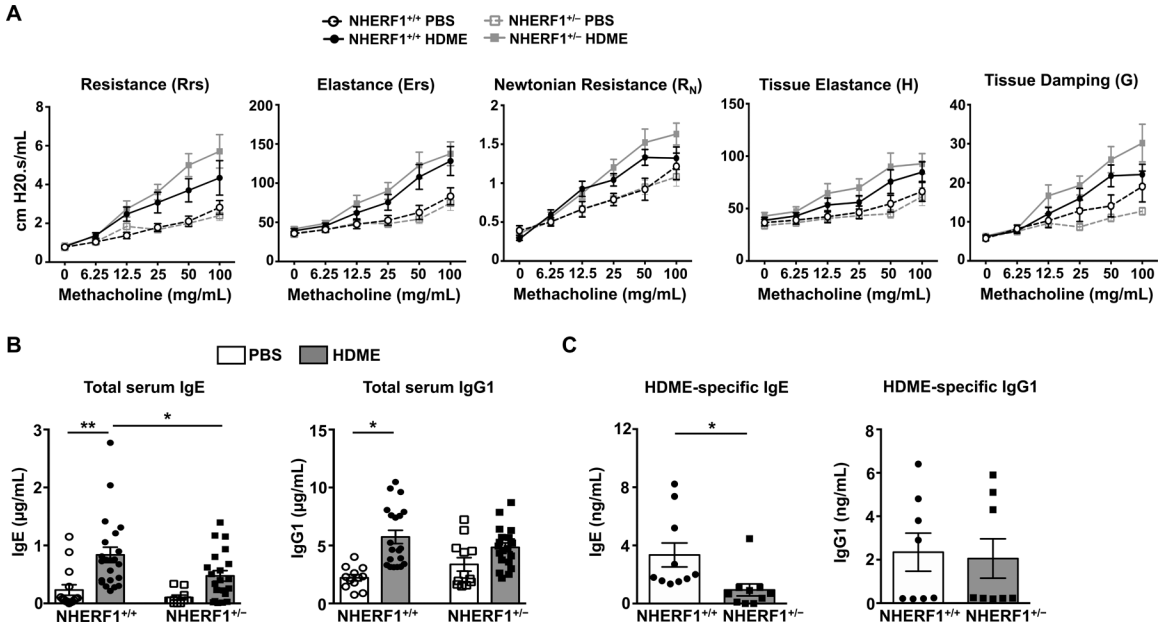

Figure 2. NHERF1 promotes IgE production but does not regulate AHR in HDME-induced mouse model of asthma.

(A) PBS control or HDME-exposed NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice were anesthetized 24 h after the last HDME challenge and analyzed for AHR. Airway resistance (Rrs), elastance (Ers), Newtonian resistance (R_N), tissue elastance (H) and tissue damping (G) after challenge with increasing doses of methacholine is shown. Data are presented as mean ± SEM and pooled from 3 experiments with a total of 9–12 mice for the PBS groups and 10–18 mice for the HDME groups. Statistical analysis was performed by two-way ANOVA with Sidak's multiple comparisons test for determining the p values. (B, C) Levels of (B) total serum IgE and serum IgG1 and (C) HDME-specific serum IgE and HDME-specific serum IgG1 in NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice treated with PBS or challenged with HMDE. Data are presented as mean ± SEM and are pooled from 3 independent experiments with a total of 8–22 (B) or 8–10 (C) mice per cohort. Statistical significance was determined Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

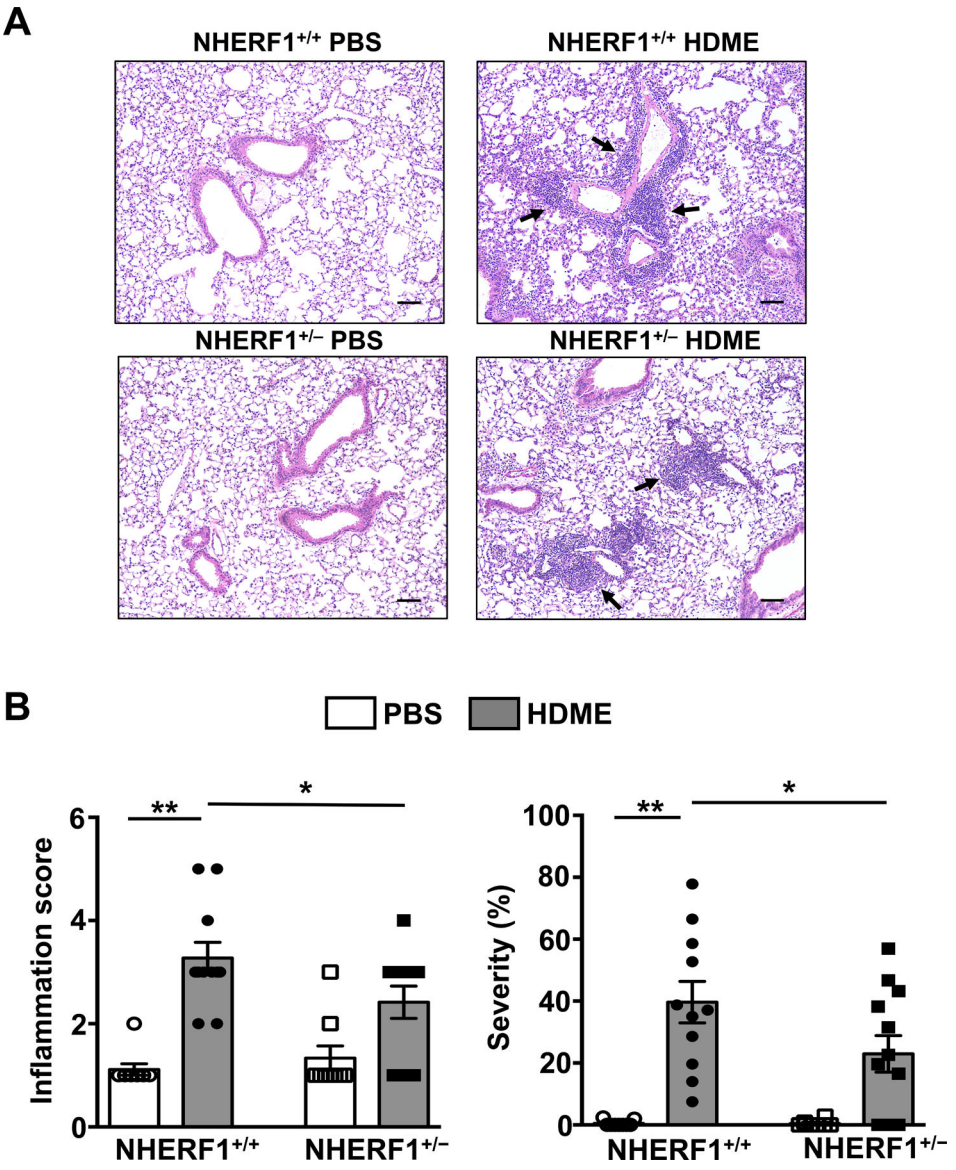

Figure 3. Lung inflammation is decreased in NHERF1$^{+/-}$ mice exposed to HDME.

(A) Hematoxylin and eosin (H&E) staining of lung section of NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice administered with PBS or HDME intranasally. Scale bar=100 μm. Representative images of the lungs at 10X magnification is shown. Black arrows indicate cellular infiltration (inflammation) around the bronchioles and blood vessels. (B) Bronchial inflammation and severity scores in PBS and HDME-treated NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice are shown. Data are presented as mean ± SEM and are pooled from 2–3 independent experiments with a total of 8–12 mice per cohort. Statistical significance was determined Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

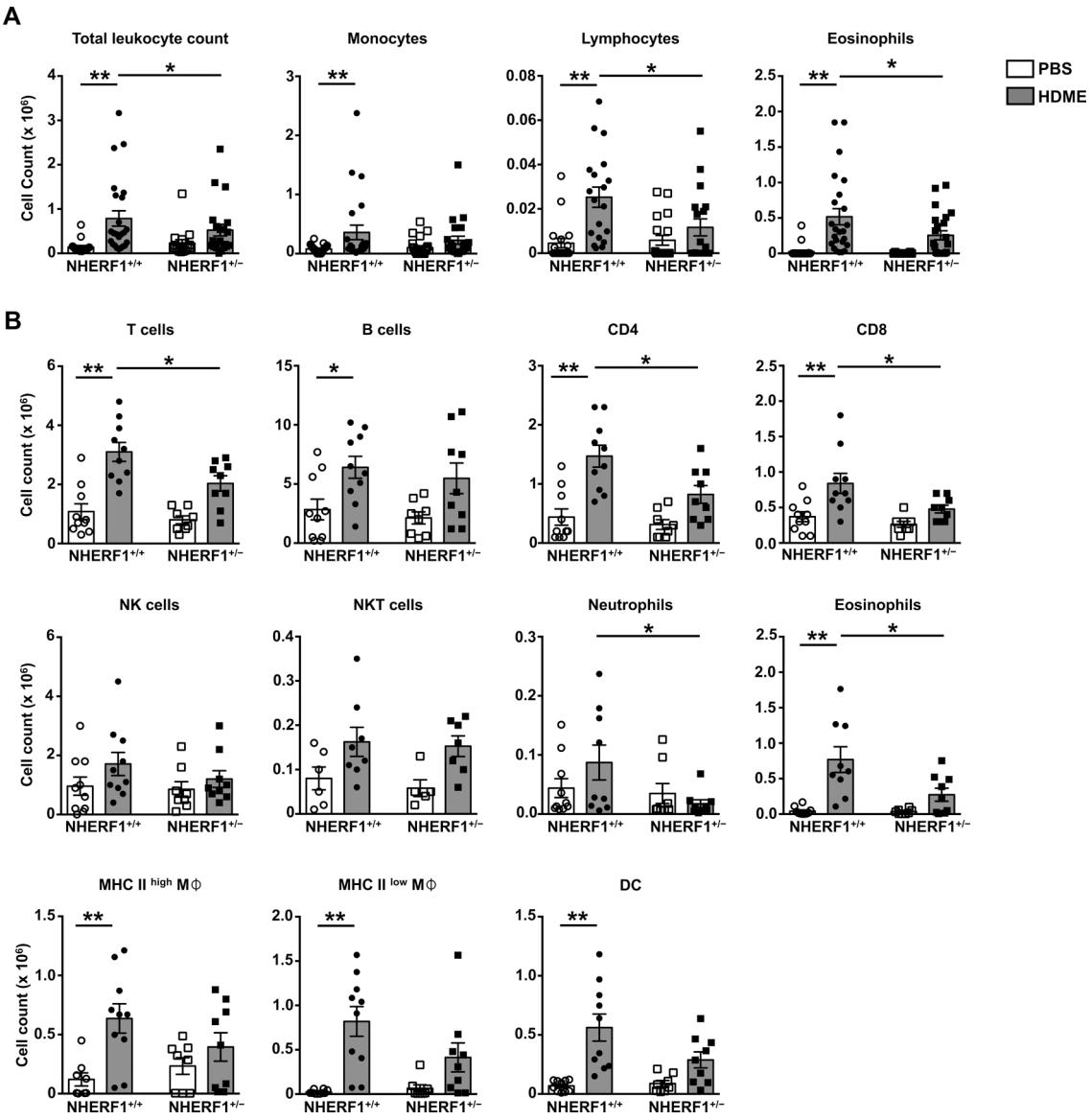

Figure 4. Reduced cellular infiltration in NHERF1$^{+/-}$ mice challenged with HDME.

(A) BALF from NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice administered with PBS or challenged with HDME intranasally were analyzed for total leukocyte counts and differential cell counts of monocytes, lymphocytes and eosinophils. (B) Lung tissues from PBS or HDME-exposed NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice were analyzed for immune cell distribution using flow cytometry. Bar graphs show various lymphocyte population (T, B, CD4, CD8, NK and NKT cells) and granulocyte population (neutrophils, eosinophils, macrophages (MΦ) and dendritic cell (DC) subsets). Data is shown as mean ± SEM pooled from 3 independent experiments with a total of 6–22 mice per cohort. Statistical significance was determined by Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

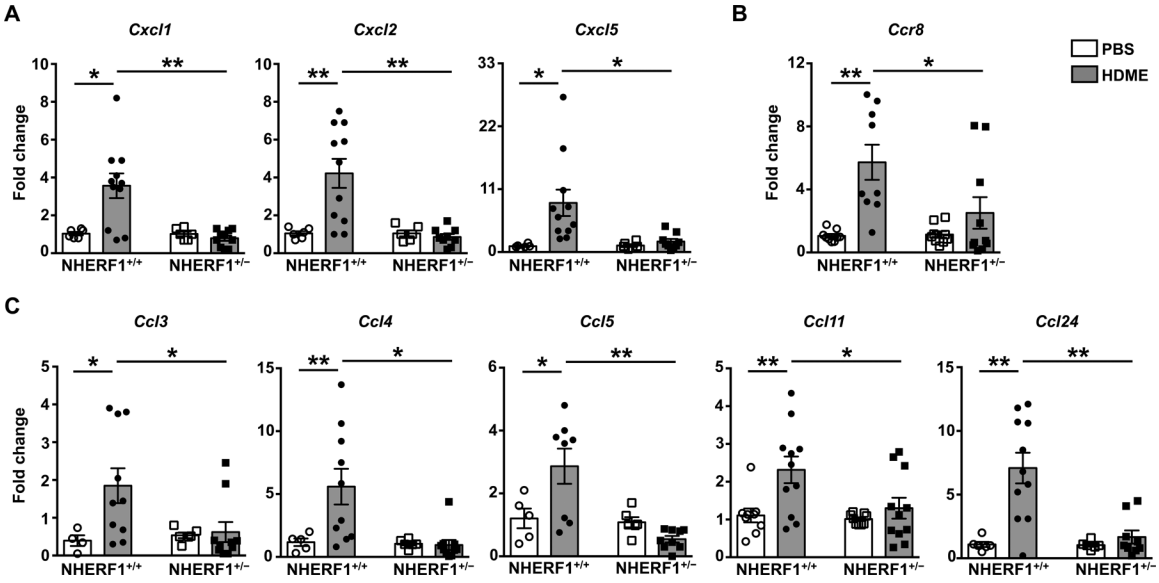

Figure 5. Attenuated chemokine and chemokine receptor expression in NHERF1$^{+/-}$ mice exposed to HDME.

Lung tissue homogenates from NHERF1$^{+/+}$ and NHERF$^{+/-}$ mice exposed to PBS or HMDE intranasally were analyzed for mRNA levels of (A) CXCL1, CXCL2 and CXCL5, (B) CCR8 and (C) CCL3, CCL4, CCL5, CCL11 and CCL24 by qPCR. Data is shown as mean fold change ± SEM and pooled from 3 independent experiments with a total of 4–11 mice per cohort. Statistical significance was determined by Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

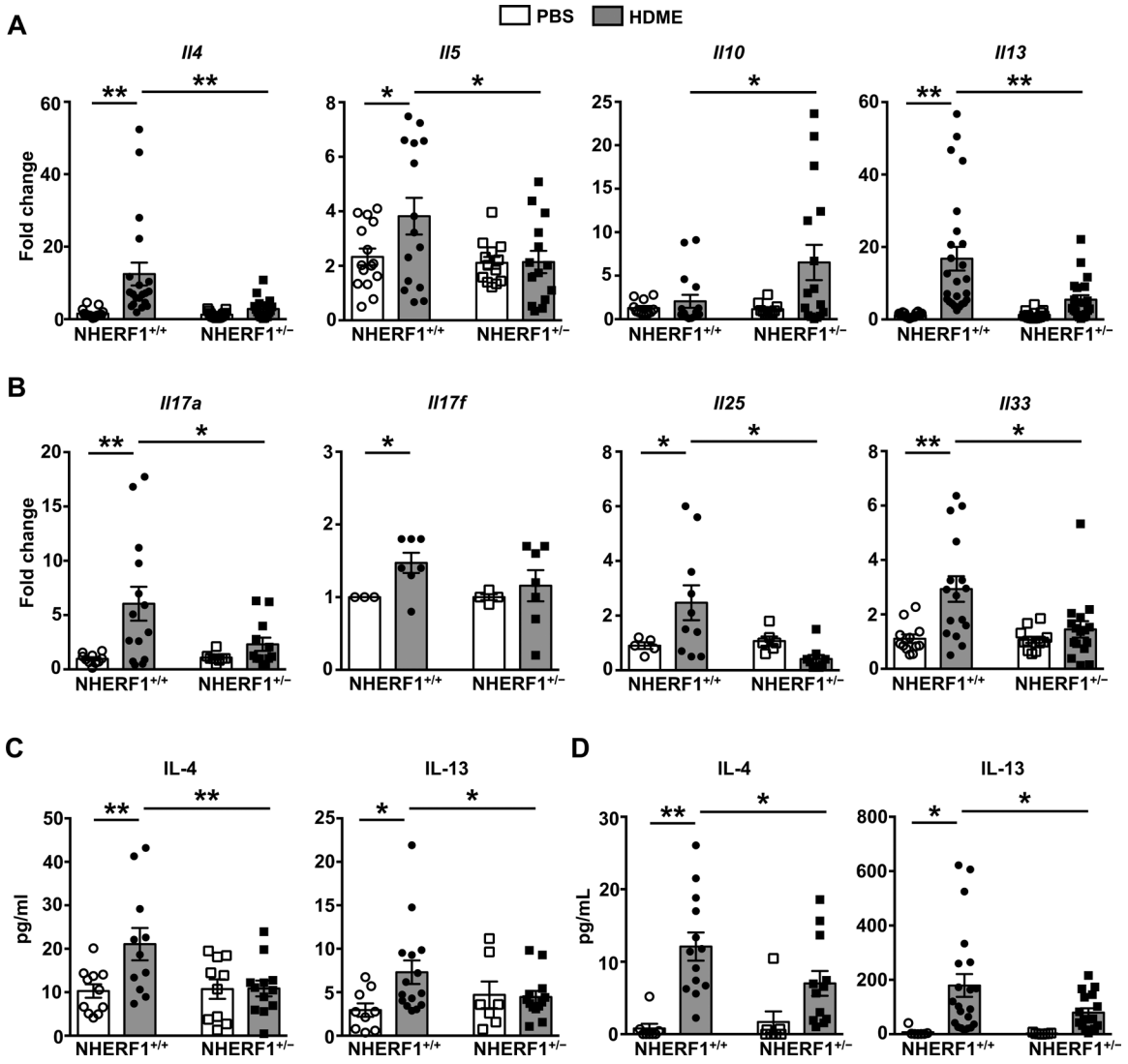

Figure 6. NHERF1$^{+/-}$ mice challenged with HDME have reduced inflammatory cytokines in lungs.

(A-C) Lungs from NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice administered with PBS or HDME intranasally were analyzed for gene expression of (A) Th2 cytokines (*Il4, Il5, Il10* and *Il13*). (B) Th17 family of cytokines (*Il17a* and *Il17f*), and alarmins (*Il25*, *Il33*). Data is shown as mean fold change ± SEM and pooled from 3 independent experiments with a total of 10–16 mice per cohort. (C) IL-4 and IL-13 cytokine levels in the BALF supernatant of NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice challenged or not with HMDE is shown. (D) Lungs lymphocytes were harvested from NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice exposed to PBS or HDME. The cells were re-exposed to HDME and IL-4 and IL-13 cytokine levels in the supernatant of were estimated. Data for (C) and (D) is shown as mean ± SEM and pooled from 3 independent experiments with a total of 3–20 mice per cohort Statistical significance was determined by Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.

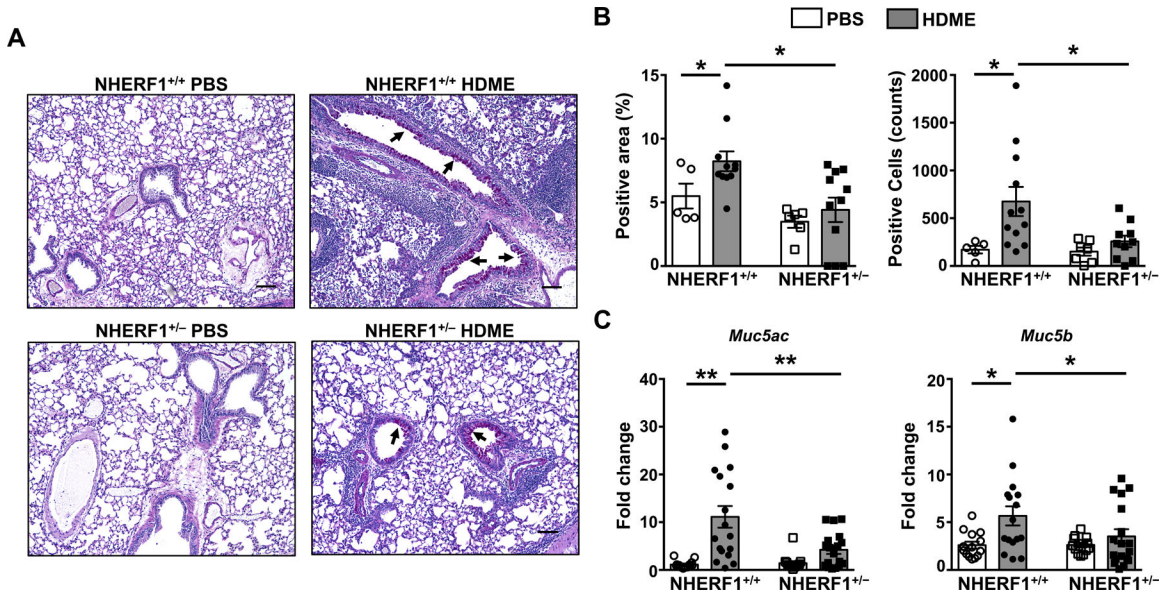

Figure 7. Reduced goblet cell hyperplasia and mucin production in the lungs of HDME-challenged NHERF1$^{+/-}$ mice.

NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice were challenged with PBS or HDME. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for PAS staining (A, B) and gene expression (C) of polymeric mucins (*Mus5ac* and *Muc5b*). Representative lung images (A, 4X magnification, arrows indicate PAS staining) and % PAS positive area and PAS positive cell counts (B) are shown. Scale bar=100μm. Data in B, and C are presented as mean ± SEM and are pooled from 3 independent experiments with a total of 5–18 mice per cohort. Statistical significance was determined using Student's unpaired *t* test. *****p≤ 0.05, ******p≤ 0.01.

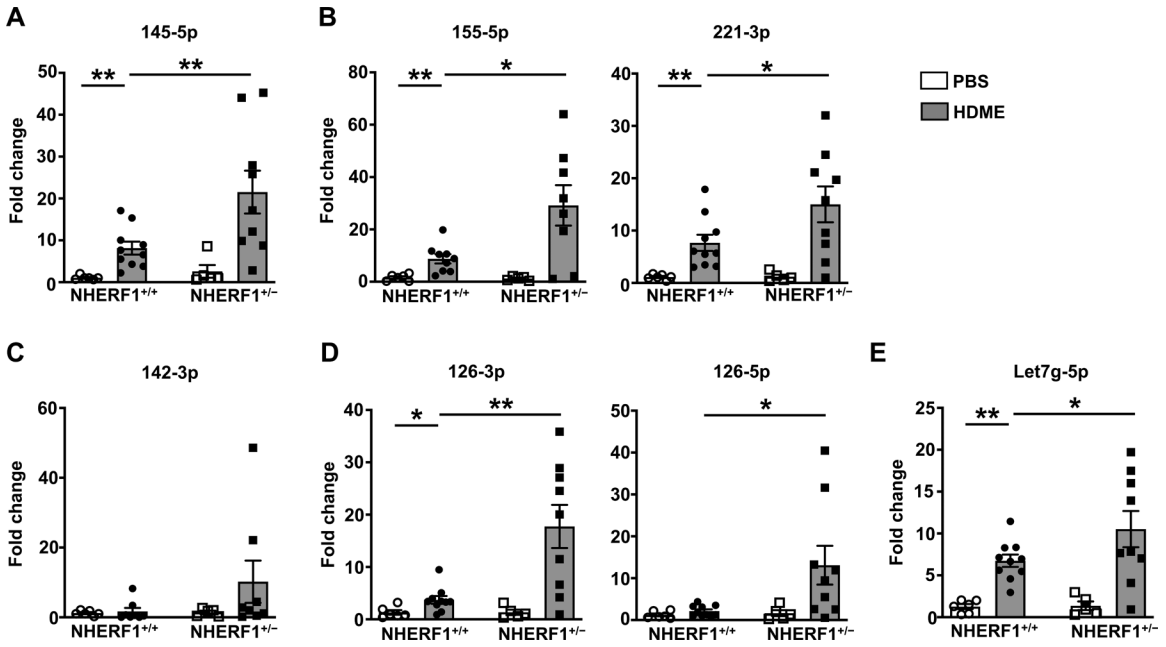

Figure 8. Expression levels of various miRNAs in the lungs of NHERF1$^{+/-}$ mice challenged with HDME.

Lung tissues of PBS- or HDME-treated NHERF1$^{+/+}$ and NHERF1$^{+/-}$ mice were analyzed for gene expression of various miRNAs as indicated in the figure. Fold change in expression of (A) 145–5p, (B) 155–5p and 221–3p, (C) 142–3p, (D) 126–3p and 126–5p, and (E) Let7g-5p in the lungs is shown. Fold change for each genotype was normalized to respective PBS control mice. Data is presented as mean ± SEM and are pooled from 3 independent experiments with a total of 5–10 mice per cohort. Statistical significance was determined using Student's unpaired *t* test. *p≤ 0.05, **p≤ 0.01.