

Deletion of *Arno* Reduces Eosinophilic Inflammation and Interleukin-5 Expression in a Murine Model of Rhinitis

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Abstract

Background: ARF nucleotide-binding site opener (ARNO) is a guanine nucleotide-exchange factor for ADP-ribosylation factor proteins. ARF nucleotide-binding site opener also binds MyD88, and small-molecule inhibition of ARNO reduces inflammation in animal models of inflammatory arthritis and acute inflammation. However, whether genetic deletion of *Arno* in mice reduces pathologic inflammation has not yet been reported. Furthermore, its role in the nasal cavity has yet to be investigated. **Objective:** To generate *Arno* knockout mice and to determine whether genetic loss of ARNO reduces eosinophilic inflammation in the ovalbumin (OVA) murine model of rhinitis. **Methods:** *Arno* knockout mice were generated and wild type and knockout littermates were subjected to the OVA-induced mouse model of rhinosinusitis. Eosinophilic inflammation was assessed through immunofluorescent quantification of EMBP⁺ eosinophils in the septal mucosa and cytokine expression was assessed by quantitative polymerase chain reaction. **Results:** *Arno* knockout mice are viable and fertile without any noted deficits. *Arno* wild type and knockout mice subjected to the OVA-induced model of rhinitis demonstrated an average of 314.5 and 153.8 EMBP⁺ cells per mm² septal tissue, respectively ($P < .05$). Goblet cells per mm of basal lamina were assessed via Alcian blue and there was no statistically significant difference between *Arno* wild type and knockout mice. Ovalbumin-induced expression of interleukin-5 (IL-5) was significantly reduced in *Arno* knockout mice ($P < .05$). There was no statistically significant reduction in IL-4, IL-13, or eotaxin-1 expression. **Conclusions:** These data demonstrate that deletion of *Arno* reduces eosinophilic inflammation and IL-5 expression in an OVA-induced model of rhinitis.

Keywords

ARNO, cytohesin-2, eosinophil, interleukin-5, ovalbumin, rhinitis

Introduction

Allergic rhinitis (AR) is a prevalent immunoglobulin E (IgE)-mediated condition of the nasal cavity.^{1,2} Characteristic symptoms of AR include itching, sneezing, nasal congestion, and rhinorrhea. Allergic rhinitis is classified based on the overall occurrence of the symptoms (perennial vs seasonal), the frequency (intermittent vs persistent), and the severity.^{1,2} The estimated direct costs of AR ranges from \$1.9 to \$3.4 billion of which nearly half is attributed to prescription medications.³ Commonly used treatments include intranasal steroids, antihistamines, immunotherapy, and others.^{4–6} Allergic rhinitis in patients and in animal models is also characterized by a release of multiple cytokines and chemokines including interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13), eotaxin-1, and others.^{1,7} Furthermore, there is often an accumulation of

eosinophils in the nasal cavity which may further drive the disease pathophysiology.⁸

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ARF nucleotide-binding site opener (ARNO), also known as cytohesin-2, is a guanine nucleotide-exchange factor for ADP-ribosylation factor GTPases.⁹ ARF nucleotide-binding site opener is a member of the cytohesin family, which are characterized by a heptad repeat coiled coil, Sec7, and pleckstrin-homology domains.¹⁰⁻¹² The cytohesin family have known functions in cell migration, endocytic trafficking, receptor signaling, and cell adhesion.^{10,13-16} ARF nucleotide-binding site opener has been shown to bind MyD88 (an adaptor protein important for many innate immune response signaling pathways) and inhibition of ARNO with the small-molecule inhibitor SecinH3 has been shown to reduce pathologic inflammation in the collagen-induced mouse model of arthritis and Carrageenan air-pouch model of acute inflammation.^{17,18} However, whether genetic deletion of *ARNO* in mice reduces pathologic inflammation has not yet been reported. Furthermore, its role in the nasal cavity has yet to be investigated.

The ovalbumin (OVA)-induced model of allergic inflammation is a commonly used murine model of rhinitis.⁷ In this model, mice are typically first sensitized by OVA via intraperitoneal injection combined with an adjuvant.¹⁹ After sensitization, mice are repeatedly challenged with intranasal instillation of OVA. This technique results in release of inflammatory cytokines and chemokines as well as local nasal eosinophil accumulation.⁷ The goal of this study was to generate *Arno* knockout mice and to determine whether genetic loss of *Arno* reduces eosinophilic inflammation and inflammatory cytokine release in the OVA murine model of rhinitis. Our rationale also included that should a reduction in eosinophilic inflammation or inflammatory cytokine release be observed, that this study may serve as a springboard to investigation of the role of ARNO in lower airway eosinophilic inflammation, cytokine signaling in the nasal cavity, and potential clinical applicability through small-molecule inhibition.

Methods

Generation of ARNO Knockout Mice

Mice carrying mutations in the *Cytl2* (*Arno*) gene were generated from embryo-derived stem cells purchased from the European Mouse Mutant Cell Repository (EUCOMM). The cell line, designated EPD0140_3_D11, was heterozygous for an allele designated by EUCOMM as *Cytl2tm1a(EUCOMM)Wtsi*. This allele contained a selection cassette flanked by FRT sites linked to a loxP site inserted into the fourth intron of the *Cytl2* gene and an additional loxP in the sixth intron. Embryo-derived stem cells were injected into mouse blastocysts (C57Bl/6) by the University of Utah Transgenic Animal Core Facility to generate chimeric animals that were in turn mated with FLP-expressing mice. Progeny animals lacking the reporter cassette but containing the 2 loxP sites were used as founders for a colony of *Cytl2* conditional mice. Crossing of these mice with germ-line expressing Cre mice generated animals carrying the null allele of *Cytl2*.

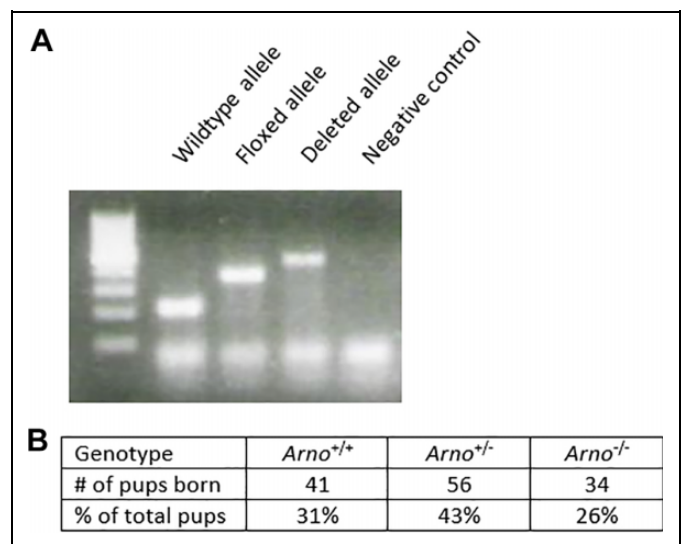


Figure 1. A, Polymerase chain reaction (PCR) genotyping demonstrates *Arno* wild type, floxed, and deleted alleles. B, Tabulation of 15 litters demonstrates Mendelian inheritance and viability of *Arno*^{-/-} mice.

Genotyping Protocol

Arno (*Cytl2*) primers used were oDLKT233: AGAAA-CAAGCCACATTCACCTCAG, oDLKT235: GTACTCT-GATCAGATCTGTAAGCTC, and oDLKT255: CTGCTAG TGTCCCTGCAAGGTG. Primers oDLKT233 and oDLKT235 amplified ARNO^{+/+} and ARNO FL/FL alleles generating fragments of ~217 bp (+/+) and ~374 bp (FL/FL), respectively. The ARNO^{-/-} was detected with primers oDLKT233 and oDLKT255 generating a band length of ~488 bp. All primers are used within one reaction to detect each band. Polymerase chain reaction samples were composed of 12 µL Promega GoTaq Green Master Mix (Cat# M7123), 6 µL H₂O, 5 µL primer total, and 2 µL DNA from tail sample per reaction. Thermocycler settings were stage 1) 94 °C for 3 minutes; 94 °C for 30 seconds; stage 2) 57 °C for 30 seconds; 72 °C for 30 seconds; for 35 cycles; and stage 3) 72 °C for 3 minutes; 4 °C indefinite.

Ovalbumin Sensitization

Studies were approved by the Johns Hopkins Institutional Animal Care and Use Committee. Mice were transferred from the University of Utah to Johns Hopkins (A.P.L.). *Arno* heterozygous (+/-) mice were bred producing littermate *Arno*^{+/+} and *Arno*^{-/-} mice. Allergic sinonasal inflammation was induced as previously described.^{7,20} In brief, mice were sensitized with an intraperitoneal injection of 900 µg of OVA (grade V; Sigma-Aldrich) and 77 µL of alum adjuvant (Thermo Scientific). One week later, mice received a second intraperitoneal injection of 450 µg of OVA and 77 µL of alum. Beginning day 14 after the initial OVA sensitization, mice were anesthetized with isoflurane and given a daily nasal instillation of 500 µg of OVA in 20 µL of phosphate-buffered saline (PBS) for 14 days.

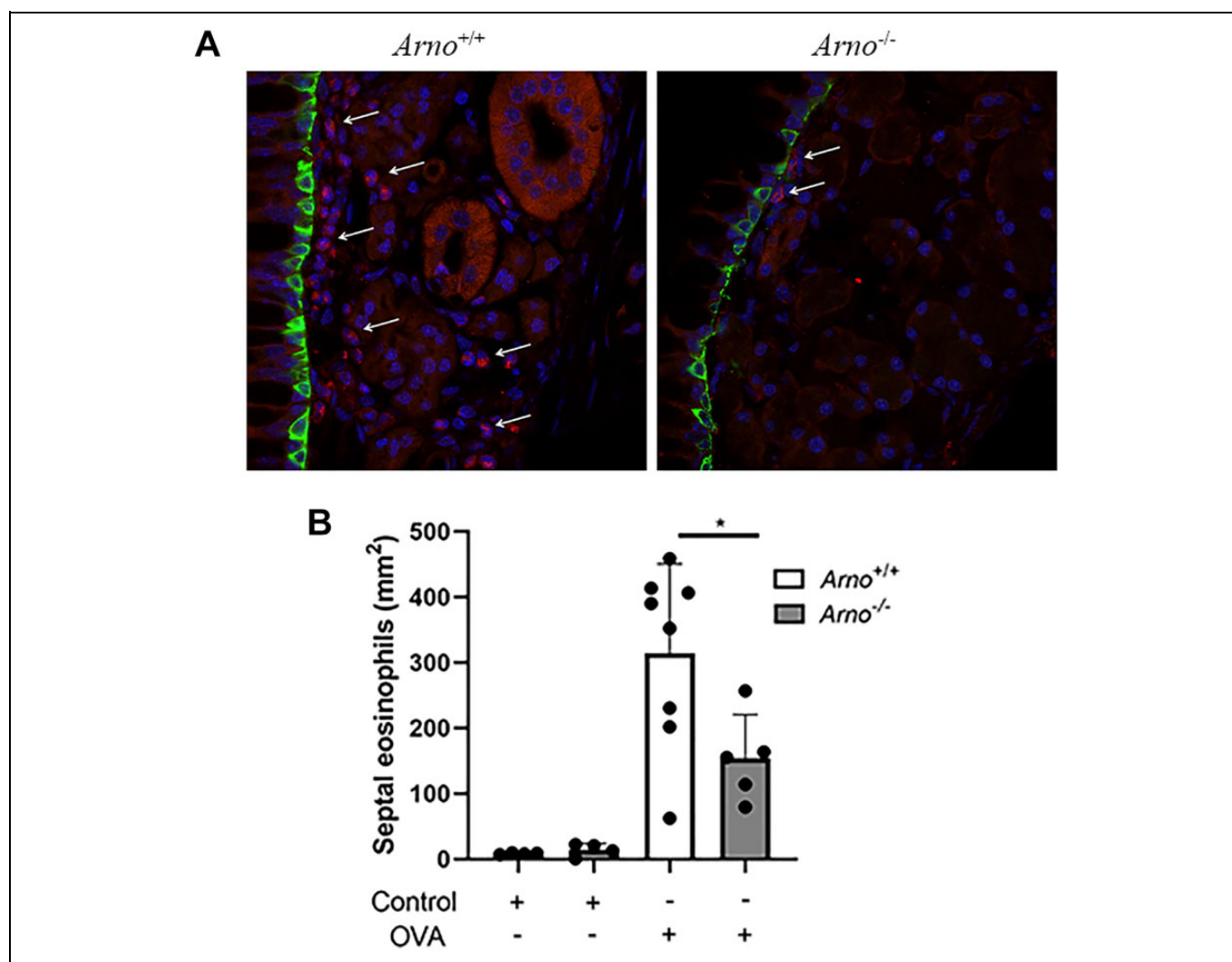


Figure 2. A, *Arno*^{+/+} mice (left) and *Arno*^{-/-} mice (right) were subjected to the OVA-induced model of allergic sinonasal inflammation. A significant decrease in EMBP⁺ cells in the nasal septum was observed in *Arno*^{-/-} mice. Representative images are displayed. EMBP (red), KRT-5 (green), and DAPI (blue). B, Quantification demonstrates a significant decrease in EMBP⁺ cells in *Arno*^{-/-} mice. For control treated mice N = 4. For OVA-treated mice N ≥ 5. *P < .05. DAPI, 4',6-diamidino-2-phenylindole; EMBP, eosinophilic major basic protein; OVA, ovalbumin.

Histopathology

Murine tissues were prepared for histologic and immunofluorescence analysis as previously described.^{20,21} In brief, after cardiac perfusion with 4% paraformaldehyde, mouse heads were fixed, decalcified, equilibrated in sucrose, and cryoembedded in Tissue-Tek OCT (Sakura). Sectioned tissue was used for immunofluorescence analysis of eosinophilic major basic protein (EMBP) to assess eosinophil accumulation or Alcian blue-nuclear fast red staining to assess goblet cell proliferation. For immunofluorescence studies, sections were blocked and permeabilized overnight followed by antigen retrieval with 10 mM sodium citrate and 0.05% Tween-20. Sections were then incubated overnight at 4 °C with EMBP (sc-33938, Santa Cruz Biotechnology) and Krt5 (PRB-160P, Covance). The slides were washed in PBS, incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen) and later

counterstained with 4',6-diamidino-2-phenylindole (Invitrogen). Confocal images were collected with a LSM700 confocal laser microscope (Zeiss). EMBP⁺ cells in sections of septal mucosa and data were presented as mean with standard deviation.

RNA Extraction and Quantitative Polymerase Chain Reaction

RNA extraction and quantitative polymerase chain reaction were performed as previously described.²⁰ In brief, septal and turbinate mucosa were removed and placed in RNeasy lysis solution (Qiagen). Total RNA was isolated from mucosal homogenates and reverse transcription was performed using the Omniscript RT kit (Qiagen) according to the manufacturer's protocol. Quantitative polymerase chain reaction was

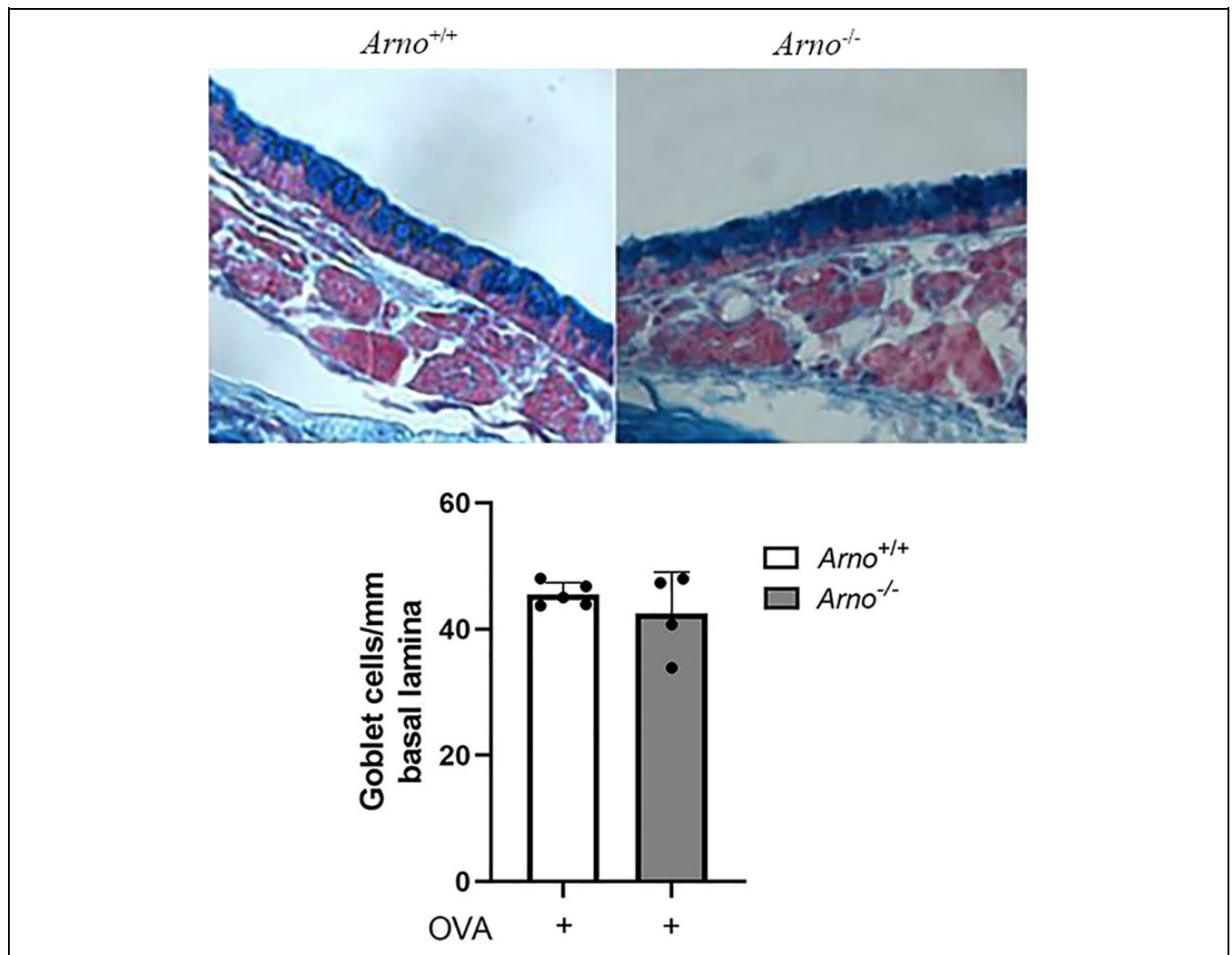


Figure 3. *Arno*^{+/+} mice (left) and *Arno*^{-/-} mice (right) were subjected to the OVA-induced model of allergic sinonasal inflammation. Tissue sections were stained with Alcian blue-nuclear fast red. Representative images are displayed. Quantification demonstrates no statistically significant decrease in goblet cell proliferation in *Arno*^{-/-} mice. N ≥ 4. OVA, ovalbumin.

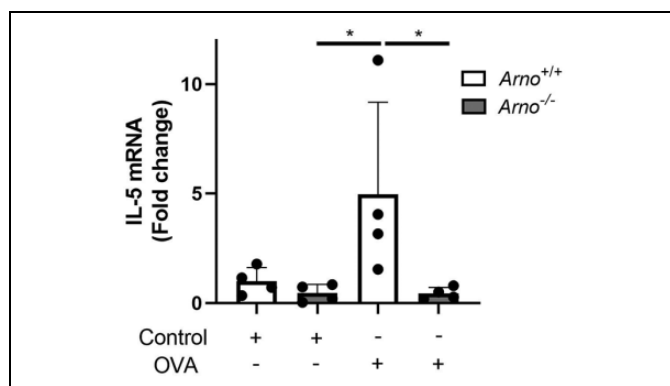


Figure 4. *Arno*^{+/+} and *Arno*^{-/-} mice were subjected to the OVA-induced model of allergic sinonasal inflammation and IL-5 expression assessed demonstrating a statistically significant decrease in *Arno*^{-/-} mice. N = 4 per group. Error bar represents standard deviation. *P < .05. IL, interleukin; OVA, ovalbumin.

performed using the Applied Biosystems StepOnePlus. Expression levels of 18S, IL-4, IL-5, IL-13, and eotaxin-1 were quantified in each sample in duplicate using the TaqMan gene expression system (Applied Biosystems). Amplicon expression was normalized to 18S. Relative changes in gene expression were calculated as $2^{-\Delta\Delta C_t}$.

Statistical Analysis

Statistical analyses were performed using analysis of variance followed by an appropriate multiple comparisons test. Tukey's multiple comparisons test was used when variation was similar among groups, and Dunnett's T3 multiple comparisons test was used when variation among groups was significant. Data that deviated from a normal distribution were log10 transformed before statistical analysis. Statistical significance was defined

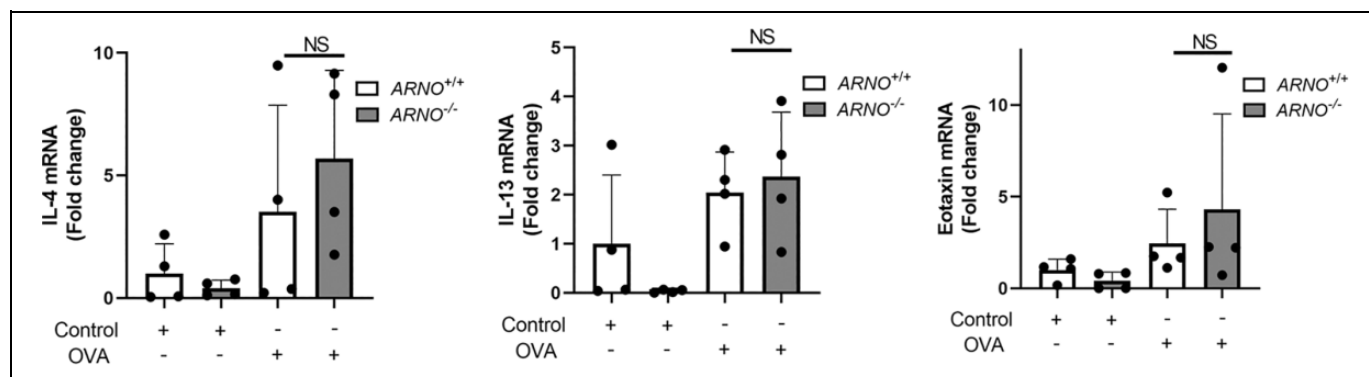


Figure 5. *Arno*^{+/+} and *Arno*^{-/-} mice were subjected to the OVA-induced model of allergic sinonasal inflammation and IL-4, IL-13, and eotaxin-1 expression assessed demonstrating no statistically significant decrease in *Arno*^{-/-} mice. N = 4 per group. Error bar represents standard deviation. IL indicates interleukin; NS, not significant; OVA, ovalbumin.

as $P < .05$. Results were analyzed and presented with the use of Prism 8 (GraphPad).

Results

Arno^{-/-} mice were generated and were noted to be viable and born at a Mendelian ratio (Figure 1). *Arno*^{-/-} mice did not demonstrate any notable deficits. Littermate *Arno*^{+/+} and *Arno*^{-/-} mice were subjected to the OVA-induced murine model of rhinitis and immunofluorescence performed to quantify EMBP⁺ eosinophils in nasal septal tissue. Wild type animals demonstrated an average of 314.5 EMBP⁺ cells per mm² septal tissue (Figure 2). In comparison, *Arno* knockout mice demonstrated an average of 153.8 EMBP⁺ cells per mm² septal tissue (51% reduction, $P < .05$) (Figure 2). The OVA-induced murine model of rhinitis is also characterized by goblet cell hypertrophy. To assess goblet cell hypertrophy, tissue sections were also subjected to Alcian blue-nuclear fast red staining. Although there was a significant increase in goblet cell hypertrophy in OVA-treated *Arno*^{+/+} mice, there was no significant reduction in goblet cell hypertrophy in OVA-treated *Arno*^{-/-} mice (Figure 3).

Interleukin-5 is a key inflammatory cytokine that regulates eosinophil activation, survival, and tissue accumulation.²² Thus, we assessed IL-5 expression in sinonasal tissue to determine whether a change in IL-5 could mechanistically explain the decrease in eosinophil accumulation in *Arno*^{-/-} mice. There was a statistically significant decrease in OVA-induced IL-5 expression in the sinonasal tissue of *Arno*^{-/-} mice (Figure 4). To determine whether this change in expression applied to additional inflammatory cytokines and chemokines, we also assessed IL-4, IL-13, and eotaxin-1 expression. Interestingly, there was no significant change in expression in these cytokine and chemokines between *Arno* wild type and knockout mice (Figure 5).

Discussion

Allergic rhinitis is a prevalent IgE-mediated condition of the nasal cavity with characteristic symptoms including itching, sneezing, nasal congestion, and rhinorrhea.^{1,2} Allergic rhinitis

in patients and in animal models is also characterized by a release of multiple cytokines and chemokines including IL-4, IL-5, IL-13, eotaxin-1, and the accumulation of eosinophils.^{1,7} Inhibition of ARNO has been previously shown to reduce pathologic inflammation in mouse models of arthritis and acute inflammation.¹⁷ However, whether genetic deletion of *Arno* in mice reduced pathologic inflammation had not yet been previously reported. In this study, we generated a targeted deletion of *Arno* in mice and demonstrated that *Arno*^{-/-} mice are less susceptible to OVA-induced IL-5 expression and eosinophil accumulation. Furthermore, this is the first study to demonstrate the effects of ARNO inhibition on nasal inflammation in the nasal cavity. Interestingly, while there was no reduction in IL-4, IL-13, or eotaxin-1 expression, there was a significant reduction in IL-5 in the sinonasal mucosal tissue in the absence of ARNO. Interleukin-5 is a key inflammatory cytokine that regulates eosinophil activation, survival, and tissue accumulation.²² Thus, the reduction in eosinophil accumulation may be explained by the decreased IL-5 expression observed in this study. Although ARNO has been linked to IL-1 β signaling, a direct link between ARNO and IL-5 has not been described and is an area of future study. ARF nucleotide-binding site opener has a well-known function in cell migration and eosinophils are known to produce IL-5. Thus, another potential explanation is that ARNO may be important for eosinophil migration, and the observed decrease in IL-5 may be a by-product of the reduction in accumulated eosinophils.

Sinonasal inflammatory upper airway diseases such as AR and chronic rhinosinusitis affect millions worldwide. However, allergic inflammatory disease of the lower airway such as asthma affects >350 million people worldwide.²³ Asthma may have many severe symptoms including airway hyper-responsiveness, wheezing, and dyspnea.²³ Given the close relationship of the upper and lower airway, it would be interesting to investigate whether a similar reduction in eosinophilic inflammation would be observed in an allergic lower airway inflammation model in *Arno*^{-/-} mice.²⁴

There are limitations to this study. First, cytohesin family members are known to regulate cell adhesion and migration.¹⁶

It would be interesting to assess in the future whether loss of ARNO directly leads to decreased eosinophil migration. However, given the low numbers of eosinophils under normal conditions, to directly test a direct effect on eosinophil migration may require IL-5 stimulated expansion of *Arno*^{-/-} hematopoietic cells, and it is unclear how these cells would respond to IL-5 stimulation. However, this would be an interesting area of further study. It would also be interesting in the future to determine whether the effect of ARNO inhibition is through ARF signaling and whether *Arf* knockout mice demonstrate similar decreases in allergic eosinophilic inflammation.^{16,17} Further insights may also be gained by repeating these studies in additional models of AR such as papain, house dust mite, and particulate matter. Lastly, from a clinical applicability standpoint, it would be interesting to test small-molecule inhibitors of the ARNO and ARF signaling pathways in these mouse models of allergic inflammation.^{17,18}

Conclusions

The data presented here demonstrate that deletion of *Arno* reduces eosinophilic inflammation and IL-5 expression in an OVA-induced model of rhinosinusitis. Future studies will help to determine the applicability of these results to other allergic models of the upper and lower airway. Other areas of future interest include assessing for any connection in the IL-5 signaling pathway and ARNO, determining the impact of ARNO in eosinophil cell migration, and use of small-molecule inhibitors in this and other mouse models to assess for potential translational clinical applicability.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: N.R.L. holds stock in Navigen Pharmaceuticals, is a patent coinventor targeting signaling mechanisms related to ARNO and was a consultant for Cooltech Inc.

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References

1. London NR Jr, Ramanathan MJr. The role of the sinonasal epithelium in allergic rhinitis. *Otolaryngol Clin North Am*. 2017;50(6):1043-1050.
2. Wise SK, Lin SY, Toskala E, et al. International consensus statement on allergy and rhinology: allergic rhinitis. *Int Forum Allergy Rhinol*. 2018;8(2):108-352.
3. Dierick BJH, van der Molen T, Flokstra-de Blok BMJ, et al. Burden and socioeconomics of asthma, allergic rhinitis, atopic dermatitis and food allergy. *Expert Rev Pharmacoecon Outcomes Res*. 2020;20(5):437-453.
4. Meng Y, Wang C, Zhang L. Advances and novel developments in allergic rhinitis. *Allergy*. 2020.
5. Hossenbaccus L, Linton S, Garvey S, Ellis AK. Towards definitive management of allergic rhinitis: best use of new and established therapies. *Allergy Asthma Clin Immunol*. 2020;16:39.
6. Cox L. Approach to patients with allergic rhinitis: testing and treatment. *Med Clin North Am*. 2020;104(1):77-94.
7. Mendiola M, Tharakan A, Chen M, Asempta T, Lane AP, Ramnathan MJr. Characterization of a novel high-dose ovalbumin-induced murine model of allergic sinonasal inflammation. *Int Forum Allergy Rhinol*. 2016;6(9):964-972.
8. An YF, Suo LM, Xue JM, et al. Role of Fc gamma RI in antigen-dependent eosinophil activation in patients with allergic rhinitis. *Am J Rhinol Allergy*. 2020;35(1):86-97.
9. Nawrotek A, Zeghouf M, Cherfils J. Allosteric regulation of Arf GTPases and their GEFs at the membrane interface. *Small GTPases*. 2016;7(4):283-296.
10. Das S, Malaby AW, Nawrotek A, et al. Structural organization and dynamics of homodimeric cytohesin family Arf GTPase exchange factors in solution and on membranes. *Structure*. 2019;27(12):1782-1797.
11. Luong P, Hedl M, Yan J, et al. INAVA-ARNO complexes bridge mucosal barrier function with inflammatory signaling. *Elife*. 2018;7:e38539.
12. Salem JC, Reviriego-Mendoza MM, Santy LC. ARF-GEF cytohesin-2/ARNO regulates R-Ras and alpha5-integrin recycling through an EHD1-positive compartment. *Mol Biol Cell*. 2015;26(23):4265-4279.
13. Mohanan V, Nakata T, Desch AN, et al. C1orf106 is a colitis risk gene that regulates stability of epithelial adherens junctions. *Science*. 2018;359(6380):1161-1166.
14. Ito A, Fukaya M, Saegusa S, et al. Pallidin is a novel interacting protein for cytohesin-2 and regulates the early endosomal pathway and dendritic formation in neurons. *J Neurochem*. 2018;147(2):153-177.
15. Chardin P, Paris S, Antonny B, et al. A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature*. 1996;384(6608):481-484.
16. Jones CA, Nishiya N, London NR, et al. Slit2-Robo4 signalling promotes vascular stability by blocking Arf6 activity. *Nat Cell Biol*. 2009;11(11):1325-1331.
17. Zhu W, London NR, Gibson CC, et al. Interleukin receptor activates a MYD88-ARNO-ARF6 cascade to disrupt vascular stability. *Nature*. 2012;492(7428):252-255.
18. Hafner M, Schmitz A, Grune I, et al. Inhibition of cytohesins by SecinH3 leads to hepatic insulin resistance. *Nature*. 2006;444(7121):941-944.
19. Liang MJ, Fu QL, Jiang HY, et al. Immune responses to different patterns of exposure to ovalbumin in a mouse model of allergic rhinitis. *Eur Arch Otorhinolaryngol*. 2016;273(11):3783-3788.

20. London NR Jr., Tharakan A, Mendiola M, et al. Deletion of Nrf2 enhances susceptibility to eosinophilic sinonasal inflammation in a murine model of rhinosinusitis. *Int Forum Allergy Rhinol.* 2019; 9(1):114-119.
21. London NR Jr., Tharakan A, Mendiola M, et al. Nrf2 activation via Keap1 deletion or sulforaphane treatment reduces ova-induced sinonasal inflammation. *Allergy.* 2019;74(9):1780-1783.
22. Brussino L, Heffler E, Bucca C, Nicola S, Rolla G. Eosinophils target therapy for severe asthma: critical points. *Biomed Res Int.* 2018;2018:7582057.
23. Athari SS. Targeting cell signaling in allergic asthma. *Signal Transduct Target Ther.* 2019;4:45.
24. Stachler RJ. Comorbidities of asthma and the unified airway. *Int Forum Allergy Rhinol.* 2015;5(suppl 1):S17-S22.