

Nrf2 activation via *Keap1* deletion or sulforaphane treatment reduces Ova-induced sinonasal inflammation

To the Editor,

The human airway plays an essential role in oxygen exchange and is subjected to airborne environmental, allergen, and infectious exposures. These agents incite oxidative stress either directly or indirectly through recruitment of inflammatory cells which release reactive oxygen species.¹ Oxidative stress has been linked to disease severity of lower airway chronic inflammatory disorders such as asthma and COPD; however, its role in upper airway (sinonasal) chronic inflammatory disorders is less clear.^{1,2} Interestingly, increased expression of cytoprotective enzymes has been reported in chronic rhinosinusitis (CRS) suggesting that oxidative stress may play a role in CRS pathophysiology.³

Nuclear erythroid 2 p45-related factor (Nrf2) is a transcription factor that upon activation invokes an antioxidant response pathway via nuclear translocation and upregulation of cytoprotective genes.⁴ Disruption of the Nrf2 pathway in mice has been reported to enhance susceptibility to ovalbumin (Ova)-induced asthma.⁵ Interestingly, activation of Nrf2 has recently been reported to stabilize sinonasal epithelial barrier function in vitro.^{4,6} We sought to determine whether sinonasal barrier stabilization through Nrf2 pathway activation could stabilize barrier function in vivo and reduce allergen-induced inflammation in a mouse model of rhinosinusitis.

Under normal circumstances, Nrf2 is sequestered in the cytoplasm and inhibited through binding Kelch-like ECH-associated protein 1 (Keap1).⁷ Disruption of this interaction results in Nrf2 activation and nuclear translocation. Therefore, we tested whether Nrf2 activation via tamoxifen-inducible *Keap1*^{flox/flox; ER-cre} mice may reverse Ova-induced sinonasal barrier dysfunction. Indeed, deletion of *Keap1* significantly reduced sinonasal barrier breakdown as assessed by serum albumin accumulation in lavage fluid and zonula occludens (ZO-1) cell surface localization on immunofluorescence (Figure 1A-B). Furthermore, genetic activation of the Nrf2 pathway through *Keap1* deletion significantly reduced Ova-induced accumulation of eosinophils (Figure 1C-D) and goblet cell hyperplasia (Figure 1E-F). Details regarding the materials and methods may be found in this article's online supporting information (Data S1). Activation of Nrf2 and stabilization of the sinonasal epithelial barrier can also be achieved through stimulation with the small molecule sulforaphane (SFN).⁴ We therefore hypothesized that SFN treatment may limit Ova-induced sinonasal barrier disruption and sought to assess preclinical therapeutic potential. Wild-type mice were subjected to the Ova-induced model of chronic sinonasal inflammation and treated with SFN at a dose found to increase Nrf2 downstream target expression (Figure

S1-S2). SFN administration significantly reduced Ova-induced accumulation of serum albumin in nasal lavage fluid as well as disruption of cell surface ZO-1 localization (Figure 2A-B). SFN administration also reduced disruption of cell surface epithelial cadherin (E-cadherin) localization although this improvement appeared to a lesser degree than ZO-1 (Figure 2C). SFN administration was also found to reduce eosinophil accumulation in sinonasal tissue (Figure 2D-E). Furthermore, Nrf2 activation via SFN treatment reduced eotaxin-1 levels in the nasal lavage fluid of these mice (Figure 2F).

These data demonstrate that Nrf2 activation via genetic and therapeutic approaches can stabilize barrier function and reduce sinonasal inflammation in a mouse model of rhinosinusitis. The Nrf2 pathway therefore represents a potential therapeutic target in CRS and chronic sinonasal inflammatory disorders.⁸ These results are in line with previous reports in the lower airway that place Nrf2 activity as a key regulator of allergen-induced inflammation.^{5,9} Interestingly, while activation of Nrf2 through either *Keap1* deletion and SFN treatment profoundly reduced sinonasal barrier dysfunction in vivo (Figure 1A, Figure 2A), the effect on eosinophil accumulation in *Keap1* tamoxifen-inducible knockout mice was much less than SFN treatment (Figure 1D, Figure 2E). The explanation for this finding is unknown, but may perhaps be related to incomplete *Keap1* knockout in this tamoxifen-inducible system.

It remains unknown the mechanism whereby Nrf2 activation stabilizes sinonasal barrier function and promotes cell surface localization of ZO-1 and the adherens junction protein E-cadherin. A potential explanation given the known function of Nrf2 in a cytoprotective response includes an overall reduction of oxidative stress. If this were the sole mechanism, one may therefore hypothesize that any pathway or means in reduction of oxidative stress may have a similar effect in this mouse model of rhinosinusitis. Additional possibilities include an undefined effect on cell junction protein cell surface localization or an indirect mechanism. Previous studies in vitro have demonstrated that SFN can stabilize human sinonasal epithelial cell dysfunction in the presence of multiple stimuli including particulate matter, cigarette smoke extract, and house dust mite antigen.^{4,6} Future studies are necessary to clarify the mechanism whereby Nrf2 stabilizes barrier function.

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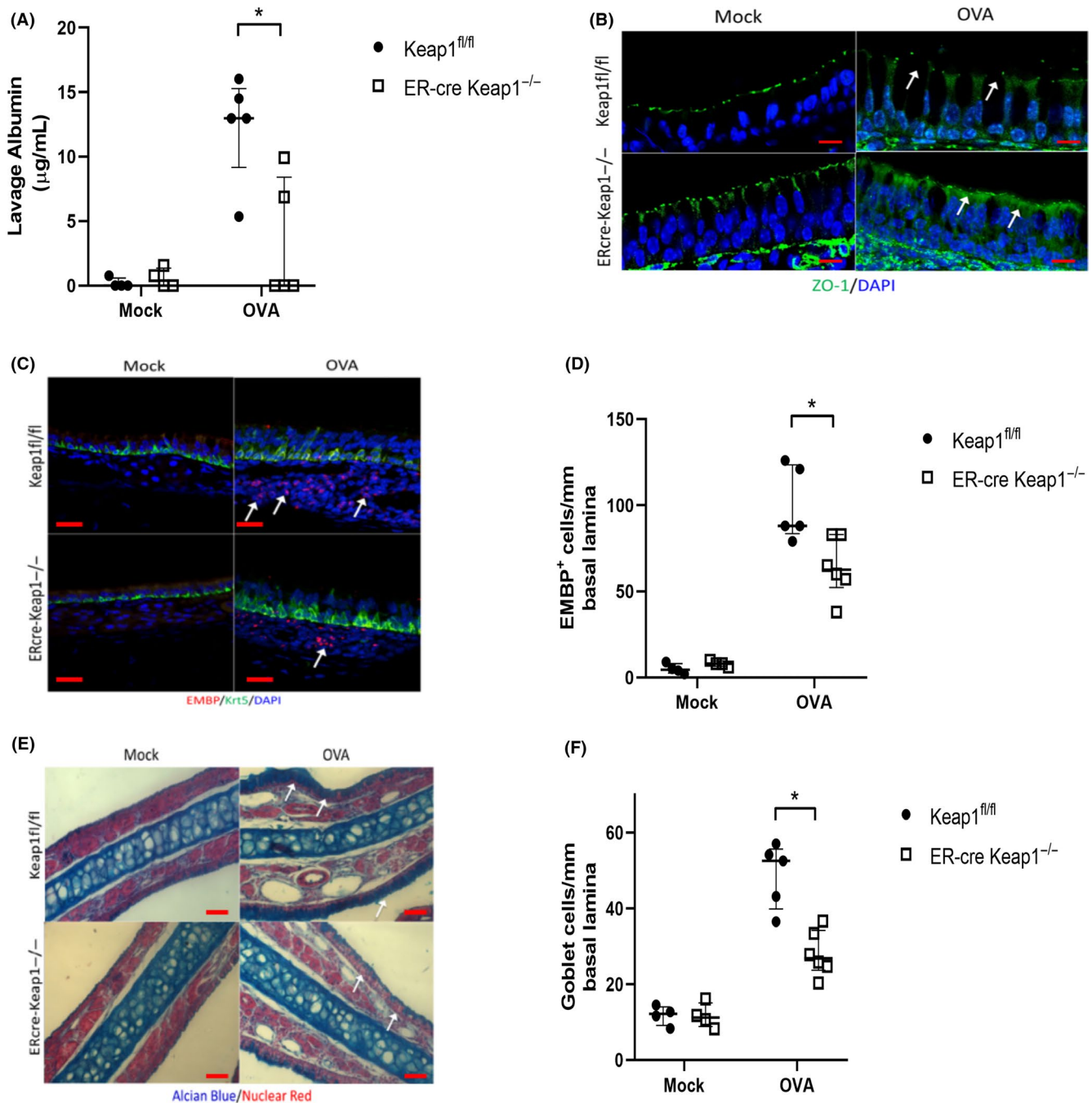


FIGURE 1 Keap1^{-/-}; ER-cre mice demonstrate reduced barrier dysfunction in response to chronic Ova exposure. A, Analysis of nasal lavage fluid for serum albumin ($n = 4-6$ per group). Mock is vehicle control. B, Immunofluorescence for ZO-1 (green) demonstrates reduced barrier disruption. DAPI (blue). Images are at 100x magnification and scale bar is 10 μm . C, Immunofluorescence for eosinophil major basic protein (EMBP, red) and Keratin-5 (Krt5, green). White arrows indicate areas of increased eosinophil accumulation. Images are at 40x magnification and scale bar is 20 μm . D, Eosinophil counts per mm of basal lamina from imaging of the entire nasal septum ($n = 4-6$ per group). Goblet cell via alcian blue stain (E-F) counts per mm of basal lamina ($n = 4-6$ per group). DAPI (blue). Images are at 20x magnification and scale bar is 50 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, data are represented as median with interquartile ranges

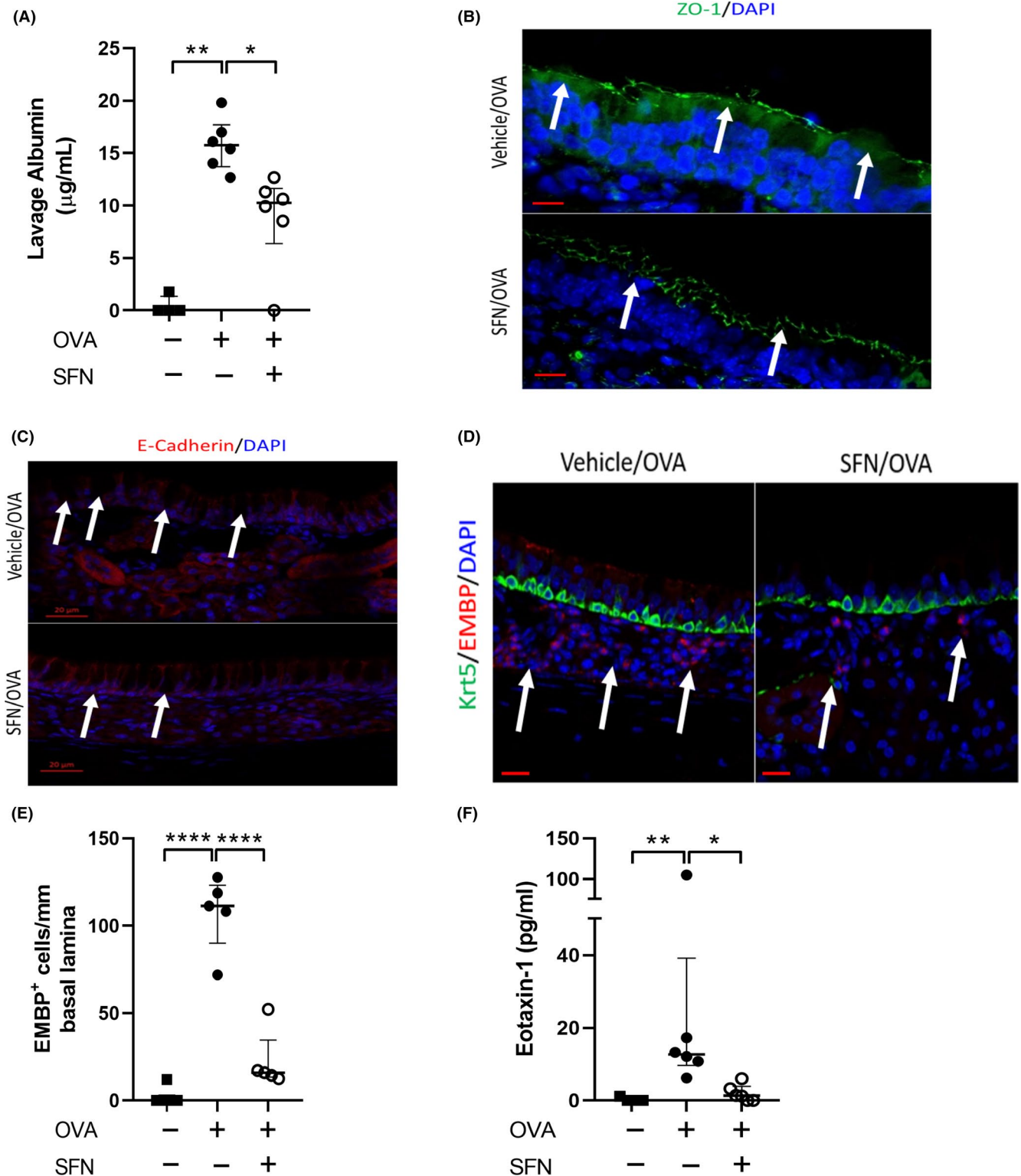


FIGURE 2 Nrf2 activation via sulforaphane treatment reduces Ova-induced sinonasal barrier dysfunction. A, Analysis of nasal lavage fluid for serum albumin ($n = 4-6$ per group). B, Immunofluorescence for ZO-1 (green) demonstrates reduced barrier disruption. Images are at 100x magnification and scale bar is 10 μm . C, Immunofluorescence for E-cadherin (red) demonstrates reduced barrier disruption. Images are at 25x magnification and scale bar is 20 μm . D, Immunofluorescence for eosinophil major basic protein (EMBP, red) and Keratin-5 (Krt5, green). White arrows indicate areas of increased eosinophil accumulation. Images are at 40x magnification and scale bar is 20 μm . E, Eosinophil counts per mm of basal lamina from imaging of the entire nasal septum ($n = 4$ per group). F, Analysis of nasal lavage fluid via ELISA for eotaxin-1 ($n = 4-6$ per group). DAPI (blue) * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, data are represented as median with interquartile ranges

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CONFLICT OF INTERESTS

NRL is a patent co-inventor for methods treating vascular barrier dysfunction licensed to Navigen Pharmaceuticals which is unrelated to the Nrf2 pathway. NRL holds a small amount of stock in Navigen Pharmaceuticals which is currently of no value. NRL is a consultant for Cooltech Inc. The remaining authors declare no competing financial interests.

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ORCID

Nyall R. London  <https://orcid.org/0000-0002-2902-4407>

Nyall R. London Jr¹ 

Anuj Tharakan¹

Michelle Mendiola¹

Mengfei Chen¹

Alex Dobzanski¹

Thomas E. Sussan²

Michael Zaykaner¹

Andrew H. Han¹

Andrew P. Lane¹

Venkataramana Sidhaye²

Shyam Biswal²

Murugappan Ramanathan Jr¹

¹Johns Hopkins Department of Otolaryngology - Head and Neck Surgery, Baltimore, Maryland

²Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

Correspondence

Murugappan Ramanathan Jr, Johns Hopkins Department of Otolaryngology - Head and Neck Surgery, Baltimore, MD.

Email: mramana3@jhmi.edu

Nyall R. London, Jr and Anuj Tharakan contributed equally to this work

REFERENCES

1. Bowler R. Oxidative stress in the pathogenesis of asthma. *Curr Allergy Asthma Rep.* 2004;4:116-122.
2. Loukides S, Bakakos P, Kostikas K. Oxidative stress in patients with COPD. *Curr Drug Targets.* 2011;12:469-477.
3. Mrowicka M, Zielinska-Blizniewska H, Milonski J, Olszewski J, Majsterek I. Evaluation of oxidative DNA damage and antioxidant defense in patients with nasal polyps. *Redox Rep.* 2015;20:177-183.
4. London NR, Tharakan A, Rule AM, Lane AP, Biswal S, Ramanathan M. Air pollutant mediated disruption of sinonasal epithelial cell barrier function is reversed by activation of the Nrf2 pathway. *J Allergy Clin Immunol.* 2016;138(6):1736-1738.
5. Rangasamy T, Guo J, Mitzner WA, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med.* 2005;202(1):47-59.
6. London NR, Tharakan A, Lane AP, Biswal S, Ramanathan M. Nuclear erythroid 2-related factor 2 activation inhibits house dust mite-induced sinonasal epithelial cell barrier dysfunction. *Int Forum Allergy Rhinol.* 2017;7(5):536-541.
7. Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999;13(1):76-86.
8. Steelant B, Seys SF, Boeckxstaens G, Akdis CA, Ceuppens JL, Hellings PW. Restoring airway epithelial barrier dysfunction: a new therapeutic challenge in allergic airway disease. *Rhinology J.* 2016;54(3):195-205.
9. Sussan TE, Gajghate S, Chatterjee S, et al. Nrf2 reduces allergic asthma in mice through enhanced epithelial cytoprotective function. *Am J Physiol Lung Cell Mol Physiol.* 2015;309(1):L27-L36.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

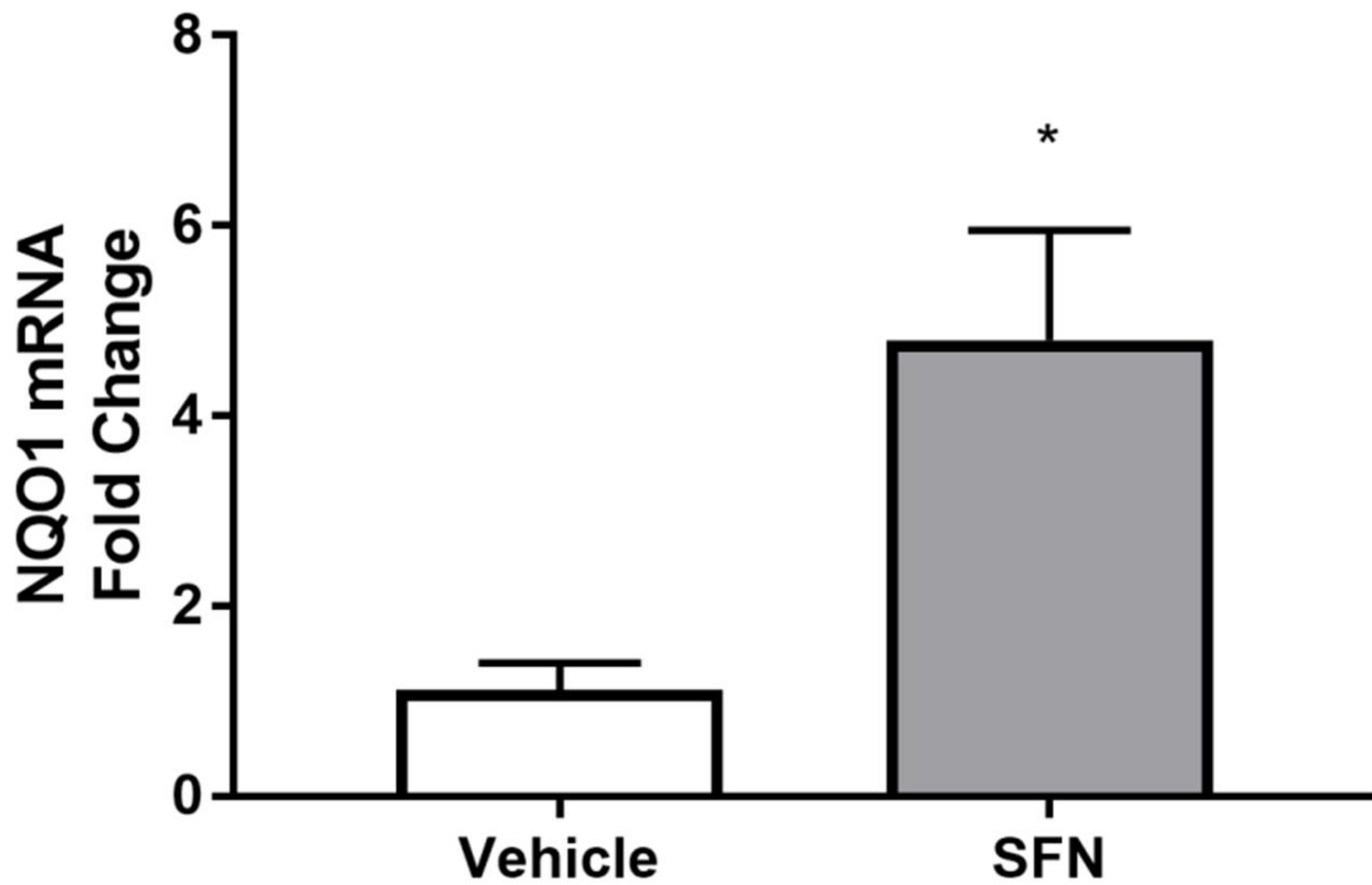
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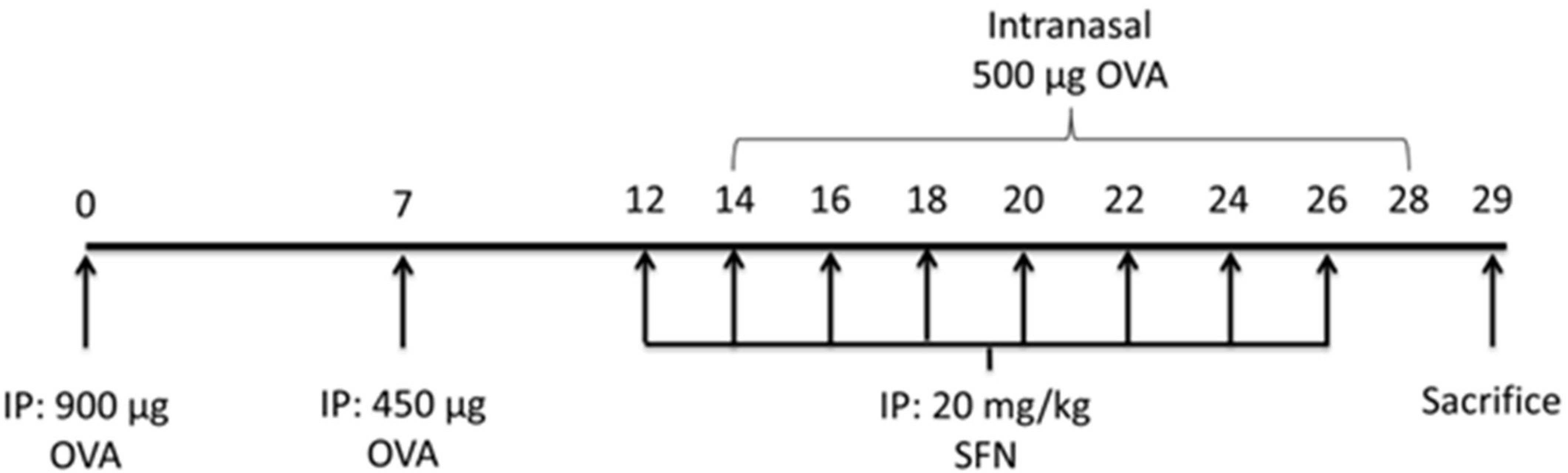
Spectrogram for childhood asthma detection and analysis

1 | INTRODUCTION

Asthma is defined as a chronic inflammation of the lower airways and can easily result in bronchoconstriction and even dyspnea. Every year, hundreds of million people around the world suffer from asthma and are unable to work or attend school due to their uncontrolled symptoms.¹ Wheezing is an obvious breathing sound that portrays airflow interference between the lungs and the bronchus

and is often considered a primary feature of an acute asthma attack, along with coughing, shortness of breath, and chest tightness. Acute exacerbation or asthma attacks using wheezing sounds have been detected by physicians both with and without stethoscopes. Designs for breathing sound monitoring systems and real-time wheeze detection systems have also been reported.² Trubel *et al* used mono-frequent forced oscillation to measure oscillatory airway resistance and calculate variability in order to separate asymptomatic children





Materials and Methods

Animals

All animal procedures were approved by the Johns Hopkins Institutional Animal Care and Use Committee. Wildtype C57BL6/J mice were used for SFN studies. CMV-ERT2cre-Keap1^{flox/flox} mice were generated by crossing Keap1^{flox/flox} mice with CAG-CreERT2+ mice (The Jackson Laboratory) on a mixed background. Littermate controls were used for all experiments. Cre mediated deletion of Keap1 exons 2-3 was induced by intraperitoneal administration of 1 mg of tamoxifen per day for 5 consecutive days. Deletion was confirmed by PCR analysis using primers: forward 5'-GAGTCCACAGTGTGTGGCC-3', reverse 5'-GAGTCACCGTAAGCCTGGTC-3'. Animals were housed in a specific pathogen-free facility with single ventilated cages and received an Ova-free diet.

Ovalbumin Sensitization

Mice were sensitized by intraperitoneal injection of 900 µg of Ovalbumin (Ova, grade V; Sigma Chemical Co.) and 77 µL of Imject alum adjuvant (Thermo Scientific) in phosphate buffered saline (PBS). On day 7, mice received additional IP injections of 450 µg of Ova in 77 µL of Alum adjuvant and PBS. Beginning day 14, mice were anesthetized with isofluorane and challenged with 500 µg of Ova in 20 µL of PBS distributed equally between the two nostrils. Intranasal challenges were administered daily for 14 days (Supplementary Figure 2). Control groups were sensitized similarly with IP injections, but were challenged intranasally with equivalent volumes of vehicle control PBS (Mock). For SFN studies, sensitized animals were randomly distributed into Vehicle/Saline, Vehicle/Ova, or SFN/Ova groups. Sulforaphane was dissolved in ethanol and diluted into PBS immediately prior to being administered by intraperitoneal injection at a dose of 20 mg/kg. Vehicle treated mice were injected with equivalent volumes of 1% ethanol diluted in PBS. Treatments were administered every other day starting on day 12 (Supplementary Figure 2). Activation of Nrf2 was

confirmed by qRT-PCR for the Nrf2 target gene NQO1 (Mm01253561_m1) from sinonasal mucosal tissue.

Histopathology

After perfusion with 4% paraformaldehyde (PFA) mouse heads were fixed with 4% PFA, decalcified overnight in TBD2 (Thermo Scientific), equilibrated in sucrose, and cryoembedded in Tissue-Tek OCT (Sakura). Sectioned tissue was used for Alcian blue-nuclear fast red staining or immunofluorescence analysis. Immunofluorescence staining was performed on 12 μ m sections. Sections were blocked and permeabilized overnight in 10% Normal donkey serum and 0.1% Triton X-100 in PBS. Antigen retrieval was performed by boiling sections in 10 mM sodium citrate and 0.05% Tween-20. After incubation with primary antibodies overnight at 4 °C. Eosinophil major basic protein (EMBP, sc-33938) antibodies were obtained from Santa Cruz Biotechnology, Krt5 from Covance (PRB-160P), 488 conjugated ZO-1 antibody from Invitrogen, and E-cadherin from Cell Signaling Technologies (24E10). The slides were washed in PBS and then incubated with Alexa Fluor® 488/568 conjugated secondary antibodies (Invitrogen). Sections were then washed, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Confocal images were collected with a LSM700 confocal laser scanning microscope (Zeiss). EMBP positive cells in sections of nasal respiratory tissue were counted. Data were presented as median with interquartile range from five images per mouse.

Quantification of nasal lavage fluid proteins

Nasal lavage fluid was collected on day 29, the day after the last intranasal instillation of OVA (Supplementary Figure 2) by insertion of a flexible catheter into the opening of the pharynx and 500 μ l of phosphate buffered saline was flushed through both nares. Nasal lavage fluid was then centrifuged at 6000rpm for 10 minutes. Lavage fluids were analyzed for serum albumin by Albumin Blue Fluorescent Assay Kit (Active Motif) or Eotaxin-1 via ELISA.

Statistical analysis

All values are shown as median with interquartile range. Eosinophil and goblet cell counts were compared by one-way ANOVA followed by Tukey post-hoc test. Eotaxin-1 was compared by Kruskal-Wallis multiple test. Statistical significance was considered to be $P < 0.05$. Results were analyzed with the use of the statistical software Prism 6 (GraphPad, La Jolla, CA).