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Nrf2 reduces allergic asthma in mice through enhanced airway epithelial cytoprotective function

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Sussan TE, Gajghate S, Chatterjee S, Mandke P, McCormick S, Sudini K, Kumar S, Breysse PN, Diette GB, Sidhaye VK, Biswal S. *Nrf2* reduces allergic asthma in mice through enhanced airway epithelial cytoprotective function. *Am J Physiol Lung Cell Mol Physiol* 309: L27–L36, 2015. First published May 8, 2015; doi:10.1152/ajplung.00398.2014.—Asthma development and pathogenesis are influenced by the interactions of airway epithelial cells and innate and adaptive immune cells in response to allergens. Oxidative stress is an important mediator of asthmatic phenotypes in these cell types. Nuclear erythroid 2-related factor 2 (*Nrf2*) is a redox-sensitive transcription factor that is the key regulator of the response to oxidative and environmental stress. We previously demonstrated that *Nrf2*-deficient mice have heightened susceptibility to asthma, including elevated oxidative stress, inflammation, mucus, and airway hyperresponsiveness (AHR) (Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S. *J Exp Med* 202: 47–59, 2005). Here we dissected the role of *Nrf2* in lung epithelial cells and tested whether genetic or pharmacological activation of *Nrf2* reduces allergic asthma in mice. Cell-specific activation of *Nrf2* in club cells of the airway epithelium significantly reduced allergen-induced AHR, inflammation, mucus, Th2 cytokine secretion, oxidative stress, and airway leakiness and increased airway levels of tight junction proteins zonula occludens-1 and E-cadherin. In isolated airway epithelial cells, *Nrf2* enhanced epithelial barrier function and increased localization of zonula occludens-1 to the cell surface. Pharmacological activation of *Nrf2* by 2-trifluoromethyl-2′-methoxychalone during the allergen challenge was sufficient to reduce allergic inflammation and AHR. New therapeutic options are needed for asthma, and this study demonstrates that activation of *Nrf2* in lung epithelial cells is a novel potential therapeutic target to reduce asthma susceptibility.

ovalbumin; oxidative stress; inflammation; airway hyperresponsiveness; Th2

ASTHMA IS A COMPLEX AIRWAY disorder characterized by reversible airflow obstruction, airway hyperresponsiveness (AHR), airway inflammation, excessive mucus production, and elevated levels of IgE and T helper type 2 (Th2) cytokines (9, 44, 58). Asthmatic symptoms are often triggered by inhaled exposure to allergens or other environmental factors, including airborne pollutants, infections, or chemicals, that cause wheezing, chest tightness, shortness of breath, and coughing. Inhaled agents first encounter the airway epithelium, which provides a first line of defense to suppress activation of innate and adaptive immune cells. Despite substantial progress in understanding of the mechanisms responsible for asthma pathogenesis, prevalence of asthma in the US has continued to rise over the past several decades, with over 8% of the US population (25.7 million individuals) currently having a diagnosis of asthma (1). Despite the availability of medications to control the disease, there are 2.1 million emergency department visits, 480,000 hospitalizations, and over 3,300 deaths from asthma in the US each year (34).

The transcription factor nuclear erythroid 2-related factor 2 (*Nrf2*) is a key regulator of cytoprotective proteins, including antioxidants, xenobiotic detoxification enzymes, and proteins in the proteasomal pathway (17, 54). Under nonstressed conditions, *Nrf2* persists at low levels in the cytoplasm where it is bound to its inhibitor Kelch ECH associating protein 1 (*Keap1*), which facilitates its ubiquitination and proteolytic degradation (18). However, in the presence of environmental stress, *Nrf2* releases from *Keap1*, translocates to the nucleus, and binds to the antioxidant response element found in the promoter of all *Nrf2*-dependent genes, resulting in activation of an adaptive cytoprotective response that detoxifies environmental stressors and exhibits numerous immuno-modulatory effects (20). This *Nrf2*-dependent response consists of 650 direct inducible target genes that coordinate to attenuate environmental and oxidative stress in multiple ways (30), such as *1*) providing direct antioxidants (35, 36); *2*) encoding enzymes that directly inactivate oxidants (15); *3*) increasing levels of glutathione synthesis and regeneration (33); *4*) stimulating NADPH synthesis (14, 54); *5*) enhancing toxin export via the multidrug response transporters (14); *6*) inhibiting cytokine-mediated inflammation (37); and *7*) enhancing the recognition, repair, and removal of damaged proteins (27).

Using an ovalbumin (OVA) allergen-induced asthma model, our laboratory previously reported that *Nrf2*-deficient mice exhibit increased AHR, eosinophilic airway inflammation, oxidative stress, mucus hyperplasia, and Th2 cytokine secretion,

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compared with wild-type controls (39). Additionally, dendritic cells from *Nrf2*-deficient mice display enhanced surface expression of activation markers, increased oxidative stress, and heightened Th2 responses, compared with dendritic cells from wild-type mice after ex vivo stimulation with allergen (29, 40, 57). Furthermore, Nrf2 protein levels and *Nrf2*-regulated antioxidant responses are markedly reduced in airway smooth muscle cells from severe asthmatic subjects, compared with normal subjects, suggesting that the *Nrf2* response may be impaired in asthmatic subjects (32). Taken together, this evidence suggests that *Nrf2* signaling is a major determinant of susceptibility to allergic asthma, and augmenting *Nrf2* signaling could be a promising approach for treatment of allergic asthma.

The airway epithelium is an important determinant of asthma susceptibility, as it provides several key functions, including maintenance of the epithelial barrier, mucociliary clearance, wound repair, and secretion of cytokines, antioxidants, surfactants, antiproteases, and antibacterial agents. Emerging evidence suggests that the allergic inflammatory responses that are typical of asthma are driven by altered epithelial cell function, and asthmatic subjects exhibit impaired barrier function (52), defective antioxidant pathways (45), aberrant repair (11), and enhanced epithelial-derived cytokine secretion (16). *Nrf2* has been implicated in several of these phenotypes, and targeting *Nrf2* in airway epithelium could play a vital role in modulating the asthmatic response.

The goals of our present study were to determine whether activation of *Nrf2* attenuates OVA-induced asthmatic phenotypes in mice and to further elucidate the role of *Nrf2* in the airway epithelium after allergen exposure. Here, we demonstrated that activation of *Nrf2*, via either genetic deletion of *Keap1* or pharmacological activation of the pathway, suppresses OVA-induced asthma, and we showed that *Nrf2* increases cytoprotective responses in the airway epithelium to reduce allergic asthma.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). *Keap1*-floxed (*Keap1*$^{fl/fl}$) mice on a C57BL/6 background were generated as previously described (4, 25). Briefly, LoxP sites were inserted into the *Keap1* gene, flanking exons 2 and 3. The *Keap1* transcript lacking exons 2 and 3 codes for a truncated nonfunctional Keap1 protein that contains the NH_2-terminal BTB domain essential for *Keap1* dimerization but lacks the redox-sensitive IVR domain and the *Nrf2*-binding Kelch domains. Tamoxifen-inducible *CMVCre-Keap1*$^{fl/fl}$ mice were generated by crossing *Keap1*$^{fl/fl}$ mice with *CAG-CreERT2*$^{+}$ mice. To induce Cre-mediated deletion of *Keap1*, tamoxifen-inducible *CMVCre-Keap1*$^{fl/fl}$ mice and *Keap1*$^{fl/fl}$ mice were treated with tamoxifen (1 mg·mouse^{-1}·day^{-1}; ip injection). Deletion of *Keap1* was determined as previously described (23), and activation of *Nrf2* was confirmed by measuring expression of its downstream target NADPH quinone oxidoreductase 1 (*Nqo1*) by quantitative PCR (TaqMan, Applied Biosystems). *CC10-Keap1*$^{-/-}$ mice, in which cre is controlled by the club cell-specific 10-kDa protein (CC10) promoter, were generated as previously described (4). Briefly, *Keap1*$^{fl/fl}$ mice were bred with *CCtCre*$^{+}$ transgenic mice that express cre under the control of the CC10 promoter. Mice were further crossed to produce *Keap1*$^{\Delta 2-3/\Delta 2-3}$; *CCtCre*$^{+}$ mice (*CC10-Keap1*$^{-/-}$). These mice did not display any gross structural alterations in the lungs in the absence of stress. CCtCre^{+} transgenic mice were developed and characterized previously and show specific activation of cre in the airway epithelium, with no effect on endogenous CC10 expression (46). All mice were housed under controlled conditions for temperature and humidity, using a 12:12-h light-dark cycle. All experimental protocols were performed in accordance with the standards established by the US Animal Welfare Acts, as set forth in National Institutes of Health guidelines and in the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Animal exposures. Mice were sensitized to Grade V OVA (Sigma Aldrich) via intraperitoneal injection of 20 μg OVA plus 2 mg Alum (Imject Alum, Thermo) in 200 μl PBS. A second sensitization was performed on *day 14* using 100 μg OVA plus 2 mg Alum. Mice were challenged every 48 h starting at *day 21* with intratracheal administration of either 100 μg OVA in 50 μl PBS or PBS alone. Mice were harvested 48 h after the fourth challenge. For tamoxifen-inducible deletion of *Keap1*, mice were treated with tamoxifen for 5 consecutive days before the first sensitization. 2-Trifluoromethyl-2′-methoxychalone (TMC) was synthesized as previously described (26). Mice were treated with TMC or vehicle by gavage 4 h before each challenge.

AHR. Mice were anesthetized with ketamine-xylazine, intubated, and paralyzed with 0.21 mg intramuscular succinylcholine chloride (Hospira). Mice were ventilated at 150 breaths/min at a tidal volume of 0.2 ml. AHR was induced by a 10-s inhalation of 30 mg/ml acetyl-β-methylcholine (Sigma Aldrich), and dynamic airway pressure (cmH_2O·s) was followed for 5 min.

Inflammation and cytokine secretion. Inflammatory cells were quantified in bronchoalveolar lavage (BAL) fluid, as previously described (49). Differential cell counts were quantified on cytospin preps stained with Diff-Quik stain (Siemens). IL-4, IL-5, and IL-13 were measured in BAL fluid by ELISA (R&D Systems).

Mucus secretion. Left lungs from OVA-exposed *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice were inflated to a pressure of 25 cmH_2O with 0.6% melted agarose, cooled, cut into four pieces, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with periodic acid Schiff (PAS) kit (Sigma Aldrich) without counterstain. The sections were blinded and scored by an independent observer.

Oxidative stress. Lipid peroxidation was measured in lung homogenates after mixing with SDS lysis buffer (100 mM NaCl, 500 mM Tris, pH 8.0, 10% SDS) at a ratio of 1:1, followed by addition of thiobarbituric acid in 10% acetic acid. Samples were boiled and then mixed with *n*-butanol at a ratio of 1:1, and absorbance was measured at 532 nm. Malondialdehyde content was compared with a standard and normalized to protein content. Protein carbonyls were measured in lung homogenates that were treated with 1% streptomycin sulfate followed by dinitrophenylhydrazine in 2 N HCl. After 1 h, 20% cold trichloroacetic acid was added at a ratio of 1:1. Precipitated protein was resuspended in 6 M guanidine hydrochloride, and protein carbonyls were determined from the absorbance at 370 nm using the molar absorption coefficient of 22,000 M^{-1}·cm^{-1}.

Epithelial barrier function. Tracheal epithelial cells were isolated as previously described (28) and plated on 12-well inserts (Falcon). Paracellular permeability was assessed via addition of FITC-coupled dextran beads (4 kDa, 10 mg/ml; Calbiochem) to the upper chamber, and fluorescence in the basal media was determined at 20 min. Transepithelial electrical resistance (TEER) was assessed by an epithelial voltohmmeter (World Precision Instruments). Zonula occludens-1 (Zo-1) immunofluorescence was measured after staining epithelial monolayers or paraffin lung sections with an anti-Zo-1 rabbit polyclonal antibody (61–7300, Life Technologies). E-cadherin immunofluorescence was performed using a rabbit polyclonal E-cadherin antibody (H-108 Santa Cruz Biotechnology).

Statistical analyses. The Student's unpaired *t*-test was used to determine statistical significance between each group. One-way ANOVA followed by post hoc analysis using Tukey test was used for comparisons of multiple groups. Values are presented as means ± SE.

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RESULTS

Genetic activation of Nrf2 reduces OVA-induced asthma. As a result of our previous study demonstrating that *Nrf2*-deficient mice have increased asthmatic phenotypes (39), we presently examined whether activation of *Nrf2* attenuates asthma. *Keap1*-deficient mice are postnatally lethal. Therefore, to genetically activate *Nrf2*, we generated *Keap1*-floxed mice (*Keap1*$^{fl/fl}$) that contain a tamoxifen-inducible CMV-cre recombinase. Tamoxifen treatment resulted in increased expression of *Nqo1*, an *Nrf2*-dependent gene that serves as a marker for *Nrf2* activation, in lungs shortly after treatment (data not shown), and this elevated expression was sustained at 36 days after treatment (Fig. 1*A*). Mice that were sensitized and challenged with OVA exhibited elevated methacholine-induced airway pressure-time index, which is a measure of AHR, increased eosinophilic inflammation, and increased secretion of Th2 cytokines (IL-4 and IL-13) and the eosinophilic chemokine IL-5 (Fig. 1). *Tam-Keap1*$^{-/-}$ mice showed significant reductions in OVA-induced AHR (Fig. 1*B*), eosinophilic inflammation (Fig. 1*C*), and secretion of IL-4 and IL-13 in BAL (Fig. 1*D*), compared with *Keap1*$^{fl/fl}$ controls. We also observed a trend toward reduced IL-5 in *Tam-Keap1*$^{-/-}$ mice, but this was not significant by ANOVA. Additionally, we observed mild infiltration of neutrophils and lymphocytes in the *Keap1*$^{fl/fl}$ mice, which were significantly reduced in *Tam-Keap1*$^{-/-}$ mice (Fig. 1*C*). Thus genetic activation of *Nrf2* attenuates asthmatic phenotypes.

Pharmacological activation of Nrf2 reduces the asthmatic response. To further identify the role of *Nrf2* in reducing asthmatic phenotypes, we treated mice with the *Nrf2* activator, TMC, before OVA challenges. Based on previous characterization of TMC (26) and our own preliminary dosing experiments (not shown), 400 mg/kg TMC via gavage gave maximal induction of *Nrf2* target gene expression at 6 h after either a single treatment or multiple daily treatments, with no obvious toxicity. Expression returned to baseline within 24 h. To test whether TMC decreased susceptibility to our asthma model, mice were given TMC/vehicle 4 h before each OVA challenge. Lungs were harvested in a small subset of mice ($N = 3$) at 2 h after OVA challenge (6 h after vehicle/TMC treatment) to confirm that treatment with TMC by gavage increased expression of the *Nrf2* target gene *Nqo1* in our experimental mice (Fig. 2*A*). This increase in *Nqo1* expression was not statistically significant ($P = 0.10$), but the fold induction was similar to our preliminary dosing experiments. Treatment with TMC 4 h before each OVA challenge decreased OVA-induced AHR

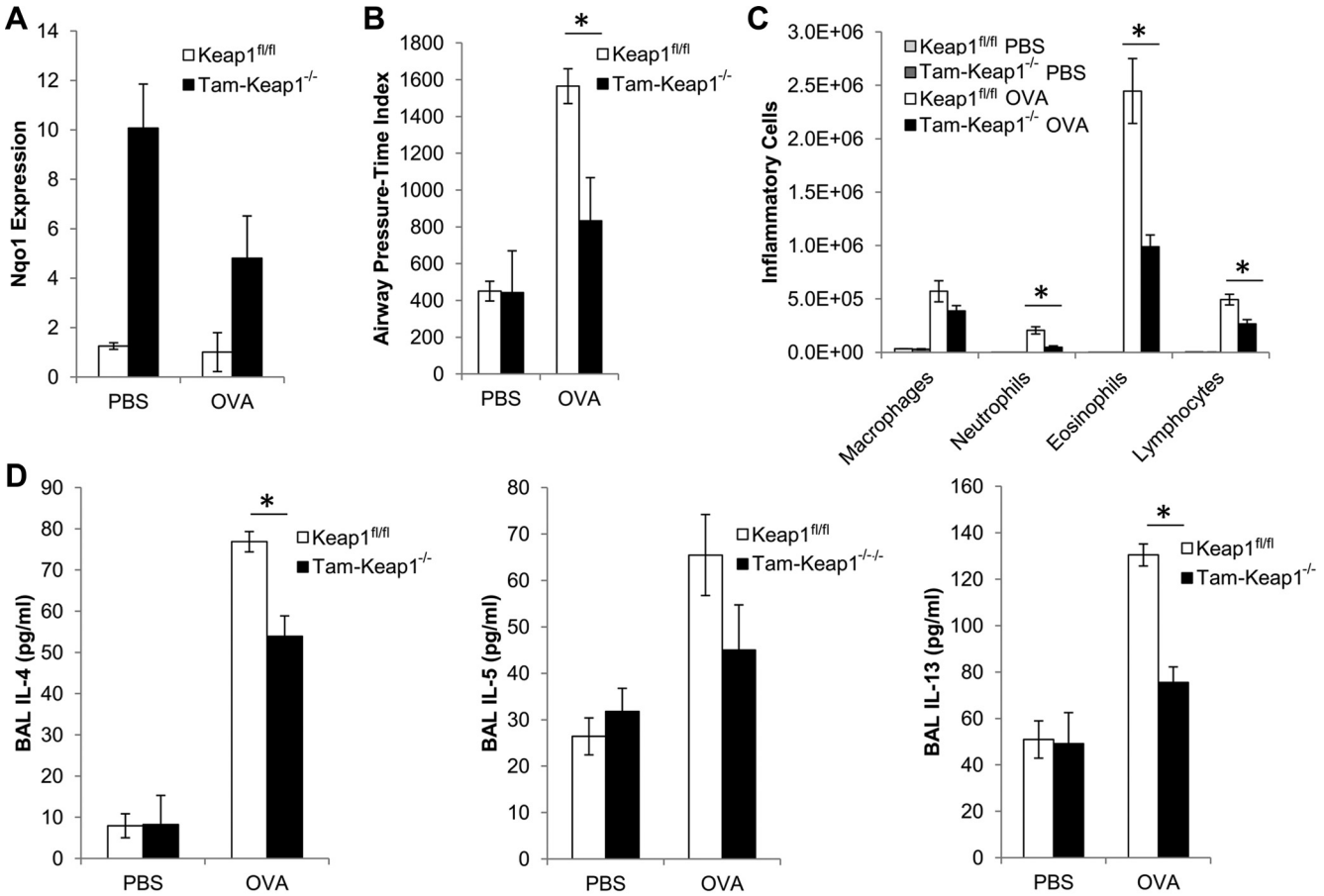

Fig. 1. Genetic activation of nuclear erythroid 2-related factor 2 (*Nrf2*) reduces ovalbumin (OVA)-induced asthmatic phenotypes. *A*: NADPH quinone oxidoreductase 1 (*Nqo1*) gene expression, as a marker of *Nrf2* activity, was measured in lungs of Kelch ECH associating protein 1-floxed (*Keap1*$^{fl/fl}$) and *Tam-Keap1*$^{-/-}$ mice that were treated with tamoxifen (Tam) before sensitization. These same mice were used in *B–D*. *B*: airway hyperresponsiveness (AHR) was assessed by airway pressure-time index (APTI) ($cmH_2O \cdot s$) after inhalation of methacholine. Inflammation (*C*) and cytokines (*D*) were quantified in the bronchoalveolar lavage (BAL). Values are means ± SE; $N = 6–9$ mice per group. $^*P < 0.05$ by ANOVA.

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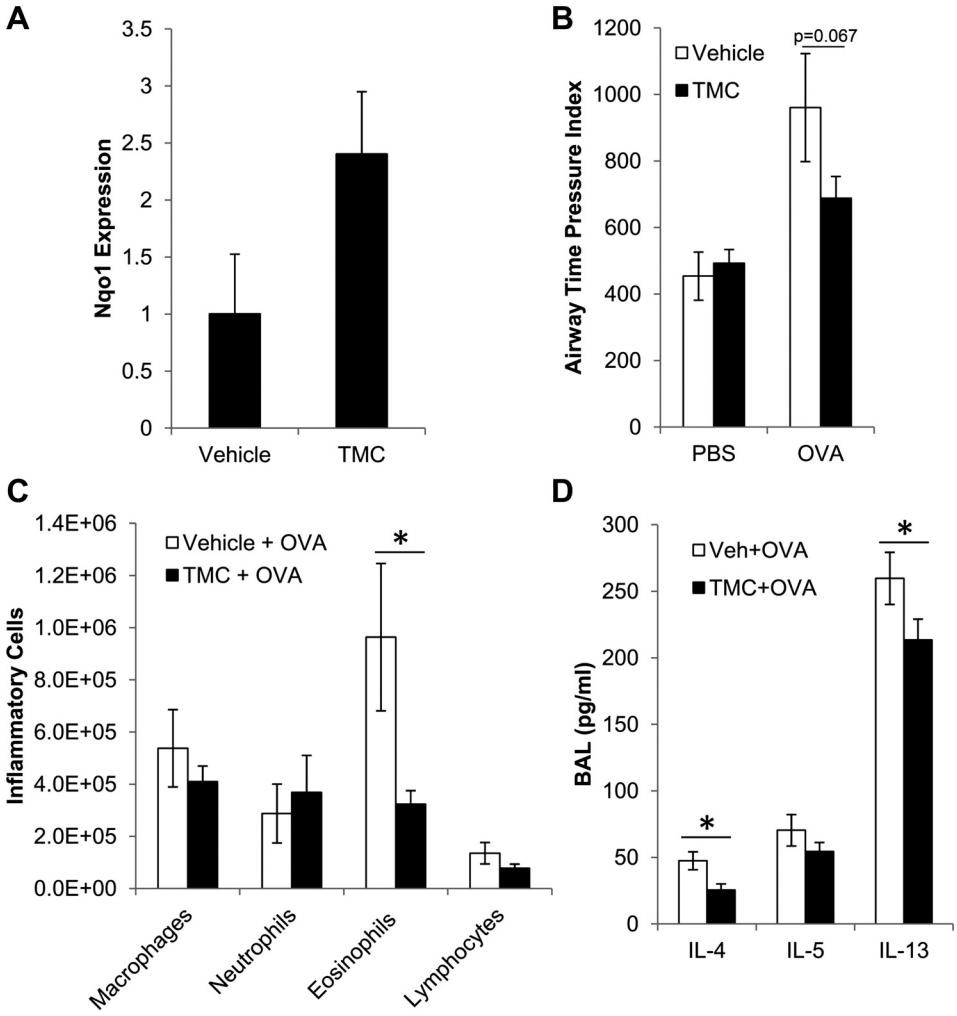

Fig. 2. Pharmacological activation of *Nrf2* during the challenge phase reduces OVA-induced asthmatic phenotypes. *A*: *Nqo1* expression was measured by quantitative PCR in lungs at 6 h after treatment with 2-trifluoromethyl-2′-methoxychalone (TMC) or vehicle by gavage ($N = 3$). AHR (*B*), inflammation (*C*), and cytokines (*D*) were measured in BAL fluid of C57BL/6 mice that were treated with 400 mg/kg TMC or vehicle by gavage 4 h before each challenge. Values are means ± SE; $N \geq 5$ mice per group. $*P < 0.05$ by Student's *t*-test.

(Fig. 2*B*), although this decrease did not quite reach statistical significance. TMC also significantly decreased OVA-induced eosinophilic inflammation (Fig. 2*C*) and secretion of IL-4 and IL-13 (Fig. 2*D*). Secretion of IL-5 was decreased slightly, but not significantly. Although genetic activation of *Nrf2* (Fig. 1) resulted in greater induction of *Nqo1* and stronger reduction of asthmatic phenotypes, compared with pharmacological activation of *Nrf2*, these data still demonstrate that pharmacological activation of *Nrf2* during the challenge phase reduces asthmatic phenotypes and suggests that *Nrf2* can suppress the susceptibility to allergen challenges.

Activation of Nrf2 in airway epithelium attenuates asthma. While asthma has traditionally been considered a disease of exaggerated allergic pathways, an emerging paradigm proposes that asthma is primarily an epithelial disorder, and that its origin and clinical manifestations have more to do with altered epithelial physical and functional barrier properties (16). Our laboratory previously generated mice that contain activated *Nrf2* specifically in club cells of the airway epithelium (*CC10-Keap1*$^{-/-}$), and we demonstrated that these mice exhibit reduced oxidative stress and inflammation in the lungs after exposure to cigarette smoke (4). Thus we assessed the role of epithelial-derived *Nrf2* activation on asthma responses. *CC10-Keap1*$^{-/-}$ mice exhibited significant reductions in OVA-induced AHR (Fig. 3*A*), eosinophilic inflammation (Fig. 3*B*), and secretion of IL-4, IL-5, and IL-13 (Fig. 3*C*), compared with *Keap1*$^{fl/fl}$ controls. These reductions in asthmatic features were similar in magnitude to the reductions observed in the *Tam-Keap1*$^{-/-}$ mice (Fig. 1). Additionally, mucus secretion was assessed by PAS staining of paraffin sections from lungs of *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice after OVA sensitization and challenge ($N = 5$). Staining was ranked and scored by a blinded observer, and representative images presented in Fig. 3*D* depict the median-scored sample from each genotype. Airways from *CC10-Keap1*$^{-/-}$ mice showed decreased PAS staining compared with that from *Keap1*$^{fl/fl}$ mice (Fig. 3*D*). Thus *Nrf2* regulates club cell function to reduce asthma in mice.

Nrf2 regulates club cell function. Club cells are nonciliated secretory cells that are a major cell type of the airway epithelium. Club cells have an array of functions, including formation of the epithelial barrier, detoxification of oxidative stress, and secretion of epithelial-derived cytokines (16). These functions are important determinants of asthma susceptibility, and we investigated the role of *Nrf2* in club cell function. Mice that were challenged with OVA exhibited oxidative stress in their lungs, as indicated by elevated levels of protein carbonyls and lipid peroxidation (Fig. 4*A*). However, protein carbonyls were significantly reduced in lungs of *CC10-Keap1*$^{-/-}$ mice, compared with *Keap1*$^{fl/fl}$ mice after OVA challenges (Fig. 4*A*), and

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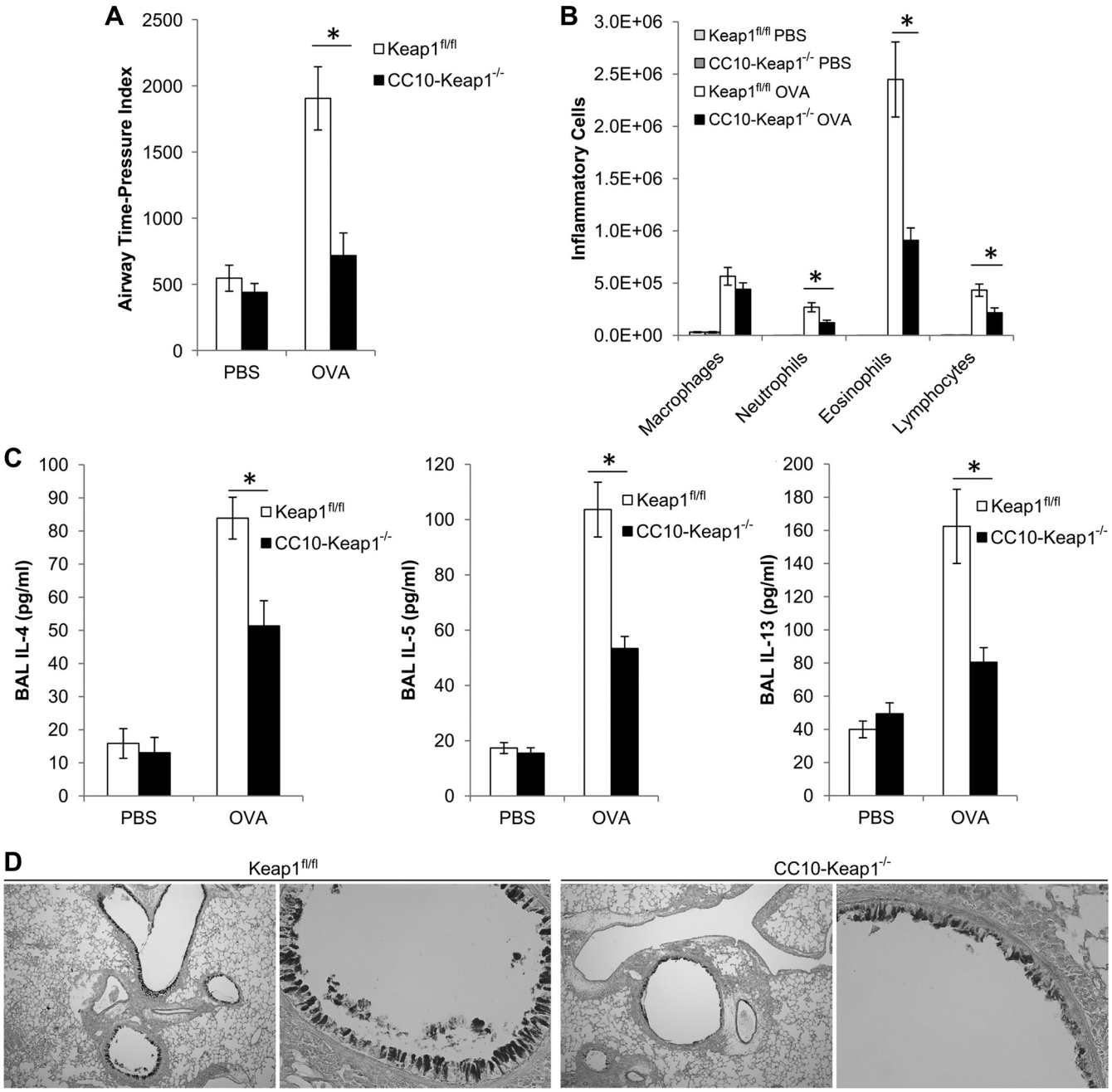

Fig. 3. Activation of *Nrf2* in club cells attenuates OVA-induced asthmatic phenotypes. *A*: AHR was assessed by APTI ($cmH_2O \cdot s$) in *Keap1*$^{fl/fl}$ and club cell-specific 10-kDa protein (CC10)-*Keap1*$^{-/-}$ mice in response to inhalation of methacholine. Inflammation (*B*) and cytokines (*C*) were quantified in the BAL. Values are means ± SE; N = 11–12 mice per group. *$P < 0.05$ by ANOVA. *D*: mucus secretion was assessed by periodic acid Schiff staining of airways from OVA-exposed *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice (N = 5 mice per group). Representative images were captured at ×40 (*left*) and ×200 (*right*) magnification.

lipid peroxidation showed a trend toward reduction in *CC10-Keap1*$^{-/-}$ mice. We also measured albumin concentration in the BAL fluid, which is an indicator of protein permeability across the airway epithelium. Airway challenges with OVA increased albumin levels in the cell-free BAL fluid, and this concentration was significantly decreased in *CC10-Keap1*$^{-/-}$ mice compared with *Keap1*$^{fl/fl}$ mice (Fig. 4*B*), suggesting that epithelial barrier function is enhanced by *Nrf2* after allergen challenge. Thus our data demonstrate that *Nrf2* regulates oxidative stress and barrier function in airway epithelial cells.

Asthmatic subjects have reduced expression of the tight junction proteins Zo-1 and E-cadherin, leading to impaired epithelial barrier function. To determine whether tight junction formation is regulated by *Nrf2*, we quantified Zo-1 and E-cadherin in airways of OVA-challenged *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice. *CC10-Keap1*$^{-/-}$ mice showed increased levels of Zo-1 in the airways compared with background levels in the adjacent smooth muscle, which was significantly higher than in *Keap1*$^{fl/fl}$ mice (Fig. 4*C*). Notably, airways from *Keap1*$^{fl/fl}$ mice contained uneven protein expression of Zo-1,

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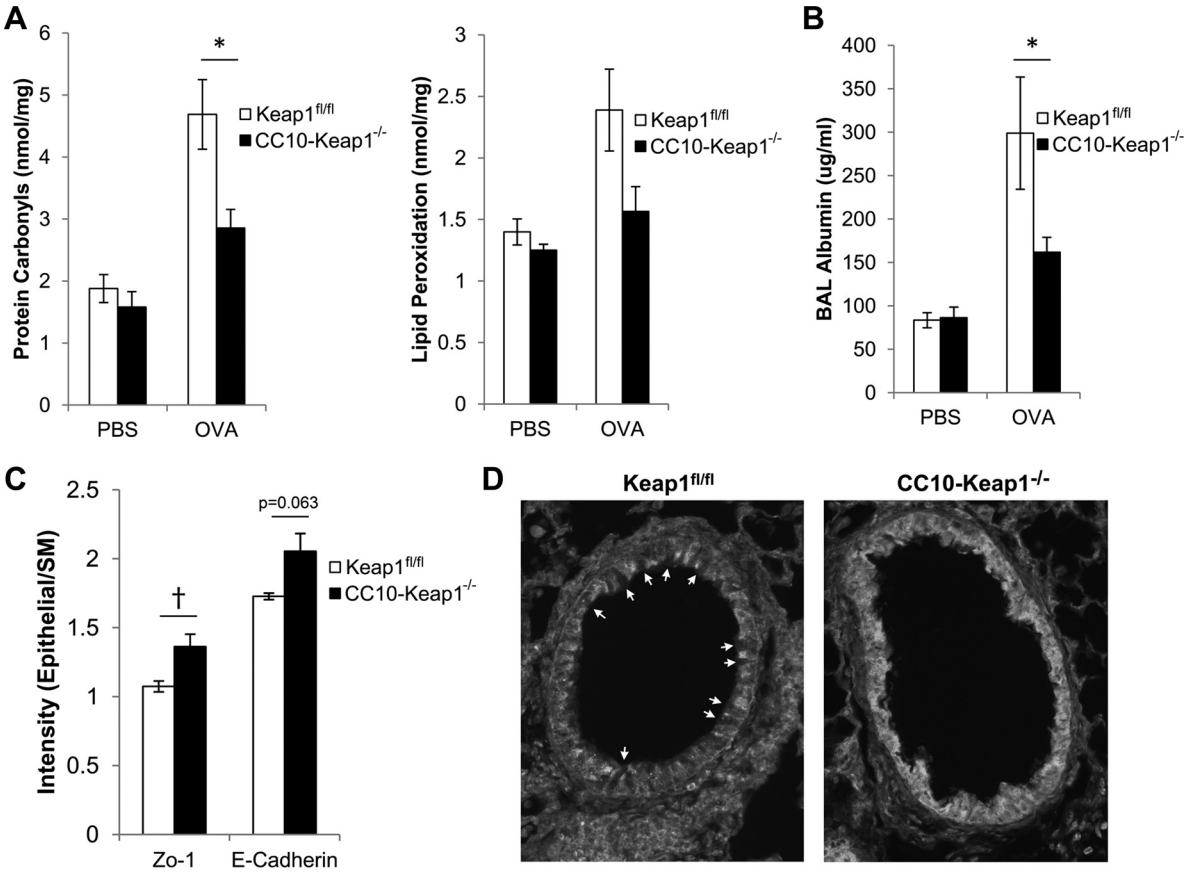

Fig. 4. *Nrf2* regulates club cell function. *A*: oxidative damage to macromolecules was measured via quantification of protein carbonyls and lipid peroxidation in lung homogenates of *CC10-Keap1*$^{-/-}$ and *Keap1*$^{fl/fl}$ mice. *B*: airway leakiness was assessed via quantification of albumin in the cell-free BAL fluid by the Albumin Blue Fluorescent Assay Kit (Active Motif). Values are means ± SE; $N = 7–8$ mice per group. *C*: Zonula occludens-1 (Zo-1) and E-cadherin intensity were quantified in the airway epithelium of OVA-sensitized/challenged *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice, and normalized to background levels using Image J. Quantification was performed on a minimum of 4 airways per mouse. $*P < 0.05$ by ANOVA. $†P < 0.05$ by Student's *t*-test. *D*: representative ×200 images of airways from OVA-sensitized/challenged *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ mice immunostained for Zo-1. Arrows indicate epithelial cells that do not express Zo-1. SM, smooth muscle.

with many cells that were nearly absent for Zo-1 (Fig. 4*D*, arrows), whereas airways from *CC10-Keap1*$^{-/-}$ mice showed greater overall Zo-1 levels and more consistent staining within the airways. Additionally, E-cadherin was elevated slightly in airways of *CC10-Keap1*$^{-/-}$ mice, which was nearly significant (Fig. 4*C*). Thus *Nrf2* enhances tight junction formation in airways of mice.

Nrf2 enhances airway barrier function. To further examine the role of *Nrf2* in airway epithelial barrier function, tracheal epithelial cells from *Keap1*$^{fl/fl}$ and *Tam-Keap1*$^{-/-}$ mice were cultured on transwell inserts to form a complete monolayer. Barrier function was assessed by quantification of TEER and paracellular diffusion of FITC-dextran across the monolayer. Confirmation of *Nrf2* activation in *Tam-Keap1*$^{-/-}$ mice was verified by elevated expression of *Nqo1* (Fig. 5*A*). Epithelial monolayers from *Tam-Keap1*$^{-/-}$ mice exhibited enhanced TEER (Fig. 5*B*) and decreased FITC-dextran diffusion (Fig. 5*C*) compared with *Keap1*$^{fl/fl}$ control mice, indicating increased barrier function. The epithelial barrier is mediated by surface expression of tight junction proteins. We stained epithelial cells for the tight junction protein, Zo-1, and observed increased localization of Zo-1 at the cell surface of *Tam-Keap1*$^{-/-}$ cells compared with *Keap1*$^{fl/fl}$ cells (Fig. 5*D*). However, Zo-1 gene expression was not dependent on *Nrf2* (data not shown). We also observed increased barrier function (TEER) in *CC10-Keap1*$^{-/-}$ epithelial cells, compared with *Keap1*$^{fl/fl}$ (Fig. 5*E*). Therefore, activation of *Nrf2* in epithelial cells enhances airway epithelial barrier function.

DISCUSSION

Our present study extends our previous findings to demonstrate that activation of *Nrf2* reduces asthmatic phenotypes in mice. We utilized multiple approaches to activate *Nrf2*, including genetic activation, via tamoxifen-induced deletion of *Keap1*, and pharmacological activation, via treatment with the *Nrf2* activator TMC. Previous studies demonstrate that patients with severe asthma, chronic obstructive pulmonary disease (COPD), or idiopathic pulmonary fibrosis contain deficient activity of *Nrf2* (3, 12, 32, 51), which suggests that the normal *Nrf2* response is not maintained in chronic pulmonary diseases. Thus targeting the *Nrf2* response may be a viable potential therapy for asthma and other chronic conditions. Indeed, activation of *Nrf2* has shown beneficial effects in a variety of disease models, including emphysema (49), bacterial exacerbations of COPD (13), viral infection (5), chronic kidney

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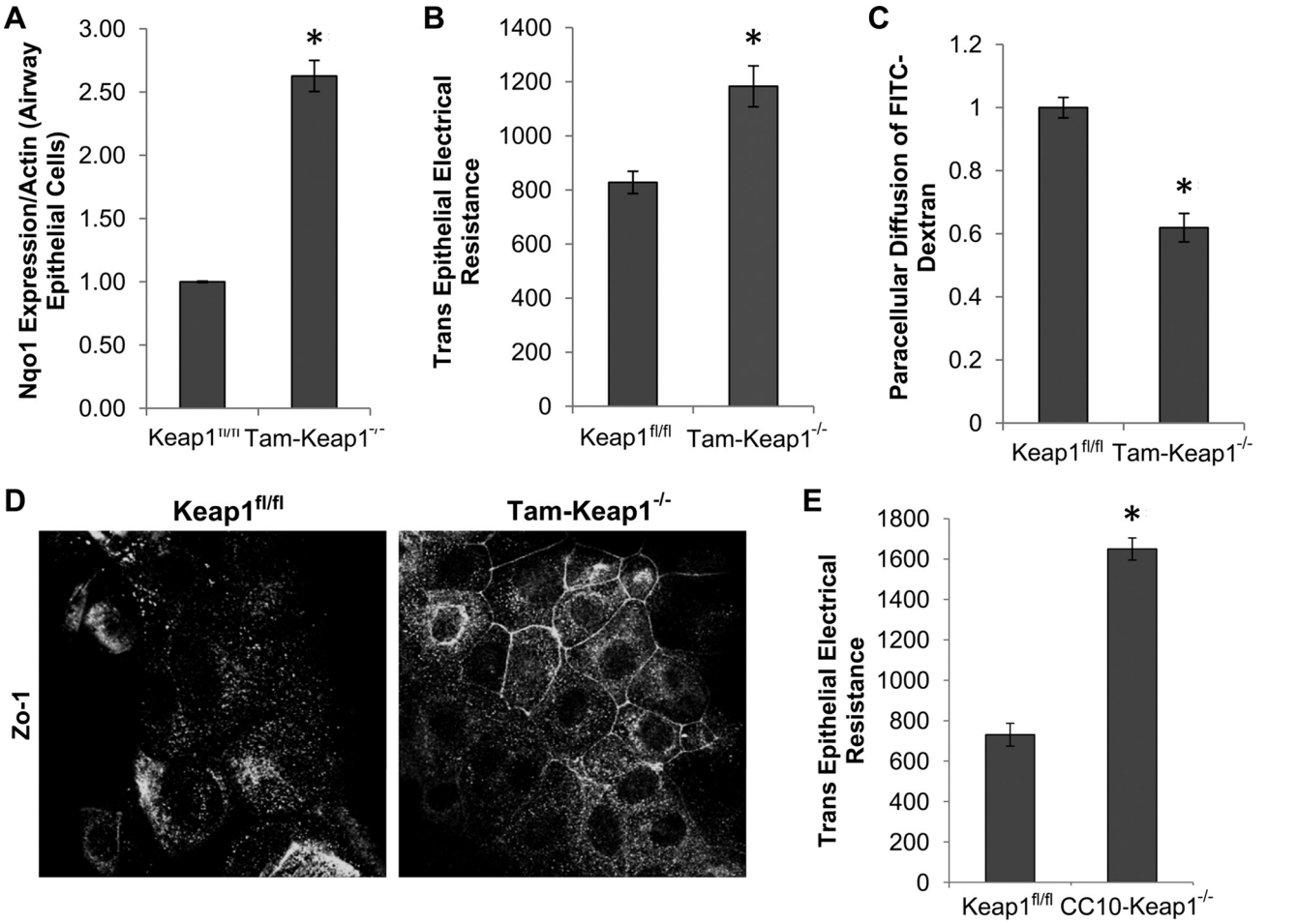

Fig. 5. *Nrf2* enhances barrier function of airway epithelial cells. *A*: *Nqo1* gene expression, as a marker of *Nrf2* activity, was measured in epithelial cell monolayers of Tam-*Keap1*$^{-/-}$ and *Keap1*$^{fl/fl}$ mice that were treated with Tam before harvesting of tracheas. *B*: after cells formed a confluent monolayer [confirmed by stable transepithelial electrical resistance (TEER) readings], TEER was measured across the monolayer daily for 5 days, and the average of all days was represented as a single replicate. *C*: FITC-dextran was added to the apical side of the confluent monolayer, and paracellular diffusion was quantified after 20 min. *D*: Zo-1 localization was measured by immunofluorescence. *E*: TEER was measured in *Keap1*$^{fl/fl}$ and *CC10-Keap1*$^{-/-}$ epithelial monolayers. Values are means ± SE; $N = 6$ mice per group. $*P < 0.05$ by Student's *t*-test.

disease (2), sepsis (55), and radiation injury (23). Additionally, dimethyl fumarate, which is an activator of *Nrf2*, is currently used as a therapy for multiple sclerosis. However, cancer cells often hijack this pathway to confer resistance to chemotherapy and radiotherapy (47), while *Nrf2*-deficient mice are protected from atherosclerosis (48). Thus there is tremendous therapeutic potential for *Nrf2* activators, but there are potential consequences that must be explored further.

Sensitization and progression of asthma are determined by a balance between the functions of epithelial cells, innate immune cells, and adaptive immune cells. We demonstrated that activation of *Nrf2* after sensitization was sufficient to reduce allergic inflammation. While this does not preclude *Nrf2* from having a role during sensitization, these data indicate that *Nrf2*-mediated reduction in asthma occurs at the challenge phase, which may be due to acute cytoprotective responses. Other studies demonstrate that *Nrf2* alters dendritic cell function and skews the Th2 response (29, 57), suggesting that *Nrf2* may also function during sensitization. We explored the role of *Nrf2* in airway epithelial cell function, which is an important component of asthma susceptibility, and we showed that *Nrf2* has a strong protective role in the airway epithelium of sensitized and challenged mice. Emerging evidence suggests that damage to the airway epithelium is a driving force of asthma susceptibility, and our data shows that *Nrf2* plays a prominent protective role in this tissue.

Club cells are a major cell type of the airway epithelium, and their primary functions include maintenance of the epithelial barrier, epithelial wound repair, and secretion of antioxidants, antibacterial compounds, and cytokines in response to cellular stress (16). The role of *Nrf2* as a critical mediator of the antioxidant response has been well studied. Previous studies demonstrate that *Nrf2* deficiency exacerbates inflammation and oxidative stress in a variety of disease models, including emphysema (38), COPD exacerbations (13), acute lung injury (31, 42), pulmonary fibrosis (6, 22), and sepsis (53). Alternatively, activation of *Nrf2* in these disease models reduces oxidative stress and inflammation (24, 43, 49). In our present study, intratracheal OVA challenges triggered oxidative stress in wild-type mice; however, protein carbonyls, a marker of oxidative stress, was significantly reduced in *CC10-Keap1*$^{-/-}$ mice. This finding demonstrates that activation of *Nrf2* in club

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cells is sufficient to alter the redox status of the lung to substantially limit OVA-induced oxidative damage, which is consistent with the role of *Nrf2* that has been established in other disease models. However, this study does not preclude other cell types from having an *Nrf2*-dependent effect on asthma susceptibility. In fact, previous studies suggest that *Nrf2* activity in dendritic cells and smooth muscle may also alter asthma susceptibility (32, 57).

Oxidative stress has been shown to play a significant role in the pathogenesis of asthma. Markers of oxidative stress are elevated in asthmatic subjects, compared with healthy subjects, while antioxidant levels, including superoxide dismutase and glutathione peroxidase, are reduced in airway epithelium of asthmatic subjects (7, 19). Replenishing antioxidants by exogenous administration of *N*-acetylcysteine reduces asthmatic inflammation in *Nrf2*$^{-/-}$ mice, suggesting that oxidative stress is an important contributor (39). Oxidative stress also plays a role in contraction of airway smooth muscle (50). Furthermore, oxidative stress can cause damage to airway epithelium and disrupt the physical barrier that normally precludes entry of inhaled allergens into the submucosa. While antioxidant therapies may have some benefit in attenuating asthmatic phenotypes, activation of *Nrf2* is likely to be a more effective therapy. Unlike antioxidant-based therapies that stoichometrically scavenge individual oxidants, *Nrf2* targets hundreds of genes to mount a coordinated and effective response against inhaled stressors.

The epithelial cell layer of the conducting airways acts as a physical barrier to exclude inhaled antigens from penetrating into the airway wall. We demonstrated that *Nrf2* reduces airway leakiness in vivo. Both in vitro and in vivo studies have shown that the airway epithelium of individuals with asthma is leakier than in normal subjects (21, 59). To further address the role of *Nrf2* in barrier function, we performed ex vivo experiments using tracheal epithelial cells from *Tam-Keap1*$^{-/-}$ and *CC10-Keap1*$^{-/-}$ mice and demonstrated that activation of *Nrf2* in epithelial cells enhances epithelial barrier function and causes an increase in surface expression of the tight junction protein Zo-1. We also demonstrated that *Nrf2* increases Zo-1 staining in airways of OVA-challenged mice. Epithelial cells from asthmatic subjects have reduced expression of tight junction proteins, including Zo-1 and E-cadherin (8, 56), and our study demonstrates that activation of *Nrf2* may restore these levels after allergen challenge. It remains unclear whether this *Nrf2*-dependent enhanced barrier function is directly caused by suppression of oxidative damage. Oxidative stress causes a rapid increase in phosphorylation of tight junction proteins, including Zo-1 and E-cadherin, leading to a redistribution of the Zo-1-occludin and E-cadherin-β-catenin complexes from the intercellular junctions and decreased TEER (41). This is consistent with our observations of *Nrf2*-induced increases in Zo-1 surface expression, which correlate with decreased oxidative stress. We did not observe *Nrf2*-dependent changes in mRNA expression of Zo-1 in isolated tracheal epithelial cells (data not shown), which suggests that Zo-1 is regulated at the protein level. Furthermore, the antioxidant glutathione was shown to increase tight junction protein levels in a rat transgenic model of human immunodeficiency virus (10), suggesting that suppression of oxidative stress is an important component of the *Nrf2*-dependent increase in epithelial barrier integrity. Since tight junction proteins are expressed by numerous cell types in addition to club cells, it is possible that *Nrf2* activation alters epithelial barrier integrity in a variety of tissues.

In conclusion, *Nrf2* reduces asthmatic features in mice, and activation of *Nrf2* in airway epithelial cells is sufficient to mediate this protective response. The airway epithelium is easily accessible from the standpoint of therapeutic interventions, and this presents a viable therapeutic target for reduction of asthma symptoms. We showed that pharmacological activation of *Nrf2*, via oral TMC treatment, reduces AHR and eosinophilic inflammation in the airways after allergen challenges, but further research is warranted to determine whether *Nrf2* reduces asthmatic phenotypes in response to other pollutants or infections. Additionally, one limitation of this study was that TMC was administered before allergen challenges, as opposed to after onset of asthmatic symptoms. Thus this strategy would mimic a controller medication rather than a rescue medication. We did not investigate whether TMC was effective after challenge with antigen. Recently, the *Nrf2* activator dimethyl fumarate received Food and Drug Administration approval for treatment of multiple sclerosis, and the *Nrf2* activator sulforaphane was shown to be safe in a recent phase II clinical trial for COPD. Future studies are needed to assess the effectiveness of these and other *Nrf2* activating compounds in patients with asthma.

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DISCLOSURES

S. Biswal and the Johns Hopkins University hold intellectual property on the development of TMC. Cureveda LLC has licensed this intellectual property. S. Biswal is cofounder of, has equity in, and serves as a scientific consultant for Cureveda LLC. These potential individual and institutional conflicts of interest have been reviewed and managed by the Johns Hopkins University School of Public Health.

AUTHOR CONTRIBUTIONS

Author contributions: T.E.S., P.N.B., G.B.D., V.K.S., and S.B. conception and design of research; T.E.S., S.G., S.C., P.M., S.M., K.S., and S.K. performed experiments; T.E.S., S.C., P.M., S.M., and K.S. analyzed data; T.E.S., S.C., V.K.S., and S.B. interpreted results of experiments; T.E.S. prepared figures; T.E.S. drafted manuscript; T.E.S., S.G., S.C., P.M., S.M., K.S., S.K., P.N.B., G.B.D., V.K.S., and S.B. approved final version of manuscript; V.K.S. and S.B. edited and revised manuscript.

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