Nrf2 Regulates Chronic Lung Inflammation and B-Cell Responses to Nontypeable *Haemophilus influenzae*

Amit A. Lugade¹, R. Robert Vethanayagam², Mehrab Nasirikenari³, Paul N. Bogner⁴, Brahm H. Segal^{1,2}, and Yasmin Thanavala¹

Departments of ¹Immunology, ²Medicine, ³Molecular and Cellular Biology, and ⁴Pathology, Roswell Park Cancer Institute, Buffalo, New York

Nrf2 is a leucine zipper transcription factor that protects against oxidant-induced injury. Nontypeable *Haemophilus influenzae* is responsible for frequent disease exacerbations in patients with chronic obstructive pulmonary disease and is responsible for causing otitis media in young children. We hypothesized that Nrf2 would limit inflammatory responses to nontypeable *H. influenzae*. The objective of this study was to assess the role of Nrf2 in chronic lung inflammation and regulation of immune responses to nontypeable *H. influenzae* in mice. Wild-type (C57BL/6) mice and Nrf2^{-/-} mice were instilled by oropharyngeal aspiration of 1×10^6 colony-forming units of live, nontypeable *H. influenzae* (NTHI) twice a week for 4 to 16 consecutive weeks to generate a chronic inflammatory milieu within the lungs that models chronic bronchitis. Nrf2^{-/-} mice had increased lymphocytic airway inflammation compared with WT mice after NTHI challenge. Although the extent of NTHI-induced peribronchovascular inflammation did not significantly differ between the genotypes, plasma cell infiltration was significantly more abundant in Nrf2^{-/-} mice. Most strikingly, Nrf2^{-/-} mice generated significantly enhanced and persistent levels of serum antibodies against P6, a key outer membrane protein of NTHI. Lung dendritic cells from Nrf2^{-/-} mice challenged with NTHI had increased activation markers compared with dendritic cells from similarly treated WT mice. Nrf2 regulates NTHI-induced airway inflammation characterized by lymphocytic and plasma cell infiltration and the activation of lung dendritic cells and B-cell responses in mice. Nrf2 may be a potential therapeutic target in limiting the bacterial infection-induced airway inflammation that drives exacerbations of chronic obstructive pulmonary disease.

Keywords: Nrf2; nontypeable *Haemophilus influenzae*; lung inflammation; B-cell responses

The lung is an interface where inhaled microbes and antigens interact with host defense cells. The inflammatory response must be calibrated to control inhaled microbes while avoiding excessive lung inflammation. Chronic obstructive pulmonary disease (COPD) is a spectrum of lung diseases that includes chronic bronchitis and emphysema. Infections by bacterial respiratory pathogens play a central role in the pathogenesis of COPD (1). Understanding mechanisms that regulate the development and persistence of chronic pulmonary inflammation and immune responses induced by respiratory pathogens may lead to better treatments for COPD.

CLINICAL RELEVANCE

Patients with chronic obstructive pulmonary disease (COPD) exhibit chronic immune cell-mediated inflammation in the lung. However, little is known about what drives the inflammatory process or the mechanisms controlling the inflammatory damage. We demonstrated that Nrf2, a transcriptional factor that induces antioxidant responses, limits experimental chronic bronchitis to nontypeable *Haemophilus influenzae*, a major cause of COPD exacerbations. Nrf2 limits plasma cell peribronchovascular inflammation, lung dendritic cell activation, and antibody responses to an outer membrane protein of nontypeable *H. influenzae*. Our study demonstrates a key role of Nrf2 in orchestrating the nature of immune responses to nontypeable *H. influenzae* and raises the potential for Nrf2 as a therapeutic target in controlling the bacterial infection-induced bronchitis that characterizes COPD exacerbations.

Morbidity and mortality among patients with COPD are related in large part to acute exacerbations, which on average occur one to three times per year. Exacerbations of COPD are associated with the acquisition of new strains of respiratory bacterial pathogens (2). Nontypeable *Haemophilus influenzae* (NTHI) is a major cause of acute sinopulmonary infections, with a particular propensity to cause exacerbations of COPD. NTHI strains are the most common pathogenic bacteria isolated from the airways of patients with COPD as colonizers and during episodes of exacerbation (3). Knowledge gained about how host innate and adaptive immune cells interact with these bacterial pathogens will be crucial to our understanding of COPD pathogenesis and also to developing novel therapeutics.

Nuclear erythroid factor-2 (Nrf2) is a cap-n-collar basic leucine zipper transcription factor that protects against oxidant-induced injury. Nrf2 is induced by a number of stimuli, including reactive oxidants (4). Upon activation, Nrf2 detaches from its cytosolic inhibitor Keap1, translocates to the nucleus, and binds to the antioxidant response element in the promoter of target genes, leading to their transcriptional induction (5). In resting cells, Nrf2 resides in the cytosol bound to the inhibitor Keap1 (6). Typically, cullin3 directs the ubiquitination and subsequent proteasome-dependent degradation of Nrf2 (7–9). Oxidation or adduction of specific cysteine residues on the adapter protein Keap1 induces a conformational change that inhibits its ability to bind to cullin3, thereby abrogating Nrf2 ubiquitination and allowing accumulation of transcriptionally active Nrf2 in the nucleus (8, 10). Nrf2-deficient mice (Nrf2^{-/-}) have increased inflammation and injury compared with wild-type (WT) mice in several experimental models (5), including LPS-induced shock (11), allergen-driven airway inflammation (12), and smoking-induced lung injury (13).

The amplification of inflammatory processes in patients with COPD is recognized as a crucial feature of the disease. We asked whether Nrf2 would have a role in limiting lung in-

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Correspondence and requests for reprints should be addressed to Yasmin Thanavala, Ph.D., Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. E-mail: yasmin.thanavala@roswellpark.org.

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flammation induced by NTHI and in modulating innate and adaptive immunity. To elucidate the role of Nrf2 in modulating chronic lung inflammation, we evaluated airway and peribronchovascular inflammation induced by prolonged exposure to NTHI in WT and Nrf2^{-/-} mice. We have established that chronic inflammation generated in lungs of mice with biweekly instillation of live NTHI replicates several aspects of histological lung inflammation observed in patients with COPD (14, 15). To enhance the clinical relevance of our studies, we used an NTHI strain isolated from the sputum of a patient with COPD and bronchitis. Although this murine model does not encapsulate structural lung damage such as emphysematous changes associated with COPD, it provides insight into inflammatory responses to respiratory bacterial pathogens that induce COPD exacerbations. Using this established mouse model of NTHI-induced bronchitis, we found that Nrf2^{-/-} mice had increased airway lymphocytic inflammation compared with WT mice. NTHI-challenged Nrf2^{-/-} mice had increased B-cell peribronchovascular inflammation and substantially augmented antibody responses to P6, an outer membrane protein of NTHI, compared with WT mice. Our studies therefore show that Nrf2 limits NTHI-induced bronchitis and regulates B-cell responses and raises the potential for Nrf2 activation as a therapeutic target in controlling COPD.

METHODS

Mice

Nrf2^{-/-} mice were generated from C57BL/6 and C57BL/129 intercrosses as described elsewhere (16) and were backcrossed nine generations in the C57BL/6 lineage. Nrf2^{-/-} mice were kindly provided by Michael Freeman (Vanderbilt University School of Medicine, Nashville, TN) through an MTA from Jefferson Chan (University of Southern California School of Medicine, Irvine, CA) and bred by homozygous mating at Roswell Park Cancer Institute. Six-week-old female Nrf2^{-/-} mice and WT C57BL/6 mice (purchased from NCI) were used in all experiments. Mice were maintained under specific pathogen-free conditions. All procedures performed on animals were IACUC approved and complied with all state, federal, and National Institutes of Health regulations. Groups of five to seven mice per genotype were used in all experiments, and each experiment was performed twice.

Initiation of Lung Inflammation

NTHI strain 1479 was used in all experiments. The preparation of bacterial suspensions and oropharyngeal administration are described in the online supplement. Mice received biweekly instillations of sterile PBS or 1×10^6 colony-forming units of live NTHI for 4 or 16 consecutive weeks before analysis.

Bronchoalveolar Lavage

After mice were killed, bronchoalveolar lavage fluid (BALF) was collected, and cell count and differential were measured as described in the online supplement. Cytokine levels in BAL supernatants were measured by sandwich ELISA.

Lung Histology Analysis

Lungs were prepared for histology as described in the online supplement. Anti-CD3 (clone 145-2C11) and anti-B220 (clone RA3-6B2) were used for immunohistochemistry to measure B-cell subsets. Lung pathology was evaluated by a pathologist (P.N.B.). A scoring schema was developed to quantify the extent of inflammation and immune cell infiltration in the lungs of mice exposed to NTHI chronically (14). Identity of the slides was blinded during two independent scoring sessions by the pathologist and a consensus score of 0 to 3 was given for each of the parameters evaluated.

Cytokine ELISPOTs

The frequency of cytokine-secreting P6-specific T-cells from the spleen and draining lymph nodes (cervical) of mice challenged with NTHI or

PBS (vehicle control) was evaluated by ELISPOT as described in the online supplement. Lymphocytes were cocultured with syngeneic antigen-presenting cells (APCs) pulsed with 1 μ M P6₄₁₋₅₅ peptide from the outer membrane protein P6 of NTHI. After an 18-hour culture, the plates were washed extensively, and cytokines were detected with biotinylated antibodies followed by addition of streptavidin-HRP. Spots were developed with tetramethylbenzidine substrate and enumerated microscopically.

B-cell ELISPOTs and Serum Anti-P6

The frequency of immunoglobulin-secreting P6-specific B-cells from the bone marrow of NTHI-exposed mice was evaluated by ELISPOT as described (17) (see online supplement). Serum titers of P6-specific antibodies were measured by indirect ELISA as described (17, 18).

Flow Cytometry

Generation of single-cell lung suspensions and preparation of cells for flow cytometry are described in the online supplement. Cells were incubated with PE-Cy5 anti-CD11c (clone N418), FITC anti-CD19 (clone MB19-1), PE anti-toll-like receptor (TLR) 2 (clone 6C2), PE anti-CD86 (clone GL1), APC anti-major histocompatibility complex (MHC) class II IA/IE (clone M5/114.15.2), and FITC anti-B cell activating factor receptor (BAFF-R) (clone eBio7H22-E16). Flow cytometric analysis was performed on a FACS Calibur flow cytometer (BD Immunocytometry Systems, San Jose, CA).

Statistical Analysis

Testing for differences between means was determined using Student's *t* test or one-way or two-way ANOVA with post-test comparisons. Categorical variables were compared by Fisher's exact test. Analysis was performed using Prism (v5; GraphPad, La Jolla, CA).

RESULTS

Enhanced Lymphocytic Airway Inflammation in Lungs of Nrf2^{-/-} Mice Chronically Exposed to NTHI

We assessed whether Nrf2 would have a role in limiting NTHI-induced bronchitis. BALF of WT and Nrf2^{-/-} mice challenged with NTHI or PBS vehicle was evaluated for total immune cell numbers and differential count (Table 1). In both genotypes, macrophages were the only immune cell present in the BALF after 4 weeks of PBS instillation. WT and Nrf2^{-/-} mice displayed an increase in BALF leukocytosis at 16 weeks compared with 4 weeks of NTHI exposure. At both time points, BALF leukocytosis was significantly greater in Nrf2^{-/-} compared with WT mice. Lymphocytes accounted for 33 and 44% of immune cells in WT mice after 4 and 16 weeks, respectively, of NTHI exposure, whereas these cells accounted for a greater proportion of BALF infiltration in Nrf2^{-/-} mice (53 and 65%, respectively). The combination of high lymphocytic accumulation and low neutrophil presence in the BALF after NTHI exposure is indicative of a chronic inflammatory environment. The proportion of BALF neutrophils and macrophages was similar between the two genotypes. These results point to a role for Nrf2 in limiting lymphocytic airway inflammation in NTHI-induced bronchitis.

We asked whether BALF concentrations of inflammatory cytokines would differ between WT and Nrf2^{-/-} mice exposed to NTHI for 4 or 16 weeks. Consistent with increased airway leukocytosis, BALF from Nrf2^{-/-} mice had increased levels of IL-4, IL-6, IL-17, and TNF- α compared with WT mice at both time points (Figure 1). In contrast, BALF collected from the lungs of WT and Nrf2^{-/-} mice instilled with PBS expressed only minimal levels of all proinflammatory cytokines measured. Taken together, these studies show that Nrf2 restrains airway inflammation and proinflammatory cytokine responses to chronic NTHI exposure.

We next evaluated histological lung inflammation after NTHI exposure. In WT and Nrf2^{-/-} mice, peribronchial and

TABLE 1. IMMUNE CELL COMPOSITION IN BRONCHOALVEOLAR LAVAGE FLUID

	Total cell count ($\times 10^3$)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)
PBS				
WT	21 $\pm$ 3	21 (100)	0 (0)	0 (0)
Nrf2 ^{-/-}	18 $\pm$ 2	18 (100)	0 (0)	0 (0)
NTHI 4 wk				
WT	289 $\pm$ 73	173 (60)	20 (7)	96 (33)
Nrf2 ^{-/-}	516 $\pm$ 102*	211 (41)*	31 (6)	274 (53)*
NTHI 16 wk				
WT	1,415 $\pm$ 265	663 (47)	123 (9)	628 (44)
Nrf2 ^{-/-}	2,145 $\pm$ 332*	531 (29)*	125 (6)	1,389 (65)*

Definition of abbreviations: NTHI = nontypeable *Haemophilus influenzae*; PBS = phosphate-buffered saline.

* $P < 0.01$, one-way ANOVA comparison of WT versus Nrf2^{-/-}.

perivascular lymphocytic inflammation was observed after 4 consecutive weeks of NTHI administration (Figure 2). The extent of peribronchovascular inflammation was greater in Nrf2^{-/-} compared with WT mice using a semiquantitative scoring system (Table 2), but this difference was not statistically significant. However, the proportion of plasma cell infiltration was significantly greater in Nrf2^{-/-} compared with WT mice (Table 2).

Immunostaining showed airway and peribronchial B-cell infiltration in WT and Nrf2^{-/-} mice (Figure 3); however, in the Nrf2^{-/-} mice, the B-cell infiltrates were more organized and had a tendency to form expansile nodular aggregates. As early as 4 weeks after NTHI exposure, CD3⁺ cells were observed surrounding airways and bronchovascularity in both genotypes, although the levels of these cells were slightly increased in Nrf2^{-/-} mice as compared with WT mice. The pattern of B-cell (B220⁺) infiltration was markedly different between WT and Nrf2^{-/-} mice. B220⁺ cells were observed within and surrounding the airways and bronchovascularity in WT mice. In Nrf2^{-/-} mice, B-cell infiltration was more extensive and formed structures resembling lymphoid follicles. The degree of interstitial and pleural inflammation was greater in Nrf2^{-/-} compared with WT mice, but these differences did not reach statistical significance. Together, these results show that Nrf2 limits airway and peribronchovascular B-cell inflammation after chronic NTHI exposure.

Enhanced Adaptive Immune Response to NTHI in Nrf2^{-/-} Mice

Based on our observations that Nrf2 regulates lymphocytic airway and peribronchovascular inflammation after NTHI, we determined whether Nrf2 also regulates T-cell and antibody responses to NTHI. The outer membrane protein P6 of NTHI

Figure 1. Increased levels of proinflammatory cytokines in bronchoalveolar lavage fluid (BALF) of Nrf2^{-/-} mice. Concentrations of cytokines in BALF of mice exposed to PBS (open bars) or nontypeable *Haemophilus influenzae* (NTHI) (filled bars) were determined by sandwich ELISA. * $P < 0.01$; two-way ANOVA with Bonferroni post-test comparison was used to evaluate the effect of genotype (WT versus Nrf2^{-/-}) and duration of NTHI exposure (4 wk versus 16 wk). Average $\pm$ SEM ($n = 10$).

Figure 2. Exuberant immune cell infiltration in lungs of *Nrf2*^{-/-} mice. Mice were exposed to NTHI for 4 consecutive weeks. H&E-stained lung sections were analyzed for inflammation by light microscopy. Regions of lymphoid and plasmacytic inflammatory accumulation around airways (aw) and bronchovascular bundles (bv) are noted by arrows. Epithelial cell damage is noted by arrowheads. Bars represent 100 μ m. Representative images of H&E sections taken from 14 mice per group.

activates human macrophages, resulting in the production of IL-8 and TNF- α , which likely enhances recruitment of neutrophils to airways and plays a role in COPD exacerbations (19). P6 is a highly conserved and immunogenic protein and is considered a promising target for vaccine development (20). P6 is therefore an ideal antigen to measure whether chronic exposure to the bacteria results in the generation of specific antigen-driven lymphocyte responses. Our model of chronic bacterial exposure permits analysis of the NTHI-specific T- and B-cell compartments to determine whether the absence of *Nrf2* affects the generation and function of these cells.

An immunodominant T-helper peptide from the outer membrane protein P6 (18) was used to measure the frequency of cytokine secreting antigen-specific T-cells in the lymphoid organs of WT and *Nrf2*^{-/-} mice exposed to NTHI (Figure 4).

T-cells from the spleens and draining lymph nodes (cervical) of both genotypes were capable of secreting the cytokines IL-2, IFN- γ , IL-4, and IL-17 in response to 18 hours of stimulation with the P6_{41–55} peptide. This suggests that there was no bias in the cytokine profile of the T-cells from the lymphoid organs of WT or *Nrf2*^{-/-} mice as they secreted the signature cytokines of the Th1, Th2, and Th17 subsets. An increase in the frequency of IL-4-secreting and IL-17-secreting P6-specific T-cells was observed in *Nrf2*^{-/-} mice compared with WT mice. The frequency of IL-2-secreting and IFN- γ -secreting P6-specific T-cells was similar between the two genotypes. Appreciable numbers of P6-specific T-cells were not detected in PBS-administered mice. These results point to *Nrf2* having a role in regulating T-cell phenotypes in response to NTHI.

Because of the extensive lung infiltration of B-cells noted in *Nrf2*^{-/-} mice, we evaluated whether the absence of *Nrf2* would influence B-cell responses to NTHI. We measured the frequency of P6-specific Ig-secreting cells in bone marrow using B-cell ELISPOTs (Figure 5A). An increase in the frequency of anti-P6 Ig-secreting cells was observed after 4 weeks of exposure to NTHI in *Nrf2*^{-/-} mice as compared with similarly treated WT mice. P6-specific B-cells were not detected in mice administered PBS vehicle. Differences in the frequency of P6-specific B-cells from WT and *Nrf2*^{-/-} mice were observed for the three most common IgG subclasses (IgG1, IgG2a, and IgG2b). These cells were present as early as 4 weeks but increased after 16 weeks of chronic NTHI exposure (data not shown). The anti-P6 Ig-secreting cells made antibodies of IgG1 and IgG2a subclasses, revealing a lack of bias in the nature of the B-cell response to NTHI similar to that revealed by the T-cell cytokine ELISPOT.

B-cell ELISPOTs show the frequencies of antigen-specific Ig-secreting lymphocytes but do not provide information on the level of antibody responses. To further probe the role of *Nrf2* in regulating NTHI antigen-specific antibody responses, we quantified endpoint serum antibody titers over the course of a 16-week exposure to NTHI in WT and *Nrf2*^{-/-} mice (Figure 5B). There was a dramatic increase in the levels of P6-specific antibodies in *Nrf2*^{-/-} compared with WT mice. The differences in serum antibody levels between the two genotypes was apparent as early as 4 weeks after NTHI exposure, the time in which increased frequencies of antibody-secreting B-cells were

TABLE 2. PATHOLOGICAL EVALUATION OF LUNG INFLAMMATION*

	Peribronchovascular Inflammation [†]			Plasma Cell Frequency [‡]		Pleural Inflammation [§]		Interstitial Inflammation [¶]	
	Marked	Moderate	Mild	10–50%	1–10%	Moderate	Mild	Moderate	Mild
4 wk									
WT	2/14	9/14	3/14	5/14	9/14	6/14	8/14	9/14	5/14
<i>Nrf2</i> ^{-/-}	5/14	8/14	1/14	12/14	2/14	10/14	4/14	11/14	3/14
16 wk									
WT	0/4	4/4	0/4	2/4	2/4	0/4	4/4	2/4	2/4
<i>Nrf2</i> ^{-/-}	3/4	1/4	0/4	4/4	0/4	0/4	3/4	3/4	1/4

Definition of abbreviation: WT = wild type.

* Frequency of mice with observed lung inflammation.

[†] Severity of peribronchovascular inflammatory cell accumulation. Marked (score of 3): average of > 15 cell cuff thickness and/or ≥ 3 peribronchovascular well developed lymphoid follicles. Moderate (score of 2): average of 5–15 cell cuff thickness. Mild (score of 1): average < 5 cell cuff thickness.

[‡] Plasma cell frequency in peribronchovascular infiltrates.

[§] Severity of inflammation in lung pleura. Moderate (score of 2): patchy pleural thickening by inflammatory cells and/or diffuse pleural inflammation without thickening. Mild (score of 1): patchy pleural involvement by inflammatory cells without pleural thickening.

[¶] Severity of inflammation in interstitial space. Moderate (score of 2): patchy interstitial involvement of inflammatory cells with or without inflammatory nodules. Mild (score of 1): focal interstitial involvement of inflammatory cells without inflammatory nodules.

Figure 3. Enhanced accumulation of CD3⁺ lymphocytes and B220⁺ lymphocytes in lungs of Nrf2^{-/-} mice. Lung sections of WT and Nrf2^{-/-} mice exposed to NTHI for 4 consecutive weeks were analyzed by immunohistochemistry (IHC) using anti-CD3 and anti-B220 in formalin-fixed lungs and zinc-fixed lungs, respectively. Brown precipitates of 3,3'-diaminobenzidine indicate regions of positive immunostaining. Nuclei are counterstained with hematoxylin. IHC staining on lung sections with isotype-matched antibody for anti-CD3 and anti-B220 are provided in the *bottom row*. Bars represent 100 μ m. Representative images of IHC sections taken from five mice per group.

also observed in the bone marrow. Additionally, the levels of anti-P6 reached a plateau by Week 5 in WT mice (1:4,000 titer), whereas titers of anti-P6 in Nrf2^{-/-} mice continued to increase, reaching a plateau by 8 weeks (1:13,000 titer) of NTHI exposure. Thus, our studies show that the absence of Nrf2 dramatically affects the function of B-cells after NTHI exposure, resulting in augmented P6-specific antibody production.

Lung APCs of Nrf2^{-/-} Mice Display Activated Phenotype after NTHI Exposure

The augmented lymphocytic airway inflammation, B-cell lung infiltration, and antigen-driven lymphocyte responses in Nrf2^{-/-} mice compared with WT mice after NTHI challenge prompted us to ask whether the activation status of APCs was also

regulated by Nrf2. At 4 weeks after NTHI exposure, single-cell lung suspensions were generated, and activation of APCs was assessed. Due to the lack of inflammation elicited in the WT and Nrf2^{-/-} mice instilled with PBS, we were unable to recover sufficient numbers of immune cells to perform flow cytometric analysis. Because of the high number of immune cells present within the lungs of NTHI-exposed mice, we assessed the inflammatory phenotype in individual mice without pooling samples. Since TLR2 recognizes and responds to the lipoprotein P6 in the outer membrane of NTHI (21), we evaluated whether the expression pattern of TLR2 on lung APCs was modulated by Nrf2 after 4 weeks of exposure to NTHI (Figure 6). Expression of MHC class II and the T-cell costimulatory molecule CD86 was also assessed to determine whether differences between the two genotypes existed in the activation status of APCs. Given the increased levels of anti-P6 Ig levels and the accumulation of B-cells in the lungs of Nrf2^{-/-} mice, we sought to determine whether phenotypic alterations could account for the enhanced B-cell activation in Nrf2^{-/-} mice exposed to NTHI. For this reason, surface expression of the B-cell costimulatory marker BAFF-R was evaluated to determine the level of lung B-cell activation after chronic NTHI exposure (22).

After 4 weeks of NTHI exposure, CD19⁺ cells accounted for 38% of lung-infiltrating immune cells in WT mice, and the frequency of this immune cell population was elevated in the lungs of Nrf2^{-/-} mice (44%). B-cells (CD19⁺) from NTHI-challenged WT and Nrf2^{-/-} mice expressed CD86 and TLR2 at similar levels by mean fluorescent intensity (MFI), although the frequency of these cells was greater in Nrf2^{-/-} mice as compared with WT mice (CD86⁺ WT: 33% versus Nrf2^{-/-}: 54%; $P < 0.01$ and TLR2⁺ WT: 23% versus Nrf2^{-/-}: 47%; $P < 0.01$). B-cells in Nrf2^{-/-} mice displayed substantially increased levels of surface MHC class II (Nrf2^{-/-} MFI = 908; $P < 0.01$) as compared with B-cells in WT mice (MFI = 395). This difference was observed not only at the level of MHC class II expression but also in the frequency of Nrf2^{-/-} B-cells that expressed MHC class II (WT: 68% versus Nrf2^{-/-}: 92%; $P < 0.01$). The proportion of B-cells expressing BAFF-R and the level of B-cell BAFF-R expression (MFI) were greater in NTHI-challenged Nrf2^{-/-} compared with WT mice (BAFF-R⁺ WT: 49% versus Nrf2^{-/-}: 58%; and BAFF-R MFI WT: 29 versus Nrf2^{-/-}: 56; $P < 0.01$).

In contrast to the differences in CD19⁺ B-cell populations, WT and Nrf2^{-/-} mice exhibited similar frequencies of lung-infiltrating dendritic cells (DCs) within the immune cell population (WT: 13% versus Nrf2^{-/-}: 15%). However, the DCs from Nrf2^{-/-} mice displayed much greater surface expression of CD86 and TLR2 as compared with WT mice (CD86 MFI WT: 99 versus Nrf2^{-/-}: 173; $P < 0.01$ and TLR2 MFI WT: 95 versus Nrf2^{-/-}: 386; $P < 0.01$). TLR2 expression on Nrf2^{-/-} CD11c⁺ DCs was almost 2.5-fold greater on average than on WT CD11c⁺ DCs. MHC class II expression on Nrf2^{-/-} DCs was also increased compared with DCs from WT mice (MFI WT: 640 versus Nrf2^{-/-}: 1058; $P < 0.01$).

Figure 4. Frequency of P6-specific T-cells is increased in Nrf2^{-/-} mice. Cytokine ELISPOTs were performed on whole-cell populations from spleen and draining lymph nodes (cervical) of mice exposed to PBS or NTHI for 4 weeks. Comparisons were made between NTHI-challenged WT and Nrf2^{-/-} mice. * $P < 0.01$, one-way ANOVA. Average $\pm$ SEM ($n = 10$).

A Anti-P6 B cells in bone marrow **B Anti-P6 Ig levels in serum**

Figure 5. Enhanced P6-specific B-cell response in Nrf2^{-/-} mice following chronic NTHI exposure. (A) B-cell ELISPOTs performed on whole-cell bone marrow populations demonstrated increased frequency of antibody-secreting P6-specific cells in Nrf2^{-/-} mice compared with WT mice at 4 weeks after NTHI challenge. †*P* < 0.01, one-way ANOVA. (B) Serum P6-specific antibody was increased in Nrf2^{-/-} mice compared with WT mice after chronic NTHI exposure. **P* < 0.01, two-way ANOVA with Bonferroni post-test comparison of WT versus Nrf2^{-/-}. Average ± SEM (*n* = 10).

0.01). Lung DCs did not express BAFF-R, whose expression is restricted to B-cells and a subset of T-cells (22). Our studies therefore show an important role of Nrf2 in limiting DC activation after NTHI exposure, which likely influences downstream T- and B-cell responses.

DISCUSSION

Taken together, our studies show an important role of Nrf2 in limiting immune responses to NTHI in mice. Repeated exposure of the lung to NTHI leads to the induction and persistence of peribronchovascular lymphocytic infiltrates and facilitates their continued exposure to NTHI antigens in the lung environment. We have demonstrated that Nrf2 regulates innate immune responses that include maturation of dendritic cells and the production of proinflammatory cytokines that prime subsequent effector T- and B-cell responses. Nrf2 was required to limit airway inflammation and peribronchovascular B-cell infiltration after NTHI challenge. Nrf2 likely mediates this anti-inflammatory effect through multiple targets at the level of innate immune cell activation and T- and B-cell effector functions. Our results support a model in which Nrf2-inducible genes counter-regulate the lung inflammatory response induced by NTHI, thereby protecting against injurious inflammation.

Smoking is a major risk factor for COPD, whereas COPD exacerbations are commonly associated with respiratory bacterial infections (2). Previous studies have suggested that Nrf2 likely affects COPD pathogenesis through modulation of responses to smoke and infections. Lungs of patients with COPD have diminished activity of Nrf2-regulated pathways (23, 24). Cigarette smoke induces Nrf2 activation in human alveolar macrophages, and Nrf2 expression is decreased in the macrophages of older current smokers and patients with COPD (25). Nrf2 is protective in smoking-induced lung injury in mice (13, 26–28). In addition, NTHI challenge in smoke-exposed mice exacerbates lung inflammation, supporting a model in which the interaction of inhaled irritants and bacterial infection augment lung injury (29). These studies and ours raise the possibility that Nrf2 activation could be beneficial in controlling smoking and the bacterial infection-induced lung inflammation that drives COPD (30).

We observed that TLR2 expression was increased in lung DCs from Nrf2^{-/-} compared with WT mice after NTHI challenge. The presence of the TLR2 ligand lipoprotein P6 in the NTHI outer membrane may contribute to the increased expression of TLR2 on lung DCs because these cells are repeatedly exposed to and stimulated by the bacteria. The effect of Nrf2-regulated, NTHI-induced TLR2 expression on

Figure 6. Enhanced activation of lung dendritic cells in Nrf2^{-/-} mice compared with WT mice exposed to NTHI for 4 weeks. Surface expression of CD86, major histocompatibility complex (MHC) class II, toll-like receptor (TLR)2, and B cell activating factor receptor (BAFF-R) on B-cells (CD19⁺) and dendritic cells (CD11c⁺) from single-cell suspensions of lungs of WT (black histograms) and Nrf2^{-/-} (gray histograms) mice were analyzed by flow cytometry. Representative plots from 10 animals of each genotype are shown, and averages from these animals are provided in each plot. The percentage of cells expressing marker ± SEM and mean fluorescent intensity ± SEM above isotype staining (open histograms) are provided in each plot. **P* < 0.01, one-way ANOVA.

DCs on downstream signaling and activation of T- and B-cells merits further study. In addition to modulating antigen-driven immune responses that we have demonstrated, Nrf2 can also regulate innate antibacterial host defense after lung injury (31).

BALF of NTHI-challenged Nrf2^{-/-} mice exhibited increased levels of IL-4, IL-6, IL-17, and TNF- α compared with BALF from WT mice. These cytokines likely contribute to the amplification of leukocyte recruitment to the lung. In addition, IL-6 may be involved in P6-specific effector T-cell differentiation because this cytokine, in combination with TGF- β , has been shown to prime naive T-cell differentiation into Th17 cells in mice (32). We also observed increased Th2 (IL-4⁺) and Th17 lymphocytes in the spleens and draining lymph nodes in NTHI-challenged Nrf2^{-/-} compared with WT mice. Th2 cells can promote allergy, whereas Th17 cells regulate neutrophilic and macrophage inflammation in the lung and likely play a role in the pathogenesis of asthma and COPD (33).

Nrf2 likely modulates the cross-talk between DCs, T-cells, and B-cells that calibrate the immune response to generate protective antibody responses against pathogens while averting allergy and autoimmunity (34–36). Nrf2 has been shown to regulate DC activation *in vitro*. Rangasamy and colleagues showed that Nrf2 inhibits maturation of murine DCs by ragweed extract, a common allergen (37). Nrf2-deficient DCs had increased display of costimulatory molecules and primed Th2 responses after exposure to ambient particulate matter as compared with similarly treated WT DCs (38). Activation of Nrf2 in DCs was associated with increased Th1 responses in aged mice (39), demonstrating that Nrf2-mediated effects on DCs are context dependent. In our model, we have shown that Nrf2 influences lung DC activation *in vivo*.

Previous studies have documented that T-cells, B-cells, DCs, and macrophages organize into lymphoid follicles close to the airways and in the lung parenchyma (15, 40, 41). The chemokine receptor CXCR3 is expressed by T-cells and B-cells in secondary lymphoid structures in other parts of the body; therefore, it was important when Kelsen and colleagues demonstrated that lung lymphoid follicle cells from patients with COPD over-express CXCR3 and that its ligands, IP10 and Mig, are expressed by lung epithelial cells and airway epithelium (42). In a landmark study, Hogg and colleagues showed that the numbers of small airways containing lymphoid follicles increases in patients with severe COPD and thus may represent a pathological correlate of disease severity (15). Recently it has also been shown that the numbers of B-cells in the lymphoid follicles (in the parenchyma and bronchial walls) is increased in patients at different stages of COPD severity (43). Polverino and colleagues recently showed that expression of B-cell activating factor of TNF family (BAFF) was increased in lungs of patients with COPD and correlated with COPD severity (44). These findings in humans support the hypothesis that B-cells are pathogenic in COPD. It has been speculated that B-cells may contribute to autoimmune responses that accelerate tissue destruction and emphysema (45).

Lung lymphoid follicles have been shown to participate in antigen-specific antibody and cellular responses (46). Nrf2 is expressed in B-cells (47, 48), and B-cell differentiation is associated with oxidative stress and activation of antioxidative pathways, including Nrf2 (49). In the present study, we noted a pattern of B-cell (B220⁺) infiltration that was markedly different between WT and Nrf2^{-/-} mice. B220⁺ cells were observed within and surrounding the airways and bronchovascular in WT mice. In Nrf2^{-/-} mice, B-cell infiltration was more extensive and formed distinct structures resembling lymphoid follicles. Our studies clearly demonstrate augmented antibody responses to P6 in the absence of Nrf2 and that B-cells

isolated from the lungs of Nrf2^{-/-} mice had increased expression of the B-cell activation marker BAFF-R compared with WT mice after chronic NTHI exposure, further supporting a role for Nrf2 in limiting B-cell responses. Our observations of increased plasma cell accumulation in peribronchovascular infiltrates and substantially augmented P6-specific antibody responses in Nrf2^{-/-} compared with WT mice after NTHI challenge raise the possibility that Nrf2 may regulate B-cell effector responses through modulation of redox-sensitive pathways.

Consistent with our findings with the NTHI model, Nrf2-deficient mice have shown increased allergen-induced airway inflammation (12). Depending on the experimental model, Nrf2-deficient mice demonstrated increased (50–52) and decreased (53) susceptibility to autoimmunity. Li and colleagues showed that aged female Nrf2^{-/-} mice were prone to developing a lupus-like illness associated with autoantibody generation (54). These findings and our current study highlight the role of Nrf2 as a regulator of pathogen-specific antibody responses and potentially of damaging autoreactive immunoglobulins.

The results of the current study establish an important role for Nrf2 in calibrating the inflammatory response to NTHI in a chronic bronchitis model. Nrf2 had broad regulatory effects on innate and T- and B-cell effector functions. Our work identifies Nrf2 as a potential therapeutic target for NTHI-induced bronchitis. In addition, the dramatic effect of Nrf2 in dampening P6-specific immunoglobulin responses raises the possibility that Nrf2 might be a limiting factor in sustaining protective antibody responses after natural infection or vaccination, which could have broad implications for immune-based therapeutics.

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