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Inhaled Birch Pollen Extract Induces Airway Hyperresponsiveness via Oxidative Stress but Independently of Pollen-Intrinsic NADPH Oxidase Activity, or the TLR4–TRIF Pathway

Karim H. Shalaby,* Alexandra Allard-Coutu,* Michael J. O’Sullivan,* Emily Nakada,* Salman T. Qureshi,[†] Brian J. Day,[‡] and James G. Martin*

Oxidative stress in allergic asthma may result from oxidase activity or proinflammatory molecules in pollens. Signaling via TLR4 and its adaptor Toll–IL-1R domain–containing adapter inducing IFN- β (TRIF) has been implicated in reactive oxygen species–mediated acute lung injury and in Th2 immune responses. We investigated the contributions of oxidative stress and TLR4/TRIF signaling to experimental asthma induced by birch pollen exposure exclusively via the airways. Mice were exposed to native or heat-inactivated white birch pollen extract (BPEX) intratracheally and injected with the antioxidants, N-acetyl-L-cysteine or dimethylthiourea, prior to sensitization, challenge, or all allergen exposures, to assess the role of oxidative stress and pollen-intrinsic NADPH oxidase activity in allergic sensitization, inflammation, and airway hyperresponsiveness (AHR). Additionally, TLR4 signaling was antagonized concomitantly with allergen exposure, or the development of allergic airway disease was evaluated in TLR4 or TRIF knockout mice. N-acetyl-L-cysteine inhibited BPEX-induced eosinophilic airway inflammation and AHR except when given exclusively during sensitization, whereas dimethylthiourea was inhibitory even when administered with the sensitization alone. Heat inactivation of BPEX had no effect on the development of allergic airway disease. Oxidative stress–mediated AHR was also TLR4 and TRIF independent; however, TLR4 deficiency decreased, whereas TRIF deficiency increased BPEX-induced airway inflammation. In conclusion, oxidative stress plays a significant role in allergic sensitization to pollen via the airway mucosa, but the pollen-intrinsic NADPH oxidase activity and TLR4 or TRIF signaling are unnecessary for the induction of allergic airway disease and AHR. Pollen extract does, however, activate TLR4, thereby enhancing airway inflammation, which is restrained by the TRIF-dependent pathway. *The Journal of Immunology*, 2013, 191: 000–000.

Allergic asthma is a chronic airway disease triggered by an aberrant inflammatory response to inhaled allergens, resulting in airway inflammation, airway hyperresponsiveness (AHR) to inhaled bronchoconstrictors, and eventual progression to airway remodeling. Allergic asthma is characterized

by a Th2-predominant T cell response. There is emerging interest in identifying common intrinsic properties of allergens, or allergen-associated danger signals that impart allergenicity to certain proteins. Oxidative stress has been implicated in airway diseases, such as asthma (1) and may be a critical mechanism evoking allergic sensitization upon initial allergen exposure and/or amplifying inflammatory responses upon secondary allergen exposure. Markers of oxidative stress have been detected in the lungs of patients with asthma caused by the release of reactive oxygen species (ROS) and reactive nitrogen species from airway epithelial cells (2), upon direct exposure to allergens or environmental pollutants, such as cigarette smoke, diesel exhaust (3), and ozone, as well as from inflammatory cells responding to these environmental stresses. Lower levels of the antioxidant, glutathione (4), as well as reduced activity of major antioxidant enzymes, such as superoxide dismutase (5), are also reported in asthmatics, and associated with airflow obstruction, AHR, and airway remodeling.

Oxidative stress may result not only from activation of inflammatory cells and structural cells, but also pollens, such as ragweed and birch, that have intrinsic NADPH oxidase activity and have been shown to rapidly trigger the production of ROS within the airway epithelium upon topical exposure (6). Using a systemic sensitization of mice with ragweed and aluminum hydroxide adjuvant, followed by subsequent intranasal exposure to ragweed, Boldogh et al. (6) found that a pollen-intrinsic capacity to generate oxidative stress amplified allergic airway inflammation. This effect could be prevented by concomitant intranasal administration of antioxidants (7). To date, it has not been investigated whether pollen-intrinsic NADPH oxidase activity is important in eliciting

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BPEX, birch tree pollen extract; BPEX^H, heat-inactivated BPEX; DMTU, N,N'-dimethylthiourea; DPI, diphenylene iodonium; Ers, respiratory system elastase; HDM, house dust mite allergen extract; 4-HNE, 4-hydroxynonenal; i.t., intratracheal; KO, knockout; LPS-RS, *Rhodobacter sphaeroides* LPS; MCh, acetyl- β -methylcholine; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; Rrs, respiratory system resistance; TRIF, Toll–IL-1R domain–containing adapter-inducing IFN- β ; wt, wild-type.

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allergic sensitization. The TLR4 has also been implicated in mediating inflammatory responses in experimental allergic asthma. Der p 2, an Ag found in house dust mite allergen extracts (HDM), was demonstrated to mimic a component of the TLR4 signaling complex, MD2, both structurally and functionally (8). Importantly, TLR4 expression and signaling specifically on airway structural cells were shown to be necessary for the establishment of inflammation and AHR in response to inhaled HDM (9). TLR4 functionality on stromal cells was also found to be critical for the development of Th2-mediated airway disease induced by inhaled LPS-laden OVA (10). Thus, there is evidence to indicate that TLR4 plays a significant role in compromising mucosal tolerance to elicit allergic sensitization, and/or in driving inflammatory responses to Ags via the airways.

The pathways of oxidative stress and TLR4 activation may also be interlinked. For example, studies in mice have revealed a role for TLR4 in mediating ozone-induced airway disease (11, 12). Recently, intradermal injection of the cysteine protease papain was shown to induce the production of ROS in the skin epithelium and dermal dendritic cells of mice, eliciting a potent antioxidant (N-acetyl cysteine)-sensitive Th2 and basophil response via the TLR4 pathway and its adaptor, Toll–IL-1R domain-containing adapter inducing IFN- β (TRIF) (13). The TLR4–TRIF pathway was suggested to be activated by oxidized phospholipids produced in the lungs of humans and animals infected with Severe Acute Respiratory Syndrome virus, anthrax, or H5N1 and implicated in triggering TLR4–TRIF-mediated acute lung injury induced by acid or inactivated H5N1 avian influenza virus (14). Interestingly, pollens such as white birch and ragweed contain readily releasable cysteine (as well as serine) proteases, the role of which is undefined in the allergic airway response (15). In addition, pollens also release bioactive lipids such as the PG-like phytoprostanes (16). These are formed via a series of auto-oxidation steps, initiated by free radical attack of α -linolenic acid. Notably, concentrations of linolenic and linoleic acid in pollen are high, lending support to the possibility of the respiratory tract encountering biologically significant concentrations of oxidized derivatives of these fatty acids (17). Whether these factors, as well as the protease activity of pollens, are capable of triggering TLR4 signaling via TRIF, and the relevance of this pathway to the development of allergic airway disease have not been examined. Thus, we hypothesized that oxidative stress, potentially activating the TLR4–TRIF pathway, is important in mediating allergic sensitization via the airway mucosa, and promoting experimental asthma to inhaled birch pollen allergen. We aimed to determine whether oxidative stress has a role in mediating airway disease when animals are exposed to pollen exclusively via the airways, in the absence of systemic sensitization with an exogenous adjuvant, such as alum. Secondly, we wished to delineate whether pollen-intrinsic NADPH oxidase activity and oxidative stress were critically involved in the initial stages of sensitization, or played a greater role in driving aspects of allergic inflammation during secondary allergen exposure of already sensitized animals. Thirdly, we examined the role of TLR4–TRIF signaling in the processes of sensitization and responses to challenge. We determined that oxidative stress indeed mediates mucosal sensitization to inhaled pollen leading to allergic airway disease, as well as the inflammatory response resulting from secondary allergen exposure, but independently of pollen-intrinsic NADPH oxidase activity. Allergic airway disease and AHR also develop independently of TLR4 and TRIF signaling, although pollen-induced airway inflammation is enhanced by TLR4 activation and dampened by TRIF-dependent pathways.

Materials and Methods

Animal treatments

Six- to 8-wk-old, female BALB/c mice were purchased from Charles River, Canada. TLR4 knockout (KO) mice on a BALB/c background were bred by S. Qureshi in the Animal Care Facilities of the McGill University Health Centre. TRIF KO mice on a C57BL/6J background, as well as wild-type (wt) controls, were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in a conventional or specific pathogen-free animal facility under a 12-h light/dark cycle with free access to food and water. Animals received *Betula populifolia*, white birch pollen extract (BPEX; Greer Laboratories, Lenoir, NC), exclusively via intratracheal (i.t.) administration under isoflurane anesthesia; anesthetized mice were administered 100 μ g BPEX into the posterior pharynx in a volume of 60 μ l sterile PBS (18). In this manner, animals were sensitized on days 0 and 7 and challenged on days 14 and 15. Alternatively, control mice received only PBS on each of these days. Experimental procedures were approved by the McGill University Animal Care Committee. The endotoxin concentration of BPEX, assessed by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ), was 150 EU/mg protein (<1/10th of the level in OVA).

Antioxidant administration concomitant with allergen exposure, sensitization, or challenge

To study the role of oxidative stress in eliciting and/or promoting airway disease to inhaled BPEX, either the antioxidant N-acetyl-L-cysteine (NAC; Sigma-Aldrich) or N,N'-dimethylthiourea (DMTU; Sigma-Aldrich) was administered i.p. 1 h prior to each i.t. BPEX exposure, prior to the sensitizations (days 0 and 7) only, or prior to the challenges (days 14 and 15) only. NAC was administered at a dose of 150 mg/kg in 0.2 ml sterile PBS [comparable to previously reported doses that have effectively inhibited oxidative stress in mice (7, 19, 20)]. Control animals were injected with an equal volume of sterile PBS (pH 7.2) or acidified PBS (pH 2.4) to exclude the possibility that the acidity of the injected NAC solution (pH 2.4) influenced the development of BPEX-induced allergic airway disease. We also tested 150 mg/kg NAC administered topically and concomitantly with the allergen, in the same i.t. 60 μ l bolus with BPEX. The antioxidant DMTU, a potent scavenger of oxygen metabolites (21, 22), was alternatively administered i.p. at a dose of 100 mg/kg in 0.5 ml sterile PBS (pH 7.2), as previously described to prevent chlorine oxidant-induced lung injury (23) (control animals received 0.5 ml sterile PBS).

Assessment of BPEX NADPH oxidase activity

To confirm the presence of NADPH oxidase activity in BPEX, aliquots prepared at 100 μ g/60 μ l sterile PBS, stored at -20°C , were thawed, and 100 μ g BPEX was incubated at 37°C for 30 min with 2 mM NBT, in the presence or absence of 1 mM NADPH, in a total volume of 0.4 ml PBS. Alternatively, the NADPH oxidase inhibitor diphenylene iodonium (DPI; 100 μ M), was added during the incubation of BPEX with NBT and NADPH, or BPEX was heat inactivated at 95°C for 15 min prior to this incubation. Solutions were then centrifuged at $8000 \times g$ for 4 min and washed once with 1 ml distilled water, after which the pellet was allowed to dissolve within 5 min in 0.4 ml methanol. Following centrifugation, the supernatants were plated and read using the Infinite M1000 PRO microplate reader (Tecan, Männedorf, Switzerland) at an absorbance of 530 nm.

Sensitization or challenge with heat-inactivated birch pollen extract

To investigate the role of birch pollen-intrinsic enzymatic, particularly NADPH oxidase activity, in mediating allergic sensitization via the airway mucosa, as well as in the secondary exposure to allergen, animals received i.t. administrations of heat-inactivated BPEX (BPEX^H; 95°C , 15 min) either on days 0 and 7, or on days 14 and 15. The same groups of animals received intact BPEX on days 14 and 15, or on days 0 and 7, respectively, and thus, enzymatic activity of the pollen extract was abolished during either the sensitization or challenge, respectively. Control animals received PBS on days 0 and 7 and intact BPEX on days 14 and 15, or were given intact BPEX throughout all of the exposures.

Airway TLR4 antagonism concomitant with allergen exposure, sensitization, or challenge

To study the effect of blocking TLR4 signaling during each airway exposure to pollen extract, in some experiments, PBS or BPEX (100 μ g) was delivered i.t. to wt mice in combination with 1 μ g TLR4-antagonizing LPS, *Rhodobacter sphaeroides* LPS (LPS-RS; Invivogen, San Diego, CA), on

days 0, 7, 14, and 15. A dose of 1 μ g TLR4-antagonizing LPS has been previously shown to prevent the induction of HDM-induced airway disease (9). To investigate the role of TLR4 signaling exclusively during the sensitization or challenge phase, some mice received BPEx with LPS-RS only on days 0 and 7 (group identified as α TLR4 BPEx/BPEx), or 14 and 15 (BPEx/ α TLR4 BPEx).

Assessment of allergen-induced airway hyperresponsiveness

On day 17, 48 h after the final allergen challenge, mice were anesthetized with an injection of xylazine hydrochloride (10 mg/kg, i.p.), followed by sodium pentobarbital (32 mg/kg, i.p.). Mice were tracheostomized using a 19G metal cannula and ventilated with a small animal ventilator (Flex-iVent; SCIREQ, Montreal, Canada) at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end-expiratory pressure of 3 cmH₂O. The mice were paralyzed with pancuronium bromide (1 mg/kg, i.p.) and subjected to lung inflations to a transrespiratory pressure of 30 cm H₂O to standardize volume history prior to the measurement of baseline respiratory mechanics. A 1.2-s, 2.5-Hz single-frequency forced oscillation maneuver was performed at 10-s intervals, and respiratory system resistance (Rrs) and respiratory system elastance (Ers) were calculated with commercial software (SCIREQ). Doubling concentrations of acetyl- β -methylcholine (MCh; Sigma-Aldrich) from 15.6 to 250 mg/kg were delivered to the mouse as an aerosol using a 4-s nebulization period synchronized with inspiration, at a nebulization duty cycle of 50%. Allergen-induced AHR was assessed by MCh-induced bronchoprovocation by recording the peak Rrs and Ers for each dose of MCh administered.

Airway inflammation

On day 17, following lung function measurements, bronchoalveolar lavage (BAL) was performed using saline containing 10% FBS. The recovered cell pellet was used to measure the total number of cells in the BAL, and cytopins were prepared and stained with Diff-Quik stain (Diff-Quik method; Medical Diagnostics, Düringen, Germany) for differential cell counts. Cytokines in BAL supernatants were assayed using an ultrasensitive Th1/Th2 multiplex and SECTOR Imager 2400 (Meso Scale Discovery, Gaithersburg, MD), according to the manufacturer's instructions. The lower limit of detection for each cytokine was as follows: IL-4, 0.87 pg/ml; IL-5, 0.70 pg/ml; IL-12p70, 5.3 pg/ml; KC, 2.9 pg/ml; IL-10, 11 pg/ml. Eotaxin was measured in BAL supernatants using a human eotaxin 1 ELISA that is 100% cross-reactive with mouse eotaxin 1 (PeproTech Canada, Dollard-des-Ormeaux, QC, Canada; lower limit of detection of 23 pg/ml).

Measurement of BPEx-specific serum IgE

On day 17, blood was collected from mice by exsanguination in serum separator tubes and left at room temperature to clot. Samples were centrifuged at 4000 $\times$ g for 5 min and the serum was collected and stored at -20°C . BPEx-specific serum IgE was measured by ELISA using a modified commercial assay (BioLegend, San Diego, CA) such that the plates were coated overnight with 200 μ g/ml BPEx rather than anti-IgE capture Ab and samples were diluted 10 times in assay diluent prior to their incubation. We tested two concentrations (200 or 400 μ g/ml) of BPEx to coat the plates as well as IgG-precipitating agarose beads. Both concentrations of BPEx gave

similar results, and IgG precipitation did not alter the sensitivity of the assay. An internal positive control sample was included.

Assessment of allergen-induced oxidative stress

To confirm that exposure of animals to birch pollen exclusively via the airways resulted in measurable oxidative stress, an end product of lipid peroxidation, 4-hydroxynonenal (4-HNE), was quantified by gas chromatography-mass spectrometry, as previously described (24), in snap-frozen lungs harvested either 30 min after a single i.t. BPEx exposure to assess immediate allergen-induced oxidative stress occurring prior to the recruitment of inflammatory cells to the lungs, or 24 h after final allergen challenge (day 16).

Statistical analysis

Airway responses to MCh bronchoprovocation were analyzed in GraphPad Prism Version 5 by two-way ANOVA, followed by Bonferroni posttests comparing all experimental groups with each other. One-way ANOVA and posthoc Newman-Keuls' tests were used for all other analyses, such as BAL inflammatory cell and cytokine data, involving three or more groups, or unpaired Student *t* test was used in the case in which only two experimental groups were compared. Data were log transformed prior to statistical analysis when not normally distributed.

Results

Oxidative stress is important in sensitization and the secondary response to inhaled allergen

To examine whether oxidative stress associated with sensitization and challenge with BPEx was necessary for the development of AHR, NAC was administered i.p. prior to each i.t. BPEx exposure on days 0, 7, 14, and 15. Mice receiving BPEx following PBS (sham) i.p. injection exhibited significant AHR to methacholine compared with mice that received just PBS inhalations (Fig. 1A, 1B). Note that PBS + NAC-treated animals (i.e., animals injected with NAC 1 h prior to PBS inhalations) had equivalent methacholine and airway inflammatory responses to animals treated with PBS only. Mice injected with NAC 1 h prior to each BPEx exposure (BPEx + NAC) had significantly lower airway responses (lower Rrs and Ers values) compared with BPEx mice. Furthermore, BPEx + NAC mice had no significant difference in Rrs compared with PBS + NAC mice (Fig. 1A), and only had an elevated Ers at the highest dose of MCh (Fig. 1B), indicating a slightly augmented peripheral airway response. Thus, the administration of NAC prior to each allergen exposure essentially prevented the development of AHR.

To assess whether oxidative stress specifically during secondary allergen exposure was necessary for the induction of AHR by BPEx, we administered NAC prior to challenges only, on days 14

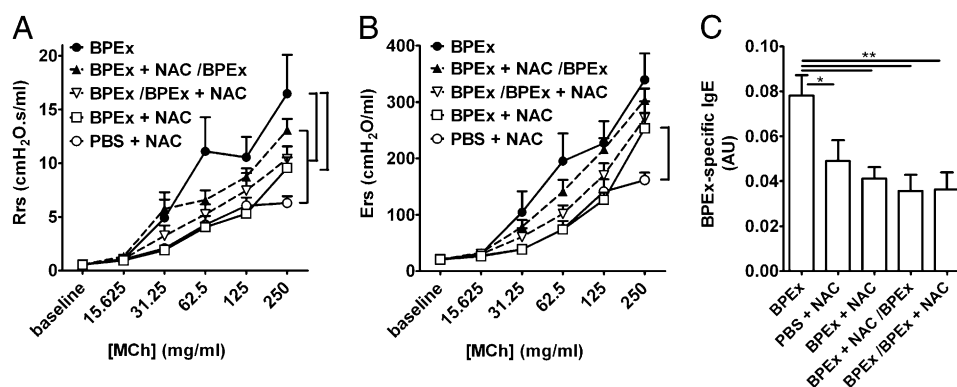


FIGURE 1. NAC antioxidant administration during challenge, but not sensitization, prevents inhaled allergen-induced AHR. Airway responses [Rrs (**A**) and Ers (**B**)] were measured in response to increasing doses of aerosolized MCh. Animals were injected i.p. with PBS 1 h prior to i.t. BPEx (BPEx), or alternatively, received 150 mg/kg NAC i.p. 1 h prior to i.t. PBS or BPEx (PBS + NAC or BPEx + NAC, respectively). Some mice were injected with NAC prior to BPEx sensitization only (days 0 and 7; BPEx + NAC/BPEx), or challenge only (days 14 and 15; BPEx/BPEx + NAC). The effect of NAC injection prior to BPEx exposures on the induction of specific serum IgE levels (**C**) was determined by ELISA ($n = 8$ –11 animals per group pooled from at least three independent experiments). For clarity, not all statistically significant differences between groups are indicated. * $p < 0.05$, ** $p < 0.01$.

and 15. This BPEX/BPEX + NAC group also had significantly reduced Rrs and Ers responses compared with BPEX mice (Fig. 1A, 1B). The BPEX/BPEX + NAC group also showed no difference in Rrs and only an elevated Ers at the highest MCh dose, compared with PBS + NAC mice, indicating that the administration of NAC during the challenge, or secondary exposure to allergen in pre-sensitized mice, was sufficient to achieve almost complete inhibition of AHR. In contrast, NAC administration exclusively prior to BPEX sensitization on days 0 and 7 (BPEX + NAC/BPEX) did not significantly reduce airway responsiveness compared with BPEX mice. Also, both Rrs and Ers were significantly elevated in these mice compared with PBS + NAC mice. Thus, NAC administration during allergic sensitization alone did not prevent the development of AHR to inhaled BPEX.

BPEX-specific serum IgE was significantly augmented in BPEX-exposed mice compared with PBS-exposed mice or mice that received NAC prior to each BPEX exposure (Fig. 1C). Furthermore, NAC injection during sensitization or challenge alone was sufficient to prevent the BPEX-induced augmentation in IgE, indicating that oxidative stress in both sensitization and challenge was necessary for the induction of IgE. All groups that received NAC had comparable BPEX-specific serum IgE levels to PBS-exposed mice.

Total BAL inflammatory cell numbers were significantly lower in PBS + NAC and BPEX + NAC mice compared with BPEX mice, indicating that the injection of NAC prior to each BPEX exposure reduced airway inflammation (Fig. 2A). NAC administration throughout the course of BPEX exposures, or exclusively during the challenge period (days 14 and 15), significantly reduced BAL eosinophilia, whereas NAC injection during the sensitization phase alone failed to do so (Fig. 2B). Compared with sham (PBS)-treated mice, however, all groups had airway eosinophilia, indicating that NAC administration only partially inhibited eosinophilic inflam-

mation. Neutrophil numbers were significantly elevated in all groups compared with PBS + NAC, and, unlike eosinophilia, BAL neutrophilia was unaffected by NAC administration (Fig. 2C). Lymphocytic inflammation, however, was influenced by oxidative stress in both sensitization and challenge phases, as NAC injection during either of these periods significantly, albeit partially, reduced the influx of lymphocytes to the airway lumen (Fig. 2D). Thus, NAC administration during allergic sensitization influenced only later lymphocytic airway inflammation, without impact upon the development of eosinophilic and neutrophilic inflammation. When administered during the secondary inflammatory response, NAC particularly affected eosinophilic airway inflammation given that the proportion of BAL eosinophils was >20% in BPEX mice, compared with ~10% in BPEX/BPEX + NAC mice, and the latter group also had a significantly lower percentage of BAL eosinophils compared with BPEX + NAC/BPEX mice (Supplemental Fig. 2A). The i.t. BPEX instillation resulted in the induction of a number of proinflammatory cytokines in the mouse BAL fluid, including IL-4, IL-5, IL-12, and KC (Fig. 2E–H). Systemic NAC injection coinciding with all BPEX exposures completely abrogated the induction of IL-4. However, BAL IL-5, IL-12, and KC, although significantly inhibited by NAC administration, remained slightly elevated compared with PBS + NAC mice. BPEX-induced TNF- α production in the airways was insensitive to NAC (data not shown). Overall, these data indicate that i.p. NAC administration significantly blunted aspects of allergic sensitization and the development of airway disease, although its effects on specific outcomes were selective or partial.

NAC was also administered i.t. in the same bolus with BPEX either during sensitization (days 0 and 7) or challenge (days 14 and 15). Otherwise, these animals received BPEX alone during challenge or sensitization, respectively. All animals exhibited respira-

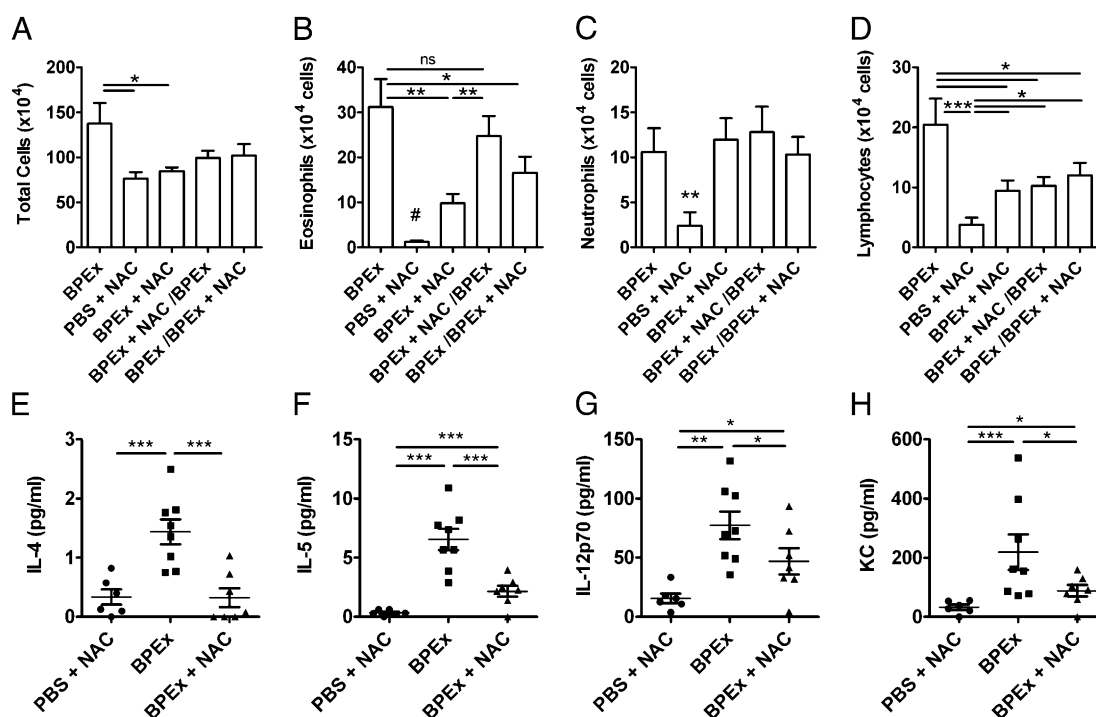


FIGURE 2. Differential effects of NAC administration during sensitization or challenge on allergen-induced airway inflammation. Total BAL inflammatory cells (**A**), eosinophils (**B**), neutrophils (**C**), and lymphocytes (**D**) were determined following i.t. PBS or BPEX exposure that had been preceded by i.p. NAC injection 1 h prior to all exposures, sensitization only, or challenge only. Proinflammatory cytokines, IL-4 (**E**), IL-5 (**F**), IL-12 (**G**), and KC (**H**), were measured by an ultrasensitive multiplex assay in the BALs of mice that received either PBS or NAC prior to i.t. BPEX and control mice that received PBS and NAC ($n = 7-8$ animals per group pooled from at least three independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. #Significant difference from all other groups, $p < 0.001$.

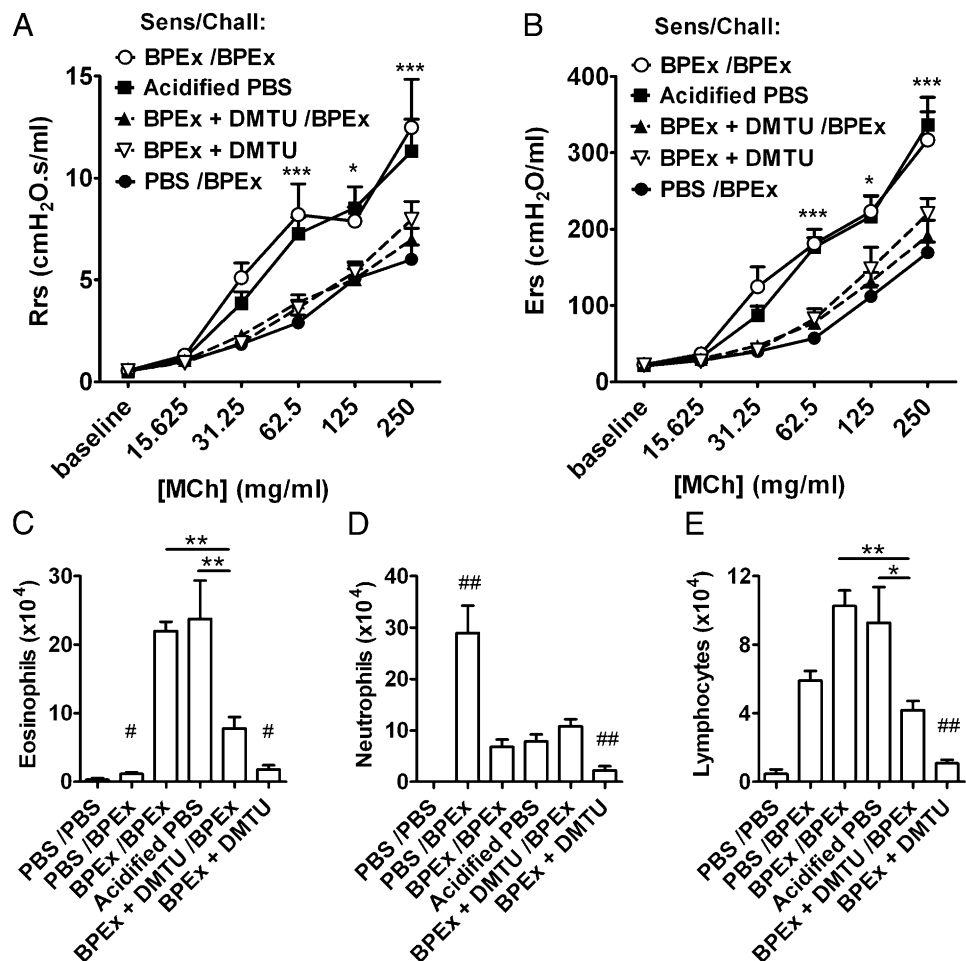
tory distress and died within 72 h of i.t. NAC exposure. The pH-adjusted (pH 7), sterile-filtered NAC was better tolerated, but augmented BPEX-induced airway inflammation, particularly when administered with BPEX challenge, thus demonstrating irritant effects upon the airways at this dose (Supplemental Fig. 1). To confirm that the acidity of the i.p.-injected NAC solution was not influencing the proinflammatory airway response to inhaled BPEX, acidified PBS (pH 2.4) was injected 1 h prior to BPEX exposures, which did not affect the development of AHR or airway inflammation compared with the i.p. injection of just sterile PBS (pH 7.2) before BPEX exposures (BPEX/BPEX) (Fig. 3). We also confirmed that exposure to BPEX on days 0 and 7 is necessary for sensitization, leading to the development of airway disease upon secondary allergen exposure on days 14 and 15, as control animals that received PBS on days 0 and 7 and BPEX on days 14 and 15 (PBS/BPEX) exhibited significantly lower airway responses to MCh compared with animals that received BPEX throughout both sensitization and challenge (BPEX/BPEX) (Fig. 3A, 3B). Sham-sensitized animals also had lower BAL eosinophilia (Fig. 3D) and lymphocytosis (Fig. 3F), but dramatically elevated neutrophilia (Fig. 3E), compared with BPEX/BPEX animals. Furthermore, an alternative antioxidant, DMTU, was administered i.p. 1 h before allergen sensitizations (days 0 and 7) only or all BPEX exposures. Interestingly, DMTU had a more potent inhibitory effect than NAC on BPEX-induced airway disease given that, unlike NAC, its administration during sensitization alone (BPEX + DMTU/BPEX) was sufficient to prevent the subsequent development of AHR (Fig. 3A, 3B) and significantly attenuated BAL eosinophilia (Fig. 3C) compared with mice that received sham treatments before

sensitization and challenge. BAL eosinophil numbers in BPEX + DMTU/BPEX mice remained somewhat elevated compared with mice that were sham sensitized (PBS/BPEX) or received DMTU throughout all BPEX exposures (BPEX + DMTU) (Fig. 3C), whereas neutrophil numbers were not reduced compared with sham-treated, BPEX-sensitized and challenged mice (Fig. 3D). As with NAC, DMTU administration during sensitization alone significantly attenuated BAL lymphocyte numbers to the same levels as PBS/BPEX mice, but not BPEX + DMTU mice or mice that received only sham sensitizations and challenges (PBS/PBS). Finally, DMTU administration with sensitization and challenge, unlike NAC, completely abrogated AHR and reduced all inflammatory cell types in the BALF to levels that were indistinguishable from those of PBS/PBS mice, indicating that oxidative stress contributes significantly to mucosal sensitization to inhaled pollen allergen as well as the inflammatory response resulting from secondary allergen exposure, which can be blocked by antioxidants.

BPEX-intrinsic NADPH oxidase activity does not contribute to allergic sensitization via the airway mucosa or airway disease

BPEX-intrinsic NADPH oxidase activity and its capacity to generate ROS were confirmed by NBT assay. BPEX generated significant ROS production only in the presence of NADPH substrate (Fig. 4A). ROS production by BPEX was diminished in the presence of the NADPH oxidase inhibitor, DPI, and completely abrogated by prior heat inactivation of BPEX (BPEX^H). ROS levels in the presence of DPI or BPEX^H were not significantly different from levels in PBS. Thus, in the absence of cellular material, the BPEX-intrinsic capacity to generate ROS was entirely NADPH oxidase dependent.

FIGURE 3. DMTU antioxidant administration, even during sensitization alone, is sufficient to prevent inhaled allergen-induced AHR and inflammation. Airway responses to aerosolized MCh (A, B) and BAL eosinophils (C), neutrophils (D), and lymphocytes (E) were measured after i.t. PBS or BPEX exposure. Animals were injected i.p. with sterile PBS (pH 7.2), acidified PBS (pH 2.4), or 100 mg/kg DMTU (pH 7.2) 1 h prior to each i.t. BPEX exposure (BPEX/BPEX, acidified PBS, or BPEX + DMTU, respectively). Some mice received sham sensitizations of i.t. PBS on days 0 and 7 (PBS/BPEX) or were injected with DMTU prior to BPEX sensitization only (BPEX + DMTU/BPEX) (PBS/BPEX control had $n = 5$ mice per group; otherwise, $n = 8$ animals per group pooled from at least three independent experiments). A small number of control mice that received both sham sensitizations and challenges (PBS/PBS; $n = 4$) was also included for reference, but excluded from statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. #Significant difference from all other groups except those with the same symbol, $p < 0.05$, ##, significant difference from all other groups, $p < 0.05$.



To investigate the importance of this property of BPEx in sensitization via the airway mucosa, or in promoting the inflammatory response to secondary allergen exposure, animals received BPEx^H during either sensitization or challenge and otherwise received intact BPEx (Fig. 4B, 4D–F). Neither sensitization of animals with BPEx^H, nor challenge with BPEx^H altered airway responsiveness or inflammation compared with BPEx/BPEx animals. Thus, the enzymatic activity of BPEx, or NADPH oxidase activity, made no contribution to allergic sensitization via the airway mucosa or,

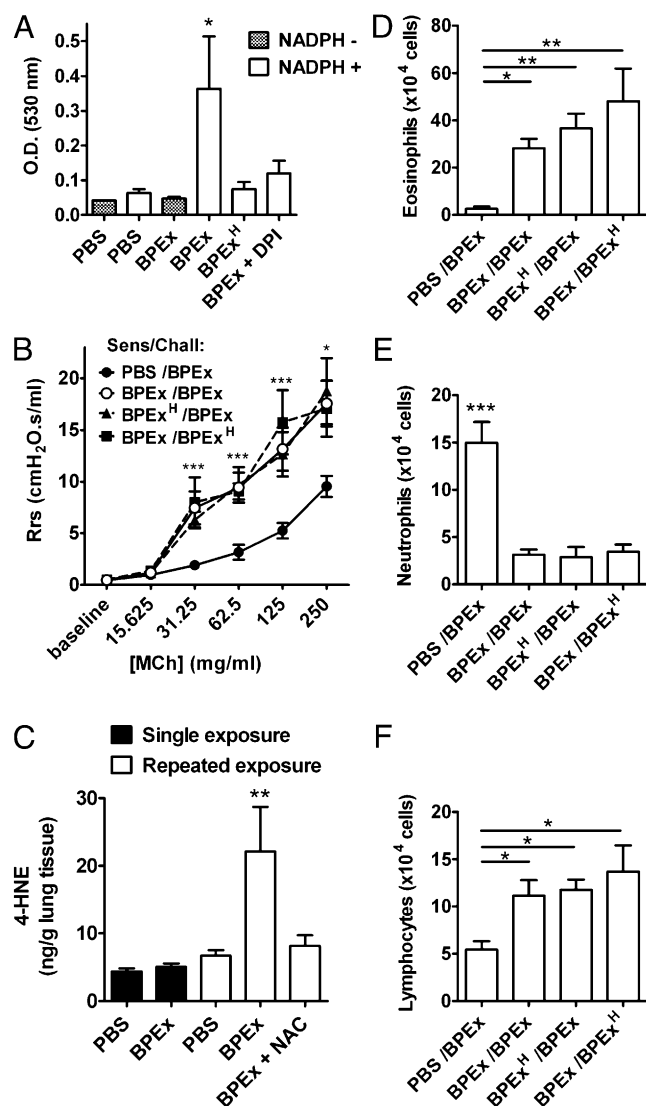


FIGURE 4. Pollen-intrinsic NADPH oxidase activity does not mediate mucosal sensitization to BPEx or the inflammatory response to secondary allergen exposure. Pollen-intrinsic NADPH oxidase activity was measured by NBT assay and verified by incubation of BPEx with the NADPH oxidase inhibitor DPI, or by prior heat inactivation (BPEx^H). (A) (Data pooled from four independent experiments). Animals were sensitized or challenged with BPEx^H and otherwise received intact BPEx to study the contribution of pollen-intrinsic NADPH activity in sensitization or challenge to the development of AHR (B) or BAL eosinophilia (D), neutrophilia (E), or lymphocytosis (F) ($n = 6-7$ animals per group pooled from three independent experiments). The 4-HNE, a marker of oxidative stress, was measured in lung tissues of mice within 30 min of a single i.t. PBS or BPEx exposure, after repeated PBS or BPEx exposure (sensitization and challenge), or after repeated BPEx exposure preceded by i.p. NAC injections (C) ($n = 5-8$ animals per group from two independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

alternatively, to the promotion of airway disease upon secondary allergen exposure in animals sensitized with intact BPEx.

Furthermore, we sought evidence of early oxidative stress in the lungs occurring within 30 min of a single i.t. BPEx exposure, prior to the recruitment of inflammatory cells and potentially elicited by the pollen-intrinsic oxidase activity, through quantification of 4-HNE. However, at this early time point, no measurable increase in lung 4-HNE was found in BPEx- compared with PBS-exposed mice (Fig. 4C). In contrast, lung 4-HNE levels were significantly augmented on day 16, following BPEx sensitization and challenge, as compared with PBS-exposed, or NAC-injected mice. Thus, repeated BPEx inhalation was confirmed to induce significant oxidative stress in the lungs, which was completely prevented by systemic antioxidant administration prior to each allergen exposure.

Oxidative stress-mediated AHR to inhaled pollen allergen is independent of TLR4

To investigate the role of TLR4 signaling in the development of allergic airway disease exclusively via airway exposure to BPEx, responses to MCh were assessed in mice in which the TLR4 receptor was antagonized, using LPS-RS, concomitantly with each exposure to BPEx (identified as α TLR4) (Figs. 5, 6, and 7). Administration of α TLR4-LPS with PBS (α TLR4 PBS) had no effect on airway responsiveness compared with animals that just received PBS (Fig. 5A, 5B). Both of these groups, however, had significantly lower Rrs and Ers responses to MCh compared with animals receiving BPEx alone (BPEx), or α TLR4-LPS with BPEx (α TLR4 BPEx). Unlike NAC administration, TLR4 antagonism did not prevent BPEx-induced AHR, and, in fact, resulted in significantly higher Rrs and Ers ($p < 0.001$, $p < 0.05$, respectively) at

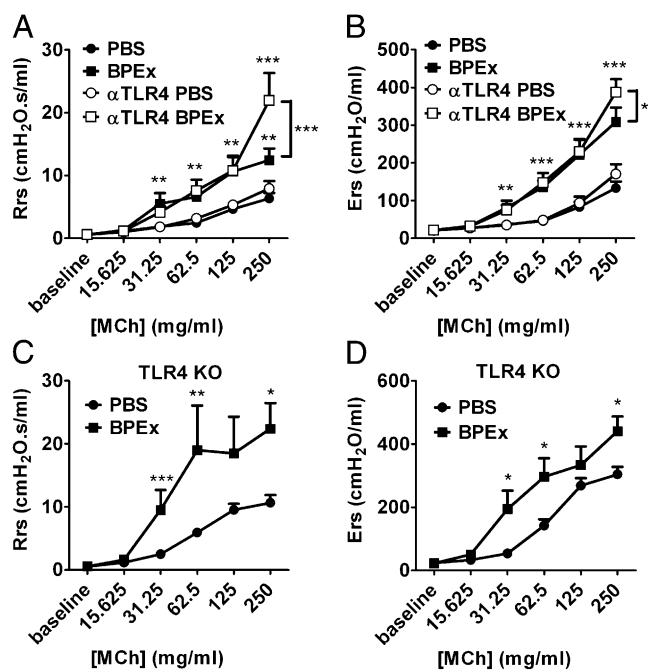


FIGURE 5. TLR4 is not necessary for the development of AHR to inhaled BPEx. The effect of TLR4 antagonism coinciding with i.t. BPEx exposures on airway responses of animals to increasing doubling doses of aerosolized MCh was measured (A, B). Animals received PBS or BPEx i.t., with or without 1 μ g TLR4-antagonizing LPS-RS (α TLR4 PBS or α TLR4 BPEx) ($n = 8-12$ animals per group pooled from at least three independent experiments). Airway responses to MCh were also assessed in TLR4 KO mice sensitized and challenged i.t. with BPEx, as compared with PBS (C, D) ($n = 7$ animals per group pooled from two independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

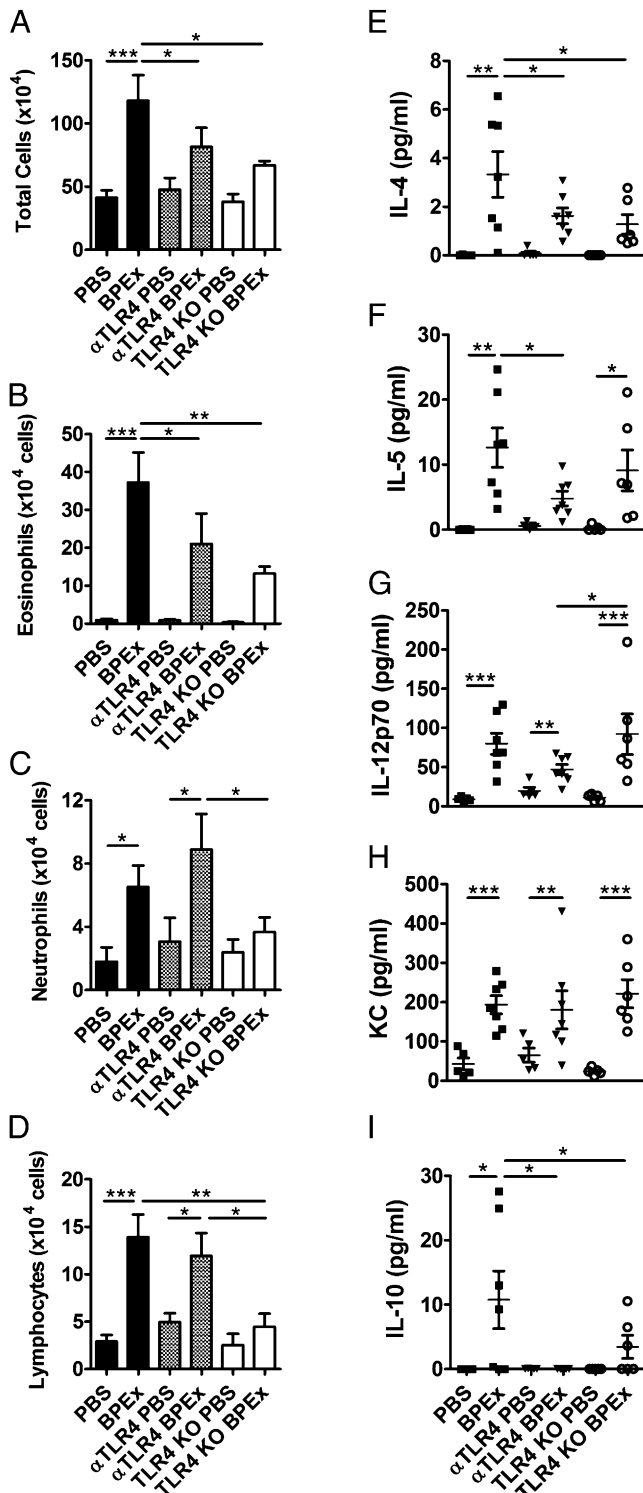


FIGURE 6. TLR4 contributes to the inflammatory response to inhaled BPEX. Total BAL inflammatory cells (**A**), eosinophils (**B**), neutrophils (**C**), and lymphocytes (**D**) were determined following i.t. BPEX exposure of TLR4-antagonized or TLR4-deficient (TLR4 KO) mice ($n = 7$ –12 animals per group pooled from at least two independent experiments). Proinflammatory cytokines, IL-4 (**E**), IL-5 (**F**), IL-12 (**G**), KC (**H**), and IL-10 (**I**), were measured in the BAL fluid of PBS- or BPEX-exposed TLR4-antagonized or TLR4-deficient (TLR4 KO) mice ($n = 5$ –7 animals per group pooled from at least two independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the highest dose of MCh, compared with BPEX animals (Fig. 5A, 5B). TLR4 antagonism concomitant with BPEX sensitization or challenge alone produced no differences in AHR (Fig. 7A, 7B).

Furthermore, i.t. BPEX still produced significant AHR in TLR4 KO mice compared with PBS administration (Fig. 5C, 5D).

TLR4 amplifies airway inflammation caused by inhaled pollen allergen

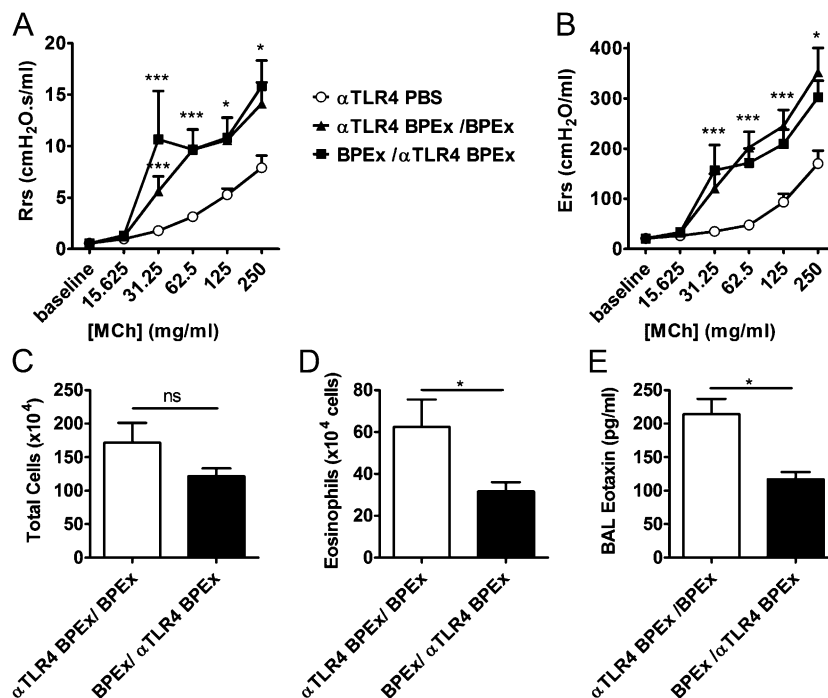
Pollen-induced airway inflammation was significantly blunted in the absence of functional TLR4, as total BAL fluid inflammatory cell numbers were lower as a result of TLR4 antagonism, as well as in TLR4 KO mice, compared with wt BPEX mice (Fig. 6A). Eosinophil numbers in the BAL fluid were significantly lower as a result of TLR4 antagonism, as well as in TLR4 KO mice exposed to BPEX, but still remained elevated compared with the respective negative controls (PBS-exposed mice) (Fig. 6B). The percentage of eosinophils in the BAL fluid was also significantly, albeit partially, reduced from nearly 30% in wt mice to 20% in the absence of functional TLR4 (Supplemental Fig. 2B). Interestingly, BPEX-induced neutrophil and lymphocyte recruitment to the airway lumen was absent in TLR4 KO mice, but remained significant in α TLR4 BPEX mice in which TLR4 was antagonized concomitantly with BPEX exposures (Fig. 6C, 6D); BPEX-challenged TLR4 KO mice had significantly lower neutrophil and lymphocyte numbers compared with α TLR4 BPEX mice. Thus, functional TLR4 is critical for neutrophil and lymphocyte recruitment to the airway lumen and plays a considerable role in promoting airway eosinophilia following birch pollen inhalation. Finally, TLR4 antagonism concomitant with BPEX challenge resulted in significantly lower eosinophilia compared with TLR4 antagonism exclusively with BPEX sensitization, although no difference was observed in any of the other inflammatory cell types (Fig. 7C, 7D and data not shown). This was accompanied by reduced BAL eotaxin levels as compared with that resulting from TLR4 antagonism exclusively with sensitization (Fig. 7E). Thus, TLR4 signaling during secondary allergen exposure, but not sensitization, contributed to eosinophilic airway inflammation.

As in the earlier experiments (Fig. 2E–H), i.t. BPEX instillation significantly augmented IL-4, IL-5, IL-12, and KC levels in the mouse BAL fluid (Fig. 6E–H). Surprisingly, the Th1-inducing cytokine IL-12 was still induced by BPEX in the absence of functional TLR4 and was even elevated in TLR4 KO mice, compared with α TLR4 BPEX mice (Fig. 6G). Another proinflammatory molecule that might have been expected to be inhibited in the absence of TLR4 signaling, KC was also still induced in TLR4 KO mice exposed i.t. to BPEX (Fig. 6H). Induction of IL-1 β , TNF- α , IFN- γ , and IL-2 was not significantly affected by the absence of TLR4 (data not shown). BAL fluid IL-5 levels were lower in α TLR4 BPEX mice compared with BPEX mice, whereas IL-5 was induced to a similar degree in wt and TLR4 KO mice exposed to BPEX (Fig. 6F). Thus, the BAL inflammatory cell and cytokine data show that the induction of the indicated proinflammatory cytokines by inhaled birch pollen was partially independent of TLR4 signaling. Only allergen-induced IL-4 and IL-10 were significantly reduced in TLR4 KO mice, or as a result of TLR4 antagonism (Fig. 6E, 6I).

TRIF tempers airway inflammation caused by inhaled pollen allergen

Elevated mRNA expression of the TRIF-inducible type I IFN, IFN- β , was detected in lung tissues of BALB/c mice harvested 30 min after a single BPEX exposure (Supplemental Fig. 2C). TRIF KO mice exposed to BPEX developed AHR comparably to their wt C57BL/6J counterparts (Fig. 8A). However, in contrast to TLR4 deficiency, which resulted in reduced airway inflammation, TRIF deficiency caused total BAL inflammatory cells (data not shown), specifically macrophages, eosinophils, and lymphocytes (Fig. 8B–E), to be

FIGURE 7. Functional TLR4 is particularly important during the secondary inflammatory response to inhaled BPEX, rather than sensitization, for the development of eosinophilic airway inflammation. Airway responses to aerosolized MCh (**A**, **B**), total BAL inflammatory cells (**C**), eosinophils (**D**), and BAL eotaxin levels (**E**) were assessed in mice that received TLR4-antagonizing LPS-RS concomitantly with either sensitization only (α TLR4 BPEX/BPEX) or challenge only (BPEX/ α TLR4 BPEX) ($n = 8$ animals per group pooled from at least three independent experiments). * $p < 0.05$, *** $p < 0.001$.



significantly augmented in response to BPEX, compared with wt C57 BPEX-exposed mice. This was accompanied by increases in the proinflammatory cytokine IL-12 and chemokines KC and eotaxin in the BAL fluid of TRIF KO mice (Fig. 8F–I, Supplemental Fig. 2D). Thus, TRIF deficiency resulted in exacerbated airway inflammation.

Discussion

Allergenic proteins possess inherent properties or are associated with danger signals that target receptors mediating allergic inflammatory responses (25). Importantly, these danger signals and receptors may be allergen specific, or may confer allergenicity to a broad range of environmental proteins. Oxidative stress has been implicated in the pathogenesis of allergic airway disease, and there is evidence of its association with innate immunity, specifically the TLR4 pathway, in mediating airway disease. We wished to determine the role of these mechanisms in evoking mucosal sensitization to pollen and/or promoting allergic airway disease upon secondary allergen exposure. We have found that oxidative stress is crucial in both of these phases of response to birch pollen allergen in the airway mucosa, leading to airway inflammation and hyperresponsiveness, whereas the pollen-intrinsic NADPH oxidase activity was not relevant to either mucosal sensitization or amplification of the secondary response, as administration of heat-inactivated BPEX during either of these periods did not reduce subsequent airway responses and inflammation. Although TLR4 signaling does not contribute to AHR induced by inhaled pollen extract, it significantly augments airway eosinophilia, neutrophilia, and lymphocytosis. In contrast, signaling via its adaptor TRIF tempers the inflammatory response to inhaled pollen extract. Finally, oxidative stress appears to influence a broader spectrum of BAL fluid cytokines that are induced by inhaled pollen, including Th1, Th2, and other inflammatory mediators, compared with TLR4 signaling.

There is evidence in the mouse (20), rat (26), and guinea pig (27) that, following i.p. sensitization with the experimental allergen OVA and adjuvant alum, the administration of antioxidant, such as NAC (or its derivatives), around the time of OVA challenge can prevent the development of airway inflammation. A few studies have also shown that AHR can be prevented (28, 29). Boldogh

et al. (6) demonstrated that intrinsic NADPH oxidase activity in ragweed pollen extract generates ROS in the airway lining fluid and epithelium within minutes of allergen challenge of mice, prior to the recruitment of inflammatory cells and independent of IgE, mast cells, and adaptive immunity. This pollen-intrinsic NADPH oxidase activity was responsible for the augmentation of Ag-induced eosinophil recruitment to the airways and systemic IgE, which could be prevented by the administration of NAC during allergen challenge (7) (AHR was not evaluated in this case). Thus, studies to date have shown in animals that have been sensitized systemically with allergen and adjuvant that NAC administration during allergen challenge is effective in inhibiting allergic airway disease, suggesting that oxidative stress is important in amplifying airway disease independently of sensitization. However, whether oxidative stress is a mechanism that is key to allergic sensitization in the first place, and via the natural route of the airway mucosa, has not been addressed. Using white birch, a pollen known to possess intrinsic NADPH oxidase (30) and cysteine protease activity (15), which may also contribute to ROS production, we investigated whether, in the context of sensitization via the airway mucosa, oxidative stress would play an equally important role in augmenting airway inflammation and promote AHR. We determined that oxidative stress is critical to the development of AHR and airway inflammation to inhaled pollen extract, as administration of either NAC or DMTU antioxidant 1 h prior to each BPEX exposure prevented AHR and blunted BAL eosinophilia and lymphocytosis, although DMTU demonstrated more potent and complete suppression of airway inflammation compared with NAC, including BAL neutrophilia. NAC treatment also prevented the BPEX-induced upregulation of numerous inflammatory mediators in the BAL fluid, including KC, IL-12, IL-4, and IL-5, although not all measured mediators.

Given that we did not sensitize animals by i.p. injection with adjuvant, we expected pollen-induced oxidative stress to be critical for allergic sensitization. ROS production in the dermal epithelium elicited by the cysteine protease papain triggers the expression and production of thymic stromal lymphopoietin, a cytokine that promotes mast cell and basophil responses and, importantly, stimulates dendritic cells to prime Th2 responses (13). Recently,

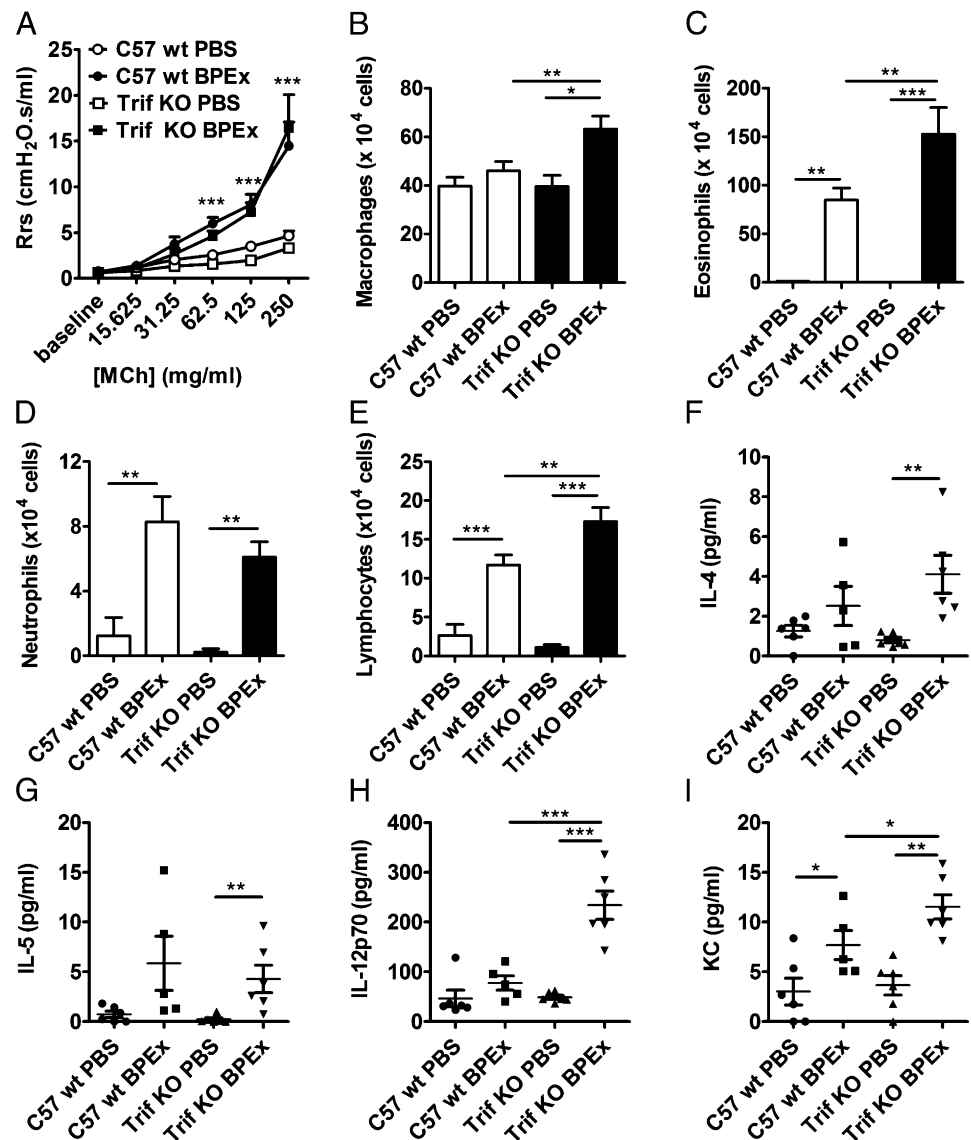


FIGURE 8. TRIF signaling does not influence AHR, but tempers allergen-induced airway inflammation. Airway responses to aerosolized MCh (**A**), macrophages (**B**), eosinophils (**C**), neutrophils (**D**), and lymphocytes (**E**) were measured after i.t. PBS or BPEx exposure of C57 wt or TRIF KO mice ($n = 6$ –16 animals per group pooled from at least three independent experiments). Proinflammatory cytokines, IL-4 (**F**), IL-5 (**G**), IL-12 (**H**), and KC (**I**), were quantified in the BAL fluid of PBS- or BPEx-exposed C57 wt or TRIF KO mice ($n = 5$ –6 animals per group pooled from at least three independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IgE cross-linking on mast cells has been shown in vitro to elicit ROS-mediated and NAC-sensitive induction of the leukotriene B₄ receptor, BLT2, which promotes Th2 responses (31). Furthermore, pollen-associated oxidative stress stimulates dendritic cell maturation and proinflammatory cytokine production (32, 33) and has been reported to promote NAC-sensitive Th2 responses or effector T cell responses of mixed cytokine profile (13, 33, 34). Other components of pollen grains besides the intrinsic NADPH oxidase activity, such as the PGE₂-resembling pollen-derived E₁ prostanes, have also been shown to contribute to this (16, 35). Therefore, we expected sensitization to birch pollen via the airway mucosa to be mediated by oxidative stress. We confirmed that the i.t. BPEx sensitizations on days 0 and 7 were indeed necessary for the development of airway disease, as PBS-sensitized, BPEx-challenged mice failed to develop AHR, BAL eosinophilia, and lymphocytosis in comparison with BPEx-sensitized and challenged mice. Although NAC administration coinciding exclusively with sensitization attenuated only lymphocyte recruitment to the airways and systemic BPEx-specific serum IgE, not AHR and BAL eosinophilia, similar DMTU treatment prevented the development of AHR and significantly inhibited BAL eosinophilia and lymphocytosis, although not as substantially as DMTU treatment coinciding with both sensitization and challenge. Taken together,

administration of these antioxidants during sensitization alone was sufficient to prevent the later development of serum allergen-specific IgE, airway inflammation, and AHR, indicating that oxidative stress plays a substantial role in allergic sensitization via the airway mucosa. Similar to our current findings, other groups have reported only partial and selective inhibitory effects of NAC (19, 32, 36, 37). For example, a study examining the adjuvant effect of ambient particulate matter on intranasal sensitization to OVA demonstrated partial suppression by i.p. NAC injection of BAL eosinophilia, but no effect on serum IgE responses (despite using double the dose of NAC administered in our study [320 mg/kg]) (36). Also, the stimulatory effects of ragweed extract on dendritic cell activation in vitro were reportedly inhibited to varying degrees by NAC and depended on the underlying redox status of the cells (32). Furthermore, NAC administration failed to prevent the exacerbated OVA-induced neutrophil recruitment to the airways of Nrf2 KO mice, a strain with reduced antioxidant status (37). Despite the regulation of KC by NAC in our study, neutrophil recruitment to the airways and allergen-induced TNF- α production were unaffected, suggesting that other neutrophil chemoattractants are insensitive to NAC. These data indicate that compounds with properties similar to DMTU may be more effective therapeutically than NAC in neutrophil-abundant airway

diseases, such as chronic obstructive pulmonary disease or severe asthma.

The discrepancy in the effects of NAC and DMTU may be related to their differential capacities to function as scavengers of ROS, to restore intracellular glutathione levels, and to activate the Nrf2-regulated defense system involving numerous antioxidant genes. These properties may also be cell specific given that human bronchial epithelial cells, for example, were suggested to be unable to convert NAC to glutathione, possibly due to compromised deacetylation of NAC to the glutathione precursor (38). Moreover, NAC is polar and known to suffer from limited cell membrane permeability, as well as bioavailability due to rapid metabolism, whereas DMTU is lipid soluble, highly diffusible across membranes, and has a relatively long serum $t_{1/2}$ (28–36 h) (21, 39–41). Therefore, in addition to its potent capacity to scavenge hydroxyl radicals, the most biologically reactive-free radical (42), DMTU might have enhanced intracellular accessibility and activity compared with NAC. Overall, our data support that distinct inflammatory processes may be variably sensitive to specific antioxidants. We also observed that i.t. administration of NAC together with BPEX, unexpectedly, was toxic or proinflammatory to the animals. Delivery of NAC into the airways has been reported to have the potential to induce bronchospasm and irritant effects (43, 44), and i.p. administration is far more common. In one study, direct delivery of NAC into the airways was efficacious only when administered in a liposomal formulation, and liposomal NAC has been demonstrated to be more effective than conventional NAC, irrespective of the route of administration (41, 45). Furthermore, heat inactivation of BPEX had no effect either in sensitization or challenge on the subsequent development of AHR or airway inflammation, indicating a negligible role for birch pollen-intrinsic enzymatic activity, and specifically NADPH oxidase activity, in mucosal sensitization or amplification of the secondary inflammatory response. We also found no measurable increase in lung 4-HNE levels immediately following a single BPEX exposure, but only after repeated BPEX sensitization and challenge, indicating that oxidative stress resulting directly from the pollen-intrinsic NADPH oxidase (or other enzymatic) activity is minimal compared with that which is generated by the recruitment of proinflammatory cells with repeated allergen exposure. This does not preclude that allergens with greater oxidative stress capacity, such as ragweed, or reduced antioxidant status of the animal, would not enhance the significance of allergen-intrinsic oxidative stress upon allergic sensitization.

There is considerable evidence indicating that oxidative stress can promote airway disease by activating innate immune signaling pathways (46, 47). Hollingsworth et al. (11) demonstrated that TLR4 KO mice fail to develop AHR, but still exhibit neutrophilic inflammation after ozone exposure, whereas another study has implicated TLR2, TLR4, and MyD88 in the development of AHR caused by ozone, and MyD88, in particular, regulating ozone-induced airway neutrophilia (48). These TLRs have been proposed to be activated by lipid ozonation products or surfactant-derived oxidized lipids. Recently, ozone-induced fragmentation of hyaluronan, a component of pulmonary extracellular matrix, has been confirmed to prime TLR4 responses (49, 50). Similar to allergic airway disease, ozone-induced airway inflammation is characterized by Th2-type inflammation (IgE, IL-4, and IL-13) (51), as well as IL-17 and NK T cell responses (52). NADPH oxidase-induced ROS reportedly also amplifies TLR4 signaling in sepsis (53). Thus, we hypothesized that oxidative stress-mediated AHR and airway inflammation elicited by inhaled birch pollen may require signaling through the TLR4 pathway. Mice were given TLR4-antagonizing LPS-RS concomitantly with BPEX exposure,

at a dose described by Hammad et al. (9), to have inhibited house dust mite allergen-induced experimental asthma. Unlike HDM-induced AHR, pollen-induced AHR still developed despite TLR4 antagonism, as well as in TLR4 KO mice. We conclude that TLR4 is not necessary for the development of AHR caused by inhaled pollen-induced oxidative stress. Our data demonstrate that pollen-induced oxidative stress mediates airway disease via mechanisms that differ from those that are activated by ozone, or intradermal papain injection, indicating that oxidative stress-induced airway disease is specific to the distinct nature or magnitude of the stress. Perhaps this is dependent upon the specific oxidized phospholipids that are generated and their capacity to trigger TLR4, as well as the TLR4 coreceptors that are engaged. For example, ozone-induced hyaluronan fragments interact with CD44 to activate TLR4, whereas LPS requires the CD14 molecule (54, 55).

Despite the lack of effect on AHR, TLR4 does contribute to airway inflammation induced by inhaled pollen, augmenting eosinophilic inflammation and IL-4 and IL-10 cytokine production, in agreement with other studies that have described a role for TLR4 in promoting allergic Th2-type inflammation (9, 10, 13). The absence of functional TLR4 did not affect the induction of a number of Th1-inducing and proinflammatory cytokines by pollen despite blunting eosinophilic, neutrophilic, and lymphocytic airway inflammation, suggesting a selective dissociation of airway cytokine expression levels from the recruitment of these cells. Our data from TLR4 KO mice indicate that neutrophil and lymphocyte recruitment to the airways in response to inhaled pollen is entirely dependent on TLR4 signaling, whereas TLR4 antagonism did not significantly influence these inflammatory responses. The apparent disparity between pharmacological TLR4 antagonism and genetic ablation of TLR4 function may be due to a submaximal antagonism of TLR4 by the dose of LPS-RS used. It is also noteworthy, however, that Tan et al. (10) reported that whereas TLR4 expression on hematopoietic cells was necessary and sufficient for Th1-type, and contributed to Th2-type inflammatory responses to inhaled LPS-laden OVA, TLR4 expression on non-hematopoietic cells was important for the development of Th2 allergic responses, not Th1, to the same Ag. Thus, in contrast to the TLR4 KO mice that completely lack functional TLR4 in both compartments, it is possible that LPS-RS delivered i.t. exclusively or primarily antagonizes TLR4 on airway epithelial cells, depending on whether it can cross this barrier. In this manner, the administration of LPS-RS perhaps may largely affect the Th2-type and eosinophilic inflammatory response, without a significant effect on neutrophilic and Th1 inflammation.

Little is known regarding the role of TLR4–TRIF signaling in asthma pathogenesis. TRIF signaling via TLR3 has been demonstrated to mediate dsRNA exacerbation of OVA-induced airway inflammation (56). However, whether TRIF signaling is involved in the development of airway disease solely to allergen has not been investigated to date. As in TLR4 KO mice, AHR was not affected in TRIF KO mice. Unexpectedly, TRIF deficiency exacerbated BPEX-induced airway macrophage, eosinophilic, and lymphocytic inflammation, as well as the secretion of proinflammatory mediators IL-12, KC, and eotaxin compared with wt mice. Thus, whereas TLR4 activation is proinflammatory, TRIF signaling appears to dampen pollen-induced airway inflammation. These findings suggest that MyD88-dependent signaling may promote Th2 and eosinophilic inflammation to inhaled pollen extract, whereas TRIF signaling may be anti-inflammatory, or may competitively or negatively regulate TLR4/MyD88 pathways; however, the precise mechanisms that account for these observations remain to be resolved. Consistent with the present observations is recent *in vivo* evidence that TRIF may be anti-inflammatory in noninfectious

lung disease (57) and that TRIF can negatively regulate TLR4-MyD88-induced activation of DCs, NK, T, and B cells, as well as proinflammatory cytokines and chemokines, including IL-12 (58). Furthermore, IFN- β gene deficiency in mice was shown to exacerbate OVA-induced Th2 and eosinophilic airway inflammation (59). Whether TRIF-biased adjuvants, such as monophosphoryl lipid A, may be advantageous in the treatment of pollen-induced airway inflammation remains to be investigated.

Commercially available pollen extracts are delipidated to facilitate the extraction process and to permit resuspension in aqueous media (60). The significance of this modification relative to natural exposure to pollens is unclear, as the fraction of pollen that is spontaneously releasable in the airways is most likely more substantial in relation to physiological exposure than the nonaqueous fraction (16). However, CD1d-restricted human $\gamma\delta$ T cells have been demonstrated in vitro to recognize, and become activated by, lipid constituents of pollen (61). Also, concentrations of linolenic and linoleic fatty acids that, upon oxidation, may potentially trigger the TLR4 pathway, are 15 times higher in the organic than aqueous fractions of pollen (17). Thus, it cannot be excluded that the TLR4 pathway may have a greater influence toward the development of airway disease in natural pollen exposure, compared with the inhalation of processed and extracted pollen. In contrast, the sonication of pollen during the extraction process releases protein Ags, proteases, and other pollen constituents that may not be readily released in natural exposure to pollen grains.

In conclusion, our data demonstrate that oxidative stress is important in both allergic sensitization to birch pollen extract via the airway mucosa and the secondary response to inhaled allergen, whereas TLR4 and TRIF signaling are not necessary for mucosal sensitization to pollen leading to airway disease, namely AHR, but modulate the inflammatory response. The TLR4 pathway significantly contributes to airway inflammation caused by inhaled pollen extract, whereas TRIF signaling limits the extent of the inflammatory response. Thus, only select aspects of the inflammatory response to inhaled pollen induced by oxidative stress, such as airway eosinophilia, lymphocytosis, and IL-4, may indeed be mediated via TLR4. Finally, our data indicate that intrinsic NADPH oxidase activity, or other enzymatic activity, is not a universal mechanism conferring allergenicity to pollens.

Disclosures

The authors have no financial conflicts of interest.

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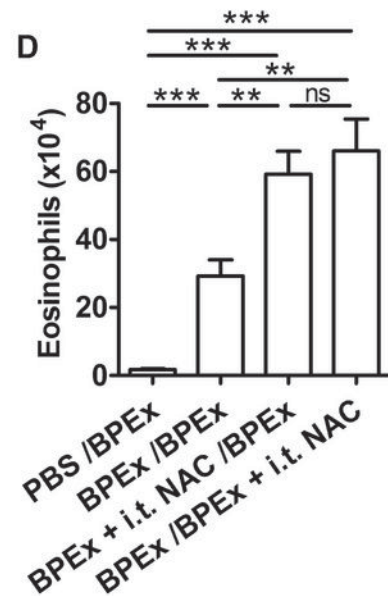
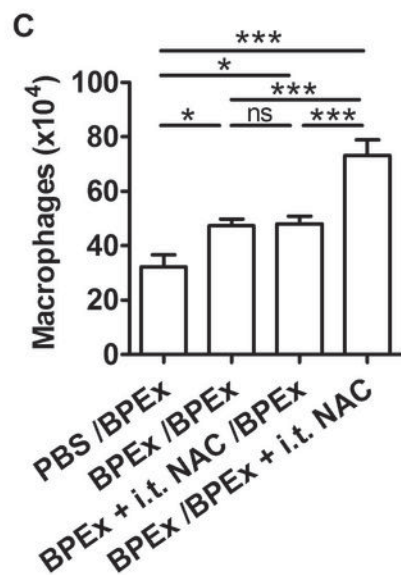
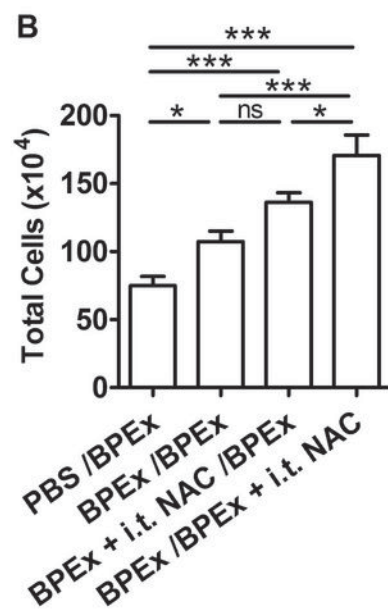
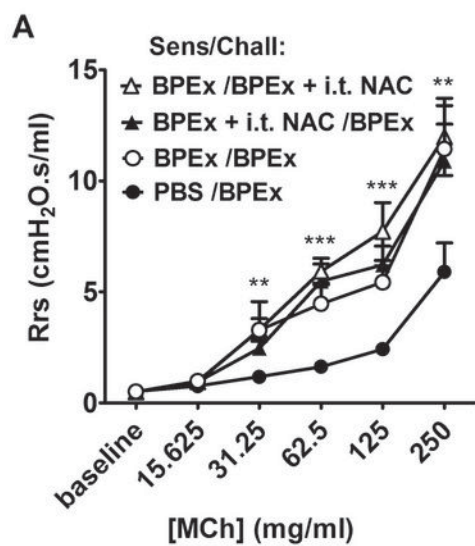
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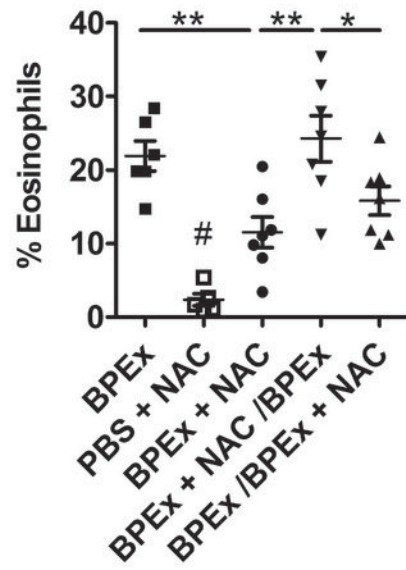
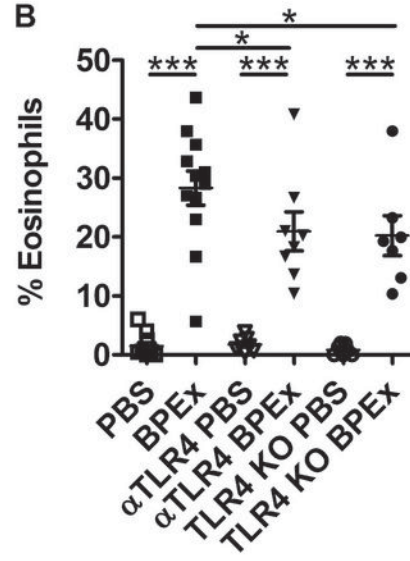
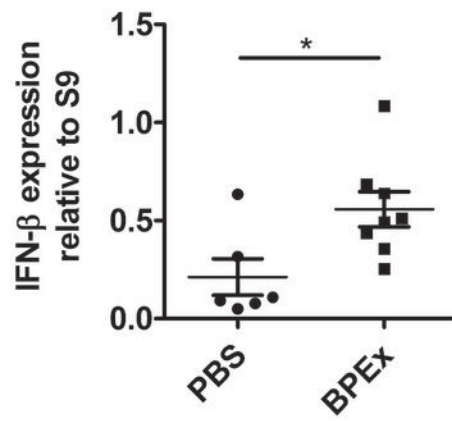
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Supplementary Figure Legends

Supplementary Figure 1. Intratracheal administration of 150 mg/kg NAC irritates the airways causing enhanced airway inflammation. Airway responses (Rrs) to increasing doses of aerosolized MCh (A), total BAL cells (B), BAL macrophages (C) and eosinophils (D) of Balb/c mice that received 150 mg/kg of pH-adjusted, sterile-filtered NAC intratracheally, concomitantly with either BPEX sensitization (BPEX + i.t. NAC /BPEX) or challenge (BPEX /BPEX + i.t. NAC) (n=8-9 animals per group pooled from four independent experiments; * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).

Supplementary Figure 2. BPEX-induced airway eosinophilic inflammation is partially dependent upon oxidative stress and TLR4 and is also regulated by TRIF-dependent mechanisms. Frequency of eosinophils among total BAL cells on day 17, 48 hrs after final intratracheal PBS or BPEX exposure preceded by i.p. NAC injection (150 mg/kg) prior to all BPEX exposures, sensitization only, or challenge only (A). Frequency of BAL eosinophils following intratracheal PBS or BPEX exposures of TLR4-antagonized or TLR4 KO mice (B) (n=5-12 animals per group pooled from at least two independent experiments). mRNA expression of the TRIF-inducible type I interferon, IFN- β (normalized to the house-keeping gene, S9) in lung tissues of wild-type Balb/c mice harvested 30 mins after a single PBS or BPEX exposure (C) (n=6-8 animals per group from two independent experiments). Eotaxin 1 protein levels in BAL fluid of wild-type or Trif KO C57Bl/6J mice on day 17, 48 hrs after final BPEX challenge (D) (n=9-10 animals per group pooled from at least 3 independent experiments).



A**B****C****D**