

Nitrogen Dioxide Promotes Allergic Sensitization to Inhaled Antigen¹

Mieke Bevelander,* Jana Mayette,* Laurie A. Whittaker,* Sara A. Pavaglio,* Christine C. Jones,* Justin Robbins,[†] David Hemenway,[†] Shizuo Akira,[‡] Satoshi Uematsu,[‡] and Matthew E. Poynter^{2*}

Allergen sensitization and allergic airway disease are likely to come about through the inhalation of Ag with immunostimulatory molecules. However, environmental pollutants, including nitrogen dioxide (NO₂), may promote adaptive immune responses to innocuous Ags that are not by themselves immunostimulatory. We tested in C57BL/6 mice whether exposure to NO₂, followed by inhalation of the innocuous protein Ag, OVA, would result in allergen sensitization and the subsequent development of allergic airway disease. Following challenge with aerosolized OVA alone, mice previously exposed via inhalation to NO₂ and OVA developed eosinophilic inflammation and mucus cell metaplasia in the lungs, as well as OVA-specific IgE and IgG1, and Th2-type cytokine responses. One hour of exposure to 10 parts per million NO₂ increased bronchoalveolar lavage fluid levels of total protein, lactate dehydrogenase activity, and heat shock protein 70; promoted the activation of NF-κB by airway epithelial cells; and stimulated the subsequent allergic response to Ag challenge. Furthermore, features of allergic airway disease were not induced in allergen-challenged TLR2^{-/-} and MyD88^{-/-} mice exposed to NO₂ and aerosolized OVA during sensitization. These findings offer a mechanism whereby allergen sensitization and asthma may result under conditions of high ambient or endogenous NO₂ levels. *The Journal of Immunology*, 2007, 179: 3680–3688.

The incidence of allergic asthma has risen steadily over the last 20 years and now represents a significant health and financial burden. Reasons for this rise remain unclear, but increases in ambient pollutant levels, such as the oxidant, nitrogen dioxide (NO₂),³ correlate with the increased incidence of allergic sensitization, asthma, and wheeze (1, 2). NO₂ is a toxic-free radical gas that is a component of air pollution (1, 3) and is also formed in the lung during inflammation (4). NO₂ has been implicated in the aggravation of asthma, particularly in children (1, 5–8). We and others have demonstrated that NO₂ inhalation is injurious to the lung and can augment the degree of allergic airway inflammation and prolong allergen-induced airway hyperresponsiveness in rodent models of asthma (9, 10).

Traditional mouse models to replicate features of human allergic asthma achieve allergen sensitization by i.p. injection with the Ag,

OVA, along with the adjuvant, aluminum hydroxide (Alum), to skew the generation of an Ag-specific Th2-biased adaptive immune response (11). Sensitized mice are then challenged with inhaled Ag, resulting in features of allergic asthma (12, 13), including Th2 cytokines in the lungs, Ag-specific Th2-driven Igs (IgE and IgG1) in the serum, mucus cell metaplasia, airway eosinophilia, and airway hyperresponsiveness to inhaled methacholine. These recall responses to the Ag are well documented in mice and equally well recognized as triggers in human allergic asthma. Despite modeling many of the cardinal features of human asthma, this well-established murine model does not allow for the study of sensitization to inhaled Ags, a necessary first step in the development of allergic asthma. The generation of an Ag-specific Th2 immune response is both necessary and sufficient (14) to confer sensitization in allergic asthma. IL-4 promotes Ig class switching by B cells to produce IgE; IL-5 promotes eosinophil growth, survival, and recruitment; and IL-13 promotes mucus production and airway hyperresponsiveness, all hallmarks of asthma.

Host organisms have evolved in the presence of immunostimulatory microbial products and recognize these molecules through TLRs to modulate immunity against the intact microorganism (15). A number of endogenous molecules have also been reported to stimulate TLRs, especially TLR2, and induce intracellular signaling (16–18). TLR2 is expressed by structural lung cells and leukocytes, signaling through which promotes inflammation, dendritic cell (DC) maturation, and Th2 polarization (19–24), leading to allergic sensitization and the development of asthma-like disease in mice (25–27). Examples of endogenous molecules that stimulate TLR2 include heat shock protein (HSP)70 (28, 29), HSP60 (30), high mobility group box 1 (31–33), low m.w. hyaluronic acid fragments (34), and molecules with hydrophobic domains (35), which are normally sequestered intracellularly, preventing them from activating cells of the innate immune system. For TLR2-induced signaling,

*Vermont Lung Center and Department of Medicine and [†]School of Engineering, University of Vermont, Burlington, VT 05405; and [‡]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Received for publication January 22, 2007. Accepted for publication June 29, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health, National Center for Research Resources Centers of Biomedical Research Excellence, Grant RR15557 (to M.E.P. and L.A.W.) and by the National Institutes of Health/National Institute on Environmental Health Sciences Transition to Independent Positions Grant K22 ES011652 (to M.E.P.).

² Address correspondence and reprint requests to Dr. Matthew E. Poynter, University of Vermont, Department of Medicine, Division of Pulmonary Disease and Critical Care Medicine, 149 Beaumont Avenue, HSRF 220, Burlington, VT 05405. E-mail address: matthew.poynter@uvm.edu

³ Abbreviations used in this paper: NO₂, nitrogen dioxide; Alum, aluminum hydroxide; BAL, bronchoalveolar lavage; BALF, BAL fluid; C_T, threshold cycle; DC, dendritic cell; HEPHA, high-efficiency particle air; HSP, heat shock protein; LDH, lactate dehydrogenase; PAS, periodic acid-Schiff; ppm, parts per million; RT, room temperature.

MyD88 is the critical adaptor molecule that promotes downstream intracellular signaling events (36), including activation of NF- κ B and MAPK (15, 37).

Because allergic sensitization does not develop when purified Ag alone is inhaled (38), immunostimulatory signals must also be necessary. Because immunostimulatory signals, including those that activate TLR2, are generated endogenously as a consequence of tissue injury (39), we hypothesized that NO₂ exposure may promote development of allergic sensitization if encountered with inhaled Ag. To test this hypothesis, we developed a mouse model of NO₂ exposure, consisting of as little as 1 h of 10 parts per million (ppm) NO₂, followed by exposure to aerosolized 1% OVA. Mice were subjected to the same sensitization regimen 1 wk after and challenged with 1% OVA alone an additional week later. Following OVA challenge, mice elicited changes consistent with the phenotypic alterations in allergic asthma. These include airway eosinophilia, mucus cell metaplasia, IgE and IgG1, airway hyper-responsiveness, and Ag-specific T cells that produce Th2 cytokines following *in vitro* restimulation. All of the features were similar to those elicited following OVA challenge of mice that were Ag sensitized by i.p. injection of OVA and Alum. Interestingly, exposure to NO₂ can precede exposure to Ag by at least 2 days and elicit an allergic response. Lung cellular damage induced by NO₂ exposure promotes the release of intracellular proteins that may stimulate airway epithelial cells to activate the transcription factor NF- κ B. Allergen sensitization following NO₂ exposure was found to require functional TLR2 and MyD88, suggesting that endogenous agonists capable of stimulating TLR2 modulate the response to NO₂. Our results demonstrate that NO₂, a common component of polluted air, as well as a molecule generated endogenously during inflammatory responses, is capable of stimulating the generation of an atopic immune response, resulting in allergic asthma-like features. Therefore, NO₂ exposure, possibly through the release of immunostimulatory molecules, can promote the development of asthmatic responses to Ags that are not inherently immunogenic, thereby increasing the repertoire of Ags that act as asthma triggers.

Materials and Methods

Mouse and experimental model of Ag sensitization and challenge

C57BL/6J mice were purchased from The Jackson Laboratory. TLR2^{-/-} and MyD88^{-/-} mice on the C57BL/6 background were bred at the University of Vermont. All mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility, and the Institutional Animal Care and Use Committee granted approval for all studies. As a positive control for the induction of allergic airway disease, mice were sensitized by administering OVA (20 μ g, grade V; Sigma-Aldrich) with Alum (2.25 mg, Imject Alum; Pierce) in 100 μ l of total volume via i.p. injection (i.p. Alum/i.p. OVA) on days 0 and 7. In the experimental group, mice were exposed to NO₂, as described below, for 1 h and were then exposed to aerosolized 1% OVA in PBS for 30 min (NO₂/OVA) on days 0 and 7. As controls, mice were administered Alum i.p. (i.p. Alum/none), were exposed to room air (0 ppm NO₂) and then 1% OVA aerosol (none/OVA), were exposed only to NO₂ (NO₂/none), or were administered Alum i.p. and immediately exposed to aerosolized 1% OVA for 30 min (Alum i.p./OVA aerosol) on days 0 and 7. For the data presented in Fig. 8, mice in the experimental groups were exposed to 30 min of 1% OVA aerosol either before (48 h, 24 h, 6 h, or immediately) or after (immediately, 6 h, 24 h, or 48 h) exposure to NO₂. All mice were challenged using three exposures to aerosolized 1% OVA for 30 min on days 14, 15, and 16 and were analyzed on day 18. Mice were euthanized by a lethal dose of pentobarbital via i.p. injection.

In vivo NO₂ inhalation

A 50-L Tedlar bag was filled with 1 part pure NO₂ to ~3 parts dry air, thus diluting the NO₂ and allowing the dimer to dissociate to NO₂. This mixture was then pumped through a regulating valve using a stainless steel/Teflon diaphragm pump, into a mixing chamber, where it was introduced into a stream of dry air entering the inhalation chamber. A calibrated NO ana-

lyzer, equipped with an NO₂ thermal converter, was used to measure NO₂ in the gas phase via chemiluminescence, according to manufacturer's instructions (ECO PHYSICS). As controls, groups of mice were exposed to high-efficiency particle air (HEPA)-filtered room air in an identical chamber for the same amount of time as the NO₂-exposed mice. In some instances, the NO₂ exposure regimen was followed by 30 min of exposure to aerosolized 1% OVA. An identical regimen was repeated 1 wk later. For the studies presented in Fig. 9, mice in the experimental groups were exposed to 1 h of 10 ppm NO₂ and were assessed 0.5, 6, 24, or 48 h later.

Pulmonary function assessment to measure airway hyperresponsiveness

Mice anesthetized with 90 mg/kg pentobarbital and tracheotomized were mechanically ventilated for the assessment of pulmonary function using the forced oscillation technique, as previously described (9). Briefly, a tracheotomy tube was inserted and then connected to the inspiratory and expiratory ports of a volume-cycled ventilator (*flexiVent*; SCIREQ). Mice were ventilated at a rate of 160–200 breaths/min, with a tidal volume of 0.2 ml, using a computer-controlled volume ventilator with 3 cm H₂O positive end-expiratory pressure. Data from regular ventilation was collected to establish the baseline values for each animal. Pressure, flow, and volume were used to calculate the peak response for airway resistance after challenge with inhaled doses of saline or aerosolized methacholine (Sigma-Aldrich) in saline, ranging from 3.125 to 50 mg/ml in half-log increments. The percentage change from baseline after each methacholine dose is reported.

Bronchoalveolar lavage (BAL)

BAL fluid (BALF) was immediately collected from euthanized mice by instillation and recovery of 800 μ l of 0.9% NaCl plus protease inhibitor mixture (Sigma-Aldrich) into the lungs through the tracheal cannula using a tuberculin syringe. The BALF was centrifuged, and the total cells in the pellet were resuspended in PBS and enumerated by counting with an Advia 120 Hematology System (Bayer HealthCare). For cytopspins, 2×10^4 cells were centrifuged onto glass slides at 800 rpm. Cytopspins were stained using the Hema3 kit (Biochemical Sciences), and differential cell counts were performed on at least 500 cells. BALF supernatants were collected for protein (Bio-Rad) and lactate dehydrogenase (LDH; Promega) quantitation from undiluted samples, as previously described (9). HSP70 levels in BALF supernatants were quantitated by ELISA using a technique adapted from Njemini et al. (40). Briefly, plates were coated overnight at 4°C with 5 μ g/ml anti-HSP70-heat shock cognate 70 (SPA-820; StressGen Biotechnologies) in 0.1 M carbonate buffer (pH 9.6). Wells were washed and blocked for 4 h at room temperature (RT) with PBS/0.05% Tween 20 with 1% BSA. After washing, undiluted BALF or dilutions of human rHSP70 (ESP-555; StressGen Biotechnologies) in 0.9% saline (as a standard) were added to appropriate wells and incubated overnight at 4°C. After washing, 10 ng/ml anti-HSP70/HSP60 was added to the wells and incubated at 4°C overnight. The wells were again washed, and anti-mouse peroxidase-conjugated Ab (1:2500) was added to each well and incubated at RT for 1 h. Plates were washed and developed using reagents from R&D Systems, and ODs were read using a Bio-Tek Instruments PowerWave_{xt} at 450 nm with background subtraction at 570 nm. Control wells in which the anti-HSP70/HSP60 coating or detection Ab was omitted displayed no reactivity with Ig in BALF, because no color development was measured.

Serum collection and Ig analysis

Following euthanasia, ~300 μ l of blood was collected via cardiac puncture of the right ventricle using a 26-g needle attached to a 1-ml syringe into serum separator tubes (BD Biosciences) and centrifuged, and serum was kept frozen at -80°C. For IgG1 and IgG2a, indirect ELISAs were performed. Ninety-six-well plates were coated overnight at 4°C with 2 μ g/ml OVA in PBS (pH 7.2–7.4), washed with 0.05% Tween 20 in PBS, and blocked for 2 h at 4°C with 1% BSA in PBS. Plates were washed, and serum diluted in blocking solution was applied to the wells in triplicate at dilutions of 1/8–1/4096 and incubated overnight at 4°C. Plates were washed, and 2 μ g/ml biotinylated secondary Abs (BD Pharmingen) in 1% BSA/PBS were incubated in the plates at RT for 1 h. Plates were washed, and 0.05 U/ml streptavidin/peroxidase (Roche) was incubated in the plates at RT for 1 h. For determination of OVA-specific serum IgE by two-step sandwich (capture) ELISA, plates were coated with 2 μ g/ml anti-mouse IgE mAb (BD Pharmingen clone R35-72) in PBS for 1 h at RT. Plates were washed and serum samples were added in duplicate in PBS/1% BSA for 1 h at RT. Plates were washed and incubated with a 1/2500 dilution of digoxigenin-coupled OVA (Roche) in PBS/1% BSA for 1 h at RT. Plates were washed and incubated with a 1/2000 dilution of anti-digoxigenin-Fab

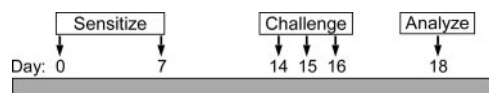


FIGURE 1. Methods of Ag sensitization and challenge. Eight-week-old female C57BL/6J mice were allergically sensitized by administration of adjuvant (Alum) and Ag (OVA) via i.p. injection or by exposure to 1 h of NO₂ (5–25 ppm), followed immediately by inhalation of 1% OVA aerosol for 30 min on days 0 and 7. Control groups in which mice were only exposed to aerosolized OVA, i.p. OVA, Alum, NO₂, or i.p. Alum plus aerosolized OVA were also used in these studies. Mice were then challenged with aerosolized OVA for 3 days (days 14, 15, and 16) and examined 48 h later (day 18).

coupled to peroxidase (Roche) in PBS/1% BSA for 30 min. Plates were washed, developed using reagents from R&D Systems, and stopped with 1 N H₂SO₄, and ODs were read using a Bio-Tek Instruments PowerWave_x at 450 nm with background subtraction at 570 nm. Data for each Ig isotype are reported as OD values from identical dilutions in the linear range of the readings (1:10 for OVA-specific IgE, 1:1000 for OVA-specific IgG1, and 1:50 for OVA-specific IgG2a).

Histopathology and morphometry

Following euthanasia and BAL, the left lobe of the lungs was instilled with 4% paraformaldehyde in PBS (4% paraformaldehyde) for 10 min at a pressure of 25 cm H₂O and placed into 4% paraformaldehyde at 4°C overnight for fixation of the tissue. Fixed lungs were then mounted in paraffin, and 7-μm sections were cut, affixed to glass microscope slides, deparaffinized, stained with periodic acid-Schiff (PAS), coverslipped, and examined by light microscopy. Sections were morphometrically assessed for inflammatory cell infiltration and PAS (mucin) positivity of the airways. Multiple airways of similar size with a length:diameter ratio of <2:1 were assessed per section, and the type, number, and location of inflammatory cells, as well as the percentage of PAS-positive airway epithelial cells, were recorded.

NF-κB immunostaining

Following euthanasia, lungs were instilled with PBS for 5 min at a pressure of 25 cm H₂O, placed into Tissue-Tek OCT Compound (Sakura Finetek), frozen in liquid nitrogen-chilled isopentane, and stored at –80°C until sectioning. Sections (10 μm) were immunostained for the RelA subunit of NF-κB and for nuclei, and visualized by confocal microscopy, as previously described (41).

Semiquantitative and quantitative RT-PCR

Total RNA isolated from lungs by RNeasy columns (Qiagen) was DNase treated and reverse transcribed into cDNA using SuperscriptII (Invitrogen Life Technologies). Semiquantitative RT-PCR was performed using PCR Supermix (Invitrogen Life Technologies) and intron-spanning primers designed and validated for mouse *Gob-5*, an indicator of mucus cell metaplasia (42), and the housekeeping gene *β-actin*. PCR conditions were denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. For *Gob-5* and *β-actin*, 25 and 30 cycles were performed, respectively. Real-time quantitative RT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and intron-spanning primers and probe (Applied Biosystems) designed and validated for mouse *Muc5AC*, and the housekeeping gene *HPRT*. Forty cycles of PCR were performed on an Applied Biosystems Prism 7900HT Sequence Detection System (Applied Biosystems), using universal cycling conditions as follows: denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The level of *Muc5AC* expression was normalized to *HPRT* levels, and relative *Muc5AC* mRNA levels were determined according to the comparative threshold cycle (C_T) method (Applied Biosystems Prism 7700 Sequence Detection System, User Bulletin No. 2; Applied Biosystems). Briefly, the C_T was determined for *Muc5AC* and *HPRT* in each sample, and the ΔC_T was calculated for each sample by subtracting the C_T of *HPRT* from the C_T of *Muc5AC*. The $\Delta\Delta C_T$ values were calculated by subtracting the ΔC_T of the experimental samples from the controls. The $\Delta\Delta C_T$ values were transformed into absolute values using the following equation: $2^{-\Delta\Delta C_T}$.

Preparation, stimulation, and ELISA analysis of single-cell CD4⁺ lymphocyte suspensions

Single-cell suspensions were generated from spleens by passing the tissues through a 70-μm mesh, and lymphocytes were enriched by separation with

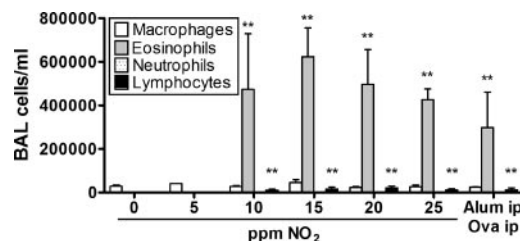


FIGURE 2. As little as 10 ppm NO₂ plus Ag exposure during sensitization promotes eosinophilic airway inflammation following allergen challenge. On days 0 and 7, mice were exposed to HEPA-filtered room air containing 0–25 ppm NO₂ for 1 h, followed by inhalation of aerosolized OVA. Positive control mice were Ag sensitized by i.p. injection of Alum and OVA. Mice were challenged on days 14–16 with aerosolized OVA, and on day 18, BALF was collected and total and differential cell types were enumerated. Values are means ($\pm$ SEM) of four mice/group. Data were analyzed by a two-tailed unpaired Student's *t* test and results are compared with 0 ppm NO₂. **, $p \leq 0.01$.

Lymphocyte Separation Medium (MP Biomedicals). CD4⁺ T cells were isolated by positive selection using CD4 magnetic microbeads (Miltenyi Biotec), according to the manufacturer's protocol. Isolated CD4⁺ T cells were >95% pure, as assessed by CD4 surface staining and FACS analysis. CD4⁺ T cells (4×10^6 cells/ml) were activated with 100 μg/ml OVA in the presence of syngeneic APCs (4×10^6 cells/ml). APCs were obtained by splenic T cell depletion by negative selection using Abs to CD4 (GK1.5), CD8, and Thy-1, and treatment with rabbit complement and mitomycin C, as previously described (43). Following 96 h of stimulation, supernatants were collected and analyzed for IL-2, IL-4, IL-5, IL-13, and IFN-γ using reagents and instructions from R&D Systems. ODs from triplicate samples and duplicate standards were read using a Bio-Tek Instruments PowerWave_x at 450 nm with background subtraction at 570 nm.

Statistical analysis

Data were analyzed by two-tailed unpaired Student's *t* test or by two-way ANOVA, followed by comparison between groups by two-tailed unpaired Student's *t* test with Welch's correction. Statistical calculations were performed using GraphPad Prism 4 for Windows. A *p* value smaller than 0.05 was considered statistically significant.

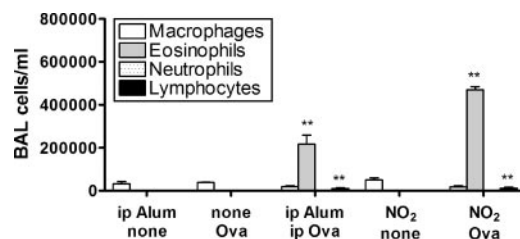


FIGURE 3. NO₂ plus Ag promotes eosinophilic airway inflammation following allergen challenge. On days 0 and 7, mice were exposed to adjuvant (top line on x-axis) and/or Ag (bottom line on x-axis), as follows: i.p. Alum alone (ip Alum/none), aerosolized OVA alone (none/OVA), the positive control of i.p. injection of Alum and OVA (i.p. Alum/i.p. OVA), 10 ppm NO₂ alone (NO₂/none), or the experimental condition of 10 ppm NO₂ plus OVA aerosol (NO₂/OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18, BALF was collected, and total cells, as well as macrophages, eosinophils, neutrophils, and lymphocytes were enumerated. Values are means ($\pm$ SEM) of four mice/group and are representative of three independent experiments. **, $p \leq 0.01$ compared with values in i.p. Alum alone, aerosolized OVA alone, and the 10 ppm NO₂ alone groups.

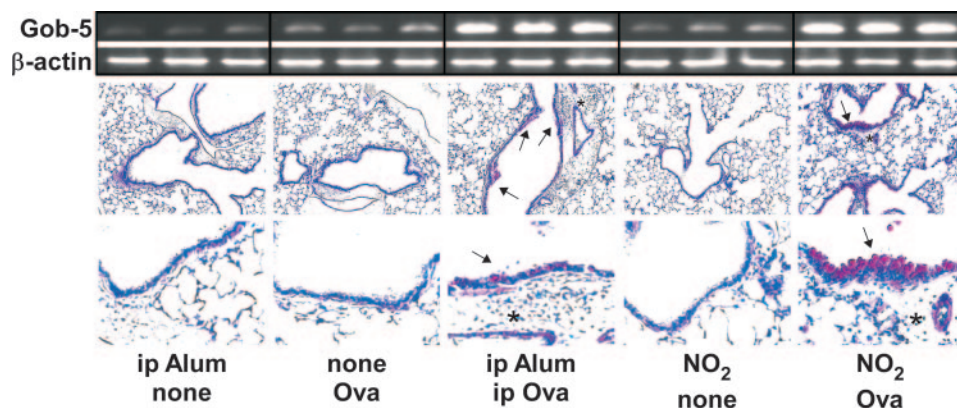


FIGURE 4. NO₂ plus Ag promotes mucus cell metaplasia following allergen challenge. On days 0 and 7, mice were exposed to adjuvant and/or Ag, as follows: i.p. Alum alone (i.p. Alum/none), aerosolized OVA alone (none/OVA), the positive control of i.p. injection of Alum and OVA (i.p. Alum/i.p. OVA), 10 ppm NO₂ alone (NO₂/none), or the experimental condition of 10 ppm NO₂ plus OVA aerosol (NO₂/OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18 lavaged lungs were collected for RNA analysis of *Gob-5* and β -actin mRNA expression by RT-PCR or were inflation fixed, sectioned, and PAS stained. Arrows indicate mucus-positive (PAS⁺) cells; *, indicates perivascular inflammation. Data are representative of three independent experiments.

Results

NO₂ induces allergic sensitization to inhaled Ag, resulting in airway eosinophilia following allergen challenge

We have previously demonstrated that exposure of allergically inflamed mice to NO₂ prolongs airway eosinophilia and hyperresponsiveness to methacholine (9). Because elevated levels of ambient NO₂ have been correlated with increased incidence of asthma, we sought to determine whether NO₂ exposure could cause allergic sensitization to an inhaled Ag that is otherwise non-immunogenic and whether challenge with inhaled Ag alone would promote the development of cardinal features of allergic asthma. Therefore, on day 0, we exposed C57BL/6J mice to 1 h of 0–25 ppm NO₂, immediately followed by inhalation of aerosolized OVA (Ag) for 30 min. The same regimen was repeated 1 wk later on day 7 (see Fig. 1). As a positive control, mice were administered OVA plus Alum via i.p. injection on days 0 and 7, which represents a widely used model for allergic sensitization, in which the sensitization largely takes place in the spleen and lymph nodes draining the peritoneum instead of in the relevant draining lymph nodes of the lung. All mice were Ag challenged with aerosolized OVA on days 14, 15, and 16 and were analyzed 48 h following the final aerosolized Ag challenge (day 18), at which time BAL was performed and airway inflammation was determined by BAL cell count. As anticipated, in mice administered Alum and OVA i.p., eosinophilic airway inflammation was pronounced and lymphocytic inflammation was evident (Fig. 2). In contrast, mice that had been administered aerosolized OVA following inhalation of room air with 0 ppm NO₂ on days 0 and 7 and that were subsequently challenged with aerosolized OVA on days 14, 15, and 16 displayed no eosinophilic or lymphocytic airway inflammation on day 18. Consistent with previous reports (38), these results demonstrate that inhalation of OVA alone is insufficient to promote allergic sensitization to this Ag. However, in mice that had been exposed for 1 h to room air containing 25, 20, 15, or 10 ppm NO₂, followed by exposure to aerosolized OVA on days 0 and 7, eosinophilic and lymphocytic airway inflammation was evident on day 18, following OVA challenge on days 14, 15, and 16, and was of a magnitude similar to mice that had been exposed to the conventional sensitization regimen of Alum plus OVA via the i.p. route (Fig. 2). Mice exposed to 5 ppm NO₂, however, did not display lymphocytic or eosinophilic inflammation following OVA challenge. The mice exposed to 5 ppm NO₂ during sensitization on days 0 and 7,

in fact, appeared identical with the mice exposed to 0 ppm NO₂, demonstrating that 1 h of exposure to 10 ppm NO₂ is the minimum dose necessary to promote eosinophilic airway inflammation following aerosolized Ag challenge (Fig. 2). Therefore, we used a regimen of 1 h of 10 ppm NO₂ for the remainder of our studies.

Because exposure to 10 ppm NO₂ followed by inhalation of aerosolized OVA appears to be sufficient to promote allergic sensitization leading to airway eosinophilia following Ag challenge, we performed additional studies to evaluate the manifestation of other features of allergic airway disease reminiscent of asthma. As is shown in Fig. 3, additional control groups, including administration of i.p. Alum without OVA, aerosolized OVA alone, NO₂ alone, or i.p. Alum plus aerosolized OVA (data not shown) during the sensitization regimen, did not promote alterations in the numbers or types of leukocytes recovered in BAL following aerosolized OVA challenge (day 18). Only Alum plus OVA i.p. or NO₂ plus aerosolized OVA promoted eosinophilic and lymphocytic airway inflammation following subsequent OVA challenge.

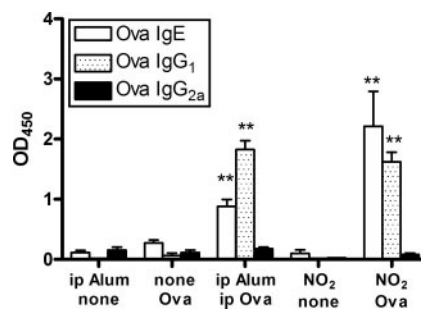


FIGURE 5. NO₂ plus Ag induces Ag-specific Igs. On days 0 and 7, mice were exposed to adjuvant (top line on x-axis) and/or Ag (bottom line on x-axis), as follows: i.p. Alum alone (ip Alum/none), aerosolized OVA alone (none/OVA), the positive control of i.p. injection of Alum and OVA (i.p. Alum/i.p. OVA), 10 ppm NO₂ alone (NO₂/none), or the experimental condition of 10 ppm NO₂ plus OVA aerosol (NO₂/OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18, serum was collected and OVA-specific IgE was measured using a capture ELISA, whereas IgG₁ and IgG_{2a} were measured using an indirect ELISA. Values are means ($\pm$ SEM) of six mice/group and are representative of three independent experiments. **, $p \leq 0.01$ compared with values in i.p. Alum alone, aerosolized OVA alone, and the 10 ppm NO₂ alone groups.

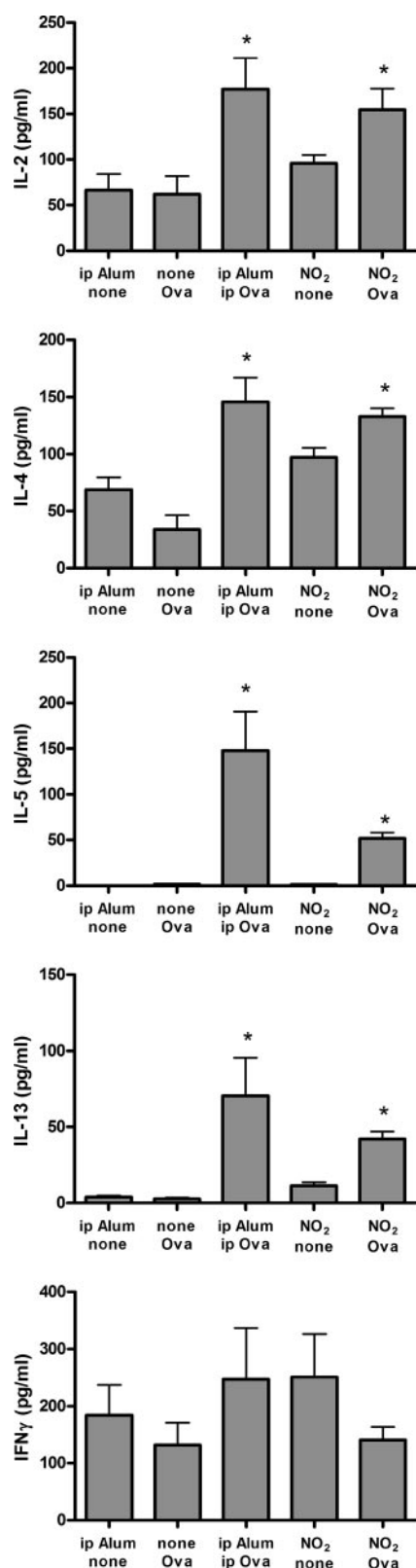


FIGURE 6. NO₂ plus Ag induces Ag-specific CD4⁺ cells that produce Th2 cytokines in vitro. On days 0 and 7, mice were exposed to adjuvant (top line on x-axis) and/or Ag (bottom line on x-axis), as follows: i.p. Alum alone (i.p. Alum/none), aerosolized OVA alone (none/OVA), the positive control of i.p. injection of Alum and OVA (i.p. Alum/i.p. OVA), 10 ppm NO₂ alone (NO₂/none), or the experimental condition of 10 ppm NO₂ plus OVA aerosol (NO₂/OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18, CD4⁺ cells were isolated from spleens by positive selection. CD4⁺ cells were cultured in the presence of OVA

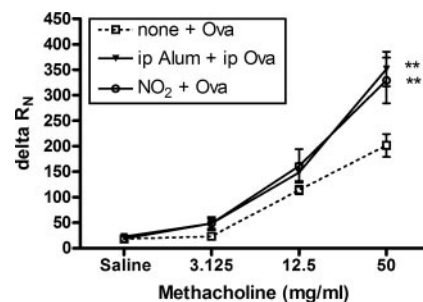


FIGURE 7. NO₂ plus Ag promotes airway hyperresponsiveness following allergen challenge. On days 0 and 7, 8-wk-old female C57BL/6J mice were exposed to aerosolized OVA alone (none + OVA), the positive control of i.p. injection of Alum and OVA (i.p. Alum + i.p. OVA), or the experimental condition of 10 ppm NO₂ plus OVA aerosol (NO₂ + OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18, pulmonary responsiveness to inhaled methacholine was measured using the *flexiVent*. Values are means ($\pm$ SEM) of four to six mice/group. Differences in the curves for the three groups of mice were analyzed by two-way ANOVA ($p \leq 0.01$), and differences at each dose of methacholine between the none + OVA and the i.p. Alum + i.p. OVA or NO₂ + OVA groups were compared using two-tailed unpaired Student's *t* test with Welch's correction. **, $p \leq 0.01$.

None of the sensitization and challenge regimens significantly altered the numbers of macrophages or neutrophils recovered in the BAL.

Priming with NO₂ and Ag induces mucus cell metaplasia following allergen challenge

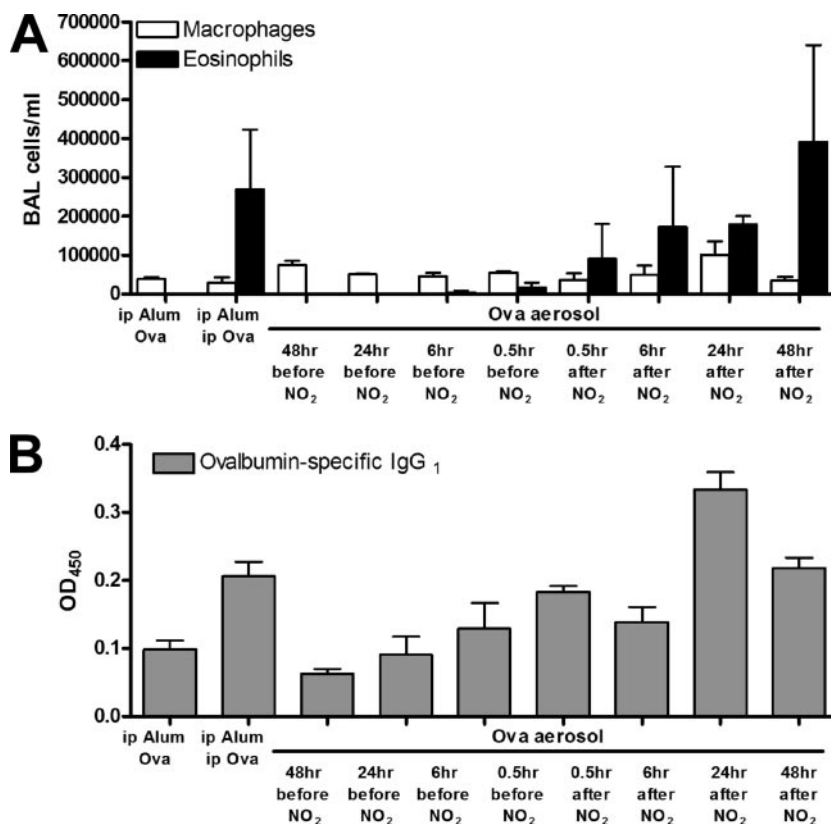
Because airway mucus cell metaplasia and mucus hypersecretion are commonly observed in allergic asthma, we examined whether expression of the mucus-associated gene, *Gob-5*, and mucus cell metaplasia were present in the lungs of mice challenged with aerosolized OVA. Only in the Alum plus OVA and the 1 h of 10 ppm NO₂ plus aerosolized OVA-exposed mice was expression of *Gob-5* augmented and were PAS⁺ cells (arrows) observed (Fig. 4). In addition, only in these groups of mice were peribronchiolar and perivascular inflammatory cells evident (*), which upon higher magnification were identified primarily as lymphocytes and eosinophils.

Sensitization with NO₂ and OVA results in Th2-induced Ig production and a Th2-biased CD4⁺ T cell adaptive immune response

Because allergic sensitization and allergy itself are associated with the generation of Ag-specific Abs, we examined the serum from mice and measured OVA-specific IgE and IgG1, the isotype switch to which is controlled by the Th2 cytokine, IL-4. Only in mice sensitized by Alum plus OVA i.p., or by the NO₂ regimen, were there significant elevations in the levels of these OVA-specific Abs (Fig. 5). We also measured OVA-specific IgG2a, the isotype switch to which is controlled by IFN- γ , but saw no elevations in this Ab in the mice allergically sensitized by the NO₂ regimen, whether they were C57BL/6 (Fig. 5) or BALB/c (data not shown).

and splenic APCs from untreated C57BL/6J mice. Conditioned medium was collected at 96 h and analyzed for IL-2; the Th2 cytokines IL-4, IL-5, and IL-13; and the Th1 cytokine IFN- γ by ELISA. No cytokines were detected in the conditioned medium from APCs alone or CD4⁺ T cells plus APCs in the absence of OVA. Values are means ($\pm$ SEM) of four mice/group. *, $p \leq 0.05$ compared with values in i.p. Alum alone, aerosolized OVA alone, and the 10 ppm NO₂ alone groups.

FIGURE 8. The magnitude of the response to Ag challenge elicited following sensitization to allergic airway disease by exposure to NO₂ depends on when Ag is delivered. On days 0 and 7, mice were exposed to aerosolized 1% OVA either 48 h, 24 h, 6 h, or immediately before exposure to HEPA-filtered room air containing 10 ppm NO₂ for 1 h, or immediately, 6 h, 24 h, or 48 h following NO₂ exposure. Negative control mice were administered Alum i.p. and were exposed to aerosolized OVA. Positive control mice were Ag sensitized by i.p. injection of Alum and OVA. Mice were challenged on days 14–16 with aerosolized OVA, and on day 18, BALF was collected for the enumeration of total and differential cell types (A) and serum was collected for the measurement of OVA-specific IgG1 (B). Values are means ($\pm$ SEM) of three to four mice/group.



Therefore, we speculated that NO₂-promoted allergic sensitization is generating a Th2-biased immune response to OVA, much like what is observed following i.p. Ag sensitization with Alum plus OVA. To definitively test this hypothesis, CD4⁺ T cells from the spleens of Ag-sensitized and challenged mice or control mice were isolated by positive selection and incubated for 96 h in the pres-

ence of OVA and APCs from naive syngeneic mice, and cytokines were then measured by ELISA. As is shown in Fig. 6, only in mice sensitized by Alum plus OVA i.p. or the NO₂ regimen were the levels of IL-2, IL-4, IL-5, and IL-13 elevated, without augmented production of the Th1 cytokine, IFN- γ . Therefore, we conclude that the 1 h of 10 ppm NO₂ exposure followed by OVA inhalation

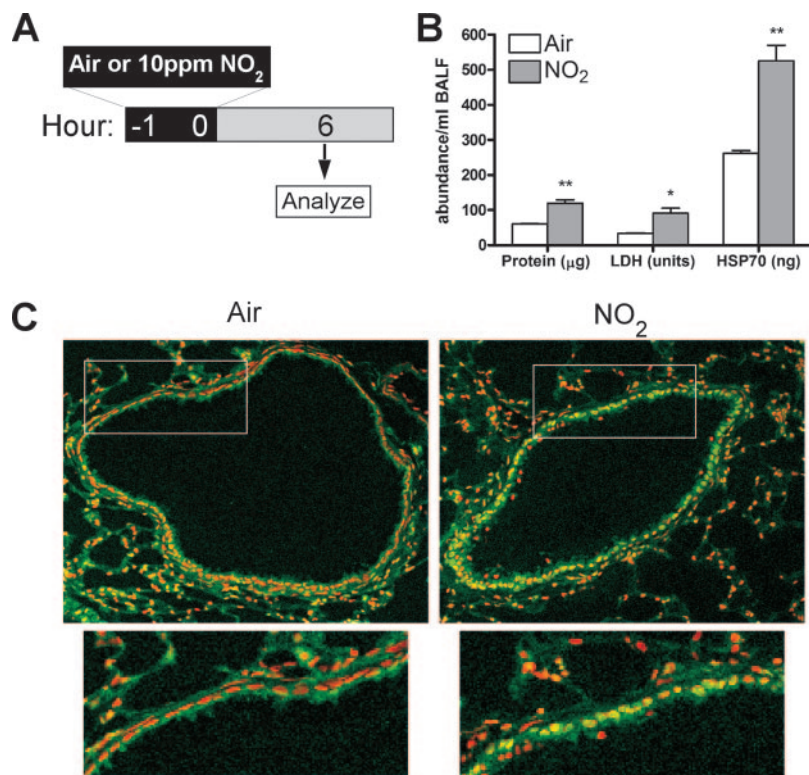


FIGURE 9. NO₂ inhalation induces lung damage and NF- κ B activation in airway epithelium. Female C57BL/6 mice were exposed to HEPA-filtered room air or 10 ppm NO₂ for 1 h, and mice were studied between 6 h thereafter (A). BALF was analyzed for total protein, LDH activity, and HSP70 content (B). Values are means ($\pm$ SEM) of three to six mice per group. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with values in the air-exposed groups. Lungs were immunostained for NF- κ B RelA (green) and nuclei (red). Nuclear RelA (bright green/yellow) is indicative of NF- κ B activation (C). Immunostaining data are representative of three mice per group.

regimen promotes a Th2 immune response and the development of many features of allergic asthma following Ag challenge with inhaled OVA, similar to that observed when mice are allergically sensitized using a conventional regimen of i.p. Alum plus OVA.

NO₂ sensitization and Ag challenge lead to airway hyperresponsiveness to methacholine

Although allergic asthma is indeed associated with Ag-specific IgE, eosinophilic airway inflammation, and mucus cell metaplasia, which are controlled by the Th2 cytokines IL-4, IL-5, and IL-13, respectively, the definitive feature of allergic asthma is airway hyperresponsiveness to agonists such as methacholine. To measure whether the 1 h of 10 ppm NO₂-promoted allergic sensitization regimen induces airway hyperresponsiveness to inhaled methacholine following Ag challenge, mice from this group were compared with negative control mice, which during the sensitization phase inhaled OVA following exposure to room air without NO₂, and positive control mice, which were administered Alum plus OVA i.p. during the sensitization phase. All mice were challenged three times with aerosolized OVA and analyzed 48 h following the third challenge. As is shown in Fig. 7, compared with the negative control mice, the mice exposed to the NO₂-promoted allergic sensitization regimen demonstrated a lower threshold of responsiveness to methacholine and an increased overall responsiveness to the highest dose of methacholine tested. In fact, the airway hyperresponsiveness to inhaled methacholine was indistinguishable from that induced by the i.p. Alum plus OVA regimen. Similar results were observed in BALB/c mice (data not shown). Altogether, these data support the notion that NO₂ exposure can promote the generation of an Ag-specific Th2 immune response to an inhaled Ag that is otherwise not immunogenic, and that this Th2-biased immune response manifests in the development of asthma-like pathophysiologies indistinguishable from those induced by an established model of allergic asthma in which mice are sensitized via i.p. administration of Alum plus OVA.

OVA inhalation following NO₂ exposure induces allergic sensitization

The generation of a Th2 immune response is incumbent upon stimulation of naive CD4⁺ T cells, which is accomplished by APCs, especially DCs. DCs have been reported to be capable of undergoing maturation several days following exposure to Ag (44). Therefore, we speculated that exposure of mice to aerosolized OVA before exposure to NO₂ would allow resident lung APCs to engulf the Ag and subsequently undergo maturation following NO₂ inhalation, thereby promoting allergic sensitization and subsequent airway eosinophilia and Th2-biased Ig isotypes. To test this hypothesis, we subjected mice to allergen inhalation either before or after 1 h of 10 ppm NO₂ exposure. To our surprise, our results demonstrate that exposure to NO₂ following allergen inhalation does not promote the subsequent development of airway eosinophilia (Fig. 8A) or serum OVA-specific IgG1 (Fig. 8B). On the contrary, NO₂ exposure can precede the inhalation of Ag by several days and still induce allergic sensitization, as evidenced by airway eosinophilia and OVA-specific serum IgG1 levels following subsequent allergen challenge. This finding suggests that exposure to NO₂ several days before inhalation of an allergen may be sufficient to induce allergic sensitization, implicating that the health effects mediated by NO₂ and allergen exposure may manifest even if they are not encountered simultaneously.

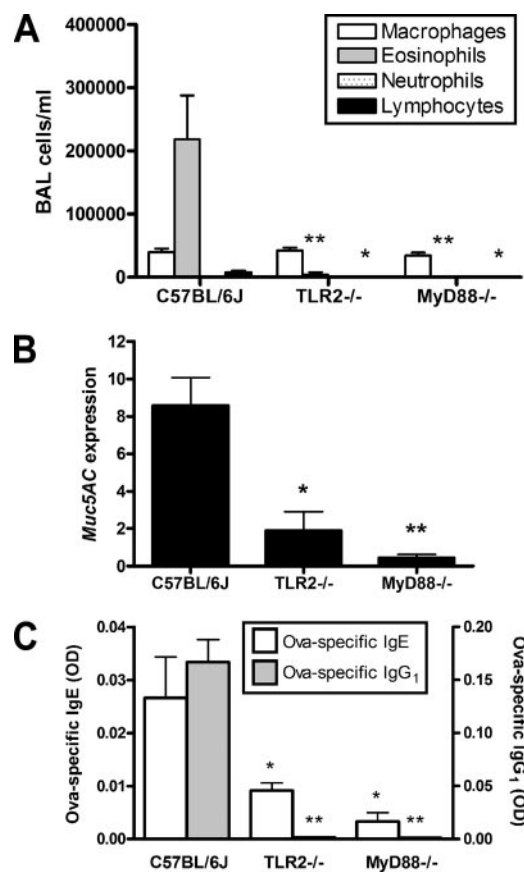


FIGURE 10. TLR2^{-/-} and MyD88^{-/-} mice are not responsive to NO₂-promoted allergic sensitization. On days 0 and 7, 8-wk-old female C57BL/6J, TLR2^{-/-}, and MyD88^{-/-} mice were exposed to 10 ppm NO₂ plus OVA aerosol (NO₂ plus OVA). All mice were challenged on days 14–16 with aerosolized OVA, and on day 18, BAL cells (A), whole lung Muc5AC mRNA expression (B), and serum OVA-specific IgE and IgG₁ (C) were measured using capture and indirect ELISAs, respectively. Values are means (±SEM) of six mice per group. *, *p* ≤ 0.05; **, *p* ≤ 0.01 compared with values in C57BL/6J mice.

NO₂ exposure promotes release of intracellular proteins and activation of NF-κB

To investigate some of the early events that may promote allergic sensitization to Ags subsequent to NO₂ inhalation, mice were exposed to air or to 10 ppm NO₂ for 1 h and then placed in room air (Fig. 9A). To assess tissue damage, BAL was performed 6 h following air or NO₂ exposure, and total protein, LDH activity, and HSP70 were measured. Protein, LDH, and HSP70 were significantly elevated in the BALF from the NO₂-exposed mice (Fig. 9B) relative to those mice exposed to normal room air. These findings demonstrate that NO₂ inhalation causes lung damage and promotes the release of molecules that are normally sequestered within cells (LDH and HSP70). Of importance, HSP70 is an endogenous molecule capable of stimulating innate immune responses through TLR2 or TLR4 (28–30). One of the intracellular signaling cascades induced by HSP70 is activation of the transcription factor, NF-κB, a critical modulator of innate and adaptive immune responses. To determine whether exposure to 1 h of 10 ppm NO₂ is capable of activating NF-κB in the lung, frozen sections were immunostained for the transcriptionally active subunit of NF-κB, RelA, which is normally localized to the cytoplasm, but which is retained in the nucleus upon NF-κB activation. The RelA subunit of NF-κB (green) is normally excluded from the nucleus (red) of

cells in the airways from air-exposed mice (Fig. 9C, *left*). However, inhalation of NO₂ induces activation of NF- κ B in the airway epithelium 6 h following exposure, as demonstrated by the increased nuclear localization (yellow) (Fig. 9C, *right*).

NO₂-induced allergic sensitization requires TLR2 and MyD88

Having demonstrated that mice allergically sensitized by exposure to NO₂ plus OVA develop cardinal features of allergic asthma, we examined whether TLR2 or MyD88, which facilitate recognition of endogenous molecules released by damaged cells (28–35), might be required for the response. Therefore, C57BL/6, TLR2^{-/-}, and MyD88^{-/-} mice were experimentally sensitized by exposure to 10 ppm NO₂ for 1 h, followed by exposure to 1% OVA. As positive controls, C57BL/6, TLR2^{-/-}, and MyD88^{-/-} mice were sensitized by i.p. injection of OVA and Alum. The same regimens were repeated 1 wk later, and mice were challenged with aerosolized OVA alone another week later and were studied 48 h after the final OVA challenge (see Fig. 1). We found that TLR2^{-/-} and MyD88^{-/-} respond like C57BL/6 mice to the Alum/OVA sensitization regimen, manifesting robust eosinophilic airway inflammation, mucus cell metaplasia, and OVA-specific IgE and IgG1 (data not shown). However, compared with the C57BL/6J mice, TLR2^{-/-} and MyD88^{-/-} mice were not responsive to the NO₂-induced sensitization regimen because they developed significantly less eosinophilic airway inflammation (Fig. 10A), *Muc5AC* up-regulation (Fig. 10B), and serum levels of IgE and IgG1 (Fig. 10C) upon allergen challenge. These results demonstrate the requirement of TLR2 and MyD88 to promote allergic sensitization following NO₂ and OVA exposure.

Discussion

The incidence of asthma is elevated in populations living or working in heavily polluted areas, where ambient NO₂ levels are high. In addition to the role of exogenous NO₂, respiratory viral infections in childhood are associated with the development of asthma (45) and the endogenous production of NO₂. Therefore, understanding the mechanisms by which NO₂ exposure contributes to allergic asthma has important domestic and global public health implications. In these studies, we demonstrate that exposure to low levels of NO₂ (as little as 10 ppm for 1 h) induces allergic Th2 sensitization to inhaled Ag and promotes the development of asthma-like pathophysiologies. Our results are in accordance with the observation that OVA inhalation alone is insufficient to trigger the innate immune response such that a subsequent Th2-biased adaptive response is generated (38), probably because OVA is an innocuous Ag that, unlike a number of allergens, does not itself possess inherent enzymatic or sufficient innate immune-stimulating activities. In addition, our results support the hypothesis that NO₂ acts as an effective inhaled adjuvant that accentuates the adaptive immune response to otherwise innocuous Ags. Through the manner in which the inhalations were performed, the Ag was never exposed to the NO₂, which is important because recent studies suggest that gaseous air pollutants can enhance the effects of allergens (46, 47). In addition, in our system, the mice have no NO₂-induced inflammation at the time of OVA challenge, which occurs a full week after the last NO₂ exposure.

Our work presented in this study is the first to demonstrate in a mouse model that NO₂ can promote allergic sensitization and the development of allergic asthma features. Earlier studies in a Brown Norway rat model, in which experimental animals were exposed to much higher (87 ppm) doses of NO₂, also demonstrated allergic sensitization and the promotion of a number of aspects of asthma-like disease (48). Concentrations of NO₂ used in rodent studies vary widely (0.2–170 ppm), as do the durations of exposure (1

h–28 days), whereas human studies have been performed using 2 ppm (49). Levels of NO₂ in areas heavily congested with roadway traffic can be as high as many hundred ppb (50), whereas the U.S. Occupational Safety and Health Administration ceiling limit for occupational exposure is 5 ppm. Our use of 10 ppm NO₂ for 1 h was chosen to represent an acute, moderate exposure to elicit minor lung damage without pain or distress.

Our data suggest that NO₂-promoted cellular damage induces the elaboration of immunostimulatory molecules that activate TLR2. A number of endogenous molecules that are normally sequestered intracellularly, preventing them from activating cells of the innate immune system, have been reported to stimulate TLR2. Exposure of these endogenous danger signals alerts the innate immune system of potential threats and promotes responses to clear damaging pathogens or damaged tissue (39). Importantly, TLR2 agonists can induce Th2 immune responses and promote experimental asthma (25–27). Intracellular components, including protein and LDH, are recovered in bronchiolar lavage fluid of mice exposed to NO₂, suggesting that the release of these or other endogenous molecules may be capable of stimulating pulmonary immune responses via TLR2. Although HSP70 was elevated in the BALF from NO₂-exposed mice and is known to the innate immune response via TLR2 (28, 29), we currently can only speculate about the identity of the critical NO₂-generated molecule(s) capable of promoting allergic sensitization. In addition, we cannot exclude the possibility that oxidation/nitration could affect cell membrane properties and the cellular localization of cell surface receptors. This process could generate ligands for specific receptors, initiating cell signaling within the lung or at distant sites.

Sensitization to an inhaled Ag requires the stimulation of naive CD4⁺ T cells in the tissue-draining lymph nodes. Pulmonary DCs are a specialized cell population that sample Ags in the airway and lung tissue, undergo maturation, migrate to the draining lymph nodes and present Ag in the context of MHCII, and express costimulatory molecules necessary for naive CD4⁺ T cell stimulation. Although numerous DCs are present in the lung of naive mice under normal conditions (51, 52), they do not initiate a stimulatory adaptive immune response following inhalation of purified Ag to which the animal has not been sensitized. One of the consequences of NO₂ exposure and NF- κ B activation in the airway epithelium could be the augmented expression of chemokines that promote the release of inflammatory cytokines or chemokines and the subsequent recruitment of inflammatory cells to the airway. Indeed, our previous studies implicate airway epithelial NF- κ B activation as an important contributor to pulmonary inflammation (41, 53). Whether expression of TLR2 and MyD88, or the activation of NF- κ B, are required in lung structural or hemopoietic-derived cells will require future study.

Environmental exposure to NO₂ may promote allergen sensitization, resulting in allergic airway disease in response to otherwise innocuous inhaled Ags, even when the inhalation of Ag occurs as much as several days following exposure to NO₂. Whether allergic sensitization can take place with even more time between the NO₂ and Ag exposures remains unknown. Our novel murine model of NO₂-induced allergic sensitization supports a causal role of NO₂ in the pathogenesis of asthma and provides a powerful tool to further define mechanisms underlying allergic sensitization.

Acknowledgment

We thank Dr. Mercedes Rincon (University of Vermont) for many helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References

- Shima, M., and M. Adachi. 2000. Effect of outdoor and indoor nitrogen dioxide on respiratory symptoms in schoolchildren. *Int. J. Epidemiol.* 29: 862–870.
- De Marco, R., A. Poli, M. Ferrari, S. Accordini, G. Giammanco, M. Bugiani, S. Villani, M. Ponzio, R. Bono, L. Carrozzi, et al. 2002. The impact of climate and traffic-related NO₂ on the prevalence of asthma and allergic rhinitis in Italy. *Clin. Exp. Allergy* 32: 1405–1412.
- Krishna, M. T., A. J. Chauhan, A. J. Frew, and S. T. Holgate. 1998. Toxicological mechanisms underlying oxidant pollutant-induced airway injury. *Rev. Environ. Health* 13: 59–71.
- Gaston, B., J. M. Drazen, J. Loscalzo, and J. S. Stamler. 1994. The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med.* 149: 538–551.
- McConnell, R., K. Berhane, F. Gilliland, S. J. London, H. Vora, E. Avol, W. J. Gauderman, H. G. Margolis, F. Lurmann, D. C. Thomas, and J. M. Peters. 1999. Air pollution and bronchitic symptoms in Southern California children with asthma. *Environ. Health Perspect.* 107: 757–760.
- Neas, L. M., D. W. Dockery, J. H. Ware, J. D. Spengler, F. E. Speizer, and B. G. Ferris. 1991. Association of indoor nitrogen dioxide with respiratory symptoms and pulmonary function in children. *Am. J. Epidemiol.* 134: 204–219.
- Strand, V., S. Rak, M. Svartengren, and G. Bylin. 1997. Nitrogen dioxide exposure enhances asthmatic reaction to inhaled allergen in subjects with asthma. *Am. J. Respir. Crit. Care Med.* 155: 881–887.
- Strand, V., M. Svartengren, S. Rak, C. Barck, and G. Bylin. 1998. Repeated exposure to an ambient level of NO₂ enhances asthmatic response to a nonsymptomatic allergen dose. *Eur. Respir. J.* 12: 6–12.
- Poynter, M. E., R. L. Persinger, C. G. Irvin, K. J. Butnor, H. van Hirtum, W. Blay, N. H. Heintz, J. Robbins, D. Hemenway, D. J. Taatjes, and Y. Janssen-Heininger. 2006. Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. *Am. J. Physiol.* 290: L144–L152.
- Gilmour, M. I. 1995. Interaction of air pollutants and pulmonary allergic responses in experimental animals. *Toxicology* 105: 335–342.
- Lindblad, E. B. 2004. Aluminium compounds for use in vaccines. *Immunol. Cell Biol.* 82: 497–505.
- Taube, C., A. Dakhama, and E. W. Gelfand. 2004. Insights into the pathogenesis of asthma utilizing murine models. *Int. Arch. Allergy Immunol.* 135: 173–186.
- Kips, J. C., G. P. Anderson, J. J. Fredberg, U. Herz, M. D. Inman, M. Jordana, D. M. Kemeny, J. Lotvall, R. A. Pauwels, C. G. Plopper, et al. 2003. Murine models of asthma. *Eur. Respir. J. Suppl.* 22: 374–382.
- Corry, D. B., G. Grunig, H. Hadeiba, V. P. Kurup, M. L. Warnock, D. Sheppard, D. M. Rennick, and R. M. Locksley. 1998. Requirements for allergen-induced airway hyperactivity in T and B cell-deficient mice. *Mol. Med.* 4: 344–355.
- Kawai, T., and S. Akira. 2005. Pathogen recognition with Toll-like receptors. *Curr. Opin. Immunol.* 17: 338–344.
- Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516–521.
- Li, M., D. F. Carpio, Y. Zheng, P. Bruzzo, V. Singh, F. Ouaz, R. M. Medzhitov, and A. A. Beg. 2001. An essential role of the NF- κ B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *J. Immunol.* 166: 7128–7135.
- Beg, A. A. 2002. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. *Trends Immunol.* 23: 509–512.
- Kiura, K., H. Kataoka, M. Yasuda, N. Inoue, and K. Shibata. 2006. The diacylated lipopeptide FSL-1 induces TLR2-mediated Th2 responses. *FEMS Immunol. Med. Microbiol.* 48: 44–55.
- Mandron, M., M. F. Aries, R. D. Brehm, H. S. Tranter, K. R. Acharya, M. Charveron, and C. Davrinche. 2006. Human dendritic cells conditioned with *Staphylococcus aureus* enterotoxin B promote TH2 cell polarization. *J. Allergy Clin. Immunol.* 117: 1141–1147.
- Zanin-Zhorov, A., R. Bruck, G. Tal, S. Oren, H. Aeed, R. Hershkovich, I. R. Cohen, and O. Lider. 2005. Heat shock protein 60 inhibits Th1-mediated hepatitis model via innate regulation of Th1/Th2 transcription factors and cytokines. *J. Immunol.* 174: 3227–3236.
- Agrawal, S., A. Agrawal, B. Dougherty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol.* 171: 4984–4989.
- Re, F., and J. L. Strominger. 2001. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem.* 276: 37692–37699.
- Re, F., and J. L. Strominger. 2004. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J. Immunol.* 173: 7548–7555.
- Yamamoto, K., I. Kawamura, J. Ito, and M. Mitsuyama. 2006. Modification of allergic inflammation in murine model of rhinitis by different bacterial ligands: involvement of mast cells and dendritic cells. *Clin. Exp. Allergy* 36: 760–769.
- Chisholm, D., L. Libet, T. Hayashi, and A. A. Horner. 2004. Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities. *J. Allergy Clin. Immunol.* 113: 448–454.
- Redecke, V., H. Hacker, S. K. Datta, A. Fermin, P. M. Pitha, D. H. Broide, and E. Raz. 2004. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J. Immunol.* 172: 2739–2743.
- Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and H. Wagner. 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J. Biol. Chem.* 277: 15107–15112.
- Asea, A., M. Rehli, E. Kabling, J. A. Boch, O. Bare, P. E. Auron, M. A. Stevenson, and S. K. Calderwood. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem.* 277: 15028–15034.
- Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker, and H. Wagner. 2001. Endocytosed HSP60s use Toll-like receptor 2 (TLR2) and TLR4 to activate the Toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem.* 276: 31332–31339.
- Park, J. S., D. Svetkauskaite, Q. He, J. Y. Kim, D. Strassheim, A. Ishizaka, and E. Abraham. 2004. Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J. Biol. Chem.* 279: 7370–7377.
- Park, J. S., F. Gamboni-Robertson, Q. He, D. Svetkauskaite, J. Y. Kim, D. Strassheim, J. W. Sohn, S. Yamada, I. Maruyama, A. Banerjee, et al. 2006. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am. J. Physiol.* 290: C917–C924.
- Yu, M., H. Wang, A. Ding, D. T. Golenbock, E. Latz, C. J. Czura, M. J. Fenton, K. J. Tracey, and H. Yang. 2006. HMGB1 signals through Toll-like receptor (TLR) 4 and TLR2. *Shock* 26: 174–179.
- Scheibner, K. A., M. A. Lutz, S. Boodoo, M. J. Fenton, J. D. Powell, and M. R. Horton. 2006. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J. Immunol.* 177: 1272–1281.
- Seong, S. Y., and P. Matzinger. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat. Rev. Immunol.* 4: 469–478.
- Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
- Kawai, T., and S. Akira. 2006. TLR signaling. *Cell Death Differ.* 13: 816–825.
- Ostroukhova, M., C. Seguin-Devaux, T. B. Oriss, B. Dixon-McCarthy, L. Yang, B. T. Amores, T. E. Corcoran, and A. Ray. 2004. Tolerance induced by inhaled antigen involves CD4⁺ T cells expressing membrane-bound TGF- β and FOXP3. *J. Clin. Invest.* 114: 28–38.
- Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296: 301–305.
- Njemini, R., M. Lambert, C. Demanet, and T. Mets. 2003. Elevated serum heat-shock protein 70 levels in patients with acute infection: use of an optimized enzyme-linked immunosorbent assay. *Scand. J. Immunol.* 58: 664–669.
- Poynter, M. E., R. Cloots, T. van Woerkom, K. J. Butnor, P. Vacek, D. J. Taatjes, C. G. Irvin, and Y. M. Janssen-Heininger. 2004. NF- κ B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J. Immunol.* 173: 7003–7009.
- Nakanishi, A., S. Morita, H. Iwashita, Y. Sagiya, Y. Ashida, H. Shirafuji, Y. Fujisawa, O. Nishimura, and M. Fujino. 2001. Role of *gob-5* in mucus overproduction and airway hyperresponsiveness in asthma. *Proc. Natl. Acad. Sci. USA* 98: 5175–5180.
- Allard, J. B., M. E. Poynter, K. A. Marr, L. Cohn, M. Rincon, and L. A. Whitaker. 2006. *Aspergillus fumigatus* generates an enhanced Th2-biased immune response in mice with defective cystic fibrosis transmembrane conductance regulator. *J. Immunol.* 177: 5186–5194.
- Pierre, P., S. J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R. M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 388: 787–792.
- Everard, M. L. 2006. The relationship between respiratory syncytial virus infections and the development of wheezing and asthma in children. *Curr. Opin. Allergy Clin. Immunol.* 6: 56–61.
- Franze, T., M. G. Weller, R. Niessner, and U. Poschl. 2005. Protein nitration by polluted air. *Environ. Sci. Technol.* 39: 1673–1678.
- Franze, T., M. G. Weller, R. Niessner, and U. Poschl. 2003. Enzyme immunoassays for the investigation of protein nitration by air pollutants. *Analyst* 128: 824–831.
- Siegel, P. D., N. H. Al-Humadi, E. R. Nelson, D. M. Lewis, and A. F. Hubbs. 1997. Adjuvant effect of respiratory irritation on pulmonary allergic sensitization: time and site dependency. *Toxicol. Appl. Pharmacol.* 144: 356–362.
- Pathmanathan, S., M. T. Krishna, A. Blomberg, R. Helleday, F. J. Kelly, T. Sandstrom, S. T. Holgate, S. J. Wilson, and A. J. Frew. 2003. Repeated daily exposure to 2 ppm nitrogen dioxide up-regulates the expression of IL-5, IL-10, IL-13, and ICAM-1 in the bronchial epithelium of healthy human airways. *Occup. Environ. Med.* 60: 892–896.
- Han, X., and L. P. Naehre. 2006. A review of traffic-related air pollution exposure assessment studies in the developing world. *Environ. Int.* 32: 106–120.
- Holt, P. G., S. Haining, D. J. Nelson, and J. D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153: 256–261.
- Gong, J. L., K. M. McCarthy, J. Telford, T. Tamatani, M. Miyasaka, and E. E. Schneeberger. 1992. Intraepithelial airway dendritic cells: a distinct subset of pulmonary dendritic cells obtained by microdissection. *J. Exp. Med.* 175: 797–807.
- Poynter, M., C. Irvin, and Y. Janssen-Heininger. 2003. A prominent role for airway epithelial NF- κ B activation in lipopolysaccharide-induced airway inflammation. *J. Immunol.* 170: 6257–6265.