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Uricase Inhibits Nitrogen Dioxide–Promoted Allergic Sensitization to Inhaled Ovalbumin Independent of Uric Acid Catabolism

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Nitrogen dioxide (NO₂) is an environmental air pollutant and endogenously generated oxidant that contributes to the exacerbation of respiratory disease and can function as an adjuvant to allergically sensitize to an innocuous inhaled Ag. Because uric acid has been implicated as a mediator of adjuvant activity, we sought to determine whether uric acid was elevated and participated in a mouse model of NO₂-promoted allergic sensitization. We found that uric acid was increased in the airways of mice exposed to NO₂ and that administration of uricase inhibited the development of OVA-driven allergic airway disease subsequent to OVA challenge, as well as the generation of OVA-specific Abs. However, uricase was itself immunogenic, inducing a uricase-specific adaptive immune response that occurred even when the enzymatic activity of uricase had been inactivated. Inhibition of the OVA-specific response was not due to the capacity of uricase to inhibit the early steps of OVA uptake or processing and presentation by dendritic cells, but occurred at a later step that blocked OVA-specific CD4⁺ T cell proliferation and cytokine production. Although blocking uric acid formation by allopurinol did not affect outcomes, administration of ultra-clean human serum albumin at protein concentrations equivalent to that of uricase inhibited NO₂-promoted allergic airway disease. These results indicate that, although uric acid levels are elevated in the airways of NO₂-exposed mice, the powerful inhibitory effect of uricase administration on allergic sensitization is mediated more through Ag-specific immune deviation than via suppression of allergic sensitization, a mechanism to be considered in the interpretation of results from other experimental systems. *The Journal of Immunology*, 2016, 197: 000–000.

Nitrogen dioxide (NO₂) is a byproduct of combustion, both indoor and outdoor, a component of air pollution, and has been linked to the development and severity of respiratory disease, including allergic asthma (1, 2). NO₂ can also be endogenously generated during infection (3, 4) and can be absorbed all along the respiratory tract. The inhalation and generation of reactive nitrogen species in the lung can lead to oxidative damage of the epithelium and airway acidification and, subsequently, impact the pulmonary immune response (5). Animal

models demonstrated that NO₂ exposure can contribute to airway epithelial damage, lung tissue fibrosis, and allergic sensitization, depending on the duration of exposure and dose of NO₂ provided (6–9). We demonstrated previously that a single 1-h 15-ppm dose of NO₂ activates airway epithelial NF-κB (8) and when administered just prior to aerosolized OVA Ag inhalation is sufficient to sensitize mice to OVA (8), resulting in a mixed Th2/Th17 response that requires TLR2 and MyD88 (8), is mediated through CD11c⁺ APCs (10), and requires caspase-1 and IL-1 signaling for the Th17 response (11, 12).

The necessity for caspase-1 activity and IL-1 signaling in our NO₂-promoted allergic sensitization model suggests some contribution of the NLRP3 inflammasome in the pulmonary response to inhaled NO₂ (12), a notion supported by the finding that IL-1α neutralization did not impede NO₂-promoted allergic sensitization (12). IL-1β is transcribed as an ~30-kDa precursor protein that typically requires caspase-1 cleavage to be secreted in its mature, active form (13). Caspase-1 activity is governed by inflammasome oligomerization, and the NLRP3 inflammasome was linked to Th2 and Th17 polarization in several allergic asthma models (14–16), although not in all (17).

Uric acid (UA) is the most abundant circulating antioxidant in humans and other mammals, accounting for half of the serum antioxidant capacity (18). Typically a byproduct of purine catabolism, UA is a small, heterocyclic compound that in its precipitated crystal form is the culprit behind gout (19), wherein it functions as a damage-associated molecular pattern (DAMP) to instigate proinflammatory responses. In gout, high concentrations of UA crystals activate the NLRP3 inflammasome, causing the production of IL-1β and attendant joint inflammation (20, 21). UA may also be generated locally by the enzyme xanthine

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Abbreviations used in this article: ACN, acetonitrile; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow–derived dendritic cell; DAMP, damage-associated molecular pattern; E, elastance; FA, formic acid; G, tissue damping; H, tissue resistance; HSA, human serum albumin; LCMS, liquid chromatography–mass spectrometry; NO₂, nitrogen dioxide; PEG, polyethylene glycol; R, resistance; R_N, Newtonian airway resistance; SRM, selected reaction monitoring; UA, uric acid; *Xdh*, xanthine dehydrogenase; Zrs, respiratory input impedance.

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dehydrogenase (*Xdh*), which is expressed by lavaged cells of house dust mite-exposed mice (22) and is transcriptionally regulated by NF- κ B (23). The clinically and experimentally used adjuvant, Alum, promotes Ag-specific adaptive immune responses, including those that are Th2 dominated and used to model allergic asthma (24–26) through the local production of UA (25), and also promotes the accumulation of UA in the lavageable airspaces that contributes to allergic sensitization and asthma exacerbation (24, 27). Given that NO₂ exposure can lead to cell and tissue damage, as well as that it can interact directly with UA (28), we hypothesized that UA may be present and functional subsequent to NO₂ inhalation, contributing to NO₂-promoted allergic sensitization.

Materials and Methods

Mice

Six- to eight-week-old female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female OTII-transgenic mice (C57BL/6 background; bred at the University of Vermont from stocks originally from The Jackson Laboratory) were 6–8 wk old at the beginning of experimentation. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care–approved facility, maintained on a 12-h light/dark cycle, and provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee. All mice were euthanized with sodium pentobarbital (150 mg/kg by i.p. injection; Wilcox Pharmacy, Rutland, VT).

In vivo NO₂ exposure and NO₂-promoted allergic sensitization and challenge

For NO₂ exposure, a single 1-h dose of 15 ppm NO₂ was administered (12), and mice were analyzed at several time points thereafter. Comparisons were made between mice exposed to NO₂ or subjected to time in a similar exposure chamber through which HEPA-filtered room air was flowed. For NO₂-promoted allergic sensitization, a single 1-h exposure to 15 ppm of NO₂ on day 1 was followed by 30 min of nebulized 1% OVA, Fraction V (Sigma-Aldrich, St. Louis, MO) in saline, on days 1, 2, and 3 (29). All mice were OVA challenged on days 14, 15, and 16, as described (29). Analyses were performed at 48 h after the final OVA challenge, on day 18.

Uricase, human serum albumin, and allopurinol doses and delivery methods

Recombinant *Candida* uricase produced in *Escherichia coli* was purchased from Sigma-Aldrich and delivered intranasally to isoflurane-anesthetized mice at 10 U/mouse in 40 μ l of sterile saline. For some studies, uricase was inactivated by exposing 40 μ l/tube of 250 U/ml solutions in sterile saline to 254-nm UV light generated by a UV cross-linker (Stratalinker 1800; Stratagene, San Diego, CA) at a distance of 18 cm for 180 min. Human serum albumin (HSA; RMBIO, Missoula, MT) was delivered intranasally to isoflurane-anesthetized mice at 2 mg/mouse (equivalent to the protein content of 10 U uricase) in 40 μ l of sterile saline. Allopurinol (Sigma-Aldrich) was freshly dissolved in saline at 2.5 mg/ml and delivered s.c. at 25 mg/kg (30) 1 h before and 6 and 24 h after NO₂ inhalation, as well as 48 h after NO₂ inhalation in the allergic airway disease study.

Assessment of airway responsiveness to methacholine

Mice were anesthetized i.p. with sodium pentobarbital (90 mg/kg), the trachea was cannulated, and the mice were connected to a flexiVent computer-controlled small animal ventilator (SCIREQ, Montreal, QC, Canada) and ventilated at 200 breaths/min with a 0.25-ml tidal volume. Next, the mice were paralyzed with an i.p. injection of pancuronium bromide (0.8 μ g/kg). The animals were stabilized over ~10 min of regular ventilation at a positive end-expiratory pressure of 3 cm of H₂O. A standard lung volume history was established by delivering two total lung capacity maneuvers to a pressure limit of 25 cm of H₂O and holding for 3 s. Next, two baseline measurements of respiratory input impedance (Zrs) were obtained, followed by inhalation of aerosolized PBS (control) for 10 s, achieved by an in-line piezoelectric nebulizer (Aeroneb; Aerogen, Galway, Ireland). Zrs was measured every 10 s for 3 min (18 measurements in total). This complete sequence of maneuvers and measurements was repeated for aerosol exposures to four ascending doses of aerosolized methacholine (12.5, 25, 50, and 100 mg/ml). Data were fit to the single-compartment model (7) to provide values for resistance (R), reflecting constriction in the lungs, and elastance (E), reflecting the elastic rigidity of

the lungs. Data were also fit to the constant phase model to provide values reflecting Newtonian airway resistance (R_N), tissue damping (G), and tissue resistance (H). R, E, R_N, G, and H were calculated for each mouse by averaging three measurements at each methacholine dose: the peak value and those immediately preceding and following it. The average values ($\pm$ SEM) in each of the mouse groups, at each incremental methacholine dose, are reported.

Bronchoalveolar lavage fluid collection and processing

Lungs were lavaged with 1 ml Dulbecco's PBS (Sigma-Aldrich). Bronchoalveolar lavage fluid (BALF) was centrifuged at 400 $\times$ g, the supernatant was collected and frozen, and the total cells in the pellet were resuspended in PBS and enumerated by counting with an ADVIA 120 Hematology System (Bayer HealthCare, Leverkusen, Germany). Differential analysis was performed by cytospin and H&E stain from ~200 cells/slide.

In vitro Ag restimulation and cytokine quantitation

Following mechanical disruption of the spleen through a 70- μ m mesh filter, splenocytes were isolated using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH), as previously described (31). Cells were counted with an ADVIA 120 Hematology System, and 4 $\times$ 10⁶ cells/ml were cultured in RPMI 1640 supplemented with 10% FBS (Cell Generation, Fort Collins, CO), penicillin/streptomycin, L-glutamine, folic acid, and 2-ME and treated with 200 μ g/ml OVA, UV-inactivated uricase, or HSA. Supernatants were collected after 96 h of incubation at 37°C in 5% CO₂. Analysis of cytokine content from cell supernatants was performed using ELISA kits for IL-5, IL-13, IL-17A, and IFN- γ (R&D Systems, Minneapolis, MN).

Quantitative RT-PCR

Total RNA was extracted from snap-frozen whole lungs or the single large lung lobe using a PrepEase RNA isolation kit (USB, Cleveland, OH) and reverse transcribed to cDNA using an iScript Kit (Bio-Rad, Hercules, CA). Primers were designed for mouse *Xdh* (5'-AGGGGATTCGGACCTTTG-3' and 5'-GCAGCAGTTTGGGTTGTTTC-3'), *Muc5ac* (5'-CCATGCA-GAGTCTCAGAACA-3' and 5'-TTACTGGAAGGCCCAAGCA-3'), *Cxcl1* (5'-AAGCCAACCACTCCCATGAC-3' and 5'-TGCGAAAGCAT-CAACAACAC-3'), *Csf3* (5'-GAGCAGTTGTGTGCCACCTA-3' and 5'-GCTCAGGTCTAGGCCAAGTG-3'), *Ccl20* (5'-CGTCGTCTCTTCCTT-GCTTT-3' and 5'-TTGACAAGTCCACTGGGACA-3'), and *Saa3* (5'-CAGGATGAAGCCTTCCATTG-3' and 5'-CATGACTGGGAACAACA-GGA-3') using the National Center for Biotechnology Information's Primer-BLAST and synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative RT-PCR was performed using SYBR Green Supermix (Bio-Rad) and normalized to *Gapdh* (5'-ACGACCCCTTCATTGACCTC-3' and 5'-TTCACACCCATCACAACAT-3') or *Actb* (5'-TCCTTCGTTGCCGG-TCCACA-3' and 5'-CGTCTCCGGAGTCCATCACA-3') using the $\Delta\Delta C_T$ method, as previously described (32).

Uricase enzyme activity and UA quantitation by Amplex Red assay

Uricase activity and UA content were measured using an assay kit from Molecular Probes (Eugene, OR), according to the manufacturer's instructions.

UA quantitation by liquid chromatography–mass spectrometry

UA (purity >99%) was purchased from Sigma Aldrich. [1,3-¹⁵N₂]-UA (purity >98%), which was used as the internal standard, was purchased from Cambridge Isotope Laboratories (Andover, MA). Acetonitrile (ACN), formic acid (FA), KOH, and methanol were obtained from Fisher Scientific (Pittsburgh, PA). A stock solution of UA was prepared at a concentration of 1.8 mM in 0.3 M KOH. Solutions of various dilutions (20, 10, 5, 1, 0.5, and 0 μ M) were prepared in ACN/H₂O (1:1). The internal standard ¹⁵N₂-UA was prepared at a concentration of 2.0 mM in 0.3 M KOH and diluted in ACN/H₂O (1:1) to a concentration of 15 μ M. All solutions were kept at –20°C prior to analysis. Equal volumes (250 μ l) of UA and ¹⁵N₂-UA standards solutions were mixed for preparing the calibration curves for concentration calculation of UA in the unknown samples. A 250- μ l aliquot of biological fluid was mixed with 250 μ l of 15 μ M ¹⁵N₂-UA. A total of 500 μ l of ACN was added, vortexed for 1 min, and centrifuged for 15 min at 4500 $\times$ g for protein precipitation. The supernatant was removed and dried in a new vial using a SpeedVac concentrator (Savant; Thermo Scientific, Waltham, MA). The dried samples were dissolved in 100 μ l ACN/H₂O (1:1) for liquid chromatography–mass spectrometry (LCMS) analysis. LCMS analyses were carried out with a Shimadzu HPLC (Shimadzu, Kyoto, Japan) connected to an ABI 4000 QTRAP triple quadrupole mass

spectrometer (AB SCIEX, Redwood City, CA) equipped with an electrospray ionization interface. The operation of the LCMS and data analysis were performed using AB SCIEX Analyst Software. The second quadrupole was operated in a collision-induced dissociation tandem mass spectrometry mode for performing data collection by selected reaction monitoring (SRM). Liquid chromatography analyses were performed using a Hypersil GOLD HILIC column (100×1 mm, $1.9 \mu\text{m}$ particles; Thermo Scientific). The mobile phase A was $\text{MeOH-H}_2\text{O}$ (3:1) containing 0.1% FA; mobile phase B was ACN containing 0.1% FA (B). The mobile phase flow was $80 \mu\text{l/min}$, and the injection volume was $1 \mu\text{l}$. The HPLC gradient began at 25% A and was linearly ramped to 50% A over the next 15 min, held for 1 min, and then reversed to the original composition of 25% A over 5 min, after which it was kept constant for 10 min to re-equilibrate the column. LCMS measurements were performed via SRM using negative electrospray ionization mode. The m/z ion selection was 167/124 for UA and 169/125 for $^{15}\text{N}_2\text{-UA}$. The declustering potential and collision energies were -65 V and -22 eV, respectively. Peak areas were integrated for the UA and $^{15}\text{N}_2\text{-UA}$ SRM peaks, and a standard curve was produced for the SRM area ratio of (UA 167/124)/($^{15}\text{N}_2\text{-UA}$ 169/125). Using this calibration curve, the UA content was calculated from each sample's measured (UA 167/124)/($^{15}\text{N}_2\text{-UA}$ 169/125) SRM area ratio.

Serum collection and Ig analysis

Following euthanasia, $\sim 300 \mu\text{l}$ of blood was collected via cardiac puncture of the right ventricle using a 25-g needle attached to a 1-ml syringe, transferred into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ), and centrifuged; serum was kept frozen at -80°C . For IgG1 and IgG2c, indirect ELISAs were performed. Ninety-six-well plates were coated overnight at 4°C with $2 \mu\text{g/ml}$ OVA, UV-inactivated uricase, or HSA 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 serially diluted in blocking solution was applied to the wells in triplicate and incubated overnight at 4°C . Plates were washed and $2 \mu\text{g/ml}$ biotinylated anti-mouse IgG1 or IgG2c secondary Abs (Pharmingen) in 1% BSA/PBS were incubated in the plates at room temperature for 1 h. Plates were washed, and 0.05 U/ml streptavidin/peroxidase (Roche) was incubated in the plates at room temperature for 1 h. Plates were washed, developed using reagents from R&D Systems, and stopped with 1 N H_2SO_4 , and optical densities were read using a BioTek Instruments PowerWave (Winooski, VT) at 450 nm, with background subtraction at 570 nm.

Dendritic cell cultures

The mouse DC2.4 cell line (33) was kindly provided by Dr. Brent Berwin (Dartmouth College). For the analysis of OVA uptake and processing, DC2.4 cells, plated at 1×10^6 cells/ml, were incubated with $10 \mu\text{g/ml}$ Alexa Fluor 488–conjugated OVA or DQ-OVA (Molecular Probes), respectively, along with 0, 10, 50, or $250 \mu\text{g/ml}$ UV-inactivated uricase and analyzed on an LSR II FACS flow cytometer (BD Biosciences, San Jose, CA) at the specified times. Data were analyzed using FlowJo software (Tree Star, Ashland, OR). For analysis of surface displacability of Ag in MHC class II, DC2.4 cells were cultured with $10 \mu\text{g/ml}$ OVA and 0, 10, 50, or $250 \mu\text{g/ml}$ UV-inactivated uricase for 24 h, washed three times with cold PBS, and treated with $33 \mu\text{M}$ FITC-conjugated OVA_{323–339} peptide (AnaSpec, Fremont, CA) in PBS for 6 h at 4°C ; FITC fluorescence was measured using a BioTek Instruments Synergy HTX plate reader.

Flow cytometry analysis of CD4^+ T cell proliferation

For the generation of bone marrow–derived dendritic cells (BMDCs), bone marrow was flushed from the femurs and tibiae of C57BL/6J mice and cultured on six-well plates at 1×10^6 cells/well (3 ml/well) in RPMI 1640 containing 10% serum and 5% conditioned media from X63-GMCSF myeloma cells transfected with murine GM-CSF cDNA (kindly provided by Dr. Brent Berwin). Medium was replaced on days 2 and 4, and the adherent and lightly adherent BMDCs, predominantly $\text{CD11b}^+\text{CD11c}^+$, were collected on day 6. CD4^+ T cells were negatively selected from the spleens and peripheral lymph nodes of OTII TCR-transgenic mice using the CD4 isolation kit, according to the manufacturer's instructions (STEMCELL Technologies, Vancouver, BC, Canada). Transgenic CD4^+ T cells were labeled with CFSE, according to the manufacturer's protocol (Molecular Probes), washed, and incubated at 1×10^6 cells/ml along with BMDCs plated at 1×10^5 cells/ml, $10 \mu\text{g/ml}$ OVA, and 0, 10, 50, or $250 \mu\text{g/ml}$ UV-inactivated uricase for 72 h. The loosely adherent T cells were harvested and analyzed by flow cytometry.

J774 cell cultures

J774 macrophages, purchased from the American Type Culture Collection (Manassas, VA), were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 1% L-gluta-

mine (Life Technologies), and $1 \times$ Primocin (InvivoGen, San Diego, CA). For experiments, cells were plated at 2.5×10^5 cells/well in $250 \mu\text{l}$ of medium in a 48-well plate and allowed to grow overnight. The following day, the medium was removed, fresh medium was added, and cells were treated for 18 h with the indicated agonists. Cell supernatants were harvested, spun down at $3300 \times g$ for 10 min to pellet cellular debris, transferred to new tubes, and analyzed for TNF content by ELISA (BD Biosciences).

Statistical analyses

Data were analyzed by the two-tailed unpaired t test, one-way ANOVA, or two-way ANOVA followed by the Bonferroni post hoc test using GraphPad Prism 6 for Windows (GraphPad). Data are presented as mean $\pm$ SEM. A p value <0.05 was considered statistically significant.

Results

NO_2 exposure induces UA

We exposed female C57BL/6 mice to 1 h of 15-ppm NO_2 and measured UA 2, 24, or 48 h later using an Amplex Red–based enzymatic assay system. By 24 h, UA was elevated in the lavageable airspace (BALF), and this secretion persisted at 48 h (Fig. 1A). An additional study was performed to measure BALF levels of UA by the more direct and specific LCMS method. Mice were again exposed to 1 h of 15-ppm NO_2 and analyzed 6, 20, 48, or 68 h later. Again, UA levels in the lavageable airspaces continued to increase over time following NO_2 exposure (Fig. 1B). UA can be generated by the enzyme *Xdh*, and other investigators reported that *Xdh* levels correspond to UA release (22). However, our analysis of gene expression from the lungs of mice exposed to NO_2 and analyzed 2, 24, or 48 h after exposure indicated no significant changes in *Xdh* expression (Fig. 1C), suggesting that lung damage induced as a consequence of NO_2 exposure (34) causes leakage of UA-containing plasma into the lavageable airspaces rather than the increases in UA coming about as a consequence of local production.

Uricase treatment inhibits NO_2 -induced allergic sensitization

Having established that NO_2 induced the robust release of UA into the BALF, we sought to determine whether this DAMP played a mechanistic role in our model of NO_2 -promoted allergic sensitization. Mice were sensitized and challenged according to the scheme shown in Fig. 2A. Briefly, after a single exposure to 15-ppm NO_2 for 1 h, mice were administered 1% w/v OVA for 30 min for 3 d. Some groups received 10 U of intranasal uricase, as used by other investigators (22), on all 3 d of the sensitization phase. Two weeks later, all mice were challenged with nebulized OVA for 3 d, and mice were analyzed 48 h later. Indicative of having developed asthma-like disease, mice sensitized via NO_2 and OVA (NO_2/OVA) displayed increased lung R and E in response to inhaled methacholine challenge (methacholine responses from naive control mice are depicted by the horizontal lines), whereas mice subjected to the NO_2/OVA protocol and administered inhaled uricase during sensitization did not (Fig. 2B). Similar results were seen for R_N , G, and H (Supplemental Fig. 1A). Furthermore, NO_2/OVA -sensitized mice developed a mixed Th2/Th17 BALF inflammatory profile, as indicated by the presence of eosinophils and neutrophils (Fig. 2C). In contrast, mice that received uricase during their NO_2/OVA sensitization displayed significantly reduced levels of BALF eosinophils and neutrophils. When gene-expression levels from the lungs of these mice were compared, uricase treatment diminished the expression of the mucin gene *Muc5ac* and the chemokines *Cxcl1* and *Ccl20*, whereas levels of the neutrophil-maturation cytokine *Csf3* were unchanged (Fig. 2D). When restimulated in vitro with OVA, splenocytes from NO_2/OVA -sensitized mice released IL-13 and IL-17A into the cell medium, levels of which were significantly decreased from cells of mice that received uricase, whereas the low levels of IFN- γ were unaffected by uricase treatment (Fig. 2E). Finally, mice treated with

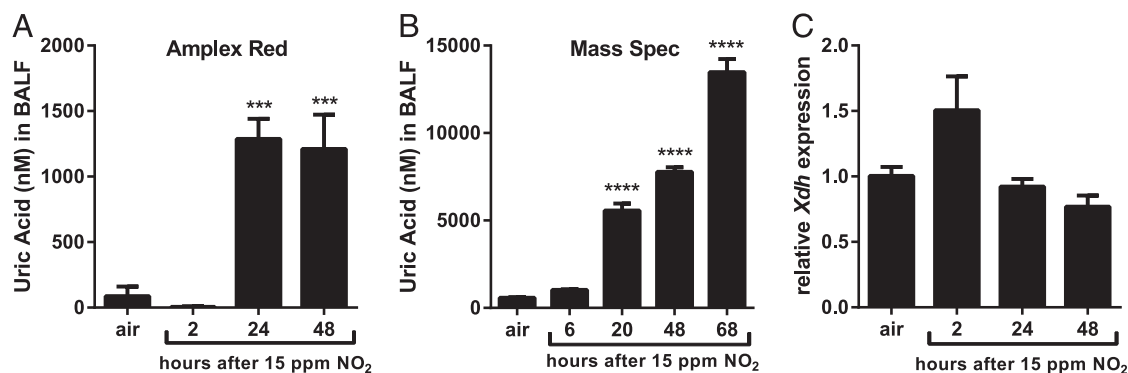


FIGURE 1. NO₂ exposure induces UA accumulation in the lung. Mice were exposed to room air or 15 ppm NO₂ for 1 h and were analyzed for UA content in BALF by Amplex Red (A) or LCMS (B) assays or were analyzed for *Xdh* gene expression in the lungs by quantitative RT-PCR (C), at several times thereafter ($n = 3-4$ mice per group). Data are representative of experiments performed twice. *** $p \leq 0.001$, **** $p \leq 0.0001$ versus air.

uricase during sensitization did not develop substantial titers of OVA-specific IgG1 in the serum compared with the robust levels from NO₂/OVA-sensitized mice (Fig. 2F). Overall, it appeared that uricase treatment during sensitization was sufficient to abrogate the parameters of Th2 and Th17 disease associated with our NO₂/OVA model. However, contrary to our expectations, we measured the presence of a substantial anti-uricase IgG1 response in those mice subjected to uricase treatment (Fig. 2G), suggesting that the uricase itself was immunogenic or that the in vivo NO₂ exposure was creating an environment conducive to generating an anti-uricase immune response.

Uricase instillation induces acute pulmonary inflammation

It was unclear from our data whether uricase was inhibiting allergic sensitization by removing UA that was generated in response to NO₂ or by initiating a specific immune reaction that would overshadow the response to OVA. Therefore, we tested whether a single dose of uricase administered to mice provoked an innate immune response in the lung. Uricase-treated mice, analyzed 24 h after administration, displayed neutrophilia in the BALF (Fig. 3A), as well as robust increases in the lung gene expression of *Cxcl1*, *Csf3*, and *Saa3* (Fig. 3B), indicative of an acute-phase and proinflammatory response. We next sought to determine whether enzymatic activity of uricase was responsible for its proinflammatory activity. Although boiling promoted the aggregation of uricase into clumps unsuitable for delivery to the lung, exposure to UV light significantly and substantially decreased uricase activity (Fig. 3C), without causing clumping. When active uricase or UV-inactivated uricase was subsequently administered via intranasal instillation into mice and inflammation was measured 24 h later, there was an absence of neutrophils in those animals that received UV-inactivated uricase (Fig. 3D). Furthermore, lung expression of *Cxcl1*, *Csf3*, and *Saa3* was increased only in response to the active enzyme (Fig. 3E).

UV-inactivated uricase inhibits NO₂-promoted allergic sensitization

Having determined that intranasal instillation of UV-inactivated uricase did not induce an innate proinflammatory response in the lung in comparison with the active enzyme, we sought to determine whether only active uricase could inhibit NO₂-promoted allergic sensitization. Accordingly, mice were subjected to NO₂ and OVA inhalation, either alone or accompanied by the administration of uricase or UV-inactivated uricase (Fig. 4A). Naive mice were used as negative controls. NO₂/OVA-promoted allergic sensitization resulted in robust eosinophilia and neutrophilia in the lavageable airspaces that was abrogated by treatment with uricase or UV-inactivated uricase (Fig. 4B). Similarly, the methacholine hyperresponsiveness displayed by mice subjected to the NO₂/OVA protocol was abrogated by the

administration of uricase or UV-inactivated uricase (Fig. 4C, Supplemental Fig. 1B). In addition, uricase activity was not required for the elimination of IL-5, IL-13, and IL-17A secretion from splenocytes restimulated with OVA (Fig. 4D). However, uricase and UV-inactivated uricase treatment led to the release of IL-5, IL-13, and IL-17A from splenocytes restimulated with UV-inactivated uricase (Fig. 4E). Likewise, uricase and UV-inactivated uricase capably decreased OVA-specific IgG1 and IgG2c levels in serum (Fig. 4F), while driving the production of uricase-specific Igs (Fig. 4G).

Uricase itself elicits an Ag-specific adaptive immune response

Having established that uricase skews the cytokine and Ig responses in our model of NO₂-promoted allergic asthma and that this effect is independent of enzyme activity, we explored the possibility that uricase itself can serve as an Ag against which mice will develop allergic responses. One group of mice was sensitized with the NO₂/OVA regimen and received UV-inactivated uricase during the sensitization period. Another group of mice was administered uricase on days 0, 1, and 2 without being subjected to NO₂ or OVA inhalations during the sensitization period. These two groups of mice were challenged 2 wk later with UV-inactivated uricase, instead of OVA (Fig. 5A), and were compared with naive mice never exposed to any of the inhaled agents. All sensitized mice developed asthma-like disease after challenge with UV-inactivated uricase, with eosinophilia and, especially, robust neutrophilia in BALF compared with naive controls (Fig. 5B). Additionally, splenocytes from sensitized mice restimulated with UV-inactivated uricase secreted IL-5, IL-13, IL-17A, and IFN- γ (Fig. 5C). Analysis of serum Igs revealed only very low levels of IgG1 specific for OVA (Fig. 5D) but pronounced uricase-specific IgG1 and IgG2c production in mice sensitized and challenged with either uricase-exposure regimen (Fig. 5E). Interestingly, most of the measured responses were indistinguishable in the mice exposed to the NO₂/OVA + UV-inactivated uricase or uricase-sensitization regimens and challenged with UV-inactivated uricase, suggesting that uricase itself functions as a potent immunogen in the setting of inhalational exposure, a situation that may override its UA-catabolizing activity to inhibit NO₂-promoted allergic sensitization to inhaled OVA.

UV-inactivated uricase does not inhibit the uptake, processing, or display of OVA Ag by dendritic cells, but it does impede OVA-specific CD4⁺ T cell proliferation and cytokine production

DC2.4 mouse dendritic cells were treated with Alexa Fluor 488-labeled OVA or DQ-OVA and increasing concentrations of UV-inactivated uricase. At 2, 4, 7, and 24 h, cells were collected, washed, and analyzed by flow cytometry for Alexa Fluor 488

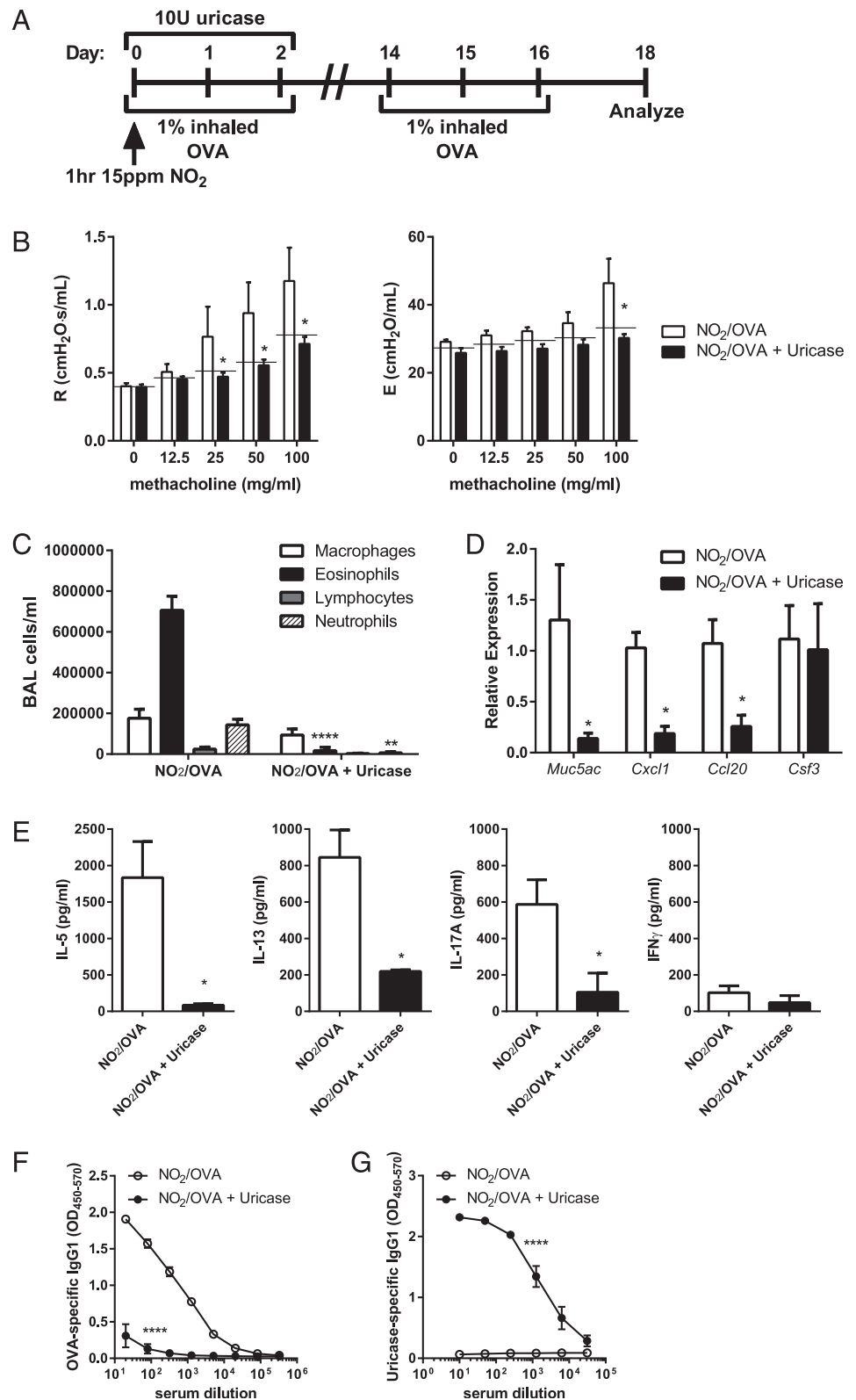


FIGURE 2. Uricase treatment inhibits NO₂-promoted allergic airway disease. Mice were subjected to NO₂-promoted allergic sensitization and administered saline or 10 U of uricase by intranasal instillation. Mice were challenged with three daily doses of 1% inhaled OVA and analyzed 48 h later (**A**) for methacholine responsiveness [(**B**) responsiveness of naive mice is indicated by horizontal lines], inflammatory cells in BALF (**C**), gene expression in the lungs (**D**), cytokine production from OVA-restimulated splenocytes (**E**), and OVA-specific (**F**) or uricase-specific (**G**) IgG1 in serum ($n = 8$ mice per group). Data are representative of experiments performed twice. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ versus NO₂/OVA.

fluorescence, indicating uptake of labeled Ag, or BODIPY fluorescence, indicating proteolysis during Ag processing that separated the quencher from the fluorescent dye. Treatment with UV-inactivated uricase, even at concentrations 25 times that of OVA, which is analogous to the relative levels in the airways when uricase is administered as an inhibitor of NO₂-promoted allergic sensitization, had no significant effects on OVA Ag uptake (Fig. 6A) or processing (Fig. 6B). In addition, when DC2.4 cells were cultured with OVA

and increasing concentrations of UV-inactivated uricase for 24 h and incubated at 4°C with FITC-conjugated OVA₃₂₃₋₃₃₉ peptide for 6 h to displace peptide presented on the cell surface by MHC class II molecules (35), uricase had no significant effect on surface fluorescence (Fig. 6C), supporting the notion that uricase does not affect the relative affinity of MHC class II for peptide. However, coculture experiments with BMDCs incubated with OVA and increasing concentrations of UV-inactivated uricase, along with CFSE-labeled

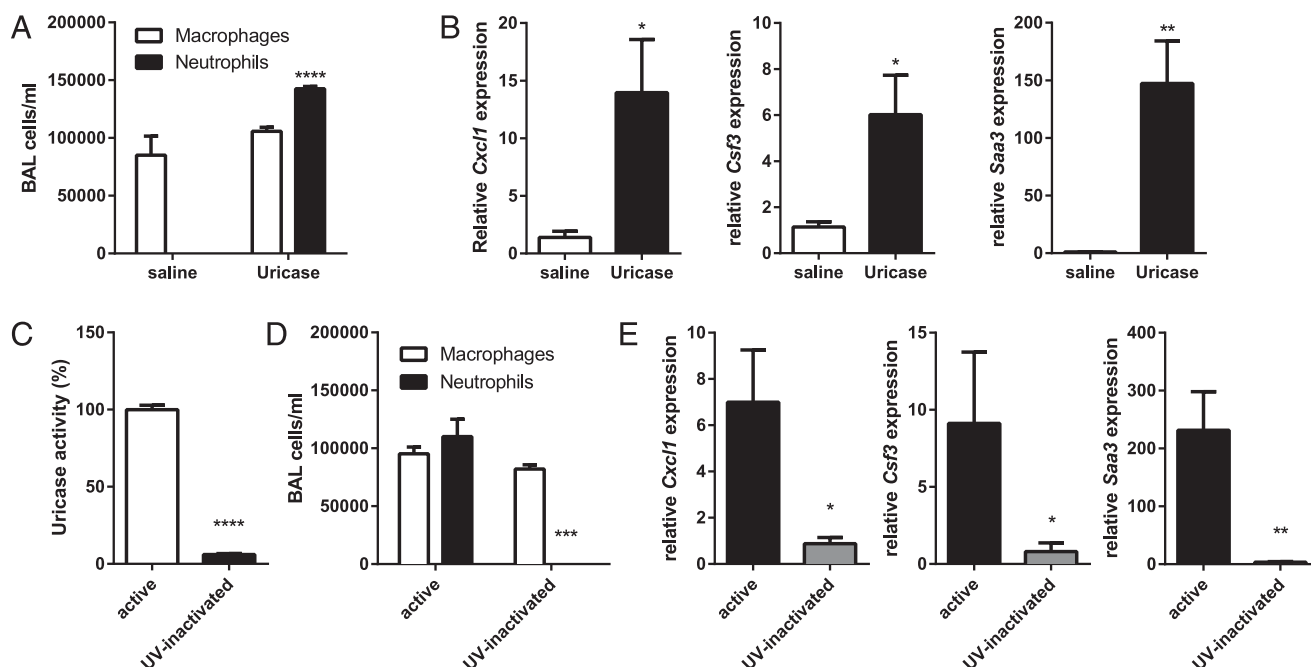


FIGURE 3. Uricase enzyme activity exerts proinflammatory effects in the lung. Mice were administered saline or 10 U of uricase by intranasal instillation and evaluated for inflammatory cells in BALF (A) or gene expression in the lungs (B) 24 h later. (C) Triplicate samples of uricase were left untreated or exposed to UV light, after which enzyme activity was measured by Amplex Red assay. Female C57BL/6J mice were administered 10 U of uricase or UV-inactivated uricase by intranasal instillation and evaluated for inflammatory cells in BALF (D) or gene expression in the lungs (E) 24 h later. $n = 7-8$ mice per group. Data are representative of experiments performed twice. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ versus saline (A and B) and versus active uricase (C-E).

OVA peptide-specific OTII CD4⁺ T cells, revealed a profound effect of UV-inactivated uricase on T cell proliferation (Fig. 6D). Furthermore, secretion of the Th2 cytokines IL-13 and IL-5, as well as secretion of IL-17A, from these CD4⁺ T cells was also repressed with increasing concentrations of UV-inactivated uricase relative to OVA, indicating a failure of the OVA-specific CD4⁺ T cell response in the presence of uricase lacking enzymatic activity (Fig. 6E).

Allopurinol does not inhibit NO₂-promoted allergic sensitization

We observed that NO₂ exposure causes the accumulation of UA in the lavageable airspaces and that uricase treatment prevents Ag challenge-induced asthma-like disease, although perhaps through a mechanism distinct from its enzymatic activity. Therefore, we examined whether inhibiting the formation of UA through the administration of allopurinol would diminish circulating UA levels and its accumulation in BALF following NO₂ exposure. Allopurinol administration before and after a 1-h exposure to 15 ppm NO₂ (Fig. 7A) decreased serum UA levels (Fig. 7B) and diminished by one half the levels of UA in the lavageable airspaces 48 h following NO₂ exposure (Fig. 7C). Having established the capacity of allopurinol to affect circulating and NO₂-induced UA accumulation, we next administered allopurinol to test whether UA is indeed a mediator of NO₂-promoted allergic sensitization. Mice underwent NO₂/OVA sensitization, during which they were treated s.c. with vehicle (saline) or 25 mg/kg allopurinol (Fig. 7D). Following OVA challenge, all mice developed asthma-like disease. Allopurinol treatment had no impact on inflammatory cell numbers or types recruited to the lavageable airspaces (Fig. 7E), the levels of IL-5, IL-13, IL-17A, or IFN- γ produced by splenocytes restimulated with OVA (Fig. 7F), or the quantities of OVA-specific IgG1 or IgG2c in the serum (Fig. 7G). These results indicate that the capacity of uricase to diminish OVA-specific immune responses in our NO₂-promoted allergic disease model is not a consequence of decreasing UA levels.

An excess of clean Ag inhibits NO₂-promoted allergic sensitization

Because enzymatic activity was not responsible for the inhibitory effect of administering uricase to the lung during NO₂-promoted allergic sensitization, and despite the demonstration that UV exposure renders uricase noninflammatory in vivo, the fact that uricase is a *Candida*-derived protein generated in *E. coli* meant that bacterial contaminants may participate in skewing the adaptive immune response upon administration. Therefore, we sought to use a very clean protein to determine whether administering similar concentrations of an innocuous protein would mitigate the OVA-specific immune response in a manner similar to that of uricase or UV-inactivated uricase. If so, the effect would be due to the large amount of "inhibitory" protein administered during NO₂-promoted allergic sensitization rather than enzymatic activity or contaminants. First, we used J774 macrophage cells to determine their response to uricase, UV-inactivated uricase, and a preparation of HSA (intended for use in pharmaceutical manufacturing). In comparison with 100 ng/ml LPS, 50 μ g/ml uricase or 50 μ g/ml UV-inactivated uricase elicited low, but significant, production of TNF from J774 cells, whereas 50 μ g/ml HSA elicited no detectable levels of TNF, similar to unstimulated cells (Fig. 8A). We next sought to determine whether HSA could inhibit NO₂-promoted allergic sensitization. Accordingly, mice were subjected to NO₂ and OVA inhalation, accompanied by the administration of saline or an amount of HSA protein equivalent to the protein content of uricase or UV-inactivated uricase used in previous experiments (Fig. 8B). Mice administered HSA displayed reduced methacholine responsiveness, similar to the levels from naive mice (horizontal lines), compared with the mice exposed to saline vehicle along with NO₂ and OVA (Fig. 8C, Supplemental Fig. 1C). Furthermore, although NO₂/OVA-promoted allergic sensitization resulted in robust eosinophilia and neutrophilia in the lavageable

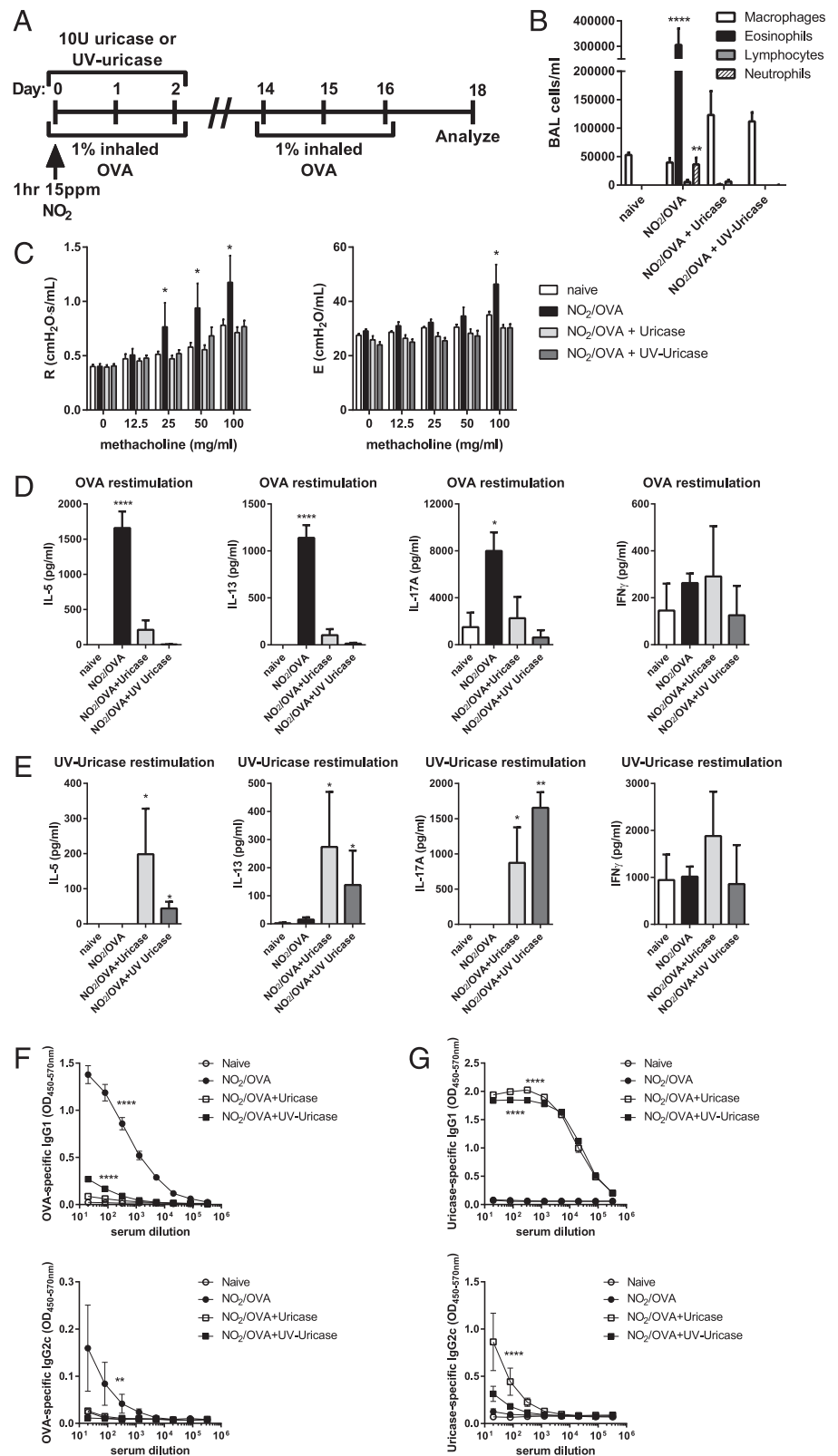


FIGURE 4. Enzymatically inactive uricase treatment inhibits NO₂-promoted allergic airway disease. Mice were left untreated (naive) or subjected to NO₂-promoted allergic sensitization and were left untreated or administered 10 U of uricase or UV-inactivated uricase by intranasal instillation. Mice were challenged with three daily doses of 1% inhaled OVA and analyzed 48 h later (**A**) for inflammatory cells in BALF (**B**), methacholine responsiveness (**C**), cytokine production from OVA-restimulated (**D**) and UV-inactivated uricase-stimulated splenocytes (**E**), and OVA-specific (**F**) or uricase-specific (**G**) IgG1 and IgG2c in serum ($n = 8$ mice per group). Data are representative of experiments performed twice. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$ versus naive.

airspaces, these responses were significantly decreased by HSA treatment (Fig. 8D). Additionally, HSA administration inhibited IL-5, IL-13, and IL-17A secretion from splenocytes restimulated with OVA (Fig. 8E) but did not induce HSA-specific cytokine production (Fig. 8F). However, although HSA significantly decreased OVA-specific serum IgG1 levels (Fig. 8G), it also promoted the production of HSA-specific IgG1 (Fig. 8H), indicating

that an anti-HSA response was generated in vivo. These results support the conclusion that the administration of large amounts of clean Ag during NO₂-promoted allergic sensitization is sufficient to skew the response away from the OVA Ag to which the mice were initially sensitized, manifesting in substantially blunted OVA-specific recall responses upon challenge and diminished OVA-driven allergic airway disease.

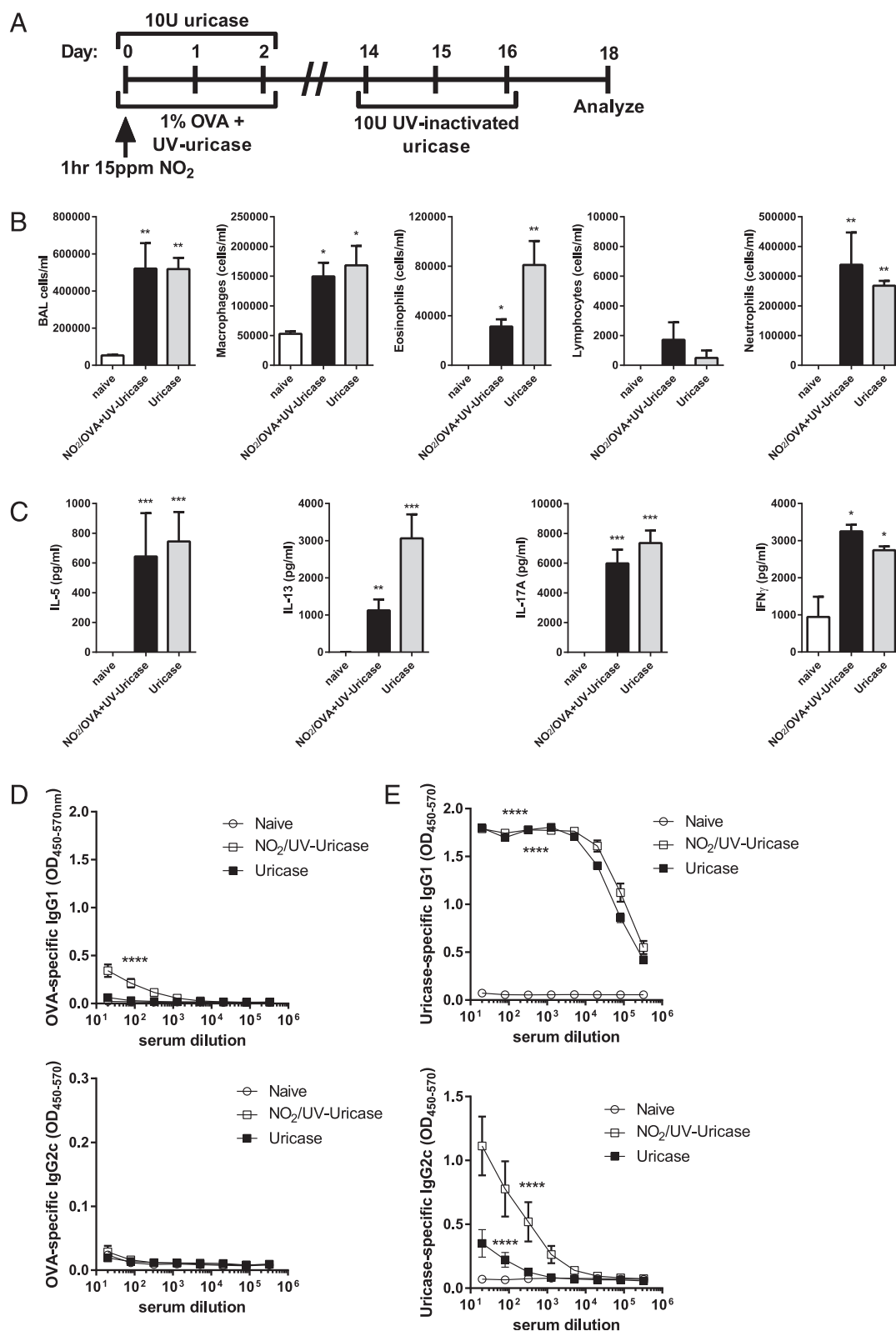


FIGURE 5. Uricease inhalation promotes a uricase-specific immune response. Mice were subjected to NO₂-promoted allergic sensitization and administered 10 U of UV-inactivated uricase by intranasal instillation or were administered 10 U of uricase by intranasal instillation without exposure to NO₂ or OVA. Mice were challenged with three daily doses of 10 U of UV-inactivated uricase and analyzed 48 h later (**A**) for inflammatory cells in BALF (**B**), cytokine production from UV-inactivated uricase-stimulated splenocytes (**C**), and OVA-specific (**D**) or uricase-specific (**E**) IgG1 and IgG2c in serum ($n = 4-8$ mice per group). Data are representative of experiments performed twice. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ versus naive.

Discussion

As an air pollutant and an endogenously generated oxidant, the detrimental effects of NO₂ on respiratory disease include induction

of acute lung injury, impairment of innate immune defenses, silo-fillers disease, induction of allergic responses, and exacerbation of asthma (36). Because avoiding the persistent levels of NO₂ present

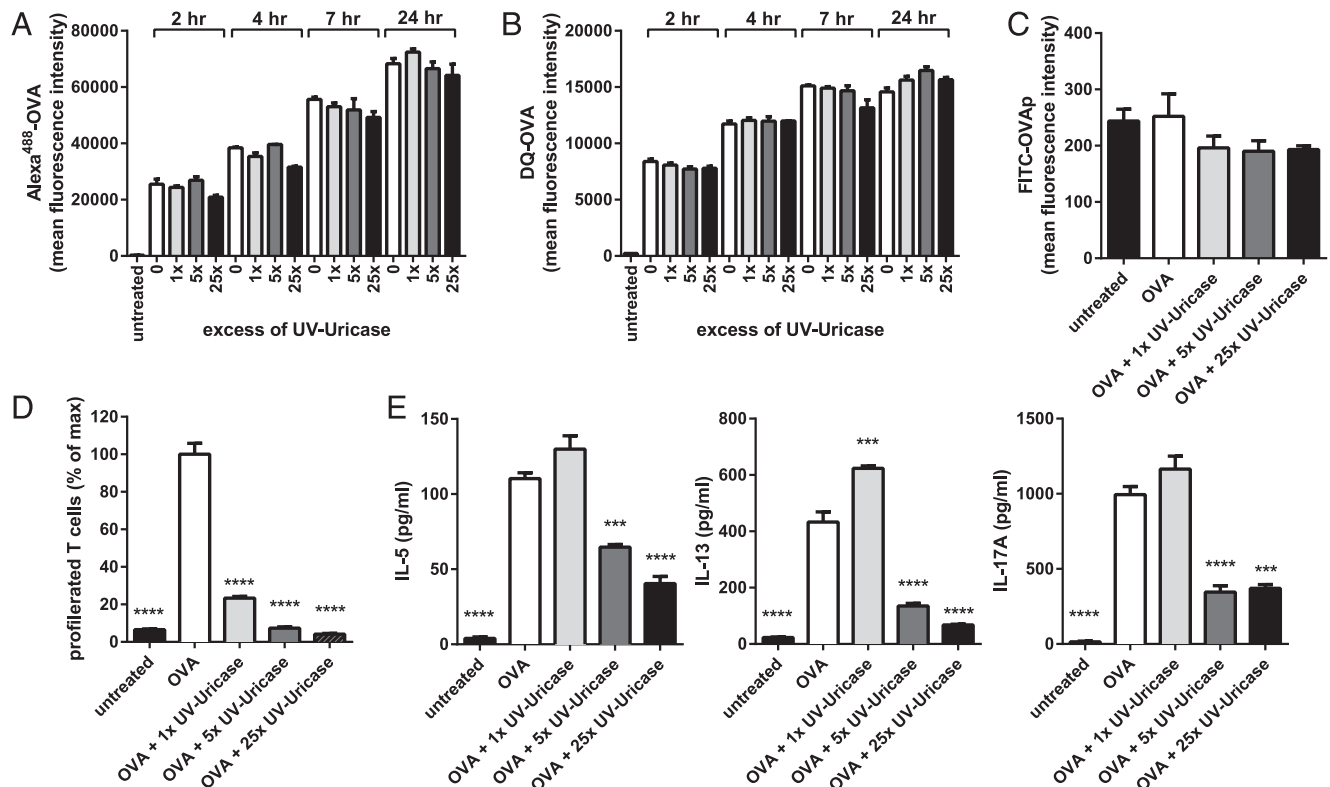


FIGURE 6. Enzymatically inactive uricase inhibits CD4⁺ T cell proliferation and cytokine production. DC2.4 cells were left untreated or were administered 10 μ g/ml Alexa Fluor 488-labeled OVA or DQ-OVA in the absence or presence of increasing concentrations of UV-inactivated uricase based on weight (10, 50, or 250 μ g/ml). At the indicated time points, BMDCs were washed, fixed, and analyzed by flow cytometry for Alexa Fluor 488 (**A**) or unquenched DQ-OVA (**B**) fluorescence. (**C**) DC2.4 cells were cultured for 24 h with OVA in the absence or presence of increasing concentrations of UV-inactivated uricase, incubated for 6 h with FITC-conjugated OVA₃₂₃₋₃₃₉ peptide, and assessed for FITC fluorescence. BMDCs cocultured with CFSE-labeled OVA-specific OTII CD4⁺ T cells were left untreated or treated with 10 μ g/ml OVA in the absence or presence of increasing concentrations of UV-inactivated uricase based on weight (10, 50, or 250 μ g/ml). After 72 h, T cell proliferation (**D**) and cytokine abundance in culture supernatants (**E**) were measured. $n = 4$ in each culture condition. Comparisons are versus 0 uricase [at each time point in (**A**) and (**B**)] or versus OVA (**C**–**E**). *** $p \leq 0.001$, **** $p \leq 0.0001$.

in ambient air is not possible for many affected populations (37), there remains an unmet need to understand the mechanisms of NO₂ pathogenesis in the induction of respiratory and systemic disease. Part of the lung injury induced by NO₂ is oxidative modification of airway surface liquid, damage to epithelium, and leakage of plasma components into the airspaces (3, 38). Plasma is an abundant source of UA, an antioxidant that is superbly capable of interacting with and detoxifying NO₂ (39, 40). However, UA may possess other qualities that make it a possible pathogenic mediator of tissue injury. We reported previously that heat shock protein 70, a DAMP protein capable of activating TLR2 and TLR4, was elevated in the lavageable airspaces of NO₂-exposed mice and that TLR2 and its intracellular adaptor MyD88, but not TLR4, were required for the generation of an Ag-specific adaptive immune response to NO₂-promoted allergic sensitization (8). A number of damage-associated molecules function to stimulate innate immune responses to clear areas of injury and avoid infection, and the presence of UA in the airways was reported to be sufficient and necessary to promote adaptive immune responses that lead to asthma-like disease, as well as to asthma exacerbation (22). A part of the mechanism through which UA functions is via stimulation of dendritic cells, activation of the NLRP3 inflammasome, and secretion of IL-1 β (20, 24). We (12) reported that NLRP3 inflammasome activation participates in the induction of the adaptive immune response subsequent to NO₂ and Ag inhalation and that IL-1R is critical for generation of the ensuing Th17 response. Consequently, we sought to determine whether UA was present in the airways of NO₂-exposed mice and whether it is a

participant in the generation of the Ag-specific immune response in NO₂-promoted allergic airway disease.

In addition to using an Amplex Red-based assay, we implemented the direct and highly specific LCMS approach, accompanied by an internal stable isotope standard, to unambiguously quantify the UA content in BALF. We found increased levels of UA in BALF as long as 68 h after NO₂ exposure: the levels were ~1% of the levels (1.89 $\pm$ 1.80 mg/dl = 112.43 μ M) reported in the plasma of female C57BL/6J mice (41). A modest amount of plasma leak subsequent to NO₂ exposure could easily account for these levels of UA, because levels as high as 10% of those in the plasma were reported subsequent to ventilator-induced lung injury, with similar concentrations seen in BALF of patients with acute lung injury (42). Interestingly, the levels of UA increase over times that correspond to the optimal time for delivery of Ag into the airways to promote allergic sensitization (8), as well as the increased abundance of inflammatory dendritic cells in the lungs and the delivery of Ag to draining lymph nodes subsequent to NO₂ exposure (10). It initially appeared that airway UA was functioning as a mediator of allergic sensitization, because its breakdown through the administration of uricase during the NO₂-promoted allergic-sensitization regimen nearly completely prevented the OVA-induced recall response that causes methacholine hyperresponsiveness, eosinophilic inflammation, Th2/Th17 cytokine production, and Ag-specific Ig production (8, 10–12), similar to what was reported in settings of alum-promoted (27) and house dust mite-promoted (22) Ag sensitization. However, these studies did not investigate the presence of a uricase-specific immune

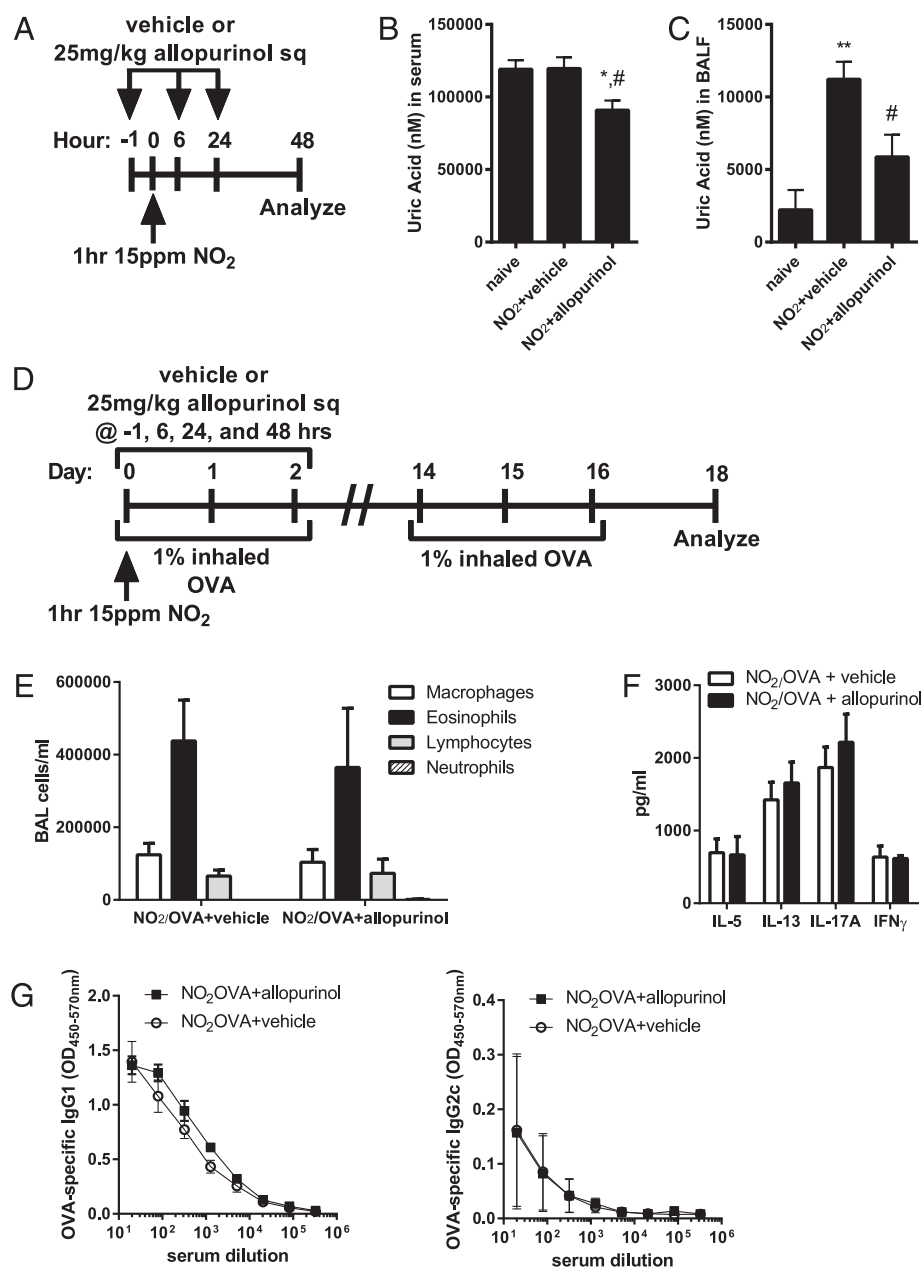


FIGURE 7. Allopurinol treatment does not inhibit NO₂-promoted allergic airway disease. **(A)** Mice were administered saline vehicle or 25 mg/ml allopurinol and exposed to 1 h of 15 ppm NO₂ (or air control) at the indicated times. Forty-eight hours after the NO₂ or air exposure, serum **(B)** and BALF **(C)** were collected and analyzed for UA content by Amplex Red assay. **(D)** Mice were subjected to NO₂-promoted allergic sensitization and s.c. administered vehicle (saline) or 25 mg/kg allopurinol at the indicated times relative to NO₂ exposure. Mice were challenged with three daily doses of 1% inhaled OVA and analyzed 48 h later for inflammatory cells in BALF **(E)**, cytokine production from OVA-stimulated splenocytes **(F)**, and OVA-specific IgG1 and IgG2c in serum **(G)**. $n = 5$ mice per group (A–C); $n = 4$ mice per group (D–G). # $p \leq 0.05$ versus NO₂+vehicle, * $p \leq 0.05$, ** $p \leq 0.01$ versus naive.

response, which we observed following uricase inhalation. We found that the uricase-specific immune response was generated even when uricase was enzymatically inactive and incapable of generating an innate immune response.

Independent of enzymatic activity and its consequences, uricase displays a potent capacity to inhibit the proliferation of, and cytokine production from, OVA-specific CD4⁺ T cells in a manner distinct from interfering with Ag uptake or processing and presentation by dendritic cells. Based on our studies, it is unlikely that uricase interferes with the presentation of the OVA_{323–339} peptide in the context of MHC class II that is required for the activation of OTII CD4⁺ T cells, a condition that would inhibit proliferation and cytokine production. Nevertheless, the effect of uricase or HSA in vivo to inhibit OVA-specific CD4⁺ T cell activities could be different from the in vitro experimental systems that we used. In experimental settings, uricase may be functioning, in part (or in whole in some instances), as an abundant Ag that skews the adaptive immune response away from that induced by other concomitantly present Ags. Our results showing

that the administration of an ultra-clean and nonproinflammatory preparation of the protein Ag HSA, at a concentration identical to that of uricase or UV-inactivated uricase, was able to inhibit the generation of NO₂-promoted allergic airway disease as effectively as uricase clearly support this mechanism. This mechanism is analogous to the concept of original antigenic sin (43), wherein subsequent immune responses to a similar set of Ags are predicated by those experienced previously. In the setting described in this study, the immune response generated during exposure to both OVA and uricase is dominated by that to uricase, such that subsequent exposure to OVA gives the impression that the OVA response was inhibited, whereas the truth is that a uricase response was promoted. Although this effectively prevents OVA-driven pathology, the development of asthma-like disease in our model of NO₂-promoted allergic sensitization, it assures that the response to subsequent uricase exposures could be deleterious. Interestingly, uricase is used for the treatment of tumor lysis syndrome in the setting of chemotherapy (an immunosuppressive situation) and is indicated for the treatment of severe gout, although it is not

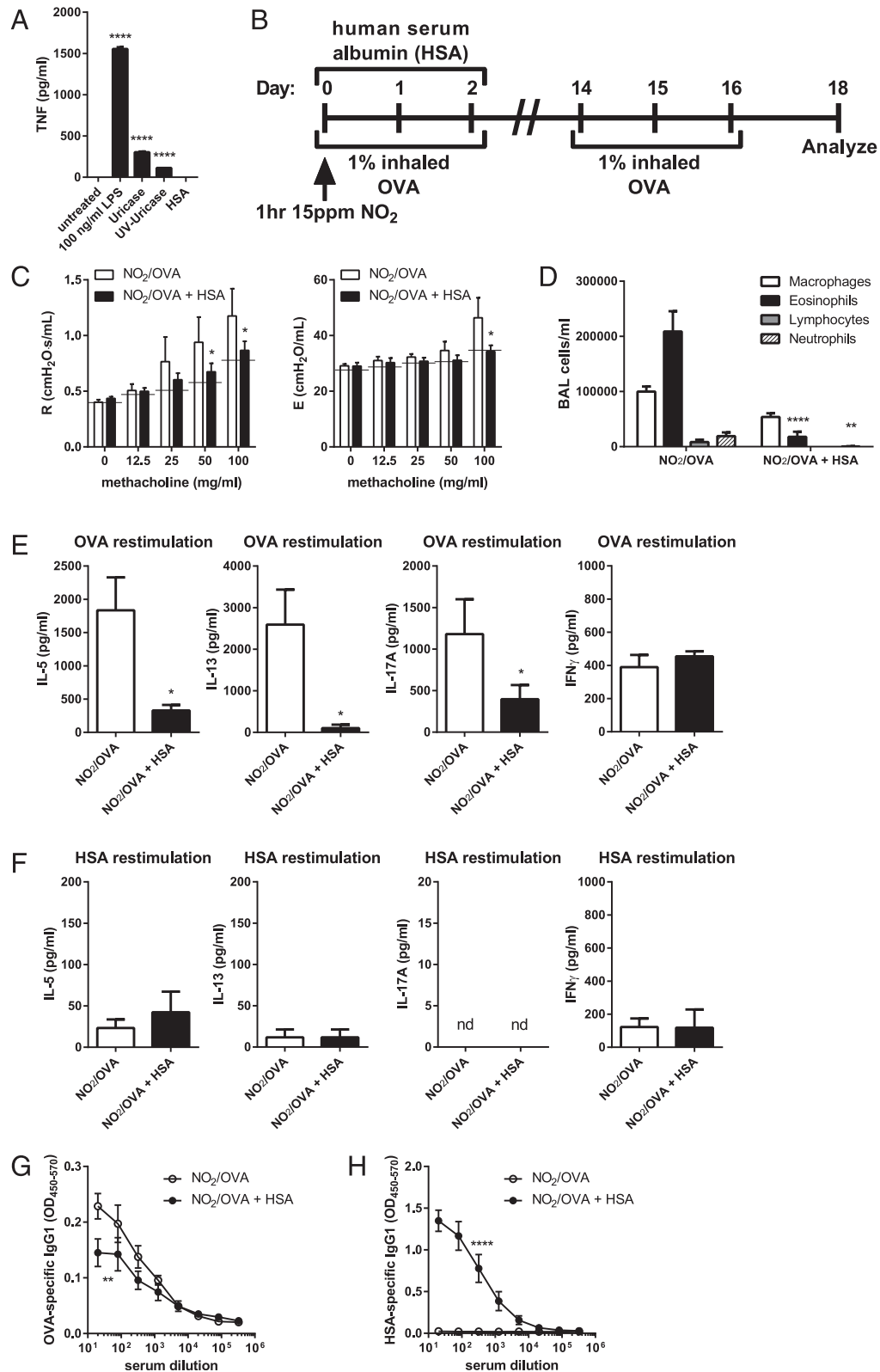


FIGURE 8. Treatment with a clean protein inhibits NO₂-promoted allergic airway disease. **(A)** J774 macrophages were left untreated or exposed to the indicated agonists for 18 h, after which TNF was measured from culture supernatants. Mice were subjected to NO₂-promoted allergic sensitization and were left untreated or administered 2 mg of HSA (an amount of protein equivalent to that in 10 U of uricase or UV-inactivated uricase) in 40 μ l by intranasal instillation. Mice were challenged with three daily doses of 1% inhaled OVA and analyzed 48 h later **(B)** for methacholine responsiveness **(C)**; responsiveness of naive mice is indicated by horizontal lines, inflammatory cells in BALF **(D)**, cytokine production from OVA-restimulated **(E)** and HSA-restimulated splenocytes **(F)**, and OVA-specific **(G)** or HSA-specific **(H)** IgG1 in serum ($n = 8$ mice per group). * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ versus untreated (A) or between groups (C–E, H). nd, not detected.

widely used by clinicians because of adverse effects, including immunogenicity. Strategies to attenuate the immunogenicity of uricase include the addition of polyethylene glycol (PEG), as used in an approved clinical formulation, although substantial immunogenicity remains (44). In fact, Abs to PEG-conjugated porcine uricase, pegloticase, were present in the vast majority of patients receiving the drug, titers of which were associated with increased pegloticase clearance and a loss of the UA-lowering response, as well as an increased risk for infusion reactions and anaphylaxis (45). Therefore, it is not surprising that similar immune-stimulating effects of uricase were observed in our studies using a non-PEGylated form.

Although it is unclear from the uricase studies whether UA is a marker or a mediator of the events involved in NO₂-promoted allergic airway disease, the allopurinol experiments indicate that UA is likely only a marker. Our results demonstrate that allopurinol significantly decreased serum UA levels and decreased by half the amount of UA in the lavageable airspaces following NO₂ exposure. We agree that the ability of allopurinol to decrease serum UA levels is modest and that it remains possible that a quantity of UA sufficient to participate in NO₂-promoted allergic sensitization is still present in the airways following NO₂ exposure, even in the setting of allopurinol administration. Despite this caveat, competitive inhibition of *Xdh* enzymatic activity with allopurinol was unable to prevent NO₂-promoted allergic sensitization, making it unlikely that induction of this enzyme was responsible for the elevated UA levels present in the airway following NO₂ exposure.

Nonetheless, *Xdh* and the UA transporter, multi-drug resistance protein 4, are expressed by human airway epithelial cells exposed to house dust mite extract or particulate matter, factors that can promote allergic sensitization in a manner inhibitable by uricase treatment (22, 46). Why UA is a potent inducer of adaptive immunity in some circumstances, whereas in other cases it is not, may be due to subtleties of experimental design and interpretation or to situational differences in abundances or activities. Our results clearly issue a warning of caution in interpreting results of studies involving uricase as a tool for evaluating the importance of UA in the generation of adaptive immune responses, as well as for those studies in which the administration of large amounts of proteins is able to seemingly mitigate adaptive immune responses to other Ags.

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Disclosures

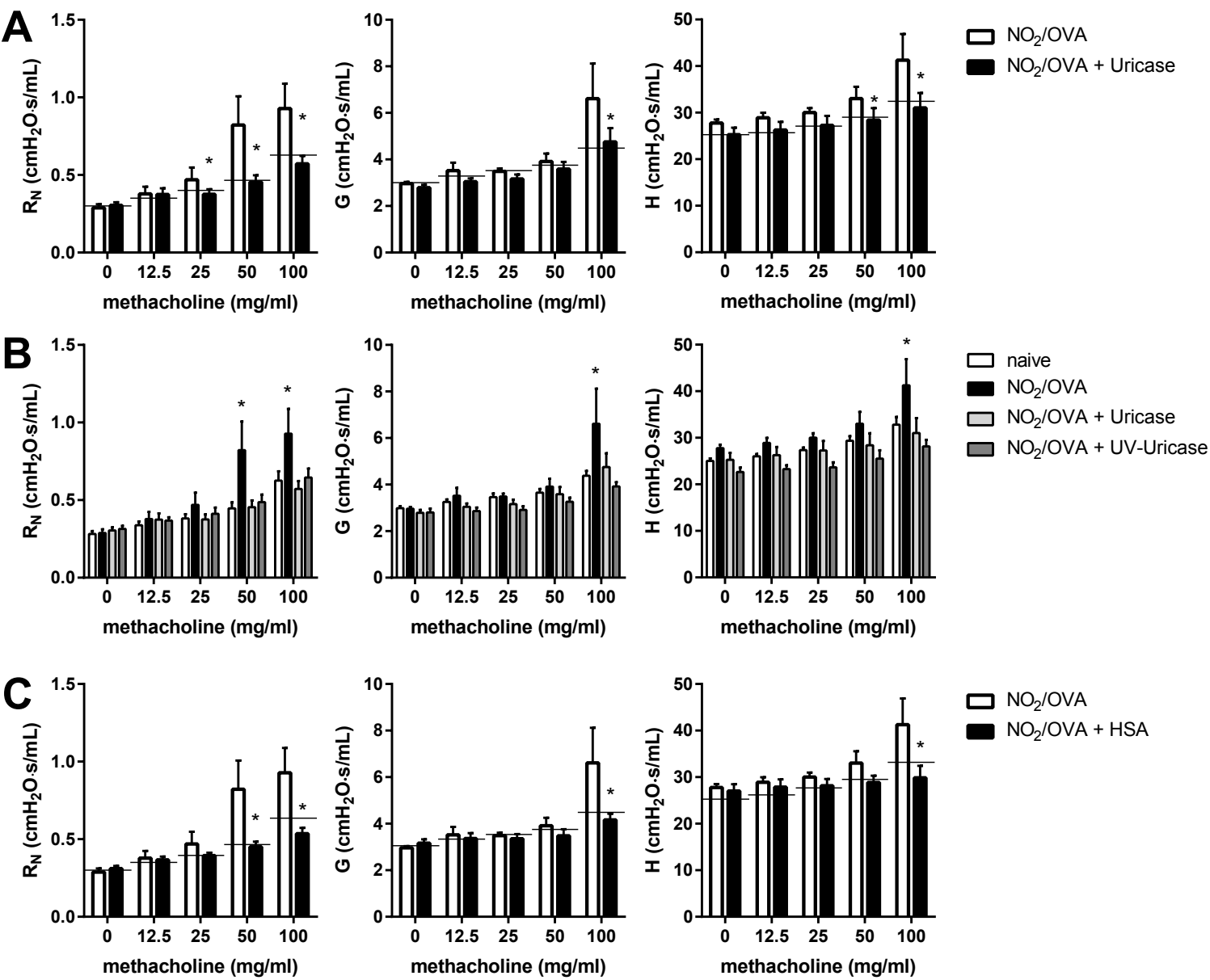
The authors have no financial conflicts of interest.

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Supplemental Figure 1



Supplemental Figure 1. Uricase, inactive uricase, and human serum albumin inhibit methacholine responsiveness in NO_2 -promoted allergic airway disease.

Mice were subjected to NO_2 -promoted allergic sensitization and administered saline or 10U of uricase (A and B), 10U UV-inactivated uricase (B), or 2 mg human serum albumin (C) by intranasal instillation. Mice were challenged with three daily doses of 1% inhaled ovalbumin and analyzed 48 hours later by the constant-phase model of the lung for Newtonian airway resistance (R_N), tissue damping (G), and tissue resistance in response to ascending doses of methacholine. N = 8 mice per group. Comparisons are versus NO_2/OVA (A and C) or naive (B).