

Ozone impairs endogenous compensatory responses in allergic asthma

Kevin Ho^a, David Weimar^a, Gina Torres-Matias^{a,b}, Hyunwook Lee^a, Saaleha Shamsi^a, Emily Shalosky^a, Michael Yaeger^{a,b}, Hannah Hartzler-Lovins^{a,c}, Katelyn Dunigan-Russell^a, Daria Jelic^a, Caymen M. Novak^a, Kymberly M. Gowdy^a, Joshua A. Englert^{a,1}, Megan N. Ballinger^{a,*,1}

^a Division of Pulmonary, Critical Care, and Sleep Medicine, Department of Internal Medicine, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, United States of America

^b Biomedical Sciences Graduate Program, The Ohio State University, Columbus, OH, United States of America

^c Molecular, Cellular and Developmental Biology Graduate Program, The Ohio State University, Columbus, OH, United States of America

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ABSTRACT

Asthma is a chronic inflammatory airway disease characterized by acute exacerbations triggered by inhaled allergens, respiratory infections, or air pollution. Ozone (O₃), a major component of air pollution, can damage the lung epithelium in healthy individuals. Despite this association, little is known about the effects of O₃ and its impact on chronic lung disease. Epidemiological data have demonstrated that elevations in ambient O₃ are associated with increased asthma exacerbations. To identify mechanisms by which O₃ exposure leads to asthma exacerbations, we developed a two-hit mouse model where mice were sensitized and challenged with three common allergens (dust mite, ragweed and *Aspergillus fumigatus*, DRA) to induce allergic inflammation prior to exposure to O₃ (DRA–O₃). Changes in lung physiology, inflammatory cells, and inflammation were measured. Exposure to O₃ following DRA significantly increased airway hyperreactivity (AHR), which was independent of TLR4. DRA exposure resulted in increased BAL eosinophilia while O₃ exposure resulted in neutrophilia. Additionally, O₃ exposure following DRA blunted anti-inflammatory and antioxidant responses. Finally, there were significantly less monocytes and innate lymphoid type 2 cells (ILC2s) in the dual challenged DRA–O₃ group suggesting that the lack of these immune cells may influence O₃-induced AHR in the setting of allergic inflammation. In summary, we developed a mouse model that mirrors some aspects of the clinical course of asthma exacerbations due to air pollution and identified that O₃ exposure in the asthmatic lung leads to impaired endogenous anti-inflammatory and antioxidant responses and alterations inflammatory cell populations.

1. Introduction

Asthma is a chronic inflammatory airway disease characterized by acute exacerbations in response to inhaled allergens or respiratory infections. Atopic asthma, distinguished from non-atopic asthma by eosinophilic inflammation, makes up approximately half of all asthma cases worldwide (Knudsen et al., 2009). Asthma exacerbations are characterized by an acute worsening of lung function that in the most severe cases can be life-threatening. A common trigger for atopic asthma is air pollution, specifically increases in ambient O₃ (Guarnieri and Balmes, 2014). O₃ is an air pollutant that induces pulmonary inflammation and

oxidative stress. Epidemiologic studies have demonstrated an increase in asthma-related symptoms during times of high air pollution (Khatri et al., 2009).

Despite this epidemiological association, little is known about the underlying mechanisms by which O₃ induces allergic asthma exacerbations. Early human studies of O₃ exposure in healthy volunteers revealed increased peripheral blood and bronchoalveolar neutrophilia (Bosson et al., 2013; Schelegle et al., 1991), but the significance of this finding and whether patients with a preexisting lung disease (e.g. asthma) have a similar response is unknown. Murine models of acute O₃ exposure have consistently demonstrated neutrophilic airway

* Corresponding author at: Division of Pulmonary, Critical Care, and Sleep Medicine, The Ohio State University Wexner Medical Center, 201 Davis Heart and Lung Research Institute, 473 West 12th Avenue, Columbus, OH 43210, United States of America.

E-mail address: megan.ballinger@osumc.edu (M.N. Ballinger).

¹ denotes equal contribution

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inflammation (Che et al., 2016; Johnston et al., 2005), but its link to asthma exacerbation in the context of chronic allergic inflammation is still unclear. One of the major barriers to exploring the association between O₃ and asthma is the lack of mouse models of allergic inflammation that can mirror the course of patients with atopic asthma who suffer from exacerbations due to air pollution. Existing asthma models are limited by the use of a single antigen for sensitization (leading to possible tolerance) (Kierstein et al., 2008; Goplen et al., 2009), short duration of allergen exposure, or the use of less clinically relevant antigens such as ovalbumin (OVA) (Li et al., 2019; Kumar et al., 2016). Bronchoalveolar lavage results from these mouse models have varying degrees of lymphocytosis, neutrophilia, and eosinophilia, which differs from the expected eosinophilic and neutrophilic response based on prior human studies (Bosson et al., 2013; Schelegle et al., 1991).

Here, we present a novel two-hit murine model where mice were exposed to O₃ following the induction of allergic inflammation with a mixture of clinically relevant environmental allergens (Common Asthma Triggers, 2020). We hypothesized that O₃ exposure in this model would induce airway remodeling, goblet cell hyperplasia, inflammatory cell recruitment to the lungs, airway hyperreactivity (AHR), and the expression of anti-inflammatory cytokines and antioxidants compared to control mice or mice exposed to either allergens or O₃ alone.

2. Materials and methods

2.1. Mice

C57BL/6 J (stock number: 000664) or TLR4^{-/-} (stock number: 029015) male mice (8–10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were randomly assigned to treatment groups. The animals were housed in specific pathogen-free conditions within The Ohio State University Animal Care Facility (Columbus, OH). All animal experiments were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

2.2. Allergic inflammation model

The DRA triple-allergen mixture was composed of extracts of 2.5 mg/ml of dust mite (*Dermatophagoides farinae*), 25 mg/ml of ragweed (*Ambrosia artemisiifolia*), and 2.5 mg/ml of *A. fumigatus* (Greer Laboratories) as previously described (Lee et al., 2019; Park et al., 2013). The DRA mixture contained 3624 EU/ml of endotoxin. Mice were anesthetized with ketamine-xylazine before all intranasal exposures. On days 0 and 5, mice were sensitized with 20 μ l (200 μ g) of the DRA allergen mixture. Mice were then challenged with 20 μ l of DRA on days 12, 14 and 17.

2.3. O₃ challenge

Mice were exposed to room air (control) or O₃ at 2 ppm for 3 h in a custom-designed plexiglass chamber (Howenstine et al., 1991). As described by Hatch et al. (Hatch et al., 2013), this was done to ensure O₃-induced toxic effects in mice, which is comparable to an O₃ concentration of 400 ppb for humans. Ozone was generated by directing 100% oxygen through an ultraviolet light generator that was mixed with room air inside the chamber. Temperature (24.9–25.5 °C), humidity (68.6–75.0 RH), and ozone concentration were monitored continuously. For mice in the DRA-O₃ group, the O₃ challenge occurred on day 18. Mice exposed to O₃ alone were challenged the day preceding the methacholine challenge.

2.4. Measurement of airway hyperreactivity

For mice in the DRA-O₃ group, airway measurements were taken 24 h following O₃ exposure. For the remaining mice, measurements were taken the day following the last exposure (DRA or O₃). Mice were anesthetized with pentobarbital (100 mg/kg intraperitoneally), a tracheostomy tube was inserted, and then were paralyzed with pancuronium bromide (0.8 ml/kg intraperitoneally) before initiation of methacholine challenge. Mice were connected to a FlexiVent FX small-animal ventilator (SCIREQ) and increasing concentrations of methacholine were administered via an Aeroneb laboratory nebulizer (2.5 mg/ml, 5 mg/ml, 10 mg/ml, 25 mg/ml, and/or 50 mg/ml). The forced oscillatory technique was used to perform sequential quick-prime 3 maneuvers following two deep inflations to normalize volume history. Data obtained from these maneuvers were analyzed using the constant phase model to calculate the airway resistance (R_n) and other measurements of lung function using flexiWare software (version 8) (Chung et al., 2020). The average of the top three R_n values at each methacholine dose were reported as the peak response to methacholine.

2.5. Bronchoalveolar lavage (BAL) cell counts

Lungs were lavaged with 1 ml of PBS and cells were counted using the Countess automated cell counter (Life Technologies). Cytospins were prepared and stained with HEMA 3 (Thermo Scientific). Following staining, differential cell counts were performed on 100 cells in a blinded fashion.

2.6. mRNA Extraction, cDNA synthesis, and qPCR

mRNA was extracted from lung tissue homogenates by using a Direct-zol RNA miniprep Plus Kit (Zymo Research) according to the manufacturer's instructions. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). Gene expression was measured by quantitative PCR (qPCR) in a QuantStudio 3 Real-time PCR system using Fast PowerUp SyBR Green Master Mix (Applied Biosystems) unless otherwise noted. A list of forward and reverse primers used for qPCR are provided in Supplemental Table 1. TaqMan probe and primers were utilized to measure *Muc5ac* and *Muc5b* expression. Data were analyzed by the 2^{- $\Delta\Delta$ Ct} method with 18S expression as an endogenous control.

2.7. Measurement of cytokines

Measurements of TSLP, IL-33, IL-1 β and IL-10 were quantified in undiluted whole lung homogenates using ELISA kits (R&D systems) following manufacturer's protocols. Muc5AC ELISA (Novus Biological) was performed according to the manufacturer's instructions.

2.8. Flow cytometry cell preparation, cellular gating strategies and UMAP analysis

Cell were isolated from whole lungs of mice by collagenase digestion as previous described (Ballinger et al., 2006). Cells were incubated with Fc receptor block anti-mouse CD16/32 antibody (BD biosciences) followed by specific antibodies listed in Supplemental Table 2. Lung cells were then fixed in 2% paraformaldehyde and analyzed on a Cytex Aurora Spectral Flow Cytometry. The UMAP FlowJo Plugin was used to dimensionally reduce and visualize data sets. A total of 15,000 CD45+ live events from each sample were concatenated into a single FCS file, with the use of keywords to preserve the group identity. The UMAP algorithm was run on the single FCS file and the resulting plot is a 2-dimensional visual representative of all of the CD45+ live cells present in the various treatment groups. Each dot on the UMAP represents a cell and cells with similar expression profiles for the various markers used in the flow analysis will be groups closer together. The cellular

populations highlighted in Fig. 6A were identified by specific gating schemes for the whole lung digest are presented in Supplemental Fig. 4 and 5 and based on those previously described by our group (Reader et al., 2020) and Yu et al. (Yu et al., 2016)

2.9. Lung histology

The upper right lobe was fixed in 10% formalin prior to processing and embedding in paraffin. H&E and PAS staining were conducted by the Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University. Whole lung images were obtained using Aperio ImageScope. Histopathologic analysis was performed by a board certified veterinary pathologist from Experimental Pathology Laboratories utilizing an established grading methodology to measure interstitial inflammation and goblet cell metaplasia (Reece et al., 2021). Briefly the grades assigned to mucin were based on the number of goblet cells and their pattern of distribution in the main bronchus, proximal branches, preterminal bronchioles, and terminal bronchioles and the scoring was as follows: 1: represents no goblet cells; 2: scattered goblet cells in the main bronchus $\pm$ proximal branches; 3: increased number of goblet cells, confluent in some places; 4: same as 3 but goblet cell extending distally, and 5: same as 3 but extending distally into terminal bronchioles. The grading criteria for increased alveolar macrophages was as follows: 1: Alveolar macrophages with increased cytoplasm and up to 2 alveolar spaces per 1.5 mm² field contain multiple macrophages; 2: alveolar macrophages with increased cytoplasm, alveolar spaces frequently contain multiple macrophage and multinucleated macrophages present; 3: 25–50% of alveolar spaces contain 1 or more macrophages; 4: >50% of alveolar spaces contain 1 or more macrophages. The grading criteria for alveolar inflammation was as follows: 1: <10% of alveoli contain mixed inflammatory cell infiltrates; 2: between 10 and 25% of alveoli contain mixed inflammatory cell infiltrates; 3: between 25 and 50% of alveoli contain mixed inflammatory cell infiltrates; 4: >50% of alveoli contain mixed inflammatory infiltrates. A summary incidence and severity of microscopic changes is located in Supplemental Table 3.

2.10. Statistics

For laboratory-based experiments, statistical analysis was performed using Graphpad Prism 9. Data sets were tested for normality via a Shapiro-Wilk test. All data are presented as mean $\pm$ SEM except where noted otherwise. Student's *t*-tests were used to compare two groups with normally distributed data. A student's *t*-test on log-transformed data was used to compare two groups with log-normally distributed data. Mann-Whitney tests were used to compare ranks if the data were not normally distributed. For analysis of qPCR data, the statistical comparison was performed on log-transformed fold change data. For comparison among multiple groups, all data were normally or log-normally distributed and an ANOVA was performed on log-transformed or transformed data as appropriate. For experiments with two-independent variables, a two-way ANOVA was performed after testing data for normality as described. If a significance was determined by ANOVA, a Tukey post-hoc test for individual group comparisons was performed. A *p*-value of <0.05 was considered statistically significant. Outliers were identified using non-linear regression via the ROUT method with a *Q* threshold of 1%.

3. Results

3.1. DRA-O₃ asthma exacerbation model recapitulates the inflammatory characteristics of allergic inflammation and O₃ exposure

To create a background of allergic inflammation, mice were treated with DRA over a three-week period. Following DRA sensitization, mice were exposed to O₃ to mimic the effects of this specific pollutant on

asthmatics with allergic asthma (Fig. 1A). There were four treatment groups: 1) control 2) O₃ alone 3) DRA alone 4) DRA followed by O₃ (DRA–O₃). Total number of cells present in the airways were increased in mice with allergic inflammation but O₃ exposure following DRA sensitization did not further increase BAL cell numbers (Fig. 1B). BAL protein levels were measured as a marker of barrier permeability and were increased in DRA and O₃ exposed groups (Fig. 1C). Inflammatory cell populations within the BALs of the different treatment groups were assessed using cytospin slides. Groups exposed to DRA (DRA alone, DRA followed by O₃) had significantly increased BAL eosinophils compared to control and O₃ exposure alone (Fig. 1D and Supplemental Fig. 1A). BAL samples from groups treated with O₃ (O₃ alone, DRA followed by O₃) had significantly higher BAL neutrophils compared to control and DRA exposure alone (Fig. 1E and Supplemental Fig. 1B). There was no significant difference in BAL eosinophilia between DRA alone compared to DRA–O₃ exposure and no significant difference in BAL neutrophilia between O₃ alone and DRA–O₃. Control and O₃ treatment alone had a higher percentage of macrophages than DRA-treated groups (Fig. 1F) but there was no difference in the total number of macrophages (Supplemental Fig. 1C). In summary, these data suggest that the combination of DRA sensitization and O₃ exposure leads to a mixed inflammatory phenotype consisting of both eosinophilic and neutrophilic inflammation.

3.2. Sequential exposure to DRA and O₃ induces inflammation within lung tissue

Lung tissue was examined and assessed for different features of airway inflammation and remodeling using an established methodology (Reece et al., 2021). A summary of the incidence and severity of microscopic changes in the lungs in found in Supplemental Table 3. Mice exposed to DRA (in combination with O₃ or alone) had evidence of increased perivascular (Fig. 2A–B) and peribronchiolar (Fig. 2A,C) interstitial inflammation as well as alveolar inflammation (Fig. 2A,D), which was characterized by a mixed inflammatory cell infiltrate. There were increased alveolar macrophages present in the alveolar spaces as well as increased cytoplasm and multinucleated macrophages present in the DRA and DRA–O₃ groups compared to O₃ or untreated (Fig. 2E). O₃ exposure alone at the dose and timepoint studied induced minimal histologic injury (Fig. 2A). This analysis suggests that our 2-hit model induced histopathologic changes resembling the chronic airway inflammation found in human asthmatics.

3.3. O₃ exposure in the setting of allergic inflammation does not alter goblet cell hyperplasia

Mucus overproduction can result in cough and airway obstruction during asthma exacerbations. Goblet cell hyperplasia is thought to be responsible for this excessive mucus production and secretion (Curran and Cohn, 2010). Examination of lung tissue following PAS staining revealed increased goblet cell hyperplasia in DRA-treated groups (Fig. 3A). Exposure to O₃ following DRA sensitization and challenge also resulted in increased goblet cell hyperplasia, but it was not significantly different than DRA alone (Fig. 3B). *Muc5ac* and *Muc5ab* have been implicated in physiologic and pathophysiologic mucus secretion. There was no difference in lung tissue *Muc5ac* mRNA levels between all of the groups (Fig. 3C). A single O₃ exposure alone did not increase mRNA levels (Fig. 3D) or protein expression (Fig. 3E) of *Muc5ac*, but DRA-exposed groups did have significantly higher *Muc5ac* mRNA levels compared to controls (Fig. 3D). There was a significant decrease in *Muc5ac* protein expression (Fig. 3E) in the DRA–O₃ group compared to the mice that received DRA alone. These data suggest that DRA exposure increases *Muc5ac* expression and goblet cell hyperplasia and that a single exposure to O₃ does not significantly alter mucus production.

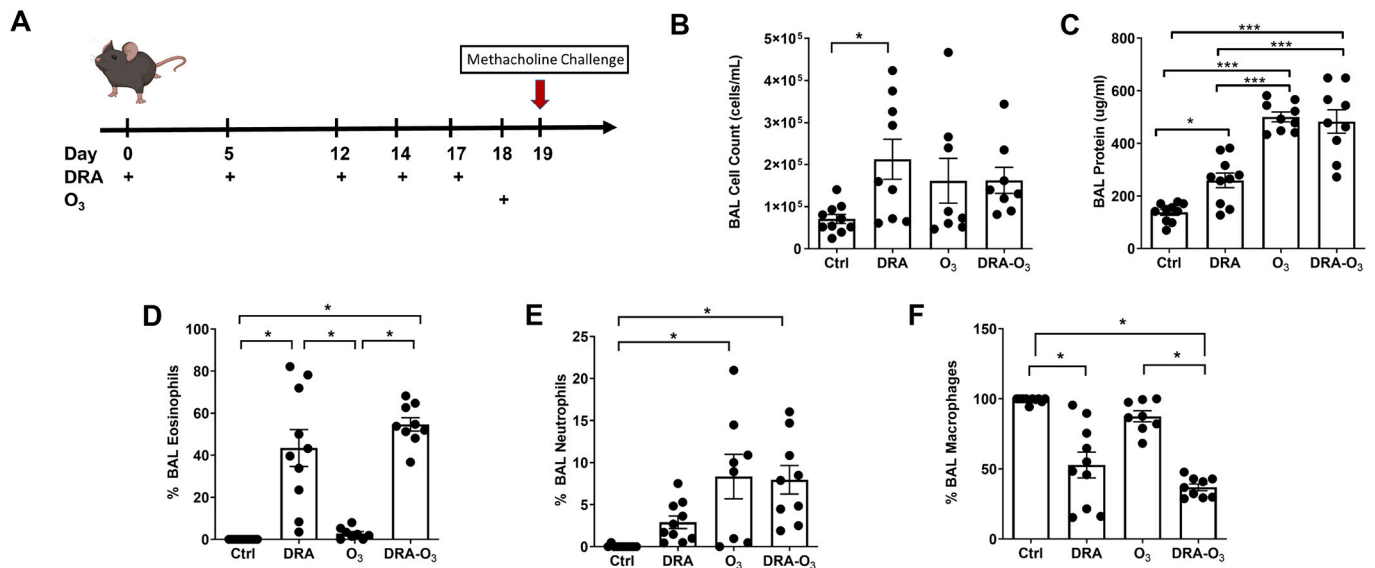


Fig. 1. DRA- O_3 exposure results in mixed eosinophilic and neutrophilic inflammation with increased lung injury. WT male mice were either untreated (Control (Ctrl), $n = 10$), challenged with DRA treated alone (DRA, $n = 10$), challenged with O_3 alone (O_3 , $n = 8$), or challenged with both DRA and O_3 (DRA- O_3 , $n = 9$). (A) A schematic of the timing for the DRA sensitization and challenges as well as O_3 challenge is provided. Mouse image is from Biorender. DRA- O_3 mice were sensitized with DRA over 17 days to induce allergic inflammation, followed by O_3 exposure on day 18. BAL was collected after methacholine challenge and the total number of cells in the BAL fluid was measured (B). (C) Total protein in the BAL fluid was measured by BCA assay. Differential cell counts were performed to determine the percentage of (D) eosinophils, (E) neutrophils, and (F) macrophages. Error bars shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

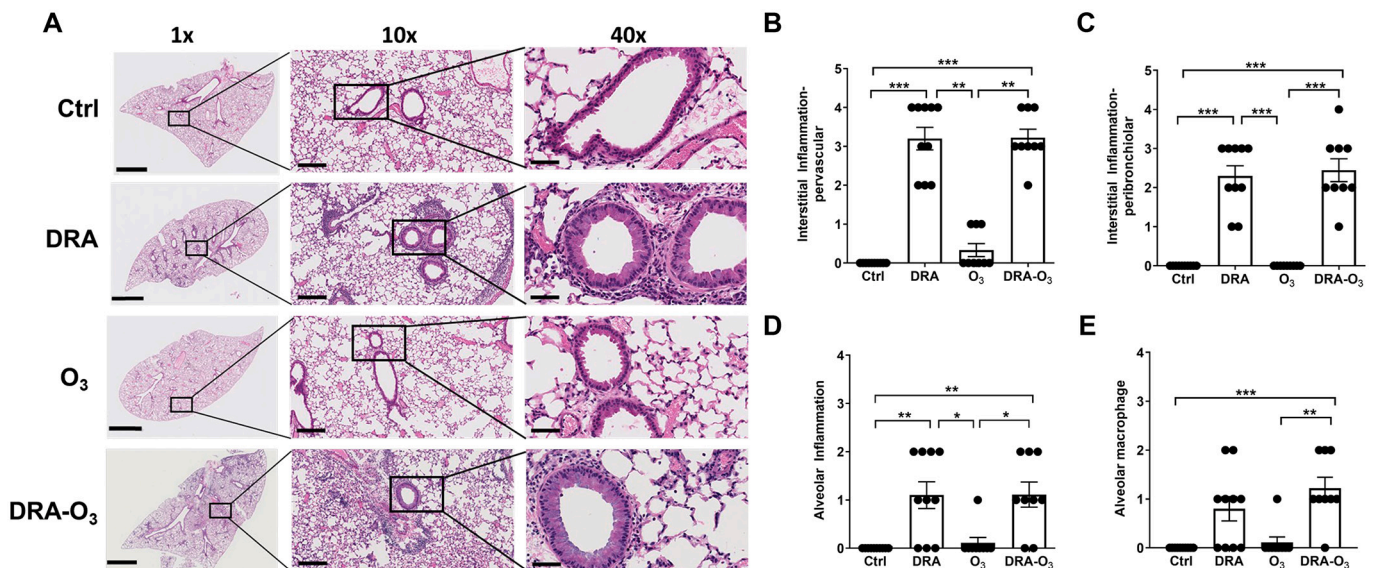


Fig. 2. Sequential exposure to DRA and O_3 induces histologic inflammation. WT male mice were challenged with either DRA- O_3 ($n = 9$), DRA ($n = 10$), or O_3 ($n = 9$), and whole lung tissue was collected and compared against lung tissue isolated from control mice ($n = 10$). (A) Whole lung histologic sections were H&E-stained following methacholine challenge. These are representative images from each treatment group. Scale bar in 1 $\times$ section is 2 mm. The insert box in 1 $\times$ section represents the location that is shown in the subsequent 10 $\times$ and 40 $\times$ images. Scale bar is 200 μ m for 10 $\times$ and 50 μ m for 40 $\times$. All lungs were examined by a veterinarian pathologist and DRA-treated groups had increased (B) perivascular interstitial inflammation, (C) peribronchial interstitial inflammation, (D) alveolar inflammation, and (E) alveolar macrophages compared to untreated control mice. Supplemental Table 1 contains grading and numerical explanations for each individual mouse. Error bars shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4. DRA- O_3 exposure increases airway hyperreactivity compared to either injury alone in a TLR4 independent fashion

Increased airway resistance is a physiologic hallmark of asthma exacerbations. To test for airway hyperreactivity (AHR) in our model, mice were given increasing doses of nebulized methacholine prior to measurements of airway resistance. As expected, mice exposed to each individual injury had increased airway resistance following methacholine

challenge, but this was similar to the effect seen in control mice. DRA- O_3 exposure led to a significant increase in AHR in response to nebulized methacholine compared to other treatment groups (Fig. 4A). There were no significant differences in the tissue damping (G) or tissue elastase (H) across the groups (Supplemental Fig. 2A and 2B). Previous data have demonstrated that O_3 -induced airway hyperreactivity and inflammation were dependent upon activation of the TLR4 signaling pathway (Williams et al., 1985; Li et al., 2011). To test whether changes in AHR and

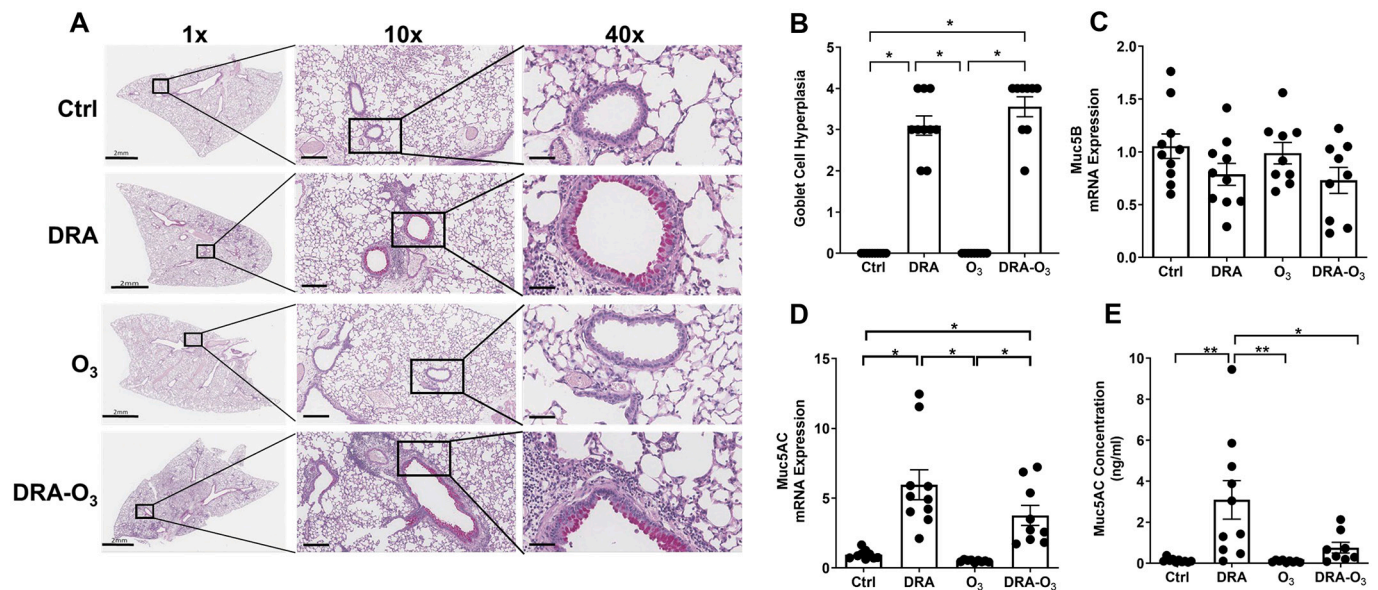


Fig. 3. DRA and DRA-O₃ exposure results in increased goblet cell hyperplasia. WT male mice (n = 10) were challenged with either DRA-O₃ (n = 9), DRA (n = 10), or O₃ (n = 9), and whole lung tissue was collected and compared against lungs isolated from control mice. (A) Whole lung histologic sections were PAS-stained following methacholine challenge. These are representative images from each treatment group. Scale bar in 1× section is 2 mm for Ctrl, DRA, O₃; and 3 mm for DRA-O₃. The insert box in 1× section represents the location that is shown in the subsequent 10× and 40× images. Scale bar is 200 μm for 10× and 50 μm for 40×. (B) Quantification of goblet cell hyperplasia was assessed on the H&E slides but data is shown here. mRNA levels of (C) *Muc5b* as well as mRNA (D) and protein (E) expression of *Muc5ac* was assessed in whole lung tissue. Error bars shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

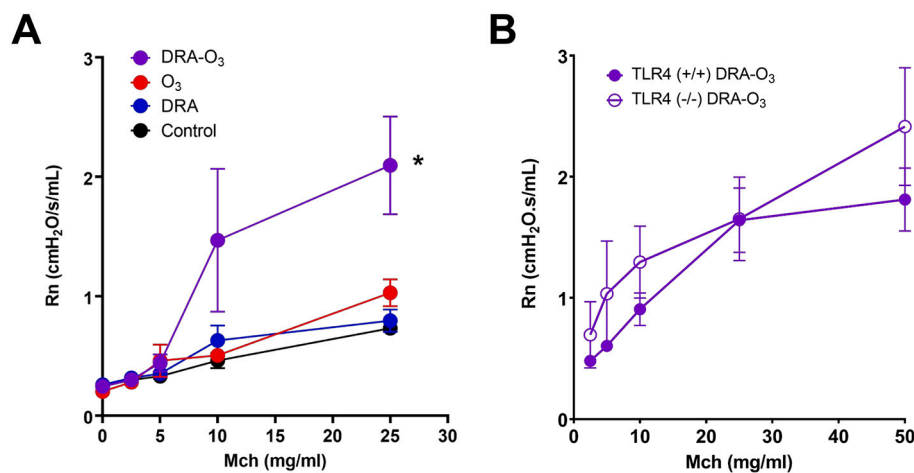


Fig. 4. DRA-O₃ exposure increases airway hyperactivity compared to either injury alone in a TLR4 independent fashion. (A) WT mice were challenged with either untreated (control, n = 10), DRA-O₃ (n = 9), DRA (n = 10), or O₃ (n = 9) and then challenged with escalating doses of methacholine (Mch). Airway hyperactivity, as assessed by measuring airway resistance (Rn) using a flexiVent small-animal ventilator. (B) WT (TLR4^{+/+}, n = 8) or knockout (TLR4^{-/-}, n = 7) were exposed to DRA and then O₃. Mice were challenged with escalating doses of methacholine. Error bars shown as mean ± SEM. *P < 0.05.

lung inflammation were TLR4 dependent in our 2-hit model, we subjected wild type (TLR4^{+/+}) and TLR4 knockout (TLR4^{-/-}) mice to O₃ exposure following DRA sensitization and challenge. Both wild-type and TLR4 knockout mice had similar increases in airway resistance following methacholine challenge (Fig. 4B). Next, we measured lung inflammatory cell recruitment and found similar levels of neutrophils, eosinophils, macrophages, and lymphocytes following DRA-O₃ challenge (Supplemental Fig. 3A and 3B). These data demonstrate that the DRA-O₃ model recapitulates the increased airway resistance that is characteristic of asthma exacerbations and that this increase in AHR is not dependent on expression of TLR4.

3.5. Sequential exposure to DRA-O₃ alters expression of anti-oxidant enzymes and anti-inflammatory cytokines

Production of Th2 cytokines has been shown to regulate the development of allergic asthma. TSLP, initially isolated from thymic stromal

cell line, is a cytokine that has been implicated as a major factor in the development of asthma, especially Th2-related phenotypes. TSLP protein expression in the lung homogenate was significantly higher in mice treated with DRA alone compared to all other treatment groups, including DRA-O₃ (Supplemental Fig. 4A). Several different cytokines or chemokines have been implicated in eosinophil recruitment (Schmid-Grendelmeier et al., 2002; Johnston and Bryce, 2017; Yi et al., 2018). We measured protein expression of IL-33 and found it to be unexpectedly decreased in the combination DRA-O₃ group (Supplemental Fig. 4B). In contrast, there were no differences in expression of the pro-inflammatory cytokine IL-1β (Supplemental Fig. 4C) between the DRA and DRA-O₃ challenged groups.

Cells have various anti-inflammatory and anti-oxidant mediators to attenuate injury and respond to exogenous stresses. IL-10 is an anti-inflammatory cytokine that inhibits inflammation by regulating cytokine and chemokine expression from activated macrophages (Lang et al., 2002). IL-10 protein expression was increased following DRA exposure

alone, but O₃ exposure following DRA sensitization impaired the compensatory increase in IL-10 (Fig. 5A). A single O₃ exposure alone did not alter IL-10 expression in lung tissue. Cellular antioxidants play a key role in protecting cells from injury following exposure to O₃ or other types of oxidant injury. Specifically, glutathione peroxidases play a protective role following O₃ exposure or allergic inflammation (Bao et al., 2017). In our model, DRA as well as DRA prior to O₃ exposure decreased expression of glutathione peroxidase-1 (GPX-1, Fig. 5B) and glutathione peroxidase-2 (GPX-2, Fig. 5C) levels compared to control mice or O₃ alone. These data suggest that allergic inflammation from DRA sensitization and challenge impairs the endogenous antioxidant response to O₃. Similarly, O₃ exposure in the setting of allergic inflammation impairs the compensatory increase in IL-10 induced by DRA alone. Together these data demonstrate the complex regulation of endogenous anti-oxidant and anti-inflammatory pathways in our 2-hit model.

3.6. O₃ exposure in the setting of allergic inflammation impairs recruitment of monocytes and decreases type 2 innate lymphoid cells

To interrogate the mechanisms by which O₃ exposure following allergic inflammation impairs IL-10 release, different immune cell populations within the lung microenvironment were profiled via flow cytometry. Whole lung tissue was collected from the various treatment groups and collagenase digested to obtain a single cell suspension. Differences of lung leukocytes were distinguished using antibodies against various cell surface markers (Supplemental Table 2) and then specific cellular populations were identified using established gating strategies (Supplemental Figs. 5A and 6A). To visualize the different immune cell populations within the lung, uniform manifold approximation and projection (UMAP) was used to dimensionally reduce and visualize the data sets (Fig. 6A). Mice exposed to allergen challenge with DRA alone had significantly higher numbers of lung macrophages including tissue resident alveolar macrophages, monocyte-derived alveolar macrophages, and interstitial macrophages compared to control mice or mice exposed to O₃ alone (Supplemental Fig. 5B and Fig. 6B). Mice exposed to DRA also had higher levels of monocytes including classical and inflammatory monocytes (Fig. 6B-D) that are known to produce counter-regulatory cytokines such as IL-10. Interestingly, mice exposed to O₃ following DRA challenge did not have a compensatory increase in these monocyte populations and had similar numbers of these cells compared to control mice. We also measured the number of other cells known to produce IL-10, such as T-cells, B-cells, and NK cells, and there was no significant differences in the absolute number of these cells between DRA and DRA-O₃ treated mice (Supplemental Fig. 6). Alternatively, mice exposed to DRA-O₃ had decreased type 2 innate lymphoid cells (Fig. 6E), which may be important given that a subset of these cells has been shown to produce IL-10 in response to allergic inflammation (Seehus et al., 2017). In summary, these data suggest that O₃ may

exacerbate airway hyperreactivity by decreasing immune cells that are responsible for the production of counter-regulatory cytokines during allergic inflammation.

4. Discussion

Asthma, specifically allergic asthma, is a major health concern. Although new therapeutics have been developed for allergic phenotypes of asthma, few established murine models of allergic inflammation have focused on preventing asthma exacerbations triggered by air pollution. Clinically asthma exacerbations are characterized by increased symptoms (e.g. cough, wheezing, dyspnea) and impaired lung function. Experimentally asthma exacerbations are typically modeled by exposing laboratory animals to a known asthma trigger (e.g. environmental allergen, viral infection, etc) in the setting of pre-existing inflammation. This second hit typically leads to increased inflammation, impaired lung function, or both. To model how air pollution can lead to asthma exacerbations, we developed a novel model where mice with allergic inflammation due to sequential DRA exposure were exposed to ozone. Using this model, we found differences in the recruitment of inflammatory cells, airway hyperreactivity, and compensatory anti-oxidant and anti-inflammatory pathways. By exposing mice to O₃ in the background of mixed eosinophilic and neutrophilic inflammation, we induced an acute worsening in airway hyperreactivity. This suggests that O₃ has a deleterious effect on mice with allergic inflammation.

Other groups have reported two-hit allergic mouse models involving O₃ exposure (Last et al., 2004; North et al., 2011), but there is inconsistency regarding the type of inflammation and how this correlates to human disease. Previous work has shown that a combination of common environmental allergens induced a mixed inflammatory phenotype, and this is important because eosinophilic inflammation is a pathophysiologic hallmark of atopic asthma in patients (Wardlaw et al., 1988) and these cells are not always present in murine models. Li et al. (Li et al., 2019) showed that O₃ exposure following allergic inflammation induced by ovalbumin (OVA) led to increased mucus production, airway inflammation, and increased airway hyperreactivity. However, the BAL inflammatory profile consisted of lymphocytes instead of eosinophils. Last et al. studied the effects of a prolonged O₃ exposure over several weeks following OVA sensitization and found a mixed inflammatory infiltrate that consisted of macrophages but did not examine airway hyperreactivity (Last et al., 2004). These findings provide insight into the effects of O₃ on allergic inflammation but are limited by the use of OVA (Kumar et al., 2016). Our study examines the effects of O₃ following intranasal sensitization and challenge with DRA, which is known to contain common antigenic triggers for asthmatics (Common Asthma Triggers, 2020). Our findings are consistent with the work of Keirstein et al who showed that mice with eosinophilic lung inflammation due Aspergillus exposure alone had increased airway hyperactivity following subsequent O₃ exposure (Keirstein et al., 2008). These

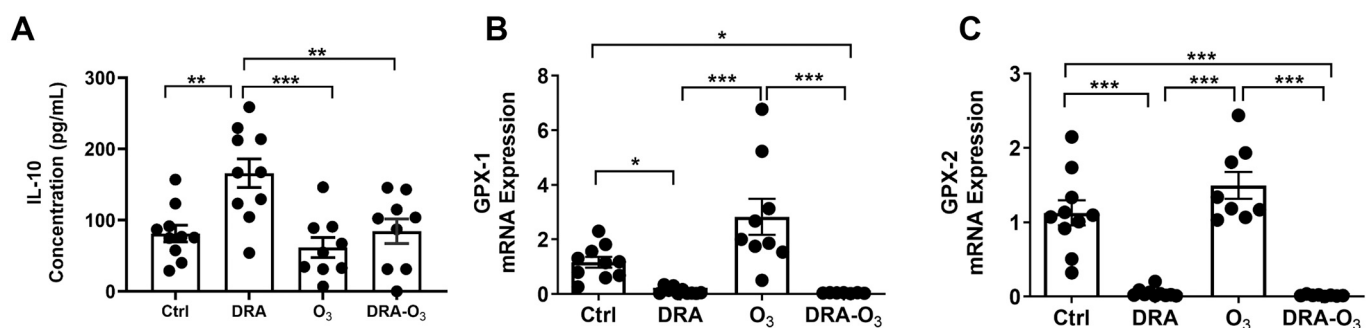


Fig. 5. Ozone exposure following DRA challenge impairs endogenous anti-oxidant and anti-inflammatory responses. WT male mice were untreated (n = 10) or challenged with DRA-O₃ (n = 9), DRA (n = 10), or O₃ (n = 9), and whole lung tissue was collected and compared against whole lung isolated from control mice. (A) IL-10 was assessed by ELISA. Expression of (B) GPX-1 and (C) GPX-2 was measured by qPCR. Error bars shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

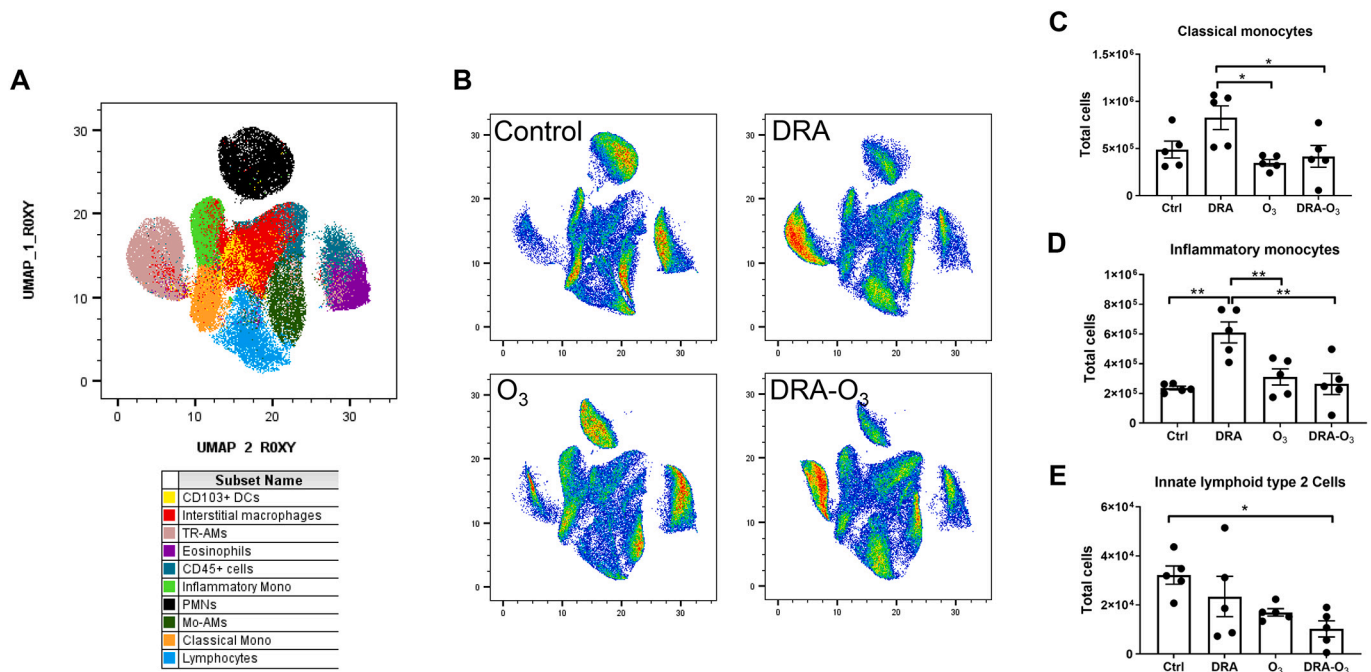


Fig. 6. Ozone exposure following DRA challenge impairs endogenous anti-oxidant and anti-inflammatory responses. WT male mice were either untreated (control, $n = 5$) or challenged with DRA ($n = 5$), O₃ ($n = 5$) or DRA-O₃ ($n = 5$) and the populations of immune cells were determined by flow cytometry. **A)** Combined UMAP multidimensional reduction of all Live CD45+ cells with specific cellular populations highlighted by flow gating strategies. **(B)** UMAP of individual treatment groups displayed in a heat map where increased cell numbers are shown in hot (red-yellow) colors and lower cell numbers are shown in cool (blue) colors. Total number of classical monocytes (C), inflammatory monocytes (D) or innate lymphoid type 2 cells (E) were measured in each group ($n = 5$ mice/group). Error bars are shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

data suggest that O₃ exposure in the setting of allergic inflammation can lead to impaired lung function which is a hallmark of asthma exacerbations. To our knowledge, we are the first to report a two-hit mouse model where mice were exposed to O₃ following the induction of eosinophilic inflammation with DRA.

Signaling via a variety of different pathogen recognition receptors regulates expression of cytokines. Toll-like receptor (TLR) signaling pathways are involved in the initiation of inflammatory responses to a variety of pathogens, allergens and environmental exposures within the lungs. However, the role of these pathways can be difficult to investigate because both structural cells and immune cells express different receptors and expression of these receptors can change throughout disease progression. In addition, TLRs can exert dual roles during asthma exacerbations with some receptors causing tolerance or sensitization, while others promote inflammation, particularly early in life (Hammad et al., 2009). Our model demonstrates that the O₃-induced exacerbation of DRA-induced allergic inflammation is independent of TLR4 signaling pathways (Fig. 4B). Additionally, there was no difference in expression of TLR4-induced pro-inflammatory cytokines (i.e. IL-1 β and IL-33) in our dual hit model (Supplemental Fig. 4C). Previous work has shown that other TLRs (Whitehead et al., 2020; Oakes et al., 2013) play a role in both asthma and O₃ lung inflammation. In addition, recent work (Hussein et al., 2020) has shown that TLR4 heterodimerizes with other TLR receptors to modulate environmental lung injury. Additional work is needed to examine how TLR receptors other than TLR4 play a role in ozone induced asthma exacerbations.

There are several possible mechanisms to explain the physiologic and histopathologic findings observed in our DRA-O₃ model. One potential mechanism of lung injury is that allergic inflammation from DRA exposure impairs endogenous anti-oxidant pathways that normally protect the lung when it is exposed to high levels of O₃. Multiple studies have shown the importance of the anti-oxidant response in protecting the lung from oxidative stress, particularly O₃-induced lung injury. Bao et al. (Bao et al., 2017) showed that exposure to O₃, OVA, or OVA

followed by O₃ increased levels of GPX compared to control. Previous data demonstrated that injurious exposures alone (O₃ or OVA) or in combination (O₃-OVA) (Lang et al., 2002) caused elevated levels of anti-oxidant enzymes. In our model following O₃ exposure alone, we observe significant increases in GPx-1 and GPx-2 (Fig. 5B and C), but there was no increase in anti-oxidants in whole lung homogenates following DRA or DRA-O₃. Similarly, IL-10, an anti-inflammatory cytokine, is critically involved in lung injury and asthma (Nie et al., 2012; Ogawa et al., 2008). There was significantly elevated IL-10 in the lung homogenates after DRA exposure alone but not O₃ group (Fig. 5A). There was no difference in the amount of IL-10 in the DRA-O₃ treated group. Together, this suggests that O₃ in the setting of allergic inflammation inhibits endogenous cytoprotective responses.

Our study characterized the how O₃ exposure in the setting of allergic inflammation altered the composition of immune cells in both the BAL and whole lung digests. To our knowledge, our study is the first to comprehensively profile immune cell populations within the lung after a 2-hit model using O₃ exposure as a trigger. Although there was a significant increase in the number of T cells in the lungs of mice exposed to both DRA and DRA-O₃ challenge (Supplemental Fig. 6B), there was no difference in total CD4+ or CD8+ T cells between these two groups. Previous work has demonstrated different CD4+ T cell subsets regulate allergic inflammation, and additional studies are underway to determine if there are different Th cell subsets in the different treatment groups. Innate lymphoid type 2 cells (ILC2s) are involved in allergic inflammation as well as O₃-induced eosinophilic inflammation and goblet cell hyperplasia (Yang et al., 2016a), however, ILC2s have not been examined in our current model. Our data demonstrates a significant decrease in ILC2 after DRA-O₃ (Fig. 6E). This is consistent with the fact that there were lower levels of IL-33, a known activator of ILC2s (Yang et al., 2016a), in mice subjected to our 2-hit model (Supplemental Fig. 4B). Sex hormones regulate the number, function and phenotype of ILC2s, and C57Bl/6 male mice have less ILC2s compared to female mice (Yang et al., 2016b). Our study also found a significant increase in mast cells

after DRA-O₃ exposure. Mast cells are known to be localized within the smooth muscle bundles of asthmatic patients and play an important role in inflammation, bronchoconstriction and mucus secretion. In addition to degranulation, mast cells also produce a variety of cellular mediators (Elieh Ali Komi and Bjerner, 2019), such as TSLP, IL-33, IL-4, and IL-13, which were found in our 2-hit model and have an important role in the development of ozone-induced lung injury (Kong et al., 2021). Additional work is in progress to determine the role of ILC2s and mast cells in this model.

As shown previously (Takeda et al., 2013), our work demonstrated that mice exposed to DRA alone had a significant increase in macrophages and monocytes in the lung (Fig. 6C and D, Supplemental Fig. 5B). The origin of various lung macrophage subpopulations within the lung microenvironment regulates allergic inflammation (Lee et al., 2015), with circulating monocytes promoting allergic inflammation and resident myeloid cells dampening the immune response. Different subsets of monocytes regulate immune responses in the lung, and markers to distinguish these subpopulations vary according to species (Zaslona et al., 2014). In humans, expression of the surface markers CD14, LPS binding protein, and CD16, an Fc-receptor, distinguished classical (CD14 + CD16⁻), non-classical (CD14^{dim}CD16⁺) and intermediate (CD14 + CD16⁺) monocytes. In mice, other surface markers can distinguish classical/patrolling (CD11b + Ly6c^{lo}) versus inflammatory (CD11b + Ly6c^{hi}) monocytes. In murine studies, it has been shown that Ly6c^{lo} monocytes are present at baseline, and the presence of Ly6c^{hi} monocytes increases in the setting of injury or inflammation. There was a significant decrease in the expression of both the classical and inflammatory monocytes after DRA-O₃ exposure. Although previous work demonstrated that O₃ exposure resulted in novel macrophage subpopulations being recruited to the lung microenvironment (Yang et al., 2014), 24 h after exposure there was no increase in macrophage or monocyte subpopulations within the lung tissue. Alternatively, exposure to the mixed allergens results in significant increases in all myeloid subpopulations within the lung. These studies suggest an important role for macrophages within the lung during environmental exacerbations of lung disease.

There are several limitations to this study. First, the concentration of O₃ we used is several times higher than O₃ levels during days with high air pollution, which may limit the clinical applicability of the study. However, 2 ppm for 3 h is equivalent to a O₃ concentration of 400 ppb in humans (Hatch et al., 2013) and has been used by other groups suggesting our results are generalizable. Studies are ongoing to currently investigate this model using a range of O₃ exposures more in line with the NAAQS guidelines. Second, the ideal DRA concentration and administration timing is currently unknown. Because we used a single timepoint, allergic inflammation may not have peaked before O₃ exposure and methacholine challenge. This is consistent with data from other groups that found peak neutrophil inflammation following O₃ exposure occurred at 12 h; whereas the peak macrophage inflammation occurs at 48 h post O₃ (Yang et al., 2016b; Tighe et al., 2011). Despite this limitation, we were still able to observe significant histopathologic changes with our DRA protocol and increased AHR with O₃ exposure following DRA sensitization. Third, we did not test for allergen-derived proteases and the effect this would have in the sensitization of mice in our triple antigen model. Previous work has demonstrated that proteases, such as serine proteases and metalloproteinases, can disrupt lung epithelial cell barriers driving the allergenicity and inflammatory responses and additional work is needed to determine their role in our triple antigen model (Matsumura, 2012; Gaspar et al., 2020). Fourth, we used C57/BL6 mice, which are known to have more of a proinflammatory phenotype at baseline and are more resistant to type 2 airway inflammation. Performing these experiments in other wild-type strains that are more susceptible to allergic inflammation would provide additional information regarding this phenotype (Birukova et al., 2019). Finally, we performed all of our experiments with male mice as an initial first step. Other groups have described sex differences in mouse models of allergic

inflammation (Yang et al., 2016b; Teufelberger et al., 2020) and O₃-induced lung injury (Fuentes et al., 2019; Cabello et al., 2015; Fuentes et al., 2018). Examining whether there are sex differences in our 2-hit model will be an important next step.

In summary, O₃ is a common environmental trigger for asthma exacerbations, but the underlying mechanisms aggravating these underlying chronic lung disease is unclear. Using a novel, two-hit murine model, we demonstrated that O₃ exposure following the induction of allergic inflammation resulted in increased AHR and induced histopathologic changes that mirror human allergic asthma exacerbations. O₃ exposure in the setting of allergic inflammation impaired the endogenous release of IL-10 that accompanies DRA exposure alone. DRA exposure impaired the upregulation of anti-oxidant enzymes that normally occurs following O₃ exposure. Future studies will be needed to determine the mechanisms responsible for these findings. Using models such as the one we describe may help to identify novel therapeutic targets for asthmatic patients to prevent exacerbations in response to environmental pollutants.

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Impact statement

These studies demonstrate that allergic asthma can be exacerbated by an environmental pollutant, such as ozone. Ozone-induced asthma exacerbation is due to an impaired endogenous anti-inflammatory and antioxidant response as well as a differential recruitment of immune cells into the lung.

CRediT authorship contribution statement

Kevin Ho: Investigation, Data curation, Writing – original draft, Writing – review & editing. **David Weimar:** Investigation, Data curation. **Gina Torres-Matias:** Investigation. **Hyunwook Lee:** Investigation. **Saaleha Shamsi:** Investigation. **Emily Shalosky:** Investigation. **Michael Yaeger:** Investigation. **Hannah Hartzler-Lovins:** Investigation. **Katelyn Dunigan-Russell:** Investigation. **Daria Jelic:** Investigation, Data curation. **Caymen M. Novak:** Investigation. **Kymberly M. Gowdy:** Conceptualization, Methodology. **Joshua A. Englert:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Megan N. Ballinger:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2022.116341>.

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