Ozone Generation Method Impacts Lung Toxicity and Oxidant Signaling

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Abstract

Ozone (O_3) is a criteria pollutant that is anticipated to increase over the next decade because of climate-related activity. Varying amounts of nitrogen oxides (NOx) are produced as byproducts during O_3 generation from oxygen depending on the method of production, including the source and oxygen purity. A review of the current literature confirms a lack of consistent monitoring and reporting of potential nitrogen species produced with different methods of experimental O_3 generation. The lack of consistent monitoring and reporting is potentially a factor that can explain divergence of reported experimental O_3 exposure outcomes from different research groups. In the present report, we compare the effects of O_3 generation from either a filtered air (FA-ozone) or a pure oxygen (Oxy-ozone) source on NOx generation and measures of O_3-induced lung injury. We also consider if this impacts mixed exposures with O_3 and ultrafine carbon black (CB) based on if the O_3 was generated from a filtered air (FA-ozone-CB) versus a pure oxygen (Oxy-ozone-CB) source. Comparing FA-ozone versus Oxy-ozone, we observed increased lung inflammation and injury in the FA-ozone group. In the FA-ozone-CB group, compared with the Oxy-ozone-CB group, the FA-ozone-CB inhalation exposure resulted in the formation of a greater amount of NOx and induced protein nitrotyrosine in the lungs. Moreover, the FA-ozone-CB group had evidence of eosinophil recruitment not observed in the Oxy-ozone-CB group. Overall, this suggests that the source of oxygen for O_3 generation impacts experimental outcomes. Furthermore, measurement and reporting of nitrogen species in O_3 exposure should be considered.

Keywords: ozone; carbon black; inhalation exposure; nitrogen oxides; inflammation

Ozone (O_3) is a criteria pollutant that is anticipated to increase over the next decade because of climate-related activity (1). O_3 is one of the most reactive gaseous components of air pollution and is associated with both acute and chronic health impacts in susceptible as well as healthy subjects. Apart from acute irritant effects on the mucosal membranes, O_3 is also reported to induce exacerbation of respiratory and a variety of other chronic illnesses (2–4).

In experimental exposure studies, O_3 is typically generated using corona discharge or ultraviolet (UV) radiation. However, these methods have varying potential to produce nitrogen oxides (NOx), depending on whether pure oxygen or room air is used as a gas source. Unfortunately, studies focused on O_3-induced biological outcomes mostly ignore these nitrogen species generated by different gas sources in their experimental

(Received in original form December 19, 2024; accepted in final form June 9, 2025)

Supported by National Institutes of Health National Institute of Environmental Health Sciences grants R01ES031253 (S.H.), R01ES03125304S1 (S.H.), and R01ES034350 (R.M.T.).

Author Contributions: Conceptualization and design: S.H. Performance of experiments: A.R., M.H.H.M., W.T.G., N.O.T., A.C.R., V.K., M.V., and Q.W. Analysis and interpretation: W.T.G., R.M.T., Q.W., and S.H. Drafting and review of manuscript: all authors.

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This article has a data supplement, which is accessible at the Supplements tab.

Artificial Intelligence Disclaimer: No artificial intelligence tools were used in writing this manuscript.

Am J Respir Cell Mol Biol Vol 74, Iss 1, pp 18–26, January 2026

Originally Published in Press as DOI: 10.1165/rcmb.2024-0633MA on June 9, 2025
Internet address: www.atsjournals.org

design. Studies using filtered air/room air or oxygen to generate O_3 show a differential magnitude and sometimes distinct outcomes from O_3 exposure (5). This is a potentially important effect modifier. A careful literature review indicates a lack of consensus guidelines on the optimal gas source for O_3 generation for studies aimed at evaluating the health effects of air pollution.

In addition, O_3 is just one component of air pollution, and, therefore, mixed-pollutant responses and the source of their generation must also be considered in experimental air pollution studies. The source and purity of air is important in both individual as well as mixed exposures, and, therefore, there is a strong rationale for considering O_3 generation methodology in single and mixed-pollutant exposures because of the impact of inadvertent NOx generation. Given that particulate matter and O_3 are the most common components of air pollution, experimental particle and O_3 mixed exposures closely represent the inherent particle and gas mixture of air pollution. The use of an ultrafine particle and gas mixed inhalation exposure system offers added relevance compared with individual particle and gas exposures by: *1*) providing the surface for the chemical reaction between various components of air pollution; *2*) providing a potential carrier system for transport of gaseous and other components of air pollution to the deeper lung parts; and *3*) providing a system for studying interactions between different components of air pollution. Our recent work demonstrated a more-than-additive impact of ultrafine carbon black (CB) and O_3 coexposure, resulting in a significantly greater pulmonary inflammation and lung function decline through an oxidant-alarmin pathway (6).

In the present work, we sought to investigate whether the O_3 generation method impacts biological outcomes using two independent exposure scenarios (i.e., pure O_3 and an ultrafine CB and O_3 mixed exposure) and two sources or purities of oxygen source (O_3 generation from room air containing 21% oxygen vs. pure medical grade oxygen).

Methods

Murine Model and Exposure Conditions

Male C57BL/6J mice (8–10 wk old) were purchased from Jackson Laboratory. All animal procedures, including housing, sedation, euthanasia, and experimentation, were approved by the West Virginia University Animal Care and Use Committee. Mice were divided into the following groups: *1*) FA: filtered room air; *2*) FA-ozone: 2 ppm O_3 generated using compressed filtered room air; *3*) Oxy-ozone: 2 ppm O_3 generated using pure medical grade oxygen; *4*) RA-ozone-CB: an aerosol of ultrafine CB (10 mg/m^3) and 2 ppm O_3 generated using compressed filtered room air; and *5*) Oxy-ozone-CB: an aerosol of ultrafine CB (10 mg/m^3) and 2 ppm O_3 generated using pure medical-grade oxygen. Experimental exposures were performed for 3 hours. Mice were killed 24 hours after exposure by intraperitoneal injection of Fatal-Plus (250 mg/kg) and then evaluated for exposure-related outcomes.

Whole-Body Inhalation Exposure System

A whole-body inhalation exposure system (HPAG; IEStechno) used previously (6, 7) for mixtures of CB aerosols and O_3 gas was modified to use either clean room air (~21% O_2) or medical grade O_2 as a feed for the O_3 generator. A diagram of the system is shown in Figure 1. A total of 50 ml of bulk CB dust (Printex 90, 14 $\pm$ 4 nm primary particle diameter, donated from Evonik) was placed on the drumhead of a high-pressure acoustic generator. The subwoofer within the HPAG created acoustic energy, which deagglomerated the dust. Dried and filtered compressed air was passed through a mass flow controller (MFC) and then through the HPAG, where the aerosolized CB was captured in the airstream. The compressed air was also pushed through a separate MFC and into the high-pressure port of a venturi pump (JS-60 M; Vaccon), which functioned as both diluent air and a further stage for particle deagglomeration, because the low-pressure port of the venturi was pulling the air–CB mix from the HPAG. A corona discharge generator (HTU500AC; Ozone Solutions) was used to produce the O_3 gas. The generator input feed gas was passed through an MFC by either: *1*) a tank of pure medical-grade O_2 (Oxy); or *2*) the compressed filtered room air source (FA) used in other areas of the exposure system. The CB aerosols were then mixed with the O_3 gas (mean mixing residence time of ~5.5 min) before being passed into a stainless-steel exposure chamber with up to 36 mice being individually housed within a stainless-steel cage rack. Animal comfort was maintained by regulating the chamber temperature (20–22°C) and relative humidity (50–70%) (HMT330; Vaisala). Chamber and HPAG pressure (Model 264; Setra Systems, Inc.) were also monitored and controlled during exposures. The chamber exhaust was high-efficiency particulate air and charcoal filtered before passing through an MFC attached to the house vacuum. A graphical user interface displayed and recorded relevant exposure data. A data acquisition card (USB-6343; National Instruments) processed analog inputs and outputs. The system could be used to expose mice to varying concentrations of CB alone, O_3 alone, or CB–O_3 mixtures. Multiple control systems were automatically used to regulate system conditions. Separate custom proportional-derivative software controllers were tuned for each of the conditions of interest. The pressure within the HPAG was held at 0.5 inches of H_2O to optimally aerosolize and deagglomerate the aerosols. This was done by varying the flow through the HPAG MFC. The pressure within the exposure chamber was also maintained at a constant level (0.0 inches of H_2O) by adjusting the exhaust flow MFC. This ensured that no leaks would be present in or out of the chamber, which also served as an additional protective measure for technician safety. Real-time readings of the aerosol mass concentration were relayed to the controller, and the acoustic energy was modulated to maintain a constant user-defined aerosol concentration. O_3 levels were also sustained by automatically adjusting the input flow of compressed room air or oxygen feeding the O_3 generator. To evaluate exposures, aerosol and chemicals for given conditions were sampled and analyzed with real-time as well as off-line assessments.

Aerosol and Gas Characterization

An aerosol mass monitor (pDr-1500; Thermo Environmental Instruments Inc.) sampled this mixed air to provide a real-time estimate of the CB mass concentration. The particle count concentration was also monitored continuously with a condensation particle counter (CPC 3075; TSI Inc.). Real-time measurements were conducted for both O_3 (Model 202; 2B Technologies, Inc.) and the NOx gases (Model 405; 2B Technologies, Inc.) during exposures. Gravimetric measurements were conducted by sampling air onto closed-faced polytetrafluoroethylene 37-mm filters with a 0.45-μm pore size (SKC Inc.) during exposures to calculate the gold standard average mass concentration of the aerosols. This gravimetric measurement was

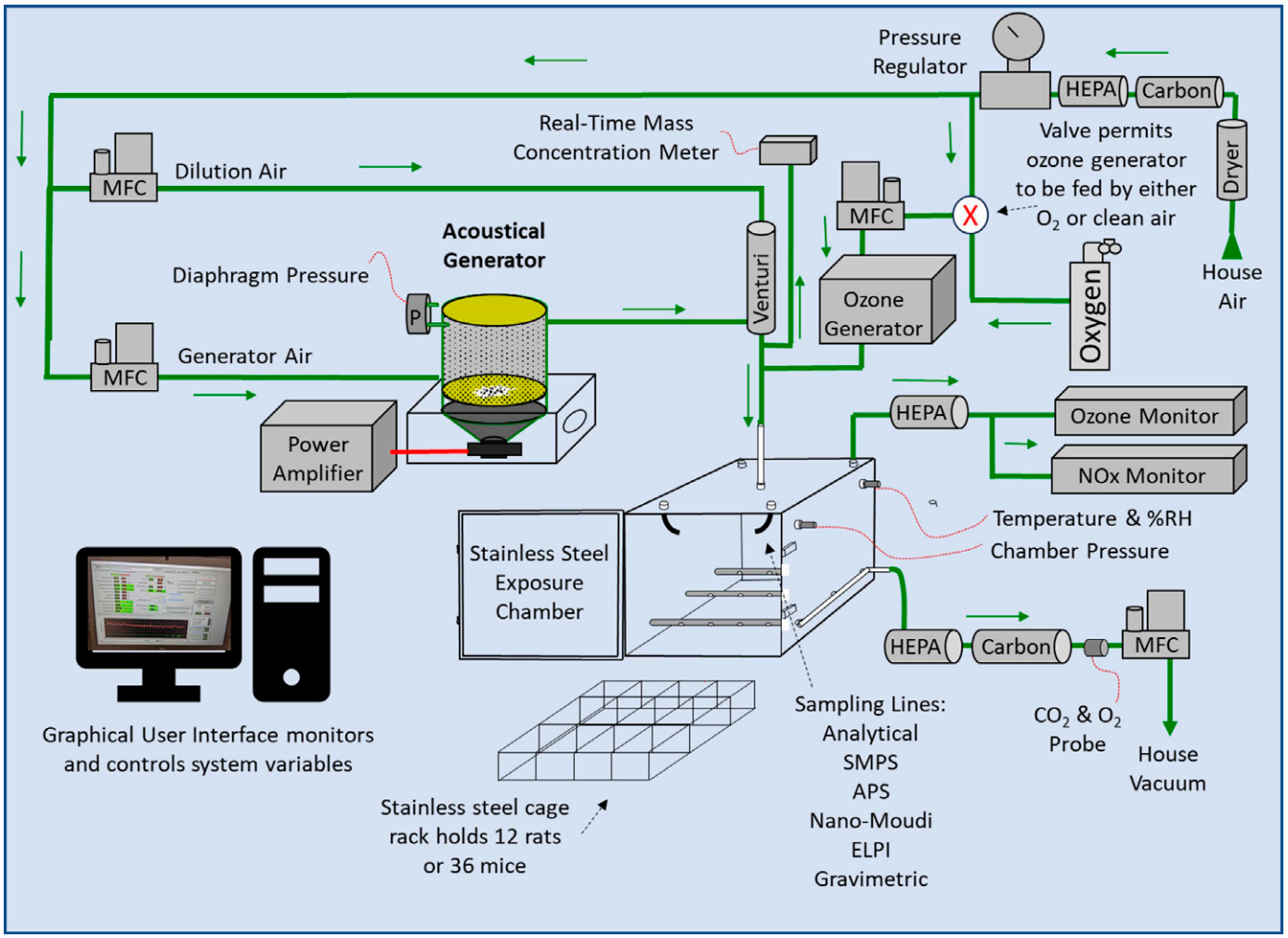


Figure 1. Inhalation exposure system. Schematic of the whole-body acoustic generator–based inhalation exposure system for animal exposure to particulates (carbon black [CB]), ozone [O_3], or CB + O_3 in a reliable and repeatable manner. HEPA = high-efficiency particulate air; MFC = mass flow controller; NO_X = total nitrogen oxide; RH = relative humidity.

also used for the daily calibration of the pDr-1500 real-time device. Aerosol size distributions were measured with multiple instruments for the CB–O_3 particles. A high-resolution electrical low-pressure impactor (ELPI+; Dekati) counted particles across a wide size range (6 nm–10 μm). A combination of a scanning particle mobility sizer (SMPS 3938; TSI Inc.; range, 13–743 nm) and an aerodynamic particle sizer (APS 3321; TSI Inc.; range, 370 nm–20 μm) was also used to determine the count size distribution over a similar range. The aerosol mass size distribution was measured with a nano micro-orifice uniform deposit impactor (MOUDI 115R; MSP Corp.).

Chemical Characterization

Real-time total chemical assessment provides evolution for a broad range (i.e., ⩾900) of "volatiles" during exposure over time. Total airborne chemical exposure was analyzed using PID (photoionization detection; ION Science Ltd.) for real-time assessment. Chemical speciation was performed off-line with a headspace sampler coupled to a gas chromatography/mass spectrometer (GC-MS, Model# 7697A, Model #8890, Model# 7000, respectively; Agilent Technologies Inc.), with suitable methods for volatile organic and volatile carbonyl compounds.

Fourier-Transform Infrared Spectroscopy

The Fourier-transform infrared spectroscopy (FTIR) measurements were performed using a DIGILAB FTS 7000 FTIR spectrometer equipped with a GladiATR Attenuated total reflectance module from PIKE Technologies. Spectra were collected over a wavenumber range of 4,000–500 cm^{-1}, performing 128 scans at a resolution of 4 cm^{-1}. All measurements were conducted at room temperature in ambient conditions.

Methods for BAL fluid collection and analyses, immunoblotting for 3-nitrotyrosine (3-NT) levels, and real-time PCR analyses are provided in data supplement.

Cytokine Assessment

Multiplex analysis of cytokine and chemokine levels in the BAL fluid at 24 hours after exposure was performed using Eve Technologies (#RD27) Mouse Cytokine/Chemokine 32-Plex Discovery Assay Array #MD32 (Millipore Sigma) using the Luminex 200 system (Luminex). Detailed methods are provided in data supplement.

Ferric Reducing Ability of Serum

Ferric reducing ability of serum assay on mouse serum was performed as described by us previously (6).

Electron Paramagnetic Resonance Spectroscopy

The oxidizing potential of mouse BAL fluid was measured by electron paramagnetic resonance spectroscopy using 1-hydroxy-3-carboxymethyl-2,2,5,5-tetramethyl-pyrrolidine spin probe (Enzo Life Science) as described previously and detailed in the supplemental methods (6, 8).

Results

Aerosol and Gas Characterization

Realtime monitoring of CB + O_3 aerosols demonstrated stable exposure to CB and O_3 during the exposure period (Figures 2A and 2B). Detailed physicochemical analyses demonstrated no significant differences in aerosol mass and size distributions, and chemical assessments (total and speciation) between compressed FA and Oxy-generated CB + O_3 aerosols (i.e., FA-ozone-CB vs. Oxy-ozone-CB; Figures 2C–2E and Table E1 in the data supplement). Both volatile organic ($\leqslant$0.02 μg/ml) and carbonyl chemicals ($\leqslant$0.05 μg/ml) were below detection limits for all exposure conditions. Low average background levels of NO (6.31 $\pm$ 1.35), NO_2 (4.58 $\pm$ 0.79), and overall NOx (10.89 $\pm$ 1.44) were detected in our inhalation facility (Figure 2F). Real-time measurement of these nitrogen species demonstrated an increase in NO (8.51 $\pm$ 1.61), NO_2 (11.41 $\pm$ 2.27), and overall NOx (19.93 $\pm$ 3.56) in FA-ozone-CB exposures, whereas Oxy-ozone-CB exposure levels of NO (5.17 $\pm$ 1.76), NO_2 (4.85 $\pm$ 0.79), and overall NOx (10.02 $\pm$ 1.88) were similar to background (Figures 2F and 2G). In the case of O_3 generation without CB (i.e., FA-ozone and Oxy-ozone), there was no significant increase in NO, NO_2, and NOx over background levels (Figure E1). Particle count concentrations were not significantly different between FA-ozone-CB and Oxy-ozone-CB (2.1×10^5/ml and 2.2×10^5/ml particles, respectively) (Figure 2I). All significant absorbance peaks were attributed to carbon- and oxygen-related bonds after FTIR using attenuated total reflectance (Figure 2J). Notably, all O_3–CB samples (i.e., FA-ozone-CB and Oxy-ozone-CB) exhibit pronounced carbon–oxygen bonds, indicating a strong interaction or reaction facilitated by the O_3 treatment. FA-ozone-CB samples demonstrated qualitatively a stronger signal but did not result in any unique functional groups.

Lung Inflammation, Injury, Redox Imbalance, and NO_2 Production in CB + O_3 Coexposure

CB + O_3 exposures, irrespective of the source of oxygen (i.e., FA-ozone-CB and Oxy-ozone-CB), increased the total cells and neutrophils in the lavage of mice; however, only FA-ozone-CB induced a significantly greater number of eosinophils than Oxy-ozone-CB (Figure 3A, inset). Real-time PCR analyses demonstrated significant induction of *IL-5* only in the case of FA-ozone-CB (Figure 3B). BAL multiplex/ELISA assays demonstrated significant GCSF, KC, IL-6, Eotaxin, and LIF release that was increased to a similar extent in the FA-ozone-CB and Oxy-ozone-CB exposures (Figure 3C). However, BAL TSLP, IL-33, TNF-α, IL-13, and VEGF were only significantly increased by Oxy-ozone-CB exposure, and IP-10 was increased only after FA-ozone-CB. FA-ozone-CB induced significant cytotoxicity, as evidenced by increased BAL LDH levels (Figure 3D). In FA-ozone-CB versus Oxy-ozone-CB, we observed significantly higher free radical generation and decreased serum antioxidants as assessed by electron paramagnetic resonance spectroscopy and ferrous reducing ability of serum assay, respectively (Figures 3E and 3F). A significantly greater induction of 3-nitrotyrosine indicating protein tyrosine nitration was observed only in the FA-ozone-CB exposures (Figure 3G).

Validation Using O_3-Only Exposure

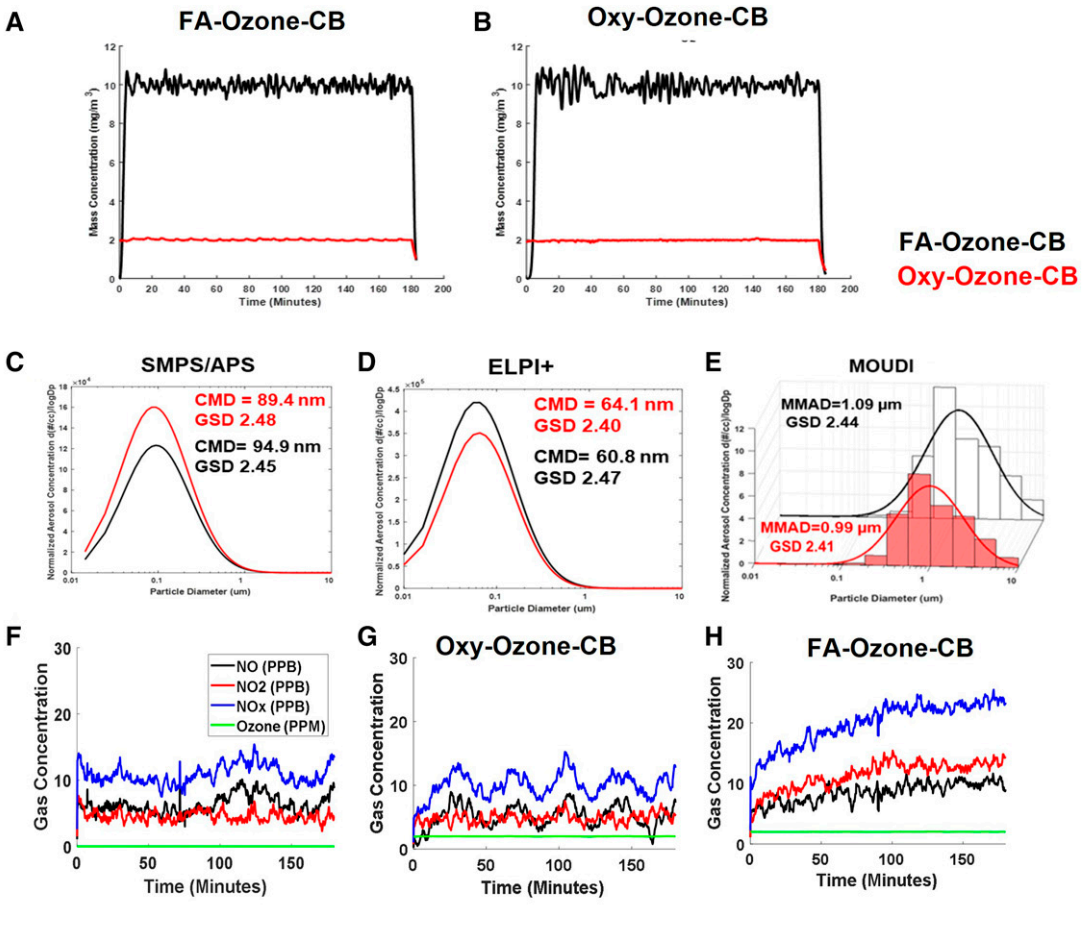
Next, we validated these findings in an O_3 exposure model without the addition of carbon black. O_3 exposure irrespective of the gas source (i.e., FA-ozone and Oxy-ozone exposures) increased the total cells and neutrophils in the lavage of mice; however, FA-ozone induced a significantly greater number of neutrophils than Oxy-ozone (Figure 4A). Similarly, BAL total proteins and LDH levels were significantly elevated in FA-ozone compared with Oxy-ozone (Figures 4B and 4C).

Discussion

In this manuscript, we detail a robust methodology for air pollution component aerosolization and whole-body inhalation exposure. Moreover, we describe unique inflammatory signatures when pure oxygen versus filtered room air is used to generate O_3. We report here increased NOx, NO, and NO_2 formation; eosinophil infiltration in the lungs; and 3-NT formation in mixed CB and O_3 exposures when O_3 generation was performed using filtered room air (i.e., FA-ozone-CB) versus when the O_3 was generated using a pure oxygen source (i.e., Oxy-ozone-CB). In addition, in the case of individual O_3 exposure, we report significantly greater neutrophil counts, LDH, and total proteins in the BAL of mice exposed to filtered room air–generated O_3 (i.e., FA-ozone) compared with pure oxygen–generated O_3 (i.e., Oxy-ozone). Our results suggest increased oxidant/free radical production potentials, nitrosylated tyrosine-containing proteins, lung inflammation, and injury when filtered room air is used for O_3 generation (FA-ozone and FA-ozone-CB) compared with pure O_2-based O_3 generation (Oxy-ozone and Oxy-ozone-CB).

This work was aimed at highlighting potential differences in experimental exposure systems that could be a driver of interlaboratory differences in exposure responses and also could be used to address specific experimental questions. We highlight this using our exposure system that can be interchangeably used for O_3 or ultrafine particle + O_3 exposure with or without the presence of NOx based on the methods of O_3 generation. We successfully validated the system in which we are measuring nitrogen species (NOx, NO, NO_2) while performing O_3 or mixed O_3 and CB inhalation exposures. This is potentially important for translational modeling of human exposures, as increased mortality, emergency room visits, and acute exacerbations of respiratory disorders have been reported when mixed effects of pollutants including particulate matter $\leqslant$2.5 μm in aerodynamic diameter, O_3, and NO_2 were evaluated (9–11). This system also offers the added advantage of real-time monitoring as well as automated feedback loop–based correction of exposure levels to the set point using our validated software. This is a considerable improvement over the majority of the exposure systems currently used in studies, as they require manual adjustment during the exposure.

Our findings confirmed an extensive inflammatory response in the lungs irrespective of the source of oxygen in both ozone-only and mixed-exposure scenarios.



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Exposure	Particle count Particles/cc (SD)
Background/Air	1.3 (0.29)
Oxy-Ozone	4 (4.1)
FA-Ozone	9.2 (32)
Oxy-Ozone-CB	$2.2x10^5$ ($1.1x10^4$)
RA-Ozone-CB	$2.1x10^5$ ($3x10^4$)

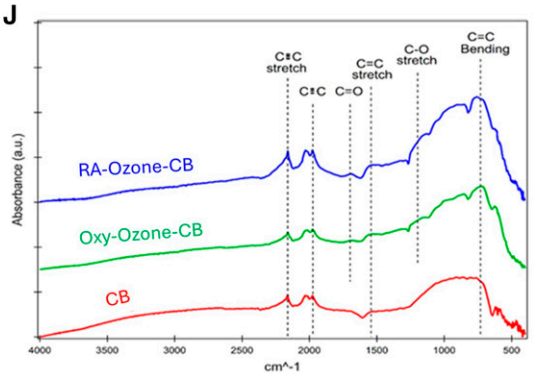

Figure 2. Aerosol monitoring and characterization. Real-time CB and O_3 chamber concentrations when (*A*) filtered air (FA) or (*B*) pure oxygen (Oxy) were used as source of oxygen. Aerosols of CB + O_3 (10 mg/m^3 CB + 2 ppm O_3) were generated using Oxy-ozone-CB or FA-ozone-CB. Size distribution of the same aerosols based on (*C*) count mobility and aerodynamic diameter (SMPS/APS), (*D*) count charge and aerodynamic diameter (ELPI+), and (*E*) mass aerodynamic diameter (MOUDI). Real-time NO, NO_2, and NO_X measurements at (*F*) background level, (*G*) Oxy-ozone-CB, and (*H*) FA-ozone-CB feed. (*I*) CPC particle counts during the whole exposure period (mean ± SD). (*J*) Fourier-transformed infrared (FTIR) spectroscopy of particles collected on Teflon filters from the exposure chamber for FTIR analysis. APS = aerodynamic particle sizer; CMD = count median diameter; ELPI+ = high-resolution electrical low-pressure impactor; GSD = geometric standard deviation; MMAD = mass median aerodynamic diameter; MOUDI = micro-orifice uniform deposit impactor; SMPS = scanning particle mobility sizer.

We observed increased eosinophils only in the BAL of FA-ozone-CB–exposed mice. Lung tissue and airspace eosinophils are frequently observed in allergic airway disease, suggesting a potential to aggravate allergic conditions, although not proven in the present study. Given that air pollution has been suggested as a model of nonatopic asthma, it is possible that this kind of exposure could drive exacerbations of a nonatopic asthma phenotype that has been described in other studies (12). Consistent

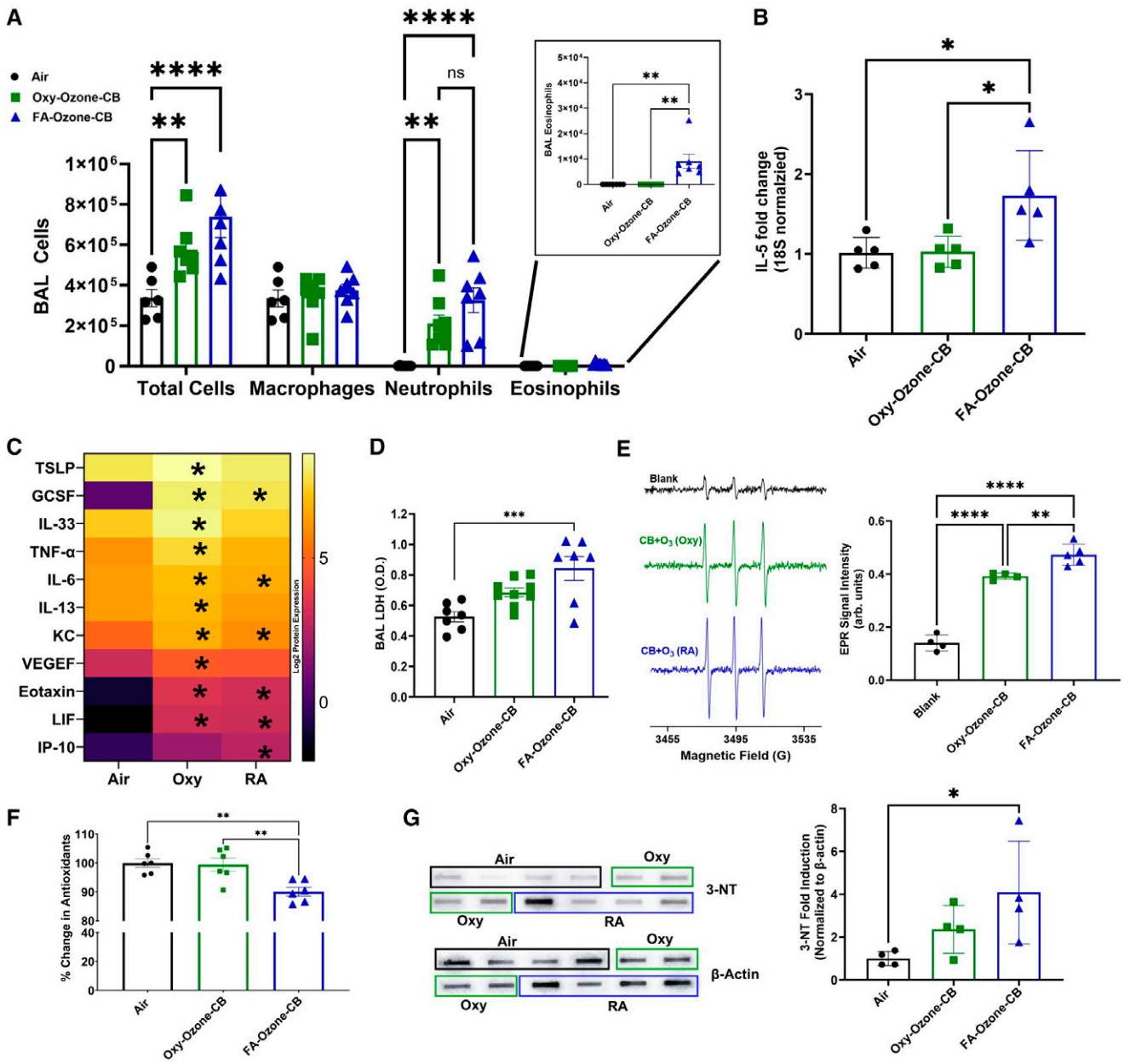

Figure 3. Pulmonary inflammation and injury after exposure to an aerosol of CB + O_3. BAL (*A*) total cells, macrophage, and neutrophil count. (*B*) *IL-5* mRNA expression by real-time PCR. (*C*) Multiplex/ELISA assay for BAL protein concentration of inflammatory mediators. (*D*) LDH release and (*E*) BAL oxidant (CM• radical) generation. Representative BAL electron paramagnetic resonance spectra and signal quantification. (*F*) serum antioxidant level assessment by ferric reducing ability of serum assay, and (*G*) lung tissue immunoblot and quantification for 3-NT at 24 hours after 3 hours of exposure to 10 mg/m^3 CB + 2 ppm O_3 aerosols generated by Oxy-ozone-CB or FA-ozone-CB. Data are presented as mean ± SEM from 4–7 mice per group and analyzed by either one-way or two-way ANOVA followed by Tukey's multiple comparisons *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. 3-NT = 3-nitrotyrosine; ns = non-significant; RA = filtered room air.

with this, eosinophil recruitment after repeated O_3-only exposure has been described in the nose and in the lungs (13, 14). In addition, we previously observed that a single O_3 exposure led to increased eosinophils in mouse lung tissue. Our observation adds to this body of literature. However, eosinophilia is frequently observed in other lung diseases beyond asthma, suggesting that this finding may not relate to asthma pathogenesis. Future studies will need to consider this in more detail and define causal roles for eosinophils. Consistent with this, we observed increased asthma-associated cytokines (i.e., IL-13, TSLP, IL-33, and TNF-α) in the Oxy-ozone-CB exposure, where there was no evidence of increased eosinophils. As elevations in these cytokines have been associated with worsened asthma severity (15–18), it is possible that these could also drive worsened

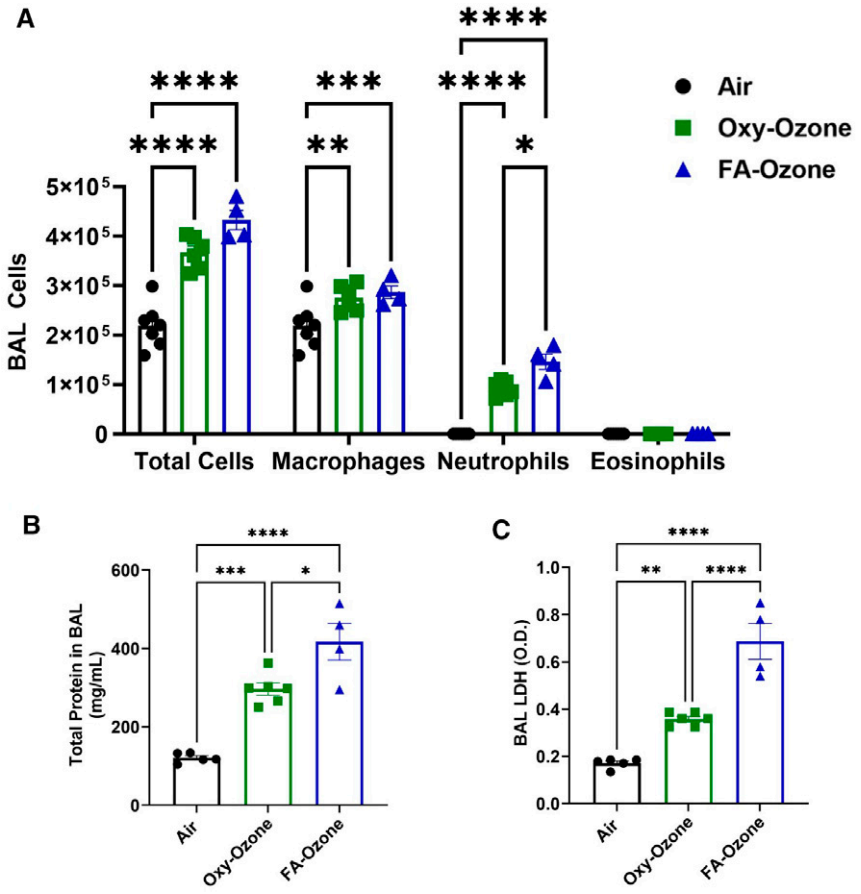

Figure 4. Pulmonary inflammation and injury after exposure to ozone alone. BAL (*A*) total cells, macrophage, and neutrophil counts; (*B*) total proteins; (*C*) and LDH at 24 hours after 3 hours of exposure to 2 ppm O_3 generated by Oxy-ozone or FA-ozone. Data are presented as mean ± SEM from 4–7 mice per group and analyzed by either Student's *t* test or one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

asthma. Alternatively, these cytokines also have been observed to modulate chronic lung inflammatory disorders, so it is possible that their increase is unrelated to asthma but may influence other chronic lung diseases. The cause and mechanisms of our observations related to different sources of O_3 generation were beyond the scope of the present study. However, this technical advance provides insight that could be used in future studies and to consider human-relevant translational exposure conditions.

Redox imbalance is one of the main mechanisms of O_3-induced biological responses (19, 20). Protein tyrosine nitration is a marker for oxidative/nitrosative stress and is involved in the pathogenesis of acute and chronic inflammatory lung diseases (21–23). O_3 exposure leads to the formation of reactive oxygen and nitrogen species in the lungs, especially in the lung macrophages that play a key role in a variety of biological reactions, including the formation of 3-NT modification of proteins (24–26). We previously reported increased oxidant generation in the lungs after O_3 alone and CB + O_3 mixed exposures (6, 7, 27, 28). Our observation of increased 3-NT–modified protein levels in the lungs of FA-ozone-CB–exposed mice confirms an eventual greater impact on protein structure and function by this exposure compared with Oxy-ozone-CB. Although the presence of NOx is well recognized in environmental settings, and the possibility of its presence in experimental exposures is always considered probable, the available literature does not provide any experimental evidence of its existence in experimental studies. We detected NO_2 as the potential difference between the two exposures, whereas other analyzed components, including gaseous pollutants and particle physicochemical characteristics, did not show significant differences (Figure 2). A list of tested compounds is provided in the supplement (Table E1). Although this is an extensive list, the formation of other compounds, especially at different exposure conditions, cannot be ruled out. However, as the chemistry of environmental reactions is complex, the formation of other chemicals in the environment is probable, as multiple sources and/or precursors (such as sulfur dioxide and volatile organic compounds) exist in the environment that can modify such reactions. A rapidly changing climate has resulted in more frequent episodes of smog, which is a classical representation of complex/heterogeneous chemistry in the environment forming complex aerosols (29). Moreover, in the environment, the reaction between O_3 and UV occurs in the presence of varying amounts of NOX, whereas in experimental settings, we are detecting it as a byproduct of the reaction. Thus, even smaller levels of nitrogen species are meaningful and may translate into higher outdoor levels.

Multiple studies have demonstrated that the adverse effects caused by air pollution follow a no-threshold dose–response curve and that people exposed to concentrations that are lower than those recommended by guidelines might still have an increased risk of morbidity and mortality (30–32). Although the chamber levels of NO_2 were low, the possibility that it is contributing to/exacerbating O_3-induced responses or sensitizing to O_3 cannot be ruled out without further experimentation. Each 1-ppb increase in annual NO_2 concentrations increased the annual risk of death by 0.003% (0.003–0.004) and translated into 1,176 attributable deaths (998–1,353) in a U.S. Medicare cohort (33). Our results are in agreement with a time series study that demonstrated synergistic effects of O_3 and NO_2 on the emergency room visits at a lag of 0–5 days (34). In addition, oxidant capacity (i.e., sums of NO_2 and O_3 [O_x]) and redox weighted oxidant capacity (O_x^{wt}) were identified as their proxies and demonstrated a significant positive correlation with emergency room visits at the entire concentration range. Similarly, a high concentration, duration of exposure, and endpoint-dependent synergistic response was observed when rats were exposed to both NO_2 and O_3 (35, 36). It is noteworthy that

interactive outcomes between O_3 and NO_2 ranging from synergism (37–39) to additive or more-than-additive outcomes (40, 41) have been consistently reported. Interestingly, we observed an increase in chamber NO_2 only when particles were present and not when O_3-only exposure was performed. Despite this, greater neutrophilia and lung injury was quantified in FA-ozone compared with Oxy-ozone. This could suggest other unknown mechanisms/processes beyond NO_2 that will require more in-depth evaluations. Air pollution exposure in the outdoor environment is always in the presence of particles. Thus, the presence of particles creates a better exposure model. O_3 and NO_2 are known to react and form various chemical species, including $NO_3^{\bullet}$ radical, N_2O_5, and HNO_3 (35, 36). Thus, the absence of a detectable increase in the levels of NO_2 does not rule out that these species were not generated during FA-ozone exposure; rather, an increase in the case of FA-ozone-CB indicates a significantly greater induction in production, where particles are potentially changing the reaction kinetics in such a way that NO_2 is available for detection. Finally, this also points toward the impact of these gases on particle chemistry, resulting in differential surface chemistry/reactivity of particles. Future mechanistic studies can shed light on these processes.

Although we are sure of the technical soundness and validity of our exposure model and system, we recognize that the biological assessments do have some limitations that are in part due to the original technical focus of the work rather than the biological mechanism focus. Some of these limitations include our choice of a relatively high dose of O_3 and CB, the use of CB and not black carbon, and the use of only one sex of mice. We believe that these limitations should be addressed in a more mechanistic-focused study and not the present technical advances article format. Follow-up studies comparing compressed laboratory air with pure oxygen–derived O_3 with the same levels of nitrogen species can further clarify the role of differential levels of nitrogen species in lung toxicity. In addition, a meta-analysis of O_3 studies after stratifying them based on source for O_2 would be informative to further emphasize the need for consistent reporting. In addition, although we have done extensive characterization of aerosols, we cannot rule out that there might be other chemical constituents that might be present differently between FA and Oxy conditions and recommend further in-depth and more sensitive chemical analyses of the air samples. The reaction between NO_2 and O_3 is irreversible and lead to the formation of $NO_3^{\bullet}$ radical as well as N_2O_5 (nitrogen pentoxide) and HNO_3 (nitric acid) (36). In addition, nitrous and nitric acid formation occurs when NO_2 reacts with the components of lung epithelial lining fluid (42). We did not measure these chemicals, and a multitude of photochemical reactions occur in the outdoor air. Thus, the possibility of additional chemical species playing a role in the observed biological outcomes needs to be tested in future mechanistic studies.

In summary, for the reproducibility of experimental studies, it is important to maintain consistency and purity in O_3 generation, which can be achieved by using pure oxygen as the gas feed for the O_3 generator. However, in the environment, O_3 is generated by the interaction of UV light with the varying amounts of particles and NOx also present at the same time. Thus, for environmental relevance, the use of filtered room air could be a more appropriate model. Therefore, the choice of using pure oxygen or filtered air as a source for O_3 generation should be based on whether the study is aimed at experimentally identifying the hazard of O_3 or trying to recreate real-world exposure scenarios. Considering the importance of O_3 research in the context of climate change, understanding potential differences in exposure models that significantly affect outcomes and interpretations is critical for the scientific community to understand. Finally, studies describing experimental O_3 generation for biological experiments should clearly describe the source of oxygen and consider NOx exposure measurements. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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