OZONE AS A DRIVER OF LUNG INFLAMMATION AND INNATE IMMUNITY, AND AS A MODEL FOR LUNG DISEASE

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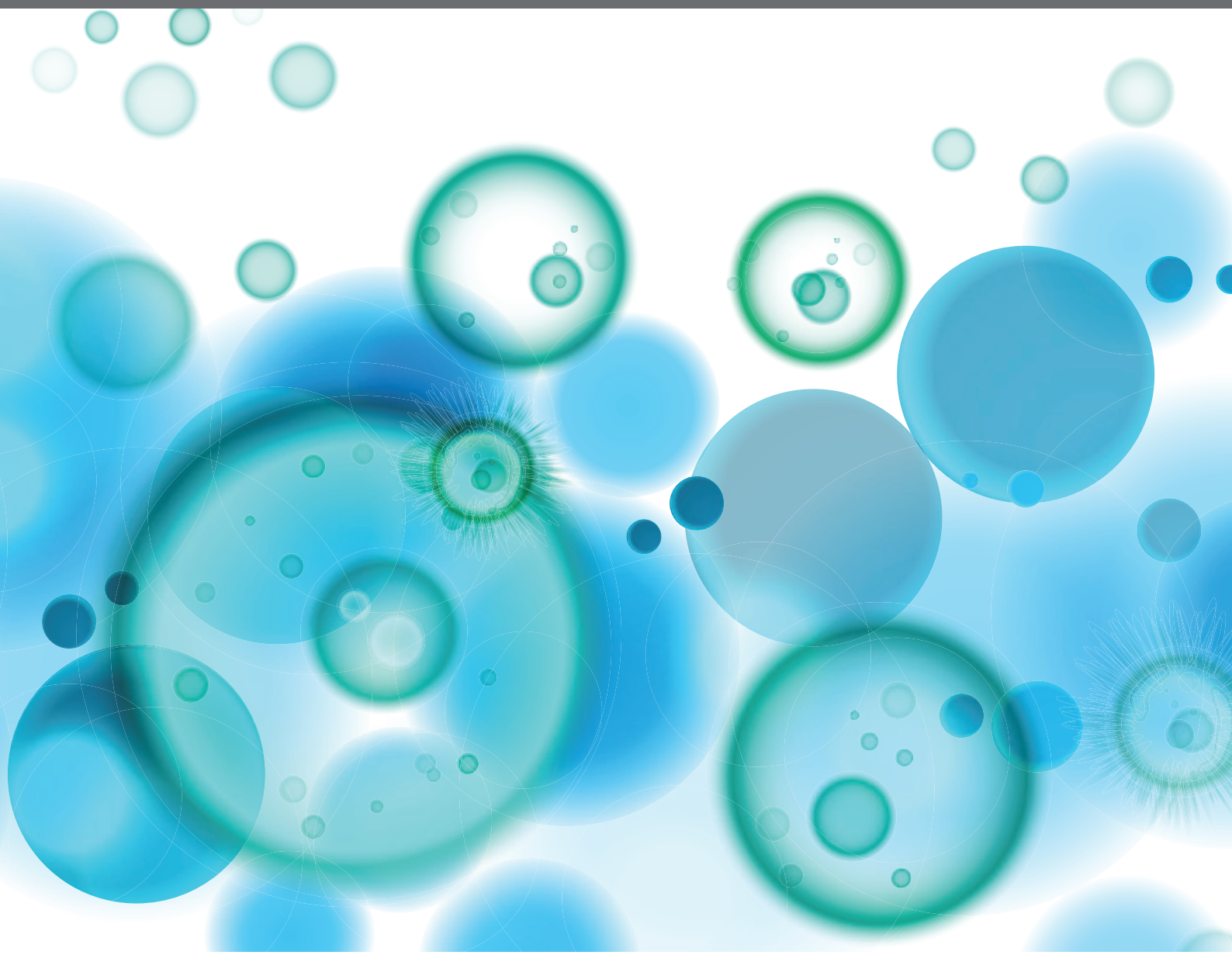

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OZONE AS A DRIVER OF LUNG INFLAMMATION AND INNATE IMMUNITY, AND AS A MODEL FOR LUNG DISEASE

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EDITORIAL
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Editorial: Ozone as a Driver of Lung Inflammation and Innate Immunity and as a Model for Lung Disease

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Keywords: ozone, air pollution, chronic obstructive pulmonary disease, emphysema, lung inflammation, corticosteroid insensitivity, oxidative stress

Editorial on the Research Topic

Ozone as a Driver of Lung Inflammation and Innate Immunity and as a Model for Lung Disease

The articles in this series will remind readers of the increasing importance of ozone as an important component of air pollution that contributes to mortality and disease progression (1–3).

Ground level ozone is created by chemical reactions in the presence of sunlight between oxides of nitrogen (NOx) and volatile organic compounds (VOC) that are both emitted as pollutants from cars, power plants, industrial boilers, refineries, chemical plants, and other sources. Levels of ozone are most likely to reach unhealthy levels on hot sunny days in urban areas, although high levels may also be observed during winter months. Ozone can also be transported long distances by wind into non-urban areas.

The effects of ozone on health are relatively well-known. Exposure to ozone can cause difficulty to breathe, shortness of breath and discomfort on breathing, cough and sore-throat presumably due to an inflammation and damage to the upper and lower airways. Ozone exposure can aggravate lung conditions such as asthma and COPD, causing acute deterioration of these conditions that necessitate emergency treatments (4, 5). As such, ozone exposure is likely to cause increased school absences, days off work, medication use, visits to doctors and emergency rooms, and hospital admissions.

Ozone is highly reactive, eliciting rapid and dose-dependent disruption of the respiratory barrier. It impacts many cell types in the lung and activates specific signaling cascades, eliciting responses including cellular damage, enhanced apoptosis, cytokine production, recruitment of inflammatory cells, and subsequent tissue repair.

Oxidative stress is a conserved mechanism that contributes to numerous environmental lung injuries. Ozone, as a principal mediator of oxidative stress in both the intracellular and extracellular compartments, has become a clinically-relevant model to understand the mechanisms underlying biological responses to oxidative stress (6, 7). Oxidation products are either directly toxic and can cause injury to lung tissue or they can function as exogenous ligands *via* binding to cell surface receptors and thereby triggering intracellular inflammatory and/or apoptotic signaling pathways (8). Thus, ozone-induced oxidant stress modifies several known cell-signaling mechanisms: activation of innate immune signaling pathways, upregulation of antioxidant genes, and enhanced release of damage-associated molecular pattern molecules (DAMPs) (9, 10). Oxidative stress also decreases the clearance of pathogens by impairing antimicrobial function of effector cells including suppressing alveolar macrophage phagocytosis, enhancing macrophage and neutrophil apoptosis, and increasing the susceptibility of epithelial cells to influenza infection.

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This Research Topic collection on ozone extends our knowledge on ozone of the recent decade. We summarize the contents of each of these 9 articles.

Dr. J. J. Zhang et al. starts this collection stating that ozone is responsible for hundreds of thousands of premature deaths and tens of millions of asthma-related emergency visits annually. To combat ozone pollution globally, an urgent reduction in fossil fuel consumption is required to cut NO_x and VOCs and greenhouse gas emissions. Preventive and therapeutic strategies are to alleviate the detrimental effects of ozone especially for susceptible individuals need to be further explored. The efficacy of antioxidants, and the major pathogenic pathways need to be investigated to identify molecular target.

Dr. C. Michaudel et al. revisits respiratory epithelial damage and inflammation in mice and extended previous knowledge showing that a single ozone (1h, 1ppm) causes within hours epithelial cell death with IL-1α and IL-33 expression followed by neutrophilic inflammation and repeated exposure over 6 weeks in mice induced chronic respiratory pathology with chronic inflammation and emphysema dependent on aryl hydrocarbon and IL-17/IL-22 expression.

Dr. M. Sokolowska et al. add new insights on the respiratory barrier disruption of epithelial tight junctions and cell death followed by ROS activation, airway hyperreactivity (AHR), myeloid cell recruitment and remodeling. High ROS levels activate a novel PGAM5 phosphatase dependent cell-death pathway, called oxeiptosis. Chronic ozone exposure leads to progressive and irreversible loss of alveolar epithelial cells and alveoli resulting in reduced gas exchange space typical of emphysema.

Dr. Wiegman et al. review oxidative stress in the lung upon acute exposure and chronic ozone exposure the activation of oxidative pathways causes AHR, cell death tissue remodeling, emphysema and chronic inflammation mimicking cigarette smoke induced COPD. There is derangement of the integrity of cell membranes and organelles of the respiratory epithelial cells causing a stress response with the release of mitochondrial reactive oxygen species (ROS), DNA, and proteases. Mitochondrial ROS and DNA activate NLRP3 inflammasome and the DNA sensors cGAS and STING accelerating inflammation enhancing alveolar septa destruction, remodeling, and fibrosis. Inhibitors of mitochondrial ROS, NLRP3 inflammasome, DNA sensor and cell death pathways may represent novel therapeutic targets.

Dr. Flayer et al. report on the effect of ozone inhalation impairing glucocorticoid responsiveness in a mouse model of allergic asthma induced by sensitization and challenge with Aspergillus fumigatus. Ozone exposure counteracted the effects of budesonide on airway inflammation, airway hyperreactivity, and surfactant protein D (SP-D) production. Asthmatics who are exposed to high ambient ozone levels may become less responsive to glucocorticoid treatment particularly during acute exacerbations of asthma.

Dr. Mumby et al. report on acute ozone exposure in man resulting in sputum neutrophilia, respiratory irritation and may be associated with systemic inflammation and chronic exposure amplifies these effects. In asthmatic subjects, ozone induces a greater number of genes in bronchoalveolar macrophages than healthy responders with up-regulation of inflammatory and immune pathways and the enhanced expression of repair programs. Models of ozone exposure recapitulate the inflammatory effects seen in humans and enable the elucidation of key transcriptional pathways that drive ozone effects on airways.

Dr. Enweasor et al. review oxidative stress in neutrophil recruitment and their resistance to glucocorticosteroids and innate lymphoid cells (ILC). Furthermore, a role of ILC type 2 cells was reported in the ozone inflammatory response, but also of ILC type 1 such as NK cells as well as NKT cells. Anti-CD1d mAb administration blocked NKT cell activation and ozone-induced AHR asthma. NKT cells mediate a unifying pathogenic mechanism for several distinct forms of asthma.

Dr. Shore investigates the metabolic effects of ozone on glucose intolerance and hyperlipidemia, characteristics of the metabolic syndrome. Further, the role of stress hormones and exacerbation by obesity and diabetes. The intestinal microbiome is critical in the regulation of metabolism is well known and a link of the gut microbiome and pulmonary inflammation in response to ozone has been uncovered including heightened AHR

Dr. Noutsios et al. review surfactant protein A (SP-A) in the airway and the interaction with alveolar macrophages (AM), the guardian cells of innate immunity in the lungs. SP-A regulates many of its functions under basal condition and in response to infection and oxidative stress. They investigated the effect of hSP-A variants on the AM gene expression profile in response to Klebsiella pneumoniae infection and differences in AM gene expression of TP53, TNF, and cell cycle.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Major Effect of Oxidative Stress on the Male, but Not Female, SP-A1 Type II Cell miRNome

George T. Noutsios[1†], Nithyananda Thorenoor[1], Xuesheng Zhang[1], David S. Phelps[1], Todd M. Umstead[1], Faryal Durrani[1] and Joanna Floros[1,2]*

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Pulmonary surfactant protein A (SP-A) plays an important role in surfactant metabolism and lung innate immunity. In humans there are two proteins, SP-A1 and SP-A2, encoded by *SFTPA1* and *SFTPA2,* respectively, which are produced by the alveolar type II cells (T2C). We sought to investigate the differential influence of SP-A1 and SP-A2 in T2C miRNome under oxidative stress (OxS). SP-A knock out (KO) and hTG male and female mice expressing SP-A1 or SP-A2 as well as gonadectomized (Gx) mice were exposed to O_3-induced oxidative stress (OxS) or filtered air (FA). Expression of miRNAs and mRNAs was measured in the T2C of experimental animals. (a) In SP-A1 males after normalizing to KO males, significant changes were observed in the miRNome in terms of sex-OxS effects, with 24 miRNAs being differentially expressed under OxS. (b) The mRNA targets of the dysregulated miRNAs included *Ago2, Ddx20, Plcg2, Irs1, Elf2, Jak2, Map2k4, Bcl2, Ccnd1*, and *Vhl*. We validated the expression levels of these transcripts, and observed that the mRNA levels of all of these targets were unaffected in SP-A1 T2C but six of these were significantly upregulated in the KO (except *Bcl2* that was downregulated). (c) Gondadectomy had a major effect on the expression of miRNAs and in three of the mRNA targets (*Irs1, Bcl2*, and *Vhl*). *Ccnd1* was upregulated in KO regardless of Gx. (d) The targets of the significantly changed miRNAs are involved in several pathways including *MAPK* signaling pathway, cell cycle, anti-apoptosis, and other. In conclusion, in response to OxS, SP-A1 and male hormones appear to have a major effect in the T2C miRNome.

Keywords: alveolar epithelium, surfactant protein A, ozone, sex differences, *MAPK*

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INTRODUCTION

Ambient ozone (O_3)-induced oxidative stress (OxS) is one of the major environmental factors contributing to the occurrence and development of upper and lower airway disease, including chronic rhinosinusitis (CRS) (1), asthma, and chronic obstructive lung disease (COPD) (2, 3). In the distal lung, the alveolar epithelial cells provide the first line of defense against environmental pathogens such as O_3, pollutants, bacteria, viruses, and allergens by producing a number of protective factors (4). In addition to secreting pulmonary surfactant to reduce the alveolar surface tension, they produce chemokines and cytokines that regulate alveolar inflammatory responses as well as proteinases and proteinase inhibitors (5). Upon environmental stress such as that of

OxS, O_3 increases the production of reactive oxygen species (ROS) (6) that disrupt the alveolar epithelial cell barrier function by the dissociation of the tight junctions of alveolar epithelium (7), thereby allowing the entrance of opportunistic bacteria such as *Pseudomonas aeruginosa* to infect the alveolar epithelium. Exposure to O_3 also reduces pulmonary surfactant secretion (8). The alveolar epithelium initiates a self-repair process by recruitment, proliferation, and differentiation of new epithelial cells to maintain the structural and functional traits that are required to maintain a normal respiratory function (9).

The alveolar epithelium is comprised of two different cells types, the alveolar type I cells (T1C) and the type II cells (T2C) that are in close proximity with the alveolar macrophages (AM) that reside in the alveolar space. The T1C are large, flat cells with a thin attenuated cytoplasm that line 90% of the alveolar surface. This distinct shape enables them to facilitate O_2/CO_2 gas exchange by minimizing the diffusion distance between the alveolar surface and the blood (10). The T2C cover ∼10% of the alveolar surface and their main function is to produce and secrete pulmonary surfactant, a phospholipid and protein mixture, which lowers the surface tension in the alveolus during the respiration process. T2C possess unique secretory organelles, called lamellar bodies, which contain surfactant lipids and surfactant proteins A (SP-A), SP-B, and SP-C. Also, T2C play a very important role in the epithelium repair process after lung injury and are considered the progenitor cells of the alveolar epithelium. Upon epithelial damage T2C proliferate as new T2C and they differentiate into T1C repairing the scarred epithelial surface (9, 11, 12).

SP-A is the most abundant protein in pulmonary surfactant and has both surfactant-related functions and innate immunity functions (13–17). SP-A knock-out mouse studies have revealed important host defense functions of SP-A, where KO mice are more vulnerable to bacterial infections compared to the mice that express SP-A (18–22). Also, SP-A has been shown to have regulatory effects on the proteome, function, cell shape, and activation state of AM (21, 23–26). OxS stress increases the production of reactive oxygen species (ROS) (6) and these in turn damage the alveolar epithelium (27), oxidize SP-A, and compromise innate immune functions (23, 28–33). In humans however, unlike in rodents, there are two different genes, *SFTPA1* and *SFTPA2*, encoding SP-A1 and SP-A2 proteins, respectively. We have previously shown that SP-A1 and SP-A2 differentially affect the proteomic expression in AM (34, 35), the AM function (36–38), surfactant secretion (39), structure of surfactant monolayers (40, 41) and more recently we have shown that SP-A1 and SP-A2 differentially regulate the AM miRNome and antioxidant pathways in the AM (42), lung function mechanics (43), and survival after *K. pneumoniae* infection (44). MicroRNAs (miRNAs) have also been shown to differentially affect SP-A1 and SP-A2 expression (45), and also contribute to the maintenance of the T2C phenotype (46).

In the present study, using humanized transgenic (hTG) mice, where each expresses either SP-A1 or SP-A2, we sought to investigate the differential influence of SP-A1 and SP-A2 on the T2C miRNome under the effect of OxS. We found that the T2C miRNome is regulated in response to OxS and that O_3 exposure has a major effect on the male SP-A1 miRNome. We also show that sex hormones play a role in T2C miRNome under the studied conditions.

Abbreviations: *Ago2*, argonaute 2; AM, alveolar macrophages; *Bcl2*, B-cell lymphoma 2; Ccnd1, cyclin D1; COPD, chronic obstructive lung disease; CRS, chronic rhinosinusitis; *Ddx20*, dead-box helicase 20; eIF2, eukaryotic initiation factor 2; FA, filtered air; Gx, gonadectomized; FDR, false discovery rate; hTG, humanized transgenic; *Irs1*, Insulin Receptor Substrate 1; *Jak2*, Janus Kinase 2; KO, SP-A knock-out; *Map2k4*, Mitogen-Activated Protein Kinase 4; *MAPK*, mitogen-activated protein kinases; miRNAs, microRNAs; NGx, non-gonadectomized; O3, ozone; OxS, oxidative stress; *Plcg2*, Phospholipase C Gamma 2; ROS, reactive oxygen species; *SFTPA1*, gene encoding SP-A1; *SFTPA2*, gene encoding SP-A2; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; T1C, alveolar type I cells; T2C, alveolar type II cells; *Vhl*, Von Hippel-Lindau protein.

METHODS

Oxidative Stress Animal Model

Twelve weeks old humanized transgenic (hTG) C57BL/6 mice (males and females) each carrying human SP-A1 ($6A^2$), SP-A2 ($1A^0$) (47), as well as SP-A knock-out (KO) were used in the present study. Females were synchronized for 7 days as described previously (42) to stimulate estrus. A total of $n = 52$ mice (36 for miRNA study, 16 for gonadectomy analysis). Protocols involving animal procedures were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine.

Animals were exposed to 2 ppm ozone (O_3) or filtered air (FA) (control) at 25°C as described previously (42, 48). We used 3 mice per group. i.e., 3 males, 3 females, 3 SP-A KO, 3 hTG SP-A2, 3 hTG SP-A1, 3 for O_3, and FA exposure ($n = 36$). All O_3 and FA exposures were conducted in parallel as described (49). Mice were sacrificed 4 h post exposure. Each animal was analyzed individually, and we did not pool any samples. Summary of the experimental workflow is depicted in **Figure 1**.

Mouse Alveolar Type II Cells Isolation

Mouse type II cells were isolated based on a modified method that was described previously (50). Briefly, mice were anesthetized with intraperitoneal injection of 87.5 mg/kg ketamine and 12.5 mg/kg xylazine and exsanguinated by cutting the inferior vena cava. Cardiac perfusion of the lung was performed with 10 mL of normal saline solution followed by endotracheal intubation and infusion of the lungs with 3 mL solution of 50 U/ml dispase II (Sigma-Aldrich, St. Louis, MO) in HBSS 1x and sealed with 0.5 mL of 1% solution of low melting agarose (Sigma-Aldrich). The lungs were removed from the thoracic cavity and lung lobes digested in 15 mL tube containing 2 mL of dispase II for 45 min at 37°C with constant shaking at 150 rpm. Digested lungs were dissected and homogenized in 7 mL of complete DMEM solution, supplemented with 10 μL DNase I (5,000 Kunitz U/ml Sigma-Aldrich). The lung epithelial cells were filtered through a 100 and 40 μm strainer, passed through 20 μm- nylon mesh, cells were collected by centrifugation at 130 xg for 8 min, and resuspended in 10 mL of DMEM/25 mM HEPES/10% FBS/1x AB/AM. Negative selection of T2C was performed by incubating the cell suspensions in 10-cm cultured dishes coated with 42

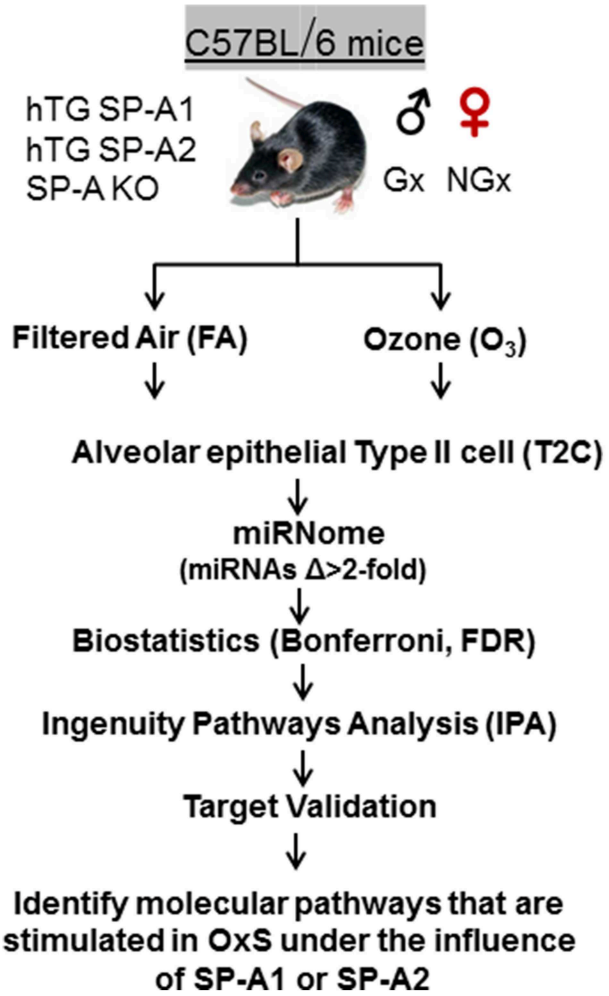

FIGURE 1 | Experimental workflow of the present study. hTG, humanized transgenic that each carries a single SP-A1 ($6A^2$) or SP-A2 ($1A^0$) variant; SP-A-KO, knock out; Gx, gonadectomized; NGx, non-gonadectomized; FA, filter air; O_3, ozone; T2C, Alveolar epithelial Type II cells; Δ, difference; FDR, false discovery rate; IPA, ingenuity pathway analysis; OxS, oxidative stress.

μg anti-mouse CD45 (targeting hematopoietic cells) and 16 μg anti-mouse CD16/32 (BD Pharmingen, San Jose, CA) (targeting alveolar macrophages) at 37°C, 10% CO_2 for 2 h. Non-attached cells were centrifuged, washed with 1x PBS (Gibco, Waltham, MA) and counted. A fraction was used to prepare cytospins, cells were stained, and a differential cell count was performed. T2C purity was 95% as assessed by Papanikolaou staining. The remaining T2C pellet was resuspended in 500 μL solution of DMEM supplemented with 40% fetal bovine serum (Gibco) and 10% DMSO (Sigma Aldrich, St. Louis, MO) and T2C were cryopreserved in liquid nitrogen until further use.

Gonadectomy and Ozone Exposure

Male and female SP-A1 and KO mice were gonadectomized (Gx) and exposed to O_3 (2 ppm) for 3 h and were sacrificed 4 h post OxS as described (51). The differentially expressed miRNAs from Gx samples were identified by RNA sequencing as described previously (42, 52). The miRNAs identified from Gx mice were selected for analysis and changes in miRNA expression in SP-A1 mice were calculated by normalizing to KO as described previously (42). Samples from 16 animals (8 males and 8 females for SP-A1 and KO) were individually analyzed.

Isolation of miRNAs, qRT-PCR, and Statistical Analysis

Total RNA from the isolated mouse T2C was prepared using QIAzol Lysis Reagent (Qiagen, Valencia, CA) and the miRNA-enriched fraction was purified and used to generate cDNA, and then served as a template for real-time qPCR. Expression profiles of the 372 most abundantly expressed and best-characterized miRNAs in miRBase were then studied as described previously (42). The expression of miRNAs from FA and O_3 exposed T2C samples from SP-A1, SP-A2, and KO mice were analyzed as described previously (42). The variability across the 3 samples was assessed by p-values ($p < 0.0166$) and miRNAs with significantly changed levels were studied further ($p < 0.0166$). Bonferroni correction applied for sex, treatment, and genotype variability. The miRNA:gene target interactions were identified and reported in a format which enables direct transfer of results to genomic databases cataloging validated miRNA-target interactions as described previously (53, 54).

Gene Expression Analysis

To assess the expression of levels of *Ago2, Ddx20, Plcg2, Irs1, Elf2, Bcl2, Jak2, Map2k4, Bcl2, Ccnd1*, and *Vhl* genes at mRNA level in the male non-gonadectomized (NGx) and gonadectomized (Gx) KO and SP-A1 T2C, we performed qRT-PCR as described previously (42). The specific RT2 qPCR Primer assay was purchased from Qiagen. Cell samples were obtained from 3 separate animals/treatment (FA and O_3), and each sample was analyzed in triplicate/animal and quantified relative to *Gapdh* mRNA expression.

RESULTS

SP-A1 and SP-A2 Differentially Regulate the T2C miRNome

The expression levels of the hTG SP-A1 and SP-A2 T2C miRNomes were determined in males and females that were exposed to FA or O_3 and compared to the corresponding KO T2C. The miRNome levels are presented as volcano plots to show the fold change regulation differences between levels of miRNAs in hTG and KO mice, as well as their statistical significance (**Figures 2**, **3**).

After FA exposure, which serves as control, in our experimental model, we observed in **Figure 2A**, a very tightly packed cluster of data points with few data points exceeding the cutoff for significance (Bonferroni corrected $p < 0.0166$), indicating that there are only a few differences between FA-exposed SP-A1 males and KO males. In **Figure 2B**, when the same comparison is made with female mice, we observed a very similar pattern to that of males, with only two miRNAs to exceed the significance threshold. Following O_3 exposure the SP-A1

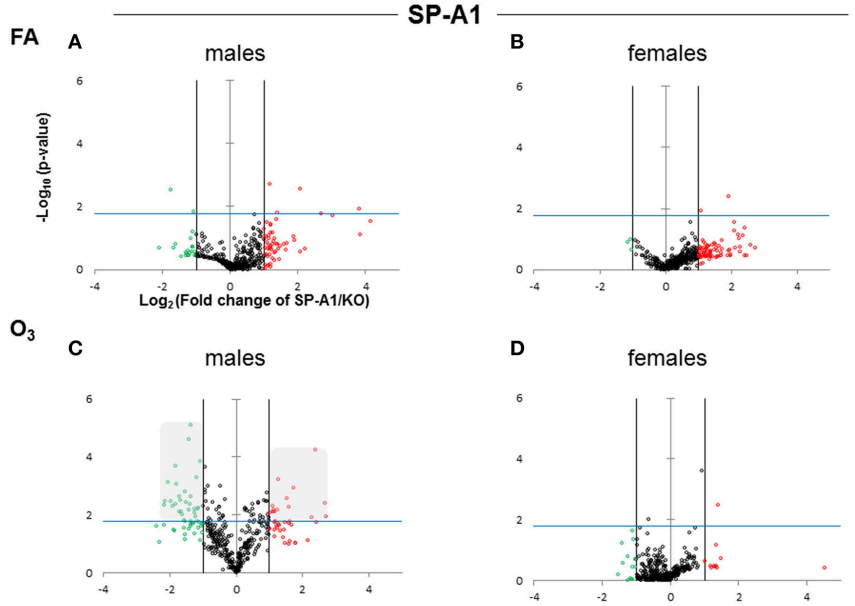

FIGURE 2 | Volcano plots indicating the statistical significance of SP-A1 T2C miRNome expression levels compared to SP-A KO under FA or O_3 exposure for males and females. The x-axis plots the $\log_2$ of the fold-changes, while the y-axis plots the $-\log_{10}$ of their *p*-values based on *t*-test of the replicate raw Ct data (section Materials and Methods). Each plot has three vertical lines. The middle vertical line that is graded corresponds to zero changes. The lines on either side represent ≥2-fold differences. Dots in the volcano plots above the blue horizontal line identify fold-changes with statistical significance of at least the Bonferroni corrected $p < 0.0166$. The red and green dots represent miRNAs that were upregulated ≥2 fold and downregulated ≥2 fold, respectively, compared to KO. Black dots signify miRNAs that were regulated <2-fold times (i.e., $x \leq 2$, x is the fold change). **(A)** Male SP-A1 mice compared to KO exposed to FA; **(B)** Female SP-A1 mice compared to KO exposed to FA; **(C)** Male SP-A1 mice compared to KO after O_3 exposure; **(D)** Female SP-A1 mice compared to KO after O_3 exposure. The shaded gray area in **(C)** shows show the differences in the miRNAs that are highly and significantly regulated.

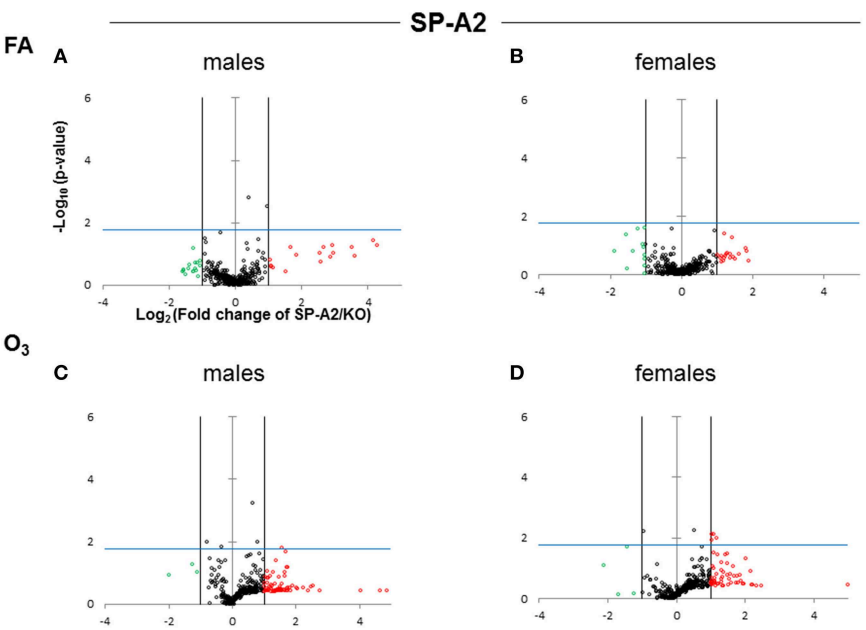

FIGURE 3 | Volcano plots (as described in **Figure 2**) indicating the statistical significance of SP-A2 T2C miRNome expression levels compared to SP-A KO under FA or O_3 exposure for males and females. **(A)** Male SP-A2 mice compared to KO exposed to FA; **(B)** Female SP-A2 mice compared to KO after FA exposure; **(C)** Male SP-A2 mice compared to KO after O_3 exposure; **(D)** Female SP-A2 mice compared to KO after O_3 exposure.

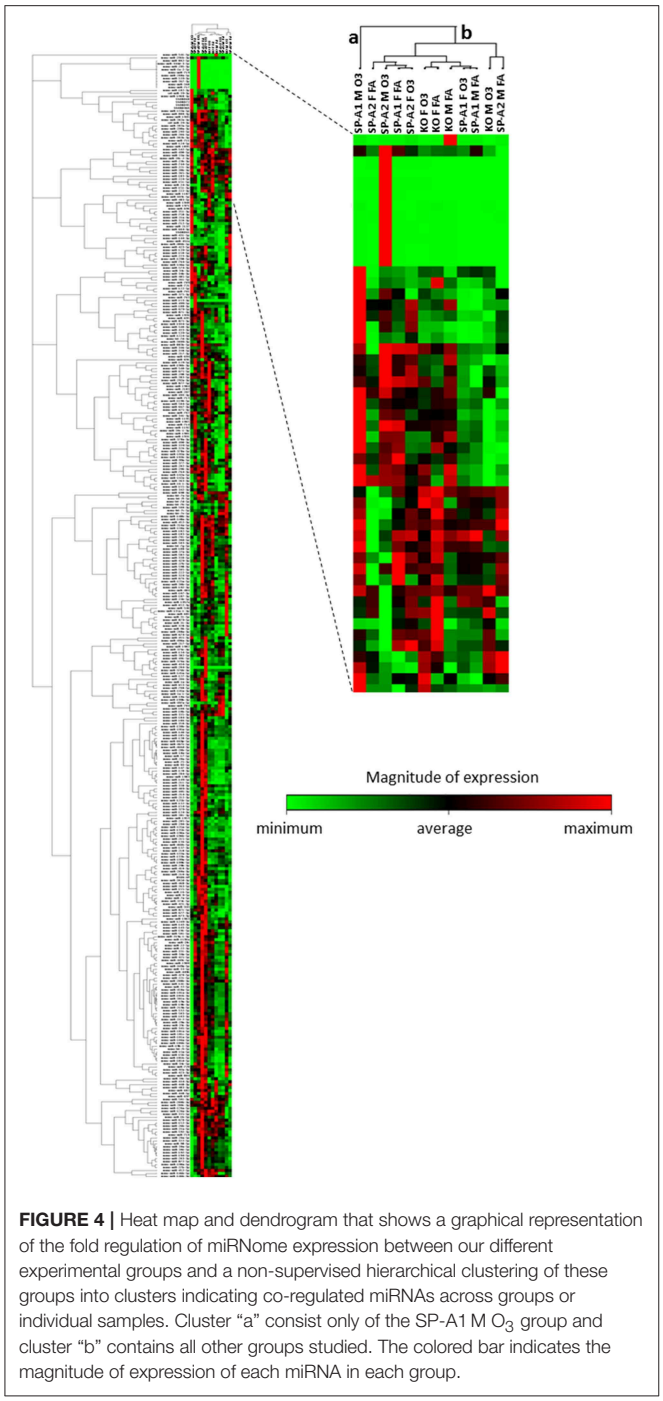

FIGURE 4 | Heat map and dendrogram that shows a graphical representation of the fold regulation of miRNome expression between our different experimental groups and a non-supervised hierarchical clustering of these groups into clusters indicating co-regulated miRNAs across groups or individual samples. Cluster "a" consist only of the SP-A1 M O_3 group and cluster "b" contains all other groups studied. The colored bar indicates the magnitude of expression of each miRNA in each group.

male mice (**Figure 2C**) show a very different picture. There are many more differences (both in magnitude and in significance) between O_3 exposed SP-A1 and KO males. SP-A1 females on the other hand show minimal changes compared to females KO (**Figures 2C,D**, respectively).

When we compared the SP-A2 hTG males and females after FA and OxS we do not see any robust differences as those seen with the SP-A1 male hTG (**Figures 3A–D**). It is immediately obvious that the pattern seen after OxS for the SP-A1 males is unique (shaded area of **Figure 2C** compared to the rest of the panels of **Figure 2** and all panels of **Figure 3**) in the volcano plots analysis. This indicates that in response to OxS the male T2C miRNome of the SP-A1 mice is more responsive compared to the rest of the hTGs and exhibits a higher number of changed miRNAs that reach the Bonferroni corrected significance threshold $p < 0.0166$ (compare shaded area).

Oxidative Stress Has a Major Effect on the Male T2C miRNome

We performed a non-supervised hierarchical clustering of our entire dataset to display a heat map with a dendrogram indicating co-regulated genes across groups or individual samples (**Figure 4**). We found two distinct clusters a and b, with clade a being the one of SP-A1 male T2C miRNome, while the rest of our experimental animals clustered together in group b. A two-way ANOVA test for sex and treatment effects showed that the F-stat for the SP-A1 mice regarding the sex effect is $F = 161.91$ with F crit $= 3.84$ and $p = 2.79 \times 10^{-5}$. The F-stat for the interaction between the two factors (sex and treatment) was $F = 16.69$ with F crit $= 3.84$ and $p = 4.63 \times 10^{-5}$. The same analysis for the SP-A2 mice miRNome did not show that sex, treatment, or the combination of these two factors were significantly different (F-stat for SP-A2 mice was $F = 1.24$ lower than the F crit $= 3.84$ and p-value not significant $p = 0.265$). These data show that in the T2C miRNome, there is a difference between sexes in response to O_3 exposure as a function of SP-A variants.

mRNA Targets of the Male SP-A1 T2C miRNome Associate With Cell Cycle, Apoptosis, and MAPK Pathway

To better understand and integrate the T2C miRNome data, we performed Ingenuity Pathway Analysis (IPA) for the T2C miRNAs whose expression was significantly altered by OxS. Only miRNAs that were shown to pass the corrected Bonferroni $p < 0.0166$ and a false discovery rate (FDR)-adjusted $q < 0.05$ were used to ensure that sex, treatment, gene, and array variability do not lead to false discoveries. Fifty-four miRNAs met the above criteria and were used for IPA. This analysis identified several mRNA transcripts, the expression of which could be affected by approximately half of the miRNAs selected for IPA. The targets identified include the following transcripts *Ago2, Ddx20, Plcg2, Irs1, Elf2, Jak2, Map2k4, Bcl2, Ccnd1*, and *Vhl* mRNAs. The levels of miRNAs that targeted the above molecules and were significantly changed in SP-A1 males in response to OxS are shown on **Table 1**.

Next, we performed qRT-PCR to assess the expression levels of *Ago2, Ddx20, Plcg2, Irs1, Elf2, Jak2, Map2k4, Bcl2, Ccnd1*, and *Vhl* genes in the male KO and SP-A1 T2C (**Figure 5**). To our surprise, we observed that in response to OxS the levels of *Ago2, Elf2, Jak2, Map2k4, Ccnd1,* and *Vhl* were significantly upregulated in the KO T2C but remained unaffected in the SP-A1 T2C. Also, the *Bcl2* gene was significantly downregulated only in the KO T2C. The above molecules are involved in several pathways including mitogen activated protein kinases (*MAPK*) signaling pathway, cell cycle, and apoptosis.

TABLE 1 | Levels and statistical significance of the male SP-A1 T2C miRNAs in OxS and shown by IPA to be directly associated with genes *Ago2, Ddx20, Plcg2, Irs1, Elf2, Bcl2, Jak2, Map2k4, Blc2, Ccnd1*, and *Vhl*.

Mature miRNA ID	Fold regulation	*p*-value	FDR (*q*-value)	Target gene(s)	PMID	Validation experiments*
miR-124-3p	−2.6902	0.006674	0.038944405	*Ago2, Ccnd1, Eif2*	27577603	HITS-CLIP
miR-135a-5p	−2.9089	0.001569	0.037656	*Jak2*	30854107	Luciferase
miR-141-3p	−3.3007	0.007623	0.038944405	*Map2k4*	28454307	WB, IHC, qRT-PCR
miR-143-3p	−3.4508	0.000843	0.029428364	*Bcl2*	29581736	WB
miR-143-5p	−2.5534	0.00237	0.038944405	*Bcl2*	20878132	qRT-PCR
miR-148a-3p	−2.193	0.00597	0.038944405	*Ppara* and indirectly *Bcl2*	26001136	Luciferase
miR-153-3p	−4.1674	0.000733	0.0281472	*Bcl2*	30537994	Luciferase
miR-190a-5p	−3.1766	0.003205	0.038944405	*Ddx20*		*in silico* report
miR-19b-3p	−3.5226	0.004329	0.038944405	*Ccnd1*	29455644	WB
miR-204-3p	2.2073	0.007978	0.038944405	N/A		
miR-208a-3p	−3.7777	0.004875	0.038944405	*Ddx20*		*in silico* report
miR-20b-5p	−2.2419	0.00638	0.038944405	Indirect effect on *Bcl2*	30816540	Luciferase
miR-219a-5p	−16.1578	0.000011	0.002112	*Plcg2*	20956612	qRT-PCR
miR-223-3p	6.3634	0.003918	0.038944405	*Irs1*	29286159	qRT-PCR
miR-26a-5p	−2.3634	0.006167	0.038944405	Indirect effect on *Htr1a*	30766477	Luciferase
miR-29b-3p	−2.6192	0.004297	0.038944405	*Ago2*		IP
miR-301a-3p	−3.0775	0.010054	0.045186977	Indirect effect in *Ago2*	28332581	qRT-PCR
miR-302a-3p	2.2073	0.007978	0.038944405	Indirect effect in Ccd1	28510621	Luciferase
miR-302a-5p	2.2073	0.007978	0.038944405	Indirect effect in Ccd1	28510621	Luciferase
miR-34b-3p	2.1409	0.004946	0.038944405	N/A		
miR-499-5p	−2.6842	0.000025	0.0032	*Sox6*	31076992	qRT-PCR
miR-539-3p	2.2073	0.007978	0.038944405	*Ntrk3*	21143953	Luciferase
miR-708-5p	−2.3546	0.008364	0.039182049	*Vhl*	21852381	qRT-PCR
miR-758-3p	3.3301	0.001181	0.032393143	Indirectly *Bcl2*	31138034	qRT-PCR

*PMID, unique identifier number used in PubMed for miRNA; –, indicates downregulation; FDR, false discovery rate; HITS-CLIP, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation; WB, western blot; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; IP, immunoprecipitation; N/A, non-applied; *, last column shows the validation techniques used for each miRNA as noted in the PMID.*

Effect of Gonadectomy and OxS on the Expression of miRNAs in SP-A1 Male and Female Mice

To study the effect of sex hormones on the expression of miRNAs after OxS, we performed miRNA expression analysis in T2C from gonadectomized (Gx) SP-A1 and KO male and female mice and compared it with that of non-gonadectomized (NGx) mice after O_3 exposure.

For this analysis, we used 120 miRNAs that were identified in both NGx (males and females) and Gx (males and females) groups. Of these, in the NGx (males vs. females) group, 89 miRNAs had their levels significantly changed (fold change ≥2) after FA exposure (**Figure 6A**). In the Gx group (male vs. female) compared to the corresponding NGx (male vs. female) group, expression of 9 miRNAs (10.1%) was significantly increased (fold change ≥2), and expression of 61 miRNAs (68.53%) was significantly decreased (fold change ≥2) (**Figure 6A**). In response to OxS, the level of 56 miRNAs was significantly altered (≥2 fold) in NGx (male vs. female) groups (**Figure 6B**). Following, comparison of the Gx group (male vs. female) to the corresponding NGx (male vs. female) group, the expression of 5 miRNAs (8.9%) was significantly increased (≥2 fold), and the expression levels of 33 (58.9%) miRNAs was significantly decreased (≥2 fold) (**Figure 6B**). Of the 89 (**Figure 6A**) and 56 (**Figure 6B**) miRNAs differentially expressed in Gx males vs. females after FA and O_3 exposure, 24 miRNAs (26.96%) are specific to FA exposure and 16 miRNAs (17.97%) are specific to O_3 exposure (**Figure 6C**). Moreover, a one-way ANOVA pertaining to the gonadectomy effect on the miRNA expression showed a significant difference with F stat = 120.5 with F crit = 3.88 and $p = 5.95 \times 10^{-23}$ (see **Supplementary File 1**) indicating that sex hormones play a role.

Furthermore, we monitored the expression levels of the target genes discussed above after gonadectomy in both SP-A1 and KO mice (**Figure 7**). We found that Gx had a major effect in three genes (*Irs1, Bcl2, Vhl*) in KO mice. *Irs1* was upregulated in Gx KO only. No significant change was observed for IRS1 in the NGx KO (**Figure 4**). *Bcl2* and *Vhl* showed decrease and increase expression, respectively, compared to control *Gapdh* mRNA (**Figure 7**); both of these had shown the reverse in NGx (**Figure 5**). Of interest, *Ccnd1* was upregulated in KO regardless of Gx. These data indicate that sex hormones play an important role in the observed miRNA sex differences by affecting regulation of miRNAs, as most of the miRNAs that were increased in NGx were decreased in Gx.

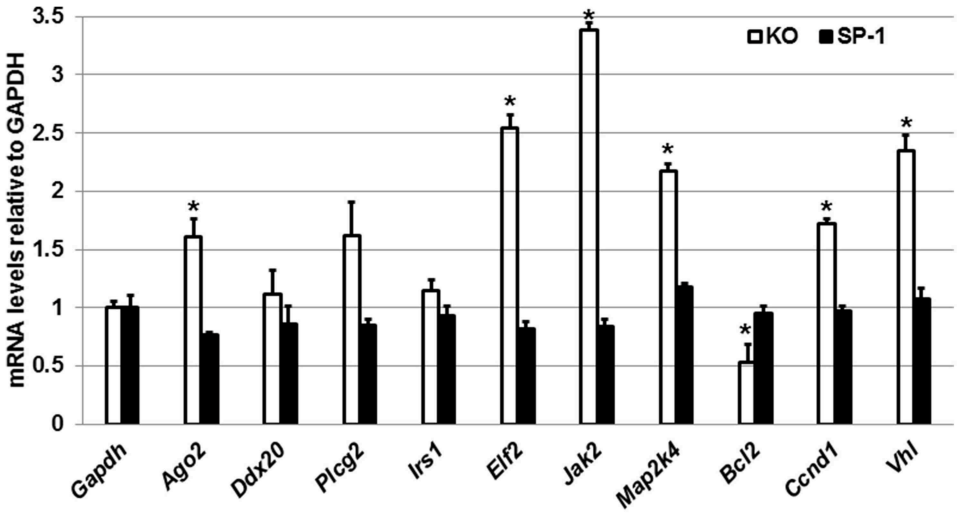

FIGURE 5 | Effect of OxS in the T2C transcriptome of males. mRNA levels of *Gapdh, Ago2, Ddx20, Plcg2, Irs1, Elf2, Bcl2, Jak2, Map2k4, Blc2, Ccnd1*, and *Vhl* genes shown to be targeted by the T2C miRNome were measured in KO and SP-A1 male mice 4 h post O_3 exposure. mRNA levels were measured by qRT-PCR and normalized to *Gapdh*. In KO the *Jak2, Elf2, Vhl, Mapk2, Ccnd1,* and *Ago2* were significantly upregulated by 3.4-, 2.5-, 2.3-, 2.2-, 1.7-, and 1.7- fold ($p < 0.05$), respectively, while the *Bcl2* was significantly downregulated by 0.5-fold. The levels of those mRNAs did not change in the SP-A1 mice. *means $p < 0.05$.

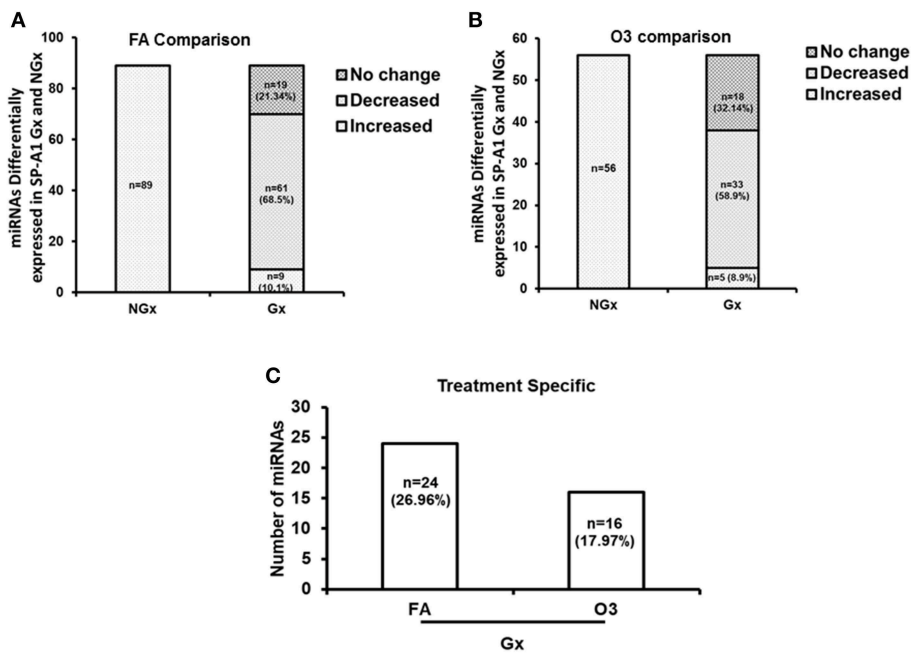

FIGURE 6 | The effect of gonadectomy and OxS on T2C miRNA expression profiles of SP-A1 mice. **(A)** The differentially expressed miRNAs in SP-A1 non-gonadectomized (NGx) and gonadectomized (Gx) mice were identified after normalizing to corresponding NGx and Gx KO. NGx shows the miRNAs ($n = 89$) that changed significantly (≥2-fold) in FA when males were compared to females. Gx depicts the comparison of Gx values (male vs. female) to NGx (male vs. female). Out of the same 89 miRNAs (found to have their levels increased in NGx), 9 miRNAs (10.1%) showed a significant increase (≥2-fold) and 61 miRNAs (68.53%) showed a significant decrease (≥2-fold). **(B)** NGx shows the miRNAs ($n = 56$) that changed significantly (≥2-fold) in OxS when males were compared to females. Gx depicts the comparison of Gx values (male vs. female) to NGx (male vs. female). Out of the same 56 miRNAs (found to have their levels increased in NGx), 5 miRNAs (8.9%) showed a significant increase (≥2-fold) and 33 miRNAs (58.9%) showed a significant decrease (≥2-fold). **(C)** Depicts the comparison of the 89 and 56 differentially expressed miRNAs identified between Gx males and females. Out of 89 miRNAs studied, 24 miRNAs (26.96%) are significantly increased in FA (≥2-fold), and 16 miRNAs (17.97%) are significantly increased in OxS (≥2-fold).

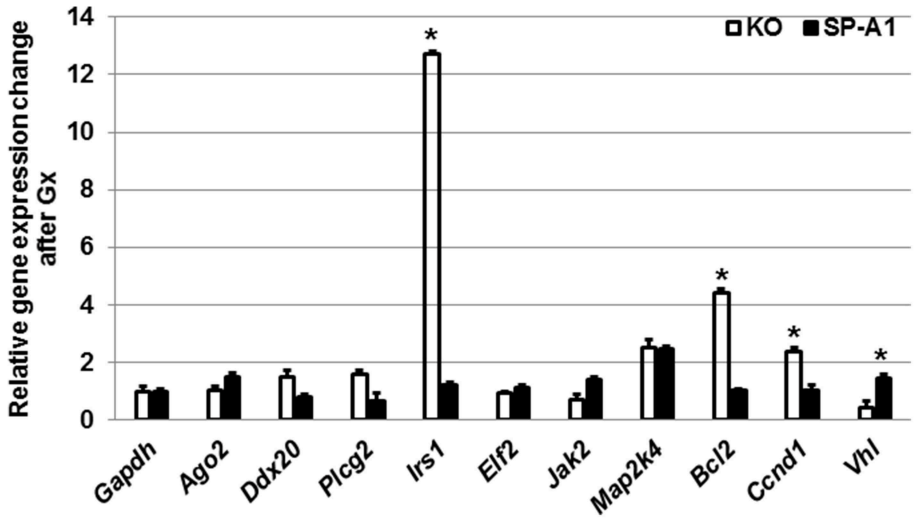

FIGURE 7 | The effect of gonadectomy and OxS on genes that are targeted by the T2C miRNome. Genes were measured by qRT-PCR in KO and SP-A1 gonadectomized (Gx) male mice 4 h post O_3 exposure and normalized to housekeeping gene *Gapdh*. The graph shows the relative expression changes of mRNAs Gx mice compared to non-gonadectomized. In the Gx KO the *Irs1, Bcl2*, and *Ccnd1* expression levels were increased 13-, 4.5-, and 2.8-fold ($p < 0.05$), respectively. In the Gx SP-A1 the *Vhl* gene was significantly increased in SP-A1 by 1.8-fold. *means $p < 0.05$.

DISCUSSION

We have previously shown a differential effect of SP-A1 and SP-A2 proteins on AM function (31, 37, 38), AM proteome (34, 35) and AM miRNome (42) as well as sex differences after OxS. In the present study, we investigated the effect of OxS on mouse T2C under the influence of either SP-A1 or SP-A2 and compared it to KO mice as well as studied the role of sex hormones in the T2C miRNome of the SP-A1 hTG mice. We found significant changes after O_3 exposure in SP-A1 males but not in the other animals. When a non-supervised hierarchical clustering analysis on the entire dataset was performed, we observed that the O_3-exposed SP-A1 male miRNome clustered separately from the rest of the experimental animals showing that OxS has a major effect on the male SP-A1 T2C miRNome. Also, a two-way ANOVA analysis showed that there is an interaction between the male sex hormones and the SP-A1 gene under the effect of OxS. Gonadectomy had a major effect on the expression of the T2C miRNome compared to non-gonadectomized mice. Our miRNome analysis in the T2C that was subjected to OxS was based on strong validation methodologies. Ingenuity Pathway Analysis (IPA), pairs miRNAs/mRNA targets based not only on the gold standard *in silico* predicted algorithms (miRBase, miRTarBase, miRWalk, Targetscan, etc.) but also on experimental data from the published literature. With IPA we showed that miRNAs that were changed significantly >2-fold in male SP-A1 T2C mice, targeted genes that are involved in the MAPK signaling pathway, cell cycle, and anti-apoptosis. We monitored/validated experimentally via qRT-PCR which of the predicted mRNA targets are responding to the effect of OxS. Gene expression analysis of the target mRNAs of interest surprisingly showed that the OxS affected predominantly the KO mouse while the SP-A1 mouse showed no significant shifts in the expression levels of the same genes. Gonadectomy of male SP-A1 and KO mice prior to O_3 exposure led to significant changes in the expression levels of three genes (*Irs1, Bcl2,* and *Vhl*) in KO, whereas the expression of *Ccnd1* remained increased in KO regardless of Gx. These data indicate that sex differences are in part attributable to circulating gonadal hormones (51), which are believed to influence the innate immune responses. The specific roles of these hormones and the underlying mechanisms of regulation remain yet to be explored.

The mRNA targets of the SP-A1 T2C miRNAs that were changed significantly in the non-gonadectomized males under OxS included genes being involved in the *MAPK* signaling, apoptosis, and cell cycle. Although the activation of the *MAPK* pathway by OxS has been described before in other systems and tissues (55–57), in the present study our data show that there are miRNAs that may regulate genes of the *MAPK* signaling pathways in the respiratory alveolar epithelial T2C. The validation experiments on the mRNA targets showed that the mRNAs of interest (that were targeted by significantly changed miRNAs, **Figure 2C**) are particularly responsive in the male KO but not responsive in SP-A1 except the *Bcl2* which showed increased levels in SP-A1. The increase of *Bcl2* in SP-A1 indicates that the SP-A1 T2C may be protected against apoptosis. Previously, we have shown that AM of SP-A2 hTG mice were also protected from apoptosis under OxS (42), indicating a differential effect of the two SP-A genes, *SFTPA1* and *SFTPA2*, in T2C and AM, respectively, with regards to apoptosis. However, the fact that most of the target genes in SP-A1 remained unaffected after OxS is puzzling. We speculate that this may in part be due to: (1) The time point used (4 h after OxS) for study is not optimal to assess mRNA levels of target genes in SP-A1; (2) the target genes may recover from OxS faster in SP-A1 than in KO; (3) SP-A1 may protect other molecules from harmful effects of OxS

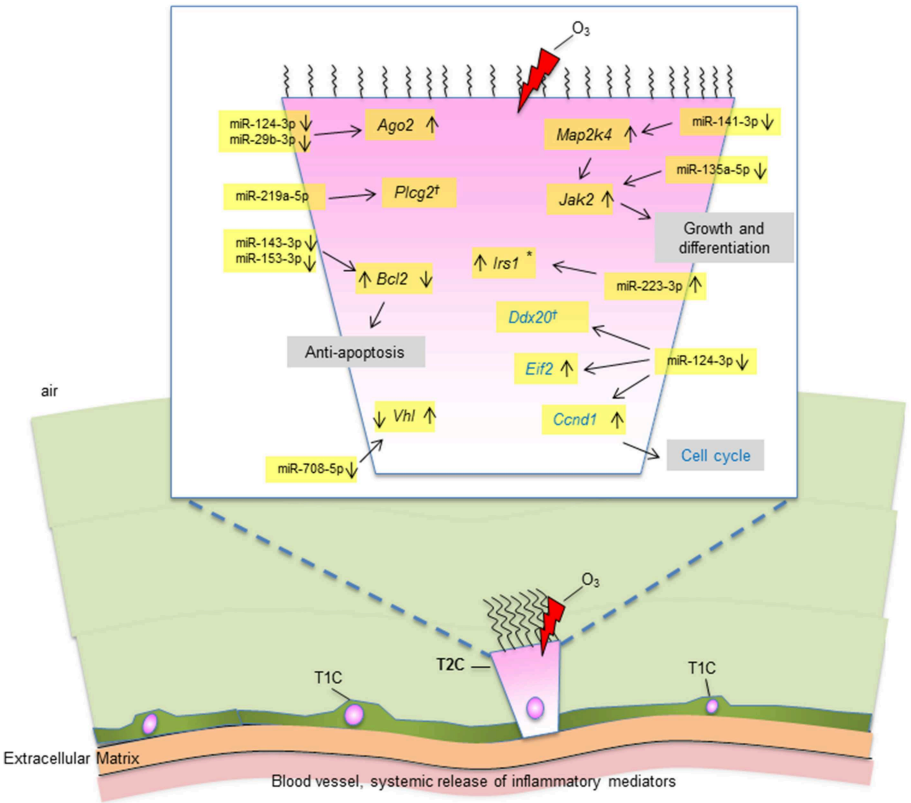

FIGURE 8 | A diagrammatic representation of miRNAs and their targets in T2C under OxS. Targets with significantly changed levels in KO compared to SP-A1 are members of several pathways as shown in figure. These include *MAPK* and *JAK2* signaling pathways, cell cycle, anti-apoptosis, and other. The miRNAs and the mRNA levels of their target genes studied in the present study are highlighted in yellow. Up (↑) or down (↓) on the right of the target gene indicate increase or decrease in KO in non-gondectomized (NGx) mice. Up (↑) or down (↓) on the left of the target gene indicate increase or decrease in gondectomized (Gx) mice. The expression of 3 genes (*Irs1, Bcl2, And Vhl*) changed after gonadectomy: *Bcl2* and *Vhl*, respectively showed increase and decrease in KO, which is the reverse of what is seen in the NGx KO. *Irs1* increased in Gx KO only, no change in NGx (*). The expression of *Ccnd1* was increased in both NGx and Gx KO indicating hormone-independent expression. The expression of *Plcg2* and *Ddx20* did not change significantly after OxS in either NGx or GX KO (†).

by being more readily oxidized by scavenging ROS (48); (4) SP-A1 may protect the T2C by affecting functions modulated by molecules that are not regulated by miRNAs. An example of this may be its role in surfactant structural organization (41) and potentially lung function. Another interesting observation was the significant upregulation of Von Hippel-Lindau (*Vhl*) mRNA, targeted by miR-708-5p (58), in the gonadectomized (Gx) SP-A1 T2C when the experimental male mice were exposed to O_3. It is known that *Vhl* is part of a degradation complex that removes damaged or unnecessary proteins and helps maintain the normal functions of cells (59). In particular, this degradation complex is known to degrade proteins when oxygen levels are lower than normal, such as hypoxia (60) and possibly in OxS. The increase in *Vhl* in Gx SP-A1 mice (but not in NGx) indicates that sex hormones may play a role in its expression in SP-A1 mice.

The increase of several targets in KO but not in SP-A1 may point to deficiencies in KO and that these increases may reflect a more active gene regulation to overcome the effects of OxS. These include, the mitogen-Activated Protein Kinase 4 (*Map2k4*) and Janus Kinase 2 (*Jak2*), which are targeted by miR-141-3p (61) and miR-135a-5p (62) and shown here to change by >2-fold; both targets are involved in the *MAPK* signaling. These could transmit the OxS distress signal across the cell membrane to the DNA in the nucleus triggering a number of different functions such as apoptosis, cell differentiation and proliferation. The argonaute 2 (*Ago2*) mRNA, which is targeted by miR-124-3p and miR-29b-3p (63), was increased significantly in the KO. *Ago2* is an important enzyme in the biogenesis of miRNAs that plays a role in the formation of the RNA-induced silencing complex (64). Of interest, cyclin D1 (*Ccnd1*) was upregulated in Gx and NGx KO males indicating independence of circulating hormones, and possibly a dependence on the presence or absence of SP-A, no change in *Ccnd1* was observed in SP-A1 T2C either in Gx or NGx males. The eukaryotic initiation factor 2 (*eIF2*) was upregulated in NGx KO but not in the Gx KO mice indicating a role of hormones in its expression. These mRNAs (*Ccnd1* and *elF2*) are targeted by miR-124-3p. This molecule is known to regulate global and specific mRNA translation in response to stress-related signals (such as OxS) (65).

Collectively, our data show that OxS has a major effect on the male SP-A1 T2C miRNome. The targets of the significant

miRNAs are implicated in several pathways that include the *MAPK* signaling pathway (*Mapk, Jak2, Irs1*), cell cycle (*Ccnd1*), anti-apoptosis (*Bcl2*), protein degradation (*Vhl*), and other. These observations diagrammatically are depicted in **Figure 8**. The limitations of the present study are that we studied a single time point and we did not look at the protein levels of the targeted mRNAs. Our publication on the alveolar macrophages (AM) miRNome showed that in response to OxS, it was SP-A2 that had a major effect on the male AM with pro-inflammatory, anti-apoptotic, and anti-oxidant pathways playing a role (42). The two studies together indicate that there is a differential role of SP-A1 and SP-A2 in the alveolar cells. The SP-A1 findings in the present study are consistent with our previous observations where SP-A1 is shown to play a role in the surfactant structural organization (41), and may also play an important role on the integrity and function of T2C. SP-A2 on the other hand has been shown to not only affect the AM male miRNome but also exhibit a higher innate immune activity (31, 36, 38).

We conclude that dysregulation of either SP-A1 or SP-A2 may affect the innate immunity and/or surfactant structure and potentially lung function. Both processes are essential for normal lung function and derangement of the regulation of either gene may be a problem in pulmonary diseases, including OxS.

ETHICS STATEMENT

All protocols used in this study were evaluated and approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health on the care and use of laboratory animals.

AUTHOR CONTRIBUTIONS

GN performed experiments, run statistics, analyzed and synthesized the data, contributed to the manuscript writing. NT performed experiments, analyzed and synthesized the data, contributed to the manuscript writing. XZ and TU performed maintenance and breeding of mouse lines, exposed mice to experimental conditions (FA & O_3) and isolated alveolar type II cells. FD performed all gonadectomy experiments. DP contributed to data analysis and synthesis and manuscript writing. JF designed and provided oversight to the entire project, involved in data analysis, integration, and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01514/full#supplementary-material

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REVIEW
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Transcriptional Effects of Ozone and Impact on Airway Inflammation

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Epidemiological and challenge studies in healthy subjects and in individuals with asthma highlight the health impact of environmental ozone even at levels considered safe. Acute ozone exposure in man results in sputum neutrophilia in 30% of subjects particularly young children, females, and those with ongoing cardiopulmonary disease. This may be associated with systemic inflammation although not in all cases. Chronic exposure amplifies these effects and can result in the formation of asthma-like symptoms and immunopathology. Asthmatic patients who respond to ozone (responders) induce a greater number of genes in bronchoalveolar (BAL) macrophages than healthy responders with up-regulation of inflammatory and immune pathways under the control of cytokines and chemokines and the enhanced expression of remodeling and repair programmes including those associated with protease imbalances and cell-cell adhesion. These pathways are under the control of several key transcription regulatory factors including nuclear factor (NF)-κB, anti-oxidant factors such as nuclear factor (erythroid-derived 2)-like 2 NRF2, the p38 mitogen activated protein kinase (MAPK), and priming of the immune system by up-regulating toll-like receptor (TLR) expression. Murine and cellular models of acute and chronic ozone exposure recapitulate the inflammatory effects seen in humans and enable the elucidation of key transcriptional pathways. These studies emphasize the importance of distinct transcriptional networks in driving the detrimental effects of ozone. Studies indicate the critical role of mediators including IL-1, IL-17, and IL-33 in driving ozone effects on airway inflammation, remodeling and hyperresponsiveness. Transcription analysis and proof of mechanisms studies will enable the development of drugs to ameliorate the effects of ozone exposure in susceptible individuals.

Keywords: gene expression, immune cell recruitment, acute ozone exposure, chronic ozone exposure, pro-inflammatory signaling

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INTRODUCTION

It has been known since the mid-1980s that ozone exposure induces airway inflammation and reduces lung function (1). As highlighted elsewhere, ozone is a powerful gaseous oxidant and toxic air pollutant that is inhaled and thereby affects the lung (2). The highly reactive oxidative agent ozone, or catena-trioxygen (O_3), is consumed by protective processes within the epithelial lining fluid to produce secondary oxidation products that activate the airway epithelium to enhance inflammatory signaling pathways and induce several pro-inflammatory and immune factors (2). In this Review, we will discuss the effects of ozone on the activation of pro-inflammatory pathways in airway epithelial cells. We then discuss the epidemiological effects of ozone and that of controlled ozone-exposure on clinical and immune features in healthy subjects and in at-risk

subjects including asthmatics and patients with other airways diseases and those in the youngest and oldest populations. Finally, we discuss how acute and chronic ozone-exposure models may provide insight into the clinical effects of ozone. The data also highlights the importance of acute vs. chronic exposure and also the effect of the timing of samples collection following ozone exposure for the analysis of ozone effects.

MECHANISMS IMPLICATED IN OZONE ACTIONS ON THE AIRWAY EPITHELIUM

There are differences in the responses of cells and animals to particulate matter (PM) and ozone (3). Both PMs and ozone can activate membrane receptors, intracellular kinases particularly NF-κB and phosphatases, and transcription factors that regulate inflammatory responses. Understanding these mechanistic processes will allow both preventative sand therapeutic strategies to be designed for subjects exposed to ozone for prolonged periods.

Mechanistically, inhaled ozone does not enter cells but reacts with components of the airway lining fluid to generate other reactive oxygen species (ROS) to enhance local oxidative stress, inflammation, and epithelial cell injury (4) (**Figure 1**). Recent data also highlights a pro-inflammatory role for ozone-derived oxysterols (epoxycholesterol-α and -β and secosterol A and B) (5). These form in the airway lining fluid and form lipid-protein adducts with the liver X receptor (LXR) resulting in suppression of cholesterol synthesis pathways. Ozone-induced lung injury pulmonary inflammation has been linked to altered expression of pro-resolving lipid mediators (SPMs) such as 14-HDHA and 17-HDHA and the SPM protectin DX (PDX) in the lung of male C57Bl/6J mice (6). Exogenous administration of 14-HDHA and 17-HDHA prior to ozone exposure decreased proinflammatory cytokine and chemokine expression, and decreased BAL macrophages and PMNs. Ozone also enhances the expression of several eicosanoids and oxidized lipids including 20-hydroxyeicosatetraenoic acid (20-HETE) in the BAL of ozone-exposed animals. These oxidized lipids may mediate the effects of ozone on AHR *in vivo* as demonstrated by effects on precision cut lung slices (7). In contrast, prostaglandin E2 (PGE2) may not be important since knockout of microsomal prostaglandin E synthase-1 (mPGES-1) did not impact on ozone-induced AHR or inflammation (8).

Key Role of Oxidative Stress in Ozone Actions

The critical role of oxidative stress in ozone function was highlighted by the fact that lipid oxidation, epithelial proliferation, bronchial mucous cell hyperplasia, and mucus hypersecretion were greater in Nrf2-/- than in wild type mice (9). Furthermore, the potent and selective Nrf2 activator 3-(pyridin-3-ylsulfonyl)-5-(trifluoromethyl)-2H-chromen-2-one (PSTC) restored ozone-induced glutathione depletion with no off-target inhibition of IL-1β-induced NF-κB translocation in human bronchial epithelial cells (HBECs) (10). Interestingly, the serum innate immune protein, mannose-binding lectin (MBL), helps drive ozone-induced proinflammatory events. Ozone (0.3 ppm)-exposed MBL-deficient (Mbl-/-) mice produced significantly reduced levels of BAL inflammatory markers including eosinophils, neutrophils, CXCL2 and CXCL5, and gene array analysis revealed differences in the NRF2 signaling networks (11).

In addition, high doses of the anti-oxidant N-acetylcysteine (NAC) prevents and reverses ozone-induced lung damage *in vivo* (12). NAC given prophylactically prevented ozone-induced BAL inflammation (macrophages) and airway smooth muscle (ASM) mass whilst NAC given therapeutically reversed AHR in addition to reducing ASM mass and the number of apoptotic cells.

Differences Between *in vivo* and *in vitro* Effects of Ozone on Cellular and Mitochondrial ROS

There are controversies regarding the role of ROS in mediating ozone functions both *in vitro* and *in vitro*. It is possible that mitochondrial damage leading to oxidant stress may play an important role in the pathogenesis of airflow obstruction and emphysema following ozone exposure (13). Mice exposed to ozone have mitochondrial dysfunction reflected by decreased mitochondrial membrane potential ($\Delta\Psi m$), increased mitochondrial oxidative stress, and reduced mitochondrial complex I, III, and V expression. This was associated with airway inflammation and AHR (14). Pharmacological reversal of mitochondrial dysfunction by the mitochondria-targeted antioxidant MitoQ reduced inflammation and AHR. In another study, a similar mitochondrial-directed anti-oxidant, MitoTEMPO, reduced chronic (6 week) ozone-induced (2.5 ppm, 3 h, twice weekly) lung inflammation (BAL cells and IL-1β, KC/CXCL1 and IL-6) and oxidative stress but had no effect on emphysema (Lm) scores. In contrast, inhibition of the NLPR3 inflammasome, which is activated by mitochondrial ROS (mtROS) and other stimuli, suppressed ozone-induced inflammation, oxidative stress, emphysema, airway remodeling, and airflow limitation (**Figure 1**).

Further evidence for a role of mitochondrial dysregulation in ozone-induced inflammation and AHR was shown by restoration of defective mitochondria using induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) *in vitro* and *in vivo* (15). iPSC-MSCs protected against oxidative stress-induced mitochondrial dysfunction in human ASMCs and in mouse lungs while reducing airway inflammation and AHR.

Toll-Like Receptors (TLRs) and Other Cell Surface Receptors Mediating Ozone-Induced Inflammation

Ozone also impacts on AHR and neutrophilic inflammation though activation of Toll-like receptor (TLR) 2 and TLR4 (16). In addition, there is a priming effect of ozone on the innate immune response as exemplified by enhanced TLR2 and TLR4 expression and functional responses to their respective agonists (17, 18). Ozone alone enhanced TLR4, TLR2, and TLR1 expression on macrophages which may account for the ability of subsequent challenge of ozone-exposed mice

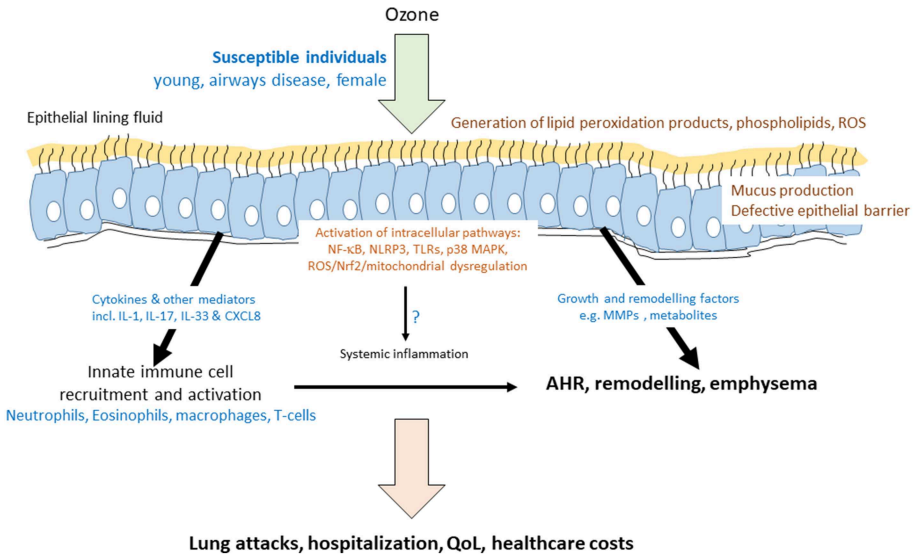

FIGURE 1 | Mechanisms of ozone-induced transcriptome activation in the airway. Schematic diagram indicating that ozone dissolves in the epithelial lining fluid of susceptible individuals to produce reactive oxygen species (ROS) and modified lipids. These act on the epithelial cells to activate a number of key intracellular and cell surface pathways leading to the induction of the mRNA for cytokines, growth factors, and remodeling enzymes. As a result, there is an acute effect on the recruitment and activation of innate immune cells and on epithelial barrier function and mucus production. These acute effects of ozone may impact on the levels of inflammatory mediators and cells in the blood. Airway inflammation may be directly, or indirectly, associated with airway hyperresponsiveness (AHR). Prolonged ozone exposure has a greater remodeling effect and can result in emphysema. Together, both acute and chronic ozone exposure results in increased hospitalisations due to lung exacerbations or attacks, decreased quality of life (QoL) in at-risk individuals and a large healthcare cost for the individual and for society.

to Pam3CYS, a synthetic TLR2/TLR1 agonist, to enhance lung IL-6 and KC/CXCL1 and reduce TNFα and MIP1α expression. Transcriptomic and bioinformatic analysis of lung tissue revealed significant effects on innate immune pathways. Of interest, hyaluronan, a damage-associated molecular pattern essential for the full range of ozone effects seen *in vivo*, acts through the TLR4/MyD88-TIRAP pathway (19, 20). The primary receptor for hyaluronic acid (HA), which is important in lung injury and is elevated following ozone exposure, is CD44. Genetic experiments using CD44 deficient mice or those lacking inter-alpha-trypsin inhibitor, which facilitates HA binding, do not demonstrate AHR following ozone exposure. In addition, pharmacologic pretreatment of ozone-exposed mice with HA-binding peptide protects against ozone-induced AHR whilst HA itself enhances the effect of ozone in a CD44-dependent manner (21). Together, these results suggest that the extracellular matrix is important in ozone-induced AHR.

Genetic deletion of surfactant protein-D (SFTPD), a collectin important for suppressing macrophage inflammatory responses alters the response to ozone (22). In SFTPD(-/-) mice, there was increased presence of lung injury and oxidative stress and the increased numbers of BAL macrophages were enlarged and foamy. SFTPD(-/-) mice also presented with a worsening of central airway and parenchymal mechanics consistent with the loss of parenchymal integrity. Ozone modulation of SFTPD regulates an IFNγ/IL-12 feedback loop which amplifies dendritic cell homing to mediastinal lymph nodes (23).

Intracellular Signaling Pathways Mediating Ozone Actions

There is evidence that p38 mitogen-activated protein kinase (MAPK) pathway mediates ozone-enhanced airway inflammation and remodeling as well as AHR in murine models of asthma (24). These responses to ozone are relatively corticosteroid insensitive but combination of dexamethasone with a p38 MAPK inhibitor (SB239063) suppressed ozone effects on inflammation and AHR suggesting that p38 MAPK activation is involved in the corticosteroid insensitivity seen in this model. SB239063 alone prevented ozone-induced airway resistance (Raw), lung compliance (CL), and BAL IL-13 levels when given in the context of an ovalbumin challenge (25). In addition, the anti-oxidant α-tocopherol alone reduced BAL eosinophils and macrophages and peribronchial inflammation. Both drugs also prevented increases in ozone-induced AHR, BAL IFNγ, and IL-6 expression and perivascular lung inflammation. This effect was enhanced using a combination of both drugs indicating a role for both p38 MAPK and oxidative stress in ozone-induced exacerbations of asthma.

The p38 MAPK pathway is also involved in the mechanism of action of the novel gaseous transmitter hydrogen sulfide (H_2S), which partially reversed ozone-induced lung inflammation, oxidative stress and emphysema (26, 27). Other pathways targeted by H_2S include the Akt pathway and the NLRP3-caspase-1 system. Other MAPK pathways may also be important since the effect of OBC on lung inflammation was dependent on the activation of MAP4K4 in CD4(+) T cells (28).

A number of other signaling pathways have been implicated in ozone-induced inflammation, tissue damage and AHR. The spleen tyrosine kinase (Syk) inhibitor NVP-QAB-205 significantly reduced AHR in an ovalbumin-exposed and challenged mouse model of asthma and restored the enhanced response to PM2.5/ozone back to normal levels (29). Furthermore, IL-10 overexpression protects mice from the detrimental inflammatory, NF-κB-mediated effects of ozone (30). The EGFR kinase inhibitor PD153035, given prophylactically, prevents ozone-induced EGFR (Y1068) phosphorylation in the lung sections and significantly attenuates lung inflammation (31). The effects of therapeutic administration on inflammation and lung function should be measured.

The airway remodeling induced by ozone exposure may involve the presence of neutrophil-derived neutrophil gelatinase-associated lipocalin (NGAL) (32). The effects seen *in vivo* on the suppression of E-cadherin and up-regulated α-SMA expression were replicated by NGAL exposure in 16HBE cells acting through the WNT/glycogen synthase kinase-3β (GSK-3β) pathway.

Ozone also impacts on cardiorespiratory conditions including hypoxia-associated pulmonary hypertension (HPH) which often occurs in COPD (33). Male C57BL/6 mice exposed to 3 weeks hypoxia (10.0% O_2) followed by ozone (4 h, 1 ppm) resulted in increased inflammation, oedema, and AHR. Fasudil, a Rho kinase inhibitor, reduced pulmonary endothelial barrier damage suggesting that enhanced pulmonary vascular pressure may contribute to lung injury, inflammation, and oedema following ozone exposure (34). In addition, the hypoxia induced transcription factor HIF-1α mediates MIF effects on ozone-induced BAL cell counts, cytokine, and AHR (35).

EPIDEMIOLOGICAL IMPACT OF OZONE IN HEALTHY AND AT RISK SUBJECTS

Controlled human exposure to ozone causes decrements in lung function, increased lung neutrophilia and increased airway levels of pro-inflammatory cytokines (4). It is not surprising, therefore, that air pollution is a known asthma trigger and has been associated with short-term asthma symptoms, airway inflammation, decreased lung function, and reduced response to asthma rescue medications and increases the risk of asthma hospitalizations and healthcare utilization (36). In addition, traffic-related pollutants may be causally related to childhood asthma (36).

Ozone impacts equally upon both FEV_1 and FVC and therefore does not result in a decrease in FEV_1/FVC. This effect is age-dependent with an increased risk in susceptible populations particularly the very young, pregnant women and individuals with an existing cardiopulmonary disease (37). Acute airway injury and inflammation also results from ozone exposure including enhanced localization of neutrophils and increased cytokine and chemokine expression (1). An early observational study in 38 asthmatics and 13 healthy control subjects in metropolitan Atlanta showed that ozone induced more symptoms of upper airway disease in asthmatics (38). High ozone levels were associated with greater airflow obstruction, lower asthma quality of life scores, more eosinophilia, and higher exhaled nitric oxide (NO) levels in asthmatics. These associations were enhanced in atopic participants irrespective of asthma status. Peak Expiratory Flow (PEF) levels were also negatively associated with weekly average ozone levels for 605 children in Turkey without upper respiratory tract complaints (39).

Significant increases in sputum neutrophilia is only seen in ∼30% asthmatics (40). This mimics other markers of sputum neutrophil activation and exhaled breath malondialdehyde (MDA) levels. There was a significant correlation between the ozone-induced fall in FEV_1 and sputum neutrophil numbers. In addition, there was evidence for sputum eosinophilia post ozone exposure (40). Furthermore, ozone significantly increased neutrophil numbers and myeloperoxidase levels in bronchoalveolar lavage and produced a 4-fold increase in bronchial mucosal mast cell numbers (41). Asthmatic patients not previously treated with inhaled corticosteroids (ICS) and those with a lower FEV1 were more likely to respond to ozone (**Figure 1**). Lower baseline airway inflammation, younger age and greater airway hyperresponsiveness (AHR) was associated with airway neutrophilia in response to ozone (42). It is possible, therefore, that the lung function and inflammatory responses to ozone are different.

Ozone Increases the Risk of Emergency Room Visits

The risk of increased emergency room visits in subjects with acute respiratory infections, asthma, chronic obstructive pulmonary disease (COPD), and pneumonia in response to environmental ozone exposure across 17 US states was also age related (43). There is evidence that ozone exposure causes systemic inflammation as measured by blood club cell protein (CCP)-16 levels (44). Recent studies examining data from 6,488 subjects from the National Health and Nutrition Examination Survey (NHANES) between 2005 and 2006 demonstrated a positive association between ozone exposure and emergency room visits in asthmatic patients (adjusted OR 1.07, 95% CI: 1.02–1.13). However, this effect was less than that seen with $PM_{2.5}$ and NO_2 exposure (45). In addition, time-series analyses of ozone exposure and emergency room visits across 17 States in the USA demonstrated a significant increase in the rate of admission per 20 ppb increase in ozone across all age groups. The effect was greater in adults compared to children and in subjects with asthma and COPD (43). Finally, short term exposure of asthmatics to increasing levels of ozone in the Chinese province of Hubei resulted in asthma mortality (46).

It is evident that ozone exposure also impacts upon the health status of patients with other respiratory diseases such as idiopathic pulmonary fibrosis (IPF) and adult respiratory distress syndrome (ARDS). For example, chronic exposure to ozone enhances the risk of ARDS among older adults (>65 years of age) in the USA (47). Increases of 1 ppb in annual average ozone levels was associated with increases in annual hospital admission rates for ARDS of 0.15% (95% CI, 0.08–0.22) which was evident even in low-pollution regions (annual average ozone level < 45 ppb) below the current annual US National Ambient Air Quality

Standards. Furthermore, long term exposure (3 years) to ozone was significantly ($P < 0.01$) associated with the development of ARDS following acute trauma even at exposure levels generally below European and USA air quality standards (48).

In a French IPF cohort (COhorte FIbrose, COFI), increasing ozone levels measured close to the patient's home was associated with acute exacerbations (AE) which are associated with high mortality (49). However, in this study ozone did not affect mortality although this was increased by increased exposure to PM2.5 and PM10. In an earlier study, AEs were significantly associated with increases in ozone exposure in the 6 weeks prior to hospital admission (50). Although air pollution *per se* is associated with AEs, disease progression and mortality in IPF, ground level ozone exposure over 40 weeks does not affect lung function (51). Overall, the data suggest that elevated levels of ozone increase respiratory tract complaints with a greater effect seen in susceptible adults and in children.

Ozone also impacts on the health of non-respiratory patients and the risk of mortality is increased in sepsis patients exposed to environmental ozone (52). The mechanisms for this effect was unclear, however, in 3,820 non-current smoking subjects from the Framingham Heart study, there was a negative correlation between ozone exposure and systemic monocyte chemoattractant protein (MCP)-1 levels as a marker of systemic inflammation (53). It is of interest that many of these exposures that affect AEs, for example, are below current National Guidelines which has huge public health implications.

Controlled Exposure to Ozone: Effects on Lung Function and Immune Cell Activation

Early studies examining the acute effect effects of O3 (220 ppb, 2.25 h) exposure under laboratory conditions on respiratory physiology in healthy subjects revealed an acute effect on lung function parameters such as FEV1 and FVC and effects on AHR to methacholine (Mch) and on respiratory epithelial permeability at 1-day postexposure (54). The responder profiles to these different readouts differed in that either the acute decreases in FEV1 or the development of AHR or epithelial permeability at 1 day post ozone did not always coincide suggesting that these are separate and independent phenotypic effects seen with O3 exposure (54).

Controlled exposure studies support these epidemiological reports. The recent Multicenter Ozone Study in Older Subjects (MOSES) examined the respiratory responses to low concentrations of ozone in 87 healthy adults aged 60 years (55). Exposure to filtered air rapidly increased mean FEV_1 and FVC values but ozone attenuated these increases in a dose-dependent manner. This effect persisted for 22 h after exposure. Ozone enhanced plasma CC16 levels and sputum neutrophilia in a dose-dependent manner after 4 h and sputum neutrophil counts correlated with airway biopsy counts (56). Sputum neutrophilia returned to normal levels 18 h post-ozone exposure (55, 56). Worryingly, concentrations of ozone close to the National Ambient Air Quality Standard (0.06 ppm) decreased lung function and increased airway inflammation in healthy young adults (57). This response was independent of a reduction in the expression of the anti-oxidant gene glutathione S-transferase mu 1 (GSTM1).

Acute ozone exposure (2 h, 0.4 ppm) of 9 healthy adults enhanced sputum neutrophils, monocytes and macrophages after 6 h with a significant upregulation of innate immune (mCD14, CD11b, CD16) and antigen presenting (CD86, HLA-DR) markers (58). Ozone also enhanced sputum interleukin (IL)-6 and induced a transient decline in lung function but had no effect on exhaled NO. This suggests that ozone can prime innate immune cells that may be important for susceptible subjects subsequently exposed to inhaled allergens.

It is unclear whether ozone acts independently of other environmental factors. In some studies ozone alone enhances plasma and sputum inflammatory cells and mediators (CC16, IL-6, and matrix metalloproteinase (MMP)9) (59) with no effect seen with carbon black ultrafine particles (cbUFP). However, other studies show no effect of ozone alone (60). Overall, the effect of ozone on immune cell numbers and activation are dependent upon the dose and timing of exposure and the samples collection point. This makes comparison of the studies more complex.

Sputum induction on 27 healthy adults before and after ozone exposure (0.4 ppm, 2 h) gave distinct transcriptional responses according to whether there was a significant increase in sputum neutrophils (61). These ozone responders had activated innate immunity (increased expression of CD16, CD11b, and CD80 and elevated IL-8 and IL-1β expression) with reduced immune cell trafficking pathways (**Figure 1**). These distinct signatures may explain the effects of ozone in sensitive individuals. Healthy subjects who respond to ozone also demonstrate significantly enhanced sputum levels of the miRNAs miR-132, miR-143, miR-145, miR-199a*, miR-199b-5p, miR-222, miR-223, miR-25, miR-424, and miR-582-5p (62). The predicted targets of these miRNAs included inflammatory and immune-response pathways.

Gene arrays of BAL cell mRNA isolated after exposure of combined healthy and asthmatic subjects to various doses of ozone were subjected to pathways analysis (63). This highlighted enhanced inflammation and repair pathways particularly those involved in chemokine/cytokine secretion, activity, and receptor binding; metalloproteinase and endopeptidase activity; adhesion, locomotion, and migration; and cell growth and tumorigenesis regulation. The response in asthmatics was 1.7–3.8-fold greater than that seen in healthy subjects. The highest differentially expressed up-regulated gene was osteopontin and treatment of an airway epithelial cell line with polymeric osteopontin enhanced wound closure in an α9β1 integrin-dependent manner.

Bioinformatic analysis of gene expression profiles has indicated that the impact of ozone exposure on generating acute lung injury (ALI) in animal models is significantly correlated with signatures seen in 3 human lung injury bronchoalveolar lavage datasets ($r = 0.33$–0.45, $p < 10^{-16}$) (64). Signatures relate to neutrophils, cytokine and chemokine activation and kinase activation pathways and there is a significant overlap of 181 potential drug targets common between human and animal models.

Metabolomics analysis of bronchoalveolar lavage fluid (BALF) from healthy subjects following a 2 h exposure to ozone (0.3 ppm) whilst undertaking light exercise could detect 28 differentially

expressed metabolites after 1 h indicative of increased glycolysis and a feedback antioxidant response (65). In contrast, at 24 h post ozone exposure 41 differentially expressed metabolites were observed. These were associated with enhanced proteolysis and lipid membrane turnover indicating repair of airway tissues (65). Interestingly, whilst many human ozone exposure models include light exercise, the effect of ozone on exhaled oxidative stress markers were attenuated by physical activity in healthy adolescents (66).

SEX SPECIFIC RESPONSES TO OZONE

Sex-specific differences exist in the incidence and prognosis of respiratory diseases with women having a greater risk of adverse health outcomes from air pollution than men (**Figure 1**). However, the underlying mechanisms for this are unknown although sex-specific patterns of immune gene expression and regulatory networks have been proposed (67). The expression of 84 inflammatory genes in murine lungs 4 h after ozone exposure (2 ppm, 3 h) was examined and linked to lung histology and bronchoalveolar lavage (BAL) cell counts (68). Inflammatory gene mRNA levels in female mouse lungs were significantly lower than in males for 20/72 genes analyzed following ozone exposure (3 h at 2 ppm) whilst only 4/72 were higher. Down-regulated genes included TLRs (TLR1, TLR3, TLR6), cytokines (IL1α, IL23α), chemokines (CXCL5, CCCL12, CCL11, CCL25), cytokine and chemokine receptors (CCR3, CCR2, CXCR4, IL1RAP), as well as other immune mediators, enzymes, receptors and transcription factors (C3AR1, CD14, CD40, RIPK2, PTGS2, NF-κB, LY96). Up-regulated pathways included macrophage activation (CXCL2, CCL19), Toll-like-receptor activation (MYD88), and modulation of IL-6 responses (C4B). Bioinformatic analysis indicated that immune cell adhesion and movement and pattern recognition receptor functions were enriched in females whereas the inflammatory response, cell-to-cell signaling and interaction, and cellular movement pathways were enriched in males. Overall, the results indicate a decrease in innate immune responses in females compared to males in response to ozone exposure (68).

The same group also examined miRNA lung expression and found that the baseline expression of miR-222-3p and miR-466 k were up- and down-regulated, respectively, as different in the lungs of male and female C57BL/6 mice which pathway analysis indicated controlled the expression of cell-to-cell signaling and interaction, cellular growth, proliferation, and gene expression (67). Exposure of male and female C57BL/6 mice to 2 ppm of ozone or filtered air for 3 h resulted in sex differences in the expression of 9 miRNAs including miR-130b-3p, miR-17-5p, miR-294a-3p, and miR-338-5p which are involved in inflammation and targeted key regulators of the immune response including IL-6, SMAD2/3, and TMEM9. The top networks included pathways associated with cell cycle, cellular development, growth, proliferation, and movement along with cell death and survival.

The detrimental effects of ozone in the lung have been attributed to bronchial-alveolar epithelial damage and defects in the bronchial-blood barrier but whether these effects are different between males and females is unclear. 3 h exposure to 2 ppm ozone up-regulated lung IL-6 levels in both males and females but the expression of IL-6R in the lung was only elevated in females (69). This was associated with a significant increase in STAT3-Y705 phosphorylation in both females and males whilst JAK expression and phosphorylation differed between sexes. In addition, NF-κB (p105/p50) and AKT1 protein levels were significantly increased only in ozone-exposed females. Furthermore, these responses varied across the oestrous cycle (69).

Ozone exposure following an infection results in greater survival in males compared to females whereas survival after infection alone is greater in females (70). Using a multianalyte immunoassay to measure 59 analytes in the lung at 4 h indicated that females had a greater oxidative stress response to ozone than males indicated by enhanced lung expression of EGF, fibrinogen, haptoglobin, IL-17, Interferon γ inducible protein (IP)-10, KC/CXCL1, MCP-5, M-CSF, MIP-2, RANTES, SCF, and TNF-α. This enhanced response was inversely correlated with the poorer survival (70). It is surprising how few studies take gender into account when modeling the effects of environmental pollutants.

Previous work from the Shore group has demonstrated that short-chain fatty acids (SCFAs) which represent the end products of bacterial fermentation are important in driving the airway response to ozone following changes in the mouse gut microbiome (71). The same group have reported that these ozone-induced responses are sex-dependent. Indeed, acute ozone exposure to ozone resulted in greater AHR in male compared to female mice which disappeared with antibiotic treatment (71). Female pups kept in the same cages as male pups also exhibited a greater AHR in response to ozone exposure which was mimicked by addition of the SCFA propionate to the drinking water. The data indicates that microbiome-derived SCFAs are processed in a sex-dependent manner to modify ozone-induced AHR.

OZONE MAY AFFECT INHALED AND ORAL DRUG RESPONSES

Acute inhalation of ozone in rodents modulates the expression of circulating stress hormones including adrenalin and corticosterone/cortisol and adrenalectomy attenuates ozone-induced lung injury and inflammation. This may account for the variable sensitivity seen to ozone *in vivo*. This also suggests that there is a potential feedback loop that may be impacted by exogenous drugs (β2-agonists and ICS) used to treat patients with asthma and COPD which affects the response to environmental ozone (72, 73).

High levels of IL-17A have been associated with a relative corticosteroid (CS)-insensitivity in mice (74). Chronic ozone exposure (2.5 ppm; 3 h twice weekly for 6 weeks) enhanced numerous inflammatory mediators including IL-17A, which was associated with neutrophil infiltration into the lung (75). Prophylactic treatment with a combination of dexamethasone (2 mg/kg) and an anti-murine IL-17A monoclonal antibody (IL-17mAb) reduced chronic ozone-induced neutrophilia and total

inflammatory cells, inhibited BAL protein levels of IL-8, IL-17A, and TNF-α and the mean linear intercept (Lm) as a marker of emphysema. Combined administration also significantly elevated BAL interferon (IFN)γ levels and glucocorticoid receptor (GR) expression whilst supressing NF-κB and p38 MAPK phosphorylation (75). The study highlighted the critical role of IL-17A in reducing CS responses as a result of ozone exposure.

Many studies implicate oxidative stress in the pathophysiology of COPD and the relative corticosteroid-insensitivity observed in most of these patients (76, 77). Repeated ozone exposure (3 ppm, 3 h) twice a week for 6 weeks induces many features of COPD including emphysema and lung/airway inflammation (78). This was associated with enhanced expression of caspase-3 and apoptosis protease activating factor-1 in macrophages and in the airway and alveolar epithelium. IL-13, KC/CXCL1, caspase-3, and IFNγ mRNAs were increased whereas heme oxygenase-1 (HO-1) mRNA decreased. These effects of chronic ozone exposure were relatively insensitive to high dose (2 mg/kg) dexamethasone given prophylactically (79). These effects were generally unaffected by the CS-upregulated protein DUSP1/MKP-1 although a small impact effect on remodeling was observed.

Interestingly, there is also a link between ozone exposure and endogenous CS responses in animal models as evidenced by the effect of the 11β-hydroxylase inhibitor metyrapone (80). Ozone (0.8 ppm, 4 h) exposure evoked a metyropone-sensitive 2-fold increase in plasma corticosterone without affecting epinephrine levels. The effects of ozone on ghrelin or plasminogen activator inhibitor-1 were unaffected by metyrapone. The effect of chronic ozone on endogenous CS responses should be investigated.

MURINE MODELS OF OZONE EXPOSURE REFLECT HUMAN EPIDEMIOLOGICAL AND CHALLENGE MODELS

The effects of acute and chronic ozone exposure in man have been confirmed in animal models and details of the transcriptomic effects elucidated in these rodent models and in human cellular models (81). Acute ozone exposure (1 ppm, 1 h) in the mouse caused respiratory epithelial disruption with protein leak, BAL neutrophil recruitment, lung inflammation and AHR. Chronic exposure (1.5 ppm, 2 h, twice weekly for 6 weeks) amplified these acute effects of ozone and furthermore resulted in collagen deposition, greater epithelial injury with reduced epithelial barrier height, distended bronchioles, and enlarged alveolar space indicative of peribronchiolar fibrosis and emphysema (**Figure 1**).

Repeated exposure of C57BL/6 mice to ozone (0.8 ppm, 4 h/day) for 9 days resulted in the presence of an asthma-like phenotype with airway eosinophilia, mucus cell metaplasia and activation of type 2 innate lymphoid (ILC2) cells (82). Interestingly, ozone (3 ppm, 2 h) induced airway eosinophilia and IL-5 expression as well as neutrophilia in BALB/c but not C57BL/6 mice (83). ILC2s isolated from BALB/c mice exposed to ozone expressed greater mRNA levels for IL5 and IL13 than seen in C57BL/6 mice. Anti-Thy1.2 treatment abolished ozone effects on AHR that were restored by addition of ILC2s. Thus, the role of ILC2 cells in ozone-induced AHR and airways inflammation are strain dependent.

The effects of ozone on lung inflammation and AHR are biphasic and are mediated, at least in part, by distinct eosinophil populations (84). The acute 1-day effect of ozone is associated with the presence of mature airway-resident eosinophils in close proximity to parasympathetic nerves causing AHR. Four days after exposure, newly divided eosinophils were recruited to airways in a TNFα-dependent manner to suppress ozone-induced AHR. In this case, only airway eosinophils correlated with AHR indicating the importance of inflammation in distinct airway compartments.

As in man, the effects of ozone are age-dependent with adolescent and young adult animals being more susceptible to changes in ventilation and pulmonary injury/inflammation (85). Older rats had an inability to induce adaptation of ventilatory function in response to repeated ozone exposure and augmentation of lung function was most prevalent in young adult animals exposed to subchronic ozone. For example, BAL γ-glutamyl transferase activity and lung inflammation were only significantly enhanced after acute ozone in adolescent and young adult rats. The susceptibility of children to the detrimental effects of ozone exposure may be due to the need for airway and lung growth to continue after birth (86). Neurokinin signaling is involved in ozone-induced cell death and in male infant rhesus monkeys ozone increased neurokinin pathway expression in the conducting airways. In addition, ozone exposure at an early age resulted in enhanced inflammation and AHR.

Asthma exacerbations are often triggered by air pollution including ozone and in a murine model of asthma, ozone exposure resulted in enhanced AHR and greater neutrophilic inflammation associated with increased BAL levels of TNF-α, IL-13, and HA (87). Ozone also decreased epithelial cell density and increased mucus production. Furthermore, In BALB/c mice sensitized to ovalbumin, combined ozone (4 h, 0.5 ppm) and diesel exhaust particles (DEP, 4 h, 2 mg/m^3) exposure once a week for 4 weeks exacerbated AHR (88). However, in this instance, enhanced AHR was not linked to pulmonary inflammation. This mimics the situation in man where there is often a disconnect between inflammation and AHR (54) (see above).

EFFECTS OF OZONE ON INNATE IMMUNE CELLS IN THE AIRWAY

Spleen-derived macrophages and inflammatory mediators are important in ozone toxicity (89). Splenectomy resulted in decreases in pro-inflammatory macrophages (CD11b+Ly6CHi) in the lung and down regulation of CCR2, CCL2, and CCL4, but increases in anti-inflammatory (CD11b+Ly6CLo) macrophages compared to ozone-exposed wild type mice. The numbers of CD11b+Ly6G+Ly6C+ granulocytic (G)-myeloid derived suppressor cells (MDSC)s were also reduced in ozone-exposed splenectomised mice. Importantly, changes in lung macrophage subpopulations and in lung G-MDSCs correlated with reduced ozone toxicity, BAL protein content and 4-hydroxynonenal expression. Ambient ozone exposure resulted

in impaired antibacterial host defense, in part related to disruption of epithelial barrier and effective phagocytosis of pathogens (**Figure 1**). The functional response to ambient ozone is dependent on many components of the innate immune signaling (90).

Inverted formin-2 (INF2) is a significant quantitative trait locus that contributes to ozone-induced neutrophilic inflammation in mice (91). Transcriptomic and bioinformatic analysis of ozone-exposed INF2(-/-) and wild type mice revealed key roles for major histocompatibility complex (MHC) class II genes and the TNF gene cluster in ozone-induced lung neutrophilia.

The influx of distinct macrophage and monocyte subpopulations into the lung during acute ozone (0.8 ppm, 3 h)-induced lung injury was associated with CCR2 expression (92). There was a more predominant effect on proinflammatory CCR2+ and CD11b+LY6CHi and NOS2+ macrophages. However, there was also an effect on the timing of the recruitment of anti-inflammatory mannose receptor+ macrophages and CD11b+LY6CLo macrophages.

Ozone-induced γδ T cells are critical for the induction of alternatively activated M2 macrophages and the resolution of inflammation after ozone exposure (93). It is probable that secretion of IL-17A by γδ T-cells alters macrophage polarization and the clearance of apoptotic cells.

Furthermore, ozone exposure regulates the expression of galectin-3 (GAL3), which is a lectin that controls macrophage activity (94). Ozone increased the numbers of proinflammatory (GAL3+, NOS2+) and anti-inflammatory (MR1+) macrophages within the lungs. NOS2+ macrophage numbers were reduced in GAL3(-/-) mice whilst MR1+ macrophages were increased. GAL3(-/-) mice had reduced numbers of ozone-induced LY6Chi macrophages but no effect on LY6Clo macrophages was seen. In addition, the numbers of granulocytic (G) and monocytic (M) myeloid-derived suppressor cells (MDSC) were altered in GAL3(-/-) mice with G-MDSC being reduced and M-MDSCs being increased. These changes in immune cell populations correlated with tissue damage after ozone exposure.

Ozone-induced lung expression of GR-1+ macrophages expressing high levels of MARCO and CX3CR1 are not derived from circulating CCR2+ cells but from lung resident macrophages (95). This cell population was reduced in CXCR1(-/-) mice which had an enhanced AHR and inflammatory response to ozone. This suggests that this subset of lung macrophages protect the host from ozone (96).

EFFECT OF OZONE ON GENE EXPRESSION IN ANIMAL AND CELLULAR MODELS

Acute (1 week) and chronic (3 and 6 weeks) exposure to ozone (3 ppm, 3 h, twice weekly) elevated total BAL cell counts associated with increased chemokine and cytokine expression. This increased inflammation was associated with Lm (mean linear intercept) scores after chronic exposure (97). The expression and activity of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a marker of anti-oxidant activity, was present after 1 week but was lost at 3 and 6 weeks, at which time the expression and activation of HIF-1α was seen. HIF-1α-induced genes such as histone deacetylase (HDAC)2, vascular endothelial growth factor (VEGF), kelch-like ECH-associated protein (KEAP)1, and macrophage migration inhibitory factor (MIF) were also up-regulated at 3 and 6 weeks ozone exposure. This suggests that the loss of the antioxidative stress response and activation of the HIF-1α pathway contribute to the inflammatory response and emphysema observed in ozone-exposed mice.

Further analysis of the transcriptomic data from this model using gene set variation analysis (GSVA) (98, 99) and the online freeware R Bioconductor (https://www.bioconductor.org/) (**Figure 2**). GSVA is a non-parametric, unsupervised method for estimating the variation of sets of genes or pathways across a dataset and indicates that there is enrichment of gene signatures associated with fibrosis in this model that are reversed by co-treatment with N-acetyl cysteine (NAC). This approach also enables the analysis of other activated cells and pathways such as CD8+ T-cells, glycolysis and the HIPPO pathway that impact upon the mechanisms of chronic (6 week) ozone exposure on lung pathophysiology. However, not all models of repeated ozone challenge enhance inflammation, AHR and mucus hypersecretion in murine models of asthma (100). In this model, repeated ozone exposures reduced ovalbumin-induced AHR but did enhance mucus hypersecretion potentially via effects on immune dysregulation.

The effect of acute ozone exposure (0.25, 0.5, or 1.0 ppm for 4 h) on gene expression profiles were examined in male Wistar Kyoto rats. Acute inflammatory response genes along with genes involved in cell adhesion and migration, steroid metabolism, cell cycle control, and cell apoptosis/growth were up-regulated in an NF-κB/RELA, SP1, and TP53-dependent manner (101). Ozone treatment of rats (2 ppm, 3 h) enhances the expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and of HO-1 in alveolar macrophages (AM) in a time-dependent manner (102). Ozone also induced markers of apoptosis (cleaved caspase-9) and of autophagy (beclin-1) in AM and enhanced BAL MMP-2 and MMP-9 expression. Enhanced NF-κB-associated inflammatory-type AM (MCP-1, NOS2, and COX-2) was also seen along with markers of anti-inflammatory/wound repair macrophages (arginase-1, YM1, and GAL3). Overall, this indicates that both proinflammatory/cytotoxic and anti-inflammatory/wound repair macrophages are activated in response to ozone in the rat. The authors suggested that these effects were downstream of ozone interactions with the airway lining fluid to oxidatively modify local lipids and proteins.

Exposure of BALB/c mice to ozone (2.5 ppm) for 6 weeks induced a chronic inflammatory process similar to that seen in COPD (78). These features included alveolar enlargement and damage linked to epithelial cell caspase 3-induced apoptosis, enhanced inflammation (IL-13, KC/CXCL1, and IFNγ), increased protease (MMP-12) expression and airway wall remodeling (enhanced collagen deposition). In C57BL/6J mice exposed to ozone before subsequent challenge with *Escherichia coli* LPS resulted in greater levels of total protein and

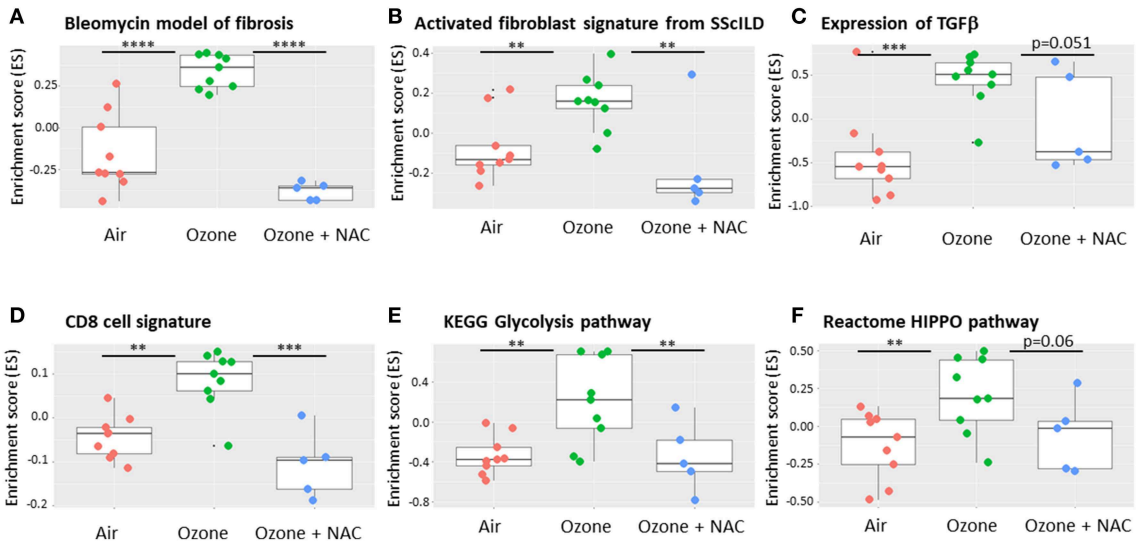

FIGURE 2 | Effect of ozone and N-acetyl cysteine (NAC) on the enrichment of gene signature for fibrotic pathways within murine lungs. Using a bioinformatic technique called gene set variation analysis (GSVA) it is possible to interrogate transcriptomic arrays or RNA-sequencing data for the presence of gene signatures that are unique for specific pathways and or cell types. These signatures may be available from the literature or from online resources or be self-generated. The results show the enrichment scores (ES), a summary of the mRNA expression of all the genes in each signature across the whole data with a range of −1 to +1. Six weeks ozone exposure (2.5 ppm, 3 h/2 days a week) enriches for markers of fibrosis including bleomycin exposure **(A)**, activated fibroblasts **(B)** and transforming growth factor (TGF)β gene expression **(C)** compared with air. The analysis also reveals enrichment for individual cell types such as CD8+ T-cells **(D)** and key remodeling pathways such as the glycolysis **(E)** and HIPPO **(F)** pathways. The ES for each of these signatures is reversed by co-treatment with N-acetyl cysteine (NAC, 100 mg/kg i.p.). Data is obtained from the experiments reported in Yang et al. (83). Data are presented as individual data points with box-and-whisker plots showing median and interquartile range. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 10^{-5}$.

of proinflammatory cytokines in lung lavage fluid and amplified the LPS-response in lung tissue (90). This was associated with enhanced systemic IL-6 expression. The enhanced LPS response following ozone exposure was due to enhanced macrophage TLR4 expression.

Ozone also affects epithelial cell tight junctions *in vivo* by suppressing the expression of claudins (CLDNs) (103). Chronic ozone exposure increased CLDN3, CLDN4, ROS, Nrf2, and Keap1 protein expression but decreased lung CLDN14 protein expression. This suggests that ozone may affect tight junction formation, at least in part, through a ROS-dependent mechanism. Ozone inhalation also suppresses mitochondrial gene expression, enhances mtDNA damage and affects mtDNA copy number indicating that the loss of pulmonary function and inflammation are linked with the loss of mtDNA integrity and DNA repair capability (104) (**Figure 1**).

The diabetes-prone KK strain of mice subjected to ozone (0.5 ppm, 5 h/day for 13 weekdays) had a further impaired insulin response linked to reduced plasma insulin and leptin levels (105). Ozone exposure for 3 weeks resulted in enhanced lung and systemic inflammation with increased numbers of monocytes/macrophages in both blood and visceral adipose tissue. Activation of CD4+T cells was also detected along with up-regulation of inflammatory (CXCL-11, IFNγ, TNFα, IL-12, and NOS2) and oxidative stress (COX4, COX5a, SCD1, NRF1, and NRF2) genes in visceral adipose tissue. The data indicates that repeated ozone exposure exacerbates insulin resistance, oxidative stress, and activates the innate immune system in susceptible animals.

Gene arrays using RNA from ozone (0.75 ppm, 2 h)-exposed primary HBECs indicated up-regulation of pro-inflammatory (e.g., IL-6, IL-8) and vascular function (e.g., PTGS2) pathways (106). In contrast, nitrogen dioxide-exposed cells gave a large oxidative stress response signal highlighting their differing mechanistic responses. In order to understand the mechanisms underlying the epidemiological evidence for an association between black carbon (BC) and ozone exposure with adverse health effects. The effects of BC and ozone-oxidized BC (OBC) on transcriptomic profiles in human lung epithelial A549 cells were studied (107). Only a few oxidative stress-related genes were co-regulated by BC and OBC whereas ∼40% of inflammatory genes and 33% of autophagy-related genes were identical. This highlights differences in the toxic mechanisms between different environmental pollutants but does indicate a degree of co-operation in modulating inflammation above oxidative stress.

A combination of structural cells including epithelial cells and resident immune cells such as macrophages are critical for the lung innate immune response to ozone. Co-culture of human airway epithelial cell lines and BAL macrophages from healthy subjects have been exposed to ozone (108). Macrophages had elevated levels of alternative activation markers, enhanced cytotoxicity and reduced phagocytosis compared to those treated in monoculture. Co-culture also affected the ability of macrophages to

regulate HA expression. These results highlight the importance of cell-cell communication and particularly epithelial-derived factors in modulating ozone-induced macrophage immunophenotypes.

Exposure of primary airway epithelial cells from juvenile rhesus monkeys to ozone resulted in attenuated IL-6 and IL-8 expression but this effect was altered if the animals had also been challenged with LPS (109). In accordance with these results, the expression of miRNAs such as miR-149 that control IL-6 expression were also dysregulated. The authors suggested that early-life exposure to ozone reprogrammed the innate immune response of airway epithelial cells to subsequent bacterial challenges in later life.

CYTOKINE MEDIATORS OF OZONE EFFECTS

IL-33 is critical in mediating ozone effects in murine models (**Figure 1**). For example, co-exposure of mice to BC and to OBC enhances inflammation and lung damage compared to individual exposures (107). Neutralizing antibodies against IL-33 prevented BC/OBC-induced lung damage and inflammation through MAPK- and PI3K-AKT-dependent pathways (110). Interestingly, anti-IL-6 antibodies had no effect on these ozone-induced parameters although anti-IL-33 did suppress IL-6 expression.

Furthermore, ST2- and IL-33-deficient mice exposed to ozone (1 ppm, 1 h) gave a further reduction in epithelial cell injury, myeloid cell recruitment, and inflammation (111). In contrast, the tight junction proteins E-cadherin and zonula occludens 1 (ZO-1) and neutrophil ROS and AHR are diminished. ST2 neutralization mimicked these effects and highlighted the importance of IL-33/ST2 signaling in epithelial barrier function and inflammation following ozone exposure. Interestingly, the enhanced effects of ozone exposure on inflammation and AHR seen in obese mice was also dependent on IL-33 (112). This was due to the combined effect of obesity and ozone to promote T2 cytokine production from ST2+ γδ T cells and ILC2 cells.

Administration of an anti-IL17 mAb to chronic ozone-exposed mice results in suppression of BAL KC/CXCL1, BAL total cell and neutrophil counts and of AHR (113). Anti-IL-17 also reduced p38 MAPK activation and highlighted the important role of IL-17 in ozone-induced inflammation, lung injury and AHR. This result using an anti-IL-17 mAb augments previous data using IL-17A and IL-1R1 knockout mice which demonstrated that ozone-induced IL-17A and neutrophilic airway inflammation was downstream of the caspase-1-IL-1 pathway (114). Prolonged (7 h) low-dose (0.7 ppm) ozone exposure resulted in neutrophilic airway inflammation, accompanied by an increased production of IL-1β, IL-18, IL-17A, Granulocyte-colony stimulating factor (G-CSF), INFγ-inducible protein 10 (IP-10) in BAL which were attenuated in IL-17(-/-) mice. Furthermore, IL1R1(-/-) mice as well as mice given a caspase inhibitor *in vivo* show decreased IL-17A expression and airway inflammation. Although IL-17A is important for ozone-induced AHR it does not appear to be critical for the lung destruction seen in IL-17A(-/-) mice (115). These mice also failed to show an effect of IL-17A on airway inflammation.

The IL-1/inflammasone axis has been associated with ozone-induced airway inflammation in allergic asthmatic patients (116) and both IL-1α(-/-) knockout mice and blocking antibodies provide evidence for a role for IL-1 in ozone-induced epithelial cell barrier function, inflammation, and AHR (117). These prevented ozone-induced effects via IL-1R1 and the adaptor protein MYD88. IL-1 release is downstream of inflammasome activation and evidence suggests that ozone-enhanced lung oxidative stress causes inflammasome activation and IL-1 release eventually leading to alveolar destruction/emphysema and respiratory failure (118). In contrast, ozone appears to suppress NLRP3-mediated inflammation by enhancing NRF2 activity in rats (119).

CONCLUSION

Ozone induces the production of reactive oxygen species (ROS) within epithelial lining fluid, which in turn activates ROS-related intracellular signaling pathways within airway epithelial cells resulting in enhanced expression of inflammatory and remodeling factors. Analysis of transcriptomic profiles in human and mouse tissues and cells have indicated the key role of specific immune and inflammatory pathways in driving the detrimental effects of ozone exposure. These data have indicated treatable mechanisms (p38 MAPK, TLRs, NRLP3, HIPPO) and mediators (IL-1, IL-17, and IL-33) that may be suitable for ameliorating the effects of ozone. Biologics may provide a suitable means of prolonged treatment in susceptible individuals where ozone exposure is high and enduring.

Future analysis of ozone effects needs to incorporate more global assessments of inflammation, AHR, and epithelial damage. The advent of increasingly affordable bulk and single-cell RNA-sequencing will provide insight into ozone actions particularly when applied to lung/airway samples from both healthy subjects and patients with disease. Recent epidemiological analysis of ozone effects in adults and children should be linked to real-time monitoring of individual exposures linked to cell/tissue sampling. Appreciation of the mechanisms by which ozone exerts its effects suggest that potent drugs that target TLRs or redox-sensitive kinases to restore immune skewing induced by ozone as well as restoration of metabolic perturbations may be of use when exposures cannot be reduced to "safe" levels. Indeed, it is likely that even current levels that are considered safe may have adverse effects on both healthy subjects and patients with different airways diseases. This will require public health changes and government interventions on a global level. This is likely to become particularly important with temperature changes associated with climate change. Ozone has profound effects on the airway, particularly in sensitive subjects, and although drugs may be developed that prove effective it is better to prevent the exposure at all ages.

AUTHOR CONTRIBUTIONS

IA and KC conceived the topic. IA and SM drafted the manuscript. IA, KC, and SM edited and revised the manuscript.

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Ozone Inhalation Attenuated the Effects of Budesonide on *Aspergillus fumigatus*-Induced Airway Inflammation and Hyperreactivity in Mice

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Inhaled glucocorticoids form the mainstay of asthma treatment because of their anti-inflammatory effects in the lung. Exposure to the air pollutant ozone (O_3) exacerbates chronic airways disease. We and others showed that presence of the epithelial-derived surfactant protein-D (SP-D) is important in immunoprotection against inflammatory changes including those induced by O_3 inhalation in the airways. SP-D synthesis requires glucocorticoids. We hypothesized here that O_3 exposure impairs glucocorticoid responsiveness (including SP-D production) in allergic airway inflammation. The effects of O_3 inhalation and glucocorticoid treatment were studied in a mouse model of allergic asthma induced by sensitization and challenge with *Aspergillus fumigatus* (*Af*) *in vivo*. The role of O_3 and glucocorticoids in regulation of SP-D expression was investigated in A549 and primary human type II alveolar epithelial cells *in vitro*. Budesonide inhibited airway hyperreactivity, eosinophil counts in the lung and bronchoalveolar lavage (BAL) and CCL11, IL-13, and IL-23p19 release in the BAL of mice sensitized and challenged with *Af* ($p < 0.05$). The inhibitory effects of budesonide were attenuated on inflammatory changes and were completely abolished on airway hyperreactivity after O_3 exposure of mice sensitized and challenged with *Af*. O_3 stimulated release of pro-neutrophilic mediators including CCL20 and IL-6 into the airways and impaired the inhibitory effects of budesonide on CCL11, IL-13 and IL-23. O_3 also prevented budesonide-induced release of the immunoprotective lung collectin SP-D into the airways of allergen-challenged mice. O_3 had a bi-phasic direct effect with early (<12 h) inhibition and late (>48 h) activation of SP-D mRNA (*sftpd*) *in vitro*. Dexamethasone and budesonide induced *sftpd* transcription and translation in human type II alveolar epithelial cells in a glucocorticoid receptor and STAT3 (an IL-6 responsive transcription factor) dependent manner. Our study indicates that O_3 exposure counteracts the effects of budesonide on airway

inflammation, airway hyperreactivity, and SP-D production. We speculate that impairment of SP-D expression may contribute to the acute O_3-induced airway inflammation. Asthmatics exposed to high ambient O_3 levels may become less responsive to glucocorticoid treatment during acute exacerbations.

Keywords: asthma, allergy, ozone, budesonide, surfactant protein-D

INTRODUCTION

In the era of novel biologicals being introduced into the clinic, glucocorticoids remain the main choice of asthma treatment due to their significant anti-inflammatory, immunosuppressive, and immunomodulatory effects (1, 2). A subset of patients however is refractory to glucocorticoids (3–7), making their asthma difficult to control (7). Glucocorticoid insensitivity can be a primary genetic trait but more commonly it is acquired during acute inflammatory exacerbations of airway disease (2, 4–6, 8). Epidemiologic studies indicate a causal link between air pollution and worldwide increases in asthma prevalence and severity. Inhalation of O_3, an ubiquitous, oxidizing, and toxic air pollutant induces acute exacerbations with proinflammatory mediator release, neutrophilic granulocyte influx and obstruction of airways (9–15) and substantially worsens asthma morbidity and mortality (16, 17). Data obtained from studies on mice (18), dogs (19) rhesus macaques (20), healthy volunteers (21), and asthma patients (22, 23) have been controversial on whether glucocorticoids are effective to inhibit O_3-induced exacerbation of airway inflammation and airway hyperreactivity in asthma. Further, the mechanisms of increased susceptibility of the asthmatic airways to O_3 and how glucocorticoid action is affected by inhalation of this air pollutant remain unclear.

Individual susceptibility suggests that genetic predisposition is involved in O_3 responsiveness (24). This is corroborated by strain dependence of the inflammatory response to O_3 observed in mice (14, 15, 25). In addition, increasing evidence supports that a failure of protective immune mechanisms also likely plays an important role in shaping the O_3 effects in the lung. Surfactant protein-D (SP-D), an epithelial cell product of the airways is a critical factor in the maintenance of pulmonary immune homeostasis. We have originally raised the importance of changes in SP-D expression in resolving allergen and O_3-induced airway inflammation (26) by demonstrating that a differential ability of Balb/c and C57BL/6 mice to respond to allergen (27) or O_3 (28), was inversely associated with the amount of SP-D recovered from the airways of these mouse strains (28, 29). Accordingly, genetically low SP-D producer or SP-D deficient mice were highly susceptible *to* and had a prolonged recovery period *from* airway inflammation after allergen or O_3 exposure (28, 30, 31). O_3-inhalation induced exacerbation of Th2-type airway inflammation in allergen challenged mice was also associated with the appearance of abnormal oligomeric molecular forms of SP-D indicating that oxidative damage can cause conformational change with a potential loss of its immunoprotective function (32, 33).

While our lab and others showed that glucocorticoids are necessary for expression of SP-D in epithelial cells (34–37), we also demonstrated a feedback regulation between SP-D and the Th2 cytokines IL-4/IL-13 (30) as well as IL-6 (28), respectively. Interestingly, we found no glucocorticoid response elements in the proximal promoter region of the SP-D gene (*sftpd*) however, this region contains an evolutionarily conserved STAT3/6 response element in a prominent proximal location. Pertinent to this, IL-4/IL-13 (activators of STAT6) as well as IL-6 (activator of STAT3) directly upregulated SP-D synthesis in airway epithelial cells *in vitro* and in mice *in vivo* (28, 30). Lastly, there are indications that STAT3 can be directly phosphorylated by H_2O_2 (the molecular product of O_3 when mixed in water) treatment of airway epithelial cells *in vitro* (38).

We hypothesized that exposure to O_3 interferes with the effects of glucocorticoids on *Af*-induced airway inflammation and hyperreactivity and, that O_3 and glucocorticoid treatment have antagonistic effects on SP-D expression and function in the lung. To study these hypotheses we utilized our *in vivo* mouse model of combined *Af* + O_3 exposure and *in vitro* human airway epithelial cell cultures.

MATERIALS AND METHODS

In vivo Studies

Balb/c mice were obtained from the Jackson laboratories (Bar Harbor, ME) and bred in-house. All experiments were performed on 8–10 weeks old mice. Experiments where mice were sensitized and challenged with *Af* and exposed to air or O_3 were carried out as previously described (30, 39, 40). In brief, mice were sensitized with 20 μg *Af* and alum by intraperitoneal injection (i.p.) on days 0 and 7, then challenged with 25 μg *Af* by intranasal (i.n.) instillation on day 13. In **Figure 1**, mice were treated with vehicle (Dimethyl sulfoxide, DMSO) or budesonide (0.25 or 2.5 mg/kg) i.n. at the time of *Af* challenge. 48 h post challenge, lung function (enhanced pause, Penh) was measured using the Buxco® system.

In **Figures 2–5**, mice followed the *Af* sensitization and challenge protocol as described, however 84 h post challenge/budesonide they were exposed to 3 ppm O_3 or air for 2 h. Animals were studied 96 h post *Af* challenge (12 h post O_3). These time points were selected to mimic O_3-induced exacerbation of allergic changes, because by 96 h post *Af*

Abbreviations: O_3, Ozone; SP-D, Surfactant protein-D; sftpd, Surfactant protein-D gene; Af, *Aspergillus fumigatus*; BAL, Bronchoalveolar lavage; AHR, Airway hyperreactivity; Human primary type II airway epithelial (hAECII) cells; Cu I, Curcurbitacin I; CCL-, C-C motif chemokine; IL-, Interleukin-; CXCL-, Chemokine (CXC motif) ligand; STAT3, Signal transducer and activator of transcription 3; qPCR, Quantitative, real-time polymerase chain reaction; ELISA, Enzyme-linked Immunosorbent assay; DMSO, Dimethyl sulfoxide; R, Lung Resistance; G, Tissue Damping; i. n., Intranasal; i.p., Intraperitoneal.

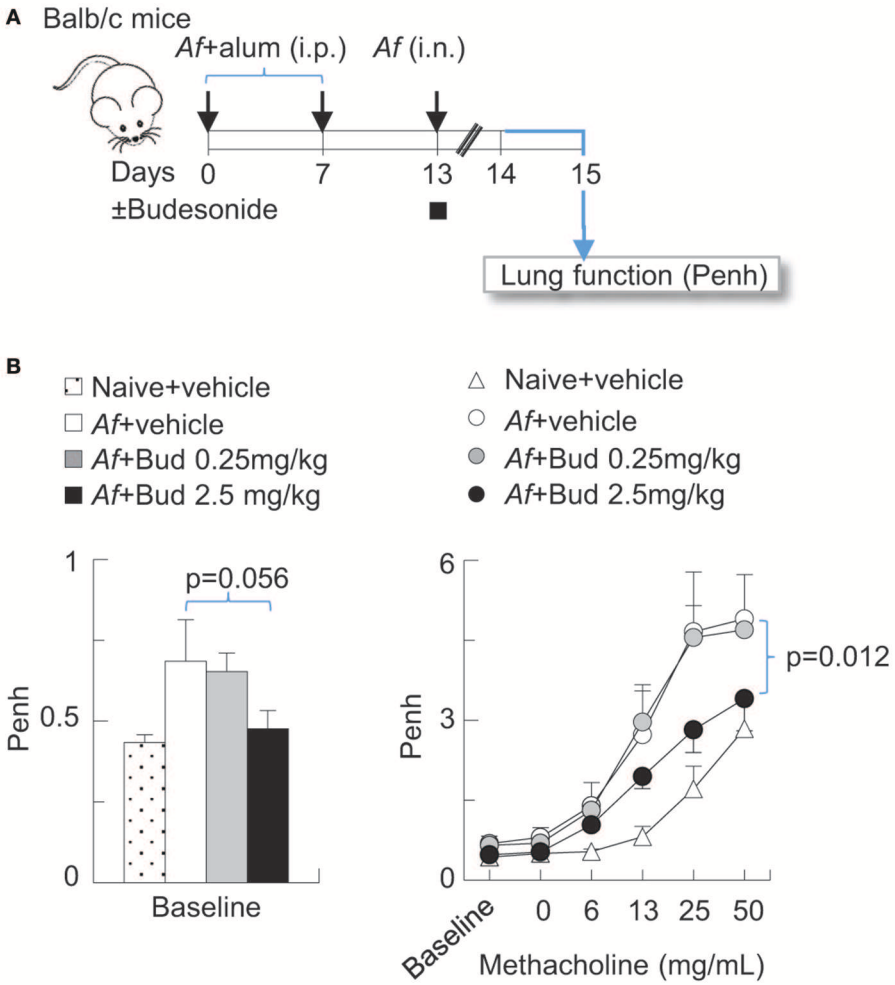

FIGURE 1 | Budesonide inhibited airway hyperreactivity induced by *Af* sensitization and challenge in Balb/c mice, in a dose-dependent manner. **(A)** Balb/c mice were sensitized to 20 μg *Aspergillus fumigatus* (*Af*), with alum (i.p.) on days 0 and 7. On day 13, mice were challenged with 25 μg *Af* (i.n.) and administered 0 (DMSO vehicle only), 0.25 or 2.5 mg/kg budesonide. 48 h post-*Af* challenge mice were studied. **(B)** Lung function (enhanced pause, Penh) was measured by the Buxco® system. Baseline measurements represent data collected over a 10 min period (left panel). Methacholine dose response was established to increasing concentrations of nebulized methacholine. Mean ± SEM of $n = 4–18$ (left panel: Dunett's multiple comparison; right panel: Two-way ANOVA with Tukey's multiple comparison).

challenge airway inflammation subsides while O_3 exposure induced inflammation peaks 12 h post exposure (**Figures 2A,B**) (33, 40). That a 3 ppm inhaled dose in rodents results in O_3 concentration in the lungs relevant to human exposure levels has been experimentally validated by others, using oxygen-18-labeled O_3 ($^{18}O_3$). Hatch et al. showed that exposure to $^{18}O_3$ (0.4 ppm for 2 h) caused 4–5-fold higher $^{18}O_3$ concentrations in humans than in rats, in all of the BAL constituents measured (41). Rats exposed to 2.0 ppm, had still less $^{18}O_3$ in BAL than humans exposed to 0.4 ppm. The species discrepancies between the recoverable O_3 levels in the lung are not entirely clear. It is thought however that as rodents are obligate nose breathers (while humans breathe through their nose and mouth), this reduces the delivered dose of O_3 to the lungs of rodents. Further, Slade et al. found that after exposure to O_3, mice react by a rapid decrease of core temperature, a species and strain specific characteristics (42). The recoverable $^{18}O_3$ in the lung tissue was negatively associated with the extent of hypothermia that significantly altered O_2 consumption and pulmonary ventilation, explaining at least partly, the interspecies differences seen in O_3 susceptibility. In addition, in pilot studies we also performed a careful assessment of the biological effects on a range of 0.5–6.0 ppm O_3 exposure. Doses lower than 3 ppm did not elicit a significant inflammatory response that would be commensurate with what is seen in humans, in regards to BAL or peripheral blood neutrophilia, upon O_3 inhalation for 2 h. Higher than 3 ppm doses caused observable respiratory distress especially in Balb/c mice. The O_3 dose we used here therefore represents a level of exposure that is well-tolerated by both Balb/c and C56BL/6 mice and that causes a significant airway inflammatory response.

Lung function was measured using the Flexivent® system (Scireq, Montreal, Canada) in response to increasing

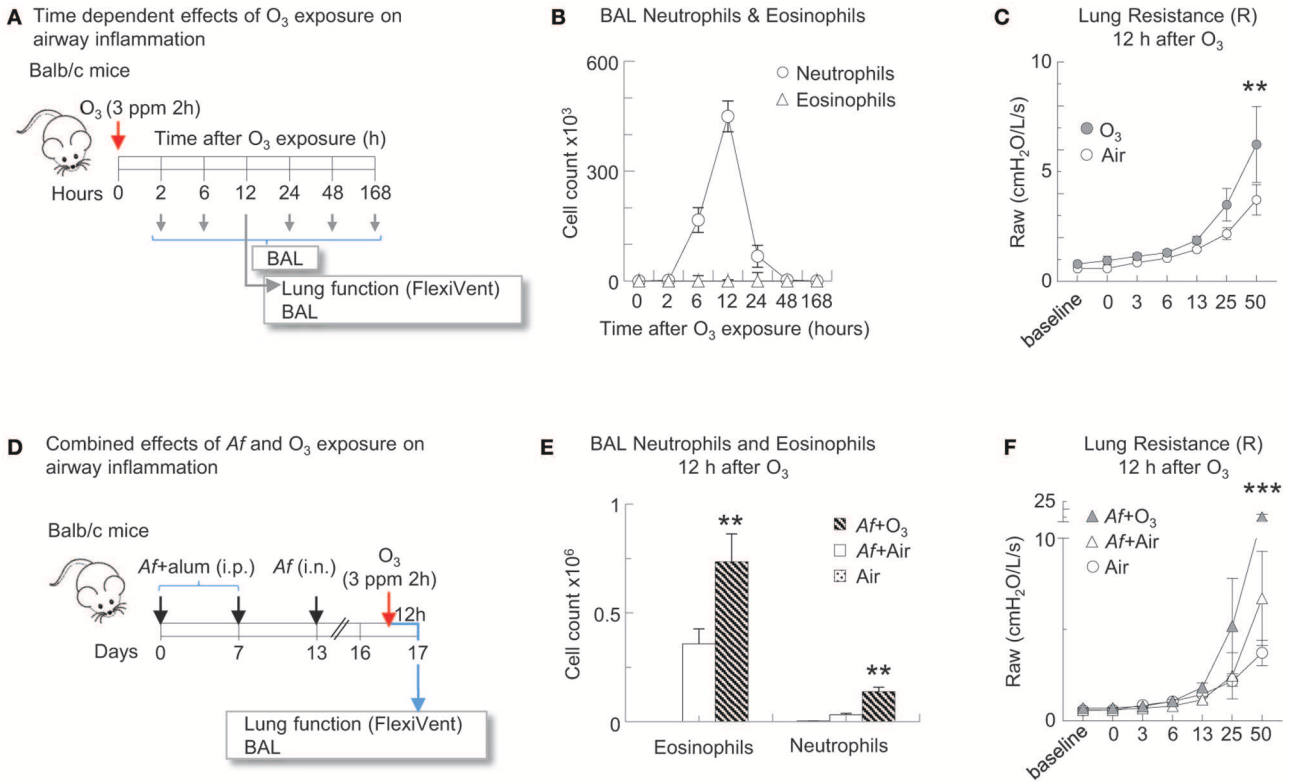

FIGURE 2 | O_3 induced airway inflammation and hyperreactivity and enhanced allergic airway changes in mice sensitized and challenged with *Af*. **(A)** Balb/c mice were exposed to air or 3 ppm O_3 for 2 h and studied at the indicated time points for airway inflammation. 12 h after O_3 exposure, lung function to methacholine was measured (Flexivent®) prior to BAL. **(B)** BAL neutrophils and eosinophils were quantified by differential cell counts on cytospin preparations multiplied by the total cell counts recovered from the BAL (Countess®). **(C)** O_3 exposed mice and air exposed controls were studied for methacholine responsiveness 12 h later. Mean ± SEM of $n = 6$ $^{**}p < 0.01$ Two-way ANOVA with Tukey's multiple comparison's test (air vs. O_3 exposure). **(D)** Balb/c mice were sensitized to 20 μg *Aspergillus fumigatus* (*Af*), with alum (*i.p.*) on days 0 and 7. On day 13, mice were challenged with 25 μg *Af* (*i.n.*). 82 h post-*Af* challenge, mice were exposed to air or 3 ppm O_3 for 2 h, then 12 h later (96 h post-*Af* challenge), lung function was measured (Flexivent®) and BAL was harvested. **(E)** BAL neutrophils (live $Ly6G^+CD11b^+$ cells) and eosinophils (live $CD11c^-$Siglec-F^+ cells) were quantitated by FACS analysis. The absolute numbers of eosinophils and neutrophils were calculated by multiplying the percentage of cells determined by flow cytometric gating with the total numbers of cells/lung (Countess®). Mean ± SEM of $n = 6$; $^{**}p < 0.01$ Student's *t*-test (air vs. O_3). **(F)** Lung function (airway resistance, Raw) was measured as indicated. Mean ± SEM of $n = 6$; $^{***}p < 0.001$ Two-way ANOVA with Tukey's multiple comparison's test (air vs. O_3 exposure).

concentrations of inhaled methacholine. BAL and lung cells were harvested to study inflammatory cells by flow cytometry. Following collection of BAL, 10 mL ice-cold PBS was injected into the right ventricle to perfuse the lung. The lung lobes were then carefully removed and snipped into small pieces before undergoing digestion with Liberase TL (Millipore Sigma, Burlington, MA) for 40 min at 37°C on a shaker. Digested whole lung homogenate was filtered through a 70 μm cell strainer to create a single cell suspension for flow cytometric analysis. In cell-free BAL supernatant, a custom mouse magnetic Luminex assay was utilized to study cytokines and chemokines while SP-D was measured by western blot, native gel electrophoresis (structure) or sandwich ELISA (quantity). All mouse procedures were reviewed and approved by the University of California, Davis, and University of Pennsylvania Institutional Animal Care and Use Committees.

Flow Cytometry

BAL and lung cells were harvested and single cell suspensions were prepared as previously described for analysis by flow cytometry (40). Fluorescently-conjugated monoclonal antibodies were purchased from Biolegend (San Diego, CA), BD Biosciences (San Jose, CA), or eBioscience (San Diego, CA). Single cell suspensions were incubated with antibodies targeting surface markers for 20 min at 4 degrees C in the dark. In the BAL samples, the following antibodies were used: APC-Cy7-CD11c, PE-Siglec F, PE-Cy7-CD11b, PerCP-Cy5.5-Ly6G. In the lung digest suspensions, neutrophils, and eosinophils were identified using APC-Cy7-CD11c, PE-Siglec F, and PerCP-Cy5.5-Ly6G. Live/dead Aqua was used in the panels throughout the study to exclude dead cells. Flow cytometry was performed on a Fortessa (BD Biosciences, San Jose, CA) and data was analyzed using FlowJo software (Ashland, OR).

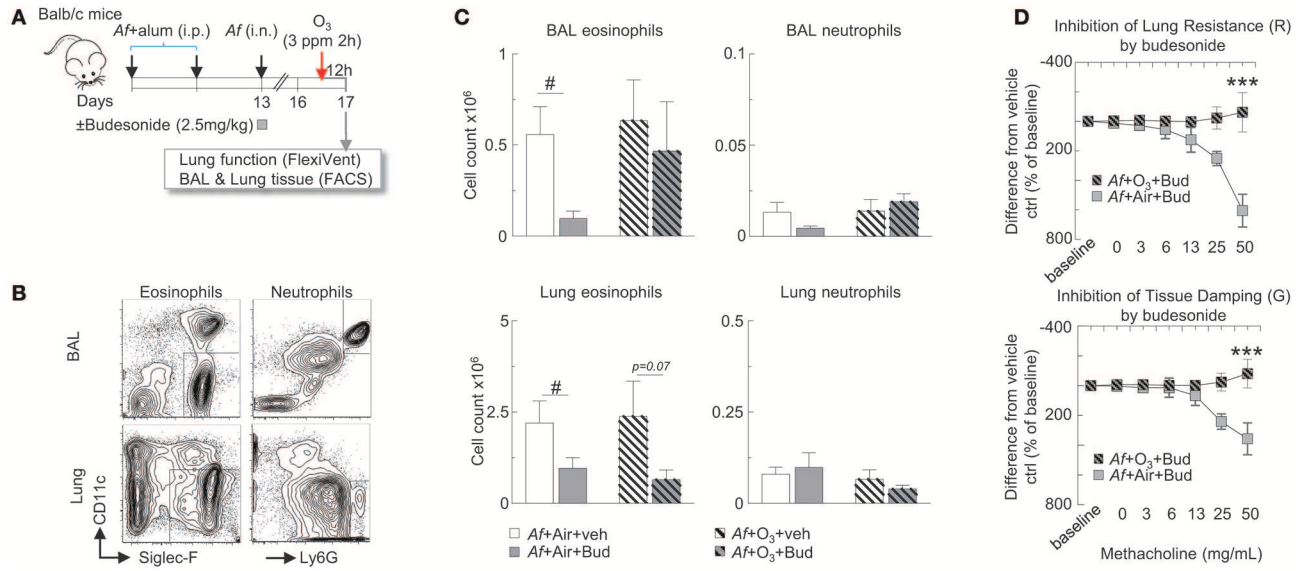

FIGURE 3 | The inhibitory effects of budesonide on *Af*-induced airway inflammation were attenuated and on airway hyperreactivity were completely abolished by O_3 exposure in sensitized and challenged mice. **(A)** Balb/c mice were sensitized to 20 μg *Aspergillus fumigatus* (*Af*), with alum (*i.p.*) on days 0 and 7. On day 13, mice were challenged with 25 μg *Af* (*i.n.*) and administered 2.5 mg/kg budesonide or vehicle (DMSO). 82 h post-*Af* challenge, mice were exposed to air or 3 ppm O_3 for 2 h, then 12 h later (96 h post-*Af* challenge), lung function was measured (Flexivent®), and BAL and lungs were harvested. **(B)** Representative gating of BAL and lung neutrophils (live $Ly6G^+CD11b^+$ cells) and eosinophils (live $CD11c^-Siglec\text{-}F^+$ cells). **(C)** The absolute numbers of eosinophils and neutrophils were calculated by multiplying the percentage of cells determined by flow cytometric gating with the total numbers of cells/lung or BAL (Countess®). Mean ± SEM of $n = 6$; #$p < 0.05$ Student's *t*-test (vehicle vs. budesonide). **(D)** Lung resistance and tissue damping results were calculated as % change from baseline. The difference between the vehicle and budesonide treatment is depicted (by subtracting the individual % change from baseline values from the vehicle treatment average at each methacholine concentration). Mean ± SEM of $n = 6$–14; ***$p < 0.001$ (*Af*+Air+Bud vs. *Af*+O_3+Bud) Two-way ANOVA with Tukey's multiple comparison's test.

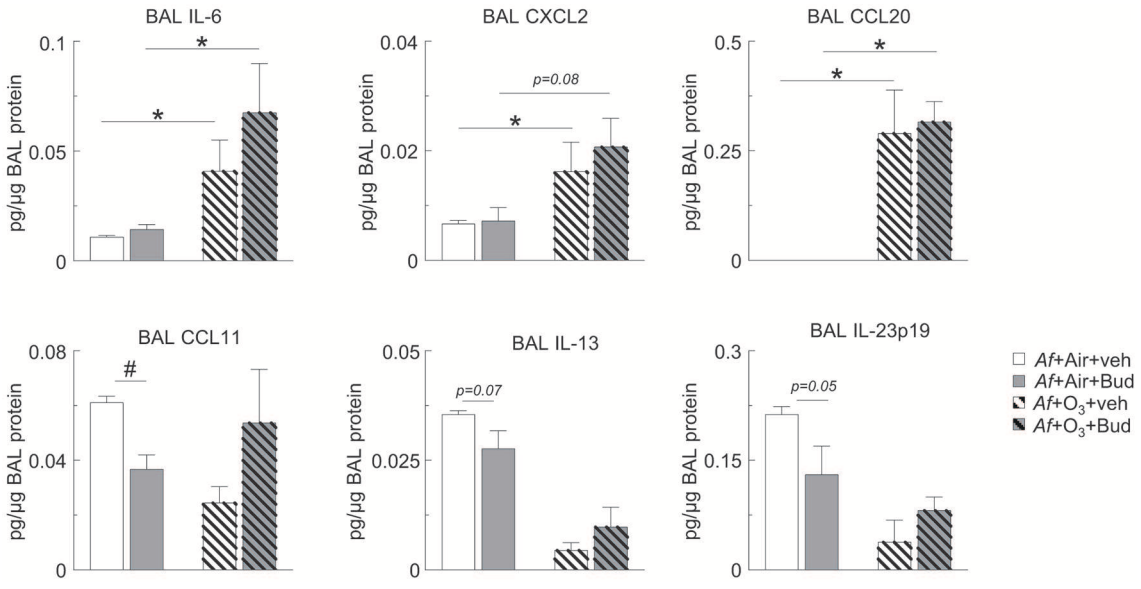

FIGURE 4 | O_3 upregulated BAL IL-6, CXCL2, and CCL20 in a budesonide-resistant manner and reversed the inhibitory effects of budesonide on CCL11, IL-13, and IL-23 expression in mice sensitized and challenged with *Af.* Cell-free BAL supernatant was assessed for the indicated cytokines and chemokines by a R&D mouse magnetic Luminex Assay (pg cytokine/μg BAL protein). Mean ± SEM of $n = 3$; *$p < 0.05$, #$p < 0.05$ Student's *t*-test [vehicle vs. budesonide (#) or air vs. O_3 (*)].

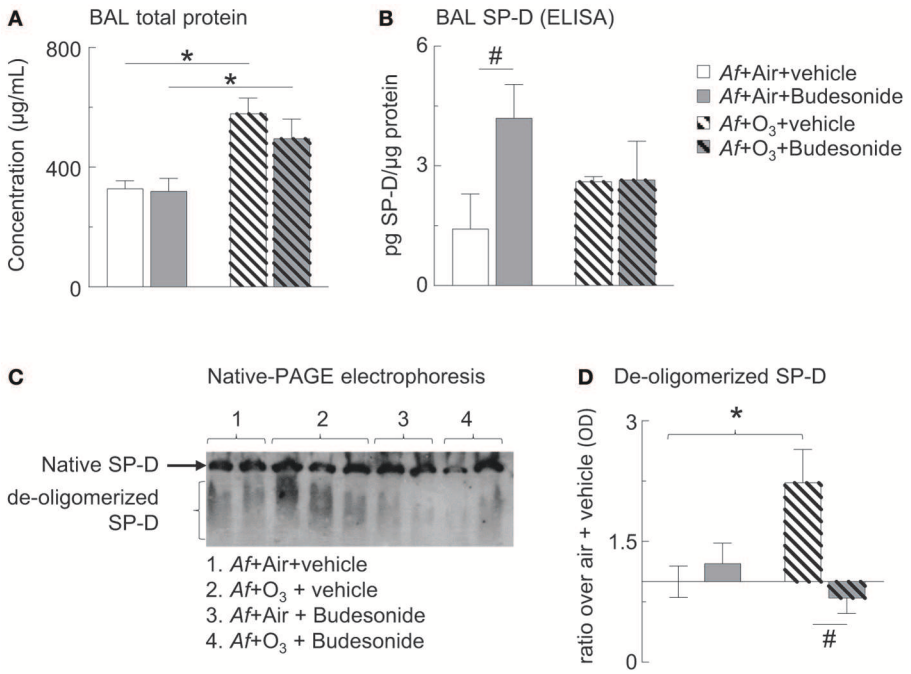

FIGURE 5 | O_3 inhibited budesonide-induced SP-D expression in *Af*-challenged mice and caused SP-D de-oligomerization in the BAL. **(A)** Cell-free BAL supernatant was assessed for total protein by BCA assay. **(B)** BAL SP-D was measured by ELISA. **(C)** Native-PAGE western blot was used to study SP-D structure. Native (intact) SP-D is the band that due to its molecular size remain on the top of the gel. Due to the variability of migratory capabilities of the de-oligomerized SP-D components, these appear as a "smear" throughout the gel. **(D)** SP-D optical density by Image J analysis; ratio over the mean value of "air+vehicle" control group data. Mean ± SEM or $n = 5$–6; *$p < 0.05$ air vs. O_3, #$p < 0.05$ vehicle vs. budesonide, Two-way ANOVA with Tukey's multiple comparison's test.

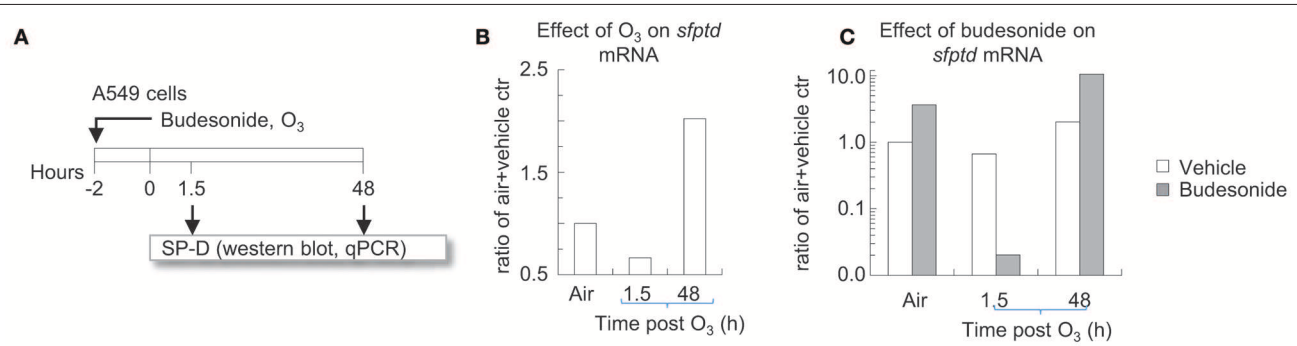

FIGURE 6 | Time dependent effects of O_3 on budesonide-induced *sftpd* mRNA in A549 cells *in vitro*. **(A)** A549 cells were incubated for 2 h with budesonide or vehicle (DMSO) and exposed to air or 300 ppb O_3. 1.5 or 48 h after exposure/treatment ended, cells were harvested and processed for RNA extraction. **(B,C)** *sftpd* mRNA was measured by qPCR. Fold over air+vehicle values are shown. Representative of three independent experiments.

Luminex Assay

Cytokines and chemokines were assayed in the BAL via a custom Magnetic Mouse Luminex Assay (R&D System, Minneapolis, MN). C-C motif chemokine 11 (CCL11), Interleukin-23p19 (IL-23p19), IL-13, IL-6, Chemokine (CXC motif) ligand 2 (CXCL2), and CCL20 were measured in the premixed kit. BAL fluid was first concentrated using a 2 mL, 3 k Amicon Ultra Centrifugal Filter (Millipore Sigma, Burlington, MA) spun at 3,000 g for 30 min. The kit was performed following the instructions from the manufacturer.

BAL SP-D Analysis

Total protein concentration was measured by the BCA Assay (Thermo Fisher Scientific, Waltham, MA). BAL SP-D was assayed by sandwich ELISA using our in-house generated monoclonal and polyclonal antibodies as previously described (33). BAL SP-D was also measured by native gel electrophoresis (33) to assess the tertiary structure of SP-D, which is critical to maintain its anti-inflammatory functions (43, 44). Proteins were transferred to a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA). Goat anti-mouse SP-D (1:2,000, R&D

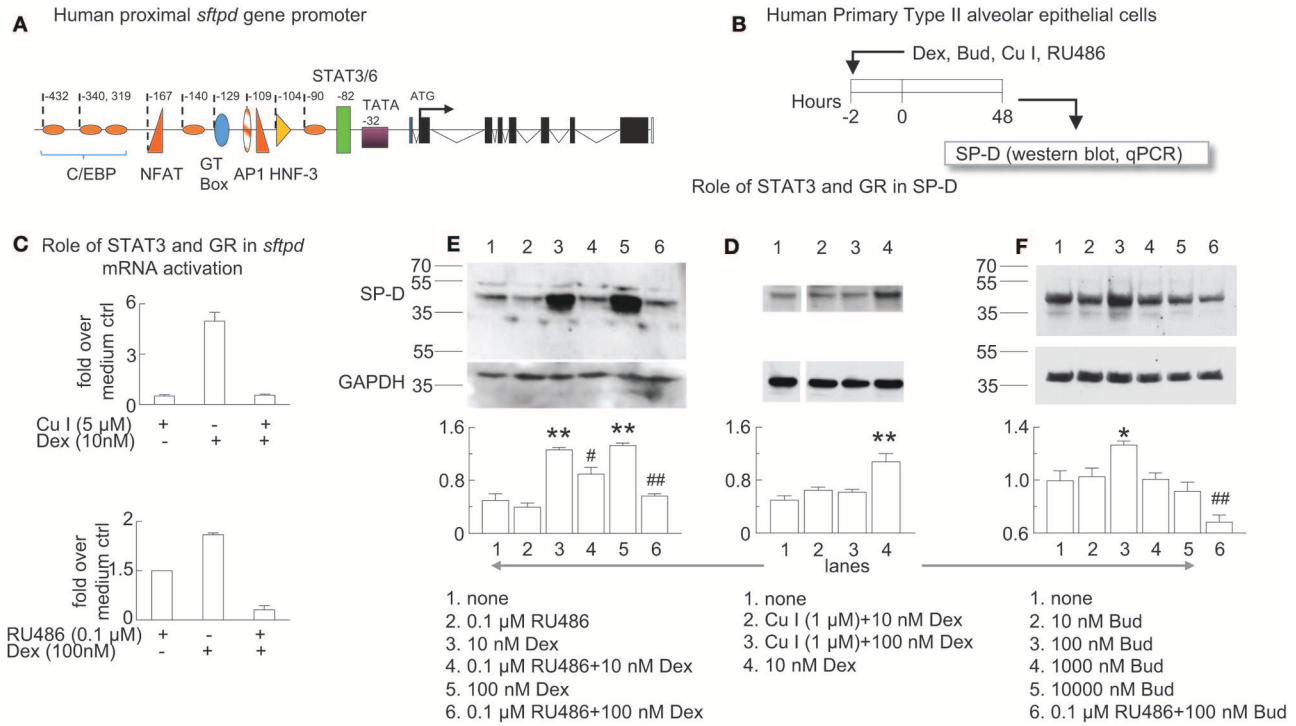

FIGURE 7 | Glucocorticoid receptor-induced *sftpd* mRNA and protein expression is facilitated by STAT3/6 binding. **(A)** Schematic of the proximal promoter region of the human SP-D gene (*sftpd*) lacking glucocorticoid response elements. The approximate positions of C/EBP (orange ovals) and STAT3/6 (green rectangle) binding are depicted. Negative values indicate the number of base pairs relative to the start of transcription. **(B)** Human Primary Type II alveolar epithelial (hAECII) cells were incubated for 2 h with dexamethasone (Dex), curcurbitacin I (Cu I), and RU486 as indicated. 48 h later, cells were harvested and SP-D was studied by western blot (protein, relative expression to GAPDH) and qPCR (*sftpd* mRNA). **(C)** qPCR of *sftpd* (fold over no dexamethasone). **(D–F)** SP-D protein was studied by western blot, compared to control GAPDH. Optical density of SP-D and GAPDH by Image J analysis; GAPDH was subtracted from SP-D density then the ratio over the mean value of "no treatment" group was calculated. Mean ± SEM of $n = 6$ **(C)** or $n = 3$ **(D–F)**. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. "no treatment"; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the same concentration of Dex or Bud alone; One-way ANOVA with Bonferroni's multiple comparison's test **(D–F)**. Western blots are representative of three independent experiments.

Systems, Minneapolis, MN) was the primary antibody while donkey anti-goat antibody coupled to horseradish peroxidase (1:10,000 GE Healthcare Life Sciences, Marlborough, MA) was the secondary antibody. SP-D signal was detected using the ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) on film (ECL Hyperfilm, GE Healthcare Life Sciences, Marlborough, MA). Image J (National Institutes of Health, Rockville, MD) analysis was used to determine the optical density of SP-D bands.

In vitro Studies

Human primary type II airway epithelial (hAECII) cells were acquired from normal human lung tissues from NDRI (National Disease Research Interchange). A549 cells were purchased from ATCC (Manassas, Virginia). Dexamethasone, budesonide, curcurbitacin I (Cu I), and RU486 was purchased from Millipore Sigma (Burlington, MA). A549 cells are a human type II alveolar epithelial cell line used by our laboratory and others (45–47) to model functions including expression of mRNA for SP-D. We used these readily available cells to establish conditions of SP-D mRNA expression upon treatment with ozone and budesonide (**Figure 6**). The budesonide effects were then recapitulated in primary hAECII cells (**Figure 7**). A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA). Primary hAECII cells were cultured in DMEM-H21 plus F-12 Ham's (1:1) supplied with 5% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine. D-valine (Invitrogen) prevented growth of fibroblasts. hAECII cells were treated with budesonide/dexamethasone for 2 h, with or without RU486 and Cu I added to the culture. A549 cells were treated with budesonide and exposed to air or O_3 (300 ppb) for 2 h, which was generated as previously described (31). DMSO was used as the vehicle control in the *in vitro* studies. At the time points indicated in the figure, cells were harvested for analysis of *sftpd* mRNA (qPCR) or SP-D protein (western blot). Cells were harvested in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) for mRNA analysis by qPCR or RIPA buffer (Thermo Fisher Scientific, Waltham, MA) for protein analysis by western blot.

For the western blots, total intracellular protein was measured by the BCA assay, then 20 μg protein was loaded for each lane. The primary antibody was goat anti-SP-D (1:500). The secondary antibody was HRP anti-goat IgG (1:1,000). For control antibodies, the primary was rabbit anti-GAPDH (1:1,000) and

the secondary was HRP anti-rabbit (1:5,000). All antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Image J (National Institutes of Health, Rockville, MD) analysis was used to determine the optical density of SP-D bands. For the qPCR, RNA was extracted from the TRIzol by chloroform layering and isopropanol precipitation, then reverse transcribed into cDNA via the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). qPCR was performed on the recovered cDNA using SYBR green reagents (Applied Biosystems, Foster City, CA) on a ViiA 7 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Fold change was calculated using the ΔΔCt method, first normalizing values to GAPDH. Human SP-D primer with sequence of 5′-ACACAGGCTGGTGGACAG-3′ (sense); 5′-TGTTGCAAGGCGGCATT-3′ (anti-sense) were used to produce 61 bp products.

Statistical Analysis

All statistics were performed using Prism v7 software (GraphPad Inc., La Jolla, CA). Data are expressed as mean ± SEM and are representative of at least 2 independent experiments. A Student's *t*-test was used to compare vehicle vs. budesonide or air vs. O_3. A One-way ANOVA with Tukey's multiple comparison's test was used in the budesonide dose response experiment **(Figure 1)**. A Two-way ANOVA with Tukey's multiple comparison's test was used when comparing all experimental groups. A $p < 0.05$ was considered statistically significant.

RESULTS

Budesonide Inhibited Airway Hyperreactivity Induced by *Af* Sensitization and Challenge in Balb/c Mice in a Dose-Dependent Manner

Inhaled glucocorticoids improve lung function and airway inflammation in allergic asthma (1, 48, 49) but their effectiveness in acute asthma exacerbations are subject of on-going investigations (49–51). We developed a model in which mice were sensitized (i.p.) and challenged with *Af* at the time of budesonide administration (i.n.) (**Figure 1A**). To establish the dose-dependent effects of budesonide on methacholine responsiveness we used Penh (enhanced pause), a non-invasive measure of airway obstruction, because it enabled us to obtain data from multiple individual animals simultaneously and complete a study using a large number of mice. These results showed that sensitization and challenge to *Af* significantly increased baseline Penh and methacholine responsiveness and that budesonide significantly inhibited airway hyperreactivity to methacholine at 2.5 mg/kg dose (**Figure 1B**). For the subsequent experiments presented in this paper we used this budesonide dose and confirmed its inhibitory effects on allergic airway hyperreactivity by the invasive FlexiVent® system (**Figures 2C,F**, **3D**). These data also provided the basis for the subsequent studies on the effects of O_3 on allergic airway inflammation and glucocorticoid responsiveness.

O_3 Induced Airway Inflammation and Hyperreactivity and Enhanced Allergic Airway Changes in Mice Sensitized and Challenged With *Af*

To establish the time course of O_3 induced airway inflammation, Balb/c mice were exposed to air or 3 ppm O_3 for 2 h and studied at several time points afterwards (**Figure 2A**). Neutrophil influx into the airways peaked 12 h after O_3 exposure (**Figure 2B**). O_3 exposed mice and air exposed controls were studied for methacholine responsiveness at the 12 h time point. O_3 induced a significant increase in methacholine responsiveness compared with air exposed controls ($p < 0.01$) (**Figure 2C**). To investigate the effects of O_3 on allergic airway inflammation Balb/c mice were sensitized and challenged with *Af*. In this model the acute inflammatory changes resolve by 96 h after allergen challenge. We therefore studied the mice at this time point, but we also exposed them to either air or O_3 12 h before (**Figure 2D**). BAL neutrophils and eosinophils were quantitated by FACS analysis (the gating strategy is shown in **Figure 3B**). O_3 exposure clearly enhanced numbers of eosinophils and neutrophils in the airways of *Af* sensitized and challenged mice in comparison with air exposure ($p < 0.01$; **Figure 2E**). In addition, lung resistance to methacholine challenge was also significantly amplified in the O_3 exposed animals ($p < 0.001$; **Figure 2F**). These results confirm our previous findings (39) and strongly indicate that O_3 induces airway hyperreactivity on its own and that it enhances airway changes in allergen sensitized and challenged animals.

The Inhibitory Effects of Budesonide on *Af*-Induced Airway Inflammation Were Attenuated, and on Airway Hyperreactivity Were Completely Abolished by O_3 Exposure in Sensitized and Challenged Mice

Experiments in dogs (19) our previous studies in healthy volunteers (52) and investigations in mild asthmatics (53) showed that glucocorticoid treatment inhibited O_3-induced inflammation in the airways. However, how would O_3 alter the inhibitory effects of budesonide on allergic airway inflammation and hyperreactivity has not been documented.

To study the hypothesis that O_3 impairs the anti-inflammatory effects of budesonide, Balb/c mice were sensitized and challenged with *Af* as described, and were intranasally treated with 2.5 mg/kg budesonide or vehicle. 82 h post-*Af* challenge mice were exposed to air or 3 ppm O_3 for 2 h, then 12 h later (96 h post-*Af* challenge), lung function was measured (Flexivent®), and BAL and lungs were harvested (**Figure 3A**). BAL eosinophils (live Siglec-F^+CD11c^- cells) and neutrophils (live $Ly6G^+CD11b^+$ cells) and lung eosinophils (live $CD45^+CD11c^-$Siglec-F^+) and neutrophils (live $CD45^+CD11c^-Ly6G^+$) were analyzed by FACS from single cell suspensions as shown in **Figure 3B**. Budesonide significantly suppressed eosinophil (not neutrophil) numbers both in the BAL and the lung in air exposed but not in O_3 exposed mice (**Figure 3C**). Strikingly, inhibition of lung resistance (upper

panel) and tissue damping (lower panel) by budesonide seen in air exposed mice (gray plain squares) was completely abolished in O_3-exposed mice (gray hatched squares, $p < 0.001$, **Figure 3D**). These data indicated that the inhibitory effects of budesonide on *Af*-induced airway inflammation were attenuated and on airway hyperreactivity were completely abolished by O_3 exposure in sensitized and challenged mice.

O_3 Upregulated BAL IL-6, CXCL2 and CCL20 in a Budesonide-Resistant Manner and Reversed the Inhibitory Effects of Budesonide on CCL11, IL-13, and IL-23 Expression in Mice Sensitized and Challenged With *Af*

Since O_3 mitigated the inhibitory effect of budesonide on airway eosinophilia and airway hyperreactivity, we wanted to investigate the underlying mediator profile. We measured BAL CCL11 (eotaxin, an eosinophil chemoattractant), IL-23p19, IL-6, CXCL2 (pro-neutrophilic mediators), CCL20 (a lymphocyte chemoattractant), and IL-13 (known to prime smooth muscle cells for airway hyperreactivity). O_3 strongly induced BAL IL-6, CXCL2, and CCL20 in a budesonide-independent manner (**Figure 4**). Meanwhile, budesonide significantly reduced BAL CCL11 showed a trend for reduction of IL-13 ($p = 0.07$) and IL-23p19 ($p = 0.05$) in the BAL of air exposed, but not O_3 exposed mice (**Figure 4**, lower panels). These data suggested that O_3 induced pro-neutrophilic factors regardless of budesonide treatment, and that the suppressive effects of budesonide on eosinophilia and airway hyperreactivity-inducing factors was attenuated by O_3.

O_3 Caused SP-D De-oligomerization and Inhibited Budesonide-Induced SP-D Expression in the BAL

We and others previously showed that SP-D plays an important role in suppressing proinflammatory mediator release in allergen or O_3-induced airway inflammation (28–30, 54) and that production of SP-D required the presence of glucocorticoids in airway epithelium (34–37). Further, we found that O_3-induced airway inflammation in allergen challenged mice resulted in abnormal oligomeric molecular forms of SP-D indicating that oxidative damage can cause conformational changes with a potential inactivation of SP-D's immunoprotective function (28, 32, 33). Here we wanted to investigate how the combination of allergen with O_3 exposure would alter the glucocorticoid effects on SP-D expression.

Assessment of total BAL protein levels showed that those were returned to normal 96 h after *Af* challenge, indicating inflammatory resolution in air exposed mice. In O_3 exposed mice however, BAL protein levels were significantly elevated indicating acute inflammation that was not prevented by budesonide treatment (**Figure 5A**). As expected on the basis of previous investigations (34–37) budesonide significantly increased BAL SP-D in air exposed mice. Importantly, this budesonide effect on SP-D expression was lost in O_3 exposed mice (**Figure 5B**). By native gel electrophoresis, structurally intact SP-D was found at the top of the gel and did not separate from the well, while de-oligomerized SP-D was resolved as a smear (**Figure 5C**). Native SP-D density was not statistically different between the groups studied (**Figure 5C**). O_3 caused de-oligomerization of SP-D in the BAL of mice sensitized and challenged with *Af*. This change was prevented by budesonide treatment (gray hatched bar, **Figure 5D**). Our data suggested that budesonide induction of SP-D is inhibited by O_3. We speculate that in budesonide treated mice SP-D was indirectly protected from de-oligomerization possibly as a result of inhibition of eosinophils (the main source of iNOS and nitric oxide) in the lungs of mice (**Figure 3C**).

Time Dependent Effects of O_3 on Budesonide-Induced *sftpd* mRNA in A549 Cells *in vitro*

We previously showed that IL-6 directly induced SP-D in type II alveolar epithelial cell cultures (28). This is interesting in light of O_3 while strongly inducing IL-6 (**Figure 4**), did not increase SP-D 12 h after exposure, but in fact it prevented the stimulatory effects of budesonide on SP-D release in the airways of mice (**Figures 5A,B**). To better understand the mechanisms of how budesonide and O_3 regulate SP-D expression we used A549 cells, a readily available human type II alveolar epithelial cell line that models functions such as expression of the SP-D gene (*sftpd*, **Figure 6A**). To confirm our findings, the budesonide effects were then recapitulated in primary human type II alveolar epithelial cells (hAECII, **Figure 7**). O_3 exposure of A549 cells inhibited *sftpd* expression 1.5 h later, but by 48 h post exposure this effect was reversed into an induction of the *sftpd* gene (**Figure 6B**). Budesonide induced *sftpd* mRNA in A549 cells exposed to air. O_3 completely prevented the budesonide induction of the *sftpd* gene 1.5 h later. However, by 48 h O_3 and budesonide synergistically increased *sftpd* mRNA (**Figure 6C**). These results are in line with our previous *in vivo* study on Balb/c mice (28) and suggest that O_3 acts on sftpd transcription in a bi-phasic manner with an early phase inhibition (<12 h) and a late phase activation >48 h. Based on these and our previous findings on IL-6 we speculated that budesonide and O_3 may interact on a common signaling pathway involved in SP-D transcription in airway epithelial cells.

Glucocorticoid Receptor-Induced *sftpd* mRNA Transcription Is Facilitated by STAT3/6 Binding

The proximal promoter region of the human SP-D gene (*sftpd*) has binding elements for C/EBP, NFAT, AP1, HNF-3, and STAT3/6 that all contribute to transcription of SP-D (**Figure 7A**) (55). Dexamethasone induced lung SP-D in mice at the level of transcription in the absence of a full glucocorticoid response element in the proximal promoter region of *sftpd*. Zhang and colleagues previously reported that STAT3 can act as a co-activator in glucocorticoid receptor signaling (56). To test if the glucocorticoid receptor works in concert with the STAT3/6 binding element to induce SP-D we studied primary hAECII cells using a specific inhibitor of STAT3 (cucurbitacin Cu I) (57) and the glucocorticoid receptor (RU486). We treated

human primary type II alveolar epithelial (hAECII) cells with budesonide and dexamethasone *in vitro* and studied SP-D mRNA (qPCR for *sftpd*) and protein (western blot for SP-D) expression (**Figure 7B**). We used dexamethasone in the *in vitro* experiments as a positive control because it is a well-characterized glucocorticoid that induces SP-D (35). Indeed, dexamethasone induced *sftpd* expression in human primary type II aleveolar epithelial cells that was abolished in the presence of Cu I or RU486 (**Figure 7C**). Similarly, Cu I and RU486 inhibited dexamethasone and budesonide-induced SP-D protein in hAECII cells (**Figures 7D–F**). Optical density analysis by Image J analysis confirmed that antagonism of the glucocorticoid receptor or STAT3 impaired dexamethasone-induced SP-D (**Figures 7D,E**) and that budesonide induced SP-D in a glucocorticoid receptor dependent manner (**Figure 7F**). These data suggested that glucocorticoid-induced SP-D synthesis was dependent on glucocorticoid receptor and STAT3 activation.

DISCUSSION

We report here the effects of O_3 on intranasal budesonide treatment in allergic airway inflammation and hyperreactivity, implicate the alterations in SP-D expression in the O_3-induced airway changes and propose the involvement of STAT3 in glucocorticoid signaling during *sftpd* transcription. Our study raises the significance of air pollution in the regulation of respiratory immunity and treatment responsiveness in asthma.

Inhaled glucocorticoids are currently the main choice for asthma treatment because they can profoundly improve lung function, alleviate airway inflammation and airway hyperreactivity (1, 48, 49) but their effectiveness in acute asthma exacerbations is subject of on-going debate (49–51, 58–60). Studies on asthma exacerbations caused by exposure to air pollutants are limited (61, 62) and the available experimental data on animals (18–20) and humans (21–23) are unclear on whether inhaled corticosteroids are effective to treat O_3-induced airway inflammation and/or airway hyperreactivity in asthma. We wanted therefore to further investigate the effects of budesonide on O_3-induced exacerbation of allergic airway changes. We found that in the *Af* sensitization and challenge model airway hyperreactivity to methacholine was inhibited by budesonide at 2.5 mg/kg dose. To mimic asthma exacerbation, we sensitized and challenged Balb/c mice with *Af,* waited for 4 days for the acute inflammation to subside and then exposed the mice to O_3. Our results show that O_3 exposure induced airway hyperreactivity on its own and significantly enhanced lung resistance to methacholine and the numbers of eosinophils and neutrophils in the airways of *Af* sensitized and challenged mice confirming previous findings (39). To study the hypothesis that O_3 impairs the anti-inflammatory effects of budesonide, mice were intranasally treated with 2.5 mg/kg budesonide or vehicle. Budesonide significantly suppressed eosinophil (not neutrophil) numbers both in the BAL and the lung in air exposed but not in O_3 exposed mice. Strikingly, inhibition of lung resistance by budesonide (seen in air exposed mice) was completely abolished by O_3 exposure.

In various experimental conditions budesonide was previously shown to inhibit mediators relevant to O_3-induced airway changes such as IL-6 (63), CCL11 (64), CXCL2 mRNA in the lung (65) and IL-13-induced *ex vivo* airway hyperreactivity (66), while CCL20 was actually stimulated by budesonide in asthmatic airway epithelial cells (67) and there is no data in the literature on the effects of budesonide on IL-23p19. O_3 upregulated BAL IL-6, CXCL2, and CCL20 in a budesonide-resistant manner and reversed the inhibitory effects of budesonide on CCL11, IL-13, and IL-23p19 expression in mice sensitized and challenged with *Af.* Induction of CCL20 by O_3 is interesting because CCL20 was thought to be responsible for recruitment of neutrophils into the airways conveying budesonide resistance (67). The role of CCL20 in O_3-induced resistance to the budesonide effects however would need further confirmation. The reduction seen in BAL CCL11 and IL-23p19 of the budesonide treated air exposed animals corresponded with decreased BAL eosinophil and neutrophil counts, while reduced IL-13 matched the observed inhibition of airway hyperreactivity in the same animals. Since O_3 exposure prevented these budesonide effects, it is possible that these mediators are directly involved in the immunologic and physiologic response to combined *Af* and O_3 exposure. On the other hand, O_3 induced IL-6, CXCL2, and CCL20 was not altered by budesonide treatment and thus may be implicated in the observed neutrophilic inflammation caused by O_3 under allergic conditions. Our results are significant because they reproduce a glucocorticoid resistant airway inflammation and the hallmark characteristics of severe neutrophilic asthma exacerbation (68, 69).

SP-D plays an important role in suppressing proinflammatory mediator release in allergen or O_3-induced airway inflammation (29, 30, 54). Levels of SP-D expression in the lung are correlated to disease severity in asthma (70, 71). Therapeutics that boost SP-D expression are thought to improve asthma symptoms (70–72). Indeed production of SP-D requires the presence of glucocorticoids in airway epithelium (34–37). Our previous work showed that O_3 exposure induced the expression of SP-D in the BAL >48 h later, as a protective mechanism (28, 33, 39) but O_3-induced airway inflammation in allergen challenged mice also led to appearance of abnormal oligomeric molecular forms of SP-D indicating that oxidative stress can cause conformational changes that can inactivate SP-D's immunoprotective function (28, 32, 33). Such de-oligomerization was due to S-nitrosylation of SH bonds responsible for holding the dodecameric SP-D together (43, 44, 73). S-nitrosylation of SP-D requires NO, resulted from increased iNOS activity produced by the large numbers of activated inflammatory cells, particularly eosinophils in the allergen and O_3-exposed lung (33, 43, 44, 73). Here we wanted to know if the combination of allergen with O_3 exposure would alter the glucocorticoid effects on SP-D expression and whether budesonide treatment would affect O_3-induced SP-D de-oligomerization. As expected on the basis of previous investigations (34–37, 72) budesonide significantly increased BAL SP-D in air exposed mice. Importantly, this budesonide effect on SP-D expression was lost in O_3 exposed mice. O_3 in addition caused de-oligomerization of SP-D in the BAL of mice

sensitized and challenged with *Af*. This change was prevented by budesonide treatment. We speculate that in budesonide treated mice SP-D was indirectly protected from de-oligomerization possibly as a result of inhibition of eosinophils (the main source of iNOS and nitric oxide) in the lungs of mice. Taken together, our data suggested that budesonide induction of SP-D is inhibited by O_3 revealing a novel mechanism by which O_3 antagonizes the therapeutic benefits of this inhaled glucocorticoid. We propose that budesonide enhances SP-D expression thereby amplifying its local therapeutic effects in asthma.

We previously showed that IL-6 directly induced SP-D in type II alveolar epithelial cell cultures (28). This is interesting in the light that O_3 while strongly inducing IL-6, did not increase SP-D 12 h after exposure, but in fact it prevented the stimulatory effects of budesonide on SP-D release in the airways of mice. To better understand the mechanisms of how budesonide and O_3 regulate SP-D expression we used A549 cells, a readily available human type II alveolar epithelial cell line that models functions such as expression of the SP-D gene (*sftpd*). To confirm our findings, the budesonide effects were then recapitulated in primary human type II alveolar epithelial cells. O_3 exposure of A549 cells inhibited *sftpd* expression 1.5 h later, but by 48 h post exposure this effect was reversed into an induction of the *sftpd* gene. Budesonide induced *sftpd* mRNA in A549 cells exposed to air. O_3 completely prevented the budesonide induction of the *sftpd* gene 1.5 h later. However, by 48 h O_3 and budesonide synergistically increased *sftpd* mRNA. These results are in line with our previous *in vivo* study on Balb/c mice (28) and suggest that O_3 acts on *sftpd* transcription in a bi-phasic manner with an early phase inhibition ($<$12 h) and a late phase activation $>$48 h. Based on these and our previous findings on IL-6 we speculated that budesonide and O_3 may interact on a common signaling pathway involved in SP-D transcription in airway epithelial cells.

Two groups independently established that glucocorticoids induced SP-D mRNA protein *in vitro* and *in vivo* (34, 35). These pioneering studies showed that hydrocortisone and dexamethasone stimulated both *sftpd* mRNA and SP-D protein *in vitro* and *in vivo* in the fetal rat lung. Since the proximal promoter region of the SP-D gene does not contain complete binding elements for the glucocorticoid receptor, it was hypothesized that glucocorticoids indirectly induced expression of the *sftpd* gene or work in concert with other binding elements. The proximal promoter region of the human SP-D gene has binding elements for C/EBP, NFAT, AP1, HNF-3, and STAT3/6 that all contribute to transcription of SP-D (55). Interestingly, Zhang et al. reported that STAT3 (an IL-6 responsive transcription factor) can act as a co-activator in glucocorticoid receptor signaling (56) and H_2O_2-treatment directly phosphorylated STAT3 in airway epithelial cells (38). We tested the role of STAT3 and the glucocorticoid receptor in SP-D mRNA (*sftpd*) and protein expression. Dexamethasone induced *sftpd* expression in human primary type II aleveolar epithelial cells was abolished by blockade of either the glucocorticoid receptor or STAT3. We established here that dexamethasone induced *sftpd* mRNA and SP-D protein via the glucocorticoid receptor and critically, STAT3. Recent evidence suggests that O_3-induced glucocorticoid insensitivity involves p38 MAPK, MKP-1, and IL-17A. Inhibition of p38 MAPK prevented the decreased the inhibitory effects of dexamethasone on O_3 stimulated inflammation and IL-17A (18) and inhibition of IL-17A reduced dexamethasone insensitivity in a mouse model of chronic O_3 exposure (74). Here we showed for the first time that STAT3 is involved in glucocorticoid-induced SP-D synthesis. Cooperation between the glucocorticoid receptor and STAT3 may be crucial for SP-D synthesis in airway epithelial cells.

There are likely many pathways that contribute to the BAL SP-D levels *in vivo*, including but not limited to budesonide treatment, O_3 exposure, and BAL IL-6 expression. Since glucocorticoids are known to have numerous side effects and after chronic administration patients can become refractory, novel asthma therapeutics to induce SP-D may seek to directly activate STAT3 signaling (5).

While prior work suggested that O_3 may impair the effectiveness of budesonide, here we studied the potential role for SP-D in this pathway. We propose a novel SP-D-mediated mechanism for the anti-inflammatory and functional effects of budesonide on the lung. A better understanding of how air pollutants such as O_3 might affect asthma treatment will lead to improved therapeutic approaches.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of California, Davis and University of Pennsylvania Institutional Animal Care and Use Committees. The protocol was approved by the University of California, Davis, and University of Pennsylvania Institutional Animal Care and Use Committees.

AUTHOR CONTRIBUTIONS

CF conducted the *in vivo* SP-D and luminex experiments, assisted with flow cytometry and Flexivent analysis, and wrote the manuscript. MG conducted the *in vivo* and *in vitro* experiments (performing the Flexivent and flow cytometry experiments) and assisted with flow cytometry and Flexivent analysis. ZJ conducted the *in vitro* experiments (isolation, purification and culture of AECII cells, Western blot, and qRT-PCR analysis for SP-D expression in the cells after treatment). JH, BK, and IR assisted with *in vivo* experiments. AH lead all aspects of the study and edited the manuscript.

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REVIEW
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Acute Respiratory Barrier Disruption by Ozone Exposure in Mice

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Ozone exposure causes irritation, airway hyperreactivity (AHR), inflammation of the airways, and destruction of alveoli (emphysema), the gas exchange area of the lung in human and mice. This review focuses on the acute disruption of the respiratory epithelial barrier in mice. A single high dose ozone exposure (1 ppm for 1 h) causes first a break of the bronchiolar epithelium within 2 h with leak of serum proteins in the broncho-alveolar space, disruption of epithelial tight junctions and cell death, which is followed at 6 h by ROS activation, AHR, myeloid cell recruitment, and remodeling. High ROS levels activate a novel PGAM5 phosphatase dependent cell-death pathway, called oxeiptosis. Bronchiolar cell wall damage and inflammation upon a single ozone exposure are reversible. However, chronic ozone exposure leads to progressive and irreversible loss of alveolar epithelial cells and alveoli with reduced gas exchange space known as emphysema. It is further associated with chronic inflammation and fibrosis of the lung, resembling other environmental pollutants and cigarette smoke in pathogenesis of asthma, and chronic obstructive pulmonary disease (COPD). Here, we review recent data on the mechanisms of ozone induced injury on the different cell types and pathways with a focus on the role of the IL-1 family cytokines and the related IL-33. The relation of chronic ozone exposure induced lung disease with asthma and COPD and the fact that ozone exacerbates asthma and COPD is emphasized.

Keywords: inflammation, cell death, interleukins, mucus, tight junctions, innate immunity

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INTRODUCTION

Human ozone (O3) exposure represents a major health issue (1, 2) playing an important role in the pathogenesis of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). Ozone causes acute epithelial airway wall injury, inflammation, and airway hyperreactivity (AHR). Ozone elicits irritation of the airways with cough, bronchoconstriction, and inflammatory cell infiltration with loss of respiratory function. AHR represents a complex response of the airways to the release of bronchoconstrictive mediators and cholinergic stimulation, and is a hallmark of ozone exposure which is shared with allergic asthma. Furthermore, increased ozone exposure, especially occurring during thunderstorms, provokes severe exacerbations of asthma and may even contribute to the asthma-related deaths (3–7). A recent epidemiologic study revealed that even a short-term exposure to ambient air pollution such as PM2.5, O_3, and NO_2 significantly

increased the risk of asthma mortality (8). Chronic ozone exposure leads to a progressive loss of the gas exchanging alveoli, a phenomenon known as emphysema, usually associated with chronic inflammation, fibrosis, and terminal respiratory failure, observed in patients with chronic obstructive pulmonary disease (COPD) and severe asthma (9). Of note, the pathogenesis of chronic lung diseases is complex and comprises the effects of various environmental particulates, toxins, chemical sand pollutants, detergents, respiratory viruses, microbial dysbiosis as well as allergen exposure, and is influenced by diverse genetic and epigenetic factors (10–15).

The respiratory airway epithelium forms a physical barrier and first line of defense of mucosal immunity (16, 17). Tight junctions (TJ) and adherens junctions (AJ), fluid, mucus, surfactant proteins, and motility of cilia are critical for the barrier control and innate response (18). Ozone impairs the function of critical proteins of the epithelial barrier (19), which will be discussed later. In addition, there is increased proliferation of the airway epithelial cells following exposure to ozone, likely as a result of direct oxidative epithelial damage (20).

Inflammatory cytokines such as members of the IL-1 family, including IL-1α, IL-1β, IL-18, IL-33, and IL-36 (21–23) as well others and several chemokines are upregulated upon ozone exposure and play major roles in the inflammatory and pathogenic response. IL-1 is involved in the inflammatory response (24), while IL-33 may have protective effects in ozone-induced inflammation as discussed below. We review here the most recent findings on ozone involvement in bronchiolar epithelial barrier dysfunction, acute lung injury, inflammation, resolution, and defective repair (20).

RESPIRATORY BARRIER INTEGRITY

The integrity of the epithelial barrier depends on tight junctions (TJ) and adherens junctions (AJ), which insure apicobasal cell polarity, but also mucus, fluid, and function of the cilia (18, 25–27). Tight junctions comprise the claudin family, occluding, and tricellulin. In addition, several scaffolding proteins, such as zonulae occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein 1, and others have been identified in the tight junctions (28, 29). E-cadherin, as well as TJs were reduced in patients with asthma (30–32). Common respiratory viruses, such as human rhinovirus (HRV) (33, 34) or respiratory syncytial virus (RSV) (35) disrupt and impair airway epithelial barrier and delay healing of infected epithelium (36), through NADPH oxidase-1 and ROS-dependent mechanisms (33, 37, 38). Disruption of tight junctions with leak of the epithelium allows systemic access of irritants, pathogen, and allergens (15, 39), as well as the drainage of host proteins, lipid mediators, or cells into the airway lumen, where they may perpetuate inflammatory response, acting back on epithelium. Depending on the dose of allergen and airway inflammation, the Zo-1 and Cld-18 proteins expression are decreased in eosinophilic asthma, but it is even more pronounced in mixed and neutrophilic asthma phenotype (27). Epithelial barrier is impaired not only in the lower airways of patients with asthma (32), but also in the nasal mucosa of allergic rhinitis due to house dust mite (HDM) (40) displaying reduced occludin and ZO-1 levels. Ozone exposure disrupts tight junction proteins and hence the function of the respiratory barrier as reported recently (27, 41, 42).

DIRECT DISRUPTION OF EPITHELIAL BARRIER BY OZONE

Ozone causes immediate damage of the bronchiolar epithelial cell barrier with cell stress, desquamation, and death with leak of protein and DNA into the airspace within 1–2 h. Since several mediators of inflammation are not yet detectable at this time, we postulate that ozone induced ROS has a direct effect on essential components of the bronchiolar cell integrity with reduced cilia function, tight junctions, mucus, and surfactant protein production (43). This first phase of ozone induced damage of the airway epithelium is followed by a second phase at of bronchiolar epithelial injury and cell death with protein leak and influx of neutrophils, ROS expressing myeloid cells, IL-1α and IL-33 production by epithelial and myeloid cells. Thus, the data suggest that ozone causes a biphasic response, an immediate direct injury via ROS and a second damage by myeloid cells, moving to and exacerbating epithelial cell damage. Indeed, neutrophil depletion by antibody against granulocytes attenuates the second phase. Similar disruptions of the respiratory barrier as shown for ozone has been described before by other air pollutants such nitrogen dioxide, disulfide, particle, chemicals, and cigarette smoke- all of which can cause chronic pulmonary diseases, but usually require longer exposure time and at higher concentrations than ozone (38, 44). A schematic view of the initial events at the respiratory barrier and early repair process is given in **Figure 1**.

DISRUPTION OF TIGHT JUNCTIONS

Upon a single ozone exposure, we found enhanced expression of E-cadherin, ZO-1, and claudin-4 using immunofluorescence (43). By contrast, the epithelial E-cadherin, ZO-1, and Cld-4 expression are reduced in the absence of IL-33 (43). Epithelial E-cadherin expression is also reduced in IL-33/ST2 receptor deficient mice, suggesting a protective effect of IL-33. At the transcriptional level, an increased Cld-4 level (4–6 h) or E-cadherin (4–8 h) expression were found. Therefore, the data suggest that IL-33/ST2 signaling has a protective effect. This IL-33-dependant epithelial protection is probably an early defense mechanism in physiological condition. However, in

Abbreviations: AHR, airways hyperreactivity; AIFM1, mitochondrial apoptosis inducing factor 1; AJ, adherens junctions; AREG, amphiregulin; BAL, broncho alveolar lavage to quantify cell counts; BALF, broncho alveolar lavage fluid to detect protein levels; GR1, Ly-6G antibody depleting neutrophils; IL-33, interleukin-33; IL-33R/ST2, interleukin-33 receptor/ suppression of tumorigenicity 2; CXCL1/KC, keratinocyte chemoattractant; COPD, chronic obstructive pulmonary disease; LCN-2, lipocalin 2; CCL1/MCP-1, chemokine ligand 2; CCL2/MIP-2, chemokine (C-X-C motif) ligand 2; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; PGAM5, mitochondrial serine/threonine-protein phosphatase; rmIL-33, recombinant mouse IL-33; ROS, reactive oxygen species; RNS, reactive nitrogen species; TIMP-1, tissue inhibitor metalloproteinase 1; TJ, tight junctions; WT, wild type mice.

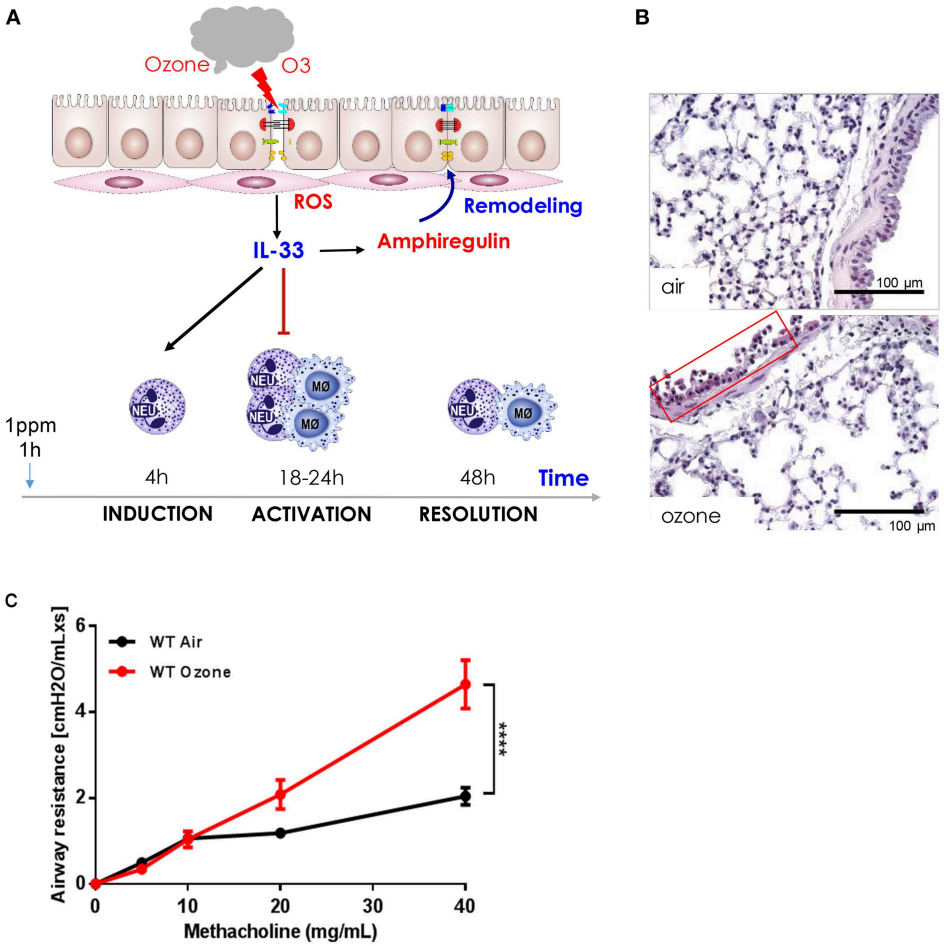

FIGURE 1 | Ozone-induced epithelial barrier dysfunction, AHR, ROS production, cell recruitment, cell desquamation, remodeling, and repair in mice. **(A)** Scheme showing ozone/ROS-induced disruption of bronchiolar epithelium, cell integrity, tight junctions with protein leak, and cell death with the release of IL-33. Neutrophils and macrophages are recruited within 18 h and macrophages release protective amphiregulin (AREG) with resolution of inflammation and remodeling (43). **(B)** Micrograph of ozone-induced disruption of bronchiolar epithelium with desquamation, cell death, and focal inflammation at 18 h after exposure (1 h, 1 ppm), scale bar 100 µm. **(C)** Airways hyperreactivity (AHR): ozone enhances methacholine induced bronchoconstriction measured as resistance (RL) using invasive plethysmography 24 h after exposure (1 h, 1 ppm, unpublished data).

the settings of allergic inflammation, excess of pathogenic Th2 cells and ILC2 in the epithelial wall, reacting to IL-33, lead to increased inflammation, and symptoms of the disease. Interestingly, in the chronic airway inflammation impairment of the barrier consists of decreased expression of several TJ proteins, but also a subsequent increase of TJs, such as CLD-4 (27). Recent reports confirm that ozone exposure impairs the function of epithelial barrier tight junction proteins (27, 41, 42).

Within 24 h after ozone exposure basal cells proliferate and at 48 h the bronchiolar epithelium is restored with firm tight junctions, mucus, and cilia (43). The mechanisms of inflammation including resolution of inflammation, remodeling of airways, and repair mechanisms are an area of intense research (13, 37, 45–47).

AIRWAY HYPERREACTIVITY

Ozone exposure causes bronchoconstriction measured by the pulmonary function tests in humans and airway hyperreactivity (AHR) in rodents and humans as assessed by increased reactivity of the airways to cholinergic stimulation (44, 48). Mice exposed to ozone display a dose-dependent increased of airway resistance (RL) to methacholine aerosol as measured by invasive plethysmograph (**Figure 1C**). This has also been demonstrated in humans (48).

The underlying mechanisms of AHR are due to the complex response of the airway wall including effect of oxidative stress (49, 50) activation of kinase pathways, the release of chemokines, cytokines (51, 52), and lipid mediators, increased sensitivity of bronchial smooth muscle cells either directly or through an effect

on innervation and neuropeptides (53), which is beyond the scope of this review.

The transient receptor potential cation channel subfamily V member1 (TRPV1) is upregulated by ozone in experimental asthma, which is sensitive to a TRPV1 antagonist. The TRPV1 antagonist suppresses the neuropeptide calcitonin gene-related peptide (CGRP) and thymic stromal lymphopoietin (TSLP) (54). Thus, TRPV1 expression may be an important mechanism for asthma exacerbation upon exposure to ozone and other environmental pollutants. Ozone induced AHR and lung inflammation is characterized by increased neutrophils recruitment in the airways and lung (54), although the role of neutrophils in inducing AHR is controversial (55, 56). Interestingly, NKT cell-deficient ($CD1d^{-/-}$ and $J\alpha18^{-/-}$) mice are resistant to ozone-induced AHR and have reduced neutrophils. Further, anti-CD1d antibody blockade of NKT cell activation prevented ozone-induced AHR. NKT cells producing IL-17 causes AHR, which was prevented in IL-17A deficient mice or by anti-IL-17 antibody blockade (57, 58). ROS mediated AHR and inflammation further depends on danger activated proteins such as HMGB1, HSPs, RAGE, and others activating the molecular pattern recognition receptors Toll-Like Receptors (TLR) 2 and 4 and the adaptor protein, MyD88 (59). However, the list of endogenous mediators activating AHR includes lipid mediators, prostaglandins and leukotrienes in response to ozone is incomplete (51, 60, 61).

These effects of ozone on bronchoconstriction and AHR raise the possibility that ozone may be involved in underlying a specific type of asthma, namely the neutrophilic inflammatory phenotype of asthma (62, 63). However, the evidence linking neutrophilic asthma to exposure to ozone as a constituent of pollution is unclear, although an increase in exacerbations of asthma in patients with asthma following a peak increase in levels of environmental ozone has been reported (64).

REACTIVE OXYGEN SPECIES AND CELL DEATH VIA OXEIPTOSIS

Ozone generates reactive oxygen species (ROS), mitochondrial damage with oxidant stress activating the NLRP3 inflammasome and contributes to AHR, airflow obstruction, and emphysema (2, 65). ROS has a dual effect on cell integrity: at low concentration, it has a beneficial, cytoprotective effect, while at high concentration it is cytotoxic causing cell death. KEAP1 acts as a ROS sensor, which triggers at high ROS levels as found upon ozone exposure a novel caspase-independent cell-death pathway known as oxeiptosis (66, 67) (**Figure 2**). We found that ROS-induced cell-death depends on interactions of cellular ROS sensor KEAP1 and the phosphatase PGAM5 with antioxidant function and AIFM1, a pro-apoptotic factor. At high ROS concentration PGAM5 is released from the complex and activates the pro-apoptotic factor AIFM1 inducing apoptotic pathway. PGAM5 deficient mice have enhanced lung inflammation with proinflammatory cytokines upon ozone-exposure. Furthermore, in Influenza A virus infection, viral load and lung inflammation were increased in PGAM5 deficient mice, which succumbed to infection (68, 69). Oxeiptosis represents a different, non-canonical cell-death pathway, which is novel, ROS-sensitive, caspase independent, cell-death pathway important for protection against inflammation induced by ROS or viral pathogens (69). The molecular events of oxeiptosis are depicted in **Figure 2.** Other cell-death pathways include inflammasome-mediated apoptosis and pyroptosis, but also necroptosis and ferroptosis. Their relative roles of these pathways need to be further investigated in the ozone model.

INFLAMMATORY CELLS RECRUITED IN THE LUNG

Cell and tissue damage induce inflammatory cell recruitment including neutrophils, macrophages, innate lymphoid cells, and more. Neutrophils play a critical role in acute and chronic inflammation (70). Neutrophils migrate within and through the vessels depending polarization within activated venules activate platelets present in the bloodstream (71). Neutrophils scan for activated platelets resulting in the redistribution of receptors via the selectin ligand PSGL-1 that drive neutrophil migration allowing the interaction with the endothelium and the circulation before inflammation proceeds. Thus, a close interaction between neutrophils and platelets drives neutrophil migration to sites of injury or cell death. We have described the inflammatory response upon ozone exposure (24). Upon a single ozone exposure at 1 ppm for 1 h the analysis at 4 h after ozone exposure revealed desquamation of epithelial cells with increased CXCL1, CCL2/MIP-2, and IL-6 production, which was followed by increased macrophages and neutrophils in BALF at 6 h. At 24 h, the inflammatory response was further enhanced in the absence of ST2 or IL-33 with predominant neutrophils and interstitial macrophages in the lung. We investigated the role in the second inflammatory phase by GR-1 antibody depletion of neutrophils and other myeloid cells. GR-1 cell depletion with anti-GR1 antibody reduced protein leak, myeloid and epithelial cell in BALF and reduced parenchymal injury and inflammation with reduced MMP9 and increased amphiregulin expression as sign of tissue repair. Therefore, neutrophils contribute to tissue injury and repair as reported in other inflammatory models (72).

Besides T lymphocytes not discussed here, innate lymphocytes (ILC) are involved in the early immune response (54, 73, 74). Tissue-resident ILC-2 is involved in both physiologic and pathologic responses, yet their physical tissue niches are poorly described (75). ILC2 are recruited upon ozone exposure, but there is little information available on their roles in inflammation and airway hyperreactivity (6, 43, 76, 77).

Natural killer T cells expressing IL-17 may induce airway hyperreactivity, which depended on IL-17 expression as mentioned before (57). Platelets are likely involved in ozone induced neutrophilic inflammation, but no studies address their role in ozone induced tissues injury. The role of platelets has been recently studied in allergic inflammation (78).

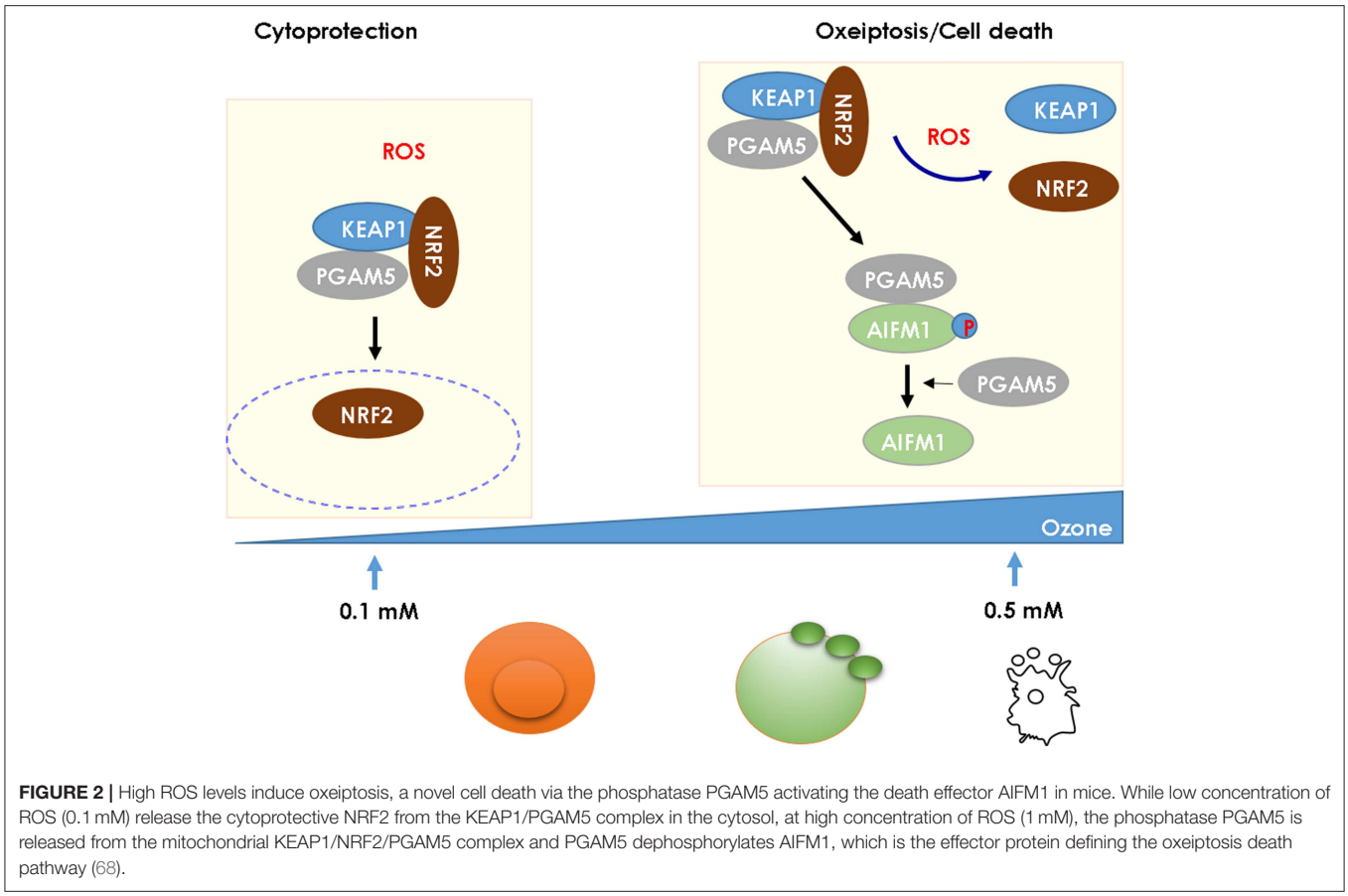

FIGURE 2 | High ROS levels induce oxeiptosis, a novel cell death via the phosphatase PGAM5 activating the death effector AIFM1 in mice. While low concentration of ROS (0.1 mM) release the cytoprotective NRF2 from the KEAP1/PGAM5 complex in the cytosol, at high concentration of ROS (1 mM), the phosphatase PGAM5 is released from the mitochondrial KEAP1/NRF2/PGAM5 complex and PGAM5 dephosphorylates AIFM1, which is the effector protein defining the oxeiptosis death pathway (68).

Fibroblast, stromal cells, pericytes, and endothelial cells are potential targets of ozone (79, 80), which is an area for further investigations. A recent study defined a perivascular fibroblast-like stromal cells producing IL-33 and TSLP which regulate ILC2s and type 2 immunity (75).

Two major previous morphological investigations on the effects of a 4 h exposure to 3 ppm ozone on rat lungs are worth mentioning (81, 82). The epithelial damage was located centrally showing cell death of bronchiolar epithelium and alveolar pneumonocytes. Lesions in capillary endothelium with endothelial swelling were widespread with ring-like formations of endothelial membranes peripherally with alveolar and interstitial edema. These results show that ozone damage is in the centro-acinar regions and affects both endothelial and epithelial cells.

INTERLEUKINS WITH INFLAMMATORY OR PROTECTIVE ROLES

Upon ozone exposure, several interleukins (IL) are increased and may regulate inflammation. Here we focus on two IL-1 family members, IL-1a and IL-33 (23, 83–85), which are known as alarmins, released upon cell stress and death. An overview of IL1 members and IL-33 and their receptors is shown in **Figure 3**.

INTERLEUKIN-1 ENHANCING INFLAMMATION

A role of interleukin-1 (IL-1)-associated cytokines has been reviewed recently (24). We investigated inflammation after a single ozone exposure (1 ppm for 1 h) using IL-1α-, IL-1β-, and IL-18-deficient mice or neutralizing anti-IL-1α antibody to investigate their role in epithelial cell death. IL-1α was increased within 1 h after ozone exposure. Epithelial injury, inflammation and AHR were IL-1α-dependent. Further, we found that IL-1α signaling via IL-1R1/MyD88 was type I alveolar epithelial cell dependent as demonstrated by cell specific MyD88 deletion in mice (86). By contrast, inflammation and epithelial injury were less reduced in absence of IL-1β and IL-18. In conclusion, the IL-1α induced tissue damage and inflammation is mediated by IL-1R/MyD88 signaling in epithelial cells. Interestingly, in more chronic disease settings, IL-1β is increased in the lungs of mice with neutrophilic phenotype of asthma and in patients with neutrophilic asthma (27). Additionally, in humans IL-1β may lead to impairment of epithelial barrier function and increase of mucus production (27). Therefore, IL-1α or IL-1 receptor may represent a therapeutic target to attenuate ozone-induced lung inflammation and airway hyperreactivity. The use of neutralizing IL-1β antibody and IL1-RA antagonist is in fact used for different experimental conditions.

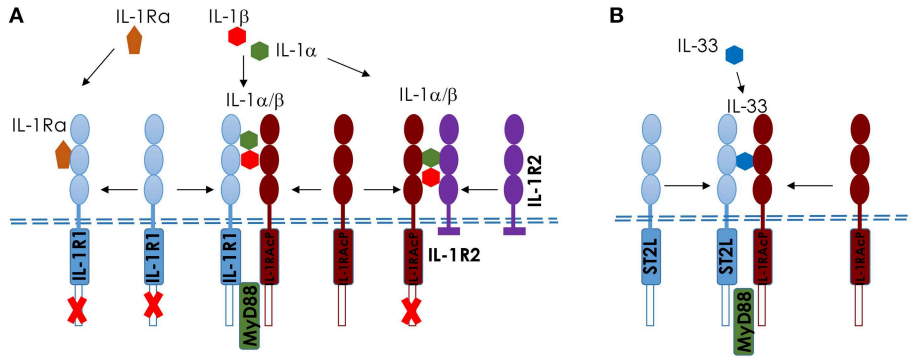

FIGURE 3 | Comparison of IL-1 and IL-33 and their membrane receptors in mice. Scheme of IL-1 **(A)** and IL-33 **(B)**, showing the different ligands and receptors. **(A)** IL-1αβ binds to IL-1R1 and induces a conformational change in the extracellular domain of IL-1R1, enabling its interaction with IL-1RAcP, which is required for intracellular signaling, including MyD88-dependent activation. IL-1Ra competes with IL-1αβ for the binding to IL-1R1; the binding of IL-1Ra to IL-1R1 prevents IL-1αβ binding, the recruitment of IL-1RAcP and the activation of intracellular signaling pathways. IL-1R2 acts as a decoy receptor on the cell surface to IL-1RAcP. **(B)** Interleukin−33 (IL−33) binds to its transmembrane receptor suppression of tumorigenicity 2 (ST2) and induces a conformational change that allows ST2 to interact with IL−1 receptor accessory protein (IL−1RAcP). Activation of ST2 and IL−1RAcP leads to the Toll/IL−1 receptor (TIR) domains clustering and the recruitment of signaling adaptor myeloid differentiation primary response protein 88 (MYD88) (23).

INTERLEUKIN-33 ATTENUATING INFLAMMATION

IL-33 is another alarmin released rapidly upon ozone and may have a protective role. IL-33 has homeostatic functions and is involved in injury and repair. The alarmin IL-33 is expressed at steady state in tissue cells and released upon airway epithelial injury and repair during inflammatory process (87, 88). IL-33 has functions in both innate and adaptive immune response (85). IL-33 binds to ST2 chain known as IL-33R or IL-1RL1 (89) which associates with IL-1RAcP (23). The full length, 35 kDa IL-33 is cleaved by different proteases and is produced by neutrophils, macrophages, or mast cells that full length IL-33 to several active moieties that are about 30-fold more active than the full length form (84, 90, 91). IL-33 receptor ST2 is was first detected in high endothelial venules in lymphoid tissues in mice (92), but is found in most innate immune cells including mast cells (85), innate lymphoid cells (85), myeloid and dendritic cells (93), and to a lesser extent on Th2 cells (85). Further, nuclear IL-33 may associate with chromatin *in vivo*, but the function of nuclear IL-33 is still unresolved.

IL-33 expression was increased in epithelial and macrophages and other myeloid cells in mice upon ozone exposure. In the absence of IL-33 or IL-33R/ST2, epithelial cell injury with protein leak and myeloid cell recruitment and inflammation was further increased, while tight junction proteins E-cadherin and ZO-1, ROS expression in neutrophils, and AHR were diminished (43). ST2 antibody neutralization recapitulated the enhanced ozone induced neutrophilic inflammation, while the administration of rmIL-33 reduced neutrophil recruitment in IL-33 deficient mice (69). These data demonstrate that ozone causes an immediate barrier injury, which precedes myeloid cell mediated inflammatory injury under the control of the IL-33/ST2 axis. Thus, IL-33/ST2 signaling appears to be critical for the maintenance of intact epithelial barrier and inflammation.

SURFACTANT PROTEINS AND MUCUS

Surfactant proteins mucus are the first line protection protecting the respiratory epithelium. Surfactant protein-D (SP-D), produced by the airway epithelium, is multimeric protein sensitive to oxidative stress. SP-D directly inhibits extracellular DNA trap formation by eosinophils. Allergic airway sensitization and ozone exposure augmented eosinophilia and nos2 mRNA (iNOS) activation in the lung tissue with modification of SP-D in the airways. Thus, the regulatory feedback between SP-D and eosinophils appears to be destroyed by the NO-rich oxidative lung tissue environment in asthma exacerbations (94).

THERAPEUTIC TARGETS

The protective role of antioxidants against ROS/RNS induced injury and inflammation environmental pollutants has been reviewed (38, 95). A comprehensive analysis of oxidative stress in ozone induced lung injury and mitochondrial dysfunction allowed to identify potential druggable pathways (2, 38, 95, 96). Indeed, several interventions attenuate ozone induced inflammation such N-acetylcysteine (97), hydrogen disulfide (98), MIF antagonist (99) as well as blockade of IL-1α (86) or IL-17A (100), recombinant IL-33 (69), L-arginine promoting DNA repair (101), Taurine (102). Also, blocking ROS-induced airway inflammation by apocynin, an NADPH inhibitor has been targeted in asthma and COPD in human clinical trials (47, 103). Furthermore, DNA released upon cell death and is highly inflammatory. In particle-induced lung injury, enzymatic degradation of DNA by DNase I reduced inflammation (10, 11). The list therapeutic targets are not exhaustive, but new mechanistic insights may lead to antagonists that are more efficacious. However, reduction of airborne pollution, especially high levels of ozone and smog, would be the most efficacious measure to prevent chronic respiratory disease.

CONCLUSIONS

Chronic ozone exposure causes chronic lung inflammation, emphysema, and interstitial fibrosis with progressive loss of lung function in man and rodents. Furthermore, ozone enhances the development of chronic lung diseases such as allergic and non-allergic asthma, COPD, and emphysema. Recent studies demonstrated the importance of IL-1α, IL-33, and IL-17A axis in ozone induced lung injury and inflammation, and a role of PGAM5 emerged, which defines a novel cell death pathway known as oxeiptosis. Understanding the fundamental mechanisms of injury and defective repair likely related to DNA damage, and defining critical targets require further investigations including clinical and epidemiological studies. Chronic progressive lung diseases could be prevented to a great extent by reducing environmental air pollution, tobacco, and wood fire smoke and smog containing ozone exposure.

AUTHOR CONTRIBUTIONS

MS, BR, and DT wrote the review. KC, VQ, and CA inspired the work and corrected. All authors read the contribution.

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Ozone Pollution: A Major Health Hazard Worldwide

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Oxides of nitrogen (NO_x) and volatile organic compounds (VOCs) released into the atmosphere can react in the presence of solar irradiation, leading to ozone formation in the troposphere. Historically, before clean air regulations were implemented to control NO_x and VOCs, ozone concentrations were high enough to exert acute effects such as eye and nose irritation, respiratory disease emergencies, and lung function impairment. At or above current regulatory standards, day-to-day variations in ozone concentrations have been positively associated with asthma incidence and daily non-accidental mortality rate. Emerging evidence has shown that both short-term and long-term exposures to ozone, at concentrations below the current regulatory standards, were associated with increased mortality due to respiratory and cardiovascular diseases. The pathophysiology to support the epidemiologic associations between mortality and morbidity and ozone centers at the chemical and toxicological property of ozone as a strong oxidant, being able to induce oxidative damages to cells and the lining fluids of the airways, and immune-inflammatory responses within and beyond the lung. These new findings add substantially to the existing challenges in controlling ozone pollution. For example, in the United States in 2016, 90% of non-compliance to the national ambient air quality standards was due to ozone whereas only 10% was due to particulate matter and other regulated pollutants. Climate change, through creating atmospheric conditions favoring ozone formation, has been and will continue to increase ozone concentrations in many parts of world. Worldwide, ozone is responsible for several hundreds of thousands of premature deaths and tens of millions of asthma-related emergency room visits annually. To combat ozone pollution globally, more aggressive reductions in fossil fuel consumption are needed to cut NO_x and VOCs as well as greenhouse gas emissions. Meanwhile, preventive and therapeutic strategies are needed to alleviate the detrimental effects of ozone especially in more susceptible individuals. Interventional trials in humans are needed to evaluate the efficacy of antioxidants and ozone-scavenging compounds that have shown promising results in animal studies.

Keywords: ozone, climate change, air quality standards, cardiovascular health effects, respiratory health effects, mitigation strategies

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SOURCES AND CHEMISTRY

Ozone, the triplet oxygen (O_3), is formed from the reaction between dioxygen (O_2, the normal oxygen molecule) and a singlet oxygen (O, oxygen atom) in the presence of a third-body molecule able to absorb the heat of the reaction. The highly reactive and short-lived singlet oxygen (O) can be generated via the photolysis of nitrogen dioxide (NO_2) or ionization of O_2. Background ozone is present in both the stratosphere and the troposphere. Stratospheric ozone is concentrated in the tropopause (~between 8 and 15 km above the ground), a region that is called ozone layer. Stratospheric ozone is nicknamed "good" ozone, because the ozone layer plays a vital role in absorbing ultraviolet (UV-B) rays that are harmful to living beings on the earth. Since direct contact with ozone at the ground level can cause damages to living cells, organs, and species including humans, animals, and plants, tropospheric or ground-level ozone is nicknamed "bad" ozone.

There is a natural influx of ozone from the stratosphere to the troposphere, peaking normally in the spring months when the vertical air movement reaches its maximum in the northern hemisphere. This influx contributes to background levels of ground-level ozone. The predominant source of tropospheric ozone, however, is the photochemical reactions involving volatile organic compounds (VOCs) and oxides of nitrogen (NO_x), mainly comprised of NO_2 and nitric oxide (NO). In the absence of or at very low concentrations of VOCs or carbon monoxide (CO), ozone reaches a steady-state concentration depending on solar intensity, ambient temperature, and the ratio of NO_2 concentration to NO concentration. Under this condition, one NO_2 molecule is converted via photolysis into one O_3 molecule and one NO molecule; and ozone is, in turn, consumed by NO to regenerate a NO_2 molecule. This cycle results in zero accumulation of ozone concentration. However, VOCs or CO participate in a series of complex photochemical reactions to produce free radicals that compete with ozone to react with NO. The net effects include the accumulation of ozone, the oxidation of VOCs into oxygenated organic compounds, and the formation of nitrogen-containing compound, and the oxidation of CO into CO_2. Because many of the oxygenated and nitrogen-containing organic compounds are present in the condensed phase due to their low volatility, they are called secondary organic aerosols (SOAs). The whole mixture composed of ozone, SOAs, and their gaseous precursors is called photochemical smog. The production of ozone in the troposphere is depicted in **Figure 1**.

This ozone formation mechanism (**Figure 1**) explains why elevated ozone concentrations are found in an increasing number of places around the world where anthropogenic emissions of NO_x, VOCs, and CO have been increasing. The combustion of fossil fuels occurs at a high temperature favorable for NO_x formation, and worldwide increases in fossil-fuel derived energy (for electricity generation, transportation, and household cooking and heating) are responsible for increasing emissions of NO_x. Major anthropogenic sources of VOCs include vehicular exhaust, fugitive evaporation of gasoline and other gaseous fuels (e.g., natural gas and propane), biomass and fossil fuel combustion, and industrial solvent use. A recent study found that volatile chemicals released from consumer products (e.g., pesticides, coatings, printing inks, adhesives, cleaning agents, and personal care products) have emerged as a large urban source of VOCs (1). Natural vegetation emissions of certain VOCs (e.g., isoprene) also contribute to ozone formation especially at the regional scale (2–5).

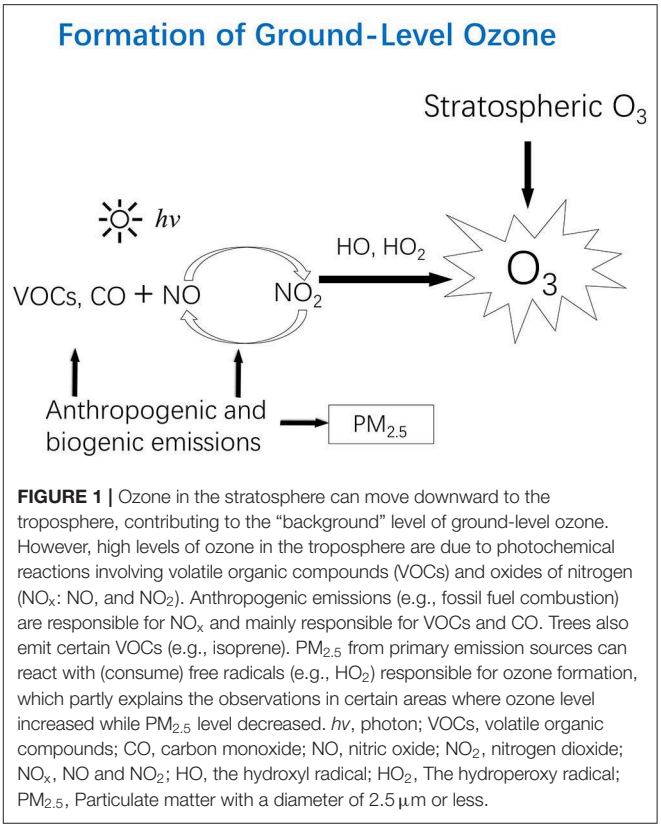

FIGURE 1 | Ozone in the stratosphere can move downward to the troposphere, contributing to the "background" level of ground-level ozone. However, high levels of ozone in the troposphere are due to photochemical reactions involving volatile organic compounds (VOCs) and oxides of nitrogen (NO_x: NO, and NO_2). Anthropogenic emissions (e.g., fossil fuel combustion) are responsible for NO_x and mainly responsible for VOCs and CO. Trees also emit certain VOCs (e.g., isoprene). $PM_{2.5}$ from primary emission sources can react with (consume) free radicals (e.g., HO_2) responsible for ozone formation, which partly explains the observations in certain areas where ozone level increased while $PM_{2.5}$ level decreased. *hv*, photon; VOCs, volatile organic compounds; CO, carbon monoxide; NO, nitric oxide; NO_2, nitrogen dioxide; NO_x, NO and NO_2; HO, the hydroxyl radical; HO_2, The hydroperoxy radical; $PM_{2.5}$, Particulate matter with a diameter of 2.5 μm or less.

Ozone formation depends on solar intensity that is directly associated with atmospheric temperature. Ironically, with a decrease in ambient concentrations of carbonaceous aerosols (e.g., soot), emitted from combustion of coal, diesel, and biomass, atmospheric visibility increases, and consequently solar intensity increases, favoring ozone formation. More importantly, particulate matter (e.g., particles with an aerodynamic diameter equal to or smaller than 2.5 μm, noted as $PM_{2.5}$) can serve as a sink of free radicals responsible for ozone formation. A recent study showed that a 40% reduction in $PM_{2.5}$ from 2013 to 2017 in the North China Plain was partly responsible for an increasing ozone trend (at 1–3 ppb per year) during the period of 2013–2017 observed in megacity clusters of eastern China (6).

IMPACT OF CLIMATE CHANGE ON GROUND-LEVEL OZONE

Ozone itself is a greenhouse gas in the atmosphere. Hence, increasing ground-level ozone contributes to global warming. On the other hand, a warming climate favors the formation and accumulation of ozone in the atmosphere mainly through

two physicochemical mechanisms. First, in certain parts of the world, a warming climate changes humidity and wind conditions, leading to decreases in the frequency of surface cyclones. The resulting more stagnant atmospheric condition decreases the dispersion of NO_x and VOCs and prolongs the time for the reactions to produce ozone. Second, ozone-forming reactions are typically enhanced by increased atmospheric temperatures. Based on these climate-induced changes in the atmospheric stability (air stagnation) and temperature, it is predicted that by the year 2050, warming alone may increase by 68% the number of ozone-standard exceedance days across the eastern United States (7). Another study predicted that changes in regional climate and globally enhanced ozone would increase ground-level ozone over most of the United States. More specially, it is predicted that the 95th percentile for daily 8-h maximum ozone would increase from 79 ppb in 2012 to 87 ppb in 2050 (8). Similarly, another predictive analysis, through integrating data from climate model outputs and historical meteorology and ozone observations across 19 urban communities in southeastern United States, estimated an increase of 0.43 ppb (95% CI: 0.14–0.75) in average ozone concentration during the 2040s compared to 2000 due to climate change alone (9).

Climate change can also prolong the ozone season. For example, high ozone concentrations usually occur in the summer in the United States. However, ozone during the fall reached the summer level in several Octobers in the 2000s and in 2010 over the southeastern United States. This was attributed to enhanced emissions of biogenic isoprene (a VOC precursor of ozone) from water-stressed plants under a drying and warming condition (10). This finding suggests that occurrences of a drying and warming fall in the future may lead to an extension of the ozone season from summer to fall in the regions with significant biogenic VOC emissions.

AMBIENT CONCENTRATIONS IN REFERENCE OF AIR QUALITY STANDARDS

Ozone is in gas phase under typical atmospheric conditions (temperature and pressure) and is commonly measured as mixing ratio, i.e., parts per million (ppm) or parts per billion (ppb). At the standard conditions for temperature (25°C) and pressure (1 atmosphere), 1 ppb ozone equals 1.97 $\mu g/m^3$. Based on its commonly recognized health effects, including causing breathing problems, triggering asthma attacks, reducing lung function, and increasing incidence of respiratory diseases, ozone is one of the regulated air pollutants in many countries and has a recommended limit by the World Health Organization (WHO). The current WHO Air Quality Guidelines for ambient (outdoor) ozone is 100 $\mu g/m^3$ (∼50 ppb) measured as 8-h maximum moving average within a day[1]. In the United States, the current National Ambient Air Quality Standards (NAAQS) for ozone include a 1-h standard (1-h maximum within a day) at 120 ppb and an 8-h standard (8-h daily max) at 70 ppb. The rationales for having two standards with different averaging times are as follows.

Historically, the NAAQS only had a 1-h standard, as a sharp peak of ozone concentration typically lasted for 1 h or a bit longer during the afternoon and evening hours in Los Angeles, California, and other large cities. This peak concentration was high enough to cause acute effects such as irritation to the eyes and the respiratory tract, lung function reduction, difficulty to breathe, and increased emergency room visits. However, this feature does not occur in most areas of the United States, because the regional transport of ozone precursors prolonged the hours of elevated ozone concentrations. Epidemiological studies have found that ozone concentrations averaged over a longer period (such as 8 h instead of 1 h) within a day are a more health-relevant indicator of ozone exposure. In fact, as of June 15, 2005, the 1-h ozone standard is no longer applied to areas designated with respect to the 8-h ozone standard, which includes most of the United States, except for portions of 10 states[2].

Since the enaction of the U.S. Clean Air Act Amendment in 1970, remarkable efforts were made to control the emissions of the two ozone precursors (and other criteria pollutants). From 1980 to 2017, total national emissions of NO_X and VOCs were reduced by 61 and 54%, respectively. Consequently, there was a 32% decrease in national average of daily maximum 8-h averages of ozone measured at 200 monitoring sites across the United States[3]. Despite this nationwide decrease and more drastic decrease in some ozone "hot spots" such as Los Angeles and Atlanta, Georgia[4,5], 100.6 million people nationwide (or nearly one in every three people) lived in U.S. counties where ozone levels exceeded the NAAQS standard of 70 ppb in 2017 (In contrast, much fewer people, 58 million in total lived in counties where $PM_{2.5}$, PM_{10}, SO_2, or lead exceeded the NAAQS)[3]. If the WHO-recommended 8-h limit of 50 ppb is used, there would be even more people living in places with ozone exceeding the health-based limit.

Although ambient ozone concentrations have showed a declining trend in the United States and similarly in Western Europe and Japan in the past decades[6], evidence suggests that global average ozone concentrations are increasing. For example, ozone measured at Mt Waliguan Observatory (a global "background" site) on the Tibetan Plateau over the period of 1994–2013 has shown an increasing trend at 0.2–0.3 ppb per year during spring and autumn (11). The springtime ozone increase was partly (∼60%) attributed to increased stratosphere-to-troposphere transport, whereas rising Asian anthropogenic emissions of ozone precursors were the key driver of increasing autumnal ozone at this site. This finding is alarming, because ozone is generally considered too reactive to be transported afar. However, this demonstrates that NO_x and VOCs emitted in more

[1]https://www.who.int/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health

[2]https://www.epa.gov/criteria-air-pollutants/naaqs-table

[3]https://www.epa.gov/air-trends/ozone-trends

[4]https://calepa.ca.gov/

[5]https://www.epa.gov/ga

[6]http://www.futureearth.org/blog/2018-feb-5/powerful-new-dataset-reveals-patterns-global-ozone-pollution

populous regions can undergo long-range transport and affect "background" ozone level. More importantly, this finding also suggests an increasing ozone trend not only in places where NO_x and VOCs were originally emitted but also along the air trajectories from precursor sources to the background site.

Although the air quality focus has been on particulate matter, especially $PM_{2.5}$, in rapidly developing economies such as China and India in the recent years, the "invisible" ozone pollution is increasingly recognized as a major health hazard. While annual $PM_{2.5}$ average concentrations showed a decreasing trend in many cities of China (12), ground-level ozone concentrations measured in some monitoring sites in China showed an increasing trend in the past several years. For example, observations made at a rural site (Dadianzi) 100 km northeast Beijing showed a steady increase in annual averages of the 8-h daily max ozone concentrations from 2004 to 2015 (13). Consistently, a recent analysis of ground-level ozone concentrations measured at nearly 1,000 sites across China also found an increasing trend of summertime ozone in northeastern China from 2013 to 2017 (6). This increase was attributed to changes in anthropogenic emissions of ozone precursors as well as reductions in $PM_{2.5}$ concentrations as described earlier. Due to the strong governmental efforts to primarily control $PM_{2.5}$ in China, anthropogenic emissions of NO_x have decreased substantially in most urban areas of China (a 21% nationwide reduction) from 2013 to 2017 (6). However, VOC emissions have remained relatively unchanged. Typically decreasing NO_x would increase ozone under VOC-limited conditions, which has been the case for many urban areas of China. All these explain that in summer months, daily air quality reports released to the public in recent years have often shown more days when the ozone standard was exceeded than when $PM_{2.5}$ or other regulated pollutants exceeded the standards in many cities of China. This trend in China appears to follow the pattern of the United States where non-compliance to ozone standard has been more frequently observed in more places than that to $PM_{2.5}$ or other pollutants.

HUMAN EXPOSURE AND DOSIMETRY

It is the fundamental principle of toxicology that "dose makes poison." It is common in air pollution epidemiologic studies to use ambient concentrations as a proxy for exposure or, more strictly speaking, dose. This approach omits inter- and intra-person differences in breathing rate and does not consider concentration differences between indoor and outdoor environments. Among common or the criteria pollutants defined by the US EPA, ozone has unique characteristics that can lead to substantial errors for using ambient ozone concentration as a proxy for dose.

First, ozone is chemically reactive and can be more effectively scavenged by building surfaces. In airtight buildings with doors and windows closed, indoor ozone levels are typically smaller than 20% of outdoor levels. In contrast, for leaky buildings and for building with windows frequently open, indoor concentrations can reach >70% outdoor concentrations. Because typically people spend the majority of time indoors, using outdoor concentration as a surrogate for ozone exposure would lead to greater overestimation of exposure for people living or working in more airtight buildings than for those living in less airtight buildings. This systematic exposure assessment error was used to explain a difference in ozone effect estimates in U.S populations living in buildings with different indoor–outdoor air exchange rates[1]. Recent advancement in small and low-cost ozone sensors makes personal monitoring or indoor monitoring more affordable and feasible. More accurate ozone monitoring can be used in future studies of ozone epidemiology and can also aid data-based personal prevention actions.

Second, outdoor ozone concentrations exhibit a substantial seasonal variation in most of the places. This adds challenges to assess the health effect of long-term exposure in epidemiological studies. Unlike using annual averages for other pollutants such as $PM_{2.5}$, warm-season averages have often been used (14, 15), assuming that the health risk associated with lower-level ozone in cold months is negligible. Accordingly, certain ozone control policies have been implemented only during photochemical smog months. In the United States, for example, gasoline is formulated with higher oxygen content (typically with increased fraction of ethanol) in warmer months to reduce VOC emissions that contribute to ozone formation. However, accumulating evidence suggests that there may not exist a threshold ozone concentration below which the risk is "zero." Therefore, completely ignoring cold months in ozone control strategy may need to be revisited.

Third, outdoor ozone typically exhibits a distinct diurnal pattern with high concentrations during afternoon and early evening hours. Hourly concentrations of ozone are usually reported at ambient monitoring stations. For regulatory purposes, these hourly data are computed as moving averages to identify maximum 1- and 8-h concentrations (based on moving averages) within a day. In epidemiological studies, using concentrations with different averaging times has different toxicological assumptions. Using 1-h max concentration is to assess the acute effect of peak exposure, whereas using 8-h daily max concentration assumes that lower concentrations during the rest of 16 h do not contribute to an adverse effect. It is also possible that either 1- or 8-h max concentrations were simply used due to the data availability in previous epidemiologic studies of short-term ozone effects (16–20). In addressing exposure to low-level concentrations (such as concentrations below the current air quality standard), however, 24-h average may be another relevant measure of daily exposure, at least for certain outcomes (21–24).

HEALTH EFFECTS EVIDENCE TO SUPPORT OZONE REGULATIONS

Following a formal process of an extensive literature review and a critical analysis, the US EPA summarized its evaluation of available evidence in the 2013 US EPA Integrated Science Assessment for Ozone[7]. Based on this assessment, the national ambient air quality standard for 8-h daily max ozone was revised from 75 to 70 ppb in 2015. The health effects evidence used to

[7] https://cfpub.epa.gov/ncea/isa/recordisplay.cfm?deid=247492

support these revisions include mainly the following, which has been well-demonstrated in recent reviews (25, 26).

- Ozone can cause adverse respiratory effects such as difficulty of breathing (e.g., shortness of breath and pain when taking a deep breath) and inflammation of the airways in the general population. These effects can aggravate lung diseases such as asthma, emphysema, and chronic bronchitis [chronic obstructive pulmonary disease (COPD)].
- Long-term exposure to ozone is likely to be one of many causes of asthma development.
- Ozone exposure is likely to cause premature deaths, and the evidence is stronger for mortality due to respiratory illnesses than for that due to other diseases.
- Children are at increased risk from ozone exposure, as children have a relatively higher dose per body mass and children's lung is still developing.

Does this revised standard imply that the effects listed above would not occur when 8-h daily max ozone concentrations are below 70 ppb? Although from a regulatory standard point, the public may be informed that it is "safe" to breathe the air when air quality meets the standards, it is easy to see that the standards are set somewhat arbitrary. For example, the WHO guideline for 8-h daily max ozone of 100 $\mu g/m^3$ ($\sim$50 ppb) is lower than the EPA standard, but the evidence to support this lower limit is similar to that in supporting a higher limit by the US EPA, with additional effects presented by the WHO as follows[1]:

- Ozone can cause coughing and sore or scratchy throat.
- Ozone exposure makes the lungs more susceptible to infection.
- Ozone continues to damage the lungs even when the symptoms have disappeared.

Although WHO also considers that ozone is a cause of COPD, this evidence was not strong enough in the 2013 EPA integrated science assessment. Other effects of ozone reported include the following. On high ozone days, there have been increased school absences, increased visits to emergency rooms, and increased hospital admissions (27–30). Long-term exposures to ozone have been associated with lower lung function and deteriorated or abnormal lung development in children (31, 32). In both the WHO guideline and the EPA ozone standard, more susceptible populations are considered. In addition to people with preexisting respiratory diseases such as asthma and COPD, children, older adults, and people who are active outdoors (especially outdoor workers) are more vulnerable to ozone exposure.

IMMUNE-INFLAMMATORY RESPONSES AND EMERGING EFFECTS

As a potent oxidizing gas, ambient ozone is well-known to cause oxidative damages to the cells and the lining fluids of the airways, thereby inducing immune-inflammatory responses in the lung. Recent findings have shown that innate immunity is implicated in ozone-induced airway inflammation, such as the involvement of innate lymphoid cells (ILCs) in mice (33, 34). Ozone exposure contributes to the increased expression of mRNA of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-lβ), interleukin-6 (IL-6), and interleukin-8 (IL-8) in human alveolar macrophages (35) and increased concentrations of IL-6, IL-8, and fibrinogenic proteins in human airway epithelial cells (36). A seminal work by Koren et al. demonstrated that an acute exposure to ozone (0.4 ppm for 2 h) resulted in 8.2-fold increase of polymorphonuclear leukocytes (PMN) in bronchial alveolar lavage (BAL) fluid and enhanced level of inflammatory mediators in the lower airways of humans (37). Krishna et al. further confirmed that ozone-induced neutrophil influx in human peripheral airways was partly mediated by IL-8 (38). Ozone exposure resulted in significant neutrophilic inflammation, reflected with increased levels of myeloperoxidase (MPO) in the supernatant of induced sputum samples from healthy subjects (39, 40).

These immune-inflammatory responses to ozone may "spill over" to the circulatory system, which may help explain emerging evidence on the cardiovascular and neuronal effects of ozone. Since the 2013 EPA assessment was released, several studies conducted in North America further confirmed significant positive associations, robust to controlling for co-pollutants, between short-term ozone exposure and one or more of the following mortality classifications: cardiovascular, dysrhythmia, cardiometabolic, and ischemic heart disease. A meta-analysis of 53 studies showed a weak but significant association between ozone and hospital admission and mortality from stroke (41). Significant associations of ozone were found with ischemic stroke occurrence in Seoul (42) and with non-myocardial infarction out-of-hospital cardiac arrests in Helsinki (43). Although Jerrett et al. (44) in the original analysis of an American Cancer Society cohort found that ozone exposure was associated with respiratory but not cardiovascular mortality, in the follow-up study using the same cohort, Turner et al. (14) found a significant association of long-term exposure to ozone with cardiovascular mortality. A recent cohort study by Lim et al. further confirmed this association between long-term exposure to ozone and increased cardiovascular mortality (45). However, there have also been epidemiological studies reporting null findings between long-term ozone exposure and cardiovascular mortality in Europe (46, 47).

Initiated in the lung, the immune-inflammatory responses to ozone may ultimately contribute to increased cardiovascular mortality and morbidity via two major pathways affecting hemostasis and autonomic tone. Increased exposure to ambient ozone has been associated with increased levels of hemostatic markers, including fibrinogen (48–50), von Willebrand factor (49), and plasminogen activator inhibitor-1 (48). Xia et al. revealed that short-term exposure to ambient ozone can elevate serum levels of ACE and ET-1, decrease their DNA methylation, and alter the lipid metabolism, which may be partly responsible for increased blood pressure and vascular endothelial disfunction (51). Day et al. found that an increase in 24-h or 2-week average exposure to ozone was associated with increased p-selectin (a soluble plasma marker of platelet activation), suggesting that ozone exposure increases the risk of thrombosis (21). Wang et al. found that increased ambient ozone exposure was associated with increased rate of carotid wall thickness progression and risk of

new plaque formation in healthy adults (52). Jia et al. showed that ambient ozone exposure within several minutes can decrease heart rate variability in the healthy elderly subjects, suggesting that a dysfunction of cardiac autonomic nervous system may be involved (53). In controlled human exposure studies, a few hours of ozone exposure resulted in changes in markers of inflammation and fibrinolysis at 300 ppb and changes in cardiac autonomic function at 110–300 ppb (54, 55). Although one study found a blunting of exercise-induced blood pressure increases (56) and another found increased systolic blood pressure in response to ozone exposure (57), other such studies found increases in diastolic blood pressure to a co-exposure of ozone and concentrated ambient PM but not to ozone alone (58, 59). In contrast, animal studies with high ozone exposures have generated more consistent findings on cardiovascular effects of ozone through altering vascular tone (60–62), mRNA for genes encoding thrombogenic factors (63), and atherogenesis (60).

Additionally, deleterious effects of ozone exposure on the central nervous system (CNS) are emerging (64, 65). Neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease, have been linked to ozone exposures in recent epidemiologic studies (66, 67). The following toxicological studies in rodents have demonstrated the CNS effects of ozone, shedding light on biological mechanisms to support the link between ozone exposure and outcomes related to CNS. Rodríguez et al. showed that ozone exposure resulted in the activation of apoptotic death in rat hippocampus mediated by endoplasmic reticulum stress (68). Bello-Medina et al. found that rats chronically exposed to ozone exhibited deficits in learning and memory loss associated with deafferentation in hippocampus-related neurons (69). Chronic exposure to low-dose ozone, on the other hand, could enhance systemic and hippocampal Th17/IL-17A immune responses, which may be partly responsible for neurodegenerative effects in rats (70).

DISCUSSION AND CONCLUSIONS

Ozone pollution is a worldwide health hazard. In many parts of the world, as described above, ozone concentrations are projected to increase, leading to increases in ozone-associated mortalities and morbidities. A study reported a 6% increase in premature deaths attributable to ozone globally from 1990 to 2010 (71), although the estimates (143,000 deaths in 1990 and 152,000 deaths in 2010) seem to be substantially lower than the estimated reported in other studies. In one study, for example, anthropogenic ozone was associated with an estimated 700,000 ± 300,000 respiratory mortalities in 2000 (72). In another study, exposure to ozone was responsible for 254,000 deaths from COPD alone in 2015 (73). Other ozone-associated mortality estimates include 316,000 respiratory deaths in China (15) and ∼23,500 in the European Union[8]. The relatively large gap in the estimates across studies is due to uncertainties associated with and inconsistence in concentration–effect relationship and ozone exposure assessment. These estimates were based on respiratory effects alone, due to uncertainties associated with the current evidence on the cardiovascular effects. It is expected that the impact would be larger when other ozone effects were considered.

Ozone exposure was associated with large morbidity estimates. An estimated 9–23 million (8–20% of total) asthma-related emergency room visits globally were attributable to ozone (74). A large multicity study in China showed that short-term exposure to ambient ozone was associated with higher non-accidental and cardiovascular mortality (20). In addition, an estimated 23.0–40.3 million respiratory-related deaths were attributable to long-term O_3 exposure in 2016 (15). In the European Union, ozone in 2010 was responsible for 19,200 cases of respiratory hospital admissions, 86,000 cases of cardiovascular hospital admissions, and over 109,000,000 minor restricted activity days[7]. Disability adjust life years (DALY) lost attributable to ozone were estimated to be 6.3 ± 3.0 million years in 2000 (58) and 4.1 (95% CI: 1·6–6·8) million years from COPD alone in 2015 (73).

Disease burden attributable to ozone is expected to continue to rise in the future for two reasons. The first is the fact that ozone concentration is on the rise in many parts of the world as described earlier. For example, an estimated increase of 0.43 ppb in average ozone concentration, during the 2040s compared to 2000 due to climate change alone, would correspond to a 0.01% increase in mortality rate in 19 urban communities in southeastern United States (9). Relative to that in 2000, there will be a 14% increase in global ozone-related mortality (75). The second is anticipated improvement in our understanding of the ozone effects beyond the lung and improved characterization of the chronic effects of long-term exposure. The improved knowledge will likely add to ozone-associated disease burden that is currently uncounted for.

There remain significant challenges in ascertaining chronic ozone exposure and effects that are currently inconclusive. Epidemiological evidence is limited to support a causal relationship between the chronic exposure and mortality or morbidity, although progresses have been made in recent years by using large datasets. For example, an analysis of national databases found a positive association between increase in long-term ozone concentrations and an increased risk of respiratory diseases and death (44). Di et al. (76) analyzed the entire US Medicare population of 60 million older adults from the years 2000 through 2012 and found a positive association between annual averages of ozone and all-cause mortality rate. Using large hospital records, increased chronic ozone exposure was associated with increased asthma hospital admissions in children (27). Long-term ozone exposure (3-year averages) has been associated with development of acute respiratory distress syndrome (ARDS) in at-risk critically ill patients, particularly in trauma patients and current smokers (77). By examining life expectancy at birth in 3,109 counties of the conterminous U.S. during 2002 to 2008 in relation to county-specific mean levels and rates of change in ozone concentrations, a study found that a 5 ppb (10 $\mu g/m^3$) increase in long-term ozone concentration was associated with 0.25 year (95% CI: −0.30 to −0.19) lower life expectancy in males and 0.21 year (95% CI: −0.25 to −0.17) in females (78).

[8]http://ec.europa.eu/environment/air/pdf/TSAP%20CBA.pdf

One of the challenges is to determine what is the best measure for long-term ozone exposure, given that ozone has distinct diurnal, and seasonal variations. A relevant question is whether repeated episodes of short-term high-level exposures can result in lasting health effects beyond the observed acute effects. Answering this question is not easy as some of the acute ozone effects are known to be reversible. What remains unknown is how much of the acute effects can be repaired or reversed between the episodes. At the meantime, people are constantly exposed to other air pollutants such as $PM_{2.5}$. The co-exposure may affect the ability to repair the damage caused by acute ozone exposures. The natural fluctuation in ambient ozone concentration hence makes it challenging to examine chronic effects of short-term and long-term exposures. However, it is imperative to address such challenges in future studies of novel study design incorporating promising technologies in monitoring and computing ozone exposures with unprecedented accuracy and precision.

The large disease burden resulting from ozone pollution warrants persistent calls for control polices worldwide, but this is more urgent in developing countries where most of the attention is paid toward $PM_{2.5}$ reductions. Based on the history of ozone control in the United States, aggregative regulatory actions to cut down anthropogenic emissions of NO_x and VOCs have not necessarily resulted in sufficient reductions in ozone concentrations in certain areas of the United States. Part of the challenges is the "non-linear" relationship of ozone production with its precursors, as reducing one of the precursors may not necessarily lead to ozone reduction. Even within a metropolitan area, the optimal ratio of NO_x to VOCs associated with minimal ozone formation changes from day to day (even hour to hour) and from upwind to downwind. Sources of VOCs can be numerous and hard to characterize. Some known sources, such as household use of consumer chemicals and biogenic emissions, are difficult to control through regulatory actions. Although the fundamental chemistry of ozone formation is clear, ozone concentrations, and spatiotemporal distributions are specific to local meteorological conditions, local sources of NO_x and VOCs, and long-range transport of ozone and associated chemical species. For all these challenges, ozone pollution in developing countries is expected to be a long-term problem, and local and national polices should be developed or strengthened to persistently combat ozone pollution.

In the United States and developed countries with relatively better air quality, following decades of controls for NO_x and VOCs emissions, further controls of anthropogenic emissions via policy, and technological tools are becoming increasingly limited. Meanwhile, predictions of ozone levels in response to changing NO_x and VOC concentrations get harder with ozone concentrations approaching their "background" level. Yet, emerging evidence does not support a threshold for adverse effects of ozone (79, 80). Or if a threshold exists, it would have to be substantially lower than the current health-based regulatory standards or guidelines.

Considering all these challenges, it is imperative to use other means to reduce the health impact of ozone. During high ozone hours, the public, especially children and those with preexisting health problems, is advised to avoid outdoor activities. Schools may be advised to cancel outdoor sports activities. Because indoor ozone levels are a small fraction of outdoor levels in airtight buildings with door/windows closed, this strategy can effectively reduce individuals' exposure to ozone. To further reduce outdoor exposure, individuals may consider wearing a face mask that can effectively scavenge ozone. Face masks rated N95 or higher can filter out $PM_{2.5}$ effectively and are widely available in the market worldwide. However, few models of face masks are designed to remove ozone. Making ozone forecasting available to the general public will enhance the effectiveness of such personal protection methods to reduce ozone exposure (81).

A wealth of data from animal studies and human studies are available in the literature to help understand pathophysiologic mechanisms by which ozone affects the lung. Relatively less is known to understand how ozone affects the cardiovascular health outcomes, although the immune-inflammatory responses initiated in the lung are thought to be the key in more downstream systemic effects. The mechanistic understanding appears to be sufficient to support the use of antioxidants or ozone scavenger to alleviate the ozone effects. For example, rodent studies confirmed that the use of N-acetylcysteine and sulfide salt can help prevent or recover the lung impairment caused by ozone (82, 83). Limited studies in humans have shown promising results. A randomized trial found that a daily supplement of vitamins C and E might provide some protection against acute nasal inflammatory response to ozone in asthmatic children (84). In a control human exposure study (2-h exposure to 400 ppb ozone vs. filtered air), healthy adults who had received a dietary antioxidant supplementation of a mixture of vitamin C, alpha-tocopherol, and vegetable cocktail exhibited a significantly smaller ozone-induced reduction in pulmonary function (85). Cohort and population-based interventional trials should be conducted in real-world settings to develop more targeted preventive or therapeutic strategies especially in vulnerable populations and individuals. This should be part of the overall strategy, along with air pollution control polices, to combat ozone pollution, a lasting worldwide health hazard.

AUTHOR CONTRIBUTIONS

JZ and YW: conception. JZ, YW, and ZF: drafting the manuscript, editing and revising the manuscript. All authors gave final approval for publishing.

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REVIEW
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The Metabolic Response to Ozone

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The respiratory effects of O_3 are well established. High ambient O_3 concentrations are associated with respiratory symptoms, declines in pulmonary function, asthma exacerbations, and even mortality. The metabolic effects of O_3 are less well appreciated. Here we review data indicating that O_3 exposure leads to glucose intolerance and hyperlipidemia, characteristics of the metabolic syndrome. We also review the role of stress hormones in these events. We describe how the metabolic effects of O_3, including effects within the lungs, are exacerbated in the setting of the metabolic derangements of obesity and we discuss epidemiological data indicating an association between ambient O_3 exposure and diabetes. We conclude by describing the role of the gut microbiome in the regulation of metabolism and by discussing data indicating a link between the gut microbiome and pulmonary responses to O_3.

Keywords: obesity, metabolome, microbiome, fatty acids, hyperglycemia

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INTRODUCTION

Ozone (O_3) is an air pollutant produced by exposure of automobile exhaust to sunlight. The respiratory effects of O_3 are well established. O_3 causes peroxidation of lipids in the nasal and airway lining liquid and epithelial cell membranes, leading to epithelial cell damage and subsequent sterile inflammation (1, 2). The inflammatory response to ozone includes production of inflammatory cytokines and chemokines as well as activation of innate lymphoid cells type 2 and subsequent release of type 2 cytokines (3–5). The details of these events vary with the concentration of O_3 and with the chronicity of exposure and the reader is referred to other reviews (1, 2) for an in depth description of these events. The net effect is that O_3 causes respiratory symptoms including cough, shortness of breath, and wheezing, as well as declines in pulmonary function. O_3 also increases the risk of pulmonary infections, of asthma exacerbations, and even of mortality, the latter mostly in patients with pre-existing cardiorespiratory conditions (6–10). What is less well-appreciated is that O_3 also has pronounced metabolic effects. Here we review data indicating that O_3 exposure impacts the function of the primary organs regulating metabolism leading to glucose intolerance and hyperlipidemia, characteristics of the metabolic syndrome. We describe how the effects of O_3, including effects on the lungs, are exacerbated in the setting of the metabolic derangements of obesity. We conclude by describing the next frontier. The gut microbiome is a key player in the regulation of metabolism. There is increasing evidence of a role for the microbiome in pulmonary responses to O_3. Whether the microbiome also contributes to the metabolic responses to O_3 remains to be established.

ACUTE EXPOSURE TO O_3 DECREASES THE METABOLIC RATE IN RODENTS

Almost four decades ago, Clemons and Garcia reported that acute O_3 exposure reduces plasma concentrations of thyroid hormones (11). Consistent with the role of thyroid hormones in setting

the metabolic rate, acute O_3 exposure also reduces core body temperature, heart rate, activity level, food consumption, and minute ventilation (12–16). These changes are proportional to the O_3 concentration administered and wane over time with repeated exposure. The reduction in minute ventilation that accompanies the reduction in metabolic rate would be expected to reduce the inhaled dose of O_3 and has consequently been viewed as protective against the toxic effects of O_3. Indeed, conditions that increase thyroid hormones, and thus increase the metabolic rate, including reductions in the ambient temperature and exogenous administration of thyroid hormones, and conditions that increase the metabolic rate, such as immaturity, increase the pulmonary inflammation and injury induced by acute O_3 exposure (14, 15, 17).

The torpor-like state described above is similar to what is observed in rodents during acute fasting (18), and acute O_3 exposure also has metabolic consequences similar to those observed during fasting: the adipose tissue initiates lipolysis mobilizing fatty acids that provide a source of energy, and the liver alters its handling of glucose. A metabolomic analysis of serum harvested from rats exposed to air or to O_3 (1 ppm) indicates that short and long-chain free fatty acids (FFAs) are uniformly elevated after O_3 exposure (19). Gene expression analysis on the livers from these rats indicated that O_3 exposure alters many genes involved in fatty acid metabolism and insulin signaling. Last et al. (20) also reported changes in genes related to lipid and fatty acid metabolism and to carbohydrate metabolism in livers from air vs. O_3 exposed mice. In addition, glucose tolerance tests performed on rats immediately after exposure to O_3 indicate hyperglycemia and impaired glucose clearance (19, 21, 22). Similarly, serum 1,5-anhydroglucitol, which is inversely related to long-term glucose control, is decreased in O_3-exposed rats (19). The effects of O_3 are concentration dependent: little effect is observed at 0.25 ppm, glucose intolerance is observed after 0.5 ppm, and both fasting hyperglycemia and glucose intolerance are observed after 1 ppm exposure (23). The latter concentration is higher than would be experienced by humans even in the most polluted of cities, but there are differences in O_3 dosimetry between rodents and humans (24). Importantly, glucose intolerance is also observed when rats are exposed to lower concentrations of O_3 for more extended periods of time (25). Serum insulin is also elevated after O_3 suggesting insulin resistance rather than impaired insulin release and experiments using euglycemic clamps verify insulin resistance (22). Acute O_3 exposure also causes reduced insulin sensitivity in liver and skeletal muscle but not adipose tissue harvested from O_3-exposed rats, as assessed by phosphorylation of AKT (21, 22).

There are sex differences in the metabolic response to acute O_3 exposure. Gordon et al. (26) reported that male rats developed the same fasting hyperglycemia and glucose intolerance after acute O_3 as described above, whereas females that were littermates of these males did not. In addition, although glucose tolerance tests performed after O_3 exposure indicated some glucose intolerance in females, the effect was much smaller than was observed in males. Interestingly, markers of O_3-induced pulmonary injury and inflammation were also lower in the female than male rats suggesting a link between the metabolic and inflammatory responses to O_3.

EVIDENCE OF METABOLIC EFFECTS OF O_3 IN HUMANS

The metabolic effects of acute O_3 exposure are not restricted to rodents. Miller et al. (27) performed a metabolomic analysis of serum collected 1 h after exposure of human subjects to filtered air or to O_3 (0.3 ppm) with a 15-min on-off exercise cycle. The results indicated O_3-induced increases in medium and long chain fatty acids and glycerol indicative of lipid mobilization from adipose tissue stores similar to what is observed in rats (19). Epidemiological studies also provide increasing evidence of an association between O_3 exposure and diabetes. For example, in a large study from Italy that included 376,157 individuals, the authors noted a positive association between average annual ambient O_3 concentrations and the risk of diabetes (28). A large (45,231 women) longitudinal analysis of African American women conducted over 16 years also indicated an association between ambient O_3 concentrations and the risk of incident diabetes (29). The association remained unaltered even after controlling for particulate air pollution, which is also associated with diabetes (30). No association between O_3 and hyperglycemia was observed in a study from the Framingham Heart cohort, even though associations were observed with $PM_{2.5}$ and NO_2, but the study was much smaller (5958 participants) and may not have been adequately powered to detect an association.

MECHANISTIC BASIS FOR THE METABOLIC EFFECTS OF O_3

Stress hormones likely account for the metabolic effects of O_3 (**Figure 1**). In rodents, serum corticosterone levels increase immediately following acute O_3 exposure (19, 31). A similar increase in cortisol is observed in human subjects after acute O_3 exposure (27). Serum concentrations of epinephrine are also increased following O_3 (19, 21, 31) and remain elevated even 18 h after cessation of exposure (21). These changes in stress hormones are thought to arise from O_3-induced stimulation of sensory afferents within the lungs and nose (32). These afferents have been shown to terminate in stress responsive regions of the brain (33). Importantly, the hyperglycemia and impaired glucose clearance observed after acute O_3 exposure are virtually abolished in rats in which either the adrenal medulla or the entire adrenal gland is surgically removed bilaterally (31). Acute O_3-induced increases in serum lipids are also ablated by removal of either the adrenal medulla or the whole adrenal gland. The data are consistent with the known effects of epinephrine and cortisol in promoting gluconeogenesis, insulin resistance, and lipolysis in the liver and adipose tissue during fasting.

It is has been reported that adrenalectomy and drugs that block either beta adrenergic receptors or glucocorticoid signaling attenuate the pulmonary inflammation and injury that occur with O_3 exposure (31, 34, 35). The genes whose expression in the lungs are impacted by O_3 are similar to

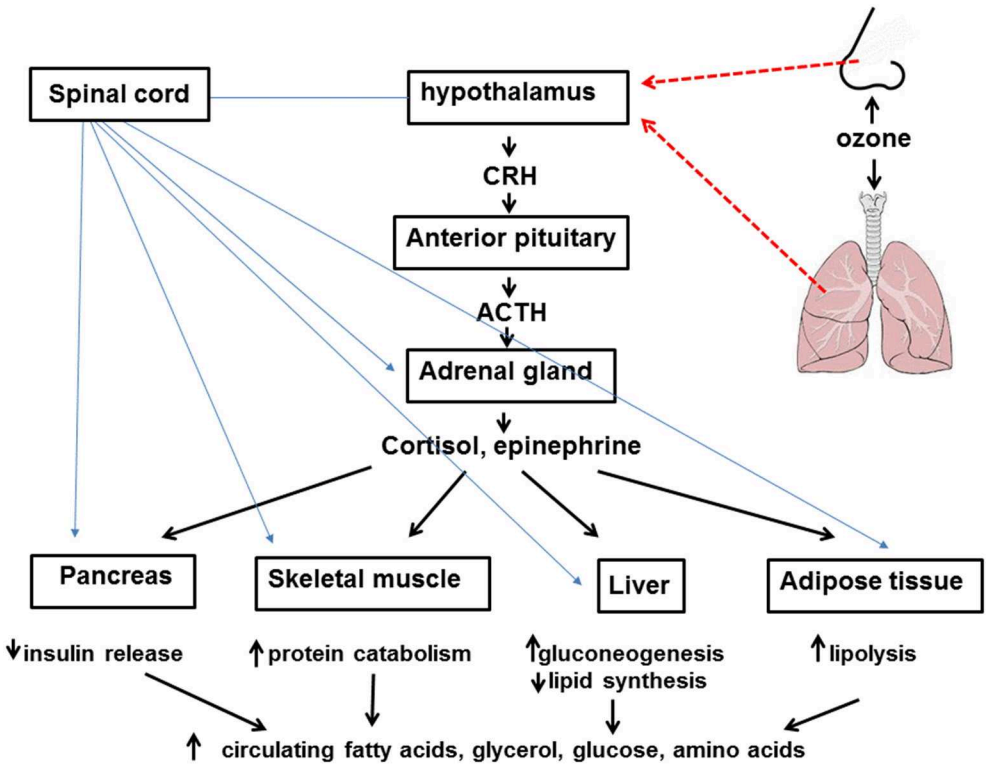

FIGURE 1 | Inhaled ozone stimulates sensory afferents in the nose and lungs (hatched red lines) ultimately leading to activation of the hypothalamus and activation of the sympathetic nervous system as well as release of corticotropin releasing hormone (CRH). CRH leads to release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which acts on the adrenal cortex to cause release of cortisol. Activation of the sympathetic nervous system (blue lines) leads to release of epinephrine from the adrenal medulla. Cortisol, epinephrine, and the sympathetic nervous system lead to attenuated insulin release from the pancreas, increased protein catabolism within skeletal muscle, reduced lipid synthesis and increased gluconeogenesis in the liver, and lipolysis within adipose tissue. The net effect of these events is increased circulating levels of fatty acids, glycerol, glucose, and amino acids.

the genes whose expression changes with glucocorticoids or with agents that, like epinephrine, induce cAMP activation (34). Furthermore, adrenalectomy substantially reduces O_3-induced changes in gene expression within the lungs (34). O_3 exposure causes activation of NF-κB in the lungs and subsequent induction of a variety of inflammatory cytokines and chemokines that contribute to neutrophil recruitment (2, 36) and these events are inhibited by exogenous administration of dexamethasone (37, 38). Therefore, it is unlikely that the effects of adrenalectomy on O_3-induced changes in gene expression (34) are the result of loss of the effects of stress hormones on activation of inflammatory genes by O_3. Instead, the observed reductions in O_3-induced neutrophil recruitment that occur after adrenalectomy or after inhibition of endogenous stress hormones (31, 34, 35) may be the result of inhibition of the effects of stress hormones on metabolic pathways. Inhibition of O_3-induced NF-κB by corticosteroids occurs at higher concentrations of these steroids than are typically released endogenously following O_3. In this context, it is interesting to note that fatty acids that are released in a stress-hormone dependent manner following O_3 (31) should have the capacity activate neutrophils via the fatty acid receptor, FFAR1 (39).

Other events may also contribute to the metabolic effects of O_3. O_3 causes an inflammatory response in the lung characterized by release of acute phase cytokine and cytokines and increases in BAL neutrophils and macrophages (5). Lung specific overexpression of a constitutively active inhibitor of κB kinase (IKK2) not only causes a similar inflammatory response in the lungs, but also induces insulin resistance, perhaps by inducing systemic and adipose tissue inflammation (40), which are thought to mediate the insulin resistance associated with obesity (41). However, in mice, inhalation of another air pollutant, $PM_{2.5}$, also causes inflammation with adipose tissue and liver, and leads to insulin resistance but the insulin resistance is not attenuated when the hepatic and adipose tissue inflammation are ameliorated by genetic deficiency in CCR2, the receptor for the macrophage chemotactic cytokines, CCL2 (42).

EFFECTS OF O_3 IN ANIMALS WITH METABOLIC SYNDROME

Given the effects of acute O_3 exposure on lipid and carbohydrate metabolism, it is interesting to consider differences in the response to O_3 under circumstances in which lipid and

carbohydrate metabolism are already compromised: metabolic syndrome and obesity. In Goto-Kakizaki rats, a model of non-obese type 2 diabetes, exposure to approximately 0.4 ppm O_3 for 4 h results in decreased LDL cholesterol and neither hyperglycemia nor glucose intolerance (43). Unfortunately, no normal rats were included in the study and the concentration used was lower than the 0.5–1.0 ppm concentrations that evoked hyperlipidemia, hyperglycemia, and glucose intolerance in normal rats (19, 21–23), so it is difficult to determine whether the differences are the result of the diabetic state or the nature of the exposure. In contrast, chronic O_3 exposure does appear to affect glucose metabolism in insulin-resistant, diabetes-prone KK mice (44). When these mice are repeatedly exposed to O_3 (0.5 ppm, 4 h a day for 13 days), the mice develop even greater insulin resistance. There is marked fasting hyperglycemia even in air-exposed KK mice and O_3 does not cause any further increases in baseline glucose but does decrease fasting insulin, suggesting impaired insulin release. Injection of insulin gradually reduces blood glucose in air-exposed KK mice, but after O_3 exposure, no such reduction is observed. Increased insulin resistance is also observed in rats and mice with diet-induced obesity as well as in normal weight mice after chronic pulmonary exposure to another air pollutant, $PM_{2.5}$ (45–47). Adipose tissue inflammation and systemic inflammation are typically observed in obese mice, and repeated exposure to O_3 exacerbates this inflammation (44): the number of activated macrophages within adipose tissue and the number of circulating inflammatory monocytes are both elevated in O_3- vs. air-exposed KK mice. Expression of adipose tissue inflammatory genes linked to insulin resistance, including TNFα, is also elevated in O_3 vs. air exposed KK mice. Pulmonary exposure to another type of air pollution, silicon dioxide nanoparticles, also augments mRNA expression of pro-inflammatory genes within adipose tissue (48). The effects of repeated O_3 exposure on adipose tissue inflammation and insulin release but not insulin sensitivity were also observed in another model of obese type 2 diabetes, KKAy mice (49). The mechanistic basis for the effects of exposure to air pollution on adipose tissue gene expression, including inflammatory gene expression are not well-understood, but it is conceivable that changes in the gut microbiome may contribute (see below).

We have established that the pulmonary inflammation and injury induced by acute O_3 exposure are also increased in obese mice. This effect of obesity was observed in *ob/ob* and *db/db* mice which are obese because of a genetically deficiency in leptin or the leptin receptor, in mice obese because of a genetic deficiency in carboxypeptidase E (Cpe), an enzyme involved in processing neuropeptides related to eating behavior, and in mice diet-induced obesity (5, 50–54). These mice are also diabetic to varying degrees. There are marked effects of obesity on the serum and urinary metabolomes of humans, rats, and mice including changes in carbohydrate, lipid, and branched chain amino acid (BCAA) metabolism (55–57). Lungs of naive obese mice also exhibit metabolic changes, including changes in lipid, phospholipid, and cholesterol metabolism (58). As described above, O_3 has substantive metabolic effects that may be linked to effects of O_3 on the lung. To determine whether O_3 also affects metabolic processes within the lungs and whether these effects of O_3 were modified by obesity, we performed a metabolomic analysis of lung tissue from *db/db* and wildtype (WT) female mice exposed acutely to air or O_3 (54). Our results indicated substantial differences in the lung metabolomes of air-exposed *db/db* and WT mice including increases in lipids and lung carbohydrates. It is possible that increases in these substances in the lungs are due to corresponding increases in the blood (57) and subsequent diffusion into the lung extracellular fluid. Acute O_3 exposure also affected the lung metabolome and there were differential effects of O_3 in *db/db* and WT mice. For example, we observed effects of O_3 on the substrates used for energy production in the lungs and these effects differed in *db/db* and WT mice. In WT mice, O_3 exposure reduced BCAA metabolites consistent with increased reliance upon BCAA catabolism for energy, but no such effect was observed in *db/db* mice. Instead, in *db/db* mice, O_3 resulted in decreased long chain acylcarnitines consistent with increased reliance upon β-oxidation for energy after O_3 exposure. Changes in lung lipids are also observed in monkeys after chronic exposure to lower concentrations of O_3 (59, 60).

As discussed above, O_3-induced increases in stress hormones appear to mediate the hyperglycemia and hyperlipidemia that occur with acute O_3 exposure. Corticosteroids also promote β-oxidation (61) and attenuate BCAA catabolism (62), similar to the effects of O_3 in *db/db* mice. In our metabolomic analysis, lung corticosterone was greater in O_3- than air-exposed mice, presumably as a result of increases in serum corticosterone, but the effect of O_3 on corticosterone was only significant in *db/db* mice (54). Thus, greater O_3-induced increases in corticosterone in *db/db* than WT mice might account for the different effects of O_3 on lung β-oxidation and BCAA metabolism observed in *db/db* vs. WT mice.

O_3 AND THE MICROBIOME: THE NEXT FRONTIER

Data from animal models indicate that the gut microbiome contributes to variety of metabolic conditions including insulin resistance and also affects metabolic processes within the liver (63–68). For example, treatment with oral antibiotics attenuates both the glucose intolerance and the adipose tissue inflammation observed in obese mice (65). Germ free mice consuming a Western style diet are protected against the development of obesity and have changes in skeletal muscle and liver that promote fatty acid metabolism (64). One way that gut microbiota regulate metabolism is via the production of metabolites that can impact their host. For example, gut microbiota modify bile acids which signal in the intestines and liver to regulate lipid metabolism (68). Hence, it is possible that the gut microbiome also contributes to the changes in metabolism as well as to the changes in hepatic gene transcription observed following acute O_3 exposure.

Data from our lab also indicate a role for the microbiome in the metabolic changes observed in the lungs after O_3 exposure. Among the lung metabolites identified in the metabolomic

profiling experiment described above were several that require bacteria for their generation in mammals (54). Notably, each of these bacterial-mammalian co-metabolites was affected by obesity, by O_3 exposure, or by the combination of obesity and O_3 exposure. It is perhaps not surprising that obesity affects metabolites of bacterial origin. The community structure of the gut microbiome is altered by obesity both in rodents and in humans [see (69) for review] and there are differences in the metabolomic profile of tissues and blood harvested from germ free vs. conventionally housed mice and from antibiotic-treated vs. control mice (70–72). Thus, gut bacteria-derived metabolites can enter the blood and most are small enough to diffuse from the blood into the lungs. It is more surprising that O_3 also affects these metabolites. One potential explanation is the effects of O_3 on the liver (19, 20), since generation of many of the bacterial-mammalian co-metabolites identified in the lungs requires a metabolic step that occurs in the liver. However, it is also conceivable that O_3 alters either the gut or the lung microbiome. O_3 also affects the nose (3), and O_3-induced changes to the nasal microbiome could also contribute to responses to O_3 by altering metabolites that stimulate nasal afferents and contribute to activation of the HPA axis (**Figure 1**).

Our data indicate that bacteria also contribute to pulmonary responses to acute O_3 exposure (73). O_3-induced airway hyperresponsiveness and O_3-induced neutrophil recruitment are reduced in male C57BL/6 mice treated with antibiotics, as well as in germ free mice. Since these changes are observed both with antibiotics that can cross the intestines and enter the blood and with antibiotics that cannot, the data suggest that the origin of the bacteria involved in these events is the gut and not the lungs. Gut bacteria generate short chain fatty acids (SCFAs) from dietary fiber and our data suggest a role for SCFAs in the effects of the microbiome on responses to O_3. We observed reductions in serum SCFAs only in mice treated with those antibiotics that attenuated responses to O_3. Furthermore, exogenous administration of SCFAs via the drinking water and diets high in fermentable fiber that increased serum SCFAs also augmented responses to O_3 (73). Together, our data support a role for the gut microbiome in pulmonary responses to O_3. Whether the gut microbiome also contributes to the metabolic changes observed after O_3 exposure and whether O_3 itself has the capacity to alter the gut microbiome remains to be established.

SUMMARY

In rodents, acute O_3 exposure causes endocrine and metabolic changes similar to those observed during fasting: stress hormones are released and act on the liver, adipose tissue, and skeletal muscle countering the effects of insulin and promoting lipolysis, thus providing a ready source of energy. However, O_3 also lowers the metabolic rate, reducing the need for energy. The net effects of these changes are hyperglycemia and hyperlipidemia, characteristics of the metabolic syndrome. Similar, albeit attenuated effects are observed in rodents after repeated exposures at lower concentrations of O_3, an exposure paradigm that perhaps better reflects human exposures to ambient O_3. Humans do not experience the torpor-like state that characterizes rodents exposed to O_3, but hyperlipidemia is also observed after acute exposure of human subjects to O_3 and there is an increasing body of epidemiological data indicating an association between O_3 exposure and diabetes. Indeed, in certain types of obese diabetic rodents, O_3 exacerbates their already compromised insulin sensitivity and also induces adipose tissue and systemic inflammation, other characteristics of the metabolic syndrome. O_3 also differentially affects both energy metabolism and inflammation within the lungs of obese diabetic vs. normal lean mice. Better understanding of the mechanistic basis for the effects of O_3 on the liver and adipose tissue is needed to protect populations already at risk of metabolic disease.

There is increasing evidence that the gut microbiome contributes to energy regulation. It remains to be established whether the gut microbiome also contributes to the derangements in energy regulation that occur after O_3 exposure, but there is evidence of a link between the gut microbiome and pulmonary responses to O_3. Better understanding of this link could result in strategies to prevent or mitigate the deleterious effects of O_3 not only on the lungs, but also on metabolic health.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Immunology

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Ozone-Induced Aryl Hydrocarbon Receptor Activation Controls Lung Inflammation via Interleukin-22 Modulation

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Airborne ozone exposure causes severe lung injury and inflammation. The aryl hydrocarbon Receptor (AhR) (1), activated in pollutant-induced inflammation, is critical for cytokine production, especially IL-22 and IL-17A. The role of AhR in ozone-induced lung inflammation is unknown. We report here that chronic ozone exposure activates AhR with increased tryptophan and lipoxin A4 production in mice. $AhR^{-/-}$ mice show increased lung inflammation, airway hyperresponsiveness, and tissue remodeling with an increased recruitment of IL-17A and IL-22-expressing cells in comparison to control mice. IL-17A- and IL-22-neutralizing antibodies attenuate lung inflammation in $AhR^{-/-}$ and control mice. Enhanced lung inflammation and recruitment of ILC3, ILC2, and T cells were observed after T cell-specific AhR depletion using the AhR^{CD4cre}-deficient mice. Together, the data demonstrate that ozone exposure activates AhR, which controls lung inflammation, airway hyperresponsiveness, and tissue remodeling via the reduction of IL-22 expression.

Keywords: ozone, AhR, ILC3, T cells, inflammation, IL-17, IL-22

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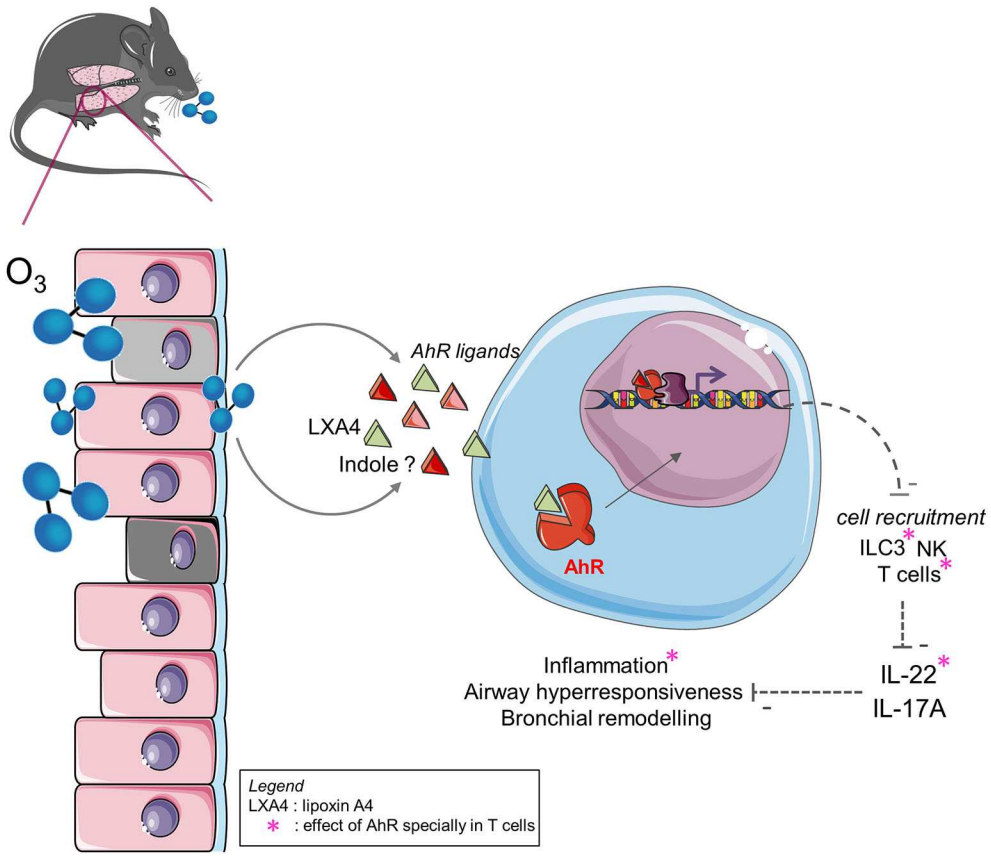
INTRODUCTION

Ozone is an abundant air pollutant that causes respiratory inflammation. Peaks of ozone correlate with severe respiratory disease, morbidity, mortality (2, 3), and hospital admissions (4–6). An increase of 10 $\mu g/m^3$ of ozone exposure for 1 h daily induces an increase of 0.26% in mortality rate (7). The consequences of ozone exposure are especially deleterious for vulnerable populations with asthma or COPD (8, 9) and exacerbate asthma (10, 11). Ozone causes severe lung tissue damage, with inflammation and emphysema, loss of lung function, and airway hyperresponsiveness in human and mice (12–14).

The aryl hydrocarbon receptor (AhR) (1) is broadly expressed in immune cells and non-hematopoietic cells (e.g., epithelial cells). AhR is implicated in pollutant metabolism and/or degradation in response to polycyclic aromatic hydrocarbons. At steady state, AhR is cytoplasmic. In the absence of ligands, AhR resides in the cytoplasm under the control of a chaperone protein complex. Upon ligand binding, the AhR complex translocates into the nucleus, the chaperones are



GRAPHICAL ABSTRACT | Ozone exposure induces tryptophan metabolites and LXA4 that activates AhR. This activation likely control cell recruitment, T_H2 and $T_H17/22$ response, airway hyperresponsiveness and tissue remodeling with IL-22 dependent manner. AhR activation in lymphocytes influences cells recruitment, IL-22 expression and inflammation.

released, and AhR heterodimerizes with AhR Nuclear Translocator (ARNT) (15). This complex binds to Dioxin Response Elements (14) on the DNA and induces gene transcription, including P450 cytochrome and cytokines (e.g., IL-17A, IL-22). Non-canonical AhR signaling pathways have also been reported, either at the genomic level, through association with other transcription factors (e.g., NF-κB), or at the non-genomic level (e.g., through the release of the Src kinase) (16, 17). AhR is known to mediate several aspects of immune response and homeostatic maintenance, and its action is dependent on the context. For example, AhR can interact with NF-κB and STAT1 to inhibit IL-6 production after LPS treatment (18). AhR activation affects different pathways, such as T cell differentiation and antioxidant response. Furthermore, AhR induces ILC3 maintenance in the gut via Cyp1a1 (19) and triggers T_H17 cells differentiation, promoting IL-17 and IL-22 production by inhibiting IFN-γ (20). The AhR activity is dependent on context, but also ligands. AhR ligands can be of endogenous (e.g., tryptophan metabolism derivatives from the microbiota) or of exogenous origin (e.g., Benzo[a]pyren, environmental pollutants, and dietary-derived compounds) (21). The response of AhR activation depends on the nature of the ligand. For example, AhR in PBMCs stimulated by TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine) induces IL-22 and IL-17A production, while stimulation by prostaglandin E2 (PGE2) has the opposite effect (22). AhR's effect on T cell polarization also depends on the nature of the ligand, as it can either promote T_H17 or Treg cell differentiation (23). Taken together, the literature reported that AhR could have a principally protective effect, for example during major depressive disorder, multiple sclerosis, rheumatoid arthritis (24), and intestinal disease (16).

IL-17A has a pro-inflammatory role in several models of inflammation, such as asthma or colitis (25, 26). IL-17A could be produced by T cells, iNKT, NK, γδ T cells, and ILC3 and could modulate the production of other pro-inflammatory cytokines (e.g., IL-6 and IL-8), chemokines, and molecules involved in tissue remodeling, such as MMPs (27). During acute ozone exposure, IL-17A is produced by γδ T cells and iNKT (28, 29) and induces neutrophil recruitment and airway hyperresponsiveness. Upon chronic ozone exposure, IL-17A

Abbreviations: AhR, Aryl hydrocarbon receptor; AhRR, AhR repressor; AREG, Amphiregulin; ARNT, Aryl hydrocarbon nuclear translocator; BAL, Broncho alveolar lavage; IDO, Indoleamine 2,3-Dioxygenase; ILC, Innate lymphoid cells; iNKT, invariant NK T cells; MPO, Myeloperoxidase; NK, Natural killer; PGE2, Prostaglandin E2; PBMC, Peripheral blood mononuclear cells; WT, Wild type (C57BL/6).

also induces inflammation, neutrophil recruitment, and airway hyperresponsiveness (30, 31). Furthermore, IL-17A induces M2 macrophage polarization and promotes apoptotic cell clearance (32). Similarly to IL-17A, IL-22 is produced by αβ T cells (T_H17, T_H22), γδ T cells, NK, and ILC3, after stimulation with IL-1β, TGF-β, or IL-23 and the transcription factor RORγt (23, 33, 34). IL-22 is a pro- or anti-inflammatory cytokine, depending on the inflammatory context. IL-22 plays a protective role when produced during epithelial or tissue damage, while IL-17A/IL-22 collaboration promotes IL-22 pro-inflammatory activity (35, 36).

In the current study, we identified an AhR ligand, the lipoxin A4, a tryptophan metabolite, which is produced after chronic exposure to ozone and induces AhR activation in the lung. AhR-deficient mice showed an increased lung inflammation and an increased cytokine production, including IL-17A and IL-22. Consistent with previous observations, we hypothesized that the protective effect of AhR is linked to the indirect repression of IL-17A and IL-22 by the reduction of cell recruitment of NK, T cells, and ILC3. Using T cell-specific AhR-deficient mice ($CD4^{cre}$ $AhR^{f/f}$), we showed that AhR present in T cells induces the indirect repression of IL-22 and IL-17A by mediation of the recruitment of T cells of IL-22^+ or IL-17^+ and ILC3 of IL-22^+ or IL-17^+. Using IL-$22^{-/-}$, IL-22Rα$^{-/-}$, and IL-22xIL-17Rα$^{-/-}$ mice, we demonstrate the pro-inflammatory role of IL-22 during chronic ozone-induced inflammation (**Graphical Abstract**). IL-22 blockade in $AhR^{-/-}$ mice restores WT mouse phenotype. Altogether, these findings suggest that AhR activation during ozone exposure may be beneficial for the host, and thus, AhR agonists or IL-22 blockade may represent potential therapeutic strategies in ozone-induced lung inflammation.

METHODS

Mice and Reagents

$AhR^{-/-}$ (from Frank Gonzalez) (37), $AhR^{flox/flox}$ (from Christopher Bradford), AhR^{CD4cre}, IL-$22^{-/-}$ (38), and IL-22×IL-17Rα$^{-/-}$ (38) mice and littermate controls of 7–9 weeks of age were used for the study. $IL22Ra1^{tm1a(EUCOMM)Wtsi}$ mice were obtained from the International Mouse Phenotyping Consortium via EMMA network. Generation of mice carrying the $Il22Ra1^{tm1c(KOMP)Wtsi}$ and $Il22Ra1^{tm1d(KOMP)Wtsi}$ alleles was achieved by breeding to StellaCre mice expressing Cre ($Dppa3^{tm1(cre)Peli}$) (39) and/or Flp (ROSA26Fki) recombinases. Colonies were maintained on a C57BL/6 genetic background.

All gene-deficient mice and WT (40) controls (C57BL/6J background) were bred and housed in our specific pathogen-free animal facility at Transgenose Institute (TAMM-CNRS, UPS 44 under agreement D-45-234-6, 2014, Orleans, France). In all experiments, 5–6 female mice per group were used, were maintained in a temperature controlled (23°C) facility with a strict 12 h light/dark cycles, and were given free access to food and water. Animal experiments were performed according to the French Institutional Committee under agreement CLE CCO 2015-1088.

Anti-IL-22 antibody (AM22.1, mouse anti-mouse, 14 μg/mouse, from Laure Dumoutier) and anti-IL-17A antibody (MM17AF3, mouse anti-mouse, 20 μg/mouse, from Jacques van Snick) were administered intranasally, once a week for 6 weeks.

Ozone Induced Airway Inflammation

Mice were exposed to ozone in a plexiglass chamber (*EMB 104, EMMS®*) at 1.5 ppm for 2 h, two times a week for 6 weeks. Ozone was created by an ozonisator (*Ozonisator Ozoniser S 500 mg, Sander®*) and a level of 1.5 ppm was controlled by a sensor (*ATI 2-wire transmitter, Analytical Technology®*). Mice were euthanized by progressive CO_2 inhalation, 24 h after last ozone exposure and BAL was collected. After a cardiac perfusion with ISOTON II (*acid-free balanced electrolyte solution Beckman Coulter, Krefeld, Germany*), lung was collected and sampled for analyses.

Broncho Alveolar Lavage (11)

After ozone exposure, BAL was performed by four lavages of lung with 500 μL of saline solution (NaCl 0.9%) each time, via a cannula introduced into mice trachea. BAL fluids were centrifuged at 300 g for 10 min at 4°C, the supernatants were stored at −20°C for ELISA analysis, and cell pellets were recovered to prepare cytospin (*Thermo Scientific, Waltham, USA*) on glass slides, followed by a staining with Diff-Quik solution (*Merz & Dade A.G., Dudingen, Switzerland*). Differential cell counts were performed with at least 400 cells.

DNA Measurement

DNA release was measured to evaluate the tissue damage in the BAL supernatant using the Quant-iT™ PicoGreen™ dsDNA assay kit (*ThermoFisher*), according to the manufacturer's instructions.

Measurement of Cytokines, Collagen, and Total Proteins

IL-22, IL-17A, MPO, and AREG in BALF were determined by ELISA (*R & D systems*, *Abingdon, UK*), while IL-4, IL-5 in lung, and TGF-β1 from BAL were measured by Luminex immunoassay using MagPix reader (Bio-Rad) according to the manufacturer's instructions. Data were analyzed with Bio-Plex Manager software (Bio-Rad). Total protein levels in BALF were analyzed with the Bio-Rad DC Protein Assay. Collagen was measured with Soluble Collagen Assay Sircol™ (*biocolor*), according to the manufacturer's instructions on BAL and lung supernatants. For the protein measurement on the lung supernatant, the same part of the lung was harvested and gridded in 1 mL of PBS with proteases inhibitor cocktail (Roche). After centrifugation, supernatant was collected and frozen.

AhR Ligand Determination

Lung supernatants were mixed with DPBS up to a total volume of 3 mL. SPE (solid-phase extraction) was then performed on Supelclean LC-8 SPE cartridges (*Supelco*) conditioned with methanol and water. The sample was loaded then washed with 3 mL of ultrapure water and dried for 15 min under vacuum (10 mmHg) before being eluted with 5 mL of an 80:20 dichloromethane:isopropyl alcohol mixture. The exudate was evaporated to dryness under a nitrogen flow and reconstituted in 200 μL of a 50:50 acetonitrile:water mixture.

LC-HRMS analyses were performed on a maXis Q-TOF mass spectrometer (*Bruker, Bremen, Germany*) coupled to an Ultimate 3000 RSLC system (*Dionex, Germering, Germany*). The column

TABLE 1 | Lung inflammation histological scoring.

Score	Cell infiltration
0	No cell infiltration
1	Moderate infiltration around vessels
2	Moderate infiltration around vessels and bronchi
3	High infiltration around vessels and bronchi

was a Kinetex C18 (150 × 2.1 mm) with a particle size of 1.7 μm (Phenomenex, Le Pecq, France), equipped with a C18 SecurityGuard Ultra (2.1 mm) guard filter (*Phenomenex*, Le Pecq, France). The mobile phase consisted of a gradient of water with 0.1% of formic acid (solvent A) and acetonitrile with 0.08% of formic acid (solvent B) as follows: 0–0.3 min 3% B, 0.3–10 min 3–90% B, 10–10.5 min 90% B, and finally, 10.5–10.6 min 90–3% B, maintained for 1.5 min before each new injection. The column was thermostated at 40°C, and the flow rate was 500 μL/min. The injection volume was 2.5 μL. The mass spectra were acquired with an ESI (electrospray Ionization) source in positive mode in the range of 50–1,650 *m/z* at a frequency of 1 Hz. The capillary voltage was set at 4.5 kV, the pressure of nebulizing gas was 2.0 bar, and the flow rate of drying gas was 9 L/min, heated at 200°C.

Histology

The left lung tissue was fixed in 4% buffered formaldehyde and paraffin embedded under standard conditions. Tissue sections (3 μm) were stained with Periodic acid Schiff (41). Histological changes were determined by a semi-quantitative severity score (0–3) for inflammatory cell infiltration (**Table 1**). The slides (one mouse per slide) were blindly examined by two independent investigators with a Nikon microscope (Nikon eclipse 80i, United States). All mice were scored (1 big and 3 small bronchi per mice).

FACS Analysis

Lungs were collected from mice, minced, digested with DNase (Sigma, 1 mg/mL) and Liberase (Roche, 5 mg/mL) during 1 h at 37°C, and red bloods cells were lysed with Lysing buffer (*BD Pharm Lyse*^{TM}, *BD Pharmingen*). Stimulation was performed with PMA (50 ng/ml, *Sigma*) and ionomycin (750 ng/ml, *Sigma*), for 2 h and 30 min. Cells were incubated with the antibodies for 25 min at 4°C in FACS buffer (PBS, 2% FCS, 2 mM EDTA). For intracellular staining, BD Cytofix/Cytoperm^{TM} was used according to the manufacturer's instructions. Cells were washed with FACS buffer and fixed by lysing buffer 1× (BD Pharmingen). The antibodies used were against mouse molecules and described in the **Table S1**. The gating strategy is presented in **Supplemental Figure 1** and was conducted as follows: epithelial cells (CD45$^-$EpCam$^+$), NK cells (CD45$^+$NKp46$^+$NK1.1$^+$), interstitial macrophages (CD45$^+$F4/80$^+$CD11c$^-$), alveolar macrophages (CD45$^+$F4/80$^+$CD11c$^+$), neutrophils (CD45+GR1+), CD4+ T cells (CD45$^+$CD3ε$^+$CD4$^+$), γδ T cells (CD45$^+$CD3ε$^+$TCRγδ$^+$), ILC2 (CD45$^+$lin$^-$ICOS$^+$ST2$^+$), and ILC3 (CD45$^+$lin$^-$RORγt$^+$CD127$^+$). The lineage staining used was composed of CD11b, CD3ε, CD45R/B220, Siglec-F, and FcεRIα. Data were acquired (∼2 million cells) with a flow cytometer (*BD FacsCanto II*) and analyzed with FlowJo software (*TreeStar, Mountain View, CA*).

Ex vivo Stimulation of Lung Cells

Lung cells were collected from mice as described above. Stimulation was performed on 10^6 cells with PMA (50 ng/ml, *Sigma*) and ionomycin (750 ng/ml, *Sigma*) for 24 h. After stimulation, supernatant was harvested and frozen at −20°C.

Immunofluorescence

Lungs were treated with paraformaldehyde (42) perfusion, and tissues were fixed for 3 days in PFA 4% and transferred in sucrose 20% for 2 weeks. Half of right lung was included in tissue-tek® O.C.T (*4583*, *tissue-tek®*) and 10 μm frozen tissue were sectioned by using a cryomicrotome (*Leica*) at −20°C. For immunofluorescence staining, slides were incubated 30 min in citrate buffer at 80°C. Slides were washed with PBS 1× after each step, followed by 45-min incubation in saturation medium (TBS 1×, 1% BSA, 10% SVF, 0.3% triton X100). The primary antibody, an anti-AhR (10 μg/mL, ab2769, Abcam), was incubated overnight at 4°C. After washing, slides were treated with pontamin (0.05%, Chicago sky Blue 6B, Sigma) for 15 min. After washing (TBS 1×), slides were incubated with a secondary antibody, a goat-anti-mouse (4 μg/mL, ab150113, *Abcam*) during 1 h at room temperature. After washing, slides were incubated with DAPI (1/5,000 dilution) for 10 min, washed, and mounted in mowiol® (*Sigma*). Lung sections were observed under a fluorescence microscope Leica (*Leica®*, CTR6000) at 400× magnification for microphotography.

Lung Function Determined by Invasive Plethysmography

Airway hyperresponsiveness was measured by using increasing concentrations of methacholine (25–200 mg/mL) using the FinePointe system (Buxco, DSI) as previously described (43).

AhR/Luciferase Reporter Assay

The H1L1.1c2 cell line was used as described before (16). Control (10% NaCl), supernatant of BAL and lung were used and incubated with the H1L1.1c2 cell line. AhR activity was calculated by subtracting the luminescence of the control (10% NaCl) from the luminescence obtained with the samples multiplied by the cytotoxicity value.

CD4+ T Cells Isolation

CD4+ T cell were isolated from the spleens of WT, AhR^{CD4cre}, and AhR$^{-/-}$ mice with Dynabeads^{TM} Untouched^{TM} Mouse CD4 Cells Kit (*ThermoFisher*). 6–7 × 10^6 CD4+ T cells were extracted from each spleen, and RNA was extracted as described below.

RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (*Sigma*) followed by a phenol/chloroform extraction. Total RNA (1 μg) was reverse-transcribed using GoScript^{TM} Reverse Transcription System (*Promega*). The mRNA levels for the genes of interest

were examined by quantitative RT-PCR using the GoTaq® qPCR Master Mix according to the manufacturer's protocol. The primer used were obtained from *Qiagen*: AhR (QT00174251), Cyp1a1 (QT00105756), and AhRR (QT00161693). Relative levels of mRNA expression were normalized to HPRT1 (QT00166768) mRNA levels using a comparative method ($2^{-\Delta\Delta Ct}$). Non-reverse-transcribed RNA samples and water were included as negative controls.

Statistical Analysis

Data were analyzed using Prism version 6 (*Graphpad Software, San Diego, USA*). The non-parametric Mann Whitney test or parametric one-way ANOVA tests with multiple Bonferroni's comparison tests were performed. Values are expressed as mean ± SEM. Statistical significance was defined at a *p*-value **** < 0.0001, *** < 0.001, ** < 0.01, and * < 0.05. Statistical differences between air and ozone mice in the same genotypes were represented by a star (or ns) just on the top of column of ozone mice, other comparisons are represented with bar between the groups compared.

RESULTS

Chronic Ozone Exposure Induces Tryptophan Metabolites Release and AhR Activation

First, we asked whether AhR is activated upon chronic respiratory ozone exposure. Thus, we investigated the expression of associated AhR genes after chronic ozone exposure (twice weekly to 1.5 ppm for 2 h for 6 weeks). We observed an increased mRNA expression of the AhR repressor (AhRR) and P450 cytochrome (Cyp1A1) in the lung homogenate (**Figure 1A**). Furthermore, an increased number of AhR^+ cells in the lung tissue by immunofluorescence (**Figure 1B**) and by flow cytometry analysis (**Figure 1C**, **Table S1**, **Supplemental Figures 1**, **2**) were observed.

Since ozone activates AhR expression, we performed functional tests on the BAL supernatant and lung homogenate from mice exposed to ozone using the H1L1.1c2 luciferase reporter cell line (16) and found an activation of the reporter cell line in the lung homogenate (**Figure 1D**). These data therefore demonstrate the presence of an AhR-activating moiety in lung supernatant of ozone-exposed mice.

Endogenous metabolites from the host and commensals are involved in AhR activation (15, 16, 22). To identify potential metabolites in our model, lungs from mice exposed to ozone were homogenized, and the supernatants were analyzed by LC-MS. Several known ligands of AhR were assessed (**Table S2**). Two peaks were observed, the first one corresponding to tryptophan, suggesting an increase of tryptophan metabolism and metabolites that activate AhR, and a second peak corresponding to the lipoxin A4 (LXA4), an anti-inflammatory molecule (44–46) (**Figure 1E**).

Therefore, ozone exposure induced AhR ligand production in the lung, and we identified LXA4 as a candidate activating AhR.

AhR Regulates Lung Inflammation, Injury, and Airway Hyperreactivity Upon Chronic Ozone Exposure

To investigate the role of AhR in ozone induced lung inflammation model, AhR-deficient mice ($AhR^{-/-}$) were exposed to ozone (1.5 ppm for 2 h over 6 weeks). Increased cell infiltration in the lung of $AhR^{-/-}$ mice was observed after ozone exposure in comparison to WT mice (**Figures 2A,B**). Indeed, in the BAL, an increased number of macrophages, neutrophils, and T cells were detected (**Figure 2A**) with augmented MPO, AREG, and collagen levels (**Supplemental Figure 3A**). More particularly, increased absolute numbers of interstitial macrophages, ILC2, ILC3, αβ T cells, γδ T cells, and NK were found in the lung of $AhR^{-/-}$ in comparison to WT mice after ozone exposure (**Figure 2B**, **Supplemental Figure 1**). Furthermore, the T_H2 cytokines and IL-4 and IL-5 levels were higher in the lung from AhR-deficient mice in comparison to WT mice (**Figure 2C**) with an increased recruitment of IL-5^+ ILC2 and/or IL-13^+ ILC2 (**Supplemental Figure 3B**).

Considering the known effect of ozone on airway hyperresponsiveness, we investigated whether chronic ozone exposure exacerbates airway hyperresponsiveness. Compared to air-exposed mice, ozone-exposed WT mice exhibit a significantly increased lung resistance (RI) in response to methacholine (**Figure 2D**), which is significantly increased in $AhR^{-/-}$ mice (**Figure 2D**). Since ozone disrupts the epithelial barrier (41, 47–49), we analyzed the desquamation of epithelial cells by microscopic assessment of the lung integrity. DNA release was used as a marker of cell death in the alveolar space, and TGF-β (50) and collagen deposition were used as indicators of repair with fibrosis in the lung. An increased number of epithelial cells, DNA, and TGF-β levels in BALF were observed (**Figure 2E**). Collagen was also augmented in the lung homogenate of WT mice, which was further increased in $AhR^{-/-}$ mice (**Figure 2E**). The microscopic investigations indicated a chronic inflammatory cell infiltration, which was enhanced in the lung of $AhR^{-/-}$ mice in comparison to WT mice (**Figure 2F**). Mucus-producing cells were increased after ozone exposure, but not different in absence of AhR (data not shown).

Taken together, these data show an increased cell recruitment, airway hyperreactivity, epithelial cell injury, inflammation, and fibrosis in AhR-deficient mice, suggesting that AhR regulates lung inflammation and epithelial damage after ozone exposure.

AhR Expressed by T Cells Is Critical to Control Ozone Induced Lung Inflammation

The effect of AhR on T cell differentiation and plasticity of T_H17 and Treg cells has been reported (23). To understand whether AhR solely expressed by T cells is sufficient to regulate ozone induced lung inflammation, AhR-deficient mice in T lymphocytes (AhR^{CD4cre}) were generated by crossing $AhR^{flox/flox}$ with $CD4^{cre}$ mice (WT^{CD4cre}), and T cell-specific AhR depletion was checked in these mice (**Supplemental Figure 4**).

Increases of ILC2, ILC3, and $CD4^+$ T cells were observed in the lung (**Figure 3B**) to same extent as in both AhR^{CD4cre} and the $AhR^{-/-}$ mice, in comparison to the $AhR^{flox/flox}$ control mice and

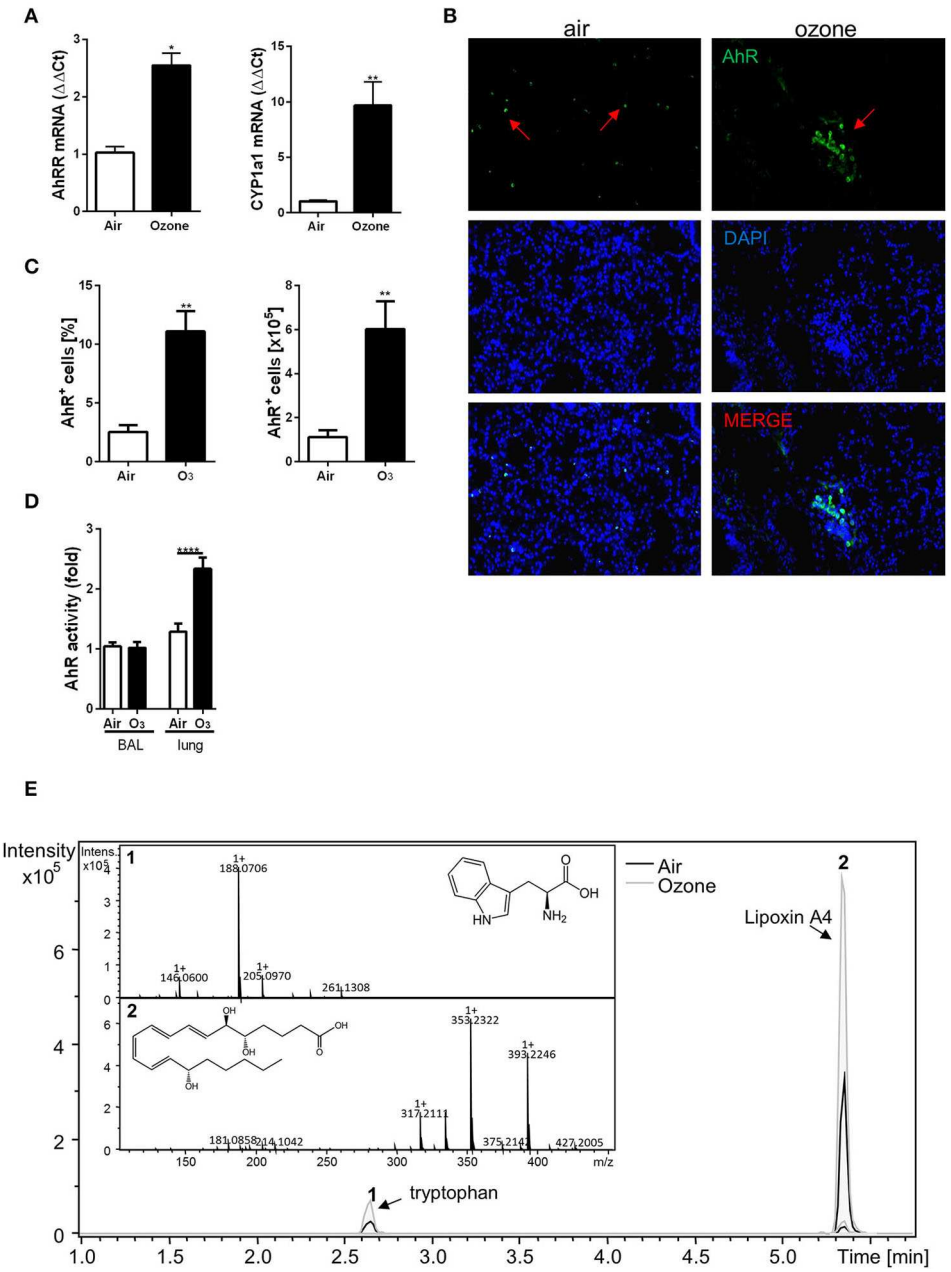


FIGURE 1 | Chronic ozone exposure induces AhR activation. Quantification of AhRR and Cyp1a1 involved in the AhR pathway activation **(A)**. AhR detection in lung, by immunofluorescence staining (magnification 400×; arrows represent AhR^+ cells) **(B)** and FACS **(C)**. AhR activity in the BAL and the lung after 6 weeks ozone exposure **(D)**. Relative abundance of tryptophan and Lipoxin A4 in lung supernatant analyzed by LC-MS **(E)**. The data are representative of one from two independent experiments with $n = 5$–6 mice per group. Values are expressed as mean ± SEM. Statistical significance was defined at a p-value **** < 0.0001, *** < 0.001, ** < 0.01, and * < 0.05.

WT mice, after ozone exposure, but not for BAL (**Figure 3A**). In contrast, interstitial macrophages and neutrophils were lower in the BAL in AhR CD4cre mice in comparison to $AhR^{-/-}$ mice, as well as ILC2 and γδ T cells in the lung (**Figures 3A,B**). Moreover with regards to T_H2 cytokine level, IL-4 from AhR^{CD4cre} mice was similar to WT mice, but not for IL-5 (**Figure 3C**).

In addition, the increased epithelial barrier damage observed in the AhR^{CD4cre} mice was similar to $AhR^{-/-}$ mice, as shown by increased DNA release in the BAL and the collagen deposition in the lung (**Figure 3D**). The epithelial cell desquamation level in the BAL was reduced in the AhR^{CD4cre} mice compared to the $AhR^{-/-}$ mice (**Figure 3D**). Microscopic observation revealed that inflammatory cell infiltration was comparable in $AhR^{-/-}$ and AhR^{CD4cre} mice (**Figures 3E,F**). To exclude any dysfunction of AhR by introducing loxP sites in the control $AhR^{flox/flox}$ mice, $AhR^{flox/flox}$ mice were compared with WT

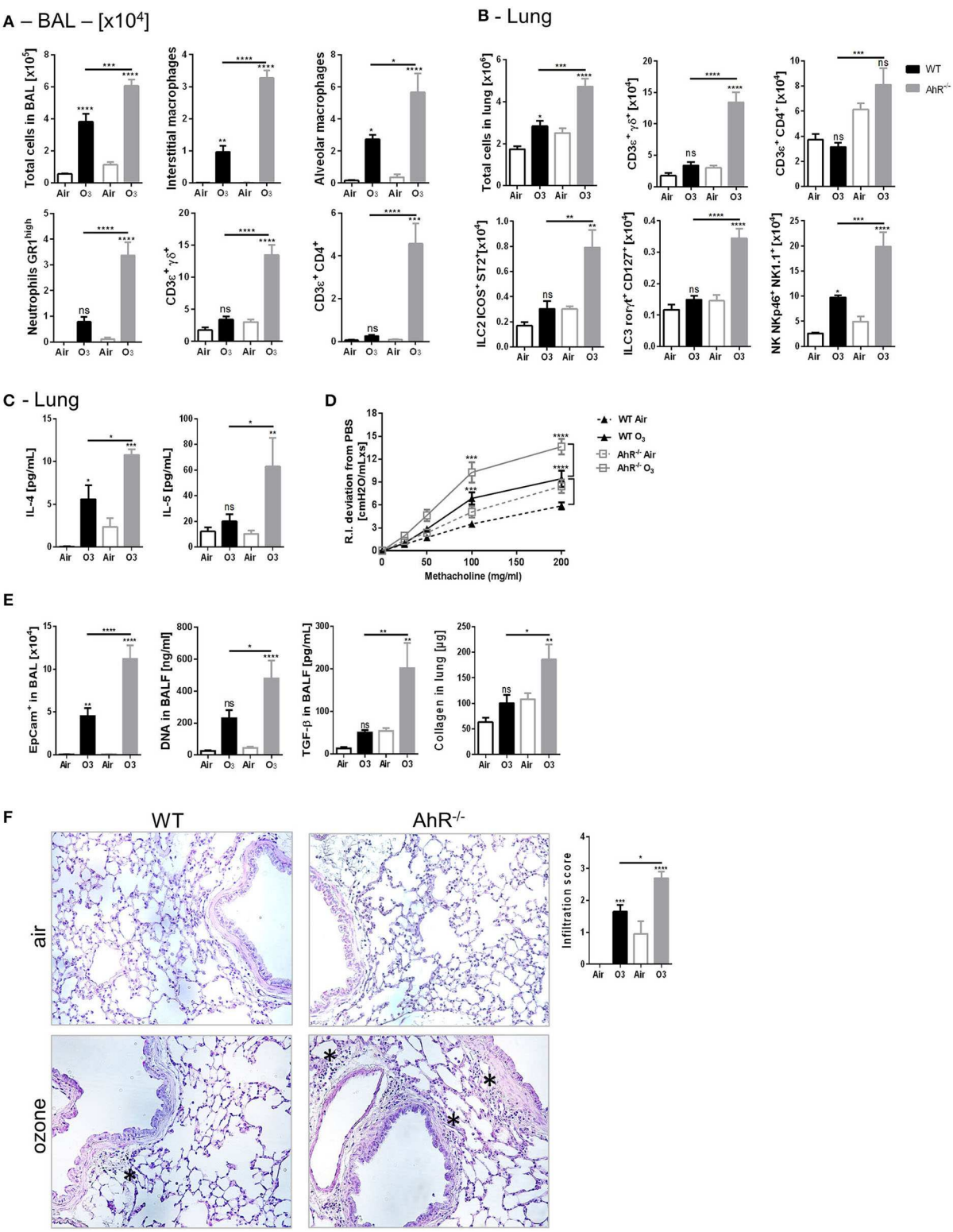


FIGURE 2 | Inflammation, AHR, and remodeling parameters in AhR deficient mice. BAL cells recruitment (total cells, macrophages, neutrophils, and T cells) **(A)**. Lung cells recruitment (total cells, neutrophils, macrophages, ILC2, ILC3, αβ, and γδ T cells) **(B)**, T_H2 cytokines (IL-4, IL-5) **(C)**, AHR **(D)**, and remodeling parameters (epithelial cells, DNA, and TGF-β in BAL; collagen in lung) **(E)**; lung histology representative pictures (400× magnification, asterisk show cell infiltration) and cell infiltration score **(F)** after ozone exposure in WT and AhR deficient mice. The data are representative for one of two independent experiments with $n = 5–6$ mice per group. Values are expressed as mean ± SEM.

mice (**Figures 3A–F**) and no relevant differences were observed between them, suggesting no impact from genetic modification.

Therefore, the data obtained from AhR^{CD4cre} mice suggest that AhR deficiency in T cells recapitulates partially the data from $AhR^{-/-}$ mice (notably in lung cells recruitment), and hence, AhR expressed in T cells is partially necessary to control ozone-induced inflammation in lung.

AhR Activation During Chronic Ozone Exposure Suppresses the Recruitment of IL-17A and IL-22 Producing Cells

AhR has a broad-spectrum of biological activity including on the production of IL-17A and IL-22 (22, 51–53). IL-17A is involved in the inflammatory process after acute (28, 29) and chronic ozone exposure (31). IL-22, in view of its protective role in the epithelial barrier, might be involved in ozone injury. Therefore, we assessed the role of AhR on IL-17A and IL-22 production during chronic ozone exposure.

First, the capacity of cells deficient for AhR to produce IL-17A and IL-22 was assessed. With *ex vivo* stimulation of isolated lung cells from WT or $AhR^{-/-}$ mice, enhanced IL-22 production was observed in WT mice, which is abrogated in $AhR^{-/-}$ mice, while the production IL-17A was increased (**Figure 4A**). These findings suggest that IL-22 and IL-17A may be differentially regulated by AhR. Moreover, increased levels of IL-17A and IL-22 were detected in the lung homogenate of the $AhR^{-/-}$ exposed mice in comparison to ozone exposed WT mice (**Figure 4B**). After 6 weeks of ozone exposure, IL-17A and IL-22 production was higher in the lung when AhR gene was missing, suggesting an impact of AhR on the recruitment of IL-22-producing cells. This increase was lost for IL-17A in $AhR^{flox/flox}$ and AhR^{CD4cre} mice, but conserved in AhR^{CD4cre} mice for IL-22 (**Figure 4B**).

To understand the mechanism involved in IL-22 and IL-17 increase, we identified the cell types expressing IL-17A and IL-22 in mononuclear cells isolated from the lungs of ozone-exposed mice. NK, ILC3, αβ, and γδ T cells expressed IL-17A or IL-22 or both IL-17A/IL-22 upon ozone exposure (**Figure 4C**). IL-17A expression was increased in NK cells, ILC3, αβ, and γδ T cells in $AhR^{-/-}$ mice, in comparison to WT mice, and an increased IL-22 expression was observed in T cells and ILC3 (**Figure 4C**). In addition, a lower expression of IL-22 in T cells and a slight decrease of IL-17A expression in NK and γδ T cells in AhR^{CD4cre} mice were observed in comparison to $AhR^{-/-}$ mice (**Figure 4C**). For double positive cells for IL-17A and IL-22, we observed an increase of NK, γδ, and αβ T cells in the $AhR^{-/-}$ mice in comparison to WT mice. A similar phenotype to the one observed in the $AhR^{-/-}$ mice was found for AhR^{CD4cre} mice for the γδ T cells. Therefore, the presence of AhR in $CD4^+$ cells is necessary for the recruitment of the $IL\text{-}17A^+$ αβ T cells and the $IL\text{-}22^+$ and $IL\text{-}17A^+$ ILC3 and partially for the $IL\text{-}22^+$ and of $IL\text{-}17A^+/IL\text{-}22^+$ γδ T cells (**Figure 4C**).

Thus, the data suggest that AhR downregulates indirectly IL-22 production via IL-22-producing cells recruitment, like NK, ILC3, and T cells, after chronic ozone exposure. For IL-17A, AhR acts directly on cell production and indirectly on cell recruitment as for IL-22.

Role of IL-17A and IL-22 Axis Upon Ozone Exposure and AhR Dependence

The possible involvement of IL-17A and IL-22 in dampening the lung inflammation in $AhR^{-/-}$ mice was then investigated. $IL\text{-}22^{-/-}$ (**Supplemental Figures 6A–F**), $IL\text{-}22R\alpha^{-/-}$, and IL-22 × $IL\text{-}17R\alpha^{-/-}$ mice (**Figure 5**, **Supplemental Figure 5**) were exposed to ozone, and different lung inflammation parameters were evaluated. Reduced cell infiltration in all gene deficient mice used was observed after chronic exposure (**Figures 5A,B**, **Supplemental Figures 6A,B**), with diminished macrophages and neutrophils recruitment in the BAL (**Figure 5A**), and NK and ILC2 cells in the lung, but not significantly (**Figure 5B**). Moreover, T_H2 cytokines, including IL-4 and IL-5, were reduced after chronic ozone exposure in $IL\text{-}22R\alpha^{-/-}$ and in IL-22 × $IL\text{-}17R\alpha^{-/-}$ mice in comparison to WT mice (**Figure 5C**). Airway hyperresponsiveness was reduced in the $IL\text{-}22R\alpha^{-/-}$ and $IL\text{-}22^{-/-}$ mice in comparison to WT mice (**Figure 5D**, **Supplemental Figure 6D**). Epithelial cells desquamation in the BAL, DNA, and TGF-β levels in BALF, as well as collagen in the lung were reduced in $IL\text{-}22R\alpha^{-/-}$ and IL-22 × $IL\text{-}17R\alpha^{-/-}$ mice (**Figure 5E**). Histological analysis of the lung tissue revealed reduced cell infiltration in parenchyma, in the $IL\text{-}22^{-/-}$, $IL\text{-}22R\alpha^{-/-}$, and IL-22 × $IL\text{-}17R\alpha^{-/-}$ mice after chronic ozone exposure, in comparison to WT mice (**Figures 5F,G**). Anti-IL-22 alone is sufficient to recapitulate the phenotype, the anti-IL-17A does not increase the effect observed with the anti-IL-22 (**Supplemental Figure 7A**). A decrease in interstitial macrophages and γδ T cells recruitment was observed in the BAL (**Supplemental Figure 7A**), as well as decreased T and NK cells in the lung (**Supplemental Figure 7B**). Lung remodeling parameters, such as epithelial cells desquamation, DNA release, and collagen production, were reduced in comparison to WT mice (**Supplemental Figures 7C,D**). Moreover, using the same neutralizing antibodies in AhR-deficient mice, we showed that the increased inflammatory parameters were dependent on IL-22/IL-17A signaling. In the BAL, IL-22 antibody neutralization was sufficient to reduce interstitial macrophages, neutrophils, and T cells, in comparison to what was observed in the AhR deficient mice (**Figure 6A**). Similar reductions of the T cells, ILC2, ILC3, and NK were observed in the lung (**Figure 6B**). Moreover, desquamation, DNA release, collagen production, and inflammatory score were reduced in AhR-deficient mice that were treated by anti-IL22 and anti-IL-17A antibodies weekly (**Figures 6C–E**).

Therefore, the data demonstrated that the absence of IL-22 reduced inflammation, suggesting a pro-inflammatory effect of this cytokine in this model. Since IL-22 expression is enhanced in absence of AhR, we hypothesized that AhR modulate inflammation including the recruitment of IL-22-producing cells, which play a major role in ozone induced lung injury.

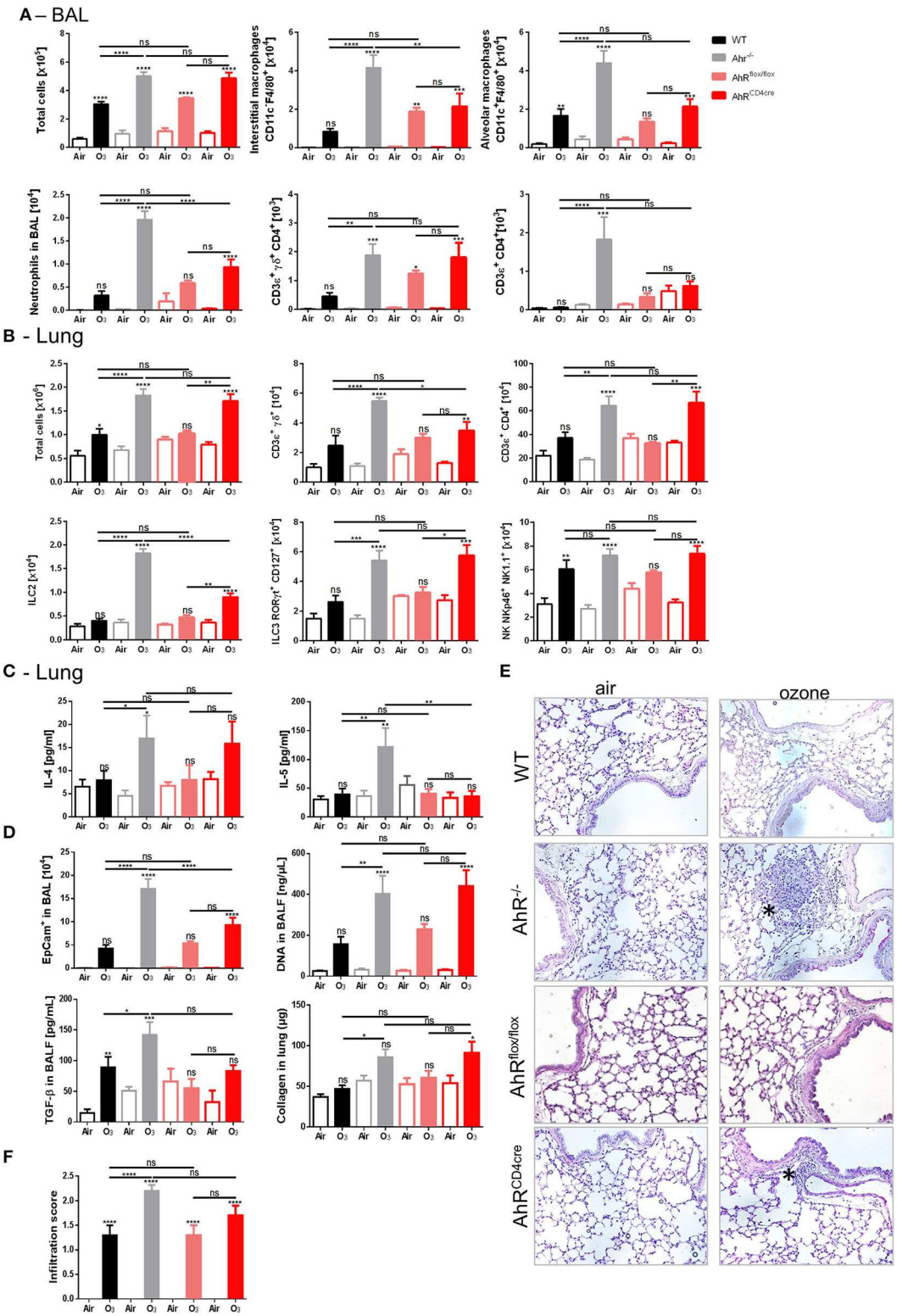


FIGURE 3 | Specific AhR depletion in T cell affects inflammatory parameters. BAL cells recruitment (total cells, macrophages, neutrophils, and T cells) **(A)**. Lung cells recruitment (total cells, neutrophils, macrophages, ILC2, ILC3, αβ, and γδ T cells) **(B)**, T_H2 cytokines (IL-4 and IL-5) **(C)** and remodeling parameters (epithelial cells, DNA, and TGF-β in BAL; collagen in lung) **(D)**; lung histology representative pictures (400× magnification, asterisks show cell infiltration) **(E)**, and cell infiltration score **(F)** after ozone exposure in WT and AhR deficient mice. The data are representative for one of two independent experiments with $n = 5–6$ mice per group. Values are expressed as mean ± SEM.

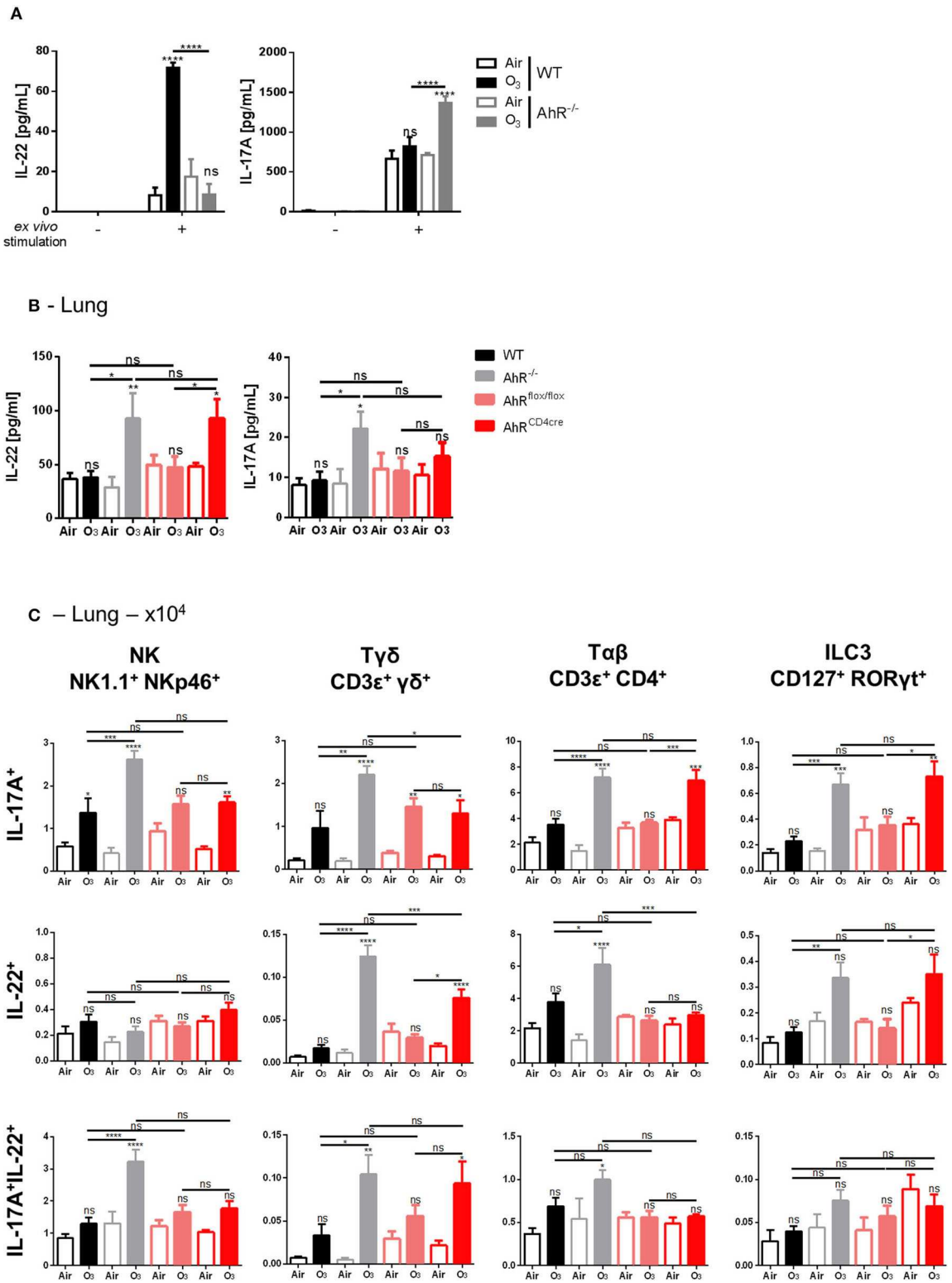


FIGURE 4 | Influence of AhR on IL-17A and IL-22 production. IL-22 and IL-17A release after *ex vivo* stimulation of lung cells **(A)**. Levels of IL-17A and IL-22 in WT and AhR deficient mice **(B)**. Absolute number **(C)** of NK ($NK1.1^+NKp46^+$), ILC3 ($CD45^+Lin^-CD127^+Ror\gamma t^+$), αβ T cells ($CD45^+CD3\,\varepsilon^+CD4^+$), γδ T cells ($CD3\varepsilon^+pan\gamma\delta^+$), $IL\text{-}17A^+$, $IL\text{-}22^+$, and $IL\text{-}17A^+IL\text{-}22^+$ producing cells. The data are representative for one of independent experiments with $n = 5$–6 mice per group. Values are expressed as mean ± SEM. Statistical significance was defined at a *p*-value **** < 0.0001, *** < 0.001, ** < 0.01, and * < 0.05.

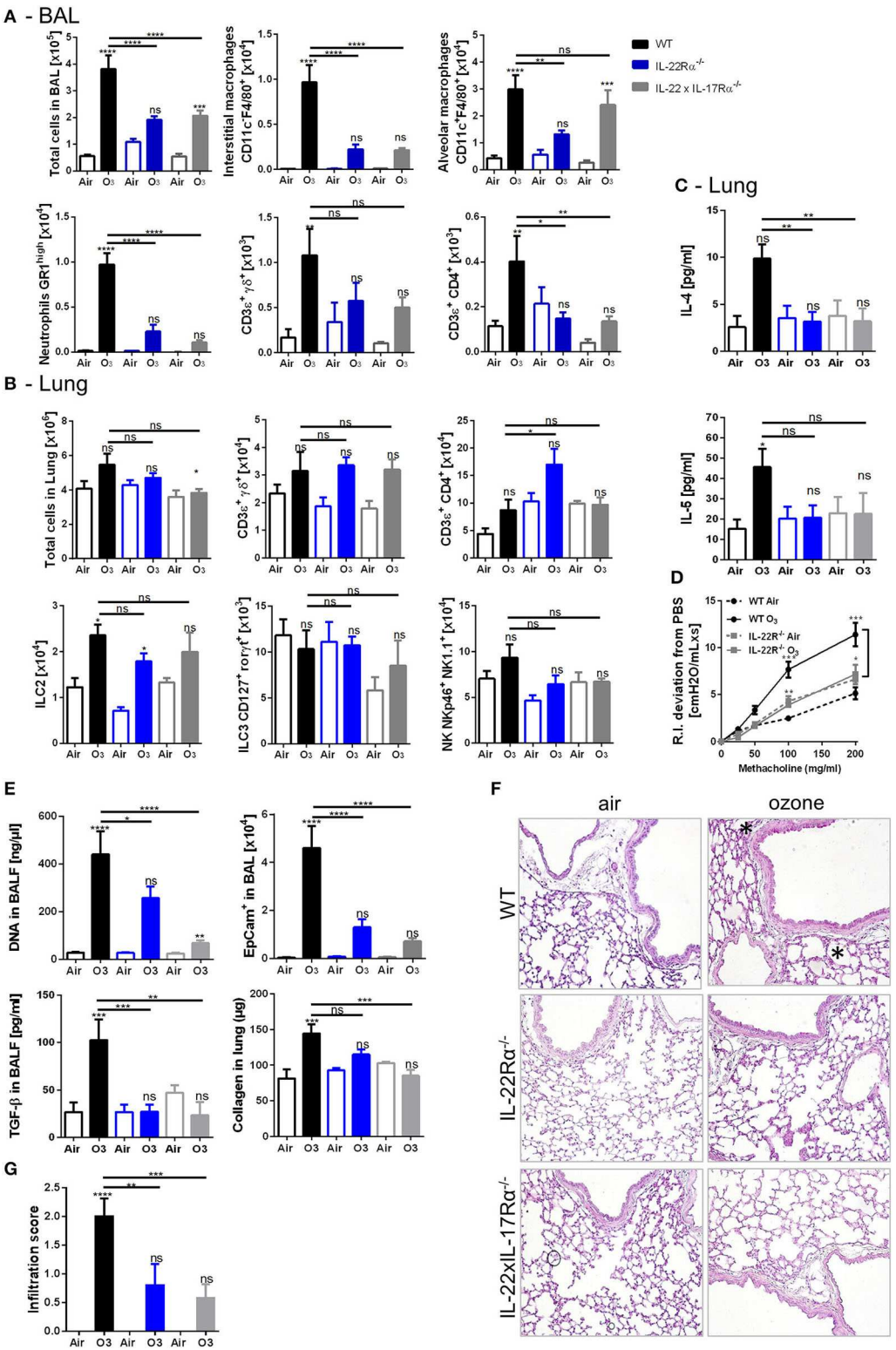


FIGURE 5 | Inflammatory parameters in IL-22R and IL-22/17Rα deficient mice. BAL cells recruitment (total cells, macrophages, neutrophils, and T cells) **(A)**. Lung cell recruitment (total cells, neutrophils, macrophages, ILC2, ILC3, αβ, and γδ T cells) **(B)**, T_H2 cytokines (IL-4, IL-5) **(C)**, AHR **(D)**, and remodeling parameters (epithelial cells, DNA and TGF-β in BAL; collagen in lung) **(E)**; lung histology (400× magnification, asterisk show cell infiltration) **(F)** and cell infiltration score **(G)** after ozone exposure in WT and AhR-deficient mice. The data are representative of one from two independent experiments with $n = 5–6$ mice per group. Values are expressed as mean ± SEM.

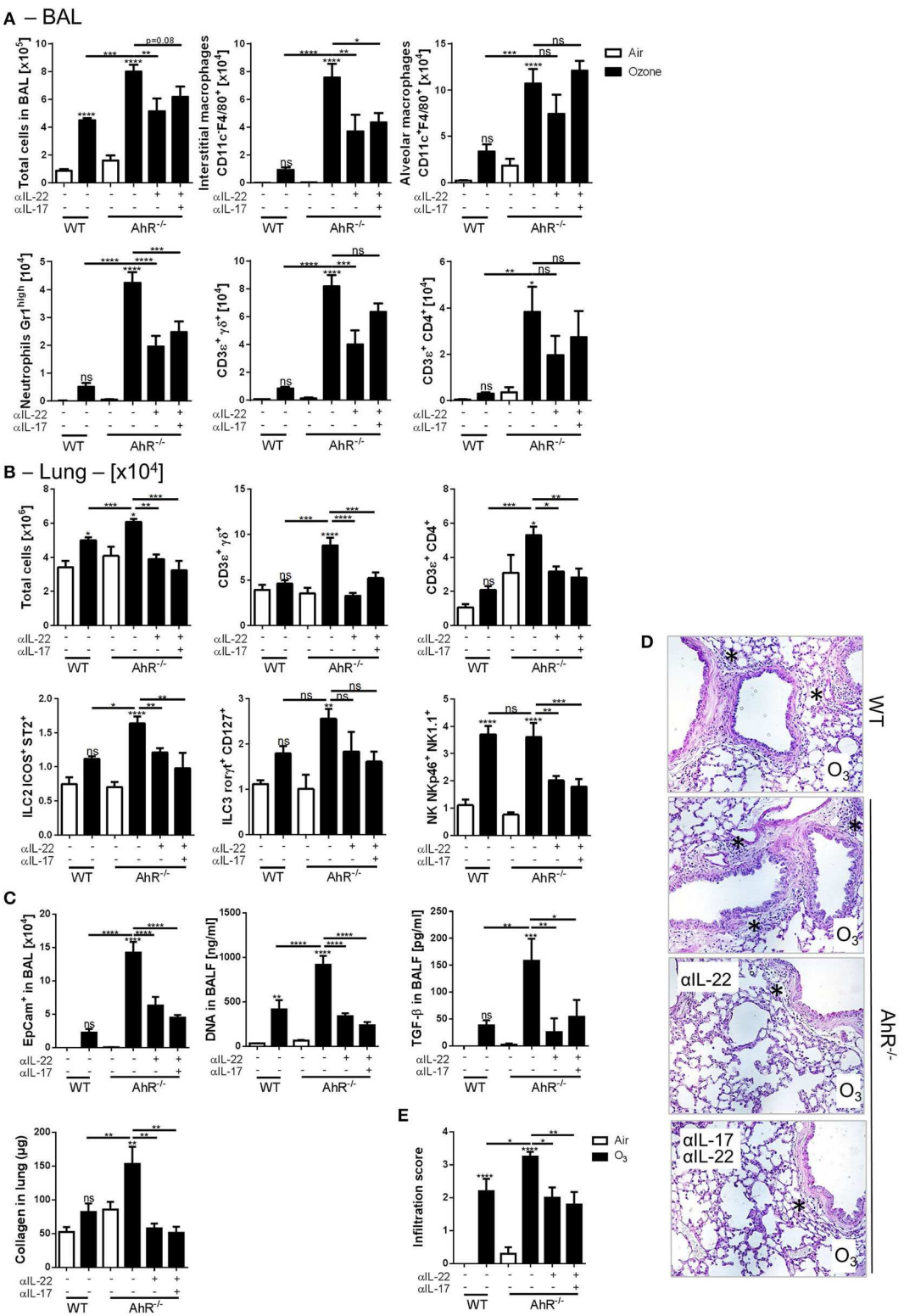


FIGURE 6 | IL-22 and IL-17A neutralization in AhR deficient mice. BAL cells recruitment (total cells, macrophages, neutrophils, and T cells) **(A)**. Lung cell recruitment (total cells, neutrophils, macrophages, ILC2, ILC3, αβ, and γδ T cells) **(B)**, and remodeling parameters (epithelial cells, DNA, and collagen in lung) **(C)**; lung histology (400× magnification; asterisks show cell infiltration) **(D)** and cell infiltration score **(E)** after ozone exposure in WT and AhR-deficient mice. The data are representative of one from two independent experiments with n = 5–6 mice per group. Values are expressed as mean ± SEM.

DISCUSSION

Environmental air pollution plays an important role in chronic respiratory diseases (54). Ozone is a major air pollutant contributing to the development of allergic and non-allergic asthma and chronic lung diseases, such as COPD and emphysema.

Here, we report that tryptophan produced upon ozone exposure leads to AhR activation and IL-22 and IL-17A production in the lung, probably through the production of tryptophan metabolites. AhR plays a protective role on lung inflammation, airway hyperresponsiveness, and tissue remodeling after chronic ozone exposure (1.5 ppm, twice a week for 2 h). The protection via AhR had previously been described in several models of lung inflammation including the COPD model induced by cigarette smoke (55, 56) and idiopathic pneumonia syndrome (1) or in the DSS-induced colitis model (57). AhR acts on several pathways, including cell cycle, cytokine production (51, 58), metabolism (20), and the maintenance of ILC3 (19). The type of immune response induced by AhR activation depends on the nature of the ligand. Several studies showed differential cytokine production due to AhR activation after PBMC stimulation with TCDD or PGE2 (22) supporting this hypothesis.

After ozone exposure, we identified two molecules released in the lung: tryptophan and LXA4, which potentially activate AhR. AhR plays a key role of tryptophan metabolism. Since the 1980s, several studies reported a role of tryptophan metabolites in the lung, including in cancer (59) and chronic inflammatory lung diseases affecting the parenchyma, such as sarcoidosis and idiopathic fibrosis (1, 60). The indole/tryptamine pathway could induce the production of several indole from tryptophan that may activate AhR. For example, in the intestine, IAld is known to induce the activation of AhR and the production of IL-22 (53). In such conditions, IAld is produced by microbiota, in particular the *Lactobacilli*. Lung/gut microbiota in the context of ozone exposure had been recently studied (61); moreover, ozone is commonly used in sterilization protocols, and it has an impact on plants (62), suggesting an impact of ozone on microbiota. Stress of the lung microbiome (63) induced by inflammatory cells and epithelial cells desquamation after ozone exposure may lead to an increased release of tryptophan in the lung that can be converted in various metabolites, but we cannot exclude an initial synthesis in gut. Moreover, we identified another AhR ligand, the lipoxin A4. The amount of lipoxin A4 was five times higher than the tryptophan itself in the lung tissue after chronic ozone exposure. This class of ligand has been described for the first time in 1999 (64). Lipoxin 44 could be produced in different cell types, as neutrophils, eosinophils, alveolar macrophages, and epithelial cells (65). Lipoxin A4 effects are mediated through its binding to FPR2/ALX membrane receptor or to AhR. Lipoxins are known to participate to inflammation resolution. In experimental asthma, lipoxins regulate NK or ILC2 activation (66), and in experimental psoriasis model induced by imiquimod, they reduce IL-17A and IL-22 production via HMGB1 modulation (67).

Here, we show that, AhR activation induced the recruitment of several immune cells, including macrophages, γδ T cells, αβ T cells, NK, ILC2, and ILC3 (**Graphical Abstract**). Upon chronic ozone exposure in AhR-deficient mice, we observed a type 2 response, as demonstrated by the production of T_H2 cytokines (IL-4 and IL-5). This response is associated with an increased IL-6 production. Airway hyperresponsiveness after chronic ozone exposure, a key feature of ozone exposure (28), is significantly enhanced in absence of AhR, as expected in a TH2 response. In the intestine, AhR maintenance is involved in the recruitment (19) of ILC3; however, the link between AhR and the ILC3 have not been investigated in the lung yet. Compared to the ILC2, ILC3 cells are very few in the lungs. But after ozone exposure, the quantity of ILC2 and ILC3 are very similar. Thus, we hypothesize that ILC3 had migrated from intestine to other tissues, particularly in the lung tissue after chronic ozone exposure. The deletion of AhR, in the $CD4^+$ cells, controls the recruitment of the ILC3 and $CD4^+$ T cells and is partly involved in the recruitment of the ILC2. Various direct or indirect mediators can drive this effect on different cell populations. Moreover, AhR may act on iNKT function (68), on the generation of Treg (69) or T_H17 cells (23), and in the balance between T_H1 and T_H2 (70) as AhR activation suppresses the T_H2 differentiation.

Furthermore, AhR regulates IL-17A and IL-22 production (**Graphical Abstract**). Here, in AhR-deficient mice, IL-17A, and IL-22 levels were increased as well as the amount of NK, ILC3, and γδ T cells expressing IL-17A and IL-22. By using AhR^{CD4cre} mice we observed that the AhR-specific deficiency in T cells influenced ILC3 numbers and increased their capability to produce IL-17A and IL-22. IL-17A is known to be a pro-inflammatory cytokine that can collaborate with IL-22 to induce inflammation (71, 72). We hypothesized that the protective effect of AhR is related to its capacity to repress the recruitment of the cells secreting IL-17A and IL-22 likely mediated the production of an endogenous ligand induced by ozone. This repression could be driven by the LXA4. Indeed, AhR deficient mice present a reduced LXA4 expression (73). As shown by Zhang et al. (31), IL-17A is pro-inflammatory in the context of chronic exposure to ozone, confirmed in our lab with utilization of $IL\text{-}17R\alpha^{-/-}$ mice (data not shown). In the $IL\text{-}22R\alpha^{-/-}$ mice, we observed a reduction of cell infiltration and T_H2 cytokines production. Moreover, airway hyperresponsiveness and tissue remodeling parameters (epithelial cells, DNA release, and collagen deposition) were reduced. Thus, our data confirm the pro-inflammatory role of both IL-17A and IL-22 in this model, but our data suggest that the phenotype observed in $AhR^{-/-}$ mice is only dependent of IL-22.

In conclusion, ozone exposure induces tryptophan metabolites and LXA4 that activates AhR. This activation likely control cell recruitment, T_H2 and $T_H17/22$ response, airway hyperresponsiveness and tissue remodeling. Altogether, our data show that AhR plays a protective role upon chronic exposure to ozone by modulating IL-22-producing

cells recruitment leading to the control inflammation (**Graphical Abstract**).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by The French Institutional Committee under agreement CLE CCO 2015-1088.

AUTHOR CONTRIBUTIONS

CM, FB, LF, IM, CC, and MS conducted the experiments. CM, BR, and DT designed the experiments. CM, BR, DT, CC, JS, VQ, HS, AC-M, and LD wrote the paper or had a critical regard on manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00144/full#supplementary-material

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REVIEW
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Oxidative Stress in Ozone-Induced Chronic Lung Inflammation and Emphysema: A Facet of Chronic Obstructive Pulmonary Disease

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Oxidative stress plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD) caused by cigarette smoke and characterized by chronic inflammation, alveolar destruction (emphysema) and bronchiolar obstruction. Ozone is a gaseous constituent of urban air pollution resulting from photochemical interaction of air pollutants such as nitrogen oxide and organic compounds. While acute exposure to ozone induces airway hyperreactivity and neutrophilic inflammation, chronic ozone exposure in mice causes activation of oxidative pathways resulting in cell death and a chronic bronchial inflammation with emphysema, mimicking cigarette smoke-induced COPD. Therefore, the chronic exposure to ozone has become a model for studying COPD. We review recent data on mechanisms of ozone induced lung disease focusing on pathways causing chronic respiratory epithelial cell injury, cell death, alveolar destruction, and tissue remodeling associated with the development of chronic inflammation and AHR. The initial oxidant insult may result from direct effects on the integrity of membranes and organelles of exposed epithelial cells in the airways causing a stress response with the release of mitochondrial reactive oxygen species (ROS), DNA, and proteases. Mitochondrial ROS and mitochondrial DNA activate NLRP3 inflammasome and the DNA sensors cGAS and STING accelerating cell death pathways including caspases with inflammation enhancing alveolar septa destruction, remodeling, and fibrosis. Inhibitors of mitochondrial ROS, NLRP3 inflammasome, DNA sensor, cell death pathways, and IL-1 represent novel therapeutic targets for chronic airways diseases underlined by oxidative stress.

Keywords: ozone, oxidative stress, inflammation, empyema, model, COPD

INTRODUCTION

Ozone is a gaseous constituent of urban air pollution that is generated by interaction of rising constituents of air pollution such as nitrogen oxide and organic compounds, induced by sunlight. Early studies have documented the effects of a short acute exposure to ozone in inducing airway inflammation and bronchial hyperreactivity in humans and in various species including mice, rats,

TABLE 1 | Mean ozone concentration targets and thresholds in Europe.

Ozone target, threshold, and mean annual values	$\mu g/m^3$	ppm
EU target (8 h mean)	120	0.060
EU information threshold	180	0.090
EU alert threshold	240	0.120
Level in United Kingdom	80	0.040
Level in France	90	0.045
Level in Germany	100	0.050
Level in Italy	130	0.065

Adapted from: Air quality in Europe – 2019 report. EU, European Union; ppm, parts per million. (https://www.eea.europa.eu/publications/air-quality-in-europe-2019).

guinea pigs, and dogs, at levels of ozone that were much higher than those measured in a highly polluted traffic-dense environment during the summer months.

More than 92% of the world's population are regularly exposed to unhealthy levels of ozone and among the world's 11 most populous countries, population-weighted seasonal ozone concentrations range from about 45 ppb in Brazil to 68 ppb in China. In Europe, the average ozone levels are reported to be highest in Italy with an annual average of 65 ppb (https://www.eea.europa.eu/publications/air-quality-in-europe-2019). Ozone concentration EU targets for the protection of human health has been set not to exceed 60 ppb but these targets are very often exceeded in several EU countries (**Table 1**). The European Air Quality Report highlights that 17 EU member states and six other reporting countries registered concentrations of ozone above the target value more than 25 times.

Over the recent years, there has been growing evidence from epidemiological and human exposure studies of the detrimental effects of ozone on respiratory health. Thus, short-term changes in ozone levels have been associated with increased mortality (1, 2), and a positive association between ozone and hospital admissions for asthma and COPD in the elderly and between ozone levels and asthma emergency visits in children (3, 4). Indeed, more recent studies have linked long-term exposure to ozone with reduced lung function and an increasing risk of developing emphysema irrespective of being a cigarette smoker (5, 6).

The effects of ozone on inflammation have already been reviewed although these have usually looked at mainly acute exposures (7). One of the long-term interests of the effect of ozone has been to determine whether exposure to ozone can represent a good model for airways disease. Acute exposure to ozone in rats or mice and later in humans have been used as a model of bronchial hyperresponsiveness underlined by a neutrophilic inflammation, the mechanisms of this effect having been reviewed (7). However, it is the chronic exposure to ozone that led to a model of emphysema and chronic airway inflammation that has been of the most interest in recent years (8). This represented the most direct proof that oxidative stress could induce features similar to that seen in the condition labeled as chronic obstructive pulmonary disease (COPD).

In this review, we will consider by what mechanisms this oxidant stress can lead to chronic bronchial inflammation and lung destruction of emphysema, and how models of oxidative stress such as exposure to ozone particularly on a long term basis can inform us on the nature of COPD and can be used to examine potentially new treatments.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

COPD is defined as a common, preventable, and treatable disease that is characterized by persistent respiratory symptoms and airflow obstruction that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases (9, 10). This is a disease with high degree of morbidity and mortality throughout the world. While initially, this was considered to be a disease mainly found or caused by cigarette smoking, it is now agreed that exposure to biomass fuel and increasingly to environmental pollution can also contribute to the pathogenesis of COPD (11).

Some of the pathological features of smoking-induced COPD consist of severe airflow obstruction which is associated partly to the inflammation in the small airways particularly by neutrophils, macrophages, and lymphocytes (12). In addition, there is airway wall remodeling of the small airways, which involves all the components of the airway wall including epithelium, lamina propria, and airway smooth muscle (13, 14). Finally, there is the presence of emphysema, with the destruction of

Abbreviations: 16HBE, human bronchial epithelial cell line; AHR, airways hyperreactivity; AIFM1, mitochondrial apoptosis inducing factor 1; AP-1, activator protein 1; ASMC, airway smooth muscle cell; ATP, adenosine triphosphate; BAL, bronchoalveolar lavage; cGAS, cyclic GMP-AMP synthase; CBS, cystathionine-beta-synthetase; CCL2, C-C motif chemokine ligand 2; CGL, cystathionine-gamma-lyase; CLDN, claudin; COPD, chronic obstructive pulmonary disease; CS, corticosteroid; CSM, cigarette smoke medium; CXCL1, chemokine (C-X-C) ligand 1; $\Delta\Psi m$, mitochondrial membrane potential; DRP1, dynamin-related protein 1; ERK, extracellular signal-regulated kinase; ETC, electron transport chain; GR, glucocorticoid receptor; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; HDAC2, histone deacetylase 2; HIF-1α, hypoxia inducible factor subunit 1α; ICAM1, intracellular adhesion molecule 1; IFNγ, interferon gamma; IL, interleukin; ILC2, innate lymphoid cells type 2; iNOS, inducible nitric oxide synthase; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem cell; ISO-1, (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; JNK, c-jun NH2 terminal kinase; KEAP1, Kelch-like ECH associated protein 1; MAPK, mitogen activated kinase; MFF, mitochondrial fission factor; MFN2, mitofusin 2; MIF, macrophage migration inhibitory factor; MIP-2, macrophage inflammatory protein 2; MKP-1, mitogen-activated protein kinase phosphatase-1; MMP-12, matrix metalloproteinase 12; MPST, 3-mercaptypyruvate sulfurtransferase; MyD88, myeloid differentiation marker 88; NAC, N-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NaHS, sodium hydrosulfide; NET, neutrophil extracellular trap; NFκB, nuclear factor κB; NK, natural killer cell; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; Nrf2, nuclear factor (erythroid-derived 2)-like 2; OVA, ovalbumin; PAF, platelet-activating factor; PGAM5, phosphoglycerate mutase family, member 5; PGE2, prostaglandin E2; ppm, parts per million; ROS, reactive oxygen species; SS-31, d-Arg-2′,6′-dimethyltyrosine-Lys-Phe-NH_2 mitochondrial antioxidant; STING, stimulator of interferon genes; TIRAP, Toll-Interleukin 1 receptor (TIR) domain containing adaptor protein; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TRPC6, canonical transient receptor potential channel 6; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

alveolar airspaces, which has been attributed to an imbalance of protease and anti-protease activity with enhanced protease activity associated with neutrophil activation (15). Airway hyperreactivity (AHR) prevalence and pathology in COPD was found to be present in one out of two COPD patients with its presence influenced by decline in lung function and smoking status (16). As such, AHR can be seen as an independent predictor and a contributing factor to COPD development.

Various pathological pathways have been proposed including increased apoptosis mechanisms (17, 18) in addition to inflammageing and autoimmunity (19). There is growing evidence that COPD may represent a cellular senescence program associated with secretion of pro-inflammatory cytokines that cause chronic inflammation, that leads to the increased levels of reactive oxygen species that can induce oxidative stress (20). Oxidative stress mechanisms can also lead to activation of pro-inflammatory pathways in addition to causing DNA damage (21).

An increasing body of evidence has been accumulating linking COPD with the process of autoimmunity, with the presence of autoantibodies in the sera of COPD patients, and with some of the antibodies correlating with particular disease phenotypes (19). Thus, it has been proposed that autoimmunity may play an important role in the pathogenesis of COPD. Interestingly, ozone-exposed mice for 6 weeks exhibited increased antibody titres to carbonyl-modified protein, as well as activated antigen-presenting cells in lung tissue and splenocytes sensitized to activation of carbonyl-modified protein (22). This is evidence of oxidative stress induced antibodies supporting an auto-immune component, as has been described in COPD with the elevation of carbonyl-modified self-protein that correlated with the severity of disease. Cigarette smoke models have also supported this effect of ozone as has been shown in matrix metalloproteinase 12 (MMP12)-generated elastin fragments serving as a self-antigen and driving the cigarette smoke-induced autoimmune processes in mice that result in a bronchitis-like phenotype and airspace enlargement (23). These elastin autoantibodies and others have been described in advanced COPD (24). The fact that autoantibodies in animal models of COPD such as after ozone exposure are capable of inducing a COPD-like disease phenotype indicate an autoimmune mechanisms perhaps involving B cells, plasma cells, and B cell-rich lymphoid follicles that are present in COPD (25).

MECHANISMS OF OZONE-INDUCED OXIDATIVE STRESS ON INFLAMMATION AND AIRWAY REMODELING

It is quite clear that ozone reacts with cellular membranes and with the epithelial lung lining fluid to generate bioactive mediators that cause oxidative stress, innate immune responses, and signaling (26, 27). When inhaled, there is direct contact between ozone and the first level of cells in the airway surface such as airway and alveolar epithelial cells, and airway macrophages. These cells release reactive oxygen species and various other inflammatory mediators including cytokines and lipids from oxidative damage to the airway epithelium (28, 29). In addition, ozone exposure has been shown to impair macrophage phagocytic and efferocytosis function (28, 30) which can cause prolonged injury and inflammation. The mechanisms underlying these changes have been previously reviewed in terms of the transcriptional effects of ozone in the lungs and airways (31, 32).

Effects of Ozone Exposure on Oxidative Stress and Intracellular Signaling Pathways

The increased oxidative stress as a direct effect of ozone is often investigated in relation to cellular dysfunction. Ozone affects several intracellular pathways in different cell types, but not always in the same way. Ozone exposure in cultured alveolar epithelial cells results in cytotoxicity but does not always affect the production of cytokines (33). However, when conditioned medium from ozone-exposed alveolar macrophages is added to alveolar epithelial cells, the cytokines chemokine (C-X-C) ligand 1 (CXCL1) and C-C motif chemokine ligand 2 (CCL2) were induced, mediated through interleukin-1α (IL-1α) (34). On the other hand, it has been reported that, although human alveolar macrophages are much more sensitive to ozone than epithelial cells, they do not produce increased amounts of IL-6, IL-8, or fibronectin following ozone exposure. This increased sensitivity of alveolar macrophages was observed in the form of increased cell death with a third of the cells lost after a low ozone exposure dose. However, whether macrophages are more sensitive to ozone *in vivo* is not known, but bronchial airway epithelial cells produce substantially more of all three proteins following ozone exposure, and both IL-6 and fibronectin are secreted vectorially toward the apical side at least in the first 4 and 24 h after ozone exposure (29). Ozone exposure has been shown to induce the expression of intracellular adhesion molecule-1 (ICAM-1) and neutrophil adhesion to human airway epithelial cells, which is mediated through the Canonical Transient Receptor Potential Channel 6 (TRPC6) (35). TRPC6 also impacts neutrophilic inflammation by nuclear factor-κB (NF-κB) activation and therefore promoting transcription of inflammatory mediators (35). Therefore, cellular interactions such as those between alveolar macrophages and, airway and alveolar epithelial cells which are the initial airway cells encountered by ozone may be important in the induction of its effects. The role of TRPC6 during oxidative stress conditions has been investigated in several cell types. Oxidative stress induces TRPC6 expression and function in podocytes (36), HEK293T cells (37), vascular myocytes (38), neutrophils (35), and macrophages (39). In a recent study, TRPC6 in bronchial epithelium cells, was shown to act as an oxidative stress sensor where the TRPC6-mediated calcium cascade leads to the activation of the extracellular signal-regulated kinase (ERK) pathway and inflammation (40). This could be a possible inflammatory response pathway in several cell types in response to ozone, but this has yet to be described in COPD.

Ozone exposure activates several types of innate immune cells which play a role in both the Th1 and Th2 inflammatory responses. While the role of neutrophils as part of the Th1 response is well-established, other innate cell such NK cells (41) and innate lymphoid type 2 (ILC2) cells (42) may also contribute.

Surfactant protein D released by exposure to ozone has been shown to stimulate interferon (IFN)-γ by NK cells to initiate IFN-γ, IL-12 feedback circuit (41). Continuous exposure to ozone has been shown to result in the conversion of an initial neutrophilic inflammation to an eosinophilic inflammation in the nasal mucosa of mice (43). This was accompanied by an overexpression of type-2 cytokines in the nasal epithelium. The role of ILC2 cells was confirmed by showing that the epithelial-derived alarmins, IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) that were increased in the nasal epithelium was dependent on ILC-2 cells, but not on adaptive T or B lymphoid cells (44). The involvement of ILC2 cells is also supported by the observation that IL-13, a product of such cells, can augment the effects of single ozone exposure, namely AHR and inflammation (45). In addition, ovalbumin sensitization and challenge can also augment ozone exposure effects (46).

Several intracellular pathways are involved in the effects of ozone exposure. Prolonged exposure to ozone induced gene expression levels of several hypoxia inducible factor-1α (HIF-1α) target genes including histone deacetylase 2 (HDAC2), vascular endothelial growth factor (VEGF), Kelch-like ECH-associated protein 1 (Keap1), and Macrophage migration inhibitory factor (MIF), in addition to a decrease in the antioxidative stress response as indicated by an increase in nuclear erythroid-related factor 2 (Nrf2) activity and protein level (47). The Toll like receptors (TLRs) and downstream adaptor proteins myeloid differentiation marker 88 (MyD88) and toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) appear to be involved in the inflammation response to ozone (48, 49). Ozone is able to induce TLR4 signaling through MyD88 in normal *Tlr4* expressing C3H/HeOuJ mice. This in contrast to C3H/HeJ mice that express a defective dominant negative mutant *Tlr4* gene (50). TLR4 can be activated by ligand binding and subsequently recruits adapter proteins including MyD88. The MyD88-dependant pathway signals through several signal transduction pathways including MAPK, NF-κB, and AP-1 to induce cytokine gene expression contributing to the inflammation response toward ozone. Heath shock protein 70 (HSP70) was identified as a downstream mediator to have a role the TLR4 mediated effects by ozone (50). The ozone-induced oxidative stress response might be involved in the activation of the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome complex (51). Both the acute and chronic exposure of mice to ozone induces lung inflammation through activation of ROS and of the NLRP3 inflammasome with caspase activation (52, 53) and release of the highly inflammatory mediator IL-1β, but also IL-1α (54), which are involved in the ozone injury response (55). Uric acid crystals cause NLRP3-dependent lung inflammation upon injury (56) and therefore it is not excluded that crystals formed during injury may contribute to ozone induced inflammation (57).

How this cytoplasmic multiprotein complex is activated upon ozone exposure remains to be determined. One of the mechanisms might involve reactive oxygen species (ROS) and oxidative stress induced danger signals as reviewed recently (58). The transcriptional effects of ozone and the subsequent impact on airway inflammation have recently been reviewed in an article in this series (31).

Reactive Oxygen Species-Mediated Barrier Disruption, Inflammation, and Emphysema

Ozone reacts with cellular membranes and with the epithelial lung lining fluid to generate bioactive mediators that cause oxidative stress, innate immune responses, and signaling (26, 27). Ozone-induced ROS at the cell membrane influences membrane integrity with the disruption of tight junction, cell stress, and death with leak of the respiratory barrier within 1–2 h. This first phase is independent of cellular ROS, inflammatory mediators, and inflammatory cells (58). These effects might be the result of ozone exposure induced changes in the expression of claudin (CLDN) proteins which are protein components of tight junctions. In mice exposed to ozone an increased expression levels of CLDN3 and CLDN4 while a reduction in CLDN14 was reported (59). These workers also showed that bronchial epithelial cell integrity was disrupted and that the trans-electrical resistance between cells was decreased leading to cell disintegration. These changes were associated with elevated ROS and increased expression of antioxidant defense system involving Nrf2 and Keap1 (59). Another potential mechanism is lipid peroxidation, which is induced by ozone in human alveolar epithelial cells (60) and in lung surfactant (61). Phospholipids and cholesterol in cell membranes and surfactant can react with ozone to form cytotoxic products which activate second messenger systems involving free arachidonic acid (62), platelet-activating factor (PAF) (63) and prostaglandin E2 (PGE2) (64). Ozone exposure induced changes to cell membrane integrity might involve several pathways, dependent on dose and duration of exposure.

The acute respiratory barrier disruption by ozone has been recently reviewed in an accompanying review in this series (65). This first phase is followed by a robust barrier injury with cell death, protein leak, and influx of ROS expressing myeloid cells including neutrophils, IL-1α and IL-33 production by epithelial and myeloid cells within 6–12 h. Thus, a biphasic response is observed, an immediate direct membrane damage by ROS and a second phase mediated by myeloid cells, which aggravates the damage of the epithelium and inflammation. In support of this hypothesis is the fact that neutrophil depletion attenuates the second phase (58). Oxidative stress peaks at ∼18 h after a single ozone exposure during the second phase with increased ROS positive neutrophils and epithelial cells (66). This increase in ROS subsequently causes cell injury, mitochondrial dysfunction, formation, and release of toxic metabolites and even DNA damage. These events have been associated with and contribute to the development of tissue destruction leading to emphysema and lung remodeling.

The toxic effect of ozone was also observed in the distal part of the lung, the alveoli, with death of alveolar epithelial cells with defective repair resulting in enlarged air spaces and emphysema (8). Emphysema upon chronic ozone exposure is commonly found in mice and is dependent on ROS-dependent inflammation (8), but this does not involve IL-17 (67).

TABLE 2 | Dose, duration, and effect of ozone exposures at different ages of rodents.

Dose	Duration	Species	Age	Effect
Acute ozone exposure				
2 ppm	8 h	Rat	Adult	Decrease ATP synthesis and mtO_2 consumption, increase lung mitochondria permeability (77)
3 ppm	8 h	Rat	Adult	Decreased mitochondrial respiration, decrease mtO_2 consumption (78)
3 ppm	8 h	Rat	Old	Decreased mitochondrial respiration, decrease mtO_2 consumption (78)
3 ppm	3 h	Mouse	Adult	Increased mitochondrial ROS, reduced membrane potential, ETC I, III, V proteins, and ATP content (79)
4 ppm	4 h	Rat	Adult	Decrease mtO_2 consumption (77)
Chronic ozone exposure				
0.5 ppm	12 h per day for 7 days	Rat	Young	Increased ROS, decreased ventilatory function (80)
0.5 ppm	12 h per day for 7 days	Rat	Adult	No effects on mitochondria O2 consumption (80)
0.5 ppm	12 h per day for 7 days	Rat	Old	Increased ROS and decreased mitochondria O2 consumption, increased ROS (80)
0.8 ppm	10–20 days	Rat	Adult	Increase number of mitochondria, increase O_2 consumption rates (81)
2.5 ppm	3 h, twice a week for 6 weeks	Mouse	Adult	Increased mitochondrial ROS, reduced membrane potential, increased ETC protein II, and IV (82)
3 ppm	3 h, twice a week for 6 weeks	Mouse	Adult	Increased mitochondrial ROS, reduced membrane potential, ETC protein and ATP content (79)

Young = <2 months age, Adult = 2–6 months of age, Old = >6 months of age. ATP, adenosine triphosphate; ETC, electron transport chain; mtO_2, mitochondrial oxygen consumption; ppm, parts per million; ROS, reactive oxygen species.

Understanding the initial physico-chemical and molecular events of ozone-induced cell membrane injury *in vitro* and *in vivo* is an area of intense research leading to cell death will be important. Inflammation and cell death are also associated with neutrophil extracellular trap (NET) formation with chromatin externalization (68) and release of extracellular DNA, which are inflammatory activating the DNA sensing pathways cGAS/STING as reviewed for lung inflammation (69). Aerosol particles such as silica and likely others including ozone cause extracellular release of nuclear and mitochondrial DNA from dying cells, which drive cGAS/STING-type I interferon dependent inflammation, which is inhibited by DNase administration or blockade of STING (70).

In ozone-exposed mice, ROS-induced cell-death was dependent on interactions of Keap1, the cellular ROS sensor and the phosphatase phosphoglycerate mutase family member 5 (PGAM5) with antioxidant function and apoptosis inducing factor mitochondria associated 1 (AIFM1), a pro-apoptotic factor. At high ROS concentration PGAM5 is released from the complex and activates the pro-apoptotic factor AIFM1 inducing apoptotic pathway resulting in a new form of cell death known as oxeiptosis (71–73). However, the contribution of the different cell death pathways such as necroptosis, apoptosis, and efferocytosis by ozone is yet resolved (74). These cell death pathways are likely to be responsible for the emphysematous process seen on oxidant-induced emphysema.

EFFECT OF OZONE EXPOSURE ON OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION

Ozone exposure in *ex vivo* and *in vivo* experimental models has focussed recently on the mitochondrial effects of ozone. Mitochondria are double membrane bound organelles that exist in most eukaryotic organisms. Mitochondria play an important role in production of adenosine triphosphate (ATP) and mitochondrial ROS production (75). Dysfunctional mitochondria influence airway contractility, gene expression, oxidative stress, cell proliferation, apoptosis and metabolism, and immune and inflammatory responses that are all implicated in airway diseases including COPD (76).

Influence of Age on Acute and Chronic Ozone Exposure

The toxic effects of ozone on mitochondria are closely dependent upon the dose and duration of ozone exposure and age of animals (**Table 2**). Short-term ozone exposure (2 ppm for 8 h or 4 ppm for 4 h) to adult rats caused decreases in mitochondrial O_2 consumption, mitochondrial ATP synthesis and oxidation of thiol groups in mitochondria in lung, and an increase in lung mitochondrial permeability (77). *In vitro* mitochondrial respiration in lung homogenates and in lung mitochondria from adult rats (2–3 months) was decreased after acute ozone exposure (15 ppm, 20 min). Subacute ozone exposure (0.8 ppm, for 10–20 days) to adult rats led to increased mitochondrial O_2 consumption in lung homogenates, increased mitochondrial number and mitochondrial respiratory activity in alveolar type II cells in the lung (81). Acute ozone exposure (3 ppm for 8 h) in both adult (4–6 months) and old rats (24–26 months) decreased mitochondrial respiration and mitochondrial O_2 consumption in isolated lung mitochondria (78). In young (3 weeks) and adult rats (6 months), a more chronic ozone exposure (0.5 ppm, 12 h, 7 days) did not affect lung mitochondrial O_2 consumption. However, in aged rats (20 months), ozone increased the mitochondrial O_2 consumption and H_2O_2 release, which means increased mitochondrial ROS activity (80). This indicates that ozone may accelerate senescence process in the elderly.

In mice, a single ozone exposure (3 ppm for 3 h) resulted in enhanced cellular and mitochondrial ROS levels and a reduced mitochondrial membrane potential ($\Delta\Psi m$) in lung.

(83). Upon acute and chronic ozone exposures, increased mitochondrial ROS levels, decreased ATP content, decreased electron transport chain (ETC) complex I enzyme activity, and reduced expression of ETC complex I, complex III, and complex V in the lungs of 1- and 6-week ozone-exposed mice (3 ppm, 3 h, twice a week) have been observed. Furthermore, ozone-induced inflammation, airway hyperreactivity, mitochondrial dysfunction, and ROS levels were reduced when mice were administered the mitochondrial antioxidant MitoQ (79). In addition, ozone induced inflammation, increased mitochondrial ROS, and expression of ETC complex II and IV in lung mitochondria in 6-week ozone exposed mice (2.5 ppm, 3 h, twice a week) was reduced by treatment with MitoTEMPO, another mitochondria-targeting antioxidant (82). Therefore, targeting the mitochondrial dysfunction to prevent or treat the ozone-induced associated inflammation, airway hyperresponsiveness, and oxidative stress could be a promising treatment strategy.

AHR is a hallmark of ozone effects on the airways (66, 67) and is exacerbated in allergen-sensitized mice (84–86). AHR is due to a direct effect of ROS on bronchial smooth muscle cells (79), but also neuronal cells (87). The effect of ROS on cell membrane integrity is likely due to a direct disruption of the cell membrane structure which needs further studies (88). This is further supported by the effect of N-acetylcysteine (NAC) in reversing established AHR after chronic exposure to ozone (89). Studies of the airway smooth muscle from mice exposed to ozone showed that the hyperresponsiveness to cholinergic contractile agents can be reproduced *in vitro* and that this oxidant stress-induced hyperresponsiveness was dependent on the activation of the p38 mitogen-activated kinase (MAPK) and inhibited by corticosteroids (52). The AHR induced by chronic ozone exposure was also dependent on IL-17 (67), as was also shown by Pichavant et al. who reported that NK cells producing IL-17 was important for the maintenance of ozone-induced AHR (86). The pulmonary inflammation induced by subacute ozone exposure has been reported to require γδ T cells and tumor necrosis factor-α (TNFα)-dependent recruitment of IL-17A + γδ T cells to the lung (90).

TABLE 3 | Effect of different treatment strategies in ozone-exposed animal models.

Compound	Target	Effect	References
Apocynin	Cellular ROS	Inhibition epithelial cell proliferation	(91)
Corticosteroids	MAPK	Inhibition of inflammation, AHR	(67)
H_2S	Cellular and mitochondrial ROS	Inhibition of inflammation, ROS, and emphysema	(92, 93)
iPSC-MSC	Lung	Inhibition of cellular and mitochondrial ROS, normal membrane potential	(83)
ISO-1	MIF cytokine	Inhibition of inflammation and AHR	(94)
MitoTEMPO	Mitochondria	Inhibition mitochondrial ROS, normal complex protein expression	(82)
MitoQ	Mitochondria	Inhibition of inflammation, AHR, mitochondrial ROS and mitochondrial dysfunction	(79)
VX-765	NLRP3 inflammasome	Inhibition of inflammation and AHR Inhibition of inflammation, AHR, emphysema	(53, 82)

AHR, airway hyperreactivity; H_2S, hydrogen sulfide; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem cell; ISO-1, (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; MAPK, mitogen activated kinase; MIF, macrophage migration inhibitory factor; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; ROS, reactive oxygen species; VX-765, Belnacasan, caspase-1 inhibitor.

THERAPEUTIC STRATEGIES TARGETING INFLAMMATION, OXIDATIVE STRESS, AND MITOCHONDRIA

Several studies have investigated the effects of novel treatment strategies relating to the effects of ozone exposure in animal models (**Table 3**). Targeting inflammation, oxidative stress, and mitochondria could be part of future treatment strategies for COPD.

Corticosteroids and ISO-1

Corticosteroids can attenuate the single exposure effects of ozone including AHR and lung inflammation (95–97). This includes the inhibition of expression of macrophage inflammatory protein 2 (MIP-2), inducible nitric oxidase synthase (iNOS) (98, 99) and NFκB (96), and the increased proliferation of the airway epithelium (91). However, chronic exposure to ozone itself can induce a state of corticosteroid insensitivity similar to what has been observed in COPD with reduced or little effect in preventing AHR, inflammation, and emphysema (94, 100). Corticosteroid insensitivity occurs in COPD through mechanisms induced by reactive oxygen species (101, 102). Several such mechanisms have been postulated, including a reduction in HDAC2 activity and expression, impaired corticosteroid activation of the glucocorticoid receptor (GR) and increased pro-inflammatory signaling pathways. Therefore, the chronic model of ozone exposure represents a good model of COPD to study the mechanisms of corticosteroid insensitivity.

Macrophage migration inhibitory factor (MIF) has been implicated as a driver of inflammation in COPD, and possibly as a driver of corticoidsteroid insensitivity in COPD. Using (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) which inhibits MIF tautomerase activity, the corticosteroid-insensitive lung inflammation and AHR after chronic ozone exposure was blocked (94). Thus, inhibition of MIF which is elevated in COPD may provide a novel anti-inflammatory approach in COPD. However, the contribution of mitogen-activated protein kinase phosphatase-1 (MKP-1) that has been proposed to underlie CS insensitivity in COPD was found to be negligible in the chronic ozone model (100). Single ozone exposure aggravated airway inflammation, airway remodeling, activation of p38 MAPK, and downregulation of

MKP-1 in ovalbumin (OVA)-sensitized and -challenged mice, an effect that was ineffectively controlled by corticosteroids (46), this also supported the role of p38 MAPK activation as a likely pathway involved in corticosteroid insensitivity (103).

Hydrogen Sulfide (H_2S)

Hydrogen sulfide (H_2S), a metabolic product of methionine, is synthesized from L-cysteine primarily by three key enzymes: cystathionine-c-lyase (CGL), cystathionine-b-synthetase (CBS) and 3-mercaptypyruvate sulfurtransferase (MPST). Identified as the third gasotransmitter, along with nitric oxide and carbon monoxide, H_2S modulates a variety of physiological functions including anti-oxidative stress, anti-senescence/aging, and anti-apoptotic effects (104–106). H_2S has been proposed as serving as a potent antioxidant through reactive oxygen species/reactive nitrogen species scavenging, or through post-translational modification of proteins by addition of a thiol (-SH) group onto reactive cysteine residues (107). In the lungs, H_2S suppresses the airway smooth muscle proliferation and cytokine release, an effect that is less effective in muscle from COPD patients (108).

In addition, H_2S content is reduced in the lungs of smokers and COPD patients (109), and H_2S attenuates nicotine-induced endoplasmic reticulum stress and apoptosis in bronchial epithelial 16HBE cells (110). H_2S is able to prevent and treat the development of inflammation, AHR, and oxidative stress in acute ozone-exposed mice (92). In addition, the ozone-induced increase in p38 MAK signaling was reduced in mice treated with H_2S indicating that this intracellular signaling pathway might be involved (92). In chronic ozone-exposed mice, H_2S is also able to prevent the inflammation, AHR, and remodeling of the lung but is not able to reverse these hallmarks of the model (93). Induction of p38 MAPK signaling is also reversed in both treatment strategies in the chronic ozone exposure model. In addition to this, the activation of the NLRP3 inflammasome and the ratio between cleaved caspase-1 to pro-caspase-1 were positively correlated with changes in lung function parameters and structural changes in the lung. H_2S was able to prevent and treat the changes observed in NLRP3 activation (93). Taken together, the main difference between the acute and chronic ozone exposure models on the treatment effects of H_2S on the ozone-induced changes suggests that H_2S treatment only affects the damage inducing pathways (oxidative stress) and not the regenerative pathways of the lung (92, 93). It is of interest that H_2S donor NaHS significantly inhibits cigarette smoke-induced mitochondrial dysfunction, oxidative damage, cell senescence, and apoptosis in alveolar epithelial A549 cells (111). These findings provide novel mechanisms underlying the protection of H_2S against ozone and cigarette smoke-induced COPD and suggest that H_2S donors targeted toward mitochondria may be beneficial in the treatment of COPD.

NLRP3 Inflammasome Inhibitor, Belnacasan (VX-765)

In addition to H_2S, Belnacasan or VX-765 inhibits NLRP3 inflammasome activation effects by inhibiting caspase-1. In acute ozone exposed mice VX-765 is able to prevent bronchoalveolar lavage (BAL) inflammatory markers, and AHR. Mitochondrial oxidative stress was reduced and this was associated with lower expression levels of dynamin-related protein 1 (DRP1) and mitochondrial fission factor (MFF), and increased expression of Mitofusin 2 (MFN2) proteins involved in mitochondrial fission and fusion, respectively (53). Similar effects were observed in chronic ozone exposed mice were VX-765 is able to prevent inflammation, emphysema, airway remodeling, and oxidative stress while it decreased the expression of the fission protein DRP1 and MFF with affecting proteins involved in fusion dynamics. (82). Mitochondrial oxidative stress and NLRP3 inflammasome were driving ozone-induced inflammation processes and targeting these specifically might have therapeutic value in COPD.

Mitochondrial-Targeted Antioxidants

Apocynin, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor decreased the proliferation in bronchial epithelium after an acute exposure to ozone but not the inflammation (91). Ozone-induced inflammation, airway hyperreactivity, mitochondrial dysfunction, and ROS levels were reduced when chronically-exposed mice were pre-administered the mitochondrial directed antioxidant, MitoQ (79). Similarly, ozone-induced inflammation, increased mitochondrial ROS, and expression of ETC complex II and IV in lung mitochondria in 6-week ozone exposed mice was reduced by treatment with mitoTEMPO, another mitochondria-targeting antioxidant (82). However, airway remodeling and airflow obstruction were not (82). Similarly, in single exposure to ozone, mitoTEMPO inhibited mitochondrial ROS without affecting inflammation and bronchial hyperresponsiveness (53).

Mucolytic/antioxidant agents such as erdosteine, carbocysteine, and NAC reduced the risk of acute exacerbations in patients with COPD (112). In the chronic ozone exposure model, preventive NAC reduced the number of BAL macrophages and airway smooth muscle (ASM) mass while therapeutic NAC reversed AHR, and reduced ASM mass and apoptotic cells (89). Thus, NAC could represent a treatment for protecting against the oxidative effects of ozone and other pollutants, as well as an agent for reducing exacerbations of COPD.

Downstream Signaling Pathways

As documented above, several intracellular pathways have been implicated in the effects of single or multiple exposures to ozone in the mouse. These pathways are related to the control of several key transcription regulatory factors including NF-κB, antioxidant factors such as Nrf2, the p38 MAPK, and priming of the immune system by up-regulating toll-like receptor expression. Thus, in the single ozone exposure model, AHR and inflammation was inhibited by a c-jun NH2 terminal kinase (JNK) inhibitor (SP600125) (113), p38 MAPK inhibitor (SD282) (46, 114) and NF-κB inhibitor (115). VX-765, an inhibitor of NLRP3 inflammasome, prevented lung inflammation and AHR caused by acute exposure to ozone (53), and also inhibited lung inflammation and emphysema from chronic exposure (82).

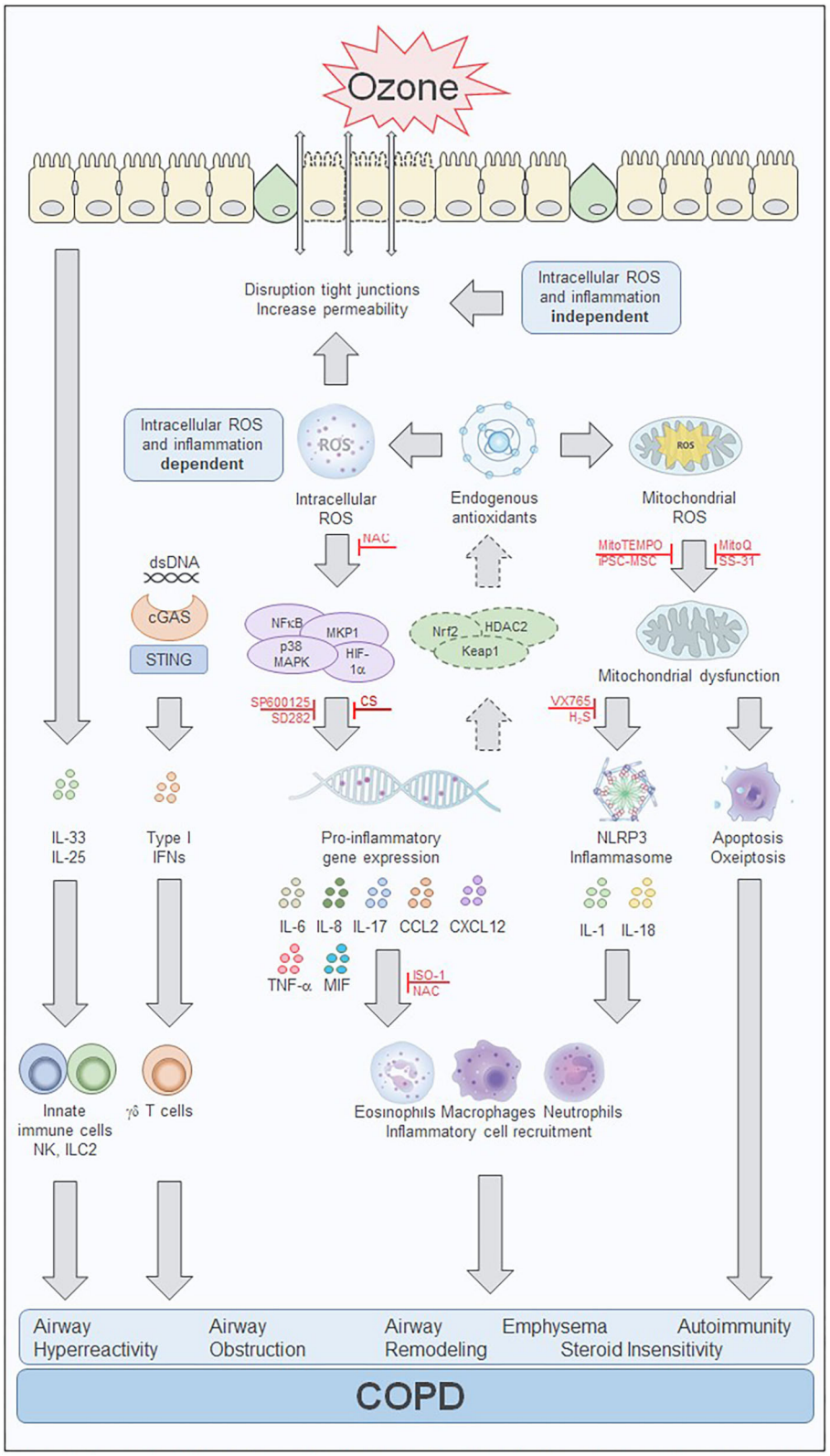

FIGURE 1 | Ozone-mediated effects on intracellular pathways involved in cell injury and inflammation. Overview of the effects of ozone in *in vitro* and *in vivo* models. The biphasic response to ozone starts with an immediate intracellular reactive oxygen species (ROS) and inflammation independent phase that is induced by

(Continued)

FIGURE 1 | extracellular oxidative stress. ROS induces membrane damage with changes in cell membrane integrity, disruption of tight junctions, epithelial cell stress, and death. Inflammatory mediators including IL-25 and IL-33 are released and attract innate immune cells such as natural killer (NK) cells and innate lymphoid type 2 (ILC2) cells. Cell death and release of dsDNA can induce an interferon (IFN) inflammation response via the cGAS/STING pathway. Processes activated during the intracellular ROS and inflammation-dependent phase of the effects of ozone include transcription factor-mediated inflammatory response and the activation of the antioxidant defense mechanism. Several inhibitors have been shown to prevent or treat the pro-inflammatory gene expression and subsequently inhibit the inflammatory response, including the JNK inhibitor, SP600125, and the p38 MAPK inhibitor, SD282. In addition, activation of the endogenous antioxidant defense system involving HADC2, Keap1, and Nrf2 may be sufficient to counteract the oxidant stress during acute ozone exposure but may be overwhelmed during chronic ozone exposure. Treatment with the antioxidant N-acetylcysteine (NAC) also reduces the inflammatory response, by scavenging of intracellular ROS with subsequent reduction of cytokine and chemokine production. Mitochondrial oxidative stress and mitochondrial dysfunction contribute to apoptotic processes and the activation of the NLRP3 inflammasome further enhancing the inflammatory response. Several treatment strategies targeting the mitochondria have been able to reduce or prevent the mitochondrial oxidative-induced dysfunction. These include several mitochondrial-targeted antioxidants such as MitoQ, MitoTEMPO, and SS-31. In addition, stem cell therapy with induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) prevented ozone-induced mitochondrial dysfunction and inflammation which may result from direct interaction and mitochondrial transfer between the iPSC-MSCs and airway cells. Treatment with the caspase-1 inhibitor VX765 and hydrogen sulfide (H_2S) prevent the activation of the inflammasome and reduces inflammation and mitochondrial oxidative stress. The MIF inhibitor ISO-1 blocks the ozone exposure induced inflammation and airway hyperreactivity and might have an impact on the corticosteroid insensitivity present in chronic ozone exposed lungs. Corticosteroids reduce inflammation induced by acute ozone exposures but fail to affect these processes in the steroid insensitive chronic ozone exposed lung. The ozone exposure driven intracellular processes contribute to the inflammatory cytokine and chemokine production, immune cell recruitment, and eventually the development of airway hyperreactivity, airway obstruction, airway remodeling, emphysema, autoimmunity, and steroid insensitivity which are hallmarks of COPD. cGAS, cyclic GMP-AMP synthase; COPD, chronic obstructive pulmonary disease; dsDNA, double stranded DNA; H2S, hydrogen sulfide; HDAC2, histone deacetylase 2; HIF-1α, hypoxia inducible factor subunit 1α; IFN, interferon; IL, interleukin; ILC2, innate lymphoid cells type 2; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem cell; ISO-1, (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; Keap1, Kelch-like ECH associated protein 1; MAPK, mitogen activated kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; MIF, macrophage migration inhibitory factor; MKP-1, mitogen-activated protein kinase phosphatase-1; NAC, N-acetylcysteine; NFκB, nuclear factor κB; NK, natural killer cell; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; SD282, p38 MAPK inhibitor; SP600125, c-jun NH2 terminal kinase (JNK) inhibitor; SS-31, d-Arg-2', 6'-dimethyltyrosine-Lys-Phe-NH2 mitochondrial antioxidant; STING, stimulator of interferon genes; TNF-α, tumor necrosis factor -α; VX765, Belnacasan, caspase 1 inhibitor.

iPSC-MSC Mitochondrial Transfer

Mitochondrial transfer from induced pluripotent stem cell-derived mesenchymal stem cell (iPSC-MSC) offered protection against oxidative stress-induced mitochondrial dysfunction in human airway smooth muscle cells (ASMC) and in mouse lungs exposed to ozone while reducing airway inflammation and hyperresponsiveness (83). Direct co-culture of ASMCs with iPSC-MSCs protected the former from cigarette smoke-induced mitochondrial ROS production, mitochondrial depolarization, and apoptosis. When ASMCs were exposed to supernatants from iPSC-MSCs or transwell inserts with iPSC-MSCs, only cigarette smoke-induced mitochondrial ROS, but not mitochondrial depolarization and apoptosis in ASMCs, were improved, indicating that soluble factors from iPSC-MSCs reduced production of mitochondrial ROS. When there was direct contact between iPSC-MSCs and ASMCs, mitochondria were transferred from iPSC-MSCs to ASMCs, possibly through formation of tunneling nanotubes, an effect that was enhanced by cigarette smoke medium (CSM) treatment. iPSC-MSCs prevented, but did not reverse, ozone-induced mitochondrial dysfunction, AHR, and airway inflammation in the mouse model of single ozone exposure, an effect resulting from direct interaction and mitochondrial transfer between iPSC-MSCs and airway cells. Therefore, transfer of mitochondria from IPSC-MSC cells to replace damaged mitochondria by oxidative stress may present a novel approach to treating conditions such as COPD.

CONCLUSION

As an important component of air pollution, ozone has been closely related to the development of COPD. The link comes from two sides: on the one hand, chronic exposure to ozone in murine model of lung inflammation and emphysema and on the other, long-term exposure to ambient air pollutant such as ozone, has been associated with increases in emphysema evaluated by computed tomographic imaging with chronic airflow obstruction. The mechanisms of ozone-induced lung and airway changes are the release of inflammatory factors such as IL-1α, IL-6, IL-8, CXCL-12, CCL2, ICAM-1, KEAP-1, and MIF, the activation of intracellular pathways such as the MAPK pathway, TLR, cell death pathways, NLRP3 inflammasome, and NF-κB, the induction of oxidative stress through a decrease in the antioxidative response and an increase in the production of ROS, with a detrimental effect on mitochondrial function such as increased mitochondrial ROS, decreased ATP content and abnormal ETC complex (**Figure 1**). Other mechanisms include the disruption of airway epithelial barrier, the development of AHR and emphysema and the state of CS insensitivity. Inhibitors of mitochondrial ROS, NLRP3 inflammasome, DNA sensor, cell death pathways and IL-1, including hydrogen sulfide, apocynin, mitochondrial-targeted antioxidants such as MitoQ and MitoTEMPO, mucolytic/antioxidant agents such as NAC, VX765, and iPS-MSCs mitochondrial transfer represent novel therapeutic options for treating oxidative stress-induced COPD.

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CW, FL, DT, and KC wrote the review. KC and BR inspired the work and corrected the manuscript. All authors have read the contribution.

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REVIEW
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Ozone-Induced Oxidative Stress, Neutrophilic Airway Inflammation, and Glucocorticoid Resistance in Asthma

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Despite recent advances in using biologicals that target Th2 pathways, glucocorticoids form the mainstay of asthma treatment. Asthma morbidity and mortality remain high due to the wide variability of treatment responsiveness and complex clinical phenotypes driven by distinct underlying mechanisms. Emerging evidence suggests that inhalation of the toxic air pollutant, ozone, worsens asthma by impairing glucocorticoid responsiveness. This review discusses the role of oxidative stress in glucocorticoid resistance in asthma. The underlying mechanisms point to a central role of oxidative stress pathways. The primary data source for this review consisted of peer-reviewed publications on the impact of ozone on airway inflammation and glucocorticoid responsiveness indexed in PubMed. Our main search strategy focused on cross-referencing "asthma and glucocorticoid resistance" against "ozone, oxidative stress, alarmins, innate lymphoid, NK and γδ T cells, dendritic cells and alveolar type II epithelial cells, glucocorticoid receptor and transcription factors". Recent work was placed in the context from articles in the last 10 years and older seminal research papers and comprehensive reviews. We excluded papers that did not focus on respiratory injury in the setting of oxidative stress. The pathways discussed here have however wide clinical implications to pathologies associated with inflammation and oxidative stress and in which glucocorticoid treatment is essential.

Keywords: asthma, oxidative stress, air pollution, ozone, glucocorticoid resistance, IL-17A, neutrophils

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INTRODUCTION: ASTHMA PHENOTYPES, GLUCOCORTICOID RESISTANCE, AND OXIDATIVE STRESS

Asthma is a highly heterogenous disease that can be classified into subsets by a number of different categories. Establishment of the appropriate subsets determines treatment approaches (1, 2). According to severity, asthma has mild, moderate, and severe forms (3–5). Asthma severity worsens during exacerbations associated with oxidative stress, the most common causes of which are viral respiratory infections and indoor/outdoor air pollution, including exposure to O_3 (6–11). Severe asthma is often more difficult to treat than the moderate or mild form of the disease (12, 13).

According to the predominant inflammatory cell type in the airways, asthma can be classified as eosinophilic, neutrophilic, mixed, or paucigranulocytic (3–5). Airway epithelial damage leads to oxidative stress, release of pro-inflammatory mediators and influx of both eosinophils and neutrophils (10). Neutrophils are the predominant inflammatory cells in severe asthma exacerbations (2, 5, 14). These cells are poorly controlled by glucocorticoids (15). Whether a causative allergen can be identified, asthma is also categorized as either allergic or non-allergic (16). Allergic (atopic) asthma is characterized by increased levels of IgE, eosinophilia, exhaled nitric oxide (NO), and Th2-type cytokines (16). Such "Th2 high" asthma can generally be treated with glucocorticoids and biologicals targeting the Th2 cytokine pathways (17). Approximately half of asthmatics however suffer from "Th2 low" asthma in which these pathogenic features cannot be identified. Thus, although Th2 low asthma patients are often resistant to corticosteroids, they cannot benefit from biologic treatment targeting the Th2 pathway either (18, 19). Especially in Th2 low asthma, corticosteroid resistance (the inability to increase FEV1 by 15% after a 7-day course of oral corticosteroids at 20 mg/day) (20) remains a significant clinical problem that continues to increase asthma morbidity and mortality (21–23).

The underlying molecular pathways of glucocorticoid resistant asthma are complex and generally associated with impaired expression and function of the glucocorticoid receptor (GR). GR-α, the classical glucocorticoid receptor isoform (24–27) has a dominant-negative inhibitor, GR-β, that does not bind corticosteroids. Overexpression of GR-β is due to abnormal activation of proinflammatory signaling pathways with emerging evidence for a contribution of oxidative stress (8, 10, 28–31). Oxidative stress is defined as an imbalance between reactive oxygen species and the capability of the biological system to detoxify the reactive intermediates or to repair the damage caused by oxidative free radicals (32, 33).

The common causes of oxidative stress potentially linked to glucocorticoid resistance in asthma are summarized in **Table 1**. Amongst the environmental causes our review is focused on inhalational exposure to the toxic air pollutant, ozone (O_3) as it was found to be a significant contributor to respiratory illness. Specifically, O_3 induces airway hyperreactivity in mouse models of asthma (6, 86, 87, 89, 91, 98–107), in Th2 low asthma in rhesus macaques (94, 108) and in healthy human subjects and patients with asthma and COPD (6, 7, 59, 109–116). Ground-level (tropospheric) O_3 is generated by the action of sunlight's UV rays from precursors (mostly air pollutants containing hydrocarbons, volatile organic compounds [VOC] and nitrogen oxides emitted during fossil fuel combustion). In cities with high O_3 levels people had an over 30% increased risk of dying from lung disease (117) and children playing outdoor sports had a three times greater chance of developing asthma (118, 119).

Against O_3-induced inflammatory injury, the lung mounts immuno-protective mechanisms such as production of the epithelial-cell derived collectin, surfactant protein D (SP-D) (101). Constitutive expression of this molecule in airway epithelial cells is promoted by glucocorticoid dependent transcription (120–122). O_3-induced oxidative stress not only destroys the biologically active tertiary molecular structure of SP-D (91, 123–125) but it also diminishes glucocorticoid responsiveness and SP-D expression in airway epithelial cells *in vivo* and *in vitro* (122). Below we discuss the potential significance of O_3-induced oxidative stress in glucocorticoid responsiveness in asthma.

TABLE 1 | Common causes of oxidative stress linked to glucocorticoid resistance in asthma.

- **Environmental exposures**
  - Allergen exposure (8, 34, 35)
  - Infections
    - bacterial (36–39)
    - fungal (34)
    - viral:
      - influenza (40–43),
      - RSV (44–47)
      - Rhinovirus (14, 48–52)
      - COVID-19 (53–56)
  - Inhalation of toxic indoor and outdoor air pollutants (57)
      - O_3 (7, 58–60)
      - Diesel exhaust (7)
      - Wildfire smoke (59, 61–63)
      - Tobacco smoke (30)
      - Comorbidities
  - Obesity (64)
  - Microbiome dysbiosis (65, 66)
  - Psychosocial stress (67–72)
  - Circadian rhythm disturbance (Shift work/jet lag) (73–81)
- **Cellular pathologies**
  - Cancer (82, 83)
  - Endoplasmic reticulum stress (84)
  - Mitochondrial dysfunction (85)
- **Molecular abnormalities**
  - NRF2 pathway dysfunction (29, 86, 87)
      - Decreased expression and function of antioxidant enzymes (eg: superoxide dismutase and catalase) (31, 35)
  - Decreased concentration of antioxidant scavenger molecules
      - Glutathione (84)
      - Surfactant protein A and D (88–91)
  - Deficiency of nonenzymatic/nutritional antioxidants (92)
      - vitamin A, C (92), E, beta-carotene
      - selenium
      - phytochemicals (66)
      - lycopene and lutein, resveratrol, flavonoids (64, 93)
      - secoisolariciresinol digluconate (94)
  - Increased production of reactive oxygen/nitrogen species (eg: ROS, RNS) (31, 35)
      - Proinflammatory signaling cascade (NF-kB, AP-1, PI3K) (58, 95–97)

O_3, ozone; RSV, respiratory syncytial virus; ROS, reactive oxygen species; RNS, reactive nitrogen species; NF-kB, nuclear factor kappa B; AP-1, activator protein 1; PI3K, phosphoinositide 3 kinase.

O_3-INDUCED AIRWAY INFLAMMATION AND GLUCOCORTICOID RESISTANCE

O_3 exposure results in accumulation of reactive oxygen species (ROS) most likely through lipid peroxidation processes of the pulmonary surfactant phospholipids (60) and cell membranes (126–128). ROS in turn rapidly activate the release of alarmins IL-1β, IL-6, IL-23, IL-33, TNF-α, and TSLP (**Figure 1A**) leading to a cascade of proinflammatory changes in structural and immune cells in the respiratory mucosal tissue (106, 116, 122, 129, 131–136). Activation of the RORγt proinflammatory signaling pathway leads to mRNA transcription of the IL-17A and IL-22 genes (**Figure 1B**) (131, 137–146). The IL-1 family of cytokines together with IL-17A and IL-22 induce influx and activation of neutrophils (129, 130). IL-33 has also been implicated in O_3 -induced airway inflammation (106, 129, 132–136). IL-33 transcription as well as release is upregulated by O_3 in the lung in a time dependent manner (106, 134). In the absence of IL-33 or the IL-33 receptor (ST2) acute O_3-exposure induced epithelial cell injury with protein leak and myeloid cell recruitment and inflammation were enhanced (134). While E-cadherin and zonula occludens 1 and reactive oxygen species expression in neutrophils and airway hyperreactivity were diminished in knockout mice. The enhancement of neutrophil influx was abolished by administration of recombinant IL-33 suggesting a protective role of IL-33 in O_3-induced epithelial barrier injury in mice.

Activated neutrophils in the airway mucosal tissue will release more ROS. Release of alarmins and influx of inflammatory cells into the airways are the pathological hallmark of severe asthma exacerbations (2, 5, 10, 14). In the healthy lung, the primary inflammatory cells recruited to the airways following O_3 inhalation are the neutrophilic granulocytes (147, 148). These cells appear in the airways within minutes and accumulate in significant numbers as early as 1–2 h after exposure (89, 106, 149). In healthy human subjects exposed to O_3 under experimental conditions, a significant airway neutrophilia was associated with a decrease in lung function (7, 147, 150) indicating the pathological significance of these cells. Interestingly, when O_3 exposure is combined with allergic sensitization in mouse models, asthmatic non-human primates (rhesus macaques) and in allergic human subjects, a marked influx of both eosinophilic and neutrophilic granulocytes is observed (10, 94, 101, 106). While neutrophilia in healthy

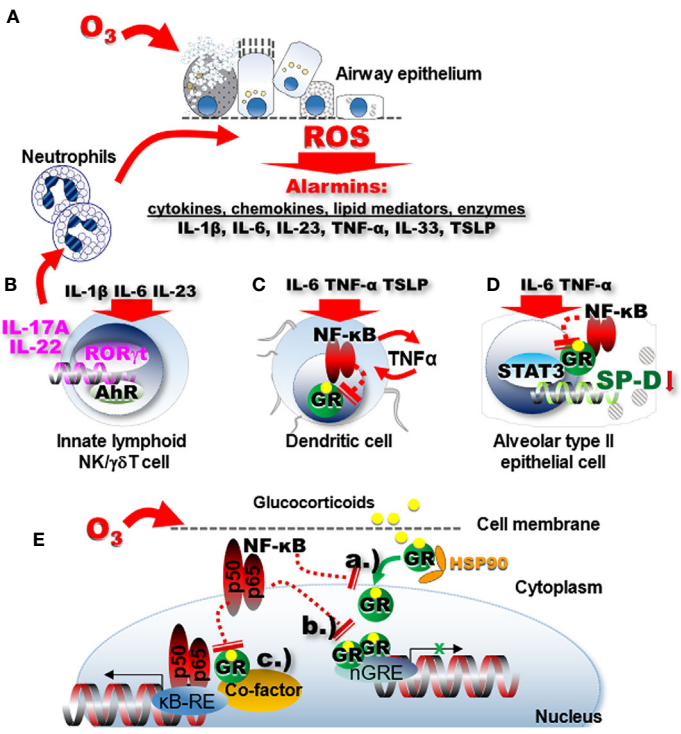

FIGURE 1 | Oxidative stress leads to impaired GR function through proinflammatory signaling. **(A)** Ozone inhalation generates ROS inducing release of alarmins through lipid peroxidation and proinflammatory activation of immune cells in the respiratory mucosal tissue. **(B)** IL-1β, IL-6, and IL-23 activate the RORγt proinflammatory signaling pathway that leads to mRNA activation of the IL-17A and IL-22 genes. The IL-1 family of cytokines and the related IL-33 together with IL-17A and IL-22 induce influx and activation of neutrophils (129, 130). In turn, activated neutrophils in the airway mucosal tissue will release more ROS. **(C)** Proinflammatory signaling activates NF-kB that in turn inhibits expression and function of the GR. Diminished GR function further activates NF-kB forming a vicious proinflammatory cycle. **(D)** The GR uses non-canonical transactivation of the surfactant protein D gene (*sftpd*) through STAT3. GR function impairment in alveolar type II epithelial cells leads to inhibition of the immunoprotective SP-D. **(E)** NF-kB inhibits expression of the GR and interferes with GR function through a.) inhibition of GR nuclear translocation b.) steric hindrance of nGRE binding, and c.) interference with transcription factor "tethering".

volunteers could be attenuated by fluticasone propionate (147), studies on mice (107), dogs (151) rhesus macaques (152), and asthma patients (153, 154) showed controversial results on the effectiveness of glucocorticoids in inhibiting O_3-induced exacerbation of asthmatic airway inflammation. Because asthmatic patients respond to O_3 with an enhanced airway neutrophilic influx compared with non-asthmatic controls (155), the observation that neutrophils are poorly responsive to glucocorticoids (15) raises a serious concern related to asthma treatment. Indeed, recent studies demonstrated that O_3 impaired the effects of glucocorticoid treatment in a mouse model of allergen-induced asthma *in vivo* as well as in human cell lines and primary epithelial cells *in vitro* (58, 122, 156). What are the underlying molecular mechanisms of O_3-induced glucocorticoid resistant neutrophilic airway inflammation in asthma?

ROLE OF ARYL HYDROCARBON RECEPTOR (AHR) SIGNALING, IL-17A, AND IL-22 IN GLUCOCORTICOID RESISTANT ASTHMA

The AhR is an intracellular, small molecule ligand-activated transcription factor that regulates gene expression of inflammation-related genes for myeloid and structural cells. AhR is a sensor of xenobiotic chemicals (such as aromatic hydrocarbons) or endogenous indole derivatives [such as kynureine (157)]. AhR mediates environmental signals and is involved in cell differentiation, cell adhesion, mucus and cytokine production (158–160). Upon ligand binding, the AhR complex translocates into the nucleus and heterodimerizes with AhR Nuclear Translocator (ARNT) to induce gene transcription. AhR is an important activator of the genes encoding cytochrome P450 and the cytokines IL-17A and IL-22. The effects of AhR on cell differentiation (including Th17 or Treg polarization) depend on the nature of the ligand and the local cytokine milieu (161, 162).

There are a number of potential mechanisms through which AhR may contribute to glucocorticoid resistance either as a promoter or as an inhibitor. *First*, glucocorticoid responsiveness of airway neutrophilia is regulated by the circadian clock molecule BMAL1 (Brain and Muscle ARNT-Like 1 or aryl hydrocarbon receptor nuclear translocator-like protein 1 [ARNTL]) (163, 164). BMAL1 function is strongly affected by environmental stressors (165) that can be mediated by AhR: Following agonist-induced activation, AhR enters the nucleus, where it can form a heterodimer with BMAL1 impairing its normal transcriptional activities (166) and promoting glucocorticoid resistance. *Second*, AhR interferes with the action of NF-κB, a pro-inflammatory transcription factor and antagonist of glucocorticoid action (see discussion below). For example, NF-κB induces AhR expression, but AhR then regulates NF-κB signaling (159) thereby enabling the glucocorticoid action. *Third*, by interacting with the function of other transcription factors, AhR promotes IL-22 (RORγt), IL-10, and IL-21 (cMaf) as well as aiolos and its own expression (through STAT3). Through aiolos, AhR inhibits expression of IL-2 (159), an inducer of glucocorticoid resistance (167). Thus, on the one hand AhR promotes Th17 cell differentiation, on the other, it induces Th17 cell plasticity into IL-10 producing protective Tr1 cells. While both IL-17A and IL-22 can elicit airway neutrophilia, IL-22 can also play a protective role when produced during epithelial or tissue damage. Recently, chronic ozone exposure induced lung inflammation, airway hyperresponsiveness and tissue remodeling was reported to be associated with increased tryptophan and lipoxin A4 (activators of AhR), and recruitment of IL-17A and IL-22-expressing cells. T cell-specific AhR deletion enhanced lung inflammation indicating that O_3 exposure activates AhR, to control airway inflammation by reduction of IL-22 expression (168).

IL-17A has been identified as a central player in the pathogenesis of severe asthma exacerbations (169). In human severe asthma patients high levels of IL-17A were found in induced sputum and bronchial biopsies (170). IL-6, the cytokine most prominently induced by O_3 in the lung (89, 171), and IL-23 (131) directly activate ROR-γt leading to IL-17A expression upon O_3 inhalation (**Figure 1B**). IL-17A signaling controls neutrophilic airway inflammation (172) mainly through stimulating the release of IL-8 and other pro-neutrophilic factors in the airways (131, 137–146) (**Figure 1B**). The importance of this cytokine in O_3 exposure-induced exacerbation of allergic airway inflammation was supported in a mouse model in which significant inhibition of IL-17A gene expression by the combined targeting of p38 MAPK activation and oxidative stress was critical in synergistically attenuating airway hyperresponsiveness, eosinophilic and neutrophilic inflammation (107).

IL-17A was also implicated in glucocorticoid resistant asthma (169, 173). For instance, Th17 cells, the main cellular source of this cytokine, were refractory to inhibition with glucocorticoids in asthma, especially, when IL-17A and IL-22 were co-expressed in these cells (169). Increased counts of dual-positive Th2/Th17 cells detected in the BAL fluid of severe asthma patients, were resistant to dexamethasone-induced cell death (169). Glucocorticoid resistance of IL-17 producing cells may be elicited by an elevated expression level of the mitogen-activated protein-extracellular signal-regulated kinase 1 (MEK1) as the MEK-ERK1/2 signaling pathway was shown to interfere with glucocorticoids (174). In a mouse model of airway inflammation, co-administration of dexamethasone with an anti-IL-17A monoclonal antibody significantly inhibited pro-neutrophilic cytokines and the p38 MAPK, NF-kB signaling pathway and reversed O_3-induced glucocorticoid insensitivity (144).

While Th17 cells were identified as the main producers of IL-17A, O_3-induced asthma exacerbation in mice did not show T cell activation or migration of T cells into the lung prior to the O_3- prompted neutrophil influx (106). These results implied that Th17 cells don't participate in IL-17A release in the early phases of the O_3-response. Mathews et al. proposed that the source of IL-17A in response to acute O_3 exposure is the γδ T cell (140). In addition, innate lymphoid cells were shown to be essential and sufficient to elicit development of O_3-induced neutrophilia (106)

and the ensuing airway hyperresponsiveness in mice. These studies suggest the importance of innate immune players in O_3-induced IL-17A pathways. Interestingly, when compared with Th2 cells ILC2s were found to be relatively steroid resistant in severe asthmatics (51, 156), although they were responsive to steroids in eosinophilic respiratory conditions (175). Increased IL-17A expression was associated with a reduction in GR-α but induced expression of GR-β in asthmatic airway epithelial cells indicating that the steroid insensitivity in severe asthmatics may be a result of a reciprocal regulation of GR-α and GR-β by IL-17 cytokines. Thus, in addition to Th cells, both IL-17A and IL-22 can be produced by ILC3, γδ T and NK cells, after stimulation with IL-1β, TGF-β, IL-6, or IL-23 and the transcription factor RORγt (168). **Figure 1B** illustrates that IL-17A-mediated neutrophilia in response to oxidative stress feeds back to a vicious cycle by releasing additional ROS into the lung tissue. Further, neutrophils have high constitutive GR-β expression that may help them resist apoptosis in response to corticosteroid treatment (25). Taken together, oxidative stress-induced IL-17A contributes to glucocorticoid resistance due to an increased activation of phosphokinase signaling pathways, reduction of GR-α, increase of GR-β in IL-17A producing innate immune and T cells thereby promoting neutrophilia.

ROLE OF THE GLUCOCORTICOID RECEPTOR (GR) IN O_3-INDUCED GLUCOCORTICOID RESISTANCE

Glucocorticoids have significant anti-inflammatory, immunosuppressive and immunomodulatory effects and remain the mainstay of asthma treatment (176). A subset of patients however is refractory to glucocorticoids (12, 177, 178), making their asthma difficult to control (179). Glucocorticoid insensitivity in rare cases, can be a primary genetic trait, but more commonly, it is acquired during inflammatory exacerbations (176). Constitutive GR expression is essential for an adequate glucocorticoid action. Corticosteroid insensitivity can be mediated by decreased function and expression of the GR. Expression of the GR gene (NR3C1) is regulated by complex transcriptional and post translational processes that are modified by airway inflammation (169, 180, 181).

How does the GR work? Glucocorticoids go through the cell membrane and bind to the GR that rearranges the stable GR-heat-shock protein (HSP)90 complex into an activated glucocorticoid-GR complex that translocates to the nucleus (**Figure 1E**). When two of these complexes form homodimers, they bind to specific glucocorticoid response elements (GRE) in the DNA sequence. GRE are located in the promoter regions of glucocorticoid-responsive genes (176). After the recruitment of co-activators or co-repressors, the GR modulates the rate of gene transcription by transactivation or transrepression. Transactivation is triggered by GRE which acts in "trans", i.e., intermolecularly (this may be considered the opposite of "cis"-acting i.e., intramolecular). On the other hand, transrepression (i.e., inhibition) is the activity of a second transcription factor through protein-protein interaction [reviewed by (182, 183)] (**Figure 1E**). The repressed molecule is usually a transcription factor whose function is to up-regulate gene transcription. Transrepression was first observed in the action of the GR to inhibit the transcriptional promoting activity of the proinflammatory transcription factors AP-1 and NF-κB. Transactivation and transrepression are both important in mediating the anti-inflammatory effects of glucocorticoids. Transactivation GRE up-regulates anti-inflammatory genes such as the NF-κB inhibitor IκBα, the AP-1 inhibitor glucocorticoid-inducible leucine zipper (GILZ) and IL-10. In a mechanism called "tethering" the GR can also interact with other transcription factors (NF-κB, AP-1, signal transducers, and activators of transcription [STAT] or CAAT Enhancer Binding Protein (C/EBP)], and modulate activation of target genes in a monomeric form (184–186). The activated monomeric GR binds to HDAC (histone deacetylase) and interferes with the activation of the κB responsive element (κB-RE) by p65 and p50 heterodimer subunits of NF-κB. Although the main function of HDACs is to modify histones and chromatin structure, HDAC isoforms can have different regulatory functions in the cytoplasm and nucleus. For instance, HDAC1 is considered to be a transcriptional co-activator (187). On the other hand, impairment of HDAC2 function is implicated in corticosteroid resistance of asthmatic and COPD patients (58, 97). Oxidative stress can lead to the reduction of HDAC2 *via* activation of phosphoinositide 3 (PI3K). PI3K induces nitric oxide levels in the asthmatic airways that further hinders the functional ability of HDAC2, as reported in asthmatic smokers (178). Moreover, treatment with theophylline, a medication that restores HDAC2 activity, glucocorticoid sensitivity is also restored (178).

GR expression levels are regulated by transcriptional and post translational mechanisms such as kinase-dependent phosphorylation as well as by homologous ligand down-regulation (by GR agonists) that can be significantly modified by increased NF-κB expression during O_3-induced oxidative stress (180). Phosphorylation-dephosphorylation is also important in the function of the transcription regulator enzyme, RNA polymerase II. The GR inhibits transcription activation through dephosphorylating RNA polymerase II (188).

Enhanced expression of NF-κB in the nuclear fraction of immune cells paralleled with an impairment of GR nuclear translocation, DNA binding and a decrease in the expression of GR (70). Mutual transrepression has been demonstrated between the GR and NF-κB as well as AP-1. In the highly inflamed airways during oxidative-stress related asthma exacerbation excessive NF-κB and AP-1 activation could be responsible for impaired GR function (27, 176, 189–191). NF-kB not only hinders GR nuclear translocation and directly interferes with GRE-mediated gene transactivation but it can also indirectly "tether" to the GR transcription complex. Importantly, while GR expression is ubiquitous, it is differentially regulated in individual cell types (192). For example cell type-specific increases in NF-κB, in airway epithelial and dendritic cells (**Figures 1C, D**), upon O_3 inhalation, may significantly inhibit GR expression and modulate allergic airway inflammation [reviewed in (72)].

Glucocorticoid resistance linked to oxidative stress through defective nuclear translocation and GRE binding (**Figure 1E**) (reviewed by Spiers et al. (28, 193, 194). That nuclear translocation of the GR is susceptible to highly pro-oxidative environments was shown by a cultured, fluorescently labeled chimeric GR. Okamoto and colleagues (193) demonstrated that nuclear translocation of GR following acute dexamethasone treatment was impaired in the presence of hydrogen peroxide. This effect was reduced by administration of exogenous antioxidants or by replacing serine for a redox-sensitive cysteine residue. The dissociation of heat shock proteins from the cytosolic GR is also impaired in a pro-oxidative environment, indicating that there may be multiple pathways involved in the cellular response to glucocorticoids (193, 194). Thus, a balanced oxidative state is critical for normal function of the GR.

GR function is also reduced when the molecule is phosphorylated. For example, the proinflammatory signaling molecule, p38MAPK can phosphorylate the GR that blocks nuclear translocation and the ability to bind to DNA leading to decreased ability of the GR to regulate transcription of anti-inflammatory genes (178). Similarly, activation of the MEK-ERK1/2 pathway was shown to antagonize the inhibitory action of glucocorticoids in Th17 cells (174).

Additional mechanisms involve increased expression of GRβ, an isomer of GRα that suppresses the ability of GRα to bind to GRE and induce anti-inflammatory genes. Increase in GRβ is caused by a rise in pro-inflammatory cytokines or through super-antigen such as staphylococcus enterotoxin-induced activation of T lymphocytes (176). Reduced GR expression was reported in asthmatic and COPD patients with insensitivity to corticosteroid treatment (189–191). GR expression can be reduced by homologous ligand down-regulation (upon administration of GR agonists) or other pathways such as transrepression of the GRα isoform by NF-κB in the inflamed tissue (27). It is unclear whether low levels of GR mRNA are due to suppression of promoter activation, decreased mRNA stability, or both (27) during oxidative stress. Importantly, the expression and function of the human GR is distinct from other species. For example, it is unclear whether transrepression of the GRα by NF-κB plays a role in corticosteroid resistance in mice as existence of the dominant negative GRβ isoform [responsible for glucocorticoid resistance (195)] could not be demonstrated in these rodents. Ligand-induced GR down regulation is seen in various tissues and cell types except in T cells (27) suggesting that innate immune and tissue cells may be more susceptible for glucocorticoid resistance. Further studies are still needed to identify the cell types ultimately responsible for mediating the effects of corticosteroid insensitivity in the lung.

AIRWAY EPITHELIAL CELL FUNCTION IS CONSTITUTIVELY REGULATED BY ENDOGENOUS GLUCOCORTICOIDS

Alveolar type II epithelial cells are the major source of pulmonary surfactant, as well as the immunoprotective lung collectins, surfactant protein (SP)-A and SP-D. SP-D, a glucocorticoid-dependent airway epithelial cell product is critical in the maintenance of pulmonary immune homeostasis (196–203). Individual susceptibility to the effects of O_3 exposure suggests that inflammatory responsiveness is genetically regulated (204). This is supported by strain dependence of the inflammatory response to O_3 observed in mice (205–207). A failure of protective immune mechanisms likely plays an important role in shaping the O_3 effects in the lung. A differential ability of Balb/c and C57BL/6 mice to respond to allergen (208) or O_3 (89), was inversely proportionate to the amount of SP-D in the lung of these mouse strains (89, 209). Further, when compared to wild-type C57BL/6 mice, the naturally low SP-D producer Balb/c or the SP-D knockout (C67BL/6) animals displayed increased susceptibility *to* and a prolonged recovery period *from* airway inflammation after allergen or O_3 exposure (89, 210–212).

In addition, O_3-induced exacerbation of Th2-type airway inflammation in allergen challenged mice was associated with the appearance of abnormal, lower order oligomeric molecular formations of SP-D. Interestingly, in asthmatic rhesus macaques, O_3 induced de-oligomerization of SP-D was restored by treatment with a flaxseed derivative anti-oxidant (94). Thus, oxidative damage can cause conformational change in the SP-D molecule resulting in a potential loss of its immunoprotective function (91, 213). Glucocorticoids were shown to be necessary for expression of SP-D in epithelial cells (120, 121, 214, 215). Interestingly however, there is no glucocorticoid response elements in the promoter region of the SP-D gene (*sftpd*). This DNA region however contains an evolutionarily conserved STAT3/6 response element in a prominent proximal location. IL-4/IL-13 (activators of STAT6) as well as IL-6 (activator of STAT3) directly upregulated SP-D synthesis in airway epithelial cells *in vitro* and in mice *in vivo* (89, 210). Between SP-D and the STAT3/6-activating IL-6 (89) as well as Th2 cytokines IL-4/IL-13 (216), respectively, negative regulatory feedback mechanisms were identified. In these, inflammatory transcriptional signaling by STAT3/6 would upregulate SP-D synthesis and release. In turn, increased amounts of this protein in the airways would suppress further inflammation through inhibition of proinflammatory cytokine transcription. Lastly, there are indications that STAT3 can be directly phosphorylated by H_2O_2 (the molecular product of O_3 when mixed in water) in airway epithelial cells *in vitro* (217). O_3 and glucocorticoid treatment had antagonistic effects on SP-D expression and function in the lung, with O_3 inhibiting glucocorticoid-induced *sftpd* transcription *in vivo* in mice and *in vitro*, in human airway epithelial cell cultures. These results indicated that glucocorticoids sustain vital functions in airway epithelium such as SP-D production, aimed at promoting immune homeostasis. This function is directly perturbed by O_3-induced oxidative stress.

ANTIOXIDANT APPROACH FOR ASTHMA TREATMENT

As we discussed, there is a marked role for oxidative stress in asthma, especially in severe exacerbations associated with

glucocorticoid resistance. Although this fact has been well established, and according to a WHO estimate, more than 80% of the Earth's inhabitants used Traditional Medicine/Complementary and Alternative Medicine (TCAM) for their primary healthcare needs (218), a large variety of nutritional, pharmacological, and environmental antioxidant clinical approaches to asthma treatment have been controversial and generally disappointing (33).

Emerging evidence from experimental models shows that successful targeting of oxidative stress in asthma is dependent on activation of NRF2 (Nuclear factor-erythroid 2 related factor 2). NRF2 is an ubiquitous master transcription factor that works through antioxidant response elements (AREs) to induce antioxidant enzyme and cytoprotective protein mRNA expression. Under baseline, "unstressed" conditions, the Kelch-like ECH-associated protein 1 (Keap1) inhibits cellular NRF2 in the cytoplasm and promotes its proteasomal degradation. NRF2 is activated by diverse stimuli such as oxidants, pro-oxidants, antioxidants, and chemopreventive agents (219). NRF2 induces cellular rescue pathways against oxidative injury, abnormal inflammatory and immune responses, apoptosis, and carcinogenesis (219). In a mouse model of asthma Sussan and colleagues used cell-specific activation of NRF2 in club cells of the airway epithelium and found a significantly reduced allergen-induced airway hyperresponsiveness, inflammation, mucus, Th2 cytokine secretion, oxidative stress, and airway leakiness and increased airway levels of tight junction proteins zonula occludens-1 and E-cadherin on the epithelial cell surface. Pharmacological activation of NRF2 during allergen challenge reduced allergic inflammation and airway hyperresponsiveness (220). Administration of the ROS inhibitors, N-acetyl cysteine or apocynin in a mouse model, had no effect on acute injury and lung inflammation but GR-1 antibody depletion of neutrophils significantly reduced ROS production in neutrophils, epithelial cells, interstitial macrophages, and eosinophils (134). In the same study, administration of IL-33 attenuated, while absence of IL-33/ST2 signaling enhanced O_3-induced airway inflammation and oxidative stress, and diminished zonula occludens-1 and E-cadherin expression highlighting the complex role this cytokine plays during lung injury (134).

In a different study, activation of NRF2 decreased the viability of the wild-type but not of the NRF2-deficient ILC2s resembling the pro-apoptotic effect of glucocorticoids albeit without the involvement of caspase 3-dependent apoptosis or necroptosis. In mice NRF2 activation decreased the number of pulmonary ILC2s and eosinophils suggesting NRF2 activation as a potential alternative strategy for steroid-resistant allergic inflammation (29). Lack of NRF2 in the lung exacerbates oxidative insults including supplemental respiratory therapy (e.g., hyperoxia, mechanical ventilation), cigarette smoke, allergen, virus, bacterial endotoxin and other inflammatory agents (e.g., carrageenin), environmental pollution (e.g., particles, O_3), and bleomycin (219, 221). Bioinformatic studies elucidated functional AREs and NRF2-directed genes that are critical components of signaling mechanisms in pulmonary protection by NRF2. Association of loss of function with promoter polymorphisms in NRF2 or somatic and epigenetic mutations in KEAP1 and NRF2 has been found in cohorts of patients with acute lung injury/acute respiratory distress syndrome or lung cancer (219).

The role of non-enzymatic antioxidants was studied in a multiple linear regression analysis that revealed significant associations of vitamin C, vitamin E, beta-cryptoxanthin, lutein/zeaxanthin, beta-carotene, and retinol with FEV1% in a large population study (93). Since removal of ROS and RNS from the cells by antioxidants could impair the action of NRF2, one might speculate that antioxidant vitamin administration with simultaneous NRF2 activation could be beneficial in oxidative stress-induced asthma exacerbation, which is a highly proinflammatory condition. To this effect, a dietary flaxseed compound (LGM2605) a synthetic form of the lignan secoisolariciresinol digluconate (SDG) was identified as both an antioxidant and an activator of NRF2. SDG demonstrated strong protective actions against different sources of oxidative damage (222, 223) supporting the potential for antioxidant approaches for asthma treatment. A cohort of asthmatic macaques from the California National Primate Research Center was identified to naturally develop airway hyperresponsiveness (224). These animals display no overt airway inflammation or Th2 cell activation and their peripheral blood mononuclear cells are unresponsive to glucocorticoids (224). Thus, these animals represent "Th2 low" glucocorticoid resistant asthmatic patients and are therefore uniquely poised for investigation of novel alternative or adjuvant approaches to glucocorticoid treatment. A 7-days treatment with LGM2605 of these macaques that received a single exposure to O_3 or air (as control) not only prevented the O_3-induced exacerbation of airway hyperresponsiveness but also significantly improved baseline lung function (94). These studies highlight the significance of oxidative stress in the effect of O_3 on airway hyperresponsiveness and support the idea that anti-oxidant treatment may be beneficial in glucocorticoid resistant, Th2 low asthma.

CONCLUSIONS

Severe glucocorticoid resistant asthma continues to increase morbidity and mortality despite the advent of new powerful biological treatments that target proinflammatory cytokines. Scientific and clinical evidence is emerging that alternative and adjuvant therapeutic approaches could significantly contribute to reducing and/or controlling severe asthmatic symptoms. Harnessing antioxidant mechanisms may have a special importance in this effort as oxidative stress has been clearly demonstrated to worsen steroid resistance in severe asthma. The pathways we discussed here are however widely applicable to clinical conditions associated with inflammation and oxidative stress and in which glucocorticoid treatment is essential. One recent example of this is the wide variability of effectiveness observed by dexamethasone treatment of severe COVID-19 patients (225–227). Our assessment of the literature raised a number of interesting questions that require future clarifications.

For example, what is the importance of the different cell types in mediating glucocorticoid resistance in asthma? Does the nature of oxidative stress depend on its etiology? What role do AhR-related mechanisms play and how does transcriptional regulation of the circadian clock figure into glucocorticoid responsiveness? Is it possible to increase expression and the protective function of molecules like SP-D? What are the effects of simultaneous molecular targeting of oxidative stress, inflammation, and NRF2 pathways? How feasible it is to translate experimental data to human studies and ultimately to clinical application? Greater understanding of how oxidative stress affects asthma and steroid resistance may lead to novel therapies that could improve the lives of millions.

AUTHOR CONTRIBUTIONS

CF drafted the ozone-related sections. CE drafted the glucocorticoid resistance-related sections. AH revised the draft, edited, and finalized the paper. All authors contributed to the article and approved the submitted version.

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