Ozone-Induced Nasal Type 2 Immunity in Mice Is Dependent on Innate Lymphoid Cells

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Abstract

Epidemiological studies suggest that elevated ambient concentrations of ozone are associated with activation of eosinophils in the nasal airways of atopic and nonatopic children. Mice repeatedly exposed to ozone develop eosinophilic rhinitis and type 2 immune responses. In this study, we determined the role of innate lymphoid cells (ILCs) in the pathogenesis of ozone-induced eosinophilic rhinitis by using lymphoid-sufficient C57BL/6 mice, Rag2−/− mice that are devoid of T cells and B cells, and Rag2−/−Il2rg−/− mice that are depleted of all lymphoid cells including ILCs. The animals were exposed to 0 or 0.8 ppm ozone for 9 consecutive weekdays (4 h/d). Mice were killed 24 hours after exposure, and nasal tissues were selected for histopathology and gene expression analysis. ILC-sufficient C57BL/6 and Rag2−/− mice exposed to ozone developed marked eosinophilic rhinitis and epithelial remodeling (e.g., epithelial hyperplasia and mucous cell metaplasia). Chitinase-like proteins and alarmins (IL-33, IL-25, and thymic stromal lymphopoietin) were also increased morphometrically in the nasal epithelium of ozone-exposed C57BL/6 and Rag2−/− mice. Ozone exposure elicited increased expression of Il4, Il5, Il13, St2, eotaxin, MCP-2, Gob5, Arg1, Fizz1, and Ym1 mRNA in C57BL/6 and Rag2−/− mice. In contrast, ozone-exposed ILC-deficient Rag2−/−Il2rg−/− mice had no nasal lesions or overexpression of Th2- or ILC2-related transcripts. These results indicate that ozone-induced eosinophilic rhinitis, nasal epithelial remodeling, and type 2 immune activation are dependent on ILCs. To the best of our knowledge, this is the first study to demonstrate that ILCs play an important role in the nasal pathology induced by repeated ozone exposure.

Keywords: ozone; innate lymphoid cells; eosinophilic rhinitis; mice

Ozone, the common oxidant air pollutant in photochemical smog, is a reactive and irritating inhaled toxicant that causes epithelial injury and acute neutrophilic inflammation in both the upper and lower airways of laboratory animals (1–3) and humans (4, 5). There is also epidemiological evidence to suggest that exposure to elevated concentrations of ambient ozone activates other inflammatory granulocytes (i.e., eosinophils) in the nasal airways of atopic and nonatopic children (6, 7).

Recently, we found that the nasal mucosa of C57BL/6 mice exposed to 0.5 ppm ozone have a dominant, but short-lived, Th1-like immune/inflammatory response (i.e., increased mRNA expression of Tnf and Il1b and neutrophilic rhinitis) after a single 4-hour exposure and Th2-like immune/inflammatory response (i.e., increased mRNA expression of Il5 and Il13 and eosinophilic rhinitis) after 9 days of 4-hour/day exposures (8). Mice repeatedly exposed to ozone also develop nasal epithelial alterations (remodeling) such as hyperplasia, hypertrophy, and mucous cell metaplasia that are similar to those reported previously in laboratory rodents (9) and nonhuman primates (3) repeatedly exposed to this gaseous outdoor air pollutant. In addition, we have found that nasal inflammatory and epithelial responses...
lesions are completely absent in similarly exposed Rag2−/−Il2rg−/− mice (also called Rag2−/−γc−/− mice) that are deficient in both recombinase activating gene (Rag) 2 and IL-2 receptor γ chain (Il2rg) and therefore are devoid of T cells, B cells, and innate lymphoid cells (ILCs) (10–12).

ILCs are recently discovered non-T and non-B lymphocytes that have been found in fat-associated lymphoid clusters and mucosa lining respiratory and digestive tracts, as well as in many other organs throughout the body (13). These lymphoid effector cells are involved in immune responses to fungal, helminthic, and viral pathogens (mainly through cytokine production) without rearrangement of antigen-specific receptors such as T or B cells (14). ILCs are classified into three types, primarily on the basis of their cytokine production profile: group 1 (ILC1s), group 2 (ILC2s), and group 3 (ILC3s). ILC1s include classical natural killer (NK) cells and T-bet-dependent, IFN-γ–producing ILCs. ILC2s produce IL-5, IL-9, IL-13, and/or amphiregulin. ILC3s comprise lymphoid tissue-inducer cells and other ILCs that produce IL-17 and/or IL-22.

It has been suggested that ILC2s play a critical role in priming local type 2 immune responses in the airways and are activated by epithelium-derived cytokines, IL-33, IL-25 (IL-17E), and thymic stromal lymphopoietin (TSLP). Increased numbers of ILC2s have been reported in the nasal mucosa of patients suffering from chronic rhinosinusitis with nasal polyps, a common upper respiratory disease characterized by elevated expression of IL-33 transcripts, high concentrations of IgE, and nasal eosinophilia (15–17). However, the pathological contributions of ILC2s in the development and exacerbation of allergic and nonallergic rhinitis have not been fully explored, especially regarding the health effects of air pollution.

Rag2−/− mice have ILC2s, as well as the other groups of ILCs, but lack functioning T and B cells (18, 19). It has been shown that Rag2−/− mice instilled intranasally with IL-25 develop type 2 inflammatory/immune responses in the pulmonary airways that include expression of Il5 and Il13 genes, eosinophil infiltration, and mucous cell hyperplasia and metaplasia (20, 21). In this study, we investigated the role of ILCs in the pathogenesis of ozone-induced nasal lesions by using C57BL/6 (wild type), Rag2−/−, and Rag2−/−Il2rg−/− mice. Animals of these three murine strains were exposed to 0 or 0.8 ppm ozone for 9 consecutive weekdays. To the best of our knowledge, this is the first study to demonstrate that ILC-sufficient C57BL/6 and Rag2−/− mice, but not ILC-deficient Rag2−/−Il2rg−/− mice, develop ozone-induced eosinophilic rhinitis concurrent with nasal epithelial remodeling and overexpression of ILC2-related mRNA. The results of this study suggest a new paradigm for the development of nasal pathology caused by repeated exposures to ozone and provide biological plausibility to the earlier epidemiologic findings that associated elevated ambient levels of ozone with airway eosinophilic inflammation in nonatopic children.

Materials and Methods

Animals
Six- to eight-week-old male C57BL/6, Rag2−/−, and Rag2−/−Il2rg−/− mice were obtained from Taconic Farms (Germantown, NY). They were housed in whole-body inhalation exposure chambers (H-1000; Lab Products, Marywood, NJ). All animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Ozone Exposure
After an acclimatization period of 1 week, mice were exposed to filtered air (0 ppm ozone; air control mice) or 0.8 ppm ozone for 1 day or 9 consecutive weekdays (4 h/d). Ozone was generated with an OREC Model O3V1-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ). Chamber ozone concentrations were monitored throughout the exposure with Dasibi 1003 AH ozone monitors (Dasibi Environment Corp., Glendale, CA). For the rationale for ozone concentration used in this study, see online supplement.

Histopathology
Two hours before necropsy, mice exposed to filtered air or ozone for 1 day were injected intraperitoneally with bromodeoxyuridine (BrdU) (Sigma Aldrich, St. Louis, MO). Heads were collected 24 hours after 1-day or 9-day exposure. After fixation and decalcification, nasal cavities were sectioned transversely at four specific anatomic locations designated T1–T4 (see Figure E1A in the online supplement) (22). Paraffin-embedded tissue sections from mice killed after 1-day exposure were stained with hematoxylin and eosin (H&E) and immunostained with an anti-BrdU antibody. Sections from mice killed after 9-day exposure were stained with H&E and Alcian blue (pH 2.5)/periodic acid Schiff (AB/PAS) and immunostained with antibodies specific for major basic protein (MBP), Ly-6B.2 alloantigen, chitinase-like 3 (YM1)/chitinase-like 4 (YM2) (23), IL-33, IL-25, and TSLP.

Nasal Morphometry
Nasal mucosa lining the lateral wall in the proximal lateral meatus of both nasal passages (T1 section) (Figure E1B) were evaluated with stereological methods and newCAST software (VisioPharm, Hoersholm, Denmark). Densities of Ly-6B.2–positive or MBP-positive cells in nasal mucosa were determined with point grids, as were the densities of IL-25 and TSLP in inflammatory cells of the nasal lamina propria. Volume densities of BrdU-labeled nuclei of the nasal epithelium, AB/PAS–positive intraepithelial mucousubstances, intraepithelial YM1/YM2 proteins, and IL-33–, IL-25–, or TSLP–positive nasal epithelium per surface density of basal lamina were calculated with point and intercept grids. Epithelial

Clinical Relevance
Understanding the potential contribution of commonly encountered air pollutants to the development or exacerbation of allergic and nonallergic rhinitis is important in the differential diagnosis, therapeutic management, and prevention of these clinically common upper respiratory diseases. Our finding, in mice, that innate lymphoid cells, and not other lymphoid cells, play a crucial role in ozone-induced eosinophilic rhinitis suggests another possible pathogenic pathway for nasal eosinophilic inflammation recognized in susceptible human populations living in air-polluted environments (e.g., atopic or nonatopic children living in communities with photochemical smog).
thickness was estimated by using H&E slides and point intercept grids.

**Quantitative Reverse Transcriptase–Polymerase Chain Reaction**

Nasal cavities were collected from mice after 9-day exposure and were stored in RNAlater (Sigma Aldrich) at −20°C until further processing. Airway mucosa lining the nasal and maxilloturbinate and lateral walls between T1 and T2 were micro dissected from both nasal passages. Total RNA isolation and complementary DNA synthesis were performed as described previously (24). Thirty-six genes were analyzed on the SmartChip Real-Time PCR System (WaferGen, Freemont, CA) and the ABI PRISM 7900HT platform (Applied Biosystems, Waltham, MA).

**Statistical Analysis**

For morphometric data, the Grubbs outlier test was performed to exclude statistically significant outliers. Differences among groups were analyzed by a two-way analysis of variance followed by a pair-wise Student’s or Welch’s t tests were performed on ΔCt values to compare air control and ozone-exposed groups in the same strain. Significance was assigned to P values <0.05.

**Results**

**Single Ozone Exposure Causes Acute Epithelial Injury in the Nasal Airways of ILC-Sufficient and ILC-Deficient Mice**

Control mice of all strains that were exposed to filtered air (0 ppm ozone) for 1 day or 9 consecutive days had no microscopic evidence of nasal histopathology (Figure E2A). As we have reported previously (8), ozone-induced nasal pathology was bilaterally symmetrical (similar location in both nasal passages) and was restricted to the mucosa lining the lateral meatus in the proximal portion of each nasal passage (T1 section) (Figure E1). At 24 hours after exposure, ozone caused acute epithelial injury in ILC-sufficient C57BL/6 and Rag2+/− mice and ILC-deficient Rag2−/−Il2rg−/− mice. It consisted of vacuolar degeneration, necrosis, and exfoliation in the nasal transitional epithelium (normally a thin pseudostratified, cuboidal epithelium with few ciliated cells and no mucus goblet cells). Morphometrically, the density of the BrdU-labeled nuclei (indicator of cells in the S phase of the cell cycle undergoing DNA synthesis) in the nasal transitional epithelium of the three mouse strains was significantly increased as compared with their respective air control mice. This increase in epithelial DNA synthesis after a single, acute exposure to ozone was statistically similar among all the strains of mice, including those with or without ILCs (Figures 1 and E2B).

**Repeated Exposure to Ozone Induces Eosinophilic Rhinitis in ILC-Sufficient Mice but Not in ILC-Deficient Mice**

Inflammatory responses were conspicuous in the nasal mucosa of ILC-sufficient C57BL/6 and Rag2−/− mice exposed to ozone for 9 days (Figure 2A). In contrast, ozone-exposed ILC-deficient Rag2−/−Il2rg−/− mice had no rhinitis. Immunostaining for MBP identified the inflammatory cells in the C57BL/6 and Rag2−/− mice repeatedly exposed to ozone as predominantly eosinophils (Figure 2B). In contrast, eosinophils in the nasal mucosa were sparse or absent in the ozone-exposed Rag2−/−Il2rg−/− mice. Morphometrically, the mucusal density of eosinophils in the ILC-sufficient mice was significantly greater than in their respective air control mice (Figure 3A). There were no differences in mucosal density of Ly-6B.2-positive cells among the study groups (data not shown). Recent studies have shown that the Ly-6B.2 antigen is expressed on monocyte subsets as well as neutrophils (25, 26). In this study, Ly-6B.2-positive cells were predominantly polymorphonuclear and

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**Figure 1.** Light photomicrographs and morphometric determination of bromodeoxyuridine (BrdU)-labeled nuclei (brown, arrows) in the nasal epithelium lining the lateral wall of the proximal nasal passages after 1-day exposure to ozone. (A) C57BL/6 mouse exposed to filtered air. (B) C57BL/6 mouse exposed to 0.8 ppm ozone. (C) Rag2−/− mouse exposed to 0.8 ppm ozone. (D) Rag2−/−Il2rg−/− mouse exposed to 0.8 ppm ozone. (E) Graphic demonstration of morphometric analysis of BrdU-labeled nuclei in the nasal epithelium. Data are expressed as mean ± SEM (n = 6/group). *P values <0.05. Scale bars, 30 μm. b, bone; bv, blood vessel in lamina propria; e, nasal epithelium; g, gland in lamina propria; N.D., not detected.
were rarely found in the mucosal lesions caused by repeated exposure to ozone.

**Ozone Induces Epithelial Remodeling in ILC-Sufficient Mice but Not in ILC-Deficient Mice**

ILC-sufficient mice exposed to ozone for 9 days had airway epithelial remodeling characterized by hyperplasia, mucous cell metaplasia, and hyalnosis (i.e., intracellular accumulation of eosinophilic protein material) in the nasal transitional epithelium of the proximal lateral meatus (Figure 2A). Nasal epithelium in C57BL/6 and Rag2^{−/−} mice repeatedly exposed to ozone was hyperplastic with a thickness of 2–4 cells as compared with an epithelial thickness of 1–2 cells in air control mice. Morphometric results were consistent with the morphology as indicated by greater (1.5×) epithelial thickness in ozone-exposed C57BL/6 and Rag2^{−/−} mice as compared with their respective air control mice (Figure 3B).

Nasal mucosa in ozone-exposed Rag2^{−/−}Il2rg^{−/−} mice as well as in air-exposed mice contained little if any AB/PAS–stained mucosubstances in the surface epithelium (Figures 2C and E2D). C57BL/6 and Rag2^{−/−} mice exposed to ozone for 9 days had significantly greater amounts of intraepithelial mucosubstances in these mucosal regions. Morphometric analysis of the volume density

**Figure 2.** Light photomicrographs of eosinophilic inflammation and epithelial remodeling in the nasal mucosa lining the lateral wall of the proximal nasal passages after repeated ozone exposure. (A) Hematoxylin and eosin stain. Exposure of 0.8 ppm ozone for 9 days caused marked rhinitis and epithelial remodeling (i.e., hyperplasia, mucous cell metaplasia, and hyalnosis) in innate lymphoid cells–sufficient C57BL/6 mice and Rag2^{−/−} mice. By contrast, innate lymphoid cells–deficient Rag2^{−/−}Il2rg^{−/−} mice exposed to ozone had no nasal lesions. Arrows indicate eosinophilic globules (hyalnosis) in the transitional epithelium. (B) Immunohistochemical stain for major basic protein in murine eosinophils. Large numbers of eosinophils (arrows) were detected in the nasal mucosa of C57BL/6 mice and Rag2^{−/−} mice after 9-day exposure to ozone. (C) Alcian blue (pH 2.5)/periodic acid–Schiff stain for acidic and neutral mucosubstances. C57BL/6 and Rag2^{−/−} mice exposed to ozone for 9 days stored Alcian blue (pH 2.5)/periodic acid–Schiff–stained intraepithelial mucosubstances in the apical aspect of nonciliated epithelial cells (arrows). (D) Immunohistochemical stain for chitinase-like 3 (YM1) and chitinase-like 4 (YM2) proteins. Air-exposed mice contained small amounts of YM1/YM2 proteins in the apical aspect of ciliated epithelial cells (arrows). YM1/YM2 proteins (arrows) were found throughout the full thickness of the nasal transitional epithelium of C57BL/6 mice and Rag2^{−/−} mice exposed to ozone for 9 days. Scale bars, 30 μm.
of mucosubstances indicated a significant increase in ozone-exposed C57BL/6 and Rag2^{-/-} mice as compared with their respective air-exposed mice and ozone-exposed Rag2^{-/-}Il2rg^{-/-} mice (Figure 3C).

As shown in Figure 1D, YM1/YM2 proteins were most conspicuous in the nasal transitional epithelium of C57BL/6 and Rag2^{-/-} mice after 9-day exposure to ozone. Morphometrically, the volume density of YM1/YM2 proteins was increased significantly in C57BL/6 and Rag2^{-/-} mice exposed to ozone as compared with corresponding air control mice and ozone-exposed Rag2^{-/-}Il2rg^{-/-} mice (Figure 3D).

**Repeated Exposure to Ozone Increases Epithelial Alarmin Expression in ILC-Sufficient Mice but Not in ILC-Deficient Mice**

We also evaluated the immunohistological expression of the alarmins, IL-33, IL-25, and TSLP, in the nasal transitional epithelium after repeated exposure to ozone. Constitutive expression of IL-33 was present in the nuclei of epithelial cells irrespective of exposure or murine strain (Figures 4A and E3A). IL-25 and TSLP were expressed in the nucleus and cytoplasm of nasal epithelial cells (Figures 4B, 4C, E3B, and E3C). Positive staining of IL-25 and TSLP was also found in inflammatory cells (mostly mononuclear cells) in the nasal mucosa of C57BL/6 and Rag2^{-/-} mice exposed to ozone.

Morphometric analysis demonstrated that ozone exposure caused greater expression of IL-33 in the nasal epithelium of ozone-exposed C57BL/6 and Rag2^{-/-} mice than in their respective air control mice (Figure 5A). Epithelial hyperplasia in C57BL/6 and Rag2^{-/-} mice exposed to ozone was considered to be a major cause of increased IL-33 expression, because (1) increased densities of IL-33 in ozone-exposed mice (1.5x greater than in air control mice) were equivalent to the increases in epithelial thickness, and (2) percentages of IL-33-positive nuclear density were not changed in C57BL/6 and Rag2^{-/-} mice after ozone exposure (Figure E4A). Ozone-exposed C57BL/6 and Rag2^{-/-} mice also exhibited greater cytoplasmic and nuclear expression of IL-25 and TSLP in the nasal epithelium (Figures 5B and 5C); however, the percentages of positive nuclei for IL-25, but not TSLP, were increased in the mice after repeated ozone exposure (Figures E4B and E4C). There were no differences in IL-33, IL-25, or TSLP expression in Rag2^{-/-}Il2rg^{-/-} mice with or without ozone exposure, although, interestingly, air-exposed Rag2^{-/-}Il2rg^{-/-} mice had increases in IL-33, IL-25, and TSLP in the nasal epithelium compared with the air control groups of C57BL/6 and Rag2^{-/-} mice.

**Repeated Exposure to Ozone Increases Nasal mRNA Expression Associated With Type 2 Immunity in ILC-Sufficient Mice but Not in ILC-Deficient Mice**

Results of qRT-PCR for the selected 36 genes, including transcripts for common immune/inflammatory cytokines, epithelial...
cell injury, and adaptation, using the SmartChip and ABI PRISM 7900HT platforms are presented in Tables E2 and E3, respectively. As shown in Figure 6, repeated exposure to ozone elicited significant overexpression of 10 genes in C57BL/6 and Rag2^{-/-} mice as compared with the respective air control group: Il4, Il5, Il13, St2 (Il1rl1), Eotaxin (Ccl11), MCP-2 (Ccl8), Gob5 (Clca1), Arg1, Fizz1 (Retnla), and Ym2 (Chil4) mRNA. In addition, there was significant underexpression of Tnf and Ifng in ozone-exposed C57BL/6 and Rag2^{-/-} mice versus respective control mice. No significant differences

Figure 4. Light photomicrographs for IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) in the nasal mucosa lining the lateral wall of the proximal nasal passages after repeated ozone exposure. (A) Immunohistochemical stain for IL-33. IL-33 expressed in the nuclei of nasal epithelium (arrows). (B) Immunohistochemical stain for IL-25. IL-25 expressed in the nuclei (arrows) and cytoplasm of nasal epithelium. IL-25–positive inflammatory cells were also observed in the nasal mucosa of C57BL/6 mice and Rag2^{-/-} mice (arrowheads). (C) Immunostain for TSLP. TSLP expressed in the nuclei (arrows) and cytoplasm of nasal epithelium. Inflammatory cells in the nasal mucosa of C57BL/6 mice and Rag2^{-/-} mice were positively stained by the TSLP antibody (arrowheads). Scale bars, 30 µm.

Figure 5. Morphometric determination of the amount of IL-33, IL-25, and TSLP in the nasal epithelium lining the lateral wall of the proximal nasal passages after repeated ozone exposure. (A) Volume density of IL-33 in the nasal epithelium (i.e., nuclei only) per length of basal lamina. (B) Volume density of IL-25 in the nasal epithelium (i.e., cytoplasm and nuclei) per length of basal lamina. (C) Volume density of TSLP in the nasal epithelium (i.e., cytoplasm and nuclei) per length of basal lamina. *P values <0.05. Data are expressed as mean ± SEM (n = 6/group).
were found between air-exposed Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice and ozone-exposed Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice.

**Discussion**

Recently, it has become clear that ILCs, in particular ILC2s, play a crucial role in type 2 immune responses to pathogens (e.g., fungi, helminths, and viruses) in the pulmonary airways (13, 27). The role(s) of ILCs in the pathogenesis of allergic rhinitis is still poorly understood, although published data suggest that ILC2s contribute to nasal polyp formation and immune responses in patients with chronic rhinosinusitis (15, 28). In the present study, the quantitative results of epithelial thickness, eosinophil density, and epithelial mucousubstances in the nasal mucosa of C57BL/6 and Rag2<sup>−/−</sup> mice exposed to ozone for 9 days were equivalent. We also found increased mRNA levels of Th2 (or ILC2) cytokines Il4, Il5, and Il13, eosinophil-chemotactic chemokine eotaxin, Th2-associated chemokine MCP-2 (29, 30), and the airway mucus-associated gene Gob5 in the nasal tissue of C57BL/6 and Rag2<sup>−/−</sup> mice after repeated exposure to ozone.

It has been reported repeatedly that mice selectively deficient in ILC2s exhibit an impaired pulmonary immune response to the protease-allergen papain, house dust mite antigen, and ovalbumin (OVA) (31, 32). These previous findings indicate that initiation of allergic airway responses under these conditions is critically dependent on ILC2s, but not Th2 lymphocytes. Likewise, our findings indicate that ILC2s are critical for eosinophilic rhinitis and the associated epithelial remodeling caused by repeated inhalation exposure to ozone. We postulate that ILC2s reside in the nasal mucosa lining the proximal lateral meatus where ozone-induced type 2 immune responses were observed. However, no microscopic methods are currently available to identify ILC2s in the nasal mucosa of mice because these cells have no unique markers for immunohistochemistry. ILC2s are usually detected by flow cytometric analysis, but this requires sufficient amounts of tissue because of the scarcity of these unique lymphoid cells (33). We have not yet been able to convincingly identify ILC2s in the murine nasal mucosa by flow cytometry. We believe this is, in part, caused by the small amount of tissue that can be harvested from the proximal nasal airways of the mouse. Future studies will have to use better techniques to identify the specific intranasal location(s) and numbers of mucosal ILC2s in both air- and ozone-exposed mice.

IL-33, IL-25, and TSLP drive activation of ILC2s, resulting in production of ILC2 cytokines in allergic inflammation (13). It has been suggested that IL-33 is released into the extracellular space during necrotic cell death (34). In our study, morphometric analysis demonstrated that IL-33 density in the nasal transitional epithelium of the proximal lateral meatus was not changed significantly in ozone-exposed mice after a single exposure (data not shown), although

![Figure 6](https://example.com/fig6.png)

**Figure 6.** mRNA expression in the proximal nasal mucosa after repeated ozone exposure. mRNA expression in the proximal nasal mucosa was analyzed for eotaxin, Il2, MCP-2, Gob5, Arg1, and Ym2 mRNA on the SmartChip Real-Time PCR System and for Il4, Il5, Il13, and Fizz1 on the ABI PRISM 7900 HT Sequence Detection System. *mRNA showing statistical significances (air versus ozone/same strain, $P$ values <0.05). Data are expressed as fold changes relative to their respective control mice ± SEM ($n = 6$/group).
necrotic changes were found occasionally in these areas. On the other hand, repeated exposure to ozone caused highly expressed IL-33 in the nasal epithelium of C57BL/6 and Rag2−/− mice. As in many previous reports (22), no clear degenerative or necrotic changes were found in the hyperplastic or metaplastic nasal epithelium of mice after repeated exposure to ozone. Recent studies have shown that airway exposure to virus or fungus can release IL-33 extracellularly without obvious epithelial cell injury (35–37). In our study, we also found increased IL-33 receptor ST2 mRNA in the nasal tissues of ozone-exposed C57BL/6 and Rag2−/− mice. Gene and protein expression of IL-33 and ST2 are increased in the nasal mucosa of patients with allergic rhinitis (38). In the present study, repeated exposure to ozone also caused increased expression of IL-25 and TSLP in the nasal epithelium (in both the cytoplasm and the nuclei) and inflammatory cells of C57BL/6 and Rag2−/− mice after 9-day exposure to ozone (Figures E5 and E6). Therefore, we speculate that all three epithelium-derived alarmins, IL-33, IL-25, and TSLP, may have been playing redundant roles in the activation of ILC2-type immune responses in the noses of ILC-sufficient mice during repeated ozone exposure. Additional studies, however, are needed to further elucidate the individual contribution of these alarmins to ozone-induced eosinophilic rhinitis.

Rag2−/−Il2rg−/− mice are alaymphoid (devoid of T and B cells and ILCs) but normally produce granulocytes including eosinophils, macrophages, and mast cells (8, 39). After a single exposure to ozone, acute injury and induced DNA synthesis (BrdU-labeled nuclei) in the nasal epithelium of Rag2−/−Il2rg−/− mice were comparable to those in C57BL/6 and Rag2−/− mice. This strongly suggested that the combined deficiency of these genes does not significantly affect the cellular defense mechanisms of the nasal epithelium against oxidative stress but does critically influence the type 2 immune activation after epithelial damage caused by ozone exposure. We do acknowledge, however, that some other, not yet identified, biological feature(s) in the Rag2−/−Il2rg−/− mice may be contributing, together with the absence of ILCs, to the dramatic loss of ozone-induced innate-type allergy in their nasal airways.

Rag2−/− mice have ILC2s, but also ILC1s (e.g., NK cells) and ILC3s (e.g., lymphoid tissue-inducer cells) (40, 41). Currently, the major biological functions of NK cells include cytolytic activity on target cells and production of type 1 (e.g., IFN-γ) and type 2 cytokines (42). NK cells have also been reported to be abundant in the nasal mucosa of patients with allergic chronic rhinosinusitis (43). Moreover, NK cells have higher cytotoxicity and expression of IL-4 and IL-13 in patients with allergic rhinitis as compared with nonatopic subjects (44). In addition, it has been reported that eosinophilia and IL-5 and IL-13 concentrations in bronchoalveolar lavage fluid were reduced in OVA-induced asthmatic models using NK cell–deficient mice (45). ILC3s play a crucial role in mediating the mucosal barrier in the gut via IL-17 and IL-22 production (13). Involvement of ILC3s in allergic rhinitis is poorly understood, although these cytokine levels in peripheral blood mononuclear cells from children with allergic rhinitis and asthma have been reported to be up-regulated (46). In our study, Il17a and Il22 mRNA in the nasal mucosa of C57BL/6 and Rag2−/− mice were not changed by ozone exposure; therefore, ILC3s are unlikely to be involved in ozone-induced eosinophilic rhinitis in mice.

Interestingly, we also observed hyalinosis and cytoplasmic accumulation of Ym1/YM2 proteins in the nasal transitional epithelium, as well as increased expression of Ym2 mRNA in the proximal nasal mucosa of C57BL/6 and Rag2−/− mice after repeated exposure to ozone. Similar staining patterns of Ym1/YM2 in the nasal respiratory and olfactory epithelium showing hyalinosis have been demonstrated previously in aged mice (47). YM1/YM2 proteins are specific to rodents, and the biological functions have not yet been fully elucidated; however, roles of YM1/YM2 in type 2 immune/inflammatory responses have been suggested. In an allergic airway disease model in mice, YM1/YM2 proteins have been found to be expressed in the airway epithelium and alveolar macrophages in an IL-13–dependent manner and to enhance airway secretion of IL-5 and IL-13 (48). An in vitro assay using purified protein has demonstrated that YM1/YM2 proteins have little chemotactic activity for eosinophils (49). Therefore, YM1/YM2 proteins may be involved in eosinophilic rhinitis induced by ozone exposure via up-regulation of type 2 cytokine production.

We also found that ozone exposure caused higher levels of Arg1 and Fizz1 mRNA in the nasal mucosa of C57BL/6 and Rag2−/− mice. YM1/YM2, Arg1, and Fizz1 are known as signature marker genes of alternative activation of macrophages, which are stimulated in a variety of tissues during Th2-polarized immune/inflammatory responses in mice (50). Arg1 has been reported to be up-regulated at the gene and protein levels in the nasal epithelium and inflammatory cells of nasal turbinates from patients with allergic rhinitis (51). Furthermore, in the lungs of mice with asthma induced by a fungal allergen or OVA, marked induction of Arg1 and Fizz1 have been detected in the airway epithelium and inflammatory cells (52, 53). In contrast to the key roles of Arg1 and Fizz1 in helminth-induced lung pathology, Arg1 or Fizz1 deficiency does not affect Th2 inflammatory response in the mouse lungs after OVA challenges (53, 54). Further evaluation is needed to determine the major cellular sources and pathophysiological roles of Arg1 or Fizz1 in eosinophilic rhinitis caused by ozone exposure.

**Conclusions**

In conclusion, we report for the first time that eosinophilic rhinitis, nasal type 2 immunity, and nasal epithelial remodeling in mice exposed to the commonly encountered ambient air pollutant, ozone, are mediated by ILCs (and most likely ILC2s), and not by T or B cells. This novel finding provides a new potential paradigm for the mode of action responsible for the epidemiologically recognized association of elevated ambient levels of ozone and eosinophilic rhinitis and eosinophil activation in nonatopic (and possibly atopic) children.

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

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