A Murine Model of Ozone-induced Nonatopic Asthma from the Collaborative Cross

To the Editor:

A better understanding of the mechanisms by which air pollutants influence the development of asthma is critically needed (1). Of particular interest is the role of ozone (O_3) because of the persistence of high ambient O_3 in population centers and the likelihood that levels will increase in the future due to climate change (2). Epidemiological studies provide evidence that exposure to O_3 is associated with the development of asthma (1), most notably the nonatopic (nonallergic) subtype of asthma (3, 4). The biologic plausibility of O_3-induced, nonatopic asthma is supported by data showing the development of key disease features in humans and animal models after O_3 exposure. Examples of this include eosinophilic airway inflammation in children without atopy (5); goblet cell hyperplasia and airway hyperresponsiveness in nonhuman primates (6, 7); mucous cell metaplasia in rats chronically exposed to O_3, which persisted for several weeks after O_3 exposures ceased (7), and, lastly, eosinophilic upper and lower airway inflammation in mice repeatedly exposed to O_3, which is dependent on type 2 innate lymphoid cells (7). The effects of repeated O_3 exposure in mice were also shown to be strain-dependent (8). These strain-dependent effects are indicative of gene–environment interaction (GxE) and provide a means by which to discover genes that mediate the response to repeated O_3 exposure.

Previous studies have examined GxE in response to a single O_3 exposure using primarily classical inbred strains and derivatives thereof (*see* Table E1 in the data supplement). Identification of GxE using mouse models has become more powerful with the advent of new mouse genetic reference populations such as the Collaborative Cross (CC). The CC is a panel of recombinant inbred strains derived from eight inbred strains (9), including three wild-derived inbred strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ), which in total captures ~90% of known genetic variation present in laboratory mice (10) (>40 million single nucleotide variants as well as structural variants). In addition, new allelic combinations in the CC produce novel, "emergent" phenotypes; for example, CC strain CC011/Unc develops colitis spontaneously (11) and CC027/GeniUnc is the only strain shown to be susceptible to peanut-induced anaphylaxis after oral peanut exposure (12). Given these findings, we sought to exploit the genetic diversity in the CC to identify a new model of O_3-induced nonatopic asthma. More specifically, our goal was to survey CC strains to identify one that exhibits heightened susceptibility to O_3 compared with previously tested strains (8) and develops hallmark traits of asthma, including airway eosinophilia, mucous cell metaplasia, and airway hyperresponsiveness.

In this study, we exposed female mice from 12 CC strains to 0.8 ppm O_3 (4 hours/day) for 9 days, based on a "repeated O_3 exposure" model (Figures 1A and E1 and Table E2) developed by Harkema and colleagues (7, 8). We used female mice only for logistic reasons and because our prior study of acute O_3 exposure-induced airway neutrophilia in five CC strains showed no demonstrable sex effects (13). Across the 12 strains evaluated here, repeated O_3 exposure caused variable degrees and types of airway inflammation, as measured by BAL differential cell counts (Figure E2). Most notably, compared with the other CC strains tested or the classical inbred strains C57BL/6NTac and BALB/cNTac (8), CC002/Unc (hereafter referred to as "CC002") exhibited extreme eosinophilic inflammation. BAL fluid from CC002 mice contained nearly 40% eosinophils versus 0–7% across the other strains (Figure 1A) and 0–2% in C57BL/6NTac and BALB/cNTac (8). Compared with eosinophils, neutrophils and lymphocytes accounted for a small fraction of the total number of leukocytes in BAL in CC002 (Figure E2). BAL protein, a marker of tissue injury, was also elevated after repeated O_3 exposure in most strains and the magnitude of CC002's response was second highest after CC025 (Figure E3). Overall, the exaggerated eosinophilic inflammation in CC002 in response to O_3 suggested it may represent a new model of nonatopic asthma; thus, we focused our follow-up experiments on this strain.

First, in a replication experiment involving more mice per exposure group, we again observed marked eosinophilia in O_3-exposed CC002 mice (Figure E4), demonstrating reproducibility. We then examined the histopathology of CC002 airways and found marked peribronchiolar and perivascular inflammatory cell infiltrates (Figure E5). Immunohistochemical staining for major basic protein confirmed the predominantly eosinophilic nature of this inflammation in large airways (Figure 1C). Alcian blue–periodic acid–Schiff–stained lung sections indicated that repeated O_3 also caused mucous cell metaplasia (Figure 1C) that on average was mild in small diameter bronchioles (Figure E6A). Morphometric assessment of Alcian blue–periodic acid–Schiff–stained mucosubstances in respiratory epithelium lining the mid-axial bronchiolar airway correlated with the semiquantitative severity scoring of mucous cell metaplasia throughout the lung lobe of exposed CC002 mice (Figure E6B). Consistent with this histopathology, *Muc5ac* (mucin 5ac) gene expression was significantly elevated in O_3-exposed CC002 mice, as was *Clca1* (chloride channel accessory 1) (Figure E7). We also observed increased expression of *Chil4* (chitinase-like 4), which is often upregulated in type 2 airway inflammation (14) (Figure E8). In addition, we found that repeated O_3 exposure caused an increase in total serum IgE (Figure E8), which is perhaps not surprising given that increased expression of total IgE has been observed after repeated O_3 exposure previously (15). Finally, we determined that repeated O_3 exposure increased total lung resistance at baseline and in response to methacholine challenge in CC002 mice (Figure 1D).

Our study demonstrates the effectiveness of using the CC to identify new mouse models of susceptibility to air pollutant-induced respiratory disease phenotypes. The discovery of CC002's exaggerated eosinophilic response to repeated O_3 exposure adds to the growing knowledge about gene-by-ozone interactions (16); our results also show that O_3 can cause hallmark asthma phenotypes, including airway hyperresponsiveness in less than two weeks in this strain. We note that because we used female mice only, we cannot rule out the possibility of sex effects in this exposure paradigm; this should be addressed in future studies (17). In total, CC002's unique response to repeated O_3 provides an

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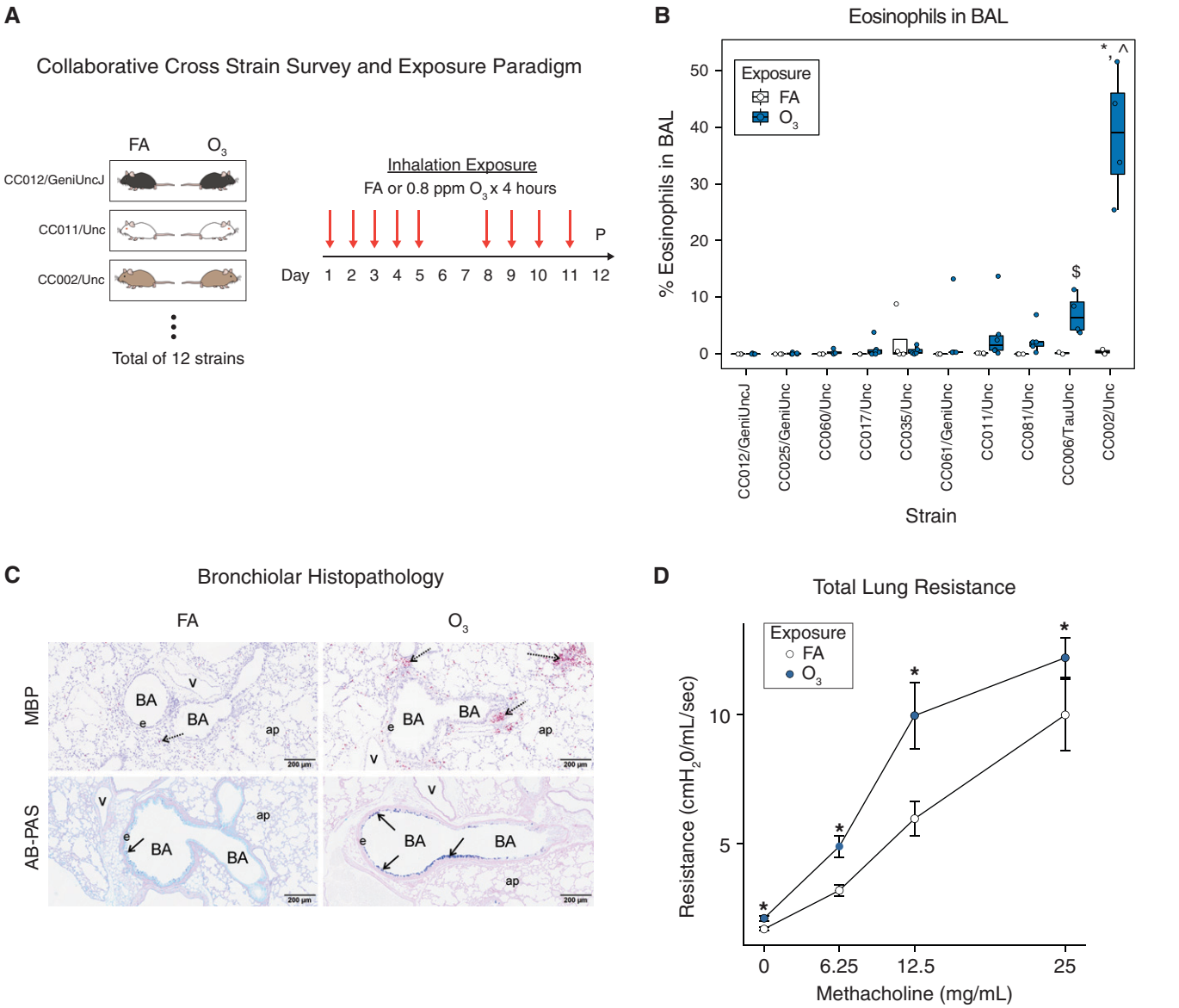

Figure 1. Repeated O_3 exposure causes eosinophilic inflammation, airway mucous cell metaplasia, and airway hyperresponsiveness in CC002 mice. (*A*) Experimental design involving repeated exposure to O_3 or filtered air (FA) across 12 Collaborative Cross (CC) strains. Mice were phenotyped 21 hours after last exposure. (*B*) Variation in O_3-induced airway eosinophils in BAL fluid in 10 out of 12 CC strains tested. See Supplement for data on other cell types and CC strains. $^*P < 0.05$ and $^\$P < 0.01$ for O_3 versus FA; $\hat{}P < 0.05$ versus other strains. FA, $N = 2$–4 per strain; O_3, $N = 4$–6 per strain. (*C*) Representative histological sections of FA controls and O_3 exposed CC002 mice showing (top) peribronchial eosinophilic inflammation, detected by immunohistochemical staining for major basic protein (MBP, red stain), and (bottom) mucous cell metaplasia in bronchiolar epithelium as detected by Alcian blue–periodic acid–Schiff (AB-PAS) staining. Scale bars, 200 μm. BA = bronchial airway, e = epithelium, ap = alveolar parenchyma, v = blood vessel. Arrows denote regions of airways featuring eosinophils or mucous cells. (*D*) Total lung resistance at baseline and after escalating doses of methacholine in O_3 exposed (vs. FA control) CC002 mice in a separate experiment. $N = 7$ per group. $^*P < 0.05$ versus FA control.

exciting opportunity to illuminate novel mechanisms that underlie the association between O_3 exposure and nonatopic asthma and motivate the use of quantitative trait locus mapping approaches to identify the genetic variants that underlie CC002's susceptibility. Because the CC002 genome contains alleles and allelic combinations not previously studied in the context of O_3 exposure, we expect this approach will likely lead to the identification of genes that have not been previously implicated in response to air pollutants. ■

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An Acceptable Concentration (0.1 ppm) of Ozone Exposure Exacerbates Lung Injury in a Mouse Model

To the Editor:

Ozone, which is present in the atmosphere, has a strong oxidation effect and has been used for disinfection and sterilization. Ozone generators have increasingly been installed at medical institutions, where they have frequently been used for infection control against coronavirus disease (COVID-19). However, an acceptable concentration of ozone (0.1 ppm) exposure has been defined on the basis of its effects on healthy people (1). Some epidemiological studies suggest that ozone exposure may be an environmental risk factor for acute respiratory distress syndrome (2), but its toxic effects in patients with respiratory dysfunction remain uncertain. The aim of this study was to compare the toxic effects of ozone exposure on an untreated mouse with its effects on a mouse with acute lung injury (ALI).

In this study, we compared the development of ALI symptoms in untreated mice with their development in a mouse model of ALI with and without exposure to ozone (0.1 ppm). Eight-week-old female BALB/c mice (Japan SLC, Inc.) were used, and ALI was developed by intranasal administration of LPS (from *Escherichia coli* O111:B4; Sigma-Aldrich) 48 hours before killing the animals (3–5). Mice were exposed to ozone 5 consecutive days before being killed. An acceptable concentration of ozone in Japan and the United States

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