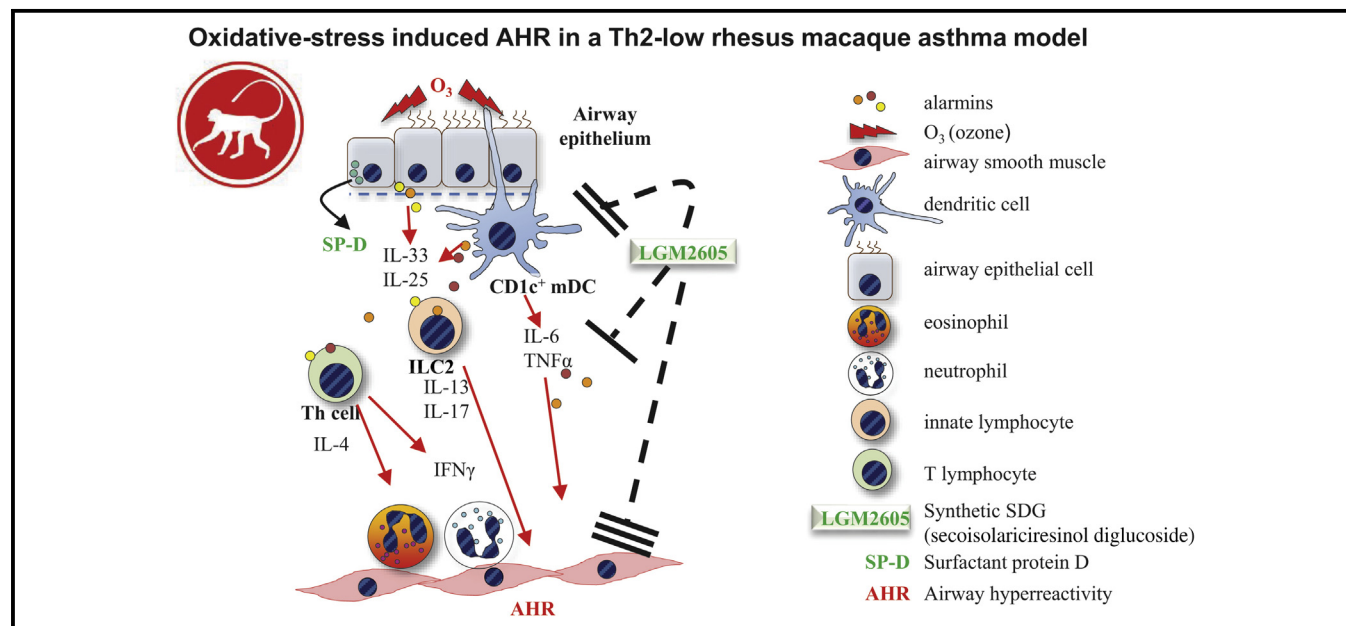


Ozone-induced enhancement of airway hyperreactivity in rhesus macaques: Effects of antioxidant treatment

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GRAPHICAL ABSTRACT



Background: Ozone (O₃) inhalation elicits airway inflammation and impairs treatment responsiveness in asthmatic patients. The underlying immune mechanisms have been difficult to study because of the lack of relevant experimental models. Rhesus

macaques spontaneously have asthma and have a similar immune system to human subjects.

Objectives: We sought to investigate mucosal immune changes after O₃ inhalation in a clinically relevant nonhuman primate asthma model and to study the effects of an antioxidant synthetic lignan (synthetic secoisolariciresinol diglucoside [LGM2605]).

Methods: A cohort of macaques (n = 17) previously characterized with airway hyperreactivity (AHR) to methacholine was assessed (day 1). Macaques were treated (orally) with LGM2605 (25 mg/kg) or placebo twice per day for 7 days, exposed to 0.3 ppm O₃ or air for 6 hours (on day 7), and studied 12 hours later (day 8). Lung function, blood and bronchoalveolar lavage (BAL) fluid immune cell profile, and bronchial brushing and blood cell mRNA expression were assessed.

Results: O₃ induced significant BAL fluid neutrophilia and eosinophilia and increased AHR and expression of *IL6* and *IL25* mRNA in the airway epithelium together with increased BAL fluid group 2 innate lymphoid cell (ILC2s), CD1c⁺ myeloid dendritic cell, and CD4⁺ T-cell counts and diminished surfactant protein D expression. Although LGM2605 attenuated some of the immune and inflammatory changes, it completely abolished O₃-induced AHR.

Conclusion: ILC2s, CD1c⁺ myeloid dendritic cells, and CD4⁺ T cells are selectively involved in O₃-induced asthma

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exacerbation. The inflammatory changes were partially prevented by antioxidant pretreatment with LGM2605, which had an unexpectedly disproportionate protective effect on AHR. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: Asthma, airway inflammation, air pollution, therapies (investigational), nonhuman primate

Air pollution was identified as one of the most significant contributors to the prevalence, severity, and chronicity of asthmatic airways inflammation. In our highly polluted neighboring San Joaquin County, the lifetime prevalence of asthma for children aged 5 to 17 years was greater than 28% in a 2013 survey (<https://www.cdph.ca.gov/Programs/CCDC/DEODC>). Asthmatic patients are particularly susceptible to airway obstruction, inflammation, and impaired responsiveness to treatment after exposure to the toxic air pollutant ozone (O₃).^{1,2} O₃ induces direct oxidative modifications of biomolecules in the respiratory tract, leading to proinflammatory activation of mucosal tissue and neutrophilic airway activation.³ The pathogenic role of oxidative stress in asthmatic patients is accepted, but studies that target oxidative burden using a variety of nutritional, pharmacologic, and environmental approaches have been controversial,⁴ and the underlying immune mechanisms responsible for the airway changes remain unclear.

Using mice, our laboratory previously showed that airway epithelial cells, dendritic cells (DCs), natural killer cells,⁵ and group 2 innate lymphoid cells (ILC2s)⁶ play highly proinflammatory roles, whereas the presence of the immunoprotective surfactant protein D (SP-D), an epithelium-derived lung collectin, is important in attenuating the O₃-induced inflammatory process.⁵⁻⁹ Establishment of the clinical relevance of these findings has been hindered because of the lack of adequate experimental systems.

We turned to study the nonhuman primate rhesus macaques because their genetic makeup is highly similar to that of human subjects, with a high degree of immune cross-reactivity and shared receptor characteristics.¹⁰⁻¹³ Macaques, like human subjects, naturally develop airway hyperreactivity (AHR), with peripheral and mucosal immunologic responses demonstrating individual variability,^{14,15} making these animals more suitable than rodents to study asthma.¹⁶ As a result of clinical asthma trials using T_H2-targeting biologics over the last few years, it has become clear that more than 50% of asthmatic patients belong to a “T_H2-low” endotype.¹⁷ New therapeutic approaches are needed for these patients because they are not responsive to T_H2-targeting strategies and also demonstrate poor glucocorticoid responsiveness. At the California National Primate Research Center (CNPRC), macaques that naturally had AHR show no T_H2 cell activation and have immune cells unresponsive to glucocorticoids.¹² Thus these animals might represent patients with “T_H2-low” asthma and are therefore uniquely poised for investigation of novel alternative or adjuvant approaches to glucocorticoid treatment.

The synthetic version of secoisolariciresinol diglucoside (LGM2605), the bioactive lignan found in flaxseed, has free radical scavenging, antioxidant, and anti-inflammatory properties¹⁸ acting through multiple pathways in mice and *in vitro* inflammatory models.¹⁸⁻²² LGM2605 inhibited human and murine myeloperoxidase activity, induced antioxidant enzymes in macrophages,¹⁸ and reduced oxidative tissue damage

Abbreviations used

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
CNPRC:	California National Primate Research Center
DC:	Dendritic cell
EC150:	Effective concentration of methacholine that increased lung resistance by 150%
GST:	Glutathione-S-transferase
GSTT:	Glutathione-S-transferase theta
hASM:	Human airway smooth muscle
HBE1:	Human bronchial epithelial
ILC:	Innate lymphoid cell
ILC2:	Group 2 innate lymphoid cell
ILC3:	Group 3 innate lymphoid cell
LGM2605:	Synthetic secoisolariciresinol diglucoside
mDC:	Myeloid dendritic cell
O ₃ :	Ozone
pDC:	Plasmacytoid dendritic cell
qPCR:	Quantitative real-time PCR
Raw:	Airway resistance to methacholine
SP-D:	Surfactant protein D
TBHP:	Tert-butyl hydroperoxide

in human precision-cut lung slices.¹⁸⁻²² Because of its synthetic availability, this compound can be administered at a precise and biologically relevant dose.

Based on our mouse models and extensive studies demonstrating the *in vitro* and *in vivo* anti-inflammatory potential of LGM2605, we hypothesized that O₃-induced exacerbation of asthma in macaques will be associated with neutrophilic and eosinophilic airway inflammation and activation of the airway mucosal innate immune system and will be amenable to antioxidant targeting with LGM2605. We aimed to (1) comprehensively define lung function and mucosal immune changes after O₃ exposure in rhesus macaques and (2) assess the effects of LGM2605. We designed a longitudinal study applying minimally invasive procedures to sequentially sample animals before and after O₃ exposure and LGM2605 administration.

METHODS

Subjects

Two- to 3-year old male and female age- and sex-matched rhesus macaques were enrolled into this study from a preselected cohort that presented with AHR within the past 12 months. Animals were relocated from outdoor field cages at the CNPRC to the experimental rooms (subject information is shown in Table E1 in this article's Online Repository at www.jacionline.org). Macaques were randomly segregated into experimental groups (LGM2605 plus air, placebo plus O₃, or LGM2605 plus O₃). During acclimation indoors, macaques were trained to accept LGM2605 or placebo in small treats (peanut butter sandwiches).

Before baseline measurements on day 1 (blood draw, bronchoalveolar lavage [BAL], bronchial brush biopsy, and pulmonary function test), animals were fasted overnight (Fig 1, A). Animals were immobilized with an intramuscular injection of ketamine (5-30 mg/kg), and a blood sample (up to 20 mL) was drawn from a peripheral vessel in accordance with veterinary staff guidelines. After blood draw, monkeys were further anesthetized with intravenous propofol (10-30 mg/kg/h), with the dose adjusted as deemed necessary by the attending veterinarian.

Macaques were then intubated with an appropriately sized cuffed endotracheal tube. Static lung mechanics and airway responsiveness to methacholine

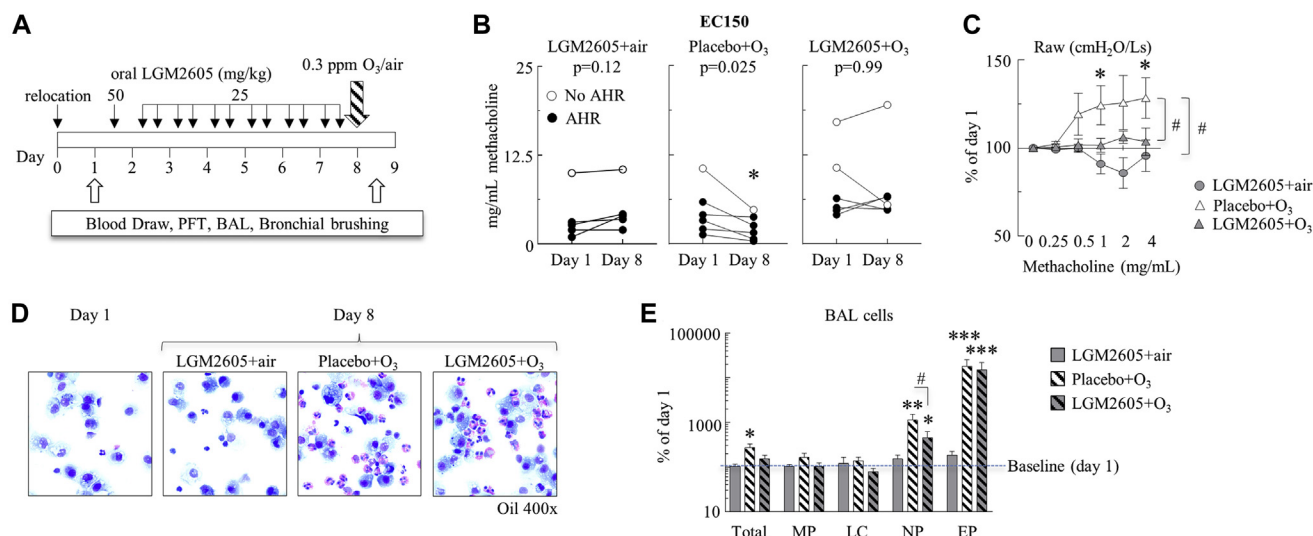


FIG 1. O₃-induced increases in AHR were prevented by LGM2605 treatment. **A**, Study timeline. Baseline measurements were performed on selected macaques after relocation indoors (day 1). Animals were given LGM2605 orally (as a small treat or in a peanut butter sandwich) daily for 7 days and then exposed to 0.3 ppm O₃ or air for 6.5 hours. Twelve hours after exposure, blood draw and bronchoscopy were repeated (day 8). PFT, Pulmonary function test. Experimental groups were as follows: LGM2605 plus air, placebo plus O₃, and LGM2605 plus O₃. **B**, EC150 on days 1 and 8 (in milligrams per milliliter; means $\pm$ SEMs of $n = 5-6$). * $P < .05$, day 1 versus day 8, Student paired t test. **C**, Methacholine dose response (Raw; percentage of day 1; each macaque served as its own control). **D**, Representative photomicrographs of Kwik-Diff–stained cytospin preparations from day 1 and day 8 BAL fluid, indicating neutrophil and eosinophil influx in O₃-exposed animals. **E**, Macrophages (MP), lymphocytes (LC), neutrophils (NP), and eosinophils (EP) were differentially counted on cytospin preparations (absolute cell counts are expressed as percentages of day 1; each macaque served as its own control). Fig 1, C and E, Mean $\pm$ SEM of $n = 5-6$. * $P < .05$, ** $P < .01$, and *** $P < .001$, day 1 versus day 8 or versus 0 mg/mL methacholine (Fig 1, C), 2-way ANOVA with Bonferroni multiple comparisons. # $P < .05$, between groups, 2-way ANOVA.

challenge were measured (additional details are shown in the [Methods](#) section in this article's Online Repository at www.jacionline.org). Fig E1 in this article's Online Repository at www.jacionline.org shows that 4 animals in our cohort were not displaying AHR on day 1, and the other 13 were. After pulmonary function tests, BAL fluid was harvested, and a bronchial brush biopsy was performed (additional details are provided in the [Methods](#) section in this article's Online Repository). Although the animals were still sedated, a single 50 mg/kg dose of LGM2605 was administered with a syringe. Macaques were then treated twice per day with 25 mg/kg LGM2605 (days 2-7). After the final dose of LGM2605, macaques were exposed to air or 0.3 ppm O₃ for 6.5 hours. Twelve hours later, blood draw and bronchoscopy were repeated (day 8). Macaques then rested indoors overnight before being returned to their native outdoor field cage or when deemed appropriate by the staff veterinarian. The University of California, Davis, Institutional Animal Care and Use Committee approved the experimental protocol.

This design was performed 6 times. Each time, 3 macaques were relocated indoors and randomly assigned to one of 3 experimental groups. This ensured that if any outdoor air quality events would affect the results of the study, they would be evenly distributed among the experimental groups. Between December 2017 and February 2018, when the study was performed, there were no significant outdoor air quality events at the CNPRC.

Source of LGM2605

LGM2605, a lignan originally derived from flaxseed, was synthesized and supplied by LignaMed (Philadelphia, Pa). The lyophilized product was dissolved in sterile water (at 0.62 mg/mL) and stored at -80°C . The stock was used to freshly make up the compound for treatment every day.

O₃ exposure

O₃ exposures were conducted in 5.1-m³ stainless steel and glass whole-body inhalation chambers that have been described previously.²³

O₃ was produced from vaporized liquid, medical-grade oxygen by electric discharge ozonizers. O₃ was mixed with high-efficiency particulate air- and activated carbon–filtered dilution air and pumped into the inlet airflow of the exposure chamber with 30 air changes per hour for an exposure concentration of 0.3 ppm that was actively maintained with a proportional control system. We selected to use exposure to 0.3 ppm O₃ for 6.5 hours because studies conducted in healthy young subjects showed respiratory effects between 0.04 and 0.6 ppm during physical activity.²⁴ Furthermore, in highly polluted urban, suburban, and rural areas in China, the hourly and 8-hourly maximum concentrations of ambient O₃ and O₃ precursor levels are frequently between 0.1 and 0.3 ppm.²⁵ We reasoned that an exposure to 0.3 ppm O₃ for a prolonged period (>6 hours) would simulate the effects of a day with poor air quality. O₃ concentration, airflow, temperature, pressure, and humidity were monitored throughout the exposure. High-efficiency particulate air- and activated carbon–filtered air was injected into the inlet airflow of the exposure chamber with 30 changes per hour for the LGM2605 plus air control group, which maintained O₃ levels of less than 0.005 ppm.

PBMC isolation

Blood was layered over Lymphoprep (STEMCELL Technologies, Vancouver, British Columbia, Canada) and centrifuged at 20°C for 20 minutes, with brake and acceleration speed set to 0. Cells were harvested from the interphase layer and washed twice with ice-cold PBS. Cells were counted with the Countess Automated Cell Counter (Thermo Fisher Scientific, Waltham, Mass) and then apportioned for downstream flow cytometry and fluorescence-activated cell sorting experiments.

BAL fluid cells and supernatants

Total cells recovered in BAL fluid were counted with the Countess Automated Cell Counter (Thermo Fisher Scientific). Fifty thousand cells were cytospun and stained with Shandon Kwik-Diff Stain (Thermo Fisher

Scientific). Differential cell counts were measured at $\times 400$ magnification under a light microscope to determine the proportion and absolute number of macrophages, lymphocytes, neutrophils, and eosinophils. The remaining cells were apportioned for flow cytometry. In the text and figures we report the absolute count for each cell type in BAL fluid (cells per milliliter of BAL fluid).

Immune and inflammatory cell morphology appeared normal in the BAL fluid samples, and macrophages displayed inflammatory alterations in response to O_3 , as expected. We noted a few small dark blue inclusions in some of the macrophage cytoplasm samples across all experimental groups and time points, the source and nature of which were unclear. We speculated that these inclusions could come from wildfire smoke-derived particles because the macaques are housed in outdoor corrals, but because there was no wildfire smoke exposure within 1 year before our study, this would be an unlikely source (unless the macrophages with inclusions persisted more than a year after the actual exposure occurred). The inclusions might also be of microbial origin through infection (although comprehensive medical assessment of our cohort did not reveal any signs of infection) or through oropharyngeal contamination of the BAL fluid samples during collection. Further investigation of this interesting phenomenon was outside the scope of the present study.

Bronchial brush biopsy specimens

Bronchial brush biopsy specimens were obtained from 6 sites per animal at airway generations 2 to 5 by using 1×6 -mm brushings and deposited into sample tubes containing PBS. The harvested cells were then placed in aliquots, snap-frozen, and stored at -80°C in TRIzol Reagent (Thermo Fisher Scientific).

Flow cytometry, quantitative real-time PCR, and Western blotting

Flow cytometry, quantitative real-time PCR (qPCR), and Western blot protocols are described in the [Methods](#) section in this article's Online Repository.

Culture of human cell lines *in vitro*

Primary human airway smooth muscle (hASM) and papilloma virus-immortalized human bronchial epithelial (HBE1) cells were kindly donated by Dr Yang Kevin K. Xiang (University of California, Davis) and Dr J. Yankaskas (University of North Carolina), respectively, to test the mechanism of action of LGM2605 *in vitro*. hASM cells were maintained in F12 (Ham) nutrient, 1% penicillin-streptomycin, 200 mmol/L L-glutamine (all from Thermo Fisher Scientific), 1 mol/L HEPES, CaCl_2 , NaOH (all from Sigma-Aldrich, St Louis, Mo), and 10% FBS (Hyclone, Logan, Utah), whereas HBE1 cells were maintained, as previously described.²⁶ Cells were grown to confluency in T75 flasks and then split into 12-well plates. At 0 hours, medium was replaced with fresh medium containing 0, 12.5, 25, or 50 $\mu\text{mol/L}$ LGM2605. Ten hours later, tert-butyl hydroperoxide (TBHP) or control H_2O was added to bring the final concentration to 0.05 or 0 mmol/L, respectively. At the 12-hour time point, the TBHP-containing medium was replaced with fresh medium containing LGM2605. At the 24-hour time point, cells were harvested in TRIzol. Details of RNA extraction and qPCR are provided in the [Methods](#) section in this article's Online Repository.

Data analysis

Statistical analysis was performed with Prism software (version 7; GraphPad Software, La Jolla, Calif). Data are expressed as means $\pm$ SEMs, unless otherwise specified, and are representative of 6 independent experiments ($n = 5$ -6 macaques per group). Two-way ANOVA with the Bonferroni multiple comparison test was used to compare experimental groups. When each animal served as its own control (percentage change or fold change), the Student paired t test was used (day 1 [before] vs day 8

[after]). Asterisks denote statistically significant changes within experimental groups (ie, day 1 vs day 8), whereas hash marks show significance between experimental groups (ie, day 8 placebo plus O_3 vs day 8 LGM2605 plus O_3). A P value of less than .05 was considered significant.

RESULTS

O_3 -induced increases in AHR were prevented by antioxidant (LGM2605) treatment

We selected 17 age- and sex-matched macaques in the present study from one of our previously characterized cohorts of animals¹³ that displayed AHR within the past year. Baseline (day 1) lung function measurements showed that 13 macaques still had "AHR" (effective concentration of methacholine that increased lung resistance by 150% [EC150], <8 mg/mL), and 4 had "no AHR" (see [Fig E1](#)). None of the 17 animals had any remarkable airway inflammation at baseline (day 1; [Fig 1, D](#)).

To investigate the effects of O_3 on lung function in macaques with pre-existing AHR and to study whether antioxidant treatment would affect the O_3 -induced changes, we developed a longitudinal experimental design. This design allowed for a before-and-after comparison of parameters in the same individual macaques to control for genetic heterogeneity and baseline variability of AHR in the outbred macaque cohort. After baseline measurements (day 1), macaques underwent 7 days of oral treatment with LGM2605 (a synthetic lignan compound with potent antioxidant properties)¹⁸⁻²² or placebo before exposure to O_3 and a repeat of measurements (day 8; [Fig 1, A](#)). The mechanism of the LGM2605 action in protecting against oxidative damage is thought to involve both free radical scavenging and induction of antioxidant enzymes.^{18-22,27,28}

To investigate the effects of LGM2605, we used a different model of radiation lung injury (described in the [Methods](#) section in this article's Online Repository). LGM2605 treatment of irradiated rhesus macaques induced dose-dependent increases in multiple nuclear factor-like 2 target antioxidant gene expression in the lung (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

Airway resistance to methacholine (Raw) inhalation was dose dependent (see [Fig E1, B](#)), and O_3 exposure significantly increased Raw after placebo but not LGM2605 treatment when day 8 data were expressed as a function of day 1 data in individual macaques ([Fig 1, C](#)). Remarkably, when monkeys were exposed to O_3 , their EC150 values on day 8 were significantly decreased compared with those on day 1 in the placebo- but not the LGM2605-treated group ([Fig 1, B](#)). Of note, our sample size design was powered for paired (before and after) statistics. Therefore although the original Raw values show similar trends when depicted as day 1 and day 8 group averages (see [Fig E3](#) in this article's Online Repository at www.jacionline.org), without controlling for individual variability, no statistically significant differences could be demonstrated, highlighting the importance of the macaques' variability in airway responsiveness. Furthermore, in animals exposed to air and treated with LGM2605, there was a trend for increased EC150 and decreased Raw values ([Fig 1, C](#)), suggesting that this antioxidant treatment might also improve baseline airway obstruction in asthmatic macaques. Taken together, these data indicated that O_3 exposure significantly increased airway responsiveness to methacholine in macaques and that antioxidant LGM2605 treatment prevented such an increase.

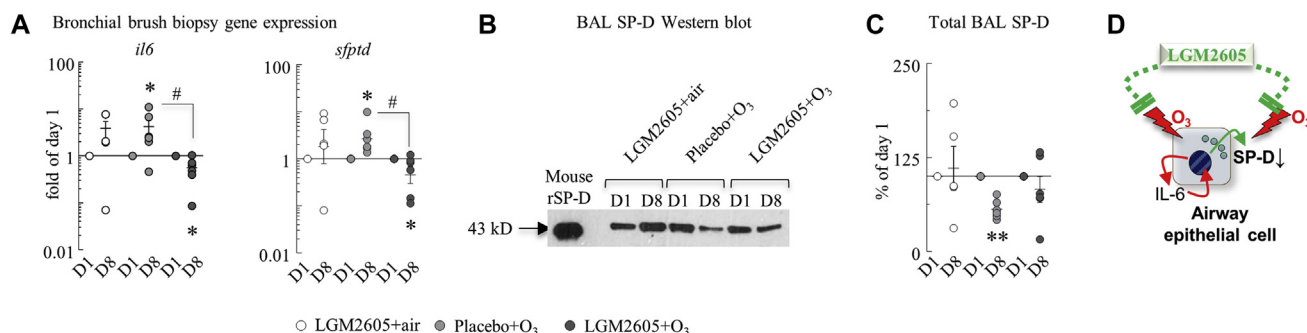


FIG 2. O₃ induced *IL6* and *SFTPD* mRNA expression in airway epithelial cells but diminished SP-D protein expression in BAL fluid. LGM2605 alleviated these effects. **A**, RNA was extracted from bronchial brush biopsy specimens, and expression of the *IL6* and *SFTPD* genes was measured by using qPCR. $\Delta\Delta$ Cycle threshold (*GAPDH* and *RPL32*), Fold of baseline/day 1; each macaque served as its own control. **B**, Representative reducing SDS-PAGE Western blot detecting nonhuman primate BAL fluid SP-D. Mouse (and human; data not shown) recombinant SP-D (*rSP-D*) was used as a loading control. **C**, Densitometric values (reducing SDS-PAGE gels [ImageJ software, National Institute of Health, Bethesda, Md]; percentage of day 1; each macaque served as its own control). **D**, Schematic depiction of the dual antagonistic effects of O₃ and LGM2605. O₃ induces IL-6 production in an autocrine manner that increases SP-D synthesis. O₃-induced oxidative damage diminishes released SP-D. LGM2605 interacts with these events. Mean $\pm$ SEM of $n = 5-6$. * $P < .05$, within groups, day 1 versus day 8, Student paired t test. # $P < .05$, between groups, 2-way ANOVA.

Previously, we showed in allergen-sensitized and challenged mice that O₃ exposure markedly amplified airway neutrophil and eosinophil counts.^{6,9,29} Similarly, in macaques in this study there were highly significant increases in total cell counts, as well as in proportions of both neutrophils and eosinophils in the airways after O₃ exposure (Fig 1, D and E) when data were expressed either as a percentage of the day 1 value (Fig 1, E) or as absolute counts (see Fig E4 in this article's Online Repository at www.jacionline.org). LGM2605 (but not placebo) had a significant and selective inhibitory effect on BAL fluid neutrophilia, but eosinophils were unaffected after O₃ exposure (Fig 1, E, and see Fig E4). In our studies on mouse models of allergen or O₃-induced airway inflammation, the extent of eosinophil³⁰ and neutrophil (unpublished observations) influx, respectively, strongly correlated with changes in lung function. Therefore the weak correlation we saw between EC150 methacholine concentrations and BAL fluid eosinophil or neutrophil cell counts of O₃-exposed macaques were unexpected (see Fig E4, B). These results suggest that LGM2605 eliminated the O₃ effects on AHR, even in the presence of large numbers of eosinophils, raising a question about the role of these cells in airway physiology.

O₃ induced *IL6* and *SFTPD* mRNA expression in airway epithelial cells but diminished SP-D protein levels in BAL fluid, whereas LGM2605 attenuated these effects

To study the molecular mechanisms involved in O₃-induced inflammation and AHR, we investigated SP-D expression at the mRNA and protein level in bronchial brush biopsy specimens and BAL fluid supernatants, respectively, from macaques. We also studied expression of *IL6* mRNA because this is a major cytokine induced by O₃ and because we previously showed in mice that IL-6 can stimulate SP-D gene expression.

In the airway epithelium O₃ induced *IL6* and *SFTPD* mRNA. This effect was reversed by LGM2605 (Fig 2, A). Native gel electrophoresis showed that O₃ exposure did not change the oligomeric SP-D structure in macaque BAL fluid (see Fig E5 in this article's Online Repository at www.jacionline.org). However, O₃ significantly reduced total BAL SP-D protein concentrations. This effect was also prevented by LGM2605 (Fig 2, B and C). Thus O₃ induced *SFTPD* gene activation, likely through autocrine effects of IL-6 on airway epithelial cells. Oxidative stress diminished SP-D protein levels that were reversed by LGM2605 (Fig 2, D).

O₃ increased ILC2 counts in BAL fluid, which were attenuated by LGM2605

We hypothesized that the effects of O₃ would involve activation of ILC2s, a previously undescribed cell population in macaques (see Fig E6 in this article's Online Repository at www.jacionline.org). Expression of the ILC2-activating cytokine genes *IL25* and *IL33* were induced by O₃ in airway epithelial cells, although changes in *IL33* mRNA expression did not reach statistical significance (Fig 3, A). LGM2605 significantly inhibited both *IL25* and *IL33* genes. O₃ increased both the proportion and absolute count of BAL fluid ILC2s in placebo- but not LGM2605-treated macaques (Fig 3, B and C). This effect was selective because other innate lymphoid cell (ILC) counts did not change on O₃ exposure or antioxidant treatment. The pie charts show that the most abundant ILCs in the macaques were group 1 ILCs, followed by ILC2s and then NCR3⁻ and NCR3⁺ group 3 innate lymphoid cells (ILC3s) in the airways. In peripheral blood there were more NCR3⁻ ILC3s than ILC2s (Fig 3, D). O₃ increased the ILC2 proportion in BAL fluid and the NCR3⁻ ILC3 proportion in peripheral blood (Fig 3, D). Furthermore, qPCR showed that O₃ reduced gene expression of fluorescence-activated cell sorting isolated ILC2 for *GATA3*, *IL13* and the neutrophilia-inducing proinflammatory cytokine

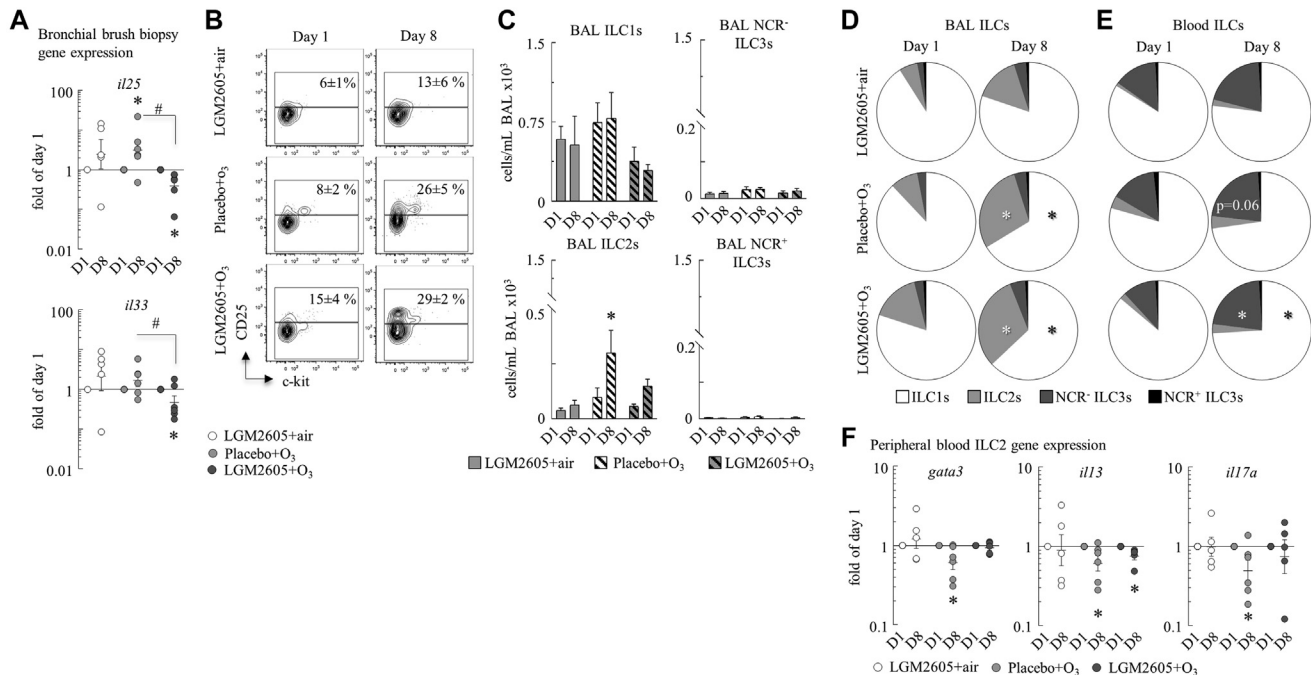


FIG 3. O₃ increased ILC2 counts in BAL fluid and NCR⁺ ILC3 counts in peripheral blood. **A**, RNA was extracted from bronchial brush biopsy specimens, and expression of the *IL25* and *IL33* genes was measured by using qPCR. $\Delta\Delta$ Cycle threshold (*GAPDH* and *RPL32*), Fold of baseline/day 1 values in each individual monkey. **B**, Blood and BAL fluid ILCs: live CD90⁺Lin⁻CD127⁺ cells. ILC2s, CD25⁺c-kit^{var} ILCs. Numbers show means $\pm$ SEMs of the percentage of ILCs that were CD25⁺ (ILC2s). **C**, Group 1 ILCs (ILC1s), ILC2s, NCR⁻ ILC3s, and NCR⁺ ILC3s were quantified (cells per milliliter of BAL fluid $\times$ 10³). ILC1s, c-kit⁻NKp44⁻ ILCs; NCR⁺ ILC3s, NKp44⁺c-kit⁺ ILCs; NCR⁻ ILC3s, NKp44⁻c-kit⁺ ILCs. **D** and **E**, Pie charts: relative proportions of ILC subsets on day 1 and day 8. **F**, RNA was extracted from blood ILC2s (live Lin⁻CD90⁺CD25⁺c-kit^{var} cells), and expression of *GATA3*, *IL13*, and *IL17A* was measured by using qPCR. $\Delta\Delta$ Cycle threshold (*GAPDH*), Fold of baseline/day 1; each macaque served as its own control. Mean $\pm$ SEM of $n = 5-6$. * $P < .05$, within groups, day 1 versus day 8 (Fig 3, A and D-F: Student paired t test; Fig 3, C: 2-way ANOVA). # $P < .05$, between groups, 2-way ANOVA.

IL17A (Fig 3, F) and that these changes were not affected by antioxidant treatment (Fig 3, F).

O₃-induced proinflammatory myeloid DC accumulation and activation were inhibited by LGM2605 in BAL fluid

Although it is not known how exactly DCs can regulate the inflammatory airway response, different DC subsets have specific functions (see Fig E7 in this article's Online Repository at www.jacionline.org).⁵ We studied the myeloid CD1⁺ and CD16⁺ and the plasmacytoid CD123⁺ DC populations. We found the most striking changes in absolute BAL fluid counts of CD1c⁺ myeloid dendritic cells (mDCs) and CD123⁺ plasmacytoid dendritic cells (pDCs) in response to O₃ (Fig 4, A), whereas there were no significant changes in CD16⁺ mDC counts from day 1 to day 8 (data not shown). Antioxidant LGM2605 treatment not only prevented the O₃-induced increases in CD1c⁺ mDC counts (Fig 4, B), it also reduced the number of these cells in air-exposed macaques (Fig 4, C). Furthermore, the number of highly activated proinflammatory TNF- α ⁺CD1c⁺ mDCs (but not tolerogenic CD123⁺ pDCs) was significantly increased in BAL fluid after O₃ exposure in placebo- but not LGM2605-treated animals (Fig 4, D). Expression of the membrane marker CD40, which is involved in antigen presentation and other cell

functions,³¹ was decreased by O₃ in CD1c⁺ mDCs but increased by antioxidant treatment in CD123⁺ pDCs (Fig 4, E). These findings were specific to the airways because LGM2605 treatment or O₃ exposure had no significant effects on DCs recovered from the peripheral blood of macaques (see Fig E8 in this article's Online Repository at www.jacionline.org).

O₃ activated BAL fluid CD4⁺ T cells

Because we found increased numbers of ILC2s and DCs in response to O₃ exposure in the macaques, we wanted to know whether T cells (see Fig E9 in this article's Online Repository at www.jacionline.org) were involved in the O₃ response. O₃ exposure increased absolute CD4⁺ T-cell counts (Fig 5, A and B) and activated (CD25⁺) CD4⁺ T-cell counts in BAL fluid but not CD8⁺ T-cell numbers or activation when macaques were treated with placebo (Fig 5, C-E). Expression (mean fluorescent intensity) of T_H1- and T_H2-type cytokines (IFN- γ and IL-4, respectively) were increased in CD4⁺ but not CD8⁺ T cells by O₃, regardless of LGM2605 treatment (Fig 5, F and G). In peripheral blood no changes were found in CD4⁺ or CD8⁺ T-cell numbers (see Fig E10 in this article's Online Repository at www.jacionline.org). Collectively, these data demonstrate that O₃ activated CD4⁺ (not CD8⁺) T cells in BAL fluid but not in the circulation. T_H cell activation was not affected by LGM2605 in macaques.

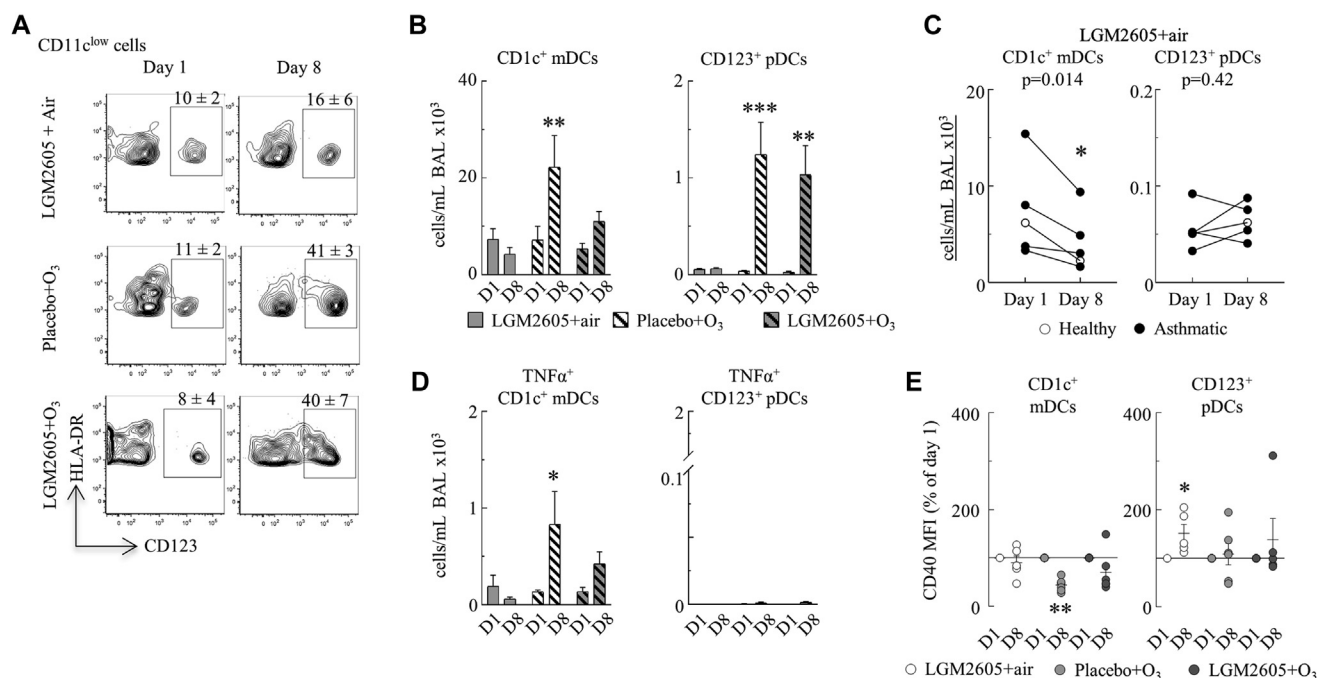


FIG 4. O₃ induced proinflammatory mDC accumulation and activation and increased antigen-presenting pDC counts in BAL fluid. **A**, CD123⁺ pDCs, Live CD45⁺CD11c^{low}HLA-DR⁺CD123⁺ cells. Mean ± SEM of CD11c^{low}HLA-DR⁺ cells that were CD123⁺ pDCs is shown. **B**, BAL fluid CD1c⁺ mDCs (live CD45⁺CD11c⁺HLA-DR⁺CD1c⁺ cells) and CD123⁺ pDCs were quantified (cells per milliliter of BAL fluid × 10³). **C**, BAL fluid CD1c⁺ mDCs and CD123⁺ pDCs were quantified in LGM2605 plus air-treated macaques (cells per milliliter of BAL fluid × 10³). **D**, TNF-α⁺CD1c⁺ mDCs and CD123⁺ pDCs (cells per milliliter of BAL fluid × 10³). **E**, CD40 expression (mean fluorescent intensity [MFI]) on DCs in BAL fluid (percentage of day 1; each macaque served as its own control). Mean ± SEM of n = 5-6. *P < .05, **P < .01, and ***P < .001, within groups, day 1 versus day 8 (Fig 4, C and E: Student paired t test; Fig 4, B and D: 2-way ANOVA).

LGM2605 induced antioxidant gene expression in the presence (but not in the absence) of TBHP in hASM *in vitro*

Our prior work suggested that the mechanism of action of LGM2605 was induction of antioxidant gene expression in cells derived from bronchial brush biopsy specimens. In the present study treatment of macaques with LGM2605 prevented O₃-induced increases in expression of the *IL6*, *SFPTD*, and *IL25* genes in cells derived from bronchial brush biopsy specimens. Because the bronchial brush biopsy specimen is comprised of a mix of airway epithelial, immune, and airway smooth muscle cells, among others, we tested the effect of LGM2605 on antioxidant gene expression (by using qPCR) in hASM and immortalized HBE1 cells.

We used TBHP to simulate O₃ exposure because TBHP is a stable form of hydrogen peroxide, a major chemical product of O₃ inhalation. hASM and HBE1 cells were treated with 0, 12.5, 25, or 50 μmol/L LGM2605 for 24 hours total, whereas TBHP or control H₂O was added to the culture medium from hours 10 to 12 (Fig 6, A). In response to TBHP exposure, the antioxidant genes *TXNLI*, *SOD1*, and *SOD2* were significantly upregulated in hASM cells (Fig 6, B). In HBE1 cells *SOD2* was upregulated and *GSTA4* was downregulated by exposure to TBHP (Fig 6, B). Expression of *TXNLI*, *SOD1*, and *SOD2* was heightened in hASM but not HBE1 cells when LGM2605 was added to the culture supernatant before and after exposure to TBHP (Fig 6, C). Together, these data suggest that hASM cells

are a unique target of LGM2605-heightened, TBHP-induced antioxidant expression.

DISCUSSION

Our goals were to characterize airway immune responses and their significance in lung function changes on O₃ inhalation in asthmatic macaques and to assess the role of oxidative stress by treating the animals with a synthetic secoisolariciresinol diglucoside, the antioxidant lignan LGM2605. We demonstrate here that O₃ significantly augmented AHR and induced airway neutrophilia and eosinophilia and increased the number and activation of airway ILC2s, TNF-α⁺CD1c⁺ mDCs, CD123⁺ pDCs, and CD4⁺ T cells in macaques. LGM2605 had a selective inhibitory effect on airway neutrophil, ILC2, and TNF-α⁺CD1c⁺ mDC counts in response to O₃ inhalation. The present work did not address whether LGM2605 would facilitate inflammatory resolution after exposure to O₃. Asthmatic macaques not exposed to O₃ had their BAL fluid total and TNF-α⁺ mDC counts reduced and showed a trend toward reduction in AHR after 1 week of LGM2605 treatment, suggesting the importance of oxidative stress in T_H2-low AHR and that this drug could be a potential therapeutic modality in asthmatic patients. LGM2605 treatment did not affect O₃-induced airway eosinophilia; however, it remarkably prevented increases in AHR, raising questions about both the role of eosinophils in mediating O₃-induced AHR and a potential specific effect of LGM2605 on airway smooth muscle

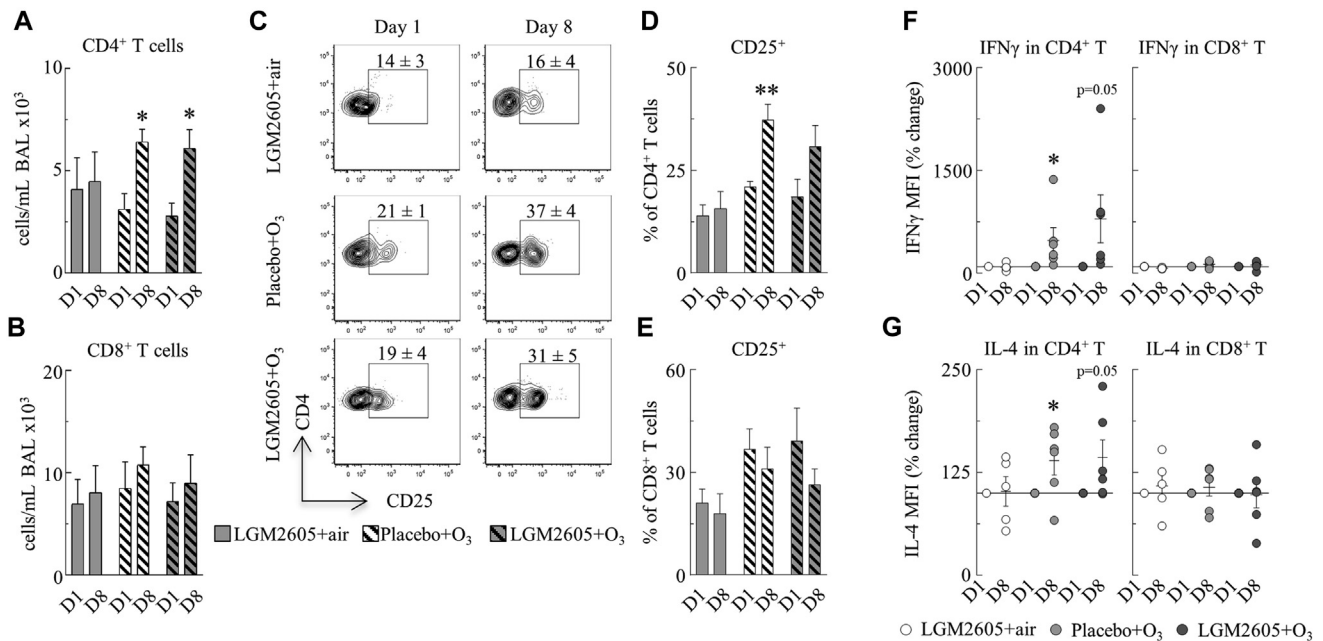


FIG 5. O₃-activated BAL fluid CD4⁺ T cells. **A** and **B**, CD4⁺ T cells (live CD45⁺CD3⁺CD4⁺CD8[−] cells) and CD8⁺ T cells (live CD45⁺CD3⁺CD4⁺CD8⁺ cells) were quantified (cells per milliliter of BAL fluid × 10³). **C–E**, Proportion of CD4⁺ and CD8⁺ T cells that were CD25⁺ (mean ± SEM in flow plots and bar graphs). **F** and **G**, IFN-γ and IL-4 expression (mean fluorescent intensity [MFI]) was measured in CD4⁺ and CD8⁺ T cells (percentage of day 1; each macaque served as its own control). Mean ± SEM of n = 5–6. *P < .05 and **P < .01, within groups, day 1 versus day 8 (Fig 5, F and G: Student paired t test; Fig 5, A–E: 2-way ANOVA).

function. Using *in vitro* human cell lines, we confirmed that in comparison with airway epithelial cells, smooth muscles were indeed highly responsive to oxidative stress, as well as to treatment with LGM2605. This is the first comprehensive description of the airway mucosal immune players involved in the nonhuman primate response to O₃ and the effects of LGM2605 on AHR (see the graphical abstract).

Previously, we showed that when naive mice are treated with O₃ alone, they have a predominantly neutrophilic airway inflammation,⁶ but when they were sensitized and challenged with an allergen before O₃ exposure, O₃ induced an eosinophil-predominant inflammatory response.²⁹ These and other investigations in mice, nonhuman primates, and human subjects suggested that O₃ affects healthy and asthmatic lungs in a highly differential manner.^{1,32,33} In the macaque studies, however, there were some discrepancies to resolve. For example, acute exposure to 0.4 or 1 ppm O₃ induced airway neutrophilia and eosinophilia but not AHR,^{34–36} whereas repeated exposures to 0.5 ppm O₃ in 11 two-week cycles elicited BAL fluid eosinophilia and AHR to histamine but not neutrophilia.^{37–40}

To reconcile these disparate findings, we carefully selected our study animals from a previously characterized age- and sex-matched cohort that had AHR within the last 12 months. In addition, we developed a longitudinal design to account for individual genetic differences and variability in patients with AHR in this outbred and heterogeneous population of macaques. In this way, we saw significant individual airway changes in response to a single exposure to O₃ at an environmentally relevant level that was not apparent previously. Furthermore, use of methacholine and not histamine for induction of lung resistance

revealed highly significant changes in AHR that previous studies might have missed.^{38,39}

However, our studies posed some new questions related to a disconnect between the changes in O₃-induced airway eosinophilia and exacerbation of AHR. Our cohort of macaques was designated “asthmatic,” with the caveat that we did not assess the reversibility of airway obstruction, and these animals did not present with T_H2-high inflammation at the baseline of the study. The cause of “T_H2-low asthma” is complex and possibly driven by multiple factors.⁴¹ The present cohort of macaques belonged to a larger population behaviorally investigated for manifestations of psychosocial stress, which is both a comorbidity and a risk factor for asthma.^{12,42,43} Indeed, approximately 60% of stressed macaques in this cohort had AHR in the absence of atopy (by skin test to common aeroallergens¹²) or T-cell activation in the periphery.¹² AHR in these animals is thought to be driven by autonomic neuronal dysregulation of the airways,^{12,13,44} leading to development of T_H2-low asthma.^{41,45} Social stress and AHR have been also associated with glucocorticoid receptor dysfunction, significantly contributing to airway changes and complicating therapeutic efforts in T_H2-low asthma.^{46–48} Lastly, oxidative pathways might be active in the absence of T_H2-type inflammation, driving the cellular and molecular components of AHR.⁴

To study the role of oxidative stress in O₃-induced airway changes, we investigated LGM2605.^{19–22} LGM2605 dose-dependently increased antioxidant gene expression in the lung tissue of macaques *in vivo* and in airway smooth muscle *in vitro*. We tested the hypothesis that this compound will protect against the effects of O₃ inhalation. Interestingly, asthmatic

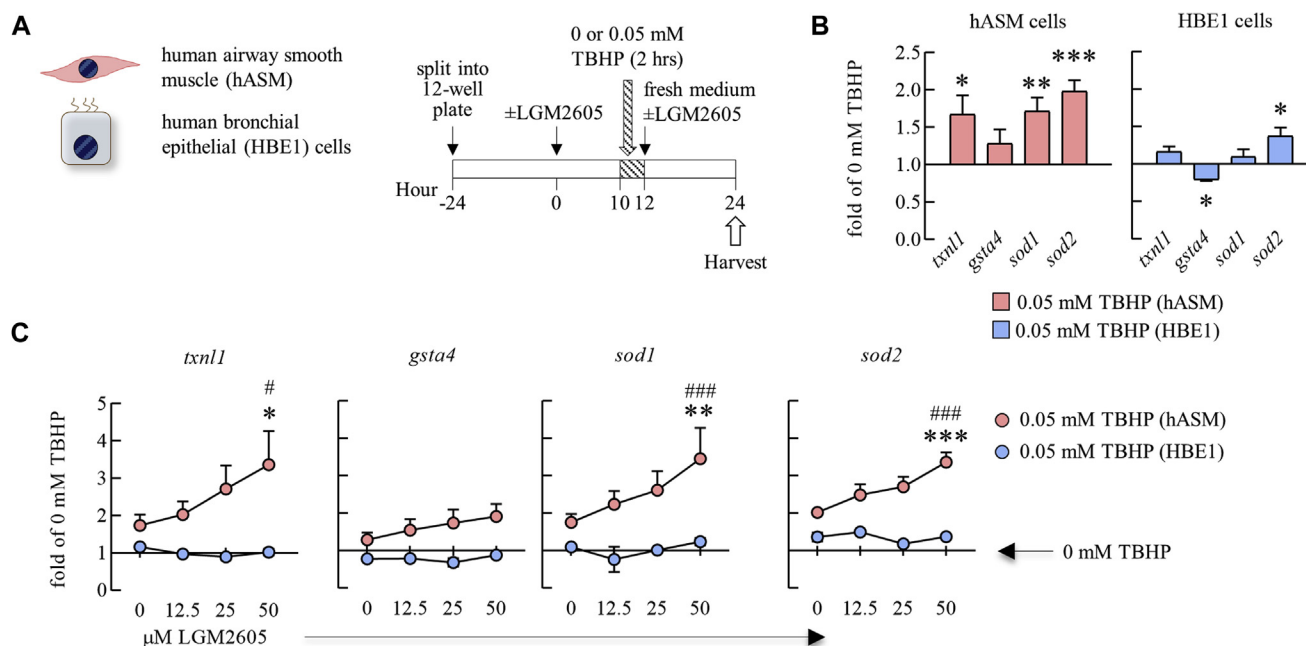


FIG 6. LGM2605 induced antioxidant gene expression in the presence (but not the absence) of TBHP in hASM *in vitro*. **A**, hASM and HBE1 cells were cultured in 12-well plates (–24 to 0 hours). At the 0-hour time point, fresh medium containing 0, 12.5, 25, or 50 μ M/L LGM2605 was added. TBHP or control H_2O was added to the wells (final concentration, 0.05 or 0 mmol/L, respectively) 10 hours later, and cells were incubated for 2 hours. At the 12-hour time point, medium containing TBHP was replaced with fresh medium containing LGM2605, and cells were harvested in TRIzol 12 hours later. **B**, Effect of TBHP on *TXNL1*, *GSTA4*, *SOD1*, and *SOD2* antioxidant gene expression in hASM and HBE1 cells (qPCR). $\Delta\Delta$ Cycle threshold (*ACTB*). Fold of 0 mmol/L TBHP. Mean $\pm$ SEM of $n = 3$ –6 (hASM cells) or $n = 2$ –3 (HBE1 cells). * $P < .05$, ** $P < .01$, and *** $P < .001$, 0 mmol/L TBHP versus 0.05 mmol/L TBHP, Student unpaired *t* test. **C**, Dose-dependent effects of LGM2605 on TBHP-induced *TXNL1*, *GSTA4*, *SOD1*, and *SOD2* antioxidant gene expression. Mean $\pm$ SEM of $n = 3$ –6 (hASM cells) and $n = 2$ –3 (HBE1 cells). * $P < .05$, ** $P < .01$, and *** $P < .001$ versus 0 μ M/L LGM2605, 2-way ANOVA. # $P < .05$, ## $P < .01$, and ### $P < .001$ versus 0 mmol/L TBHP, 2-way ANOVA.

macaques (with AHR) given LGM2605 showed a trend for improved lung function, even without O_3 exposure, corroborating the involvement of oxidative stress in the pathogenesis and potential therapeutic effects on T_H2 -low asthma and especially on AHR. This was supported by the fact that LGM2605 had a disproportionately greater protective effect on lung function than on airway inflammation in response to O_3 inhalation. In fact, this is the first study demonstrating the specific efficiency of an antioxidant lignan compound on alleviating AHR. The anti-inflammatory effects of LGM2605 were also selective and affected BAL fluid neutrophil but not eosinophil counts in O_3 -exposed macaques. This observation will require further clarifications of its significance and mechanisms because it raises uncertainty in regard to the role of eosinophils in patients with AHR.

Using HBE1 and hASM cells *in vitro*, we confirmed that LGM2605 specifically affects airway smooth muscles. In hASM but not HBE1 cells LGM2605 highly enhanced TBHP-induced antioxidant gene expression. In fact, *TXNL1* and *SOD2* were significantly upregulated by LGM2605 both in hASM cells *in vitro* and in macaques *in vivo*, suggesting these genes to be specifically important in mediating the effects of LGM2605.

Given the importance of oxidative stress in mediating the effects of O_3 , glutathione-related pathways might play a

significant role. Glutathione-S-transferase (GST) gene variants, for example, were suggested to influence the effects of O_3 inhalation in asthmatic children.⁴⁹ Fifteen nonsynonymous variants and 1 nonsense variant in glutathione-S-transferase theta (GSTT) 1 and 15 nonsynonymous variants in GSTT2 found in macaques were associated with GSTT-dependent individual drug responses,⁵⁰ suggesting a role for GST and other antioxidant gene polymorphisms in air pollutant-induced asthma, and might determine potential responsiveness to antioxidant treatment.

To investigate the possible molecular mechanisms involved in O_3 -induced inflammation and AHR, we studied the lung collectin SP-D. This immunoprotective airway epithelial molecule is constitutively expressed in the lung, but during an inflammatory response, its production is greatly amplified. SP-D suppresses proinflammatory activation of immune and inflammatory cells in the lung.^{8,51} Previously, we showed that oxidative damage to the quaternary structure of SP-D⁹ or genetic deficiency of the SP-D gene (*SFPTD*) conveys heightened susceptibility to inflammatory changes.^{5,7} IL-6, a cytokine essential in eliciting neutrophilia and AHR in response to O_3 , is also important for inducing SP-D mRNA activation.^{7,52} In the macaque airway epithelium, O_3 induced both the *IL6* and *SFPTD* genes, and this effect was reversed by LGM2605. Although O_3 did not specifically alter the oligomeric SP-D structure in macaques, it significantly reduced SP-D protein concentrations, and this effect

was also prevented by LGM2605. Thus, even though O₃ induced *SPPTD* gene activation, likely through autocrine effects of IL-6 on airway epithelial cells, it destroyed the released SP-D protein, which is known to be susceptible to oxidative damage.^{9,53} Alleviation of oxidative stress by LGM2605 prevented these changes.

ILCs are mucosa-resident cells that are among the first to respond to foreign substances and pathogens, especially in the lung. We previously showed in mice that O₃-induced AHR required cooperation between airway epithelial cells, DCs, and ILCs.^{5-7,9} ILC2s were indeed essential to mediate O₃-induced airway inflammation and AHR.⁶ We hypothesized here that the effects of O₃ would involve activation of ILC2s in macaques. Studies of ILCs in rhesus macaques are limited, and to our knowledge, ILC2s have never been examined in this species. We developed a flow cytometric method based on studies in human subjects to examine these elusive cells.⁵⁴ CD25 was used to define ILC2s in contrast to more specific ILC2 markers because of the availability of commercial reagents, although prior studies in human subjects showed that of the ILC subsets, CD25 is expressed most highly on ILC2s.⁵⁵ The genes for the ILC2-activating cytokines *IL25* and *IL33* in airway epithelial cells and the number of ILC2s in BAL fluid were markedly increased by O₃ but not when animals were treated with LGM2605. Changes in ILC2 counts were selective and localized to the airways, revealing that O₃ had compartment-specific effects on ILC populations. Intriguingly, altered expression of *GATA3*, *IL13*, and *IL17A* mRNA in blood ILC2s from O₃-exposed macaques suggests potential involvement in airway neutrophilia and hyperreactivity. We were surprised to find expression of *IL17A* to be altered in blood ILC2s because this is not a canonical cytokine for this population, although mouse models have shown that IL-25 signaling can induce IL-17A⁺ ILC2s.⁵⁶

Previously, we demonstrated the involvement of proinflammatory mDCs in the pathogenesis of O₃- and allergen-induced airway inflammation in mouse models^{5,57,58} and found that O₃ increased the number of DCs in the airways, in part because of decreased recirculation to the mediastinal lymph nodes.^{5,31} It was hypothesized that DC activation and trapping in the lung contribute to injury in response to O₃. Our macaque study corroborates this hypothesis and shows that the proinflammatory CD1c⁺ mDC population with increased TNF- α and decreased CD40 levels was accumulated in the airways of O₃-exposed macaques. We also found that antioxidant treatment alleviated these changes and intriguingly inhibited CD1c⁺ DC counts in asthmatic macaques, even without O₃ exposure, suggesting a role for oxidative stress in activation of these cells but not in CD123⁺ pDCs. That O₃ would increase CD123⁺ pDC counts in the airways was previously unknown, and this finding will require further investigation, especially because of the significance of these cells in antiviral immunity.⁵⁹ The reduction in numbers of TNF- α ⁺ mDCs by LGM2605 treatment in O₃-exposed macaques correlated to the reduction in AHR and BAL fluid neutrophilia in the same animals, highlighting the potential pathogenic role of these cells in mediating O₃ effects. Furthermore, these results complemented our findings on ILC2s, prompting us to speculate that mDCs and ILC2s can cooperate and instruct adaptive immune cells during O₃-induced airway inflammation.⁶⁰ Indeed, a recent study demonstrated that IL-33 derived from DCs induced ILC2 activation to promote airway inflammation.⁶¹

The role of T cells in O₃-induced airway inflammation and hyperreactivity is controversial. Acute exposure of human subjects to O₃ increased or decreased activated (CD25⁺) CD4⁺ T cells recovered from BAL fluid.⁶² When CD4⁺ T cells were depleted from mice before O₃ exposure, inflammation was either enhanced or reduced depending on the strain, whereas IL-4 consistently suppressed O₃-induced inflammation.⁶³ Our laboratory showed that CD4⁺ T cells were not activated on O₃ inhalation in BALB/c mice within the first 12 hours.⁶

In the present study we showed that O₃ increased IFN- γ and IL-4 expression in CD4⁺ and CD4⁺CD25⁺ T cells independent of antioxidant treatment, although some of these changes were marginally significant ($P = .05$). On the other hand, decreased expression of CD40 on DCs after O₃ and increased number of CD4⁺CD25⁺ T cells suggests a tolerogenic phenotype.⁶⁴ Because LGM2605 did not prevent increases in the numbers of these cells, it is unlikely that they play any role in improved AHR similar to our CD123⁺ pDC findings. Because neutrophils (T_H1 or T_H17 induced) and eosinophils (T_H2 induced) were in the airways after O₃, it is likely that this mixed inflammation was driven by mixed activation of T_H-type cells. Taken together, our data on ILCs, DCs, and T cells suggest that O₃ activates various arms of the immune system, likely T_H1-, T_H2-, and T_H17-type responses that play competing roles in mediating AHR and inflammation in asthmatic patients.

Our study demonstrates the role of mucosal immunity in the pathophysiology of O₃-induced AHR and reveals the significance of oxidative stress in rhesus macaques (see the graphical abstract). We propose the therapeutic potential of LGM2605 as an adjuvant treatment for AHR and airway neutrophilia in asthmatic patients.

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Clinical implications: Our study highlights the role of selective immune pathways and oxidative stress in a nonhuman primate model of asthma exacerbation caused by O₃ inhalation. LGM2605 can have a clinical potential as an adjuvant asthma therapy.

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1 **Online Repository Materials**

2 **Detailed lung function and BAL protocol**

3 Static Lung Mechanics were measured in a whole-body plethysmograph (Buxco
4 Electronics, Wilmington, NC). Sedated monkeys were connected to a 3-way valve assembly and
5 given supplemental O₂ during the procedure. The semi-automated Maneuvers XA (Buxco
6 Electronics) software controlled positive pressure inflation and negative pressure deflation to the
7 lung in order to calculate the following parameters: standard static lung volumes/capacities,
8 forced expiratory volumes and flows, quasi-static lung compliance, functional residual capacity
9 and thoracic gas volume.

10 Airway responsiveness to aerosolized methacholine challenge was then performed in a
11 head-out body plethysmograph (body box), with the intubation tube attached to a pneumatic
12 four-way valve/pneumotachograph assembly. All challenges were administered as aerosols at a
13 set inflation pressure and breathing frequency (10.0 cm H₂O and 30.0 bpm) using a compressed
14 air nebulizer (Vortran, Inc., Miniheart Model) in series with a positive pressure ventilator (Bird
15 Mark 7A respirator). Following baseline measurements and aerosolization of saline,
16 methacholine challenge began at 0.0625 mg/mL, and doubled until a maximum concentration of
17 32 mg/mL was reached. The final concentration of aerosol delivered was the concentration that
18 doubled the raw EC200 value or caused arterial oxygen saturation to fall below 70%. Pulmonary
19 mechanics were evaluated using a transfer impedance method: Briefly, the monkeys breathed
20 spontaneously through the pneumotachograph (Fleisch no. 2) while the thorax of the monkey
21 was vibrated using a pseudo-random noise waveform encompassing frequencies of 2 to 128 Hz
22 by two speakers mounted in the walls of the head-out plethysmograph (Pulmetrics Group,
23 Boston, MA). The small changes in flow produced at the mouth were measured along with the

changes in pressure inside the plethsmograph using a Microswitch transducer (model 743PC). This technique allowed the monkey to breathe spontaneously while making continuous pulmonary mechanics measurements. Concurrently, changes in lung compliance were assessed by placing either a fluid or air-filled balloon catheter in the esophagus at the level of the heart to measure transpulmonary pressure. Pressure/flow signals were collected and processed using a digital data acquisition system (PO-NE-MAH, Gould Instruments Inc) and lung compliance calculated for each dose of either methacholine.

Following PFT measurements, BAL was harvested by inserting a bronchoscope through the orotracheal tube into the trachea and then into the lung lobe and segment to be sampled. The camera attachment allowed us to define the exact location in the airway where samples were obtained by bronchial brush biopsy and lavage. The end of the bronchoscope was wedged in a segmental or subsegmental bronchus and 2cc/kg saline was instilled and aspirated back, for recovery of airway lining fluid. Approximately 50% of instilled fluid was recovered. A second aspiration was performed to obtain sufficient cells for analysis. BAL was performed immediately following the PFT. After BAL was collected, bronchial brush biopsy was performed to gain cells from the affected airways. In brief, a flexible brush was passed through the bronchoscope and the bronchial surface was gently abraded to obtain bronchial epithelial cells.

LGM2605 dose-response effects on anti-oxidant enzyme gene activation

LGM2605 has been tested in non-GLP acute and repeat dose mouse safety models with no observed side effects up to 4X when dosed p.o. or s.c. Higher doses of LGM2605 have not been tested in formal GLP safety models.

Before we performed our studies on the effects of LGM2605 on O₃ exposure of asthmatic macaques, we wanted to verify that this compound would induce anti-oxidant genes in the

macaque lung. For this purpose, RNA was extracted from stored frozen lung tissue that was obtained from macaques that underwent whole thorax irradiation (a method used by the Christofidou-Solomidou lab to generate oxidative stress) and treatment with 0, 25, or 50 mg/kg LGM2605 (by oral gavage). Briefly, animals were exposed to 11.5 Gy given by irradiation at a dose rate of 0.75 ± 0.05 Gy/min using the linear accelerator after shielding all areas with the exception of the thorax region consisting of the area from the sternum to the end of the last rib using an anterior-posterior (AP) and posterior-anterior (PA) technique by the 6 MV photon beam with approximately 50% dose contribution from both the AP and PA beams. LGM2605 was administered once per day on Day 64 through Day 70 (initiated 24 hours post radiation exposure). Animals were euthanized on Day 70. Snap frozen lung tissues from irradiated, LGM2605-treated male Rhesus macaques were processed at the University of Pennsylvania for qRT-PCR to determine gene expression changes in lung Nrf2 target genes. Findings were normalized to beta actin and GAPDH.

Flow cytometry

Flow cytometry experiments were performed on BAL and peripheral blood cells. Abs were purchased from Biolegend (San Diego, CA), BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), Beckman Coulter (Brea, CA), and Miltenyi Biotec (Bergisch Gladbach, Germany).

Single cell suspensions of isolated PBMCs were stained with the following anti-human Abs purchased from Biolegend: CD14-FITC (clone M5E2), CD16-FITC (clone 3G8), CD20-FITC (clone 2H7), CD117(c-kit)-APC/Cy7 (clone 104D2), CD25-Pacific Blue (clone M-A251), CD161-PerCP/Cy5.5 (clone HP-3G10), CD4-FITC (clone A161A1), CD8a-APC/Cy7 (clone RPA-T8), HLA-DR-APC/Cy7 (clone L243), CD11c-Pacific Blue (clone 3.9), CD123-PE (clone

6H6), TNF α -APC (clone Mab11), CD16-PerCP/Cy5.5 (clone 3G8), CD1c-Alexa Fluor 488 (clone L161), CD40-PE-Cy7 (clone HB14), IL-4-PE (clone MP4-25D2), IFN γ -PE-Cy7 (clone B27). Anti-human Abs purchased from BD Biosciences included: CD3-Alexa Fluor 488 (clone SP34-2), CD56-Alexa Fluor 488 (clone B159), CD90-APC (clone 5E10), CD45-PE-CF594 (anti-rhesus, clone D058-1283). CD336 (NKp44)-PE (clone Z231) was purchased from Beckman Coulter. CD127-PE-Vio770 (clone MB15-18C9) was purchased from Miltenyi Biotec. Full antibody panels and voltages for acquisition can be found in **Table E2**.

Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). Acquisition of all samples was performed LSR II or Fortessa (BD, Franklin Lakes, NJ). Data were analyzed using FlowJo v9.6 software (Tree Star Inc., Ashland, OR) as we previously described (27). Lineage markers for macaque ILCs included: CD3, CD14, CD16, CD19, and CD56.

qPCR

RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). qPCR was performed on 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 $\Delta\Delta C_t$ method, first normalizing values to the housekeeping gene(s) *gapdh* alone (Blood ILC2s) or the geometric mean of *gapdh* and *rpl32* (bronchial brush biopsies), then to Day 1. Primers were designed 5' to 3' as follows: *gapdh* forward GCACCACCAACTGCTTAGCAC reverse TCTTCTGGGTGGCAGTGATG. *rpl32* forward TGACGTTGTGGACCAGGAACT reverse TGACGTTGTGGACCAGGAACT. *il6* forward ATCCCCAACAAGAGGAAACGAA reverse CGTTCCAGGGCTAGGGATTT. *sfptd* forward AAGTGGGCTTCCAGATGTGG reverse CGACACTTTGGCCATTTGGG. *il33*

forward GCCTTGTGTTTCAAGCTGGG reverse TGTATGTGCTTAGAGAAGCAAGAG. *il25*
forward GGGGCAACCCAGATTAGGTGAG reverse GACGGGTATCCTTTGCTGGG. *gata3*
forward CCTCTCTGCTCTTCGCTACC reverse TCCAAAACTGGGTACGGCA. *il13*
forward CGGTCATTGCTCTCACTTGC reverse CCAGCTGTCAGGTTGATGCT. *il17a*
forward GGGAAGACCTCATTGGTGCT reverse AGGTTGACCATCACAGTCCG.

RNA derived from *in vitro* cultures of human cells were reverse transcribed using the RT2 First Strand Kit (Qiagen, Hilden, Germany). qPCR was performed on cDNA using RT² SYBR Green ROX qPCR Mastermix (Qiagen, Hilden, Germany) on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Fold change was calculated using the $\Delta\Delta C_t$ method, first normalizing values to the housekeeping gene *actb*. Primers were purchased directly from Qiagen (Hilden, Germany).

Western Blot

BAL SP-D was detected via SDS and Native-PAGE western blot. Total protein in the BAL was quantified via Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). 1 μ g protein was run under standard reducing gel electrophoresis conditions using NuPAGE™ MOPS SDS Running Buffer (Thermo Fisher Scientific, Waltham, MA). The gel was transferred to a mini Nitrocellulose membrane using the iBlot® gel transfer mini stacks (Thermo Fisher Scientific, Waltham, MA). The membrane was probed with mouse anti-SP-D 1:1000 (in-house 10F6E12 monoclonal) followed by goat anti-mouse conjugated HRP 1:3000 (Santa Cruz Biotechnology, Santa Cruz, CA), then activated via Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA).

SUPPLEMENTAL FIGURE LEGENDS

Figure E1. Variable baseline airway hyperreactivity in rhesus macaques. (A) AHR macaques were defined by EC150 cutoff of <8 mg/mL methacholine. **(B)** Lung resistance (Raw) to the indicated concentrations of methacholine in AHR and No AHR rhesus macaques. Mean±SEM of n=4-13; ***p<0.001 (within groups) #p<0.05 (between groups), Two-way ANOVA.

Figure E2. LGM2605 dose-dependently induced expression of anti-oxidant genes in the rhesus macaque lung. LGM2605 treatment of rhesus macaques (n=3) induced Nrf2-related antioxidant genes in a dose dependent manner: nuclear factor, erythroid 2 like 2 (*nfe2l2*), heme-oxygenase 1 (*ho1*), NADPH:quinone acceptor oxidoreductase (*nqo1*) glutathione peroxidase 1 (*gpx1*), Glutathione S-transferase alpha 4 (*gsta4*), Glutathione S-transferase mu 4 (*gstm4*), Thioredoxin Like 1 (*txnl1*), Thioredoxin Reductase 1 (*txnrd1*), Superoxide Dismutase 1, (*sod1*), Superoxide Dismutase 2, Mitochondrial (*sod2*). qPCR was performed on lung tissue obtained from macaques that underwent whole-thorax irradiation as part of a different study (unpublished). $\Delta\Delta C_t$ (*actb* and *gapdh*), fold of baselines values.

Figure E3. Airway responsiveness to inhaled methacholine. Lung resistance (Raw) to the indicated concentrations of methacholine [cmH₂O/(L x s) or % change from 0 mg/mL]. In % change graphs, each macaque served as its own control. Mean±SEM of n=5-6; Two-way ANOVA.

Figure E4. Absolute count of BAL cells. (A) Total BAL cells were harvested and quantified (cells/mL BAL $\times 10^3$). Macrophages (MP), lymphocytes (LC), neutrophils (NP), and eosinophils (EP) were differentially counted on cytopins, then quantified by multiplying total BAL cell count by % of each cell type (cells/mL BAL $\times 10^3$). Mean $\pm$ SEM of n=5-6; *p<0.05, **p<0.01, ***p<0.001 (within groups: day 1 vs. day 8) #p<0.05 (between groups), Two-way ANOVA. **(B)** On day 8 after O₃ exposure, EC150 showed a moderate negative correlation with the absolute count of BAL neutrophils and eosinophils.

Figure E5. BAL SP-D structural analysis by native-page. (A) Representative native-page western blot detecting non-human primate BAL SP-D. De-oligomerized SP-D was resolved as protein detected outside of the well because native SP-D does not migrate through the gel. **(B)** Structural changes in SP-D was determined by semi-quantitative densitometry on de-oligomerized SP-D bands from native-page gels (% change de-oligomerized SP-D from day 1 to day 8, each macaque served as its own control). Mean $\pm$ SEM of n=5-6; Student's *t*-test (within groups: day 1 vs. day 8).

Figure E6. Representative ILC gating strategy. (A) After size exclusions, peripheral blood and BAL ILCs were defined as live CD90⁺Lineage⁻CD127⁺ cells (lineage markers – CD3, CD14, CD16, CD20, CD56). ILC2s were defined as CD25⁺c-kit^{var} ILCs; ILC1s were defined as c-kit⁻NKp44⁻ ILCs; NCR⁺ ILC3s were defined as NKp44⁺c-kit⁺ ILCs; NCR⁻ ILC3s were defined as NKp44⁻c-kit⁺ ILCs. **(B)** An FMO is provided for the ILC2 gate. **(C)** Peripheral blood CD3⁺ T cells were gated on CD25.

Figure E7. Representative DC gating strategy. After size exclusions, peripheral blood and BAL CD1c⁺ mDCs were defined as live CD45⁺CD11c⁺HLA-DR⁺CD1c⁺ cells. CD16⁺ mDCs were defined as live CD45⁺CD11c⁺HLA-DR⁺CD16⁺ cells. CD123⁺ pDCs were defined as live CD45⁺CD11c⁻HLA-DR⁺CD123⁺ cells.

Figure E8. Blood DC counts and CD40 expression. (A) CD1c⁺ and CD123⁺ DCs were quantified in the peripheral blood (cells/mL x10³). (B) CD40 expression (MFI) on DC subsets (% change from day 1 to day 8). In % change graphs, each macaque served as its own control. Mean±SEM of n=5-6; Two-way ANOVA.

Figure E9. Representative T cell gating strategy. After size exclusions, peripheral blood and BAL CD4⁺ T cells were defined in the peripheral blood and BAL as live CD45⁺CD3⁺CD4⁺CD8⁻ cells; CD8⁺ T cells were defined as live CD45⁺CD3⁺CD4⁻CD8⁺ cells. CD25 was examined on CD4⁺ and CD8⁺ T cells as an activation marker.

Figure E10. IL-4 expression in circulating T cells. (A) Quantification of CD4⁺ and CD8⁺ T cells in the peripheral blood (cells/mL x10⁶). (B-C) IFN γ and IL-4 expression (MFI) was measured in CD4⁺ and CD8⁺ T cells (% change from day 1 to day 8). In % change graphs, each macaque served as its own control. Mean±SEM of n=5-6; *p<0.05 (within groups: day 1 vs. day 8; C: Student's paired *t*-test, A-B: Two-way ANOVA).

Group	Animal ID	Sex	Weight (kg)	Birth Date	Age (yrs)	PFT time (hours post exposure)
LGM2605+air	44145	M	5.82	3/21/2014	3.72	15.5
	44055	M	6.34	3/17/2014	3.84	15
	44307	M	7.45	4/17/2014	3.80	15.15
	45324	F	3.92	5/9/2015	2.70	15.25
	45005	F	4.36	4/2/2015	2.85	15.25
placebo+O ₃	45356	M	3.84	5/13/2015	2.57	11.5
	45461	M	3.99	6/6/2015	2.62	11.5
	44951	M	4.78	3/26/2015	2.86	13.25
	45230	F	4.11	4/28/2015	2.72	13.5
	45440	F	5.04	5/31/2015	2.67	13.25
	45439	F	4.09	5/31/2015	2.69	11.5
LGM2605+O ₃	44147	M	6.57	4/1/2014	3.69	13.5
	44365	M	7.02	4/22/2014	3.78	11.45
	44441	M	6.37	5/1/2014	3.76	11.5
	45006	F	5.02	4/2/2015	2.79	11.5
	45094	F	3.38	4/14/2015	2.76	13.25
	45473	F	3.74	6/12/2015	2.65	13.5

Table E1. Subject Information including animal ID, sex (male, M; female, F), weight (kg), birth date, age (years), and time of PFT (hours post O₃ or air exposure).

	ILC panel	DC panel	T cell panel	
Color	Antigen			Voltage
FITC	CD3	CD1c	CD4	400
	CD14			
	CD16			
	CD20			
	CD56			
PE	NKp44	CD123	IL-4	467
PE-Cy7	CD127	CD40	IFN γ	450
PE-CF594	N/A	CD45	CD45	350
PCP-Cy5.5	N/A	CD16	N/A	450
APC	CD90	N/A	CD3	500
APC-Cy7	c-kit(CD117)	HLA-DR	CD8	350
PB	CD25	CD11c	CD25	400
BV510	L/D	L/D	L/D	350

Table E2. Flow cytometry staining panels of the target for fluorescent-conjugated antibodies and the voltage used to acquire events on the flow cytometer.