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Protective Role of Eosinophils and Tumor Necrosis Factor-α after Ozone Inhalation

Allison D. Fryer, David B. Jacoby, and Sarah A. Wicher

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with a Critique by the HEI Review Committee

Research Report 191

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives balanced funding from the U.S. Environmental Protection Agency and the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 330 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides. diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in more than 260 comprehensive reports published by HEI, as well as in more than 1,000 articles in the peer-reviewed literature.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Review Committee are widely disseminated through HEI's Web site (*www.healtheffects.org*), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 191, *Protective Role of Eosinophils and Tumor Necrosis Factor-α after Ozone Inhalation,* presents a research project funded by the Health Effects Institute and conducted by Dr. Allison D. Fryer of Oregon Health & Science University, Portland, Oregon, and her colleagues. The report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Review Committee's comments on the study.

The Investigators' Report, prepared by Fryer and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

The Critique, prepared by members of the Review Committee with the assistance of HEI staff, places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.

HEI STATEMENT

Synopsis of Research Report 191

Protective Role of Eosinophils and TNFα after Ozone Inhalation

INTRODUCTION

Exposure to ozone induces deleterious responses in the airways that include shortness of breath, inflammation, and bronchoconstriction. People with asthma have increased airway sensitivity to ozone and other irritants. Dr. Allison Fryer and colleagues addressed how exposure to ozone affects the immune and physiological responses in guinea pigs. Guinea pigs are considered a useful animal model for studies of respiratory and physiological responses in humans; their response to airborne allergens is similar to that in humans and shares some features of allergic asthma.

Fryer and colleagues had previously observed that within 24 hours of exposure, ozone not only induced bronchoconstriction but also stimulated the production of new cells in the bone marrow, where all white blood cells develop. As a result of ozone exposure, increased numbers of newly synthesized white blood cells, particularly eosinophils, moved into the blood and lungs.

The central hypothesis of the current study was that newly synthesized eosinophils recruited to the lungs 3 days after ozone exposure were beneficial to the animals because they reduced ozone-induced bronchoconstriction. The investigators also hypothesized that the beneficial effect seen in normal (*nonsensitized*) animals was lost in animals that had been injected with an allergen, ovalbumin (*sensitized*). They also planned to explore the effects of inhibitors of certain cytokines (cell-signaling molecules).

Immune responses in sensitized animals are dominated by a *Th2 pattern*, which is characterized by the synthesis of cytokines (interleukin [IL]-4, IL-5, and IL-13) and the Th2 subset of $CD4^+$ T lymphocytes and the cells they activate (predominantly eosinophils, and B lymphocytes that switch to making immunoglobulin E [IgE]). Thus, sensitized animals were used as a model of allergic humans, whose immune responses tend to be dominated by IgE.

APPROACH

Fryer and colleagues exposed normal and sensitized (allergic) guinea pigs to 2 ppm ozone or filtered air for 4 hours and measured changes in cell numbers and airway responses 1 or 3 days later. They counted the numbers of eosinophils and other white blood cells (macrophages, neutrophils, and lymphocytes) in bone marrow, blood, and bronchoalveolar lung lavage fluid. The investigators also measured important physiological responses, including bronchoconstriction. Some animals were pretreated with etanercept and monoclonal anti-IL-5, which block tumor necrosis factor-α (TNFα) and IL-5, respectively. TNFα and IL-5 blockers have been used to treat patients with asthma.

What This Study Adds

- Eosinophils are white blood cells that play an important role in inflammation, allergies, and allergic asthma, and can modify the airway response to ozone inhalation. This study tested a novel hypothesis: that allergic guinea pigs react differently to ozone than normal animals because of newly synthesized eosinophils that travel from bone marrow to the lungs.
- The study confirmed that newly formed eosinophils found in the lungs 3 days after ozone exposure had a beneficial effect in normal, but not allergic, animals. Tumor necrosis factor-alpha may be involved in the regulation of eosinophil synthesis and movement.
- These findings suggest how the responses to ozone may differ in people who have allergies or asthma from those in people who do not.

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Allison D. Fryer Division of Pulmonary and Critical Care Medicine, Oregon Health & Science University, Portland, Oregon, and colleagues. Research Report 191 contains both the detailed Investigators' Report and a Critique of the study prepared by the Institute's Review Committee.

A key feature of the study was a technique to distinguish which white blood cells were synthesized after exposure from those that already existed, by injecting animals with bromodeoxyuridine (BrdU). BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells, serving as a marker of newly produced cells. Therefore, a snapshot can be obtained of the proportion of newly synthesized (BrdU-positive) versus pre-existing (BrdU-negative) cell types.

KEY RESULTS

1. *Allergic and normal animals differed in the time course of bronchoconstriction and changes in cell types after ozone exposure.* In normal animals, bronchoconstriction increased substantially at day 1 but decreased by day 3 after ozone exposure. In contrast, in allergic animals bronchoconstriction remained high at day 3. Ozone also increased the percentage of newly formed, BrdU-positive eosinophils in the bone marrow and lungs of normal but not allergic animals.
2. *Pretreatment with the TNFα blocker etanercept had complex effects, which differed between normal and allergic animals.* In normal animals, etanercept decreased ozone-induced new synthesis of eosinophils in the bone marrow and blocked eosinophil migration to the lung; it also increased bronchoconstriction at day 3 (relative to day 1 without etanercept). In allergic animals, etanercept had no effect on any cell type in the bone marrow or lung after exposure to ozone and did not change bronchoconstriction compared with allergic animals not treated with etanercept.

   Etanercept tended to increase the numbers of blood monocytes and lymphocytes in air- and ozone-exposed normal and allergic animals at day 3, but had no effect on eosinophils in blood at this time point. This was one of the few statistically significant findings in the blood of exposed animals in the study.
3. *Anti-IL-5 reduced bronchoconstriction at day 3 after exposure of allergic animals to ozone.* In contrast, bronchoconstriction was greatly increased in normal animals treated with anti-IL-5.

CONCLUSIONS

Fryer and colleagues explored the airway and cellular responses in guinea pigs exposed to ozone. The HEI Review Committee, which conducted an independent review of the study, agreed that the findings supported the authors' hypothesis (1) that exposure to ozone stimulates production of eosinophils in bone marrow, (2) that these newly formed eosinophils migrate to the lungs, and (3) that those eosinophils play a delayed but potentially beneficial role in reducing ozone-induced inflammation in the airways of healthy normal animals, but not in allergen-sensitized animals. The Committee also agreed that guinea pigs were a good model for studying responses to an allergen, because a major subtype of asthma (the high Th2 or allergic type) is associated with high levels of eosinophils in the blood.

A novel finding was that the TNFα blocker etanercept decreased ozone-induced formation of eosinophils in the bone marrow and blocked eosinophil migration to the lung in normal animals. However, because injecting etanercept had little effect on eosinophils and did not decrease bronchoconstriction in allergic guinea pigs, the potential for treating patients with allergic asthma with TNFα blockers is uncertain. This is consistent with the poor performance of TNFα blockers in clinical studies of asthma treatment.

Blocking the cytokine IL-5 with an anti-IL-5 antibody substantially decreased bronchoconstriction in sensitized animals. This suggests that therapies targeting IL-5 and eosinophils would be promising in at least some types of asthma. The Committee expressed caution toward experiments with cytokine blockers, both in animal models and humans, because such blockers are often not specific to a particular cell type and may differ at different sites in the body. Without further detailed confirmation of the effects of the blockers, interpreting these experiments can be challenging.

The Committee concluded that the study by Fryer and colleagues raises several intriguing directions for future research, including exploring ways in which newly formed eosinophils differ from preexisting ones, and how such findings apply to humans with allergy or asthma.

INVESTIGATORS' REPORT

Protective Role of Eosinophils and Tumor Necrosis Factor-α after Ozone Inhalation

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ABSTRACT

Exposure to ozone is associated with increased asthma exacerbations for days after exposure. One mechanism of ozone-induced hyperreactivity is via recruitment of eosinophils to the lungs where they release an antagonist for neuronal M_2 muscarinic receptors, increasing acetylcholine release from parasympathetic nerves. Depletion of eosinophils completely prevented airway hyperreactivity one day after ozone. However, three days after ozone exposure, depleting eosinophils had the opposite effect; it significantly potentiated ozone-induced airway hyperreactivity.

Whether ozone exposure recruits different populations of eosinophils to lungs was tested by treating guinea pigs with 5-bromo-2′-deoxyuridine (BrdU*), exposing them to 2 ppm ozone or to filtered air for four hours and measuring bronchoconstriction in response to electrical stimulation of the vagus nerves one or three days later. Inflammatory cells were counted in bronchoalveolar lavage (BAL), blood, and bone marrow and were differentiated into BrdU+ (newly divided) and BrdU− (preformed) cells.

Ozone exposure significantly increased bronchoconstriction induced by electrical stimulation of vagus nerves above that of air-exposed animals. Ozone exposure also induced a selective increase in eosinophilopoiesis in bone marrow. These newly formed eosinophils formed a 'second wave' of eosinophil influx into airways between one and three days after ozone inhalation. The appearance of these newly divided eosinophils corresponded with a change in eosinophil function from deleterious to beneficial.

To test the effect of allergy on eosinophil recruitment, some guinea pigs were sensitized to ovalbumin (not challenged) twenty-one days prior to ozone exposure. In sensitized animals ozone exposure failed to induce eosinophilopoiesis in bone marrow, and in the absence of newly divided eosinophils, ozone-induced hyperreactivity was significantly greater than in nonsensitized animals. The role of tumor necrosis factor (TNFα) was tested using an inhibitor, etanercept. Results were similar to those for sensitization; etanercept blocked ozone-induced eosinophilopoiesis and significantly potentiated ozone-induced airway hyperreactivity. There was no role for nitric oxide since L-NAME, a nitric oxide synthase inhibitor, had no effect on ozone-induced hyperreactivity or inflammatory cell recruitment when administered three days after exposure.

These data demonstrate that ozone exposure initiates eosinophil production in bone marrow and their subsequent recruitment to lungs. These newly divided eosinophils constitute a previously unrecognized population of eosinophils with beneficial properties. TNFα may be a critical cytokine in ozone-induced eosinophilopoiesis. Importantly, these beneficial eosinophils are not recruited to lungs in atopic individuals, making airway hyperreactivity worse. These data predict that treatments targeting eosinophils may be more effective in individuals that are atopic.

This Investigators' Report is one part of Health Effects Institute Research Report 191, which also includes a Critique by the Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Allison D. Fryer, Pulmonary and Critical Care Medicine, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239; e-mail: *fryera@ohsu.edu*.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

* A list of abbreviations and other terms appears at the end of this volume.

INTRODUCTION

Breathing polluted air causes coughing, wheezing, and shortness of breath. While a number of constituents of polluted air may be responsible, one with known pulmonary effects is ozone (Peden 2002; Rice et al. 2015; Schwartz 2004). Almost half of the people in the United States are exposed to high levels of ozone, and one out of every three is at risk of experiencing adverse health effects from ground-level ozone (U.S. Environmental Protection Agency 1999). Breathing ozone-polluted air causes shortness of breath and airway hyperreactivity (increased bronchoconstriction in response to any provocation in the lungs) that is self-limited in healthy individuals but is exacerbated and longer lasting in people with asthma (Peden 2002; Schwartz 2004; Sheffield et al. 2015; White et al. 1994). Air pollution and ozone also have systemic effects, which increases risk of myocardial infarction and stroke, often with a lag of two to three days after exposure (Bell et al. 2005; Henrotin et al. 2007; Ruidavets 2005).

Inhaling ozone causes lung inflammation and airway hyperreactivity. Epidemiologic and experimental data show that ozone inhalation results in immediate airway hyperreactivity that lasts up to seven days (Park et al. 2004; Seltzer et al. 1986). Additionally, ozone exposure induces acute and developing lung inflammation over one to three days that lasts up to seven days (Schultheis and Bassett 1991, 1994; Yost et al. 1999, 2005). Although neutrophils are often reported as the dominant inflammatory cell, eosinophils are also significantly increased (Yost et al. 1999, 2005), and depleting them or neutralizing eosinophil major basic protein prevents ozone-induced airway hyperreactivity one day after exposure (Yost et al. 1999). Thus, acutely, ozone-induced hyperreactivity is mediated by eosinophils.

Eosinophils are known to profoundly influence autonomic nerve function in lungs. Eosinophils are actively recruited to airway nerves in asthma and in animal models of asthma (Costello et al. 1997). When these eosinophils become activated they release eosinophil major basic protein, which has been shown to be an endogenous antagonist for M_2 muscarinic receptors on parasympathetic nerves (Jacoby et al. 1993). These M_2 muscarinic receptors normally inhibit acetylcholine release from nerves; blocking them increases acetylcholine release, potentiating bronchoconstriction as much as ten-fold (Fryer and Maclagan 1984; Fryer and Wills-Karp 1991). In animals that are made allergic to a protein and then challenged by inhalation of that protein, M_2 receptor function can be protected and airway hyperreactivity prevented by: (1) depleting eosinophils with an antibody to interleukin-5 (IL-5); (2) preventing eosinophil migration into lungs with antibody to the adhesion molecule very late antigen-4 (VLA-4); (3) preventing eosinophil recruitment to parasympathetic nerves with a C-C chemokine receptor type 3 (CCR3) receptor antagonist; or (4) preventing eosinophil adhesion to nerves with steroids (Elbon et al. 1995; Evans et al. 1997; Fryer et al. 1997, 2005). Neuronal M_2 receptor function can also be protected and airway hyperreactivity prevented with an antibody specific for eosinophil major basic protein and can be acutely reversed by neutralizing major basic protein in vivo with heparin (Evans et al. 1997; Fryer and Jacoby 1992).

After exposure to ozone, the number of eosinophils in the lungs waxes and wanes over three days (Murlas and Roum 1985; Villegas-Castrejon et al. 1999; Yost et al. 2005). We have shown that eosinophils have multiple roles in airway responses to ozone exposure and that their role changes from causing airway hyperreactivity immediately after ozone exposure to preventing hyperreactivity three days later (Yost et al. 2005). Preventing eosinophil recruitment and activation or neutralizing eosinophil major basic protein prevented ozone-induced airway hyperreactivity 24 hours after exposure to ozone. However, three days after ozone exposure these interventions not only failed to prevent hyperreactivity, but significantly potentiated it (Yost et al. 2005). Thus, eosinophils have different roles in lungs at different times after ozone exposure, and this may help to explain why depleting eosinophils is effective only in a subpopulation of people with asthma (Bel et al. 2014; Haldar et al. 2009; Nair et al. 2009; Ortega et al. 2014).

People with atopic asthma have different inflammatory cell profiles from people with nonatopic asthma, but how these different populations of inflammatory cells contribute to disease is unknown. Depletion of eosinophils with an antibody to IL-5 (AbIL5) has met with variable success in asthma therapy. This is partly because initial trials (Kips et al. 2003; Leckie et al. 2000) did not use subjects with airway eosinophilia and partly because eosinophil depletion in airway tissues may not have occurred despite depletion in BAL (Flood-Page et al. 2003). It is increasingly clear that different asthma phenotypes exist (Fajt and Wenzel 2015; Lötvall et al. 2011; Woodruff et al. 2009). One of these is characterized by excessive eosinophils; two clinical trials show that AbIL-5 is protective against asthma exacerbations in these people (Bel et al. 2014; Haldar et al. 2009; Nair et al. 2009; Ortega et al. 2014). Thus, a role has been established for eosinophils in airway hyperreactivity in eosinophilic human asthma.

Another cytokine with variable roles in asthma is TNFα. It is increased in BAL from patients with asthma (Berry et al. 2006; Cazzola and Polosa 2006; Thomas 2001) and in animal models of asthma. Blocking TNFα with etanercept

(a TNF receptor IgG_1 fusion protein), or by genetically knocking it out, suppresses antigen-induced airway inflammation (Choi et al. 2005; Hutchison et al. 2008; Kim et al. 2006) and airway hyperreactivity (Arbach et al. 2002; Nie et al. 2009, 2011; Proskocil et al. 2013). In patients with severe and steroid-resistant asthma, anti-TNFα therapy decreases airway hyperreactivity and reduces exacerbation frequency (Berry et al. 2006; Howarth et al. 2005). TNF may be involved in recruitment of eosinophils (Lukacs et al. 1995) and may be released by eosinophils (Spencer et al. 2008).

Nitric oxide is produced by nerves (Belvisi et al. 1995) and by inflammatory cells including eosinophils (Lee et al. 2013; MacPherson et al. 2001), monocytes/macrophages (Fang and Vazques-Torres 2002; Khanduja et al. 2011), and neutrophils (Sánchez de Miguel et al. 2002). It has been shown to relax airway smooth muscle reducing bronchoconstriction (Adnot et al. 1995, Tucker et al. 1990). To test whether nitric oxide is involved in vagally-mediated bronchoconstriction, guinea pigs were treated with L-NAME, a competitive antagonist of nitric oxide synthase, during airway physiology measurements.

We labeled newly divided cells with BrdU to test whether ozone induces eosinophilopoiesis in bone marrow, resulting in recruitment of different eosinophil populations to the lungs, and tested the role of TNFα and nitric oxide (Saluja et al. 2010; Spencer et al. 2008; Yoshimura et al. 2012) in ozone-induced airway hyperreactivity. We also tested whether the effect of ozone on lung eosinophils was dependent upon sensitization status.

SPECIFIC AIMS

The central hypothesis tested here is that ozone exposure initiates production of eosinophils in bone marrow and their subsequent recruitment to lungs. These newly synthesized and recruited eosinophils have a delayed but beneficial role in ozone-induced inflammation, since depleting them significantly worsens airway hyperreactivity three days after ozone exposure. Since atopic or allergic status results in different inflammatory cell profiles, we tested whether ozone exposure recruited these beneficial eosinophils to lungs in sensitized guinea pigs.

- Aim 1: Measure whether ozone exposure induces eosinophil expansion in bone marrow and subsequent migration to lungs three days later in nonsensitized and sensitized guinea pigs.
- Aim 2: Since TNFα can mediate both eosinophil recruitment and release of nitric oxide from eosinophils, and nitric oxide mediates bronchodilation, measure the roles of TNFα and of nitric oxide in ozone-induced airway hyperreactivity and associated recruitment of newly divided eosinophils in nonsensitized and sensitized guinea pigs.

Determining whether there are distinct eosinophil populations, distinguished by different physiological functions, is important in understanding how human asthma phenotypes may respond to air pollution and ozone exposure and in identifying who may benefit from eosinophil depletion or TNFα inhibition.

METHODS AND STUDY DESIGN

ANIMALS

Specific pathogen-free female Dunkin-Hartley guinea pigs were shipped from Charles River Laboratories (Kingston, NY) in filtered crates and housed in rooms with high-efficiency particulate-filtered air. Animals were exposed to either ozone (2.0 ppm) or filtered air for four hours and returned to the animal facility. One or three days later, airway physiology was measured or inflammatory cells from bone marrow, blood, and lungs were harvested. Some guinea pigs (150–200g) were sensitized to ovalbumin and exposed to air or ozone twenty-one days later. At the time of exposure all guinea pigs weighed between 350 and 400g.

At the end of each experiment, animals were killed by an overdose of barbiturate i.v. Death was confirmed by the absence of a heartbeat.

All animal procedures and experimental protocols complied with National Institutes of Health Guidelines and were reviewed and approved by the animal care and use committee of Oregon Health & Science University.

OZONE EXPOSURE

Guinea pigs were placed in individual wire cages with access to food and water. Cages were then placed within a 700-Liter stainless steel exposure chamber with laminar airflow and animals were exposed to either ozone (2.0 ppm) or filtered air for four hours. Ozone was generated by an ultraviolet light generator (Orec, Glendale, CA) and introduced into the chamber airflow at a rate of 2 L/min. Ozone concentrations within the chamber were monitored (model 1008 AH, Dasibi Environmental, Glendale, CA). The air supply within the chamber was replaced at a rate of 20 times/hour. After exposure, guinea pigs were returned to the animal facility, housed in filtered air, and given free access to food and water.

PRETREATMENTS

Sensitization

Some guinea pigs were sensitized to ovalbumin (4.2 mg i.p. on days 1, 3, and 5). Three weeks after the last ovalbumin injection guinea pigs were exposed to ozone (2.0 ppm) or to filtered air for four hours as previously described (Yost et al. 1999, 2005).

BrdU Treatment

In order to measure which inflammatory cells were newly divided after ozone exposure, some guinea pigs were treated with BrdU, a thymidine analogue that is incorporated into DNA of dividing cells. Animals received 50 mg BrdU/kg i.p. immediately prior to ozone or air exposure, and another 50 mg/kg i.p two hours after exposure. Thereafter, animals received a single daily dose of 50 mg BrdU/kg i.p. Preliminary experiments indicated that doses of BrdU greater than 100 mg/kg significantly inhibited inflammation and bronchoconstriction induced by electrical stimulation of vagus nerves (data not shown); thus, all experiments were conducted using 50 mg/kg BrdU. We determined that this BrdU dose did not inhibit airway inflammation or hyperreactivity (data not shown). For some experiments to measure physiological endpoints, some guinea pigs served as controls and were not treated with BrdU. There were no differences in physiological responses between BrdU treated and BrdU untreated groups, so data from these groups were combined.

Inhibitors and Antibodies

The role of TNFα was measured by treating guinea pigs with 3 mg/kg, i.p. of etanercept, a TNFα antagonist, three hours before exposure to ozone or air. Since the half-life of etanercept is four days, no additional doses were given.

The role of nitric oxide in ozone-induced airway inflammation and airway hyperreactivity was determined by measuring bronchoconstriction at electrical stimulation frequencies ranging from 1 to 25 Hz, inhibiting nitric oxide synthase with L-NAME (30 mg/kg i.v.), repeating measurements thirty minutes later, then comparing frequency–response curves. Time controls showed no difference between these frequency–response curves (data not shown).

Some guinea pigs were pretreated with AbIL-5 (TRFK5; 240 μg/kg i.p., given three days before ozone or air exposure) to deplete eosinophils. This dose has been shown to deplete eosinophils in BAL, lung tissue, and skin (Proskocil et al. 2008, 2015; Yang et al. 1997; Yost et al. 1999, 2005).

PHYSIOLOGICAL STUDIES

One or three days after ozone exposure, physiological studies were performed to measure airway hyperreactivity. Guinea pigs were anesthetized with urethane (1.9 g/kg i.p., Sigma Aldrich, St. Louis, MO) and both jugular veins were cannulated for drug administration. Animals were tracheotomized, mechanically ventilated with positive pressure at a constant volume (1 mL volume/100 g body weight; 100 breaths/min), and paralyzed (succinylcholine chloride 10 μg/kg i.v.; Sigma Aldrich, St. Louis, MO). A carotid artery was cannulated for measurement of heart rate and blood pressure. All animals were pretreated with guanethidine (5 mg/kg i.v.) to deplete noradrenaline. Both vagus nerves were isolated, cut, and distal ends placed on platinum electrodes and covered with mineral oil. Pulmonary inflation pressure (Ppi) is the pressure required to inflate the lungs and was measured using a pressure transducer (Becton Dickinson, Franklin Lakes, NJ) attached to a side arm of the tracheal cannula. Bronchoconstriction was measured as an increase in pulmonary inflation pressure (in mm H_2O) over baseline inflation pressure as previously described (Fryer and Maclagan 1984). Bradycardia was measured as a decrease in heart rate from baseline.

Both vagus nerves were electrically stimulated simultaneously (10 V, 0.2 millisecond pulse width, 1–25 Hz, 5-second duration) at one-minute intervals producing frequency-dependent bronchoconstriction and bradycardia. These responses are due to acetylcholine release from parasympathetic nerves onto muscarinic receptors located on airway or cardiac smooth muscle, since both responses could be abolished by atropine (1 mg/kg i.v.).

The function of M_3 muscarinic receptors on airway smooth muscle was assessed in vagotomized guinea pigs by administering i.v. methacholine. Intravenous methacholine (1–10 μg/kg), caused bronchoconstriction and bradycardia in vagotomized animals in a dose-related manner.

INFLAMMATORY CELL COLLECTION FOR ANALYSIS

Blood, bone marrow, and BAL were collected at the end of each experiment, and cells were counted and sorted based upon whether they were positive for BrdU.

Blood

Blood was collected from the arterial cannula into a heparinized syringe, 0.5 mL was lysed with 9.5 mL HCl, and total white blood cells were counted using a hemocytometer. To measure white blood cell differentials and BrdU staining, 1 mL of blood was lysed with water, centrifuged for 10 min at 300g to remove supernatant, resuspended in 2 mL phosphate buffered saline (PBS), and cytospun onto slides.

Bone Marrow

Bone marrow was harvested by isolating the left femur and flushing the marrow with 10 mL PBS. Cells were centrifuged for 10 min at 300g, resuspended in 2 mL PBS, counted with a hemocytometer to obtain total cell counts, and cytospun onto slides.

Bronchoalveolar Lavage

BAL was collected via the trachea cannula as previously described (Yost et al. 2005) with five (10 mL) aliquots PBS (room temperature). Cells were centrifuged for 10 min at 300g, resuspended in 2 mL PBS, counted with a hemocytometer to obtain total cell counts, and cytospun onto slides.

Staining for Differentials and BrdU

Slides were fixed in 70% ethanol (overnight, 4°C). Endogenous peroxidase activity was quenched by incubating slides in 3% hydrogen peroxide in cold methanol (−20°C) (10 min). Slides were washed with water and DNA was denatured using dilute HCl (reagent 2, BrdU staining kit, Invitrogen) (30 min). Slides were washed with PBS containing 0.05% tween, blocked with reagent 3 (BrdU staining kit, Invitrogen) (10 min), and incubated for 1 hour with mouse anti-BrdU biotinylated antibody (reagent 4, BrdU staining kit, Invitrogen). Slides were washed with PBS containing 0.05% tween. BrdU staining was amplified using ABC elite (Vector Laboratories) (30 min). BrdU staining was visualized with ImmPACT DAB (Vector laboratories) (10 min). To obtain differential counts, slides were then stained with Hemacolor (EMD Chemicals, Philadelphia, PA).

Sources of Antibodies and Drugs

AbIL-5 (TRFK-5) was purchased from BD Pharmagen (San Diego, CA). Etanercept was purchased from Immunex (Thousand Oaks, CA). BrdU, atropine, guanethidine, methacholine, succinylcholine, and urethane were purchased from Sigma (St. Louis, MO). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). All drugs were dissolved and diluted in 0.9% NaCl.

STATISTICAL METHODS AND DATA ANALYSIS

All data are expressed as means ± standard error of the mean (SEM).

Frequency and methacholine dose–response curves were analyzed using a two-way analysis of variance (ANOVA) for repeated measures (as responses to multiple frequencies or multiple doses of methacholine are recorded from the same animal).

Inflammatory cells, and BrdU+ and BrdU− cells in BAL, blood, and bone marrow were analyzed by one-way ANOVA comparing selected columns followed by a Bonferroni correction.

Baseline Ppi, weight, heart rate, and blood pressure were analyzed by ANOVA comparing selected pairs of columns with a Bonferroni correction.

RESULTS

EFFECT OF OZONE EXPOSURE, OVALBUMIN SENSITIZATION, AND ETANERCEPT ON PHYSIOLOGICAL PARAMETERS

Exposure to ozone increased the pressure required to inflate the lungs (baseline Ppi) in all groups shown in Table 1, regardless of treatment or number of days after exposure. Heart rate and blood pressure were not greatly affected by ozone exposure or any of these treatments (Table 1). Weight was greater in sensitized than in nonsensitized animals. However, neither changes in inflation pressure nor weight appeared to affect bronchoconstriction since they did not predict airway hyperreactivity (Figure 1), and i.v. methacholine-induced bronchoconstriction was unchanged (Appendix Figure A.1).

EFFECT OF OZONE EXPOSURE ON AIRWAY PHYSIOLOGY

In the lungs, ozone exposure significantly increased vagally-induced bronchoconstriction one and three days after a single exposure to 2 ppm ozone for four hours (Figure 1A). Bronchoconstriction after ozone exposure was greater on day one than on day three. Ozone-induced airway hyperreactivity on day three was also significantly potentiated in animals that had been sensitized to ovalbumin (Figure 1B; compare sensitized ozone; filled squares, to ozone in nonsensitized animals; grey line).

Neither ozone exposure nor sensitization affected bronchoconstriction induced by i.v. methacholine, which directly stimulates airway smooth muscle and bypasses the cut vagus nerves, demonstrating that airway hyperreactivity was mediated at the level of the parasympathetic nerves (Appendix Figure A.1 A, B). Vagally-induced bronchoconstriction was blocked by atropine (1 mg/kg i.v.) in all groups (% decrease in vagally-induced bronchoconstriction with atropine: filtered air 96.4 ± 1.2%, ozone day 1 95.7 ± 2.2%, ozone day 3 94.3 ± 0.63%, filtered air sensitized 97.0 ± 0.63%, ozone sensitized 96.5 ± 2.1%), demonstrating that it was mediated by release of acetylcholine onto muscarinic receptors. Thus, ozone exposure caused

Table 1. Baseline Data for All Groups

Treatment Group	Exposure	Weight (g)	Baseline Ppi (mm H_2O)	Heart Rate (Beats/Min)	Blood Pressure (mm Hg)	
					Systolic	Diastolic
Nonsensitized	Air	351.3 ± 5.2	102.2 ± 4.9	287.8 ± 9.8	41.0 ± 1.1	19.6 ± 0.7
	O_3 Day 1	383.5 ± 11.2	202.5*± 4.8	273.8 ± 5.5	46.0 ± 2.1	20.9 ± 1.0
	O_3 Day 3	382.6 ± 11.0	151.4*± 6.3	283.6 ± 6.0	44.8 ± 2.7	24.0 ± 1.5
Nonsensitized +Etanercept	Air	360.5 ± 3.3	105.0 ± 8.6	272.5 ± 18.7	44.5 ± 1.5	24.5#± 2.0
	O_3 Day 3	379.6 ± 4.8	172.0*± 12.4	272.0 ± 8.7	44.8 ± 1.7	21.6 ± 1.3
Sensitized	Air	406.8# ± 17.4	102.0 ± 8.6	302.0 ± 9.9	43.5 ± 2.9	20.0 ± 0.8
	O_3 Day 3	394.8 ± 3.0	158.3*± 12.7	275.0*± 4.3	44.3 ± 1.9	22.3 ± 1.6
Sensitized + Etanercept	Air	402.7 ± 16.9	95.0 ± 5.0	275.0 ± 5.0	45.0 ± 1.0	21.5 ± 0.5
	O_3 Day 3	366.5 ± 4.3	137.5 ± 19.7	277.5 ± 8.5	47.0 ± 3.1	23.0 ± 1.0

* are significantly different from air control within each group and # is significantly different from nonsensitized air controls.

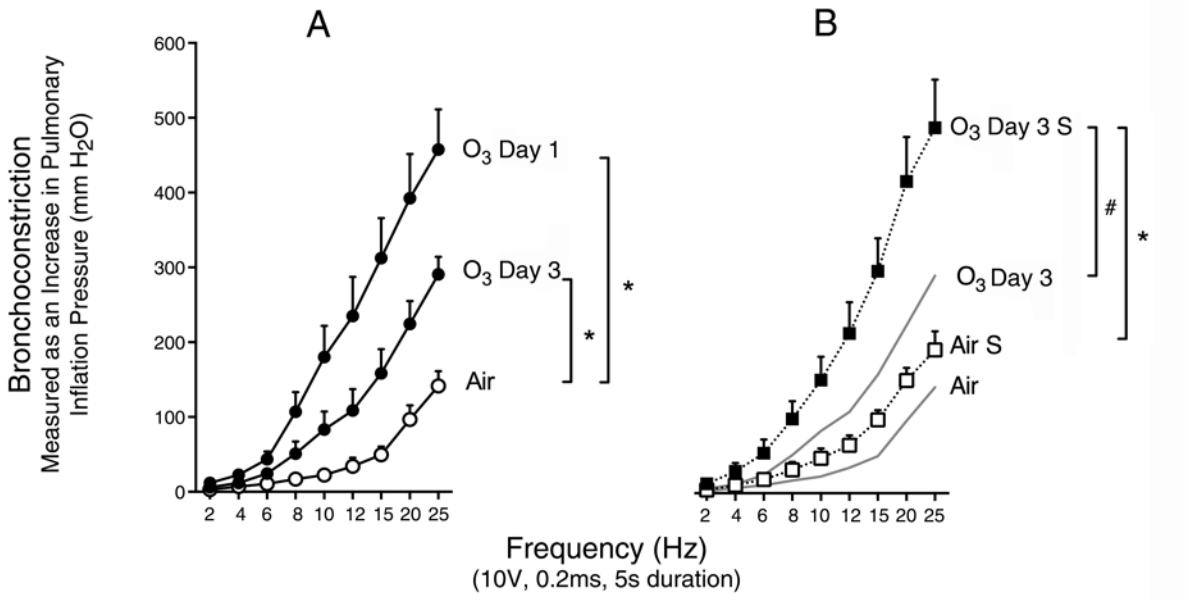

Figure 1. Vagally-induced bronchoconstriction on days 1 or 3 in ovalbumin-sensitized (S) and nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. (A) Nonsensitized: filtered air (n = 9), ozone day 1 (n = 4), or ozone day 3 (n = 7). **(B)** Sensitized: filtered air (n = 5) or ozone day 3 (n = 5). To allow statistical comparison with the nonsensitized data, the filtered air and ozone day 3 data from panel A are shown in panel B as lines without symbols. Data are mean ± SEM. Brackets show comparisons: * = $P < 0.05$; # = $P < 0.05$ by repeated measures two-way ANOVA.

airway hyperreactivity at the level of the vagus nerves lasting at least three days, confirming previous studies (Yost et al. 2005). Sensitization significantly potentiated ozone-induced airway hyperreactivity.

In the heart, electrical stimulation of the vagus nerves induced frequency-related bradycardia (Appendix Figure A.2 A, B), and i.v. methacholine induced dose-related bradycardia (Appendix Figure A.2 W, X). Neither ozone exposure nor sensitization affected bradycardia (Appendix Figure A.2 A, B, W, X). Thus, the ability of ozone exposure to potentiate vagal responses is limited to the lungs.

ROLE OF EOSINOPHILS IN OZONE-INDUCED AIRWAY HYPERREACTIVITY

Depleting eosinophils with AbIL-5 potentiated ozone-induced airway hyperreactivity in nonsensitized guinea pigs (Figure 2A), but completely inhibited ozone-induced airway hyperreactivity in sensitized animals (Figure 2B). Both time points were measured three days after exposure to ozone. This demonstrates that eosinophils have opposite effects three days after ozone exposure in sensitized vs. nonsensitized animals. In sensitized animals eosinophils mediate hyperreactivity, while in nonsensitized animals they inhibit airway hyperreactivity.

EFFECT OF OZONE EXPOSURE ON LUNG INFLAMMATION

In nonsensitized animals ozone exposure caused airway inflammation both one and three days after inhalation (Figure 3; top row, left column). The increase in inflammatory cells in BAL included both BrdU+ (Figure 3; top row, center column) and BrdU− cells (Figure 3; top row, right column).

In sensitized animals ozone-induced inflammation was potentiated (Figure 4; top row, left column). However, the increase was largely comprised of BrdU− cells. Newly formed (BrdU+) cells failed to increase in BAL after ozone exposure (Figure 4; top row, right and center columns). The same shift from newly formed cells (BrdU+) to preexisting cells (BrdU−) was also seen in airway neutrophils and macrophages (Figure 4). Sensitization alone significantly increased eosinophils in BAL even in the absence of ozone (Figure 4, left column, Air vs. AirS). Ozone exposure did not increase lymphocytes in BAL at all unless animals were sensitized first, and then both BrdU+ and BrdU− cells were increased (Figure 4).

Figures 5 and 6 show the results of ozone exposure on circulating inflammatory cells in nonsensitized and sensitized guinea pigs. Ozone exposure did not change numbers of any inflammatory cells, neither BrdU+ nor BrdU−, in blood of nonsensitized guinea pigs one or three days after exposure to ozone.

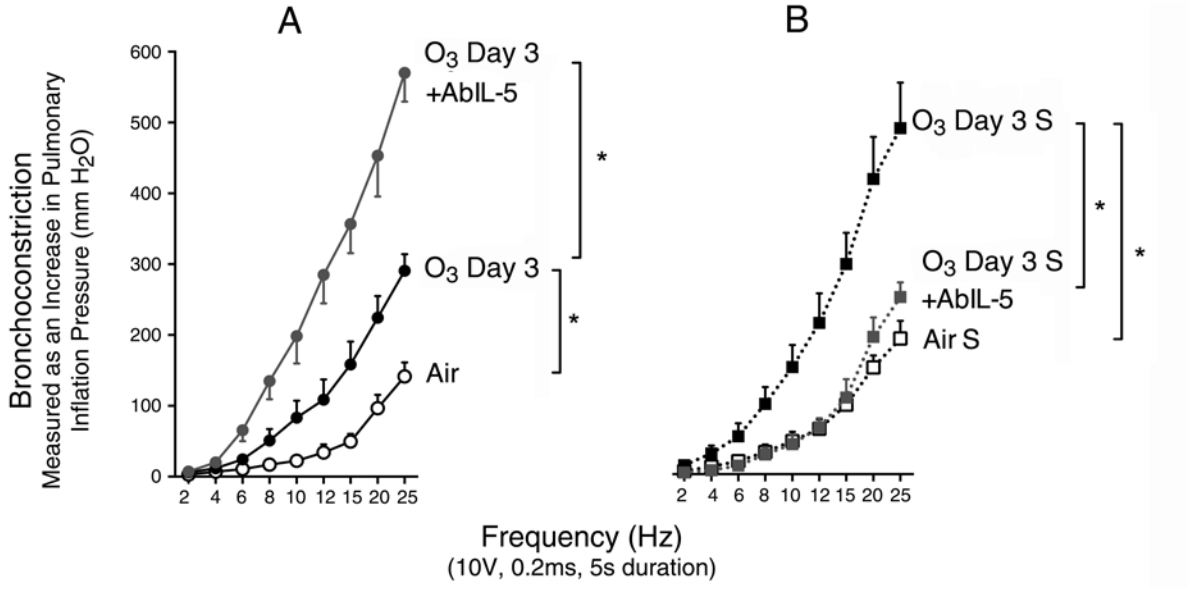

Figure 2. Vagally-induced bronchoconstriction on day 3 in ovalbumin-sensitized (S) and nonsensitized guinea pigs pretreated with AbIL-5 and exposed to 2 ppm ozone or filtered air for 4 hours. (A) Nonsensitized: filtered air (n = 9), ozone (n = 7), or ozone with AbIL-5 (n = 3); **(B)** Sensitized: filtered air (n = 5), ozone (n = 5), or ozone with AbIL-5 (n = 4). Data are mean ± SEM. Brackets show comparisons: * = $P < 0.05$ by repeated measures two-way ANOVA.

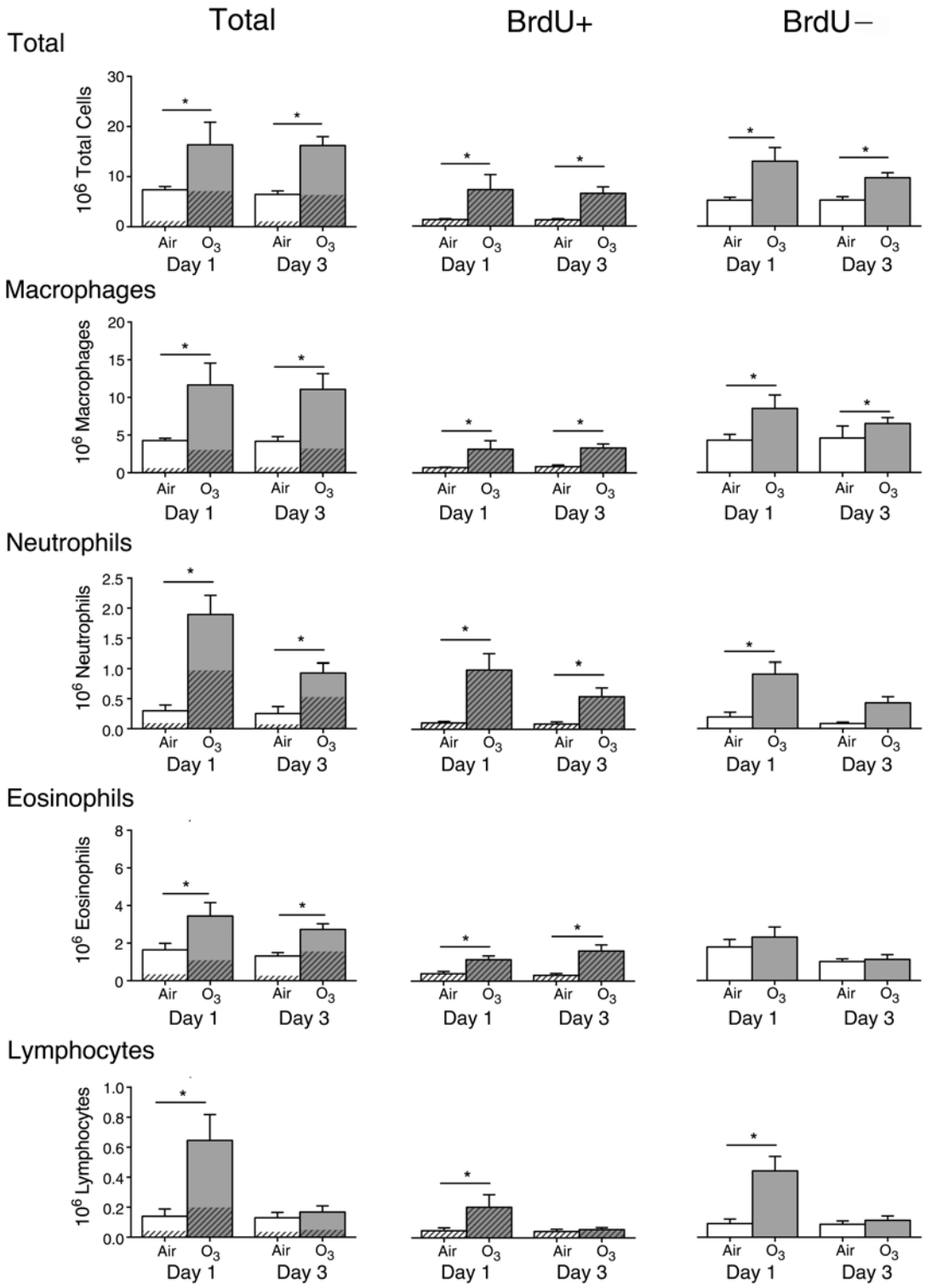

Figure 3. Newly divided inflammatory cells (BrdU+) on days 1 or 3 in BAL from nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. Data are expressed in millions of cells, mean ± SEM. Filtered air day 1 (n = 8), ozone day 1 (n = 7), filtered air day 3 (n = 9), and ozone day 3 (n = 8). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

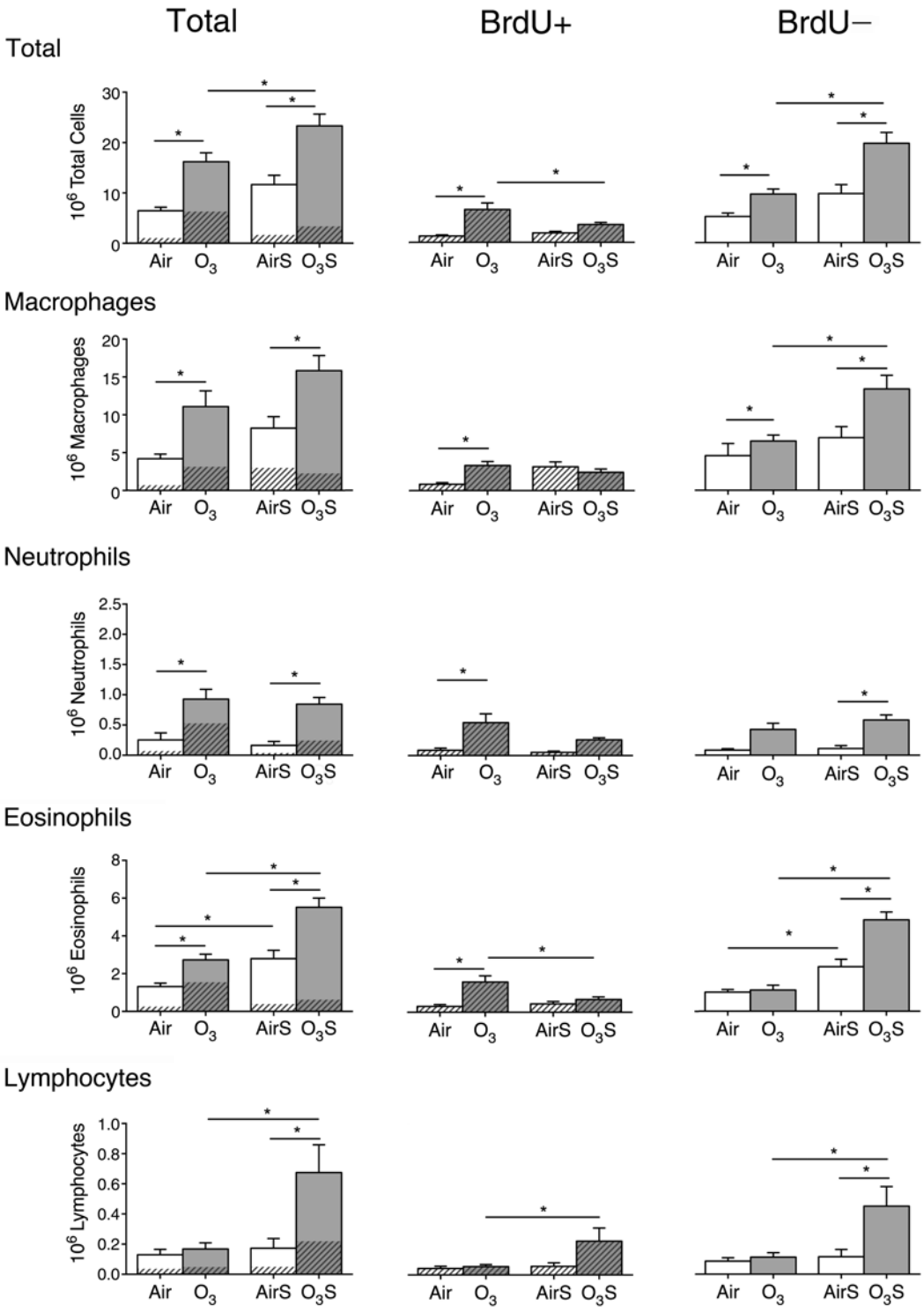

Figure 4. Newly divided inflammatory cells (BrdU+) on day 3 in BAL of ovalbumin-sensitized (S) or nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 3 (nonsensitized); the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells, mean ± SEM. Filtered air day 3 (n = 9), ozone day 3 (n = 8), sensitized filtered air day 3 (n = 8), and sensitized ozone day 3 (n = 8). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

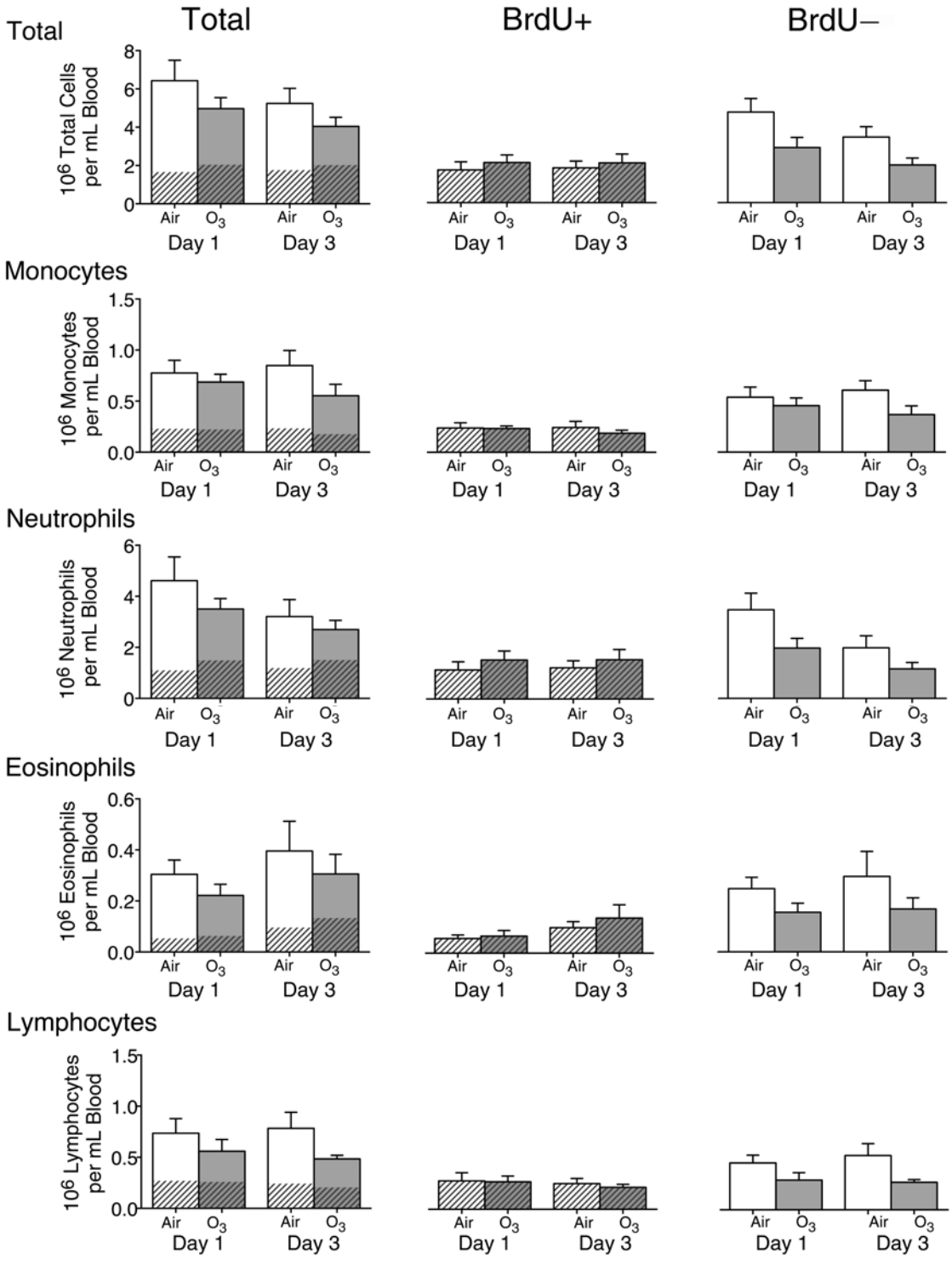

Figure 5. Newly divided inflammatory cells (BrdU+) on day 1 or 3 in circulating blood from nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. Data are expressed in millions of cells/mL blood, mean ± SEM. Filtered air day 1 (n = 8), ozone day 1 (n = 7), filtered air day 3 (n = 9), and ozone day 3 (n = 8). * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

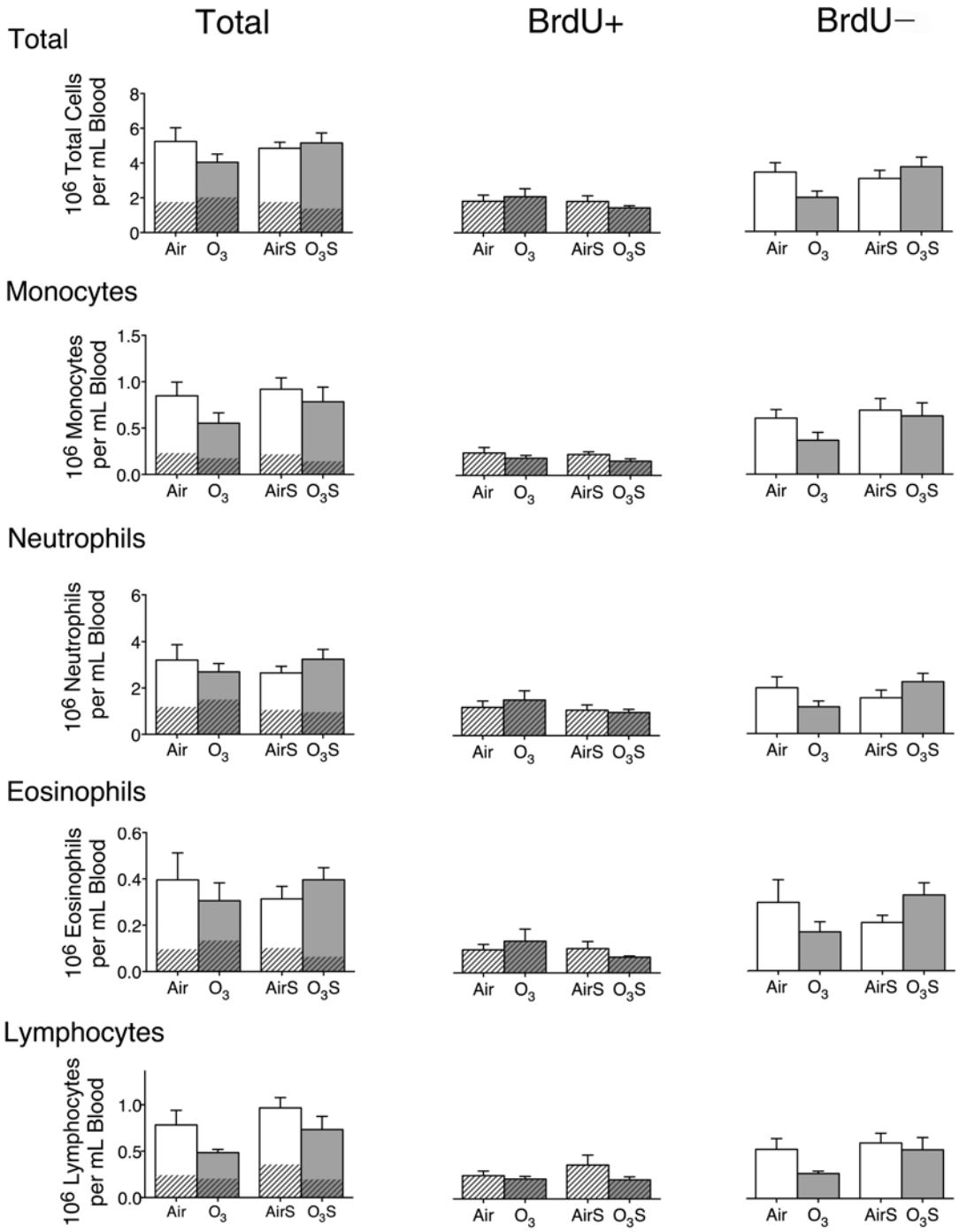

Figure 6. Newly divided inflammatory cells (BrdU+) on day 3 in circulating blood from ovalbumin-sensitized (S) or nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 5 (nonsensitized); the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells/mL blood, mean ± SEM. Filtered air day 3 (n = 9), ozone day 3 (n = 8), sensitized filtered air day 3 (n = 8), and sensitized ozone day 3 (n = 8). * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

EFFECT OF OZONE EXPOSURE ON INFLAMMATORY CELLS IN BONE MARROW

In nonsensitized animals, ozone exposure increased BrdU+ eosinophils in bone marrow from 25% in filtered air controls to 41% one day after ozone exposure, and from 35% (three days after filtered air exposure) to 63% three days after ozone exposure. The increase in BrdU+ eosinophils was significant three days after ozone exposure and was accompanied by a corresponding, significant, decrease in BrdU− eosinophils (Figure 7, eosinophils, center and right columns). In contrast ozone exposure did not increase monocytes or neutrophils in bone marrow either one or three days after ozone exposure (Figure 7, left column). Thus, more than half the eosinophils in bone marrow were newly divided three days after ozone exposure, while no other inflammatory cells in bone marrow were affected by ozone.

Sensitization alone had no effect on the percentage of cells in bone marrow, or the distribution of BrdU+ or BrdU− cells. However, the ozone-induced, significant increase in BrdU+ eosinophils seen three days after exposure to ozone in nonsensitized animals was completely blocked in sensitized animals (Figure 8, eosinophils, center column). Sensitization increased the percentage of BrdU+ eosinophils (at three days) from 35% in nonsensitized, to 40%; the addition of ozone exposure did not further

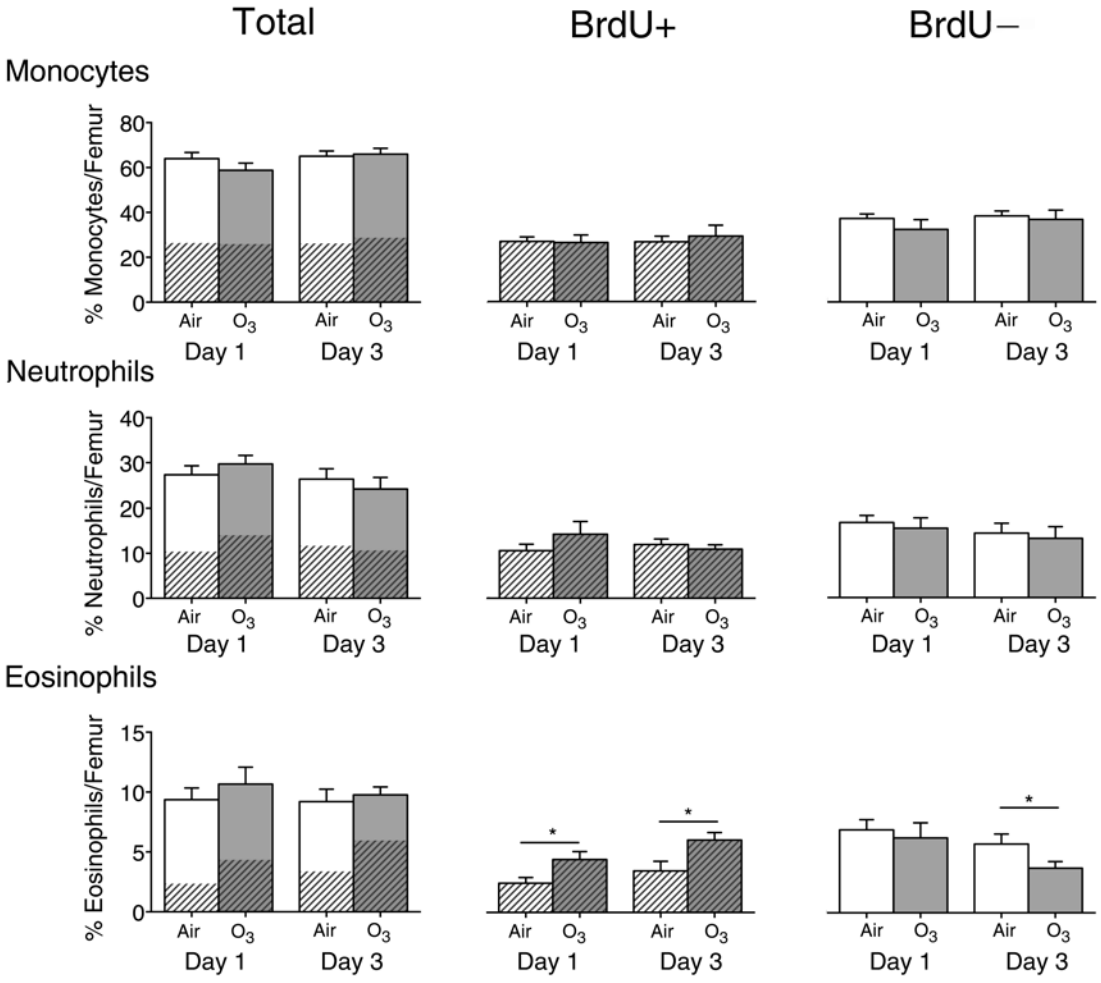

Figure 7. Newly divided inflammatory cells (BrdU+) on day 1 or 3 in bone marrow from nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. Data are expressed as percentage of total cells in the bone marrow of the left femur, mean ± SEM. Filtered air day 1 (n = 8), ozone day 1 (n = 7), filtered air day 3 (n = 9), and ozone day 3 (n = 8). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

increase BrdU+ eosinophils (42%) in sensitized animals. Thus, ozone exposure caused eosinophilopoiesis in bone marrow of nonsensitized animals but had no effect on eosinophils in bone marrow of sensitized animals.

CONTRIBUTION OF NITRIC OXIDE TO OZONE-INDUCED AIRWAY HYPERREACTIVITY

Inhibiting nitric oxide production by pretreating with L-NAME 30 minutes prior to physiological measurements did not affect ozone-induced airway hyperreactivity three days after ozone exposure (Figure 9). A time control showed that repeated frequency–response curves were not different from each other (data not shown). Inflammatory cells in bone marrow, blood, and bronchoalveolar lavage were not changed by administration of L-NAME during physiological measurements (data not shown). Nitric oxide thus appears to play no role in ozone-induced hyperreactivity.

CONTRIBUTION OF TNFα TO OZONE-INDUCED AIRWAY HYPERREACTIVITY

Blocking TNFα with etanercept in nonsensitized animals potentiated ozone-induced airway hyperreactivity (Figure 10A) that was identical in scale to hyperreactivity induced by sensitizing animals to ovalbumin (Figures 1B

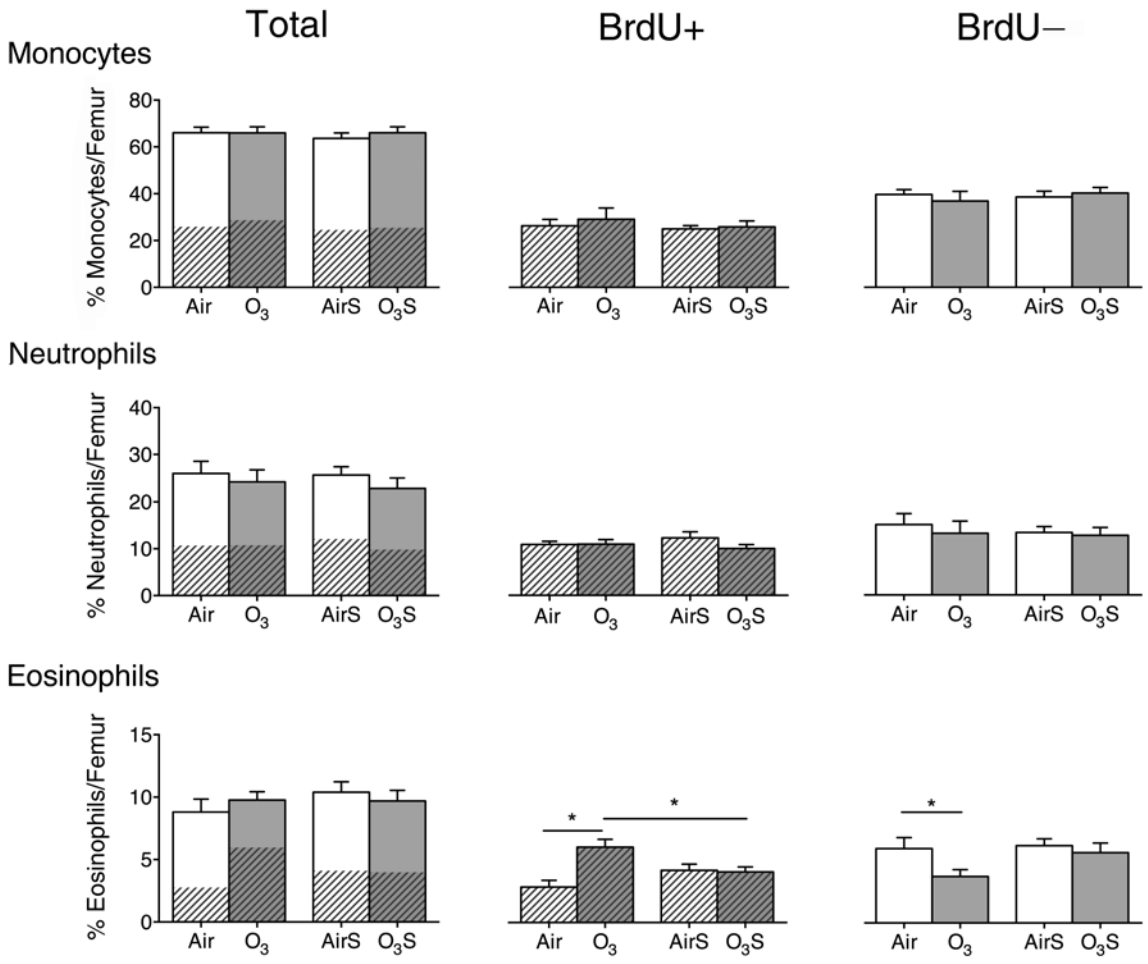

Figure 8. Newly divided inflammatory cells (BrdU+) on day 3 in bone marrow from ovalbumin-sensitized (S) or nonsensitized guinea pigs after a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 7 (nonsensitized); the data are reproduced here to allow statistical comparison. Data are expressed as percentage of total cells in the bone marrow of the left femur, mean ± SEM. Filtered air day 3 (n = 9), ozone day 3 (n = 8), sensitized filtered air day 3 (n = 8), and sensitized ozone day 3 (n = 8). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

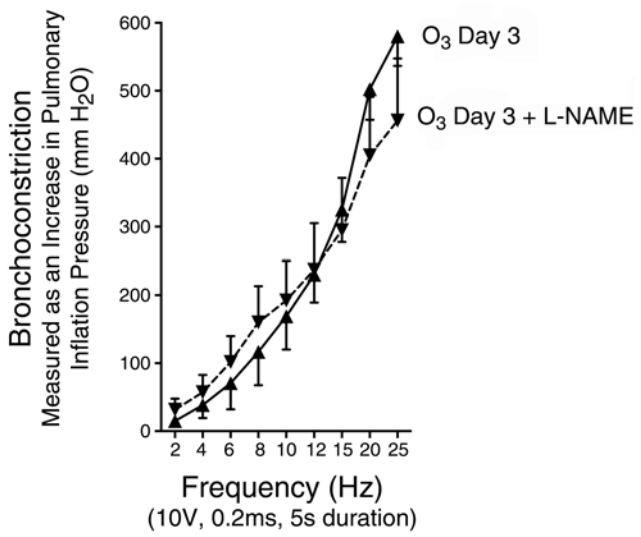

Figure 9. Effect of L-NAME on vagally-induced bronchoconstriction in guinea pigs 3 days after a 4-hour exposure to 2 ppm ozone. Upward triangles = bronchoconstriction before L-NAME; downward triangles = bronchoconstriction 30 minutes after L-NAME. Data are mean ± SEM. Ozone day 3 + L-NAME (n = 4). * = $P < 0.05$ by repeated measures two-way ANOVA.

and 10A). Etanercept had no effect on methacholine-induced bronchoconstriction (Appendix Figure A.1C), demonstrating that potentiation of airway hyperreactivity was mediated at the level of the nerves and not at the level of the airway smooth muscle. Combining etanercept and sensitization had no additional effect on ozone-induced airway hyperreactivity (Figure 10B) or on methacholine-induced bronchoconstriction (Appendix Figure A.1D) compared with sensitization alone.

In the heart, both electrical stimulation of the vagus nerves and i.v. methacholine induced frequency- and dose-related bradycardia (Appendix Figure A.2). Etanercept had no significant effect on bradycardia in either sensitized or nonsensitized animals (Appendix Figure A.2 C, D, Y, Z).

EFFECT OF ETANERCEPT ON OZONE-INDUCED LUNG INFLAMMATION IN SENSITIZED AND NONSENSITIZED GUINEA PIGS

Similar to sensitization, etanercept pretreatment blocked ozone-induced influx of BrdU+ macrophages, neutrophils, and eosinophils into the BAL (Figure 11 [top left]). However, similar to sensitization, etanercept pretreatment

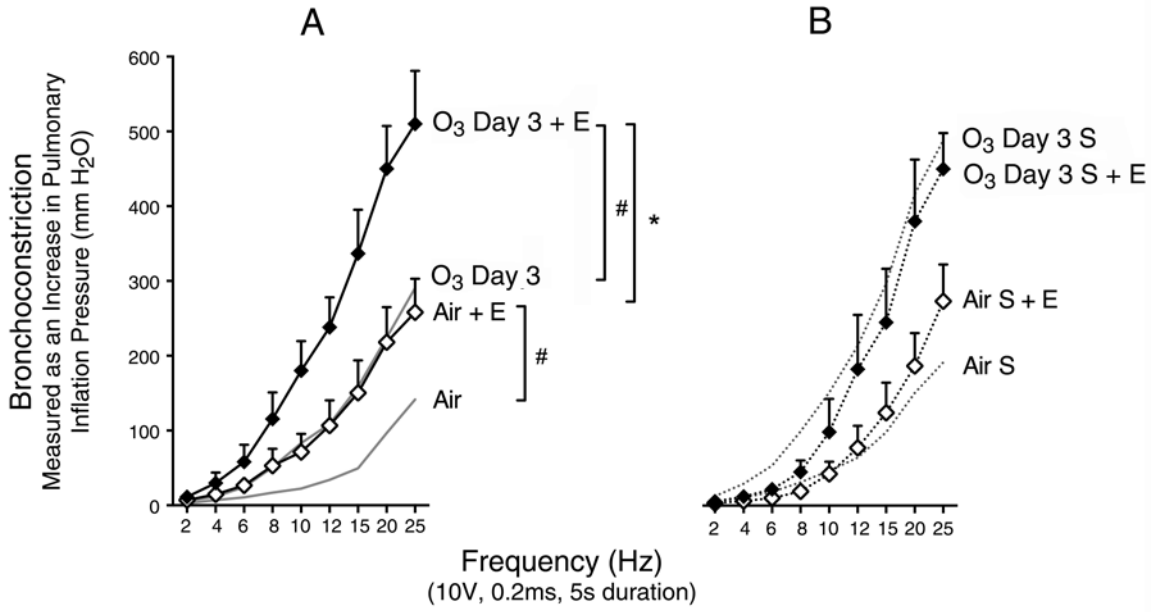

Figure 10. Vagally-induced bronchoconstriction on day 3 in ovalbumin-sensitized (S) and nonsensitized guinea pigs that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. (A) Nonsensitized: The nonsensitized filtered air and ozone day 3 data from Figure 1, panel A (lines without symbols) are compared to bronchoconstriction on day 3 for guinea pigs treated with etanercept and exposed to either filtered air or ozone. **(B)** Sensitized: The sensitized filtered air and ozone day 3 data are from Figure 1B (lines without symbols) and are compared to bronchoconstriction on day 3 measured for sensitized guinea pigs treated with etanercept and exposed to either filtered air or ozone. Data are mean ± SEM. Filtered air + etanercept (n = 5), ozone + etanercept (n = 6), sensitized air + etanercept (n = 4), and sensitized ozone + etanercept (n = 4). Brackets show comparisons: * = $P < 0.05$; # = $P < 0.05$ by repeated measures two-way ANOVA.

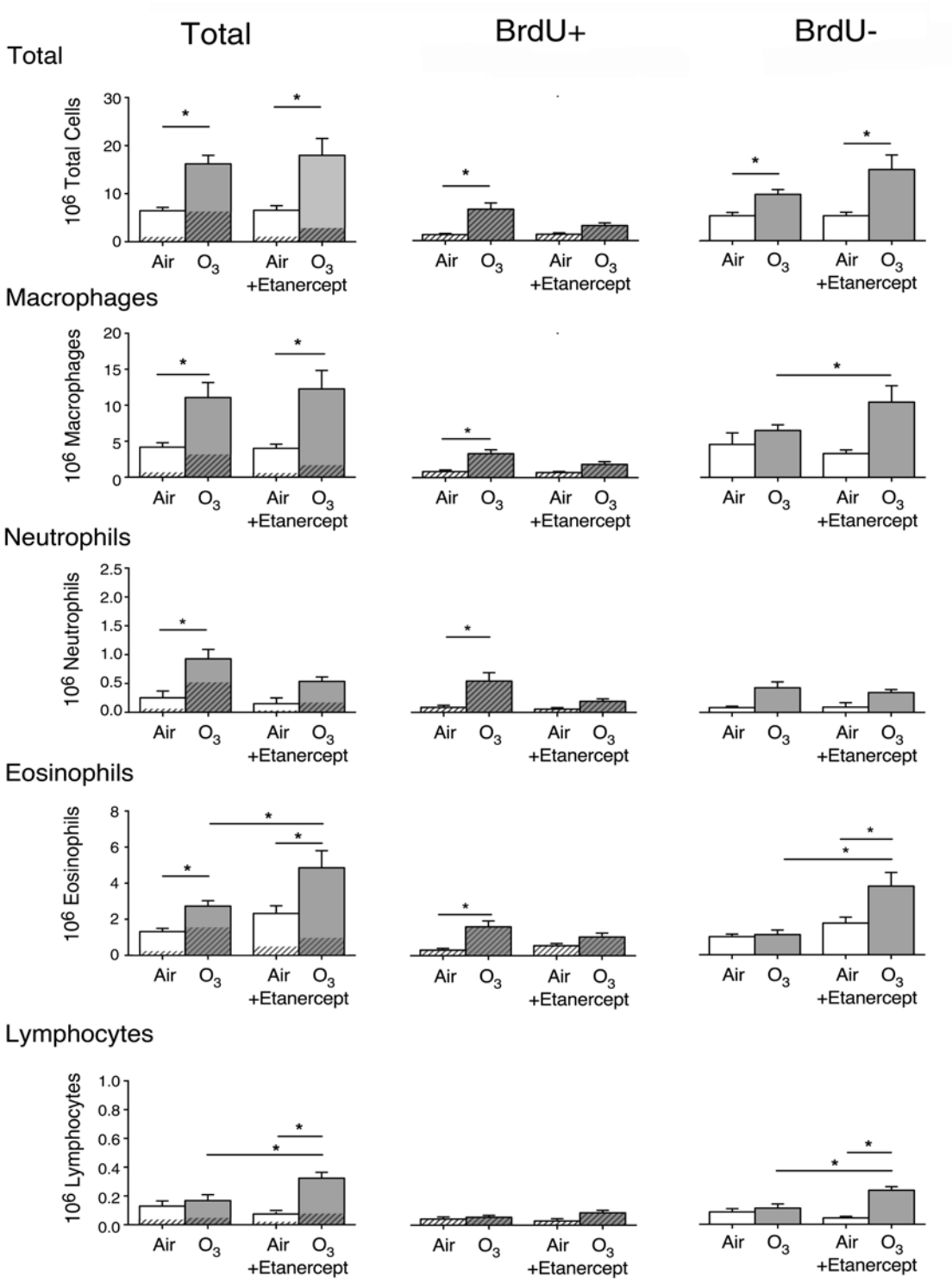

Figure 11. Newly divided inflammatory cells (BrdU+) on day 3 in BAL of nonsensitized guinea pigs that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 3; the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells, mean ± SEM. Filtered air day 3 (n = 9), and ozone day 3 (n = 8), filtered air + etanercept (n = 6), ozone + etanercept (n = 6). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

blocked the ozone-induced influx of BrdU+ macrophages, neutrophils, and eosinophils into the BAL (Figure 11, center column) while potentiating ozone-induced recruitment of BrdU− counterparts of these cells (Figure 11, right column). Etanercept showed similar effects on blocking entry of BrdU+ cells into the BAL of sensitized animals (Figure 12).

EFFECT OF ETANERCEPT ON CIRCULATING CELLS AFTER OZONE EXPOSURE IN SENSITIZED AND NONSENSITIZED GUINEA PIGS

Etanercept alone significantly increased circulating monocytes in the absence of ozone in nonsensitized guinea pigs. This increase was comprised equally of both BrdU+ and BrdU− cells (Figure 13). Etanercept also increased lymphocytes, though the increase was not significant. Ozone exposure had no additional effect on circulating monocytes or lymphocytes in etanercept-treated animals. Neither neutrophils nor eosinophils were altered by etanercept (Figure 13). Neither etanercept nor ozone exposure in the presence of etanercept significantly altered any cells in the blood of sensitized animals (Figure 14).

EFFECT OF ETANERCEPT ON OZONE-INDUCED INFLAMMATORY CELLS IN BONE MARROW

Similar to sensitization, etanercept pretreatment of nonsensitized animals blocked the ability of ozone to increase BrdU-labeled eosinophils in bone marrow (Figure 15; middle column, bottom row). Combining etanercept with sensitization had no additional effect (Figure 16). Thus, eosinophils are the only BrdU+ cells that are increased in bone marrow of nonsensitized animals three days after ozone exposure, and as with sensitization the increase is blocked by etanercept.

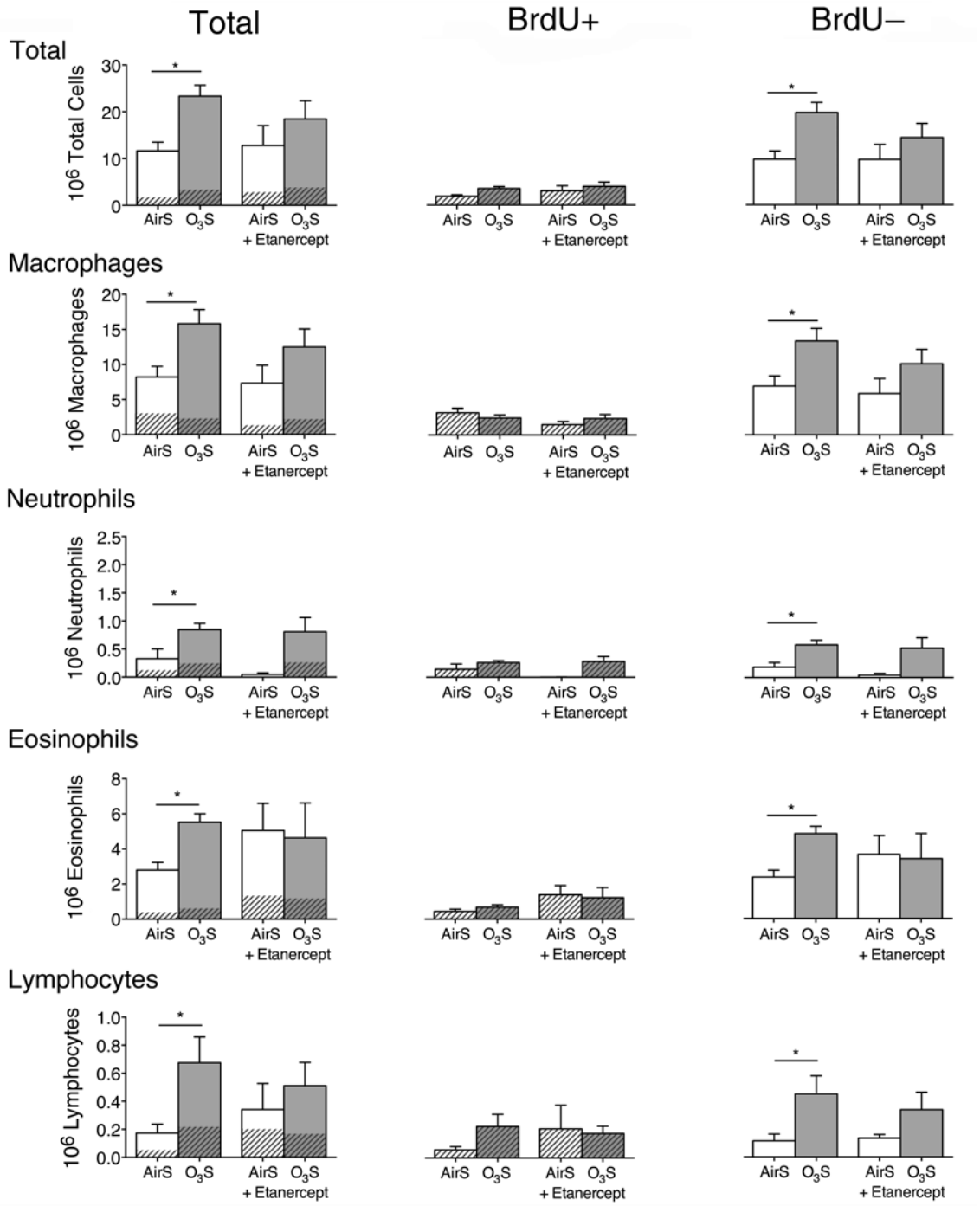

Figure 12. Newly divided inflammatory cells (BrdU+) on day 3 in BAL for ovalbumin-sensitized (S) guinea pigs that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 sensitized data in Figure 4; the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells, mean ± SEM. Sensitized filtered air day 3 (n = 8), and sensitized ozone day 3 (n = 8), sensitized filtered air + etanercept (n = 4), sensitized ozone + etanercept (n = 4). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

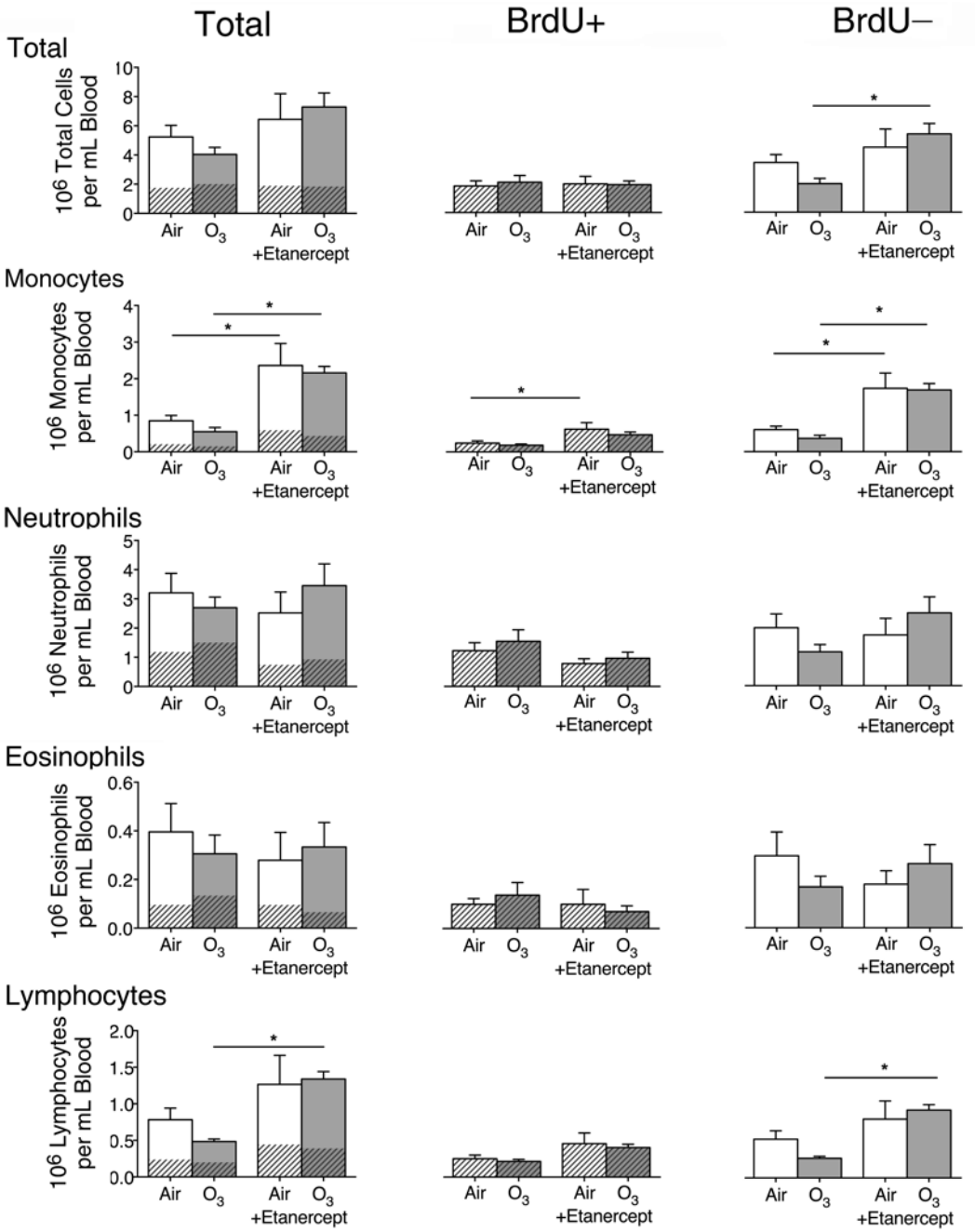

Figure 13. Newly divided inflammatory cells (BrdU+) on day 3 in circulating blood for nonsensitized guinea pigs that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 5; the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells/mL blood, mean ± SEM. Filtered air day 3 (n = 9), ozone day 3 (n = 8), filtered air day 3 + etanercept (n = 6), and ozone day 3 + etanercept (n = 6). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

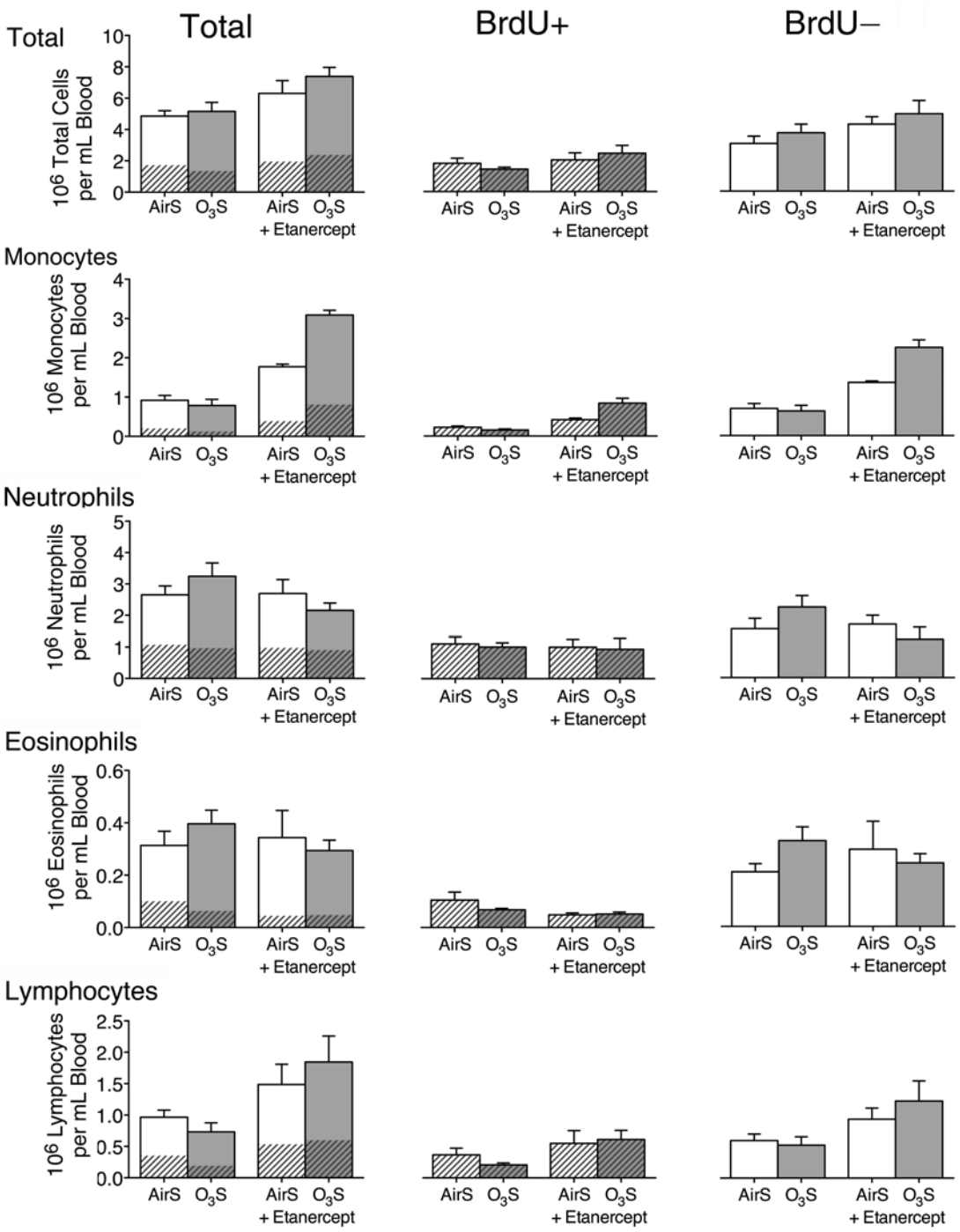

Figure 14. Newly divided inflammatory cells (BrdU+) on day 3 in circulating blood for ovalbumin-sensitized guinea pigs (S) that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 6; the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells/mL blood, mean ± SEM. Sensitized filtered air day 3 (n = 8), sensitized ozone day 3 (n = 8), sensitized filtered air day 3 + etanercept (n = 4), and sensitized ozone day 3 + etanercept (n = 4). * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

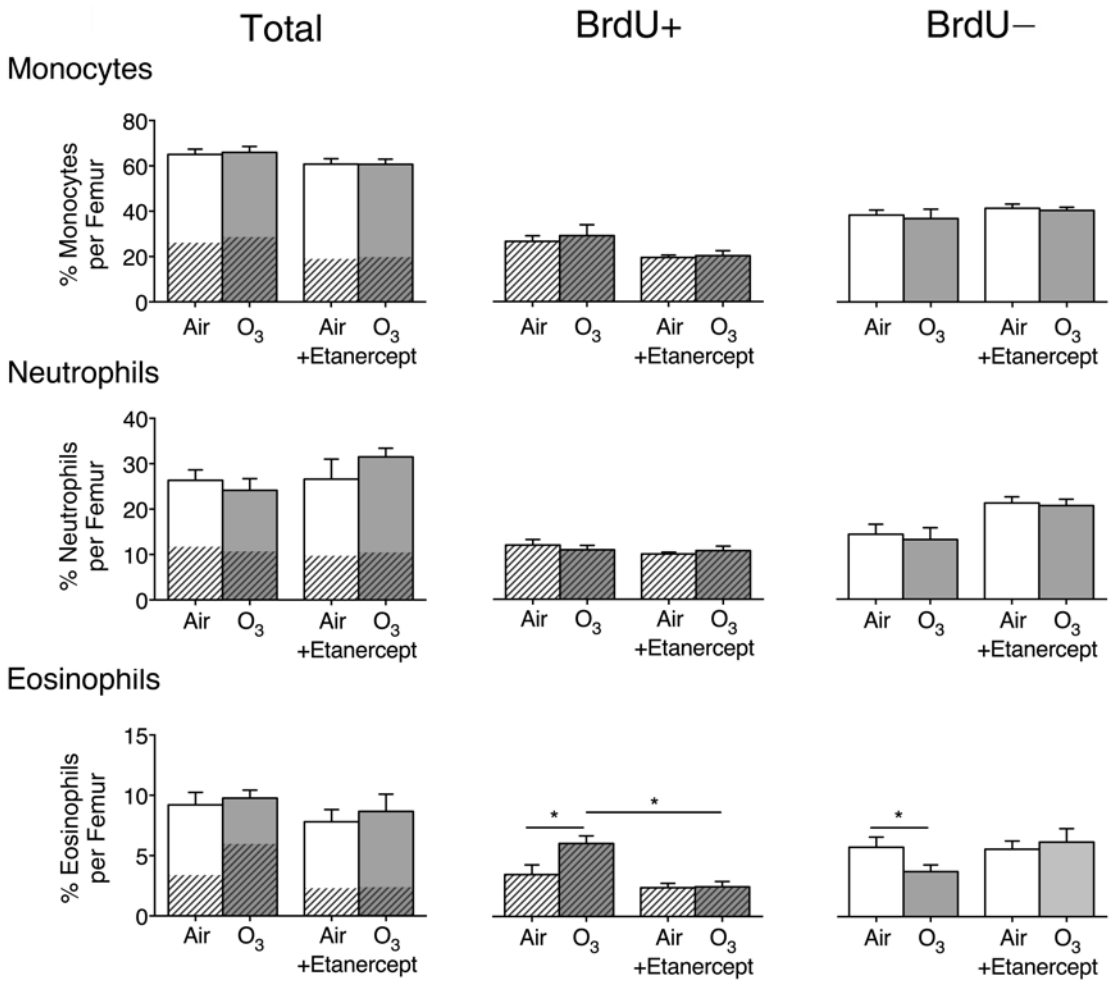

Figure 15. Newly divided inflammatory cells (BrdU+) on day 3 in bone marrow for nonsensitized guinea pigs that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 data in Figure 7 (nonsensitized); the data are reproduced here to allow statistical comparison. Data are expressed as percentage of total cells in the bone marrow of the left femur, mean ± SEM. Filtered air day 3 (n = 9), ozone day 3 (n = 8), filtered air day 3 + etanercept (n = 6), and ozone day 3 + etanercept (n = 6). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

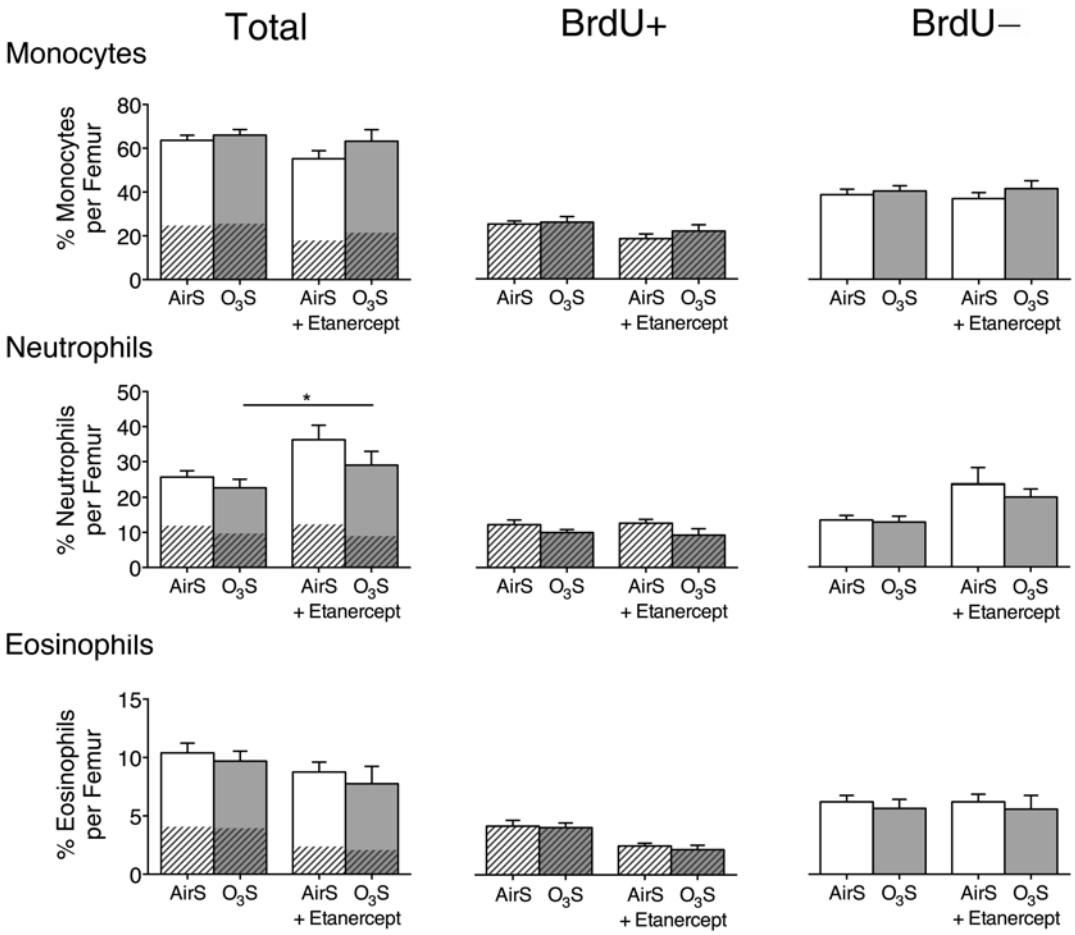

Figure 16. Newly divided inflammatory cells (BrdU+) on day 3 in bone marrow for ovalbumin-sensitized guinea pigs (S) that were treated with etanercept 3 hours before a 4-hour exposure to 2 ppm ozone or filtered air. BrdU+ newly divided cells are shown as hatched bars or hatched portions of bars; BrdU− preformed cells are shown as not hatched (white or solid color). Data from animals exposed to filtered air are shown in white bars; data from animals exposed to ozone are in grey bars. The first pair of bars in each column is identical to the day 3 sensitized data in Figure 8; the data are reproduced here to allow statistical comparison. Data are expressed in millions of cells in the bone marrow of the left femur, mean ± SEM. Sensitized filtered air day 3 (n = 8), sensitized ozone day 3 (n = 8), sensitized filtered air day 3 + etanercept (n = 4), and sensitized ozone day 3 + etanercept (n = 4). Horizontal bars show comparisons: * = $P < 0.05$ by one-way ANOVA with a Bonferroni correction.

DISCUSSION AND CONCLUSIONS

Ozone exposure increases airway eosinophils in humans, regardless of atopic status (Hernandez et al. 2010). After ozone exposure, eosinophils are recruited to parasympathetic nerves in lungs and release eosinophil major basic protein, which blocks neuronal M_2 muscarinic receptors that normally inhibit acetylcholine release from parasympathetic nerves (Yost et al. 2005). Thus after ozone exposure, acetylcholine release is increased, potentiating vagally-mediated bronchoconstriction (confirmed here in Figure 1). Depleting eosinophils completely prevents airway hyperreactivity one and three days after ozone exposure (Yost et al. 2005), supporting their central role in ozone-induced hyperreactivity. However, eosinophil populations in lungs fluctuate after ozone exposure (Murlas and Roum 1985; Villegas-Castrejon et al. 1999; Yost et al. 2005), and three days after a single exposure to ozone the role of eosinophils markedly changes, so that depletion of eosinophils now significantly exacerbates airway hyperreactivity (Yost et al. 2005). We have confirmed this beneficial role of eosinophils at this same time point after ozone exposure (Figure 2A). This rapid change in eosinophil function, from deleterious to beneficial over three days, is likely to be of great importance in understanding the role of eosinophils in lungs and, equally important, the optimal use of eosinophil-targeted treatments, such as AbIL-5.

Our data strongly suggest that this change in airway pathophysiology between one and three days after ozone exposure is due to replacement of preformed eosinophils by newly formed eosinophils, and that newly formed eosinophils are functionally different. Here, we have demonstrated that ozone inhalation has a potent, eosinophil-selective effect on bone marrow, stimulating bone marrow eosinophilopoiesis as early as one day after inhalation. This is seen as an increase in BrdU+ eosinophils in bone marrow (Figure 7). These newly formed eosinophils form a second wave of eosinophil influx into airways between one and three days after ozone inhalation that corresponds with the change in eosinophil function from deleterious to beneficial.

Examination of lung lavage reveals the majority of eosinophils moving into lungs during the first day after ozone exposure are BrdU− (i.e., preformed). In contrast, by three days after ozone exposure, many preformed, BrdU− eosinophils have been replaced by newly formed BrdU+ eosinophils (Figure 3). Replacement of old eosinophils with newly formed eosinophils corresponds to the time course of changing eosinophil effects from deleterious to beneficial.

These data, obtained in nonsensitized guinea pigs, suggest that ozone-induced airway hyperreactivity in humans without asthma or atopy would be self-limited, as deleterious (old) eosinophils are replaced by beneficial (new) eosinophils. However, as many people with asthma have atopy, we modeled this by repeating our experiments in guinea pigs sensitized to ovalbumin and found profoundly different effects of ozone exposure on both airway physiology and eosinophil populations. Table 2 summarizes the effects of ozone in sensitized and nonsensitized guinea pigs. In sensitized animals, eosinophil depletion with AbIL-5 prevented airway hyperreactivity three days after exposure to ozone, demonstrating that beneficial effects of eosinophils, seen in nonsensitized animals three days after ozone exposure (Figure 2A), had been lost with sensitization (Figure 2B). Examination of bone marrow of sensitized animals revealed that the dramatic increase in BrdU+ eosinophils after ozone inhalation in nonsensitized animals was also completely lost (Figure 8). The loss of these newly formed eosinophils was reflected in a paucity of BrdU+ eosinophils in the airways at three days (Figure 4), so that nearly all eosinophils in the lungs of sensitized, ozone-exposed animals, were preformed (BrdU−). Thus, the absence of newly formed eosinophils, which we have shown are beneficial (Yost et al. 2005; and Figure 2A), is accompanied in sensitized animals by a significant potentiation of ozone-induced airway hyperreactivity (Figure 1).

While we are not yet certain what signal produced by ozone-exposed lungs stimulates eosinophilopoiesis in bone marrow, it is known that many cytokines are released by ozone-exposed airway cells. An increase in TNFα is an early response to ozone exposure (Cho et al. 2007). It is implicated in eosinophil hematopoiesis (Askenasy 2015; Masid-de-Brito et al. 2014) and is also a product of eosinophil activation (Spencer et al. 2008). Blocking TNFα inhibits airway hyperreactivity in response to viral infection (Nie et al. 2011), organophosphate exposure (Proskocil et al. 2013), and antigen challenge (Maillet et al. 2011; Nie et al. 2009). We used a TNFα Fc fusion protein, etanercept, to block the effects of TNFα in ozone-exposed animals. Blocking TNFα in this way significantly potentiated ozone-induced airway hyperreactivity and blocked eosinophilopoiesis in bone marrow, mimicking the effects of sensitization three days after exposure to ozone. Production of new, BrdU+ eosinophils in bone marrow was suppressed in etanercept-treated animals (Figure 15), with a corresponding lack of new, BrdU+ eosinophils. This was also seen in the lungs (Figure 11). Thus, etanercept mimicked sensitization, in that it suppressed ozone-induced eosinophilopoiesis and potentiated ozone-induced airway hyperreactivity. These data suggest a novel role of TNFα in

Table 2. Summary of Results[a]

Treatment Group	Exposure / Pretreatment	Vagally Mediated Bronchoconstriction	BrdU+ Eosinophils		
			Bone Marrow	Blood	BAL
Nonsensitized	O_3 Day 1	↑↑↑	↑	–	↑
	O_3 Day 3	↑↑	↑↑	–	↑↑
	O_3 Day 3 AbIL5	↑↑↑↑	NA	NA	NA
	Air + Etanercept	↑	–	–	–
	O_3 Day 3 + Etanercept	↑↑↑↑	↓	–	↓
Sensitized	Air	↑	–	–	–
	O_3 Day 3	↑↑↑↑	↓	–	↓
	O_3 Day 3 AbIL5	↑	NA	NA	NA
	Air + Etanercept	↑	–	–	–
	O_3 Day 3 + Etanercept	↑↑↑↑	↓	–	–

[a] Results show comparisons with nonsensitized animals breathing filtered air.

NA = not tested. Dash = no change from air-exposed controls. Up arrow = significant increase relative to air-exposed nonsensitized animals. Down arrow = significant decrease relative to air-exposed nonsensitized animals. The number of arrows indicates the size of the effect (not the significance).

mitigating ozone-induced airway hyperreactivity and that TNFα is a key mediator of ozone-induced eosinophilopoiesis in nonsensitized animals.

Ozone is known to stimulate airway epithelial cells to release IL-1β, IL-1α, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and TNFα (Bayram et al. 2001; Devalia et al. 1997; Fakhrzadeh et al. 2004; McCullough et al. 2014; Nichols et al. 2001; Song et al. 2011). While IL-1β and TNFα both increase in lungs after ozone exposure, these cytokines have drastically different effects on ozone-induced airway hyperreactivity. Blocking IL-1β effects with anakinra, a synthetic IL-1 receptor antagonist, prevents ozone-induced airway hyperreactivity three days after ozone exposure (Verhein et al. 2008), suggesting that IL-1β mediates ozone-induced hyperreactivity in nonsensitized animals three days later. Conversely, blocking TNFα with etanercept significantly potentiated ozone-induced airway hyperreactivity at the same time point (Figure 10). Thus, TNFα is protective in lungs three days after ozone exposure while IL-1β mediates hyperreactivity.

Ozone itself cannot directly stimulate bone marrow since it is a reactive oxygen species, which is unlikely to pass the airway epithelium (Pryor 1992). Ozone exposure significantly increases IL-1β in bone marrow (Verhein et al. 2008). IL-1β and IL-1α induce bone marrow stromal cells to express IL-5 (Hogan et al. 2000). Eosinophil hematopoiesis occurs when eosinophil progenitor cells in bone marrow are exposed to IL-5, IL-3, and GM-CSF (Clutterbuck and Sanderson 1988; Hogan et al. 2000; Möhle et al. 1997; Sonoda et al. 1989; Stanley et al. 1994). Furthermore, in allergic asthma, inhalation of allergen stimulates eosinophil hematopoiesis, via IL-3/IL-5–induced upregulation of the alpha subunit of the IL-5 receptor expressed on eosinophil progenitor cells (Denburg et al. 1999, Wood et al. 1998). Bone marrow egress is then stimulated by IL-5 and eotaxin (Palframan et al. 1998a,b), and mediated by integrins, Mac-1 and lymphocyte function associated antigen (LFA-1), binding to intracellular adhesion molecule 1 (ICAM-1) (Pelaquini et al. 2011). Migration from blood into tissues is mediated by adhesion molecules expressed on endothelial cells, which can be induced by IL-4, IL-13, TNFα, and IL-1β (Atsuta et al. 1997; Bochner et al. 1995, Lademarco et al. 1995; Patel 1998; Raab et al. 2001; Woltmann et al. 2000), while GM-CSF and eotaxin along with RANTES and IL-8, mediate recruitment

through the endothelial and epithelial layers (Ebisawa et al. 1994a,b; Erger and Casale 1995; Liu et al. 2015). Once present in tissues, eosinophil survival is mediated by GM-CSF and IL-5 (Levi-Schaffer et al. 1998; Wardlaw 2001). Thus, inflammatory cytokines are likely to be the systemic link between ozone, eosinophil hematopoiesis in bone marrow, and subsequent eosinophil recruitment to lungs in nonsensitized animals.

We found no additive effect of TNFα blockade on sensitization. Etanercept did not further potentiate ozone-induced hyperreactivity (Figure 10B) or further suppress eosinophilopoiesis (Figure 16). Given that etanercept blocks airway hyperreactivity in animals sensitized to and challenged with an antigen (Maillet et al. 2011; Nie et al. 2009), the beneficial role of TNFα as a stimulator of eosinophilopoiesis may be lost with sensitization.

While TNFα is not normally a bronchodilator, it has been shown that TNFα can stimulate production of neuronal nitric oxide synthase (Stasko et al. 2013) that subsequently produces nitric oxide, which is a bronchodilator (Belvisi et al. 1995). Blocking nitric oxide synthase with L-NAME had no effect on ozone-induced hyperreactivity in nonsensitized animals (Figure 9) or on inflammatory cells in bone marrow, blood, or BAL three days after ozone exposure (data not shown). Thus, upregulation of neuronal nitric oxide synthase and subsequent increased release of nitric oxide are not involved in airway relaxation three days after ozone exposure.

The importance of understanding the various roles of eosinophils in airway hyperreactivity is underscored by experience with clinical trials of a humanized monoclonal antibody to IL-5, mepolizumab. This antibody, which was recently recommended for approval by the Pulmonary and Allergy Drug Advisory Committee of the FDA (approval date: June 11, 2015), effectively depletes circulating eosinophils. Initial clinical trials of mepolizumab were disappointing (Kips et al. 2003; Leckie et al. 2000), but in retrospect this should not have been surprising as patients in this trial were enrolled without regard for whether they had predominantly eosinophilic asthma. It is now known that asthma patients may have predominantly eosinophilic asthma, predominantly neutrophilic asthma, or a mixture of the two. When mepolizumab was studied in patients with sputum eosinophilia, a dramatic inhibitory effect on exacerbation rate and increased ability to withdraw steroids was demonstrated (Haldar et al. 2009; Nair et al. 2009). A subsequent trial demonstrated that circulating eosinophil counts could predict response to eosinophil depletion (Gaillard et al. 2015). Based on these studies, the initial approval of mepolizumab is likely to be for people with severe asthma and more than 150 eosinophils/mL blood at the time of treatment, or with more than 300 eosinophils/mL blood at any time in the prior 12 months.

Targeting therapy based upon eosinophil number, while an improvement over the initial all-comers trials, may still fall short of an optimal, precision medicine goal. For example, while eosinophilia is typically associated with allergies and atopy, it is now understood that many cases of severe asthma with eosinophilia are nonatopic (Pavord 2011). Our studies, in which beneficial eosinophils are seen only in nonsensitized animals, suggest that the response to eosinophil depletion in humans with asthma may depend on atopic status. Additional support for the presence of multiple eosinophil phenotypes comes from a recent paper showing that in lungs of mice sensitized and challenged with ovalbumin, cells identified as eosinophils by Siglec-F expression change from CD11c-negative to CD11c-positive as they migrate from lung tissue into the airway lumen (Abdala-Valencia et al. 2015). If our findings translate to humans, we would expect a greater beneficial effect of treatments that target eosinophils in patients with atopy, in whom we predict there are no beneficial eosinophils.

Likewise, the effect of targeting TNFα in asthma has met with variable success (Berry et al. 2006; Erin et al. 2006; Holgate et al. 2011; Howarth et al. 2005; Morjaria et al. 2008; Rouhani et al. 2005; Wenzel et al. 2009). Etanercept appears to be effective in some patients with severe corticosteroid refractory asthma (Morjaria et al. 2008). The role of TNFα in asthma is complex and likely to depend upon asthma phenotype. We found no beneficial role for etanercept in ozone-induced inflammation or airway hyperreactivity in sensitized animals, and our data suggest that the anti-inflammatory effect of TNFα (suppressing eosinophil expansion) may be deleterious in nonsensitized animals. If our data were to translate to human disease, it may suggest that TNFα blockers may be most beneficial in people with severe asthma and high TNFα who also have atopy (i.e., in whom we predict there is already a loss of beneficial eosinophils).

Our data suggest a completely new paradigm: that eosinophils newly stimulated by ozone exposure, and dependent upon TNFα, have beneficial effects and mitigate airway hyperreactivity days after ozone exposure in nonsensitized guinea pigs. However, these ozone-induced, newly divided eosinophils fail to appear in lungs of sensitized animals. Understanding the relationships among ozone, eosinophilopoiesis, TNFα, and sensitization (or atopy) may allow more a rational application of targeted therapies that are now becoming available. We suggest that, in particular, atopic status, may, by eliminating beneficial eosinophils, predict that therapies targeting eosinophils or TNFα will be more effective.

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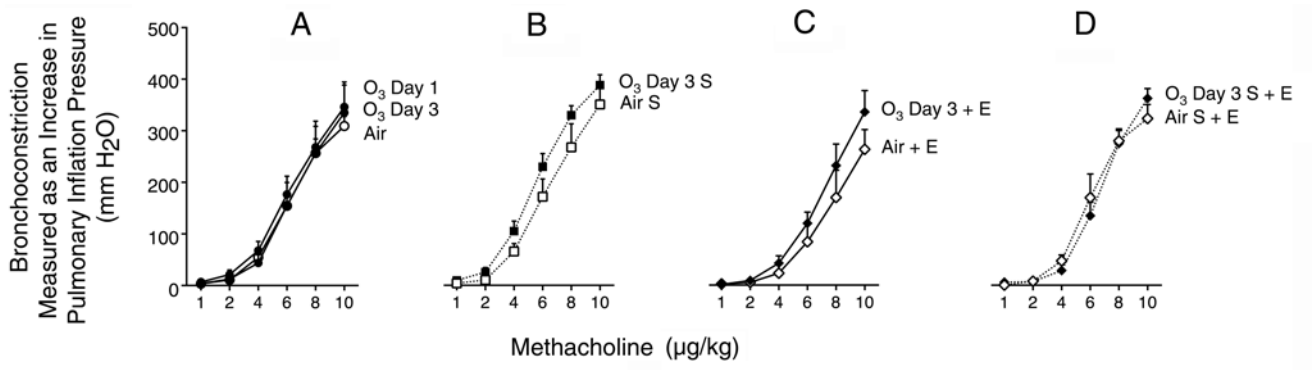
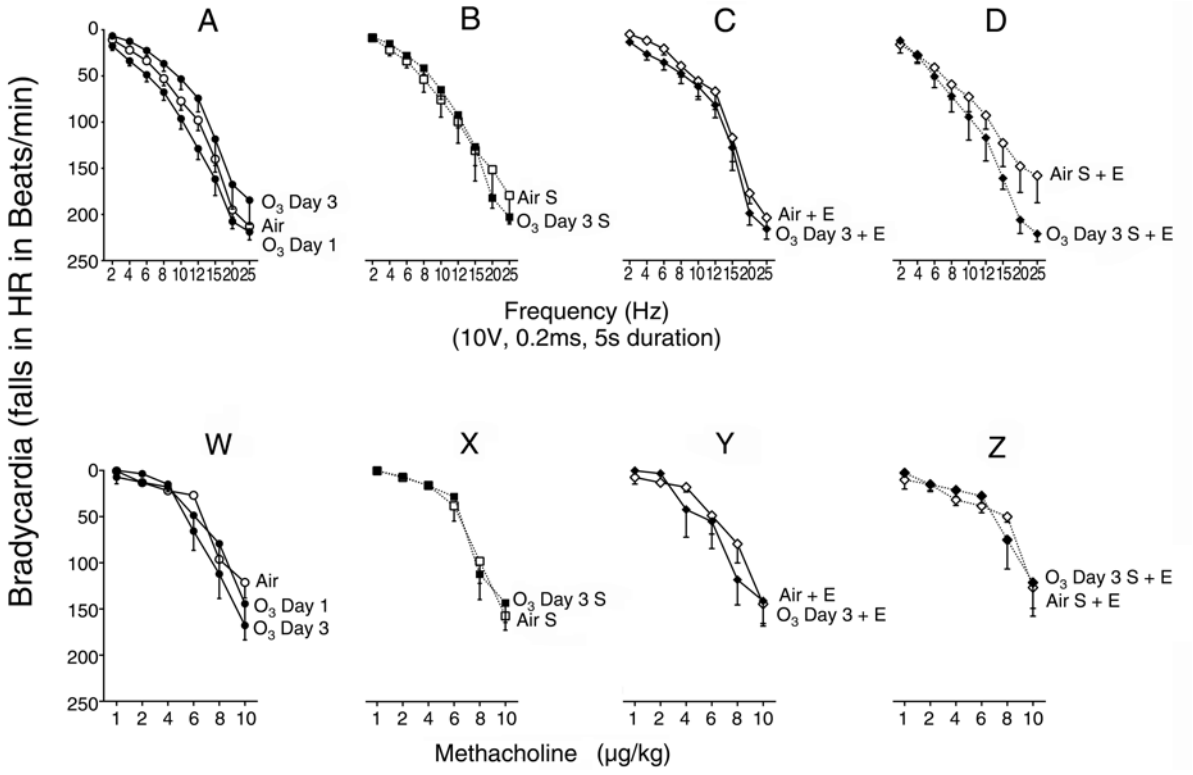
APPENDIX A. METHACHOLINE-INDUCED BRONCHOCONSTRICTION AND VAGALLY- OR METHACHOLINE-INDUCED BRADYCARDIA



Appendix Figure A.1. Methacholine-induced bronchoconstriction in ovalbumin-sensitized (S) and nonsensitized guinea pigs exposed to 2 ppm ozone for 4 hours, with or without etanercept (E). (A) Nonsensitized: filtered air (n = 9), ozone day 1 (n = 4), or ozone day 3 (n = 7); (B) Sensitized: filtered air (n = 5) or ozone day 3 (n = 5); (C) Nonsensitized: filtered air + etanercept (n = 5) or ozone day 3 + etanercept (n = 6); (D) Sensitized: filtered air + etanercept (n = 4) or ozone day 3 + etanercept (n = 4). Data are mean ± SEM. * = $P < 0.05$; # = $P < 0.05$ by repeated measures two-way ANOVA.



Appendix Figure A.2. Vagally- or methacholine-induced bradycardia in ovalbumin-sensitized (S) and nonsensitized guinea pigs exposed to 2 ppm ozone for 4 hours, with or without etanercept (E). Panels A–D show vagally-induced bradycardia; Panels W–Z show methacholine-induced bradycardia. **(A & W)** Nonsensitized: filtered air (n = 9), ozone day 1 (n = 4), or ozone day 3 (n = 7); **(B & X)** Sensitized: filtered air (n = 5) or ozone day 3 (n = 5); **(C & Y)** Nonsensitized: filtered air + etanercept (n = 5) or ozone day 3 + etanercept (n = 6); **(D & Z)** Sensitized: filtered air + etanercept (n = 4) or ozone day 3 + etanercept (n = 4). Data are mean ± SEM. * = $P < 0.05$; # = $P < 0.05$ by repeated measures two-way ANOVA.

Research Report 191, *Protective Role of Eosinophils and Tumor Necrosis Factor-α after Ozone Inhalation*, A.D. Fryer et al.

INTRODUCTION

Exposure to ozone induces deleterious responses in the airways that include shortness of breath, inflammation, and airway hyperresponsiveness (AHR*: increased bronchoconstriction following a provocation in the airways). People with asthma have increased airways sensitivity to ozone and other provocative agents.

In 2011 Dr. Allison Fryer, of Oregon Health & Science University, submitted a preliminary proposal to HEI under its Requests for Preliminary Application 10-3, *Health Effects of Air Pollution*, which encouraged novel proposals that were outside the scope of HEI's major requests for applications but were compatible with HEI's research programs and mission.

Fryer and colleagues proposed to analyze the immune and physiological responses to ozone, using guinea pigs. Because guinea pig and human lungs respond similarly to pharmacological agents, guinea pigs have been used as a relevant animal model for the study of agents that may cause respiratory and physiological responses. In addition, their response to airborne allergens is similar to the human response and shares some features of one major type of asthma, allergic asthma.

In her proposal, Fryer presented preliminary data indicating that within 24 hours of exposure, ozone not only induced AHR but also stimulated hematopoiesis in the bone marrow of guinea pigs. As a result, increased numbers of newly synthesized white blood cells — particularly eosinophils — moved into the blood and lungs. The central hypothesis of the proposed project was that newly synthesized eosinophils recruited to the lungs three days after ozone exposure were beneficial to the animals in that they limited ozone-induced AHR. Preliminary data also indicated that the beneficial effect of newly synthesized eosinophils seen in normal animals was lost in animals that had been injected (*sensitized*) with an allergen, ovalbumin.

HEI's Research Committee thought the hypothesis novel and of potential clinical relevance for understanding responses to ozone in people with allergy and asthma. The Committee asked Dr. Fryer to expand on these observations in a full application. In her full application, Fryer proposed a set of experiments to confirm the preliminary findings as well as additional in vivo experiments to explore the effects of inhibitors of cytokines — proteins released by many cells upon activation, and which act on other cells — and other products on the response to ozone. The Research Committee recommended the two-year study for funding; it was later extended to three years.

This Critique provides the HEI Review Committee's evaluation of the study. It is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report (IR) into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

OZONE-INDUCED LUNG INFLAMMATION IN HEALTHY AND ASTHMATIC INDIVIDUALS

Multiple studies with healthy human volunteers have established that short-term exposure to low concentrations of ozone — close to and even below the current National Ambient Air Quality Standard (NAAQS) (0.07 ppm, daily average) — during intermittent exercise decreases lung function (measured as forced vital capacity and forced expiratory volume in 1 second [FEV_1]) and increases AHR and airway inflammatory responses (e.g., Balmes et al. 1996; Kim et al. 2011; Torres et al. 1997). Kim and colleagues (2011) found that healthy young adults exposed to 0.06 ppm ozone for 6.6 hours with intermittent exercise showed a decreased mean FEV_1 following ozone exposure and a large increase in the percentage of neutrophils in induced sputum. Earlier studies (Balmes et al. 1996; Torres et al. 1997) established that the lung function and inflammatory responses to ozone were independent of each other. The mechanisms by which ozone induces all these acute effects in humans were reviewed recently (Bromberg 2016).

Dr. Allison D. Fryer's 3-year study, "Air pollution and systemic inflammation of autonomic nerves," began in December 2011. Total expenditures were $262,854. The draft Investigators' Report from Fryer and colleagues was received for review in June 2015. A revised report, received in December 2015, was accepted for publication in February 2016. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and the Review Committee's Critique.

* A list of abbreviations and other terms appears at the end of this volume.

People with asthma, a condition characterized by chronic airway inflammation, are considered particularly sensitive to ozone effects. The most recent survey of ozone health effects conducted by the U.S. Environmental Protection Agency (U.S. EPA) states, "there is adequate evidence for asthmatics to be an at-risk population based on the substantial, consistent evidence among controlled human exposure studies and coherence from epidemiologic and toxicological studies" (U.S. EPA 2013). Allergic asthma is one of the major subtypes of asthma, characterized by the presence of atopy (see next section) and increased eosinophils in the airways and often in the blood. Acute exposure to ozone in this population results in an expansion of eosinophil numbers in the airways (e.g., Peden et al. 1997; Vagaggini et al. 2002).

IMMUNE RESPONSES IN ALLERGY AND ASTHMA

The Th2 Pattern Response

In some humans, the immune response to airborne allergens promotes the development of the Th2 subset of $CD4^+$ T lymphocytes, which then synthesize the cytokines characteristic of this subset — interleukin (IL)-4, IL-5, and IL-13. IL-5 activates eosinophils; IL-4 and IL-13 promote the switch of B lymphocytes to synthesizing immunoglobulin (Ig) E.

Individuals who have a genetic predilection to develop this pattern of responses in which IgE is involved are referred to as *atopic*. After initial exposure (sensitization) and especially after re-exposure (challenge) to an allergen, guinea pigs and some strains of mice develop Th2-pattern and IgE-mediated responses that are similar to those that occur in people with atopy. Thus, these allergen-sensitized laboratory animals are good models for responses in people with atopy.

Note that in the developed world the Th2 and IgE pattern of response is deleterious, because of the problems associated with allergies and allergic asthma. However, in other parts of the world, where parasitic helminthic worms are prevalent, this Th2 and IgE pattern of response is beneficial — IgE and eosinophils act together to kill the parasites.

Within the subtype of allergic asthma, a high percentage (approximately 50% of all individuals with asthma) of patients are *high Th2-type*, characterized by high levels of IgE in blood; eosinophils in the blood and airways; and increased periostin, a protein induced by IL-13 action on airway epithelial cells (Jia et al. 2012). High Th2-type asthma is frequently more severe but is responsive to treatment with steroids (Fahy 2015).

TNFα in Inflammatory Conditions and Asthma

Tumor necrosis factor-alpha (TNFα), made predominantly by cells of the monocyte/macrophage lineage, is a key member of the early acute inflammatory response, but is not part of the Th2 pattern response. Inhibiting the inflammatory effects of TNFα, either by using an antibody specific for TNFα or molecules that block the action of TNFα on its receptor, has been very successful in treating patients with the debilitating and painful condition, rheumatoid arthritis. As asthma is also a chronic inflammatory condition and TNFα is increased in bronchoalveolar lavage fluid from patients with asthma (Berry et al. 2006; Cazzola and Polosa 2006; Thomas 2001), some clinical studies — discussed at the end of this Critique — have evaluated the role of blocking TNFα effects in people with asthma.

PREVIOUS STUDIES FROM FRYER AND COLLEAGUES

Fryer and colleagues have evaluated the role of eosinophils in the airway inflammatory responses of guinea pigs exposed to allergen. For example, Elbon and colleagues (1995) showed that in animals that were challenged with ovalbumin by inhalation (after sensitization to ovalbumin by intraperitoneal [i.p.] injection), anti-IL-5 inhibited the migration of eosinophils into the lung. Fryer's team has also assessed the effects of TNFα in this model. Nie and colleagues (2009) showed that pretreatment with etanercept, a TNFα receptor-IgG_1 fusion protein that was used in the current study, decreased AHR and reduced eosinophil numbers in blood and lung lavage fluid as well as around airway nerves and smooth muscle.

Fryer's group has also evaluated the effect of ozone exposure on airway responses and eosinophil function (e.g., Fryer and Jacoby 1992; Yost et al. 1999, 2005). Yost and colleagues (2005) showed that after exposure to 2 ppm ozone — the concentration used in the current study — AHR lasted up to three days, and that the numbers and possible roles of eosinophils changed in the airways over that time period. In particular, the investigators showed that eosinophils may be involved in enhancing AHR early (1 day after exposure) but may have a later protective or beneficial role (3 days after exposure). In the current study, Fryer and colleagues further explored the role of eosinophils in the response to ozone in guinea pigs.

STUDY HYPOTHESES AND SPECIFIC AIMS

The main hypotheses to be tested were that (1) exposure of guinea pigs to ozone stimulates new production of eosinophils in bone marrow, and these newly synthesized

eosinophils migrate to the lungs (confirming their preliminary findings); and (2) the newly synthesized and recruited eosinophils play a delayed but beneficial role in ozone-induced inflammation in the airways of healthy normal (nonsensitized) animals, but not in allergen-sensitized animals.

Aim 1: Measure whether ozone exposure induces eosinophil expansion in bone marrow and subsequent migration to lungs three days later in normal and sensitized guinea pigs.

Aim 2: Since TNFα can mediate both eosinophil recruitment and release of nitric oxide (NO) from eosinophils, and NO mediates bronchodilation, evaluate the roles of TNFα and NO in ozone-induced AHR and the associated recruitment of newly synthesized eosinophils in normal and sensitized guinea pigs.

STUDY DESIGN AND METHODS

GENERAL INTRODUCTION

In this study, Fryer and colleagues exposed guinea pigs to 2 ppm ozone or filtered air for 4 hours, and measured changes 1 or 3 days later in the numbers of eosinophils and other white blood cells in bone marrow, blood, and lung lavage fluid. A key analysis estimated how many of these white blood cells, particularly eosinophils, were synthesized after the exposure (newly formed) and how many already existed before the exposure. The investigators also measured important physiological responses — bronchoconstriction (that is, AHR) and bradycardia (decrease in heart rate).

As a model for atopy and allergic asthma, some animals were sensitized with ovalbumin; their responses were compared with those of normal animals. Other experiments evaluated the role of TNFα, IL-5, and NO in the response to ozone, by pretreating animals with blockers of the effects of these molecules.

MEASURING NEWLY SYNTHESIZED EOSINOPHILS

A key element of this study was the use of 5-bromo-2′-deoxyuridine (BrdU) to determine which cells had been newly synthesized. BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells, serving as a marker of newly produced cells. Therefore, a snapshot can be obtained of the proportion of newly synthesized (BrdU positive, or BrdU+) versus pre-existing (BrdU negative, or BrdU−) cell types in a particular tissue at a particular time point.

In order to detect the full complement of newly synthesized cells, the HEI Research Committee advised the investigator at the onset of her study to change the BrdU protocol she was using by switching to an osmotic minipump for more constant delivery of BrdU. Using this recommended protocol, however, Fryer was unable to repeat her preliminary observations. However, toward the end of the study, Fryer reported that she could repeat her preliminary findings if she used the original BrdU injection protocol. The Research Committee decided to give the investigator an extra year of funding, and Dr. Fryer refocused her study to conduct the experiments described in the current report. Thus, guinea pigs were injected i.p. with 50 mg BrdU/kg immediately prior to ozone or air exposure, and another 50 mg/kg i.p. two hours after exposure. Thereafter, animals received a single daily i.p. dose of 50 mg BrdU/kg until they were killed and their tissues examined.

ANIMALS AND EXPOSURES

Normal and sensitized (allergic) female guinea pigs were exposed once for 4 hours to filtered air or to 2 ppm ozone (produced by an ultraviolet light generator) in an exposure chamber. Guinea pigs were sensitized by injecting them with ovalbumin i.p. 3 times, 48 hours apart, starting 26 days before exposure.

IN VIVO TREATMENT WITH ANTIBODIES AND INHIBITORS

Fryer and colleagues conducted several experiments to evaluate the role of TNFα in the response to ozone, in which animals were injected with a single dose of the TNFα antagonist etanercept three hours before exposure to ozone or air. The investigators also conducted a limited set of experiments to examine the effects of blocking IL-5 in normal and ovalbumin-sensitized animals, in which animals were injected with a monoclonal antibody specific for IL-5 (Ab-IL5) given three days before ozone or air exposure.

The investigators also injected some normal animals with L-NAME (N_{ω}-Nitro-L-arginine methyl ester), an inhibitor of nitric oxide synthase, to evaluate the role of NO in ozone-induced AHR. Guinea pigs were injected with L-NAME 30 minutes prior to physiological measurements (discussed in the next section) on day 3 after ozone exposure. Because these animals showed no difference from control animals either in ozone-induced AHR or in cell numbers in bone marrow, blood, or lung lavage fluid, these results are not discussed further in the Critique.

PHYSIOLOGICAL MEASUREMENTS

One or three days after exposure to ozone or filtered air, Fryer and colleagues electrically stimulated (at 1 to 25 Hz) the vagus nerve in anesthetized animals to produce frequency-dependent bronchoconstriction — measured as an increase in pulmonary inflation pressure, the pressure required to inflate the lungs — and bradycardia.

To assess the function of M_3 muscarinic receptors on airway smooth muscle cells in vagotomized guinea pigs, the investigators also measured dose-dependent bronchoconstriction and bradycardia after intravenous methacholine (which directly stimulates airway smooth muscle). The investigators showed that ozone exposure and sensitization with ovalbumin did not affect methacholine-induced bronchoconstriction (or bradycardia), indicating that the airway responses that did occur were mediated at the level of parasympathetic nerves. Because there were no significant changes in bradycardia, these results are not discussed further in the Critique.

TISSUE AND CELL ANALYSES

The investigators prepared white blood cells obtained from bone marrow, peripheral blood, and lung lavage fluid from sensitized and normal guinea pigs. White cells were spun onto slides and fixed with 70% ethanol. Using a standard kit, cells that had taken up BrdU were identified by staining. Total white cells were counted, and a differential count of macrophages, neutrophils, lymphocytes, and eosinophils was made. Each of these inflammatory cell types was assessed as either BrdU+ or BrdU−.

STATISTICAL METHODS AND DATA ANALYSIS

All data were expressed as means ± standard error of the mean (SEM). Inflammatory cells and BrdU+ and BrdU− cells in lung, blood, and bone marrow were analyzed by one-way analysis of variance (ANOVA) comparing selected columns, followed by a Bonferroni correction. Frequency and methacholine dose–response curves were analyzed using a two-way ANOVA for repeated measures (as responses to multiple frequencies or dose of acetylcholine are recorded from the same animal). Baseline pulmonary inflation pressure, body weight, heart rate, and blood pressure were analyzed by ANOVA comparing selected pairs of columns with a Bonferroni correction.

HEI REVIEW COMMITTEE EVALUATION OF THE REPORT

STUDY APPROACH

In its independent review of the study, HEI's Review Committee concluded that Fryer and colleagues had successfully extended their earlier work on the biological effects of exposure to toxic agents in the guinea pig to address a novel question about the role of eosinophils in the response to ozone. The Committee recognized the relevance of the guinea pig as a model of human allergy and considered that experiments in animals that were sensitized to an allergen, ovalbumin, provided added relevance to studies of humans who are atopic; immune responses in these people would be polarized toward an allergic or Th2-type pattern involving IgE and eosinophils. In addition to their well-described characterizations of newly synthesized and pre-existing white blood cells by the use of BrdU, the investigators studied an endpoint, AHR, with clinical relevance to human airway disease. The investigators included experiments to study the role of specific cytokines in the response to ozone in both sensitized and normal animals, providing intriguing directions for possible therapies for allergic asthma.

KEY RESULTS

The investigators focused on how exposure to ozone affected AHR as well as the numbers and percentage of BrdU+ eosinophils — and, to a lesser extent, other inflammatory cell types — in bone marrow and lung lavage fluid at days 1 and 3 after exposure in normal and ovalbumin sensitized (allergic) guinea pigs. Fryer and colleagues found only a few changes in blood. The investigators also focused on responses at day 3 after exposure, because their preliminary data indicated that responses of sensitized and normal animals differed most at that time point.

- ***Normal and sensitized guinea pigs responded differently to ozone***. Ozone increased the percentage of newly formed BrdU+ eosinophils in the bone marrow and lungs of normal animals, but not in those of sensitized animals. The time course of AHR differed in the two sets of animals: In normal animals ozone-induced AHR increased substantially at day 1 but decreased by day 3. In sensitized animals, ozone-induced AHR was still high at day 3.

  •Pretreatment with the TNFα blocker etanercept had complex effects, which differed between normal and sensitized guinea pigs. Effects were found in both air- and ozone-exposed animals and on cells

other than eosinophils. Etanercept increased ozone-induced AHR in normal animals at day 3 but had no effect on the already high ozone-induced AHR in sensitized animals at the same time point.

In normal animals etanercept *decreased* the percentage BrdU+ eosinophils in total eosinophils in bone marrow at day 3 after either air or ozone exposure. This decrease in percentage BrdU+ eosinophils after ozone exposure resulted from etanercept *increasing* the total number of eosinophils in lung at this time point, but the majority of these cells were BrdU−. This decrease in percentage BrdU+ eosinophils after ozone exposure contrasts with the *increased* percentage of BrdU+ eosinophils in total eosinophils after ozone exposure in the absence of etanercept. Etanercept also *increased* the percentage of BrdU− macrophages and lymphocytes in the lungs of normal animals. In sensitized animals, etanercept had no effect on any cell type in the bone marrow or lung after exposure to either ozone or air.

Etanercept *increased* the numbers of blood monocytes and lymphocytes (both BrdU+ and BrdU−, but particularly BrdU−) in both air- and ozone-exposed and normal and sensitized animals at day 3, although not all increases reached significance. These were some of the few statistically significant findings in the blood of exposed animals in the study. Etanercept had no effect on eosinophils in blood at this time point.

- ***Pretreatment of sensitized animals with anti-IL-5 reduced AHR at day 3 after ozone exposure***. In contrast, in normal animals anti-IL-5 greatly enhanced AHR at day 3. The effects of anti-IL-5 treatment on eosinophil numbers in bone marrow, blood, or lung were not evaluated in the current study.

DISCUSSION AND INTERPRETATION

In its independent review of the study, the HEI Review Committee agreed that the results of this study supported a major feature of the investigators' hypothesis, namely, that exposure to ozone would result in bone marrow synthesis of a newly formed population of eosinophils that moves to the lungs. The Committee also agreed that the results supported another part of the study hypothesis, that this synthesis of newly formed eosinophils in the bone marrow followed by migration to the airways did not occur in animals that had been sensitized to an allergen (i.e., ovalbumin).

The study also showed that blocking the effects of two different cytokines, IL-5 and TNFα (which are produced by different cells and have very different roles in the immune response), had distinct effects in the response to ozone exposure in normal and sensitized (allergic) animals. The Committee agreed with the investigators' interpretation that TNFα may be involved in the development of eosinophils in the bone marrow and recruitment of eosinophils to the lungs in normal animals. However, for the reasons described below, a clear interpretation in the current study of the effects of etanercept — used to block TNFα — was challenging. The almost complete abolition of AHR by anti-IL-5 antibody in sensitized guinea pigs strongly suggests that IL-5 plays a major role in the eosinophil-mediated ozone-induced response in these animals.

Based on the changes in AHR and BrdU+ and BrdU− eosinophils at day 3 after ozone exposure, the Committee agreed that the investigators' interpretation of a "beneficial" effect of newly synthesized BrdU+ eosinophils was reasonable. The investigators did not speculate on the nature of this beneficial response, and the Committee thought this would be important to address in future studies. For example, do newly synthesized eosinophils produce a product that improves AHR later in the response to ozone, or do these cells fail to produce a product (or products) that exacerbates AHR?

The Committee noted that in the current study the investigators did not show that anti-IL-5 treatment depleted eosinophil numbers at any site in the body. (Depletion of eosinophils by anti-IL-5 is considered the basis for the success of anti-IL-5 therapies in people with asthma [see later in Implications for Humans]). This would be important to verify in future experiments because in previous guinea pig studies Fryer and colleagues showed that anti-IL-5 depleted eosinophils from the blood, but not from the lungs (Proskocil et al. 2015).

The Committee also thought it would be important to determine whether newly formed and pre-existing eosinophils were truly distinct subpopulations of cells. For such an investigation, it would be necessary to isolate and purify viable populations of both newly synthesized and pre-existing eosinophils — for example, by using fluorescent BrdU. Key questions could then be asked about whether the populations differed in expression of genes and proteins, and especially of cell surface proteins. Such investigations would need to be coupled with studies to determine whether the function of the isolated and purified populations differed, for example, in vitro studies on different airway cells that participate in AHR (including smooth muscle, nerves, and structural cells of the airways). These types of studies would be the first to describe true subpopulations of eosinophils, but would not be unprecedented, because this approach of linking the

expression of genes and proteins with functional studies has identified subpopulations of other cells of the innate immune system — macrophages and dendritic cells (e.g., Dorhoi and Kaufmann 2015; Merad et al. 2013).

The Committee agreed with the investigators' conclusions that exposure to ozone did not induce the synthesis of newly formed eosinophils in sensitized animals. The investigators did not speculate as to the reasons for this finding, but the Committee thought the mechanisms underlying this finding were worth exploring in future studies.

The Committee considered that one major difference between sensitized and normal animals is that allergen sensitization by its very nature expands the number of ovalbumin-specific T (and B) lymphocytes, and in particular ovalbumin-specific $CD4^+$ Th2 cells, which synthesize IL-4, IL-5, and IL-13 and activate eosinophils. Transferring purified T cell populations from sensitized animals into normal animals before exposure to ozone might show whether the sensitized T cells or one of their products blocked the hematopoietic effects of ozone on eosinophils in the bone marrow.

The Committee commented on the challenges in interpreting the results of studies that use agents that block cytokine action in vivo, noting that the action of the blocking agent is frequently not specific for a particular cell type. This arises because nearly all cytokines are made by more than one cell type and receptors for cytokines are expressed on a variety of cells. For example, IL-5 is synthesized by cells other than $CD4^+$ Th2 cells, including innate type 2 lymphoid cells — a recently identified population that plays an important role in the murine response to ozone (Kumagai et al. 2016; Ong et al. 2016) — and eosinophils themselves. In addition, receptors for IL-5 are expressed by cell types other than eosinophils: although the alpha chain of the IL-5 receptor is expressed almost exclusively on eosinophils, the beta chain is expressed on many leukocytes. Thus, injecting anti-IL-5 is likely to have more effects than simply depleting eosinophils. Exploring this type of analysis — to determine more precisely the effects of in vivo treatment on individual cell types at different sites in the body — would also be valuable in future studies.

Similarly, it is likely that the TNFα-IgG fusion protein etanercept affects more cell types than just eosinophils. Although TNFα is synthesized predominantly by cells of the monocyte-macrophage lineage, it is also synthesized by many other cell types. In addition, TNFα binds to two types of receptors, one (TNFR1) expressed on a variety of cells, the other (TNFR2) expressed only by cells of the immune system. Indeed, the current study found that etanercept also tended to increase levels of blood monocytes and lymphocytes, whether or not the animals had been exposed to ozone. Consistent with the findings of a link between etanercept and macrophage-monocytes in guinea pigs, Fryer and colleagues previously showed that etanercept affected monocyte function and AHR in animals treated with the organophosphorus pesticide parathion (Proskocil et al. 2013). How etanercept might affect monocyte (or lymphocyte) levels was not the subject of the current study.

A further question for future studies is whether there is a direct link between the changes induced by ozone in AHR and changes in inflammatory cell numbers — and BrdU+ cells — in the guinea pig airways. In a previous study (Proskocil et al. 2015), Fryer and colleagues found that some but not all organophosphorus pesticides induced AHR, but none had an effect on the numbers of inflammatory cells in lung lavage fluid or blood. Thus, it is not clear whether there is a direct link between the induction of AHR and the influx of inflammatory cells into the lung in the guinea pig.

Because the current studies were conducted at a single ozone concentration (2 ppm) and for one time period (4 hours), the Committee thought it important to address how robust the AHR and cell type findings are to other concentrations of ozone, particularly at lower concentrations closer to the current NAAQS, and other exposure durations. Repeated exposure to ozone would also be worth exploring, since multiple sequential exposures might result in a diminution of the response, which has been previously described in human studies (e.g., Jörres et al. 2000).

Implications for Humans

The study indicates that injecting anti-IL-5 into sensitized animals reduces AHR, a key marker of airway dysfunction in humans with allergy and asthma. These results are concordant with data from recent clinical trials of anti-IL-5 antibody therapy in people with high-Th2-type human asthma, characterized, among other factors, by high levels of eosinophils in the blood (Ortega et al. 2014; Yancey et al. 2016). In particular, treating patients with severe eosinophilic asthma for 32 weeks with a humanized anti-IL-5 antibody reduced asthma exacerbations compared with placebo and was associated with improvements in markers of asthma control including FEV_1 (Ortega et al. 2014). In a meta-analysis (Yancey et al. 2016), treatment with the same anti-IL-5 antibody also approximately halved exacerbations requiring hospitalization or emergency room visits compared with placebo in patients with severe eosinophilic asthma.

Targeting other Th2 cytokines has also shown recent promise in asthma treatment. For example, Wenzel and colleagues (2016) showed that a human anti-IL-4 antibody increased lung function and reduced severe exacerbations in patients with uncontrolled persistent asthma, irrespective of baseline eosinophil count.

Because in the current study etanercept had no effect on AHR or eosinophils in sensitized animals after exposure to either ozone or air, the results suggest that blocking TNFα in people who are atopic (and thus making a Th2-type and IgE pattern of response to allergen) might have little clinical benefit. These findings contrast with the investigators' earlier study, however, in which etanercept decreased AHR and eosinophil levels in guinea pigs that were both sensitized and challenged with ovalbumin (Nie et al. 2009). This earlier study provided grounds for the investigators to suggest that blocking TNFα function might be successful in treating patients with asthma, and, in the current study, that blocking TNFα function might be effective in people with atopy. (The difference in results between the two guinea pig studies from Fryer and colleagues suggests that the animals' allergen sensitization status may be an important factor in determining the response to TNFα blocking.)

To date, however, small clinical trials in people with asthma of differing severity have shown that blocking TNFα function — using etanercept or antibodies to TNFα — has little or no effect on clinical endpoints (Holgate et al. 2011; Morjaria et al. 2008; Rouhani et al. 2005; Wenzel et al. 2009). Furthermore, the safety of using TNFα blockers in people with asthma has also been questioned — an increased incidence of serious infections in one study (Wenzel et al. 2009, using an anti-TNF monoclonal antibody) and the development of transient hemiplegia (loss of motor function on one side of the body) in one patient, resulting in the curtailing of the study (Rouhani et al. 2005, using etanercept). Thus, the future of clinical trials to block TNFα function in people with asthma is uncertain.

CONCLUSIONS

Fryer and colleagues explored physiological (focused on the airways) and cellular responses at different sites in the body after exposure of guinea pigs to 2 ppm ozone for 4 hours. The findings supported the authors' hypotheses (1) that exposure to ozone stimulates the production of eosinophils in bone marrow, (2) that these newly synthesized eosinophils migrate to the lungs, and (3) that the newly synthesized and recruited eosinophils play a delayed but potentially beneficial role in ozone-induced inflammation in the airways of healthy normal but not allergen-sensitized animals. The HEI Review Committee agreed with these main conclusions. They also agreed that guinea pigs were a good model for studying responses to an allergen and in a major subtype of asthma — the high Th2 or allergic type asthma in which high levels of eosinophils are found in the blood.

A novel finding was that etanercept, an agent that blocks the in vivo action of the cytokine TNFα, decreased ozone-induced new synthesis of eosinophils in the bone marrow and blocked eosinophil migration to the lung in normal animals. Thus, TNFα may be involved in the regulation of eosinophil synthesis and movement. In sensitized animals, however, blocking TNFα had little effect on eosinophils and did not decrease AHR, a clinical endpoint used in many human studies of asthma. The current study also found that blocking the action of the cytokine IL-5 with an anti-IL-5 antibody, shown in other studies to deplete eosinophil levels, did substantially decrease AHR in sensitized animals.

In extrapolating these findings with cytokine blockers to humans, the potential for treating patients with allergic asthma with TNFα blockers is uncertain, whereas therapies targeting IL-5, eosinophils, and other components of the Th2-pattern of response seem promising. The Committee noted, however, that a caveat to performing experiments with blockers of cytokine function — both in animal models and humans — is that these blockers are frequently not specific for a particular cell type. Furthermore, the effects of such blockers on even a single cell type may be different at different sites in the body. Thus, without extensive confirmatory evidence of their effects, interpreting these experiments can be challenging.

The Committee concluded that the study by Fryer and colleagues raises several potentially intriguing directions for future research that were beyond the scope of the current study. What is the beneficial effect of newly synthesized eosinophils in cellular and molecular terms? Do the newly formed and pre-existing eosinophils represent truly functionally distinct subpopulations of eosinophils? What are the cellular and molecular characteristics that underlie the difference in responses between sensitized and normal animals? Are these findings valid at other, lower, ozone concentrations and exposure durations, or after multiple exposures? And, do these findings have implications for responses of humans with allergy, atopy, or asthma? Resolutions to these questions await further research.

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ABBREVIATIONS AND OTHER TERMS

AbIL-5	antibody specific for IL-5
AHR	airway hyperresponsiveness
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BrdU	5-bromo-2′-deoxyuridine
CCR3	C-C chemokine receptor type 3
FEV_1	forced expiratory volume in 1 second
GM-CSF	granulocyte macrophage colony stimulating factor
ICAM-1	intracellular adhesion molecule 1
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
IL	interleukin
IR	investigators' report
L-NAME	N_ω-Nitro-L-arginine methyl ester
Mac-1	macrophage-1 antigen
NAAQS	National Ambient Air Quality Standard
NO	nitric oxide
O_3	ozone
PBS	phosphate buffered saline
Ppi	pulmonary inflation pressure
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted — a chemokine also known as CCL5
SEM	standard error of the mean
Siglec-F	sialic acid-binding immunoglobulin-type lectin-F
TNFα	tumor necrosis factor-alpha
TRFK5	a rat anti-IL5 antibody that reacts with guinea pig, human and mouse IL-5
U.S. EPA	U.S. Environmental Protection Agency

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