

Newly Divided Eosinophils Limit Ozone-Induced Airway
Hyperreactivity In Non-Sensitized Guinea Pigs

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Running Title: Newly Divided Eosinophils are Beneficial

Abstract

Ozone causes vagally-mediated airway hyperreactivity and recruits inflammatory cells, including eosinophils, to lungs where they mediate ozone-induced hyperreactivity one day after exposure, but are paradoxically protective three days later. To test the role of newly divided eosinophils in ozone-induced airway hyperreactivity in sensitized and non-sensitized guinea pigs. Non-sensitized and sensitized guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU) to label newly divided cells and were exposed to air or ozone for 4 hours. One or three days later, vagally-induced bronchoconstriction was measured and inflammatory cells harvested from bone marrow, blood, and bronchoalveolar lavage. Ozone induced eosinophil hematopoiesis. One day post ozone, mature eosinophils dominate the inflammatory response and potentiate vagally-induced bronchoconstriction. However, by three days, newly divided eosinophils have reached the lungs where they inhibit ozone-induced airway hyperreactivity, since depleting them with AbIL-5 or a TNF α antagonist, worsened vagally-induced bronchoconstriction. In sensitized guinea pigs, both ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided eosinophils to lungs three days later failed to occur. Thus, mature eosinophils dominated the ozone-induced inflammatory response in sensitized guinea pigs. Depleting these mature eosinophils prevented ozone-induced airway hyperreactivity in sensitized animals. Ozone induces eosinophil hematopoiesis and recruitment to lungs where three days later, newly divided eosinophils attenuate vagally-mediated hyperreactivity. Ozone-induced hematopoiesis of beneficial eosinophils is blocked by a TNF α antagonist, or by prior sensitization. In these animals, mature eosinophils are associated with hyperreactivity. Thus, interventions targeting eosinophils, while beneficial in atopic individuals, may delay resolution of airway hyperreactivity in non-atopic individuals.

Key words

Eosinophil hematopoiesis, Ozone, Airway hyperreactivity, Sensitization, BrdU,

Abbreviations:

AbIL-5	Antibody to interleukin 5
TNF α	Tumor Necrosis Factor-alpha
AbMBP	Antibody to major basic protein
BrdU	5-bromo-2-deoxyuridine
MCh	Methacholine
O ₃	Ozone
PPI	Pulmonary inflation pressure
HR	Heart Rate
S	Sensitized
BAL	Bronchoalveolar lavage
Et	Etanercept
Eos	Eosinophil
PMN	Neutrophil
Mac	Macrophage
Lymp	Lymphocyte
GM-CSF	Granulocyte macrophage- colony stimulating factor
Ppm	Parts Per Million

Introduction

Ground level ozone is linked to respiratory and cardiovascular disease (6, 20, 34). Globally, there were 250,000 premature deaths linked to ozone exposure in 2015 alone (3) (www.stateofglobalair.org). The limits for ozone exposure set by the United States Environmental Protection Agency and World Health Organization are 0.070 and 0.06 parts per million (ppm)/hour over an 8 hour day (1, 58). Over 50% of Americans, and 92% of the world's population are regularly exposed to unhealthy levels of ozone, frequently up to 0.2 ppm per day, (2) that exceed these standards more than 25 days each year (WHO, 2014). In Shanghai, China ozone levels of as great as 0.48 ppm have been recorded (15). Asthmatics are particularly susceptible to air pollution, and high ozone days are followed by an increase in asthma-related emergency room visits and hospitalizations (28, 69). Thus, understanding the effects of ozone on the lungs is important for individuals living all over the world.

Ozone inhalation causes airway hyperreactivity in humans (8, 69), and animals (26, 50, 66, 71, 74, 80, 81) that is blocked by anticholinergics (8, 66, 67, 81). In experimental animals ozone-induced airway hyperreactivity is clearly mediated by parasympathetic nerves since vagally-induced bronchoconstriction is potentiated by ozone, while airway smooth muscle contraction to intravenous muscarinic agonists (in vagotomized animals so that contraction is independent of the vagus) is not changed (26, 75, 80).

Ozone causes lung inflammation in humans (21, 31, 32, 43) and animals (60, 71, 80, 81). In humans, airway eosinophils increase between 6-16 hours after ozone (21, 32), while eosinophil granule proteins (suggesting eosinophil activation) are elevated in bronchoalveolar lavage after ozone (21, 24, 32, 52). Eosinophils induce airway hyperreactivity by releasing major basic protein (18, 80, 81) that binds to inhibitory M_2 muscarinic receptors on parasympathetic nerves increasing acetylcholine in release and potentiating reflex bronchoconstriction (17, 18, 80, 81). In experimental animals, depleting eosinophils with AbIL-5, or neutralizing eosinophil major basic protein with either an antibody or with heparin, prevents ozone-induced airway hyperreactivity and protects or restores neuronal M_2 receptor function one-day post ozone (26, 80, 81).

Respiratory symptoms may lag behind ozone exposure (28, 42, 69) with asthma related emergency department visits and hospitalizations significantly increasing one to three days after high ozone days (28, 42, 69). We have shown that in guinea pigs eosinophils move into the lungs in two distinct waves after ozone (measured in bronchoalveolar lavage and histologically). The first wave occurs within one-day of exposure and the second occurs three days after exposure (80, 81). Blocking eosinophil recruitment and activation prevents hyperreactivity one day after ozone, demonstrating that eosinophils mediate airway hyperreactivity at this time point. Three days post ozone, the mechanism of airway

hyperreactivity is changed so that ozone induced potentiation of vagally mediated bronchoconstriction is associated with increased substance P and blocked by NK1 antagonists (74). Additionally, the role of eosinophils is also changed, since depleting eosinophils three days after ozone not only fails to prevent hyperreactivity, but significantly worsens it (81). Thus, eosinophils are harmful one day after ozone, but beneficial three days after ozone.

In the current studies, we test whether eosinophils recruited into the lungs one and three days after ozone exposure are mature or newly divided eosinophils, using 5-bromo-2-deoxyuridine (BrdU) to label dividing cells. We also tested whether prior antigen sensitization (as a model of atopy and allergy) affects ozone-induced eosinophil recruitment. Finally, we tested the role of TNF α (which is important in hyperreactivity after viral infection and antigen challenge) in ozone-induced eosinophil recruitment and airway hyperreactivity.

Methods:

Animals

Specific pathogen-free female Dunkin-Hartley guinea pigs (Charles River, Kingston, NY) were shipped in filtered crates and housed in rooms with high-efficiency particulate filtered air. All protocols involving animals followed NIH guidelines and were approved by Oregon Health & Science University Animal Care and Use Committee. Experiments were carried out in Dunkin-Hartley guinea-pigs since airway autonomic pharmacology (38, 68) and neural control of airways (14) are well characterized and similar to human. Furthermore, all previous data was performed using guinea pigs so data from these experiments can be compared to previous results.

Ozone Exposure

Guinea pigs were exposed to 2ppm ozone for 4 hours. As guinea pigs are obligate nose breathers, only 50% of the ozone they inhale reaches their lungs (49). Thus, guinea pigs were exposed to total of 4.0 ppm ozone. This concentration is higher than environmental levels, which can reach 1.04ppm over an 8-hour day (15, 34) but it is known to cause acute, reproducible airway inflammation and hyperreactivity in animals (36, 45, 76, 80, 81). Similarly, in humans ozone causes hyperreactivity (8, 30, 37, 40, 73), and inflammation in bronchoalveolar lavage (4, 5, 32, 73). In guinea pigs, lower concentrations of ozone (0.2ppm for 4 hours; total of 0.8ppm) also caused significant airway hyperreactivity (data not shown), but inflammation in bronchoalveolar lavage was not uniformly increased (78). As we were studying inflammation, the higher concentration of ozone was used in these experiments.

Guinea pigs were placed in individual wire cages with access to food and water and exposed to ozone (2.0ppm) or filtered air for 4h as previously described (26, 75, 81). One or three days later, either airway physiology was measured or inflammatory cells from bone marrow, blood, and bronchoalveolar lavage were harvested. Some guinea pigs (150-200g) were sensitized to ovalbumin (4.2 mg i.p.) 21, 19, and 17 days before exposure to air or ozone. No guinea pigs were challenged with antigen. Sensitization was confirmed since only, guinea pigs sensitized to ovalbumin developed ovalbumin specific IgE antibodies in homogenized lung and bronchoalveolar lavage (measured using guinea pig Ova specific IgE ELISA from Cusabio). At the time of exposure all guinea pigs weighed 350-400g.

Measurement of pulmonary inflation pressure

Guinea pigs were anesthetized with urethane (1.9g/kg i.p.). Heart rate and blood pressure were measured via a carotid artery cannula, and i.v. lines were placed into jugular veins. Animals were tracheotomized, mechanically ventilated with positive pressure and constant volume (1 ml volume/100 g body weight; 100 breaths/min), and paralyzed (succinylcholine chloride 10ug/kg i.v.). Both vagus nerves were cut and distal ends placed on platinum electrodes and covered with mineral oil. Guanethidine was administered (5mg/kg i.v.) to deplete

noradrenaline. Pulmonary inflation pressure (Ppi) 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 mmH₂O) over baseline inflation pressure as previously described (25, 80).

Measurement of bronchoconstriction

Both vagus nerves were electrically stimulated (10V, 0.2ms pulse width, 1-25Hz, 5s duration) at one-minute intervals producing frequency-dependent bronchoconstriction and bradycardia that were blocked by atropine (1mg/kg i.v.), demonstrating they were mediated by release of acetylcholine onto muscarinic receptors. The function of M₃ muscarinic receptors on airway smooth muscle were assessed in vagotomized guinea pigs using methacholine (1-10ug/kg i.v.).

Measurement of inflammatory cells

BrdU labeling of inflammatory cells: To measure cell division, some guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU) a thymidine analogue that is incorporated into DNA of dividing cells. Animals received BrdU (50mg/kg i.p.) immediately before ozone or air exposure, and again 2 hours after completion of exposure, and 50mg/kg i.p. once daily thereafter.

Collecting inflammatory cells: Blood was collected from the arterial cannula into a heparinized syringe. 0.5ml was lysed with HCl and white blood cells were counted using a hemocytometer. To measure white blood cell differentials and for BrdU staining, 1ml of blood was water lysed, centrifuged to remove supernatant, and re-suspended in PBS. Bone marrow was harvested by isolating the left femur and flushing the bone with 10ml PBS. Bronchoalveolar lavage was collected via the trachea cannula with five (10 ml) aliquots 1x PBS (room temperature). In all cases, cells were counted using a hemocytometer, cytopun onto slides, and differentials counted from slides.

Staining for differentials and BrdU: Slides were fixed in 70% ethanol (overnight, 4°C). Endogenous peroxidase activity was quenched with 3% H₂O₂ in cold methanol (-20°C) for 10 min. DNA was denatured using dilute HCl (reagent 2, BrdU staining kit, Invitrogen) for 30 min, and slides were washed with 0.05% tween in PBS, blocked (reagent 3, BrdU staining kit, Invitrogen) for 10 min, and incubated for 1 hour with mouse anti-BrdU biotinylated Ab (reagent 4, BrdU staining kit, Invitrogen). BrdU staining was amplified using ABC elite kit (Vector Laboratories) for 30 min and visualized with ImmPACT DAB (Vector laboratories; 10 min), and then stained with haematoxylin and eosin (Hemacolor EMD Chemicals, Philadelphia, PA) for differentials. Eosinophils were identified primarily by the presence of bi-lobed nuclei and secondarily by the presence of large red granules. Neutrophils were identified by nucleus divided into 3-5 lobes and small light pink granules.

Throughout the paper results are displayed as the total number of BrdU+ and BrdU- cells in blood and bronchoalveolar lavage. Because the total number of cells is also changing with treatment, results also include the percent of BrdU+ cells. Since the number of cells is dependent upon variables including lung volume, bone marrow volume, and isolation methodology, the ratio of BrdU+:BrdU- cells (represented by the percent of BrdU+ cells) maybe a better indication of cell populations *in vivo*.

Eosinophil Depletion

Some guinea pigs were pretreated with an antibody to IL-5 (AbIL-5) (TRFK5; 500ug/kg i.p., three days before ozone or air to deplete eosinophils (63, 80, 81) (Cat# 554393 BD Pharmagen, Piscataway, NJ). Eosinophil depletion was confirmed in these experiments by counting eosinophils in bronchoalveolar lavage, and bone marrow. Because no adverse effects have been reported following eosinophil depletion in animal or human studies(29), or in sensitized animals (61, 63) AbIL-5 was not given to control animals.

Tumor Necrosis Factor α Blockade

To inhibit TNF α , guinea pigs were pretreated with etanercept (3mg/kg i.p., Amgen, Thousand Oaks, CA), a TNF α receptor IgG fusion protein, three hours prior to exposure to ozone or air. The half-life of etanercept is 4 days (19, 72, 82) this dose has been shown to prevent airway hyperreactivity following antigen challenge (53), viral infection (54), and parathion (organophosphorus pesticide) exposure (62) in guinea pigs. Because etanercept had no effect on vagally mediated or smooth muscle induced bronchoconstriction in air-exposed control and sensitized not challenged animals in studies done within the past 6 years (53, 62) controls were not repeated in these experiments.

Drugs. 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.

Statistics. All data are expressed as mean $\pm$ SEM. Frequency and methacholine dose response curves were analyzed using a repeated measures two-way analysis of variance (ANOVA). Inflammatory cells in bronchoalveolar lavage, blood, and bone marrow were analyzed by one-way ANOVA comparing selected columns with a Bonferroni correction. Statistical probability of $P \leq 0.05$ was considered significant.

Results

Ozone increased baseline pulmonary inflation pressure from 102 ± 5 mmH₂O to 150 ± 6 mmH₂O in non-sensitized and 158 ± 13 mmH₂O in sensitized guinea pigs. This increase in baseline pulmonary inflation pressure did not affect the outcome because baseline pulmonary inflation pressure in animals treated with AbIL-5 or etanercept was 110 ± 11 mmH₂O and 172 ± 12 mmH₂O respectively, and in both treatment groups ozone caused airway hyperreactivity independent of baseline change. In guinea pigs, baseline heart rate 288 ± 10 bpm, baseline systolic blood pressure 41 ± 1 mmHg, and baseline diastolic blood pressure 20 ± 8 mmHg were not affected by any treatment.

Electrical stimulation of both vagus nerves caused frequency dependent bronchoconstriction that was significantly increased one day after ozone (Fig. 1A). In contrast, methacholine-induced bronchoconstriction was unchanged by ozone (Fig 1B), demonstrating that ozone-induced hyperreactivity is mediated by increased acetylcholine release from parasympathetic nerves. Eosinophils, neutrophils, macrophages, and lymphocytes were significantly increased in bronchoalveolar lavage by ozone (Fig 1C). These increases were comprised of both newly divided BrdU⁺, and mature BrdU⁻ cells, however BrdU⁻ cells were only significantly increased in macrophages, and combined cells (Fig 1C). Ozone did not change circulating leukocytes (Fig 5A-E). In bone marrow, ozone did not change total cells (air: $2.3 \pm 0.32 \times 10^8$ vs. ozone: $2.5 \pm 0.4 \times 10^8$ cells), monocytes or neutrophils (Fig 1D), however it selectively and significantly increased BrdU⁺ eosinophils from 26 to 44% (Fig 1D, Table 1).

Three days after ozone guinea pigs were still hyperreactive to stimulation of the vagus nerves (Fig 2A). However, hyperreactivity was significantly less than one day post ozone (Fig 1A). Sensitization alone had no effect on vagally-induced bronchoconstriction (open circles Fig 2A & 2B). Ozone still potentiated vagally-induced bronchoconstriction in sensitized guinea pigs (Fig 2B), similar to non-sensitized guinea pigs (Fig 2A). However the potentiation was significantly greater in sensitized than in non-sensitized guinea pigs (compare solid circles Fig 2A & 2B). In both sensitized and non-sensitized guinea pigs airway hyperreactivity is mediated by the vagus nerves since, methacholine-induced bronchoconstriction was not potentiated by ozone (Fig 2C, 2D). Ozone increased total inflammatory cells in bronchoalveolar lavage in both non-sensitized and sensitized animals (Fig 2E). However, while both BrdU⁺ and BrdU⁻ cells were increased in non-sensitized guinea pigs, only BrdU⁻ cells were increased in sensitized guinea pigs (Fig 2E). Ozone did not change leukocytes in blood (Fig 5), or bone marrow (air: $2.6 \pm 0.21 \times 10^8$, ozone: $1.9 \pm 0.21 \times 10^8$, air sensitized: $2.8 \pm 0.24 \times 10^8$, ozone sensitized: $2.3 \pm 0.20 \times 10^8$).

Eosinophils in bronchoalveolar lavage were significantly increased three days after ozone in both non-sensitized and sensitized guinea pigs (Fig 3A). In non-sensitized guinea pigs this increase was comprised entirely of new BrdU⁺ eosinophils (Fig 3A, Black), so that the percent

of BrdU+ cells increased from 22 to 56% (Fig 3A black, Table 1). In contrast, in sensitized guinea pigs, these BrdU+ eosinophils completely failed to appear, so the increase was entirely mature BrdU- eosinophils (Fig 3A, grey). Ozone did not change circulating eosinophils in non-sensitized or sensitized guinea pigs (Fig 5G). Similar to findings in bronchoalveolar lavage, bone marrow production of new BrdU+ eosinophils was significantly increased by ozone in non-sensitized guinea pigs from 36 to 62% (Fig 4A, black, Table 1), while ozone-induced eosinophil hematopoiesis failed to occur in sensitized animals (only 44% BrdU+, Table 1, Fig 4A, grey).

Neutrophils in bronchoalveolar lavage were significantly increased three days after ozone in both non-sensitized and sensitized guinea pigs (Fig 3B). In non-sensitized guinea pigs, this increase was comprised entirely of BrdU+ cells (Fig 3B, black), while in sensitized animals; the increase was entirely mature BrdU- neutrophils (Fig 3B, grey). Neither ozone nor sensitization changed neutrophils in blood (Fig 5H) or bone marrow (Fig 4B).

Macrophages in bronchoalveolar lavage were significantly increased three days after ozone in both non-sensitized and sensitized guinea pigs (Fig 3C). In non-sensitized guinea pigs the increase was comprised of both BrdU+ and BrdU- cells (19% to 34% BrdU+ S Table 4, Fig 3C, black), while in sensitized guinea pigs only BrdU- macrophages were increased (17% to 15% BrdU+, S Table 4, Fig 3C, grey). Three days after ozone lymphocytes in bronchoalveolar lavage were not changed in non-sensitized animals (Fig 3C, black), but both BrdU+ and BrdU- cells were significantly increased in sensitized guinea pigs (Fig 3D, grey). Neither ozone nor sensitization affected monocytes or lymphocytes in blood (Fig 5 I and J) or bone marrow (Fig 4C).

To test the role of eosinophils in airway hyperreactivity three days after ozone, guinea pigs were pretreated with AbIL-5. In non-sensitized guinea pigs, AbIL-5 significantly worsened vagally-mediated bronchoconstriction three days after ozone (Fig 2A). Conversely, in sensitized guinea pigs, AbIL-5 completely prevented ozone-induced airway hyperreactivity (Fig 2B). AbIL-5 had no effect on methacholine-induced bronchoconstriction (Fig 2C). The effects of AbIL-5 on ozone-induced airway hyperreactivity are likely due to eosinophil depletion since AbIL-5 significantly reduced eosinophils in bronchoalveolar lavage in both non-sensitized and sensitized guinea pigs three days post ozone (Fig 8) and bone marrow of non-sensitized guinea pigs (ozone: $11.6 \pm 1.5\%$, ozone+AbIL-5: $5.6 \pm 1.1\%$). Thus, in non-sensitized guinea pigs depleting BrdU+ eosinophils worsens ozone-induced airway hyperreactivity. However, in sensitized animals depleting mature BrdU- eosinophils prevents ozone-induced hyperreactivity.

Three days after ozone, etanercept significantly worsened vagally-mediated bronchoconstriction in non-sensitized guinea pigs (Fig 2A). In contrast, in sensitized guinea

pigs, etanercept had no effect on ozone-induced airway hyperreactivity (Fig 2B) three days after exposure. Methacholine-induced bronchoconstriction was not changed by etanercept in non-sensitized (Fig 2C) or sensitized animals (Fig 2D) at 3 days. One day after ozone, etanercept has no effect on vagally-mediated bronchoconstriction (S, Fig 5A).

In bronchoalveolar lavage, etanercept did not affect the ozone-induced increase in inflammation in non-sensitized or sensitized guinea pigs (Fig 2F). However, the increase in newly formed BrdU⁺ leukocytes after ozone (37% BrdU⁺, Fig 2E, black) was prevented by etanercept, so that the increase in leukocytes was made up of only BrdU⁻ cells (18% BrdU⁺, Fig 2F). In sensitized guinea pigs, etanercept did not affect total inflammatory cells in bronchoalveolar lavage of air-exposed guinea pigs (Fig 2E to 2F), or the increase in inflammatory cells after ozone (Fig 2E to 2F). Leukocytes in blood were not changed by etanercept in sensitized or non-sensitized animals (Fig 5F).

Etanercept pretreatment significantly increased eosinophils in bronchoalveolar lavage after ozone (right panel, Fig 3A). Additionally, etanercept changed the composition of these eosinophils from predominantly BrdU⁺ (56%) to predominantly BrdU⁻ (22% BrdU⁺, Table 1) (right panel, Fig 3A). In sensitized guinea pigs pretreated with etanercept, ozone failed to recruit any additional eosinophils to bronchoalveolar lavage (right panel Fig 3A). Etanercept did not affect circulating eosinophils in non-sensitized or sensitized animals (Fig 5G). However, in bone marrow etanercept prevented ozone-induced eosinophil hematopoiesis in non-sensitized guinea pigs at one (Fig 9E) and three days (right panel, Fig 4A), so that percent of BrdU⁺ cells after ozone (28% BrdU⁺, Table 1) was not different from air (31% BrdU⁺, Table 1).

In non-sensitized guinea pigs, etanercept inhibited recruitment of BrdU⁺ neutrophils to bronchoalveolar lavage (35% BrdU⁺, Table 2, Fig 3B). In contrast, etanercept did not effect neutrophil recruitment to bronchoalveolar lavage in sensitized animals (33% BrdU⁺, Table 2, Fig 3B). Neutrophils in blood (Fig 5H) and bone marrow (right panel, Fig 4B) of etanercept treated guinea pigs were not changed by ozone or by sensitization.

In non-sensitized but not sensitized guinea pigs, etanercept significantly decreased BrdU⁺ macrophages in bronchoalveolar lavage after ozone (34% to 15% BrdU⁺, Table 3, Fig 3C). Blood monocytes at baseline were increased by etanercept in both non-sensitized and sensitized animals (Fig 5I). Ozone additionally increased circulating monocytes in etanercept treated sensitized animals

Ozone significantly increased BrdU⁻ lymphocytes in bronchoalveolar lavage only in the presence of etanercept in non-sensitized animals (right panel, Fig 3D). Etanercept significantly increased circulating lymphocytes after ozone (right panel, Fig 5J). Bone marrow mononuclear

354 cells were not affected by etanercept in non-sensitized or sensitized guinea pigs (Table 3, Fig
355 4C).

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357 In the heart, bradycardia induced by electrical stimulation of both vagus nerves, or
358 intravenous methacholine was not changed by ozone, by sensitization, by AbIL-5, or by
359 etanercept (Fig 6 & 7) alone or in combination.

Discussion

Our current therapeutic approach to asthma often involves suppressing chronic inflammation (33). However, in some asthmatic children, reducing lung inflammation with steroids worsens bronchoconstriction after ozone exposure (35), suggesting that some components of lung inflammation may be protective. Beneficial effects of lung inflammation have received little attention.

Here we show for the first time that newly divided BrdU⁺ eosinophils are protective in lungs three days after ozone. Selective depletion of eosinophils at this time point significantly worsens airway hyperreactivity. The eosinophil population in bronchoalveolar lavage three days after ozone is comprised of predominantly BrdU⁺ cells (i.e. newly divided). In contrast, one day after ozone, eosinophil depletion prevents airway hyperreactivity, but at this time point eosinophils are comprised predominately of BrdU⁻ cells (i.e. mature cells). Thus, the proportion of new to old eosinophils, and their role in airway hyperreactivity, changes between one and three days after ozone. Eosinophils have previously been shown to have a protective role in viral infection (22), colon cancer (27), and inflammatory arthritis (16), here we show that when the population of eosinophils is dominated by newly divided cells, eosinophils are also protective three days after ozone.

The temporal role of eosinophils in mediating ozone-induced airway hyperreactivity suggests that distinct eosinophil phenotypes exist. While ozone significantly increased the percent of newly divided (BrdU⁺), eosinophils both one and three days later (Table 1), the percent of newly divided eosinophils in lungs at three days (56%±8.5) was significantly greater than at one day (35%±2.6). While ozone also significantly increased newly divided eosinophils in bone marrow, ozone did not increase BrdU⁺ positive neutrophils and monocytes in bone marrow. Thus, ozone significantly and specifically increased eosinophil hematopoiesis.

Blocking TNF α with etanercept had no effect on hyperreactivity or inflammation one day after ozone (Fig 9), but worsened airway hyperreactivity three days after ozone (Fig 2A). At this three day time point, blocking TNF α also prevented the ozone-induced increase in newly divided eosinophils in bone marrow (Fig 4A) and despite increasing total eosinophil numbers prevented newly divided eosinophils in bronchoalveolar lavage (Fig 3A). Thus, TNF α promotes ozone-induced eosinophil hematopoiesis of beneficial, newly divided eosinophils. Loss of these beneficial, eosinophils in bronchoalveolar lavage worsened ozone-induced airway hyperreactivity.

Atopic patients are sensitized to allergens and have different airway inflammatory responses to ozone (7, 12, 55). We sensitized guinea pigs to ovalbumin to determine whether pre-existing atopy affected ozone-induced synthesis and recruitment of protective, BrdU⁺ eosinophils to lungs. Ozone-induced airway hyperreactivity and eosinophil recruitment to lungs was greater

in sensitized (Fig 2B) than in non-sensitized guinea pigs (Fig 2A). In stark contrast to non-sensitized animals, sensitization prevented the ozone-induced production of new eosinophils in bone marrow (Fig 4A) and the recruitment of BrdU⁺ eosinophils to bronchoalveolar lavage three days after ozone (Fig 3A). In sensitized guinea pigs, BrdU⁻ eosinophils comprised the dominant inflammatory cell population in the lungs three days after ozone. Hyperreactivity was blocked when eosinophils were depleted with AbIL-5, confirming that eosinophils mediate ozone-induced airway hyperreactivity when BrdU⁻ eosinophils are the dominant population in the lungs. Blocking TNF α in sensitized animals had no additional effect on eosinophil populations in bone marrow (Fig 4A) or bronchoalveolar lavage (Fig 3A) or on ozone-induced airway hyperreactivity (Fig 2B). These data suggest that sensitization prevents TNF α from promoting production of new, beneficial eosinophils and their recruitment to the lungs three days after ozone. Thus, atopic individuals may have worsened airway hyperreactivity following ozone exposure, or delayed resolution of symptoms because of a lack of a bone marrow response to ozone and loss of beneficial, BrdU⁺, newly divided, eosinophils.

In addition to eosinophils, ozone also significantly increased lymphocytes in bronchoalveolar lavage of sensitized animals and etanercept treated non-sensitized animals (Fig 3D). There is strong data supporting a role for lymphocytes in eosinophil recruitment in atopic individuals (56, 57). Activated lymphocytes release IL-5, IL-13, IL-4, IL-1 β , IL-8 (13, 57, 79) all of which can contribute to eosinophil maturation in bone marrow, or to eosinophil recruitment and survival in lungs (46). Here we show a crucial role for IL-5 since AbIL-5 blocks recruitment of eosinophils to lungs (Fig 8). A potential source of IL-5 may be ILC2 lymphocytes, which are known to produce IL-5, since depletion of ILC2 cells in sensitized mice completely blocks ozone induced eosinophilia and airway hyperreactivity (57). Thus, ILC2 cells may be a source of IL-5 that mediates ozone induced eosinophil recruitment.

Ozone also significantly increased neutrophils in bronchoalveolar lavage although there was no effect in bone marrow (Fig 4). In humans, ozone induced lung inflammation is dominated by neutrophils (5, 39, 41, 65). While neutrophil activation releases cytokines including IL-8, which is a potent eosinophil chemoattractant (23), neutrophil depletion does not prevent ozone-induced airway hyperreactivity in dogs (44, 70) or humans (4, 73). Thus, neutrophils likely don't play a role in the inflammatory mechanisms we are studying here.

Anti-TNF α treatments are effective in patients with severe refractory asthma (10, 11, 64, 77). However, blocking TNF α is ineffective in patients with moderate or atopic asthma where it does not change eosinophilic inflammation in lungs (10, 11, 64, 77). This is consistent with our data where total eosinophils are not changed by TNF α blockade, although, the percent of ozone-induced newly divided BrdU⁺ eosinophils in bronchoalveolar lavage was decreased (Fig 3A, Table 1). Thus, blocking TNF α may exacerbate asthma by preventing newly divided eosinophil development and recruitment to lungs. Blocking TNF α is known to decrease

adhesion molecule expression (47, 48). Blocking TNF α increased circulating monocytes and lymphocytes, this is likely due to decreased adhesion molecule expression on endothelial cells resulting in retention of inflammatory cells in blood.

This study demonstrates that ozone causes airway hyperreactivity that lasts at least three days in non-sensitized and sensitized guinea pigs. In non-sensitized guinea pigs, TNF α promotes ozone-induced eosinophil hematopoiesis and recruitment to lungs three days after ozone. Newly divided, BrdU⁺ eosinophils are protective three days after ozone, since depleting them, with AbIL-5 or TNF α antagonists, significantly worsens ozone-induced airway hyperreactivity. Sensitization with ovalbumin prevents the ozone-induced increase in eosinophil hematopoiesis and subsequent recruitment of beneficial BrdU⁺ eosinophils to lungs. In sensitized guinea pigs, the ozone-induced increase in eosinophils in bronchoalveolar lavage is made up predominately of the mature BrdU⁻ eosinophils that mediate hyperreactivity. Ozone-induced airway hyperreactivity one day post ozone is mediated by release of eosinophil major basic protein, which inhibits neuronal M₂ muscarinic receptors, and increases acetylcholine release from the parasympathetic nerves, leading to increased vagally-induced bronchoconstriction (17, 18, 26, 80, 81). These effects are likely mediated by mature BrdU⁻ eosinophils. While three days post ozone, depleting eosinophils exacerbates ozone-induced airway hyperreactivity, even though it restores neuronal M₂ muscarinic receptor function (81), clearly dissociating neuronal M₂ muscarinic receptor function and hyperreactivity at this time point in non-sensitized guinea pigs. The mechanism underlying the neuro-protective effect of BrdU⁺ eosinophils following ozone is not known. Thus, when newly divided eosinophils are the dominant population in the lungs eosinophils are protective. However, when mature eosinophils are dominant population in the lungs eosinophils mediate ozone induced airway hyperreactivity.

Asthma is a heterogeneous disease including diverse inflammatory phenotypes (33). Thus, it is not unexpected that targeted therapies including AbIL-5, while effective in eosinophilic asthma, are less effective in other inflammatory phenotypes (9, 51, 59). Our data show that eosinophils are likely heterogeneous and that the presence of eosinophils alone is not sufficient to predict their role in airway hyperreactivity. Furthermore, our data show that newly divided, BrdU⁺ eosinophils have a beneficial role, and suggest that therapies directed at protection of or expansion of BrdU⁺ eosinophils may provide novel mechanisms to cure asthma or prevent exacerbations.

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Figure Legends

Figure 1. Ozone (O_3 , 2.0ppm, 4h) significantly increased vagally-mediated bronchoconstriction and lung inflammation measured 1 day later in non-sensitized guinea pigs. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bronchoconstriction that was significantly increased by ozone (closed circles, A) compared to air (open circles, A). In contrast, i.v. methacholine-induced bronchoconstriction was not similarly potentiated by ozone (B). In bronchoalveolar lavage (C), eosinophils, neutrophils, macrophages and lymphocytes were all significantly increased by ozone. These increases were comprised of BrdU+ and BrdU- cells although the increase in BrdU- cells was only significant in macrophages and combined cells. Note each column represents the total cell number and is subdivided to show BrdU- (white) and BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. In bone marrow, ozone significantly potentiated the percent of BrdU+ eosinophils (black portion, D, final column. Data expressed as in C). Data shown as mean $\pm$ SEM n=4-9 *indicates $P \leq 0.05$ in all cases. In C and D statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions.

Figure 2. Ozone (O_3 , 2.0ppm, 4h) significantly increased vagally-mediated bronchoconstriction and total inflammatory cells in non-sensitized and sensitized guinea pigs (S) measured 3 day later. Electrical stimulation of both vagus nerves (same parameters as figure 1) produced frequency dependent bronchoconstriction that was significantly increased 3 days after ozone in non-sensitized (A) and sensitized (B) guinea pigs. Antibody to IL-5 (AbIL-5) significantly potentiated ozone-induced airway hyperreactivity in non-sensitized guinea pigs (A), but significantly inhibited hyperreactivity in sensitized animals (B). Etanercept (Et) significantly increased ozone-induced hyperreactivity in non-sensitized animals (A), but had no effect in sensitized animals (B). Bronchoconstriction induced by i.v. methacholine in vagotomized animals was not potentiated by ozone (O_3), AbIL-5, or etanercept in non-sensitized (C), or sensitized guinea pigs (D). Ozone significantly increased inflammatory cells in bronchoalveolar lavage in non-sensitized (black/white) and sensitized (grey/white) guinea pigs 3 days later (E). In non-sensitized animals ozone significantly increased both BrdU+ (black) and BrdU- (white) inflammatory cells while in sensitized guinea pigs, ozone increased only BrdU- (white) inflammatory cells (E). Etanercept had no effect on total inflammatory cells in air or ozone exposed animals (F). Data shown as mean $\pm$ SEM n=4-9 *indicates $P \leq 0.05$ in all cases. In E and F statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions.

Figure 3. Ozone significantly increased eosinophils (A), neutrophils (B), and macrophages (C) but not lymphocytes (D) in bronchoalveolar lavage of non-sensitized (black/white) guinea pigs 3 days later. Note, as in figure 1 each column represents the total and is subdivided to show BrdU- (white) and BrdU+ (black or grey) cells. The percent of BrdU+ cells is included under

each column. In non-sensitized animals the ozone induced increase was comprised of BrdU+ cells. Only eosinophils were significantly potentiated by sensitization (A, compare first 2 sets of bars). In sensitized guinea pigs, ozone significantly increased eosinophils (A), neutrophils (B), and macrophages (C) and lymphocytes (D) in bronchoalveolar lavage. However in sensitized animals ozone did not increase BrdU+ cells. Etanercept potentiated ozone's ability to recruit eosinophils, macrophages, and lymphocytes in non-sensitized animals (third set of columns, black/white), and potentiated ozone's ability to recruit neutrophils in sensitized animals (fourth set of columns, grey/white). Statistics are shown as in figure 1 $P \leq 0.05$, * significantly different from air, # significantly different from untreated ozone.

Figure 4. Ozone specifically and significantly increased eosinophil hematopoiesis in non-sensitized (black/white) guinea pigs 3 days later (A). Note, as in figure 1 each column represents the total and is subdivided to show BrdU- (white) and BrdU+ (black or grey) cells. The percent of BrdU+ cells is included under each column. Ozone increased BrdU+ (black) eosinophils in bone marrow (first set of columns, black, A) in non-sensitized guinea pigs, however, this increase failed to occur in non-sensitized (second set of columns, grey, A) or etanercept treated non-sensitized (third set of columns, black, A) animals. Neutrophils (B) and monocytes (B) in were not changed by ozone, by sensitization, or by etanercept (B). Statistics are shown as in figure 1 $P \leq 0.05$, *significantly different from air, #significantly different from untreated ozone.

Figure 5. Ozone had no effect on total leukocytes, eosinophils, neutrophils, monocytes, and lymphocytes in blood 1 (A-E) or 3 (F-J) days after exposure in non-sensitized (black/white) and sensitized (grey/white) guinea pigs. Note, as in figure 1 each column represents the total and is subdivided to show BrdU- (white) and BrdU+ (black or grey) cells. The percent of BrdU+ cells is included under each column. Etanercept significantly increased circulating monocytes (I). Etanercept increased circulating monocytes (I) and lymphocytes (J) after ozone in both non-sensitized and sensitized animals. Data are mean $\pm$ SEM; n=4-9. Statistics are shown as in figure 1 $P \leq 0.05$, *significantly different from air, #significantly different from untreated ozone.

Figure 6. Vagally-induced (A) and methacholine-induced (B) bradycardia was not changed by ozone (O_3) at 1 day. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train, at 1 min intervals) produced frequency dependent bradycardia. Data shown as mean $\pm$ SEM n=4-9.

Figure 7. Vagally-induced and methacholine-induced bradycardia was not changed by ozone (O_3) sensitization (S), etanercept (Et), or AbIL-5 3 days after ozone. Electrical stimulation of both vagus nerves (same parameters as Fig 1) produced frequency dependent bradycardia that was not affected by ozone (A) sensitization (B), etanercept (A, B), or AbIL-5 (A). Methacholine-induced bradycardia was not changed by ozone (C) or sensitization (D), or etanercept (C, D). Data shown as mean $\pm$ SEM n=4-9.

Figure 8. Eosinophils (Eos) in bronchoalveolar lavage were depleted by antibody to IL-5 (AbIL-5) in non-sensitized and sensitized (S) guinea pigs 3 days after ozone (O₃). Three days after ozone combined, eosinophils (eos), neutrophils (PMN), and macrophages (MAC) were significantly increased when compared to air in both non-sensitized and sensitized guinea pigs. In non-sensitized animals AbIL-5 significantly decreased eosinophils in ozone-exposed guinea pigs. In sensitized animals, total, eosinophils (Eos), and lymphocytes (lymp) were decreased following AbIL-5 following ozone. Data are mean $\pm$ SEM; n=5-9, P $\leq$ 0.05 * significantly different from non-treated.

Figure 9. Pretreatment with etanercept (Et) had no effect on ozone-induced airway hyperreactivity. Electrical stimulation of both vagus nerves (same parameters as figure 1) produced frequency dependent bronchoconstriction that was significantly increased 1 day after ozone (open circles compared to closed circles data re-graphed from figure 1, A). Etanercept had no effect on ozone-induced airway hyperreactivity (closed triangles, A). Methacholine-induced bronchoconstriction was not changed by etanercept (B). Both BrdU+ and BrdU- neutrophils in bronchoalveolar lavage were significantly increased by ozone in etanercept treated animals (C). Etanercept had no effect on circulating inflammatory cells (D). In bone marrow, etanercept significantly decreased the percent of BrdU+ eosinophils after ozone compared to non-treated animals in figure 1 (E). Data are mean $\pm$ SEM; n=4-9. Statistics are shown as in figure 1 P $\leq$ 0.05, *significantly different from air, #significantly different from 1 day post ozone.

Table 1. Percent BrdU+ eosinophils. Data are mean $\pm$ SEM; n=4-9 P $\leq$ 0.05 *indicates significantly different from air, #significantly different from non-sensitized, and $\neq$ significantly different from sensitized ozone.

Table 2. Percent BrdU+ neutrophils. Data are mean $\pm$ SEM; n=4-9 P $\leq$ 0.05 * Significantly different from air, # significantly different from untreated.

Table 3. Percent BrdU+ macrophages. Data are mean $\pm$ SEM; n=4-9 P $\leq$ 0.05 * Indicates significantly different from air, #significantly different from non-sensitized.

Table 4. Percent BrdU+ lymphocytes. Data are mean $\pm$ SEM; n=4-9

Table 5. Percent BrdU+ Leukocytes. Data are mean $\pm$ SEM; n=4-9 P $\leq$ 0.05 * Indicates significantly different from air, #significantly different from non-sensitized.

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Table 1 Statistical comparison percent BrdU+ Eosinophils						
				Bronchoalveolar Lavage	Blood	Bone Marrow
				Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM
Non-sensitized	Control	1 day	Air	19 $\pm$ 3.6	19 $\pm$ 2.6	26 $\pm$ 4.2
			O ₃	35 $\pm$ 2.6*	33 $\pm$ 8.8	44 $\pm$ 6.1*
		3 day	Air	22 $\pm$ 5.6	28 $\pm$ 4.8	36 $\pm$ 5.3
			O ₃	56 $\pm$ 8.5*	41 $\pm$ 8.7	62 $\pm$ 5.1*#
	Etanercept	3 day	Air	23 $\pm$ 2.9	28 $\pm$ 6.1	31 $\pm$ 2.1
			O ₃	22 $\pm$ 2.4#	20 $\pm$ 1.9	28 $\pm$ 2.5#
Sensitized	Control	3 day	Air	19 $\pm$ 3.5	30 $\pm$ 4.8	40 $\pm$ 3.2
			O ₃	12 $\pm$ 1.9#	20 $\pm$ 3.3	44 $\pm$ 5.0#
	Etanercept	3 day	Air	26 $\pm$ 4.8	18 $\pm$ 4.5	29 $\pm$ 2.8
			O ₃	25 $\pm$ 4.1#	18 $\pm$ 2.1	29 $\pm$ 3.7

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Table 2 Statistical Comparison Percent BrdU+ Neutrophils						
				Bronchoalveolar Lavage	Blood	Bone Marrow
				Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM
Non-sensitized	Control	1 day	Air	37 $\pm$ 7.0	25 $\pm$ 3.4	39 $\pm$ 3.7
			O ₃	51 $\pm$ 8.7	43 $\pm$ 8.6	46 $\pm$ 7.7
		3 day	Air	28 $\pm$ 7.1	41 $\pm$ 5.1	47 $\pm$ 4.8
			O ₃	54 $\pm$ 7.7	53 $\pm$ 8.7	51 $\pm$ 7.8
	Etanercept	3 day	Air	46 $\pm$ 15.0	33 $\pm$ 4.0	47 $\pm$ 14.0
			O ₃	35 $\pm$ 5.0	28 $\pm$ 1.5#	34 $\pm$ 2.2
Sensitized	Control	3 day	Air	37 $\pm$ 6.0	44 $\pm$ 8.0	48 $\pm$ 3.6
			O ₃	31 $\pm$ 2.2	32 $\pm$ 4.5	45 $\pm$ 3.3
	Etanercept	3 day	Air	6 $\pm$ 6.3#	36 $\pm$ 5.5	37 $\pm$ 6.2
			O ₃	33 $\pm$ 5.4*	43 $\pm$ 15.0	32 $\pm$ 2.3

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Table 3 Statistical Comparison Percent BrdU+ Macrophages
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				Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-sensitized	Control	1 day	Air	17 ± 1.3	30 ± 3.8	42 ± 2.3
			O ₃	24 ± 2.8*	37 ± 5.3	46 ± 5.6
		3 day	Air	19 ± 4.3	27 ± 1.9	41 ± 3.2
			O ₃	34 ± 5.1*	35 ± 3.2*	44 ± 6.1
	Etanercept	3 day	Air	17 ± 2.4	25 ± 1.6	32 ± 1.3
			O ₃	15 ± 1.6#	22 ± 3.5#	33 ± 2.8
Sensitized	Control	3 day	Air	17 ± 2.2	26 ± 3.8	40 ± 2.2
			O ₃	15 ± 2.1#	22 ± 3.3	39 ± 3.3
	Etanercept	3 day	Air	20 ± 3.4	23 ± 1.3	33 ± 3.1
			O ₃	19 ± 1.9	27 ± 4.5	35 ± 3.1

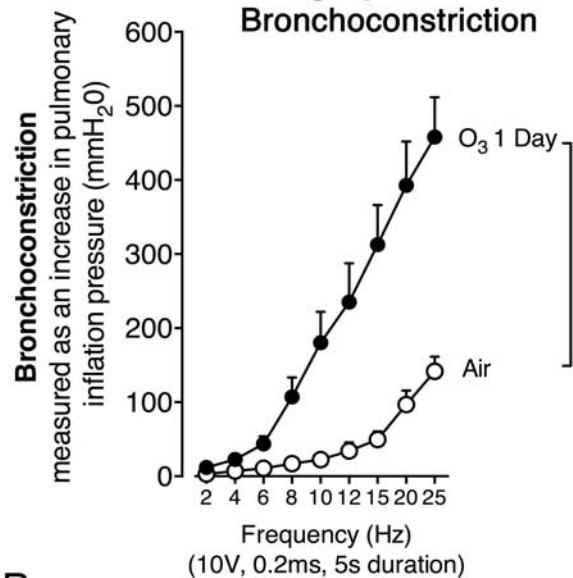
Table 4 Statistical Comparison Percent BrdU+ Lymphocytes						
				Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-sensitized	Control	1 day	Air	32 ± 7.2	32 ± 6.1	42 ± 2.3
			O ₃	21 ± 3.8	50 ± 5.7	46 ± 5.6
		3 day	Air	29 ± 3.5	33 ± 3.4	41 ± 3.2
			O ₃	34 ± 5.3	44 ± 4.7	44 ± 6.1
	Etanercept	3 day	Air	31 ± 11.0	35 ± 0.9	32 ± 1.3
			O ₃	26 ± 2.2	30 ± 1.8	33 ± 2.8
Sensitized	Control	3 day	Air	33 ± 6.0	36 ± 7.7	40 ± 2.2
			O ₃	27 ± 7.8	30 ± 4.1	39 ± 3.3
	Etanercept	3 day	Air	33 ± 18.2	35 ± 6.5	33 ± 3.1
			O ₃	34 ± 7.2	35 ± 7.1	35 ± 3.1

Table 5 Statistical Comparison Percent BrdU+ Total Leukocytes						
				Bronchoalveolar Lavage Mean ±SEM		Blood Mean ±SEM
Non-sensitized	Control	1 day	Air	20 ± 1.4		26 ± 2.9
			O ₃	31 ± 3.4*		43 ± 7.7
		3 day	Air	20 ± 3.5		35 ± 3.2
			O ₃	37 ± 4.3*		49 ± 7.5
	Etanercept	3 day	Air	20 ± 2.0		30 ± 1.8
			O ₃	18 ± 1.3#		26 ± 1.6#
Sensitized	Control	3 day	Air	19 ± 2.4		38 ± 6.3
			O ₃	16 ± 1.4#		30 ± 4.0
	Etanercept	3 day	Air	24 ± 3.0		31 ± 3.7
			O ₃	22 ± 0.9		34 ± 8.3

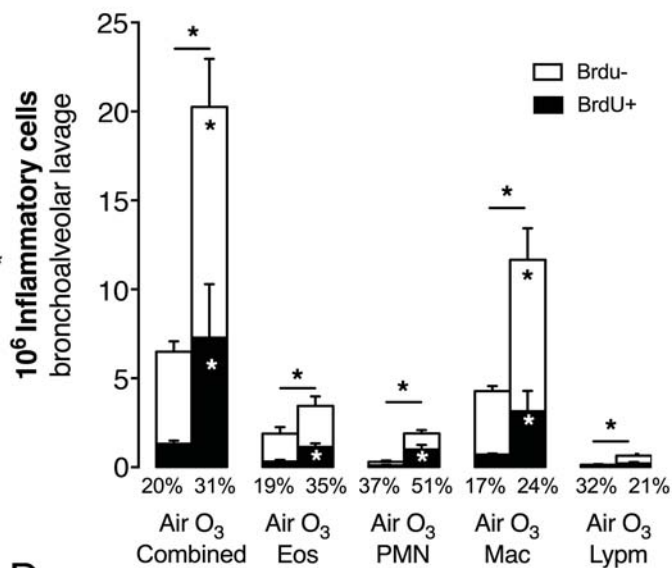
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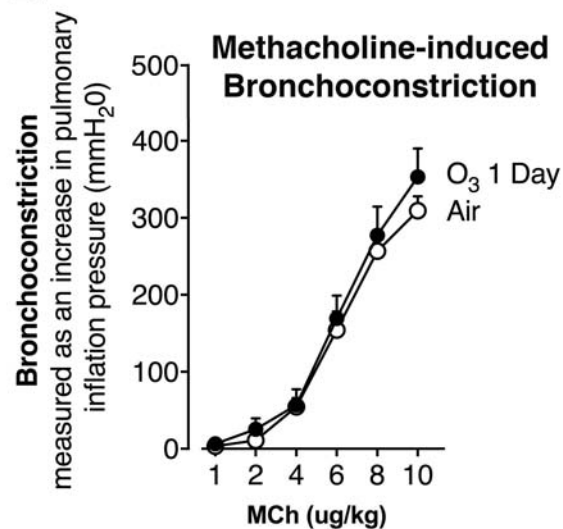
A. Vagally-induced Bronchoconstriction



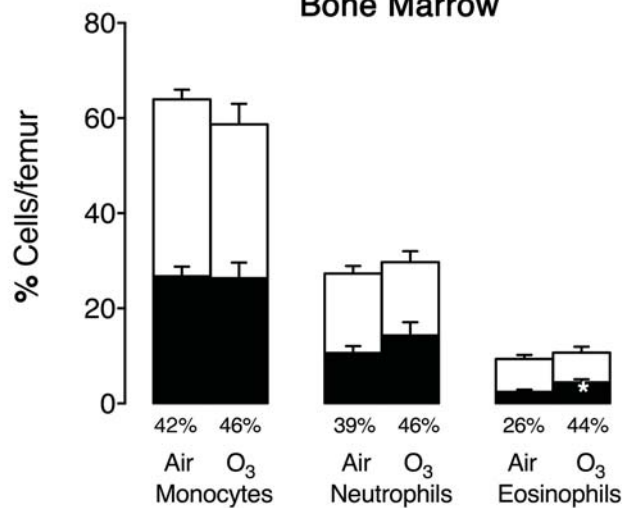
C. Brochoalveolar Lavage

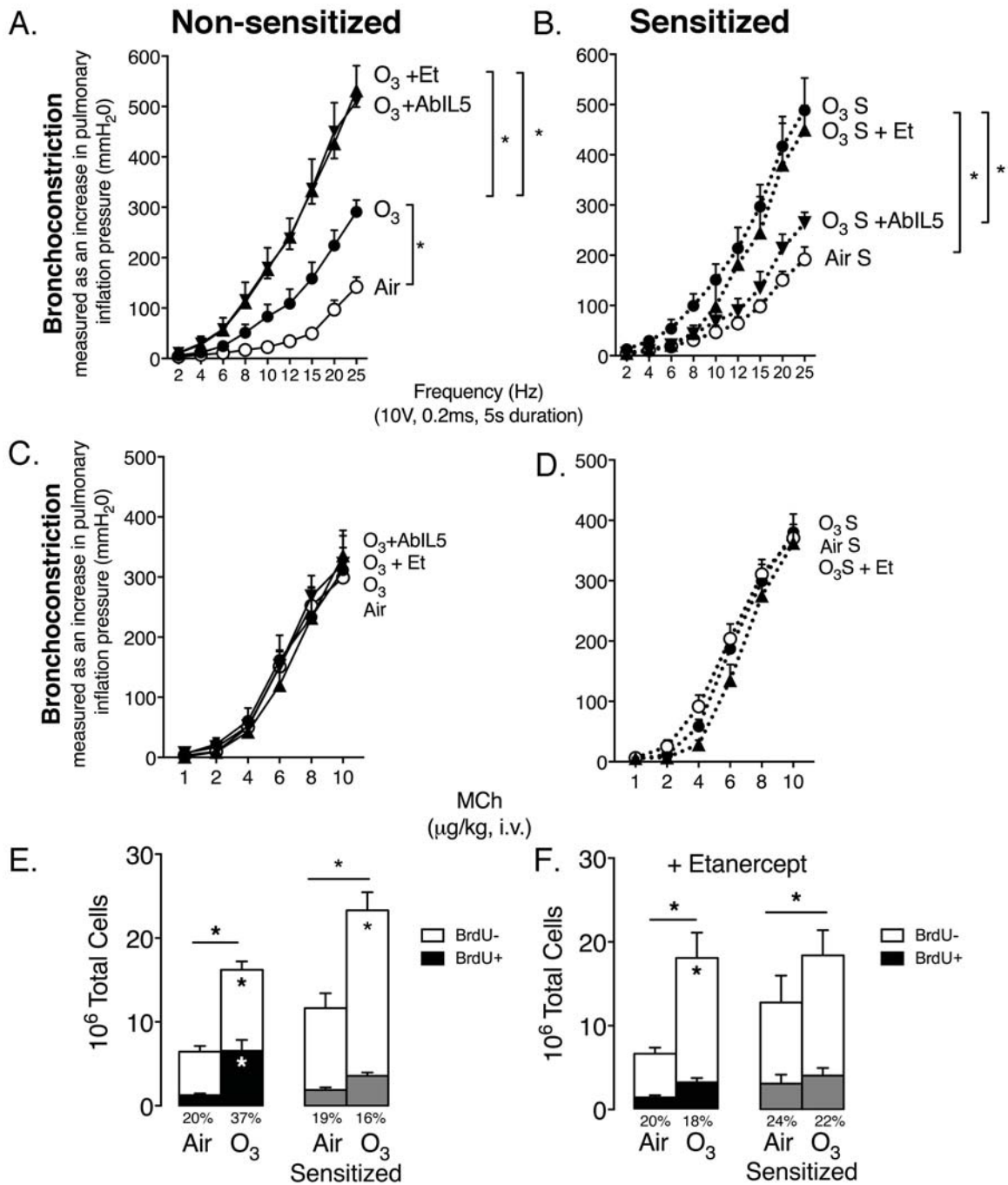


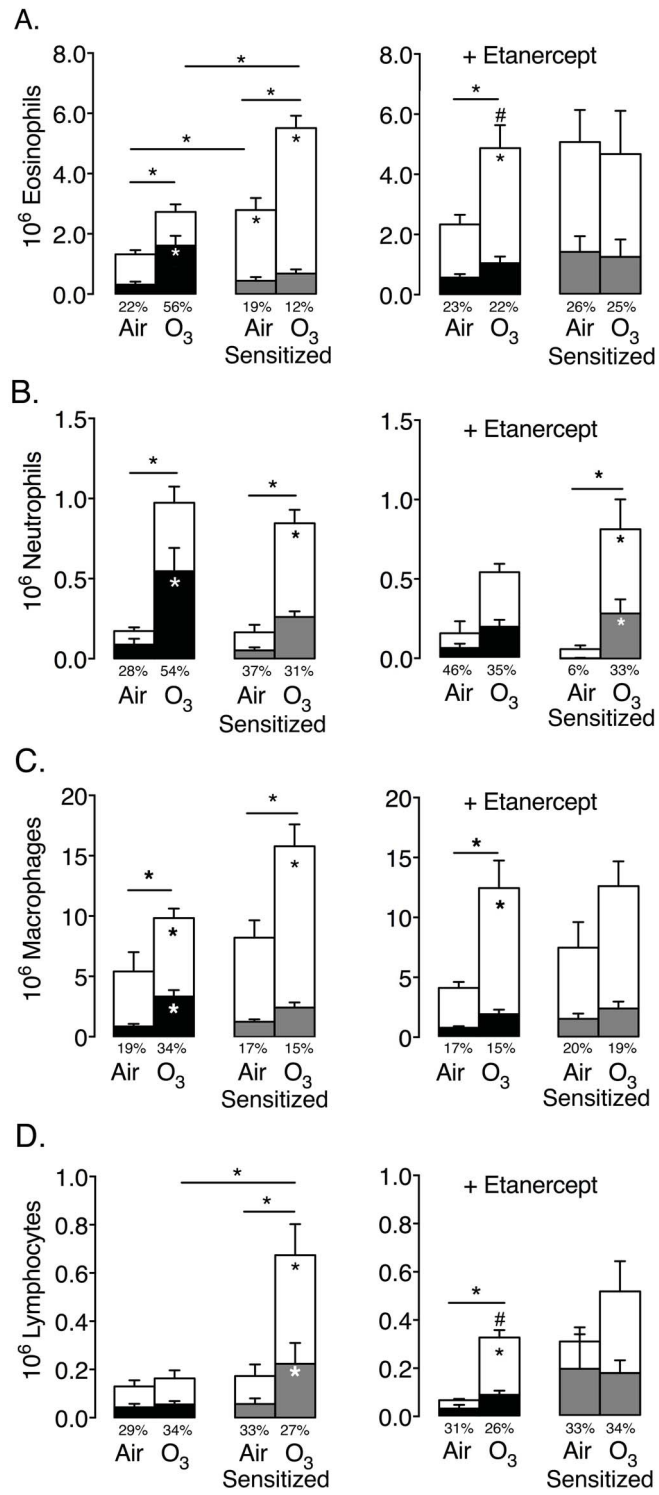
B. Methacholine-induced Bronchoconstriction



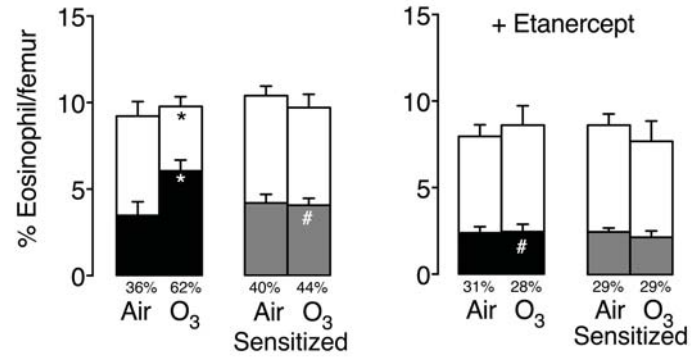
D. Bone Marrow



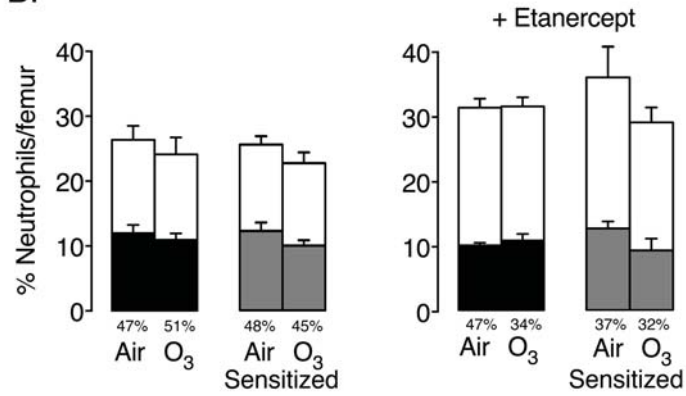




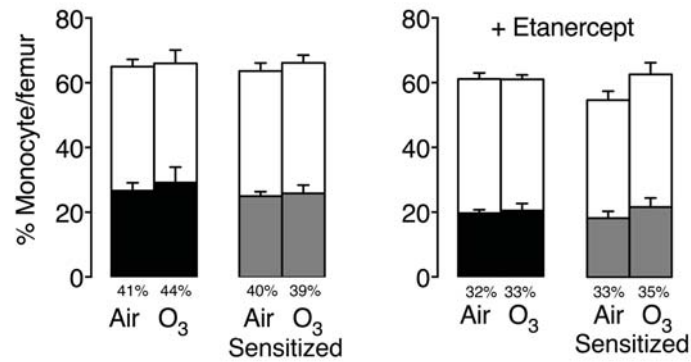
A.



B.

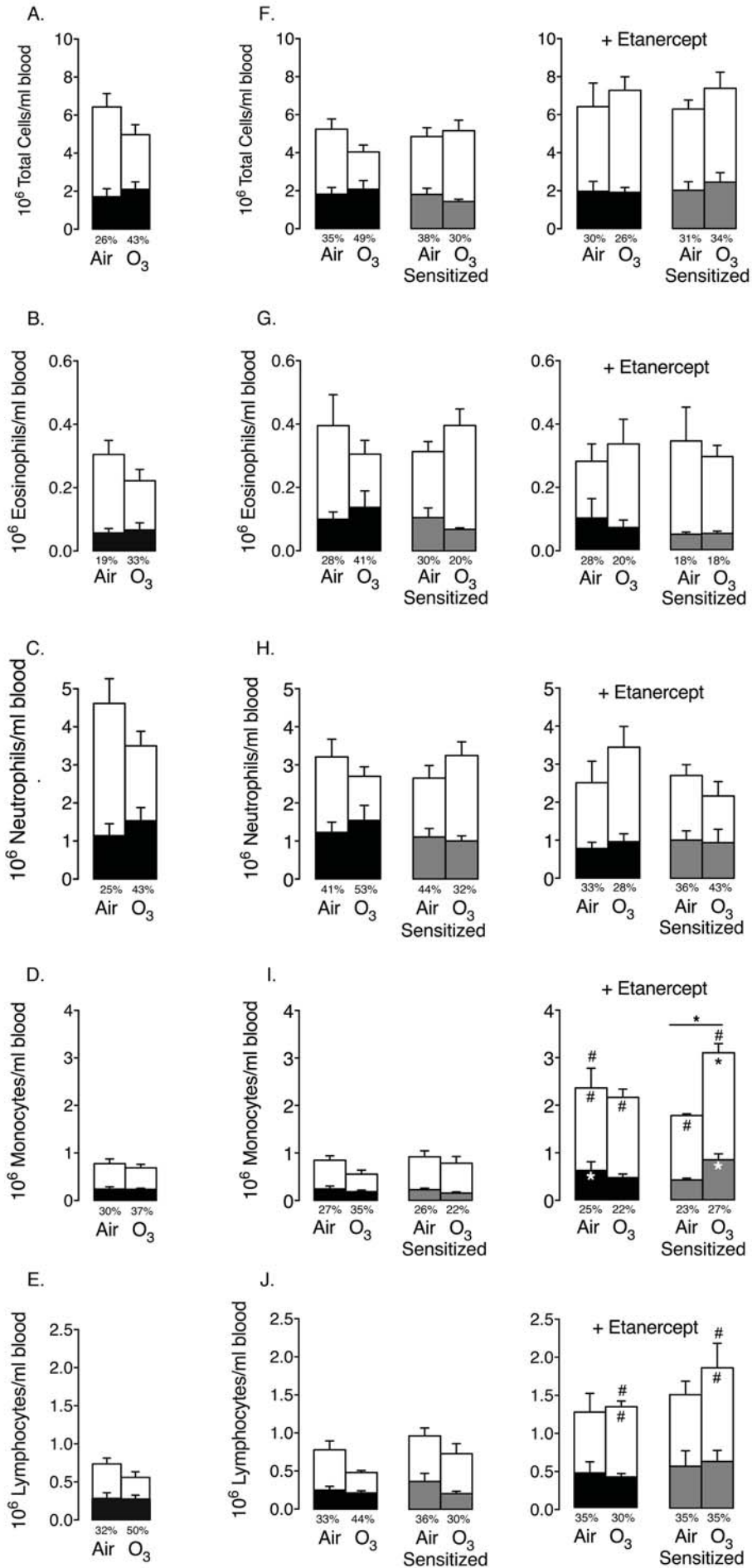


C.

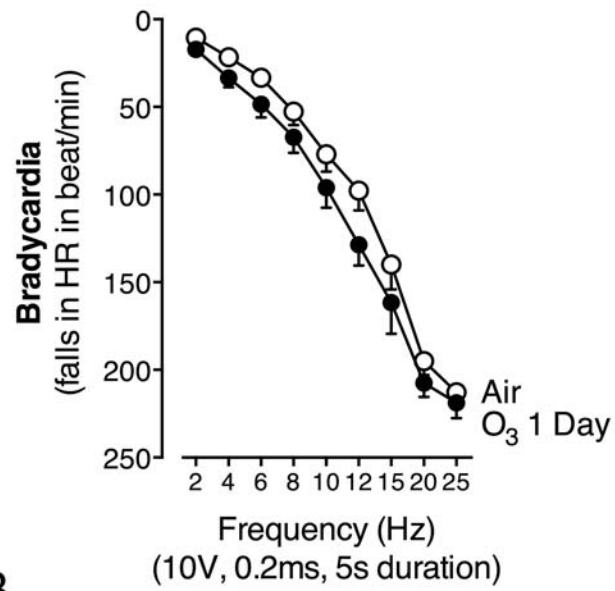


One Day Post Ozone

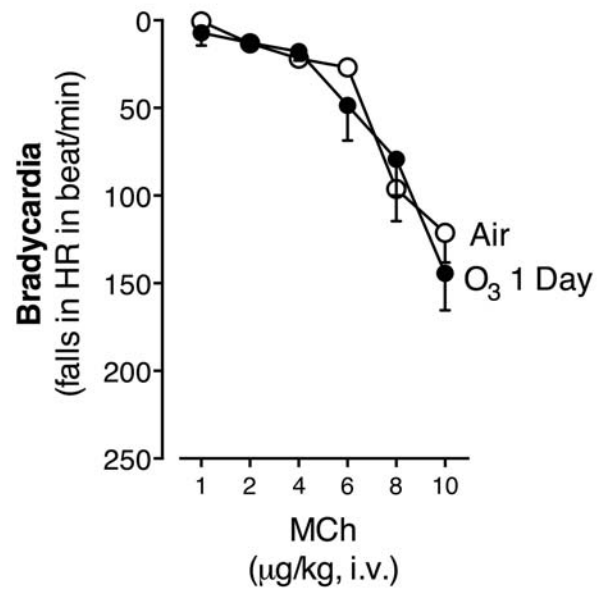
Three Days Post Ozone

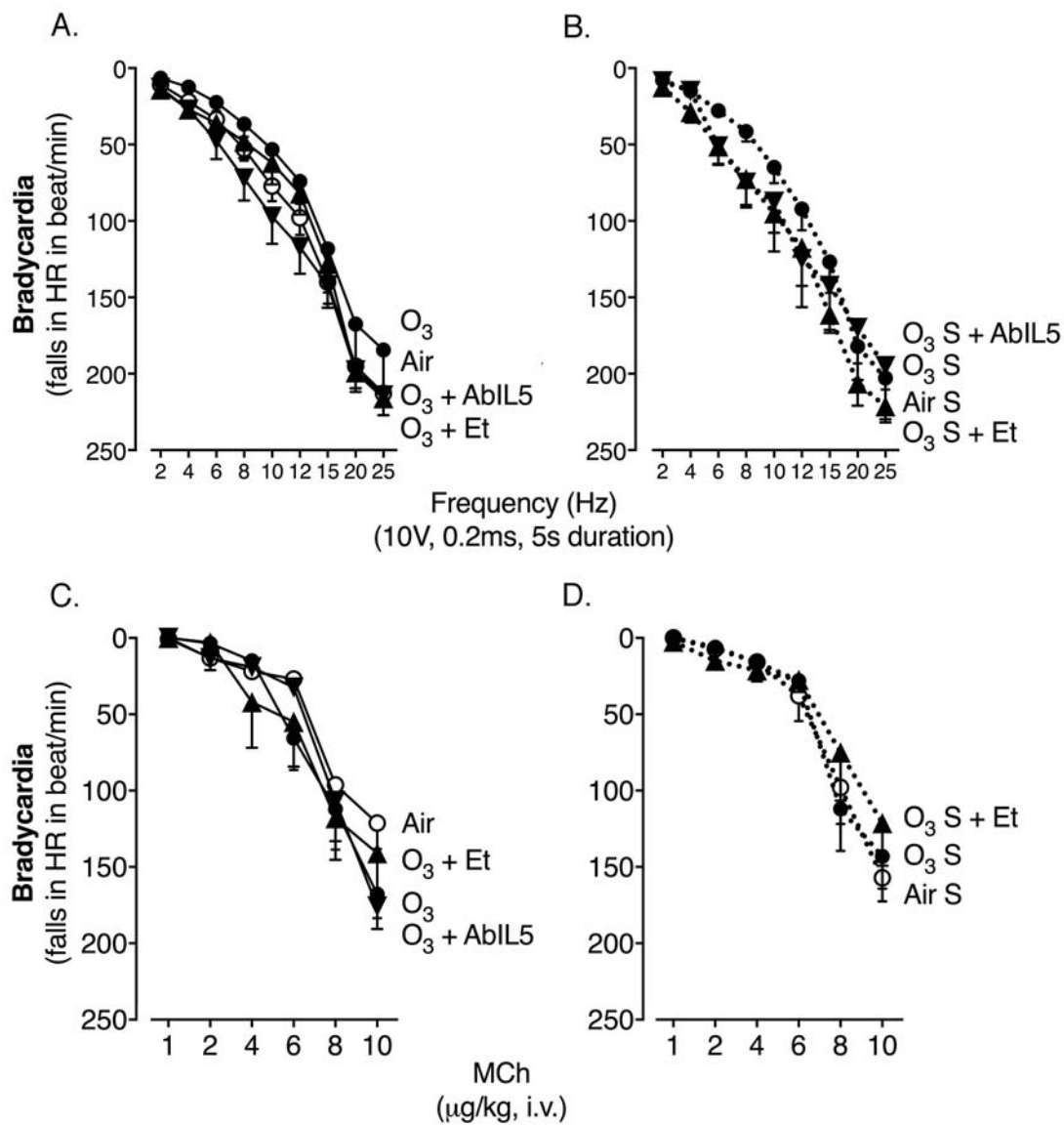


A.



B.





10⁶ Inflammatory cells

Air

O₃ 3d

O₃ 3d AbIL5

Air S

O₃ 3d

O₃ 3d S AbIL5

