OZONE INDUCED AIRWAY HYPERREACTIVITY AND EOSINOPHIL HEMATOPOIESIS DEPENDS ON TUMOR NECROSIS FACTOR-ALPHA AND SENSITIZATION STATUS IN GUINEA PIGS

By

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A DISSERTATION

Presented to the Department of Physiology and Pharmacology
&
Oregon Health & Science University School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

January 2017

School of Medicine

Oregon Health & Science University

CERTIFICIATE OF APPROVAL

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AKNOLWEDGEMENTS

First of all, I would like to thank my mentor Dr. Allison Fryer for her guidance, support, advice, and encouragement during this process. Thank you for pushing me past my own boundaries and encouraging me to grow into a better scientist. I am a stronger writer, presenter, and scientist because of you. Thank you for leading by example regarding how to have a full well rounded life and encouraging me to embrace science, but still live fully and enjoy the arts. Thank you for fostering a lab environment that was like a family to me.

To Dr. David Jacoby thank you for your mentorship, insight, and advice. I always appreciated your ability to put both scientific questions and results into perspective. Thank you for fostering a lab environment that was like a family to me.

To my all the members of my Dissertation Advisory Committee, Dr. Virginia Brooks, Dr. Marielle Gold, Dr. Ellison, and Dr. Giruad. Thank you for your time, support, suggestions and feedback. You have helped me consider different possibilities, explanations, and methods I am a stronger scientist because of you. Thank you for your advice and mentorship.

To Dr. Jackie Wirz thank you for your all of your support and encouragement during my time as a graduate student. Thank you for all of your help navigating and preparing my post doctoral application and interview. Thank you for advocating for myself and

my fellow students we appreciate you being our advocate and voice. I truly appreciate all of your advice about life, grad school, and careers. Thank you for talking me through the stress of writing and defending this dissertation. I will miss your perspective, humor, and enthusiasm.

To the members of the Fryer & Jacoby Laboratory: Dr. Becky Proskocil-Chen, Dr. Jane Nie, Dr. Matt Drake, Emily Blum, Quinn Roth-Carter, Katie Lebold, Dr. Breda Marsh Dr. Greg Scott, and Dr. Abby Rynko. You are a wonderful group of people who made coming to lab everyday a joy regardless of how experiments were working. I cherish all of you. Thank you for your help with *in vivo's*, insight, scientific discussions, editing drafts of manuscripts, and lab adventures. I will miss you all very much!

Finally, I would like to thank my friends and family. To my classmates of PMCB 2011 thank you for adventures, coffee runs, scientific feedback, friendship I could not imagine this journey with out any of you. To my Kappa Alpha Theta Sorority sisters Kelly and Charisa thank you for your support and reminding me to have a life outside of the lab. To my family, my sister Emily and brother in law Isaac, my parents Anne and Edward, thank you all for your encouragement and support.

Thank You All!

Sarah Wicher

PUBLICATIONS

Manuscripts

1. **Wicher S.A**., Jacoby, D.B, Fryer, A.D., Newly Divided Eosinophils Inhibit Ozone-Induced Airway Hyperreactivity In Non-Sensitized Guinea Pigs. Am J Physiol Lung Cell Mol Physiol. (Revisions).
2. Fryer A.D, Jacoby D.B. **Wicher, S.A.**, Protective Role of Eosinophils and TNFα After Ozone Inhalation. Health Effects Institute. *(proofs)*.

Abstracts

1. **Wicher SA**, Jacoby DB, Fryer AD (2016). Recruitment of Eosinophils to Lungs and Their Role in Vagally Mediated Airway Hyperreactivity Depends upon TNFa and Sensitization Status. American Physiological Society, Experimental Biology, San Diego, CA

2. **Wicher SA**, Jacoby DB, Fryer AD (2015) Tumor Necrosis Factor (TNF) Mediates Hematopoiesis of Beneficial Eosinophils Three Days After Ozone. International Eosinophil Society, July 2015 Chicago, Illinois, USA.

3. **Wicher SA**, Jacoby DB, Fryer AD (2013) Ozone induced airway hyperreactivity is exacerbated by blockade of eosinophil recruitment into lungs. Submitted to meeting of International Eosinophil Society, July 2013 Oxford, England.

ABSTRACT

Ozone is an environmental component of air pollution. It is a reactive oxygen species that acutely damages airway epithelium, increasing inflammatory cytokines, and neutrophil and eosinophil recruitment to lungs. In humans, ozone inhalation induces airway hyperreactivity often developing one to three days after exposure. In guinea pigs, ozone inhalation similarly causes lung inflammation and airway hyperreactivity lasting at least three days. One day after ozone, eosinophils mediate ozone-induced airway hyperreactivity, since depleting eosinophils or the eosinophil granule protein major basic protein prevents ozone-induced airway hyperreactivity. However, eosinophils move into the lungs at two distinct time points, with a second wave occurring three days after ozone. In contrast to one day, three days after ozone depleting eosinophils not only fails to prevent ozone-induced airway hyperreactivity, but significantly increases it. Thus, three days after ozone the role of eosinophils has changed from deleterious to protective. The overall hypothesis tested in this dissertation is that ozone inhalation induces eosinophil hematopoiesis in bone marrow, and that newly divided eosinophils will ameliorate airway hyperreactivity three days later in non-sensitized and sensitized animals.

To measure ozone-induced inflammatory cell hematopoiesis, non-sensitized and sensitized guinea pigs, were treated with 5-bromo-2-deoxyuridine and exposed to air or ozone for 4 hours. One or three days later, lung inflation pressure, heart rate, vagally-induced and methacholine-induced bronchoconstriction was measured and

inflammatory cells harvested from bone marrow, blood, and bronchoalveolar lavage. Some animals were pretreated with an antibody to IL-5 or etanercept, to test whether depletion of eosinophils of blockade of TNFα altered ozone induced airway hyperreactivity.

In non-sensitized guinea pigs, ozone causes airway hyperreactivity lasting at least three days, and induces eosinophil hematopoiesis and recruitment to lungs, where three days later, newly divided eosinophils are protective (Chapter III). However, in sensitized guinea pigs, ozone-induced eosinophil hematopoiesis fails to occur, and in the absence of protective, newly divided eosinophils, mature BrdU- eosinophils mediate ozone-induced airway hyperreactivity (Chapter IV). In non-sensitized guinea pigs, ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided eosinophils to lungs is mediated by TNFα, while in sensitized guinea pigs, TNFα does not play a role in ozone-induced eosinophil hematopoiesis (Chapter V).

Ozone induces asthma exacerbations and inflammation in the lungs. My findings show that eosinophils in the lungs after ozone exposure are heterogeneous, and that the presence of eosinophils alone is not sufficient to predict their role in airway hyperreactivity. The presence of multiple eosinophil populations in the lungs with distinct functions, suggest that depleting all eosinophils may not improve asthma exacerbations, and might even explain the heterogeneous clinical responses to eosinophil depletion to via anti-IL-5 therapies. Furthermore, my data demonstrate that

newly divided, BrdU+ eosinophils have a beneficial role. Thus, in asthmatics, therapies directed at protection of or expansion of BrdU+ eosinophils may provide novel mechanisms to prevent or reduce asthma exacerbations following ozone exposure.

In summary, this dissertation demonstrates that ozone induces eosinophil hematopoiesis one and three days after ozone in non-sensitized guinea pigs. These newly divided, eosinophils are protective in the lungs three days after ozone. However, sensitization prevents the ozone-induced increase in eosinophil hematopoiesis and subsequent recruitment of beneficial newly divided eosinophils to the 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. In non-sensitized guinea pigs, TNFα mediates ozone-induced eosinophil hematopoiesis and recruitment of newly divided eosinophils to the lungs. However, in sensitized guinea pigs, TNFα does not play a role in ozone-induced eosinophil hematopoiesis. These data are the first to demonstrate that newly divided eosinophils are protective in the lungs three days after ozone.

CHAPTER I.

INTRODUCTION

INTRODUCTION

Over 50 percent of Americans live in areas with unhealthy levels of ozone. Air pollution including ozone damages the lungs and the work in this dissertation describes the inflammatory response to ozone in the lungs.

A. AIRWAY ANATOMY

The lungs' primary function is respiration. In mammals, inhaled atmospheric oxygen is delivered via a series of tubes, called airways, to terminal sacs called alveoli. In the alveoli, oxygen rapidly diffuses into the bloodstream and carbon dioxide exchange also occurs, albeit in the opposite direction, from bloodstream into the alveoli. The oxygen is carried by blood to cells in the body and the carbon dioxide is then exhaled (Thompson J.M. 2002, West 2012, West et al 2007). During respiration, irritants such as ozone are inhaled and damage the lungs. This section provides normal structure and function of human lungs.

1. Anatomy of the Lungs

The respiratory system is comprised of two parts. The first part is the conducting zone, which is comprised of airways that transport air to the distal lung. The second part is the respiratory zone where gas exchange occurs (Gartner 2015, West 2012). The conducting airways listed in order from external to internal include the nasal cavity, mouth, nasopharynx, pharynx, larynx, trachea, primary bronchi, secondary bronchi,

and tertiary bronchi, bronchioles and terminal bronchioles (Gartner 2015, West 2012). The conducting airways are a system of branching tubes. With each branch, individual airway luminal diameters decrease. However, the total cross-sectional area of the respiratory system increases due to the exponential increase in airway number, and thus decreases flow velocity as air moves distally in the lungs (Gartner 2015, West 2012). The first branch occurs as the trachea divides into the right and left bronchi, and bronchi continue to divide until they reach the terminal bronchioles. The respiratory zone begins as terminal bronchioles divide into respiratory bronchioles, which have occasional alveoli budding from their walls. Respiratory bronchioles lead to alveolar ducts, which are completely lined with alveoli (West 2012). This alveolated region, where gas exchange occurs, comprises the majority of the lung (Figure 1.1) (West 2012).

The trachea is reinforced by 10-12 horseshoe-shaped cartilage rings that prevent the trachea from collapsing. Bronchi also contain cartilage, although it decreases in content and organization with each generation of bronchi, from rings to cartilage plates in the secondary bronchi. There is no cartilage in the respiratory bronchioles (Gartner 2015, West 2012). On the posterior side of the trachea, smooth muscle is attached to the horseshoe shaped cartilage rings (Amrani & Panettieri 2003). Tracheal smooth muscle runs in longitudinal and horizontal bands. In bronchi and bronchioles, smooth muscle encircles the airways between the cartilage and basement membrane (Amrani & Panettieri 2003, James & Carroll 2000). Contraction of airway smooth

muscle narrows the airway due to the spiraling layers of smooth muscle (Amrani & Panettieri 2003, James & Carroll 2000).

Epithelial cells line the conducting airways in the lung, providing a physical line of defense against environmental damage and infection. The epithelial layer in the trachea and bronchi is separated from the rest of the lung by the lamina propria. It is comprised of basal cells, goblet cells, and pseudostratified ciliated columnar epithelial cells (Gartner 2015, Grainge & Davies 2013). Basal cells are pluripotent stem cells that replenish both pseudostratified ciliated columnar epithelial cells and goblet cells. Goblet cells are located adjacent to the lamina propria (Gartner 2015, Grainge & Davies 2013, Leeson Thomas Sydney 1988). Goblet cells and submucosal glands release mucus, which trap inhaled particulate matter and microbes. Debris laden mucus is swept out of the lungs by beating cilia on epithelial cells (Corren J 2003, Thompson J.M. 2002).

In small bronchioles, the epithelium contains stem cells, called club cells, that replicate to repair damaged epithelium (Reynolds & Malkinson 2010). Additionally, club cells secrete surfactant, which reduces surface tension in the alveoli and prevents alveolar collapse (Reynolds & Malkinson 2010, West 2012). Alveolar epithelium is comprised of type I and type II alveolar cells. Type I alveolar cells are squamous epithelial cells that cover 95% of the alveolar surface. Type I alveolar cells form tight junctions which prevent extracellular fluid from seeping into the alveolar lumen

(Gartner 2015). Type II alveolar cells are located adjacent to alveoli and secrete surfactant into the alveolar lumen (Gartner 2015).

In addition to their barrier function, lung epithelial cells regulate inflammation, immunity, and wound repair. Airway epithelial cells produce cytokines and chemokines, which recruit and activate inflammatory cells and initiate repair of damaged epithelium (Bals & Hiemstra 2004, Bosson et al 2003, Grainge & Davies 2013, Schleimer et al 2007, Song et al 2011). Additionally, airway epithelial cells also express adhesion molecules, including intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) on their basement membrane. Adhesion molecules mediate trans-epithelial migration of inflammatory cells from lung into the airway lumen (Bloemen et al 1993, Sato et al 1997, Wong et al 2006). Airway epithelial cells also express immune receptors, including IL-1, tumor necrosis factor, and toll-like receptors (Bals & Hiemstra 2004, Ioannidis et al 2013, Parker & Prince 2011), and produce antimicrobial peptides and proteins as part of the lungs' immune defense. Damage to epithelium can disrupt lung physiology, increase proinflammatory cytokines and adhesion molecules that in turn facilitate inflammatory cell recruitment into tissue and epithelial repair (Arsalane et al 1995, Dokic et al 2011).

FIGURE 1.1. Airway Generations

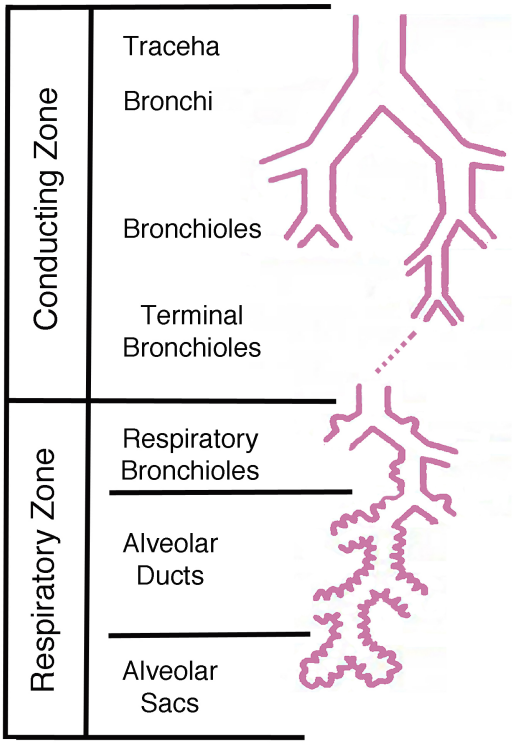


Figure 1.1. Inhaled air flows into the nose or mouth and into the lower airways of mammalian lungs. Air moves down the trachea, which divides into the right and left primary bronchi. The primary bronchi divide into bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts, and alveolar sacs. The conducting airways include the trachea, bronchi, bronchioles, and terminal bronchioles. The respiratory zone is the site of gas transfer in the lungs, and includes respiratory bronchioles, alveolar ducts, and alveolar sacs. Figure adapted from (West 2012).

2. Lung Blood Supply

Deoxygenated blood flows from the body into the right atrium of the heart and then into the right ventricle where it is pumped into the right and left pulmonary arteries. Pulmonary arteries branch into a dense capillary network in the walls of the alveoli where oxygen and carbon dioxide are exchanged. Pulmonary veins then return freshly oxygenated blood to the left atrium of the heart, which is then pumped into systemic circulation by the left ventricle via the aorta. Bronchial arteries branch from the aorta and supply oxygenated blood to the lung tissue. The blood that entered the lungs via the bronchial arteries is returned to the heart via pulmonary and bronchial veins (Leeson Thomas Sydney 1988, West 2012).

B. AIRWAY INNERVATION

Introduction

Nerves transmit information between cells that can be close together, i.e. 2 nerves, or distant, including between the central nervous system and end organs. Nerve cells are neurons and are comprised of a cell body, short broad-based protrusions called dendrites, and long thin projections called axons. Information is carried down dendrites and axons via membrane channels that open and close in response to changes in membrane potential, and transmit information between cells via release of chemicals called neurotransmitters from axons (Thompson J.M. 2002). When two neurons contact each other, the site of contact is referred to as a synapse, whereas when a nerve contacts a target cell in tissue, the site of contact is referred to as a junction (Sherrington 1987, Tansey 1997).

The lungs detect stimuli and relay information from the bronchopulmonary system to the central nervous system via afferent sensory nerves. Smooth muscle and glands within the lungs receive information from the CNS via efferent parasympathetic and sympathetic autonomic nerves.

1. The vagus nerve

The vagus nerve carries all of the parasympathetic innervation and most of the sensory innervation to the lungs (Belvisi 2002). Vagal branches, called the superior laryngeal and the recurrent laryngeal nerves, innervate the trachea. Smaller branches of the

vagus innervate the smaller airways (Belvisi 2002, Richardson 1979). The vagus nerve also innervates the heart, ureters, esophagus, stomach, and intestinal tract (Guyton 2000).

The majority of sensory nerves that innervate the lung originate in the nodose and jugular vagal ganglia and the rest originate from dorsal root ganglia (Carr & Undem 2003, Richardson 1979). Afferent fibers project to the nucleus tractus solitarius in the brain and relay signals to the efferent nerves via the central vagal reflex (Kalia & Mesulam 1980, Kummer et al 1992). Sensory reflexes include cough, mucus secretion, smooth muscle tone, and bronchoconstriction.

FIGURE 1.2. Autonomic Neurons Structure and Synapse

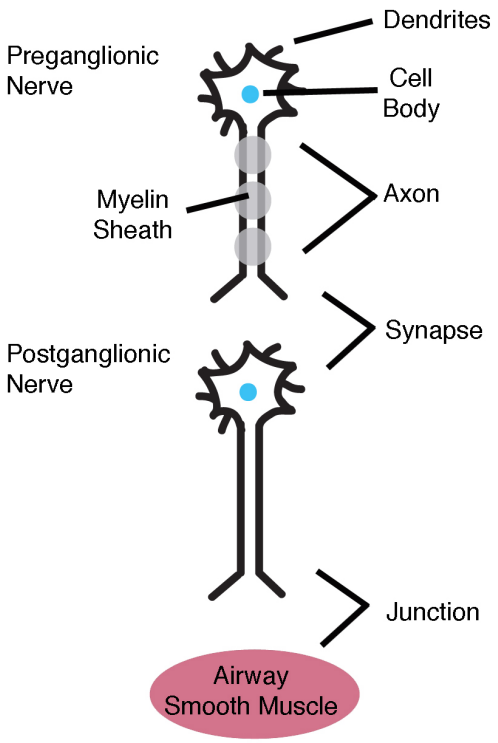


Figure 1.2. Structural features of neurons, synapses and junctions. Myelinated preganglionic nerves originate in the central nervous system and project axons that synapse with postganglionic nerves. Signals are propagated from the preganglionic nerve to the postganglionic nerve via release of neurotransmitters at the synapse. Postganglionic nerves are unmyelinated and the cell bodies are located outside of the central nervous system. Axons of postganglionic nerves extend to target tissues and form neuronal junctions. Based on terminology from (Sherrington 1987) figure adapted from (Rynko 2013).

2. Sensory Nerves

In the lungs, sensory nerves innervate airway epithelium, airway smooth muscle, blood vessels, airway ganglia, alveolar walls, airway epithelium, and submucosa (Brouns et al 2006, Kummer et al 1992, Lundberg et al 1984, Luts et al 1993). Sensory nerves in the lung detect stimuli including temperature, pH, ozone, particles, smoke, allergens, and mechanical stretch (Bevan & Yeats 1991, Green et al 1984, Kuo et al 1992, Ni & Lee 2008, Schelegle & Walby 2012). They can be classified into two main categories based on their structure and signal transduction speed. Sensory Aδ fibers are myelinated and thus rapidly conduct signals along their axons. They express rapidly adapting stretch receptors, which respond to mechanical stretch in the lung (Carr & Undem 2003, Sant'Ambrogio & Widdicombe 2001). In contrast, C-fibers are unmyelinated and thus conduct signals more slowly. C- fibers respond to inflammatory mediators and noxious stimuli (Belvisi 2002). The majority of both Aδ and C-fibers originate in the nodose and jugular vagal ganglia located near the upper end of the vagus nerve, right outside the CNS. A smaller portion of sensory nerves supplying the lung originate from dorsal root ganglia along the spinal column (Carr & Undem 2003, Richardson 1979).

Sensory nerves can signal locally in the lung through release of tachykinins, substance P, vasoactive intestinal protein, calcitonin-gene related protein and nuerokinin A (Carr & Undem 2003). These neurotransmitters have local effects mediated by nuerokinin

receptors including smooth muscle contraction and immune cell activation (Canning et al 2002, Groneberg et al 2006)

Sensory nerves also signal centrally via a reflex arc through the nucleus tractus solitaries in the central nervous system and then activation of efferent parasympathetic nerves in the vagus (Kalia & Mesulam 1980, Kummer et al 1992). Activation of the sensory reflex arc can trigger cough, increased mucus secretion, increased smooth muscle tone, and bronchoconstriction (Green et al 1984, Mazzone & Undem 2016, Sant'Ambrogio & Widdicombe 2001).

3. Parasympathetic Nerves

Parasympathetic nerves are efferent autonomic nerves that originate from the nucleus ambiguous of the solitary tract in the brainstem (Guyton 2000, Kalia 1981). Axons of preganglionic parasympathetic nerves travel within the vagus nerve and synapse onto postganglionic parasympathetic nerves whose cell bodies are located in the trachea and bronchi (Canning & Fischer 1997, Myers 2001, Richardson & Ferguson 1979). Postganglionic parasympathetic nerves project to submucosal glands, airway smooth muscle, and blood vessels (Adamko et al 1999, Baker 1986, Canning & Fischer 1997, Knight et al 1981). Both preganglionic and post-ganglionic parasympathetic nerves release the neurotransmitter acetylcholine. In the ganglia, synaptic transmission is mediated by activation of nicotinic receptors, and modified by neuronal M_1 and M_2 muscarinic receptors. Acetylcholine released from post-ganglionic nerve junctions

activates muscarinic receptors on target tissues to cause smooth muscle contraction and mucus secretion (Figure 1.2) (Belvisi 2002, Nadel & Barnes 1984).

Airway smooth muscle tone is primarily controlled by parasympathetic nerves in both humans and animals (Boushey et al 1980, Nadel 1977, Nadel & Barnes 1984). Airway smooth muscle contraction is induced through acetylcholine release. Tonic release of acetylcholine produces partial contraction of airway smooth muscle at baseline (Widdicombe 1963). This baseline contraction of airway smooth muscle is called tone (Kesler & Canning 1999). Airway tone is regulated in the conducting airways, but not the respiratory zone, due to a decrease of parasympathetic innervation of respiratory bronchioles and alveoli (El-Bermani & Grant 1975, Nadel 1977).

Increased firing of parasympathetic nerves leads to increased release of acetylcholine. Acetylcholine activates M_3 muscarinic receptors on airway smooth muscle (Kesler & Canning 1999, Nadel & Barnes 1984, O'Connor et al 1996) to induce smooth muscle contraction and bronchoconstriction. Bronchoconstriction can also be induced pharmacologically with intravenous or inhaled acetylcholine or other M_3 agonists, such as methacholine (Gambone et al 1994, Wagner & Jacoby 1999). Bronchoconstriction can also be prevented pharmacologically with muscarinic antagonists (O'Connor et al 1996).

FIGURE 1.3 Airway innervation

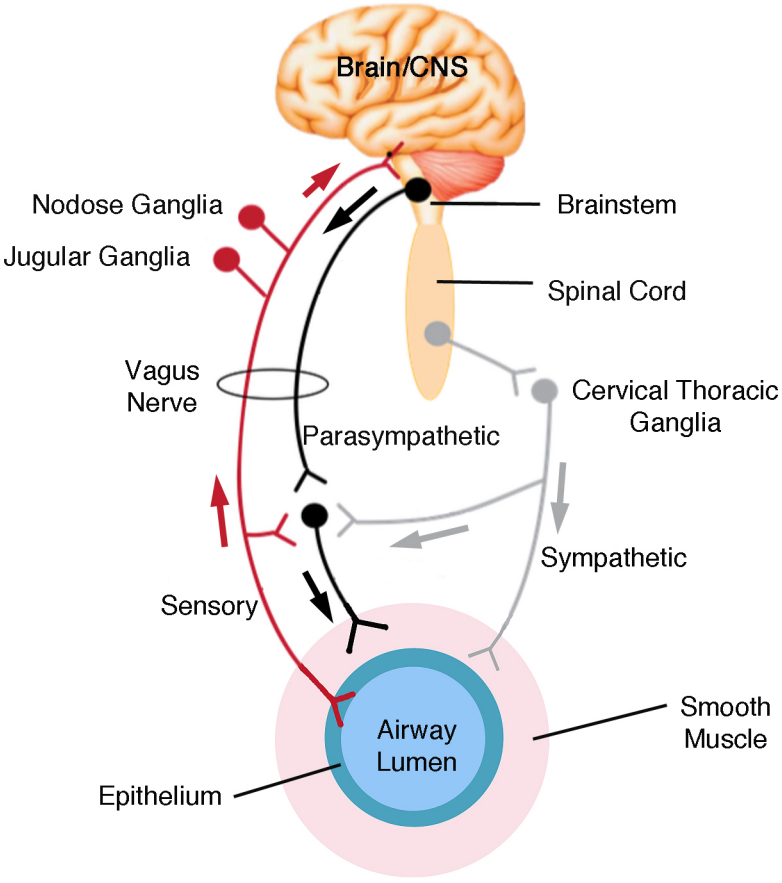


Figure 1.3. Parasympathetic nerves (black) and sensory nerves (red) travel in the vagus nerve. Afferent sensory nerves transmit information from the airways to the nodose and jugular ganglia and then to the central nervous system. Efferent parasympathetic nerves (black) transmit information down the vagus nerve from the central nervous system to the airways. Sympathetic nerves (grey) do not innervate human airway smooth muscle, but do innervate guinea pig airway smooth muscle. Figure adapted from (Verhein 2010).

FIGURE 1.4. Inhibitory M2 muscarinic receptors on parasympathetic nerves

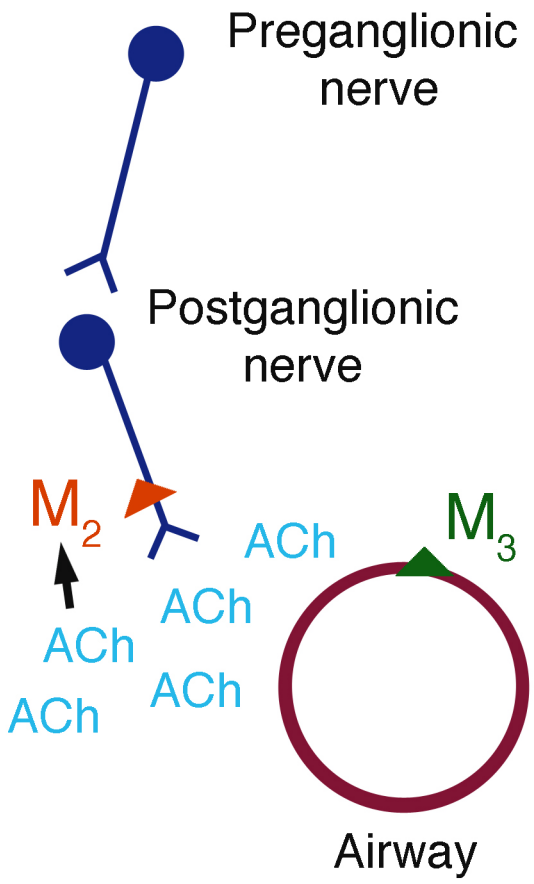


Figure 1.4. Action potentials are transmitted from preganglionic parasympathetic nerves to postganglionic parasympathetic nerves, which release acetylcholine that binds to M_3 muscarinic receptors, causing smooth muscle contraction. Acetylcholine also binds to M_2 muscarinic receptors located on parasympathetic nerves inhibiting further acetylcholine release and bronchoconstriction.

4. Sympathetic Nerves

Sympathetic nerves supplying the lungs originate from the upper six thoracic segments of the spinal cord and synapse onto postganglionic nerves located in cervical thoracic ganglia (Thompson J.M. 2002). In human lungs, sympathetic nerves innervate submucosal glands, parasympathetic nerves, and bronchial blood vessels (Kummer et al 1992), but not airway smooth muscle (Belvisi 2002, Richardson & Beland 1976, Richardson 1979). Thus, sympathetic nerve contribution to regulating airway tone in humans is negligible. It is more likely that they regulate parasympathetic nerves to decrease airway contraction indirectly. For example, activation of β_1 receptors on parasympathetic nerves can also cause bronchodilation by decreasing parasympathetic acetylcholine release (Panettieri 2015). However, in animals including guinea pigs, sympathetic innervation of airway smooth muscle can induce bronchodilation (Ainsworth et al 1982, Canning 2003, Kummer et al 1992). Sympathetic nerves release norepinephrine, which binds to α_1 β_1 and β_2 adrenoreceptors in the lung (Spina et al 1989a, Spina et al 1989b, Thompson J.M. 2002). Activation of β_2 receptors causes smooth muscle relaxation and, thus, bronchodilation (Persson & Gustafsson 1986).

5. Non-Adrenergic Non-cholinergic nerves

Non-adrenergic non-cholinergic (NANC) nerves are sensory nerves that originate in the esophagus and innervate airway smooth muscle (Belvisi 2002, Canning et al 1996). While the physiological role of these nerves is still unclear, NANC nerves can induce

smooth muscle contraction via release of substance P or nuerokinin A (Krishnakumar et al 2002, Stretton 1991), and bronchodilation through release of nitric oxide and vasoactive intestinal peptide (Belvisi et al 1995, Liu et al 1999, Palmer et al 1986, Ward et al 1995).

C. ACETYLCHOLINE AND RECEPTORS IN THE LUNGS

1.Acetylcholine

Acetylcholine is a key neurotransmitter in both the central nervous system and the peripheral nervous system. Acetylcholine was first characterized in 1914 by Sir Henry Dale (Dale 1914). Additionally, studies by Otto Loewi, in 1926, demonstrated that acetylcholine was released from the vagus nerve. In the lung, acetylcholine induces bronchoconstriction and mucus secretion. Acetylcholine is synthesized in nerves by choline acetyltransferase from choline and acetyl-coenzyme A. Vesicular cholinergic transporter transfers acetylcholine into nerve vesicles for storage (Bowman W.C 1980). Nerve activation causes vesicles to fuse with the cell membrane in the nerve terminal, leading to neurotransmitter release (Bowman W.C 1980). Once released, acetylcholine is rapidly hydrolyzed by acetylcholinesterase and butyrylcholinesterases in blood and surrounding tissues (Bowman W.C 1980, Massoulié et al 1996). Choline is taken back into the neuron through choline transporters (Bowman W.C 1980). Epithelial, endothelial, immune, and smooth muscle cells are also capable of synthesizing and releasing acetylcholine (Kawashima et al 1990, Kirkpatrick et al 2003, Proskocil et al 2004, Rinner et al 1998). However, non-neuronal cells lack the ability to store acetylcholine (Kawashima et al 2012b). Non-neuronal acetylcholine signals in an autocrine or paracrine manner to regulate mucociliary clearance, immune cell activation and cytokine release, and sensory nerve activity (Kawashima & Fujii 2000, Kawashima et al 2012b, Wessler et al 1998).

Acetylcholine receptors are classified as either nicotinic or muscarinic. Muscarinic receptors are found on most cells in the lungs including parasympathetic nerves, airway smooth muscle, epithelial cells, and inflammatory cells. Nicotinic receptors are found on inflammatory cells and parasympathetic ganglia.

2. Nicotinic Receptors

Nicotinic receptors are ligand gated ion channels composed of 5 subunits that form a pore in the membrane. Four subtypes of nicotinic receptor subunits (α, β, γ, and δ) combine to create many different nicotinic receptor isoforms that vary by cell type. Nicotinic receptors are found on parasympathetic nerves, epithelial cells, and a variety of inflammatory cells including macrophages, eosinophils, and neutrophils, parasympathetic nerves, and epithelial cells (Blanchet et al 2007, Iho et al 2003, Li et al 2012, Pena et al 2010, Racke & Matthiesen 2004). Preganglionic parasympathetic nerves relay signals to postganglionic parasympathetic nerves by activating postganglionic nicotinic receptors via release of acetylcholine.

3. Muscarinic Receptors

There are 5 muscarinic receptor subtypes (M_1, M_2, M_3, M_4, M_5) (Caulfield & Birdsall 1998, Peralta et al 1987), and they are expressed on most cell types in the lung. Muscarinic receptors are members of the G-protein coupled receptor family. Similar to other family members, they have an extracellular N-terminus, 7 transmembrane alpha helices and an intracellular C-terminus. The 7 transmembrane alpha helical domains are connected by 3 intracellular and 3 extracellular loops (Bonner et al 1987). The G protein coupling to each muscarinic receptor subtype is determined by the sequence of the 3rd intracellular loop (Peralta et al 1987, Zhang et al 1996). Muscarinic receptors are similar among species (Eglen & Watson 1996, Hall et al 1993). M_1, M_2, M_3, muscarinic receptors are expressed on smooth muscle, immune cells, nerves, glands, and endothelial cells while M_4 and M_5 are expressed on some immune cells (Haddad et al 1996, Kawashima et al 2012a, Mak & Barnes 1990, Wess 2004). All subtypes are found within the brain and CNS.

Pre-junctional M_2 muscarinic receptors limit acetylcholine release from parasympathetic nerves and are found in both humans and animals (Figure 1.4) (Fryer & Maclagan 1984, Minette & Barnes 1988, White 1995). Inflammatory mediators, such as eosinophil major basic protein, cause decreased function of neuronal M_2 muscarinic receptors and limits their ability to inhibit acetylcholine release, subsequently potentiating vagally induced bronchoconstriction (Costello et al 1997, Jacoby et al 1993, Yost et al 1999).

i. G-Protein Signaling

All 5 muscarinic receptor subtypes are coupled to G-proteins. G-proteins are heterotrimers, which include an α, β, and γ subunit. G-proteins are classified by their alpha subunit and are either $G\alpha_i$, $G\alpha_s$, $G\alpha_q$. Ligand binding to the muscarinic receptor causes a conformational change the Gα subunit exchanges GDP for GTP releasing the G protein complex from the receptor and disassociation of the Gα subunit from the βγ dimer. These subunits can then initiate signaling through second messenger systems or associate with ion channels. The Gα subunit then hydrolyses to GTP to GDP and the Gα subunit from the βγ dimer become re-associated with the GPCR.

Stimulation of M_1, M_3, and M_5 muscarinic receptors leads to excitatory responses. M_1, M_3, and M_5 muscarinic receptors are coupled to $G\alpha_q$, which stimulates phospholipase C (PLC). Phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 and DAG release intracellular calcium, which induces smooth muscle contraction (Figure 1.5) (Exton 1996, Roffel et al 1990).

Stimulation of M_2 and M_4 muscarinic receptors leads to inhibitory excitatory responses. M_2 and M_4 receptors are coupled to $G\alpha_i$, which inhibits adenylyl cyclase and decreases cyclic adenosine monophosphate (cAMP) production.

ii. Muscarinic Receptor Distribution in the Lungs

M_1 Muscarinic Receptors

M_1 muscarinic receptors are located on parasympathetic nerves where they enhance neurotransmission (Bloom et al 1987, Yang & Biggs 1991a, Yang & Biggs 1991b). M_1 receptors are also located on mucus glands where they increase mucus secretion (Culp et al 1996, Mak & Barnes 1990). M_1 muscarinic receptors on mast cells prevent release of histamine after antigen challenge (Reinheimer et al 2000). Although M_1 muscarinic receptors are expressed on epithelial cells in the lung, their function is not known (Casale & Ecklund 1988, Mak & Barnes 1990).

FIGURE 1.5. Muscarinic receptor signaling

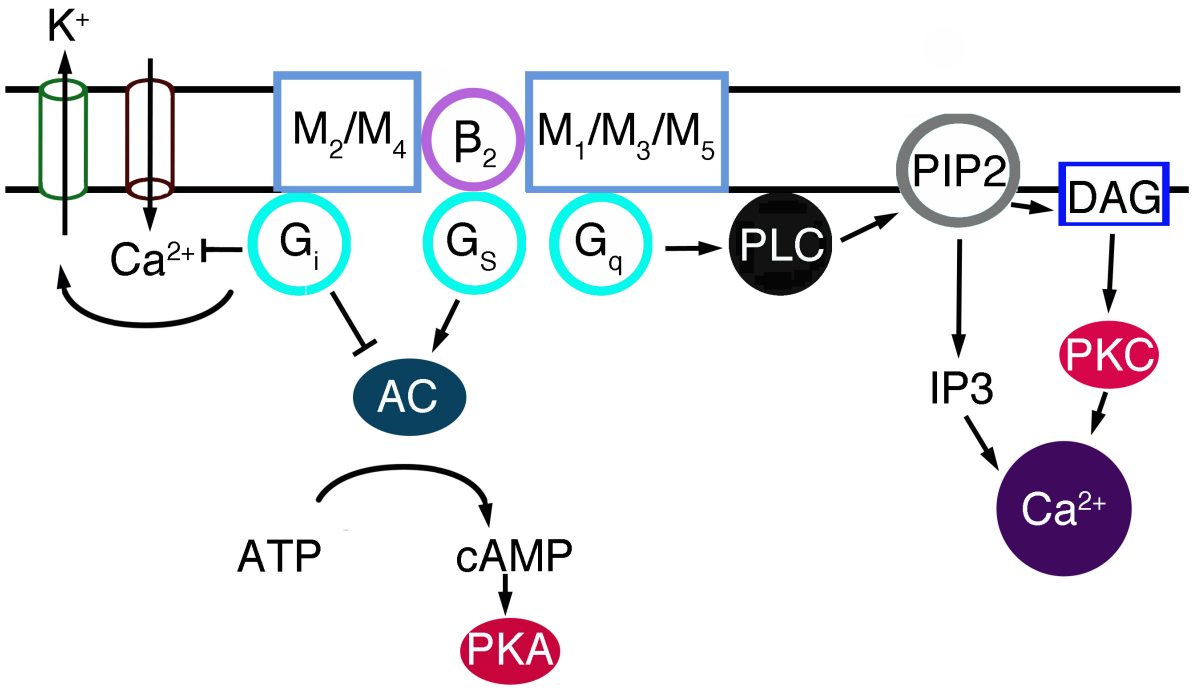


FIGURE 1.5. Muscarinic receptors M1, M3, and M5 are coupled to Gq, which activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), forming inositol 1,4,5 triphosphate (IP3) and diacyl glycerol (DAG). DAG activates protein kinase C (PKC). PKC and IP3 cause release of intracellular calcium and leads to smooth muscle contraction. Muscarinic receptors M2 and M4 are coupled to Gi, which activates potassium channels, inhibits calcium channels and also inhibits adenylate cyclase (AC). B2 receptors are coupled to Gs, which activates adenylate cyclase (AC) and converts ATP to cAMP, which activates protein kinase A (PKA). Figure adapted from (Rynko 2013).

M_2 Muscarinic Receptors

In the lungs, M_2 muscarinic receptors are expressed on airway smooth muscle and on postganglionic parasympathetic nerves (Brown et al 2013, Haddad et al 1991, Walker et al 2004). It is well established, in all species tested, that activating M_2 muscarinic receptors on parasympathetic nerves reduces acetylcholine release (Figure 1.4) (Fryer et al 1996, Fryer & Maclagan 1984), and inhibits vagally mediated bronchoconstriction (Fryer & Maclagan 1984, Minette et al 1989, Wang et al 1995, Yost et al 2005). In contrast, blocking M_2 muscarinic receptors with antagonists results in increased vagally mediated bronchoconstriction since the limits on acetylcholine release have been removed (Baker et al 1992, Fisher et al 2004, Fryer & Maclagan 1984). In animal models, loss of M_2 muscarinic receptor function is responsible for airway hyperactivity following exposure to ozone (Gambone et al 1994, Yost et al 1999, Yost et al 2005) or organophosphate pesticides (Proskocil et al 2010), and viral infection (Adamko et al 1999, Lee et al 2004, Nie et al 2011). Thus, normal M_2 function is critically important for the regulation of bronchoconstriction.

M_2 muscarinic receptor expression on smooth muscle varies by species. M_2 muscarinic receptors comprise 50% of muscarinic receptor subtypes on human airway smooth muscle and 80% on guinea pig airway smooth muscle (Haddad et al 1991, Roffel et al 1988). M_2 muscarinic receptors on airway smooth muscle limit beta agonist induced relaxation of airway smooth muscle by inhibiting adenylate cyclase (Brown et al 2013,

Walker et al 2004), and do not appear to directly contribute to bronchoconstriction (Fisher et al 2004, Struckmann et al 2003).

M_3 Receptors

M_3 muscarinic receptors are expressed on airway smooth muscle and mediate smooth muscle contraction and bronchoconstriction (Fisher et al 2004, Itabashi et al 1991, Jooste et al 2005, Mak & Barnes 1990). In blood vessels, activation of M_3 muscarinic receptors does not contract muscle, but releases nitric oxide that causes vascular smooth muscle relaxation (i.e. vasodilation) (Lung 2011, Orii et al 2010). In airway epithelial cells, M_3 receptor activation increases cilia beat frequency (Salathe et al 1997, Yang & McCaffrey 1996) and increases secretion of mucus and water from submucosal glands (Okayama et al 1993, Ramnarine et al 1996).

D. AIRWAY INFLAMMAITON

Airway inflammation is a major contributor and confounder of many airway diseases, including asthma, viral infection, and ozone. Inflammatory cells are recruited to the lungs, where they release cytokines and chemokines that can increase inflammation, decrease inflammation, and induce repair. Inflammatory cells will be discussed with an emphasis on the cells that comprise lung inflammation.

1. Macrophages

Macrophages are the dominant resident inflammatory cell in the lungs. Macrophages have many roles including phagocytosis, inflammation, and repair (Murphy 2008). Macrophages provide the first line of defense against environmental insults and infectious agents. Macrophages are phagocytic cells that engulf apoptotic cells, debris, bacteria, and viruses, and induce immune responses through release of cytokines and chemokines that activate and recruit other immune cells (Byers & Holtzman 2011, Murphy 2008). Activated macrophages initiate and sustain inflammation by releasing large amounts of cytokines including TNFα, IL-1β , and IL-12 (Fakhrzadeh et al 2004, Martinez & Gordon 2014).

Alternatively, macrophages can mediate tissue repair by releasing large amounts of IL-10, and IL-1 receptor alpha to reduce inflammation (Byers & Holtzman 2011, Fakhrzadeh et al 2004, Martinez & Gordon 2014, Mosser & Zhang 2008).

Macrophages are also involved in airway remodeling; they release growth factors such as transforming growth factor beta or platelet-derived growth factor that induce fibrosis (Kovacs & DiPietro 1994, Vignola et al 1996).

Macrophages in bronchoalveolar lavage of patients with asthma have increased activation (Borish et al 1992, Tonnel et al 1983, Vignola et al 1996). In guinea pig models of asthma, macrophage depletion prevents organophosphate-induced and virus-induced airway hyperreactivity (Lee et al 2004, Proskocil et al 2013). Thus, macrophages play an important role in inflammatory responses in the lung.

2. Neutrophils

While neutrophils are abundant in blood, they are not normally found in healthy lung (Murphy 2008). However, neutrophils are recruited to the airways during infection, and following exposure to environmental irritants including ozone (Fahy et al 1995, Holz et al 1999, Kim et al 2011, Koren et al 1989). Neutrophils are recruited to the lungs by IL-8 (Gosset et al 1997, Schelegle et al 1991). Activated neutrophils release IL-1, IL-6, IL-8, TNFα, and granulocyte macrophage colony stimulating factor (GM-CSF) (Monteseirin 2009, Roberge et al 1994). Neutrophils also release matrix metalloproteases, which are involved in airway remodeling (Cundall et al 2003). Neutrophil accumulation in the lung results in increased permeability of lung epithelium mediated by neutrophil release of IL-8 (Belperio et al 2002, Martin 2002). Additionally, ozone exposure stimulates release of neutrophil elastase that stimulates

goblet cell degranulation and development of mucus plugs, which obstruct airways and lead to increased risk of infection (Agusti et al 1998, Koren et al 1989, Nogami et al 2000). During acute asthma exacerbations, neutrophils are increased in sputum (Fahy et al 1995).

3. Lymphocytes

T cells and B cells are both important regulators of airway disease. B cells produce antigen specific antibodies (Murphy 2008), while T cells release a variety of cytokines that shape the inflammatory response. There are multiple classes of CD4+ T cells in the lungs including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and T regulatory cells (T regs).

T lymphocytes shape the inflammatory response depending on the antigen and the inflammatory cytokines produced. Th1 cytokines associated with infection include IFNγ, IL-2, and TNFα (Romagnani 2000). Th1 cytokines promote macrophage activation and cell cytotoxicity (Romagnani 2000).

Th2 cytokines are associated with allergic disease and include IL-4, IL-5, IL-9, IL-10 and IL-13 (Romagnani 2000). Th2 cytokines promote eosinophil recruitment and survival, mucus secretion, antibody class switching, and airway hyperreactivity (Bracke et al 1998, Fulkerson et al 2014, Iademarco et al 1995, Kim et al 2010, Lee et al 2008,

Mattes et al 2002). Th17 cells induce epithelial cells to release cytokines that mediate increased inflammatory cell recruitment (Murphy 2008).

In allergic asthma, there is a skewing of the cytokine profile toward Th2 (Bergqvist et al 2015, Kim et al 2010, Oeser et al 2015, Wu et al 2014). This skewing of Th2 Cytokine pathway is associated with poor asthma control (Bergqvist et al 2015).

4. Eosinophils

Eosinophils are a granulocytic immune cell known to have a major role in allergic asthma (Adamko et al 2003, Broide et al 2011, Walford & Doherty 2014). Eosinophils have a bi-lobed nucleus and contain protein-rich granules filled with major basic protein, eosinophil peroxidase, eosinophil derived neurotoxin, and eosinophil cationic protein. When eosinophils become activated, they release their preformed granule proteins in a process called degranulation. Eosinophil granule proteins are cytotoxic to both parasites and host tissues (Gleich & Adolphson 1986, Malik & Batra 2012). Eosinophils also produce a range of cytokines including TNFα, IL-1, IL-10, IL-4, and IL-5 (Melo et al 2013, Miyamasu et al 1995, Spencer et al 2009). Eosinophils are recruited to the lungs by IL-8, IL-5, eotaxin, IL4, and GM-CSF (Erger & Casale 1995, Foster et al 1996, Liu et al 2015, Wakugawa et al 2000, Yamada et al 2000). Additionally, eosinophil survival is increased with increases in IL5 and GM-CSF (Levi-Schaffer et al 1998, Nussbaum et al 2013).

Eosinophils are rarely found in the lungs of healthy individuals (Bousquet et al 1990, Horn et al 1975, Kirby et al 1987). However, in allergic asthma, eosinophils are significantly increased in bronchial biopsies and bronchoalveolar lavage (Bousquet et al 1990, Horn et al 1975, Kirby et al 1987), and are correlated with decreased lung function and airway hyperreactivity (Bousquet et al 1990, Foster et al 1996, Horn et al 1975). However, in asthmatics, therapies that specifically deplete eosinophils by targeting IL-5 failed to improve lung function (Bel et al 2014a, Garrett et al 2004, Legrand & Klion 2015, Ortega et al 2014, Ortega et al 2016).

The limited view of eosinophils singularly contributing to disease pathology has recently begun to shift (Furuta et al 2014, Jacobsen et al 2012, Lee et al 2010, Rosenberg et al 2013) as eosinophils have been shown to have important roles in immune system activation through antigen presentation (Wang et al 2007) and are critical to tissue remodeling and repair (Goh et al 2013, Gouon-Evans et al 2000, Jeziorska et al 1996, Jeziorska et al 1995, Robertson et al 2000).

It has recently been shown that mice sensitized and challenged with ovalbumin have multiple eosinophil populations in the lungs (Abdala Valencia et al 2016, Percopo et al 2017). These eosinophil populations differ both in surface marker expression and cytokine expression (Abdala Valencia et al 2016, Percopo et al 2017). Thus, the limited efficacy observed following eosinophil depletion in asthmatics may be caused by loss of a beneficial population of eosinophils in the lungs.

5. Tumor Necrosis Factor-alpha

TNFα is a proinflammatory cytokine increased in the lungs after ozone exposure (Bao et al 2013, Esther et al 2011, Wang et al 2015). Ozone induced production of TNFα is mediated by alveolar macrophages (Fakhrzadeh et al 2004, Ishii et al 1997) and epithelial cells (Nichols et al 2001, Young & Bhalla 1995). In the lungs, TNFα up-regulates adhesion molecules ICAM-1 and VLA-4 on airway epithelial cells (Atsuta et al 1997, Bloemen et al 1993) and endothelial cells (Iademarco et al 1995). Additionally, TNFα induces expression of the eosinophil chemoattractant, eotaxin, from nasal fibroblasts (Terada et al 2000). This suggests that TNFα may mediate eosinophil recruitment into the airway lumen. In guinea pigs, TNFα presumably mediates airway hyperreactivity via recruitment of inflammatory cells as blocking TNFα prevents airway hyperreactivity in animals exposed to organophosphorus pesticide (Proskocil et al 2013), infected with virus (Nie et al 2011), and challenged with antigen (Nie et al 2009).

TNFα is a member of the tumor necrosis factor receptor superfamily. TNFα is a homotrimer with a structural motif known as the TNFα homology domain. The TNFα homology domain binds to the cysteine rich domains on TNFα receptors. TNFα is synthesized as a membrane-bound transmembrane protein and is cleaved by TNFα converting enzyme to release the soluble form (Murphy 2008). Both soluble and membrane forms of TNFα are biologically active. TNFα is produced by neurons, smooth muscle cells, epithelial cells, inflammatory cells, fibroblasts, and endothelial

cells (Francis et al 2004, Nie et al 2011, Proskocil et al 2013, Sato et al 1997, Warner & Libby 1989). Additionally, TNFα has diverse effector functions, including upregulation of adhesion molecules, inflammatory cell recruitment, release of inflammatory mediators, increase body temperature, and apoptosis (Atsuta et al 1997, Bloemen et al 1993, Francis et al 2004, Iademarco et al 1995, Murphy 2008, Nagai et al 1988, Young & Bhalla 1995).

TNFα activates tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2). Both TNFR are homotrimeric transmembrane proteins (Murphy 2008). TNFR1s are expressed on most cells (Murphy 2008). However, TNFR2s are expressed predominantly on inflammatory cells (Murphy 2008). Activation of TNFRs result in several effector functions, depending on the duration and concentration of the TNFα signal. TNFα signaling can induce transcription of cytokines through activation of the transcription factor NF-κB (Figure 1.6) (Micheau & Tschopp 2003, Sedger & McDermott 2014). Alternatively, TNFα can also induce apoptosis through recruitment of TNFR1 associated death domain (Micheau & Tschopp 2003, Murphy 2008, Sedger & McDermott 2014). (Figure 1.6) (Micheau & Tschopp 2003, Sedger & McDermott 2014).

Down stream signaling is the same regardless of which TNFR is stimulated. Thus, pharmacologists have targeted TNFα rather than the two receptors. TNFα signaling is blocked by etanercept, a soluble fusion protein comprised of 2 TNFR2s fused to the

constant region of IgG molecule, that prevents TNFα from binding to TNFR1 or TNFR2 (Figure 1.8) (Sedger & McDermott 2014). Etanercept is FDA approved for the treatment of arthritis (Sedger & McDermott 2014). Etanercept is used in this dissertation to measure the effect of TNFα on ozone induced inflammatory cell recruitment to the lungs and airway hyperreactivity in non-sensitized and sensitized guinea pigs.

FIGURE 1.6 Tumor Necrosis Factor Signaling

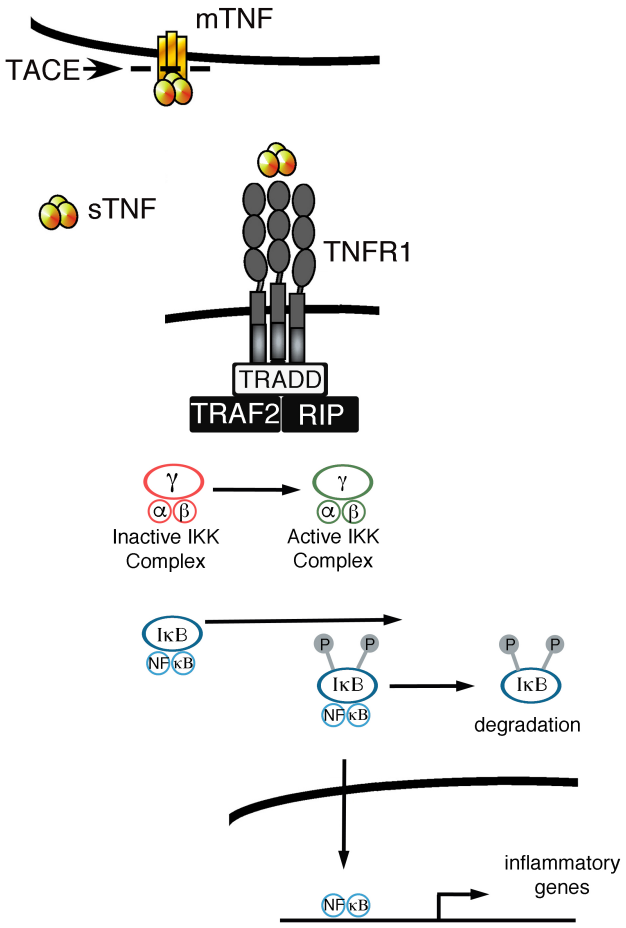


Figure 1.6 . Membrane bound tumor necrosis factor-alpha (TNFα) is cleaved by tumor necrosis factor converting enzyme (TACE), releasing soluble TNFα. TNFα binds to tumor necrosis factor receptor 1 (TNFR1), recruiting the adaptor proteins TNFR1-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein (RIP) to the cytoplasmic domain of the receptor. IKK kinase complex is activated, and phosphorylates IκB, releasing NF-κB. NF-κB is then translocated to the nucleus, to activate transcription of inflammatory genes. Figure adapted from (Rynko 2013).

FIGURE 1.7. Etanercept bound to TNFα

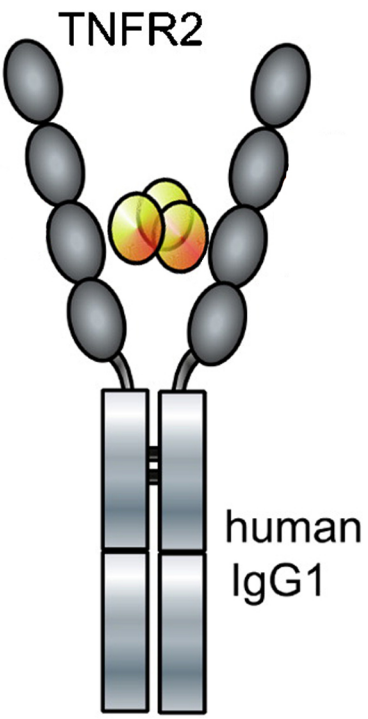


Figure 1.7. Etanercept, a tumor necrosis factor antagonist, is a receptor fusion protein composed of 2 tumor necrosis factor receptor 2 molecules fused to the constant region of human IgG. (Figure adapted from (Sedger & McDermott 2014).

E. INFLAMMATORY CELL HEMATOPOESIS

Introduction

Leukocyte hematopoiesis occurs in red bone marrow of flat bones and endosteum of long bones. Red bone marrow is highly vascularized fibrous tissue separated from mineralized bone by a layer of osteoblasts (Anthony & Link 2014, Gartner 2015). Bone marrow stroma is comprised of mesenchymal stem cells, reticular cells, and adipocytes and it forms a fibrous network that supports hematopoietic stem cells (Anthony & Link 2014). Progenitor cell expansion is stimulated by cytokine signals from the systemic circulation and bone marrow stromal cells (Anthony & Link 2014). Once mature, leukocytes egress from bone marrow via venous sinusoids (Anthony & Link 2014).

1. Eosinophils

Normal, adult humans routinely produce approximately 170,000 eosinophils per day (Gartner 2015). Eosinophil expansion is stimulated in patients with helminth infection, allergen exposure, and eosinophil-associated disorders (Hogan et al 2003, Inman et al 1999, Mori et al 2009). Eosinophils are derived from an eosinophil lineage committed progenitor that is derived from a common myeloid progenitor in humans (Mori et al 2009) or a granulocyte-macrophage progenitor in mice (Iwasaki et al 2005). Eosinophil progenitors, unipotential stem cells, are identified by expression of CD34, IL-3 receptor-alpha, IL-5 receptor-alpha, and lack CD45 expression (Gartner 2015, Iwasaki et al 2005, Mori et al 2009). Expansion and maturation of eosinophil progenitors is initiated by IL-5, TNFα, and granulocyte macrophage colony stimulating factor, and IL-

3 (Clutterbuck & Sanderson 1988, Dyer et al 2011, Fulkerson et al 2014, Gartner 2015, Lopez et al 2003, Robinson et al 1999). IL-5 propagates eosinophil maturation by inducing expression of IL-4 (Fulkerson et al 2014). In mice lacking eosinophil granule proteins, major basic protein and eosinophil peroxidase fail to undergo eosinophil hematopoiesis (Ackerman 2013, Doyle et al 2013). Thus, expression of eosinophil granule proteins is required for eosinophil hematopoiesis in the bone marrow. Eosinophils are recruited to the lungs by IL-8, IL-5, eotaxin, IL4, and GM-CSF (Erger & Casale 1995, Foster et al 1996, Liu et al 2015, Wakugawa et al 2000, Yamada et al 2000).

2. Neutrophils

In adult humans, 1 x 10^9 neutrophils/kg are released from the bone marrow each day (Rankin 2010). Mature neutrophils remain in the bone marrow for 4-6 days, forming a storage pool termed the bone marrow reserve (Furze & Rankin 2008, Rankin 2010). Thus, neutrophils can be rapidly mobilized from the bone marrow and released into the circulation in response to tissue damage or infection (Burdon et al 2008) Neutrophils develop from granulocyte macrophage progenitors that are derived from a common myeloid progenitor (Dahl 2009). Neutrophil progenitor expansion is stimulated by granulocyte colony stimulating factor and IL-6 (Dahl 2009). Migration out of the bone marrow is induced by macrophage inhibitory protein -2, and granulocyte colony stimulating factor (Burdon et al 2008, Semerad et al 2002).

3. Macrophages and monocytes

Macrophages are derived from bone marrow produced monocytes, which enter the blood stream and fully develop into macrophages once they migrate into tissue. Similar to neutrophils and eosinophils, monocytes develop in the bone marrow from granulocyte macrophage progenitor that is derived from a common myeloid progenitor (Dahl 2009). Monocyte development is induced by macrophage colony stimulating factor, granulocyte macrophage colony stimulating factor, GM-CSF and IL-3 (Dahl 2009). Monocytes are recruited from bone marrow into circulation by monocyte chemoattractant protein 3 and monocyte chemotactic protein 1 (Crane et al 2009, Tsou et al 2007).

4. Lymphocytes

T- and B-cell lymphocytes arise from common lymphoid progenitor cells (Corfe & Paige 2009, Schwarz & Bhandoola 2009). Common lymphoid progenitors develop from activation with stem cell factor and c-kit (Corfe & Paige 2009). T cell precursors migrate from the bone marrow and mature in the thymus (Schwarz & Bhandoola 2009). B-cell maturation occurs in the bone marrow and is induced by IL-7 (Corfe & Paige 2009).

In this dissertation, data shown for bone marrow monocytes include both monocytes and lymphocytes because morphologically they are indistinguishable.

F. OZONE

1. Synthesis

Ozone is present in the earth's upper atmosphere and at ground level. In the upper atmosphere, ozone forms a protective layer that prevents harmful ultra violet radiation from reaching the earth's surface. In contrast, at ground level, ozone is toxic to humans, animals, and plants. Ozone is the main component of ground-level air pollution. Ground level ozone is formed when volatile organic compounds and nitrogen oxides from vehicle exhaust react in the presence of sunlight (Carter 1994).

$$CO_2 + NO \rightarrow NO_2 + CO$$

$$NO_2 + UV \rightarrow NO + O$$

$$O_2 + O \rightarrow O_3$$

The United States Environmental Protection Agency sets the standards for acceptable levels of ozone exposure. The National Ambient Air Quality Standards for ozone is 0.070 ppm (parts per million) averaged over an 8 hour day (epa.gov). Over 50% of Americans live in counties where the daily ozone levels regularly exceed the standards set by the Environmental Protection Agency (2016). Ground level ozone is the major pollutant in developing countries (Huang 2014). In 2015, ozone levels in Shanghai, China were 0.13 ppm for 12 days this concentration exceeds guidelines set by the EPA (0.07 ppm) and WHO (0.05 ppm) (Chen 2015). While in Mexico City, Mexico ozone levels ozone levels are (Calderon-Garciduenas et al 2015) close to or exceed the

national (0.07 ppm) and international (0.05 ppm) ozone standards. Thus, ground level ozone is a global problem. Understanding the effects of ozone on the lungs will be important for individuals living all over the world.

2. Ozone exposure and lung function in humans

Ozone exposure protocols vary by ozone concentration, amount of exercise, duration of exposure, and the length of time after exposure before airway function measurements are made. The total exposure to inhaled ozone is calculated as a product of ozone concentration and duration of exposure (Gelzleichter et al 1992). Ozone concentrations between 0.12-0.2 ppm are in the ambient range, while concentrations above 0.2 ppm are considered high. Healthy individuals require exercise during ozone exposure experiments to develop measureable decreases in lung function. During aerobic exercise, individuals breathe more deeply through their mouth, bypassing the protection of the nasal passages. Deep breathing by mouth allows increased ozone exposure to the distal lungs. Thus, when ambient ozone levels are high it is recommended that individuals exercise indoors.

Ozone exposure decreases lung function in healthy and asthmatic individuals (Khatri et al 2009, Schelegle et al 2009) (Folinsbee et al 1988, Holtzman et al 1979, Kreit et al 1989). Healthy individuals additionally experience airway hyperresponsiveness to inhaled bronchoconstrictors such as histamine or methacholine after ozone exposure (Aris et al 1993, Beckett et al 1985, Folinsbee et al 1988, Golden et al 1978, Holtzman

et al 1979). Asthmatic individuals experience a greater decrease in lung function (FEV_1 and forced vital capacity) and greater increase airway hyperresponsiveness when compared to healthy individuals (Ierodiakonou et al 2016, Kreit et al 1989, Peters et al 2001).

Importantly, EPA standards are set for healthy individuals, an asthmatic develops symptoms, lower quality of life scores, worse lung function, with increased airflow obstruction at ozone levels that are lower than the current EPA standards of 0.070 ppm (Khatri et al 2009). High environmental ozone levels are associated with an increase in asthma related emergency department visits and hospitalizations which lag one to three days after ozone exposure (Gleason et al 2014, Mar & Koenig 2009, Sheffield et al 2015).

Ozone can also increase sensitivity to allergen exposure in individuals with allergic asthma (Holz et al 2002, Jenkins et al 1999, Jorres et al 1996, Molfino et al 1991). Ozone increases eosinophilic inflammation in the lungs of individuals with allergic asthma (Dokic & Trajkovska-Dokic 2013, Holz et al 2002, Khatri et al 2009, Peden et al 1997, Vagaggini et al 2002). This suggests that individuals with allergic asthma may be the population most sensitive to ozone.

Many mechanisms contribute to development of decreased lung function including inflammation, airway wall thickening, increased sensory nerve sensitivity, and changes in parasympathetic nerve function (Coulson et al 2002, Hargreave et al 1986,

McParland et al 2003, O'Byrne & Inman 2003). Of particular interest to this dissertation is the role of inflammation in ozone-induced hyperresponsiveness. Ozone-induced airway hyperresponsiveness can be blocked by atropine, a muscarinic receptor antagonist, in both healthy individuals and asthmatic individuals (Beckett et al 1985, Golden et al 1978, Holtzman et al 1979). This demonstrates that ozone-induced airway hyperresponsiveness is mediated by release of acetylcholine from parasympathetic nerves in the lungs onto M_3 muscarinic receptors.

3. Ozone-Induced Inflammation Humans

In the lungs, airway epithelium is damaged after ozone exposure by reactive oxygen intermediates (Krishna et al 1997, Leikauf et al 1995, Pryor & Church 1991). Ozone causes necrosis of ciliated cells, deciliation, and break down of secretory cells. In addition, epithelial damage is accompanied by increased vascular permeability and an influx of inflammatory cells (Laskin et al 2011). Nasal biopsies revealed that ozone induces expression of proinflammatory cytokines including IL-6, IL-8, TNFα, IL-1β and adhesion molecule ICAM1 and E-selectin, which facilitate inflammatory cell recruitment and migration into lung tissue (Arsalane et al 1995, Dokic et al 2011).

Ozone exposure increases neutrophils in healthy individuals that (Holz et al 1999) remain elevated in bronchoalveolar lavage for 24 hours (Aris et al 1993, Balmes et al 1996, Holz et al 1999, Kim et al 2011, Koren et al 1989, Krishna et al 1998, Seltzer et al 1986, Vagaggini et al 2010, Vagaggini et al 2007). While neutrophils are increased

in the lungs after ozone, neutrophil numbers in the lungs do not correlate with decreases in lung function observed following exposure (Balmes et al 1996). In patients with asthma, neutrophils increase in bronchoalveolar lavage one hour after ozone but, by 24 hours after ozone, this neutrophilic inflammation has subsided (Dokic & Trajkovska-Dokic 2013, Holz et al 1999, Newson et al 2000). Depleting neutrophils with corticosteroids does not prevent the ozone mediated decrease in lung function (Alexis et al 2008, Vagaggini et al 2007). Additionally, ozone causes neutrophil activation and release of IL-8 and neutrophil elastase (Alexis et al 2008, Vagaggini et al 2010). While IL-8 is known to mediate neutrophil activation and migration into tissue (Baggiolini et al 1989, Bickel 1993), it is also a potent eosinophil chemoattractant (Erger & Casale 1995). Thus, increased neutrophil activation in the lungs may contribute to increased eosinophilic inflammation.

Ozone increases eosinophil cationic protein in bronchoalveolar lavage of healthy individuals for the first 24 hours after exposure (Hiltermann et al 1999, Holz et al 1999, Kopp et al 1999). Ozone's effects are potentiated in individuals with mild asthma or atopy who have elevated lung eosinophils at baseline. In these individuals, ozone significantly increased eosinophil cationic protein in bronchoalveolar lavage immediately after exposure (Dokic & Trajkovska-Dokic 2013, Kirby et al 1987). Ozone's effect on the number of eosinophils in the lungs does not mirror its effect on eosinophil granule proteins. In healthy individuals, while no increase in eosinophils in bronchoalveolar lavage was measured, eosinophil cationic protein was increased

(Holz et al 1999). However, in asthmatics with eosinophils in the lungs at baseline, ozone causes a significant decrease in the number of eosinophils in bronchoalveolar lavage with in one hour after ozone (Hiltermann et al 1999, Holz et al 1999, Holz et al 2002). This suggests that ozone causes eosinophil activation and degranulation.

4. Ozone exposure and lung function in animals

Human studies indicate a concentration dependent relationship between ozone exposure, decreased lung function and increased inflammation. However, understanding how different inflammatory cell populations contribute to the development of ozone induced airway hyperreactivity is not possible using human studies. Thus, much of the work to dissect and identify the mechanism of ozone-induced hyperreactivity has been done in animal models including the work presented in this dissertation. The benefits of animal models include extensive pharmacological tools, invasive *in vivo* procedures, the ability to track movement of inflammatory cells from bone marrow to lungs, regulated environmental exposure and food intake, and quantifiable ozone exposure.

Ozone causes airway hyperreactivity in non-human primates (Joad et al 2006, Joad et al 2008, Moore et al 2012), dogs (Matsui et al 1991, Stevens et al 1995a, Stevens et al 1995b), mice (Inman et al 1999, Park et al 2004, Rivera-Sanchez et al 2004), rats (DeLorme et al 2002, Depuydt et al 1999, Koto et al 1997), and guinea pigs (Gambone et al 1994, Inoue et al 2000, Nakano et al 2000, Vargas et al 1994, Verhein et al 2013,

Yost et al 1999, Yost et al 2005). Additionally, ozone causes increased airway hyperresponsiveness to allergen exposure in sensitized guinea pigs (Chhabra et al 2010), non-human primates (Moore et al 2012, Moore et al 2014), and mice (Neuhaus-Steinmetz et al 2000).

5. Ozone exposure and inflammation in animals

Ozone stimulates guinea pig lung macrophages (Arsalane et al 1995, Pendino et al 1995) and airway epithelial cells (Inoue et al 2000, Rusznak et al 1996) to produce proinflammatory cytokines including TNFα, IL1β, IL-6, and IL-8 one day after exposure. Interestingly, macrophage soluble TNFα release is reduced by three days after exposure. Ozone also decreases macrophage phagocytosis for a minimum of three days after ozone exposure (Arsalane et al 1995). Macrophages participate in repair of ozone induced lung injury and reducing lung inflammation by phagocytizing oxidized lipids from bronchoalveolar lavage fluid (Dahl et al 2007).

Similar to healthy humans, ozone also causes neutrophilic inflammation in bronchoalveolar lavage soon after exposure in many animal species including non-human primates (Hicks et al 2010), mice (Park et al 2004, Williams et al 2008, Zhang et al 2015), rats (Kodavanti et al 2015), dogs (Li et al 1992, Stevens et al 1994, Stevens et al 1995a) and guinea pigs (Gambone et al 1994, Inoue et al 2000, Verhein et al 2011, Yost et al 1999, Yost et al 2005). Although neutrophilic inflammation is significantly increased in bronchoalveolar lavage immediately following ozone

exposure, preventing neutrophil migration into the lungs with inhaled steroids or an antibody to the adhesion molecule CD11b/CD18 did not prevent ozone-induced airway hyperreactivity (Li et al 1992, Stevens et al 1994).

Ozone increases lung eosinophils in guinea pigs (Gelzleichter et al 1992, Tan & Bethel 1992, Verhein et al 2008, Yost et al 2005), rats (Gordon et al 2016, Harkema et al 1994), mice (Jang et al 2006, Neuhaus-Steinmetz et al 2000), and non-human primates (Dokic & Trajkovska-Dokic 2013, Joad et al 2006, Schelegle et al 2003). Ozone also induces eosinophil activation in animals. Depleting eosinophil granule protein major basic protein one day after ozone prevents ozone-induced airway hyperreactivity (Yost et al 1999).

6. Role of eosinophils in ozone-induced airway hyperreactivity in guinea pigs

Ozone increases lung eosinophils in guinea pigs (Tan & Bethel 1992, Verhein et al 2008, Yost et al 2005). In guinea pigs, ozone causes airway hyperreactivity immediately following ozone exposure (Schultheis et al 1994) that persists for at least three days (Schultheis et al 1994, Verhein et al 2008, Yost et al 2005). Eosinophils are increased both one day, and again three days, after ozone. However, eosinophil numbers return to baseline two days after ozone exposure (Figure 1.9) (Yost et al 2005).

One day after ozone, depleting eosinophils with an antibody to interleukin 5 (AbIL5), or blocking eosinophil major basic protein with an antibody or with heparin prevented ozone induced airway hyperreactivity, by restoring neuronal M_2 muscarinic receptor function on parasympathetic nerves and preventing bronchoconstriction (Yost et al 1999, Yost et al 2005). Therefore one day after ozone, airway hyperreactivity is mediated by eosinophils.

In guinea pigs, two days after ozone, the number of eosinophils in bronchoalveolar lavage have returned to baseline and M_2 muscarinic receptor function is restored, but animals remain hyperreactive (Yost et al 2005). Reducing eosinophils around airway nerves with AbIL5 prevents bronchoconstriction (Yost et al 2005). Thus, two days after ozone eosinophils mediate ozone induced airway hyperreactivity.

In contrast, three days after ozone exposure the role of eosinophils in hyperreactivity has changed from mediating airway hyperreactivity to protective, in that depleting eosinophils, or preventing their migration into lung tissue with an antibody to the adhesion molecule VLA4, significantly worsens ozone-induced airway hyperreactivity (Figure 1.8) (Yost et al 2005).

Eosinophil numbers increase in bronchoalveolar lavage both one and three days after ozone. However, the role of eosinophils changes from mediating ozone induced airway hyperreactivity one day after ozone to beneficial three days after ozone (Yost et

al 2005). The second increase in eosinophils three days after ozone suggests that a second beneficial eosinophil population is recruited to the lungs three days after ozone.

What is not known is how ozone changes eosinophil populations in the lungs one and three days after ozone and what inflammatory signals mediate eosinophil recruitment to the lungs three days after ozone. The work in this thesis will explore how ozone changes eosinophil populations in bronchoalveolar lavage one and three days after ozone and what inflammatory mediators mediate eosinophil recruitment to the lungs.

FIGURE 1.8 Eosinophil depletion prevents ozone-induced airway hyperreactivity one and two days after ozone, but increases ozone-induced airway hyperreactivity three days after ozone.

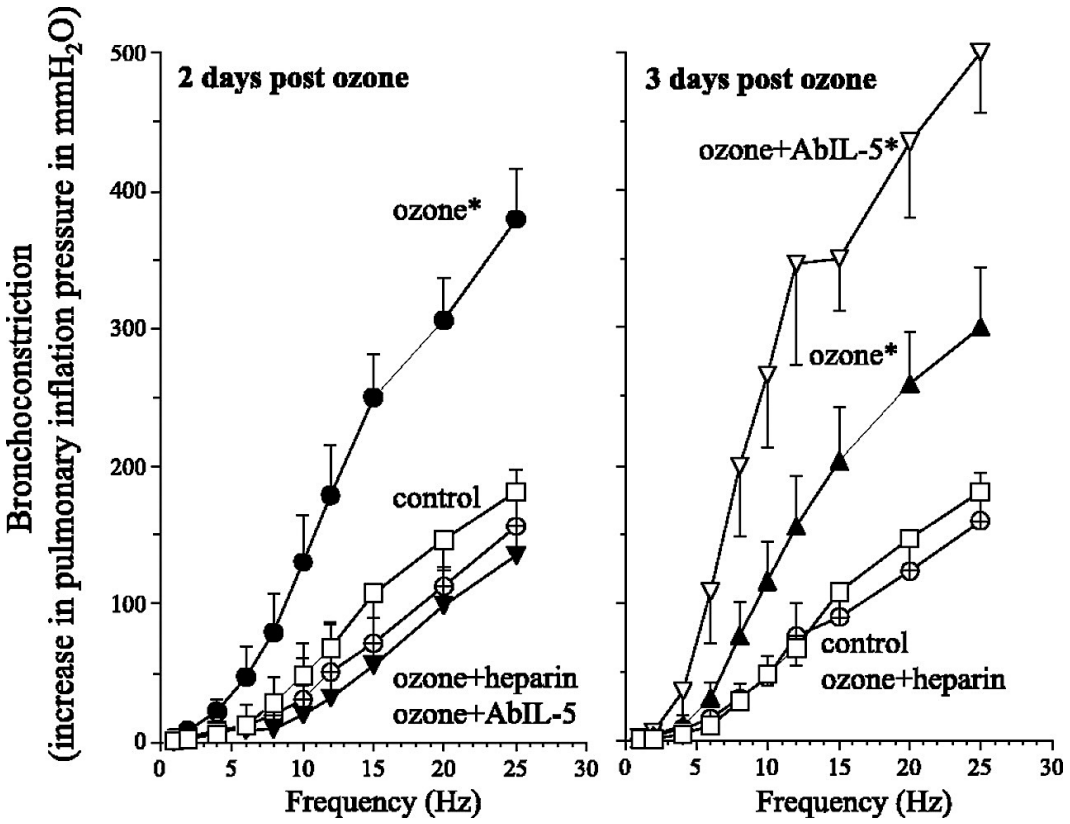


Figure 1.8 Ozone caused airway hyperreactivity two (A, black circles) and three (B, black triangles) days after exposure when compared to air exposed controls (A&B open squares). Eosinophil were depleted with an antibody to IL-5 (AbIL5), a cytokine required for eosinophil hematopoiesis, survival, and migration into tissue. Depletion of eosinophils one day (data not shown) and two days after ozone (A, black triangles) prevented ozone-induced airway hyperreactivity. Three days after ozone, eosinophil depletion significantly worsened ozone-induced airway hyperreactivity. Data from (Yost et al 2005).

FIGURE 1.9 Eosinophils increase in bronchoalveolar lavage one and three days after ozone.

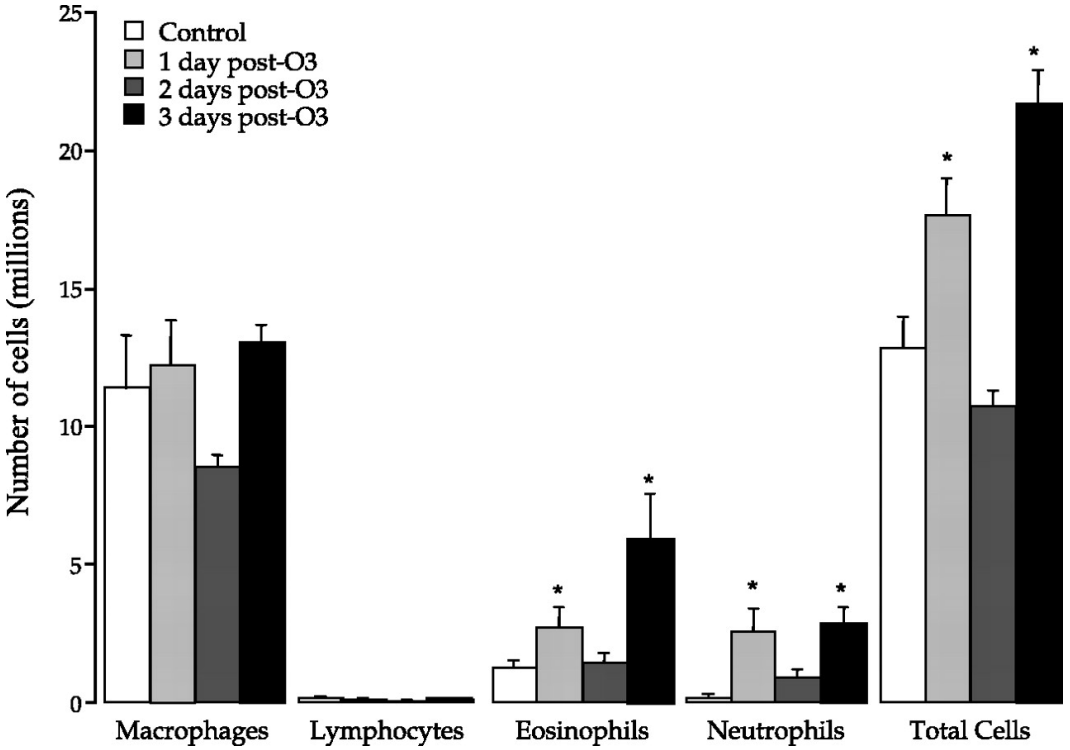


Figure 1.9 Eosinophils were increased in bronchoalveolar lavage one day after ozone (light grey) compared with air control (white). Two days after ozone (dark grey), all inflammatory cells were decreased. Three days after ozone, eosinophils were significantly increased in bronchoalveolar lavage compared with air control. Data from (Yost et al 2005).

G. HYPOTHEISIS

Over 166 million Americans live in counties where they are regularly exposed to unhealthy levels of ozone (The American Lung Association; www:lungusa.org). It is well known that ozone inhalation causes hyperreactivity and inflammation in the lungs that lasts for several days. Despite much research using human and animal exposures the role of inflammation in mediating ozone-induced airway hyperreactivity is still largely unknown.

In guinea pigs, ozone-induced airway hyperreactivity is mediated by parasympathetic nerves, since vagally-induced bronchoconstriction is potentiated by ozone, while airway smooth muscle contraction to intravenous muscarinic agonists is not changed (Gambone et al 1994, Verhein et al 2008, Yost et al 1999). Eosinophils move into the bronchoalveolar lavage in two distinct waves after ozone. The first wave occurs one day after exposure and the second occurs three days after exposure (Yost et al 1999, Yost et al 2005).

One day after ozone, eosinophils mediate airway hyperreactivity by releasing major basic protein that inhibits neuronal M_2 muscarinic receptors leading to increased acetylcholine release from parasympathetic nerves and bronchoconstriction. Conversely, three days after depleting eosinophils or preventing their migration into the lung not only fails to prevent hyperreactivity, but significantly worsens it (Yost et al

2005). This indicates that the role of eosinophils changes from detrimental at one day after ozone to protective three days after ozone.

Atopic patients are sensitized to allergens and have increased eosinophils in the lungs after ozone (Bascom et al 1990, Brown et al 2007, Holz et al 2002, Peden et al 1995). Prior sensitization increases airway hyperreactivity following ozone exposure (Schelegle et al 2003, Vargas et al 1994). Additionally, sensitization changes the mechanism of airway hyperreactivity following viral infection from an eosinophil independent mechanism to an eosinophil dependent mechanism (Adamko et al 1999). This suggests that the role of eosinophils three days after ozone may change with sensitization.

The overall hypothesis tested in this dissertation is that ozone inhalation induces eosinophil hematopoiesis in bone marrow, and that newly divided eosinophils will ameliorate airway hyperreactivity three days later in non-sensitized and sensitized animals. Specifically, these studies will determine whether ozone inhalation induces production of eosinophils in the bone marrow and/or increases eosinophil migration to the lungs in sensitized and non-sensitized guinea pigs and identify what inflammatory signals might contribute to eosinophil hematopoiesis and recruitment to the lungs three days after ozone and whether this changes with sensitization.

FIGURE 1.10 Model of Hypothesis tested in this thesis

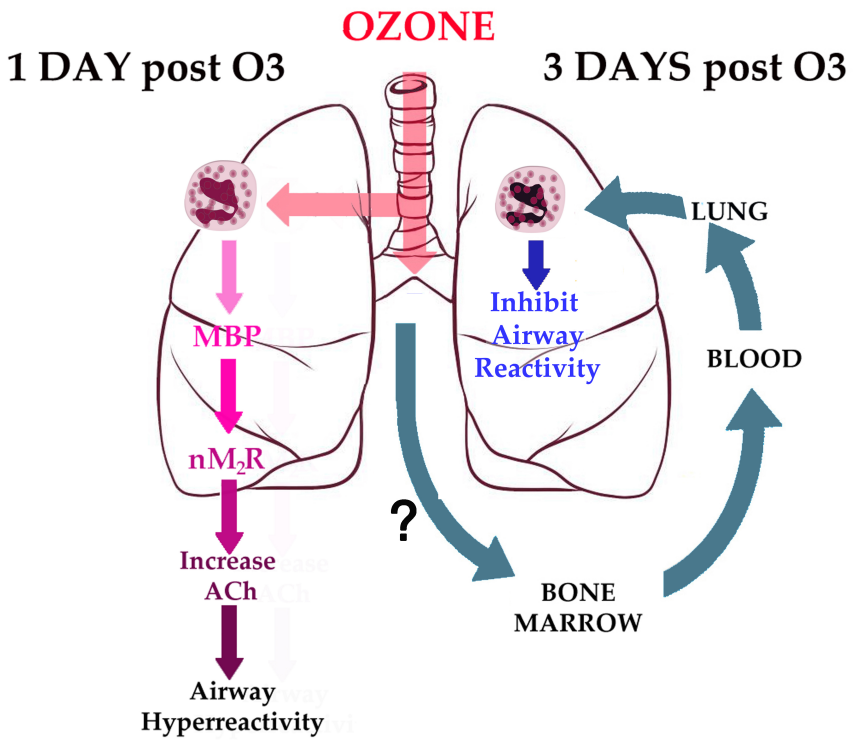


Figure 1.10 One day after ozone eosinophils release major basic protein (MBP) inhibiting neuronal M_2 muscarinic receptors (nM_2R) located on parasympathetic nerves, increasing acetylcholine release (ACh) onto M_3 muscarinic receptors located on airway smooth muscle causing airway hyperreactivity. Three days after ozone, the role of eosinophils in the lungs has changed from mediating ozone-induced airway hyperreactivity at one day to being beneficial at three days. Specifically, these studies will determine whether ozone inhalation induces eosinophil hematopoiesis in bone marrow and/or increases eosinophil migration to the lungs in sensitized and non-sensitized guinea pigs and identify what inflammatory signals might contribute to eosinophil hematopoiesis and recruitment to the lungs three days after ozone and whether this changes with sensitization.

CHAPTER II.

METHODS

A. Choice of animal model

Guinea pigs were chosen to study the effect of ozone on airway hyperreactivity and inflammatory cell populations because the anatomy and physiology of guinea pig lung is similar to that of humans. Both human and guinea pig airways are lined with pseudostratified ciliated epithelium (Dalen 1983), which contain goblet cells and mucus glands that release mucus following neuronal stimulation (Poblete et al 1993, Rogers 2001, Widdicombe et al 2001). Also, guinea pig airway epithelium is innervated by sensory nerves (Kummer et al 1992, Lundberg et al 1984). Guinea pig lungs also have significant airway smooth muscle (Wenzel & Holgate 2006), and their smooth muscle pharmacology in the lung more closely resembles humans than other animal models (Ressmeyer et al 2006). Histamine, methacholine, and leukotrienes all cause smooth muscle contraction in guinea pigs (Muccitelli et al 1987) and norepinephrine and other beta agonists induce smooth muscle relaxation in both guinea pig and human (Tanaka et al 2005).

Smooth muscle tone is controlled by parasympathetic innervation of airway smooth muscle in guinea pigs. Autonomic innervation of human and guinea pig lungs is also very similar (Canning & Fischer 1997, Kocmalova et al 2017, Undem et al 1990). In both humans and guinea pigs, parasympathetic nerves express the voltage gated sodium channel (NaV) 1.7, while mice express NaV 1.1 and/or NaV 1.3 (Kocmalova et al 2017). Blocking NaV 1.7 blocks parasympathetic nerve signaling in the lungs of humans and guinea pigs, but not mice (Kocmalova et al 2017). Parasympathetic

nerves innervate airway smooth muscle (Kesler & Canning 1999) and release acetylcholine, which activates M_3 muscarinic receptors on airway smooth muscle causing contraction (Roffel et al 1997, Ten Berge et al 1993). Acetylcholine release from parasympathetic nerves is controlled by inhibitory M_2 muscarinic receptors (Fryer & Maclagan 1984, Minette & Barnes 1988). In contrast to humans, guinea pigs have significantly greater sympathetic innervation of airway smooth muscle (Ainsworth et al 1982, Canning 2003, Kummer et al 1992).

Guinea pigs are also more immunologically similar to humans than other animal models (Padilla-Carlin et al 2008). Ozone inhalation by humans or guinea pigs causes a significant increase in the number of inflammatory cells into the bronchoalveolar lavage (Gambone et al 1994, Inoue et al 2000, Toward & Broadley 2002, Yost et al 1999, Yost et al 2005). Additionally, it causes airway hyperreactivity that persists over several days (Schultheis et al 1994).

In these experiments, specific pathogen-free female Dunkin-Hartley guinea pigs (Charles River, Kingston, NY) were used for the measurement of airway physiology and inflammation after ozone exposure. At the time of exposure, all guinea pigs weighed 350-400g. Guinea pigs were shipped in filtered crates, housed in rooms with high-efficiency particulate filtered air, and fed a normal diet. All protocols involving animals in this dissertation followed National Institute of Health guidelines, and were approved by Oregon Heath & Science University Animal Care and Use Committee.

B. Measurement of airway physiology

1. Surgical Preparation:

Guinea pigs were anesthetized with urethane (1.9 g/kg body weight i.p.), which is a non-depolarizing, central nervous system depressant that causes minimal depressant effects on the heart and lungs (Maggi & Meli 1986, Sceniak & Maciver 2006). Urethane does not suppress autonomic nerves (Kohn 1997.). Urethane causes deep anesthesia for 8-10 hours (Green 1982, Kohn 1997.) and experiments described in this dissertation generally lasted between 3-4 hours. Complete anesthesia was achieved when both blink and toe pinch responses were absent. Body temperature was maintained at 37°C with a rectal probe and a Harvard apparatus homoeothermic blanket.

Once animals were anesthetized, guinea pigs were placed on their back with the neck exposed. An incision was made at the front of the neck, exposing trachea, nerves, arteries and veins. Both jugular veins were cannulated for i.v administration of drugs. The jugular veins were isolated, and connective tissue was removed. A small hole was cut in the vein and the beveled end of cannula (PE90 tubing) was inserted into the vein toward the heart. The cannula was slid into the vein. A suture was tied around the cannula and vein to hold the cannula in place. A carotid artery was cannulated for measurement of heart rate (beats per minute) and blood pressure (mmHg) using a fluid filled transducer (heparinized PBS) (BD DTXplus; Becton Dickinson, Franklin Lakes, NJ). The carotid artery was isolated, connective tissue removed, and clamped with a

micro cross action bulldog clamp. A small hole was then cut to the artery and a cannula filled with heparinized saline was inserted into the artery. The artery was then slid into the artery. Once the artery was cannulated, the micro cross action bulldog clamp was removed and the cannula was sutured to the artery. Animals were tracheotomized, cannulated, mechanically ventilated with positive pressure constant tidal volume (1 ml volume/100 g body weight; 100 breaths/min) with an animal ventilator (Model 683; Harvard Apparatus). Guinea pigs were paralyzed with succinylcholine chloride, (5 μg/min) using an infusion pump (11 plus; Harvard Apparatus). Guinea pigs were also chemically sympatheticomized with guanethidine (5mg/kg i.v.) to deplete noradrenaline, since guinea pig smooth muscle receives some sympathetic signals (Oh et al 2006). Skin was sewed to a metal collar to create a pocket to hold mineral oil. Both vagus nerves were isolated, crushed, and the distal ends were placed on platinum electrodes and covered with warm mineral oil (37°C). The surgical set up is shown in (Figure 2.2).

Pulmonary inflation pressure (Ppi; measured in mmH_2O), the pressure needed to inflate the lungs. Ppi was measured using a pressure transducer (BD DTXplus; Becton Dickinson, Franklin Lakes, NJ) attached to a side arm of the tracheal cannula. Bronchoconstriction was measured as the increase in pulmonary inflation pressure over baseline inflation pressure (Dixon 1903).

C. Measurement of blood pressure and heart rate

Blood pressure (mmHg) was measured via a fluid filled pressure transducer attached to the carotid artery cannula. Heart rate (beats per minute) was derived from blood pressure via a tachograph on the Grass polygraph (Figure 2.1).

D. Measurement of vagally-induced bronchoconstriction and bradycardia

Both vagus nerves were electrically stimulated (10V, 0.2ms pulse width, 1-25Hz, 5s duration) at one-minute intervals using an SD9 stimulator (Grass), that produced frequency-dependent bronchoconstriction and bradycardia that were recorded on a polygraph (Grass). An example of a polygraph trace is shown in (Figure 2.3).

The stimulus parameters were chosen for parasympathetic nerve stimulation. Vagus nerves contain both sensory and parasympathetic nerves. Stimulation of vagal sensory nerves can stimulate bronchoconstriction through release of substance P(Undem et al 1990). Parasympathetic nerves require less current than sensory nerves for stimulation. Therefore, voltage was set at 10V, which allows for 50% of maximum response for parasympathetic nerves and 10% of maximum response for sensory nerves (Undem et al 1990). Pulse duration was set to 0.2ms, which induces a greater than 80% maximum response for parasympathetic nerves, and 50% maximum response for sensory nerves (Undem et al 1990). Frequency dependent bronchoconstriction and bradycardia were blocked by atropine (1mg/kg i.v.), demonstrating they were mediated

by neuronal release of acetylcholine onto M_3 muscarinic receptors on airway smooth muscle.

E. Measurement of postjunctional muscarinic receptor function

After vagal reactivity was measured, the function of postjunctional M_3 muscarinic receptors on airway smooth muscle and M_2 muscarinic receptors on cardiac muscle was measured using methacholine (1-10 μg/kg i.v.). M_3 muscarinic receptor function on airway smooth muscle was measured as an increase in bronchoconstriction (mm H_20). M_2 muscarinic function on cardiac muscle was measured as a fall in beats per min. At the end of each experiment, guinea pigs were euthanized by aortic exsanguination via the carotid artery cannula.

F. Collection and analysis of inflammatory cells

Blood

Using a heparinized syringe, 2 ml of blood was collected from a carotid artery cannula. Red blood cells were lysed by the addition of 9.5 ml 1 N HCl to 0.5ml blood. The number of inflammatory cells per milliliter of blood were counted using a hemocytometer.

For BrdU staining, 1ml of blood was water lysed in 35ml diH_2O for 30 seconds. This lysis was quenched with 4mL of 10x PBS. Cells were spun in an Eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germany) at 300 x g for 10min at room temperature

and then resuspended in 2 mL PBS. Differential cell counts were obtained by staining cytospun slides with Hemacolor (Figure 2.4 B).

2. Bronchoalveolar Lavage

Bronchoalveolar lavage was collected via the trachea cannula with five (10 ml) aliquots of PBS (room temperature). Cells were placed temporarily on ice and spun in an Eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germany) at 300 x g for 10 minutes at room temperature and then resuspended in cold 2 mL PBS 4ºC. Total inflammatory cell counts were obtained using a hemocytometer. Differential cell counts were obtained by staining cytospun slides with Hemacolor and then counting the number of each cell type (Figure 2.4 A).

3. Bone Marrow

Bone marrow was harvested from the left femur. The leg bone was isolated. The femur was then cut at the pelvis and knee, exposing the bone marrow. The bone marrow was then isolated by flushing the femur with 10 ml PBS. Cells were temporarily placed on ice and spun in an Eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germany) at 300 x g for 10min at room temperature and then resuspended in 2 mL PBS 4ºC. Total inflammatory cell counts were obtained using a hemocytometer. Differential cell counts were obtained by staining cytospun slides with Hemacolor and then counting the number of each cell type (Figure 2.4 C).

4. Cytospin Slides

For differential counts and BrdU stained inflammatory cells in bronchoalveolar lavage, blood or bone marrow were diluted to concentration of 30,000-50,000 cells per 100 µl in PBS. 100 µl of cell suspension was then cytospun onto superfrost microscope slides at 500 rpm for 10 minutes at room temperature. Cell spots were allowed to dry at room temperature. Once dry, slides were placed in 70% ethanol for fixation at 4°C overnight. The next day slides were stained for BrdU or allowed to dry and stored at 4°C for staining at a later date.

FIGIURE 2.1 Grass Polygraph

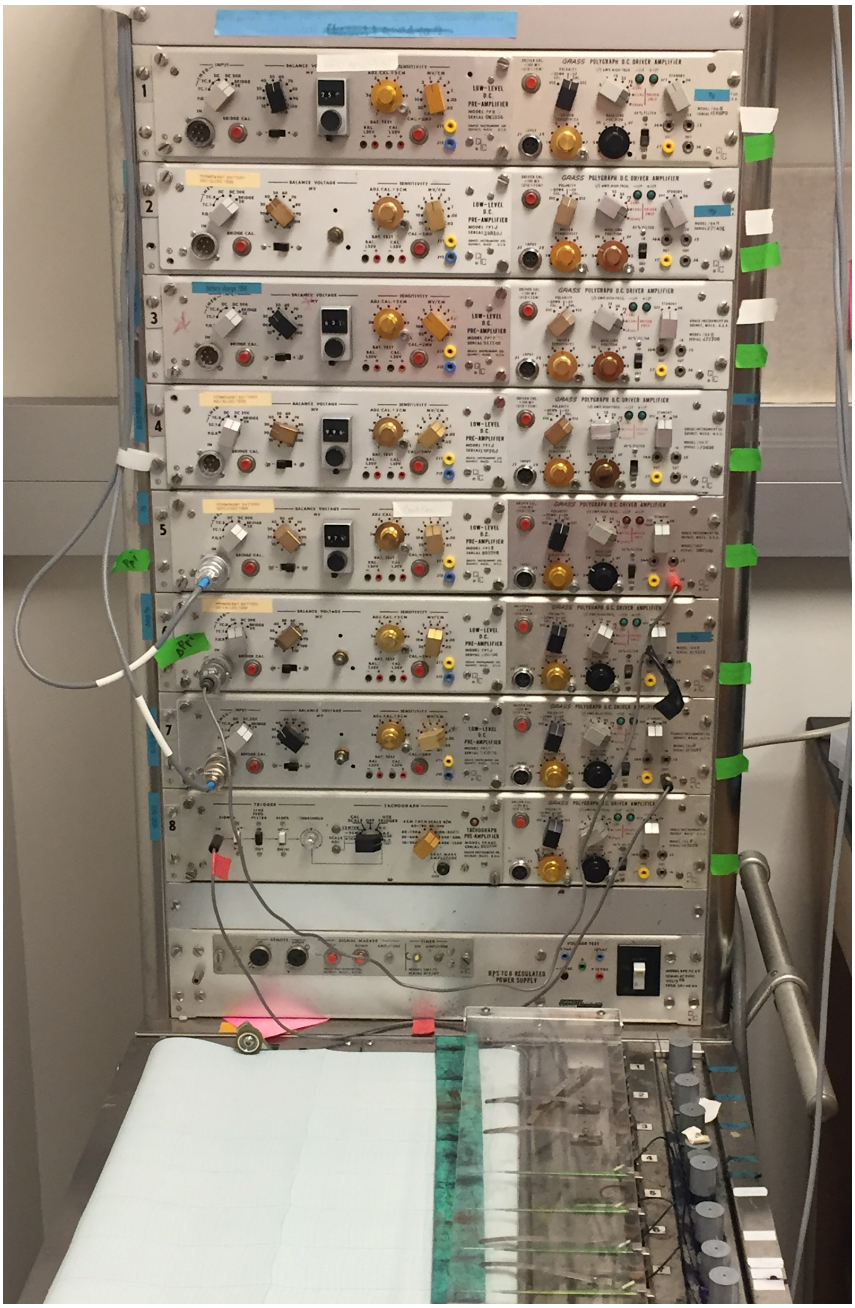


Figure 2.1 Airway physiology was measured using a grass polygraph.

FIGURE 2.2 Surgical preparation for airway physiology measurements in guinea pigs

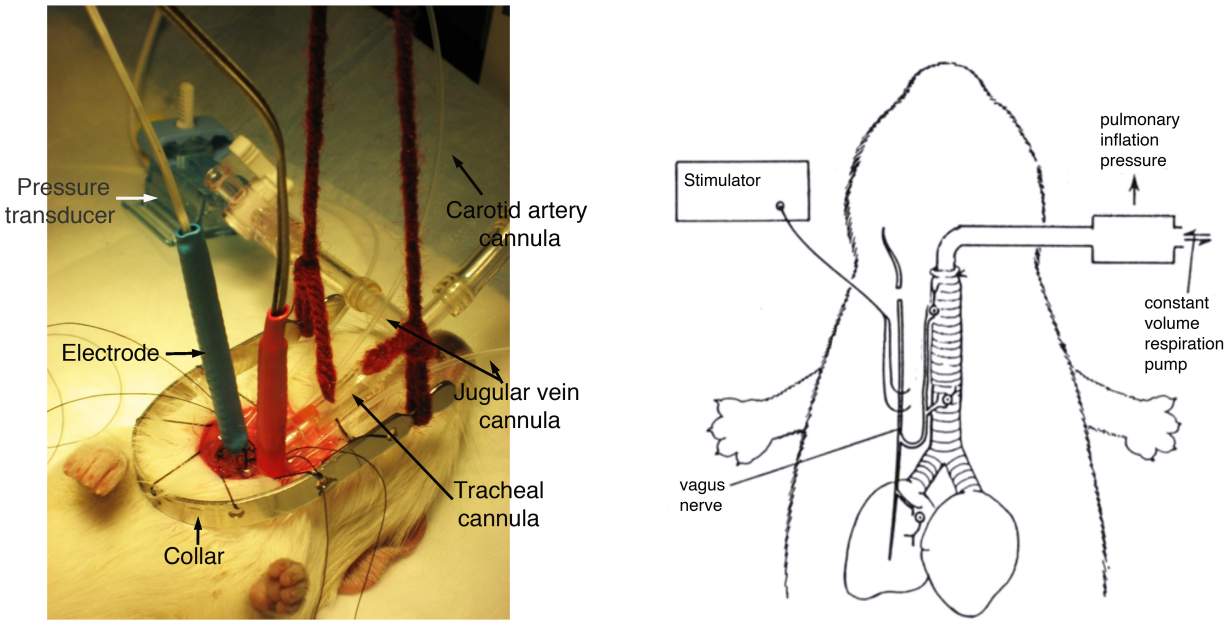


Figure 2.2. Guinea pigs were anaesthetized, paralyzed, and mechanically ventilated via trachea cannula. Pulmonary inflation pressure was measured with a pressure transducer off a sidearm of the tracheal cannula. The right and left vagus nerves were isolated and crushed. The distal end of each vagus nerve was placed on a platinum electrode, covered in mineral oil, and stimulated to induce bronchoconstriction. Drugs were administered via cannulas inserted in jugular veins. Heart rate and blood pressure were measured from another pressure transducer attached to a fluid filled cannula in a carotid artery. Figure adapted from (Verhein 2010). Schematic adapted from (Fryer 1986).

FIGURE 2.3 Example of data recorded on a polygraph, showing pulmonary inflation pressure, blood pressure, and heart rate.

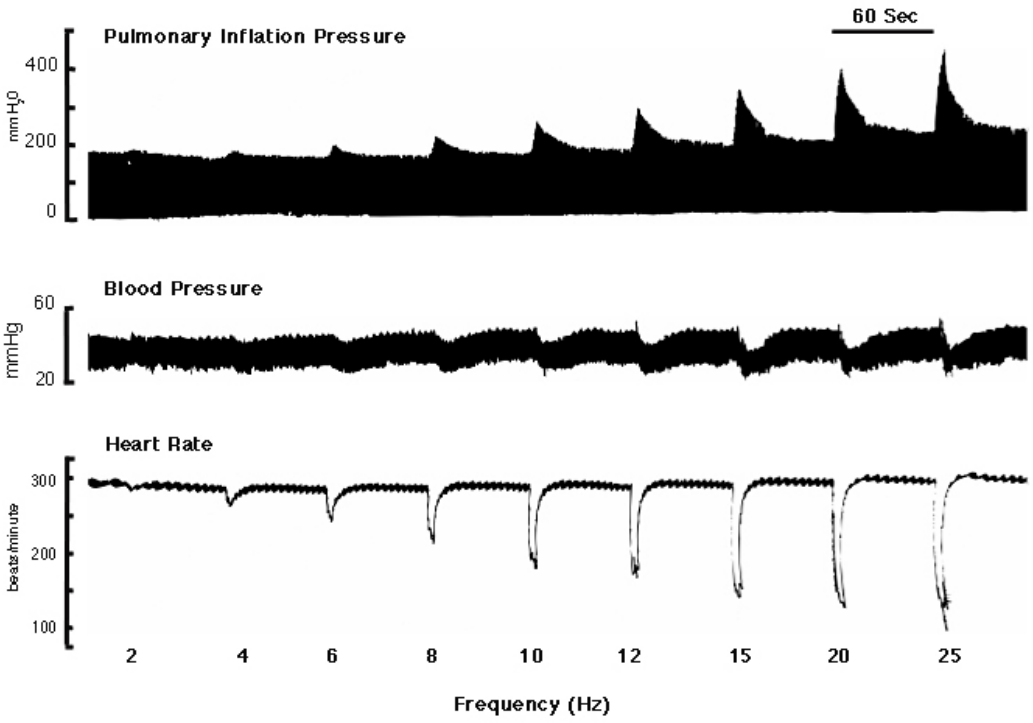


Figure 2.3. Electrical stimulation of both vagus nerves (2-25Hz, 10V, 0.2ms duration, 5 sec on, 60 seconds off) caused frequency dependent increases in pulmonary inflation pressure over baseline. Electrical stimulation of the vagus nerves also caused a frequency dependent fall in blood pressure and heart rate. Figure from (Verhein 2010).

FIGURE 2.4 Example of white blood cells from bronchoalveolar lavage, blood, and bone marrow cytospin onto slides.

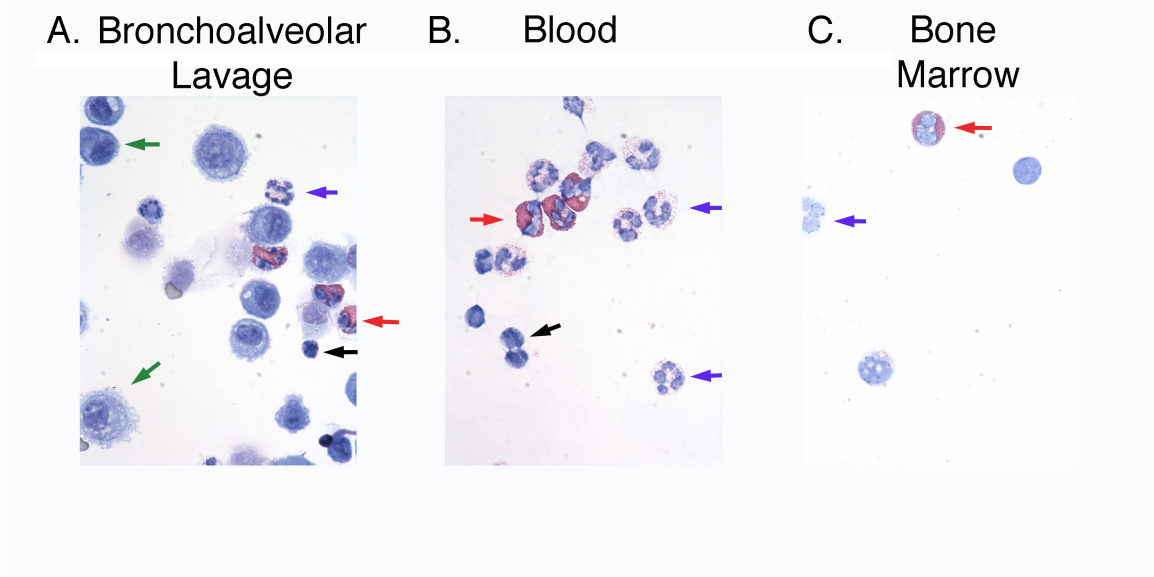


Figure 2.4 Examples of white blood cells from bronchoalveolar lavage (A), blood (B), and bone marrow (C). White blood cells were cytospun onto slides and stained with haematoxylin (blue) and eosin (red). Eosinophils are identified by bi-lobed nuclei and dense granules stained red with eosin (red arrows). Neutrophils have multi-lobed nuclei (blue arrows). Macrophages have large diffuse nuclei with large amount of cytoplasm (green arrows). Lymphocytes have dense nuclei and little to no cytoplasm (black arrows).

G. Ozone

1. Exposure

Guinea pigs were exposed to ozone or filtered air for 4 hours as described previously (Yost et al 1999). Animals were placed in individual wire cages with access to food and water in ceramic dishes. The wire cages were placed into a 700-liter stainless steel exposure chamber with laminar airflow (Figure 2.5). Ozone was generated by passing oxygen over an ultraviolet light (Orec, Glendale, CA) at a rate of 2L/min. Ozone was then pumped into the 700-liter chamber where it was mixed with HEPA filtered air. Ozone concentration in the chamber was monitored by an ozone analyzer (model 1008 AH, Dasibi Environmental, Glendale, CA). The air inside the exposure chamber was replaced 20 times per hour. Following exposure, animals were returned to the animal care facility until physiological measurements were made or inflammatory cells were harvested. The concentrations of ozone used are greater than the ozone exposure limit of 0.070 ppm averaged over 8 hours, set by the Environmental Protection Agency (The Environmental Protection Agency: https://www.epa.gov/ozone-pollution/2015-national-ambient-air-quality-standards-naaqs-ozone).

2. Control Animals

To control for potential environmental exposures discussed in more detail in section J confounding variables all animals were housed in OHSU's facility for a minimum of 5 days prior to treatment. Additionally, each shipment of guinea pigs had an un-treated air control animal. If this untreated air control guinea pig had vagally-induced

bronchoconstriction that exceeded 300 (2-25 Hz, 10 V, 0.2 ms pulse duration, 5 seconds on, 60 seconds off) the batch of animals was excluded from analysis. All of the data shown in this thesis was from animals whose batch controls were below 300 ppi.

3. Determining Ozone Concentration

Guinea pigs are obligate nose breathers, and scrub 50% of the ozone they are exposed to (Miller et al 1979). Thus, a guinea pig exposed to 2.0 ppm ozone inhales a concentration closer to 1.0 ppm. Ozone is known to cause airway inflammation and hyperreactivity (Inoue et al 2000, Matsubara et al 1995, Verhein et al 2015, Yost et al 1999, Yost et al 2005). However, no study has determined the lowest concentration of ozone that causes airway hyperreactivity and inflammation in the lungs of guinea pigs.

0.1 ppm ozone

Animals were exposed to 0.1 ppm ozone for 4 hours. One day later, airway physiology was measured and inflammatory cells in bronchoalveolar lavage were counted. This dose of ozone failed to cause airway hyperreactivity (Figure 2.6) and inflammation in the bronchoalveolar lavage (Figure 2.7) one day after ozone. This dose of ozone was not suitable for experiments, since it failed to induce airway hyperreactivity and inflammation.

0.2 ppm ozone

Animals were exposed to 0.2 ppm ozone for 4 hours. One day later, airway physiology was measured and inflammatory cells in bronchoalveolar lavage were counted. This dose of ozone caused airway hyperreactivity (Figure 2.6) but failed to increase inflammation in the bronchoalveolar lavage (Figure 2.7) one day after ozone. This dose of ozone was not suitable for experiments, since it failed to induce inflammation in guinea pigs.

2.0 ppm ozone

Animals were exposed to 2.0 ppm ozone for 4 hours. One day later airway physiology was measured and inflammatory cells in bronchoalveolar lavage were counted. This dose of ozone caused airway hyperreactivity (Figure 2.6) and inflammation in the bronchoalveolar lavage (Figure 2.7) one day after ozone. Thus, this dose of ozone was selected for experiments designed to measure ozone-induced airway hyperreactivity and recruitment of newly divided cells to the lungs.

Figure 2.5 Ozone machine

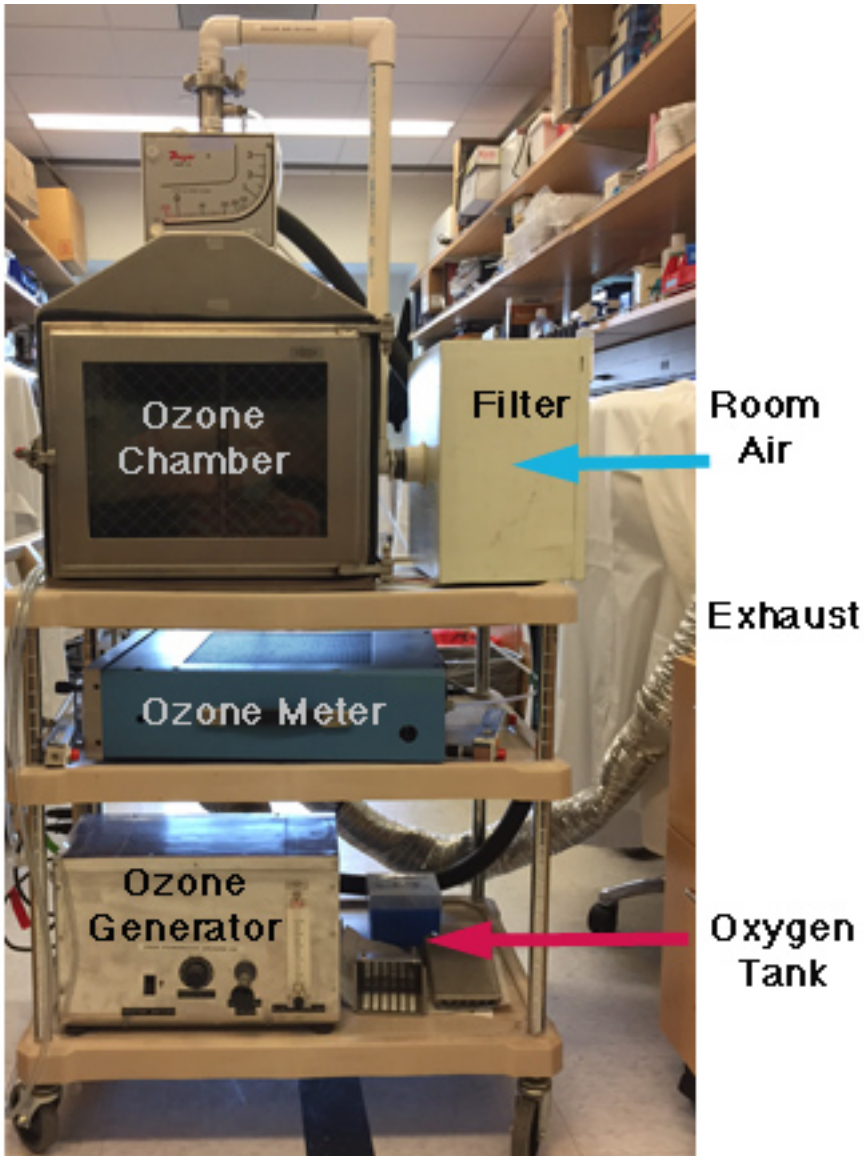


Figure 2.5. Guinea pigs are placed into stainless steel cages with access to food and water. Oxygen is pumped through the ozone generator and into the ozone chamber where it mixes with filtered room air. The concentration of ozone is monitored by the ozone meter, which pulls air from the chamber. A concentration of 0.1-2.0 ppm is maintained. The ozone chamber is exhausted through a fume hood.

FIGURE 2.6. Vagally-induced bronchoconstriction was significantly increased by 0.2 and 2.0 ppm ozone.

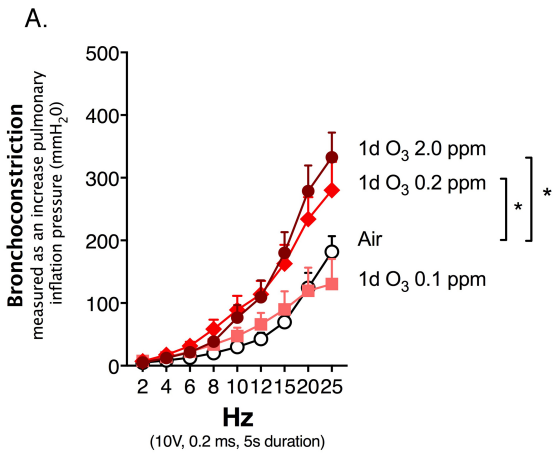


Figure 2.6 Vagally-induced bronchoconstriction was significantly increased by 0.2 and 2.0 ppm ozone (O_3). Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bronchoconstriction that was significantly increased by 0.2 ppm (diamonds) and 2.0 ppm ozone (closed circles, A) compared to air (open circles, A). Data shown as mean ± SEM n=4-7 *indicates P ≤0.05 by repeated measures 2-way ANOVA.

FIGURE 2.7. 2.0 ppm ozone increases inflammatory cells in bronchoalveolar lavage one day after ozone exposure.

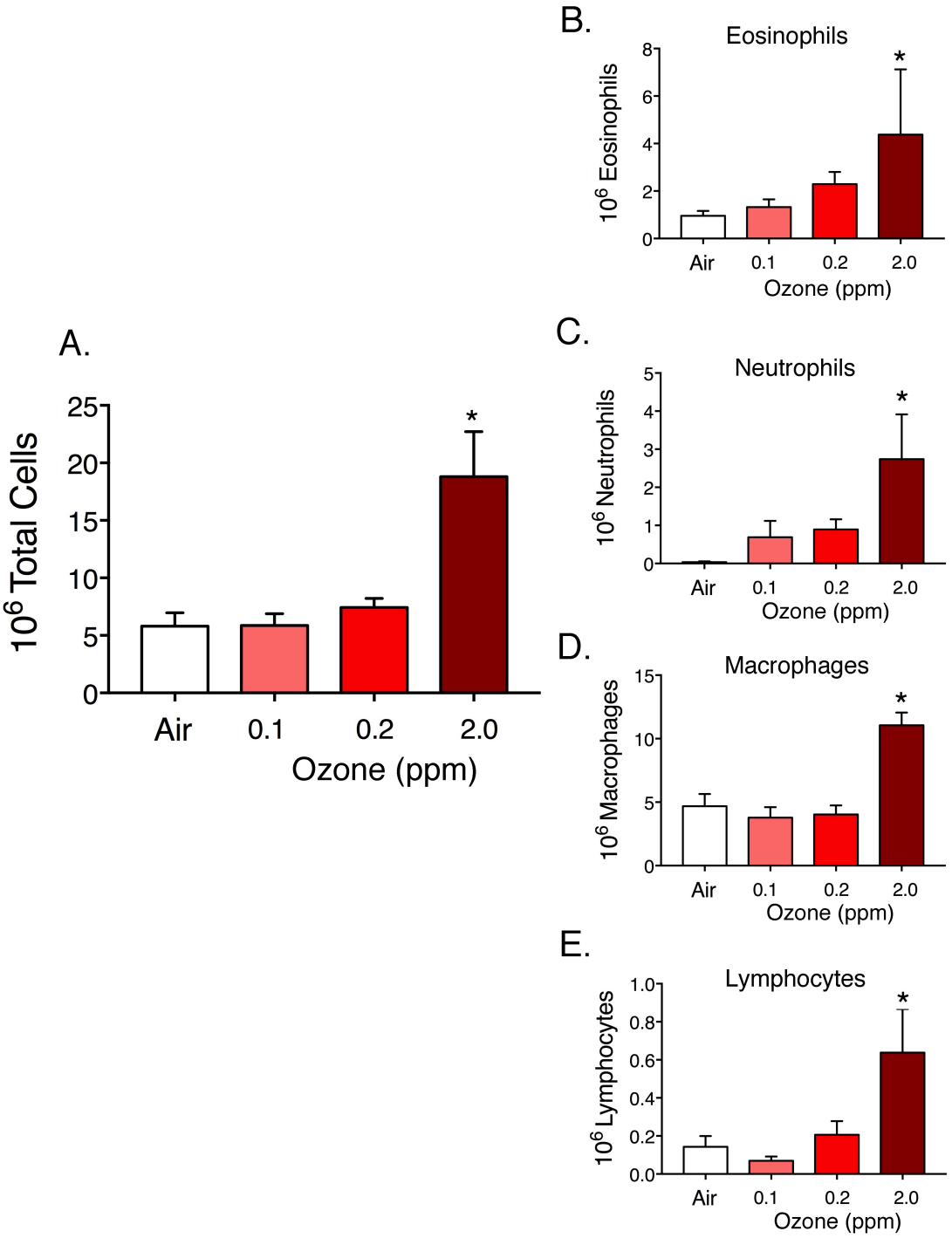


Figure 2.7 Inflammatory cells in bronchoalveolar lavage were significantly increased by 2.0 ppm ozone (O_3) 1 day after exposure. One day after exposure to 2.0 ppm ozone (4h), total inflammatory cells (A), eosinophils (B), neutrophils (C), macrophages (D), and lymphocytes (E) were significantly increased in bronchoalveolar lavage. Data shown as mean ± SEM n=4-7 *indicates $P \leq 0.05$ by one-way ANOVA comparing selected pairs of columns with a bonferroni correction.

I. Pretreatments of animals before ozone exposure

1. 5-bromo-2-deoxyuridine (BrdU)

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. BrdU (10 mg/ml) was dissolved in sterile PBS while shaking at 37°C.

i. Determining BrdU concentration

As there is little agreement in the literature over the dose and timing for BrdU (Cameron & McKay 2001, Erjefalt et al 1995, Huang et al 2012), the following experiments were carried out to identify a working concentration of BrdU in guinea pigs.

200 mg/kg +150 mg/kg/day BrdU

Animals received BrdU (100 mg/kg i.p) immediately before ozone or air and again 2 hours after completion of exposure. Daily thereafter, animals received (50mg/kg i.p.) in the morning and (100 mg/kg i.p.) in the evening. This dose of BrdU (labeled 'high' BrdU in figures below), was not suitable for experiments on hyperreactivity and inflammation because it prevented ozone induced airway hyperreactivity (Figure 2.8 B), likely because it also inhibited recruitment of total inflammatory cells (Figure 2.8 C) and of eosinophils (Figure 2.8 D) to the bronchoalveolar lavage three days after exposure.

100 mg/kg + 50 mg/kg/day BrdU

Animals received BrdU (50mg/kg i.p.) immediately before ozone or air exposure, and again 2 hours after completion of exposure. Daily thereafter, animals received (50mg/kg i.p.) in the morning. This dose of BrdU (labeled low BrdU on the graphs) was shown to label newly divided cells without impacting neural function (Figure 2.8 A,B), inflammatory cell recruitment (Figure 2.8 C) or eosinophil recruitment (Figure 2.8 D) to the bronchoalveolar lavage. Thus, this dose of BrdU was selected for experiments designed to measure ozone-induced hematopoiesis and recruitment of newly divided cells to the lungs (Figure 2.9).

FIGURE 2.8 High (*200 mg/kg +150 mg/kg/day BrdU*) dose of BrdU interfere with ozone induced airway hyperreactivity and inflammatory cell recruitment to bronchoalveolar lavage.

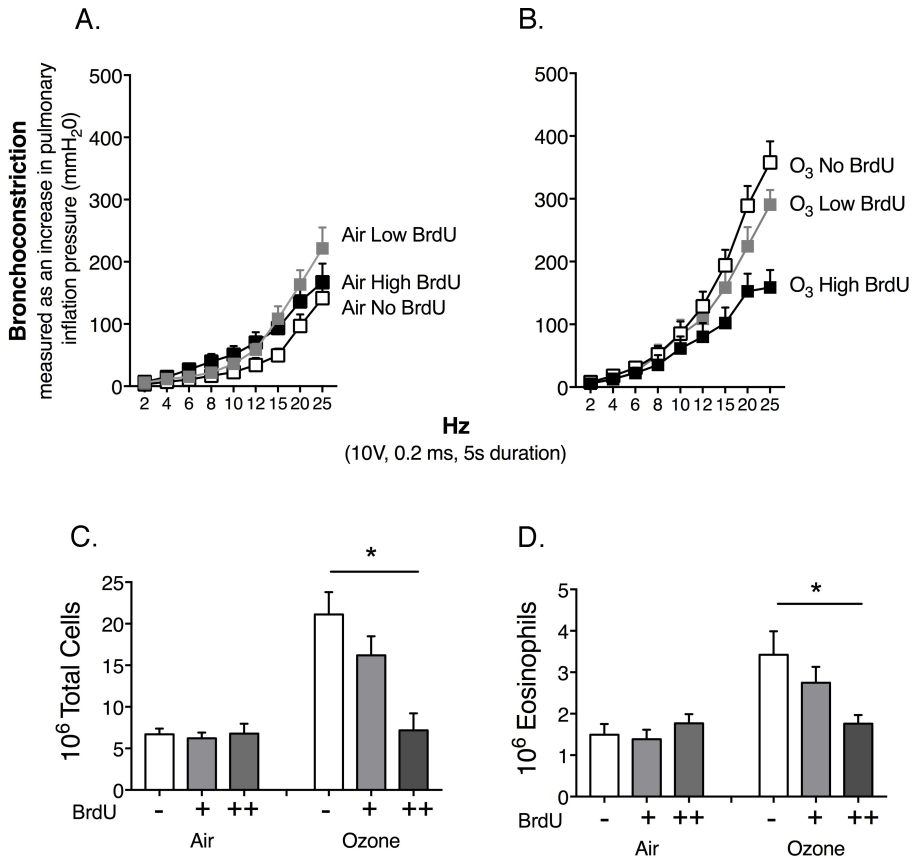


Figure 2.8 High (200 mg/kg +150 mg/kg/day BrdU) dose of BrdU inhibits vagally-mediated airway hyperreactivity and inflammatory cell recruitment to bronchoalveolar lavage three days after ozone. In air-exposed guinea pigs, BrdU has no effect on vagally mediated bronchoconstriction (A). Three days after ozone, low doses of BrdU (*100 mg/kg + 50 mg/kg/day BrdU*) have no effect on ozone-induced

bronchoconstriction, but high doses of BrdU attenuate vagally-mediated airway hyperreactivity (B, black) when compared to non BrdU treated animals (B, white). In air exposed guinea pigs, BrdU has no effect on total inflammatory cell (C) or eosinophil (D) recruitment to bronchoalveolar lavage. In contrast, 3 days after ozone high doses of BrdU (++) attenuate ozone induced increase in total inflammatory cells (C, black) and eosinophils (D, black) recruitment to bronchoalveolar lavage. Data shown as mean ± SEM n=6-11 *indicates $P \leq 0.05$ by one-way ANOVA comparing selected pairs of columns with a bonferroni correction.

FIGURE 2.9 BrdU Administration

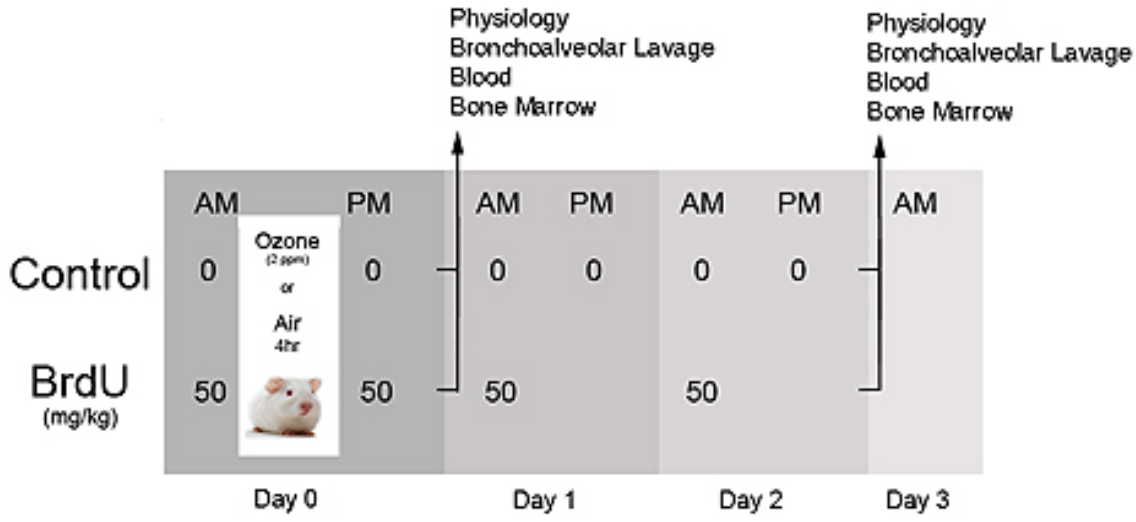


Figure 2.9 To measure ozone-induced hematopoiesis and recruitment of newly divided cells to the lungs animals received BrdU (50mg/kg i.p.) immediately before ozone or air exposure, and again 2 hours after completion of exposure. On day one airway physiology was measured. If animals were taken to the three day time point animals received two additional doses of BrdU (50 mg/kg) in the morning on day one and day two. On day three, airway physiology and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage.

2. Antibody to Interleukin 5 (AbIL5)

Some guinea pigs were pretreated with an antibody to IL5 (AbIL5) (TRFK5; 500ug/kg body weight i.p.) three days before ozone or air to deplete eosinophils. This dose was chosen because it has been shown to effectively deplete eosinophils in guinea pigs (Proskocil et al 2008, Yost et al 1999, Yost et al 2005). Eosinophil depletion was confirmed in the experiments described in this dissertation by counting eosinophils in bronchoalveolar lavage and bone marrow.

3. Sensitization to antigen

Some guinea pigs (150-200g) were sensitized to ovalbumin (4.2 mg i.p., Grade II, Sigma), dissolved in sterile saline, at 21, 19, and 17 days before exposure to air or ozone. No guinea pigs were challenged with antigen. This method has been used to understand the mechanism of airway hyperreactivity in sensitized and challenged guinea pigs. While no animals were challenged in this thesis, this sensitization protocol leads to the development of antigen specific IgE antibodies (Handley et al 1992) and airway hyperreactivity upon re-exposure to ovalbumin (Buels et al 2012, Nie et al 2009).

4. Etanercept

To inhibit TNFα, guinea pigs were pretreated with etanercept (3mg/kg i.p.), a TNFα receptor IgG fusion protein, 3 hours prior to exposure to ozone or air. The half-life of etanercept is 4 days (Dauden 2010, Sumbria et al 2013, Zhou 2005), so it was

administered only once. This dose and timing protocol has been shown to effectively prevent airway hyperreactivity following antigen challenge (Nie et al 2009), viral infection (Nie et al 2011), and organophosphorus pesticide exposure (Proskocil et al 2013) in guinea pigs. In our own laboratory, etanercept has not had any effect on bronchoconstriction induced by vagally stimulation or iv agonists, in 16 air exposed control animals in studies done within the past 6 years (Nie et al 2009, Proskocil et al 2013). Therefore, control air exposed animals were not treated with etanercept in these experiments.

I. Staining for BrdU in inflammatory cells

Cells were cytospun onto slides in 2 spots and fixed in 70% ethanol (overnight, 4°C). Endogenous peroxidase activity was quenched with 3% H_2O_2 in cold methanol (-20°C) for 10 min. Slides were washed (3 times, 2 min) with diH_2O. Cell spots were outlined with immEdge hydrophobic barrier pen. To reduce surface tension, slides were rinsed once with 0.05% Tween20 in diH_2O. BrdU labeling was detected using a BrdU staining kit (Life technologies). DNA was denatured using dilute HCl (reagent 2, BrdU staining kit, Life technologies) for 30 min, and slides were washed (3 times, 2 min each wash with 0.05% Tween20 in PBS. Slides were blocked with sodium azide (reagent 3, BrdU staining kit, Life technologies) for 10 minutes, and the upper spot was incubated for 1 hour with mouse anti-BrdU biotinylated Ab (reagent 4, BrdU staining-kit, Life Technologies). As a negative control, the lower spot remained in block (reagent 3, BrdU staining kit, Life technologies) Slides were washed (3 times, 2 min) with 0.05%

Tween20 in PBS. BrdU staining was amplified using ABC Elite kit (Vector) for 30 minutes. Slides were washed (3 times, 2 min) with 0.05% Tween in PBS. BrdU staining was visualized with ImmPACT DAB (Vector) reacted for 10 minutes. Slides were washed (3 times, 2 min) in diH_2O. Slides were then stained with Hemacolor for differential counts. Slides were allowed to dry, sealed with Cytoseal 60, and covered with a 22 x 50 mm coverslip. Examples of BrdU staining are shown in Figure 2.10.

J. Confounding variables

In every animal shipment, I always expose at least one animal to air so I have an internal control for each shipment. In the summer of 2012, in three separate shipments of guinea pigs, all air exposed, control animals were hyperreactive, i.e. bronchoconstriction induced by vagal stimulation in air-exposed animals was identical to that in ozone-exposed guinea pigs. Upon contacting the supplier, I learned that guinea pigs were routinely shipped from breeding facilities in either North Carolina or New York, but that the site was random.

Upon receiving that information, I examined public data on ozone levels from the Kingston, NY or Raleigh, NC (Data provided by North Carolina Department of Environmental and Natural Resources Division of Air Quality, and EPA AIR NOW website public data https://www.airnow.gov/index). Environmental ozone levels were high in North Carolina in June compared to New York (Figure 2.11 A). When animal shipments for that whole summer were separated by breeding facility, it was clear that

all shipments containing hyperreactive air control animals originated from North Carolina (Figure 2.11 B) and all had been shipped during the period of high ozone.

Although interesting, I did not pursue accumulating additional data on environmental exposure to ozone, and I discarded all data collected from guinea pigs originating in North Carolina. Since then, I have only used guinea pigs originating from the New York facility and hence vagally-induced airway reactivity of the air controls has been restored to historical levels.

K. Data analysis and statistics

All data are expressed as mean ± SEM. Frequency and methacholine dose response curves obtained in anesthetized animals were analyzed using a repeated measures two-way analysis of variance (ANOVA). Bronchoalveolar lavage, blood, bone marrow, and baseline data each were analyzed by one-way ANOVA comparing selected columns with a Bonferroni correction (Bewick et al 2004, Hazra & Gogtay 2016). Statistical probability of $P \leq 0.05$ was considered significant. Analyses were made with GraphPad prism.

FIGURE 2.10 BrdU Staining of cells in bone marrow and BAL.

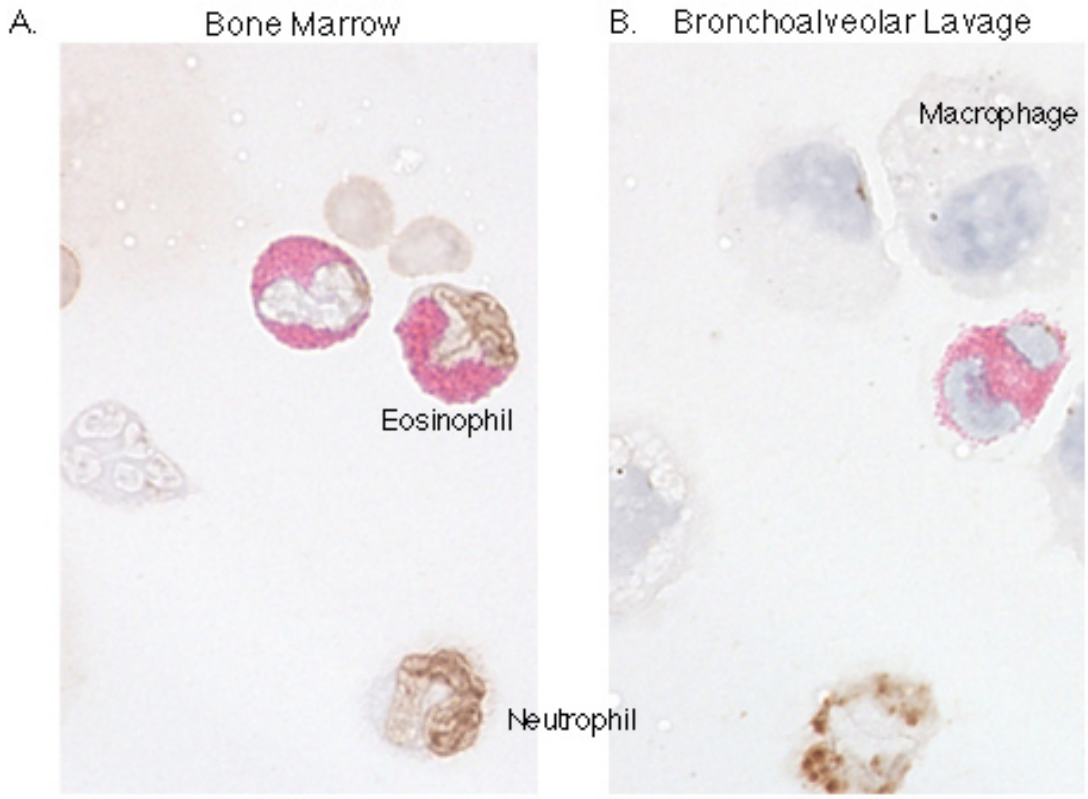


Figure 2.10 Newly divided cells have incorporated BrdU, a thymidine analogue, into the DNA of dividing cells. BrdU+ cells (with brown nucleus) are shown in bone marrow (A), and bronchoalveolar lavage (B) of guinea pigs. Eosinophils are identified by granules stained red with eosin. In A, there is a BrdU- and a BrdU+ eosinophil, and a BrdU+ neutrophil. In B, there are 2 BrdU- macrophages (at the top half), and a BrdU- eosinophil, as well as a BrdU+ neutrophil.

FIGURE 2.11 Effect of environmental ozone on airway reactivity

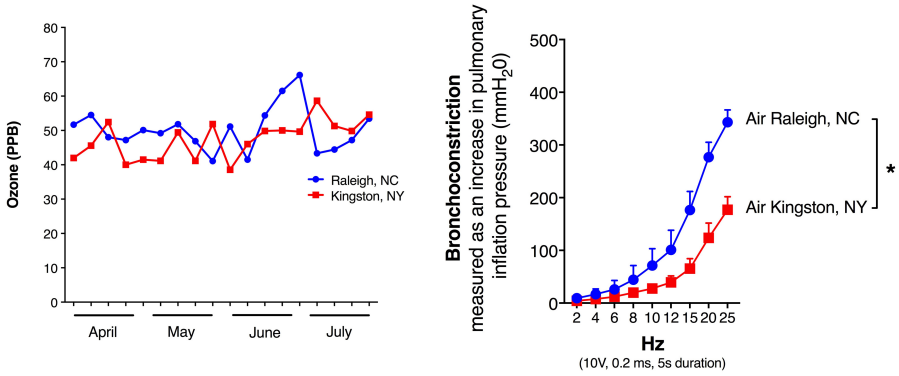


Figure 2.11 Guinea pigs were shipped to Oregon from 2 different breeding facilities, either Raleigh, North Carolina (blue), or Kingston, New York (red) (same supplier). The environmental ozone levels for Raleigh, North Carolina (A, Blue) or Kingston New York (A, Red) were measured from April to August 2012. Upon arrival, guinea pigs were exposed to air and one-day later airway reactivity was measured. Animals from Raleigh, North Carolina (B, Blue) were more responsive to vagal stimulation than animals originating from Kingston, New York (B, Red). Animals were shipped in June/July when ozone levels in Raleigh, North Carolina were high. Raleigh, NC n=3 Kingston, NY n=7, * statistically different by 2 way repeated measures ANOVA.

Table 2.1

Pharmacological Reagents		
Drug	**Product Number**	**Supplier**
Acetyl-β-methylcholine chloride	A2251	Sigma-Aldrich
Acetylcholine chloride	A5253	Acros organics
Antibody to interleukin 5 (TRFK5)	554393	BD Pharmagen
Atropine sulfate salt hydrate	A0257	Sigma-Aldrich
5-Bromo-2′-deoxyuridine	B5002	Sigma-Aldrich
Etanercept (embrel)	58406-425-01	Amgen
Guanethidine sulfate	G2494	Bosche Scientific
Ovalbumin (Grade II)	A5253	Sigma-Aldrich
Succinylcholine chloride	S-8251	Sigma-Aldrich
Urethane	U2500	Sigma-Aldrich

Table 2.2

Immuno staining reagents		
Reagent	**Product Number**	**Supplier**
BrdU staining kit	933944	Life Technologies
Cytoseal 60	8310	Richard-Allen
200 proof Ethanol	2716	Decon
Hemacolor	65944A	Harleco
ImmEdge hydrophobic pen	4100	Vector
Microscope cover glass	12-544-D	Fisher Scientific
Superfrost plus microscope slides	12-550-15	Fisher Scientific
Tween-20	BP337	Fisher Scientific
Vectastain ABC Kit	PK-6100	Vector
Vector Immpact DAB	SK-4105	Vector

CHAPTER III

OZONE INDUCES EOSINOPHIL HEMATOPOIESIS IN BONE MARROW AND RECRUITS NEWLY DIVIDED EOSINOPHILS TO THE LUNGS

ABSTRACT

Background: Ozone causes airway hyperreactivity that is mediated by the vagus nerves and lasts at least three days. Simultaneously, ozone also recruits inflammatory cells, including eosinophils to the lungs over this same time course. One day after ozone, eosinophils mediate ozone-induced airway hyperreactivity. In contrast, three days after ozone, eosinophils in the lungs are protective.

Objective: Here I tested whether ozone induces hematopoiesis of eosinophils and other inflammatory cells in bone marrow one and three days after ozone and tested whether newly divided or mature eosinophils, differently affect vagally-mediated bronchoconstriction.

Methods: Guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU) to label newly divided cells and then were exposed to air or 2 ppm ozone for 4 hours. One or three days later, vagally-induced bronchoconstriction was measured and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage and quantified.

Results: Ozone significantly increased vagally-mediated bronchoconstriction one and three days after ozone. Ozone also significantly increased eosinophils in bronchoalveolar lavage on both days. On both one and three days after ozone the increase in eosinophils was predominantly comprised of newly divided eosinophils (i.e.

these were BrdU+ eosinophils). Newly divided eosinophils were significantly increased in bone marrow one and three days after ozone; other inflammatory cell types were not similarly increased. Depleting eosinophils with antibody to interleukin 5 (AbIL5) three days after ozone significantly increased vagally-mediated bronchoconstriction.

Conclusion: Ozone causes airway hyperreactivity lasting at least three days. Ozone induces eosinophil hematopoiesis and recruitment to lungs where three days later, newly divided eosinophils are protective.

INTRODUCTON

Ozone exposure causes airway hyperreactivity that lasts at least three days in humans and animals (Gambone et al 1994, Verhein et al 2011, Verhein et al 2013, Yost et al 1999, Yost et al 2005). Ozone also causes lung inflammation in humans (Dokic & Trajkovska-Dokic 2013, Hernandez et al 2010b, Hiltermann et al 1999, Leroy et al 2015) and animals (Plopper et al 2012, Stevens et al 1995b, Yost et al 1999, Yost et al 2005).

In humans, ozone induced lung inflammation is typically characterized by neutrophilic influx (Aris et al 1993, Koren et al 1989, Krishna et al 1998) into bronchoalveolar lavage that peaks between 6 and 24 hours after ozone (Schelegle et al 1991). However, neutrophils depletion does not prevent epithelial cell damage (Pino et al 1992), or ozone-induced airway hyperreactivity (Li et al 1992, Stevens et al 1994). Thus, while neutrophils may be increased in the lungs of ozone exposed humans and animals, they do not contribute to ozone-induced airway hyperreactivity.

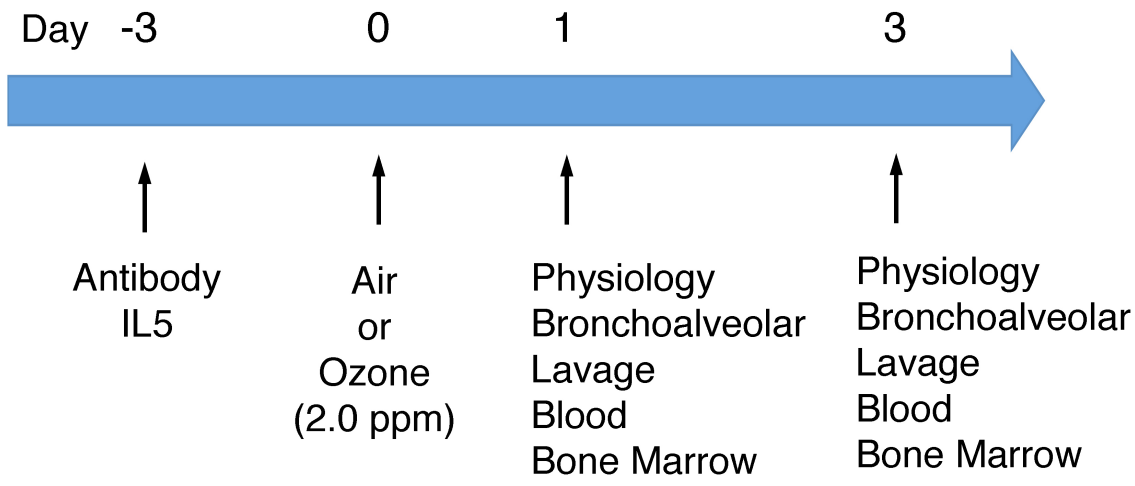
Ozone increases eosinophils in the bronchoalveolar lavage one and three days after exposure between these two days eosinophils return to baseline (Yost et al 2005). It is known that eosinophils mediate ozone-induced airway hyperreactivity one day after ozone as blocking eosinophil recruitment, activation, or neutralizing eosinophil major basic protein, each prevented ozone induced airway hyperreactivity (Gambone et al 1994, Yost et al 1999). In contrast, three days after ozone, depleting eosinophils or

preventing their migration into the lungs not only fails to prevent hyperreactivity, but also significantly worsens it (Yost et al 2005). This demonstrates that three days after ozone, the role of eosinophils in the lungs has changed from deleterious to protective in guinea pigs.

The role of eosinophils in the lungs of humans with asthma, specifically allergic asthma, is well established (Adamko et al 2003, Yamada et al 2000, Zeiger et al 2017). Although, the specific roles of eosinophils in human lung following ozone exposure is not known, the general approach to treating all airway hyperreactivity is to reduce inflammation using steroids. This is not uniformly effective in ozone exposure (Stevens et al 1994). In one instance, asthmatic children exposed to ozone actually had exacerbated bronchoconstriction when lung inflammation was reduced with steroids (Ierodiakonou et al 2016), suggesting that some component of lung inflammation may also be beneficial after ozone in humans.

Here I tested whether ozone induced eosinophilia in the lungs, is associated with hematopoiesis in bone marrow. I used BrdU to label newly divided cells and measured whether ozone increased newly divided BrdU+ eosinophils and whether BrdU+ or BrdU- eosinophils were dominate in the lungs, and differently affected vagally-induced bronchoconstriction in guinea pigs one and three days after ozone.

EXPERIMENTAL PROTOCOL



Some animals were pretreated with antibody to IL5 three days prior exposure to ozone (2.0 ppm) or air. To measure which cells were dividing after ozone or air, some guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU). Animals received BrdU (50mg/kg i.p.) immediately before ozone (2.0 ppm 4hrs) or air exposure, and again 2 hours after completion of exposure, and 50mg/kg i.p. once daily thereafter. One or three days later, physiology was measured and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage.

RESULTS

Ozone significantly increased baseline pulmonary inflation pressure from 102 ± 5 mmH_20 (air) to 203 ± 5mmH20 one and to 152 ± 6 mmH20 three days after exposure (Table 3.1). Neither baseline heart rate nor baseline blood pressure were significantly changed by ozone either one or three days after exposure (Table 3.1).

Electrical stimulation of both vagus nerves (1-25 Hz) caused frequency dependent bronchoconstriction that was significantly increased one day after a single exposure to ozone when compared to air exposed guinea pigs (Figure 3.1A). Three days after ozone, vagally-mediated bronchoconstriction was still significantly increased when compared to air exposed animals (Figure 3.1 B). However, three days post ozone vagally mediated bronchoconstriction was significantly less than one-day post ozone (Figure 3.1 green circles A to B). Ozone induced airway hyperreactivity is mediated by the vagus nerves since, in vagotomized guinea pigs methacholine induced bronchoconstriction was not changed one or three days after ozone (Figure 3.2 A&B).

In the heart, electrical stimulation of both vagus nerves (1-25 Hz) induced frequency dependent bradycardia that was not changed by ozone either one (Figure 3.3 A) or three (Figure 3.3 B) days after exposure. Similarly, intravenous methacholine also caused dose dependent bradycardia that was not changed by ozone at either time point (Figure 3.3 C- D).

Ozone significantly increased total inflammatory cells in bronchoalveolar lavage three days after ozone (Figure 3.4). This increase was comprised of a significant increase in eosinophils, neutrophils, macrophages, and lymphocytes (Figure 3.4). AbIL5 significantly reduced eosinophils in bronchoalveolar lavage three days after ozone (Figure 3.4). AbIL5 also decreased baseline pulmonary inflation pressure three days after ozone from 150 ± 6 mmH_20 to 110.4 ± 11 mmH_20 (Table 3.1). AbIL5 pretreatment did not affect baseline heart rate or blood pressure three days after ozone (Table 3.1). AbIL5 had no effect on vagally-mediated or methacholine-induced bradycardia (Figure 3.3 B & D black). In contrast, three days after ozone AbIL5 pretreatment significantly worsened vagally-mediated bronchoconstriction (Figure 3.1 B, black). Methacholine mediated smooth muscle contraction was not changed by AbIL5 three days after ozone (Figure 3.2 B), suggesting that eosinophils in lungs three days after ozone are beneficial.

To investigate newly divided inflammatory cells one and three days after ozone exposure, guinea pigs were treated with BrdU to label newly divided cells after ozone exposure. Ozone significantly increased total inflammatory cells in the bronchoalveolar lavage one and three days after ozone (Figure 3.5 A). This increase was comprised of both newly divided BrdU+ and mature BrdU- inflammatory cells (Figure 3.5 A). At both time points, eosinophils were significantly increased one and three days after ozone (Figure 3.5 B) and these increases were comprised entirely of newly divided BrdU+ cells (Figure 3.5 B, black). Neutrophils were also significantly

increased one and three days after ozone (Figure 3.5 C). One day after ozone, this increase was comprised of both newly divided, BrdU+, and mature, BrdU-, cells (Figure 3.5 C); however three days after ozone, the increase was comprised of only newly divided BrdU+ neutrophils (Figure 3.5 C). Macrophages were also significantly increased one and three days after ozone (Figure 3.5 D) and at both time points the increase was comprised of both newly divided BrdU+ and mature BrdU- cells (Figure 3.5 D). Lymphocytes were significantly increased one day after ozone, and this increase was comprised of both newly divided BrdU+ and mature BrdU- cells (Figure 3.5 E). However, three days after ozone, lymphocytes in bronchoalveolar lavage were not changed (Figure 3.5 E).

Although ozone increased inflammatory cells in the bronchoalveolar lavage, ozone did not affect the total number of circulating leukocytes in the blood, nor did it differentially affect eosinophils, neutrophils, monocytes, or lymphocytes in circulation (Figure 3.6). The number of newly divided, BrdU+ and mature BrdU- inflammatory cells in ozone-exposed animals was not different from air exposed animals in any cell type (Figure 3.6).

In bone marrow, ozone did not change the total number of leukocytes one or three days after ozone (1 day air 2.3 ± 0.32 x 10^8, 1 day ozone 2.5 ± 0.4 x 10^8, 3 day air 2.6 ± 0.21 x 10^8, 3 day ozone 1.9 ± 0.21 x 10^8). Neither did ozone increase the percent of eosinophils, neutrophils, or monocytes in bone marrow one or three days after

exposure (Figure 3.7). However, ozone significantly increased the percent of newly divided BrdU+ eosinophils one and three days after exposure (Figure 3.7 A, black). The percent of newly divided BrdU+ eosinophils significantly increased between one and three days after ozone (Figure 3.7 A). The significant increase in newly divided BrdU+ eosinophils at three days was mirrored by a significant decrease in mature BrdU- eosinophils (Figure 3.7A). The percent of newly divided BrdU+ neutrophils and monocytes were not changed one or three days after ozone exposure (Figure 3.7 B&C).

FIGURE 3.1. Ozone causes airway hyperreactivity lasting three days that is potentiated by AbIL5

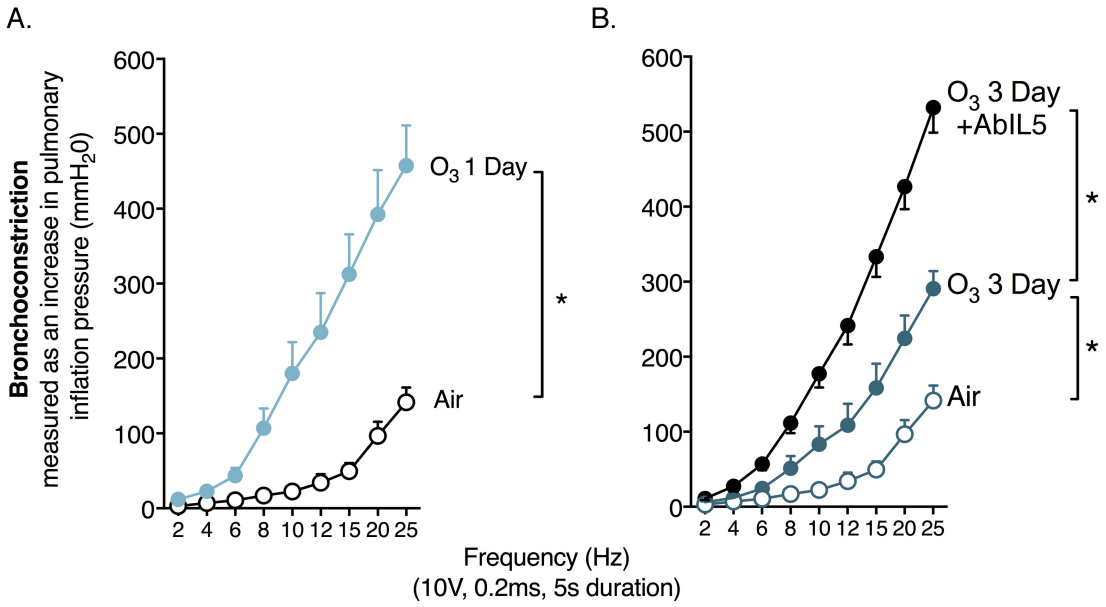


Figure 3.1. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train, at one min intervals) produced frequency dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A & B, open circles, Air). Vagally-induced bronchoconstriction was significantly increased one (A, green) and three (B, green) days after a single ozone exposure (2.0ppm, 4h) when compared to air control (note same controls were used in A&B). Pretreatment with AbIL5 significantly increased ozone-induced airway hyperreactivity three days after exposure (B, Black). Data shown as mean ± SEM, n=4-9 *indicates $p \leq 0.05$ by repeated measures two-way ANOVA.

FIGURE 3.2. Methacholine-induced bronchoconstriction is not changed by ozone or by AbIL5

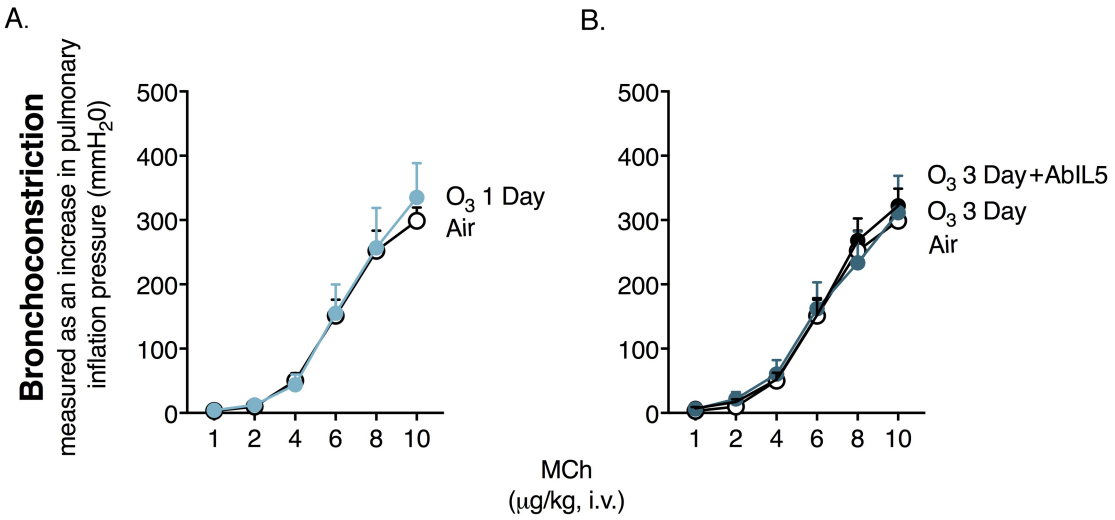


Figure 3.2. Methacholine (1-10 ug/kg i.v.) causes a dose dependent increase in bronchoconstriction (A & B, Air) in vagotomized animals. Methacholine-induced bronchoconstriction was not affected by ozone (O_3) one (A, green) or three (B, green) days after exposure. Pretreatment with AbIL5 had no effect on methacholine-induced bronchoconstriction three days after ozone (B, black). Data shown as mean ± SEM, n= 4-9 guinea pigs.

FIGURE 3.3 Vagally-induced and methacholine-induced bradycardia were not changed one or three days after ozone.

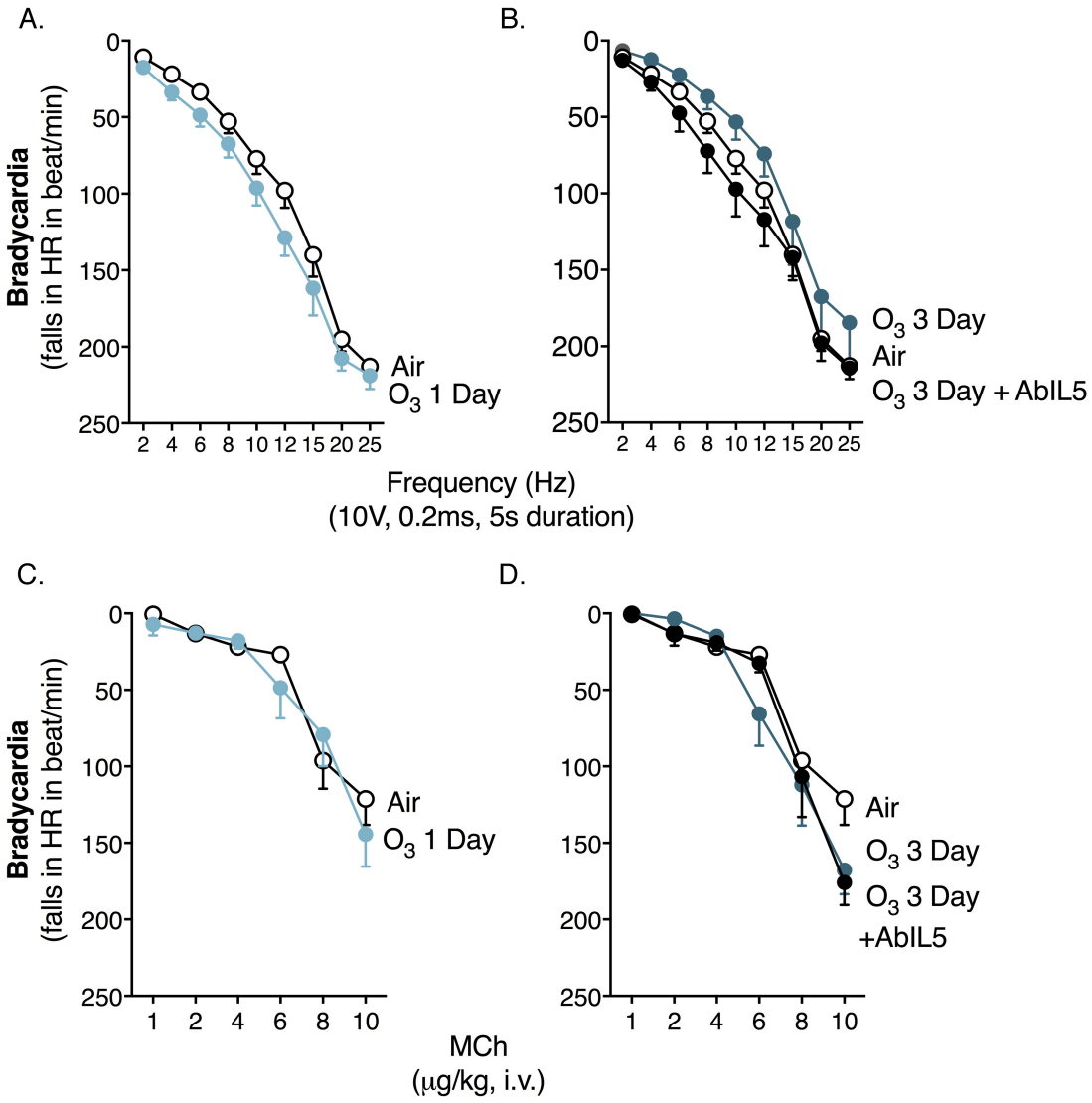


Figure 3.3. Vagally-induced (A&B) and methacholine-induced (C&D) bradycardia was not changed 1 or 3 days after ozone (O_3, 2.0ppm, 4h). Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency

dependent bradycardia that was not affected by ozone 1 (A, green) or 3 days (B, green) later. Methacholine-induced bradycardia (1-10ug/kg i.v.) was not changed one (C) or three (D) days post ozone. Pretreatment with AbIL5 had no effect on vagally-induced (B, black) or methacholine-induced (D, black) bradycardia three days after ozone. Data shown as mean ± SEM n=4-9.

FIGRUE 3.4 Antibody to IL-5 inhibited the ozone-induced increase in eosinophils in bronchoalveolar lavage three days after ozone.

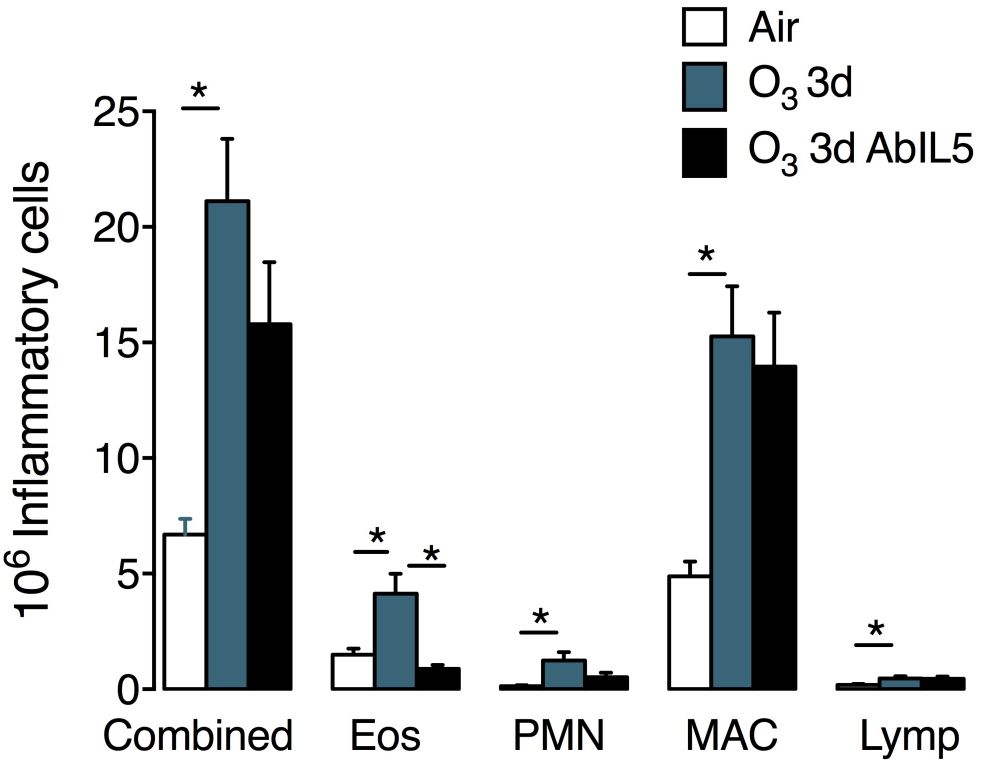


Figure 3.4 Three days after exposure, ozone (O_3) significantly increased the total number of inflammatory cells (Combined) in the bronchoalveolar lavage, which was reflected by a significant increase in eosinophils (Eos), neutrophils (PMN), macrophages (MAC), and lymphocytes (Lymp). Antibody to IL5 (AbIL5) significantly decreased eosinophils in ozone-exposed guinea pigs. Data are mean ± SEM, n=5-7 *Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$).

FIGURE 3.5 Ozone significantly increased newly divided inflammatory cells in bronchoalveolar lavage one and three days later.

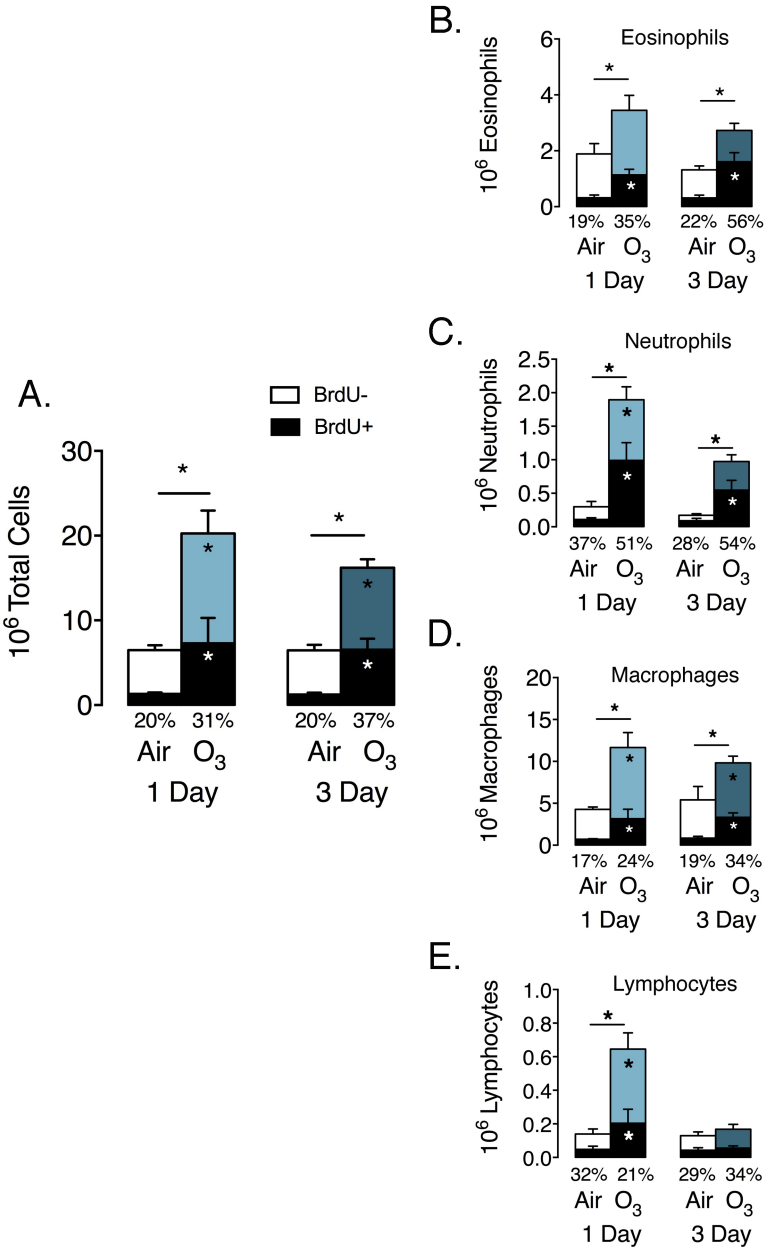


Figure 3.5 Ozone (O3, 2.0ppm, 4h) significantly increased inflammatory cells in bronchoalveolar lavage one and three days later (A). All cell types, including eosinophils (B), neutrophils (C), macrophages (D) and lymphocytes (D) were significantly increased in bronchoalveolar lavage one and three days after ozone exposure. Note in this figure, the entire column height represents the total number of cells that is then subdivided into mature BrdU- (white/green) and newly divided BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. The increase in eosinophils was comprised only of BrdU+ cells, while the increase in neutrophils, macrophages, and lymphocytes was comprised of both BrdU+ and BrdU- cells. Three days after ozone, eosinophils (B), neutrophils (C), and macrophages (D) but not lymphocytes (E) in were increased in bronchoalveolar lavage. The increase in neutrophils and eosinophils was comprised only of BrdU+ cells, while the ozone induced increase in macrophages was comprised of both BrdU+ and BrdU- cells. Data shown as mean ± SEM, n=8-9. Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions.

FIGURE 3.6 Ozone had no effect on circulating leukocytes one or three days after ozone

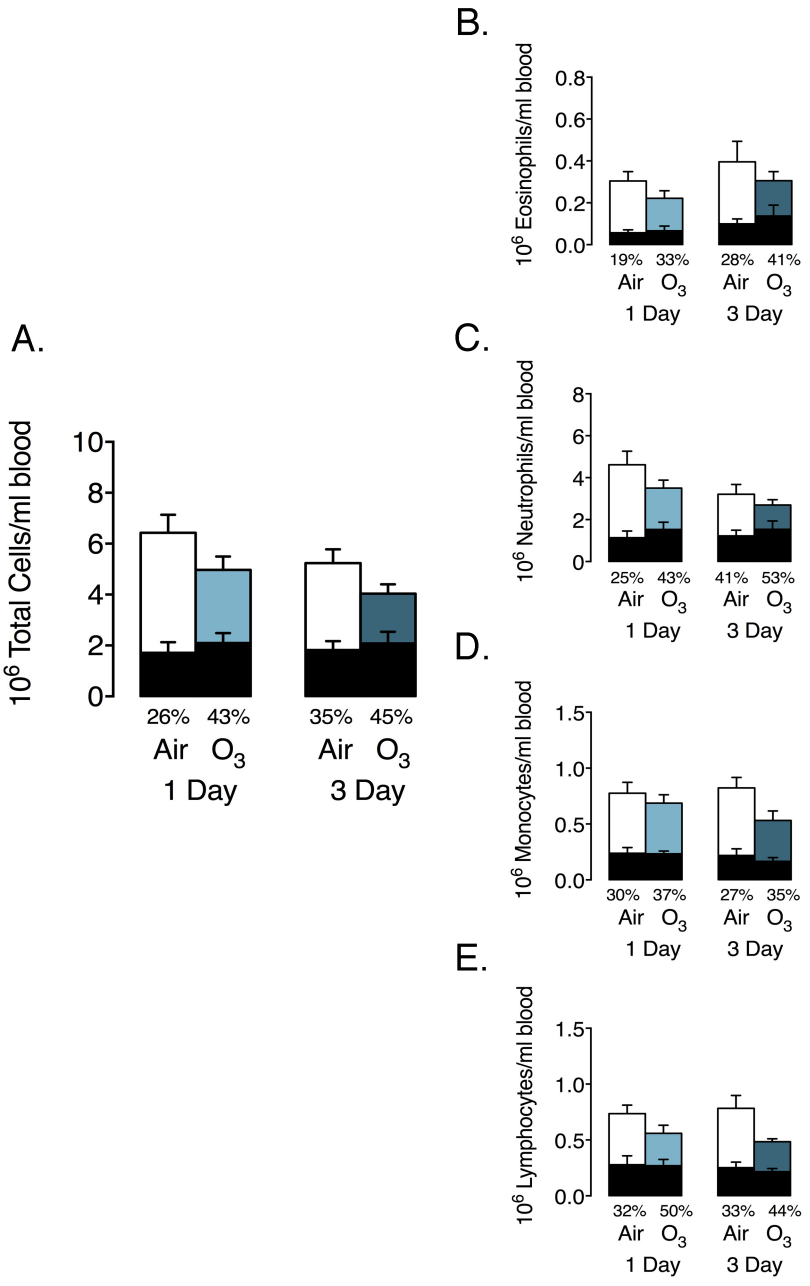


Figure 3.6 Ozone had no effect on total circulating leukocytes (A), eosinophils (B), neutrophils (C), monocytes (D), and lymphocytes (E) in blood one or three days after exposure. Note, as in figure 3.3 the overall column height represents the total cell number, which is then subdivided into mature BrdU- (white/green) and newly divided BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. Data shown as mean ± SEM, n=8-9.

FIGURE 3.7 Ozone-induced eosinophil hematopoiesis one and three days after exposure.

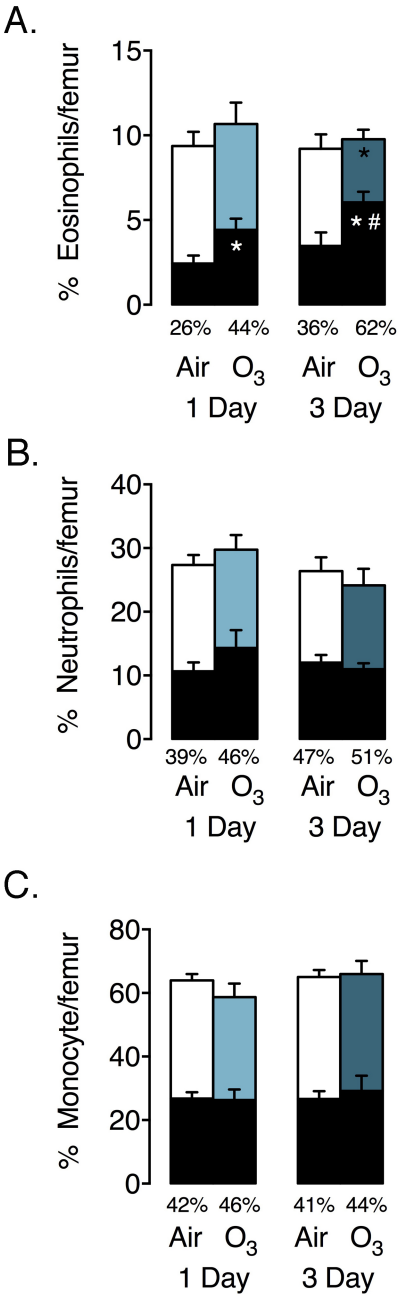


Figure 3.7. Ozone significantly increased BrdU+ (black) eosinophils in bone marrow one day after ozone. Three days after ozone, the percent of BrdU+ eosinophils was increased while the percent of BrdU- eosinophils was decreased. The increase in BrdU+ eosinophils was significantly greater three days after ozone when compared to one day after ozone (A). Neutrophils (B) and monocytes (C) in were not changed by ozone one or three days after exposure. Note as in figure 3.3, the overall column height represents the total number of cells which is then subdivided to show BrdU- (white/green) and BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. Data shown as mean ± SEM, n=8-9. Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions. *significantly different from air, #significantly different from one day ozone.

TABLE 3.1 Baseline Values

Table 3.1 Baseline values										
	Baseline PPI (mmH_2O)		**Heart Rate** (bpm)		**Systolic BP** (mmHg)		**Diastolic BP** (mmHg)		**Weight** (g)	
Air	102.2	±4.9	287.8	±9.8	41.0	±1.1	19.6	±0.7	351.3	± 5.2
O_3 1 Day	202.5	±4.8*	273.8	±5.5	46.0	±2.1	20.9	±1.0	383.5	±11.2
O_3 3 Day	151.4	±6.3*	283.6	±6.0	44.8	±2.7	24.0	±1.5	382.6	±11.0

Table 3.1 Baseline values. Values are means ± SEM. Baseline pulmonary inflation pressure was significantly increased one day and three days after a single exposure to 2.0ppm ozone (O_3; 4 hr). Baseline heart rate and blood pressure were not affected by ozone 1 or 3 days later. *Significantly different by one-way ANOVA with a Bonferroni correction ($p<0.05$).

TABLE 3.2 Percent BrdU+ Eosinophils

% BrdU+ Eosinophils		Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
1 Day	Air	19 ± 3.6	19 ± 2.6	26 ± 4.2
	O_3	35 ± 2.6*	33 ± 8.8	44 ± 6.1*
3 Day	Air	22 ± 5.6	28 ± 4.8	36 ± 5.3
	O_3	56 ± 8.5*	41 ± 8.7	62 ± 5.1*#

Table 3.2 Percent BrdU+ eosinophils. In bronchoalveolar lavage, the percent BrdU+ eosinophils were significantly increased both one and three days after ozone when compared to control. In contrast in blood, the percent of BrdU+ eosinophils was not changed by ozone at either time point. In bone marrow, the percent of BrdU+ eosinophils was significantly increased both one and three days after ozone when compared to control. The ozone-induced increase in BrdU+ eosinophils was significantly increased three days when compared to one day. Data shown as mean ± SEM, n=8-9. Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from one day ozone.

TABLE 3.3 Percent BrdU+ Neutrophils

% BrdU+ Neutrophils		Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
1 Day	Air	37 ± 7.0	25 ± 3.4	39 ± 3.7
	O_3	51 ± 8.7	43 ± 8.6	46 ± 7.7
3 Day	Air	28 ± 7.1	41 ± 5.1	47 ± 4.8
	O_3	54 ± 7.7	53 ± 8.7	51 ± 7.8

Table 3.3 Percent BrdU+ neutrophils. In bronchoalveolar lavage, blood, and bone marrow the percent of BrdU+ neutrophils was not changed one or three days after ozone. Data shown as mean ± SEM, n=8-9.

TABLE 3.4 Percent BrdU+ Macrophages

% BrdU+ Macrophages		Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
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

Table 3.4 Percent BrdU+ macrophages. In bronchoalveolar lavage, the percent BrdU+ macrophages was significantly increase both one and three days after ozone when compared to air. The percent of BrdU+ macrophages in blood, and bone marrow were not changed one or three days after ozone. Data shown as mean ± SEM, n=8-9. Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). *significantly different from air.

TABLE 3.5 Percent BrdU+ Lymphocytes

% BrdU+ Lymphocytes		Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
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

Table 3.5. Percent BrdU+ lymphocytes. In bronchoalveolar lavage, blood, and bone marrow the percent of BrdU+ lymphocytes was not changed one or three days after ozone. Data shown as mean ± SEM, n=8-9.

TABLE 3.6 Percent BrdU+ Total cells

% BrdU+ Total Cells		Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM
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

Table 3.6. Percent BrdU+ total inflammatory cells. In bronchoalveolar lavage, the percent of BrdU+ total cells was significantly increase both one and three days after ozone when compared to air. The percent of BrdU+ total cells in blood, was not changed one or three days after ozone. Data shown as mean ± SEM, n=8-9. Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). *significantly different from air.

DISCUSSION

Exposure to ozone causes airway hyperreactivity that lasts at least three days in humans (Kreit et al 1989, Peters et al 2001, Que et al 2011) and animals (Fabbri et al 1984, Gambone et al 1994, Moore et al 2012, Schelegle & Walby 2012, Stevens et al 1995b, Verhein et al 2008, Yost et al 1999, Yost et al 2005). In both human children and adults, a lag period of one to three days may precede symptom development (Chen et al 2015, Gleason et al 2014, Mar & Koenig 2009, Sheffield et al 2015). Here I confirmed, in guinea pigs, that ozone induces airway hyperreactivity one and three days after exposure.

Ozone-induced airway hyperreactivity is vagally mediated. In vagotomized animals, direct stimulation of muscarinic receptors on airway smooth muscle with methacholine caused bronchoconstriction that bypasses the vagus nerves. Methacholine-induced bronchoconstriction was not potentiated in ozone-exposed animals (Gambone et al 1994, Verhein et al 2011, Verhein et al 2008, Yost et al 1999, Yost et al 2005). I confirmed that here (Figure 3.2) Thus, I demonstrated that ozone-induced hyperreactivity is mediated by the vagus nerves since vagally mediated bronchoconstriction is significantly increased after ozone in guinea pigs (Figure 3.1).

It is well known that ozone causes lung inflammation in humans (Alexis et al 2010, Aris et al 1993, Dokic & Trajkovska-Dokic 2013, Hernandez et al 2010a, Holz et al 1999, Leroy et al 2015) and animals (Boatman et al 1974, Stevens et al 1994)

including guinea pigs (Gambone et al 1994, Schultheis et al 1994, Verhein et al 2011, Verhein et al 2008, Yost et al 1999, Yost et al 2005). Ozone-induced increase in inflammatory cells is confirmed here in guinea pigs (Figure 3.4, 3.5). A new observation is that newly divided cells comprise a significant proportion inflammatory cells in the lungs, demonstrating that ozone stimulates inflammatory cell hematopoiesis and subsequent recruitment to lungs.

Lung eosinophils were the focus of this study since their role switches from causing airway hyperreactivity one day after ozone, to protective three days later (Yost et al 1999, Yost et al 2005). One day after ozone, depleting eosinophils with AbIL5 or cyclophosphamide, blocking eosinophil migration with AbVLA4, or neutralizing eosinophil major basic protein (MBP) with either an antibody (AbMBP) or with heparin, each prevented ozone-induced airway hyperreactivity (Gambone et al 1994, Yost et al 1999, Yost et al 2005). In contrast, three days after ozone, depleting eosinophils with AbIL5 (Figure 3.4), or blocking their recruitment to the lungs(Yost et al 2005) significantly exacerbated ozone-induced airway hyperreactivity. Thus, eosinophils mediate airway hyperreactivity one day after ozone, while they are protective three days after exposure.

The temporal role of eosinophils in mediating ozone-induced airway hyperreactivity suggests that either distinct eosinophil populations exist, or that their role changes with age. There are few cell surface markers that distinguish eosinophils from other cells

(Abdala Valencia et al 2016, Percopo et al 2017). Additionally, it has been shown that eosinophil cell surface markers change as the cells migrate into lung tissue following antigen challenge (Abdala Valencia et al 2016, Percopo et al 2017). So, here I focused on eosinophil age by using BrdU to separate eosinophils into cells that had divided after ozone vs those that had divided before ozone.

One day after ozone, eosinophils in bronchoalveolar lavage are comprised predominately of BrdU- cells (i.e. mature cells). In contrast, three days after ozone, eosinophils in bronchoalveolar lavage are comprised predominantly of newly divided BrdU+ cells. The proportion of newly divided to mature eosinophils, and their role in airway hyperreactivity, changes between one and three days after ozone from mediating ozone-induced hyperreactivity at one day to protective in the lungs three days later. Thus, when mature BrdU- eosinophils are the predominant population in the lungs, eosinophils mediate ozone induced airway hyperreactivity, while when newly divided BrdU+ eosinophils are the predominant population in the lungs eosinophils are protective.

While ozone also significantly increased newly divided BrdU+ eosinophils in bone marrow, ozone did not increase the percent of BrdU+ positive neutrophils and monocytes in bone marrow. Thus, ozone significantly and specifically increased eosinophil hematopoiesis.

The ratio of newly divided BrdU+ and mature BrdU- neutrophils and monocytes were not changed in the blood or the bone marrow. However, ozone significantly increased newly divided neutrophils and macrophages in bronchoalveolar lavage both one three days after ozone. This suggests that newly divided neutrophils and macrophages may have been recruited to the lungs following ozone where they accumulate. Alternatively, inflammatory progenitors have been shown in the lungs (Denburg et al 1997, Hashimoto et al 2013). Ozone has been shown to increase macrophage maturation in the lung (Tighe et al 2016). Thus, ozone may induce maturation of neutrophils and macrophages in lung tissue where they are subsequently recruited into bronchoalveolar lavage.

Ozone is a reactive oxygen species that does not to pass the airway epithelium (Pryor 1992). Thus, ozone itself cannot directly stimulate bone marrow, but must depend upon an intermediary inflammatory signal. Ozone is known to stimulate and release a cascade of cytokines from airway epithelial cells that are capable of inducing and contributing to eosinophil hematopoiesis and/or recruitment including IL-1β, IL-1α, IL-8, granulocyte macrophage colony simulating factor (GM-CSF), and tumor necrosis factor α (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). IL-1β is also significantly increased in bone marrow after ozone exposure (Verhein et al 2008). IL-1β and IL-1α can induce the eosinophil chemotactic factor, IL-5, in bone marrow stromal cells (Hogan et al 2000). Additionally, IL-5 as well as GM-CSF stimulate eosinophil

hematopoiesis (Clutterbuck & Sanderson 1988, Hogan et al 2000). IL-1β and TNFα increase adhesion molecule expression on endothelial cells that mediate eosinophil migration into tissue (Atsuta et al 1997, Iademarco et al 1995, Raab et al 2001), while, GM-CSF, along with RANTES and IL-8 mediate eosinophil migration through endothelium and epithelium (Ebisawa et al 1994a, Ebisawa et al 1994b, Erger & Casale 1995, Liu et al 2015). GM-CSF also enhances eosinophil survival (Levi-Schaffer et al 1998). Thus, inflammatory cytokines are likely to be the systemic link between ozone, eosinophil hematopoiesis in bone marrow, and subsequent eosinophil recruitment to the lungs.

The data presented here demonstrates that exposure to ozone specifically, selectively, and significantly induced eosinophil hematopoiesis in non-sensitized guinea pigs. These newly divided eosinophils were likely immediately recruited to the lungs since, newly divided BrdU+ cells are significantly increased in bronchoalveolar lavage one and three days after ozone.

Chapter IV

SENSITIZATION PREVENTS OZONE INDUCED EOSINOPHIL HEMATOPOIESIS IN BONE MARROW AND RECRUITMENT OF NEWLY DIVIDED EOSINOPHILS TO THE LUNGS.

ABSTRACT

Background: Ozone transiently reduces lung function in healthy individuals, but in allergic asthmatics it exacerbates allergic hyperreactivity. Allergic asthmatics also have eosinophils resident in the lung tissue. In non-sensitized guinea pigs, ozone inhalation causes vagally-mediated airway hyperreactivity that lasts at least three days and recruits BrdU+ eosinophils to the lungs. Three days after ozone BrdU+ eosinophils are protective, since depleting them worsens ozone induced airway hyperreactivity.

Objective: Here I tested whether sensitization to an antigen changes the role of eosinophils three days after ozone, or ozone induced eosinophil hematopoiesis and recruitment to the lungs.

Method: Some guinea pigs were sensitized to ovalbumin 21 days before experiments began. Both non-sensitized and sensitized guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU), to label newly dividing cells, and then exposed to air or ozone for 4 hours. Three days later, guinea pigs were anesthetized, bronchoconstriction measured, and inflammatory cells harvested from bone marrow, blood, and bronchoalveolar lavage.

Results:

Ozone increased vagally-mediated bronchoconstriction in sensitized guinea pigs when compared to air controls. Ozone induced airway hyperreactivity was significantly

increased when compared to non-sensitized guinea pigs. Three days after ozone, depleting eosinophils with AbIL5 worsened vagally-induced bronchoconstriction in non-sensitized animals, but prevented vagally induced bronchoconstriction in sensitized guinea pigs. Ozone induced an increase in newly divided BrdU+ eosinophils in the bone marrow of non-sensitized, but not sensitized, guinea pigs. In bronchoalveolar lavage, ozone increased eosinophils in both non-sensitized and sensitized guinea pigs. However, in non-sensitized guinea pigs, this increase was comprised of an increase in BrdU+ eosinophils, while in sensitized guinea pigs this increase was comprised of an increase in mature BrdU- eosinophils.

Conclusions: Ozone causes airway hyperreactivity three days after exposure in non-sensitized and sensitized guinea pigs. Three days after ozone, in non-sensitized guinea pigs, BrdU+ eosinophils are protective. In contrast, in sensitized guinea pigs, mature BrdU- eosinophils mediate ozone-induced airway hyperreactivity. These data suggest that interventions targeting eosinophils may be beneficial in allergic individuals.

INTRODUCTON

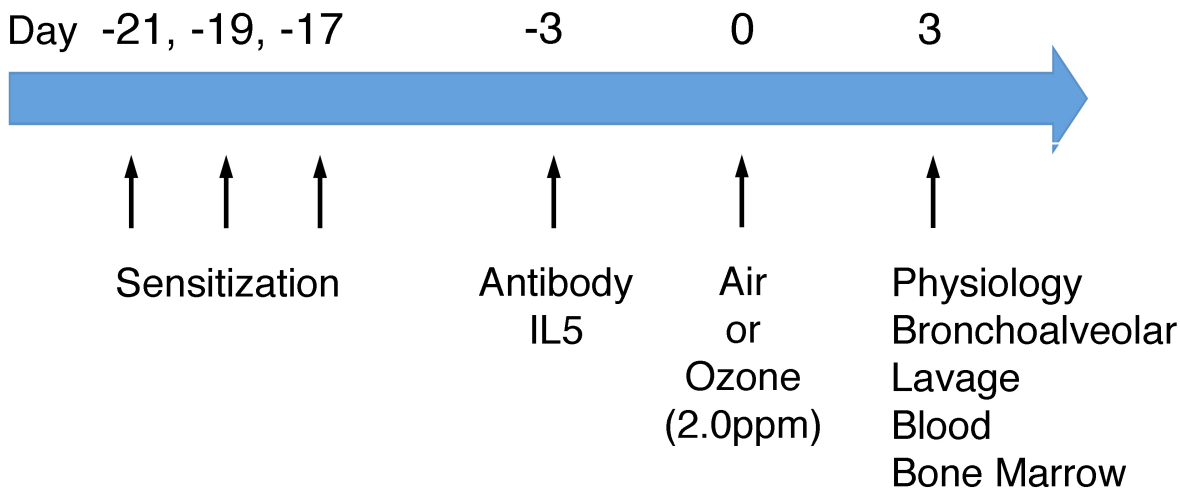
Allergic asthma is a recently characterized subdivision of this disease (Fajt & Wenzel 2015). In allergic asthmatics, ozone exposure causes airway hyperactivity (Hernandez et al 2010b, Hiltermann et al 1999, Holtzman et al 1979, Jorres et al 1996, Khatri et al 2009, Peden et al 1997). Allergic asthmatics also have increased eosinophils in circulation and increased resident eosinophils in their lungs (Hernandez et al 2012, Hernandez et al 2010a, Khatri et al 2009, Peden et al 1995, Shim et al 2015). In allergic individuals, the eosinophil granule protein, eosinophil cationic protein, can be detected in bronchoalveolar lavage immediately after ozone, demonstrating that ozone causes activation and degranulation of resident eosinophils (Dokic & Trajkovska-Dokic 2013). Eosinophils appear in the lungs of allergic patients within 6 hours of ozone exposure (Dokic & Trajkovska-Dokic 2013).

Prior sensitization increases airway hyperreactivity induced by organophosphate exposure (Proskocil et al 2008), viral infection (Robinson et al 1997), and ozone exposure (Schelegle et al 2003, Vargas et al 1994). Additionally, sensitization changed the mechanism of airway hyperreactivity following viral infection from an eosinophil independent mechanism to an eosinophil dependent mechanism (Adamko et al 1999).

Sensitized animals have eosinophilic inflammation in the lungs (Keyhanmanesh et al 2010, Proskocil et al 2008, Schelegle et al 2003), which is significantly increased by ozone (Schelegle et al 2003). Here I tested whether sensitization changes the role of

eosinophils in the lungs three days after ozone, and whether sensitization affects ozone induced eosinophil hematopoiesis and recruitment to lungs.

EXPERIMENTAL PROTOCOL



Some guinea pigs were sensitized to ovalbumin (4.2 mg i.p.) 21, 19, and 17 days before exposure to air or ozone. To measure which cells were dividing after ozone or air, some guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU). 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. Three days later, physiology was measured, and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage.

RESULTS

Ozone increased baseline pulmonary inflation pressure from 102 ± 5 mmH_20 to 150 ± 6 mmH_20 in non-sensitized guinea pigs, and from 102 ± 9 to 158 ± 13 mmH_20 in sensitized guinea pigs. In both sensitized and non-sensitized guinea pigs, vagally-mediated bronchoconstriction was significantly increased above this increase in baseline.

Ozone induced airway hyperreactivity since, three days after ozone, the vagally-mediated bronchoconstriction was significantly greater than in air-exposed guinea pigs (Figure 4.1 A). Sensitization alone had no effect on vagally-mediated bronchoconstriction, since bronchoconstriction was not different from non-sensitized air exposed animals (Figure 4.1 A& B open symbols). In sensitized guinea pigs, ozone induced airway hyperreactivity since, three days after ozone, vagally induced bronchoconstriction was significantly greater than sensitized air-exposed guinea pigs (Figure 4.1 B). However, ozone-induced airway hyperreactivity was significantly greater in sensitized guinea pigs (compare maroon squares to green circles Figure 4.1 A & B). In both sensitized and non-sensitized guinea pigs, airway hyperreactivity is mediated by the vagus nerves since, in vagotomized guinea pigs, methacholine-induced bronchoconstriction was not changed by ozone (Figure 4.2 A, B).

Baseline heart rate, and blood pressure, were not affected by ozone or by sensitization (Table 4.1). In the heart, electrical stimulation of both vagus nerves caused frequency

dependent bradycardia that was not changed by ozone in non-sensitized or sensitized guinea pigs (Figure 4.3 A & B). Similarly, methacholine-induced bradycardia was not changed by ozone or by sensitization (Figure 4.3 C & D).

To test the role of eosinophils in airway hyperreactivity three days after ozone, guinea pigs were pretreated with an antibody to IL5 (AbIL5). Three days after exposure, ozone significantly increased the total number of inflammatory cells in the bronchoalveolar lavage, which was reflected by a significant increase in eosinophils, neutrophils, macrophages, and lymphocytes in non-sensitized (Figure 4.4 green) and sensitized (Figure 4.4 maroon) guinea pigs. Three days after ozone, AbIL5 depleted eosinophils in bronchoalveolar lavage of non-sensitized (Figure 4.4 green) and sensitized (Figure 4.4 maroon) guinea pigs. AbIL5 did not affect any other cell type in bronchoalveolar lavage of non-sensitized guinea pigs (Figure 4.4 green), but decreased lymphocytes in sensitized guinea pigs (Figure 4.4 green). AbIL5 decreased baseline pulmonary inflation pressure three days after ozone from 150 ± 6 to 110.4 ± 11 mmH_20 in non-sensitized, and from 158 ± 13 mmH_20 to 106.0 ± 6 in sensitized, guinea pigs. AbIL5 pretreatment did not affect baseline heart rate and blood pressure three days after ozone in non-sensitized or sensitized guinea pigs (Table 4.1). In non-sensitized guinea pigs, AbIL5 pretreatment significantly worsened vagally-mediated bronchoconstriction three days after ozone (Figure 4.1 A, black). Conversely, in sensitized guinea pigs, AbIL5 completely prevented ozone-induced airway hyperreactivity (Figure 4.1 B,

black). Methacholine mediated smooth muscle contraction was not changed by AbIL5 three days after ozone in non-sensitized animals (Figure 4.2 A).

To investigate different populations of inflammatory cells in non-sensitized and sensitized guinea pigs three days after ozone exposure, guinea pigs were treated with BrdU to label newly divided cells after ozone exposure. Ozone increased total inflammatory cells in bronchoalveolar lavage in both non-sensitized and sensitized animals (Figure 4.5 A). However, while both BrdU+ and BrdU- cells were increased in non-sensitized guinea pigs, only mature BrdU- cells were increased in sensitized guinea pigs (Figure 4.5 A). Sensitization significantly increased eosinophils in bronchoalveolar lavage (Figure 4.5 B). This increase was comprised of mature BrdU- eosinophils. Three days after ozone, eosinophils were significantly increased in bronchoalveolar lavage in both non-sensitized and sensitized guinea pigs (Figure 4.5 B). In non-sensitized guinea pigs, this increase was comprised entirely of new BrdU+ eosinophils (Figure 4.5 B, Black), so that the percent of BrdU+ cells increased from 22 to 56% (Figure 4.5 B, black, Table 4.2). In contrast, in sensitized guinea pigs, these BrdU+ eosinophils completely failed to appear, so the increase was comprised entirely of mature BrdU- eosinophils (Figure 5.5 B, grey). Neutrophils in bronchoalveolar lavage were significantly increased three days after ozone in both non-sensitized and sensitized guinea pigs (Figure 4.5 C). In non-sensitized guinea pigs, this increase was comprised entirely of BrdU+ cells (Figure 4.5 C, black), while in sensitized animals, the increase was entirely mature BrdU- neutrophils (Figure 4.5 C, grey). Macrophages

in bronchoalveolar lavage were significantly increased three days after ozone in both non-sensitized and sensitized guinea pigs (Figure 4.5 D). In non-sensitized guinea pigs the increase was comprised of both BrdU+ and BrdU- cells (19% to 34% BrdU+ S, Table 4.4, Figure 4.5 D, black), while in sensitized guinea pigs only mature BrdU- macrophages were increased (17% to 15% BrdU+, S Table 4, Figure 4.5 D, grey). Three days after ozone, lymphocytes in bronchoalveolar lavage were not changed in non-sensitized animals (Figure 4.5 E, black), but both BrdU+ and BrdU- cells were significantly increased in sensitized guinea pigs (Figure 4.5 E, grey).

Although ozone increased inflammatory cells in the bronchoalveolar lavage, ozone did not affect the total number of circulating leukocytes in the blood, nor did it differentially affect the number of eosinophils, neutrophils, monocytes, or lymphocytes in circulation (Figure 4.6) in non-sensitized or sensitized guinea pigs. The number of newly divided, BrdU+ and mature BrdU- in ozone exposed animals was not different from air exposed animals in any of the cell types (Figure 4.6).

In bone marrow, ozone did not change the total number of leukocytes three days after ozone in non-sensitized or sensitized guinea pigs (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: $2.3 \pm 0.20 \times 10^8$). Likewise, ozone did not increase the percent of eosinophils, neutrophils, or monocytes three days after exposure in non-sensitized or sensitized guinea pigs (Figure 4.7). Bone marrow production of newly divided BrdU+ eosinophils was significantly increased by

ozone in non-sensitized guinea pigs from 36 to 62% (Figure 4.7 A, black, Table 4.2), while ozone-induced eosinophil hematopoiesis failed to occur in sensitized animals (only 44% BrdU+, Figure 4.7 A, grey). The number of newly divided BrdU+ or mature BrdU- cells in the neutrophils and monocytes were not changed three days of ozone in non-sensitized or sensitized guinea pigs (Figure 4.7 B&C).

FIGURE 4.1 Antibody to IL-5 prevents ozone induced airway hyperreactivity in sensitized animals.

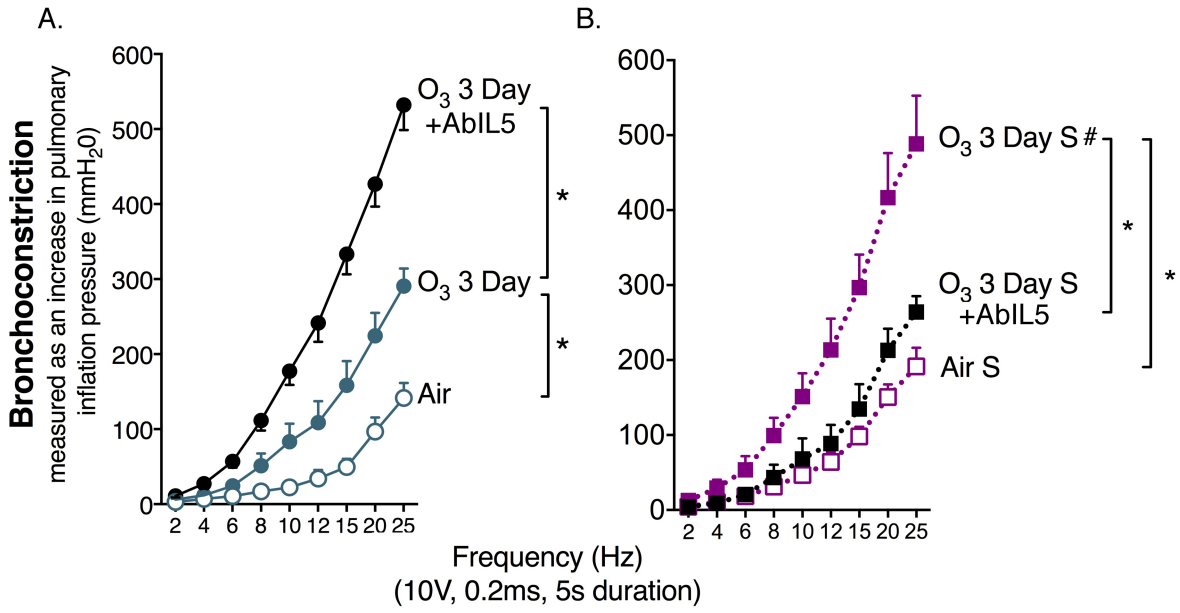


Figure 4.1 Ozone (O_3, 2.0ppm, 4h) significantly increased vagally-mediated bronchoconstriction in sensitized guinea pigs (S) measured 3 days later (B). Note vagally mediated bronchoconstriction from Figure 3.1 B is re-graphed here for statistical comparison. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A & B open symbols, Air). Vagally induced bronchoconstriction was significantly increased by three days after ozone in non-sensitized (A, green) and sensitized (B, maroon) guinea pigs. Sensitization significantly increased ozone-induced hyperreactivity when compared to non-sensitized animals (compare green circles to maroon squares, #). Pretreatment with AbIL5 significantly increased ozone-induced airway hyperreactivity 3 days after

exposure in non-sensitized guinea pigs (A, Black). In contrast, in sensitized guinea pigs, AbIL5 prevented ozone-induced airway hyperreactivity (B, Black). Data shown as mean ± SEM n=5-9, *Significantly different by repeated measures two-way ANOVA ($p<0.05$) # Significantly different from 3 day post ozone non-sensitized by repeated measures two-way ANOVA.

FIGURE 4.2. Methacholine-induced airway smooth muscle contraction was not affected by sensitization (S) or by ozone (O_3).

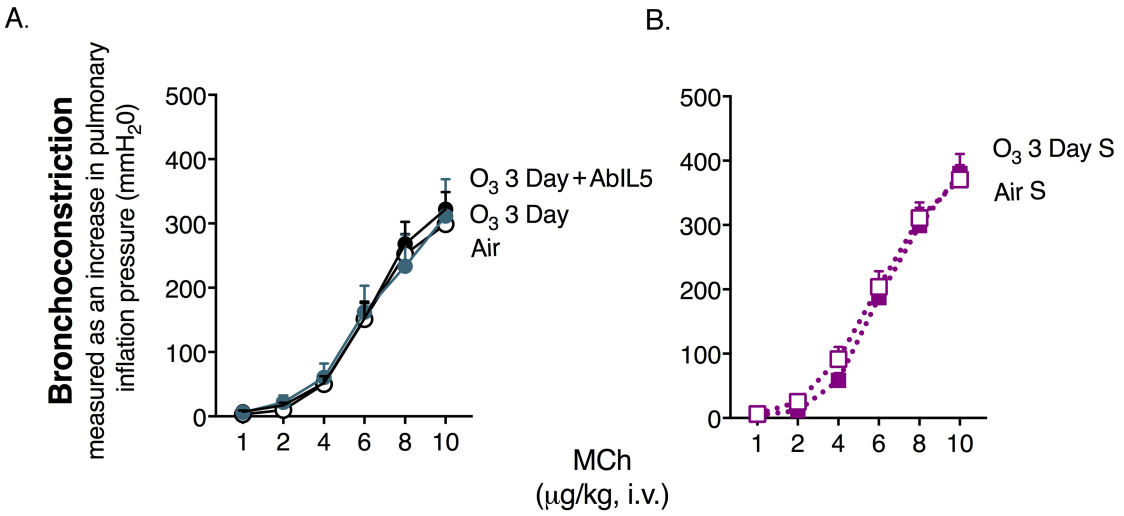


Figure 4.2 Methacholine (1-10 µg/kg i.v.) causes a dose dependent increase in bronchoconstriction (A & B, Air) in vagotomized animals. Bronchoconstriction induced by i.v. methacholine in vagotomized animals was not affected by ozone (O_3) at day three, in either non-sensitized (A, green), or sensitized (B, maroon) guinea pigs. Pretreatment with AbIL5 had no effect on methacholine-induced bronchoconstriction in non-sensitized guinea pigs (A, black). Note methacholine-induced bronchoconstriction from Figure 3.2 B is re-graphed here for statistical comparison. Data shown as mean ± SEM n= 5-9 guinea pigs.

FIGURE 4.3 Vagally-mediated and methacholine-induced bradycardia was not affected by ozone or by sensitization three days after exposure.

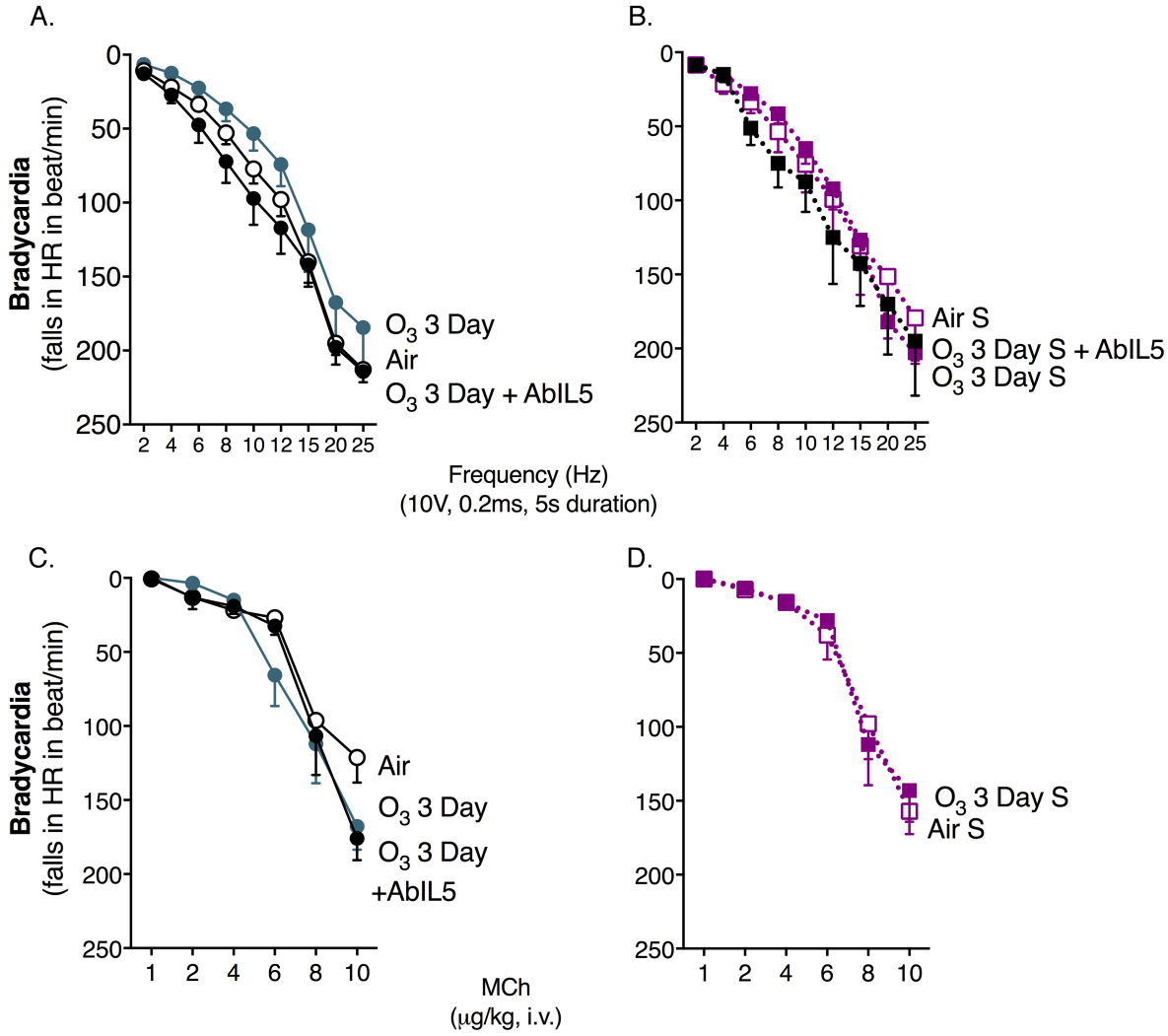


Figure 4.3. Vagally-mediated (A & B) and methacholine-induced (C & D) bradycardia was not affected by ozone (O_3), by sensitization (S), or by AbIL5. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train, at one min intervals) produced frequency dependent bradycardia. Vagally induced bradycardia was not affected by

ozone or AbIL5 in non-sensitized (A) or sensitized guinea pigs (B). Methacholine (1-10 μg/kg) induced bradycardia was not affected by ozone (C) or by sensitization (D). Data shown as mean ± SEM n= 5-9. * Significantly different from control by repeated measures two-way ANOVA ($p<0.05$).

FIGURE 4.4 Bronchoalveolar lavage eosinophils were depleted by antibody to IL-5 in non-sensitized and sensitized guinea pigs three days after ozone.

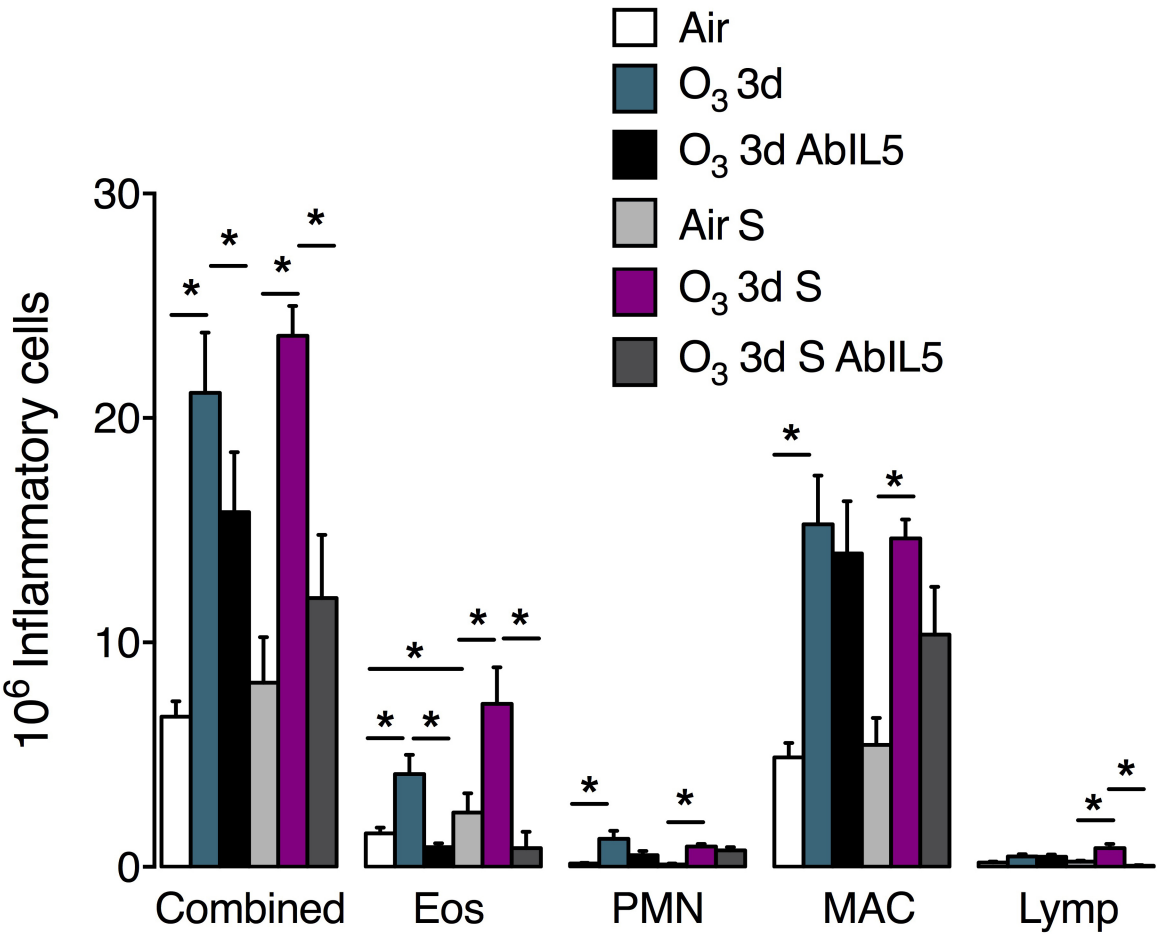


Figure 4.4 Three days after exposure, ozone (O_3) significantly increased the total number of inflammatory cells (Combined) in the bronchoalveolar lavage, which was reflected by a significant increase in eosinophils (Eos), neutrophils (PMN), macrophages (MAC), and lymphocytes (Lymp) in non-sensitized (green) and sensitized (S, maroon) guinea pigs. In non-sensitized guinea pigs, antibody to IL-5 (AbIL5) significantly decreased eosinophils in ozone-exposed guinea pigs. In sensitized guinea pigs, AbIL5 significantly decreased eosinophils and lymphocytes three days after

ozone. Data from figure 3.4 was re-graphed here for statistical comparison. Data are mean ± SEM, n=5-7 *Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$).

FIGURE 4.5. Three days after ozone total leukocytes, eosinophils, neutrophils macrophages, and lymphocytes were increased in bronchoalveolar lavage of sensitized guinea pigs.

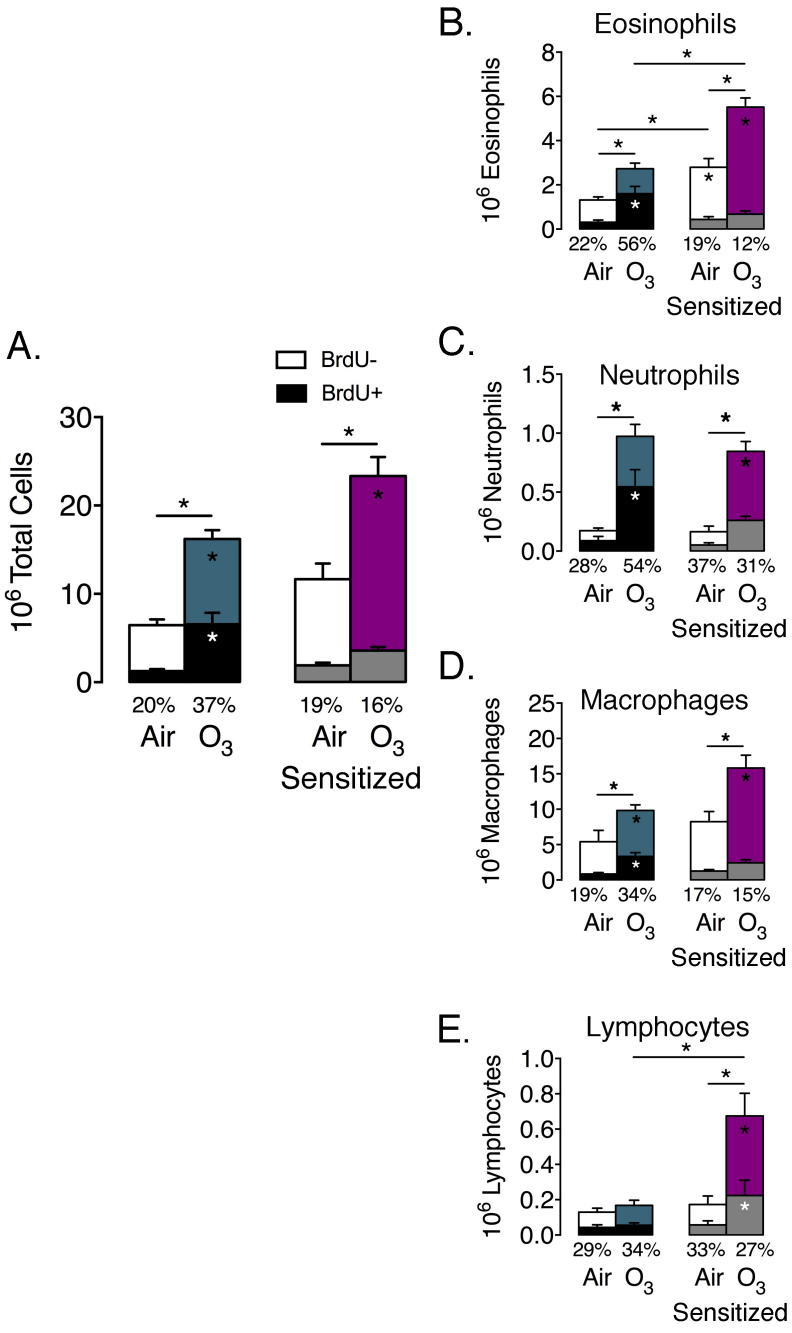


Figure 4.5 In non-sensitized guinea pigs, eosinophils (B), neutrophils (C), and macrophages (D) but not lymphocytes (E) in were increased in bronchoalveolar lavage three days after ozone. The increase in neutrophils and eosinophils was comprised only of BrdU+ cells, while the ozone induced increase in macrophages was comprised of both BrdU+ and BrdU- cells. Sensitization significantly increased eosinophils in the bronchoalveolar lavage (B). In sensitized guinea pigs, ozone significantly increased total leukocytes (A), eosinophils (B), neutrophils (C), macrophages (D), and lymphocytes (E) in bronchoalveolar lavage. This increase was comprised of BrdU- cells except for lymphocytes where the ozone induced increase was comprised of both BrdU+ and BrdU- cells. Note entire column height represents the total number of cells that is then subdivided into mature BrdU- (white/green/maroon) and newly divided BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the bronchoalveolar lavage of non-sensitized guinea pigs three days after ozone have been included from Figure 3.5 for statistical comparison. Data shown as mean ± SEM n=7-9 Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions.

FIGURE 4.6 Ozone had no effect on circulating leukocytes in sensitized guinea pigs.

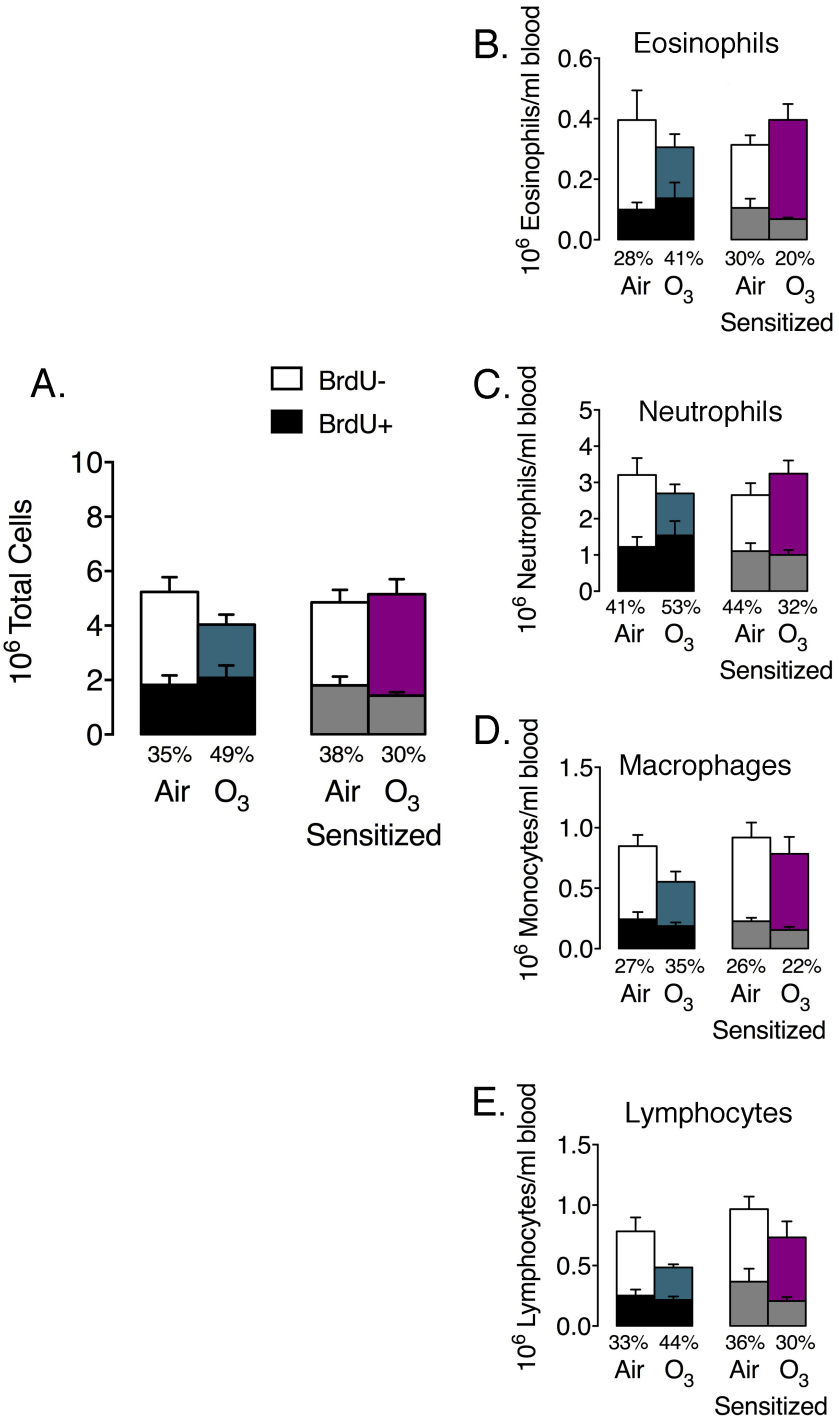


Figure 4.6 Ozone (O_3) had no effect on total circulating leukocytes (A), eosinophils (B), neutrophils (C), monocytes (D), and lymphocytes (E) in blood three days after exposure in non-sensitized (green/black) or sensitized (maroon/grey). Note as in figure 4.5, the entire column height represents the total number of cells that is then subdivided into mature BrdU- (white/green/maroon) and newly divided BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the blood of non-sensitized guinea pigs three days after ozone has been included from Figure 3.6 for statistical comparison. Data shown as mean ± SEM n=7-9.

FIGURE 4.7 Sensitization inhibited ozone-induced eosinophil hematopoiesis.

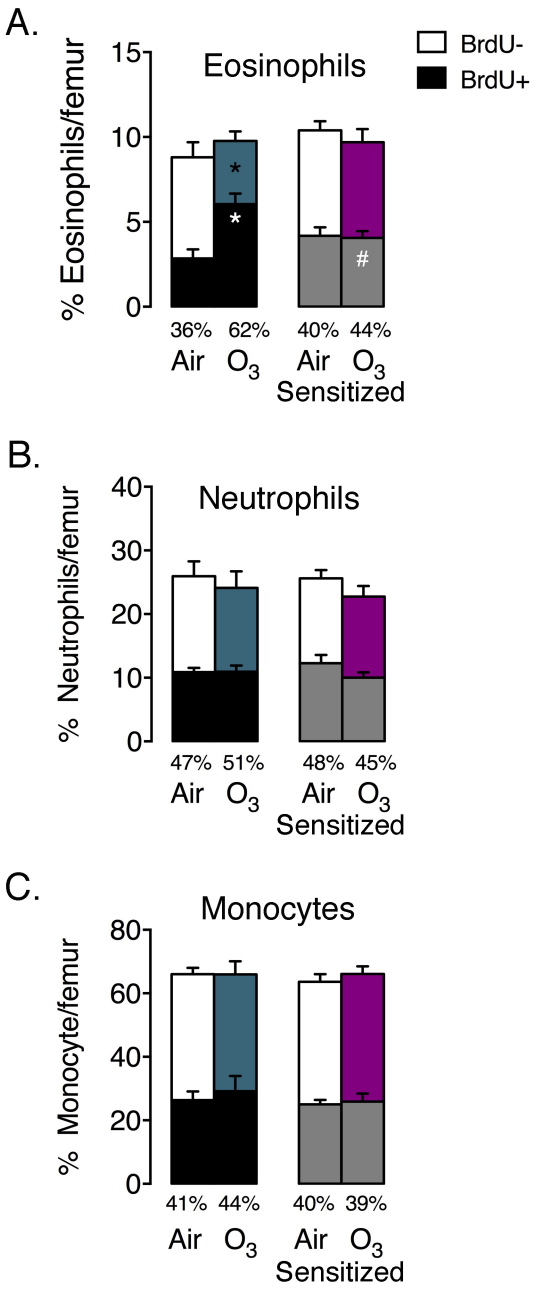


Figure 4.7 Ozone increased BrdU+ (black) eosinophils in bone marrow (black, A) in non-sensitized guinea pigs, however, this increase failed to occur in sensitized (grey, A) animals. Neutrophils (B) and monocytes (C) in were not changed by ozone, or by sensitization. Note as in figure 4.5, the entire column height represents the total number of cells that is then subdivided into mature BrdU- (white/green/maroon) and newly divided BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from bone marrow of non-sensitized guinea pigs 3 days after ozone from Figure 3.7 were included for statistical comparison. Data shown as mean ± SEM n=7-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions *significantly different from air, #significantly different from non-sensitized ozone.

TABLE 4.1 Baseline Values

			Baseline PPI (mmH_2O)	Heart Rate (bpm)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Weight (g)
Non-sensitized		Air	102.2 ±4.9	287.8 ±9.8	41.0 ±1.1	19.6 ±0.7	351.3 ± 5.2
		O_3 3 Day	151.4 ±6.3*	283.6 ±6.0	44.8 ±2.7	24.0 ±1.5	382.6 ±11.0
	AbIL5	O_3 3 Day	110.0 ±10.9#	279.3 ±8.7	47.6 ±2.0	28.0 ±1.4	388.1 ±22.5
Sensitized		Air	102.0 ±8.6	302.0 ±9.9	43.5 ±2.9	20.0 ±0.8	406.8 ±17.4
		O_3 3 Day	158.3 ±12.7*	275.0 ±4.3	44.3 ±1.9	22.3 ±1.6	394.8 ± 3.0
	AbIL5	O_3 3 Day	106.0 ±6.0≠	322.5 ±17.8	55.0 ±2.6	27.0 ±3.0	384.6 ±13.9

Table 4.1 Baseline values are means ± SEM. Baseline pulmonary inflation pressure was significantly increased three days after a single exposure to 2.0ppm ozone (O_3; 4 hr) in sensitized and non-sensitized guinea pigs. Baseline heart rate and blood pressure were not affected by ozone in sensitized or non-sensitized guinea pigs. *Significantly different by one-way ANOVA with a Bonferroni correction ($p<0.05$).

TABLE 4.2 Percent BrdU+ Eosinophils

% BrdU+ Eosinophils			Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-Sensitized	3 day	Air	22 ± 5.6	28 ± 4.8	36 ± 5.3
		O_3	56 ± 8.5*	41 ± 8.7	62 ± 5.1*
Sensitized	3 day	Air	19 ± 3.5	30 ± 4.8	40 ± 3.2
		O_3	12 ± 1.9#	20 ± 3.3	44 ± 5.0#

Table 4.2 Percent BrdU+ Eosinophils. In bronchoalveolar lavage, the percent of BrdU+ eosinophils was significantly increased three days after ozone when compared to air control. In sensitized guinea pigs, the percent of BrdU+ eosinophils in bronchoalveolar lavage was significantly decreased when compared to non-sensitized guinea pigs three days after ozone. Similarly in bone marrow, the percent of BrdU+ eosinophils was significantly increased three days after ozone when compared to air control. In sensitized guinea pigs, the percent of BrdU+ eosinophils in bone marrow was significantly decreased when compared with non-sensitized guinea pigs three days after ozone. Data shown as mean ± SEM n=7-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

TABLE 4.3 Percent BrdU+ Neutrophils

% BrdU+ Neutrophils			Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-Sensitized	3 day	Air	28 ± 7.1	41 ± 5.1	47 ± 4.8
		O_3	54 ± 7.7	53 ± 8.7	51 ± 7.8
Sensitized	3 day	Air	37 ± 6.0	44 ± 8.0	48 ± 3.6
		O_3	31 ± 2.2	32 ± 4.5	45 ± 3.3

Table 4.3 The Percent BrdU+ Neutrophils. In bronchoalveolar lavage, blood, and bone marrow the percent BrdU+ neutrophils was not changed by ozone or by sensitization three days after exposure. Data shown as mean ± SEM n=7-9.

TABLE 4.4 Percent BrdU+ Macrophages

% BrdU+ Macrophages			Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-Sensitized	3 day	Air	19 ± 4.3	27 ± 1.9	41 ± 3.2
		O_3	34 ± 5.1*	35 ± 3.2*	44 ± 6.1
Sensitized	3 day	Air	17 ± 2.2	26 ± 3.8	40 ± 2.2
		O_3	15 ± 2.1#	22 ± 3.3	39 ± 3.3

Table 4.4 Percent BrdU+ macrophages. In bronchoalveolar lavage, the percent of BrdU+ macrophages was significantly increased three days after ozone when compared to air control. In sensitized guinea pigs the percent of BrdU+ macrophages was significantly decreased when compared to non-sensitized guinea pigs three days after ozone. Data shown as mean ± SEM n=7-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

TABLE 4.5 Percent BrdU+ Lymphocytes

% BrdU+ Lymphocytes			Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-Sensitized	3 day	Air	29 ± 3.5	33 ± 3.4	41 ± 3.2
		O_3	34 ± 5.3	44 ± 4.7	44 ± 6.1
Sensitized	3 day	Air	33 ± 6.0	36 ± 7.7	40 ± 2.2
		O_3	27 ± 7.8	30 ± 4.1	39 ± 3.3

Table 4.5 The Percent BrdU+ lymphocytes. In bronchoalveolar lavage, blood, and bone marrow the percent BrdU+ lymphocytes was not changed by ozone or by sensitization three days after exposure. Data shown as mean ± SEM n=7-9.

TABLE 4.6 Percent BrdU+ Total Cells

% BrdU+ Total Cells			Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM
Non-Sensitized	3 day	Air	20 ± 3.5	35 ± 3.2
		O_3	37 ± 4.3*	49 ± 7.5
Sensitized	3 day	Air	19 ± 2.4	38 ± 6.3
		O_3	16 ± 1.4#	30 ± 4.0

Table 4.6 Percent BrdU+ total cells. In bronchoalveolar lavage, the percent of BrdU+ cells was significantly increased three days after ozone in non-sensitized guinea pigs. In sensitized guinea pigs the percent of BrdU+ cells was significantly decreased three days after ozone when compared to non-sensitized guinea pigs. The percent BrdU+ cells was not changed in blood of non-sensitized or sensitized guinea pigs. Data shown as mean ± SEM n=7-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

DISCUSSION

Allergic patients are sensitized to allergens and have increased eosinophils in the lungs after ozone (Bascom et al 1990, Brown et al 2007, Holz et al 2002, Peden et al 1995). Here, I sensitized guinea pigs to ovalbumin to test whether pre-existing allergy affected ozone-induced synthesis and recruitment of protective, newly divided BrdU+ eosinophils to lungs.

Ozone causes airway hyperreactivity in non-sensitized guinea pigs three days after ozone (Schultheis et al 1994, Verhein et al 2011, Verhein et al 2008, Yost et al 2005). I confirmed that here (Figure 4.1 A). Three days after ozone, eosinophils are protective since depleting them with antibody to IL5 or preventing their recruitment to the lungs with an antibody to the adhesion molecule VLA4 significantly worsened ozone-induced airway hyperreactivity (Yost et al 2005). Three days after ozone, newly divided eosinophils are the dominant eosinophil population in the lungs (Figure 4.5). Thus, three days after ozone, newly divided eosinophils are protective.

It is well known that ozone causes airway hyperreactivity in sensitized animals (Kierstein et al 2008, Moore et al 2012, Schelegle et al 2003). Here I show that ozone causes airway hyperreactivity three days after exposure in sensitized guinea pigs (Figure 4.1). Additionally, ozone induced airway hyperreactivity is significantly increased when compared to non-sensitized guinea pigs (Figure 4.1 A vs B). In stark contrast to non-sensitized guinea pigs, eosinophils in the lungs are no longer protective

three days after ozone in sensitized guinea pigs, since depleting them with AbIL5 prevents ozone induced airway hyperreactivity. Three days after ozone, mature BrdU- eosinophils are the dominant eosinophil population in the lungs of sensitized guinea pigs. Thus, in sensitized guinea pigs, mature BrdU- eosinophils mediate ozone induced airway hyperreactivity three days after a single exposure to ozone.

In bronchoalveolar lavage, the ozone induced increase of BrdU- eosinophils (Figure 4.5 B) may be mediated by recruitment of eosinophils already resident in lung tissue of sensitized animals. In addition to eosinophils, ozone also significantly increased lymphocytes in bronchoalveolar lavage of sensitized animals (Figure 4.5 E). Inflammation commonly associated with sensitization has been shown to activate lymphocytes to release IL-5 and IL-13 (Nussbaum et al 2013, Price et al 2010). IL-5 and IL-13 induce eotaxin expression, which results in eosinophil recruitment and accumulation in lung tissue (Nussbaum et al 2013). Additionally, eosinophil survival may be increased, as in mice sensitized to *Aspergillus fumigatus,* where survival is mediated by GM-CSF and IL5 (Kierstein et al 2008). Following ozone, IL-5 could be increased from lymphocytes (Nussbaum et al 2013), while GM-CSF, which is a product of eosinophils, is also released from airway epithelial cells after ozone (Clutterbuck & Sanderson 1988, Hogan et al 2000, Stanley et al 1994). Additionally, individuals with inhalation allergies and atopic dermatitis may have increased eosinophil survival mediated by autocrine production of IL-3, GM-CSF, and IL-5 (Wedi et al 1997).

The data presented here demonstrate that exposure to ozone specifically, selectively, and significantly induced eosinophil hematopoiesis in non-sensitized guinea pigs (Figure 4.7 A). These newly divided eosinophils were likely immediately recruited to the lungs since, BrdU+ cells were significantly increased in bronchoalveolar lavage three days after ozone and their appearance is associated with decreased hyperreactivity. Sensitization prevented the ozone induced increase in eosinophil hematopoiesis, demonstrating that sensitization altered the inflammatory response to ozone in bone marrow. Loss of BrdU+ eosinophils in the lungs resulted in potentiation of ozone-induced airway hyperreactivity in sensitized guinea pigs.

Clinical trials of mepolizumab show that not all asthmatics respond to eosinophil suppression (Flood-Page et al 2003, Hendeles et al 2004, Sehmi et al 2016), suggesting that different asthma phenotypes may be associated with different balances of beneficial and deleterious eosinophils. Both in asthma and in other chronic inflammatory disease such as atopic dermatitis, inflammatory bowel disease, and interstitial cystitis, it will be important to define both the deleterious and the beneficial effects of eosinophils, and to determine whether it is possible to switch eosinophils back to the beneficial phenotype seen in non-sensitized animals.

CHAPTER V.

TUMOR NECROSIS FACTOR-ALPHA MEDIATES OZONE INDUCED EOSINOPHIL HEMATOPOIESIS AND RECRUITMENT OF NEWLY DIVIDED EOSINOPHILS TO THE LUNGS IN NON-SENSITIZED GUINEA PIGS

ABSTRACT

Background: Ozone-induces eosinophil hematopoiesis one and three days after exposure. Three days after ozone, newly divided eosinophils are recruited to the lungs where they are protective, since depleting them with AbIL5 worsened ozone-induced airway hyperreactivity. Ozone also increases tumor necrosis factor-alpha (TNFα) in bronchoalveolar lavage.

Objective: Since TNFα induces inflammatory cell recruitment to the lungs, I tested whether it mediates ozone induced eosinophil hematopoiesis and/or recruitment to the lungs using the TNFα antagonist, etanercept. I also tested whether etanercept would affect airway hyperreactivity one and three days after ozone in non-sensitized and sensitized guinea pigs.

Methods: 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. Some animals were given the TNFα antagonist, etanercept (3mg/kg i.p.) 3 hours before ozone exposure. One or three days later, vagally-induced bronchoconstriction was measured and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage.

Results: Blocking TNFα inhibited ozone induced eosinophil hematopoiesis one and three days after ozone. Three days after ozone, recruitment of newly divided

eosinophils to the lungs were also inhibited by etanercept. Loss of newly divided BrdU+ eosinophils significantly worsened ozone-induced airway hyperreactivity; thus etanercept additionally potentiated vagally induced bronchoconstriction above that following by ozone. Etanercept had no effects in sensitized guinea pigs three days after ozone exposure.

Conclusions: In non-sensitized guinea pigs TNFα mediates ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided eosinophils to lungs three days later where they inhibit vagally mediated bronchoconstriction. TNFα does not play a role in ozone induced eosinophil hematopoiesis or recruitment in sensitized guinea pigs.

INTRODUCTION

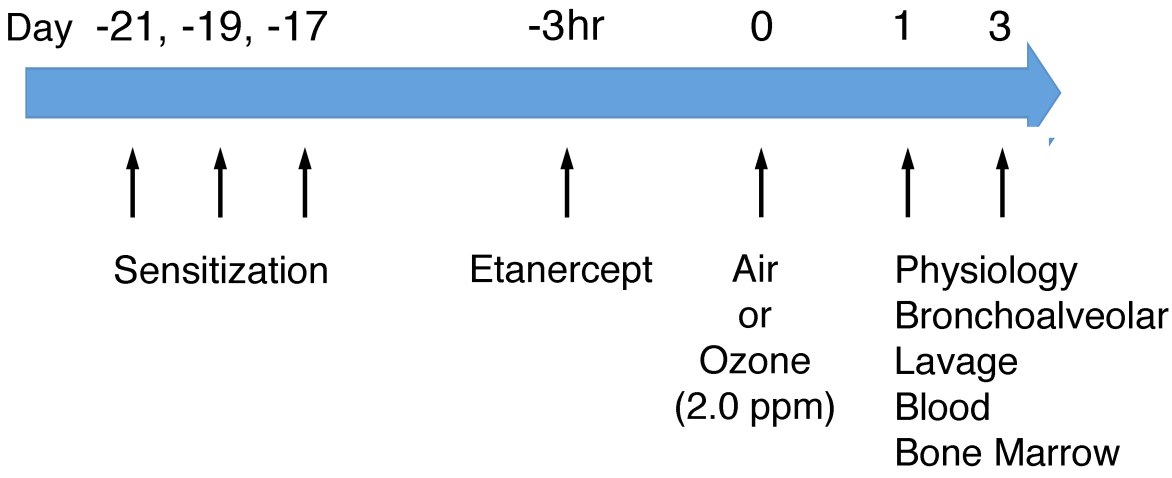
Ozone-induces eosinophil hematopoiesis one and three days after exposure (Chapter III). Three days after ozone, newly divided eosinophils are protective in the lungs, since depleting them with AbIL5 worsened ozone-induced airway hyperreactivity (Chapter III). Ozone is a reactive oxygen species, which is unlikely to pass the airway epithelium (Pryor 1992). Thus, ozone must depend upon an intermediary inflammatory signal to mediate eosinophil hematopoiesis and recruitment to the lungs.

Ozone increases TNFα in lungs of humans (Dokic et al 2011, Esther et al 2011, Rusznak et al 1996), and animals (Arsalane et al 1995, Bao et al 2013, Fakhrzadeh et al 2004, Wang et al 2015). TNFα is a proinflammatory cytokine that induces expression of IL-5 and GM-CSF (Levi-Schaffer et al 1998); these cytokines can mediate eosinophil hematopoiesis, and survival (Clutterbuck & Sanderson 1988, Levi-Schaffer et al 1998, Takatsu & Nakajima 2008, Wedi et al 1997). Addionally, TNFα stimulates inflammatory cell migration into lung tissue by increasing expression of adhesion molecules including ICAM-1 and VLA4 on endothelial cells (Atsuta et al 1997, Bloemen et al 1993, Francis et al 2004, Iademarco et al 1995, Murphy 2008, Nagai et al 1988, Sato et al 1997, Terada et al 2000).

TNFα is known to mediate airway hyperreactivity in many different models of asthma including, antigen sensitization and challenge (Nie et al 2009), organophosphate pesticide exposure (Proskocil et al 2013), and viral infection (Nie et al 2011). Here I

tested whether a TNFα blocker, etanercept, prevented ozone-induced airway hyperreactivity one and three days after ozone in non-sensitized and sensitized guinea pigs. Since TNFα can contribute to eosinophil hematopoiesis and recruitment, I also tested whether blocking TNFα inhibits ozone-induced eosinophil hematopoiesis and recruitment to the lungs.

EXPERIMENTAL PROTOCOL



Some guinea pigs were sensitized to ovalbumin (4.2 mg i.p.) 21, 19, and 17 days before exposure to air or ozone. Some animals were given the TNFα antagonist, etanercept (3mg/kg i.p.) 3 hours before ozone exposure. To measure which cells were dividing after ozone or air, some guinea pigs were treated with 5-bromo-2-deoxyuridine (BrdU). 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. One or three days later, physiology was measured and inflammatory cells were harvested from bone marrow, blood, and bronchoalveolar lavage.

RESULTS

Effect of etanercept in non-sensitized guinea pigs

Etanercept did not effect baseline pulmonary inflation pressure one or three days after ozone in non-sensitized guinea pigs (Table 5.1). Electrical stimulation of both vagus nerves (1-25 Hz) caused frequency dependent bronchoconstriction that was significantly increased one (Figure 5.1 A green circles), and three days (Figure 5.6) after ozone in non-sensitized when compared to air control. One day after ozone, etanercept pretreatment had no effect on ozone-induced airway hyperreactivity (Figure 5.1 A, blue triangles). In contrast, three days after ozone, etanercept significantly worsened vagally-mediated bronchoconstriction in non-sensitized guinea pigs (Figure 5.6 A, blue triangles to green circles). This suggests that TNFα is protective in the lungs three days after ozone in non-sensitized guinea pigs. Methacholine-induced bronchoconstriction was not affected by etanercept at either one (Figure 5.1 B) or three days non-sensitized guinea pigs (Figure 5.7 A).

In bone marrow, ozone did not change the total number of leukocytes one or three days after ozone in non-sensitized guinea pigs:

1 day air $2.3 \pm 0.32 \times 10^8$
1 day ozone $2.5 \pm 0.4 \times 10^8$
1 day air etanercept $2.8 \pm 0.32 \times 10^8$
1 day ozone etanercept $3.3 \pm 0.25 \times 10^8$

3 day air $2.6 \pm 0.21 \times 10^8$
3 day ozone $1.9 \pm 0.21 \times 10^8$
3 day air etanercept $2.5 \pm 0.28 \times 10^8$
3 day ozone etanercept $2.5 \pm 0.42 \times 10^8$

In blood, etanercept had no effect on total leukocytes in circulation, in air, or ozone at one (Figure 5.4) or three (Figure 5.10) days in non-sensitized guinea pigs. In bronchoalveolar lavage, etanercept did not change total inflammatory cells in air or exposed animals one (Figure 5.3) or three (Figure 5.9) days after ozone. One day after ozone, total inflammatory cells in bronchoalveolar lavage were not increased in etanercept treated animals. In contrast, three days after ozone, etanercept did not affect the ozone-induced increase in inflammation in non-sensitized animals (Figure 5.9 A). However, the increase in newly formed BrdU+ leukocytes after ozone (37% BrdU+, Figure 5.9 A, black) was prevented by etanercept, so that the increase in leukocytes was made up of only BrdU- cells (18% BrdU+, Figure 5.9 A).

Etanercept significantly decreased the percent of newly divided BrdU+ eosinophils in bone marrow both one and three days after ozone, suggesting TNFα mediates ozone induced eosinophils hematopoiesis in non-sensitized animals (Figure 5.5 A, 5.11 A). In blood, etanercept did not effect eosinophils in circulation in air or ozone at one (Figure 5.4 B) or three days (Figure 5.10 B) in non-sensitized guinea pigs. In bronchoalveolar lavage, etanercept had no effect on eosinophils populations in the lungs when compared to untreated animals one day after ozone, suggesting that TNFα does not mediate eosinophil recruitment into bronchoalveolar lavage one day after ozone. In contrast, three days after ozone in non-sensitized guinea pigs, etanercept pretreatment significantly increased eosinophils in bronchoalveolar lavage after ozone (Figure 5.9

B). Additionally, etanercept changed the composition of these eosinophils from predominantly BrdU+ (56%) to predominantly BrdU- (22% BrdU+, Table 5.2, Figure 5.9 B). Suggesting, that TNFα mediates recruitment of newly divided BrdU+ eosinophils to the lungs three days after ozone.

Etanercept had no effect on the percent of neutrophils in bone marrow at one (Figure 5.5) day or at three days (Figure 6.11) in non-sensitized, guinea pigs suggesting TNFα does not mediate neutrophil hematopoiesis in any group. In the blood of air controls, etanercept decreased circulating neutrophils, specifically targeting the BrdU- cells (there was a significant decrease in BrdU- cells with etanercept; (Figure 5.4 C). However, there was no effect of etanercept on circulating neutrophils in any other treatment group (Figure 5.10 C). So either TNFα inhibits migration of mature neutrophils from blood into the tissues in air exposed guinea pigs, or this data point is not real, but random. However, in bronchoalveolar lavage, ozone significantly increased neutrophils one (Figure 5.3) and three days after ozone (Figure 5.9). Etanercept prevented this increase at both time points, decreasing both BrdU+ and BrdU– cells, though it was a significant decrease only one day post ozone. TNFα is known to neutrophil induce neutrophil chemotaxis (Weppler & Issekutz 2008), so this effect is likely due to loss of TNFα induced chemotaxis.

Etanercept had no effect on the percent of monocytes in bone marrow at one or three days, suggesting that TNFα does not mediate monocyte hematopoiesis. In the blood of air controls, etanercept increased BrdU- monocytes, at one (Figure 5.4 D) and three

days (Figure 5.10 D) in both air and ozone exposed animals, suggesting that TNFα mediates monocyte recruitment into tissue. In bronchoalveolar lavage, one day after ozone, etanercept significantly decreased BrdU+ and BrdU- macrophages (Figure 5.4 D), suggesting that TNFα mediates macrophage recruitment into bronchoalveolar lavage one day after ozone. Three days after ozone, in non-sensitized guinea pigs, etanercept significantly decreased BrdU+ macrophages in bronchoalveolar lavage three days after ozone (34% to 15% BrdU+, Table 5.4, Figure 5.9 D).

In blood, ozone increased BrdU- lymphocytes one (Figure 5.4 E) and three (Figure 5.10 E) days after ozone in non-sensitized guinea pigs, suggesting that after ozone TNFα mediates lymphocyte recruitment into lung tissue. In bronchoalveolar lavage, etanercept prevented the ozone-induced increase in BrdU- lymphocytes one day after exposure (Figure 5.3 E). In contrast, three days after ozone etanercept significantly increased mature BrdU- lymphocytes in non-sensitized guinea pigs (Figure 5.9 E).

Effect of etanercept in sensitized guinea pigs

In sensitized guinea pigs, etanercept did not effect baseline pulmonary inflation pressure three days after ozone (Table 5.1). Electrical stimulation of both vagus nerves (1-25 Hz) caused frequency dependent bronchoconstriction that was significantly increased and three days (Figure 5.6) after ozone in sensitized guinea pigs when compared to air control. In sensitized guinea pigs, etanercept did not effect ozone-induced airway hyperreactivity three days after ozone (Figure 5.6 B, blue triangles to

maroon squares). Methacholine-induced bronchoconstriction was not affected by etanercept in sensitized animals (Figure 5.7 B).

In sensitized guinea pigs, ozone did not change the total number of leukocytes in bone marrow three days after ozone:

3 day air sensitized $2.8 \pm 0.24 \times 10^8$
3 day ozone sensitized $2.3 \pm 0.20 \times 10^8$
3 day air sensitized etanercept $2.5 \pm 0.30 \times 10^8$
3 day ozone sensitized etanercept $2.2 \pm 0.45 \times 10^8$

In sensitized guinea pigs, etanercept had no effect on total leukocytes in circulation, in air, or ozone at three days (Figure 5.9 A). In sensitized guinea pigs, etanercept did not affect total inflammatory cells in bronchoalveolar lavage of air-exposed guinea pigs (Figure 5.9 A), or the increase in inflammatory cells after ozone (Figure 5.9 A).

Three days after ozone, in sensitized guinea pigs etanercept had no effect on eosinophils in the bone marrow (Figure 5.11 A), suggesting that TNFα does not mediate eosinophil hematopoiesis in sensitized guinea pigs. In blood, etanercept did not effect eosinophils in circulation in air or ozone in sensitized animals (Figure 5.10 B). In sensitized guinea pigs pretreated with etanercept, ozone failed to recruit any additional eosinophils to bronchoalveolar lavage (Figure 5.9 B).

Etanercept had no effect on the percent of neutrophils in bone marrow in sensitized animals, suggesting TNFα does not mediate neutrophil hematopoiesis (Figure 5.11 B).

There was no effect of etanercept on circulating neutrophils in sensitized guinea pigs (Figure 5.10 C). Although ozone still produced neutrophilia in the bronchoalveolar lavage of sensitized guinea pigs (Figure 5.9), etanercept had no inhibitory effect on ozone-induced increase in neutrophils in lungs. Thus, in sensitized guinea pigs, TNFα does not seem to play a role in ozone induced neutrophil recruitment to the lungs.

Etanercept had no effect on the percent of monocytes in bone marrow sensitized animals, suggesting that TNFα does not mediate monocyte hematopoiesis. In the blood of air controls, etanercept increased BrdU- monocytes, at three days (Figure 5.10 D) in both air and ozone exposed animals, suggesting that TNFα mediates monocyte recruitment into tissue. In bronchoalveolar lavage, etanercept had no effect on the ozone-induced increase of macrophages in sensitized guinea pigs (Figure 5.9).

In blood, ozone increased BrdU- lymphocytes and three (Figure 5.10 E) days after ozone in sensitized guinea pigs, suggesting that after ozone TNFα mediates lymphocyte recruitment into lung tissue. In contrast, three days after ozone etanercept significantly increased mature BrdU- lymphocytes in non-sensitized guinea pigs (Figure 5.9 E).

Etanercept did not effect baseline heart rate or blood pressure in non-sensitized or sensitized guinea pigs (Table 5.1). In the heart, bradycardia induced by electrical

stimulation of both vagus nerves, or intravenous methacholine was not changed at one (Figure 5.2) or three (Figure 5.8) days by ozone, by sensitization, or by etanercept.

FIGURE 5.1. Pretreatment with etanercept had no effect on ozone-induced airway hyperreactivity one day after exposure.

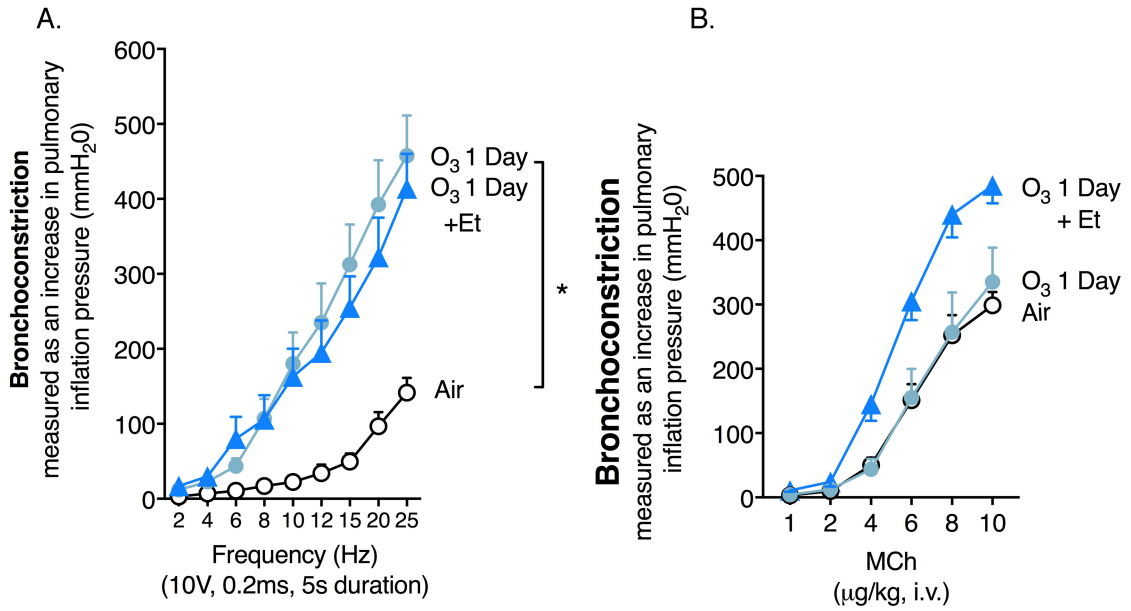


Figure 5.1. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bronchoconstriction that was significantly increased one day after ozone (open circles compared to closed circles data re-graphed from Figure 3.1 A). Etanercept pretreatment had no effect on ozone-induced airway hyperreactivity (blue triangles, A). Methacholine-induced smooth muscle contraction was not affected by ozone (data re-graphed from Figure 3.2A). Methacholine-induced bronchoconstriction was not changed by etanercept (B, triangles blue). Data shown as mean ± SEM n=4-9 *indicates $p \leq 0.05$ by repeated measures two-way ANOVA.

FIGURE 5.2. Vagally-induced and methacholine-induced bradycardia was not changed by ozone or etanercept three days later.

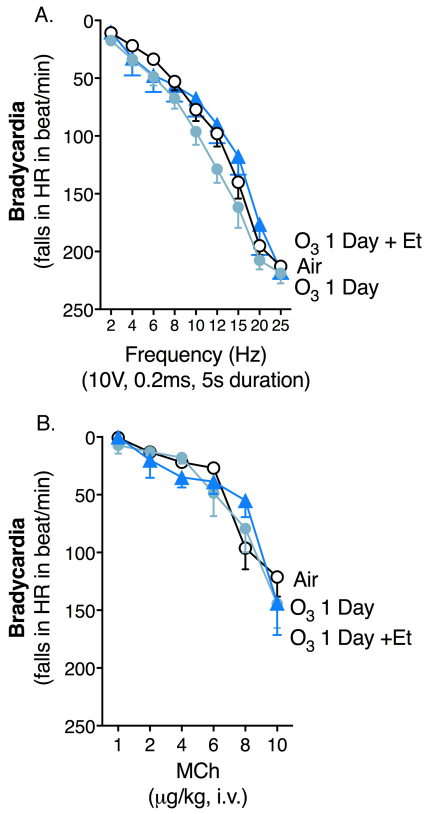


Figure 5.2. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bradycardia that was not affected by ozone (A, green circles) or by etanercept (A, blue triangles). Methacholine-induced bradycardia was also not changed by ozone (B, green circles) or by etanercept (B, blue triangles). Note: bradycardia from figure 3.3 A & C was re-graphed here for comparison. Data shown as mean ± SEM n=4-9.

FIGURE 5.3. Effect of etanercept on inflammatory cells in bronchoalveolar lavage one day after ozone exposure

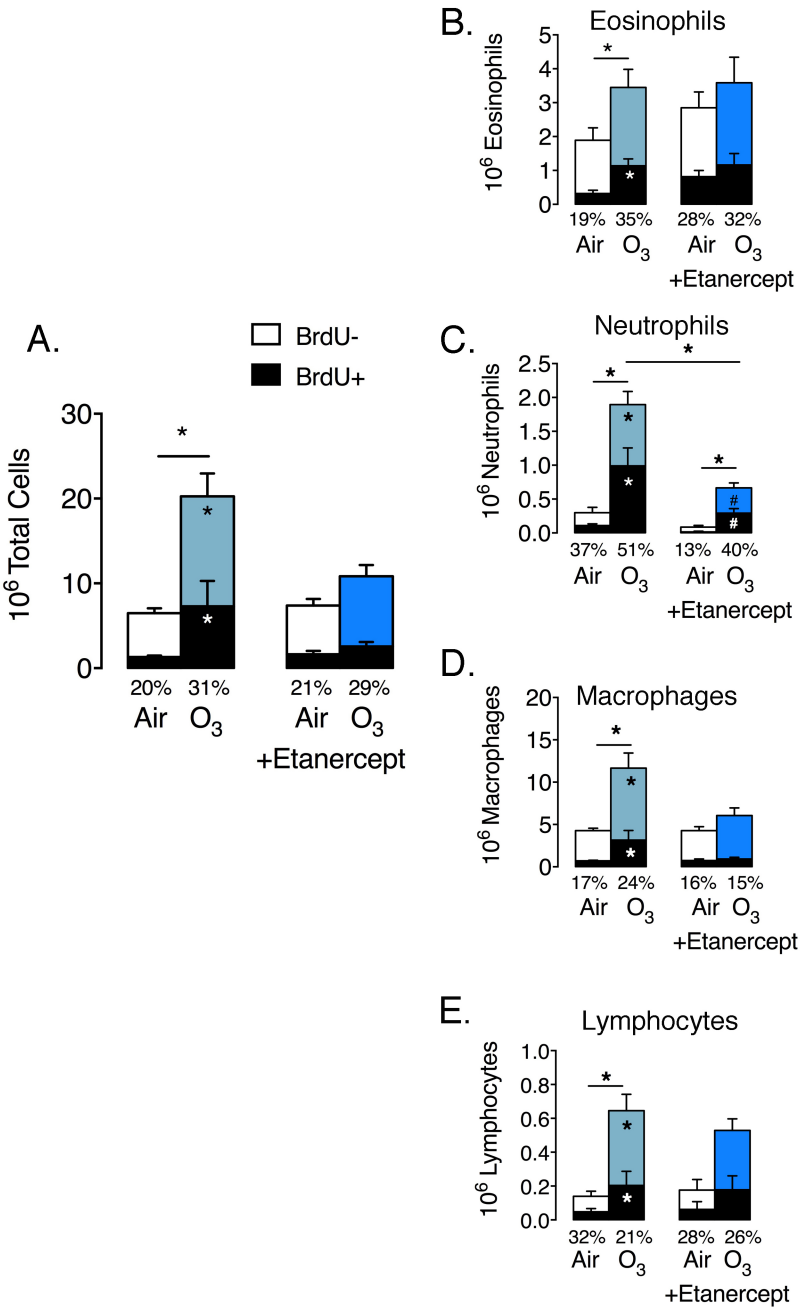


Figure 5.3. One day after ozone, total (A), eosinophils (B), neutrophils (C), macrophages (D) and lymphocytes (D) were all significantly increased in bronchoalveolar lavage. The increase in eosinophils was comprised only of BrdU+ cells, while the increase in neutrophils, macrophages, and lymphocytes was comprised of both BrdU+ and BrdU- cells. Etanercept pretreatment prevented ozone induced increase in total inflammatory cells (A, Blue/Black). Etanercept had no effect on BrdU+ or BrdU- eosinophils in bronchoalveolar lavage one day after ozone (B, Blue). In etanercept treated guinea pigs, neutrophils were significantly increased by ozone this increase was comprised of an increase in BrdU+ cells (C, Black). However, the ozone induced increase in neutrophils was significantly decreased when compared to untreated animals (C Green to Blue). Etanercept prevented the ozone-induced increase in macrophages (D, Blue) and Lymphocytes (E, Blue). Note as in Figure 4.5, the entire column height represents the total number of cells that is then subdivided into mature BrdU- (White/Green/Blue) and newly divided BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the bronchoalveolar lavage of guinea pigs 1 day after ozone (O_3) have been included from Figure 3.5 for statistical comparison. Data shown as mean ± SEM n=6-9. Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions.

FIGURE 5.4. Effect of etanercept on circulating lymphocytes one day after ozone.

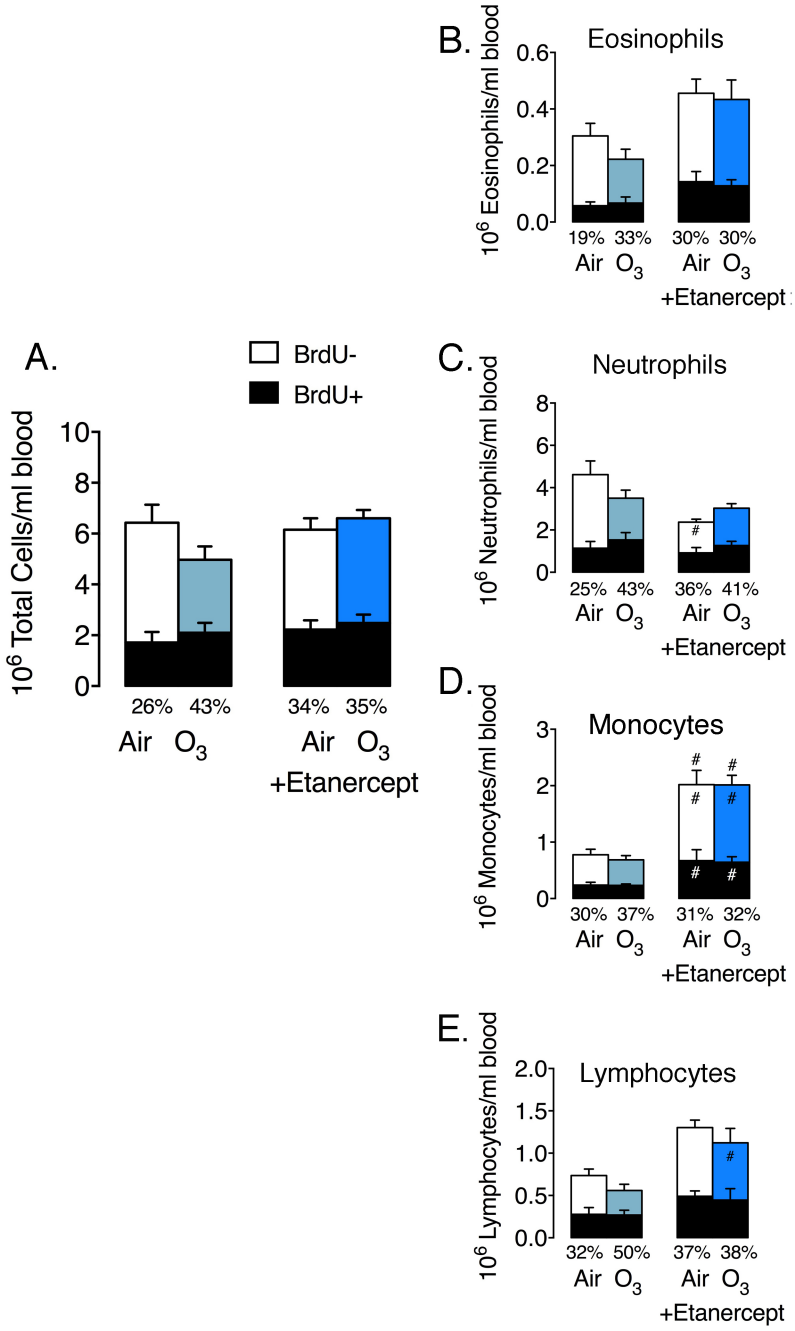


Figure 5.4. Ozone had no effect on total circulating leukocytes (A), eosinophils (B), neutrophils (C), monocytes (D), and lymphocytes (E) in blood one or exposure. Etanercept pretreatment significantly decreased BrdU- neutrophils (C, white). Etanercept significantly increased circulating monocytes (D) increase was comprised of an increase in BrdU+ and BrdU- cells. Ozone did not additionally change circulating monocytes in etanercept treated guinea pigs. Etanercept significantly increased BrdU- lymphocytes one day after ozone. Inflammatory cells from blood of guinea pigs one day after ozone have been included from Figure 3.6 for statistical comparison. Note, each column represents the total and is subdivided to show BrdU- (white/green/blue) and BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. Data are mean ± SEM; n=6-9. $P \leq 0.05$ by one way ANOVA comparing selected pairs of columns, # significantly different from untreated.

FIGURE 5.5. Effect of etanercept on inflammatory cells in bone marrow one day after ozone.

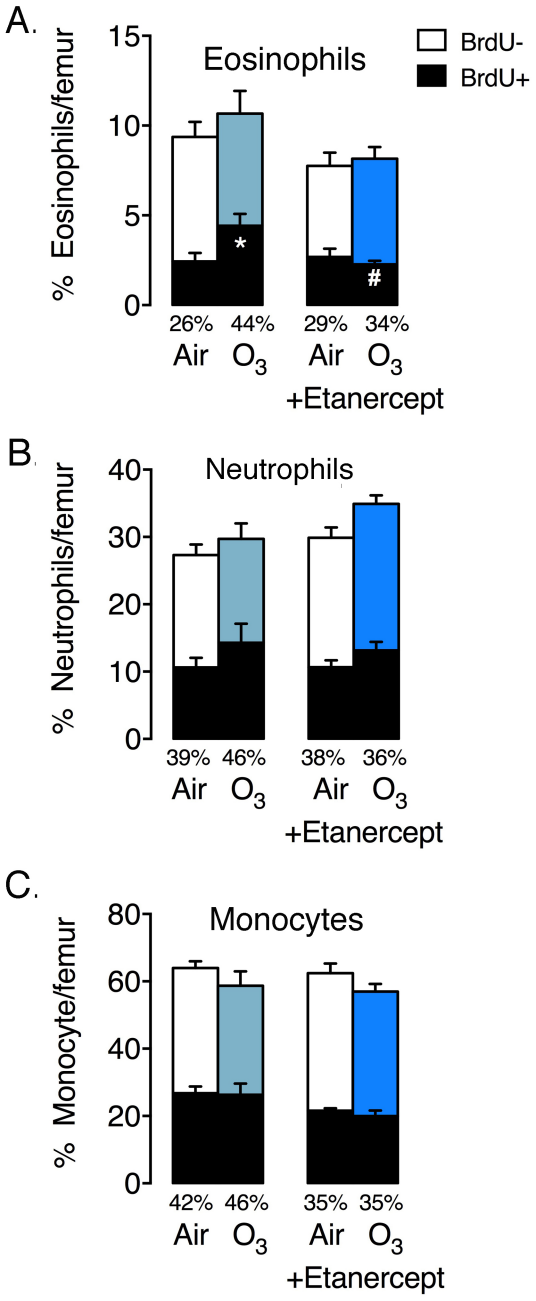


Figure 5.5. In bone marrow, etanercept significantly decreased the percent of BrdU+ eosinophils after ozone compared to non-treated animals (A). Neutrophils (B) and monocytes (C) in were not changed by ozone, or by etanercept. Note, each column represents the total and is subdivided to show BrdU- (white/green/blue) and BrdU+ (black) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the bone marrow of non-sensitized guinea pigs one day after ozone have been included from Figure 3.7 for statistical comparison. Data are mean ± SEM; n=4-9 $P \leq 0.05$ by one-way ANOVA comparing selected pairs of columns with a bonferroni correction *significantly different from air, #significantly different from 1 day post ozone.

FIGURE 5.6 Etanercept pretreatment significantly increased ozone-induced airway hyperreactivity in non-sensitized guinea pigs measured three days later.

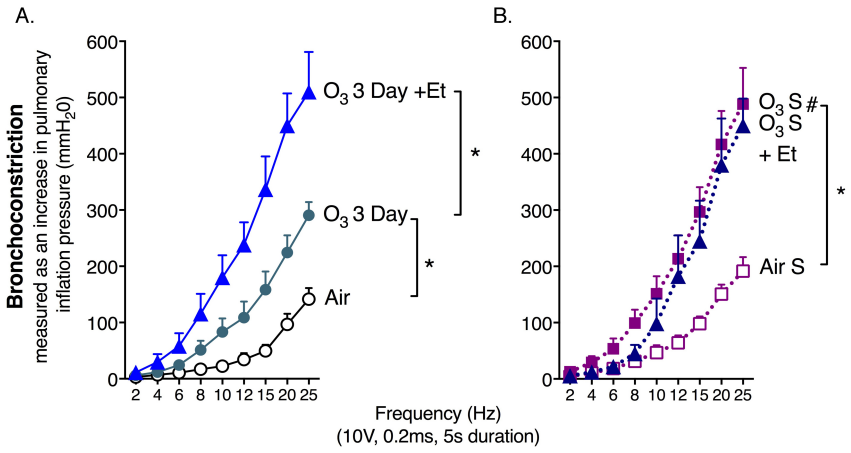


Figure 5.6 Etanercept (Et) pretreatment significantly increased ozone induced airway hyperreactivity in non-sensitized guinea pigs measured 3 days later. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bronchoconstriction that was significantly increased three days after ozone in non-sensitized (A) and sensitized (B) guinea pigs as in Figure 4.1. Vagally-mediated bronchoconstriction was significantly greater in sensitized (B) compared to non-sensitized (A) guinea pigs 3 days after ozone as in Figure 4.1. Etanercept significantly increased ozone-induced hyperreactivity in non-sensitized animals (A, blue triangles to green circles), but had no effect in sensitized animals (B, blue triangles to maroon squares). Data shown as mean ± SEM n=4-9 *indicates $p \leq 0.05$ by repeated measures two-way ANOVA. # significantly different than non-sensitized 3 day post ozone.

FIGURE 5.7 Etanercept pretreatment does not affect methacholine-induced bronchoconstriction three days after ozone in non-sensitized or sensitized guinea pigs.

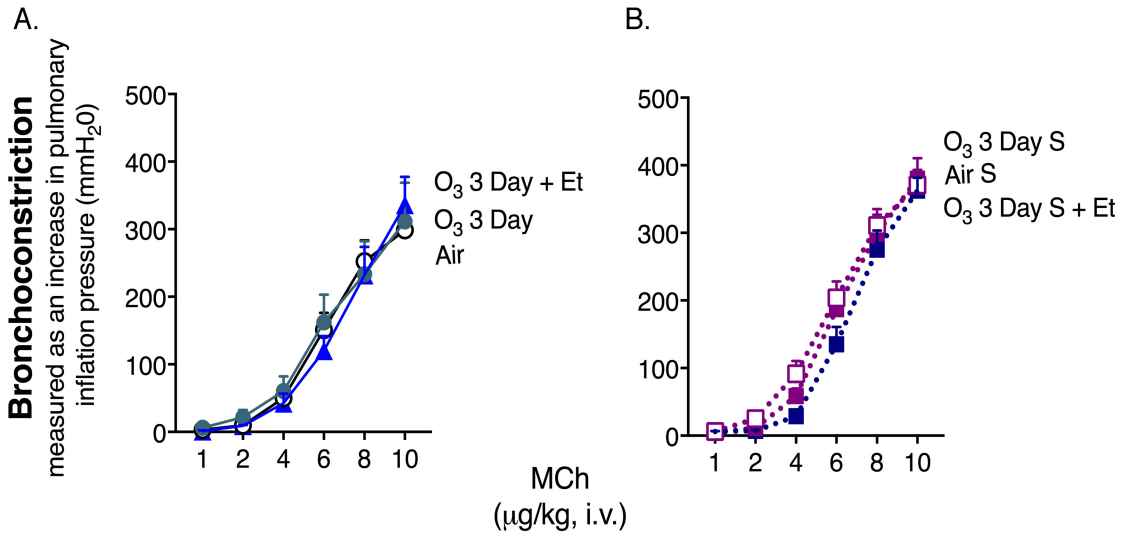


Figure 5.7 Bronchoconstriction induced by i.v. methacholine (1-10ug/kg i.v.) in vagotomized animals was not potentiated by ozone in non-sensitized (A, green) or sensitized (B, maroon) guinea pig as in Figure 4.2. Etanercept pretreatment no effect on methacholine-induced bronchoconstriction in non-sensitized (A, blue triangles) or sensitized (B, blue squares) guinea pigs. Data shown as mean ± SEM n= 4-9 guinea pigs.

FIGURE 5.8. Vagally-induced and methacholine-induced bradycardia was not changed by ozone, by sensitization, or by etanercept, three days after ozone.

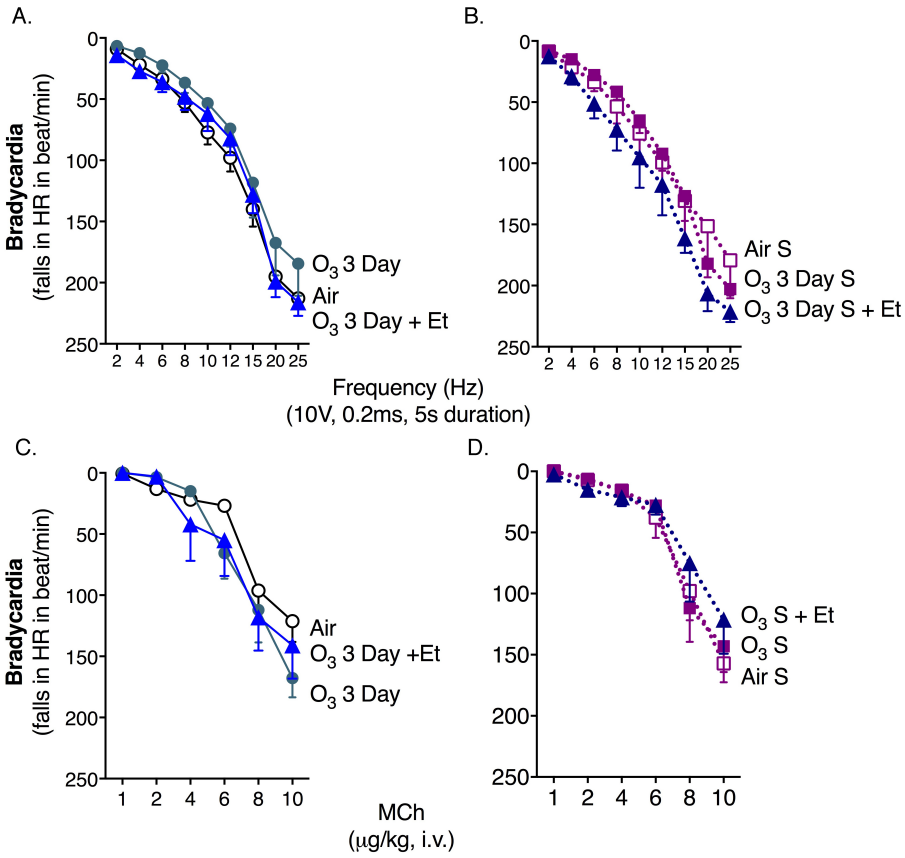


Figure 5.8. Electrical stimulation of both vagus nerves (1-25 Hz, 10V, 0.2ms, 5s train at 1 min intervals) produced frequency dependent bradycardia that was not affected by ozone (O_3; A) sensitization (S; B), or etanercept (Et; A & B blue triangles). Methacholine-induced bradycardia was not changed by ozone (C), by sensitization (D), or etanercept (C & D, Blue triangles). Note vagally-mediated and methacholine induced bradycardia in non-sensitized and sensitized guinea pigs is identical to data in Figure 4.3, but is reproduced here to allow statistical comparison. Data shown as mean ± SEM n=4-9.

FIGURE 5.9 Effect of etanercept on inflammatory cells in bronchoalveolar lavage three days after ozone exposure

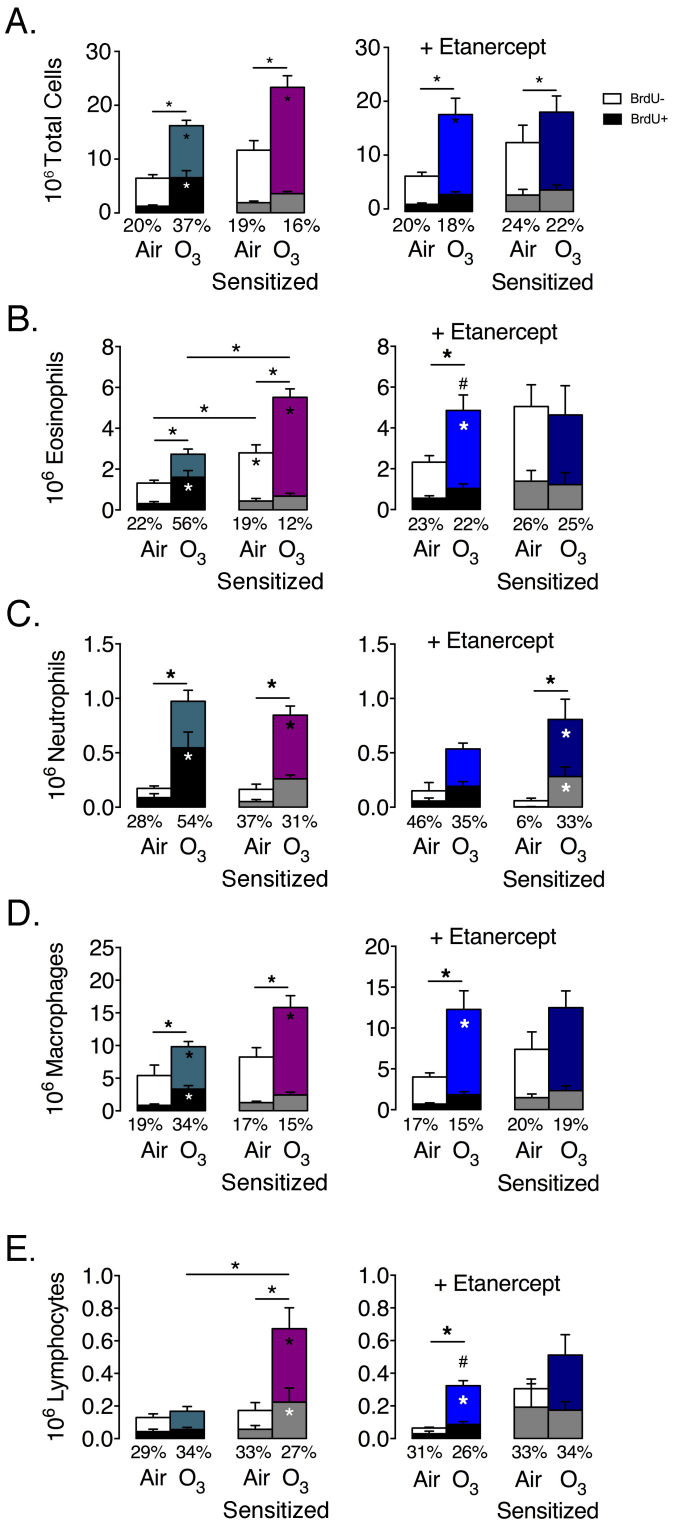


Figure 5.9. Ozone significantly increased total leukocyte (A) eosinophils (B), neutrophils (C), and macrophages (D) but not lymphocytes (E) in bronchoalveolar lavage of non-sensitized (black/white) guinea pigs 3 days later. In non-sensitized animals the ozone induced increase was comprised of BrdU+ cells. Only eosinophils were significantly potentiated by sensitization (B, compare first 2 sets of bars). In sensitized (grey/white) guinea pigs, ozone increased total (A), eosinophils (B), neutrophils (C), macrophages (D), and lymphocytes (E), however in sensitized animals ozone increased BrdU- cells. Etanercept potentiated ozone's ability to recruit eosinophils, macrophages, and lymphocytes in non-sensitized animals (third set of columns, black/blue), and potentiated ozone's ability to recruit neutrophils in sensitized animals (fourth set of columns, grey/blue). Note, each column represents the total and is subdivided to show BrdU- (white/green/maroon/blue) and BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the bronchoalveolar lavage Figure 5.5 have been re-graphed here for statistical comparison. Data shown as mean ± SEM n=4-9 Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions. *Significantly different from air, #significantly different from untreated.

FIGURE 5.10 Effect of etanercept on circulating lymphocytes non-sensitized and sensitized guinea pigs, three days after exposure.

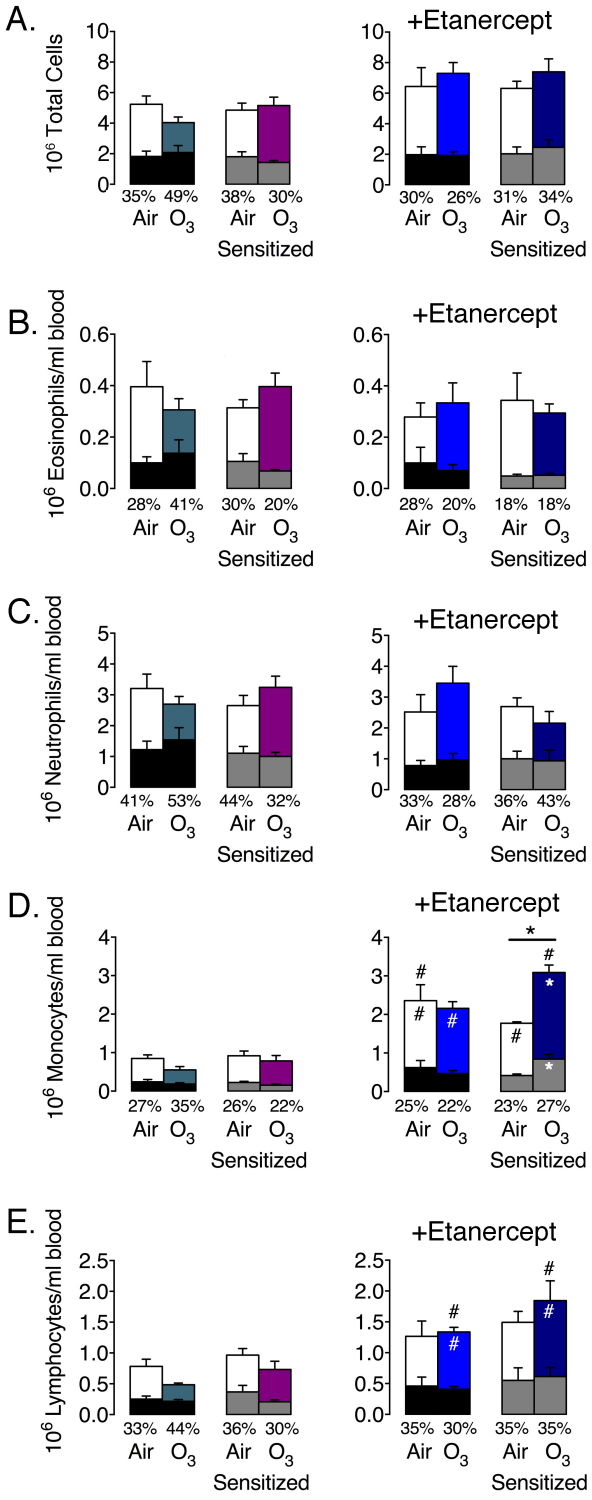


Figure 5.10. Ozone had no effect on total leukocytes (A), eosinophils (B), neutrophils (C), monocytes (D), and lymphocytes (E) in blood three days after exposure in non-sensitized (black/green) and sensitized guinea (grey/maroon) pigs. Etanercept significantly increased circulating monocytes (D) in both non-sensitized and sensitized guinea pigs. Three days after ozone, etanercept pretreatment increased circulating monocytes (D) and lymphocytes (E) in both non-sensitized (black/blue) and sensitized (grey/blue) animals. Note each column represents the total and is subdivided to show BrdU- (white/green/maroon/blue) and BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. Inflammatory cells from the blood Figure 4.6 have been re-graphed here for statistical comparison. Data shown as mean ± SEM n=4-9 Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions. *Significantly different from air, #significantly different from untreated.

FIGURE 5.11. Effect of etanercept pretreatment on ozone induced inflammatory cell hematopoiesis in non-sensitized and sensitized pigs, three days after exposure.

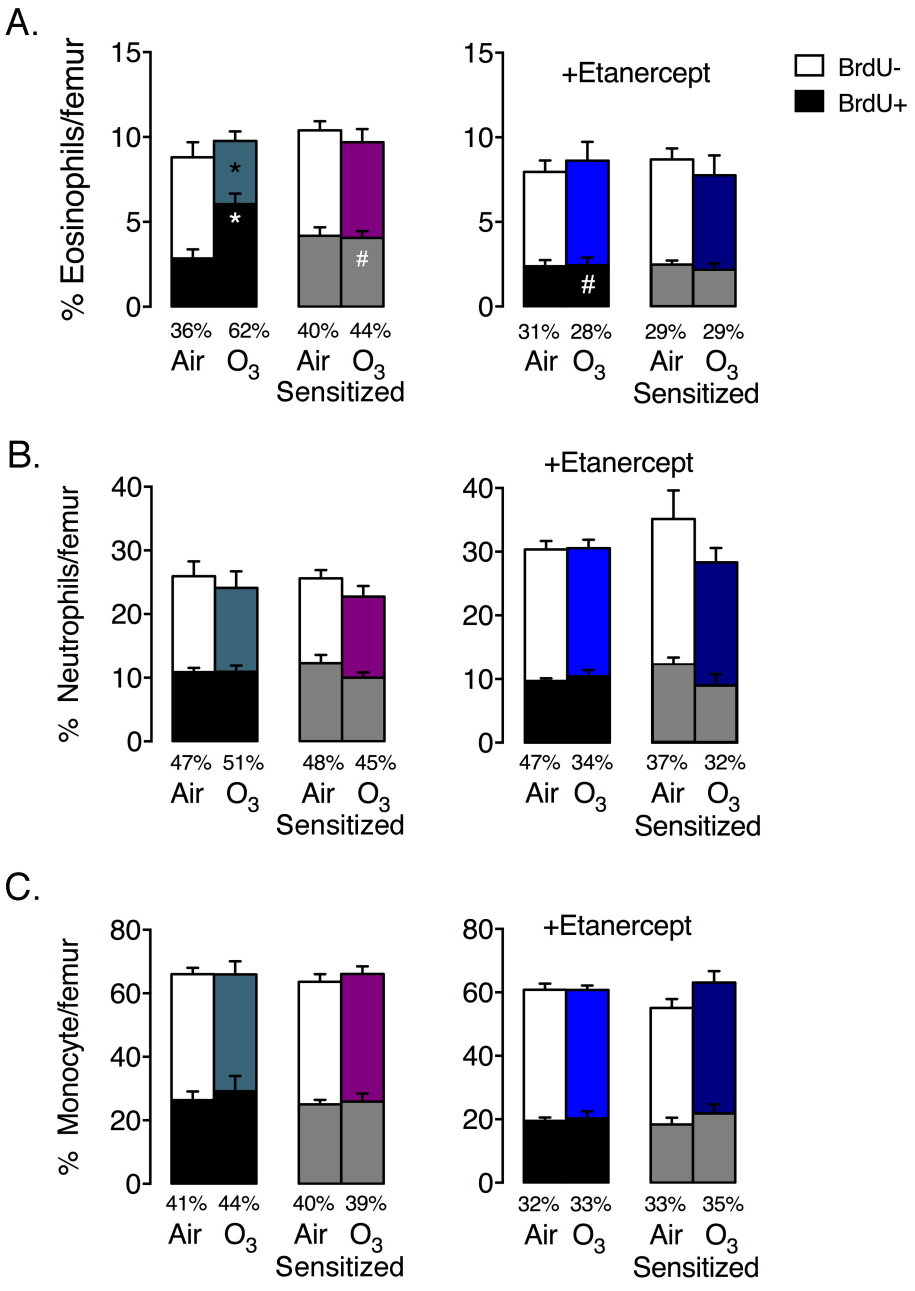


Figure 5.11. Ozone significantly increased BrdU+ (black) eosinophils in bone marrow (first set of columns, black, A) in non-sensitized guinea pigs, however, this increase failed to occur in sensitized (second set of columns, grey, A). Etanercept pretreatment prevented ozone induced increase in BrdU+ eosinophils in 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. Note as in Figure 3.5, the entire column height represents the total number of cells that is then subdivided into mature BrdU- (white) and newly divided BrdU+ (black/grey) cells. The percent of BrdU+ cells is included under each column. The first column is identical to data in figure 4.7, but is reproduced here to allow statistical comparison. Data shown as mean ± SEM n=5-9 Significantly different by one-way ANOVA with a Bonferroni correction ($p \leq 0.05$). Statistical comparisons of total cells are shown as horizontal lines above bars. Statistical comparisons for BrdU+ and BrdU- are shown within subdivisions. *Significantly different from air, #significantly different from non-sensitized ozone.

TABLE 5.1 Baseline Values

			Baseline PPI (mmH_2O)		Heart Rate (bpm)		Systolic BP (mmHg)		Diastolic BP (mmHg)		Weight (g)	
Non-sensitized	Control	Air	102.2	±4.9	287.8	±9.8	41.0	±1.1	19.6	±0.7	351.3	5.2
		O_3 1 Day	202.5	±4.8*	273.8	±5.5	46.0	±2.1	20.9	±1.0	383.5	±11.2
		O_3 3 Day	151.4	±6.3*	283.6	±6.0	44.8	±2.7	24.0	±1.5	382.6	±11.0
	Etanercept	O_3 1 Day	158.3	±12.7	297	±4.3	42.5	±2.8	19.0	±1.9	394.8	± 2.7
		O_3 3 Day	172.0	±12.4*	272.0	±8.7	44.8	±1.7	21.6	±1.3	379.6	± 4.8
Sensitized	Control	Air	102.0	±8.6	302.0	±9.9	43.5	±2.9	20.0	±0.8	406.8	±17.4
		O_3 3 Day	158.3	±12.7*	275.0	±4.3	44.3	±1.9	22.3	±1.6	394.8	± 3.0
	Etanercept	O_3 3 Day	137.5	±19.7	277.5	±8.5	47.0	±3.1	23.0	±1.0	366.5	±4.3

Table 5.1 Baseline values are means ± SEM. Baseline pulmonary inflation pressure was significantly increased one and three days after a single exposure to 2.0ppm ozone (O_3; 4 hr) in sensitized and non-sensitized guinea pigs. Baseline heart rate and blood pressure were not affected by ozone in sensitized or non-sensitized guinea pigs.

*Significantly different by one-way ANOVA with a Bonferroni correction ($p<0.05$).

TABLE 5.2 Percent BrdU+ Eosinophils

% BrdU+ Eosinophils				Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-sensitized	Control	1 day	Air	19 ± 3.6	19 ± 2.6	26 ± 4.2
			O_3	35 ± 2.6*	33 ± 8.8	44 ± 6.1*
		3 day	Air	22 ± 5.6	28 ± 4.8	36 ± 5.3
			O_3	56 ± 8.5*	41 ± 8.7	62 ± 5.1*#
	Etanercept	1 day	Air	28 ± 4.4	29 ± 2.5	29 ± 1.2
			O_3	32 ± 5.4	30 ± 1.9	34 ± 2.8
		3 day	Air	23 ± 2.9	28 ± 6.1	31 ± 2.1
			O_3	22 ± 2.4#	20 ± 1.9	28 ± 2.5#
Sensitized	Control	3 day	Air	19 ± 3.5	30 ± 4.8	40 ± 3.2
			O_3	12 ± 1.9#	20 ± 3.3	44 ± 5.0#
	Etanercept	3 day	Air	26 ± 4.8	18 ± 4.5	29 ± 2.8
			O_3	25 ± 4.1	18 ± 2.1	29 ± 3.7

Table 5.2 Percent BrdU+ Eosinophils. In bronchoalveolar lavage, the percent of BrdU+ eosinophils was significantly increased one and three days after ozone when compared to air control. In sensitized and etanercept treated animals, the percent of BrdU+ eosinophils in bronchoalveolar lavage was significantly decreased when compared to non-sensitized guinea pigs three days after ozone. Similarly in bone marrow, the percent of BrdU+ eosinophils was significantly increased one and three days after ozone when compared to air control. In sensitized and etanercept treated guinea pigs, the percent of BrdU+ eosinophils in bone marrow was significantly decreased when compared with non-sensitized guinea pigs three days after ozone. Data shown as mean ± SEM n=4-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

TABLE 5.3 Percent BrdU+ Neutrophils

% BrdU+ Neutrophils				Bronchoalveolar Lavage Mean ±SEM	Blood Mean ±SEM	Bone Marrow Mean ±SEM
Non-sensitized	Control	1 day	Air	37 ± 7.0	25 ± 3.4	39 ± 3.7
			O_3	51 ± 8.7	43 ± 8.6	46 ± 7.7
		3 day	Air	28 ± 7.1	41 ± 5.1	47 ± 4.8
			O_3	54 ± 7.7	53 ± 8.7	51 ± 7.8
	Etanercept	1 day	Air	13 ± 8.9	37 ± 4.4	38 ± 2.1
			O_3	40 ± 4.0	41 ± 3.1	36 ± 2.5
		3 day	Air	46 ± 15.0	33 ± 4.0	47 ± 14.0
			O_3	35 ± 5.0	28 ± 1.5#	34 ± 2.2
Sensitized	Control	3 day	Air	37 ± 6.0	44 ± 8.0	48 ± 3.6
			O_3	31 ± 2.2	32 ± 4.5	45 ± 3.3
	Etanercept	3 day	Air	6 ± 6.3#	36 ± 5.5	37 ± 6.2
			O_3	33 ± 5.4*	43 ± 15.0	32 ± 2.3

Table 5.3 The Percent BrdU+ Neutrophils. In bronchoalveolar lavage, blood, and bone marrow the percent BrdU+ neutrophils was not changed by ozone or by sensitization three days after exposure. In sensitized guinea pigs treated with etanercept, BrdU+ neutrophils were decreased in bronchoalveolar lavage of air exposed guinea pigs. Three days after ozone BrdU+ neutrophils were significantly increased from sensitized etanercept treated air control. Data shown as mean ± SEM n=4-9. *significantly different from sensitized air + etanercept, #significantly different from sensitized air.

TABLE 5.4 Percent BrdU+ Macrophages

% BrdU+ Macrophages				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	1 day	Air	17 ± 1.5	31 ± 2.4	35 ± 2.8
			O_3	15 ± 1.4	32 ± 2.5	35 ± 2.0
		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 5.4 Percent BrdU+ macrophages. In bronchoalveolar lavage, the percent of BrdU+ macrophages was significantly increased one and three days after ozone when compared to air control. In sensitized and etanercept treated guinea pigs, the percent of BrdU+ macrophages in bronchoalveolar lavage was significantly decreased when compared to non-sensitized guinea pigs three days after ozone. Data shown as mean ± SEM n=4-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

TABLE 5.5 Percent BrdU+ Lymphocytes

% 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	1 day	Air	28 ± 4.3	31 ± 2.4	35 ± 2.8
			O_3	26 ± 8.8	32 ± 2.5	35 ± 2.0
		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 5.5 Percent BrdU+ lymphocytes. In bronchoalveolar lavage, blood, and bone marrow the percent BrdU+ lymphocytes was not changed by ozone, by sensitization or by etanercept one or three days after exposure. Data shown as mean ± SEM n=4-9.

TABLE 5.6 Percent BrdU+ Total Cells

% BrdU+ Total Cell				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	1 day	Air	21 ± 2.4	34 ± 2.0
			O_3	29 ± 2.0	35 ± 2.7
		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

Table 5.6 Percent BrdU+ total cells. In bronchoalveolar lavage, the percent of BrdU+ cells was significantly increased one and three days after ozone in non-sensitized guinea pigs. In sensitized and etanercept treated guinea pigs, the percent of BrdU+ cell in bronchoalveolar lavage were significantly decreased three days after ozone when compared to non-sensitized guinea pigs. In bone marrow, etanercept decreased BrdU+ cells when compared to three day ozone. Data shown as mean ± SEM n=4-9 Significantly different by one-way ANOVA comparing selected pairs of columns with a Bonferroni correction ($p \leq 0.05$). *significantly different from air, #significantly different from non-sensitized ozone.

DISCUSSION

One day after ozone, inflammatory cells including eosinophils are increased in bronchoalveolar lavage (Yost et al 1999, Yost et al 2005). At this time point eosinophils mediate ozone induced airway hyperreactivity (Yost et al 1999, Yost et al 2005). Blocking TNFα with etanercept had no effect on ozone-induced airway hyperreactivity (Figure 5.1) or the ratio of newly divided BrdU+ to mature BrdU- eosinophils in the lungs one day after ozone. Thus, TNFα does not affect ozone-induced airway hyperreactivity or eosinophil recruitment to the lungs one day after ozone.

In contrast to TNFα's effect one day after ozone, my data show a novel protective role for TNFα in the lungs three days after exposure. Blocking TNFα with etanercept significantly worsened ozone-induced airway hyperreactivity at this time point (Figure 5.6). TNFα's effect appears to be mediated by a beneficial subpopulation of newly divided eosinophils, since etanercept blocked an increase in newly divided BrdU+ eosinophils in bone marrow (Figure 5.11 A) and in bronchoalveolar lavage (Figure 5.9 B). Similar effects were seen by depleting eosinophils with AbIL5, which also worsens airway hyperreactivity in non-sensitized animals three days after ozone (Yost et al 2005). I confirmed the protective role of eosinophils in the lungs three days after ozone (Figure 4.1 A).

Blocking TNFα in sensitized animals had no additional effect on eosinophil populations in bone marrow (Figure 5.11) or bronchoalveolar lavage (Figure 5.9) or on ozone-induced airway hyperreactivity (Figure 5.6 B). These data suggest that sensitization prevents TNFα from promoting production of new, beneficial eosinophils and their recruitment to the lungs three days after ozone. Allergic patients sensitized to allergens also have increased eosinophils in bronchoalveolar lavage after ozone (Bascom et al 1990, Brown et al 2007, Noah et al 1995). Thus, allergic 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, newly divided, eosinophils.

In both sensitized and non-sensitized guinea pigs, blocking TNFα increased the number of mature, BrdU-, monocytes and lymphocytes in blood. Since TNFα is known to regulate vascular endothelial adhesion molecule expression (Dokic et al 2011, Iademarco et al 1995, McHale et al 1999, Okahara et al 1994), blocking TNFα may lead to increased retention of cells in the bloodstream by down regulating adhesion molecules. However, retention of monocytes and lymphocytes in circulation after TNFα blockade did not inhibit ozone induced increase in inflammatory cells in the lungs demonstrating inflammatory cell recruitment to the lungs is not inhibited by TNFα blockade three days after ozone. While ozone increases all inflammatory cells in the lungs, only loss of newly divided, eosinophils exacerbate ozone-induced airway hyperreactivity.

In humans, anti-TNFα treatments were effective in patients with severe refractory asthma, but not those with moderate asthma or allergic asthma (Berry et al 2006, Brightling et al 2008, Rouhani et al 2005, Wenzel et al 2009). Blocking TNFα in those patients did not change eosinophilic inflammation in lungs (Berry et al 2006, Brightling et al 2008, Rouhani et al 2005, Wenzel et al 2009). Importantly, my findings indicate that measuring total eosinophils may insufficiently distinguish between mature eosinophils and beneficial, newly divided eosinophils. It is possible that blocking TNFα may worsen asthma control after ozone exposure through the loss of protective, newly divided, eosinophils.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The data presented in this dissertation provide novel findings in the field of ozone-induced airway hyperreactivity. Here I show that in non-sensitized guinea pigs, ozone induces eosinophil hematopoiesis and recruitment to lungs, where three days later, newly divided eosinophils are protective (Chapter III). However, in sensitized guinea pigs, ozone-induced eosinophil hematopoiesis fails to occur, and in the absence of protective, newly divided eosinophils, mature BrdU- eosinophils mediate ozone-induced airway hyperreactivity (Chapter IV). In non-sensitized guinea pigs, ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided eosinophils to lungs is mediated by TNFα, while in sensitized guinea pigs, TNFα does not play a role in ozone-induced eosinophil hematopoiesis (Chapter V). I have adapted Figure 1.10 to include my findings here and have included it as Figure 6.1.

Here I show that ozone, increased newly divided eosinophils in the bone marrow one and three days after ozone. Three days after ozone, the eosinophil population in bronchoalveolar lavage is predominately comprised of newly divided eosinophils. This shows that ozone-induces eosinophil hematopoiesis and subsequent of recruitment of newly divided cells to the lungs three days later (Chapter III).

Previous work demonstrated that eosinophils role changed between one and three days after ozone. One day after ozone, airway hyperreactivity was mediated by degranulation of eosinophils, release of major basic protein, blockade of inhibitory M_2

muscarinic on parasympathetic nerves, increased acetylcholine release and increased vagally mediated bronchoconstriction (Yost et al 1999). Here I show, that one day after ozone, the eosinophil population in bronchoalveolar lavage is predominately comprised of mature BrdU- eosinophils. Thus, one day after ozone mature BrdU- eosinophils mediate ozone induced airway hyperreactivity. In contrast, three days after ozone, depleting eosinophils significantly worsened ozone-induced airway hyperreactivity (Chapter III) (Yost et al 2005). Data included in this thesis demonstrate that at this three day time point, the eosinophil population in bronchoalveolar lavage is predominantly comprised of newly divided, BrdU+ eosinophils. Depleting the BrdU+ cells with AbIL5 worsens airway hyperreactivity, thus, newly divided eosinophils are protective in the lungs three days after ozone.

Prior sensitization increases airway hyperreactivity to subsequent provocations such as ozone (Inman et al 1999, Joad et al 2006, Khatri et al 2009, Vargas et al 1994). In guinea pigs, I demonstrated that ozone induced airway hyperreactivity was significantly increased in sensitized guinea pigs when compared to non-sensitized animals. Mature BrdU- eosinophils are increased in the lungs three days after ozone in sensitized guinea pigs. Depleting eosinophils with AbIL5 inhibited ozone-induced airway three days after exposure. Thus, I demonstrated that mature BrdU- eosinophils mediate ozone-induced airway hyperreactivity. Sensitization has been shown to alter the mechanism of virus-induced hyperreactivity from an eosinophil independent mechanism in non-sensitized animals to an eosinophil dependent mechanism in

sensitized animals (Adamko et al 1999). Here I show, that the role of eosinophils three days after ozone changes from beneficial in non-sensitized animals to mediating ozone induced airway hyperreactivity in sensitized guinea pigs.

Sensitized guinea pigs have increased eosinophils in bronchoalveolar lavage at baseline (Chapter IV)(Adamko et al 1999, Proskocil et al 2008, Sonar et al 2012). Ozone increased eosinophils in bronchoalveolar lavage above sensitization alone (Chapter IV) (Dokic & Trajkovska-Dokic 2013, Holz et al 2002, Jang et al 2006, Kierstein et al 2008). I demonstrated that ozone does not change populations of mature or newly divided eosinophils in the blood or the bone marrow of sensitized guinea pigs (Chapter IV). However, mature eosinophils are significantly increased in bronchoalveolar lavage three days after ozone. In sensitized animals, cytokines known to mediate eosinophil survival, IL-4, IL-5, and GM-CSF, are increased in bronchoalveolar lavage after ozone (Kierstein et al 2008, Swartzendruber et al 2012, Takatsu & Nakajima 2008). Addionally, eosinophils in allergic animals down regulates Fas-L, a gene that induces eosinophil apoptosis, following ozone exposure (Kierstein et al 2008). Thus, the accumulation of mature BrdU- cells in bronchoalveolar lavage is likely mediated by increased eosinophil survival.

TNFα is increased in the lungs after ozone exposure (Bao et al 2013, Esther et al 2011, Wang et al 2015). Blocking TNFα with etanercept had no effect on hyperreactivity or inflammation one day after ozone, but worsened airway hyperreactivity three days

after ozone. At this three day time point, blocking TNFα also prevented the ozone-induced increase in newly divided eosinophils in bone marrow, and despite increasing total eosinophil numbers prevented newly divided eosinophils in bronchoalveolar lavage. TNFα has been shown to induce eosinophil differentiation of eosinophil progenitors (Lopez et al 2003). Thus, TNFα promotes ozone-induced eosinophil hematopoiesis of beneficial, newly divided eosinophil in non-sensitized guinea pigs.

In contrast, I demonstrated that blocking TNFα in sensitized animals had no effect on ozone-induced airway hyperreactivity or on eosinophil populations in bone marrow or bronchoalveolar lavage. These data demonstrate that sensitization prevents TNFα from promoting production of new, beneficial eosinophils and their recruitment to the lungs three days after ozone.

It was recently shown, that mice sensitized and challenged with ovalbumin have multiple eosinophil populations in the lungs (Abdala Valencia et al 2016, Mesnil et al 2016, Percopo et al 2017). Mice were identified as having two resident eosinophil populations in the lungs identified Siglec F (int), CD62L (+), CD101 (low) and Siglec F (High), CD62L(-), CD101(high) (Mesnil et al 2016). Additionally eosinophils in mice have been shown to change surface receptor expression from Siglec-F(med) CD11c(-) to Siglec-F(high) CD11c(low) as they move from the lung into the airway lumen (Abdala Valencia et al 2016). A third study found that eosinophils that expressed the common neutrophil receptor gr1 expressed increased levels of IL-13 (Percopo et al

2017). These three independent descriptions of unique eosinophil populations in the lungs, suggests that newly divided BrdU+ are likely a distinct population of eosinophils and that they may release a unique combination of cytokines, which contribute to the resolution of ozone-induced airway hyperreactivity three days after ozone exposure.

New biologic therapies have been developed that reduce eosinophilic inflammation by targeting IL-5 (Bel et al 2014b, Lim & Nair 2015, Ortega et al 2014) and IL-5 receptors (Laviolette et al 2013). However, it is becoming clear that eosinophils have diverse roles in tissue repair (Goh et al 2013), remodeling (Gouon-Evans et al 2000, Jeziorska et al 1996, Jeziorska et al 1995, Robertson et al 2000), and immune regulation (Wang et al 2007). In human lungs, the presence of beneficial eosinophils is suggested by a recent study in asthmatic children where depleting inflammatory cells with steroids, not only failed to prevent ozone induced airway hyperreactivity, but actually potentiated it (Ierodiakonou et al 2016). Thus, broad depletion of eosinophilic inflammation with steroids or eosinophil targeting therapies may worsen ozone induced airway hyperreactivity and delay resolution of symptoms.

In conclusion, TNFα mediates ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided BrdU+ eosinophils to lungs three days later where they inhibit vagally mediated bronchoconstriction in non-sensitized guinea pigs. TNFα does not play a role in ozone-induced eosinophil hematopoiesis or recruitment to the lungs in sensitized guinea pigs.

The data included in this thesis and the conclusions I have drawn are important for human health since over 50 percent of people in the United States live in areas with unhealthy levels of ozone (2016). Ozone has been shown to increase asthma related exacerbations and hospitalizations with a lag of one to three days following the exposure (Mar & Koenig 2009, Sheffield et al 2015). However, the current approach to asthma treatment of depleting inflammation with corticosteroids fail to improve lung function (Ierodiakonou et al 2016, Stevens et al 1994, Vagaggini et al 2001). The findings presented in this study show, that eosinophils in the lungs after ozone exposure are heterogeneous, and that the presence of eosinophils alone is not sufficient to predict their role in airway hyperreactivity. Furthermore, my data demonstrate that newly divided, BrdU+ eosinophils have a beneficial role. Thus, in asthmatics, therapies directed at protection of or expansion of BrdU+ eosinophils may provide novel mechanisms to prevent or reduce asthma exacerbations following ozone exposure.

This dissertation demonstrates, that ozone causes airway hyperreactivity that lasts at least three days. In non-sensitized guinea pigs, TNFα promotes ozone-induced eosinophil hematopoiesis and recruitment to lungs. These newly divided, BrdU+ eosinophils have a beneficial, protective effect by three days after exposure. Sensitization status fundamentally alters the eosinophils' role. Sensitization 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 .

FIGURE 6.1. Ozone induced eosinophil hematopoiesis is dependent on Tumor Necrosis Factor-alpha and sensitization status.

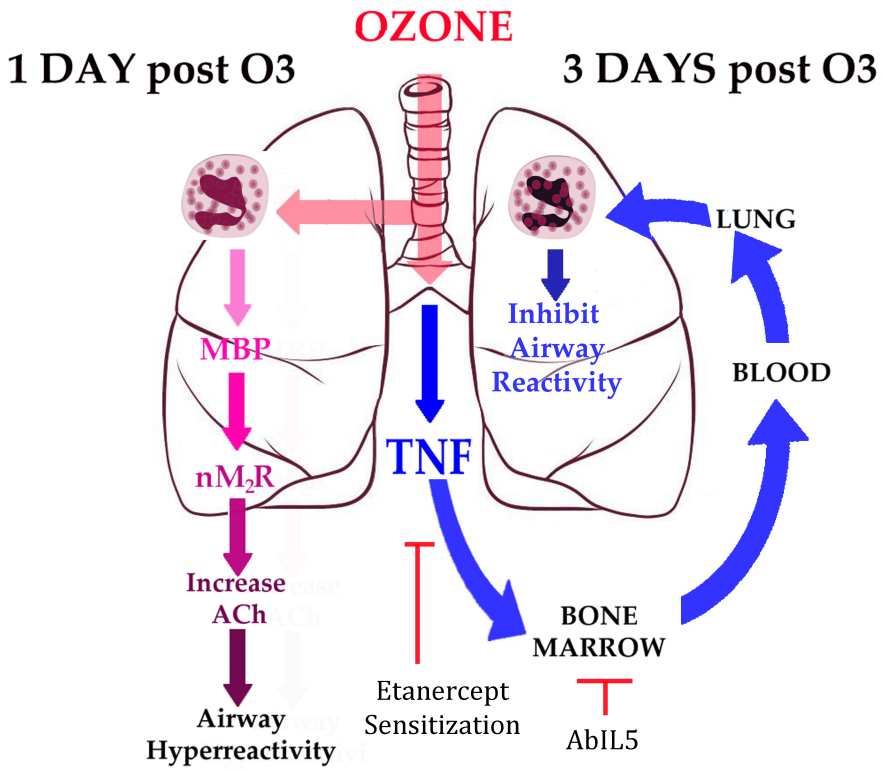


Figure 6.1 Using a guinea pig model I demonstrated that ozone induced eosinophil hematopoiesis is dependent on TNF and sensitization status. In non-sensitized guinea pigs, ozone causes airway hyperreactivity lasting at least three days, and induces eosinophil hematopoiesis and recruitment to lungs, where three days later, newly divided eosinophils are protective (Chapter III). However, in sensitized guinea pigs, ozone-induced eosinophil hematopoiesis fails to occur, and in the absence of protective, newly divided eosinophils, mature BrdU- eosinophils mediate ozone-

induced airway hyperreactivity (Chapter IV). In non-sensitized guinea pigs, ozone-induced eosinophil hematopoiesis and subsequent recruitment of newly divided eosinophils to lungs is mediated by TNFα, while in sensitized guinea pigs, TNFα does not play a role in ozone-induced eosinophil hematopoiesis (Chapter V).

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