TIOTROPIUM INHIBITS INCREASES IN NERVE-ASSOCIATED EOSINOPHILS FOLLOWING ANTIGEN CHALLENGE AND PREVENTS AIRWAY HYPERREACTIVITY IN A GUINEA PIG MODEL OF ASTHMA

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ABSTRACT

Parasympathetic nerves in the lung provide the dominant control over airway smooth muscle tone. In asthmatic humans and in animal models of allergic asthma the airways are hyperresponsive to nonspecific contractile stimuli, and this hyperresponsiveness is mediated by increased acetylcholine release from parasympathetic nerves. Airway hyperresponsiveness is associated with eosinophil recruitment to airway nerves. In animals, treatments that prevent eosinophils from associating with airway nerves prevent airway hyperresponsiveness, and in humans, treatments that reduce lung eosinophils reduce asthma exacerbations. Furthermore, surgical denervation of the airways in humans with asthma decreases airway responsiveness and abolishes sputum eosinophils, suggesting parasympathetic nerves are important both for airway hyperresponsiveness and eosinophil recruitment. The hypothesis tested in this thesis is that in allergic asthma parasympathetic nerves in the lungs regulate inflammation leading to airway hyperresponsiveness, both through release of the neurotransmitter acetylcholine and through release of eosinophil chemoattractant proteins.

Data presented in this thesis demonstrate that selective blockade of M_3 muscarinic acetylcholine receptors with tiotropium during inhaled antigen challenge prevents subsequent airway hyperresponsiveness in a guinea pig model of asthma. Surprisingly, airway hyperresponsiveness was prevented by a mechanism that does not involve inhibition of bronchoconstriction, since vagally induced bronchoconstriction was not inhibited in control animals that were not exposed to antigen. Instead, the mechanism may be anti-inflammatory as both total and nerve-associated eosinophils were reduced in lung sections. These data suggest selective M_3 muscarinic receptor antagonists may have benefits in asthma separate from inhibition of bronchoconstriction.

Data in this thesis also show that neuronal cells grown in culture produce several ligands for CCR3 chemokine receptors on eosinophils in response to tumor necrosis factor alpha, a cytokine that is increased in asthma. Human SK-N-SH cells produce mRNA transcripts and protein for CCL5 (RANTES), CCL7 (MCP-3), and CCL11 (eotaxin), while guinea pig parasympathetic neurons only produce one chemokine, CCL7. However, *in vivo* data presented in this thesis do not support the hypothesis that eosinophils are recruited to airway nerves via neuronally produced CCR3 chemokines. Instead, these data suggest a model where eosinophils are recruited to the lungs via CCR3 chemokines produced by nonneuronal cells, and this general increase in eosinophils results in a proportional increase in nerve-associated eosinophils. The results extend previous observations of nerve-associated eosinophils, since they show eosinophils associate with both cholinergic and noncholinergic nerves following antigen challenge. Thus, eosinophils may interact with parasympathetic, sensory, or possibly sympathetic neurons in the lung to mediate airway hyperreactivity.

The implications of these findings are that selective M_3 muscarinic receptor antagonists may be beneficial for long-term treatment in asthma via an anti-inflammatory mechanism. In particular, they may reduce asthma exacerbations by reducing nerve-associated eosinophils (parasympathetic, sensory, or sympathetic) and preventing subsequent increases in airway hyperresponsiveness.

CHAPTER 1.

INTRODUCTION

Introduction

A) Lung Anatomy and Function

The lung's primary function is to exchange oxygen in air with carbon dioxide in blood. Human lungs provide an enormous surface area, 50-100 m^2, for gases to diffuse between air and blood (West, 2005). Prior to gas exchange, air is warmed, humidified, and cleaned as it is passes through a series of tubes called airways from the nose or mouth to alveoli, which are thin-walled air sacs in the lungs where gas exchange actually occurs. In healthy humans, cartilage and fibrous elements maintain the patency of these airways (Gartner *et al.*, 2001). In humans with asthma, airflow is limited by increased mucus secretion into the airways and airway narrowing due to smooth muscle contraction in response to immune and neuronal signals. Asthma is the focus of my thesis research, and since asthma is a disease of the lungs, I begin this chapter with a basic description of human lung anatomy and function.

1. Conducting Airways

At a gross level, air flows sequentially from the nose or mouth into the larynx, trachea, bronchi, bronchioles, and finally alveoli during inspiration, and this order is reversed during exhalation. Air entering the trachea encounters a series of branching airways, beginning with the bifurcation of the trachea into the primary bronchi that supply the left and right lungs. Each level of branching is identified as an airway generation, and there are 8-23 generations of airways between the trachea and any given alveolus (Jeffery, 1995). Individual airways are smaller in each generation; however, the total cross-sectional area increases logarithmically with each generation (Jeffery, 1995). Thus, the net result of airway branching is a very large surface area in the distal respiratory

airways for gas to exchange across alveoli. The volume of air in the conducting airways is approximately 150 mL. Yet, this is just a fraction of the volume of air in the whole lung, since the respiratory zone (see below) is much larger with a volume of 2.5 to 3 liters (West, 2005).

Trachea

In general, the airways have several layers of tissue surrounding the lumen, the mucosa, submucosa, and adventitia that are each identifiable in histological sections (Figure 1.1). In the trachea, the mucosa lines the airway lumen and is composed of pseudostratified ciliated columnar epithelium and the lamina propria, a subepithelial connective tissue layer. Epithelium forms a protective barrier that is selectively permeable to fluids, ions, infectious agents, and other macromolecules. Tracheal epithelium is composed primarily of basal cells (stem cells that replenish other epithelial cells), goblet cells, and ciliated columnar cells, which each make up approximately 30% of the cell population (Gartner *et al.*, 2001). Goblet cells secrete mucinogen, which forms mucus when hydrated. Mucus in turn helps to trap particulate matter and is cleared from airways by beating cilia on ciliated columnar cells (Klein *et al.*, 2009; Seybold *et al.*, 1990). Mucous glands in the lamina propria and submucosa also produce mucus, which is secreted into the lumen via short ducts that open onto the epithelial surface. Leukocytes contribute to immune surveillance in these layers, and they traffic to and from the tissue via blood and lymphatic vessels in the submucosa (Jeffery, 1995). Finally, horseshoe-shaped cartilaginous rings in the adventitia support the trachea and connect to the trachealis muscle, which contracts during a cough to narrow the lumen and cause air to flow faster (Gartner *et al.*, 2001). Together, cells of the trachea work to both conduct air and protect the body from infectious agents and environmental insults.

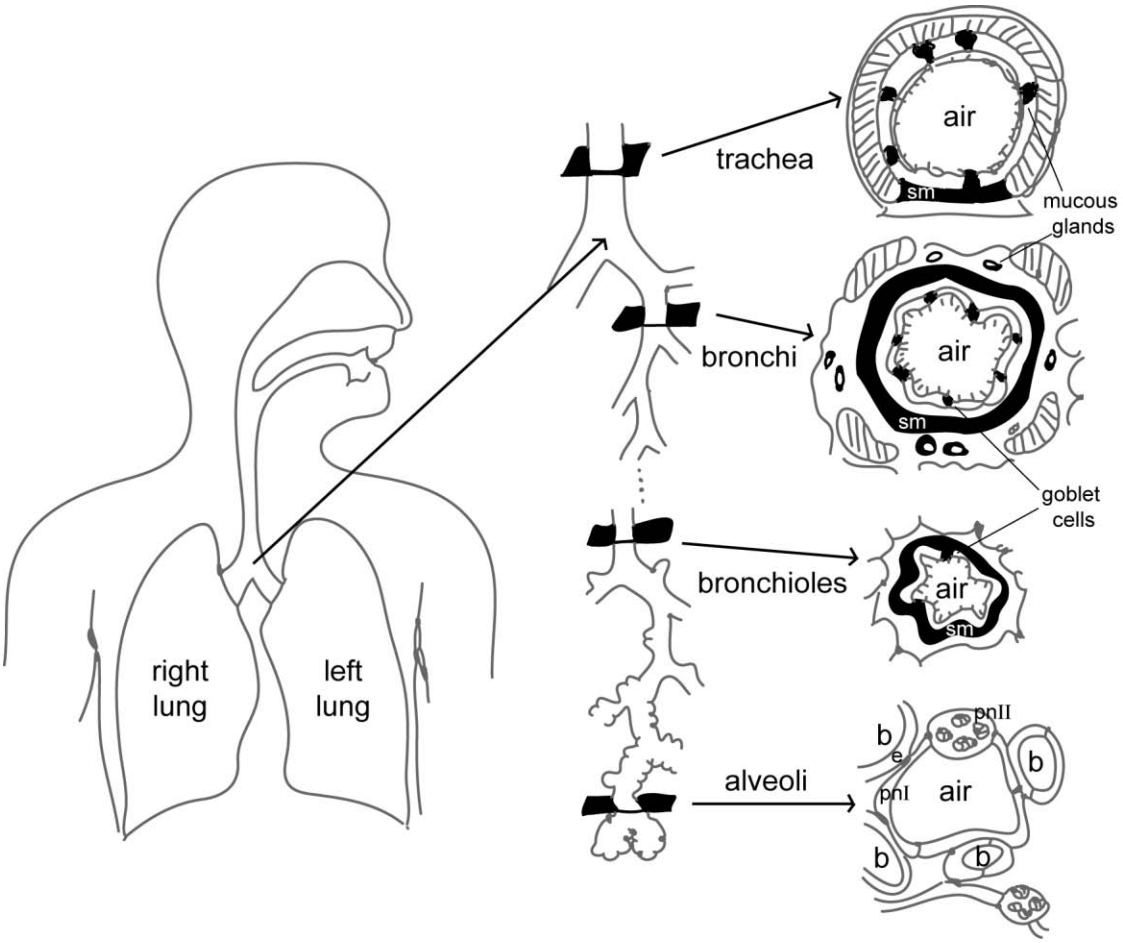

Figure 1.1 Airflow in human lungs. Air flows from the nose or mouth into the trachea, which bifurcates into the primary bronchi that supply the right and left lungs. The airways continue to branch into bronchi, bronchioles, respiratory bronchioles, and alveolar ducts, before terminating in alveolar sacs. Airway histology changes between the trachea and alveoli as described in the text. Cartilaginous rings and plates (cross-hatched), mucous glands (black circles in trachea and ovals in bronchi), goblet cells (black circles in epithelium of bronchi and bronchioles), and airway smooth muscle (sm, black strip in trachea and black rings surrounding the entire airway in bronchi and bronchioles) are all shown. In alveoli, gas diffuses across the type I pneumocyte (pnI) and capillary endothelial cell (e) into the blood (b), and type II pneumocytes (pnII) secrete surfactant.

Bronchi and Bronchioles

As airways decrease in size, the composition of cells changes (Figure 1.1). Supportive cartilage rings in the trachea are reduced to plates in bronchi that disappear in bronchioles. Mucous glands are found in cartilage-containing bronchi but are absent in bronchioles. Similarly, goblet cells decrease and then disappear in smaller bronchioles (Gartner *et al.*, 2001). Clara cells begin to appear in the epithelium of smaller bronchioles; these cells secrete bronchiolar surfactants to reduce surface tension and contain P450 enzymes that metabolize inhaled toxins (Gartner *et al.*, 2001; Tam *et al.*, 2011). In addition, smooth muscle begins to completely encircle the bronchi and bronchioles at the interface between the lamina propria and submucosa. These smooth muscle fibers surround the airways in two opposing spirals (Figure 1.2); they are particularly important in asthma because their contraction both shortens and narrows the airway limiting airflow (Jeffery, 1995). Functionally, as airways get smaller, smooth muscle control of airflow increases, mucus production is reduced, metabolism of toxins begins, and the epithelium transitions toward the thin respiratory epithelium found in alveoli.

2. Respiratory Zone

The conducting airways (trachea, bronchi, and conducting bronchioles) deliver air to the respiratory zone where gas exchange takes place.

Respiratory Bronchioles

Respiratory bronchioles mark the beginning of the respiratory zone (Figure 1.1). These bronchioles are distinguished by the presence of alveoli protruding from their walls.

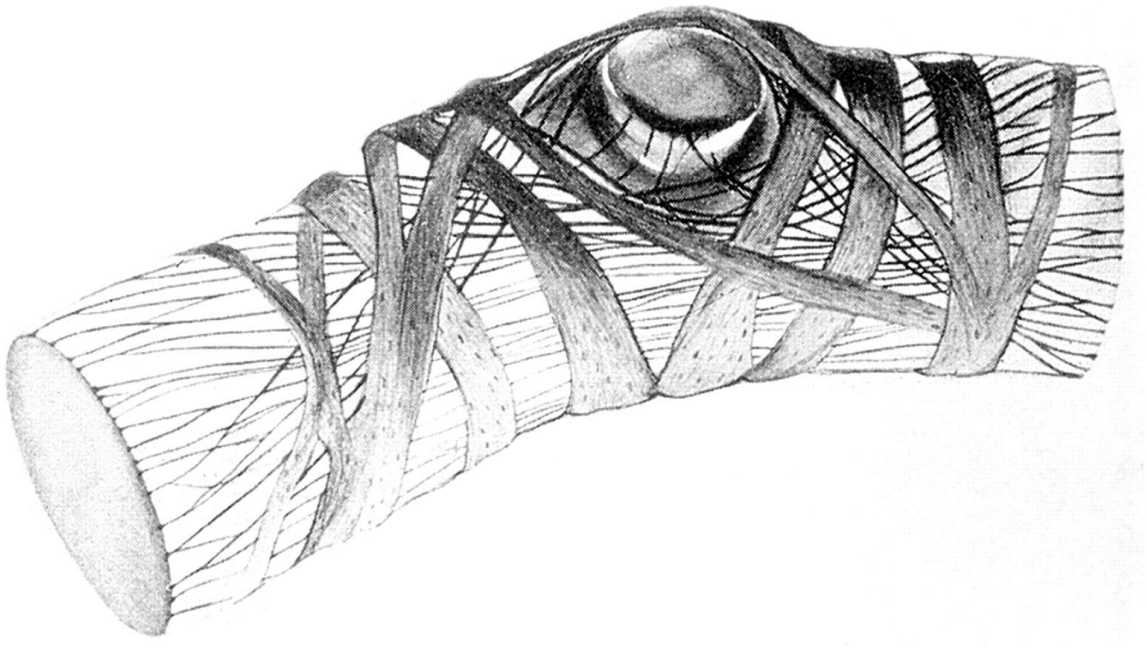

Figure 1.2 Illustration of smooth muscle spiraling around an airway. This image was originally published in WS Miller. The Lung. Springfield, IL: Charles C. Thomas; 1937. This title is in the public domain and does not require permission for use in a thesis.

Respiratory bronchioles branch several times before ending in alveolar ducts. These ducts are lined by alveoli and branch even further before terminating in two to three clusters of alveoli (Jeffery, 1995). Respiratory bronchioles are also the smallest airways with ciliated epithelium. Thus, mucociliary clearance of particulate matter out of the lungs and into the esophagus begins in the respiratory bronchioles.

Alveoli

Alveoli are small air sacs for gas exchange made up of type I pneumocytes and type II pneumocytes. Type I pneumocytes are thin, simple squamous epithelial cells that cover approximately 95% of the surface area of alveoli. They form tight junctions that prevent fluid from leaking into the airway lumen. Type II pneumocytes are more numerous but smaller and only account for about 5% of the surface area. They are responsible for secreting surfactant, which forms a thin film at the interface with the air to decrease surface tension and prevent collapse of the alveoli (Gartner *et al.*, 2001).

Networks of blood capillaries run between neighboring alveoli, and the capillary endothelial cells come into very close contact with Type I pneumocytes. Oxygen in the alveolar lumen diffuses passively across the type I pneumocyte and capillary endothelial cell into the blood, and carbon dioxide diffuses from blood into the alveolar lumen. The total distance crossed for gas exchange can be as little as 300 nm (West, 2005).

Pulmonary Blood Circulation

Oxygen from the alveoli diffuses into deoxygenated blood in the capillaries that has travelled from the right ventricle of the heart into the pulmonary artery circulation. Red blood cells passing through alveolar capillaries spend 3/4 of a second in the capillary network before draining into veins that deliver the now oxygenated blood to the left atria,

left ventricle, and then systemic circulation. All blood from the right heart travels through the pulmonary artery circulation (Gartner *et al.*, 2001; West, 2005).

Bronchial Blood Circulation

A bronchial artery circulation also supplies the lung. Bronchial arteries bring some of the oxygenated blood from the left ventricle back to the lungs via the thoracic aorta to capillary beds in the airway walls of the conducting airways (trachea, bronchi, and bronchioles). After leaving these tissues this blood passes through the bronchial veins on its way back to the right atrium of the heart (Gartner *et al.*, 2001).

3. Airway innervation

Nerves are found throughout the lung where they regulate mucus secretion, airway smooth muscle tone, and vascular tone. The nervous system detects changes in the environment and integrates this information to determine a physiological response. Each neuron is composed of a cell body, dendrites, and a single axon, and information is carried via propagation of action potentials, which result in neurotransmitter release (Guyton *et al.*, 2000). When two neurons contact each other, the site of contact and neurotransmitter release is termed a synapse. However, when a neuron releases neurotransmitter onto target cells that are not neurons, this site of contact is termed a junction (Foster *et al.*, 1897; Tansey, 1997).

In the lung, afferent sensory nerves carry information from the periphery to the central nervous system and efferent autonomic nerves (parasympathetic and sympathetic) carry information from the central nervous system to the tissues. The autonomic nerves are divided into pre and postganglionic neurons, where ganglion is the term used to define a cluster of nerve cell bodies. Preganglionic neurons have their cell bodies in the central

nervous system and their axons synapse on the cell bodies of postganglionic nerves. The axons of postganglionic nerves travel to target cells in the tissue (Guyton *et al.*, 2000).

Most of the sensory nerves and all of the preganglionic parasympathetic nerves supplying the lung are found within the vagus nerves. The left and right vagus nerves run from the medulla oblongata in the brain, where cell bodies of preganglionic parasympathetic nerves are located, through the neck and into the thoracic cavity (Kalia, 1981). Here, parasympathetic and sensory nerves branch off to supply the trachea, main bronchi, and smaller airways (Belvisi, 2002). Additional branches of the vagus nerve also supply the heart, esophagus, stomach, small intestine, colon, liver, gallbladder, pancreas, and ureters (Guyton *et al.*, 2000). Sympathetic nerves supplying the lung originate in the upper six thoracic segments of the spinal cord (Belvisi, 2002; Jeffery, 1995).

Parasympathetic nerves

Preganglionic parasympathetic nerves in the vagus nerve synapse with postganglionic parasympathetic nerve cell bodies in the trachea and bronchi, which are clustered in ganglia of 2-38 cells (Baker *et al.*, 1986; Canning *et al.*, 1997; Kalia, 1981; McAllen, 1978). The axons of postganglionic parasympathetic nerves project to airway smooth muscle, mucous glands, and blood vessels (Basbaum, 1984; Canning *et al.*, 1997; Cavallotti, 2005; Daniel *et al.*, 1986; Haberberger *et al.*, 1997; Knight *et al.*, 1981). Parasympathetic neurons primarily release acetylcholine to induce smooth muscle contraction, mucus secretion, and vasodilation as described below.

However, in some species, including humans, guinea pigs, and cats there is also inhibitory control of airway smooth muscle mediated by a separate population of parasympathetic nerves (Canning *et al.*, 1993; Canning *et al.*, 1996; Diamond *et al.*, 1980; Fischer *et al.*, 1996), and at least in guinea pigs, the cell bodies for these neurons reside in the esophagus (Canning *et al.*, 1993; Canning *et al.*, 1996). Inhibitory nonadrenergic, noncholinergic innervation results in smooth muscle relaxation (Canning *et al.*, 1993; Diamond *et al.*, 1980; Richardson *et al.*, 1976). Nitric oxide and vasoactive intestinal peptide are primarily responsible for relaxation, and these neurotransmitters are rarely coexpressed with acetylcholine (Belvisi *et al.*, 1992; Canning *et al.*, 1996; Fischer *et al.*, 1996).

Sensory nerves

Airway sensory nerve cell bodies are primarily located in the nodose and jugular ganglia within the vagus nerve (Carr *et al.*, 2003; Kummer *et al.*, 1992). Additionally, a minority of sensory nerves have their cell bodies in dorsal root ganglia outside of the spinal cord (Kummer *et al.*, 1992). Sensory nerves are pseudo-unipolar and lack dendrites (De Koninck *et al.*, 1993). Instead, the axon extends from the cell body and branches into two, with one branch innervating airway epithelium, lamina propria, airway smooth muscle, alveolar walls, blood vessels, or airway ganglia, and the other branch synapsing with neurons in the nucleus tractus solitarius of the brain (Basbaum, 1984; Davies *et al.*, 1986; Kalia *et al.*, 1980; Kummer *et al.*, 1992). Sensory nerves primarily utilize glutamate or tachykinins (substance P, neurokinin A) as neurotransmitters in the nucleus tractus solitarius (Carr *et al.*, 2003).

Sensory nerves in the lung are activated by a variety of physical and chemical stimuli and can be broadly classified as A-fibers, which are myelinated and mechanosensitive or C-fibers, which respond to noxious chemical stimuli and are not myelinated (Carr *et al.*, 2003). Sensory nerves are very important for vagal reflexes that initiate airway smooth muscle contraction and bronchoconstriction via activation of parasympathetic nerves. C-fibers also activated to release neuropeptides, including calcitonin gene-related peptide and tachkykinins, locally in lung tissue without propagation of signals to the brain. These local axonal reflexes contribute to smooth muscle contraction, vascular permeability, and mucus secretion (Belvisi, 2002; Carr *et al.*, 2003).

Sympathetic nerves

Cell bodies of preganglionic sympathetic nerves supplying the lung are found within the spinal cord, and their axons synapse with cell bodies of postganglionic nerves found outside the spinal cord in the cervical thoracic ganglia (Guyton *et al.*, 2000; Kummer *et al.*, 1992). The axons of postganglionic sympathetic nerves travel to the lung where they innervate blood vessels and submucosal glands via release of the neurotransmitter norepinephrine (Basbaum, 1984; Kummer *et al.*, 1992). Sympathetic innervation of airway smooth muscle varies widely across species. In human, there is very little direct innervation of airway smooth muscle, and sympathetic nerves have no effect on airway smooth muscle tone (Daniel *et al.*, 1986; Richardson *et al.*, 1976; Richardson *et al.*, 1979). However, in guinea pigs, cats, and dogs sympathetic nerves innervate airway smooth muscle, where they release norepinephrine to cause airway smooth muscle relaxation via β-adrenoreceptors, while in pigs they contract airway smooth muscle via α-adrenoreceptors (Ainsworth *et al.*, 1982; Cabezas *et al.*, 1971; Diamond *et al.*, 1980;

Leff *et al.*, 1985). In mice exogenous norepinephrine contracts airway smooth muscle (Martin *et al.*, 1988).

Although there is no evidence for sympathetic nerve control of airway smooth muscle tone in humans, systemic norepinephrine or exogenous β_2-adrenoreceptor agonists induce smooth muscle relaxation via β_2-adrenoreceptors that are not innervated (Billington *et al.*, 2003; Richardson *et al.*, 1976).

B) Asthma

Asthma is an obstructive lung disease diagnosed the presence of variable airway obstruction, hyperresponsiveness, inflammation, and symptoms (Reddel *et al.*, 2009). The working definition of asthma provided in the most recent expert panel report from the National Heart Lung and Blood Institute is:

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular mast cells, eosinophils, neutrophils (especially in sudden onset, fatal exacerbations, occupational asthma, and patients who smoke), T lymphocytes, macrophages, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of coughing (particularly at night or early in the morning), wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (EPR-3, 2007)*."*

In 2009 an estimated 24.6 million persons or 8.2% of the United States population had asthma, representing a 12% increase from 2001 (CDC, 2011). The estimated direct cost

of asthma in the United States was $3,259 per person per year, amounting to $56 billion per year for 2002-2007 (Barnett *et al.*, 2011).

1. Asthma symptoms

Asthma symptoms are related to reversible airflow obstruction and include wheezing (a high-pitched expiratory whistling sound), cough, shortness of breath, and chest tightness. Not every asthma patient will have every symptom. Symptoms are often episodic and triggered or worsened during exercise, viral infection, inhalation of allergens, inhalation of irritants (tobacco or wood smoke, airborne chemicals), changes in weather, strong emotional expression (laughing or crying), stress, or the menstrual cycle (EPR-3, 2007). In patients with nocturnal asthma, symptoms are worsened overnight and can be sufficient to wake the patient (EPR-3, 2007).

2. Asthma exacerbations

Asthma exacerbations are characterized by a worsening of a patient's symptoms from baseline including cough, wheezing, and difficulty breathing. They range from mild to severe. Mild exacerbations last one to two days and are just outside the normal variation for a patient (O'Byrne, 2011; Reddel *et al.*, 2009). Moderate exacerbations are "events that are troublesome to the patient, and that prompt a need for a change in treatment (Reddel *et al.*, 2009)." Severe asthma exacerbations are defined "as events that require urgent action on the part of the patient and physician to prevent a serious outcome, such as hospitalization or death (Reddel *et al.*, 2009)." Severe exacerbations usually develop over 5-7 days and once treated, resolve over 7-10 days (O'Byrne, 2011).

All asthma patients are at risk for severe exacerbations, and even patients with mild asthma have almost one severe exacerbation per year (O'Byrne, 2011). Therefore, it is important to understand how triggers such as allergens and viruses lead to airway obstruction in order to prevent exacerbations.

3. Variable airway obstruction in asthma

In asthma, airflow obstruction leads to symptoms. Airflow in the lungs is limited by bronchoconstriction and in more severe cases by edema, mucus hypersecretion, and the formation of mucus plugs in the airway lumen. Airflow limitation is variable and generally fully reversible (EPR-3, 2007). However, over time asthmatics may develop airway remodeling, structural changes including fibrosis under the airway epithelial basement membrane, mucous gland hyperplasia and mucus hypersecretion, epithelial cell injury, smooth muscle hypertrophy, and angiogenesis. In chronic asthma, these changes may result in irreversible airflow obstruction (EPR-3, 2007).

Bronchoconstriction occurs when airway smooth muscle contracts to rapidly narrow the conducting airway diameter. This decreases the volume of the conducting airways and increases resistance to airflow (Cabezas *et al.*, 1971; Kesler *et al.*, 1999; Olsen, 1965; Severinghaus *et al.*, 1955). In humans, bronchoconstriction is commonly measured with spirometry as a decrease in the volume of air that is exhaled during the first second of forced exhalation (FEV_1) (West, 2005). Bronchoconstriction is also measured as an increase in resistance, which is the difference in pressure between the alveoli and mouth per unit of airflow, using a body plethysmograph (a large airtight box)(West, 2005). Finally, airflow limitation is also readily measured as a decrease in peak expiratory airflow, using a peak flow meter (Reddel *et al.*, 2009). Bronchoconstriction is an

important measurement in the research presented in this thesis. However, I use an invasive method described in Chapter 2 to measure bronchoconstriction in guinea pigs, since the procedures used in humans rely on maneuvers that are not possible in animals.

In asthma, bronchoconstriction is inappropriately robust, causing pathologic airflow obstruction and symptoms. Bronchoconstriction occurs in response to a variety of physical and chemical stimuli including allergens, irritants, and inflammatory mediators, which act either directly at the smooth muscle or indirectly through neuronal reflexes. In particular, parasympathetic nerves provide the dominant control of airway smooth muscle tone in the lung and are critical for mediating bronchoconstriction.

Parasympathetic nerve control of airway smooth muscle tone

Airway parasympathetic nerves release acetylcholine to contract airway smooth muscle during normal breathing in humans and animals (Jammes *et al.*, 1979; Kesler *et al.*, 1999; Roberts *et al.*, 1988; Sheppard, 1982; Widdicombe, 1966). Tonic parasympathetic activity results in partial contraction of airway smooth muscle (Widdicombe, 1966; Widdicombe *et al.*, 1962). This means that during normal breathing there is baseline airway smooth muscle tone (Figure 1.3A). Thus, vagotomy, which severs parasympathetic nerves innervating the lung, decreases airway smooth muscle tone. Conversely, airway smooth muscle tone can be rescued by electrically stimulating the distal ends of the cut vagus nerves (Cabezas *et al.*, 1971; Kesler *et al.*, 1999; Olsen, 1965; Severinghaus *et al.*, 1955).

Acetylcholine released by parasympathetic nerves acts directly at muscarinic acetylcholine receptors on airway smooth muscle to cause bronchoconstriction.

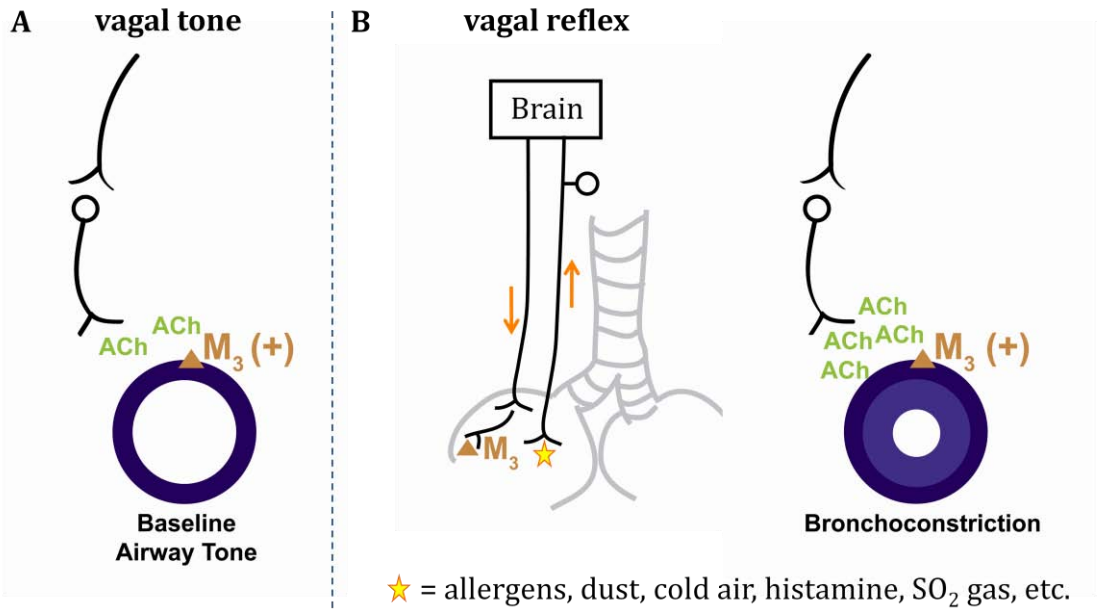

Figure 1.3 Parasympathetic nerve control of baseline airway smooth muscle tone and bronchoconstriction. (A) During normal breathing parasympathetic nerves fire tonically and release the neurotransmitter acetylcholine (ACh), which stimulates M_3 muscarinic receptors on airway smooth muscle (purple). This leads to smooth muscle contraction and determines baseline airway smooth muscle tone. (B) Bronchoconstriction is induced when sensory nerves in the lungs are activated by a variety of physical and chemical stimuli (yellow star). Sensory neurons conduct signals to the brain via the vagus nerves, and parasympathetic neurons also in the vagus nerves carry this reflex response back to the lungs. This results in increased acetylcholine release onto M_3 muscarinic receptors, increased smooth muscle contraction, and bronchoconstriction.

Therefore, as with vagotomy, muscarinic receptor antagonists decrease smooth muscle tone and prevent bronchoconstriction induced by electrical stimulation of the vagus nerves (Colebatch *et al.*, 1963; Kesler *et al.*, 1999; Severinghaus *et al.*, 1955; Sheppard, 1982). In addition, bronchoconstriction is increased by acetylcholinesterase inhibitors, which increase acetylcholine levels by preventing metabolism of acetylcholine (Colebatch *et al.*, 1963).

Parasympathetic nerve control of bronchoconstriction via vagal reflexes

Parasympathetic nerves are the efferent arm of vagal reflexes initiated by both physical (ex. dust and cold air) and chemical (ex. allergens, histamine, methacholine, and irritant gasses such as SO_2) stimuli (Gold *et al.*, 1972; Holtzman, 1980; Sheppard, 1982; Wagner *et al.*, 1999; Widdicombe *et al.*, 1962). These stimuli directly or indirectly activate afferent sensory nerves in the respiratory tract, which conduct signals to the brain via the vagus nerve (Widdicombe *et al.*, 1962). Parasympathetic neurons carry the reflex response back to the lungs via acetylcholine release that leads to bronchoconstriction (see Figure 1.3B). From a therapeutic perspective, vagal reflexes are important because muscarinic receptor antagonists are capable of blocking bronchoconstriction initiated by a variety of chemical and physical stimuli in humans (Holtzman, 1980; Sheppard, 1982).

Evidence for reflex bronchoconstriction comes from experiments where a stimulus is applied to one location in the respiratory tract and a cholinergic response is measured in a separate location innervated by the efferent arm of the reflex arc (Nadel *et al.*, 1965; Wagner *et al.*, 1999). For example, SO_2 gas applied to the larynx of tracheostomized animals causes bronchoconstriction in the trachea and bronchi, and severing either the

afferent sensory nerves that leave the larynx or the efferent vagus nerves completely prevents this reflex bronchoconstriction (Olsen, 1965).

4. Airway hyperresponsiveness in asthma

Asthma is also characterized by nonspecific airway hyperresponsiveness, which is defined as excessive bronchoconstriction to contractile stimuli. Hyperresponsiveness is thus a measure of how sensitive the airways are to contractile stimuli. Clinically, airway hyperresponsiveness is measured by inducing bronchoconstriction with methacholine (muscarinic agonist), histamine, cold air, or exercise challenge (EPR-3, 2007). Low doses or low intensity challenges that do not cause bronchoconstriction in healthy humans cause bronchoconstriction in patients with asthma. The degree of airway hyperresponsiveness is often determined based on the dose or intensity of exercise that causes a 20% increase in bronchoconstriction (provocative concentration 20% = PC_{20}).

In asthmatics, airway hyperresponsiveness is not static and can fluctuate over time. For instance, airway responsiveness increases significantly in patients with allergic asthma during ragweed season, reaching maximum levels by the end of the season. While small increases in airway responsiveness may be asymptomatic, larger increases are associated with worsening of asthma symptoms (Boulet *et al.*, 1983). Thus, although asthmatics are always hyperresponsive relative to healthy controls the degree of hyperresponsiveness can change.

It is important to note that hyperresponsiveness is not synonymous with bronchoconstriction. Episodic bronchoconstriction and symptoms in asthma result from a combination of airway sensitivity (responsiveness) and the presence and amount of contractile stimuli. Understanding mechanisms that increase airway responsiveness in

asthmatic individuals is therefore important for developing therapies to prevent exacerbations and is the focus of my thesis research. Importantly, parasympathetic nerves and vagal reflexes mediate airway hyperreactivity in asthmatic individuals.

Evidence for a role of parasympathetic nerves in airway hyperresponsiveness

The role of parasympathetic nerves and vagal reflexes in airway hyperresponsiveness in asthma patients is supported by experiments investigating contraction of airway smooth muscle from asthma patients *in vitro* and *in vivo*. Many contractile agonists act both directly at the smooth muscle and indirectly via activation of vagal reflexes to induce smooth muscle contraction. However, the increased sensitivity to contractile agonists in asthmatics is not due to increased smooth muscle sensitivity, since there is no correlation between *in vitro* contractile responses of bronchial smooth muscle isolated from asthma and nonasthma patients and methacholine responsiveness in these same patients *in vivo* (Roberts *et al.*, 1984; Whicker *et al.*, 1988). Thus, airway hyperresponsiveness occurs *in vivo,* where vagal reflexes are present, but not *in vitro,* where reflexes are absent.

Further evidence for the importance of parasympathetic nerves and vagal reflexes in airway hyperresponsiveness comes from studies with inhaled histamine. Histamine acts both directly at smooth muscle and indirectly through vagal reflexes to cause bronchoconstriction. Hyperresponsiveness to inhaled histamine is mediated by vagal reflexes in humans, since it can largely be blocked pharmacologically with a muscarinic receptor antagonist that blocks acetylcholine released from parasympathetic nerves or by blocking neurotransmission through parasympathetic ganglia with a neuronal nicotinic receptor antagonist (Holtzman, 1980). Additionally, in humans with severe asthma, histamine-induced asthma exacerbations cease when neurotransmission in autonomic

and sensory nerves supplying the airways are blocked with novocain. After surgical denervation of the airways in these same patients, exogenous histamine no longer induces asthma exacerbations (Dimitrov-Szokodi *et al.*, 1957). Therefore, airway nerves and not smooth muscle contractility mediate increased sensitivity to histamine in asthma patients.

5. Asthma phenotypes and inflammation

Inflammation is another defining characteristic of asthma. However, asthma is a heterogeneous disease and several distinct asthma phenotypes have been identified based on inflammatory cells that infiltrate the lungs and inflammatory cytokines that are increased in the lungs (Broekema *et al.*, 2011; Haldar *et al.*, 2008; Woodruff *et al.*, 2009). Inflammatory cells and inflammatory mediators are commonly measured in bronchoalveolar lavage fluid (fluid used to lavage airways), induced sputum, bronchial biopsies, or peripheral blood.

Eosinophils, neutrophils, and T helper cells are immune cells that are particularly important for defining asthma phenotypes. Eosinophils regulate inflammation at mucosal sites such as the gut and lung, and they are important in allergic inflammation (Hogan *et al.*, 2008). Although eosinophils are rarely found in the lungs of healthy humans, they are common in the lungs of asthmatics where their presence correlates with asthma severity (Bousquet *et al.*, 1990). Neutrophils are the most prevalent cell type in peripheral blood, and they have a prominent role in early immune responses to microbes where they recognize, phagocytize, and kill various microbes (Janeway, 2005). Neutrophils are also prevalent in asthma, and approximately 50% of severe exacerbations are associated with neutrophils, while the remaining 50% are associated

with eosinophils (O'Byrne, 2011). Finally, T-helper type 2 (Th2) cells release a number of cytokines, which help to drive IgE antibody production, allergic inflammation, and eosinophil responses (Janeway, 2005). Thus, the presence of eosinophils, neutrophils, or Th2 cytokines can be used to identify different asthma patient phenotypes.

When asthma phenotypes are defined based on cluster analysis of inflammatory cells, symptoms, and additional patient characteristics five asthma phenotypes are apparent (Figure 1.4)(Haldar *et al.*, 2008). The different phenotypes can be divided based on the presence of eosinophilic inflammation and daily symptoms. Patients with high levels of eosinophils fall into one of two phenotypes: 1) inflammation predominant, with high levels of eosinophils but few daily symptoms or 2) early onset atopic (allergic) asthma, with both eosinophilic inflammation and moderate-high daily symptoms. Patients with low levels of eosinophils, may or may not have high levels of neutrophils, and fall into one of three phenotypes: 1) early symptom predominant, with high symptom expression and atopy (allergy), 2) obese non-eosinophilic with high symptom expression, or 3) benign asthma, which covers patients that are well-managed and have few daily symptoms and little inflammation (Haldar *et al.*, 2008).

Asthma phenotypes are also defined by inflammation apparent at a molecular level instead of a cellular level. Asthma is often associated with Th2 cell responses and Th2 cytokines including interleukins (IL)-4, IL-5, and IL-13. Yet not all asthma patients have Th2 inflammation. In one study, only half of the asthma patients have increased expression of Th2-regulated genes in epithelial scrapings relative to healthy controls. These same patients have eosinophilia, increased expression of gel-forming mucins (MUC5AC and MUC2), increased airway responsiveness (PC_{20} to methacholine), increased serum IgE levels, and evidence for airway remodeling (thickened epithelial

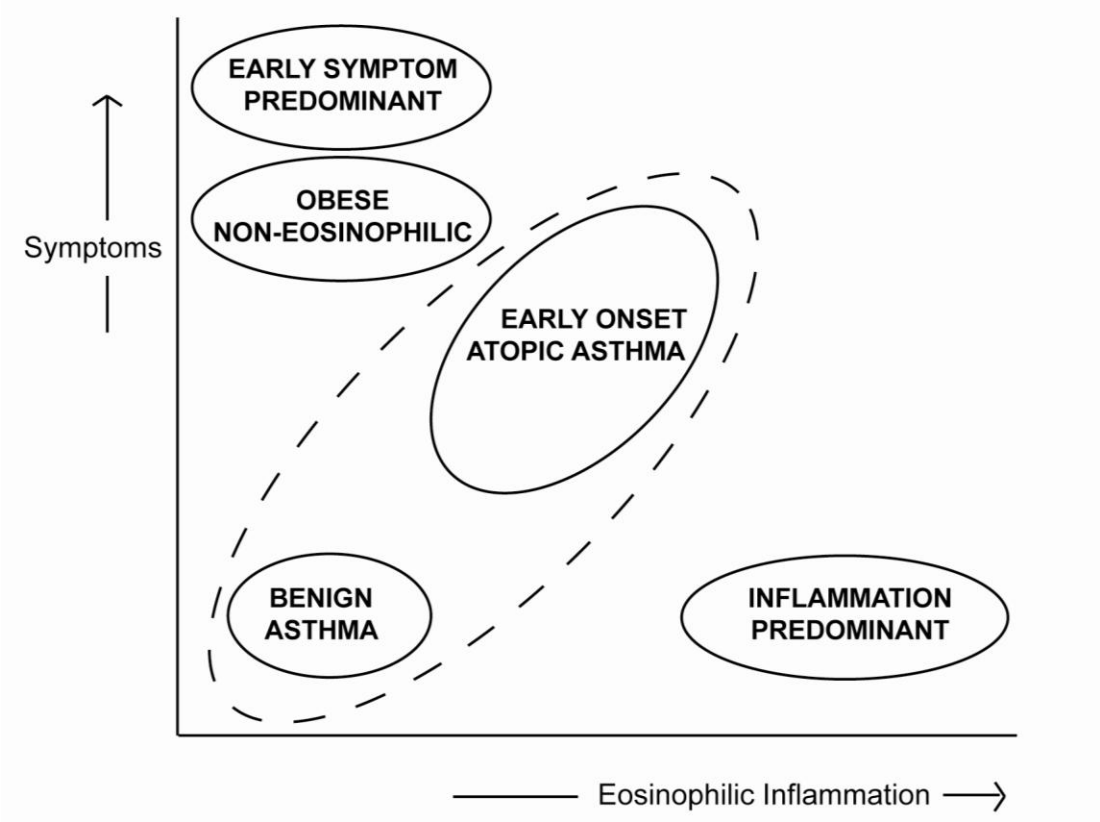

Figure 1.4 Asthma phenotypes defined by the presence of eosinophilic inflammation and expression of symptoms. Five asthma phenotypes have been identified with cluster analysis of asthma patients seen in both primary and secondary care settings. Patients with high levels of eosinophils have either inflammation predominant or early onset atopic asthma phenotypes. While patients with low levels of eosinophils have early symptom predominant, obese non-eosinophilic, or benign asthma. Patients with benign asthma are well controlled. For the remaining patients asthma phenotypes can be used to target treatments. For patients with concordant disease (dashed line), where eosinophilic inflammation correlates with symptoms treating the symptoms will be beneficial. However, patients with discordant disease may benefit from different treatments. Patients with high eosinophil inflammation and low daily symptoms benefit from additional corticosteroids, which reduce eosinophils and

decrease exacerbations. Patients with high daily symptom expression but low eosinophil levels do not benefit from corticosteroids and may benefit from other treatments instead. This image is based on a figure from Haldar et al. (2008).

basement membrane). The remaining asthma patients are still hyperresponsive relative to healthy controls, but do not have eosinophilic inflammation or increased mucin expression, and although atopic they have lower serum IgE levels than the Th2 high population (Woodruff *et al.*, 2009). Thus, asthma patients can be divided into those that have high levels of Th2 cytokines and those that do not.

Separating patients into different phenotypes is important for developing and testing treatments that target inflammation. This is underscored best by results from trials with anti-IL-5 antibody. IL-5 is a cytokine important for eosinophil differentiation and survival (Hogan *et al.*, 2008). Early anti-IL-5 trials showed reductions in blood and sputum (Kips *et al.*, 2003; Leckie *et al.*, 2000) but no effect on airway hyperreactivity (Leckie *et al.*, 2000) and led many investigators to believe eosinophil presence was not critical for asthma. However, later trials that only included patients with high levels of sputum eosinophils found that anti-IL-5 therapy reduced asthma exacerbations and allowed reductions in corticosteroid use (Haldar *et al.*, 2009; Nair *et al.*, 2009). Similarly, phenotyping studies have demonstrated that only patients with eosinophilic inflammation benefit from steroids and therefore steroids can be reduced or eliminated in patients without eosinophilic inflammation (Haldar *et al.*, 2008; Woodruff *et al.*, 2009). In addition, the antibiotic clarithromycin, known to reduce neutrophils *in vitro*, decreases neutrophil number and increases quality of life predominately in patients with neutrophilic asthma (Simpson *et al.*, 2008). Together these findings suggest that mechanisms of disease for one patient population may not apply to all asthma patients.

In my thesis research, I use a guinea pig model of asthma that best reflects allergic, eosinophilic asthma. Across asthma phenotyping studies, approximately 50% of asthma patients have eosinophilic asthma (Haldar *et al.*, 2008; Nadif *et al.*, 2009; Woodruff *et al.*,

2009); therefore, my results are likely to be relevant to this patient population. However, they may not be relevant for patient populations, such as those with high levels of neutrophils and few eosinophils.

6. Current asthma therapies

Treatments for asthma are administered to target current symptoms and to reduce future risk of asthma exacerbations (Reddel *et al.*, 2009). The expert panel for the management of asthma provides national guidelines for which drugs should be used (EPR-3, 2007). Current guidelines recommend that β_2-adrenoreceptor agonists be used as first line bronchodilators during an exacerbation, and that the muscarinic antagonist, ipratropium, be used in combination with short acting β_2-adrenoreceptor agonists in moderate-to-severe asthma exacerbations. β_2-adrenoreceptor agonists activate β_2-adrenoreceptors on smooth muscle to directly induce relaxation, while ipratropium blocks bronchoconstriction due to release of acetylcholine from parasympathetic nerves. Ipratropium is also recommended as the treatment of choice for bronchospasm in patients who cannot tolerate β_2-adrenoreceptor agonists. For prevention of exacerbations, inhaled corticosteroids are a mainstay of treatment and are believed to reduce exacerbations by reducing eosinophil number and activation (O'Byrne, 2011). Long-acting B_2-agonists are often used in combination with corticosteroids to enhance bronchodilation. Other therapies used to treat inflammation in specific patient populations include drugs that inhibit cysteinyl leukotrienes, anti-IL-5 monoclonal antibodies that reduce eosinophils, and anti-IgE monoclonal antibodies that reduce mast cell activation. A newer nonpharmacological therapy involves ablating airway smooth muscle with bronchial thermoplasty to prevent bronchoconstriction (O'Byrne, 2011).

C) Antigen challenge model of allergic asthma

Asthma exacerbations normally occur spontaneously, however, in order to study mechanisms that lead to increases in airway hyperresponsiveness an experimental model is needed. Challenge with inhaled antigen (allergen) is used in both humans and animals to study mechanisms that lead to spontaneous exacerbations in allergic asthma.

Allergies occur in humans who develop IgE antibodies directed against specific antigens on innocuous proteins. Antigens are defined as any molecule that binds specifically to an antibody and their name comes from their **anti**body **gen**eration abilities (Janeway, 2005). This generation of antibody, termed sensitization to an allergen, results in a heightened immune response upon subsequent exposure to antigen. This response can induce an asthma exacerbation in atopic (allergic) humans who also have asthma (Boulet *et al.*, 1983).

Not every antigen induces production of IgE antibodies. Instead, specific proteins are associated with IgE production. Overall, they tend to be small, highly soluble, and easily carried on dry particles, which allows them to diffuse into mucosal surfaces following inhalation. Examples include ragweed pollen and house dust mites (Janeway, 2005). In animal models of asthma, allergy is induced by immunization with allergens that induce IgE production. Ovalbumin is a well-characterized allergen used in animal models (Andersson, 1980; Elbon *et al.*, 1995; Evans *et al.*, 1997; Kumar *et al.*, 2008). Following intraperitoneal injection of ovalbumin in animals, both IgE and IgG1 antibodies develop against ovalbumin antigen (Andersson, 1980). Subsequent challenge of sensitized animals with inhaled ovalbumin results in bronchoconstriction, airway hyperresponsiveness, and infiltration of eosinophils into the airways. Thus, antigen

sensitization and subsequent challenge with inhaled antigen mimics important characteristics of allergic asthma.

1. Sensitization to Antigen

IgE antibody production in humans and animals involves several immune cell types and the adaptive immune response. Dendritic cells take up allergens and process them into peptide antigens, which they then present to naïve T helper cells in lymph nodes. This induces the T-cells to proliferate and differentiate into effector Th2 type T cells. Effector Th2 cells then recognize antigen taken up and presented by B cells. This induces effector Th2 cells to secrete Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) and to stimulate B cells to proliferate and isotype switch to IgE production. Secreted IgE antibodies bind with high avidity to FcεRI receptors on mast cells found in lung tissues (Janeway, 2005).

2. Antigen challenge

Challenge with inhaled antigen is carried out experimentally in both humans with allergic asthma and sensitized animals to model the inflammatory response that occurs following natural exposure to allergens in allergic asthma.

Early and late bronchoconstriction responses following antigen challenge

Experimental antigen challenge in humans and animals induces an immediate early bronchoconstriction response. Inhaled antigen binds IgE antibodies on mast cells and activates mast cells to release mediators that include histamine, prostaglandin D2, leukotriene C4, IL-4, and tumor necrosis factor alpha (TNFα) (Janeway, 2005). Histamine and leukotrienes in particular induce the early bronchoconstriction response that lasts for up to 2 h in humans (Figure 1.5)(Booij-Noord *et al.*, 1972; Cockcroft *et al.*, 1977; Roquet *et al.*, 1997). In sensitized and antigen challenged guinea pigs, the early

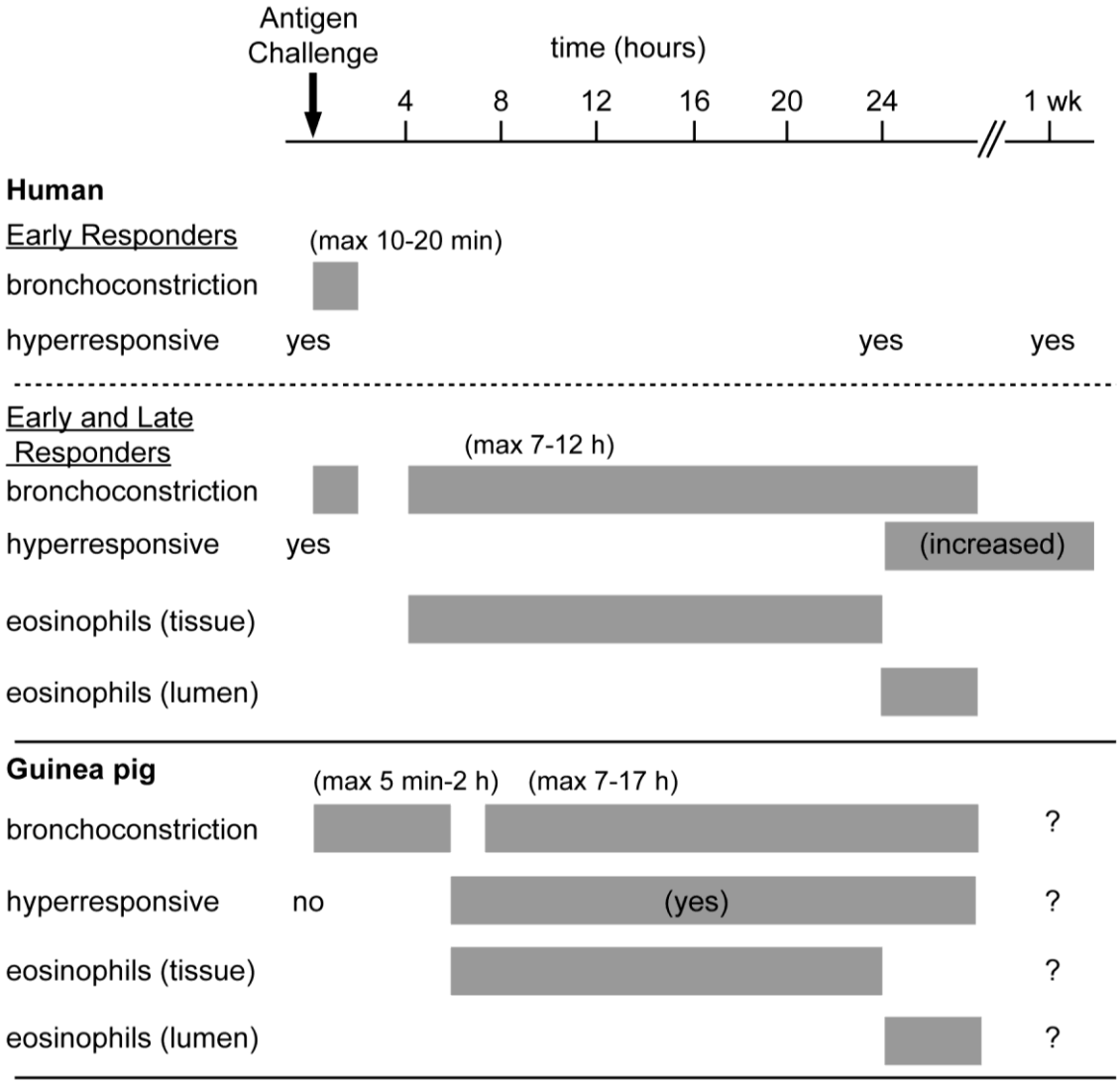

Figure 1.5 Characteristic responses to antigen challenge in humans with allergic asthma and sensitized guinea pigs. In humans with allergic asthma, challenge with inhaled antigen results in an immediate early bronchoconstriction response (early responders). Approximately 50-70% of patients have an additional late bronchoconstriction response (early and late responders). In these patients, antigen challenge also increases airway hyperresponsiveness and eosinophil recruitment to the lungs. Sensitized guinea pigs model allergic asthma patients with both an early and late bronchoconstriction response. However, the early bronchoconstriction response is

longer and the late bronchoconstriction response is delayed. Unlike humans, sensitized guinea pigs are not hyperresponsive at baseline. However, following antigen challenge airway hyperresponsiveness develops, and this mimics the increase in hyperresponsiveness seen in humans. See text for references.

bronchoconstriction response lasts for up to 6 h after antigen challenge (Figure 1.5)(Church *et al.*, 1993; Hutson *et al.*, 1988; Smith *et al.*, 2007; Toward *et al.*, 2004). Thus, guinea pigs have an immediate inflammatory response to antigen inhalation that results in bronchoconstriction and mimics responses in humans with allergic asthma.

In humans, a second late phase bronchoconstriction response begins 4 h after antigen challenge and lasts up to 33 h after challenge occurs in 47-70% of patients with allergic asthma (Figure 1.5)(Booij-Noord *et al.*, 1972; Boulet *et al.*, 1983; Cockcroft *et al.*, 1977; Roquet *et al.*, 1997). Late phase bronchoconstriction is mediated by both histamine and additional factors, since antagonists for histamine only partially reduce bronchoconstriction (Booij-Noord *et al.*, 1972). This late bronchoconstriction occurs in all guinea pigs and begins 7 h after antigen challenge but is quite variable in length (lasting 11-48 h after challenge, Figure 1.5)(Church *et al.*, 1993; Hutson *et al.*, 1988; Smith *et al.*, 2007; Toward *et al.*, 2004). In addition, a late-late bronchoconstriction response, not observed in humans, also occurs 3 days after antigen challenge in guinea pigs (Figure 1.5)(Church *et al.*, 1993; Hutson *et al.*, 1988).

Increases in airway responsiveness following antigen challenge

In the human experimental model, antigen challenge also worsens airway hyperresponsiveness, but only in patients with both early and late bronchoconstriction responses (Figure 1.5). Increased responsiveness develops within 24 h and persists for up to a week or more before returning to a pre-antigen challenge baseline (Cockcroft *et al.*, 1977; Kariyawasam *et al.*, 2007). Thus, even after the airways are no longer contracted, they remain more sensitive to contractile stimuli. These data are consistent with findings of seasonal exposure to ragweed pollen. Patients who have both early and

late bronchoconstriction responses experimentally have higher preseason IgE levels and greater increases in airway responsiveness during their allergy season than patients who are only early responders (Boulet *et al.*, 1983).

Guinea pigs are different from humans in that sensitized guinea pigs are not hyperresponsive. However, following antigen challenge, airway hyperreactivity develops. Airway hyperreactivity is observed as early as 6 h after antigen challenge and is maintained until at least 24 h after challenge (Figure 1.5) (Smith *et al.*, 2007; Toward *et al.*, 2004)(Costello *et al.*, 1998; Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 1997; Fryer *et al.*, 2006; Verbout *et al.*, 2007). Thus, guinea pigs model the increase in responsiveness that occurs following allergen exposure in patients with allergic asthma and are a relevant model for studies of airway hyperreactivity.

Eosinophil recruitment to the lung following antigen challenge

In both humans and guinea pigs, eosinophil recruitment to the lung precedes increases in airway responsiveness. In humans, eosinophils increase in airway tissue by 4 h after antigen challenge (Figure 1.5)(Brown *et al.*, 1998). By 24 h after antigen challenge, tissue eosinophils are returning toward baseline levels, and by 7 days after challenge they have returned to baseline levels (Brown *et al.*, 1998; Kariyawasam *et al.*, 2007). In guinea pigs, eosinophils peak in airway tissue 6 h after antigen challenge (Asano *et al.*, 2001; Humbles *et al.*, 1997). In both humans and guinea pigs, eosinophils increase in the airway lumen as they decrease in airway tissues, and they are significantly increased in bronchoalveolar lavage and sputum 24 h after challenge (Church *et al.*, 1993; Humbles *et al.*, 1997)(Brown *et al.*, 1998). Thus, sensitized and challenged guinea pigs respond to antigen in a similar manner to humans with allergic asthma, and in both

species, increases in tissue eosinophils are associated with increases in airway responsiveness following antigen challenge.

D) Eosinophils functions in asthma

Eosinophils are granulocytic leukocytes that have multiple means of influencing the inflammatory response to antigen challenge and potentially increasing airway hyperresponsiveness. Eosinophils make and release many preformed mediators, they present antigens to T-cells to induce cytokine release, and they are important for the survival of plasma B cells in bone marrow that secrete antibodies.

Eosinophils have a bilobed nucleus, are approximately 8 μm in diameter (Sokol *et al.*, 1987), and were named for their ability to bind eosin, a negatively charged dye, that was first recognized by Paul Ehrlich in 1879 (Kaufmann, 2008; McEwen, 1992). Eosin and other negatively charged dyes bind to highly cationic proteins that are packaged into crystalloid granules (Hamann *et al.*, 1991). These proteins include major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). Eosinophil cationic granule proteins are cytotoxic and are toxic to both helminthic parasites and bacteria (Ackerman *et al.*, 1985; Lehrer *et al.*, 1989; Linch *et al.*, 2009; Wang *et al.*, 2006). In addition, ECP and EDN are ribonucleases, and EDN has demonstrated antiviral activity in respiratory infections (Rosenberg *et al.*, 2001).

Eosinophil functions are not limited to their cationic granule proteins. Eosinophils also make and release at least 35 cytokines, chemokines, and growth factors (Table 1.1) (Hogan *et al.*, 2008). In addition, they also produce lipid mediators and neuro-mediators

Table 1.1 Eosinophil-derived mediators

Cytotoxic granule proteins	Cytokines	Growth Factors	Lipid Mediators	Neuro-mediators	Chemokines	Other
MBP	IL-2	HB-EGF-LBP	LTD4	substance P	CCL3	MMP9
EPO	IL-3	stem cell factor	LTE4	VIP	CCL5	
ECP	IL-4	PDGF	PGE1		CCL7	
EDN	IL-5	VEGF	PGE2		CCL8	
	IL-6	NGF	15-HETE		CCL11	
	IL-8	BDNF	PAF		CCL13	
	IL-10	NT3			IL-8	
	IL-12					
	IL-13					
	IL-16					
	IL-18					
	TGFα/β					
	GM-CSF					
	TNFα/β					
	IFNγ					

Abbreviations: BDNF, brain derived neurotrophic factor; CCL, CC chemokine ligand; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase; GM-CSF, granulocyte/macrophage colony stimulating factor; HB-EGF-LBP, heparin-binding epidermal growth factor-like binding protein; 15-HETE, 15-hydroxyeicosatetraenoic acid; IFN, interferon; IL, interleukin; LT, leukotriene; MBP, major basic protein; MMP, matrix metalloproteinase; NGF, nerve growth factor; NT3, neurotrophin 3; PAF, platelet activating factor; PDGF, platelet derived growth factor; PG, prostaglandin; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal polypeptide. Mediators reviewed in (Hogan *et al.*, 2008) and data for additional growth factors from (Noga *et al.*, 2003).

(common neurotransmitters) (Table 1.1)(Hogan *et al.*, 2008). Cytokines are stored in granules and can be released rapidly upon activation (Spencer *et al.*, 2009). In general, eosinophils release their products via three mechanisms classical exocytosis, piecemeal degranulation, and cytolysis (Moqbel *et al.*, 2006). With classical exocytosis, whole granules fuse with the cell membrane and release their contents (Moqbel *et al.*, 2006). In piecemeal degranulation, small vesicles bud off from the membrane of cytoplasmic granules and carry proteins to the cell membrane where they are released (Melo *et al.*, 2008). While in cytolysis, the eosinophil cell membrane ruptures and clusters of granules are left behind (Persson *et al.*, 1997). Extracellular granules continue to release cytokines in a receptor-mediated manner following cytolysis (Neves *et al.*, 2008).

In asthma patients and in antigen-challenged guinea pigs, there is evidence for significant eosinophil degranulation, since extracellular granules are visible with electron microscopy and extracellular MBP is visible with immunostaining (Beasley *et al.*, 1989; Costello *et al.*, 1997; Erjefalt *et al.*, 1997; Filley *et al.*, 1982; Jeffery *et al.*, 1992; Ohashi *et al.*, 1992). In humans, decreased eosinophil degranulation in bronchial biopsies separates asthma patients in complete remission (>3 years without symptoms, no airway hyperresponsiveness, normal lung function, no asthma drugs) from patients that are in clinical remission (airway hyperresponsiveness remains) but continue to have evidence for degranulation, suggesting eosinophil degranulation contributes to airway hyperresponsiveness in asthma (Broekema *et al.*, 2011).

Furthermore, eosinophils also regulate T-cell and B-cell functions. Eosinophils are required for the survival of antibody-producing plasma cells in bone marrow of mice (Chu *et al.*, 2011), and thus may be important for modulating IgE production in allergic asthma patient. In addition, eosinophils process allergens and present antigens to activate and

induce proliferation of naïve and effector Th2 cells (MacKenzie *et al.*, 2001; Padigel *et al.*, 2006; Wang *et al.*, 2007). Eosinophils traffic to lymph nodes draining the lungs in antigen-challenged mice and guinea pigs (Erjefalt *et al.*, 1997; Korsgren *et al.*, 1997). Activation of T cells there to release Th2 cytokines there may explain the mast-cell independent increase in mucus production and recruitment of eosinophils to lung tissues in mice that are deficient for all immunoglobulins, including IgE (Korsgren *et al.*, 1997). Combined, these data suggest eosinophils have numerous potential mechanisms for regulating inflammation in asthma and some of their products could directly interact with nerves to increase airway hyperreactivity.

E) Mechanisms of airway hyperresponsiveness in asthma

Since increases in airway hyperresponsiveness increase the likelihood of having an asthma exacerbation, it is important to understand the mechanisms leading to airway hyperresponsiveness. Data from humans indicate that airway nerves mediate airway hyperreactivity. One of the best-understood mechanisms for airway hyperresponsiveness in asthma is loss of inhibitory M_2 muscarinic receptor function on the parasympathetic nerves, which occurs in some humans with asthma (Ayala *et al.*, 1989; Minette *et al.*, 1989). Neuronal M_2 receptors normally limit acetylcholine release from parasympathetic nerves, and thus loss of negative feedback through M_2 receptors in the efferent half of vagal reflexes leads to increased acetylcholine release at the neuromuscular junction and excessive bronchoconstriction.

In animals, neuronal M_2 receptor dysfunction develops following antigen challenge (Fryer *et al.*, 1991b), ozone exposure (Schultheis *et al.*, 1994), and viral infection (Fryer *et al.*, 1991a), and is closely associated with airway inflammation. Eosinophils cluster around

the nerves in airways of sensitized guinea pigs and in humans who have died of fatal asthma (Costello *et al.*, 1997). Following antigen challenge, eosinophils are recruited to airway nerves (Fryer *et al.*, 2006) and are activated to release an endogenous M_2 receptor selective antagonist, major basic protein (Jacoby *et al.*, 1993). M_2 receptor dysfunction and hyperreactivity mediated by the vagus nerves are prevented by eosinophil depletion (Elbon *et al.*, 1995), and by neutralizing eosinophil major basic protein or removing it from M_2 receptors (Evans *et al.*, 1997; Fryer *et al.*, 1992). Furthermore, treatments that reduce eosinophil accumulation in tissues and their association with airway nerves following antigen challenge, including pretreatment with glucocorticoids, blockade of integrins used in trafficking into tissues, blockade of CCR3 chemokine receptors, and inhibition of TNFα, all prevent airway hyperreactivity (Evans *et al.*, 2001; Fryer *et al.*, 1997; Fryer *et al.*, 2006; Nie *et al.*, 2009). In sensitized guinea pigs, eosinophils also mediate M_2 receptor dysfunction following ozone exposure (Yost *et al.*, 1999) and virus infection (Adamko *et al.*, 1999).

M_2 muscarinic receptor dysfunction on parasympathetic nerves can also occur through eosinophil-independent mechanisms. In the absence of antigen sensitization, viral neuraminidases reduce agonist affinity for M_2 receptors by removing sialic acid. The muscarinic agonist, carbachol, has 10-fold lower affinity for M_2 receptors following desialation with neuraminidase (Fryer *et al.*, 1990). Additionally, interferon-γ and tumor necrosis-α, cytokines produced during the inflammatory response to inhaled antigen or virus, reduce M_2 receptor gene expression (Jacoby *et al.*, 1998; Nie *et al.*, 2009). The mechanisms by which neuronal M_2 receptor function are lost in humans with asthma are not known, but the increased association of eosinophils with nerves in the lungs of humans who died of fatal asthma (Costello *et al.*, 1997) suggests a role for eosinophils.

In humans with eosinophilic asthma, treatments that reduce tissue eosinophils (corticosteroids and anti-IL-5 treatment) reduce exacerbations (Haldar *et al.*, 2009; Haldar *et al.*, 2008; Nair *et al.*, 2009). This suggests eosinophils may increase airway hyperreactivity in these patients to mediate spontaneous exacerbations. Moreover, surgical denervation of the airways (with suturing to prevent regrowth) in patients with intractable asthma eliminates or reduces exacerbations and symptoms, often corresponding to whether one or both lungs are denervated (Dimitrov-Szokodi *et al.*, 1957). Of note, airway denervation also abolishes sputum eosinophils and reduces peripheral blood eosinophils (Figure 1.6) (Dimitrov-Szokodi *et al.*, 1957). Taken together these data support the role of eosinophil and neural interactions in promoting airway hyperreactivity in asthma.

F) Evidence for a possible role of parasympathetic nerves in the regulation of eosinophilic inflammation in asthma

Airway hyperreactivity is mediated by acetylcholine release from parasympathetic nerves in the lung, and eosinophil association with parasympathetic nerves is important for increasing airway hyperresponsiveness. The remainder of this chapter focuses on mechanisms by which parasympathetic nerves could regulate eosinophilic inflammation in asthma, both through release of acetylcholine and through release of chemoattractant proteins for eosinophils. Specifically, acetylcholine and muscarinic receptor pharmacology in the lung are discussed to provide a basis for discussing the limited effectiveness of nonselective anticholinergic drugs in asthma and the rationale for why selectve blockade of M_3 muscarinic receptors may be more effective. Then eosinophil migration to the lungs via CCR3 chemokine receptors is discussed along with evidence

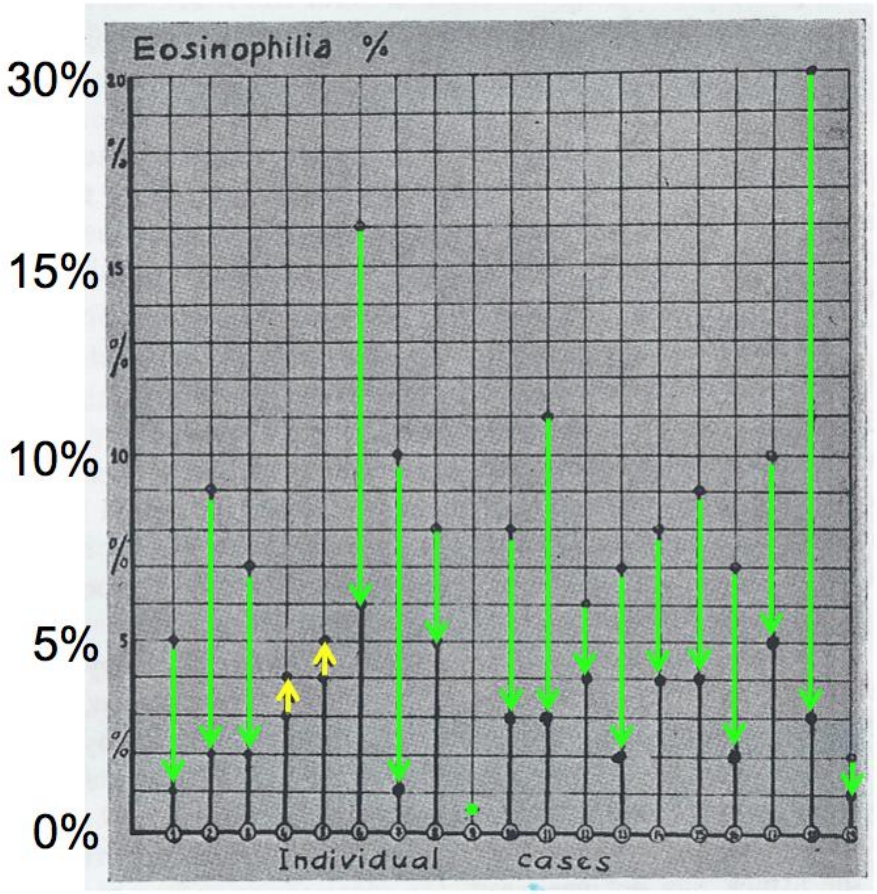

Figure 1.6 Denervation of the airways in humans with intractable asthma decreases peripheral blood eosinophils. Peripheral blood eosinophil levels are shown before and after denervation for each patient. Decreases are shown with green arrows and increases are shown with yellow arrows. This figure was modified from Dimitrov-Szokodi et al. (1957) and is reprinted with permission.

that suggests parasympathetic nerves may produce ligands for these receptors to recruit eosinophils.

G) Acetylcholine and pharmacology of muscarinic receptors in the lung

Acetylcholine is produced and released by both parasympathetic nerves and nonneuronal sources cells in the lung where it signals through muscarinic receptors to regulate bronchoconstriction, mucus secretion and clearance, vasodilation, and inflammation. There are five muscarinic receptor subtypes that control acetylcholine physiology in the lung and anticholinergic drugs available for use in asthma block all of these receptors.

1. Neuronal acetylcholine

Neuronal acetylcholine is released from parasympathetic nerves. Parasympathetic nerves that synthesize and release acetylcholine innervate all conducting airways, from the trachea to the bronchioles (Canning *et al.*, 1997), and pulmonary blood vessels (Cavallotti, 2005; Haberberger *et al.*, 1997).

2. Nonneuronal acetylcholine

Acetylcholine synthesis and release is not limited to cholinergic neurons in the lung. Both epithelial cells (Proskocil *et al.*, 2004; Reinheimer *et al.*, 1998) and endothelial cells (Haberberger *et al.*, 1997; Haberberger *et al.*, 2000) in the lung contain the cellular machinery required to synthesize and release acetylcholine including choline acetyltransferase (ChAT), which catalyzes the synthesis of acetylcholine from its precursors acetyl-coenzyme A and choline; hemicholinium-3 sensitive choline transporters, which transport choline into cells (Ferguson *et al.*, 2003); and vesicular acetylcholine transporters, which package acetylcholine into vesicles. In addition, ciliated epithelial cells express organic cation transporters OCT1 and OCT2, which can

transport acetylcholine, in their luminal membranes (Kummer *et al.*, 2006; Lips *et al.*, 2005). Airway epithelial cells in freshly isolated human bronchi contain 23±6 pmol acetylcholine per gram bronchus, which is approximately 1% of the 2600±500 pmol acetylcholine per gram bronchus contained in the whole bronchial wall (Reinheimer *et al.*, 1998).

3. Muscarinic acetylcholine receptors in the lung

Sir Henry Dale first divided the actions of acetylcholine, and other choline derivatives, into nicotinic and muscarinic, based on their similarity to responses elicited by either nicotine or muscarine (Dale, 1914). Nicotinic acetylcholine receptors are ligand-gated ion channels, and muscarinic receptors are G protein-coupled receptors (GPCR). Although nicotinic receptors are also present in the lungs and are crucial for neurotransmission between pre and postganglionic parasympathetic nerves, muscarinic receptors are a major physiological target for acetylcholine in the lungs.

Five muscarinic receptor subtypes, M_1, M_2, M_3, M_4, and M_5, are recognized by the International Union of Pharmacology (Caulfield *et al.*, 1998). M_1, M_3, and M_5 receptors typically couple to $G\alpha_q$, which stimulates phospholipase C (PLC) to release the second messengers inositol phosphate 3 (IP3) and diacylglycerol (DAG) that lead to calcium release from intracellular stores. M_2 and M_4 receptors typically couple to $G\alpha_i$, which inhibits adenyly cyclase and can also activate potassium channels and inhibit voltage-gated calcium channels. All five muscarinic receptor subtypes are expressed in the lungs. Airway smooth muscle, nerves, submucosal glands, endothelial cells, epithelium, and fibroblasts express M_1, M_2, and M_3 receptors, while inflammatory cells also express M_4, and M_5 receptors.

Subtype-specific distribution and function has been determined using pharmacological analysis, *in situ* hybridization, RT-PCR, and knockout mice. The distribution of receptor subtypes in tissues based on these assays are in good agreement. However, it is important to note that most "selective" muscarinic antagonists have at most only a 10-fold selectivity for one muscarinic receptor subtype over other subtypes, where selectivity is usually defined as 100-fold higher affinity (Caulfield *et al.*, 1998).

4. Muscarinic control of airway smooth muscle tone

In the lungs, acetylcholine causes bronchoconstriction via smooth muscle contraction (Haddad *et al.*, 1991; Roffel *et al.*, 1988; Roffel *et al.*, 1990; Stengel *et al.*, 2000; Struckmann *et al.*, 2003). Airway smooth muscle expresses both M_2 and M_3 receptors in humans, cows, guinea pigs, dogs, and mice (Fernandes *et al.*, 1992; Haddad *et al.*, 1991; Mak, 1990; Mak *et al.*, 1992; Roffel *et al.*, 1987; Struckmann *et al.*, 2003). Although M_2 receptors often outnumber M_3 receptors, physiological data indicate that M_3 receptors have the dominant role in smooth muscle contraction.

M_3 receptors signal via $G\alpha_q$ to induce smooth muscle contraction (Figure 1.7) (Billington *et al.*, 2003). Functional experiments demonstrate that contraction induced by muscarinic ligands in isolated trachea and bronchi is mediated by M_3 receptors in all species including humans (Haddad *et al.*, 1991; Roffel *et al.*, 1988; Roffel *et al.*, 1990; Struckmann *et al.*, 2003). Recently, it has also been shown that smooth muscle M_3 receptors can be activated in the absence of acetylcholine by membrane depolarization induced chemically with KCl (Liu *et al.*, 2009). However, this ligand-independent activation of M_3 receptors has only thus far been demonstrated in mice *in vitro*.

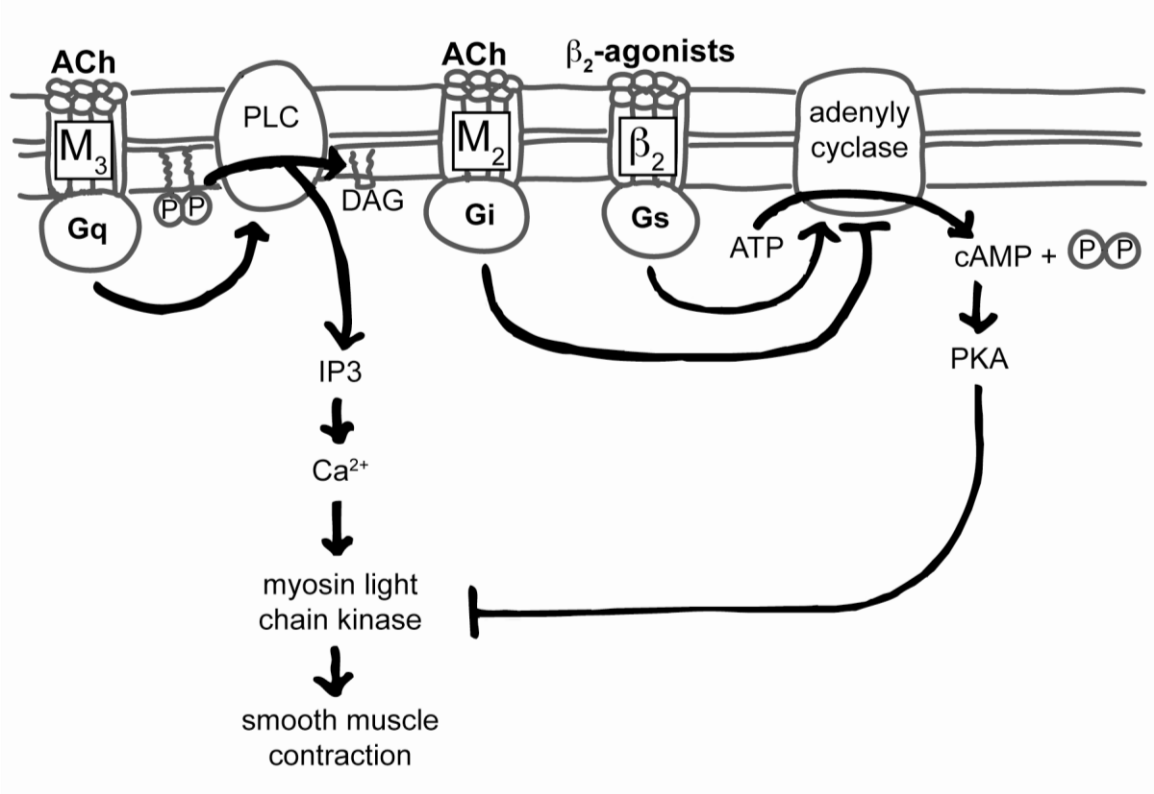

Figure 1.7 Muscarinic control of airway smooth muscle tone. Acetylcholine activates M_3 muscarinic receptors, which couple via $G\alpha_q$ to activate phospholipase C (PLC). PLC cleaves phosphoinositol 4,5-bisphosphate (PIP2) into the intracellular messengers 1,2-diacylgycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 releases calcium from intracellular stores and this leads to activation of myosin light chain kinase and smooth muscle contraction. Acetylcholine also activates M_2 muscarinic receptors, which couple via $G\alpha_i$ to inhibit adenyly cyclase activity, inhibiting relaxation mediated via adenyly cyclase. β_2 adrenoreceptor agonists activate β_2 adrenoreceptors, which couple via $G\alpha_s$ to activate adenyly cyclase. Adenyly cyclase catalyzes the production of cyclic AMP (cAMP) from ATP. cAMP activates phosphokinase A (PKA), which leads to inhibition of myosin light chain kinase and relaxation of airway smooth muscle. Thus, M_3 muscarinic receptors directly induce airway smooth muscle

contraction, β_2 adrenoreceptors induce smooth muscle relaxation, and M_2 muscarinic receptors inhibit relaxation.

M_2 receptors indirectly control airway smooth muscle contraction by functionally antagonizing $G\alpha_s$-induced relaxation (Figure 1.7). Thus activation of M_2 receptors inhibits relaxation induced by either β_2-adrenoreceptor agonists or direct activation of adenylyl cyclase with forskolin (Fernandes *et al.*, 1992). *In vitro*, airway narrowing induced by muscarine can only be completely prevented in airways from mice deficient in both M_2 and M_3 receptor genes (Struckmann *et al.*, 2003). *In vivo*, however, only M_3 receptors contribute to bronchoconstriction induced by electrical stimulation of the vagus nerves and intravenous methacholine, since bronchoconstriction is entirely absent in mice deficient for M_3 receptors (Fisher *et al.*, 2004).

5. Muscarinic control of acetylcholine release from airway parasympathetic nerves

Both pre and postganglionic parasympathetic nerves innervating the lungs express muscarinic receptors that are densest at the ganglia (van Koppen *et al.*, 1988; van Koppen *et al.*, 1987). Muscarinic receptors on parasympathetic nerves modulate synaptic neurotransmission between the pre and postganglionic nerves, and also limit release of acetylcholine by postganglionic nerves at target tissues (smooth muscle, mucous glands).

Preganglionic autonomic nerves release acetylcholine onto nicotinic receptors at their synapses with postganglionic nerves. In the lungs, muscarinic receptors modulate neurotransmission across this synapse (Myers, 2001). Preganglionic neurons contain inhibitory M_2 receptors at the synapse, which limit acetylcholine release. Thus, activating M_2 receptors during electrical stimulation of the preganglionic nerves reduces

acetylcholine release into the synapse, which in turn decreases the amplitude of nicotinic fast excitatory postsynaptic potentials recorded in bronchial ganglia (Myers *et al.*, 1996).

M_1 muscarinic receptors are also found in cell bodies of postganglionic nerves, although there are species differences regarding the importance of these receptors in modulating synaptic neurotransmission. In guinea pigs and rats there is no evidence for a physiological function of these receptors (Murai *et al.*, 1998; Myers *et al.*, 1996; Undem *et al.*, 1990). However, in rabbits and humans they appear to facilitate neurotransmission at the synapse and increase bronchoconstriction (Bloom *et al.*, 1988; Fujimura *et al.*, 1992; Lammers *et al.*, 1989).

Of greater physiological importance are inhibitory M_2 receptors on postganglionic parasympathetic nerves. These receptors are activated by acetylcholine to inhibit further acetylcholine release in a feedback mechanism that limits bronchoconstriction and mucus secretion in healthy animals and humans (Ayala *et al.*, 1989; Blaber *et al.*, 1985; Fryer *et al.*, 1996; Fryer *et al.*, 1984; Minette *et al.*, 1989; Ramnarine *et al.*, 1996). When neuronal M_2 receptors in trachea are blocked, the amount of acetylcholine released by nerve stimulation significantly increases (Baker *et al.*, 1992). Pharmacologically blocking inhibitory M_2 receptors in guinea pigs or deleting them genetically in mice significantly potentiates vagally induced bronchoconstriction *in vivo*, while selectively activating M_2 receptors inhibits vagally induced bronchoconstriction 80% (Fisher *et al.*, 2004; Fryer *et al.*, 1984). As described previously (Section E, mechanisms of airway hyperresponsiveness), blockade of these neuronal M_2 receptors is one mechanism that can lead to airway hyperresponsiveness in asthma.

6. Muscarinic control of mucus secretion

Parasympathetic nerves also stimulate mucus secretion from submucosal glands in the lungs. Mucus is an aqueous solution that includes electrolytes, mucins (large glycoproteins), enzymes, and antibacterial agents (Rogers, 2001), and is beneficial in airway defense and for trapping particles. Particles are then removed along with the mucus by ciliary clearance into the mouth and esophagus. Excessive mucus secretion contributes to airway obstruction in severe asthma.

Both constitutive and induced release of mucus occurs *in vivo* and in isolated glands *in vitro* (Baker *et al.*, 1985; Dwyer *et al.*, 1992; Gallagher *et al.*, 1975). Constitutive mucus release does not depend on cholinergic nerves (Baker *et al.*, 1985; Borson *et al.*, 1984; Gallagher *et al.*, 1975). However, vagal stimulation and exogenous acetylcholine both increase mucus secretion from submucosal glands (Borson *et al.*, 1984; Gallagher *et al.*, 1975).

Both M_1 receptors and M_3 receptors are present in human and animal submucosal glands (Mak, 1990; Mak *et al.*, 1992). M_3 receptors are responsible for both vagal and exogenous acetylcholine-induced mucin secretion (Ramnarine *et al.*, 1996). Despite their presence, a direct role for M_1 receptors has not been demonstrated in airway submucosal glands, but it has been hypothesized that these receptors may be responsible for fluid or electrolyte release by serous cells (Yang *et al.*, 1988).

7. Muscarinic control of mucociliary clearance

Activating muscarinic receptors in epithelial cells transiently increases intracellular calcium (Salathe *et al.*, 1997) and increases ciliary beat frequency (Klein *et al.*, 2009;

Salathe *et al.*, 1997; Seybold *et al.*, 1990), which would increase transport of mucus and particulates out of the lung. Muscarinic signaling also increases the velocity of liquid (Seybold *et al.*, 1990) and particle transport (Klein *et al.*, 2009) upward in isolated tracheas.

M_3 receptor mRNA is found in human airway epithelium by *in situ* hybridization (Mak *et al.*, 1992), while mRNA for both M_3 and M_1 receptors has been identified in mouse epithelia (Klein *et al.*, 2009). Experiments using muscarinic receptor gene-deficient mice demonstrate that M_3 receptors are both required and sufficient for the full increase in ciliary beat frequency and particle transport speed induced by muscarine in wild-type mice (Klein *et al.*, 2009). A role for M_3 receptors is further supported by pharmacological experiments in sheep (Salathe *et al.*, 1997).

While M_3 receptors provide the dominant control of ciliary beat frequency, M_1 and M_2 receptors can also contribute. M_1 receptors increase ciliary transport speed, but this function is only uncovered in mice that are deficient for both M_2 and M_3 receptors. M_2 receptor activation prevents increases in ciliary beat frequency initiated by M_1 receptors and also by nonmuscarinc stimuli such as ATP. Inhibition of ciliary beat frequency mediated by M_2 receptors is likely indirect, since M_2 mRNA and protein are not detectable in epithelial cells but are found in neighboring cells (Klein *et al.*, 2009).

8. Muscarinic control of vasodilation

While neuronal acetylcholine does not contribute to resting tone in pulmonary blood vessels, stimulation of the vagus nerves causes vasodilation (Laitinen *et al.*, 1987). Exogenous acetylcholine will only relax human pulmonary arteries if the endothelium is intact (Greenberg *et al.*, 1987). Acetylcholine likely acts at muscarinic receptors on

endothelial cells to stimulate production of nitric oxide, which relaxes smooth muscle (Furchgott *et al.*, 1980; Greenberg *et al.*, 1987; McMahon *et al.*, 1992). Endothelial cells in pigs express mRNA for both M_2 and M_3 receptors and data from M_3 receptor-deficient mice suggest that M_3 receptors are important for vasodilation *in vivo* (Fisher et al., 2004; Kummer et al., 1999).

9. Muscarinic regulation of inflammation

A functional role for muscarinic receptors in immune responses has been demonstrated in lung mast cells, alveolar macrophages, and airway epithelial cells. In addition, human peripheral blood lymphocytes, neutrophils, and eosinophils all express muscarinic receptors (Table 1.2) (Bany *et al.*, 1999; Dulis *et al.*, 1979; Hellstrom-Lindahl *et al.*, 1996; Ricci *et al.*, 2002; Tayebati *et al.*, 1999; Tayebati *et al.*, 2002; Verbout *et al.*, 2006). However, their role in airway physiology is unknown.

Acetylcholine inhibits histamine release from mast cells via muscarinic receptors in isolated bronchi, and pharmacological data suggest this inhibition is mediated by M_1 receptors (Reinheimer *et al.*, 1997; Reinheimer *et al.*, 2000; Wessler *et al.*, 2007). In rats, however, acetylcholine enhances rather than inhibits evoked histamine release (Reinheimer *et al.*, 2000).

Alveolar macrophages reside in the airway lumen where they phagocytose foreign substances and initiate immune responses against invading pathogens (Janeway, 2005). Acetylcholine induces release of leukotriene B_4 and other chemoattractant factors from alveolar macrophages via M_3 receptors. These mediators induce human peripheral blood monocyte, neutrophil, and eosinophil chemotaxis (Reinheimer *et al.*,

Table 1.2 Expression of muscarinic receptor subtypes by immune cells					
	M_1	**M_2**	**M_3**	**M_4**	**M_5**
Eosinophils			mRNA	mRNA	mRNA
Lymphocytes		protein binding assay	mRNA protein binding assay	mRNA protein binding assay	mRNA protein binding assay
Mast cells	functional studies				
Macrophages			functional studies		
Neutrophils			mRNA	mRNA	mRNA

Expression in human cells based on PCR, western blot, radioligand binding studies, or functional studies. Additional data for immunostaining exist; however, the selectivity of commercially available muscarinic receptor antibodies has been questioned (Jositsch *et al.*, 2009; Pradidarcheep *et al.*, 2008), so this data is not included. Data compiled from (Bany *et al.*, 1999; Hellstrom-Lindahl *et al.*, 1996; Reinheimer *et al.*, 2000; Ricci *et al.*, 2002; Sato *et al.*, 1998; Tayebati *et al.*, 1999; Verbout *et al.*, 2006).

1998). Additionally, acetylcholine may also contribute to inflammation by inducing release of chemotactic factors from airway epithelial cells (Koyama *et al.*, 1992; Koyama *et al.*, 1998).

10. Muscarinic receptors in airway remodeling

In disease, acetylcholine may also contribute to airway remodeling by acting at muscarinic receptors to increase proliferation of both fibroblasts and smooth muscle cells. In primary cultures of human fibroblasts and fibroblast cell lines, acetylcholine stimulates collagen production and proliferation (Haag *et al.*, 2008; Matthiesen *et al.*, 2007; Matthiesen *et al.*, 2006; Pieper *et al.*, 2007). While acetylcholine does not directly increase smooth muscle cell proliferation, it enhances proliferation induced by growth factors, including platelet-derived growth factor and epidermal growth factor (Gosens *et al.*, 2003; Krymskaya *et al.*, 2000). Muscarinic receptor antagonists block the proliferative effects of acetylcholine in both fibroblast and smooth muscle cells.

Human lung fibroblasts contain mRNA for M_1, M_2, and M_3 muscarinic receptors with trace levels of M_4 receptors (Haag *et al.*, 2008; Matthiesen *et al.*, 2006). It is likely that M_2 receptors are dominant, since the proliferative response in fibroblasts is pertussis toxin sensitive and can be blocked with selective muscarinic antagonists that suggest M_2 receptors are responsible (Matthiesen *et al.*, 2006). Acetylcholine-enhanced proliferation of human airway smooth muscle cells is M_3 receptor-dependent and is lost when M_3 receptor expression is decreased with cell passage *in vitro* (Gosens *et al.*, 2003).

11. Summary of muscarinic receptors in normal lung function

The contribution of M_1, M_2, and M_3 receptors to pulmonary physiology is summarized in Table 1.3. Although M_4 and M_5 receptors are expressed on inflammatory cells (Table 1.2) their function is unknown. Most lung tissues express more than one muscarinic receptor subtype, but the function of one muscarinic subtype is often dominant. Where the functions of additional muscarinic receptor subtypes are known, they either inhibit or supplement the dominant receptor's function. For example, M_2 receptors on postganglionic parasympathetic nerves inhibit acetylcholine release, and this function is inhibited by M_1 receptors in ganglia, which increase acetylcholine release by facilitating neurotransmission. While in airway smooth muscle, M_2 receptors supplement contraction mediated via M_3 receptors.

In healthy individuals, this muscarinic physiology is balanced and results in airway smooth muscle tone, vasodilation, mucus secretion, and mucociliary clearance. However, in asthma bronchoconstriction and increased mucus secretion contribute to disease symptoms. Acetylcholine released by the vagus nerves onto M_3 receptors mediates both of these physiological functions. Therefore blockade of M_3 receptors would be expected to be beneficial for reducing symptoms and improving lung function.

H) Anticholinergic drugs in the treatment of asthma

Atropine (Figure 1.8) and other naturally occurring muscarinic receptor antagonists found in plants of the Datura genus have been effectively used as bronchodilators for centuries. In western medicine, the leaves and roots of *D. stramonium* were administered in cigarettes to treat respiratory diseases starting in the 1800s (Gross *et al.*, 1984). However, while atropine effectively inhibits bronchoconstriction, it also

Table 1.3 Function of muscarinic receptor subtypes in lung

	M_1	M_2	M_3
Parasympathetic Nerves	Increase neurotransmission at ganglia	Limit acetylcholine release	
Smooth Muscle		Inhibit relaxation*	Contraction
Submucosal Glands	Unknown		Mucus secretion
Endothelial Cells		Unknown	Vasodilation*
Airway Epithelium	Increase ciliary beat frequency* (if M_2 and M_3 blocked)	Reduce ciliary beat frequency*	Increase ciliary beat frequency
Immune Function	Limit evoked histamine release from mast cells		Induce release of chemotactic factors from alveolar macrophages*
Airway Remodeling		Increase proliferation in fibroblasts	Enhance proliferation induced by growth factors in smooth muscle

Receptor subtype involvement is based on human data unless noted with *. See text for references.

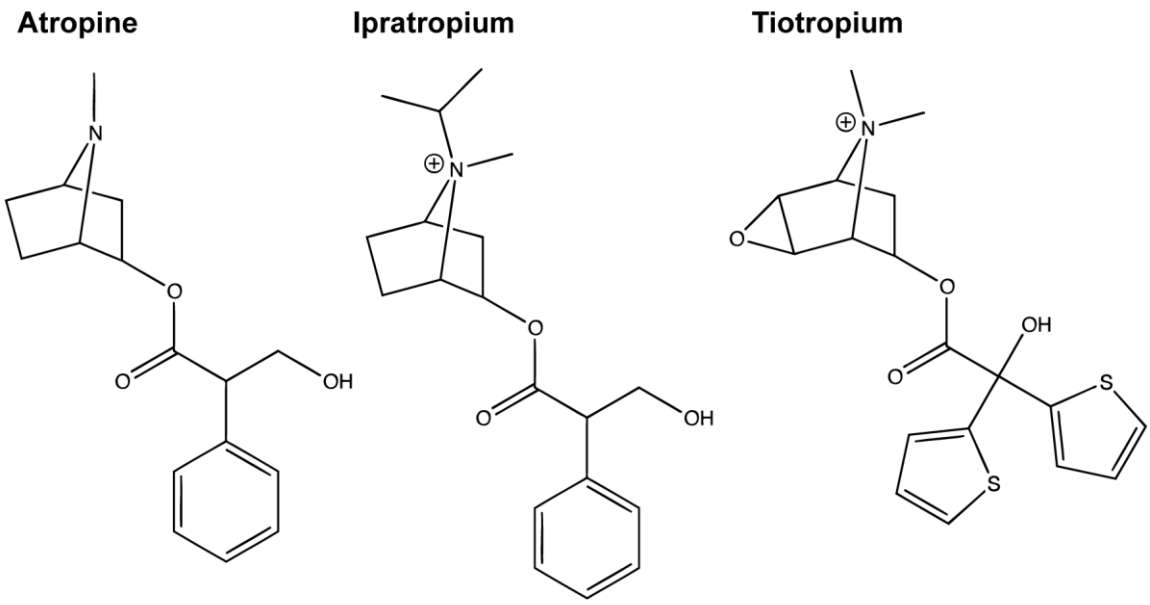

Figure 1.8 Chemical structures of muscarinic receptor antagonists used clinically to treat asthma. Chemical structures for atropine and its synthetic derivatives ipratropium and tiotropium are shown. Ipratropium and tiotropium contain a quaternary ammonium that limits bioavailability and distribution across the blood-brain-barrier. Drug structures courtesy of Dr. Bart Moulton.

crosses the blood-brain-barrier (Virtanen *et al.*, 1982) and its use is associated with side effects described below. Therefore, when beta adrenoreceptor agonists that directly relax airway smooth muscle became available they largely replaced atropine. However, since then, synthetic derivatives of atropine have been developed that contain a quaternary ammonium that limits bioavailability and prevents distribution across the blood-brain-barrier. Therefore, these next generations of drugs, which include ipratropium and tiotropium (Figure 1.8) have fewer side effects.

Atropine, ipratropium, and tiotropium are all competitive antagonists (Casarosa *et al.*, 2009), and thus inhibit bronchoconstriction by competing with acetylcholine for binding to M_3 receptors on airway smooth muscle. The pharmacological properties of atropine, ipratropium, and tiotropium are discussed below and their binding affinities and duration of binding to muscarinic receptors are summarized in Table 1.4.

1. Atropine

Atropine is a nonselective muscarinic antagonist with similar affinities for all five muscarinic receptor subtypes (Casarosa *et al.*, 2009). Relative to the quaternary ammonium derivatives, atropine is also well absorbed across the gastrointestinal tract into systemic circulation. Total absorption of atropine across the intestine is approximately 25% in rat (Levine, 1959), while bioavailability following intramuscular injection in humans is reported to be 50% (Goodman *et al.*, 2006). As a result, atropine has many undesirable side effects including dry mouth, urinary retention, and accelerated heart rate. At high doses side effects include coma, fever, and hallucinations (Brown *et al.*, 2006). Atropine, ipratropium, and tiotropium are all cleared by urinary excretion (Ensing *et al.*, 1989; FDA, 2010; Hinderling *et al.*, 1985; Kentala *et al.*, 1990).

Table 1.4 Binding affinities and duration of binding for atropine, ipratropium, and tiotropium at human muscarinic receptors

		Atropine	Ipratropium	Tiotropium
K_i (nM)[a]	M_1	0.170	0.398	0.016
	M_2	0.339	0.295	0.020
	M_3	0.209	0.263	0.010
	M_4	0.107	0.224	0.010
	M_5	0.316	0.851	0.110
Dissociation half-life (hours)[a,b]	M_1	-	0.10	10.5
	M_2	-	0.03	2.6
	M_3	0.04	0.22	27.0

[a]Antagonist affinities determined in heterologous competition binding experiments against [^{3}H]NMS. Dissociation kinetics using Motulski and Mahan method (Casarosa *et al.*, 2009). [b](Dowling *et al.*, 2006).

2. Ipratropium bromide

Ipratropium bromide is used clinically as a second-line bronchodilator behind β_2-agonists. Like atropine, ipratropium is nonselective and has similar affinities for all five muscarinic receptor subtypes (Casarosa *et al.*, 2009). The major differences between ipratropium and atropine are the inability of ipratropium to cross the blood-brain barrier and its poor absorption in the gastrointestinal tract. Ipratropium is better absorbed when administered by inhalation (Ensing *et al.*, 1989), which may be due to uptake by organic cation/carnitine transporters (OCTN) in airway epithelium. OCTN2, and to a lesser extent OCTN1, transport both ipratropium and tiotropium in a human bronchial epithelial cell line (Nakamura *et al.*, 2010). Ipratropium produces peak bronchodilation within 60-90 minutes of inhalation and its duration of action is 4-6 hours, requiring 4 times daily administration.

3. Tiotropium bromide

Tiotropium is still being evaluated for use in asthma but is approved for use in chronic obstructive pulmonary disease (GOLD, 2009). Tiotropium has a much higher affinity for muscarinic receptors and a much longer duration of binding to muscarinic receptors than either atropine or ipratropium (Table 1.4). However, tiotropium's most interesting property is its significantly greater duration of binding to M_3 receptors than M_1 and M_2 receptors, which provides tiotropium with kinetic selectivity for M_3 receptors (Casarosa *et al.*, 2009; Disse *et al.*, 1993). I take advantage of tiotropium's kinetic selectivity to selectively block M_3 receptors in Chapter 3.

Functionally, tiotropium blocks M_2 receptors on parasympathetic nerves in guinea pigs early after administration to increase acetylcholine release. However, following washout, neuronal acetylcholine release returns to baseline within 2 hours, a time point when

smooth muscle contraction via M_3 receptors is still completely blocked. M_3 receptor function only begins to return after 7 hours (Takahashi *et al.*, 1994).

Tiotropium's onset of bronchodilation in humans is very slow, reaching peak bronchodilation in 3-4 hours, but tiotropium then has a very long duration of action (1-2 days) and can be administered daily (Maesen *et al.*, 1995). The slow onset of action makes tiotropium inappropriate for a rescue medication, but the duration of action makes it useful as a once-daily bronchodilator. In clinical trials with asthma patients thus far, tiotropium effectively blocks methacholine-induced bronchoconstriction. When administered in combination with inhaled glucocorticoids, tiotropium is superior to doubling the dose of glucocorticoids in patients with uncontrolled asthma (O'Connor *et al.*, 1996; Peters *et al.*, 2010).

I) Limited effectiveness of anticholinergics in asthma

While anticholinergic drugs are recommended for acute use as rescue bronchodilators, they are not recommended for long-term management of asthma (EPR-3, 2007). Even though vagal reflexes are important for mediating airway hyperreactivity, anticholinergics have not been tested for their ability to prevent exacerbations in asthma.
This is partially because enthusiasm for pursing anticholinergics in asthma has been limited by controversy over the effectiveness of muscarinic receptor antagonists in asthma and by association the role of parasympathetic nerves.

Numerous studies in humans show limited or no benefit of atropine and ipratropium in their ability to block bronchoconstriction induced by nonspecific stimuli such as histamine, sulfur dioxide, exercise, cold air, and antigens (Casterline *et al.*, 1976; Chan-

Yeung *et al.*, 1971; Cockcroft *et al.*, 1978; Fish *et al.*, 1977; Fisher *et al.*, 1970; Nadel *et al.*, 1965; Rosenthal *et al.*, 1977; Ruffin *et al.*, 1978; Woenne *et al.*, 1978). Conversely, other studies show that atropine and ipratropium are effective, and are able to inhibit bronchoconstriction induced by these same stimuli (Chan-Yeung, 1977; Chen *et al.*, 1981; Holtzman, 1980; Nadel *et al.*, 1965; Sheppard, 1982; Widdicombe *et al.*, 1962; Yu *et al.*, 1972). With no further analysis these discrepancies indicate muscarinic receptors have limited effectiveness.

1. Dose of anticholinergics in asthma

However, these discrepancies may be explained partially by the dose of anticholinergic used and the method of delivery, since the majority of studies that failed used a single dose of inhaled muscarinic antagonist, while many of the studies that were successful used either a higher dose of antagonist or administered the antagonist intravenously.

The dose of muscarinic antagonist administered is important during an acute exacerbation because of the competitive nature of muscarinic antagonists. Doses of muscarinic receptor antagonist that are chosen for their ability to effectively inhibit tonic release of acetylcholine from parasympathetic nerves at baseline or bronchoconstriction induced by inhaled methacholine may be ineffective for inhibiting higher concentrations of acetylcholine released during an exacerbation. For example, in asthma patients, low doses of inhaled atropine inhibit baseline cholinergic tone and also prevent bronchoconstriction induced by exogenous inhaled methacholine. However, much higher doses of atropine are required to prevent cold air-induced bronchoconstriction mediated by the vagus nerves (Figure 1.9)(Sheppard, 1982). Similarly, when increasing doses of ipratropium are given by nebulization to patients admitted to the hospital during

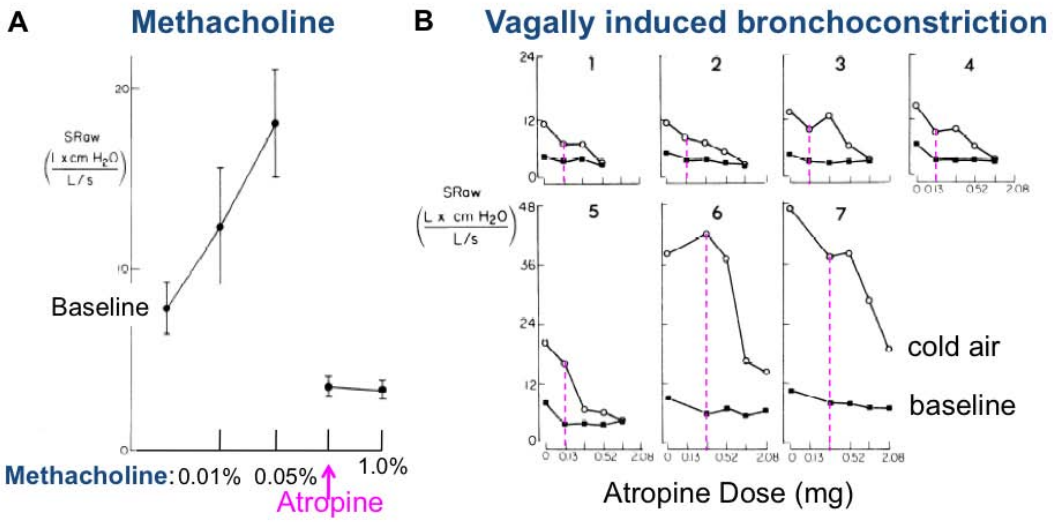

Figure 1.9 Higher doses of atropine are required to inhibit cold-air induced bronchoconstriction than baseline smooth muscle tone or inhaled methacholine. (A) Mean airway resistance (bronchoconstriction) at baseline and following inhalation of 0.01% and 0.05% methacholine are shown for seven asthma patients. The lowest dose of inhaled atropine that prevented bronchoconstriction induced with 1.0% methacholine was found for each patient, and average airway resistance following this dose of atropine (pink arrow) is plotted. This dose of atropine was sufficient to inhibit both bronchoconstriction to inhaled methacholine and baseline cholinergic tone. (B) Airway resistance before (filled circles) and after inhalation of cold air (open circles) are shown for each patient (1-7) following administration of placebo (0 mg atropine) or increasing doses of inhaled atropine. Cold air successfully induced bronchoconstriction in each patient. This bronchoconstriction was due to the action of acetylcholine at muscarinic receptors, since atropine completely blocked or reduced bronchoconstriction in all patients. However, the dose of atropine that was sufficient to block baseline airway smooth muscle tone and bronchoconstriction induced by inhaled methacholine in each patient (from A, shown as dashed pink line in B) was not adequate to block cold air-

induced bronchoconstriction. These figures are modified from (Sheppard, 1982) and do not require permission for use in a thesis.

an acute exacerbation, 500 μg is required to achieve maximum bronchodilation (Ward *et al.*, 1981). This is 10 times greater, however, than the 40-80 μg dose that blocks baseline vagal cholinergic tone and bronchoconstriction (Baigelman *et al.*, 1977; Cockcroft *et al.*, 1978). Thus, inadequate dosing may explain studies that show no benefit of muscarinic antagonists.

In addition, the method by which muscarinic receptor antagonists are administered is also important for achieving complete vagal blockade. In both humans and animals, atropine administered by inhalation does not distribute as evenly in airway tissue as atropine administered intravenously (Holtzman *et al.*, 1983; Sheppard *et al.*, 1983). Thus, with low doses of muscarinic receptor antagonist, intravenous administration may improve access to the nerves and result in more effective inhibition of bronchoconstriction.

2. Selectivity of anticholinergics in asthma

It is also possible, however, that the limited effectiveness of atropine and ipratropium is due to nonselective blockade of all muscarinic receptor subtypes. Atropine and ipratropium bind to and dissociate from all muscarinic receptor subtypes equally. While blockade of M_3 muscarinic receptors on airway smooth muscle would inhibit bronchoconstriction, blockade of other muscarinic receptors may be counterproductive. For instance, blocking inhibitory M_2 receptors on parasympathetic nerves can increase acetylcholine enough to overcome M_3 receptor blockade and increase bronchoconstriction, as has been shown in guinea pigs treated with atropine and ipratropium (Fryer *et al.*, 1987).

In particular, since asthma is an inflammatory disease and most inflammatory cells express several muscarinic receptor subtypes, nonselective blockade of all muscarinic receptor subtypes may worsen the inflammatory response. This could result in increased airway responsiveness and increased acetylcholine release from parasympathetic nerves during an exacerbation. Previous data from the Fryer and Jacoby laboratories also support this hypothesis, since nonselective blockade of all muscarinic receptor subtypes at the time of antigen challenge potentiated airway hyperreactivity 24 h later in guinea pigs (Verbout *et al.*, 2009; Verbout *et al.*, 2007). This potentiation of airway hyperreactivity was associated with increased eosinophil degranulation in airway tissues (Verbout *et al.*, 2007). Thus, nonselective blockade of all muscarinic receptor subtypes may contribute to the limited effectiveness of atropine and ipratropium in asthma. It is not known whether selective blockade of M_3 muscarinic receptors, the subtype important for smooth muscle contraction and mucus secretion, would have greater benefits in asthma.

J) Recruitment of eosinophils to lungs and nerves

Eosinophil recruitment into the lungs is associated with allergic asthma and antigen challenge models of allergic asthma. Furthermore, denervation of the airways in humans with asthma abolishes sputum eosinophils and reduces airway hyperresponsiveness. Since nerve-associated eosinophils are associated with airway hyperresponsiveness, it is important to consider mechanisms that could contribute to eosinophil migration into the lungs and their association with airway nerves.

A multi-step process is required for eosinophils to migrate from blood vessels into tissues. In order to cross the vascular endothelium eosinophils must first loosely bind and roll along the endothelial surface using selectins and integrins. Eosinophils express

L-selectin, CD162 (P-selectin glycoprotein ligand-1), and sialyl-Lewis X, which bind to CD34 and MAdCAM-1; P-selectins; and E-selectins, respectively, on the vascular endothelium of small venules (Berg *et al.*, 1993; Georas *et al.*, 1992; Sriramarao *et al.*, 1994; Symon *et al.*, 1996). When eosinophils are activated by chemokines (**chemo**tactic cyto**kines**) displayed on the endothelial cell surface by glycosaminoglycans, heparin sulfate, or chemokine binding proteins this increases their integrin affinity and the loose interactions with selectins are replaced by firm integrin-mediated adhesion (Barthel *et al.*, 2008; Rot *et al.*, 2004). In particular, VLA-4 ($\alpha 4\beta 1$ integrin) on eosinophils and VCAM-1 on endothelial cells are important for eosinophil trafficking into lung tissues, since blockade of either VLA-4 or VCAM-1 with blocking antibodies and genetic deletion of VCAM-1 all reduce lung eosinophils following antigen challenge (Abraham *et al.*, 1994; Fryer *et al.*, 1997; Gonzalo *et al.*, 1996; Nakajima *et al.*, 1994; Pretolani *et al.*, 1994; Weg *et al.*, 1993). Chemokine stimulation also induces eosinophils to change shape and migrate into the tissues along a chemokine gradient (Boehme *et al.*, 1999; El-Shazly *et al.*, 1999; Humbles *et al.*, 2002). Unidirectional migration along a soluble gradient is termed chemotaxis, while migration along an immobilized gradient is called haptotaxis (Rot *et al.*, 2004).

Chemokines are small structurally similar proteins (8-14 kDa) that can be broadly classified as homoeostatic/constitutive or inflammatory. Homeostatic chemokines are primarily involved in tissue homing and are not transcriptionally regulated following inflammation, while inflammatory chemokines that would be relevant for eosinophil recruitment in asthma are produced in response to inflammatory cytokines (Le *et al.*, 2004). Chemokines bind to and signal through GPCRs, and they can be divided into four subclasses, CC, CXC, CX3C, and XC based on the relative position of the first two consensus cysteines in their amino acid sequence. The cysteines are separated by 0-3

non-conserved amino acids (X) in the first three families while only one consensus cysteine in present in the XC subfamily. The CC and CXC subclasses are the largest, containing 28 and 17 members respectively while the CX3C class contains one member and the XC subclass contains two members (Ubogu, 2011).

1. CCR3 chemokine receptor and ligands

The search for eosinophil chemokine receptors began with the finding that a selective chemotactic factor for eosinophils was released into the lungs of guinea pigs following antigen-challenge (Bandeira-Melo *et al.*, 2001a; Kay *et al.*, 1971). This factor was originally called eosinophil chemotactic factor of anaphylaxis, and it was later discovered that the dominant contributor to this chemotactic activity was CCL11 (also known as eotaxin), which is a ligand for CCR3 receptors (Jose *et al.*, 1994b; Kay *et al.*, 1971; Sabroe *et al.*, 1998). It is now known that eosinophils express at least three functional chemokine receptors CCR1, CCR3, and CXCR4 (Daugherty *et al.*, 1996; Nagase *et al.*, 2000; Ponath *et al.*, 1996; Post *et al.*, 1995; Sabroe *et al.*, 1998). These receptors are in addition to receptors for chemoattractants, such as C5a and platelet activating factor, which are not selective for eosinophil migration. In humans, CCR3 and CXCR4 are expressed more consistently and at higher levels than CCR1 on eosinophils (Daugherty *et al.*, 1996; Nagase *et al.*, 2001). However, some individuals express CCR1 at high levels and their eosinophils respond to CCR1 agonists accordingly (Sabroe *et al.*, 1999). CCR3 and CXCR4 may serve very different purposes *in vivo*, since ligands for CCR3 tend to be inducible and are increased during inflammation, while CXCL12 (SDF-1), the primary ligand for CXCR4, is homeostatic (Nagase *et al.*, 2001).

2. CCR3 receptors

CCR3 receptors are of particular interest in asthma, since eosinophil recruitment into the lungs correlates with expression of ligands for CCR3 receptors (Brown *et al.*, 1998; Ying *et al.*, 1999). Moreover, eosinophil recruitment into the lungs of antigen-challenged mice and guinea pigs depends on CCR3 receptors. In mice, eosinophils are significantly reduced or absent in the lungs following antigen challenge of CCR3 receptor-deficient mice (Humbles *et al.*, 2002; Ma *et al.*, 2002; Pope *et al.*, 2005). In guinea pigs, blockade of CCR3 receptors significantly decreases nerve-associated eosinophils but not all tissue eosinophils, following antigen challenge (Fryer *et al.*, 2006). This suggests CCR3 receptors on eosinophils are important for eosinophil recruitment to airway nerves and that nerves may release ligands for this receptor.

CCR3 receptors are highly expressed at the cell surface of human eosinophils with somewhere between 40,000 and 400,000 CCR3 receptors per eosinophil (Daugherty *et al.*, 1996; Ponath *et al.*, 1996). Activation of CCR3 receptors causes a transient increase in intracellular calcium in humans, mouse, and guinea pig that is sensitive to pertussis toxin, indicating CCR3 receptors are $G\alpha_i$-linked GPCRs (Boehme *et al.*, 1999; Daugherty *et al.*, 1996; Ponath *et al.*, 1996; Post *et al.*, 1995; Sabroe *et al.*, 1998). Signaling downstream of this calcium response leads to chemotaxis and activation of other eosinophil functions including degranulation, leukotriene production, and vesicular transport-mediated release of cytokines (Bandeira-Melo *et al.*, 2001b; Bandeira-Melo *et al.*, 2001c; Fujisawa *et al.*, 2000). Activation of chemotaxis in particular involves phosphorylation and activation of tyrosine kinases and the MAP kinase ERK 1/2, which induce actin polymerization, cell shape change, and chemotaxis within minutes (Boehme *et al.*, 1999; El-Shazly *et al.*, 1999).

In addition to their expression on eosinophils, CCR3 receptors are also expressed by other leukoctyes involved in Th2 cytokine-mediated inflammation, including a subset of Th2 lymphocytes, mast cells, and basophils (Price *et al.*, 2003; Sallusto *et al.*, 1997; Uguccioni *et al.*, 1997). CCR3 receptors on mast cells mobilize to the cell surface following IgE receptor cross-linking and are involved in Th2 cytokine release (Price *et al.*, 2003). Functional CCR3 receptors are also expressed on airway epithelial cells, airway smooth muscle cells from asthma patients, and some dorsal root ganglia sensory nerves (Joubert *et al.*, 2005; Oh *et al.*, 2001; Stellato *et al.*, 2001).

3. CCR3 receptor ligands

There are at least nine ligands that activate human CCR3 receptors to induce chemotaxis (Table 1.5). The official chemokine nomenclature (CCL and number) is used in this thesis. However, at the beginning of each section and in figures I provide an older common name in parentheses (some chemokines have more than one common name) (IUIS/WHO, 2003). It is important to note that there are species differences in chemokine expression and not every chemokine found in humans has a homolog in animals. For example, no homolog for CCL26 (eotaxin-3) has been identified in mice. In guinea pig, only three CCR3 chemokine genes have been cloned (CCL5, CCL7, and CCL11) (Asano *et al.*, 2001; Campbell *et al.*, 1997; Jose *et al.*, 1994a). However, there are also predicted sequences for an additional two genes (CCL26 and CCL28).

Of the known CCR3 chemokines, only the eotaxins (CCL11, CCL24, and CCL26) are selective for CCR3 receptors. The remaining chemokines are promiscuous and activate at least one other chemokine receptor. CCR3 chemokine binding to additional receptors and the expression of these receptors on leukocytes is shown in Table 1.6. In addition, CCR3 chemokines have varying affinities for CCR3 receptors. Among the ligands that have been tested in binding assays, CCL11 has at least 10-fold higher affinity for CCR3

Table 1.5 Human CCR3 chemokine affinity and expression in asthma

Official name	Common name	Affinity for CCR3	Cells that express chemokine in asthma	Expression in allergic asthma
CCL5	RANTES	3.1 nM (K_D)	epithelium, endothelium, T cells, macrophages, mast cells, eosinophils	Allergic asthma: mRNA + protein ↑ Antigen challenge: protein ↑ 24 h (BAL)
CCL7	MCP-3	2.7 nM (K_D)	epithelium, endothelium, macrophages, T cells, mast cells, eosinophils, neutrophils	Allergic asthma: mRNA ↑
CCL8	MCP-2		epithelium, smooth muscle	
CCL11	Eotaxin	0.1 nM - 0.5 nM (K_D)	epithelium, endothelium, macrophages, airway smooth muscle, T cells, eosinophils, neutrophils, mast cells	Allergic asthma: mRNA + protein ↑ Antigen challenge: mRNA ↑ 2 h protein ↑ 4 h (tissue)
CCL13	MCP-4	5.8 nM (IC_{50})	endothelium, epithelium, macrophages, mast cells, T cells	Allergic asthma: mRNA ↑
CCL15	MIP-5	2.5 nM (IC_{50})		
CCL24	Eotaxin-2	3 nM (IC_{50})	epithelium, endothelium, mast cells, macrophages, T cells	Allergic asthma: mRNA ↑ Antigen challenge: protein ↑ 48 h (tissue)
CCL26	Eotaxin-3	10 nM (IC_{50})	epithelium, smooth muscle, endothelium	Antigen challenge: mRNA ↑ 24 h protein ↑ 48 h (tissue)
CCL28	MEC		Eosinophils, lung	Asthma: mRNA present

Affinities are based on radioligand binding assays with human CCR3 receptors and are reported as either K_D or IC_{50} values. Data compiled from (Berkman *et al.*, 2001; Brown *et al.*, 1998; Coulin *et al.*, 1997; Daugherty *et al.*, 1996; Fukakusa *et al.*, 2005; Ghaffar *et al.*, 1999; Iino *et al.*, 2002; Lacy *et al.*, 1999; Patel *et al.*, 1997; Ponath *et al.*, 1996; Powell *et al.*, 1996; Ravensberg *et al.*, 2005; Shinkai *et al.*, 1999; Sur *et al.*, 1996; Venge *et al.*, 1996; Wang *et al.*, 2000; White *et al.*, 1997b; Ying *et al.*, 1999). Abbreviations: RANTES, regulated on activation, normal T-cell expressed and secreted; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MEC, mucosae-associated epithelial chemokine; BAL, bronchoalveolar lavage.

Table 1.6 CCR3 ligand binding to CCR3 and other chemokine receptors

CCR3 Ligands	CCL5 CCL7 CCL8 **CCL11** CCL13 CCL15 **CCL24** **CCL26** CCL28	CCL5 CCL7 CCL15	CCL7 CCL13	CCL5 CCL8	CCL28
Chemokine Receptor	**CCR3**	**CCR1**	**CCR2**	**CCR5**	**CCR10**
Leukoctyes Expressing Receptors	**eosinophils** Th2 cells mast cells basophils	monocytes eosinophils basophils	Th1 cells basophils	Th1 cells	lymphocytes

CCR3 ligands in green only activate CCR3 receptors, while chemokines in black are promiscuous and activate additional CC chemokine receptors. Data compiled from (Daugherty *et al.*, 1996; Ponath *et al.*, 1996; Ubogu, 2011; Willems *et al.*, 2010).

receptors than other CCR3 chemokines (Table 1.5). Furthermore, some chemokines are full agonists (CCL5, CCL11) while others are only partial agonists (CCL7) at CCR3 based on intracellular calcium responses (Daugherty *et al.*, 1996). Thus, while there are nine known ligands that induce chemotaxis via CCR3 receptors they do not have equal efficacy and potency at CCR3 receptors and they may not affect eosinophil recruitment equally *in vivo*.

Eight of the nine CCR3 chemokines have been detected in the lungs of asthma patients and many are known to increase following antigen challenge (Table 1.5). Of these chemokines, CCL11 and CCL24 have been shown to positively correlate with total tissue eosinophil number and airway hyperreactivity in asthma patients (Powell *et al.*, 1996; Ying *et al.*, 1999). In both antigen-challenged humans and guinea pigs, lung CCL11 levels peak at times that correspond to when tissue eosinophils peak (Brown *et al.*, 1998; Humbles *et al.*, 1997). In mice, CCL11 and CCL24 are both involved in eosinophil recruitment to lungs following antigen challenge, since only mice that are deficient for both genes have significantly reduced tissue eosinophils (Pope *et al.*, 2005). However, while these genes are responsible for most of the eosinophil recruitment into lung tissue other CCR3 chemokines must also be involved, since even fewer eosinophils are recruited into lungs of CCR3 receptor-deficient mice (Pope *et al.*, 2005). In addition, in humans with allergic asthma chemotactic activity for eosinophils in bronchoalveolar lavage fluid is partially blocked by antibodies against CCL5, CCL11, and CCL13 (Lamkhioued *et al.*, 1997; Venge *et al.*, 1996), suggesting several chemokines contribute to eosinophilic inflammation. Therefore, any of the CCR3 chemokines may be necessary for specific localization of eosinophils to airway nerves.

Neurons outside of the lung are known to produce chemokines in response to nerve injury (Bhangoo *et al.*, 2007; Schreiber *et al.*, 2001; Taskinen *et al.*, 2000; Wainwright *et al.*, 2009a; Wainwright *et al.*, 2009b). Within the lung, immunostaining of lung sections from humans (nonasthmatic), antigen-challenged guinea pigs, and chronically challenged rhesus monkeys demonstrate that airway nerves stain for CCL11 and CCL26 protein (Chou *et al.*, 2005; Fryer *et al.*, 2006). However, it is not known whether the nerves produced these chemokines, since chemokines bind readily to glycosaminoglycans in tissues (Rot *et al.*, 2004), or whether the nerves are parasympathetic, sensory, or sympathetic.

4. Transcriptional regulation of chemokines

CCR3 chemokines are regulated by several cytokines including the Th2 cytokines IL-4 and IL-13 and tumor necrosis factor-α (TNFα) (Ghaffar *et al.*, 1999; Heiman *et al.*, 2005; Hoeck *et al.*, 2001; Hogan *et al.*, 2008; O'Gorman *et al.*, 2005). TNFα may be particularly relevant for eosinophil recruitment to airway nerves, since inhibition of TNFα signaling prevented eosinophils from associating with airway nerves following antigen challenge in guinea pigs (Nie *et al.*, 2009).

Tumor necrosis factor alpha (TNFα)

TNFα is an inflammatory cytokine that is synthesized as a transmembrane protein that assembles into homotrimers in the plasma membrane. Membrane bound TNFα can be cleaved by TNFα converting enzyme to release soluble TNFα. Both soluble and membrane bound TNFα are active. They activate TNF receptor 1 (TNF-R1), which is expressed by most cell types, and TNF receptor 2 (TNF-R2), which is primarily expressed by immune cells (Matera *et al.*, 2010). While TNF-R1 and TNF-R2 are both

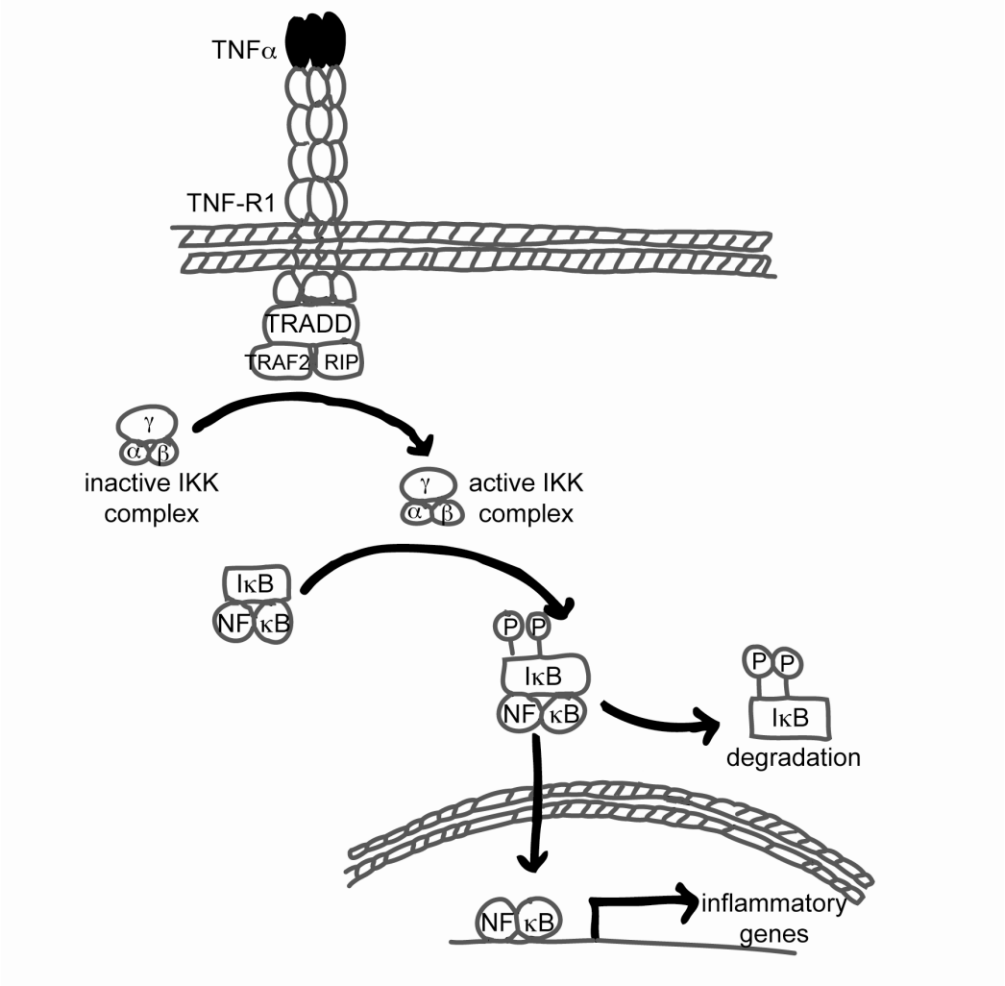

Figure 1.10 Activation of TNF-R1 by TNFα leads to nuclear localization of NF-κB and transcription of inflammatory genes. Upon TNFα binding to TNF-R1, TRADD (TNF-R1-associated death domain protein) binds to the cytoplasmic tail of TNF-R1. TRADD recruits additional proteins including TRAF2 (TNF receptor associated factor 2) and RIP (receptor interacting protein). TRAF2 recruits the IKK kinase, and it is activated by RIP kinase. The activated IKK complex phosphorylates IκB, an inhibitory protein that normally prevents NF-κB from translocating to the nucleus, and leads to its degradation. The nuclear translocation signal on NF-κB is then exposed allowing its translocation to the nucleus where it induces transcription of inflammatory genes.

activated by TNFα, they have different cytoplasmic domains and recruit different adaptor proteins. Activation of TNF-R1 is associated with cell survival and regulation of proinflammatory genes, including CCR3 chemokines, via activation of the transcription factors nuclear factor-κB (NF-κB) (Figure 1.10) and activator protein 1 (AP-1) (Hehlgans *et al.*, 2005; Keystone *et al.*, 2010; Matera *et al.*, 2010). TNF-R2 preferentially interacts with membrane bound TNFα to regulate proliferation, gene activation, and apoptosis of immune cells (Keystone *et al.*, 2010; Matera *et al.*, 2010).

TNFα mRNA and protein are increased in the airways of some asthmatics (Cembrzynska-Nowak *et al.*, 1993; Howarth *et al.*, 2005). In the lungs, TNFα is predominately expressed by mast cells and alveolar macrophages. The number of mast cells expressing TNFα is increased in bronchial biopsies from patients with severe asthma (Howarth *et al.*, 2005), and alveolar macrophages from asthma patients with high levels of Th2 cytokines express higher levels of TNFα than asthma patients without evidence for Th2 inflammation or healthy controls (Woodruff *et al.*, 2009). Additional sources of TNFα include inflammatory cells (macrophages, monocytes, dendritic cells, B cells, CD4+ cells, neutrophils, and eosinophils) and structural cells (fibroblasts, epithelial cells, and smooth muscle cells) (Heffler *et al.*, 2007).

TNFα is increased in the lung following antigen challenge in asthmatic humans, sensitized guinea pigs, and sensitized mice (Gosset *et al.*, 1991; Kelly *et al.*, 1992; Lukacs *et al.*, 1995; Watson *et al.*, 1993). TNFα levels increase soon after antigen challenge, within 60 min, and thus are released in a time frame that is relevant for potential transcriptional regulation of CCR3 chemokines, prior to eosinophil recruitment into the lungs (Kelly *et al.*, 1992; Lukacs *et al.*, 1995).

Furthermore inhalation of recombinant TNFα in mild asthmatics or instillation of TNFα into the lungs of guinea pigs causes recruitment of eosinophils into the airway lumen within 24 h (Thomas *et al.*, 2002; White *et al.*, 1997). Although eosinophils express both TNF receptors 1 and 2, TNFα does not directly activate eosinophils to induce chemotaxis (White *et al.*, 1997a; Zeck-Kapp *et al.*, 1994). Therefore TNFα-induced recruitment of eosinophils is likely mediated via secondary tissue release of chemokines.

In addition to preventing eosinophils from associating with airway nerves, pretreatment with the TNFα inhibitor, etanercept, also prevents airway hyperresponsiveness in antigen-challenged guinea pigs (Nie *et al.*, 2009). Eosinophil recruitment into the lungs following antigen challenge is also prevented by either inhibiting TNFα or genetic deletion of both TNFα receptors (see below) in mice (Cho *et al.*, 2011; Lukacs *et al.*, 1995). In humans with severe refractory asthma, inhibition of TNFα with etanercept reduces airway hyperresponsiveness and improves quality of life (Berry *et al.*, 2006; Howarth *et al.*, 2005). However, there was no significant effect of etanercept on bronchoalveolar lavage eosinophils or airway hyperresponsiveness in patients with mild asthma following antigen challenge. These differences may be explained by TNFα expression in the populations tested, since the studies with severe asthma patients screened patients for high levels of TNFα in advance and found that TNFα levels were only increased in severe but not mild asthma relative to healthy controls (Berry *et al.*, 2006; Howarth *et al.*, 2005). Thus, it may not be surprising that etanercept treatment was ineffective in mild asthma.

TNFα is known to induce production of CCR3 chemokines in nonneuronal cells including epithelial cells, smooth muscle, and fibroblasts (Ghaffar *et al.*, 1999; Heiman *et al.*, 2005; Hoeck *et al.*, 2001; O'Gorman *et al.*, 2005). Since parasympathetic neurons from guinea pigs express TNF-R1 but not TNF-R2 *in vitro* (Nie *et al.*, 2009), it is possible that TNFα induces expression of CCR3 chemokines in parasympathetic neurons via TNF-R1 to recruit eosinophils. However, it is not known whether TNFα induces expression of CCR3 chemokines in neurons or whether neuronally produced chemokines are responsible for eosinophil association with airway nerves following antigen challenge *in vivo*.

K) Central Hypothesis

In humans with asthma and in animal models of asthma, airway hyperreactivity is mediated by the vagus nerves, which contain both sensory and parasympathetic fibers. Activation of sensory nerves by a wide variety of physical and chemical substances initiates reflex responses that result in increased acetylcholine release from parasympathetic nerves and bronchoconstriction. This reflex response is more sensitive in asthmatic individuals. Muscarinic receptor antagonists that block the action of acetylcholine released from parasympathetic nerves would be expected to be beneficial in asthma, and muscarinic receptor antagonists are recommended for treatment of bronchoconstriction during an acute asthma exacerbation. However, nonselective muscarinic receptor antagonists have had limited effectiveness in asthma, and they are not currently recommended for long-term use in preventing exacerbations.

The limited effectiveness of nonselective muscarinic antagonists may be due in part to blockade of all muscarinic receptors in the lung. All five muscarinic receptor subtypes

are expressed in the lung, and blockade of some of these receptors may counteract the benefits of blocking smooth muscle M_3 receptors involved in bronchoconstriction. Blockade of muscarinic receptors on inflammatory cells may be particularly important, since nonselective blockade of all muscarinic receptor subtypes at the time of antigen challenge increased eosinophil degranulation and potentiated airway hyperreactivity 24 h after challenge in previous studies. These data suggest acetylcholine released by airway nerves may limit inflammation and airway hyperreactivity.

In addition to regulating airway inflammation to the lung via release of acetylcholine, parasympathetic nerves may also recruit eosinophils via chemoattractant proteins, since airway denervation in humans with asthma abolished sputum eosinophils. Nerve-associated eosinophils are increased in antigen-challenged guinea pigs and in humans who died of fatal asthma, while treatments that reduce eosinophils reduce airway hyperreactivity in animals and exacerbations in asthmatics. Parasympathetic nerves could recruit eosinophils via neuronal production of CCR3 chemokines, since blockade of CCR3 chemokine receptors on eosinophils significantly reduces nerve-associated eosinophils, but not all eosinophils in lung tissue.

The central hypothesis of this thesis is that *in allergic eosinophilic asthma parasympathetic nerves in the lungs regulate inflammation leading to airway hyperreactivity, both through release of the neurotransmitter acetylcholine and through release of eosinophil chemoattractant proteins.* These studies will determine whether selective blockade of M_3 muscarinic receptors at the time of antigen challenge improves subsequent airway hyperreactivity and whether parasympathetic nerves produce CCR3 chemokines in response to TNFα *in vitro* or antigen challenge *in vivo* using a guinea pig model of allergic asthma. These studies also quantify the association of eosinophils with

parasympathetic nerves following antigen challenge.

CHAPTER 2.

METHODS

Methods

Suppliers for all reagents and supplies used in this chapter are listed at the end of the chapter in Table 2.6.

A) Choice of guinea pig as an animal model of asthma

The experiments in this thesis were designed to study the role of M_3 muscarinic receptors in airway hyperresponsiveness and neuronal production of eosinophil chemokines. For preclinical studies of asthma, an *in vivo* model is required to study hyperresponsiveness, since airway hyperresponsiveness only occurs in the context of the entire lung. Several species have been used to model pathogenesis of asthma including guinea pig, mouse, rat, cat, dog, rabbit, sheep, pig, horse, and primate (Canning, 2003; Leclere *et al.*, 2011). I chose to use guinea pigs in my studies because they are small and most importantly their anatomy, physiology, and smooth muscle pharmacology resembles that of humans.

The histological anatomy of guinea pig and human lung is similar, both have pseudo-stratified tracheal epithelium (Dalen, 1983), subepithelial capillary plexus (Mazzone *et al.*, 2010), significant airway smooth muscle (Wenzel *et al.*, 2006), and submucosal glands throughout their trachea (Choi *et al.*, 2000; Widdicombe *et al.*, 2001). Guinea pig smooth muscle pharmacology is also similar to that of humans. Histamine, methacholine, and leukotrienes LTC_4, LTD_4, and LTE_4 all cause smooth muscle contraction in guinea pig with a similar potency and maximum contractile response as for human (Muccitelli *et al.*, 1987). Conversely, norepinephrine and other beta agonists induce smooth muscle relaxation in both guinea pig and human (Tanaka *et al.*, 2005).

Physiologically, smooth muscle tone is controlled by both contractile and relaxant parasympathetic innervation of airway smooth muscle in both humans and guinea pig. Finally, guinea pigs respond to inhaled antigens in a similar manner to humans with both immediate and late bronchoconstriction responses that are mediated by histamine and leukotrienes (Hutson *et al.*, 1988; Muccitelli *et al.*, 1987; Smith *et al.*, 2007), airway hyperresponsiveness, eosinophil recruitment to and degranulation in airway tissues (Costello *et al.*, 1998; Costello *et al.*, 1997; Erjefalt *et al.*, 1997a; Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 1997; Smith *et al.*, 2007), and epithelial damage (Erjefalt *et al.*, 1997b). With chronic allergen exposure airway remodeling including smooth muscle thickening, smooth muscle hypercontractility, and mucous gland hypertrophy also occurs (Bos *et al.*, 2007; Gosens *et al.*, 2005).

B) *In Vivo* Experiments

1. Guinea Pigs

Specific pathogen-free female Hartley guinea pigs (Elm Hill Labs; Chelmsford, MA) were handled in accordance with National Institutes of Health guidelines, and the Oregon Health & Science University Institutional Animal Care and Use Committee approved all protocols. All guinea pigs were housed in high-efficiency particulate-filtered air with *ad libitum* access to standard laboratory food and water.

2. Sensitization to ovalbumin

Guinea pigs (150-200 g) were sensitized to Grade II ovalbumin (20 $mg \cdot kg^{-1}$, i.p.) on days 1, 3, and 6 (Figure 2.1). Pharmacological treatments and antigen challenge (Figure 2.1) were then given starting 21 days after the last injection. Nonsensitized control animals were ordered at the same time and housed alongside sensitized animals.

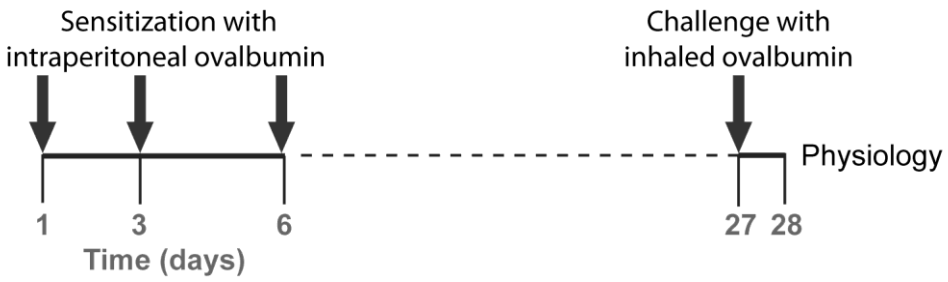

Figure 2.1 Timeline for sensitization and challenge with ovalbumin. Guinea pigs are sensitized with 20 $mg \cdot kg^{-1}$ ovalbumin (i.p.) on days 1, 3, and 6. They are allowed to develop antibodies against ovalbumin for three weeks and are challenged with inhaled ovalbumin on day 27. Airway hyperreactivity develops within 24 h of challenge and airway physiology is measured on day 28.

3. Antigen challenge with inhaled ovalbumin

Although the Fryer and Jacoby laboratories have used antigen challenge with inhaled ovalbumin for decades with low mortality, more recently a high percentage of guinea pigs have needed resuscitation or have died within one hour of antigen challenge. Therefore, I optimized antigen challenge conditions to reduce death following antigen challenge. The protocol in the lab when I began was to challenge guinea pigs in a constant flow exposure chamber (Figure 2.2) with 2.5% ovalbumin containing antifoam for 5 min or until signs of respiratory distress appeared, and to then administer a beta agonist, isoproteronol (0.5 $mg \cdot kg^{-1}$, i.p.), to ease respiratory distress from acute bronchoconstriction. Respiratory distress was easily identified as deep diaphragmatic breaths with exaggerated abdominal movements (Green, 1982) and gasping for air. This method resulted in 30%-70% mortality depending upon whether guinea pigs were treated with anticholinergics (Verbout *et al.*, 2009).

I tested the following parameters to determine which reduced death: 1) reducing the ovalbumin concentration (from 2.5% to 0.5%), 2) using higher purity ovalbumin (changed from Grade II to Grade V), 3) giving antihistamines prior to challenge (0.5 $mg \cdot kg^{-1}$ pyrilamine, i.p.), and 4) eliminating beta agonist (isoproteronol) immediately after challenge. Eliminating isoproteronol was the only factor that eliminated death. Of the animals treated with isoproteronol, 10/15 (67%) needed resuscitation or died. Of the animals that did not receive isoproteronol, 0/12 animals (0%) needed resuscitation or died. Since beta-adrenergic receptors are also found in the heart where they increase heart rate and contractility, isoproteronol may have placed stress on the heart to cause death. However, this hypothesis was not tested since guinea pig heart rate was not monitored.

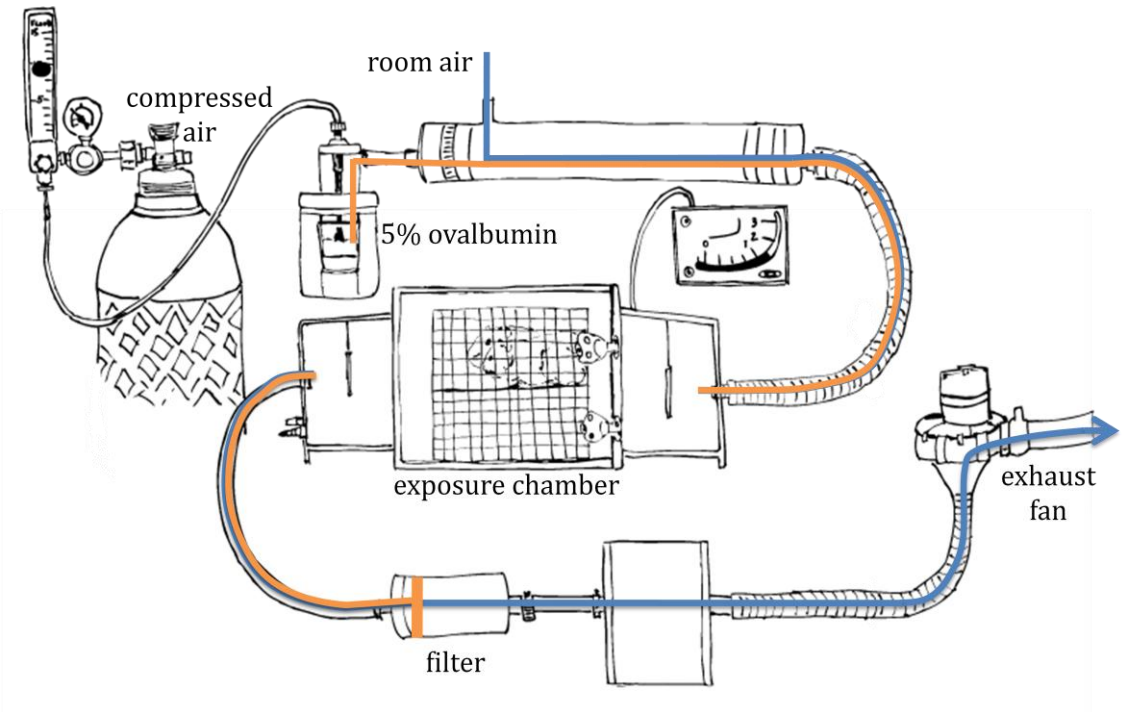

Figure 2.2 Antigen Exposure Chamber. Guinea pigs are challenged with aerosolized ovalbumin in an antigen exposure chamber. Sensitized guinea pigs are placed in a wire cage where they are exposed to aerosolized ovalbumin for 10 min or until signs of respiratory distress appear. In the optimized protocol, ovalbumin is aerosolized by bubbling compressed air into a 5% solution of ovalbumin. Aerosolized ovalbumin (orange) mixes with room air (blue) and is pulled through the exposure chamber, where the guinea pigs breathe it in, by an exhaust fan. Ovalbumin is then removed from the air by a high-efficiency particulate air filter, and clean air is released to the room.

Although eliminating isoproteronol prevented death, sensitized and challenged guinea pigs were no longer hyperresponsive relative to sensitized controls. Therefore, I confirmed that sensitized guinea pigs were allergic to ovalbumin by measuring acute bronchoconstriction to intravenous ovalbumin, which is mediated by histamine release from activated mast cells. Intravenous ovalbumin caused bronchoconstriction in 3/3 sensitized guinea pigs, while ovalbumin did not cause bronchoconstriction in a control guinea pig that was not sensitized. I then increased the amount of antigen delivered to the lungs during challenge by increasing the ovalbumin concentration to 5%, the flow of compressed air into the ovalbumin solution from 5 $L \cdot min^{-1}$ to 9 $L \cdot min^{-1}$, and the time of exposure to 10 min. I confirmed that these changes resulted in greater aerosolization of ovalbumin by weighing the ovalbumin solution before and after challenge. Increasing the amount of antigen aerosolized resulted in reproducible airway hyperresponsiveness to antigen challenge.

In the experiments included in this thesis, I exposed guinea pigs to 5% ovalbumin containing 0.2 % antifoam Y-30 emulsion in sterile PBS for 10 min, or until signs of respiratory distress appeared, in which case antigen challenge was immediately stopped. Grade II ovalbumin, which has been used historically in the laboratory, was prepared on the day of challenge by dissolving 4 g of crushed ovalbumin powder into 80 mL of sterile PBS in a 2 L beaker (5 inch diameter; autoclaved) for 2 h at room temperature. Clumps of impurities were removed by filtering through sterile gauze. Antifoam Y-30 emulsion (100 μL) was mixed into 60 mL of filtered ovalbumin solution and this was placed into the plastic cup used in the antigen exposure chamber (Figure 2.2).

4. Pretreatment with insufflated tiotropium or lactose powder

To mimic delivery of tiotropium to humans, tiotropium bromide formulated as Spiriva was administered as a powder to guinea pigs using a DP-4 dry powder insufflator (Figure 2.3), which delivers small puffs of fine powder. My source of Spiriva was HandiHaler capsules, which each contain 22.5 μg of tiotropium bromide and 5.5 mg of lactose filler. Tiotropium bromide was insufflated (i.t.) into the lungs at doses of 0.2 $\mu g \cdot kg^{-1}$ (0.05 mg Spiriva$\cdot kg^{-1}$) or 1 $\mu g \cdot kg^{-1}$ (0.25 mg Spiriva$\cdot kg^{-1}$). Since 2-5 mg of powder must be loaded into the dry powder insufflator, Spiriva was mixed with additional D-(+)-lactose monohydrate that had been sieved through a 70 μm cell strainer. Vehicle control animals received an equivalent weight$\cdot kg^{-1}$ of lactose powder.

To administer tiotropium or lactose powder, sensitized guinea pigs were anesthetized with ketamine (30 $mg \cdot kg^{-1}$, i.m.) and xylazine (5 $mg \cdot kg^{-1}$, i.m.) and supported vertically (Figure 2.3 B). A second person gently pushed up under the guinea pig's lower jaw to straighten the trachea while I used a small animal laryngoscope (Figure 2.3 C) to visualize the vocal cords and insert the insufflator delivery tube (Figure 2.3 A) into the trachea. Approximately a finger's width (1.5 cm) of delivery tube was left outside of the guinea pig's mouth to avoid inserting past the trachea. No resistance was felt as the delivery tube was inserted into the trachea, this is important because resistance indicates the delivery tube has slipped into the esophagus. I had previously determined the neck position, depth to insert delivery tube, and resistance by practicing insertion of the delivery tube into the trachea of guinea pigs that had been euthanized (for other experiments) and then dissecting open the tissue to determine the delivery tube position. To insufflate lactose or tiotropium powder into the lungs, the insufflator syringe was

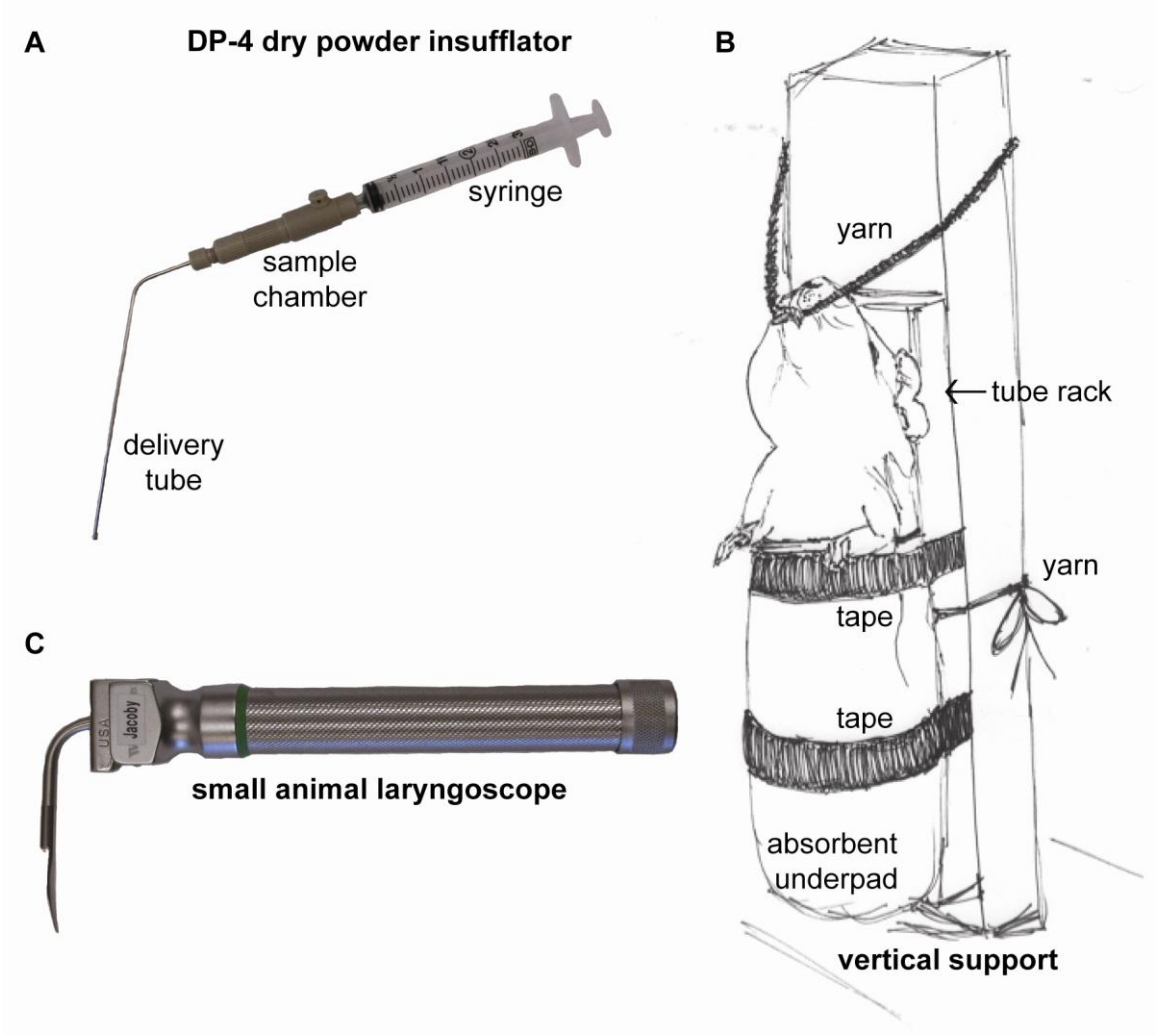

Figure 2.3 Insufflation of tiotropium and lactose into guinea pig lungs. Drugs are puffed into the lungs of anesthetized guinea pigs with a DP-4 dry powder insufflator (A). Guinea pigs are anesthetized and supported vertically (B). Guinea pigs are wrapped in an absorbent underpad and taped to a plastic tube rack at two sites (one site under the front arms, to mimic the support given when guinea pigs are held for i.p. injections, and a second site lower down). The tube rack is tied to a vertical support and yarn from the vertical support is looped under the guinea pig's front teeth to support the head. A small animal laryngoscope (C) is used to visualize the vocal cords and insert the delivery tube of the insufflator into the trachea.

plunged 2-3 times. I confirmed drug delivery by weighing the insufflator sample chamber (Figure 2.3 A) before and after insufflation. When necessary, the delivery tube was inserted a second time to insufflate any remaining drug. Separate delivery tubes were used for tiotropium and lactose vehicle, and the sample chamber was cleaned with compressed air after each use.

5. Pretreatment with i.p. atropine

Sensitized guinea pigs were treated with the nonselective muscarinic receptor antagonist, atropine in sterile saline (1 $mg \cdot kg^{-1}$, i.p.), 1 h before challenge and again 6 h after challenge as previously described (Verbout *et al.*, 2007).

6. Anesthesia and surgical preparation

Guinea pigs were anesthetized with urethane (1.7 $g \cdot kg^{-1}$ i.p.) and temperature was maintained at 37°C with a heating blanket. Urethane was chosen as the anesthetic because it is nondepolarizing and has minimal effects on peripheral nerves innervating the lung and heart (Green, 1982). Depth of anesthesia was verified by absence of a reflexive blink response when the corner of the eye was touched and absence of a reflexive kick response when the hind paw was pinched.

Both jugular veins were cannulated for intravenous drug administration (Figure 2.4), and animals were chemically sympathectomized with guanethedine (2 $mg \cdot kg^{-1}$, i.v.), paralyzed with succinylcholine chloride (5 $\mu g \cdot min^{-1}$, i.v.), and mechanically ventilated via a tracheal cannula (see below). Since, the animals were paralyzed, heart rate and blood pressure were monitored via a carotid artery cannula to ensure adequate levels of anesthesia (Figure 2.4). Guinea pigs were vagotomized by crushing both vagus nerves with suture thread, and distal portions (below the crushed area) of both vagi were placed on platinum electrodes and submerged in mineral oil (Figure 2.4).

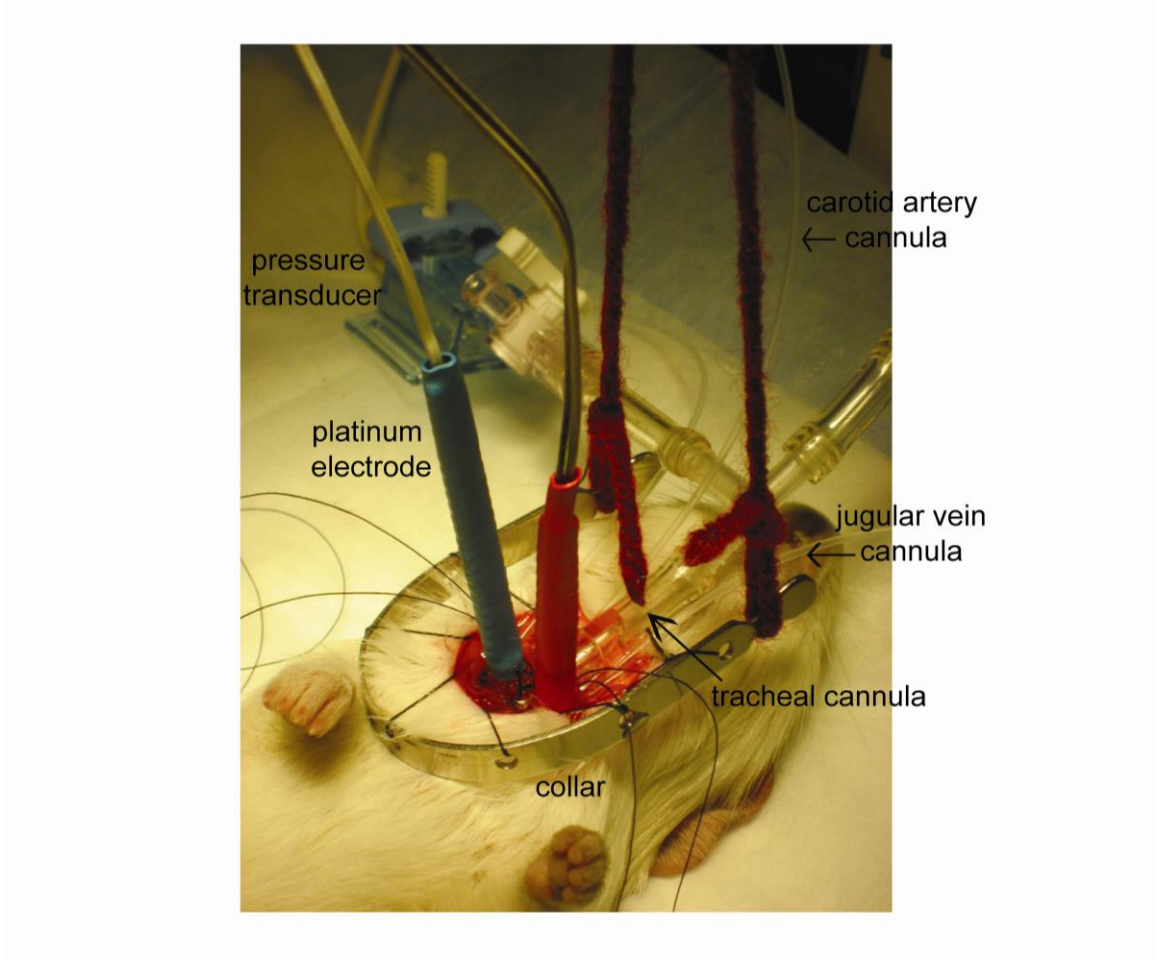

Figure 2.4 Surgical preparation for measurement of *in vivo* physiology in guinea pig. Surgery is carried out in anesthetized guinea pigs to cannulate the right and left jugular veins for intravenous drug access, a carotid artery for measurement of blood pressure and heart rate, and the trachea. The guinea pigs are paralyzed and ventilated mechanically via the tracheal cannula. Pulmonary inflation pressure is measured with a pressure transducer via a sidearm of the tracheal cannula. The right and left vagus nerves are isolated and crushed to sever CNS control. The distal nerve ends are attached to platinum electrodes, submerged in mineral oil, and stimulated electrically. A metal collar is sewn to the guinea pig to create a pocket to hold the mineral oil.

7. Measurement of bronchoconstriction

Pulmonary inflation pressure (Ppi), blood pressure, and heart rate were measured on a Grass polygraph (Model 79E, Grass Instrument Co.). Ppi was measured via a sidearm of the tracheal cannula. Electrical signals from a DTX Plus pressure transducer (Beckton Dickinson) were delivered directly into the preamplifier for one channel on the polygraph. Signal was converted via a driver and recorded as total Ppi in mm H_2O. The total Ppi driver output was also routed through a second preamplifier and driver. This signal was windowed to allow more precise measurements of changes in Ppi above baseline. Using this method, changes in Ppi as small as 2 mm H_2O could be accurately measured.

Animals were ventilated with a positive pressure, constant volume rodent ventilator (Model 683, Harvard Apparatus) at a tidal volume of 2.5 mL and 100 breaths$\cdot$min^{-1}. Since the volume of air with each breath was constant, increases in pressure measured at the level of the tracheal cannula reflected changes in pulmonary airflow resistance. Increases in pulmonary inflation pressure, however, cannot separate changes in airflow resistance due to narrowing of the airways versus decreased lung compliance (changes in elastic recoil of the lung). It is possible to determine these parameters individually by measuring transpulmonary pressure (difference in pressure between the trachea and pleural space), airflow at the trachea, and lung volumes. However, previous experiments using this method in guinea pigs have demonstrated that acute increases in pulmonary inflation pressure correspond to increases in airway resistance and not to changes in lung compliance (Fryer, 1986). Therefore, the complexity of measuring transpulmonary pressures was not required to assess changes in pulmonary airflow resistance due to bronchoconstriction.

8. Measurement of blood pressure and heart rate

Blood pressure in mm Hg was measured via a fluid filled pressure transducer attached to the carotid artery cannula, and heart rate in beats per minute was derived from the blood pressure reading via a tachograph on the Grass polygraph.

9. Measurement of vagally induced bronchoconstriction and bradycardia

Both vagus nerves were simultaneously stimulated electrically (1-25 Hz, 10 V, 0.2 ms pulse duration, for 5 s at 45-60 s intervals) to produce frequency-dependent bronchoconstriction measured as increases in pulmonary inflation pressure in mm H_2O and bradycardia measured as a fall in heart rate in beats per min (Figure 2.5). An example trace is shown in Figure 2.6.

Both nerves were stimulated simultaneously, since stimulating both nerves together causes a synergistic increase in airflow resistance. Prior work has demonstrated that stimulation of individual vagi predominantly causes ipsilateral bronchoconstriction. When airways in only one lung are constricted, ventilation is shifted to the unstimulated, contralateral lung and there is only a moderate increase in resistance. When bilateral bronchoconstriction occurs, the air has nowhere to shunt and airflow resistance can be determined more accurately (Olsen, 1965).

The stimulus parameters were chosen to favor parasympathetic nerve stimulation. Vagal nerves contain both parasympathetic and sensory nerves (see Chapter 1, Introduction), and antidromic stimulation of vagal sensory nerves can also stimulate bronchoconstriction, which is thought to be mediated by release of substance P (Undem *et al.*, 1990). Less current is required to stimulate parasympathetic nerves than is

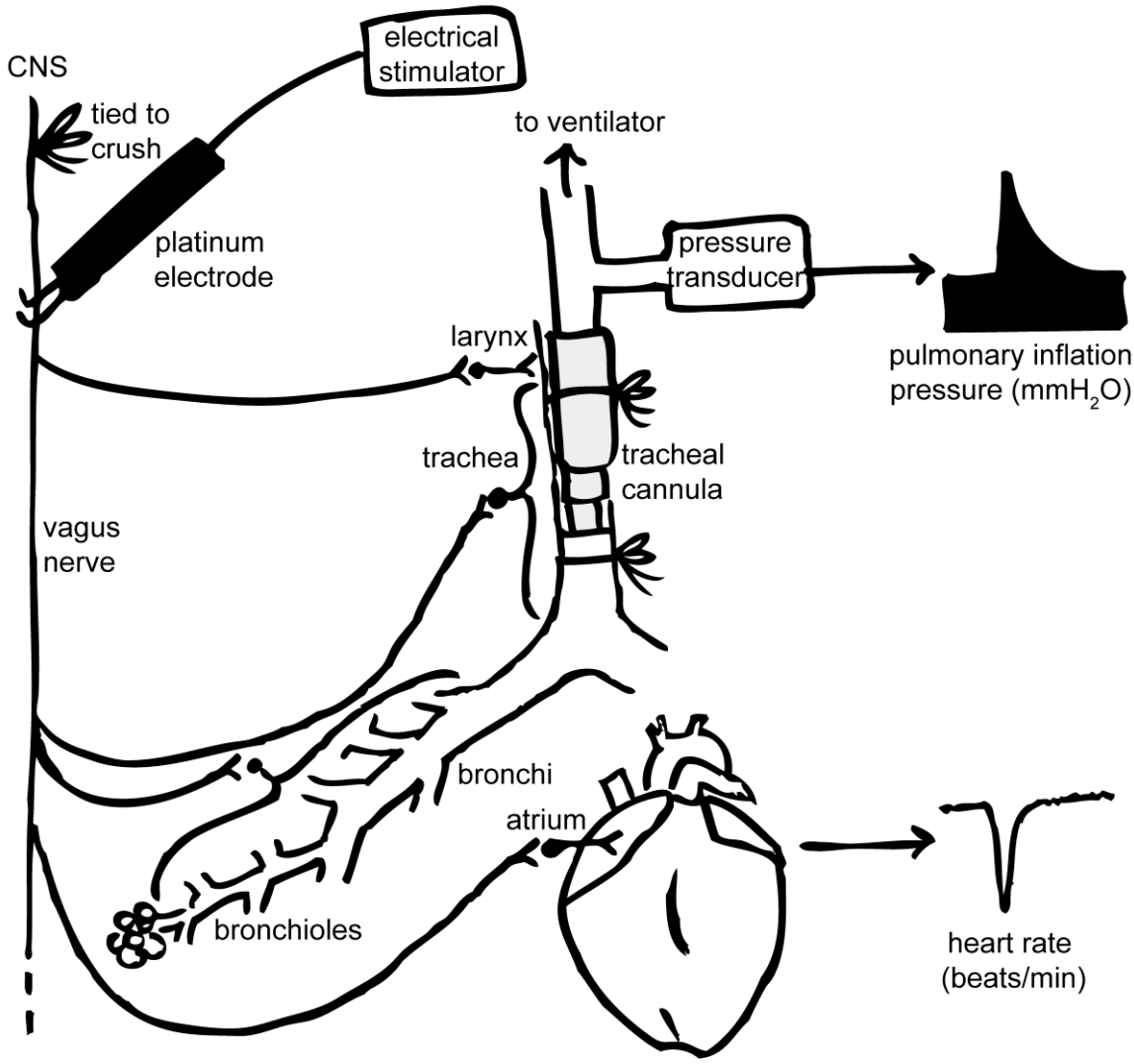

Figure 2.5 Vagus nerve stimulation induces bronchoconstriction and bradycardia. Parasympathetic nerves in the right and left vagus nerves supply postganglionic nerves that innervate the larynx, trachea, bronchi, and bronchioles. Vagal parasympathetic nerves also supply postganglionic nerves in the heart. Electrical stimulation of the vagus nerves induces bronchoconstriction in airways that is measured via a side arm of the tracheal cannula as an increase in pulmonary inflation pressure in mmH_2O. It also induces bradycardia in the heart that is measured in beats per minute and is derived from measurements of blood pressure using a tachograph.

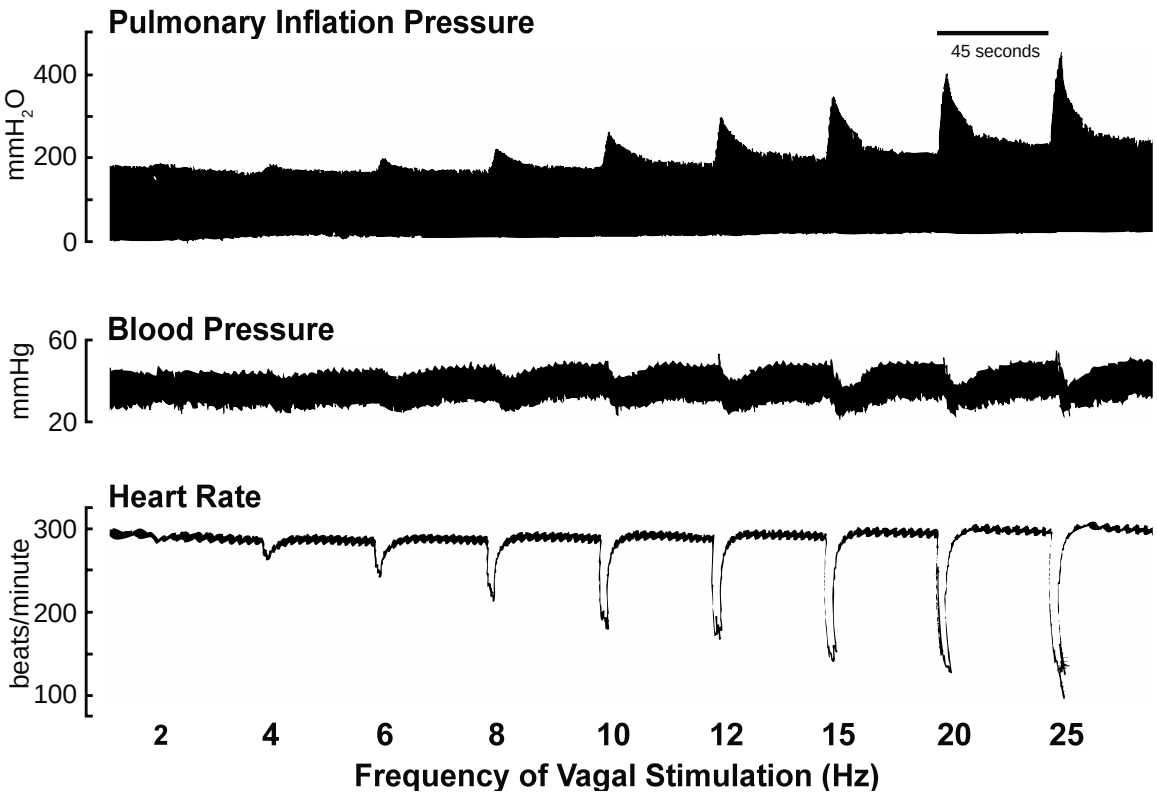

Figure 2.6 An example trace of pulmonary inflation pressure, blood pressure, and heart rate recordings. Following electrical stimulation of the vagus nerves (2-25 Hz) there are frequency-dependent increases in pulmonary inflation pressure, decreases in blood pressure, and decreases in heart rate.

required to stimulate sensory nerves. Therefore, voltage was set to 10 V, which allows 50% of the maximum response for parasympathetic nerves and only 10% of the maximum response for sensory nerves (Undem *et al.*, 1990). Pulse duration was set to 0.2 ms, which allows greater than 80% of the maximum response for parasympathetic nerves and closer to 50% of the maximum response for sensory nerves (Undem *et al.*, 1990). Atropine (1 $mg \cdot kg^{-1}$, i.v.) was given at the end of each experiment to confirm bronchoconstriction induced by vagal nerve stimulation was due to stimulation of cholinergic nerves.

10. Measurement of postjunctional muscarinic receptor function

Following measurement of vagal reactivity, acetylcholine (1-10 $\mu g \cdot kg^{-1}$) was administered to test the function of postjunctional M_2 receptors in cardiac muscle and postjunctional M_3 receptors in airway smooth muscle. M_2 receptor function was measured as a fall in heart rate in beats per min and M_3 receptor was measured as an increase in bronchoconstriction in mm H_2O.

11. Measurement of bronchoconstriction following intravenous administration of lactose and tiotropium in nonsensitized guinea pigs

Vagus nerve-induced bronchoconstriction was elicited by electrically stimulating both vagus nerves at 2 min intervals for 5 s (10 Hz, 10V, 0.2 ms pulse duration) in nonsensitized guinea pigs. Intravenous-acetylcholine (4 $\mu g \cdot kg^{-1}$)-induced bronchoconstriction was elicited in the same animals following every 3rd vagal stimulation. Once three bronchoconstrictions of similar magnitude were achieved, gallamine (3 $mg \cdot kg^{-1}$, i.v.) was administered to block neuronal M_2 receptors. Tiotropium bromide (0.3-10 $\mu g \cdot kg^{-1}$) was given at increasing doses by administering Spiriva dissolved in PBS intravenously. Each increasing dose of tiotropium was given after inhibition of bronchoconstriction reached a plateau (time determined in early

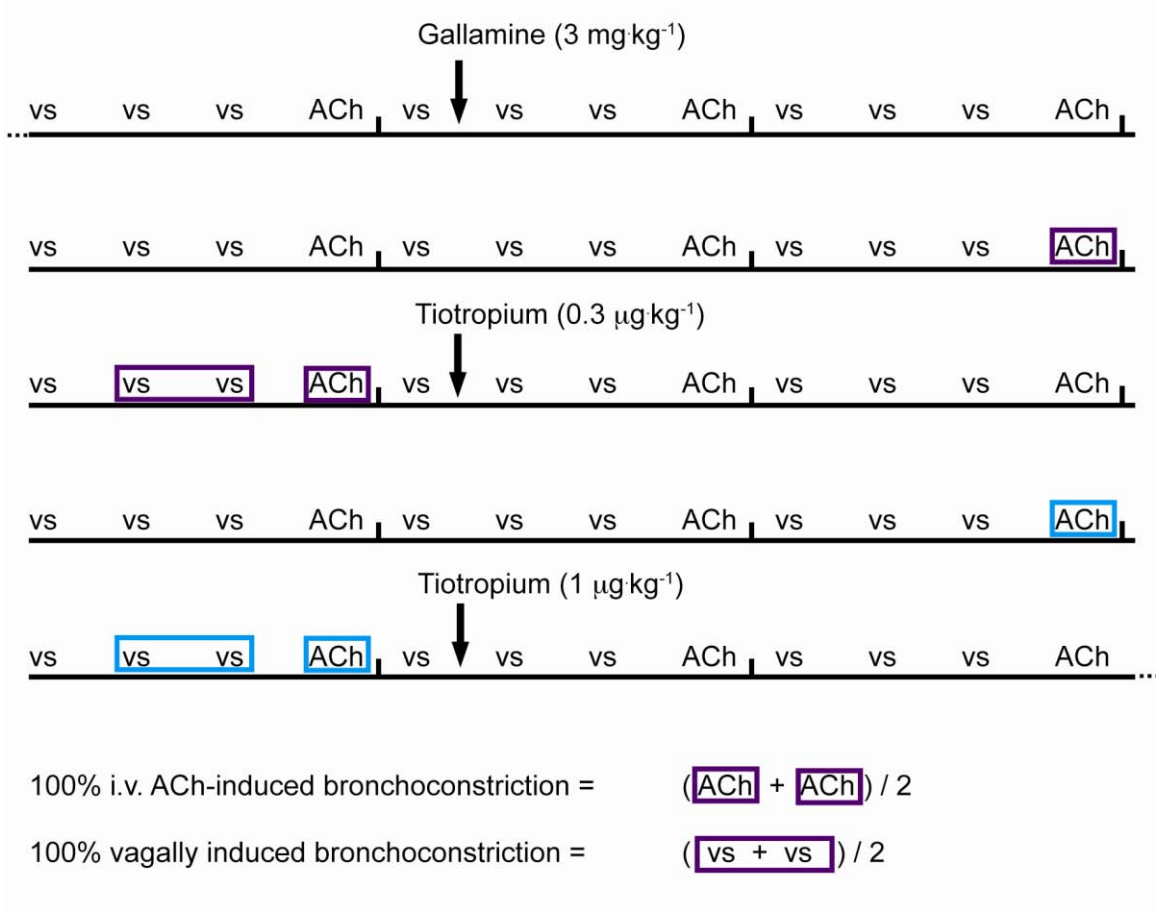

Figure 2.7 Method for measuring inhibition of bronchoconstriction with i.v. tiotropium. Vagus nerve-induced bronchoconstriction was elicited by stimulating both vagus nerves (vs) every 2 min. Intravenous-acetylcholine-induced bronchoconstriction (ACh) was elicited in the same animals following every 3rd vagal stimulation. Gallamine was given to block neuronal M_2 receptors. The last two intravenous-acetylcholine-induced bronchoconstrictions (purple) following gallamine administration were averaged and set to 100%. The last two vagally induced bronchoconstrictions (purple) were averaged and set to 100%. Following each dose of tiotropium or lactose, intravenous-acetylcholine (blue) and vagally induced (blue) bronchoconstrictions were calculated similarly and presented as a percentage of baseline values following gallamine.

experiments and maintained throughout). Time/Vehicle control animals received equivalent concentrations of intravenous lactose. Following each dose, the last two intravenous-acetylcholine-induced bronchoconstrictions were averaged and presented as a percentage of baseline following gallamine (Figure 2.7). The last two bronchoconstrictions following vagal stimulation were similarly averaged and presented as a percentage of baseline following gallamine (Figure 2.7).

12. Bronchoalveolar lavage analysis

At the end of each experiment, inflammatory cells were obtained by lavaging the lungs of anesthetized guinea pigs with five 10 mL aliquots of PBS containing 10 $\mu g \cdot mL^{-1}$ isoproteronol. Cells were washed, resuspended in PBS, and counted using a hemocytometer. Cells were also cytospun onto slides and stained with Hemacolor to obtain differential cell counts (Figure 2.8).

C) Tissue harvest and preparation for histology and PCR

1. Harvest of lung tissue from *in vivo* animals and sectioning in paraffin

Following measurement of *in vivo* physiology, guinea pigs were perfused with PBS and exsanguinated. Lungs were removed, inflated with 10 mL zinc-buffered formalin via the tracheal cannula, and fixed overnight at 4° C. Lungs were then transferred to 70% ethanol and stored at 4° C. Two transverse sections of the proximal region of each of two lobes were cut with a razor blade and delivered to the Histopathology Shared Resource core at OHSU for embedding in paraffin and sectioning (10 μm sections).

2. Harvesting whole lung RNA to use as a positive control for PCR

Whole lung mRNA was isolated as a positive control. Lung tissue was wrapped in aluminum foil and flash frozen in liquid nitrogen. Approximately 200 mg of tissue was

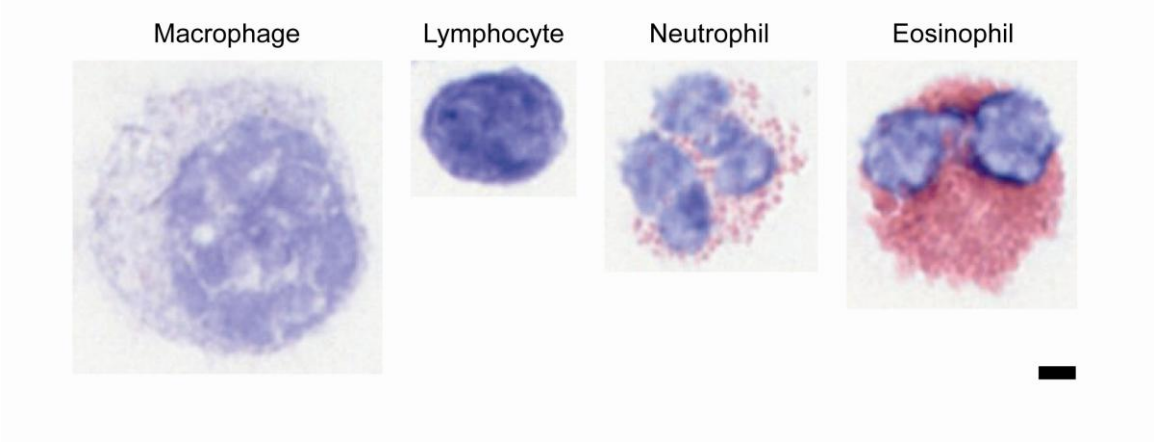

Figure 2.8 Leukocytes in guinea pig bronchoalveolar lavage fluid. Macrophages, lymphocytes, neutrophils, and eosinophils were counted on cytospin slides of bronchoalveolar lavage stained with Hemacolor. Scale bar = 5 μm.

cut off with a razor blade, weighed, and homogenized in RLT lysis buffer (RNeasy kit) with beta-mercaptoethanol.

3. Harvest of lungs and parasympathetic ganglia for cryosectioning and PCR

Guinea pigs were euthanized with an overdose of pentobarbital (150 $mg \cdot kg^{-1}$). After the animal had stopped breathing and no longer responded to pinching the feet or touching the corner of the eye the chest was opened and the lungs and trachea were perfused with saline administered via the left ventricle of the heart. The trachea, with esophagus attached, was immediately isolated from the larynx to the bifurcation with the bronchi, and placed in a sterile cell culture dish of PBS on ice. Lungs were inflated with Zamboni's fixative and removed for cryosectioning (see below).

Postganglionic parasympathetic nerves are the only nerves with their cell bodies in the trachea (see Chapter 1, Introduction). Parasympathetic ganglia were harvested from the trachea using a dissection technique developed by a graduate student, Abby Rynko, in our laboratory. Forceps and scissors were treated with RNAse Away and used to open the trachea through the cartilaginous rings and dissect away the esophagus. This was done carefully, since ganglia are found along the tracheal surface that faces the esophagus. The trachea was pinned (with dissection pins) luminal side down on sylgard 184 in a glass petri dish that had been chilled on ice. The surface of the trachea and sylguard around the trachea were dried with a kim wipe, and the trachea was stained for 15 min with 250 μL of ice cold 0.5% neutral red in sterile PBS (pH 7.4) applied to the surface. Neutral red is a vital stain that stains lysosomes, golgi apparatus, and nissl granules in neurons (Kaila *et al.*, 1998). Ganglia stained a light red color were identified under 20X-30X magnification with a Nikon SMZ 1000 dissecting microscope. Ganglia

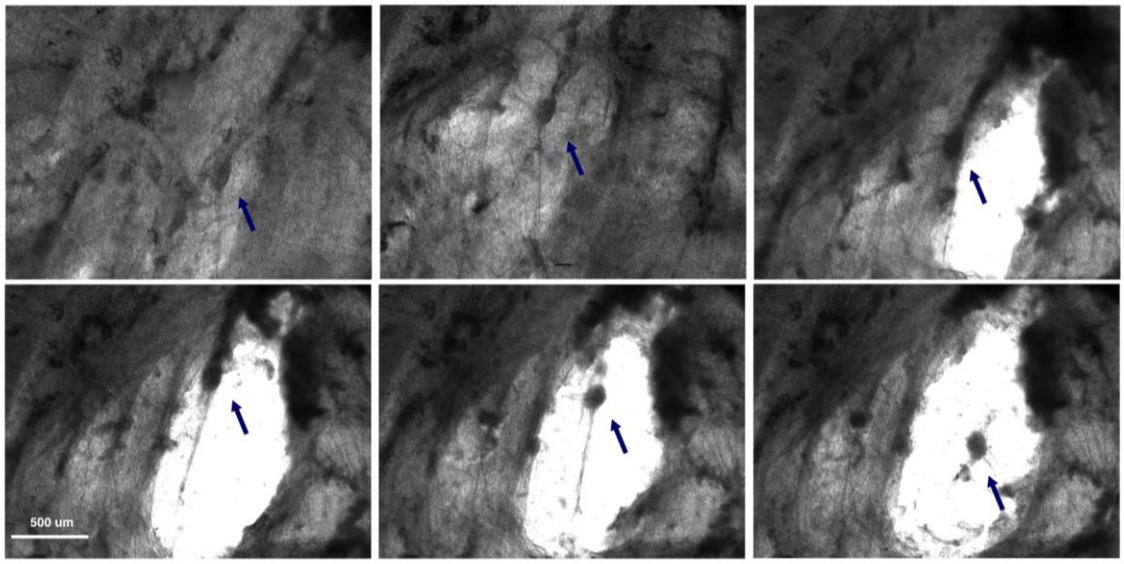

Figure 2.9 Harvest of parasympathetic ganglia from guinea pig trachea.

Parasympathetic nerve ganglia (arrow) were stained with neutral red and indentified using a dissecting microscope. Tissue near the ganglia was cleared so that ganglia and attached nerves could be pulled into an empty space and separated from surrounding tissue. Ganglia were then grasped with forceps and removed. Picture courtesy of Abby Rynko.

were dissected out of tracheal smooth muscle and adventitia using fine forceps (Figure 2.9). Tissue near the ganglia was removed so that ganglia and attached nerves could be pulled into an empty space. Ganglia were then removed to a small cell culture dish, where any remaining unstained tissue was removed in ice cold PBS, before transferring to an RNAse free microcentriuge tube on ice. Ganglia were combined and when all ganglia were harvested, they were flash frozen in liquid nitrogen and stored at -80° C. I was able to identify and harvest up to 11 ganglia from each trachea.

4. Cryosectioning lung tissue

Removed lungs (see previous section) were fixed overnight in Zamboni's Fixative at 4° C. Lungs were rinsed in PBS, and airways in each lung were lavaged three times with 10 mL PBS through a cannula inserted into the primary bronchus to remove fixative. Two transverse sections of the proximal region of the lower left lobe were cut with a razor blade. These sections were cryoprotected by rinsing in 18% sucrose overnight in PBS at 4° C and then switching to 9% sucrose and 50% Optimal Cutting Temperature Compound (OCT) in PBS overnight at 4° C before they were embedded in OCT in a cryomold and frozen at -20° C. Frozen blocks were cut at 13° C on a H/I bright cryostat (Hacker Instruments, Winnsboro, SC, Model 0TF5000) into 10 μm sections and mounted on Colorfrost Plus microscope slides, which are positively charged and adhere tissue through electrostatic interactions. Slides were stored at -80° C until used for histological analysis.

D) Histology experiments

1. Histological evaluation of nerve-associated eosinophils in paraffin sections

Airway nerves were detected with a monoclonal antibody specific for PGP9.5 and eosinophils were stained with chromotrope 2R (modified from (Evans et al., 2001)). Slides were dewaxed in xylene, rehydrated with graded alcohols, and then soaked in diH_2O. Antigen retrieval was carried out by microwaving slides for 12 min on high in antigen unmasking solution. Endogenous peroxidase activity was quenched with 3% H_2O_2 diluted in cold methanol (10 min). Sections were permeabilized with 0.05% tween 20 in PBS (2x3 min), blocked in 10% normal goat serum (30 min room temperature), incubated with mouse anti-PGP9.5 (5 $\mu g \cdot mL^{-1}$, 1 h room temperature), and incubated with biotinylated goat anti-mouse IgG (7.5 $\mu g \cdot mL^{-1}$, 30 min room temperature). Antibody staining was detected with a streptavidin-linked horseradish peroxidase substrate (Vectastain ABC kit) and Vector SG chromagen. Normal mouse IgG or no primary antibody served as controls. Slides were then stained with 1% chromotrope 2R in diH_2O for 1 min, air dried overnight, and mounted with Cytoseal 60.

While blinded to treatment, I quantified eosinophils within airway walls (large bronchioles). Overlapping 400X photographs of each airway were taken and overlaid to make a single image (Figure 2.10) for each airway using a digital camera and NIH Image J Software (version 1.42q (Rasband, 1997-2009)) with Mosaic J plugin (Thevenaz *et al.*, 2007). Total airway area between the epithelial basement membrane and surrounding alveoli was measured using MetaMorph imaging software (version 7.1.2.0, Molecular Devices, Sunnyvale, CA). Total eosinophils and nerve-associated eosinophils (within 8 μm of a PGP9.5 positive nerve, approximately the diameter of an eosinophil) were counted and expressed as eosinophils per mm^2 airway area. Values were averaged

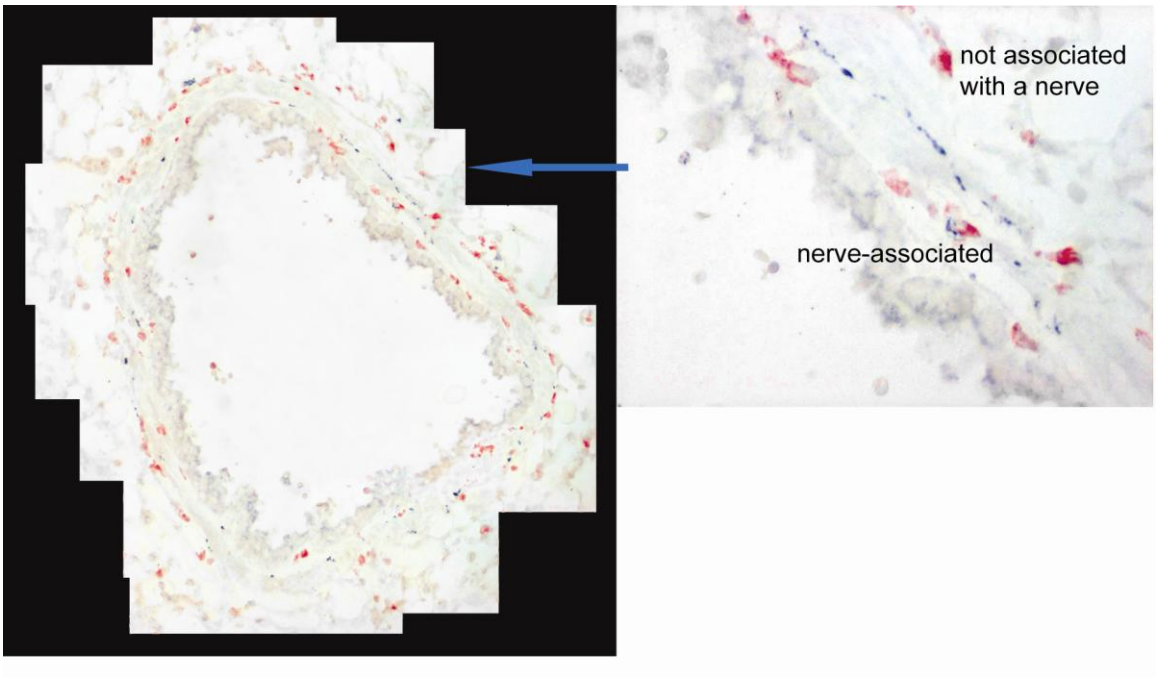

Figure 2.10 Identification of nerve-associated eosinophils in paraffin sections. Airway nerves were detected with an antibody against PGP9.5 (blue) and eosinophils were stained with chromotrope 2R (red). Total and nerve-associated eosinophils (within 8 μm of a nerve, approximate diameter of an eosinophil) were counted in each airway. Eosinophils in airway epithelium were excluded.

across three airways for each animal and these numbers were used to calculate the mean for each group.

2. Histological evaluation of cholinergic and noncholinergic nerve-associated eosinophils in cryosections

Airway nerves were detected with a polyclonal antibody specific for PGP9.5, cholinergic nerves were detected with a polyclonal antibody against choline acetyltransferase (ChAT), and eosinophils were stained with chromotrope 2R. Tissues were thawed and post-fixed in Zamboni's for 5 min at room temperature, rinsed in PBS, and permeabilized with additional fixation in a 1:1 solution of methanol and acetone for 15 min at 4° C. Sections were rinsed, outlined with a hydrophobic pen, and blocked in 10% normal donkey serum and 0.3% Triton X-100 in PBS for 1 h at room temperature in a humid chamber. Sections were incubated with primary antibodies overnight at 4° C in a humid chamber. Goat anti human ChAT (10 μg/mL) and rabbit anti human PGP9.5 (1:1000) were applied together in a solution of 2% normal donkey serum and 0.3% Triton X-100 in PBS. Sections were rinsed and incubated overnight in secondary antibodies at 4° C in a humid chamber in the dark. Donkey anti-goat 488 (1:400) and donkey anti-rabbit 405 (1:100) were applied together in a solution of 2% normal donkey serum, 0.3% Triton X-100 in PBS. Sections were rinsed and stained with fresh 1% chromotrope 2R in diH_2O for 1 min at room temperature in the dark. Each slide was rinsed and mounted with Vectashield mounting media for fluorescence under a coverslip sealed with nail polish. Normal goat IgG or no primary antibody served as controls. All slides were imaged with a Zeis LSM 710 confocal microscope at 200X magnification within 3 h of mounting.

Notes on method development: In developing the above protocol for PGP9.5, ChAT, and Chromotrope 2R staining I found that both primary and secondary staining was

better overnight at 4° C than 1 h at room temperature. Also, chromotrope 2R fluorescence begins to fade within minutes in diH_2O and in PBS, and this is why all imaging was carried out within 3 h of mounting. Further, the anti-rabbit 405 secondary used with PGP9.5 primary is not visible on a regular fluorescence microscope when used with Vectashield mounting media because there is a large amount of fluorescence from the mounting media that is also picked up by the blue filter. Staining is visible with the confocal microscope where it is easy to adjust the specific wavelengths being detected. Vectashield mounting media was necessary because I discovered that Prolong Gold, Fluoromount-G, Slow Fade Gold, and Vector hardest mounting medias are all incompatible with chromotrope 2R fluorescence. Since I needed to use the confocal microscope, the 405 secondary was also required, because the confocal does not have a light source that can excite 350. Finally, inadequate rinsing of the tissue (not lavaging the airways) to remove Zamboni's results in over fixation and reduced PGP9.5 staining and absent or reduced ChAT staining.

Image Analysis

All image analysis was carried out while I was blinded to treatment. Each tiled image was saved as a tiff and then opened with MetaMorph imaging software (Version 7.7.3.0, Molecular Devices, Sunnyvale, CA) and calibrated (1 pixel = 0.415 μm x 0.415 μm; Figure 2.11). The animal number and airway were recorded in an excel spreadsheet for image data and the image was identified as cartilaginous (coded 1) or noncartilaginous (coded 0). The red channel (eosinophils) was turned off, and the display brightness in the blue (nerves) and green channels (ChAT) was adjusted for each airway to see epithelial and smooth muscle borders. The area tool was then used to measure the area of each of three airway compartments (Figure 2.12). Epithelium was measured from the lumen to the basement membrane. The smooth muscle compartment included

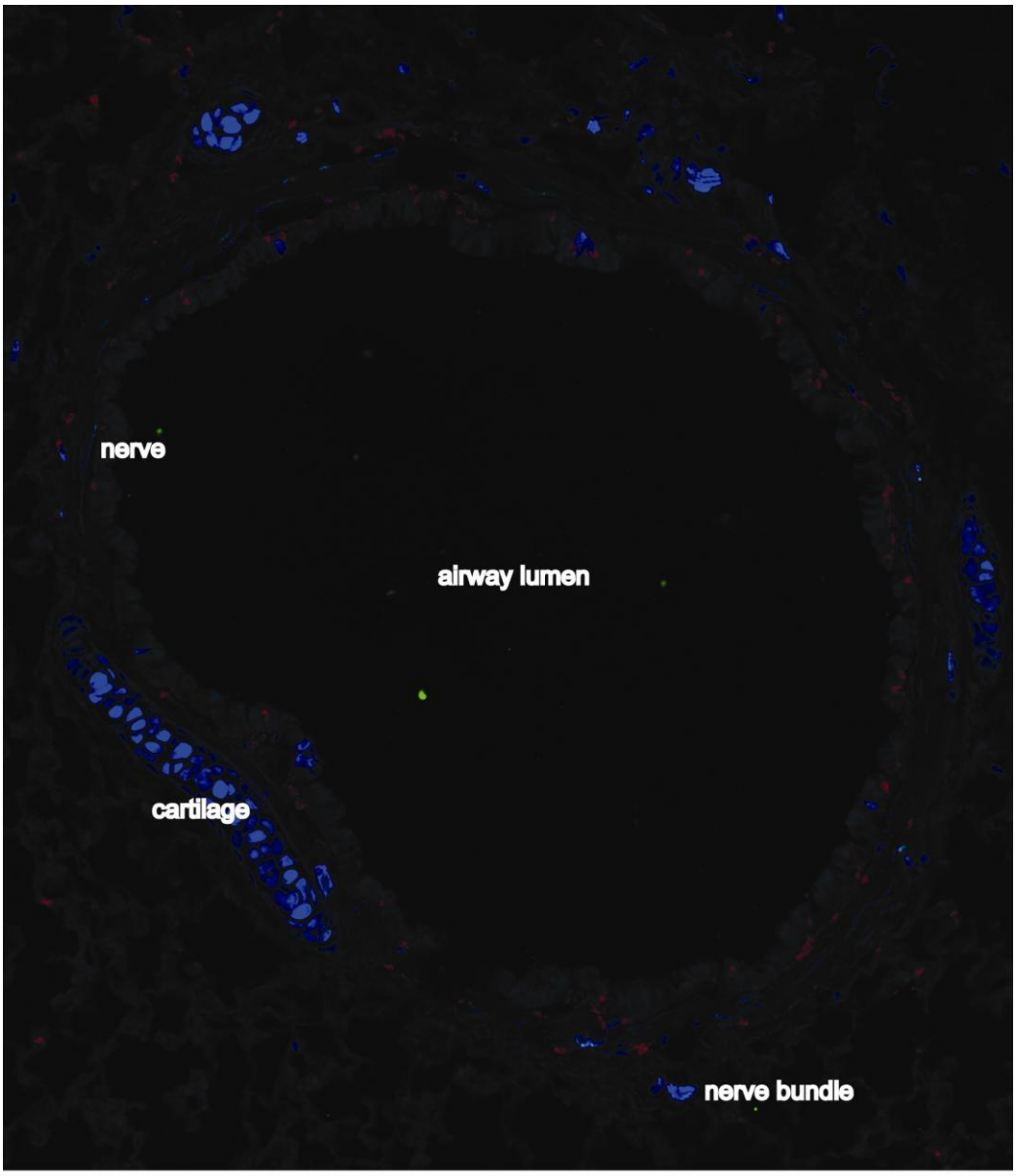

Figure 2.11 Tiled image of unadjusted cartilaginous airway. Unadjusted tiled image of a cartilaginous airway. PGP9.5 antibody staining labels nerves and cartilage in blue. ChAT antibody staining labels cells synthesizing acetylcholine in green. Chromotrope 2R dye stains eosinophils in red.

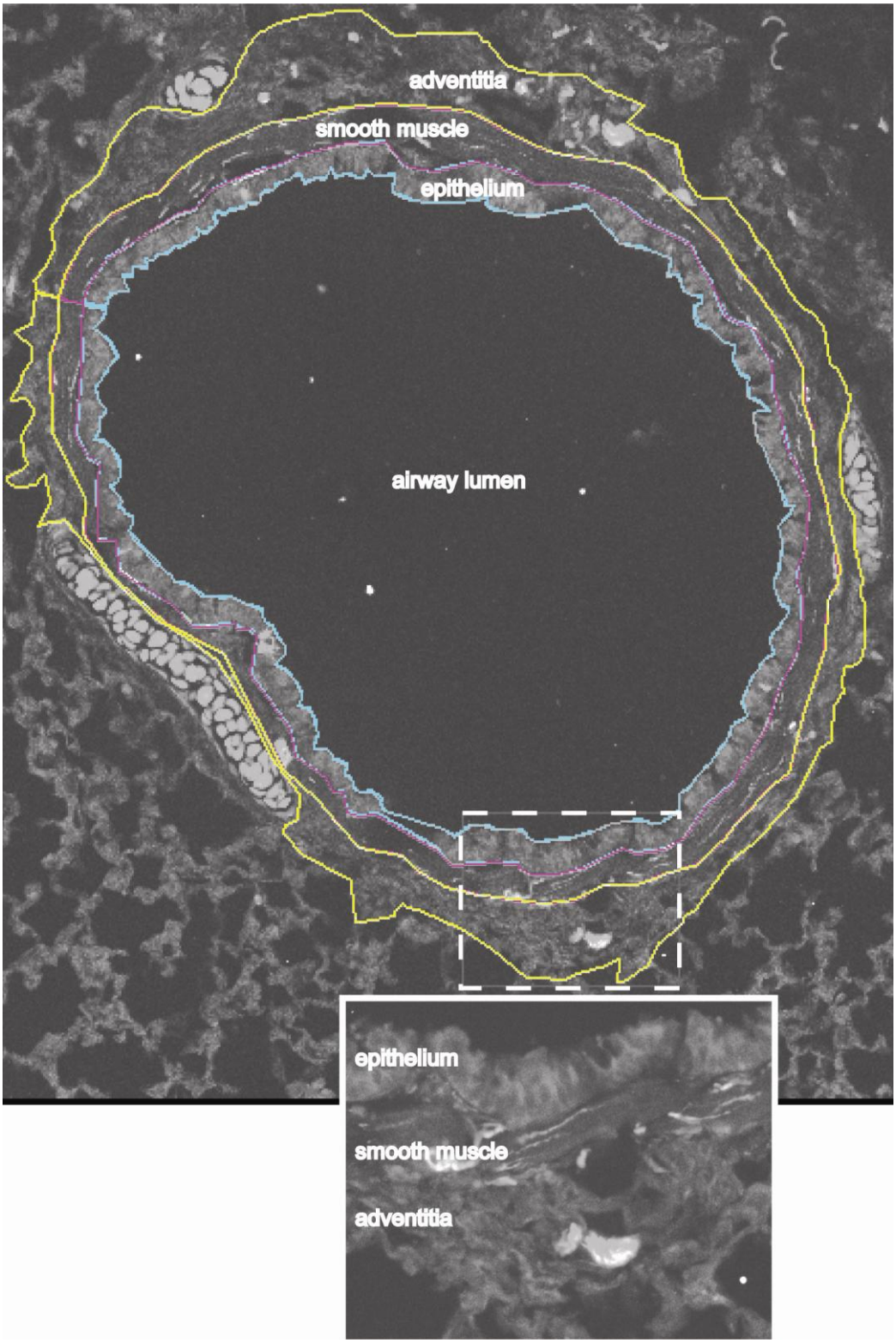

Figure 2.12 Measuring airway epithelium, smooth muscle, and adventitia areas.

Airway epithelium, smooth muscle, and adventitial areas are measured by outlining each

region as shown using Metamorph software and logging each region area to excel. This image is a grayscale version of the original after the red channel (eosinophils) has been turned off and the display has been adjusted to see borders between tissue regions.

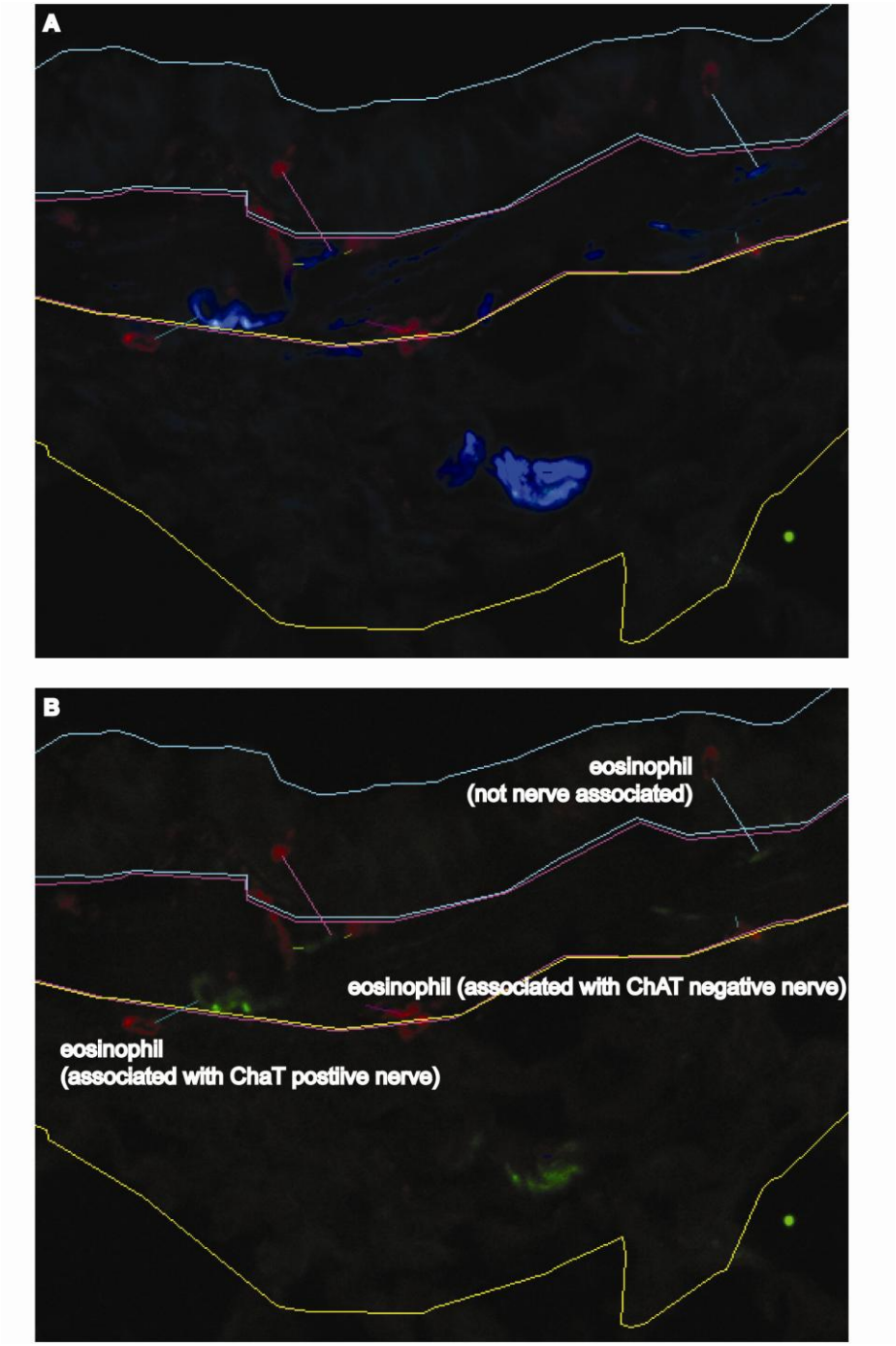

Figure 2.13 Measuring distances between eosinophils and nearest nerve. (A) A line is drawn from each eosinophil (red) to the nearest nerve (PGP9.5+, blue) and the distance is logged to excel. (B) The blue channel (PGP9.5) is turned off and the nerve is coded as cholinergic (ChAT+, green) or noncholinergic (ChAT-). Eosinophils that are less than 8 μm from a nerve are considered nerve-associated. Eosinophils are assigned

to a region (epithelium, smooth muscle, adventitia) based on their location and not the location of the nearest nerve.

subepithelial space and smooth muscle. Adventitia included airway tissue beyond the smooth muscle. For cartilaginous airways the adventitia was measured up to the cartilage but not beyond. Large blood vessels were excluded. Area data were logged to an excel spreadsheet for area along with animal number and airway. Two noncartilaginous and two cartilaginous airways were imaged for each animal.

After area region was drawn, the red channel (eosinophils) was turned on and the green channel (ChAT) was turned off, after resetting the display brightness for both the blue and green channels. I used the line tool to draw a line from each eosinophil to the nearest PGP9.5 positive nerve (blue; Figure 2.13) and logged the distance in excel. I then turned off the blue channel (PGP9.5) and turned on the green channel (ChAT) to determine whether the nerve was cholinergic (ChAT positive; Figure 2.13). In the excel spreadsheet for eosinophils, each eosinophil was identified by animal number and airway, the tissue area (epithelial, smooth muscle, adventitial) the eosinophil was located in, distance to nearest nerve, and whether the nerve was cholinergic (coded 1) or noncholinergic (coded 0).

Data Analysis

The data in my image, area, and eosinophil spreadsheets were imported into a database by Robert Buels. He wrote a custom Perl script to output eosinophil number and eosinophils per mm^2 in each region (epithelium, smooth muscle, adventitia, total) and cholinergic (<8 μm from PGP9.5 positive and ChAT positive nerve), noncholinergic (<8 μm from PGP9.5 positive and ChAT negative nerve), and nonnerve (>8 μm from any nerve) associated eosinophil number and eosinophils per mm^2 in each region. The data were sorted by treatment and by cartilaginous or noncartilaginous airways. Replicate

values were averaged for each animal and these numbers were used to calculate the mean for each treatment group.

3. Histological evaluation of CCL11 (Eotaxin) expression in cholinergic nerves

CCL11 was detected with a polyclonal antibody against guinea pig CCL11 and cholinergic nerve fibers were identified with a polyclonal antibody against ChAT. Eosinophils were additionally stained with chromotrope 2R. Immunostaining was carried out exactly as for histological evaluation of cholinergic and noncholinergic nerve-associated eosinophils in cryosections (see above). However, rabbit anti-guinea pig eotaxin antiserum A3 (gift from Dr. Peter Jose's Laboratory, Imperial College, London, England) diluted 1:100 was used instead of PGP9.5 antibody. Preimmune serum A0 served as a control for CCL11 staining.

All image analysis was carried out while I was blinded to treatment. Colocalization of CCL11 with ChAT fibers was determined by identifying each ChAT-positive fiber in the airway and turning off the green channel (ChAT) to determine whether it stained for CCL11. The percentage of ChAT-positive fibers that also stained for CCL11 was calculated for two noncartilaginous and two cartilaginous airways from each animal. Values were averaged for each animal and these numbers were used to calculate the mean for each treatment group.

4. *In situ* hybridization for PGP9.5 and chemokine mRNA

In situ hybridization was used to label PGP9.5 mRNA in cryosections from guinea pig trachea. Since sense mRNA gets translated into protein, an antisense RNA probe was designed to hybridize with PGP9.5 mRNA in the tissue. A sense RNA probe was developed as a control for nonspecific labeling. Several methods can be used to generate in situ hybridization probe. I used a PCR method, which is quick but does not

produce a large amount of probe at one time. PCR primers were designed to amplify approximately 500 bp of PGP9.5 (neuronal marker). I added a tail to one of the primers in each primer pair that contained a T7 polymerase start site. A PCR reaction was carried out using primary nerve culture cDNA (see PCR section) as a template and Amplitaq Gold polymerase to generate PCR products for the target gene with an added T7 polymerase start site (see Table 2.1 for primers). These products were run on an agarose gel, extracted with a gel extraction kit, and sequenced (see PCR section below). This PCR product was transcribed into RNA probe using RNA polymerase T7 and nucleotides containing digoxigenin-labeled UTP in the presence of Protector RNAse inhibitor. The RNA probes were isolated using a Roche mini quick spin column and a sample was run on an agarose gel where they ran faster than the PCR template with T7 polymerase site, as expected.

Trachea tissue was fixed with 4% paraformaldehyde and cryosectioned as for lung (see above). Cryosections of guinea pig were air dried for 1 hr at room temperature and then postfixed in 4% paraformaldehyde. Sections were rinsed in PBST (see Table 2.5) and then permeabilized with proteinase K (10 μg/mL in PBS) for 9 min at room temperature. Sections were rinsed, re-fixed with 4% paraformaldehyde for 5 min to maintain tissue structure after protein degradation, and then rinsed again. Sections were immersed in 0.1 M triethanolamine and acetic anhydride for 15 min and then rinsed. Sections were blocked with 100 μL of prewarmed (60° C) hybridization solution (see Table 2.5) per slide and covered with a coverslip cut from an autoclave bag (or parafilm). Slides were placed in a slide box used as a humid chamber. The entire box was rinsed with RNAse Away and placed vertically so that slides were horizontal when placed inside. A kimwipe soaked with 50% formamide in 5X saline sodium citrate buffer (SSC) was placed at the bottom of the humid chamber beneath a blank slide to maintain humidity. The humid

Table 2.1 Primers used to generate PCR product with incorporated T7 RNA polymerase site for guinea pig PGP9.5 *in situ* hybridization probe development

Primer Pairs	**5' Primer**	**3' Primer**
Antisense PGP9.5	GACTGGAGGACGAGGCTCTG	ATTG**TAATACGACTCACTATAGGG**ACTGGT GCCATGGTTCACTG
Sense PGP9.5	ATTG**TAATACGACTCACTATAGGG** ACTGGAGGACGAGGCTCTGG	ACTGGTGCCATGGTTCACTG
Bold = T7 RNA polymerase start site		

chamber was sealed with vinyl tape and left in an incubator at 65° C for 1 hour. 1 μL of digoxigenin-labeled antisense or sense (control) probe was added to hybridization solution for each slide and preheated to 85° C for 3 minutes to denature any self-hybridization. Coverslips were removed and 100 μL of prewarmed hybridization solution with digoxigenin-labeled probe was added to each slide. Slides were covered again and placed overnight in the humid chamber (sealed with vinyl tape) at 65° C. Coverslips were removed with forceps in a solution of 5X SSC prewarmed to 65°C. Slides were washed for 30 min in 50% formamide in 1X SSC at 65° C and then 10 min in TNE (see Table 2.5) at 37° C. Sections were incubated in TNE containing 20 μg/mL RNAse A to destroy any unhybridized RNA probe and then washed for 10 min in TNE at 37° C, followed by 20 min in SSC at 65° C and 2 x 20 min in 0.2X SSC at 65°C. After this step the RNA probe is hybridized and sections no longer need to be treated in an RNAse free manner. To detect the probe anti-digoxigenin immunostaining was carried out. Sections were washed in MABT (see Table 2.5) at room temperature and outlined with a hydrophobic pen before blocking with 20% heat-inactivated sheep serum in MABT for 1 h at room temperature in a humid chamber for immunostaining. Sections were incubated in primary antibody, anti-digoxigenin linked to alkaline phosphatase (1:2000) in 2% heat-inactivated sheep serum in MABT overnight at 4° C in a humid chamber. The next day, slides were rinsed in MABT and then in NTMT (see Table 2.5) at room temperature. Coloration solution containing NBT and BCIP substrates for alkaline phosphatase in NTMT was applied to each slide and tissues were allowed to develop in the dark. Slides were checked periodically until staining developed. For the PGP9.5 probe, purple precipitate was visible in ganglia at 4 h. The coloration reaction in sections incubated with antisense and sense probe at the same time by rinsing 2 x 5 min in PBS and then post-fixing in 4% paraformaldehyde for 30 min. When mounted with mounting

media containing DAPI, I was able to confirm that cells staining for PGP9.5 contained nuclei.

E) Nerve cell culture experiments

For studies of neuronal production of eosinophil chemokines, I used primary cultures of guinea pig parasympathetic nerves to pursue mechanisms for *in vivo* observations in guinea pig. I also used the SK-N-SH human neuroblastoma cell line to study the potential of neuronal cells to produce chemokines that are found in humans but not guinea pigs. Neuroblastoma cells are thought to originate from sympathetic neuroblasts and tend to have an adrenergic phenotype (Thiele, 1998). These cells are commonly used in our laboratory because they express muscarinic receptors and allow study of neuronal muscarinic receptor expression. I used these cells to screen for the potential of nerve cells to make CCR3 chemokines in response to TNFα stimulation, since they express TNFα receptor I, as do parasympathetic nerves in culture (Nie *et al.*, 2009).

1. Growth and treatment of SK-N-SH cells with human TNFα to measure changes in CCR3 chemokine mRNA and protein

Maintenance of SK-N-SH cells

Laboratory stocks of passaged SK-N-SH cells were removed from liquid Nitrogen and thawed for approximately 1 minute at 37°C in a water bath. Warmed SK-N-SH cell culture media (see Table 2.5) was added to the thawed cells, and they were cultured in a 75 cm^2 cell culture flask at 37°C with 5% CO_2 in a cell culture incubator. Media was replaced with fresh warmed media approximately every 3 days. When cells reached confluency they were treated with trypsin for 3 min at 37°C to detach cells. Trypsinized cells were collected in 10 mL of SK-N-SH media and centrifuged at 1000 rpm for 10 min

in an eppendorf centrifuge 5810 to remove trypsin. Supernatant was aspirated away and cells were resuspended in SK-N-SH media, counted in a hemocytometer, and diluted to seed a new maintenance culture or to seed an experiment.

Dose response to human TNFα

SK-N-SH cells were seeded in 6-well cell culture plates at a density of 15 x 10^4 cells per well. Cultures were grown for 3 days at 37°C with 5% CO_2 to approximately 85% confluency. For dose response experiments, media was aspirated away and replaced with SK-N-SH media supplemented with 0-50 $ng \cdot mL^{-1}$ recombinant human TNFα. Cells were maintained in culture with TNFα for 24h. After 24 h cells were lysed in RLT lysis buffer (RNeasy kit) with beta-mercaptoethanol and RNA was isolated from the lysed cells (see PCR section below).

Time course for treatment with TNFα

Cells were treated with 150 $ng \cdot mL^{-1}$ human TNFα for 0, 4, 8, 24, or 48 h. Supernatants were harvested and cells were lysed and harvested for RNA isolation. Supernatants were centrifuged for 7 min at 150 g to reduce any cellular debris, aliquoted, and stored at -80°C for protein analysis.

2. Growth and treatment of guinea pig parasympathetic nerve cultures to measure changes in CCR3 chemokine mRNA

Isolation and growth of guinea pig parasympathetic nerves in culture

Guinea pigs were killed with an overdose of pentobarbital (150 $mg \cdot kg^{-1}$, i.p.). The trachea and primary bronchi were extracted using sterile forceps and scissors and placed in a sterile beaker of 5x penicillin-streptomycin. In a tissue culture hood, connective tissue was removed, and the trachea was opened along the ventral surface

by cutting through the cartilage rings. Epithelium was wiped off the trachea with a moistened sterile cotton swab, and excess cartilage was trimmed away from the trachealis muscle. The smooth muscle and remaining cartilage were cut into small pieces using scissors in a beaker of sterile filtered 0.05% Type XI collagenase in HBSS with Ca^{2+} and Mg^{2+} and 1X penicillin-streptomycin. Tissue was transferred to a 25 cm^2 cell culture flask with 0.05% collagenase (10 mL per trachea) and incubated in a 37° C shaking water bath for 4 hours. An equal volume of 5% FBS in DMEM with L-glutamine was added to neutralize the collagenase, and cells were centrifuged for 10 minutes at 300g. The supernatant was discarded and the cell pellet, containing neurons, was resuspended in 10% FBS in a 1:1 mixture of DMEM with L-glutamine and Ham's F-12 with L-glutamine. These cells were preplated on a 100 mm x 20 mm tissue culture dish overnight at 35.5°C with 5% CO_2 to remove adherent cells. The following day (Day 0) supernatant from the preplate was centrifuged at 300g for 10 minutes. Supernatant was discarded and the pellet was resuspended in serum free media (SFM, see Table 2.5). Cells were seeded on matrigel coated 12-well plates or 35 mm x 10 mm tissue culture dishes and cultured overnight at 35.5°C with 5% CO_2. Media was replaced on Day 1 with SFM + 2 μM cytosine arabinoside to kill dividing cells and further enrich for neurons. On Day 4 cells were returned to SFM without cytosine arabinoside and this media was then changed every other day until the cells were treated (Days 5-7) and harvested.

Preliminary experiments treating with murine TNFα, murine IFNγ, and human IL-4

In preliminary experiments, parasympathetic nerve cultures grown in 12-well plates were treated with 2 $ng \cdot mL^{-1}$ murine TNFα, 2 $ng \cdot mL^{-1}$ murine TNFα + 1000 $U \cdot mL^{-1}$ murine IFNγ, 10 $ng \cdot mL^{-1}$ human IL-4, or 2 $ng \cdot mL^{-1}$ murine TNFα + 10 $ng \cdot mL^{-1}$ human IL-4 in SFM for 2 or 6 h. The same treatments were also given in SFM with 10% FBS for 6 h to cultures that were first treated for 24 h with 10% FBS in SFM. Cells were lysed in RLT lysis

buffer (RNeasy kit) with beta-mercaptoethanol and RNA was isolated from the lysed cells (see PCR section below).

Dose response to guinea pig TNFα

Recombinant guinea pig TNFα became commercially available after the preliminary experiments were completed. Nerves from two guinea pig tracheas were cultured together and grown in six 35 mm x 10 mm dishes for each experiment. Cells were cultured in SFM or SFM containing guinea pig TNFα (0.05-5.0 $ng \cdot mL^{-1}$) for 4h and then nerve cell bodies were harvested for PCR analysis (see below). Doses were chosen based on EC_{50} concentrations provided by the supplier. The number of nerve clusters was counted in advance, and treatments were divided between dishes so that approximately 10 nerve clusters would get the same treatment. It was not possible to treat each culture with every dose of TNFα. Treatments were staggered to allow time to harvest the nerve cell bodies.

3. Harvesting guinea pig parasympathetic nerve cell bodies grown in culture to isolate neuronal mRNA

Even after preplate steps and cytosine arabinoside treatment to kill dividing cells many nonneuronal cells survive in primary cultures of guinea pig parasympathetic nerves. This makes it difficult to interpret what cell type is responding to treatment. I used a method developed in our laboratory by Dr. Zhenying Nie and Dr. Kirsten Verhein to harvest parasympathetic nerve cell bodies for RNA isolation. This method takes advantage of guinea pig parasympathetic nerves growing in clusters with their neurites extending out. Cultured parasympathetic nerve cell bodies were isolated using an inverted microscope and a capillary tube pipette attached to a 1 mL syringe with connective tubing. The capillary tube pipette was prepared by pulling a thin walled glass capillary tube in a Bunsen burner flame. A set of fine forceps was used to break the tip

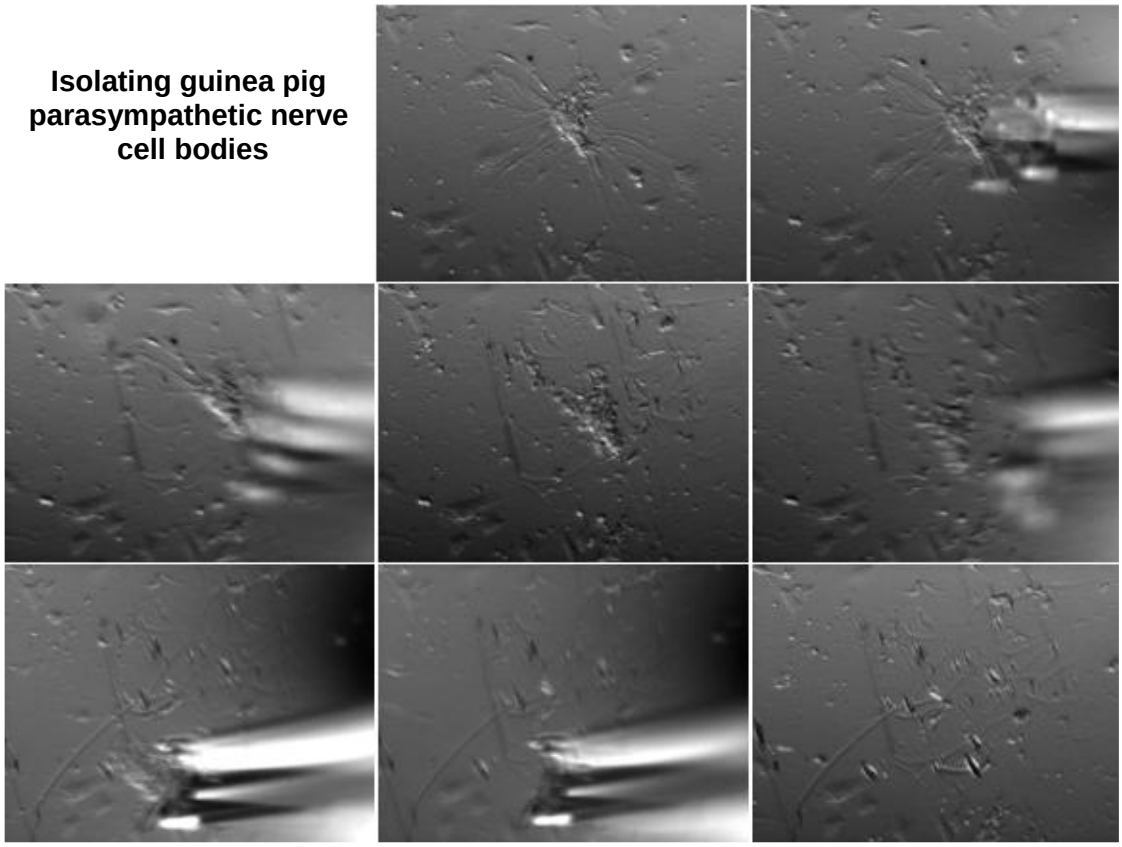

Figure 2.14 Isolating guinea pig parasympathetic nerve cell bodies in culture. Guinea pig parasympathetic nerves in culture grow in clusters with their neurites extending outward. A glass capillary tube is used to first sever the neurites attached to the matrigel and to then suck up the neuronal cell bodies using suction applied with a syringe. Most contaminating cells in the culture are left behind.

off, leaving a sterile opening finer than the original opening. Nerves were harvested from one cell culture dish at a time using a new capillary pipette for each dish. Cell culture media was removed and replaced with 2 mL of ice cold PBS. Nerve clusters were located using the inverted microscope and cell bodies were isolated by first detaching neurites from matrigel (by cutting the neurites with the glass pipette) and then applying suction with a syringe to suck up the cell bodies (Figure 2.14). Care was taken to suck up as little extra media as possible. Cells were ejected into an RNAse-free microcentrifuge tube. All nerve clusters from each dish (or treatment if more than one dish was needed) were combined in one tube and stored on ice until ready for RNA isolation (see PCR section below).

F) Measurement of RNA with real-time quantitative PCR

1. RNA isolation and cDNA generation

Isolating RNA from SK-N-SH cells, preliminary parasympathetic nerve culture cell lysates, and homogenized whole lung and generation of cDNA with Superscript III

RNA was isolated using a Qiagen RNeasy kit. The optional DNAse step was included to remove genomic DNA and RNA was eluted in 40 μL of 10 mM Tris pH 8. The concentration of eluted RNA was determined by measuring absorbance at 260 nM on a spectrophotometer (Thermo Spectronic, BioMate 3). Isolated RNA (2 μg per 20 μL reaction) was reverse transcribed into cDNA using Superscript III reverse transcriptase and random hexamer primers in a Px2 Thermal Cycler (Thermo Electron Corporation). RNA was denatured at 65° C for 5 min, cooled to 4°C for 1 min when reverse transcriptase was added, primers annealed at 25°C for 10 min, cDNA synthesis was carried out at 50°C for 50 min, and synthesis was terminated at 70°C for 15 min. cDNA and remaining RNA were stored at -80°C.

RNA isolation from cultured parasympathetic nerve cell bodies and parasympathetic ganglia and cDNA generation with cells-to-ct kit

Cultured guinea pig parasympathetic nerve cell bodies and parasympathetic ganglia harvested from guinea pig tracheas were centrifuged at 2,000 rpm for 7 min at 4° C (accuspin microR Centrifuge). Supernatant was removed so that no more than 5 μL of liquid remained. The Power SYBR Green Cells-to-CT kit was used to isolate RNA and generate cDNA. Briefly, cells were lysed in 50 μL lysis solution containing DNAse I at room temperature for 5 min, the lysis reaction was stopped with stop solution at room temperature for 2 min, and samples were then stored at -80°C until cDNA was generated. Parasympathetic ganglia were also homogenized by hand with an RNAse free disposable pestle during the lysis step. Reverse transcription reactions (50 μL reaction volume) were set up using 10 μL of the lysis cells. Reactions where reverse transcriptase enzyme was omitted and water added in its place were set up to control for amplification of genomic DNA instead of cDNA generated from RNA in the PCR step. Reverse transcription was carried out at 37°C for 1 h followed by a 5 min reverse transcriptase inactivation step at 95°C and a 4°C hold step in a Veriti, 96 well thermal cycler (Applied Biosystems, Carlsbad, CA).

2. Primer design, testing, and sequencing

Primers used for PCR reactions in this thesis were ordered from Integrated DNA Technologies and are listed in Table 2. I used Primer 3 (http://frodo.wi.mit.edu/primer3) to design all primer sets not already available in the laboratory. Primers were designed against gene reference sequences obtained from NCBI. When possible, primer combinations crossed at least 1 kb of intron. For real-time PCR, primers were designed to amplify 100-250 bp products. A maximum of 3 nucleotide repeats were allowed in

any primer, GC content ranged from 45-55%, and the 3' nucleotide was clamped as a G or C. Guinea pig primers were tested on whole lung cDNA. Human primers were tested on cDNA from SK-N-SH cells treated with TNFα or human tracheal epithelial cells treated with virus (for other projects in the laboratory). Amplification of the desired product was confirmed by running the PCR products on an agarose gel (1-2% in TAE, Table 2.5) to confirm only one product was amplified, isolating the PCR product from the gel with a QIA quick gel extraction kit, and sequencing it. Sequencing was done by the OHSU MMI DNA service core.

3. Real-time quantitative polymerase chain reaction (PCR)

Real-time polymerase chain reaction (PCR) was used to measure changes in target gene mRNA expression. Amplification of RNA with PCR was detected using SYBR green, a DNA-binding dye that fluoresces when bound to double-stranded DNA. The threshold cycle number (ct), PCR cycle at which fluorescence signal meets or exceeds a threshold intensity, was measured in each sample (Fraga *et al.*, 2008). Threshold levels were set so they were in the exponential phase where approximate doubling (depends on primer efficiency) of PCR product occurred with each cycle.

The standard curve method for relative quantification of PCR product was used to measure changes in target gene expression due to treatment because this method does not assume 100% efficiency of each primer set (Bookout *et al.*, 2006). In this method a dilution series of cDNA samples is constructed for the gene of interest and an endogenous reference gene, which is used to control for the amount of input RNA. Ct versus log nanogram of input standard RNA (quantity determined before reverse transcription) are graphed and a linear regression is fit to the standard curve data for both the target gene and the reference gene (18S ribosomal RNA was used as the

Table 2.2 Oligonucleotide primers used in PCR reactions

Gene	5' primer sequence	3' primer sequence	Positive Control
Human			
CCL5	CCATATTCCTCGGACACCAC	ACACACTTGGCGGTTCTTTC	SK-N-SH cells
CCL7	GAAAGCCTCTGCAGCACTTC	TGGTGGTCCTTCTGTAGCTC	SK-N-SH cells
CCL8	GTTTCTGCAGCGCTTCTGTG	CTTCCTTGGGACATTGGATG	Epithelial cells
CCL11	AACCACCTGCTGCTTTAACC	TCCTGCACCCACTTCTTCTT	SK-N-SH cells
CCL13	TCTCTGCAGTGCTTCTGTGC	GAAGATGACAGCCTTCTGGG	none
CCL15	CATGCTTGTTGCTGTCCTTG	ACACGGGATGCTTTGTGAG	Epithelial cells
CCL24	GCCTTCTGTTCCTTGGTGTC	TGTACCTCTGGACCCACTCC	Virus infected epithelial cells
CCL26	GGAGGAGTTTGGGAGAAACC	TGTGGCTGTATTGGAAGCAG	none
CCL28	GTGACTTGGCTGCTGTCATC	GCCGTATGTTTCGTGTTTCC	Epithelial cells
Guinea Pig			
CCL5	TGTGTCATCCTCACCACTGC	ACACACTTGGCGGTTCTTTC	Guinea pig lung
CCL7	TCATTGCAGTCCTTCTGTGC	CATTTGCTGCTGGTGATCC	Guinea pig lung
CCL11	AAGTCTCCACAGCGTTTCTG	TCTTCAGTCGCTGAAAGGAG	Guinea pig lung
PGP9.5	CAACAACCGAGACAAACTGG	TGTCATCTACCCGACACTGG	Guinea pig lung
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	SK-N-SH and guinea pig lung

I did not design the 18S, human CCL11, human CCL24, and human CCL26 primers. cDNA from human tracheal epithelial cells served as a positive control.

reference gene). The relative amounts of target gene and reference gene in each sample are interpolated from these curves. Target gene mRNA levels was normalized to 18S levels and expressed in arbitrary units. The numbers are arbitrary because they are relative to the total amount of input RNA and not to a known amount of target gene mRNA (Bookout *et al.*, 2006).

Sample cDNA was diluted and used in real-time quantitative PCR with Quantitect SYBR Green for cDNA samples prepared with superscript III or Power SYBR Green for cDNA prepared with the cells-to-ct kit. Real-time PCR was carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems), see Tables 2.3 and 2.4 for conditions. All samples were run in duplicate. A no RT control (reverse trancriptase left out of cDNA reaction) was included to ensure that PCR products represented amplification of cDNA generated from RNA and not genomic contamination. A no template control was included for each primer set when water was added to the reaction instead of cDNA to ensure there was no PCR product or DNA contamination in any of the reagents. Amplification of the correct product was verified by the presence of one peak of expected size (determined during primer testing and sequencing) in the derivative plot of the dissociation curve. SYBR green detects any double stranded DNA including primers and unintended PCR products. At the end of each PCR, the temperature is increased in 1° C intervals and the abrupt loss of fluorescence signal when each PCR product dissociates is measured. There is an inflection in the dissociation curve profile when a PCR product dissociates (this is the melting temperature) that appears as a peak in a plot of the derivative of the dissociation curve (Fraga *et al.*, 2008).

Table 2.3 Real-time PCR conditions for 18S and CCR3 chemokines using Quantitect SYBR Green

Temperature	Time	Purpose	Cycles
95°C	15 min	activate polymerase	1
95°C	20 sec	denaturation	35
58°C	30 sec (for 18S) 2 min (for chemokines)	primer annealing	
72°C	30 sec	elongation	
95°C	15 sec	denaturation	1
60°C-95°C	1 min (each temperature)	dissociation curve	1
95°C	15 sec	denaturation	1
60°C	15 sec	stabilize products	1

Table 2.4 Real-time PCR conditions for 18S, PGP9.5, and CCR3 chemokines using Power SYBR Green

Temperature	Time	Purpose	Cycles
95°C	10 min	activate polymerase	1
95°C 59°C	15 sec 1 min	denaturation primer annealing and elongation	40
95°C	15 sec	denaturation	1
60°C-95°C	1 min (each temperature)	dissociation curve	1
95°C	15 sec	denaturation	1
60°C	15 sec	stabilize products	1

G) Measurement of chemokines in SK-N-SH cell supernatants by ELISA

CCL5, CCL7, and CCL11 protein levels were measured in supernatants from SK-N-SH cells using human CCL5/RANTES, human CCL7/MCP-3, and human CCL11/Eotaxin DuoSet ELISA development kits. 1x8 strip wells were coated with capture antibody (1 $\mu g \cdot mL^{-1}$ mouse anti-human RANTES, 1 $\mu g \cdot mL^{-1}$ mouse anti-human MCP-3, or 2 $\mu g \cdot mL^{-1}$ mouse anti-human eotaxin) overnight at room temperature. Wells were rinsed with 0.05% Tween 20 in PBS. Then blocked with 1% bovine serum albumin in PBS at room temperature for 1 h. Wells were rinsed again and 100 μL of sample, blocking solution, or standards diluted in blocking solution were incubated for 2 h at room temperature. Wells were rinsed and incubated with detection antibody (10 $ng \cdot mL^{-1}$ biotinylated goat anti-human RANTES, 100 $ng \cdot mL^{-1}$ biotinylated goat anti-human MCP-3, or 100 $ng \cdot mL^{-1}$ goat anti-human Eotaxin) for 2 h at room temperature. Wells were rinsed and then incubated with streptavidin conjugated to horseradish peroxidase (1:200) for 20 min in the dark. Wells were rinsed again and incubated with SureBlue Reserve TMB Microwell Peroxidase substrate solution for 10 min in the dark. Color development was stopped with 1 N HCl and the optical density for each well was read immediately at 450 nm and 570 nm on a Versa Max tunable Microplate reader with Softmax Pro5 software (Molecular Devices, Sunnyvale, CA). A five-parameter logistic regression equation was fit to the standard curve with relative median fluorescence intensity graphed on the y-axis and standard concentration on the x-axis (this equation contains parameters for estimated response at zero concentration, slope factor, mid-range concentration, estimated response at infinite concentration, and an additional parameter for asymmetry). Backcalculations of standards were compared to actual concentrations and were found to be within 86-113% of actual values for CCL5, 93-110% of actual values for CCL7, and 68-122% of actual values for CCL11.

Table 2.5 Cell culture media and buffer solutions

PBST	1 X PBS with 0.1% Tween 20
Hybridization solution	10 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% Dextran Sulfate, 1X Denhardt's, 200 $\mu g \cdot mL^{-1}$ yeast tRNA, 50% formamide
NTMT	100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20
Serum Free Media (SFM)	50% DMEM/ 50% Ham's F-12, 0.05% BSA, 10 $\mu g \cdot mL^{-1}$ insulin, 6.7 $ng \cdot mL^{-1}$ Na Selenite, 20 $\mu g \cdot mL^{-1}$ transferrin, 100 $ng \cdot mL^{-1}$ 2.5S NGF, 1 X penicillin-streptomycin
SK-N-SH cell culture media	10% fetal bovine serum, 1X non essential amino acids, 1X sodium pyruvate, in Minimum Essential Medium Eagle
TAE	20 mM Tris base, 20 mM acetic acid, 1 mM EDTA
TNE	10 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA
MABT	100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5

Table 2.6 Reagents and Supplies

Pharmacological Reagents	
Drugs	**Supplier**
Acetylcholine chloride	Acros Organics
Atropine sulfate	Sigma
Gallamine triethiodide	Sigma
Guanethedine sulfate	Bosche Scientific
DL isoproteronol hydrochloride	Sigma
Ketamine hydrochloride	JHP Pharmaceuticals
D-(+)-lactose monohydrate	Mallinckrodt Baker Inc.
Ovalbumin (Grade II)	Sigma
Ovalbumin (Grade V)	Sigma
Pentobarbital sodium	Sigma
Pyrilamine maleate	Sigma
Spiriva HandiHaler capsules	Boehringer Ingelheim Pharmaceuticals
Succinylcholine chloride	Sigma
Urethane	Sigma
Xylazine HCl	Vedco

Immunostaining Reagents	
Reagent	**Supplier**
Goat anti-ChAT (AB144P)	Millipore
Rabbit anti-eotaxin A3	Dr. Peter Jose, Imperial College, London
Mouse anti-PGP9.5 (7863-2004)	AbD Serotec
Rabbit anti-PGP9.5 (7863-0504)	AbD Serotec
Donkey anti-goat 488 (A11055)	Invitrogen
Donkey anti-rabbit 405 (711-475-152)	Jackson Immuno Research
Biotinylated goat anti-mouse IgG (BA-9200)	Vector
Acetone	Fisher
Antigen unmasking solution	Vector
Chromotrope 2R	Sigma

Colorfrost Plus microscope slides	Fisher
Cryomold (25 mm x 20 mm x 5 mm)	Sakura
Cytoseal 60	Richard-Allan
200 proof ethanol	Decon
Fluoromount-G mounting media	Southern Biotech
ImmEdge hydrophobic pen	Vector
Methanol	Fisher
Normal donkey serum	Jackson Immuno Research
Normal goat IgG	R&D Systems
Normal goat serum	Vector
Normal mouse IgG	Invitrogen
Optimal cutting temperature compound	Sakura
10X PBS	Fisher
Prolong gold antifade reagent	Invitrogen
Rabbit preimmune serum A0	Dr. Peter Jose, Imperial College, London
Slow fade gold antifade reagent	Invitrogen
Sucrose	Sigma
Triton X-100	Fisher
Tween 20	Fisher
Vectashield hardset	Vector
Vectashield mounting media for fluorescence (H-1000)	Vector
Vectashield mounting media for fluorescence with DAPI (H-1200)	Vector
Vectastain ABC kit	Vector
Vector SG chromagen	Vector
Xylene	Fisher
Zamboni's fixative	American MasterTech
Zinc-buffered formalin	Anatech

Commercially available reagents and kits	
Reagent	**Supplier**
Glacial acetic acid	EMD

Antifoam Y-30 emulsion	Sigma
70 μm cell strainer	BD Biosciences
DP-4 dry powder insufflator	Penn-Century
Hemacolor	EMD Chemicals
Kimwipe	Kimtech
Neutral red	Fisher Scientific
PBS	Mediatech
Small animal laryngoscope	WelchAllyn
Sterile gauze (12 ply)	Fisher
Sylguard 184 elastomer base	Dow Corning
Sylguard 184 curing agent	Dow Corning
***In situ* hybridization reagents**	
Acetic anhydride	Sigma
Anti-digoxigenin linked to alkaline phosphatase	Roche
BCIP	Roche
Denhardt's solution 50X concentrate	Sigma
Dextran sulfate sodium salt	Sigma
Diethyl pyrocarbonate	Sigma
Digoxigenin RNA Labeling Mix	Roche
0.5M EDTA pH 8	Gibco
Formamide	Sigma
Magnesium chloride	Fisher
Maleic acid	Fisher
Mini quick spin RNA columns	Roche
NBT	Roche
Paraformaldehyde (16%)	Electron microscopy sciences
Protector RNAse inhibitor	Roche
Proteinase K	Qiagen
RNA polymerase T7	Roche
RNAse away	Molecular BioProducts
SDS	Fisher
Sheep serum	Sigma

Sodium chloride	Fisher
Sodium citrate tribasic dihydrate	Sigma
20X SSC	Sigma
Triethanolamine	Sigma
Trizma base	Sigma
Tris hydrochloride	Invitrogen
Vinyl tape	VWR
Yeast tRNA	Invitrogen
RT-PCR reagents	
Agarose gel loading dye (6X)	Boston BioProducts
Amplitaq gold	Applied Biosystems
Track It 100 bp DNA Ladder	Invitrogen
DNase I	Qiagen
Ethidium bromide	Fluka Biochemicals
2-mercaptoethanol	Sigma
Power SYBR Green Cells-to-Ct Kit	Ambion
QIA quick gel extraction kit	Qiagen
Quantitect SYBR green PCR kit	Qiagen
RNAse-free disposable pestle	Nalg Nunc
Rneasy Mini kit	Qiagen
SuperScript III reverse transcriptase	Invitrogen
Ultra Pure agarose	Invitrogen
ELISA reagents	
Human CCL5/RANTES DuoSet ELISA development kit	R&D Systems
Human CCL7/MCP-3 DuoSet ELISA development kit	R&D Systems
Human CCL11/Eotaxin DuoSet ELISA development kit	R&D Systems
SureBlue Reserve TMB Microwell Peroxidase substrate solution	KPL
EIA/RIA 1x8 strip wells	CoStar

Cell culture reagents	
Reagent	**Supplier**
Bovine serum albumin	Fisher
Collagenase type XI	Sigma
Cytosine arabinoside	Sigma
DMEM with L-glutamine	Mediatech
Fetal bovine serum (SH30070)	Hyclone
Ham's F-12 with L-glutamine	Mediatech
HBSS with Ca^{2+} and Mg^{2+}	Mediatech
Insulin-transferrin-selenite	Mediatech
Matrigel	BD Biosciences
Minimum essential medium eagle	Cellgro
2.5S nerve growth factor	Harlan Bioprods BT
Non essential amino acids	Cellgro
Penicillin-streptomycin solution	Mediatech
PBS	Mediatech
Rat transferrin	Jackson ImmunoResearch
SK-N-SH neuroblastoma cells	ATCC
Sodium pyruvate	Cellgro
0.25% trypsin-EDTA	Invitrogen
60 mm x 15 mm cell culture dish	Corning
35 mm x 10 mm cell culture dish	Corning
100 mm x 20 mm cell culture dish	Corning
25 cm^2 cell culture flask	Corning
75 cm^2 cell culture flask	Corning
6 well cell culture dish	Corning
12 well cell culture dish	Corning
Glass capillary tube	Warner Instruments
Cotton-tipped applicators	Fisher
SteriFlip – 50 mL disposable vacuum filtration system 0.22 nM	Millipore

Cytokines	
Cytokine	**Supplier**
Murine IFNγ (315-05)	Peprotech
Human Il-4 (IL004)	Chemicon
Human TNFα (T0157)	Sigma
Guinea pig TNFα (5035TG-025)	R&D Systems
Murine TNFα (315-01A)	Peprotech

CHAPTER 3.

NON-BRONCHODILATING MECHANISMS OF TIOTROPIUM PREVENT AIRWAY HYPERREACTIVITY IN A GUINEA PIG MODEL OF ALLERGIC ASTHMA

ABSTRACT

Background and purpose: Asthma is characterized by reversible bronchoconstriction and airway hyperreactivity. Although M_3 muscarinic receptors mediate bronchoconstriction, nonselective muscarinic receptor antagonists are not currently recommended for chronic control of asthma. We tested whether selective blockade of M_3 receptors, at the time of antigen challenge, blocks subsequent development of airway hyperreactivity in antigen-challenged guinea pigs.

Experimental approach: Ovalbumin-sensitized guinea pigs were pretreated with 1 $\mu g \cdot kg^{-1}$ of a kinetically selective M_3 receptor antagonist, tiotropium, or 1 $mg \cdot kg^{-1}$ of a nonselective muscarinic receptor antagonist, atropine, and challenged with inhaled ovalbumin. 24 h later animals were anesthetized, paralyzed, ventilated, and vagotomized. We measured vagally mediated bronchoconstriction and intravenous acetylcholine induced bronchoconstriction.

Key results: Electrical stimulation of both vagus nerves induced frequency-dependent bronchoconstriction in sensitized animals that was significantly increased after antigen challenge. Antigen-induced hyperreactivity was completely blocked by tiotropium pretreatment but only partially blocked by atropine pretreatment. Surprisingly, although tiotropium blocked bronchoconstriction induced by intravenous acetylcholine, it did not inhibit vagally induced bronchoconstriction in sensitized controls, suggesting that tiotropium does not block hyperreactivity by blocking receptors for vagally released acetylcholine. Rather, tiotropium may have worked through an anti-inflammatory mechanism, since it inhibited eosinophil accumulation in lungs and around nerves.

Conclusions and implications: These data confirm that testing M_3 receptor blockade with exogenous acetylcholine does not predict vagal blockade. Our data also suggest that selective blockade of M_3 receptors may be effective in asthma via mechanisms that are separate from inhibition of bronchoconstriction.

Data in this Chapter have been accepted for publication as

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Figures 3.10 and 3.11 are unique to this thesis and were not included in this publication.

INTRODUCTION

In the lungs, airway smooth muscle tone is controlled by acetylcholine released from parasympathetic nerves. Acetylcholine stimulates M_3 muscarinic receptors on airway smooth muscle to induce smooth muscle contraction, resulting in bronchoconstriction. Parasympathetic nerves not only maintain airway tone, but they also mediate reflex bronchoconstriction (Canning, 2006). Diverse chemical and physical stimuli, including inhaled antigens, histamine, and cold air, activate afferent sensory nerves in the lungs to initiate a reflex that increases acetylcholine release from parasympathetic nerves and bronchoconstriction (Canning, 2006; Gold *et al.*, 1972; Sheppard, 1982).

Humans with asthma are hyperreactive to bronchoconstrictor stimuli. Exposure to antigens (antigen challenge) causes inflammation and airway hyperreactivity that develop within 24 h and persist for up to a week in both humans with asthma and sensitized animals (Brown *et al.*, 1998; Chung *et al.*, 1985; Cockcroft *et al.*, 1977). In antigen-challenged animals, airway hyperreactivity is mediated by increased acetylcholine release from the vagus nerves (Evans *et al.*, 1997; Evans *et al.*, 2001; Verbout *et al.*, 2007). Thus, anticholinergics, which block the action of acetylcholine at M_3 muscarinic receptors, would be expected to be beneficial in asthma. However, anticholinergics are not currently recommended for long-term control of asthma (EPR-3, 2007). This is because many studies that used the anticholinergics atropine or ipratropium in humans with asthma found they were unable to effectively block bronchoconstriction (Fryer *et al.*, 2008). This may be due, in part, to the low doses of

anticholinergics used, since benefits were found in studies with higher doses (Sheppard, 1982; Ward *et al.*, 1981).

Another possible explanation is that atropine and ipratropium are not selective for M_3 muscarinic receptors over other muscarinic receptor subtypes. M_1-M_3 muscarinic receptors are expressed on structural cells in the lungs, including smooth muscle, nerves, mucous glands, epithelial cells, fibroblasts, and endothelial cells, and all five muscarinic receptors (M_1-M_5) are found on inflammatory cells (Wessler *et al.*, 2008). Nonselective blockade of all muscarinic receptor subtypes could counteract the inhibition of bronchoconstriction through M_3 muscarinic receptor blockade in asthma. For example, M_2 muscarinic receptors found on prejunctional parasympathetic nerves are stimulated by acetylcholine to limit further release of acetylcholine from the nerves onto airway smooth muscle (Fryer *et al.*, 2008). Blocking these inhibitory M_2 receptors can increase acetylcholine enough to overcome M_3 receptor blockade and increase bronchoconstriction, as has been shown in guinea pigs treated with atropine and ipratropium (Fryer *et al.*, 1987).

Experiments in this paper were designed to test whether selectively blocking M_3 muscarinic receptors at the time of antigen challenge reduces subsequent airway hyperreactivity. Tiotropium bromide, a kinetically selective muscarinic receptor antagonist, was used to block M_3 muscarinic receptors in sensitized guinea pigs at the time of antigen challenge, and airway hyperreactivity was measured 24 h later. The results show that selective blockade of M_3 muscarinic receptors prevents airway hyperreactivity through a mechanism that is separate from direct inhibition of bronchoconstriction, and that this blockade is associated with inhibition of the inflammatory response to antigen challenge.

EXPERIMENTAL DESIGN

Sensitization and challenge with antigen

Guinea pigs were sensitized to ovalbumin (20 $mg \cdot kg^{-1}$, i.p) on days 1, 3, and 6 as described in Chapter 2, Methods. Starting 21 days after the last injection, some sensitized animals were challenged with an aerosol of 5% ovalbumin for 10 min or until signs of respiratory distress appeared as described in Chapter 2, Methods.

Treatments

Tiotropium is a kinetically selective M_3 receptor antagonist that dissociates more slowly from M_3 (human $t_{½}$ = 27-34.7 h) muscarinic receptors than M_2 (human $t_{½}$ = 2.6-3.6 h) or M_1 (human $t_{1/2}$ = 10.5-14.6 h) muscarinic receptors (Casarosa *et al.*, 2009; Disse *et al.*, 1993). In order to selectively block M_3 receptors at the time of challenge, tiotropium was administered with a dry powder insufflator 24 h prior to antigen challenge (see Chapter 2). This allowed tiotropium to dissociate from M_2 and M_1 receptors while remaining predominantly bound to M_3 receptors at the time of antigen challenge. Lactose powder administered with a dry powder insufflator served as a vehicle control for tiotropium (see Chapter 2, Methods).

Atropine is a short-acting nonselective muscarinic receptor antagonist. Atropine (1 $mg \cdot kg^{-1}$, i.p.) was administered 1 h before and 6 h after challenge as previously described (Verbout *et al.*, 2007) to block all muscarinic receptors during antigen challenge and during the early response post-antigen challenge (see Chapter 2, Methods).

To determine the effect of selectively blocking M_3 receptors with tiotropium at the time of challenge on airway hyperresponsiveness physiological measurements (see Chapter 2) were made in seven groups of animals. Three groups of animals were sensitized: 1) sensitized controls; 2) sensitized animals treated with lactose; and 3) sensitized animals treated with 1 $\mu g \cdot kg^{-1}$ tiotropium. *In vivo* physiology was measured 48 h after tiotropium or lactose administration in these animals. Four groups of animals were sensitized and challenged: 1) sensitized and challenged animals; 2) sensitized animals treated with lactose as a vehicle control and challenged 24 h later; 3) sensitized animals treated with 1 $\mu g \cdot kg^{-1}$ tiotropium and challenged 24 h later; 4) sensitized animals treated with atropine and challenged 1 h later. *In vivo* physiology was measured 24 h after challenge with inhaled ovalbumin in these groups, which corresponds to 48 h after tiotropium or lactose administration and 25 h after the first injection of atropine.

Physiological measurements were also made at the time of challenge (24 h after treatment with lactose or tiotropium) in four groups of animals: 1) sensitized controls (anesthetized with ketamine and xylazine); 2) sensitized animals treated with lactose (vehicle control); 3) sensitized animals treated with 0.2 $\mu g \cdot kg^{-1}$ tiotropium; and 4) sensitized animals treated with 1 $\mu g \cdot kg^{-1}$ tiotropium.

Intravenous tiotropium or lactose

To determine whether the route of tiotropium administration affects blockade of vagally induced bronchoconstriction cumulative doses of tiotropium or lactose vehicle were administered intravenously to nonsensitized guinea pigs. Blockade of vagus nerve-induced bronchoconstriction and intravenous-acetylcholine-induced bronchconstriciton were measured in the same animals as described in Chapter 2, Methods.

Measurements

Measurement of vagally induced bronchoconstriction and bradycardia, postjunctional muscarinic receptor function, inhibition of bronchoconstriction following intravenous administration of lactose and tiotropium, and inflammation in bronchoalveolar lavage was measured as described in Chapter 2, Methods. Histopathological evaluation of total and nerve-associated eosinophils in paraffin sections was also carried out as described in Chapter 2, Methods.

Statistical analysis

Values are means ± standard error of the mean (SEM). Baseline pulmonary and cardiovascular parameters were compared to the sensitized, no treatment group using one-way ANOVA with Bonferroni's correction for multiple comparisons. Frequency-response curves to nerve stimulation, dose-response curves to intravenous acetylcholine, intravenous tiotropium, or intravenous lactose were compared using two-way ANOVA for repeated measures with Bonferroni's correction. Bronchoalveoloar lavage leukocyte and tissue eosinophil data were log-transformed to equalize variances and then analyzed using one-way ANOVA with Bonferroni's correction. A Kruskal-Wallis test was used to compare lymphoctye means in Figure 8 because variances were not equalized with log transformation. Significant p-values are reported as *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Statistical data were analyzed with GraphPad Prism (version 5, GraphPad Software, Inc, La Jolla, CA).

RESULTS

Effect of M_3 muscarinic receptor blockade at the time of antigen challenge on subsequent airway hyperreactivity

To test the effect of selectively blocking M_3 muscarinic receptors during antigen challenge on development of airway hyperreactivity, tiotropium bromide was insufflated into the lungs of ovalbumin-sensitized guinea pigs. Tiotropium (1 $\mu g \cdot kg^{-1}$) was administered 24 h prior to antigen challenge to allow it to dissociate from M_1 and M_2 receptors, while still blocking M_3 muscarinic receptors at the time of antigen challenge. In separate experiments, atropine (1 $mg \cdot kg^{-1}$, i.p.) was used to block all muscarinic receptor subtypes during antigen challenge and during the early response post antigen challenge. Since atropine is short-acting (elimination $t_{½}$ = 2.4 h, (Kentala *et al.*, 1990)), it was administered 1 h before and 6 h after challenge. All physiological measurements were made 24 h after antigen challenge.

Antigen challenge did not change baseline pulmonary inflation pressure, heart rate, or blood pressure measured in vagotomized guinea pigs 24 h later relative to sensitized guinea pigs (Table 3.1). Similarly, baseline pulmonary and cardiac parameters were not changed in those sensitized or sensitized and challenged animals pretreated with tiotropium, lactose vehicle, or atropine (Table 3.1). Tiotropium, however, caused a small increase in baseline pulmonary inflation pressure in sensitized guinea pigs.

Antigen challenge of sensitized guinea pigs caused airway hyperreactivity that was mediated by the vagus nerves (Figure 3.1). Electrical stimulation of both vagus nerves induced frequency-dependent bronchoconstriction in vagotomized, sensitized guinea

pigs (Figure 3.1A) that was significantly potentiated 24 h after antigen challenge (Figure 3.1B). Pretreatment with lactose vehicle did not change this vagally induced bronchoconstriction in either sensitized (Figure 3.1A) or sensitized and challenged (Figure 3.1B) guinea pigs. Pretreatment with tiotropium did not reduce vagally induced bronchoconstriction in sensitized guinea pigs (Figure 3.1A). However, tiotropium pretreatment completely prevented the development of airway hyperreactivity in sensitized and challenged guinea pigs (Figure 3.1B). In contrast, atropine pretreatment only partially inhibited airway hyperreactivity following antigen challenge (Figure 3.1B). Thus, tiotropium pretreatment prevented subsequent vagally mediated airway hyperreactivity in antigen-challenged animals without reducing bronchoconstriction in sensitized control animals.

Acetylcholine released from parasympathetic nerves in the lungs stimulates M_3 muscarinic receptors on airway smooth muscle to induce bronchoconstriction. Smooth muscle M_3 receptor function was measured with exogenous intravenous acetylcholine. All of these animals were vagotomized to remove the confounding effects of reflex-induced bronchoconstriction. Antigen challenge did not increase intravenous-acetylcholine-induced bronchoconstriction (filled circles, Figure 3.2B) relative to sensitized controls (open circles, Figure 3.2A). Neither insufflated lactose nor atropine pretreatment had any affect on intravenous-acetylcholine-induced bronchoconstriction (Figure 3.2). Tiotropium inhibited intravenous-acetylcholine-induced bronchoconstriction in both sensitized (Figure 3.2A) and sensitized and challenged (Figure 3.2B) guinea pigs 48 h after administration. Thus, some airway smooth muscle M_3 receptors are still blocked 48 h after tiotropium treatment, while no M_3 receptors are blocked 18 h after atropine treatment.

M_3 receptor blockade at the time of antigen challenge

Since vagally induced bronchoconstriction was not blocked in sensitized guinea pigs 48 h after tiotropium treatment (Figure 3.1A), we tested whether smooth muscle M_3 receptors were blocked at the time of antigen challenge. Either lactose (vehicle control) or one of two different doses of tiotropium (0.2 $\mu g \cdot kg^{-1}$ or 1 $\mu g \cdot kg^{-1}$) was insufflated into the lungs of ovalbumin-sensitized guinea pigs, and physiological measurements were made 24 h later. Baseline pulmonary inflation pressure, heart rate, and blood pressure were not significantly changed by either dose of tiotropium or by lactose vehicle relative to sensitized guinea pigs that received no additional treatment (Table 3.2).

Bronchoconstriction induced by intravenous acetylcholine in sensitized guinea pigs was not changed by pretreatment with insufflated lactose vehicle (Figure 3.3A). However, intravenous-acetylcholine-induced bronchoconstriction was inhibited in a dose-dependent manner in sensitized animals pretreated with tiotropium (Figure 3.3A). The higher dose of tiotropium significantly decreased bronchoconstriction, by approximately 75%, demonstrating that 1 $\mu g \cdot kg^{-1}$ tiotropium still blocked M_3 muscarinic receptors on airway smooth muscle 24 h after administration, which was the time of antigen challenge in Figures 1 and 2.

Electrical stimulation of both vagus nerves caused frequency-dependent bronchoconstriction that was not inhibited by lactose vehicle in sensitized guinea pigs (Figure 3.3B). Surprisingly, and in contrast to intravenous acetylcholine, neither dose of tiotropium inhibited bronchoconstriction induced by electrical stimulation of both vagus nerves in sensitized guinea pigs (Figure 3.3B). Since vagally induced bronchoconstriction could be blocked with intravenous atropine (data not shown), it must

have been mediated by acetylcholine release onto muscarinic receptors. These data suggest that insufflated tiotropium did not block M_3 receptors at the neuromuscular junction with parasympathetic nerves.

Determining whether the route of tiotropium administration affects blockade of vagally induced bronchoconstriction

It is possible that insufflated tiotropium was unable to inhibit bronchoconstriction induced by electrical stimulation of the vagus nerves (Figure 3.3B) because the delivery method (insufflation) prevented tiotropium from distributing to M_3 muscarinic receptors at the junction with parasympathetic nerves. Therefore, we tested whether intravenous administration of tiotropium blocked equally M_3 muscarinic receptors stimulated by intravenous acetylcholine and vagally released acetylcholine. Reproducible baseline bronchoconstrictions to intravenous acetylcholine (4 $\mu g \cdot kg^{-1}$) and electrical stimulation of the vagus nerves (10 Hz) were measured in vagotomized, nonsensitized guinea pigs treated with gallamine to block neuronal M_2 muscarinic receptors. Cumulative doses of intravenous tiotropium inhibited bronchoconstriction in a dose-dependent manner (Figure 3.4). The lowest dose of tiotropium (0.3 $\mu g \cdot kg^{-1}$) significantly inhibited intravenous-acetylcholine-induced bronchoconstriction by more than 50%, whereas only the highest dose of tiotropium (10 $\mu g \cdot kg^{-1}$) significantly inhibited vagally induced bronchoconstriction (relative to the appropriate intravenous lactose controls). At a minimum, intravenous tiotropium was 10 fold more potent for inhibition of intravenous-acetylcholine-induced bronchoconstriction than for vagally induced bronchoconstriction. Thus, lower doses of intravenous tiotropium were required to block M_3 muscarinic receptors involved in exogenous acetylcholine-induced bronchoconstriction than were required to block endogenous acetylcholine-induced bronchoconstriction.

For these experiments, lactose (filler in Spiriva) was used as a control and was given to guinea pigs in separate experiments intravenously (dashed lines, Figure 3.4). We found no difference between the effect of lactose on intravenous-acetylcholine-induced bronchoconstriction and vagally induced bronchoconstriction. Yet we did find that overall bronchoconstriction appears to be inhibited in the lactose controls. Whether this inhibition is due to time, since the experiments lasted close to 2 hours, or to lactose concentration was not tested. Regardless, the inhibitory effect of tiotropium was substantially greater than lactose.

Determining whether tiotropium blocked cardiac M_2 receptors

In the heart, acetylcholine stimulates M_2 muscarinic receptors to cause bradycardia. Neither insufflated tiotropium nor insufflated lactose vehicle affected bradycardia induced by intravenous acetylcholine or by electrical stimulation of the vagus nerves relative to untreated sensitized controls 24 h after insufflation into the lungs (Figure 3.5), which would be at the time of antigen challenge. These data indicate that cardiac M_2 receptors were not blocked by tiotropium at the time of antigen challenge.

Similarly, in the antigen challenge model, bradycardia induced by either intravenous acetylcholine or electrical stimulation of the vagus nerves in sensitized (Figure 3.6, A and C) and sensitized and challenged (Figure 3.6, B and D) guinea pigs was unchanged by antigen challenge, lactose pretreatment, tiotropium pretreatment, or atropine pretreatment (Figure 3.6). Thus, cardiac M_2 muscarinic receptors were not blocked by tiotropium or atropine at the time physiological measurements were made (48 h after tiotropium and 18 h after atropine administration).

Effect of M_3 receptor blockade during antigen challenge on airway inflammation

We tested whether tiotropium pretreatment had any effect on airway inflammation. Insufflating powder into the lungs, regardless of whether it contained tiotropium, increased total bronchoalveolar cells (Figure 3.7). Both 24 h (Figure 3.7) and 48 h (Figure 3.8) after lactose or tiotropium powder administration, neutrophils were significantly increased relative to untreated sensitized controls. Antigen challenge did not further increase this neutrophilic inflammation (Figure 3.8).

Antigen challenge increased eosinophils in bronchoalveolar lavage of sensitized guinea pigs regardless of whether they received no treatment, lactose, tiotropium, or atropine pretreatment. However, this was only statistically significant following correction for multiple comparisons in animals treated with lactose 24 h prior to challenge (Figure 3.8).

In lung tissue, quantitative analysis of eosinophils in airway smooth muscle and adventitia and around airway nerves demonstrated that antigen challenge of sensitized guinea pigs increased total and nerve-associated eosinophils in lactose vehicle pretreated animals (Figure 3.9). In tiotropium pretreated guinea pigs, antigen challenge did not significantly increase either total eosinophils or nerve-associated eosinophils (Figure 3.9). Thus, tiotropium inhibited eosinophilic inflammation following antigen challenge in airway tissues. In the absence of lactose, there was a similar increase in total airway eosinophils from 264 ± 33 eosinophils/mm^2 in sensitized guinea pigs to 437 ± 73 eosinophils/mm^2 in sensitized and challenged guinea pigs. Additionally, eosinophils specifically associated with airway nerves were also increased from 67 ± 13 eosinophils/mm^2 in sensitized guinea pigs to 134 ± 27 eosinophils/mm^2 in sensitized and

challenged animals. In the presence of atropine, there were 379±48 total airway eosinophils/mm^2 and 126±28 eosinophils/mm^2 associated with nerves in sensitized and challenged guinea pigs.

Table 3.1 Baseline Pulmonary and Cardiovascular Parameters for Data in Vagotomized Guinea Pigs for Figures 1-2, 6, and 8-9.

Pretreatment	n	Pulmonary Inflation Pressure (mm H_2O)	Heart Rate (beats/min)	Blood Pressure (mmHg)	
				Systolic	Diastolic
Sensitized					
None	6	87±4	291±3	51±4	29±4
Lactose	6	103±6	291±10	49±0.4	28±2
Tiotropium	7	107±5 *	284±8	44±5	26±3
Sensitized and Challenged (24 h after challenge of sensitized guinea pigs)					
None	7	106±2	278±9	54±2	29±1
Lactose	6	98±4	299±13	50±6	32±5
Tiotropium	6	90±3	293±8	51±2	30±3
Atropine	7	97±5	293±10	49±2	28±2

Values are means ± SEM. n, number of guinea pigs. All baseline parameter means were compared to the sensitized no treatment group; statistically significant changes are noted with *.

Table 3.2 Baseline Pulmonary and Cardiovascular Parameters in Vagotomized Guinea Pigs for Data in Figures 3, 5, and 7.

Pretreatment	n	Pulmonary Inflation Pressure (mm H_2O)	Heart Rate (beats/min)	Blood Pressure (mmHg)	
				Systolic	Diastolic
Sensitized					
None	3	107±9	315±10	41±7	19±2
Lactose	3	100±0	317±16	41±4	26±3
Tiotropium, 0.2 $\mu g \cdot kg^{-1}$	4	108±6	305±9	43±3	26±5
Tiotropium, 1 $\mu g \cdot kg^{-1}$	4	125±16	293±4	43±2	24±1

Values are means ± SEM. n, number of guinea pigs. None of the baseline parameter means were statistically different from the sensitized, no treatment group.

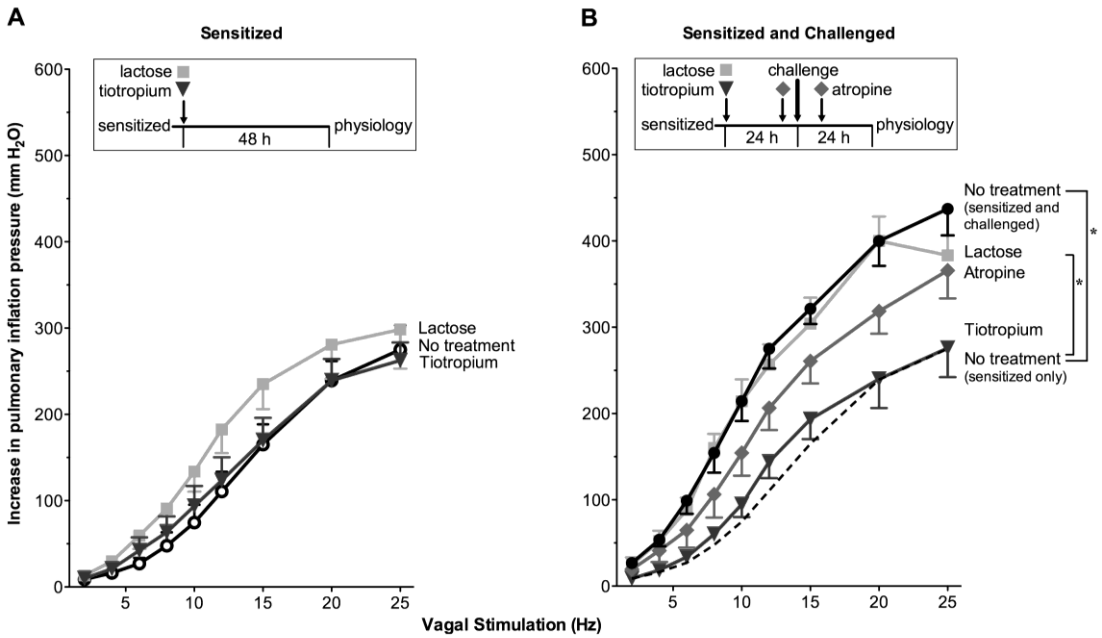

Figure 3.1 Selectively blocking M_3 receptors with tiotropium at the time of antigen challenge prevents development of subsequent airway hyperreactivity but does not inhibit bronchoconstriction. Electrical stimulation of both vagus nerves (2-25 Hz, 10V, 0.2 msec, 5 sec pulse train) produced frequency-dependent bronchoconstrictions (measured as an increase in pulmonary inflation pressure) in vagotomized, sensitized, guinea pigs (A, open circles; also shown in B as a dashed line) that were potentiated 24 h after antigen challenge (B, filled circles). Lactose vehicle did not change vagally induced bronchoconstriction 48 h after administration in either sensitized (A, squares) or sensitized and challenged guinea pigs (B, squares). Tiotropium (1 $\mu g \cdot kg^{-1}$, i.t.) did not change vagally induced bronchoconstriction in sensitized guinea pigs (A, triangles) measured 48 h after administration but did prevent potentiation of vagally induced bronchoconstriction in sensitized and challenged guinea pigs (B, triangles). Unlike tiotropium, atropine (1 $mg \cdot kg^{-1}$, i.p.) administered 1 h before and 6 h after challenge only partially prevented the potentiation of vagally induced bronchoconstriction in sensitized

and challenged guinea pigs (B, diamonds). Data are expressed as means ± SEM, n=6-7. All frequency response curves were compared to the sensitized, no treatment group (open circles) and statistically significant changes are noted with *.

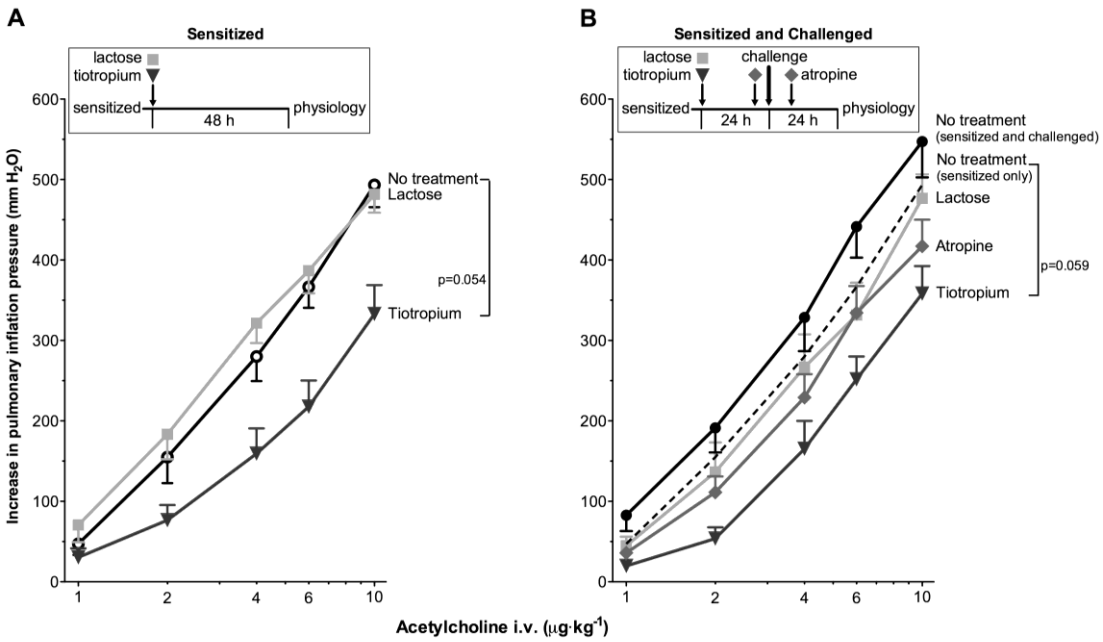

Figure 3.2 Tiotropium still blocks some M_3 muscarinic receptors on airway smooth muscle 48 h after administration in both sensitized (A) and sensitized and challenged (B) guinea pigs. In vagotomized, sensitized, guinea pigs i.v. acetylcholine caused dose-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A, open circles; also shown in B as a dashed line) that was not changed by antigen challenge (B, filled circles). Bronchoconstriction was not changed by lactose vehicle 48 h after administration (A and B, squares). Atropine (1 $mg \cdot kg^{-1}$, i.p.) administered 1 h before and 6 h after antigen challenge also did not change i.v.-acetylcholine-induced bronchoconstriction 24 h after challenge (B, diamonds). In contrast, tiotropium (1 $\mu g \cdot kg^{-1}$, i.t.) inhibited i.v.-acetylcholine-induced bronchoconstriction in both sensitized (A, triangles) and sensitized and challenged (B, triangles) guinea pigs when measured 48 h after administration. Data are expressed as mean $\pm$ SEM, n=6-7. All dose response curves were compared to the sensitized, no treatment group (open circles). p-values approaching significance are reported following Bonferroni correction for six comparisons.

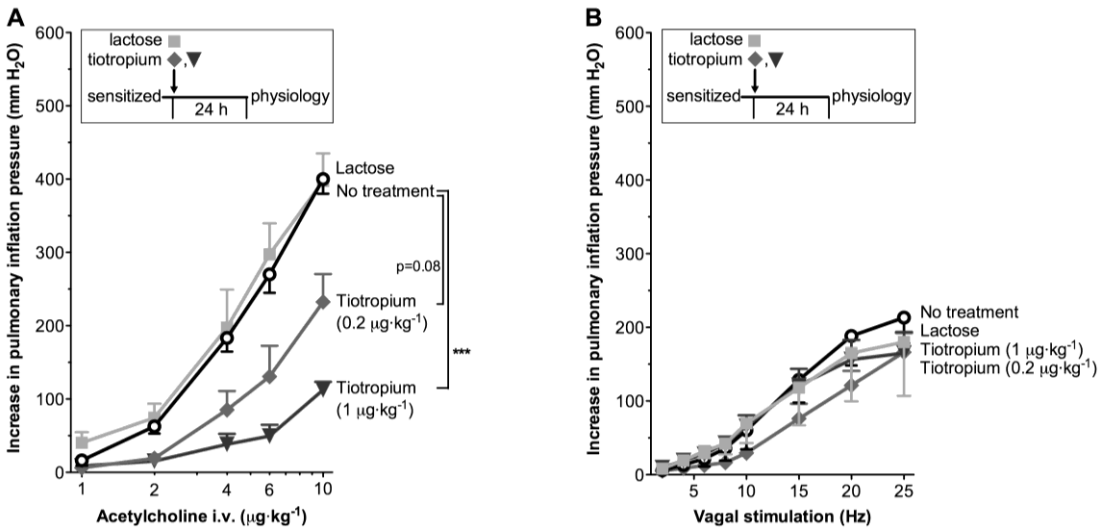

Figure 3.3 Tiotropium blocks smooth muscle M_3 receptors 24 h after administration when tested with intravenous acetylcholine but not vagally released acetylcholine. In vagotomized, sensitized, guinea pigs i.v. acetylcholine caused dose-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A, open circles), and electrical stimulation of both vagus nerves caused frequency-dependent bronchoconstriction (B, open circles; 2-25 Hz, 10V, 0.2 msec, 5 sec pulse train). Bronchoconstriction was not changed by lactose vehicle 24 h after administration (A and B, squares). Tiotropium, 0.2 $\mu g \cdot kg^{-1}$ i.t. (diamonds) and 1 $\mu g \cdot kg^{-1}$ i.t. (triangles), inhibited bronchoconstriction induced by i.v. acetylcholine (A) but not bronchoconstriction induced by vagal stimulation (B) 24 h after administration. Data are expressed as mean ± SEM, n=3-4. All dose and frequency response curves were compared to the no treatment group (open circles); statistically significant changes are noted with *.

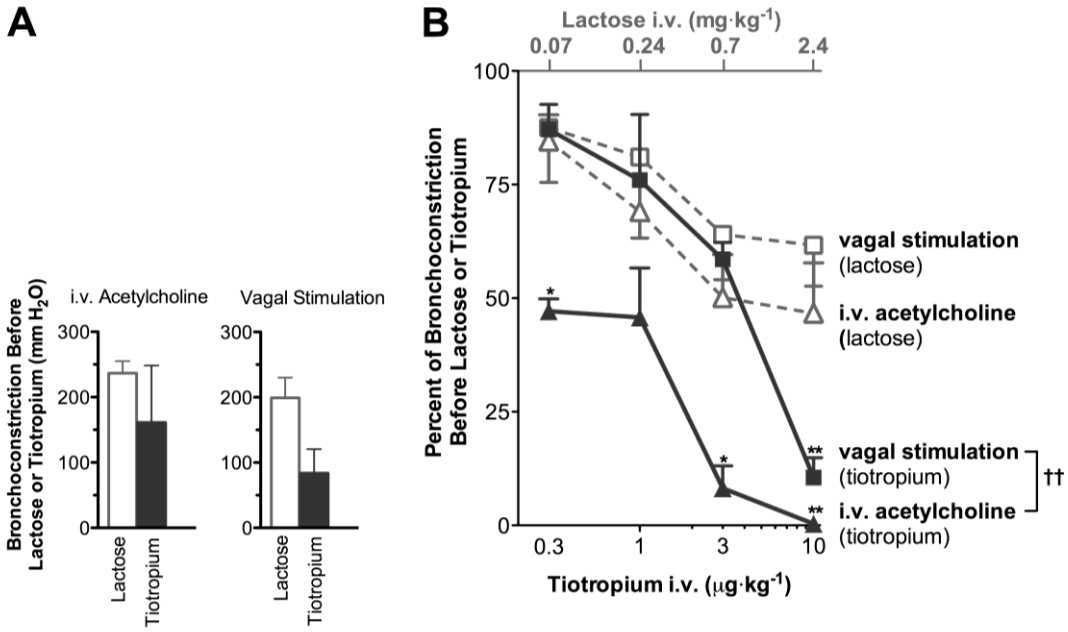

Figure 3.4 Intravenous administration of tiotropium blocks bronchoconstriction induced by intravenous acetylcholine at lower doses than are required to block bronchoconstriction induced by electrically stimulating the vagus nerves.

Bronchoconstriction, induced by either electrically stimulating both vagus nerves (squares; 10 Hz, 10V, 0.2 msec, 5 sec pulse train, at 2 min intervals) or by i.v. acetylcholine (triangles; 4 $\mu g \cdot kg^{-1}$, every 6 min between vagal stimulations), was measured in vagotomized guinea pigs that were not sensitized or challenged. Bronchoconstriction before intravenous drug administration is graphed on the left as an increase in pulmonary inflation pressure in mm H_2O (A), and on the right as a percentage of this bronchoconstriction (B). All animals received gallamine (3 $mg \cdot kg^{-1}$, i.v.) to block neuronal M_2 muscarinic receptors. Cumulative doses of tiotropium (filled symbols) inhibited bronchoconstriction in a dose-dependent manner, but it was significantly more effective against i.v.-acetylcholine-induced bronchoconstriction (B, filled triangles) than it was against vagally induced bronchoconstriction (B, filled

squares). Lactose (B, open symbols) was administered as a control and concentrations are shown on the upper axis. Bronchoconstriction was also inhibited with lactose, although the effect was not significantly different between i.v.-acetylcholine-induced bronchoconstriction (B, open triangles) and vagally induced bronchoconstriction (B, open squares). Data are expressed as means ± SEM, n=3. Inhibition of i.v.–acetylcholine-induced bronchoconstriction was compared to inhibition of vagally induced bronchoconstriction for each treatment (lactose and tiotropium); significance is noted with †. Inhibition by tiotropium of bronchoconstriction induced by vagal stimulation or i.v. acetylcholine was also compared at each dose to inhibition in the lactose control; significance is noted with *.

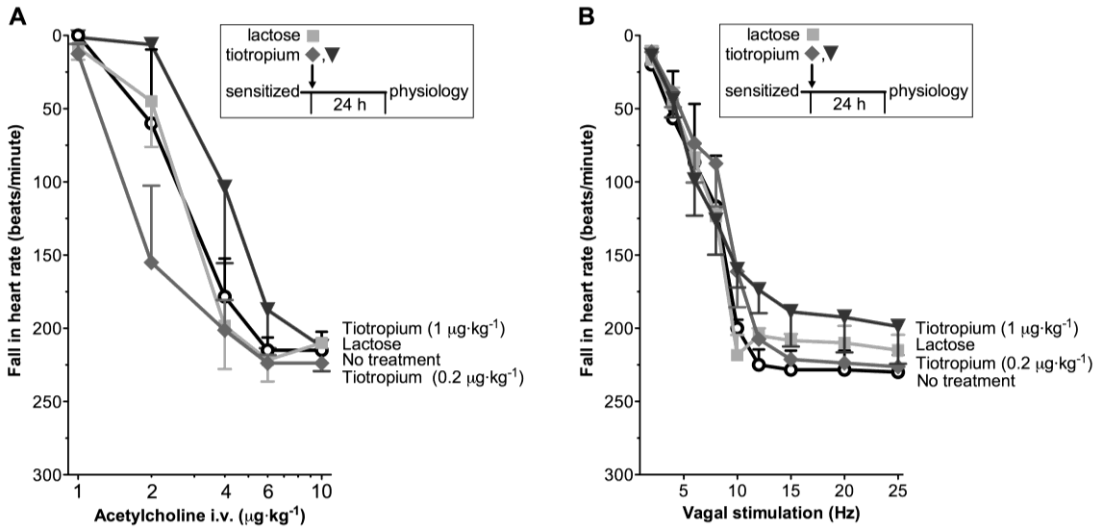

Figure 3.5 Tiotropium does not block M_2 muscarinic receptors in the heart 24 h after administration. In vagotomized, sensitized, guinea pigs i.v. acetylcholine caused dose-dependent bradycardia measured as a fall in heart rate in beats per minute (A, open circles). Electrical stimulation of both vagus nerves caused frequency-dependent bradycardia (B, open circles; 2-25 Hz, 10V, 0.2 msec, 5 sec pulse train). Neither lactose vehicle (squares), 0.2 $\mu g \cdot kg^{-1}$ i.t. tiotropium (diamonds), nor 1 $\mu g \cdot kg^{-1}$ i.t. tiotropium (inverted triangles) affected bradycardia induced by i.v. acetylcholine (A) or vagal stimulation (B) 24 h after administration. Data are expressed as mean $\pm$ SEM, n=3-4. None of the dose and frequency response curves were statistically different from the no treatment group (open circles).

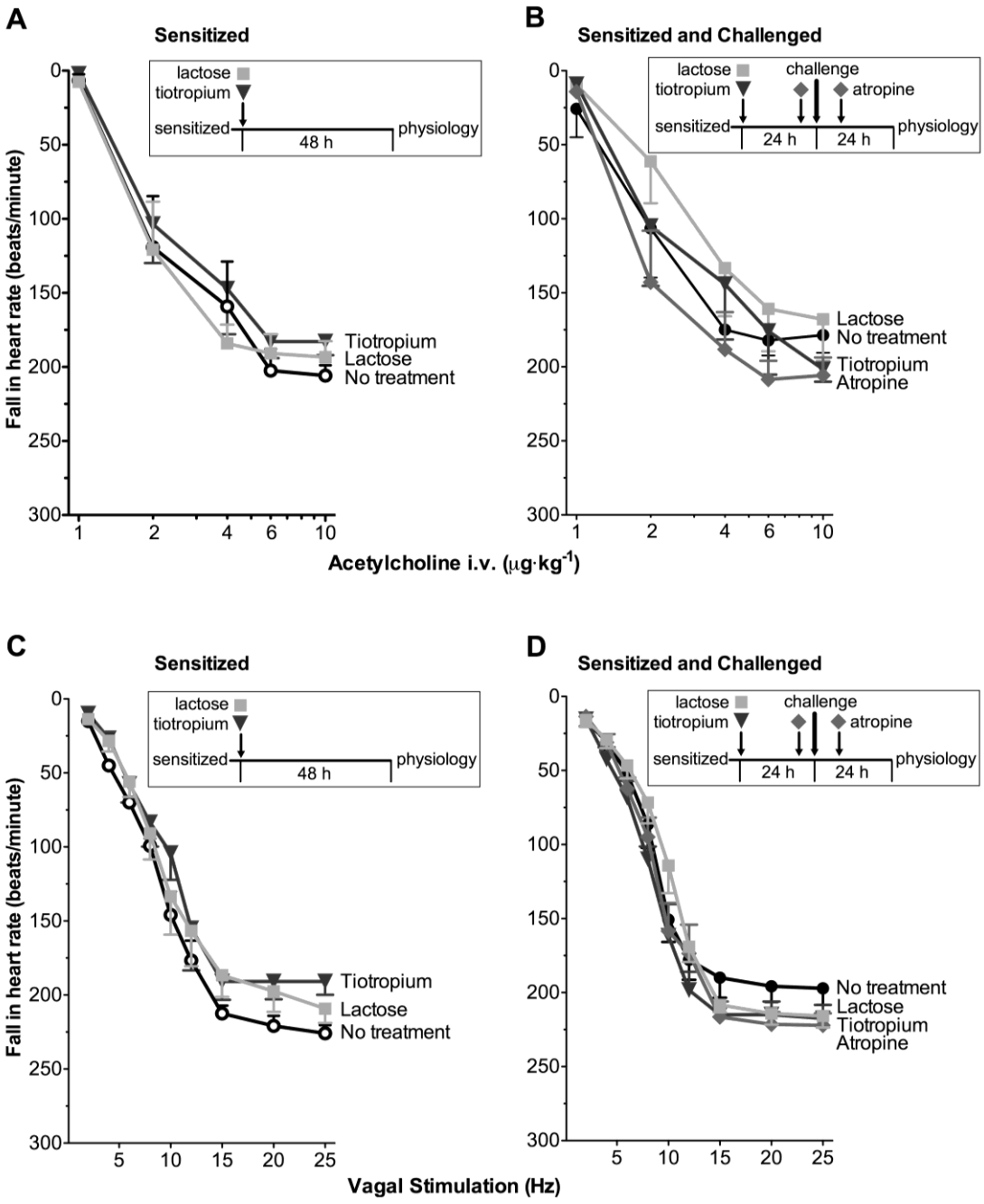

Figure 3.6 Lactose, tiotropium, and atropine pretreatment each had no affect on bradycardia in sensitized or sensitized and challenged guinea pigs. In vagotomized, sensitized, guinea pigs i.v. acetylcholine caused dose-dependent bradycardia (A, open circles) and electrical stimulation of both vagus nerves (2-25 Hz, 10V, 0.2 msec, 5 sec pulse train) caused frequency-dependent bradycardia (C, open

circles), both measured as a fall in heart rate in beats per minute. Antigen challenge did not change bradycardia (B and D, filled circles). Neither lactose (squares) nor tiotropium (1 $\mu g \cdot kg^{-1}$, i.t., triangles) changed bradycardia measured 48 h after administration in sensitized (A and C) or sensitized and challenged guinea pigs (B and D). Similarly, atropine (1 $mg \cdot kg^{-1}$, i.p., diamonds) administered 1 h before and 6 h after antigen challenge also did not change bradycardia in sensitized and challenged animals. Data are expressed as means $\pm$ SEM, n=6-7. None of the dose and frequency response curves were statistically different from the sensitized, no treatment controls (open circles).

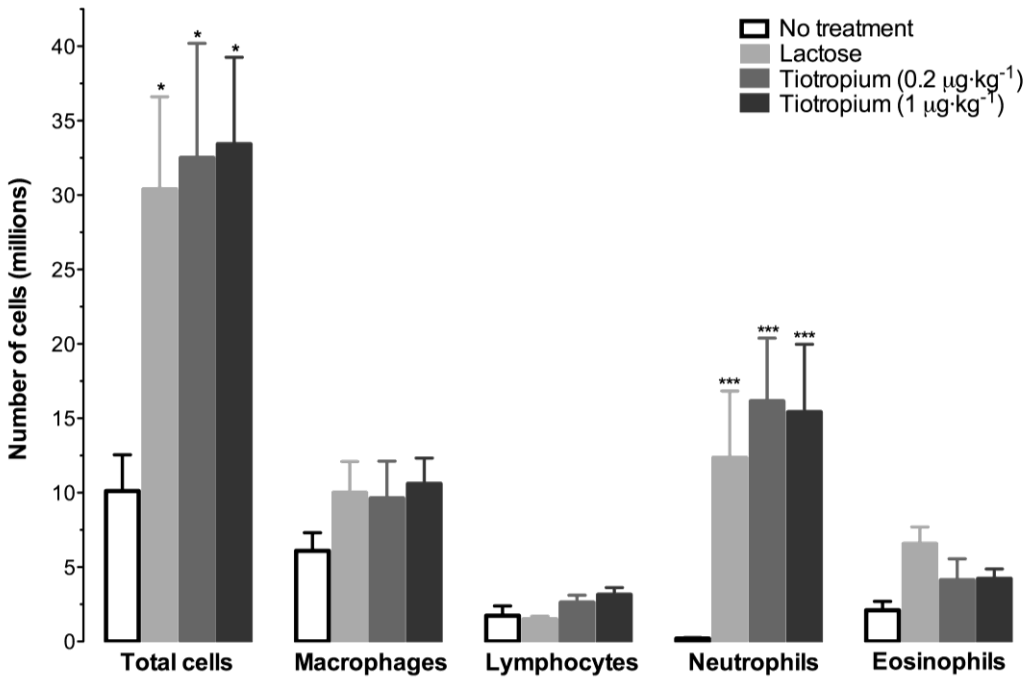

Figure 3.7 Neutrophils in bronchoalveolar lavage were increased 24 h after insufflation of lactose powder regardless of whether it also contained tiotropium. Inflammatory cells were counted in bronchoalveolar lavage of sensitized guinea pigs (open bars). Lactose vehicle (light gray), 0.2 $\mu g \cdot kg^{-1}$ i.t. tiotropium (medium gray), and 1 $\mu g \cdot kg^{-1}$ i.t. tiotropium (dark gray) all increased total and neutrophil cell numbers in sensitized guinea pigs. Data are expressed as mean $\pm$ SEM, n=3-4. Mean leukocyte numbers were compared to the no treatment group (open circles); statistically significant changes are noted with *.

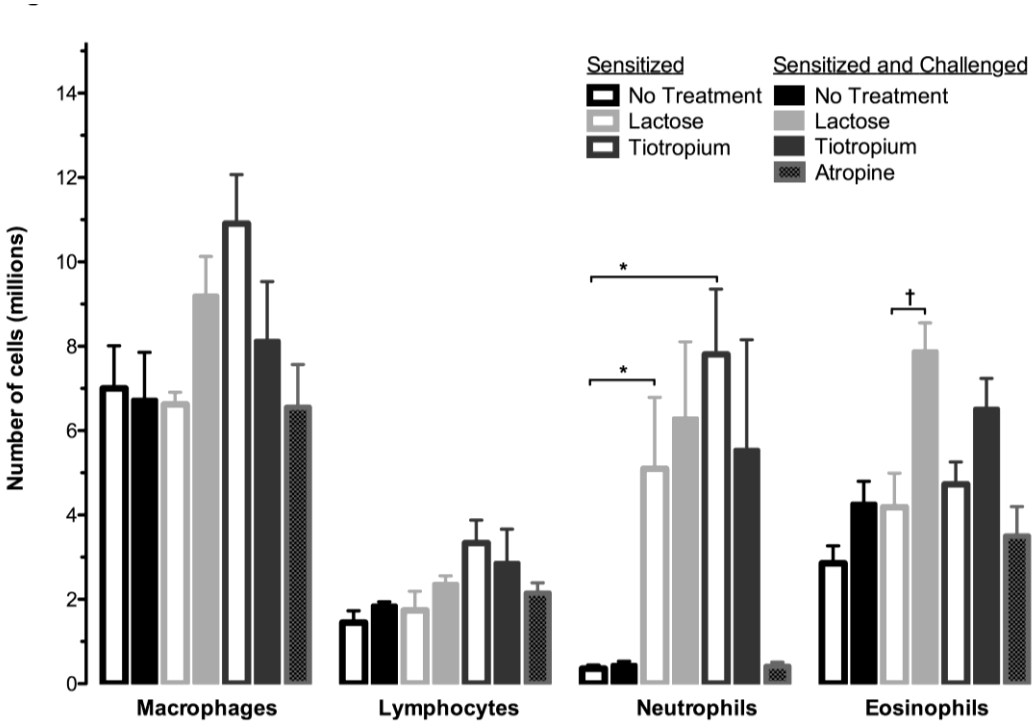

Figure 3.8 Neutrophils in bronchoalveolar lavage remain increased 48 h after powder insufflation. Inflammatory cells were counted in bronchoalveolar lavage of sensitized guinea pigs (open bars) and sensitized and challenged guinea pigs (filled bars). Neutrophils increased following pretreatment with lactose vehicle (open light gray) or 1 $\mu g \cdot kg^{-1}$ i.t. tiotropium (open dark gray) relative to sensitized controls that received no treatment (open black). Antigen challenge did not increase neutrophils further in animals that received no pretreatment (filled black), lactose (filled light gray), tiotropium (filled dark gray), or atropine (1 $mg \cdot kg^{-1}$, i.p., checkered gray) pretreatment. Antigen challenge did increase eosinophils, and this was significant in animals pretreated with lactose vehicle (light gray). Values are means $\pm$ SEM, n=5-7. Mean leukocyte numbers in sensitized animals pretreated with lactose or tiotropium were compared to sensitized animals that received no treatment; significant changes are noted with *. Each antigen-challenged group was also compared to the appropriate unchallenged control group; significance is noted with †.

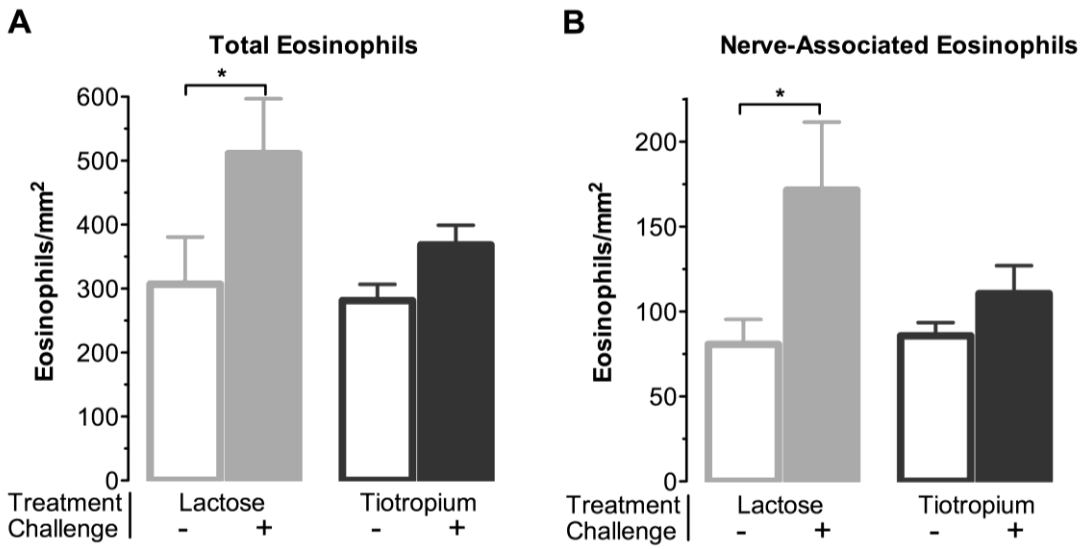

Figure 3.9 Tiotropium reduces total and nerve-associated eosinophils in the lungs of antigen-challenged guinea pigs. Total (A) and nerve-associated (B, within 8 μm of a nerve) eosinophils were counted per mm^2 airway smooth muscle and adventitia in tissue sections. Nerves were labeled with antibody to protein gene product 9.5, and eosinophils were stained with Chromotrope 2R. Eosinophils are found in the lungs and around nerves in sensitized guinea pigs pretreated with lactose vehicle (open light gray bars) or tiotropium (1 $\mu g \cdot kg^{-1}$, i.t., open dark gray bars). Antigen challenge increased total and nerve-associated eosinophils in sensitized guinea pigs pretreated with lactose (filled light gray bars). In contrast, in sensitized guinea pigs that received tiotropium 24 h prior to challenge, total and nerve-associated eosinophils were not significantly increased following antigen challenge (filled dark gray bars). Data are expressed as means ± SEM, n=6-7. Comparisons were made between all groups; statistically significant changes are noted with *.

DISCUSSION

The data presented here demonstrate that selectively blocking M_3 muscarinic receptors during antigen challenge prevents subsequent development of airway hyperreactivity in guinea pigs. Muscarinic receptor antagonists with 100-fold higher affinity for M_3 receptors over other muscarinic receptor subtypes are not readily available; therefore, we used tiotropium, a clinically used (GOLD, 2009; Peters *et al.*, 2010; Vogelmeier *et al.*, 2011), kinetically selective, M_3 receptor antagonist. Tiotropium was administered 24 h prior to antigen challenge to take advantage of its slow dissociation from M_3 receptors relative to M_2 and M_1 receptors (Casarosa *et al.*, 2009; Disse *et al.*, 1993). Functional studies in guinea pig trachea show M_3 receptor function begins to return 9 h after tiotropium washout, while M_2 receptor function returns fully within 2 h (Takahashi *et al.*, 1994). In our study, we found no evidence for pharmacological blockade of M_2 muscarinic receptors 24 h after tiotropium administration, since intravenous-acetylcholine-induced bradycardia was not reduced. However, as previously shown for guinea pig (Villetti *et al.*, 2006) tiotropium still blocked M_3 receptors on airway smooth muscle when tested with intravenous acetylcholine.

Baseline heart rate was not changed with antigen challenge or pretreatments. However, there was a significant 20 mm H_2O increase in baseline pulmonary inflation pressure in sensitized guinea pigs pretreated with tiotropium. This increase is unlikely to be physiologically significant, since greater increases (150 mm H_2O) following ozone do not suppress bronchoconstriction (Verhein *et al.*, 2008). I was unable to determine whether tiotropium pretreatment reduced vagal tone in our animals, since baseline pulmonary inflation pressure was measured after vagotomy.

The Fryer and Jacoby laboratories have demonstrated that airway hyperreactivity develops within 24 h of antigen challenge in sensitized guinea pigs (Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 2006; Verbout *et al.*, 2007). This was confirmed in those experiments where bronchoconstriction induced by electrical stimulation of both vagus nerves was significantly increased in sensitized and challenged guinea pigs relative to sensitized controls. Airway hyperreactivity was mediated by the vagus nerves, since airway smooth muscle contraction to intravenous acetylcholine, which bypasses the nerves in vagotomized animals, was not similarly increased. Tiotropium pretreatment completely prevented hyperreactivity in antigen-challenged guinea pigs, but did not block smooth muscle M_3 muscarinic receptors innervated by the vagus nerves. This suggests that tiotropium prevents airway hyperreactivity in antigen challenged guinea pigs through a mechanism that is separate from inhibition of bronchoconstriction (summarized in Figure 3.10).

Eosinophilic inflammation correlates with asthma severity (Bousquet *et al.*, 1990). In patients with eosinophilic asthma, reducing lung eosinophils significantly decreases asthma exacerbations and allows decreased steroid use (Haldar *et al.*, 2009; Nair *et al.*, 2009). In antigen-challenged guinea pigs, hyperreactivity is mediated by eosinophil recruitment to airway nerves and subsequent activation and release of eosinophil major basic protein (Costello *et al.*, 1997; Evans *et al.*, 1997; Evans *et al.*, 2001; Verbout *et al.*, 2007). Tiotropium reduced eosinophil accumulation in airway tissue and around airway nerves following antigen challenge. This could be a mechanism for preventing airway hyperreactivity (Figure 3.10), since airway hyperreactivity is also prevented by treatments that inhibit eosinophil association with nerves or inhibit deposition of eosinophil major basic protein on nerves (Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et*

al., 2006; Nie *et al.*, 2009). It is worth noting that bronchoalveolar neutrophils were increased by powder insufflation into the lungs. However, increased neutrophils were not associated with hyperreactivity, so the presence of neutrophils alone is not sufficient to cause airway hyperreactivity.

Tiotropium has already been shown to reduce airway remodeling, including smooth muscle thickening, smooth muscle hypercontractility, and mucous gland hypertrophy. It also reduces Th2 cytokines and lung eosinophils associated with chronic antigen challenge (Bos *et al.*, 2007; Gosens *et al.*, 2005; Ohta *et al.*, 2010). Furthermore, tiotropium's anti-inflammatory effects are not limited to antigen challenge, since tiotropium also reduces cytokine production and neutrophil accumulation following cigarette smoke exposure in mice (Wollin *et al.*, 2010). Together these studies support my data that tiotropium may prevent vagally mediated airway hyperreactivity by reducing inflammation.

Since smooth muscle M_3 receptors innervated by the nerves were not blocked at the time of antigen challenge, it is possible that tiotropium prevented airway hyperreactivity by blocking acetylcholine released from non-neuronal sources such as epithelial cells or macrophages (Wessler *et al.*, 2008). Tiotropium could reduce eosinophil trafficking into tissue by blocking M_3 receptors on airway epithelial cells or alveolar macrophages that normally induce release of eosinophil chemoattractants (Buhling *et al.*, 2007; Koyama *et al.*, 1998; Sato *et al.*, 1998). Tiotropium could also reduce eosinophil accumulation by blocking M_3 muscarinic receptors on eosinophils (Verbout *et al.*, 2006). But, the role of M_3 muscarinic receptors on eosinophils is unknown. In addition, tiotropium could block M_3 receptors on smooth muscle cells not innervated by nerves to inhibit pro-inflammatory cytokine release (Gosens *et al.*, 2009) following antigen challenge.

In contrast to selectively blocking M_3 receptors, blockade of all muscarinic receptors with atropine during antigen challenge was much less effective in preventing airway hyperreactivity, suggesting that blockade of all muscarinic receptors may counteract the benefit of blocking M_3 receptors. For example, M_1 muscarinic receptors on human airway mast cells inhibit evoked histamine release (Reinheimer *et al.*, 2000), and it may be important to maintain the activity of these inhibitory muscarinic receptors. Alternatively, differences in atropine and tiotropium kinetics might explain differences in prevention of airway hyperreactivity, since tiotropium, but not atropine, blocked some smooth muscle M_3 receptors when airway responsiveness was measured. However, this seems unlikely because neither drug blocked smooth muscle M_3 receptors innervated by vagal nerves (Figure 3.1A and (Verbout *et al.*, 2007)).

The Fryer and Jacoby laboratories have previously shown that atropine at the time of antigen challenge makes airway hyperreactivity significantly worse (Verbout *et al.*, 2009; Verbout *et al.*, 2007). Although, atropine did not potentiate hyperreactivity in this study, it was not completely protective either. Thus, tiotropium, but not the non-selective atropine, prevented development of hyperreactivity. Lack of atropine-induced hyperreactivity in our study may be explained by the additional use of antihistamines and isoproteronol (β-agonist) in the previous studies, while neither drug was used here.

During the course of these studies, I also observed that insufflated tiotropium blocked bronchoconstriction induced by intravenous acetylcholine but did not block bronchoconstriction induced by vagally released acetylcholine in sensitized guinea pigs. These data are consistent with previous studies using atropine in humans with asthma and in anesthetized dogs (Holtzman *et al.*, 1983; Sheppard *et al.*, 1983; Sheppard,

1982) that demonstrate there are several variables contributing to the efficacy of a competitive muscarinic antagonist. These include dose of antagonist, route of antagonist administration, and regional differences in agonist concentration. In these previously published studies, low doses of inhaled atropine blocked bronchoconstriction induced by inhaled acetylcholine (or methacholine) and also maximally decreased baseline vagally mediated cholinergic tone. However, much higher doses of atropine (8-16 fold) were required to block bronchoconstriction induced by either indirect (cold air) or direct (electrical) stimulation of the vagus nerves (Holtzman *et al.*, 1983; Sheppard, 1982). Thus, similar to atropine, I show that doses of insufflated tiotropium that block exogenous acetylcholine do not block vagally induced bronchoconstriction. This is important because our data and these papers confirm that blockade of exogenous acetylcholine-induced bronchoconstriction is not an adequate measure of blockade of acetylcholine from its physiological source, the vagus nerves. Furthermore, these data demonstrate that inhibition of vagally induced bronchoconstriction is not necessary to prevent subsequent development of airway hyperreactivity.

The difference in atropine's ability to inhibit bronchoconstriction induced by inhaled acetylcholine and vagally released acetylcholine only occurs when atropine is administered by inhalation (Holtzman *et al.*, 1983; Sheppard *et al.*, 1983). Intravenous atropine distributes evenly to all airway smooth muscle muscarinic receptors and equally blocks bronchoconstriction induced by exogenous and endogenous acetylcholine (Holtzman et al., 1983). In contrast, in our study, intravenous tiotropium did not equally block exogenous acetylcholine and vagally induced bronchoconstriction; significantly higher doses were required to block vagally induced bronchoconstriction. These data indicate that the dose of tiotropium is critical for bronchodilation, independent of delivery route.

Tiotropium is a competitive antagonist (Casarosa *et al.*, 2009), but its slow dissociation kinetics make it functionally irreversible at M_3 receptors. My data suggest a model (Figure 3.11) where tonic neuronal stimulation maintains high local concentrations of acetylcholine at smooth muscle M_3 receptors, while M_3 receptors further away from the nerves are exposed to lower endogenous acetylcholine concentrations. Tiotropium would have to compete with acetylcholine for receptor sites and this would require more tiotropium around nerves where agonist concentrations are high. Thus, even though insufflated tiotropium blocked bronchoconstriction induced by exogenous agonists, it would require a higher dose to block vagally induced bronchoconstriction.

In summary, the data presented here and summarized in Figure 3.10 demonstrate that selectively blocking M_3 muscarinic receptors with tiotropium at the time of antigen challenge prevents subsequent vagally mediated airway hyperreactivity. Importantly, airway hyperreactivity is prevented by a dose of tiotropium that is unable to inhibit vagally induced bronchoconstriction. Furthermore, our data suggest that the mechanism may be anti-inflammatory and that selective blockade of airway M_3 muscarinic receptors may be an effective treatment for asthma separate from bronchodilation.

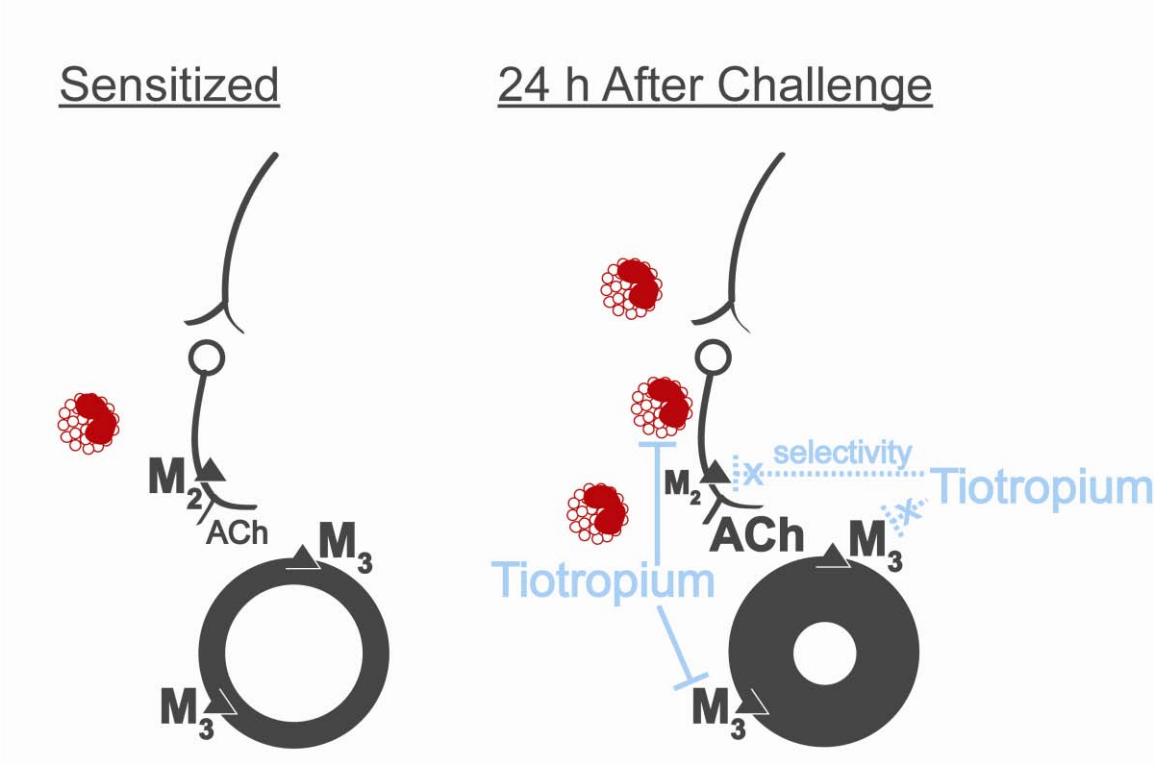

Figure 3.10 Selective blockade of M_3 receptors 24 h after challenge prevents airway hyperreactivity through a non-bronchodilating mechanism. In sensitized guinea pigs (left), parasympathetic nerves release acetylcholine onto smooth muscle M_3 receptors to induced smooth muscle contraction and bronchoconstriction. Acetylcholine also stimulates neuronal M_2 receptors to inhibit further release of acetylcholine. While eosinophils are present in the lung their presence does not result in airway hyperresponsiveness. Following antigen challenge, airway hyperreactivity develops (right). Eosinophils (red) are recruited into the lung where they associate with the nerves and contribute to airway hyperreactivity. Eosinophil major basic protein is an antagonist for neuronal M_2 receptors, and blockade of these receptors is one mechanism that results in increased acetylcholine release from nerves. Tiotropium administered 24 h prior to challenge prevents the development of airway hyperreactivity 24 h after challenge. Tiotropium did not block neuronal M_2 receptors at the time of challenge due

to kinetic selectivity nor M_3 receptors innervated by the nerves, due to dose. However, tiotropium did inhibit smooth muscle M_3 receptors that were not innervated by the nerves and also reduced total and nerve-associated eosinophils in the airways. Therefore, tiotropium may have prevented airway hyperreactivity through an anti-inflammatory mechanism.

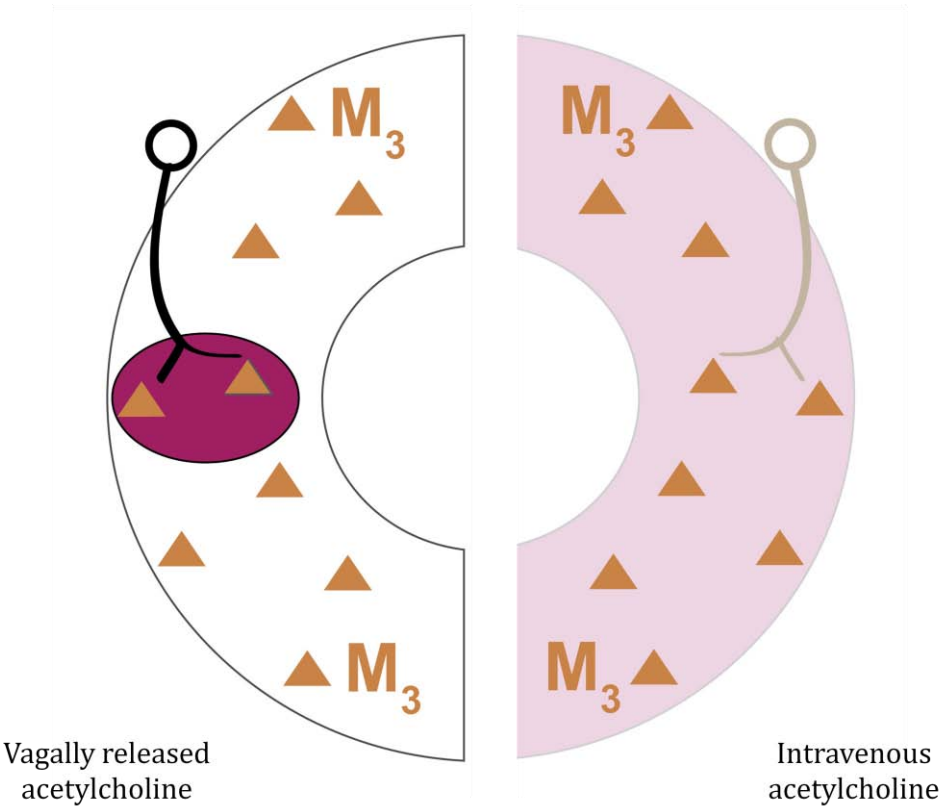

Figure 3.11 Model for tiotropium binding to smooth muscle M_3 receptors *in vivo*. Some M_3 receptors in airway smooth muscle (orange triangles) are directly innervated by parasympathetic nerves while other M_3 receptors are not. Tonic neuronal stimulation maintains high local concentrations of acetylcholine (dark pink) at M_3 receptors innervated by the nerves. Tiotropium compete with acetylcholine for receptor sites, and this would require more tiotropium near nerves where agonist concentrations are high. Since tiotropium's dissociation kinetics make it pseudo-irreversible, differences in tiotropium binding due to local acetylcholine concentrations would be apparent when bronchoconstriction is induced by electrically stimulating the nerves to release acetylcholine (dark pink on the left) versus intravenous acetylcholine (light pink on the right). When the nerves are stimulated only those muscarinic receptors close to the nerves are tested, where the least tiotropium binding occurs, and bronchoconstriction is not blocked (left). Intravenous acetylcholine stimulates all muscarinic receptors

including those distant from the nerves, where the most tiotropium binding occurs, and therefore bronchoconstriction is inhibited.

CHAPTER 4.

NEURONAL PRODUCTION OF CCR3 CHEMOKINES IN RESPONSE TO TNFα OR ANTIGEN CHALLENGE IN GUINEA PIGS

ABSTRACT

Background and purpose: Eosinophils are recruited to airway nerves in humans who have died of fatal asthma and in animal models of asthma. In guinea pigs, eosinophil association with airway nerves and airway hyperresponsiveness following antigen challenge are both blocked by either a CCR3 receptor antagonist or inhibition of TNFα. I tested whether TNFα or antigen challenge induced expression of ligands for CCR3 in nerves.

Experimental approach: Human SK-N-SH neuroblastoma cells and guinea pig parasympathetic nerve cultures were treated with TNFα and expression of nine known ligands for CCR3 were measured by real-time PCR (mRNA) and ELISA (protein). Parasympathetic ganglia and lungs were isolated from guinea pigs that were nonsensitized, ovalbumin-sensitized, or sensitized and challenged with inhaled ovalbumin (2 h, 6 h, and 24 h after challenge). PCR and immunostaining were used to measure mRNA in tracheal ganglia and protein in cholinergic nerves in the lung for the CCR3 chemokine CCL11. In addition, immunostaining was used to measure eosinophil association with cholinergic and noncholinergic nerves following antigen challenge.

Key results: TNFα induced expression of CCL5, CCL7, and CCL11 mRNA and protein in SK-N-SH cells and only CCL7 mRNA in cultured parasympathetic ganglia. CCL11 mRNA was detected in parasympathetic ganglia isolated from a few animals and low levels of CCL11 protein were colocalized with approximately 60% of cholinergic nerve fibers. Eosinophils increased in general following antigen challenge but were not selectively recruited to either cholinergic or noncholinergic nerves.

Conclusions and implications: Neuronal cells are capable of producing ligands for CCR3 in response to TNFα. However, the data do not support selective recruitment of

eosinophils to nerves via neuronal production of ligands for CCR3 following antigen challenge. Following antigen challenge, more eosinophils associate with both cholinergic and noncholinergic nerves, suggesting eosinophils may contribute to airway hyperreactivity in asthma via interactions with either population.

Collaboration: This project was begun as a collaboration with Dr. Cole Nelson in our laboratory. The initial screening for CCR3 chemokine mRNA expression in SK-N-SH cells was carried out by Dr. Cole Nelson, using primers that were already available in the laboratory or that I designed. Additionally, one of three experiments in Figure 4.2 (D, E, and F) was carried out and analyzed by Dr. Cole Nelson.

INTRODUCTION

Asthma is a heterogeneous disease characterized by reversible periods of bronchoconstriction, airway hyperreactivity, and inflammation. Airway hyperreactivity is mediated by parasympathetic nerves and is associated with eosinophil recruitment to airway nerves as described in Chapter 1, Introduction. Since eosinophils are increased around airway nerves in both humans who die of fatal asthma and in antigen-challenged guinea pigs and since chemokines are involved in eosinophil recruitment into airway tissues (see Chapter 1, Introduction), I investigated whether parasympathetic nerves secrete chemokines to recruit eosinophils.

CCR3 is the predominant chemokine receptor on eosinophils although some human eosinophils also express functional CCR1 or CXCR4 (Daugherty *et al.*, 1996; Nagase *et al.*, 2001; Nagase *et al.*, 2000; Powell *et al.*, 1996; Sabroe *et al.*, 1999). Blockade of CCR3 receptors on eosinophils reduces eosinophil association with airway nerves and prevents airway hyperresponsiveness following antigen challenge in guinea pig (Fryer *et al.*, 2006). Moreover, eosinophil recruitment into lungs of CCR3-deficient mice is drastically reduced following antigen challenge (Humbles *et al.*, 2002; Ma *et al.*, 2002; Pope *et al.*, 2005). CCR3 can be activated by nine known chemokine ligands. Of these ligands, the eotaxins (CCL11, CCL24, and CCL26) are specific for CCR3 while CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4), CCL15 (MIP-5), and CCL28 are promiscuous, activating other chemokine receptors in addition to CCR3 (Ubogu, 2011). Many CCR3 ligands are increased in the airways of patients with asthma and in animals following antigen challenge (Brown *et al.*, 1998; Humbles *et al.*, 1997; John *et al.*, 2005; Jose *et al.*, 1994; Li *et al.*, 1997; Santiago *et al.*, 2008; Sur *et al.*,

1996; Ying *et al.*, 1999). Several are found colocalized with airway nerves in the lungs of humans (CCL11), antigen-challenged guinea pigs (CCL11), and antigen-challenged monkeys (CCL26) (Chou *et al.*, 2005; Fryer *et al.*, 2006). Since blockade of CCR3 receptors significantly reduces eosinophil recruitment to airway nerves and since MBP release onto parasympathetic nerves contributes to airway hyperresponsiveness, I tested whether parasympathetic neurons in the lung recruit eosinophils via production of CCR3 chemokines.

TNFα is a cytokine increased in the lungs of some asthma patients (Howarth *et al.*, 2005) and found at significantly higher levels in alveolar macrophages from asthma patients with high Th2 levels and high eosinophil levels (Woodruff *et al.*, 2009). TNFα instilled into the lungs of both guinea pigs and patients with mild asthma results in eosinophil recruitment that is indirect, since TNFα itself does not cause eosinophil chemotaxis (Thomas *et al.*, 2002; White *et al.*, 1997). TNFα is released within 60 min following antigen challenge (Kelly *et al.*, 1992) and is necessary for eosinophil association with airway nerves and airway hyperresponsiveness in guinea pigs, since pretreatment with a TNFα inhibitor, etanercept prevents both (Nie *et al.*, 2009). Etanercept also reduces airway hyperresponsiveness in patients with severe refractory asthma (Berry *et al.*, 2006; Howarth *et al.*, 2005) although is no effect in mild asthma (Rouhani *et al.*, 2005), which likely reflects differences in TNFα expression between the two populations tested. In mice, eosinophils are significantly reduced following antigen challenge in TNF receptor-deficient mice relative to wildtype (Cho *et al.*, 2011). TNFα has been shown to induce CCR3 chemokine production in other cell types including epithelium, smooth muscle, and fibroblasts (Ghaffar *et al.*, 1999; Heiman *et al.*, 2005; Hoeck *et al.*, 2001; O'Gorman *et al.*, 2005). Since TNFα is increased following antigen

challenge and is important for eosinophil association with airway nerves, I tested whether TNFα induces production of CCR3 chemokines in nerves. I used a human neuroblastoma cell line, primary cultures of guinea pig parasympathetic nerves, and also quantified the association of eosinophils with parasympathetic nerves *in vivo*.

The data presented here suggest that neuronal cells are capable of producing several CCR3 chemokines including CCL5, CCL7, and CCL11 in response to TNFα in culture. Yet, *in vivo*, parasympathetic nerves do not appear to produce high levels of CCR3 chemokines. Here I show that eosinophils are not selectively recruited to nerves following antigen challenge, but instead are increased in general, resulting in increased eosinophil association with both cholinergic and noncholinergic nerves that is therefore likely not due to neuronal production of chemokines.

EXPERIMENTAL DESIGN

In vitro experiments

SK-N-SH human neuroblastoma cells were treated with 0-50 $ng \cdot mL^{-1}$ human TNFα for 24 h or with 150 $ng \cdot mL^{-1}$ TNFα for 0-48 h as described in Chapter 2, Methods.

Primary cultures of guinea pig parasympathetic nerves were treated with 2 $ng \cdot mL^{-1}$ murine TNFα, 2 $ng \cdot mL^{-1}$ murine TNFα + 1000 $U \cdot mL^{-1}$ murine IFNγ, 10 $ng \cdot mL^{-1}$ human IL-4, or 2 $ng \cdot mL^{-1}$ murine TNFα + 10 $ng \cdot mL^{-1}$ human IL-4 in preliminary experiments as described in Chapter 2, Methods.

Primary cultures of guinea pig parasympathetic nerves were also treated with recombinant guinea pig TNFα (0.05-5.0 $ng \cdot mL^{-1}$) for 4 h as described in Chapter 2, Methods.

In vivo experiments

Parasympathetic ganglia and lung tissue were harvested from guinea pigs that were nonsensitized, sensitized to ovalbumin (20 $mg \cdot kg^{-1}$, i.p.), or sensitized and challenged with inhaled ovalbumin (2 h, 6 h, or 24 h after challenge) as described in Chapter 2, Methods.

Measurements

Measurement of RNA with real-time quantitative PCR and measurement of chemokines in SK-N-SH cell supernatants by ELISA were performed as described in Chapter 2, Methods. Histological evaluation of cholinergic and noncholinergic nerve-associated

eosinophils in cryosections and histological evaluation of CCL11 (Eotaxin) expression in cholinergic nerves were performed as described in Chapter 2, Methods. *In situ* hybridization was also performed as described in Chapter 2, Methods.

Statistics:

Values are presented as means ± standard error of the mean (SEM), means ± standard deviation (SD), or medians. Means were compared using one-way ANOVA with Bonferroni's correction for multiple comparisons. In Figures 4.1 A and B means were log transformed to equalize variances before ANOVA was performed. In Figures 4.1, 4.2 (A-E), and 4.4 comparisons were made to the untreated group (0 $ng \cdot mL^{-1}$ or 0 h). In Figures 4.10-4.13 and Figures 4.15-4.16 comparisons were made to the sensitized control group. A Kruskal-Wallis test was used to compare medians in Figure 4.2 F, since there was no variation in the 0 h group. In Figure 4.10, showing eosinophil number and area density in noncartilaginous airways, one animal was excluded from the 24 h time point for statistical analysis only because the values were >2 SD from the mean without its inclusion. Significant p-values are reported as *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Statistical data were analyzed with GraphPad Prism (version 5, GraphPad Software, Inc, La Jolla, CA).

RESULTS

TNFα induces CCL5, CCL7, and CCL11 in SK-N-SH neuroblastoma cells

Of the nine known CCR3 ligands (CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, and CCL28), transcripts for three of them, CCL5 (RANTES), CCL7 (MCP-3), and CCL11 (Eotaxin) were detected and induced by human TNFα in SK-N-SH neuroblastoma cells. TNFα (0.15-150 $ng \cdot mL^{-1}$) induced mRNA expression in a dose (Figure 4.1) and time (Figure 4.2 A-C) dependent manner. CCL5 and CCL7 protein were present in the supernatant of untreated cells and increased significantly in cell supernatants over 48 h following treatment with TNFα (Figure 4.2 A and B). CCL11 protein was below the limit of detection in untreated cell supernatants but was detectable by 4 h and significantly increased by 48h (Figure 4.2 F). Thus, human neuroblastoma cells express three CCR3 chemokines, CCL5, CCL7, and CCL11.

Chemokine expression in primary cultures of guinea pig parasympathetic nerves

Cultures of guinea pig parasympathetic nerves, although enriched for nerves, contain many contaminating cells. To determine whether any chemokines would be detected in nerves, preliminary experiments were carried out to look for expression of CCL5, CCL7, and CCL11 in mRNA isolated from all cells, including contaminating cells. Cultures of guinea pig neurons from nonsensitized animals were treated with either murine TNFα, murine TNFα + murine IFNγ, human IL-4, or murine TNFα + human IL-4 for 2 h or 6 h. CCL5 and CCL7 mRNA were expressed in untreated cultures but did not increase following any treatment (Figure 4.3 A and B). CCL11 mRNA expression was not quantifiable. In contrast, in cultures of guinea pig parasympathetic neurons from sensitized animals, CCL5 and CCL7 mRNA expression were doubled with treatments

containing TNFα (Figure 4.3 C). The exception being that CCL5 was not increased by TNFα + IL-4. CCL11 expression still was not quantifiable. To test whether serum might contain cytokines or growth factors found in cultures of nerves from sensitized guinea pigs, cultures from nonsensitized guinea pigs were treated with FBS in addition to cytokines. CCL5 and CCL7 mRNA expression increased following treatments with TNFα (Figure 4.3 D). In the presence of serum, CCL11 expression was quantifiable but not increased following treatment with cytokines (Figure 4.3 D). Thus, in preliminary experiments CCL5 and CCL7 were increased by TNFα in nerve cultures from sensitized animals and in cultures from nonsensitized animals grown with serum.

TNFα induces CCL7 mRNA in isolated nerve cell bodies from guinea pig parasympathetic nerve cultures

Parasympathetic nerve cell bodies were isolated from parasympathetic nerve cultures following treatment with guinea pig TNFα (0.05-5 $ng \cdot mL^{-1}$). Of the three CCR3 chemokines cloned in guinea pig, only CCL7 mRNA was quantifiable in parasympathetic nerve cell bodies, and it was induced by TNFα (Figure 4.4). This was only significant at the lowest TNFα dose (0.05 $ng \cdot mL^{-1}$) in neurons cultured from nonsensitized guinea pigs and at the two highest doses (1.5 and 5 $ng \cdot mL^{-1}$) in neurons cultured from sensitized guinea pigs. CCL5 and CCL11 could sometimes be detected but were never reliably expressed or quantifiable. Thus, only CCL7 was quantifiable in parasympathetic nerves.

Expression of CCR3 chemokine mRNA in parasympathetic nerves *in vivo* following antigen challenge

In tissue sections of guinea pig trachea, mRNA was detected in parasympathetic nerve ganglia with an antisense *in situ* hybridization probe against the neuronal marker

PGP9.5, but not nerve fibers (Figure 4.5). However, very few sections contained ganglia thus I decided that *in situ* hybridization was not a practical way of determining whether parasympathetic nerves expressed chemokines *in vivo*.

As an alternative, I isolated parasympathetic ganglia stained with a vital dye (neutral red) from the tracheas of nonsensitized, sensitized, and sensitized and challenged (2 h, 6 h, or 24 h after challenge) guinea pigs. PGP9.5 mRNA was detected in ganglia from all animals by PCR, confirming neuronal mRNA could be measured (Figure 4.6 B). CCL11 mRNA was detected in parasympathetic ganglia from 0-2 animals per treatment group (Figure 4.6 A) at levels that were too low to quantify with real-time PCR. CCL5 and CCL7 were never detected in mRNA from ganglia. However, these primers amplify CCL5 and CCL7 mRNA in whole lung (see Chapter 2, Methods).

Expression of CCL11 protein in cholinergic nerves *in vivo*

Immunofluorescence was used to detect colocalization of CCL11 and ChAT positive nerve fibers in noncartilaginous (bronchioles) and cartilaginous airways (bronchi). Lung sections were analyzed from nonsensitized, sensitized, and sensitized and antigen challenged guinea pigs 2 h, 6h, and 24 h after challenge. The most intense CCL11 staining was found in airway epithelium (Figure 4.7 and 4.8) although other tissues including smooth muscle and nerves stained positively for CCL11 when compared to preimmune serum controls (Figure 4.9). Approximately 60% of ChAT positive fibers were also CCL11 positive (Figure 4.10). ChAT positive fibers did not stain more intensely than surrounding tissues, so that without the ChAT staining it was not possible to identify nerve fibers (Figure 4.7 and 4.8). The percentage of ChAT positive nerve fibers colocalized with CCL11 did not increase with sensitization or antigen challenge in

either noncartilaginous or cartilaginous airways (Figure 4.10). Thus, CCL11 protein is found colocalized with parasympathetic nerves fibers *in vivo*.

Eosinophils stained with the dye chromotrope 2R were found just beneath and in airway epithelium, which stains brightly for CCL11, and in adventitia. Strikingly, very few eosinophils were seen in airway smooth muscle (Figure 4.9). In general, eosinophils could be found in two concentric circles on either side of the smooth muscle (Figure 4.7, 4.8, and 4.9).

Determining whether eosinophils are recruited to parasympathetic nerves or other nerves following antigen challenge

Since CCL11 protein expression in parasympathetic nerves was not obviously greater than in surrounding tissue, I tested whether eosinophils were recruited to cholinergic parasympathetic nerves or other (sensory, sympathetic, or noncholinergic parasympathetic) nerves following antigen challenge.

Eosinophils are present in noncartilaginous (Figure 4.11) and cartilaginous (Figure 4.12) airways. In nonsensitized control animals, the total number of eosinophils in noncartilaginous and cartilaginous airways is similar (Figure 4.11 A and Figure 4.12 A). However, the density of total eosinophils is higher in noncartilaginous airways than cartilaginous airways (Figure 4.11 B and 4.12 B). Following sensitization neither number nor density of eosinophils increase in noncartilaginous airways (Figure 4.11). In cartilaginous airways, the mean number of eosinophils per airway and number of eosinophils normalized to airway area is doubled in sensitized animals (Figure 4.12), although this was not statistically significant. Antigen challenge of sensitized animals increased eosinophils by 6 h after challenge in both noncartilaginous (Figure 4.11) and

cartilaginous airways (Figure 4.12); however, this was not always statistically significant. Eosinophils numbers were maximal at 6 h, and by 24 h after challenge eosinophils were returning toward baseline values.

The distance of each eosinophil to the nearest nerve was measured and the median distance of eosinophils to the nearest nerve in each airway determined (Figure 4.13 and 4.14). Eosinophils did not move closer to nerves following sensitization or sensitization and challenge in either noncartilaginous (Figure 4.13) or cartilaginous (Figure 4.14) airways.

The percentage of eosinophils associated with cholinergic nerves (within 8 μm of a ChAT+ and PGP9.5+ nerve), noncholinergic nerves (within 8 μm of a ChAT- and PGP9.5+ nerve), or no nerve (> 8 μm from a nerve) were measured (Figure 4.15) to determine whether eosinophils were selectively recruited to cholinergic or noncholinergic nerves. In noncartilaginous airways of nonsensitized animals, 30% of eosinophils were associated with cholinergic nerves, 20% were associated with noncholinergic nerves, and 50% were not associated with nerves (Figure 4.16 A, C, and E). In cartilaginous airways of nonsensitized animals, 10% of eosinophils were associated with cholinergic nerves, 20% were associated with noncholinergic nerves, and 70% were not associated with any nerve (Figure 4.16 B, D, and F). The percentage of cholinergic or noncholinergic nerve-associated eosinophils was not significantly changed by sensitization or antigen challenge (2 h, 6 h, or 24 h after challenge) in either noncartilaginous or cartilaginous airways (Figure 4.16). Thus, eosinophils were not selectively recruited to either cholinergic nerves or noncholinergic nerves.

Finally, the total number of eosinophils associated with cholinergic nerves, noncholinergic nerves, and no nerves were determined (Figure 4.17). Both cholinergic and noncholinergic nerve-associated eosinophils were increased by 6 h after antigen challenge as were non-nerve associated eosinophils. This was statistically significant for noncholinergic nerves in cartilaginous airways 6 h after challenge. 24 h after antigen challenge eosinophil numbers were returning toward baseline. Thus, there was a trend toward the number of eosinophils associated with airway nerves increasing after antigen challenge and this was maximal 6 h after challenge in both noncartilaginous and cartilaginous airways.

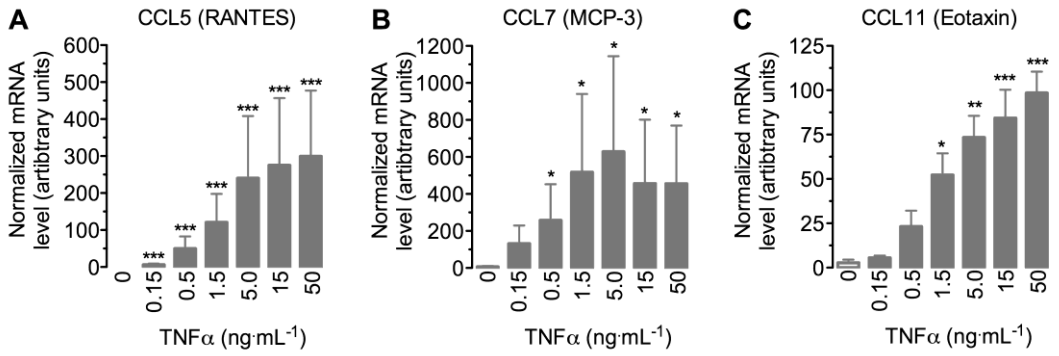

Figure 4.1 Neuronal expression of CCL5 (RANTES), CCL7 (MCP-3), and CCL11 (eotaxin-1) mRNA is increased by TNFα. CCL5 (A), CCL7 (B), and CCL11 (C) transcript levels in human SK-N-SH cells were increased by exposure to human TNFα (0.15-50 $ng \cdot mL^{-1}$) for 24 h. Data are expressed as chemokine mRNA normalized to 18S RNA and are presented as mean ± SEM (n=3). Comparisons were made to the 0 $ng \cdot mL^{-1}$ group; statistically significant changes are note with *.

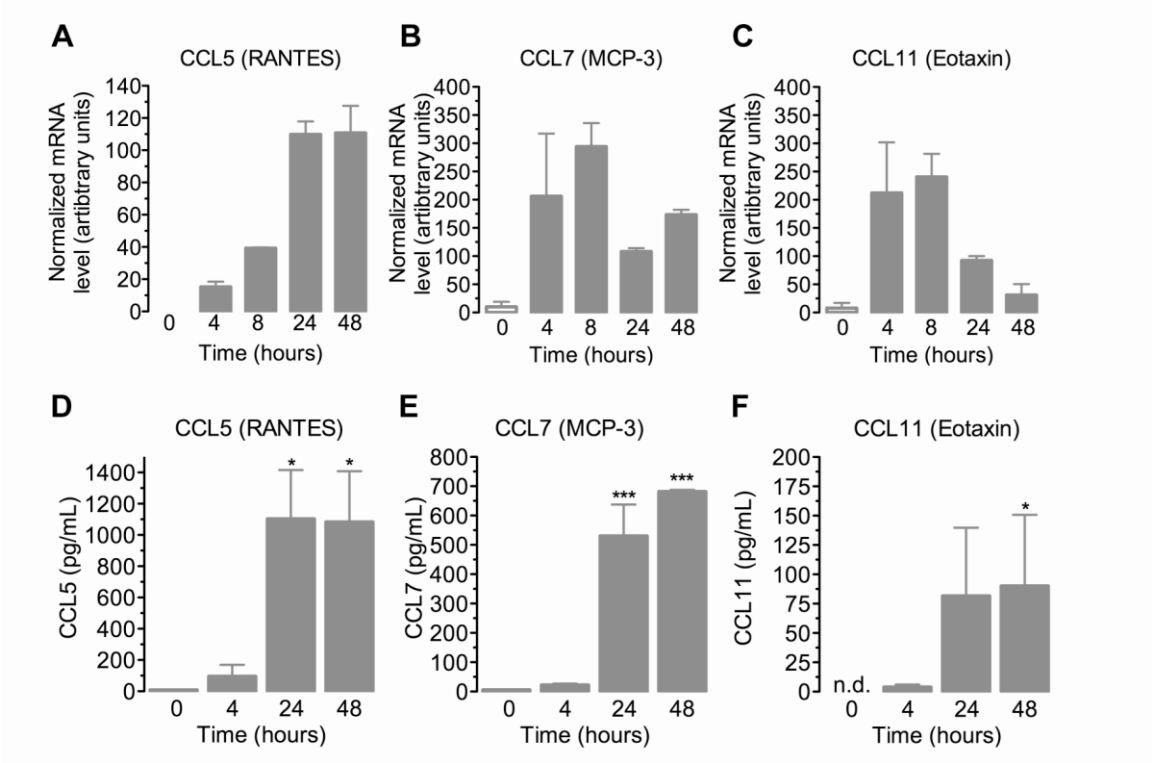

Figure 4.2 Neuronal expression of CCL5 (RANTES), CCL7 (MCP-3), and CCL11 (eotaxin) mRNA and protein are increased by TNFα over time. CCL5 (A), CCL7 (B), and CCL11 (C) transcript levels in human SK-N-SH cells were increased by exposure to human TNFα (150 $ng \cdot mL^{-1}$) over time. CCL5 mRNA peaked at 24 h. CCL7 and CCL11 mRNA peaked by 8 h and then decreased. CCL5 (D), CCL7 (E), and CCL11 (F) protein were all secreted in response to TNFα reaching maximal levels at 24 h. Data are expressed as chemokine mRNA normalized to 18S RNA presented as mean ± SD (n=2) or chemokine protein in pg/mL presented as mean ± SEM (n=3). Comparisons were made to the 0 h treatment group for protein (D-F); statistically significant changes are noted with *. n.d. = not detected.

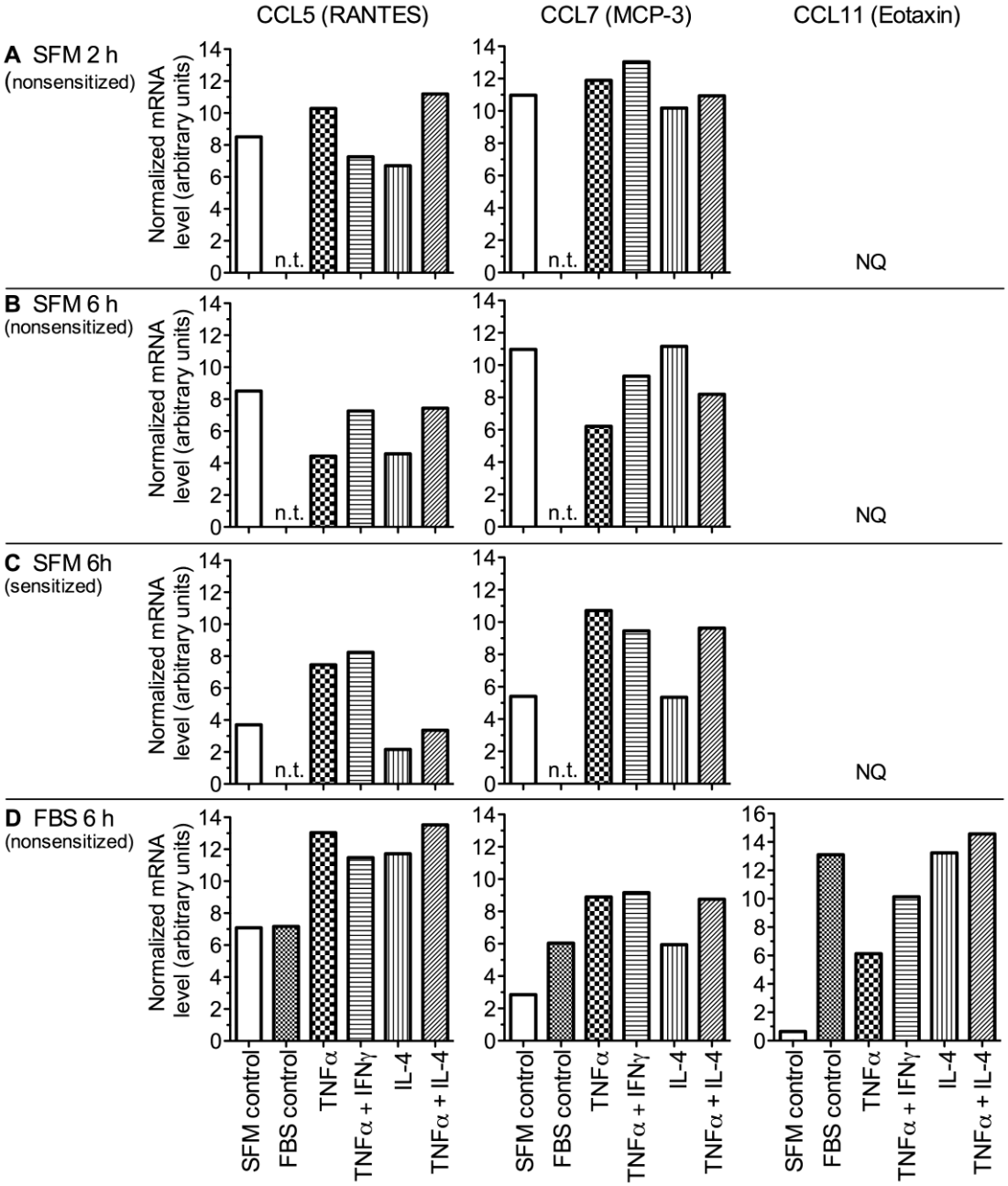

Figure 4.3 CCL5 (RANTES), CCL7 (MCP-3), and CCL11 (Eotaxin) mRNA are expressed in primary cultures of guinea pig trachea enriched for parasympathetic nerves. CCL5, CCL7, and CCL11 transcript levels were measured following treatment with murine TNFα (2 ng·mL^{-1}), murine TNFα + murine IFNγ (1000 U·mL^{-1}), human IL-4 (10 ng·mL^{-1}), or murine TNFα + human IL-4. CCL5 and CCL7 mRNA transcripts were

not increased by any treatment in cultures of neurons harvested from nonsensitized animals grown in serum free media (SFM) and treated for either 2 h (A) or 6 h (B). In contrast, CCL5 and CCL7 expression appeared to increase in cultures of neurons harvested from sensitized guinea pigs that were treated for 6h (C) if the treatment included TNFα. The exception was that TNFα + IL-4 did not increase CCL5 expression. CCL5 and CCL7 mRNA expression also appeared to increase following treatments that included TNFα in cultures of neurons harvested from nonsensitized animals that were grown with 10% fetal bovine serum (FBS; D). Additionally, IL-4 alone may have increased CCL5 expression. CCL11 mRNA expression was increased by culturing with FBS in one of two experiments. In most experiments CCL11 could be detected but not quantified (NQ). Data are expressed as chemokine mRNA normalized to 18S RNA and are presented as means. n=1 (A-C) and n=2 (D); n.t.= not tested.

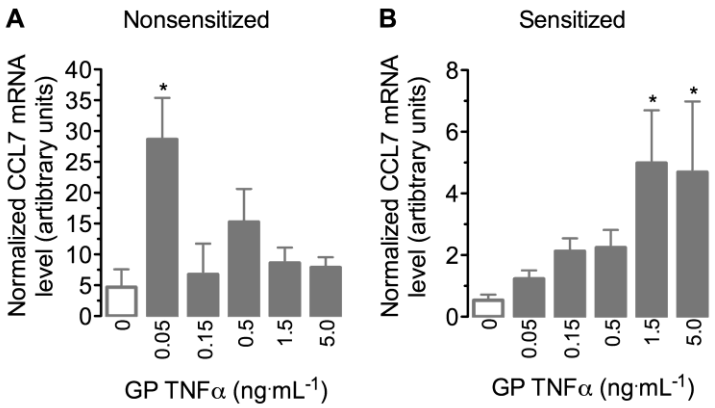

Figure 4.4 TNFα increases CCL7 (MCP-3) mRNA expression in isolated nerve cell bodies from cultured guinea pig parasympathetic nerves. CCL7 (MCP-3) mRNA expression was increased 4 h after treatment with guinea pig TNFα (0.05-5.0 ng·mL^{-1}) in cultured parasympathetic nerves from nonsensitized (A) and sensitized (B) guinea pigs. Data are expressed as CCL7 mRNA normalized to 18S RNA and are presented as mean ± SEM (n=2-6). Comparisons were made to control (0 ng·mL^{-1} TNFα); statistically significant changes are noted with *.

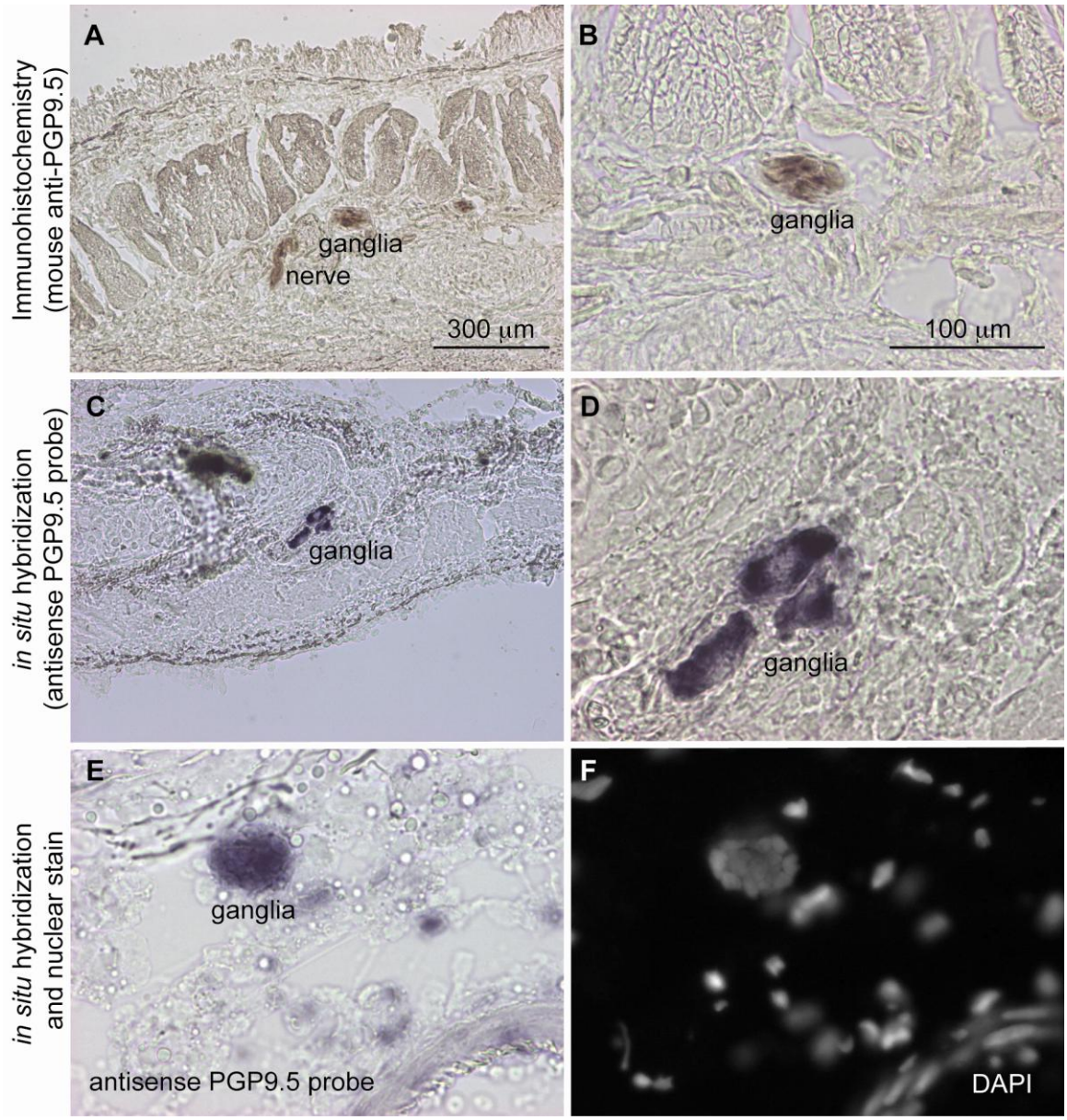

Figure 4.5 mRNA is present by *in situ* hybridization in guinea pig tracheal parasympathetic ganglia. Parasympathetic ganglia and nerve fibers were labeled with an antibody against PGP9.5 (neuronal marker) in guinea pig trachea (A and higher magnification in B). Ganglia, but not fibers, were labeled following *in situ* hybridization with an antisense probe against PGP9.5 (C and higher magnification in E). Colocalized *in situ* hybridization signal (E) and DAPI signal in nuclei (F) confirm *in situ* hybridization labeling of cell bodies. A and C are magnified 100X. B and D-F are magnified 400X.

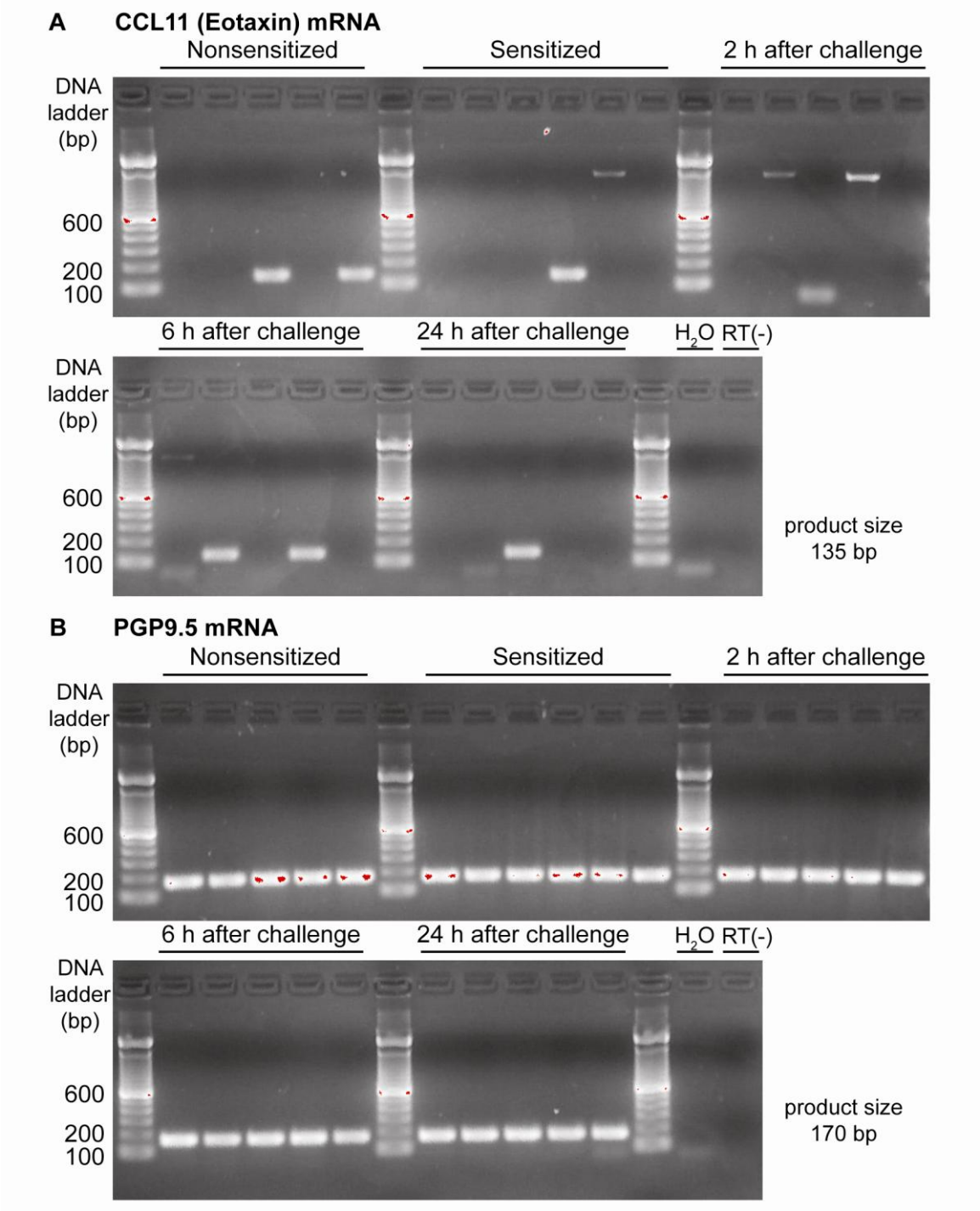

Figure 4.6 Some tracheal parasympathetic nerve ganglia express CCL11 (Eotaxin) mRNA. Ganglia were harvested from nonsensitized, sensitized, and sensitized and challenged guinea pigs (2, 6, and 24 h after challenge). Each lane shows PCR products for a single animal. (A) CCL11 transcripts were detected in some guinea pigs, and

transcript expression and frequency did not appear to be change by sensitization or antigen challenge. (B) PGP9.5 transcripts were detected in every animal confirming neuronal mRNA had been isolated.

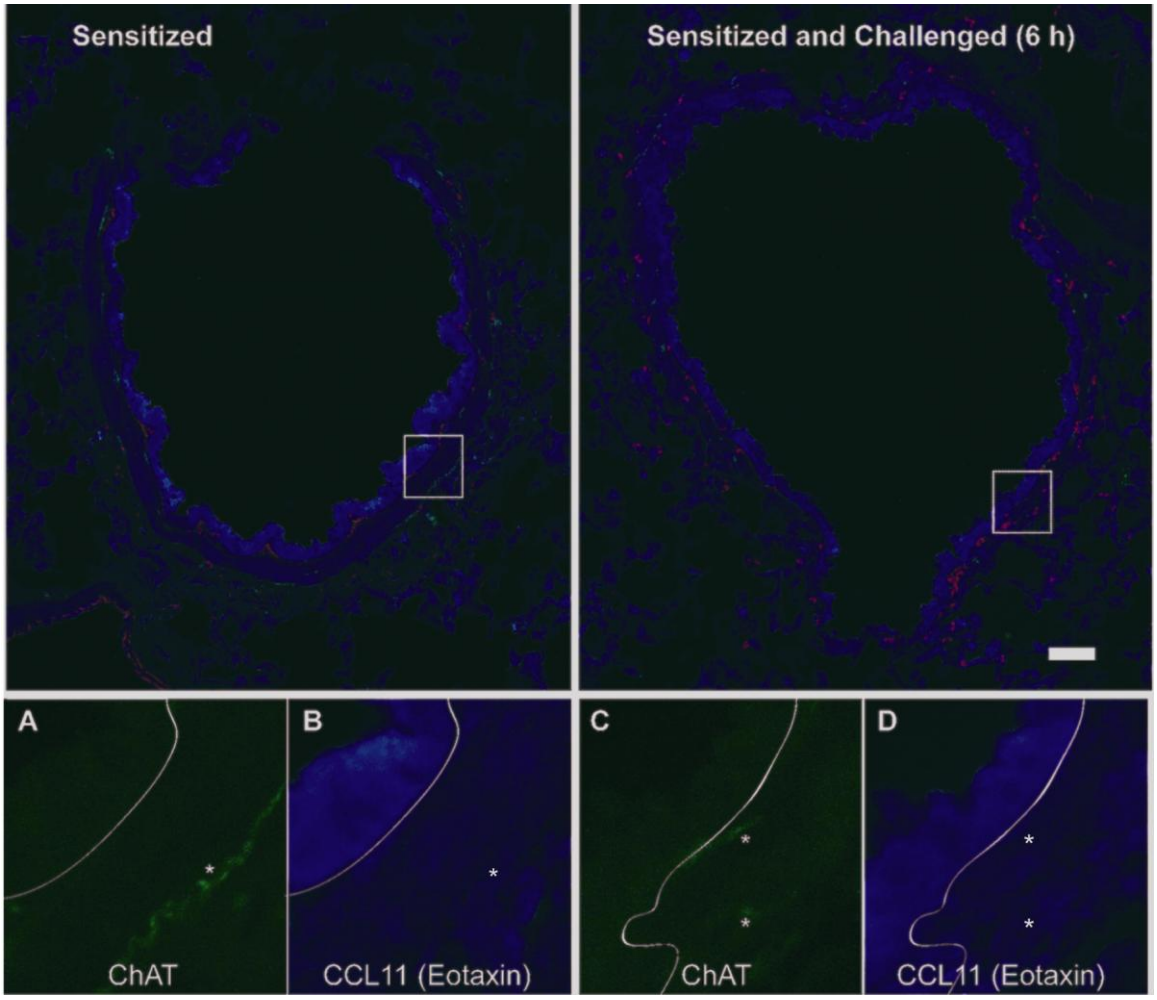

Figure 4.7 Cholinergic nerves colocalize with low levels of CCL11 (Eotaxin) in noncartilaginous airways. Sensitized animals express CCL11 (blue) at high levels in airway epithelium and at lower levels in smooth muscle and nerves. A few eosinophils (red) are found beneath the airway epithelium. In a magnified view, cholinergic nerve fibers (A, green and *) are found in regions with low levels of CCL11 protein (B). 6 h after antigen challenge eosinophils increase in and just beneath the airway epithelium and on the adventitial side of airway smooth muscle. Very few eosinophils are found within airway smooth muscle. CCL11 is still highly expressed in airway epithelium. In a magnified view, cholinergic nerve fibers (C) are expressed in regions with low levels of CCL11 (D). In the magnified view, lines show epithelial borders. Scale bar = 50 μm.

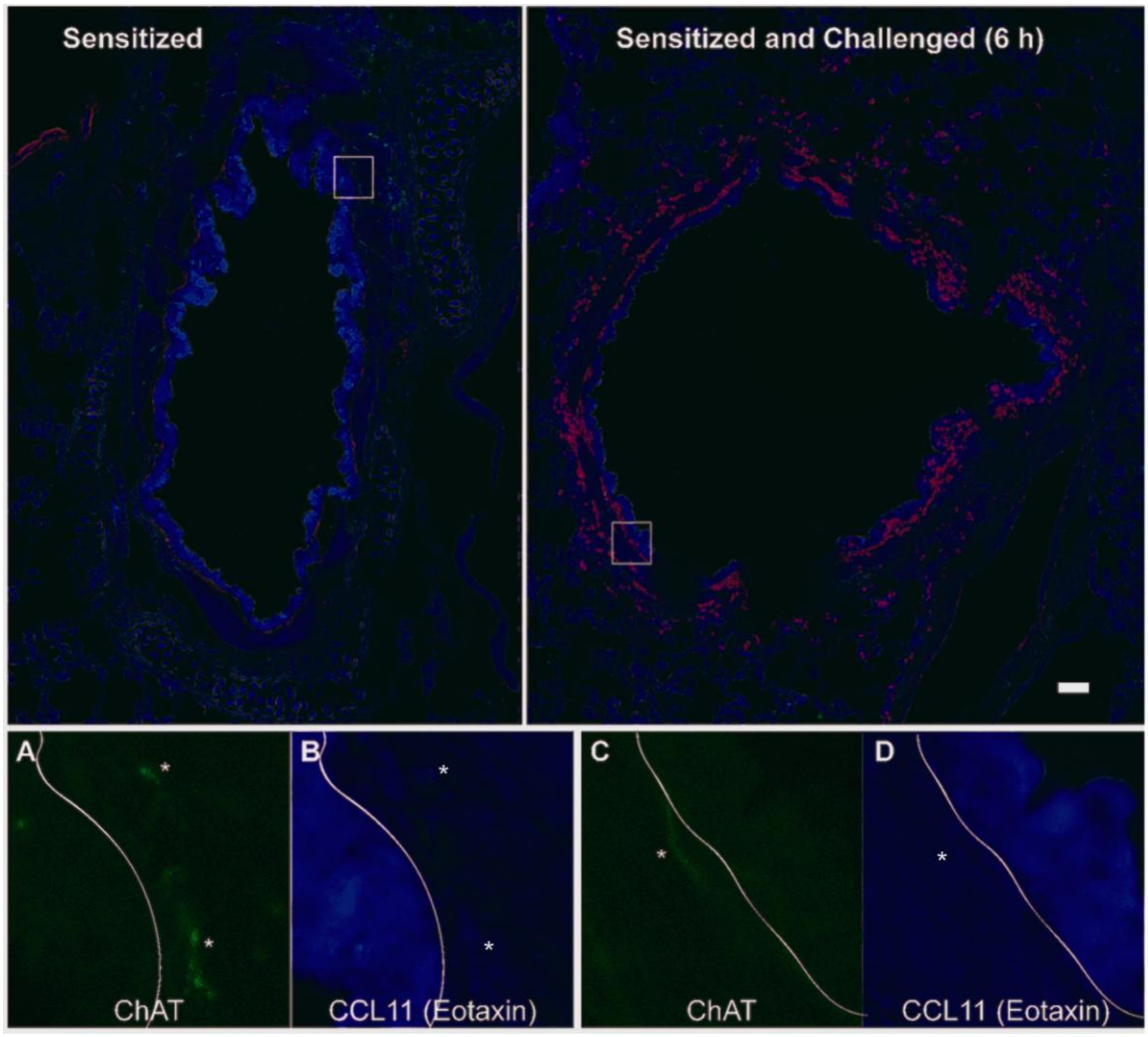

Figure 4.8 Cholinergic nerves colocalize with low levels of CCL11 (Eotaxin) in cartilaginous airways. Sensitized animals express CCL11 (blue) at high levels in airway epithelium and at lower levels in smooth muscle and nerves. A few eosinophils (red) are found beneath the airway epithelium. Cholinergic nerve fibers (A, green, and *) are found in regions with low levels of CCL11 protein (B). 6 h after antigen challenge eosinophils increase in and just beneath the airway epithelium and on the adventitial side of airway smooth muscle. Very few eosinophils are found within airway smooth muscle. CCL11 is still highly expressed in airway epithelium and cholinergic nerve fibers (C) are found in regions with low levels of CCL11 (D). In the magnified view, lines show epithelial borders. Scale bar = 50 μm.

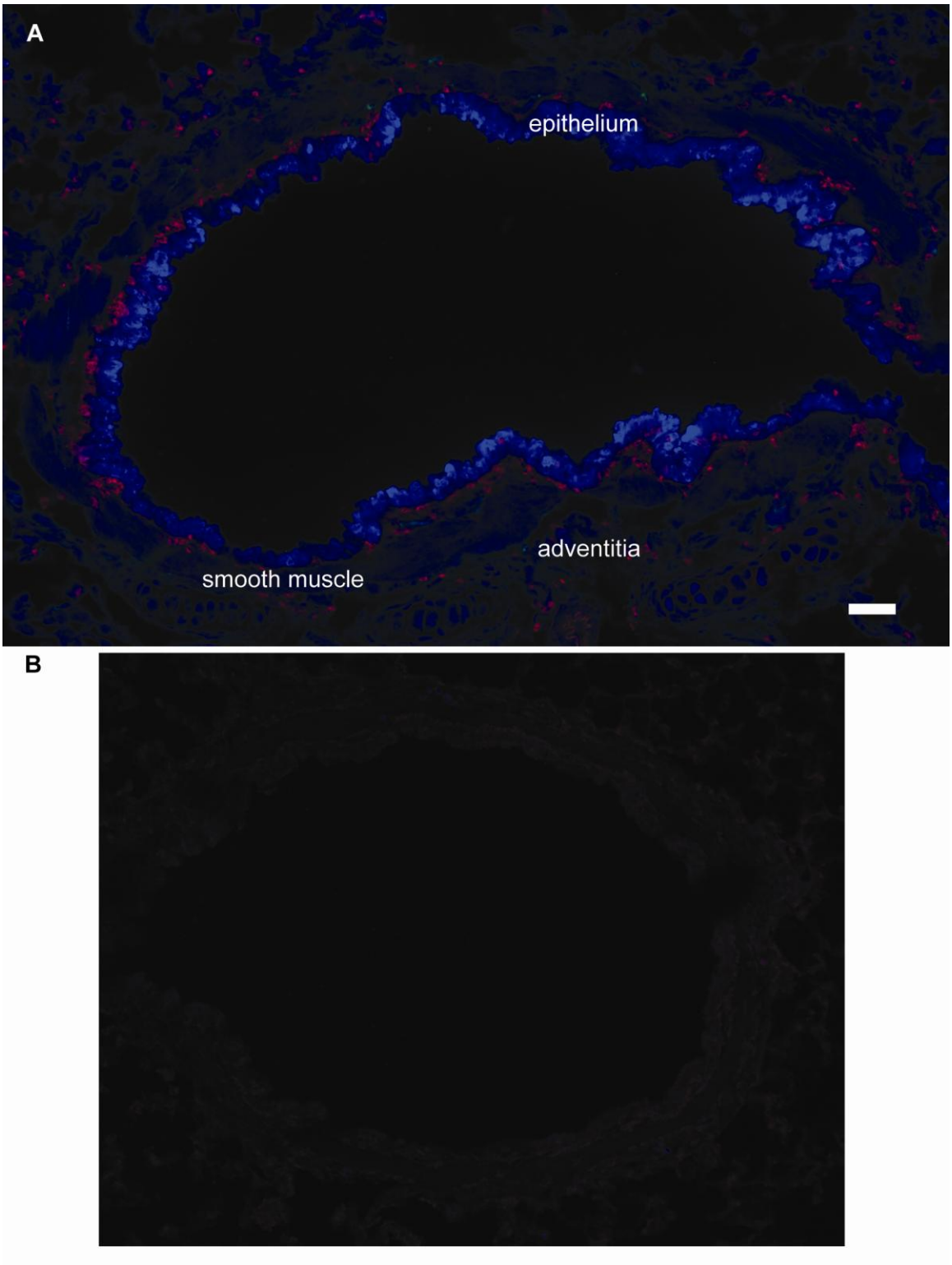

Figure 4.9 Eosinophils accumulate beneath and within airway epithelium expressing CCL11 (Eotaxin) protein. (A) CCL11 staining (blue) is strongest in airway

epithelium. However, CCL11 staining was also observed in other tissues including airway smooth muscle and adventitia. Eosinophils labeled with chromotrope 2R (red) accumulate just beneath and within airway epithelium and in adventitia. Very few eosinophils are found within airway smooth muscle. (B) There is virtually no staining in the preimmune serum control for CCL11. Scale bar = 50 μm.

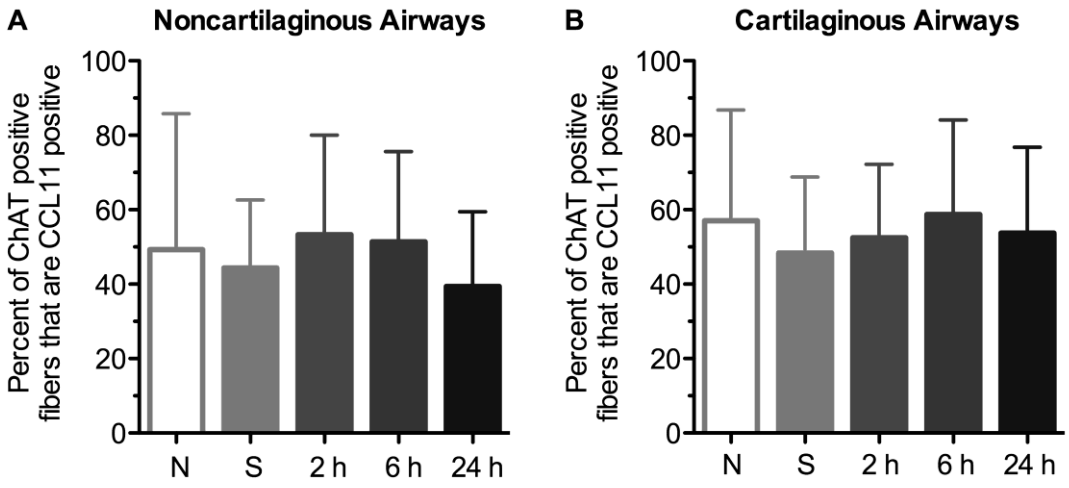

Figure 4.10 The percentage of cholinergic nerve fibers colocalized with CCL11 does not increase following sensitization or antigen challenge. The percentage of cholinergic nerve fibers (ChAT+) that were also positive for CCL11 was calculated for nonsensitized (N) animals in noncartilaginous (A) and cartilaginous airways (B). This number did not increase following sensitization (S) or antigen challenge of sensitized guinea pigs (2 h, 6 h, and 24 h after challenge). Data are expressed as mean ± SEM (n=3). None of the means were significantly different from the sensitized control group.

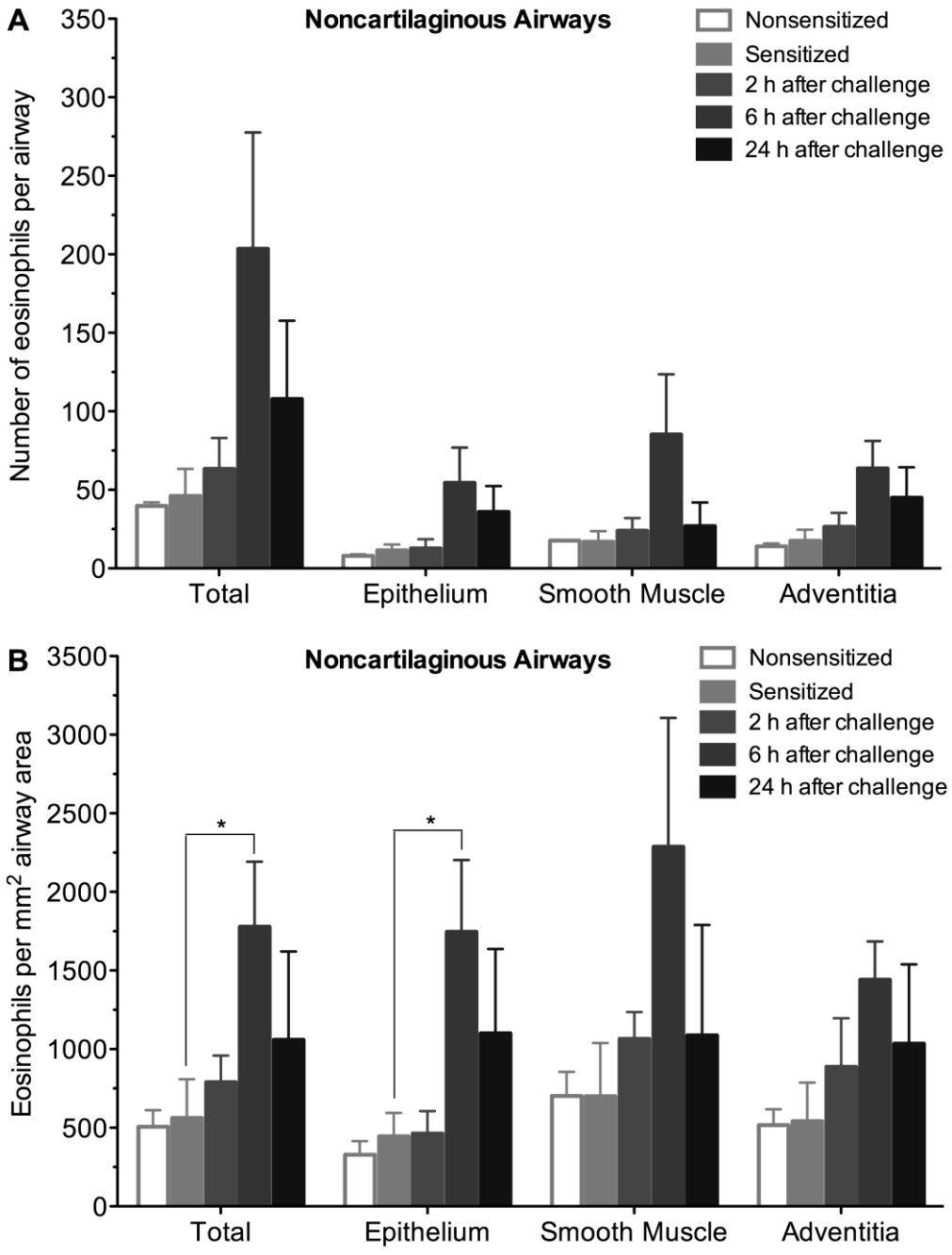

Figure 4.11 Eosinophils increase in noncartilaginous airways after antigen challenge. Eosinophils are found in airways of nonsensitized animals. Sensitization does not change the number of eosinophils (A) nor the number of eosinophils normalized to airway area (B). Eosinophils are recruited into the airways following

sensitization and antigen challenge (2 h, 6 h, and 24 h after challenge). Maximum increases in eosinophils are found 6 h after challenge. Similar eosinophil numbers and densities are found within each region (epithelium, smooth muscle, and adventitia). Data are expressed as mean ± SEM; n=3-5. Comparisons were made to the sensitized group; statistically significant changes are noted with *.

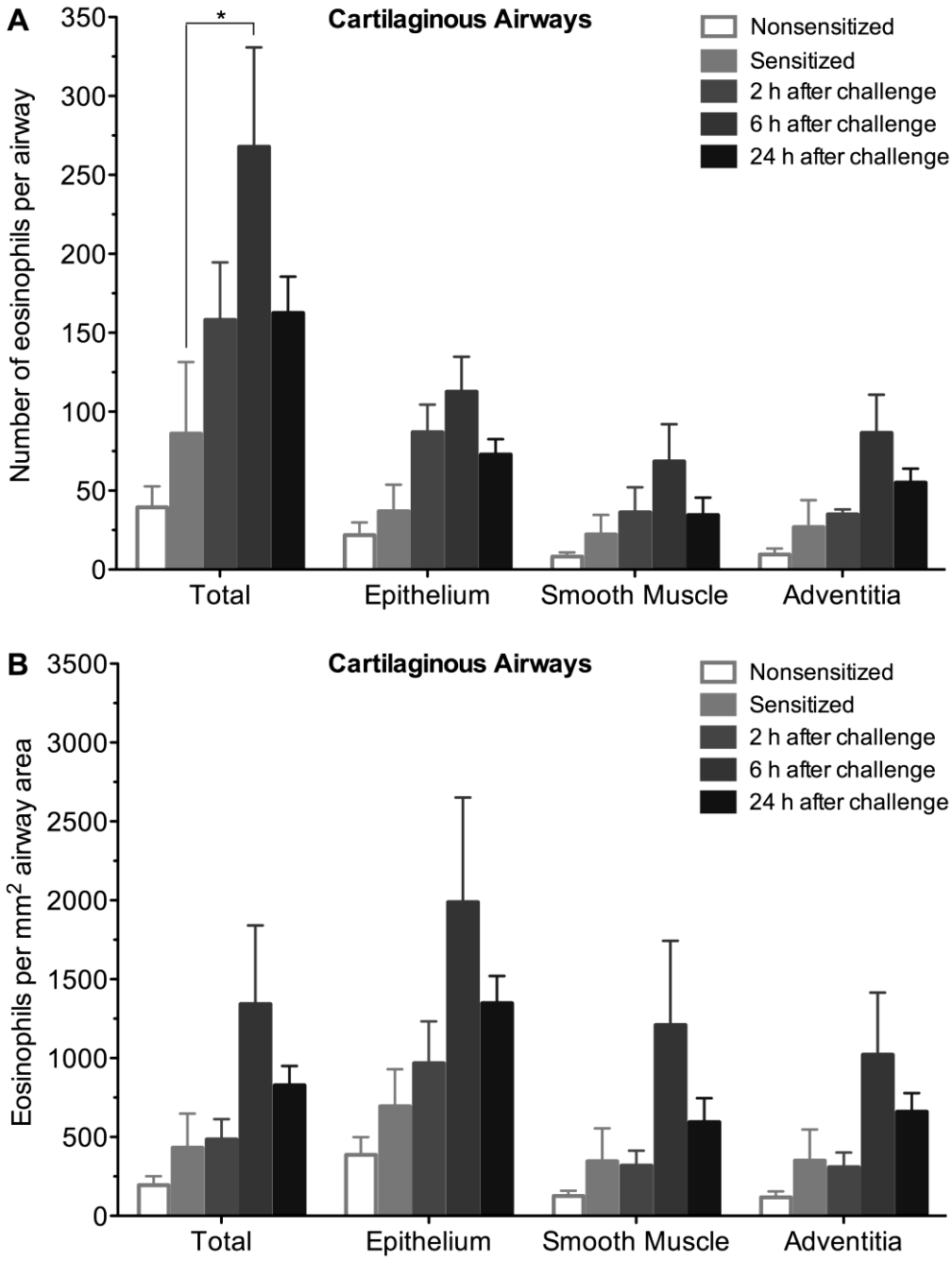

Figure 4.12 Eosinophils increase in cartilaginous airways after sensitization and sensitization and challenge. Eosinophils are found in airways of nonsensitized animals. Sensitization increases the number of eosinophils (A) and the number or eosinophils normalized to airway area (B). Eosinophils are further recruited into the

airways following sensitization and antigen challenge (2 h, 6 h, and 24 h after challenge). Maximum increases in eosinophils are found 6 h after challenge. The greatest number and density of eosinophils are found within airway epithelium. However, eosinophils increase in all regions (epithelium, smooth muscle, and adventitia) following antigen challenge. Data are expressed as mean ± SEM; n=3-5. Comparisons were made to the sensitized group; statistically significant changes are noted with *.

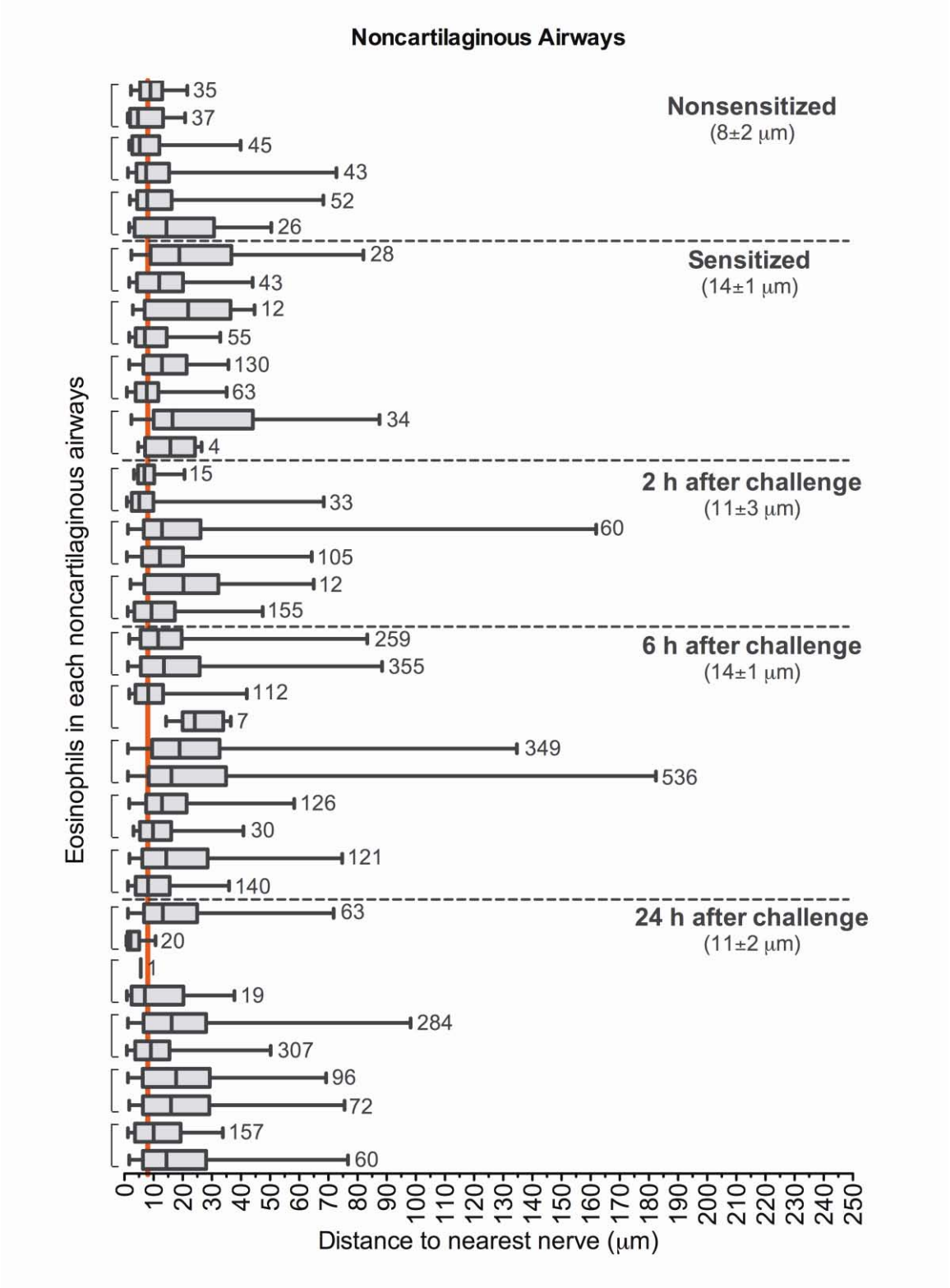

Figure 4.13 Eosinophils do not move closer to nerves following sensitization or sensitization and challenge in noncartilaginous airways. Distance from each

eosinophil to the nearest nerve was measured, and the number (at end of whisker) and distribution of eosinophils relative to nerves is graphed for each noncartilaginous airway. Data are shown for nonsensitized animals, sensitized animals, and sensitized and antigen challenged animals 2, 6, and 24 h after challenge. Eosinophils in each airway are graphed and replicate airways are graphed next to each other. The average median distance ± SEM (listed under treatment) to the nearest nerve is not significantly different for any treatment. Eosinophils associated with airway nerves are less than 8 μm (red line) from a nerve. n=3-5; whiskers show minimum and maximum distance.

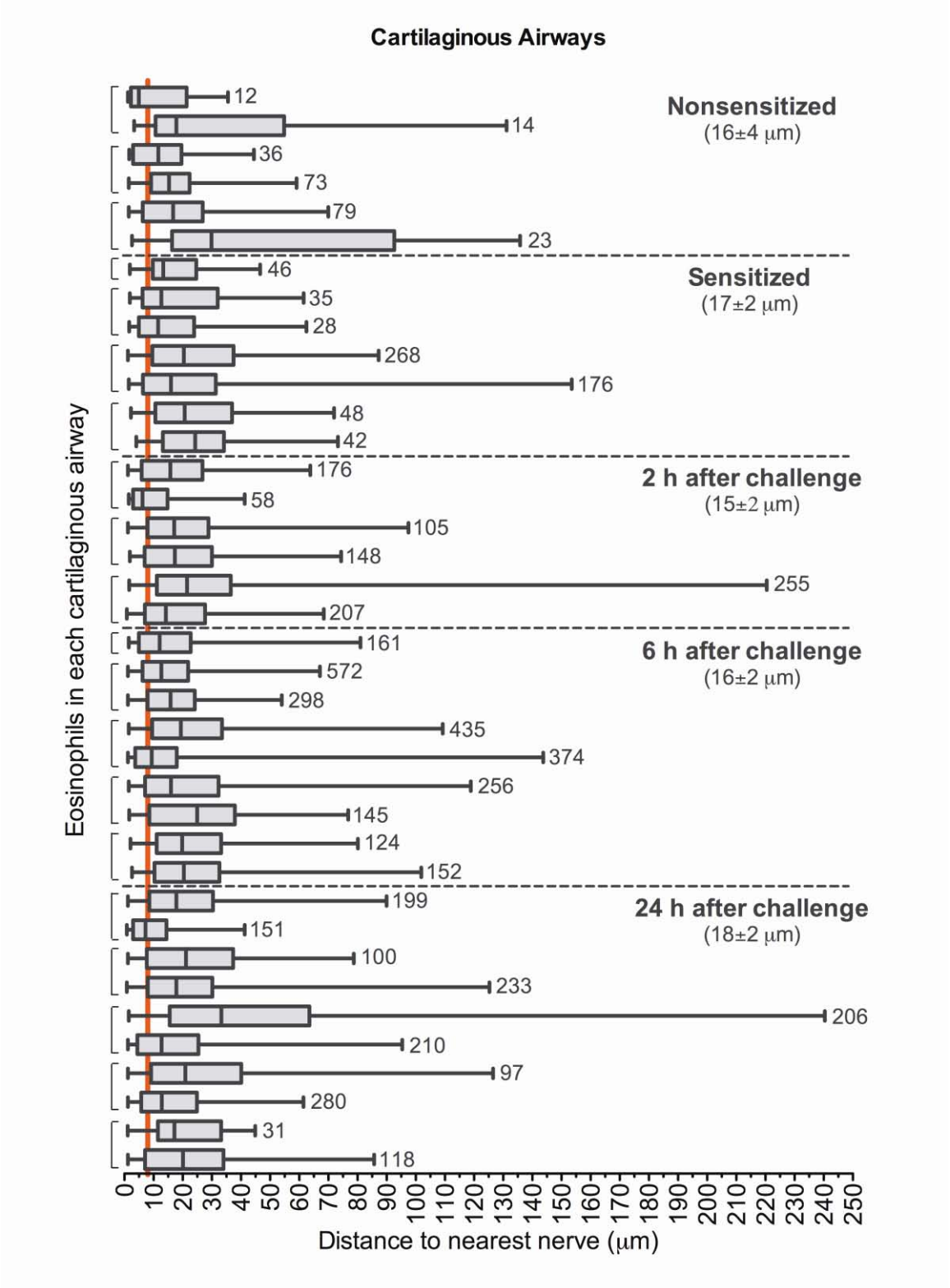

Figure 4.14 Eosinophils do not move closer to nerves following sensitization or sensitization and challenge in cartilaginous airways. Distance from each eosinophil

to the nearest nerve was measured, and the number (at end of whisker) and distribution of eosinophils relative to nerves is graphed for each cartilaginous airway. Data are shown for nonsensitized animals, sensitized animals, and sensitized and antigen challenged animals 2, 6, and 24 h after challenge. Eosinophils in each airway are graphed and replicate airways are graphed next to each other. The average median distance ± SEM (listed under treatment) to the nearest nerve is not significantly different for any treatment. Eosinophils associated with airway nerves are less than 8 μm (red line) from a nerve. n=3-5; whiskers show minimum and maximum distance.

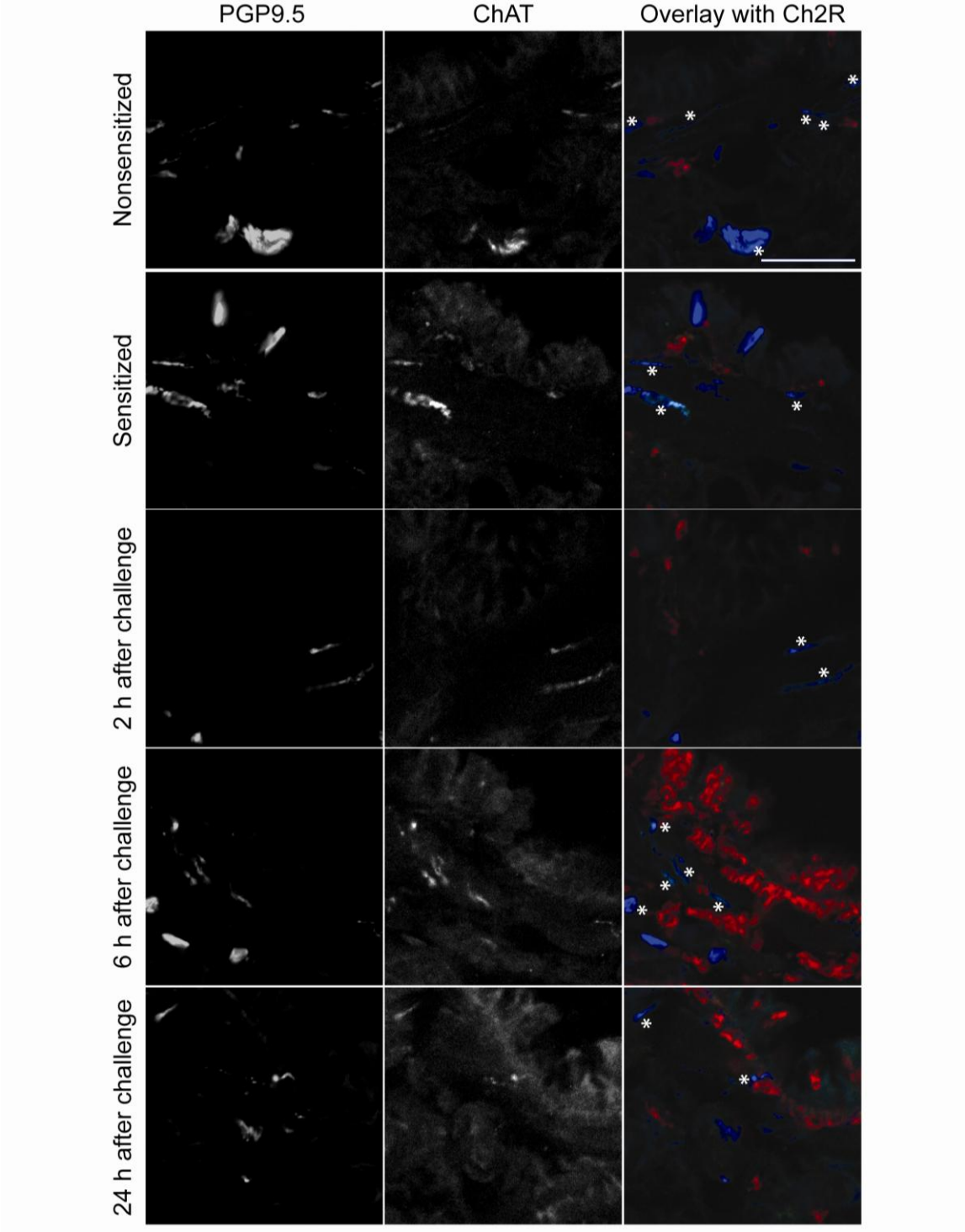

Figure 4.15 Airway eosinophils increase after antigen challenge and associate with both cholinergic and noncholinergic nerves. Eosinophil association with nerves was measured in cartilaginous airways from nonsensitized, sensitized, or sensitized and

challenged guinea pigs. Lungs were harvested 2, 6, or 24 h after antigen challenge. Eosinophils labeled with a dye chromotrope 2R (Ch2R; red) were increased 6 h and 24 h after antigen challenge and they associated with both noncholinergic nerves labeled with an antibody against PGP9.5 (blue in overlay) and cholinergic nerves labeled with antibodies against PGP9.5 and choline acetyltransferase (ChAT; stars in overlay). All images are magnified 200X. Scale bar = 50 μm.

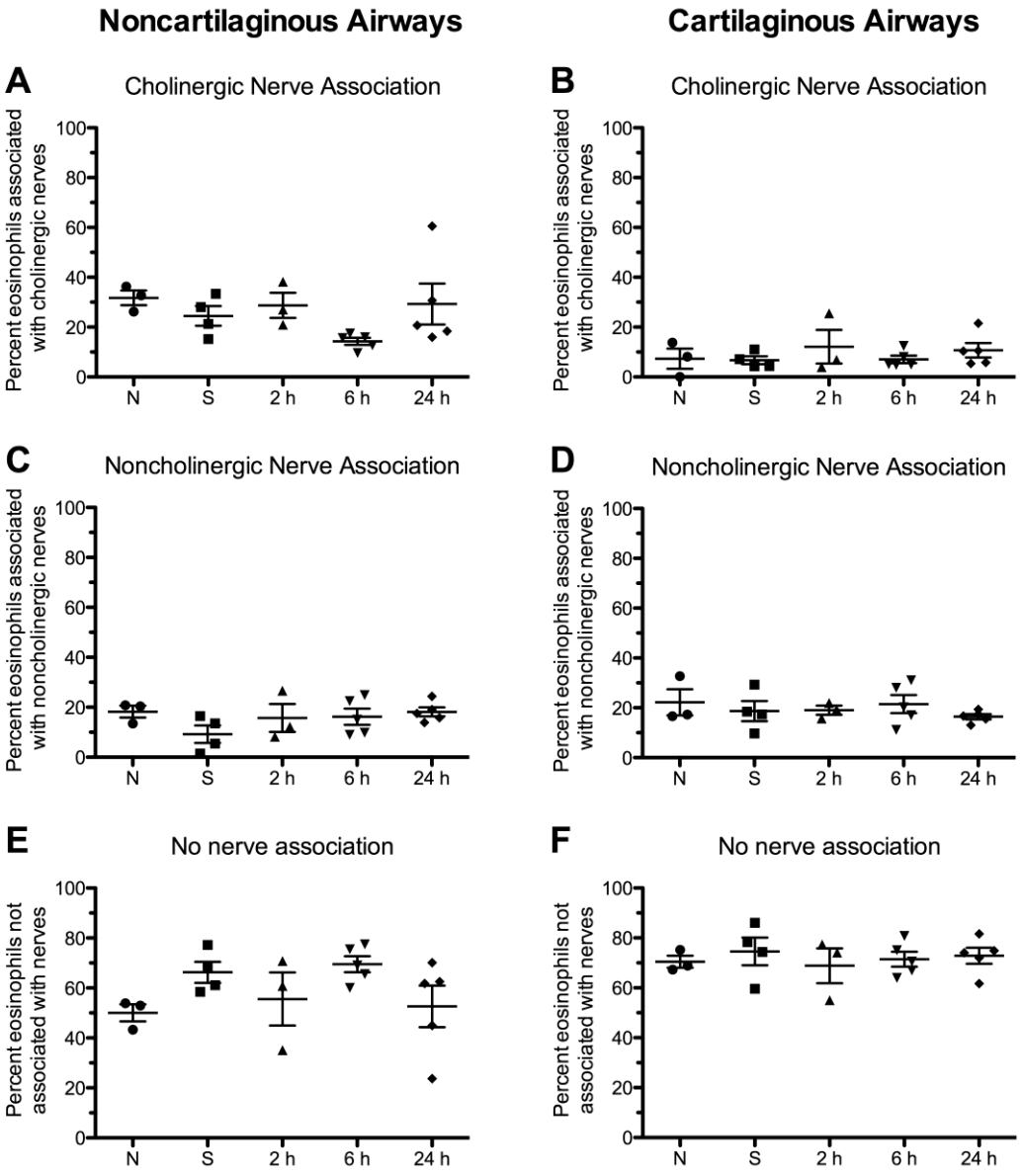

Figure 4.16 Eosinophils are not selectively recruited to cholinergic or noncholinergic nerves in airways following sensitization or antigen challenge. Eosinophils are associated with both cholinergic (A and B; ChAT+ and PGP9.5+) and noncholinergic (C and D; ChAT- and PGP9.5+) nerves in airways of nonsensitized (N) guinea pigs. Eosinophils must be within 8 μm (approximate diameter of an eosinophil) of a nerve to be associated. Neither sensitization (S) nor antigen challenge of sensitized

guinea pigs (2 h, 6 h, or 24 h after challenge) changed the percentage of eosinophils associated with nerves in noncartilaginous (A and C) or cartilaginous (B and D) airways. The percentage of eosinophils not associated with nerves (E and F) was similarly not changed. Each point represents the average of 2 airways from one animal. Mean and SEM are shown; n=3-5. None of the means were significantly different from the sensitized control group.

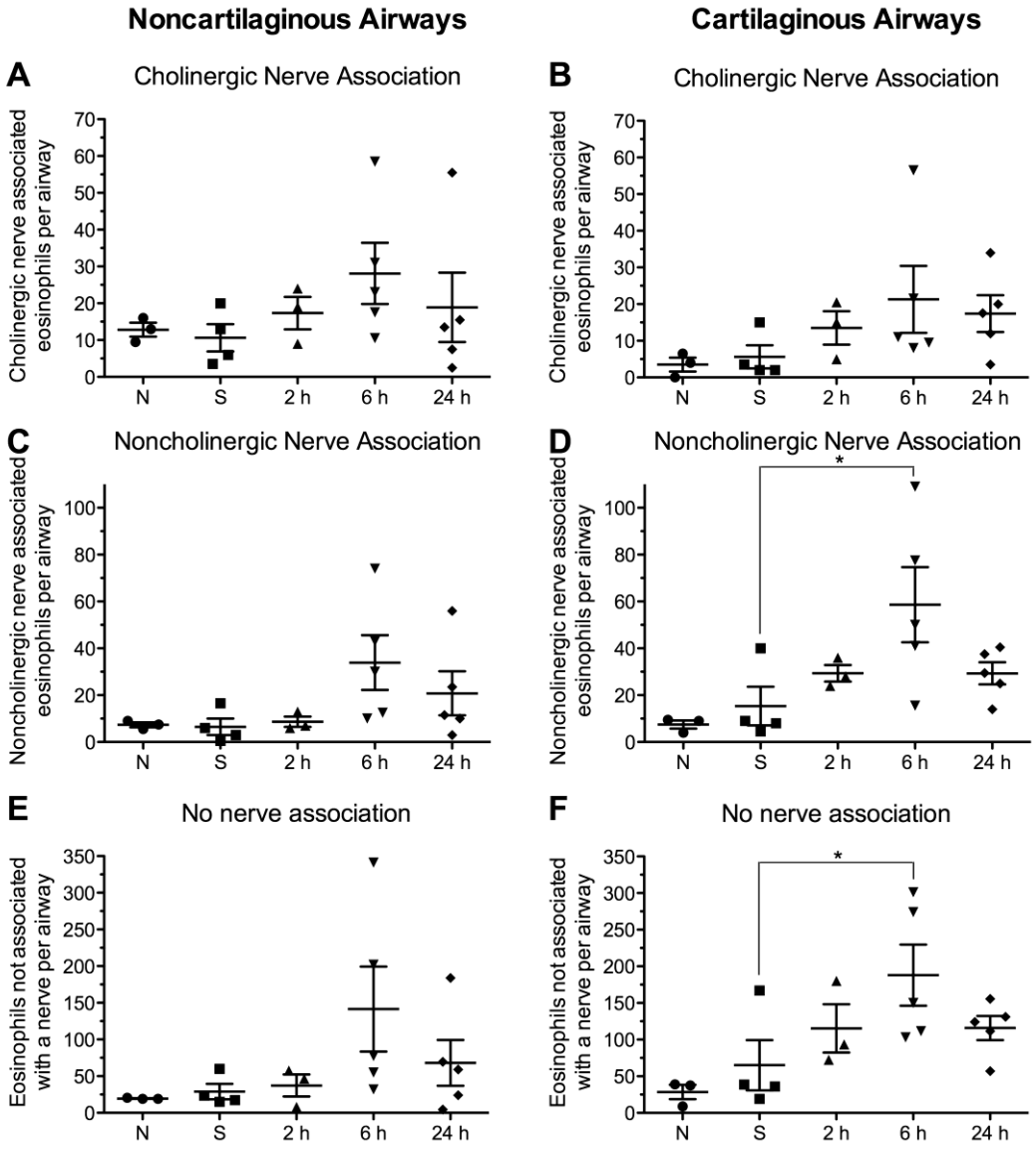

Figure 4.17 Antigen challenge increases the number of eosinophils associated with cholinergic and noncholinergic nerves. Eosinophils are associated with both cholinergic (A and B; ChAT+ and PGP9.5+) and noncholinergic (C and D; ChAT- and PGP9.5+) nerves in airways of nonsensitized (N) guinea pigs. Eosinophils must be within 8 μm (approximate diameter of an eosinophil) of a nerve to be associated. Sensitization (S) did not increase the number of nerve-associated eosinophils. However, challenge of sensitized guinea pigs (2 h, 6 h, or 24 h after challenge)

increased the number of eosinophils associated with nerves in noncartilaginous (A and C) and cartilaginous (B and D) airways. This was significant 6 h after challenge for noncholinergic nerves in cartilaginous airways. The number of eosinophils associated with nerves (E and F) also increased following antigen challenge. Each point represents the average of 2 airways from one animal. Mean and SEM are shown; n=3-5. Comparisons were made to the sensitized group; statistically significant changes are noted with *.

DISCUSSION

The data presented here demonstrate that TNFα induces expression of three of nine known CCR3 chemokines, CCL5, CCL7, and CCL11, in SK-N-SH neuroblastoma cells and only one chemokine, CCL7, in cultured parasympathetic nerves. However, *in vivo* data presented here do not support the hypothesis that eosinophils are recruited to nerves via neuronally produced CCR3 chemokines following antigen challenge. Instead, eosinophils appear to be recruited to the lungs via CCR3 chemokines produced by nonneuronal cells following antigen challenge. In the lungs, they associate with both cholinergic and noncholinergic nerves and thus may contribute to airway hyperreactivity via interactions with either population.

TNFα induced maximal expression of CCL5, CCL7, and CCL11 mRNA and protein between 4 and 24 h, a time frame that is relevant for eosinophil recruitment into airways following antigen challenge. TNFα-induced inflammatory genes fall into three broad expression classes that depend on both transcriptional control and mRNA stability, which is largely determined by AU-rich elements in the 3' UTR that target mRNA for degradation (Barreau *et al.*, 2005; Hao *et al.*, 2009). Group I genes are turned on within 30 min, have very unstable transcripts, and have inhibited expression with continued TNFα exposure (Hao *et al.*, 2009). Time courses in the current study miss any very transiently expressed group I chemokines. Group II genes, are turned on quickly and are continually transcribed but have unstable transcripts resulting in plateaued expression by 2 h. CCL7 is a known group II gene (Hao *et al.*, 2009), and the plateau in mRNA expression by 4 h in SK-N-SH cells is consistent with this. Since CCL7 mRNA

expression is decreased at 24 h, the dose response in SK-N-SH cells at this time point may reflect both transcript induction and mRNA instability. The data presented here suggest further that CCL11 is also a group II gene, since mRNA plateaus within 4 h of TNFα stimulation. Lastly, group III genes are induced more slowly but their transcripts are much more stable allowing continual increases in expression, which peak at 12 h or later (Hao *et al.*, 2009). CCL5 is a known group III gene (Hao *et al.*, 2009), and the dose and time course expression of CCL5 mRNA in SK-N-SH cells are consistent with this.

Human SK-N-SH neuroblastoma cells were used to screen for TNFα-induced CCR3 chemokine expression because they express TNF receptors (Nie *et al.*, 2009) and all nine known CCR3 ligands are found in humans. However, SK-N-SH cells may not be representative of airway parasympathetic nerves, since they have a sympathetic origin and are also a tumor cell line (Thiele, 1998). Tumor cells, including neuroblastoma cells, often produce chemokines, including CCL5 and CCL7 (Chuluyan *et al.*, 1998; Geminder *et al.*, 2001; Somasundaram *et al.*, 2009). Therefore, it was important to determine whether parasympathetic nerves also generate CCR3 chemokines in response to TNFα. Of the known CCR3 chemokines, only CCL5, CCL7, and CCL11 have been cloned in guinea pig (Asano *et al.*, 2001; Campbell *et al.*, 1997; Jose *et al.*, 1994). Guinea pig parasympathetic neurons in culture only expressed quantifiable levels of CCL7 mRNA, although a later time point may have allowed better detection of CCL5, since it is a group III gene that was occasionally detected but was not quantifiable. This was not considered an essential experiment because in guinea pig, unlike human, CCL5 is not chemotactic for eosinophils (Campbell *et al.*, 1997; Ponath *et al.*, 1996).

In both preliminary experiments where all cultured cells were harvested and in experiments where just the nerve cell bodies were isolated, TNFα only appeared to induce CCL7 in a dose-dependent manner in cultures from sensitized animals. It is possible that sensitization increases TNF receptor expression on nerves or that sensitization changes other cells (ex. lymphocytes) in the culture that may indirectly influence nerves. Although baseline CCL7 mRNA levels in untreated parasympathetic nerves were different for cultures from nonsensitized and sensitized animals, they are not directly comparable. This is because expression levels are measured relative to total input RNA and not total copies of CCL7 (see Chapter 2, Methods).

In vivo, parasympathetic ganglia harvested from guinea pig trachea did not express detectable levels of CCL7 or CCL5. Instead, only CCL11 was detected in parasympathetic ganglia from 0-40% of animals. This chemokine was often detected in primary cultures of guinea pig parasympathetic nerves, but was only ever quantifiable in one preliminary experiment with serum added. The promoter of CCL11, unlike other chemokine genes regulated by TNFα, has a STAT6 site immediately adjacent to the NF-κB site, and TNFα-induced CCL11 expression depends on STAT6 also being present (Hoeck *et al.*, 2001). Serum likely contains cytokines such as IL-4 and IL-13 that signal through STAT6. The inconsistent expression of CCL11 mRNA in ganglia suggests two possible explanations. First, parasympathetic nerves express CCL11, but at low levels that are very close to the limit of detection. Second, parasympathetic nerves are not synthesizing CCL11 mRNA, and ganglia only express CCL11 if nonneuronal cells expressing CCL11 mRNA remain attached to the ganglia. My data cannot distinguish between these two options. Future experiments could include development of a panel of primers not found in nerves, to test for expression of RNA from potential contaminating

cells, including epithelial cells, smooth muscle, and fibroblast. If CCL11 expression correlates with expression of a marker for another cell type this would suggest strongly suggest CCL11 expression was due to mRNA from nonneuronal cells.

Since only CCL11 mRNA was detected in primary cultures of guinea pig parasympathetic nerves, an antibody against guinea pig CCL11 was used to look for protein expression. While approximately 60% of cholinergic parasympathetic nerves colocalized with CCL11 protein by immunostaining, this does not prove that nerves produce CCL11. Chemokines are secreted proteins that are positively charged and bind to negatively charged glycosaminoglycans throughout the tissue to maintain chemokine gradients (Rot *et al.*, 2004). In airway tissues from humans with asthma, antigen-challenged humans, and antigen-challenged guinea pigs, *in situ* hybridization demonstrates that CCL11 mRNA is produced predominantly by airway epithelial cells, but also by endothelial cells, alveolar macrophages, smooth muscle cells, lymphocytes, mast cells, and eosinophils (Brown *et al.*, 1998; Li *et al.*, 1997; Ying *et al.*, 1999). The data presented here indicate parasympathetic nerves may contribute to CCL11 protein in the tissue. However, since CCL11 staining colocalized with cholinergic nerve fibers was not brighter than that found in surrounding tissue, this data also suggests parasympathetic nerves do not set up a chemokine gradient of CCL11 protein to recruit eosinophils.

Previous studies demonstrating eosinophil association with airway nerves have not been able to distinguish between neuronal cell populations. In addition, eosinophils in airway epithelium have previously been excluded because airway epithelium is not innervated by parasympathetic nerves (Costello *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 2006; Nie *et al.*, 2009; Verbout *et al.*, 2007). Since mRNA and protein data from guinea pig

parasympathetic ganglia and lung sections did not support parasympathetic nerves as a major source of CCR3 chemokines, I asked whether eosinophils were recruited to cholinergic nerves or noncholinergic nerves. Choline acetyltransferase is an enzyme used to synthesize acetylcholine and is found in cholinergic parasympathetic nerves, which are physiologically important in asthma because they stimulate smooth muscle contraction. However, not all parasympathetic nerves are cholinergic and sensory nerves and sympathetic nerves also innervate the airways of guinea pigs.

I found that sensitization with ovalbumin doubles eosinophils in cartilaginous airways (bronchi) but not in noncartilaginous airways (bronchioles) relative to nonsensitized controls, although this was not statistically significant. Antigen challenge increases eosinophils in both noncartilaginous and cartilaginous airways in a time-dependent manner and this was statistically significant some of the time. Few eosinophils move into the airways 2 h after challenge; however, by 6 h after challenge eosinophils are maximally increased. 24 h after challenge, which is the time point analyzed in previous experiments that quantify nerve-associated eosinophils, eosinophil numbers are returning toward baseline values. This pattern of eosinophil accumulation is consistent with previous time-course studies that assess early time points in guinea pigs and humans. In guinea pigs, eosinophil peroxidase activity in lung homogenates peaks 6 h after challenge (Asano *et al.*, 2001; Humbles *et al.*, 1997). Following antigen challenge of humans with allergic asthma, tissue eosinophils peak between 4 and 24 h after challenge (Brown *et al.*, 1998). General tissue eosinophil recruitment also correlates well with CCL11 expression. Measured by ELISA in guinea pig lung tissue and by the number of cells expressing CCL11 (mRNA or protein) in human bronchi, CCL11 peaks around 6 h after antigen challenge and returns to baseline by 24 h after challenge, when tissue eosinophils are also reduced (Asano *et al.*, 2001; Brown *et al.*, 1998; Humbles *et*

al., 1997). In asthma patients that are not challenged, CCL11 and CCL24 mRNA expression correlate with tissue eosinophils, and CCL11 correlates further with airway hyperresponsiveness (Ying *et al.*, 1999). CCL5, CCL7, and CCL13 mRNA are all increased in asthma, but do not correlate with tissue eosinophils (Powell *et al.*, 1996; Ying *et al.*, 1999).

Previous work demonstrates that eosinophils traffic from tissue into the airway lumen 24 h after challenge, where they increase significantly in bronchoalveolar lavage (Brown *et al.*, 1998; Humbles *et al.*, 1997). This is not the only mechanism that can account for a decrease in tissue eosinophils between 6 and 24 h though. Evidence for eosinophil degranulation is seen following antigen challenge in airway tissues from humans with asthma and guinea pigs by electron microscopy (Beasley *et al.*, 1989; Erjefalt *et al.*, 1997a). Degranulation was not assayed here but may be partially responsible for the decrease in eosinophils. In addition, eosinophils are also able to present antigens to T-cells (Wang *et al.*, 2007), and in guinea pig eosinophils are increased in lymph nodes 5 h after challenge (Erjefalt *et al.*, 1997a). Thus, airway tissue eosinophils may also be reduced due to eosinophil trafficking to lymph nodes.

Although tissue eosinophils were increased as expected following antigen challenge, the data presented here do not support selective recruitment of eosinophils to either cholinergic or noncholinergic airway nerves. Eosinophils did not move closer to airway nerves following antigen challenge in noncartilaginous or cartilaginous airways, and the proportion of eosinophils associated with either cholinergic or noncholinergic nerves also did not change. Instead, eosinophils increased in general, and were distributed proportionally to cholinergic and noncholinergic nerves. In general, eosinophils did not move into the smooth muscle, but instead accumulated along the borders where they

were often within 8 μm of cholinergic (found in smooth muscle and adventitia) and noncholinergic nerves (found in smooth muscle, epithelium, and adventitia). This pattern of eosinophil distribution is not unique to guinea pig. In cross sections of bronchi from humans with asthma, leukocytes in general and eosinophils in particular are found with a similar distribution that generally excludes airway smooth muscle (Haley *et al.*, 1998). This distribution may be explained by the location of blood vessels supplying airways. A dense capillary plexus is found on either side of airway smooth muscle, in the subepithelial space and in the adventitia (Lim *et al.*, 2002; Widdicombe, 1993). Eosinophils entering airway tissue via small post-capillary venules (Lim et al., 2002) would see a concentration gradient of CCL11 favoring chemotaxis toward the epithelium. Another chemokine may regulate adventitial eosinophils.

Even though eosinophils do not appear to be selectively recruited to nerves via neuronally produced chemokines there is a strong trend toward more eosinophils associating with nerves after challenge. This is a substantial increase when two-dimensional data presented here are translated to the three-dimensional surface of all conducting airways. Furthermore, eosinophils associate with both cholinergic and noncholinergic nerves. This suggests eosinophils could have effects at sensory, sympathetic, and parasympathetic nerves that contribute to increases in airway hyperreactivity and exacerbations in asthma. Surgical denervation of all three-nerve populations in humans with asthma resulted in improvements in all but one patient and abolished sputum eosinophils (Dimitrov-Szokodi *et al.*, 1957). Furthermore, neuronal reflexes and not neuronally produced chemokines may actually be necessary to establish or maintain eosinophilia. In mice, depletion of neuropeptides from sensory

nerves with capsaicin significantly reduces NF-κB phosphorylation, CCL11, IL-5, and bronchoalveolar lavage eosinophils following antigen challenge (Rogerio et al., 2011).

Once in the lungs, eosinophils can be induced to release cytokines, chemokines, and neurotrophins in addition to their granule proteins (Hogan *et al.*, 2008). Airway nerves express the integrin ICAM, and activation of eosinophils by CCR3 chemokines following adhesion to ICAM may stimulate eosinophils to release factors, such as MBP that mediate airway hyperreactivity (Evans *et al.*, 1997; Fujisawa *et al.*, 2000; Nie *et al.*, 2007). *In vitro*, eosinophils release factors that increase sensory nerve growth and branching (Foster *et al.*, 2011). Increased nerve growth could be another mechanism contributing to airway hyperreactivity in asthma.

In addition, chemokines produced by nerves or other cells may also directly stimulate nerves to contribute to airway hyperresponsiveness. Sensory nerves from dorsal root ganglia express a variety of functional chemokine receptors, including CCR3. Stimulation of these receptors *in vitro* with chemokines results in mobilization of intracellular calcium and excitatory responses, lowering the threshold for membrane depolarization or directly depolarizing the nerve (Oh *et al.*, 2001). *In vivo*, pain hypersensitivity following nerve injury correlates with upregulation of chemokine receptors on sensory nerves and prolonged expression of inflammatory chemokines (Bhangoo *et al.*, 2007). Chronic antigen exposure in rhesus monkeys induces CCL26 in large airway nerves (Chou *et al.*, 2005). Neuronal CCL26 in this model is not important for eosinophil recruitment, since MBP is not found in or near these nerves (Chou *et al.*, 2005). It is possible, that with chronic antigen exposure nerves in the lung also upregulate chemokine receptors.

A limitation to the data presented in this chapter is although many changes were reproducible they failed to reach statistical significance. This is partially due to the number of comparisons being made. It is harder to achieve significance with more comparisons, since p-values for pairwise comparisons must be adjusted for the total number of comparisons. However, it is also partially due to the variability in measured responses. In particular, eosinophil numbers 6 h after challenge were highly variable, and this variability is probably physiologically relevant since this time point occurs when eosinophils may be increasing, decreasing, or maximal in any given guinea pig.

In summary, neuronal cells are capable of producing CCR3 chemokines in response to TNFα, and some parasympathetic nerves colocalize with low levels of CCL11 *in vivo*. While, it is possible that parasympathetic nerves express chemokines that have not been cloned in guinea pig or only occur in humans, the current data do not support neuronal production of chemokines as a mechanism for eosinophil recruitment following antigen challenge. Instead, these data suggest a model where CCR3 chemokines produced by epithelial or other nonneuronal cells increase eosinophils in airways after antigen challenge, and this general increase in eosinophils results in more nerve-associated eosinophils (Figure 4.18). This study extends previous observations of nerve-associated eosinophils, since it shows eosinophils associate with both cholinergic and noncholinergic nerves, and it suggests interactions with both populations may be important for airway hyperreactivity.

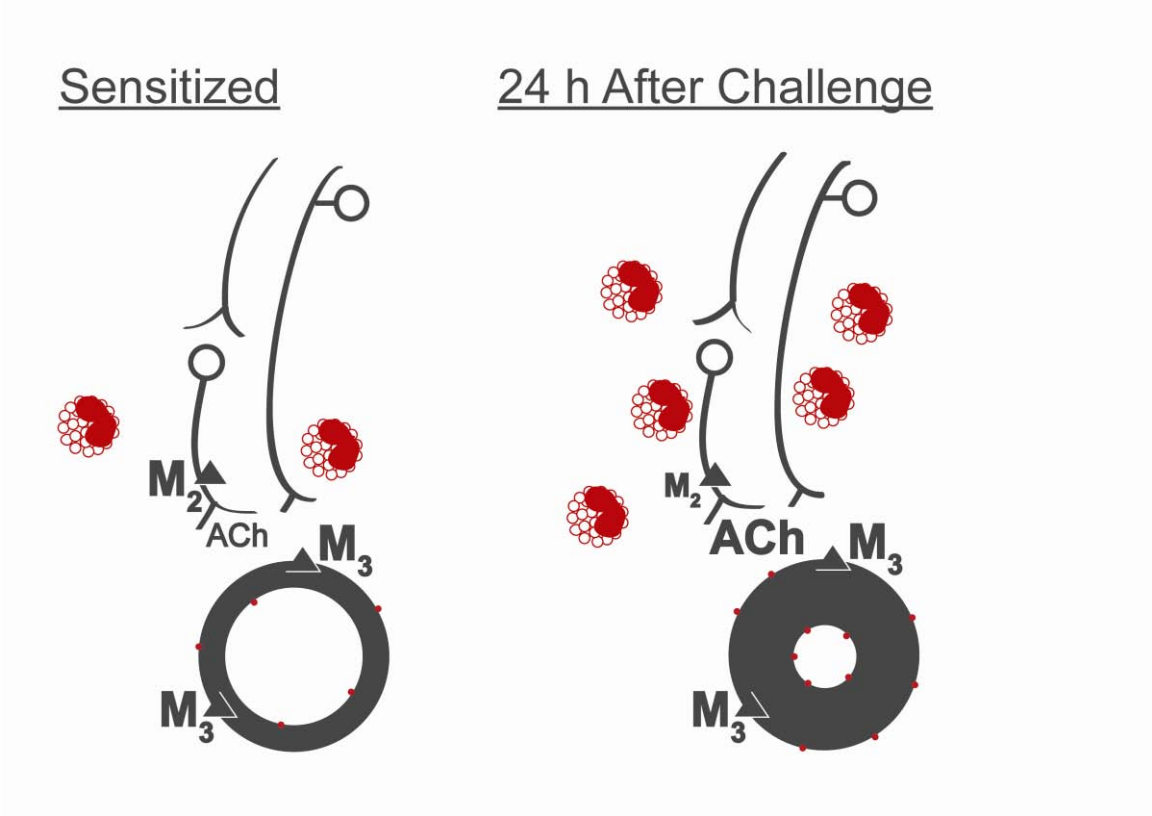

Figure 4.18 Eosinophils associate with both cholinergic and noncholinergic nerves following antigen challenge. In sensitized guinea pigs (left), eosinophils (red) are found on either side of airway smooth muscle (gray circle). In sensitized animals, some airway eosinophils associate with nerves, both cholinergic (parasympathetic) nerves that release acetylcholine and also noncholinergic nerves, such as sensory nerves. Following antigen challenge (right), eosinophils increase in the lungs following expression of CCR3 ligands by nonneuronal cells. Eosinophils increase around cholinergic and noncholinergic nerves suggesting they interact with both populations to cause vagally mediated airway hyperreactivity.

CHAPTER 5.

GENERAL DISCUSSION

GENERAL DISCUSSION

Airway hyperresponsiveness is a characteristic feature of asthma. While asthma patients by definition are hyperresponsive, their airways are not always constricted more than the airways of healthy humans. Instead, bronchoconstriction is triggered and occurs during an exacerbation. Airway hyperresponsiveness is associated with the recruitment of eosinophils to the lungs and their association with airway nerves. In animal models of asthma, treatments that prevent esoinsophils from associating with airway nerves prevent subsequent airway hyperreactivity, while in humans treatments that reduce eosinophils also reduce asthma exacerbations. Thus, understanding airway hyperresponsiveness and the factors that contribute to increases in airway responsiveness is important for developing treatments to prevent exacerbations, which pose the greatest risk to asthma patients.

In my thesis research I used a guinea pig model of allergic asthma to study airway hyperresponsiveness and eosinophil recruitment to airway nerves. The major findings of my research are that selective blockade of M_3 muscarinic receptors at the time of inhaled antigen challenge prevents airway hyperreactivity in a guinea pig model of allergic asthma and that this may be due to inhibition of nerve-associated eosinophils (Chapter 3). Also, eosinophils associate with both cholinergic and noncholinergic nerves following antigen challenge suggesting they may interact with either nerve population to induce airway hyperreactivity (Chapter 4).

My thesis research specifically addresses the role of parasympathetic nerves in asthma. Airway hyperreactivity in humans and animals is mediated by the vagus nerves, which

contain both parasympathetic and sensory nerve fibers. Sensory nerves are activated by contractile stimuli to initiate vagal reflexes that end with acetylcholine release from parasympathetic nerves onto M_3 receptors on airway smooth muscle. Nonselective muscarinic receptor antagonists, which block these receptors, are recommended for treatment of bronchoconstriction during an exacerbation, however, they are not recommended for long-term treatment to prevent exacerbations due to their limited effectiveness in asthma (EPR-3, 2007). Since nonselective blockade of all muscarinic receptor subtypes increased eosinophil degranulation and potentiated airway hyperreactivity in previous experiments (Verbout *et al.*, 2007), I tested the hypothesis that selective blockade of M_3 receptors at the time of challenge would improve subsequent airway hyperreactivity. My data demonstrate that selective blockade of M_3 receptors at the time of antigen challenge prevents subsequent airway hyperreactivity in a guinea pig model of allergic asthma (Chapter 3). Moreover, vagally mediated airway hyperreactivity is prevented by a mechanism that does not involve inhibition of bronchoconstriction and may be due to anti-inflammatory effects of blocking M_3 receptors (Chapter 3).

I showed that tiotropium, used in a kinetically selective manner for M_3 receptors, completely prevents airway hyperreactivity in guinea pigs that are sensitized and antigen challenged. Tiotropium reduces vagally induced bronchoconstriction and returns airway responsiveness to the same level seen in unchallenged sensitized control animals (Chapter 3). Since tiotropium pretreatment does not reduce vagally induced bronchoconstriction in unchallenged sensitized control animals, I have shown that hyperreactivity in sensitized and challenged guinea pigs is prevented by a mechanism that does not involve inhibition of bronchoconstriction (Chapter 3). There are two reasons this is important. First, these data suggest that selective M_3 receptor

antagonists may have benefits for asthma treatment independent of their ability to acutely inhibit bronchoconstriction. Second, these data suggest exacerbation frequency may be an important outcome to measure in clinical trials with selective anticholinergics. If M_3 selective muscarinic antagonists also prevent increases in airway hyperresponsiveness in humans with asthma, this could reduce the development of exacerbations and decrease their frequency.

I have shown that tiotropium prevents airway hyperreactivity in antigen-challenged guinea pigs, which best represent atopic patients with eosinophilic asthma triggered with allergens. Asthma is a heterogeneous disease, however, and recent phenotyping studies demonstrate that approximately 50% of asthma patients have eosinophilic inflammation (Haldar *et al.*, 2008; Nadif *et al.*, 2009; Woodruff *et al.*, 2009). In addition, multiple stimuli increase airway hyperresponsiveness, including inhaled antigens, respiratory viruses, and environmental triggers such as ozone and pesticides (Adamko *et al.*, 1999; Evans *et al.*, 1997; Proskocil *et al.*, 2008; Yost *et al.*, 1999). Therefore, future studies should determine whether selective blockade of M_3 receptors also prevents airway hyperreactivity triggered by stimuli other than antigens and in animal models that represent noneosinophilic asthma phenotypes. Testing prevention of airway hyperreactivity following respiratory virus infection would be particularly important, since many asthma exacerbations are associated with viral infections (O'Byrne, 2011). Furthermore, airway hyperreactivity following infection with sendai virus in guinea pigs is eosinophil-dependent in sensitized animals but eosinophil-independent in nonsensitized animals (Adamko *et al.*, 1999), which would allow investigation of eosinophilic and noneosinophilic phenotypes. It is possible that selective muscarinic antagonists may only benefit a subpopulation of asthma patients, such as those with eosinophilic asthma.

Assuming selective muscarinic receptor antagonists would benefit humans with eosinophilic asthma my data also have implications for the clinical use of tiotropium. Based on tiotropium's dissociation kinetics, tiotropium could be administered in the morning to achieve M_3 selective blockade in the middle of the night. This could be particularly important for a subset of patients who have nocturnal asthma and are awakened by their symptoms in the middle of the night. There is an influx of eosinophils into the distal airways and alveoli of these patients that peaks around 4 AM (Kraft *et al.*, 1995; Kraft *et al.*, 1999) and bronchoconstriction is associated with higher vagal activity at 4 AM (Morrison *et al.*, 1989). Selective blockade of M_3 receptors during the night may then be beneficial for two reasons. First, it would be important to leave available neuronal M_2 receptors, which normally limit acetylcholine release from the nerves. Second, selective blockade of M_3 receptors may also limit eosinophil influx into the airways, since tiotropium reduces eosinophil recruitment following antigen challenge (Chapter 3 and (Bos *et al.*, 2007; Ohta *et al.*, 2010)).

One limitation of my studies is the lack of tiotropium dissociation kinetics from M_4 and M_5 receptors. In addition, tiotropium's selectivity for M_3 over M_1 receptors is based solely on *in vitro* experiments in human cells (Casarosa *et al.*, 2009; Disse *et al.*, 1993). However, tiotropium does dissociates fully from neuronal M_2 receptors within 2 h in isolated guinea pig trachea indicating it is very unlikely that tiotropium blocked M_2 receptors at the time of challenge (24 h after administration) in my experiments (Takahashi *et al.*, 1994). Pharmaceutical companies are currently developing affinity-selective muscarinic receptor antagonists with greater than 100-fold selectivity for one muscarinic subtypes over all of the other subtypes (Sagara *et al.*, 2006). As these affinity-selective drugs become available for research use, it will be important to determine whether selectivity for M_3 receptors over all other muscarinic subtypes or selectivity for M_3 receptors over a

subset of muscarinic receptors is important. Since nonselectively blocking all muscarinic receptors at the time of antigen challenge with atropine only partially reduced subsequent airway hyperresponsiveness in my experiments, it seems likely that blockade of at least one other muscarinic receptor subtype will work against the benefits of blocking M_3 receptors (Chapter 3).

The outcome measured in most human asthma studies with muscarinic antagonists is inhibition of baseline vagal tone or bronchoconstriction induced by exogenous acetylcholine. However, effective blockade of baseline vagal tone or blockade of bronchoconstriction induced by inhaled acetylcholine does not ensure adequate blockade of acetylcholine release from parasympathetic nerves during an exacerbation. This has previously been shown for atropine (Sheppard, 1982) and ipratropium (Cockcroft *et al.*, 1978; Ward *et al.*, 1981) in humans. I have extended this observation to tiotropium in guinea pig, since doses of insufflated tiotropium that inhibit intravenous-acetylcholine-induced bronchoconstriction do not inhibit vagally induced bronchoconstriction in guinea pigs (Chapter 3). These data indicate that anticholinergic doses should instead be determined by blockade of reflex-induced bronchoconstriction, such as that induced by inhaled histamine or cold air, for both animal studies and asthma trials.

The dose of anticholinergics necessary to inhibit physiologically relevant bronchoconstriction in asthma patients is important. When doses of muscarinic antagonists that appear to be efficacious, based on blockade of baseline vagal tone or bronchoconstriction induced by exogenous agonists, fail to block reflex-induced bronchoconstriction the importance of parasympathetic nerves in disease is questioned. Much of the controversy over the ability of anticholinergics to inhibit acute

bronchoconstriction induced by antigen, exercise, psychogenic factors, and viruses is likely explained by differences in the dose of anticholinergics used (see Chapter 1). The idea that parasympathetic nerves are not important in asthma also occurs because doses of muscarinic antagonist that are not effective for asthma exacerbations are often used with great clinical benefit in patients with chronic obstructive pulmonary disease (COPD) (GOLD, 2009). The major difference between these diseases is that in COPD baseline vagal tone is one of the only reversible components of airway obstruction (Fryer *et al.*, 2008), and low doses of anticholinergics are effective for blocking baseline tone.

There are several potential explanations for why tiotropium administered by insufflation blocked intravenous-acetylcholine-induced bronchoconstriction but not vagally induced bronchoconstriction in Chapter 3. One possible mechanism is that tiotropium does not reach smooth muscle cells or M_3 receptors innervated by parasympathetic nerves. Parasympathetic nerves directly innervate only a subset of smooth muscle cells. In human bronchi there are only 11 nerve varicosities containing neurotransmitter for every 100 smooth muscle cells as determined by electron microscopy (Daniel *et al.*, 1986). However, I showed that intravenous administration of tiotropium, which increases access to airway smooth muscle, blocks intravenous-acetylcholine-induced bronchoconstriction at lower doses than vagally induced bronchoconstriction. These data suggest that distribution of tiotropium in tissue alone cannot explain the difference in blockade of vagally and intravenous-acetylcholine-induced bronchoconstriction.

This led me to propose a new model in which variations in local acetylcholine concentration contribute to the level of tiotropium binding by differential competition for M_3 receptor occupancy (Figure 3.11). In regions of smooth muscle directly innervated by nerves, high local acetylcholine concentrations compete with tiotropium for binding at M_3

receptors, resulting in less tiotropium binding and little inhibition of vagally induced bronchoconstriction. In contrast, in other regions of smooth muscle, low local acetylcholine concentrations compete less with tiotropium for binding at M_3 receptors, resulting in more tiotropium binding and inhibition of intravenous-acetylcholine-induced bronchoconstriction. Since tiotropium binding is pseudo-irreversible, these regional differences in M_3 receptor blockade were revealed when I measured blockade of vagally induced bronchoconstriction and intravenous-acetylcholine-induced bronchoconstriction.

The intravenous tiotropium experiment assumes equal access of tiotropium to all smooth muscle muscarinic receptors. This assumption may not be correct. I later observed that eosinophils rarely migrate into smooth muscle in the lung, and the literature suggested this was because eosinophils enter the lung via capillary beds that exist on either side of airway smooth muscle but not within smooth muscle (Chapter 4). This means that although intravenous tiotropium would have better access to smooth muscle compared to insufflated tiotropium, intravenous tiotropium would still have to distribute from the outside of the smooth muscle inwards. Many parasympathetic nerves are found within the smooth muscle and tiotropium may not have had equal access to M_3 receptors innervated by these nerves. Furthermore, intravenous administration was used to give both exogenous acetylcholine and tiotropium. Therefore, the same population of M_3 receptors were blocked by tiotropium and tested by exogenous acetylcholine, and this population may not have included M_3 receptors innervated by parasympathetic nerves.

My data therefore do not rule out unequal access of insufflated tiotropium to smooth muscle. This could be tested further by giving cumulative doses of insufflated tiotropium acutely and determining whether there is a greater difference in the dose of insufflated tiotropium required to inhibit intravenous-acetylcholine-induced and vagally induced

bronchoconstriction than there is when tiotropium is administered intravenously. Regardless of mechanism, the important outcome is that higher doses of tiotropium are needed to block vagally released acetylcholine whether this is to overcome distribution limitations or high local acetylcholine concentrations. I have already demonstrated that it is feasible to block vagally induced bronchoconstriction by increasing tiotropium concentrations (Chapter 4).

Raising anticholinergic doses to a level that effectively inhibits bronchoconstriction during an exacerbation in asthma patients may not be possible, as side effects of the drugs will also increase. The greatest side effect associated with currently recommended doses of ipratropium and tiotropium is dry mouth because anticholinergics block muscarinic receptors in the salivary gland, which stimulate salivary secretions (Brown *et al.*, 2006; Littner *et al.*, 2000). Other potential side effects of nonselective anticholinergics include increased heart rate, increased urinary retention, and decreased gut motility (Brown *et al.*, 2006). Muscarinic signaling is also prevalent in the central nervous system and drugs like atropine, which cross the blood-brain barrier also have effects on cognitive function (Brown *et al.*, 2006; Sheppard, 1982). These side effects could be serious enough to outweigh any benefit derived from increasing the dose to a level that would block bronchoconstriction during an exacerbation.

The concern over side effects is supported by a recent meta-analysis of trials with chronic tiotropium treatment in COPD. There is a 46% increased risk of mortality, or 1 extra death for every 124 patients treated, primarily associated with cardiovascular events in patients administered 5 μg tiotropium with the Respimat Soft Mist Inhaler (Singh *et al.*, 2011). The risk doubles if 10 μg tiotropium is administered with the same

device (Singh *et al.*, 2011). In contrast, a higher dose of tiotropium (18 μg) administered as a powder with a Handihaler device does not increase the risk for mortality (Michele *et al.*, 2010). With the Respimat inhaler more tiotropium makes it into systemic circulation, since peak plasma concentrations and the amount of tiotropium excreted in the urine in the first 12 h are significantly higher (van Noord *et al.*, 2009). This increase in systemic drug concentration may account for the increased mortality.

One of the surprising implications of my research is that inhibition of bronchoconstriction may not be necessary to have benefits of anticholinergics in asthma, since selective blockade of M_3 muscarinic receptors was beneficial in antigen-challenged guinea pigs even though bronchoconstriction was not blocked. My data suggest that tiotropium may have worked to prevent airway hyperreactivity through an anti-inflammatory mechanism, since tiotropium decreased total and nerve-associated eosinophils in the tissues following antigen challenge (Chapter 3).

I also showed that following antigen challenge of sensitized guinea pigs, eosinophils are recruited into the lung where they associate with both cholinergic and noncholinergic nerves (Chapter 4). The greatest number of eosinophils was found 6 h after challenge (Chapter 4). Increased eosinophils are still present 24 h after challenge (Chapter 3 and 4), but are decreased from 6 h (Chapter 4). The decrease in eosinophils between 6 and 24 h after antigen challenge may reflect eosinophil degranulation, which could lead to airway hyperreactivity through blockade of M_2 receptors on parasympathetic nerves with eosinophil-released MBP.

Classically degranulation is detected with either electron microscopy or immunofluorescence (Beasley *et al.*, 1989; Costello *et al.*, 1997; Erjefalt *et al.*, 1997a; Verbout *et al.*, 2007). It would be interesting to determine whether there is evidence for increased extracellular MBP 24 h after antigen challenge compared to 6 h after challenge. This was not feasible with immunostaining in the study presented in Chapter 4 because cryosections fixed with Zamboni's fixative are incompatible with available antibodies that work in guinea pig.

Previous data demonstrate that blockade of CCR3 chemokine receptors reduces nerve-associated eosinophils and prevents vagally mediated airway hyperreactivity following antigen challenge (Fryer *et al.*, 2006). CCL11, a CCR3 chemokine, has previously been detected in airway nerves from antigen-challenged guinea pigs and in human (nonasthmatic) airway nerves by immunohistochemistry (Fryer *et al.*, 2006). The second hypothesis that I tested was that parasympathetic nerves may recruit eosinophils via release of CCR3 chemokines in asthma. I found that human neuroblastoma cells and guinea pig parasympathetic nerves are able to produce CCR3 chemokines in response to TNFα (Chapter 4). However, despite neuronal expression of CCR3 chemokines *in vitro*, three lines of evidence argue against the hypothesis that eosinophils are recruited to parasympathetic nerves via neuronally produced chemokines *in vivo*. First, parasympathetic ganglia from only a few animals express CCL11 mRNA and cholinergic nerve fibers only colocalize with low levels of CCL11. The cholinergic nerve fibers do not stain more intensely than surrounding tissues, which might have been expected if they produced a chemokine gradient. Second, eosinophils do not move closer to airway nerves following antigen challenge. Lastly, eosinophils are not selectively recruited to either cholinergic or noncholinergic nerves, since the proportion of

eosinophils associated with cholinergic nerves or noncholinergic nerves does not change following antigen challenge.

My data instead support a model where eosinophils are recruited to airways via CCR3 chemokines produced by other cells. Airway epithelium may be responsible for eosinophil recruitment, since it is a significant source of CCL11 and many eosinophils are found in or near airway epithelium (Chapter 4 and (Brown *et al.*, 1998; Li *et al.*, 1997; Ying *et al.*, 1999)). The airway epithelium is also known to produce CCR3 chemokines CCL5, CCL7, CCL13, CCL24, CCL26, and CCL28 in asthma patients or following acute or chronic antigen challenge in various species (Chou *et al.*, 2005; John *et al.*, 2005; Powell *et al.*, 1996; Ying *et al.*, 1999).

Once in the lung, a portion of the recruited eosinophils end up associated with airway nerves (within 8 μm of a nerve). My data extend previous findings, and demonstrate that eosinophils associate with both cholinergic and noncholinergic nerves following antigen challenge (Chapter 4). Since blockade of CCR3 receptors inhibits nerve-associated eosinophils and airway hyperreactivity (Fryer *et al.*, 2006), my data suggest that eosinophils could interact with either sensory or sympathetic nerves in addition to parasympathetic nerves to increase airway hyperreactivity in asthma. Thus, rather than specific recruitment of eosinophils to nerves, eosinophil recruitment to the whole lung increases and this results in more eosinophils near airway nerves, where they can modulate nerve function.

My data do not rule out the possibility that airway nerves, including parasympathetic nerves, may still produce additional CCR3 chemokines that have not been cloned in guinea pig. Only three of the nine known CCR3 chemokine (CCL5, CCL7, and CCL11)

genes in humans have been cloned in guinea pig. If airway nerves are producing additional chemokines, they may be utilized for a purpose other than setting up a chemokine gradient to recruit eosinophils, since my data do not support selective recruitment of eosinophils to nerves.

One potential function that neuronally produced or eosinophil produced chemokines could have is to directly activate airway nerves to mediate airway hyperreactivity. This has been proposed for pain hypersensitivity models where both chemokine receptors and chemokines are expressed chronically by nerves following nerve injury (Bhangoo *et al.*, 2007). Airway sensory nerves in particular may express CCR3 receptors, since approximately 15% of cultured sensory nerves from dorsal root ganglia express functional CCR3 receptors (Oh *et al.*, 2001). Activation of these receptors increases the excitability of these neurons (Oh *et al.*, 2001). If CCR3 is expressed by airway nerves (sensory or parasympathetic) or is induced following antigen challenge, chemokines that are normally associated with eosinophil recruitment could also directly stimulate nerves to contribute to airway hyperresponsiveness. Therefore, future experiments should determine whether airway nerves express CCR3 receptors or whether neuronal CCR3 receptors are induced following antigen challenge.

Since eosinophils likely increase around airway nerves via chemokines produced by nonneuronal cells, tiotropium could have reduced eosinophilic inflammation in the lung by blocking chemokine production by nonneuronal cells. Acetylcholine activates M_3 muscarinic receptors on both airway epithelial cells and macrophages to release chemotactic factors for eosinophils and neutrophils (Koyama *et al.*, 1992; Koyama *et al.*, 1998; Sato *et al.*, 1998). M_3 receptors on these cells would see the highest concentration of tiotropium following insufflation into the lungs making them good

candidates for tiotropium's actions. Also, epithelial cells are a significant source of acetylcholine in the lung although they produce much less acetylcholine than airway nerves (Proskocil *et al.*, 2004; Reinheimer *et al.*, 1998). Since vagally induced bronchoconstriction was not inhibited by tiotropium (Chapter 3) it is possible that epithelial acetylcholine may be involved in the development of airway hyperresponsiveness. Intriguingly glucocorticoids, which are well known to reduce airway eosinophils, also reduce epithelial acetylcholine measured by HPLC in rats (Reinheimer *et al.*, 1998).

It is also possible that tiotropium could inhibit eosinophilic inflammation by blocking M_3 receptors on inflammatory cells other than macrophages and epithelial cells. This could be through a direct mechanism since eosinophils express M_3 receptors (Verbout *et al.*, 2006), however, their function is not known. The mechanism could also be indirect. Lymphocytes express M_3 receptors (Hellstrom-Lindahl *et al.*, 1996; Ricci *et al.*, 2002; Tayebati *et al.*, 1999; Tayebati *et al.*, 2002), and it's possible that acetylcholine activates M_3 receptors on these cells to increase Th2 cytokine release, since tiotropium reduces Th2 cytokines and lung eosinophils following chronic antigen challenge in mice (Ohta *et al.*, 2010). This could be important, since IL-5 regulates eosinophil growth and differentiation in bone marrow and IL-4 and IL-13 are both capable of regulating CCR3 chemokines (Hogan *et al.*, 2008). To determine whether M_3 receptors on leukocytes are necessary for eosinophil recruitment into lung, M_3 receptor-deficient mice (Yamada *et al.*, 2001) could be used to create bone marrow chimeras. This would involve irradiating wildtype mice to kill their bone marrow and then reconstituting their bone marrow with cells isolated from either wildtype or M_3 receptor-deficient mice (Coligan, 2001). If M_3 receptors on inflammatory cells are necessary for eosinophil recruitment, mice

reconstituted with cells deficient for M_3 receptors would have reduced eosinophil recruitment following antigen challenge.

In conclusion, the experiments in this thesis lead to a new model of eosinophil recruitment and airway hyperreactivity following antigen challenge. In this model, eosinophils are recruited to the lung via CCR3 chemokines produced by nonneuronal cells where they lead to vagally mediated airway hyperreactivity via interactions with parasympathetic, sensory, and possibly sympathetic nerves. M_3 muscarinic receptors are necessary for eosinophil recruitment to airways following antigen exposure and for subsequent development of airway hyperreactivity; however, it is not clear whether M_3 receptors important for airway hyperreactivity are located on smooth muscle, epithelial, or inflammatory cells in the lungs. Combined, these data suggest M_3 muscarinic receptor selective antagonists may be beneficial for long-term treatment in asthma patients with eosinophilic asthma via an anti-inflammatory mechanism.

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