The Role of TNF-α and IL-1β in Virus-induced M2 Receptor Dysfunction

By

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CERTIFICATE OF APPROVAL

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PUBLICATIONS

Manuscripts

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2. Hammarlund, E., Thomas, A., Poore, E.A., **Rynko, A.**, Mori, T., Chen, Z., Slifka, M.K. Durability of Vaccine-Mediated Immunity Against Tetanus and Diphtheria Toxins. (In Preparation).

3. Hoffmann, T. J., Simon, B. J., Zhang, Y. and Emala, C. W. Low Voltage Vagal Nerve Stimulation Reduces Bronchoconstriction in Guinea Pigs Through Catecholamine Release. *Neuromodulation.* 2012 Nov-Dec;15(6):527–36. – **Rynko, A.** and Jacoby, D.B. Public commentary that supplements the article.

Abstracts

1. **Rynko, A.**, Fryer, A.D., Jacoby, D.B. Blocking IL-1β Prevents M2 Muscarinic Dysfunction in Parainfluenza Infected Guinea Pigs. *J. Immunol.* 2013 190:

120.13.

2. **Rynko, A.**, Fryer, A.D., Jacoby, D. B. Etanercept Blocks Parainfluenza Downregulation Of M2 Muscarinic Receptor mRNA In Parasympathetic Nerves In Vivo. *Am J Respir Crit Care Med.* 2012 185: A2150.

3. **Rynko, A.**, Fryer, A.D., Jacoby, D.B. Parainfluenza And Influenza A Virus Can Not Be Detected In Sensory Neurons Of Infected Mice. *Am J Respir Crit Care Med.* 2011 183: A5546.

ABSTRACT

Respiratory virus infections are associated with the majority of asthma attacks in children and adults. Virus infection alters the neural control of the airways. Parasympathetic nerves release the neurotransmitter acetylcholine onto airway smooth muscle, inducing contraction. M_2 muscarinic receptors on parasympathetic neurons normally function to limit the release of the acetylcholine. However, virus infection induces M_2 receptor dysfunction, which causes more acetylcholine to be released, leading to bronchoconstriction. While virus-induced M_2 receptor dysfunction and airway hyperreactivity is well documented, little is known about the cellular mediators and mechanisms mediating these effects.

While certain respiratory virus can infect airway neurons, I used mice to demonstrate that influenza and parainfluenza virus infect the airway epithelium, but do not infect airway neurons. I also demonstrated that airway neurons are not infected with parainfluenza virus in guinea pigs (Chapter III). This suggested other cellular mediators released during infection induce changes in neuronal control.

Previous work has demonstrated a role for inflammatory mediators in airway hyperreactivity. In animal models, blocking TNF-α prevents M_2 receptor dysfunction and airway hyperreactivity after antigen or virus challenge. Blocking IL-1β can prevent airway hyperreactivity in ozone or antigen-challenged animals. In addition,

direct virus or TNF-α treatment decreases M_2 receptor expression in cultured parasympathetic neurons. Given that virus infection affects M_2 receptor function on parasympathetic nerves, I developed a novel dissection technique in guinea pigs to show that parainfluenza virus infection decreased M_2 receptor expression in parasympathetic neurons *in vivo*. This effect was prevented by blocking either TNF-α or IL-1β (Chapter IV). Using a guinea pig model, I also demonstrated blocking IL-1β during parainfluenza virus infection prevented M_2 receptor dysfunction (Chapter V). *In vitro*, I determined IL-1β directly decreases M_2 receptor expression in human and guinea pig parasympathetic neurons, and TNF-α is the major contributing factor of IL-1β production (Chapter VI).

In summary, I have demonstrated that influenza and parainfluenza virus do not infect sensory neurons or parasympathetic ganglia. I also developed a new dissection technique and showed parainfluenza virus infection indirectly decreased M_2 receptor expression in parasympathetic neurons, which is mediated by TNF-α and IL-1β. Moreover, the IL-1β-mediated the loss of M_2 receptor expression happens *in vivo* and *in vitro*. Furthermore, I demonstrated that blocking IL-1β *in vivo* prevented the loss of inhibitory M_2 receptors during parainfluenza virus infection. The data presented in this thesis provide novel findings in the field of virus-induced M_2 receptor function and airway hyperreactivity. These studies provide a new drug target for future therapies and suggest that blocking IL-1β can be a potentially effective treatment for virus-induced asthma exacerbations.

CHAPTER I.

INTRODUCTION

INTRODUCTION

Viral infections cause 80% of asthma exacerbation in children (Johnston *et al.*, 1995) and 50% in adults (Atmar *et al.*, 1998). This thesis focuses on respiratory viral infections, airway inflammation, and altered parasympathetic nerve control by determining the role of TNF-α and IL-1β in neuronal M_2 muscarinic receptor dysfunction in order to better understand virus-induced asthma exacerbations.

A. AIRWAY ANATOMY

Respiratory viruses affect the respiratory system, which is designed to facilitate gas exchange between air and blood. Therefore, this section provides the normal function of the lungs and structural features necessary for blood oxygenation.

1. Anatomy of human airways

The respiratory system contains an upper and lower region. The upper respiratory system consists of nasal cavity, pharynx, and larynx, which is termed the extrapulmonary region, as these airways lie outside the lungs. The trachea is also part of the extrapulmonary region and bifurcates into two primary bronchi, one to each lung. The bronchi continues to divide into smaller airway passages, getting more numerous as they divide (Figure 1.1). Inspired air travels though the extrapulmonary region into the intrapulmonary region of the lungs, which is all

generations from secondary bronchi to the alveolar sacs. The conducting airways consist of the trachea, bronchi, bronchioles and terminal bronchioles, thus containing both intrapulmonary and extrapulmonary regions. From the terminal bronchioles, air moves into the respiratory zone of the lungs, which contain the respiratory bronchioles, alveolar ducts, and alveolar sacs. Gas is exchanged in the alveolar region of the respiratory zone, which takes up most of the lung (West, 2008).

2. Structural and cellular components of human airways

The conducting airways have several structural and cellular components that aid in respiration. The trachea is supported by C-shaped cartilaginous rings, which keep the trachea from collapsing. The bronchi contain cartilage that start to dissipate as the bronchi divide into bronchioles. Along the posterior side of the C-shaped rings of the trachea are transverse and longitudinal bands of non-striated smooth muscle. The intrapulmonary bronchi and bronchioles also have smooth muscle in circles around the airways between the cartilage and basement membrane. When airway smooth muscle contracts, it both shortens and narrows the airways due to the spiral positioning of the bands (Jeffery, 1995).

The conducting airways are lined with epithelial cells, which have several functions. The epithelium acts as a barrier of protection against harmful environmental insults

and infectious diseases. Epithelial cells are often the first to respond to infectious agents and aid in leukocyte recruitment through the release of inflammatory mediators, such as chemokines and cytokines (Murphy *et al.*, 2008). Epithelial cells can also release antimicrobial agents, such as defensins, in response to infections (Hiemstra, 2001; Ashitani *et al.*, 2002; Hiratsuka *et al.*, 1998). In addition, epithelial cells express important innate immune receptors, including interlekin-1 (IL-1), tumor necrosis factor (TNF), and toll-like receptors (TLR) (Bals and Hiemstra, 2004). They also express cell surface proteins and receptors that can be exploited by viruses for cell entry (Grove and Marsh, 2011).

The epithelium of the trachea is comprised of pseudostratified ciliated columnar epithelial cells, goblet cells, and basal cells (Leeson *et al.*, 1988). As the bronchi divide and get smaller, the epithelial cells get shorter and cuboidal (Jeffery, 1995). Basal cells lie near the basement membrane that anchor the epithelium to the extracellular matrix and are stem cells that replenish epithelial cells (Randell, 2006; Rock *et al.*, 2010; Leeson *et al.*, 1988). Goblet cells and submucosal glands, which release mucus into the airway lumen via ducts, are interspersed in the epithelium of the trachea, primary and secondary bronchi. Mucus acts to trap particles and pathogens, which are propelled out of the lungs through movement of epithelial cell cilia (Jeffery, 1995). Mucus production from glands is mostly mediated through parasympathetic and sensory nerve control, with slight stimulation from sympathetic nerves (Wine, 2007; Phipps *et al.*, 1982). Parasympathetic nerve

stimulation of mucus production from goblet cells is thought to be mediated indirectly through smooth muscle contraction and epithelial damage, as parasympathetic nerves do not innervate the airway epithelium (Evans and Koo, 2009). Within the epithelium of the of the small bronchioles are Clara cells that secrete surfactant, which reduces surface tension by disrupting the attractive forces of water molecules water and prevents alveolar collapse (Jeffery, 1995). Clara cells also function as progenitor cells that multiply and regenerate bronchial epithelial cells, often after lung injury (Randell, 2006; Reynolds and Malkinson, 2010). The alveoli epithelium contains two types of cells, type I pneumocytes and type II pneumocytes. Type I pneumocytes are squamous epithelial cells covering over 90% of the surface and are held together by tight junctions to prevent leakage of fluids. Type II pneumocytes are less abundant, occurring individually or in small groups, and release surfactant to prevent the alveoli sacs from collapsing (Leeson *et al.*, 1988).

Although there are no cilia on epithelial cells in the alveoli, alveolar macrophages can aid in protection at these sites. Macrophages can migrate quickly into the alveolar space and bronchioles from connective tissue, pleura, vessels, and bronchial airways to engulf pathogens (Leeson *et al.*, 1988). They are then pushed up out of the airways via cilia in the bronchioles to the pharynx where they are eliminated by swallowing or expectoration (Leeson *et al.*, 1988). Leukocytes can also traffic to these areas via blood and lymphatic vessels (Leeson *et al.*, 1988).

There are two sets of lymphatic vessels: the pleural set, which lies in the pleura, and the pulmonary set, which runs along bronchi, pulmonary arteries and veins (Leeson *et al.*, 1988). In addition, there are bronchus-associated-lymphoid tissues (BALT) in the bronchi, nasal-associated lymphoid tissue (NALT), and larynx-associated lymphoid tissue (LALT) in the larynx in the nasopharyngeal duct that are lymphoid nodules associated with the epithelium that participate in mucosal immunity (Jeffery, 1995; Debertin *et al.*, 2003; Kracke *et al.*, 1997; Pabst, 2010; Bienenstock *et al.*, 1973a; Bienenstock *et al.*, 1973b).

3. Blood supply to human lungs

The blood supply to the lungs consists of a pulmonary and bronchial double supply system. The right ventricle of the heart carries deoxygenated blood into the right and left pulmonary arteries, which terminates into a dense network of capillaries in the alveoli wall where oxygen is exchanged. Pulmonary veins then carry oxygenated blood via red blood cells to the systemic circulation and then the left atrium and left ventricle of the heart. The bronchial supply system has bronchial arteries that arise from the aorta and left ventricle of the heart. These arteries supply oxygenated blood to tissue of bronchi, bronchioles, terminal bronchioles, and connective tissue of the lung. The two systems communicate at the terminal bronchioles, where most of the blood from the bronchial arteries is return to the pulmonary veins. Deoxygenated blood then flows into the right atrium of the heart (Leeson *et al.*, 1988; West, 2008).

FIGURE 1.1. Airway structure

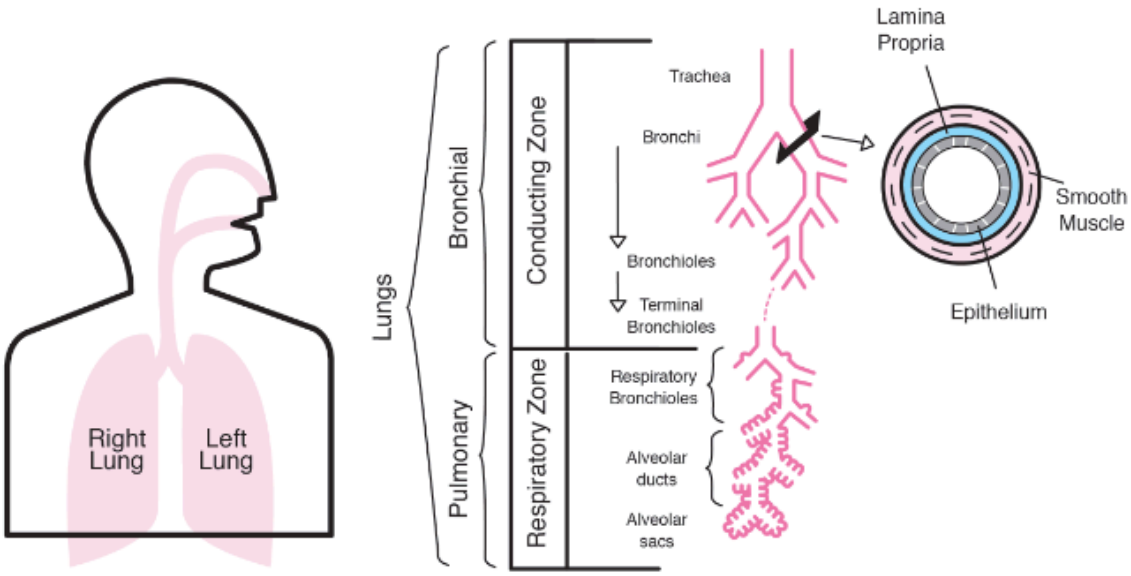

Figure 1.1. Inspired air flows into the mouth or nose and into the lower airways. The air moves down the trachea, which bifurcates to gives rise to the primary bronchi. The primary bronchi continues to divide giving rise to more bronchi, bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts, and alveolar sacs. The conducting airways consist of the trachea, bronchi, bronchioles, and terminal bronchioles. The respiratory zone, where gas exchange happens, includes the respiratory bronchioles, alveolar ducts, and alveolar sacs. A cross-section of bronchi shows airway smooth muscle, airway epithelium and airway lumen. Figure adapted from (West, 2008).

B. AIRWAY INNERVATION

The nervous system is comprised of vast network of neurons that communicate between one and other by sending and receiving information from the outside environment and internal organs. Jan Purkinje was the first to describe a nerve cell and had the first image of a brain cell in 1839 (Raine, 2011). Further understanding of a neuron with the structure and neurobiological components was later described by Otto Deiters, Camoillo Golgi, and Santiago Ramon y Cajal in the late 19th century (Peters *et al.*, 1991). Cajal was the first to identify a neuronal synapse, which is a place of contact between two nerve cells where they communicate through electrical or chemical signals (neurotransmitters) (Peters *et al.*, 1991; Sherrington, 1897). Neurons have cell bodies, dendrites, and an axon. The axon extends to target tissue where it sends information via action potentials to a point of contact with a target cell, termed a neuronal junction (Guyton and Hall, 2000; Fraczek *et al.*, 2008). Nerves can extend over vast distances and can be encapsulated in a myelin sheath by Schwann cells to increase conduction velocity and save energy (Figure 1.2) (Kalat, 2007).

The autonomic nervous system is an important part of the peripheral nervous system that regulates physiological responses by sensing stimuli and changes in the environment. The autonomic nervous system controls mucus secretion, smooth muscle tone, circulation, vascular tone, and bronchoconstriction (airway narrowing) (Nadel, 1977; Cabezas *et al.*, 1971; Woolcock *et al.*, 1969; Spencer and D, 1964;

Widdicombe, 1963a). In the lungs, sensory nerves sense stimuli and send information to the brainstem via the vagus nerves as part of the afferent arm of the central vagal reflex (Figure 1.3). Information is brought back down via autonomic nerves (parasympathetic and sympathetic) via the efferent arm of the reflex. The autonomic nerves are the involuntary division of the nervous system that controls digestion, pupillary dilation, respiration, and heart rate (Langley, 1898; Guyton and Hall, 2000). The autonomic nerves are divided into two parts, preganglionic and postganglionic neurons. The preganglionic parasympathetic neurons have ganglia that originate in the medulla oblongata of the brainstem and send information down each vagus nerve through the neck and into the thoracic cavity. The vagus nerve contains most of the afferent sensory and autonomic nerves innervating the lung. The vagus nerve branches off and innervates the trachea, main bronchi and bronchioles (Berthoud and Neuhuber, 2000). In the thoracic cavity, the vagus nerve branches into superior and recurrent laryngeal nerves to synapse onto the postganglionic nerves in the trachea and main bronchi (Kalia, 1981). The postganglionic nerves have axons that then extend and regulate the function of target tissue.

1. Sensory nerves

The sensory nerves respond to a variety of stimuli in the environment, including mechanical stretch, temperature, pH, allergen, smoke, and pollutants (Carr and Undem, 2003; Gold *et al.*, 1972; Coleridge *et al.*, 1993; Giesbrecht *et al.*, 1993; Lee *et*

al., 1989; Coleridge and Coleridge, 1984; Ho *et al.*, 2001). The sensory nerves spread throughout the lung and innervate airway epithelium, submucosa, smooth muscle, blood vessels, alveolar walls, and airway ganglia (Brouns *et al.*, 2006; Luts *et al.*, 1993; Lundberg *et al.*, 1984; Springall, 1995; Sheldrick *et al.*, 1995).

The majority of the sensory nerves originate from the nodose and jugular ganglia at the base of the skull, and the rest come from the thoracic and cervical dorsal-root ganglia in the spinal cord (Kummer *et al.*, 1992; Carr and Undem, 2003). They comprise approximately 80% of the nerve fibers in the vagus nerve (Agostoni *et al.*, 1957). The afferent fibers project to the nucleus tactus solitarius of the brain (Kummer *et al.*, 1992) and relay information to the efferent parasympathetic, sympathetic, and non-adrenergic non-cholinergic nerves via the central vagal reflex (Nadel *et al.*, 1965; Kalia, 1981). The elicited reflex responses include cough, mucus secretion, smooth muscle tone, and bronchoconstriction (Wagner and Jacoby, 1999; Kesler and Canning, 1999; Coleridge and Coleridge, 1994a).

Sensory nerves can be classified into two main categories: Aδ fibers and C fibers. C-fibers are chemosensitive and respond to noxious stimulus and inflammatory mediators (Belvisi, 2003). They are also unmyelinated, with slow conduction velocities and small cell bodies. Sensory Aδ fibers are, mechanosensative, thinly myelinated and have much faster conduction velocities with large cell bodies. Aδ

fibers also have rapidly adapting stretch receptors that detect changes in lung volumes (Pack and DeLaney, 1983).

Sensory nerves release neuropeptides in the lung to induce local reflex arcs, without sending information to the brain, which mediate mucus secretion, vascular permeability, and smooth muscle contraction (Belvisi, 2002; Coleridge and Coleridge, 1994b; Carr and Undem, 2003; De Swert and Joos, 2006). Sensory nerves release tachykinins, which include substance P, neurokinin A (NKA), and neurokinin B (NKB) (De Swert and Joos, 2006). These neuropeptides all bind to NK_1, NK_2, and NK_3 receptors, although substance P has higher affinity for NK_1, NKA for NK_2, and NKB for NK_3 (Maggi, 2000; Almeida *et al.*, 2004). They can also release other neuropeptides such as vasoactive intestinal protein (VIP) and calcitonin-gene related protein (Holzer, 1988). Neuropeptides are also capable of inducing inflammation in the airways (Barnes, 2001; De Swert and Joos, 2006).

2. Parasympathetic nerves

The preganglionic parasympathetic nerves have cell bodies that originate in the nucleus ambiguus of the solitary tract in the brainstem (Kalia, 1981; Richardson and Ferguson, 1979; McAllen and Spyer, 1978). The nerve axons travel within the vagus nerve, including the superior laryngeal nerve and the recurrent laryngeal nerves, and synapse onto postganglionic nerves near the airway wall (Myers, 2001). These

small clusters of cells (ganglia) are located in the trachea and bronchi (Baker *et al.*, 1986; Canning and Fischer, 1997; Myers, 2001), and have axons that project down to airway smooth muscle, blood vessels, and mucus glands (Daniel *et al.*, 1986; Haberberger *et al.*, 1997; Knight *et al.*, 1981; Canning and Fischer, 1997). At the synapse between the pre and postganglionic nerve, the neurotransmitter acetylcholine (ACh) is released onto nicotinic receptors on postganglionic nerves. This initiates excitatory action potentials and release of ACh from nerve endings at neuronal junctions in target tissue. ACh, which is the main neurotransmitter used by parasympathetic nerves, was first characterized by Sir Henry Dale in 1914 (Dale, 1914), and is discussed in more detail in the next section (C) on the topic of acetylcholine and muscarinic receptors in the lung.

Parasympathetic nerves are the main controllers of airway smooth muscle tone in humans and animals (Nadel and Barnes, 1984; Nadel, 1977; Nadel, 1971; Boushey *et al.*, 1980). Contraction of airway smooth muscle is induced through tonic ACh release (Widdicombe, 1966; Widdicombe, 1963b). This tonic release produces a baseline partial contraction of airway smooth muscle, called tone (Kesler and Canning, 1999). Regulation of airway smooth muscle tone happens in the conducting airways, but not in the respiratory zone, as parasympathetic nerves do not innervate or control respiratory bronchioles and alveoli (Nadel, 1971).

Parasympathetic nerves mediate bronchoconstriction through the release of ACh onto muscarinic receptors on airway smooth muscle. ACh release can be induced by electrical stimulation of the distal ends of the vagus nerves after vagotomy to cause bronchoconstriction *in vivo* or through electrical field stimulation of isolated tracheal rings *in vitro* (which is used for some experiments in this thesis) (Kesler and Canning, 1999; Olsen *et al.*, 1965; Cabezas *et al.*, 1971; Severinghaus and Stupfel, 1955). Bronchoconstriction can be inhibited by vagal sectioning or through pharmacologic ganglion blockade to prevent smooth muscle contraction (Karczewski and Widdicombe, 1969; Widdicombe, 1966; Severinghaus and Stupfel, 1955). Bronchoconstriction can also be prevented pharmacologically with atropine, which is a muscarinic receptor antagonist (Colebatch and Halmagyi, 1963; Severinghaus and Stupfel, 1955; Nadel and Widdicombe, 1963; Sheppard *et al.*, 1982; Kesler and Canning, 1999; Colebatch and Halmagyi, 1963). The parasympathetic nerves also induce blood vessel dilation and mucus secretion from glands (Haberberger *et al.*, 1997; Widdicombe, 1963a; Spencer and D, 1964; Belvisi, 2002).

Parasympathetic nerve activation and neurotransmitter release can be regulated by cell mediators released from immune cells, such as histamine (Kikuchi *et al.*, 1984; Colebatch *et al.*, 1966), serotonin (Sheller *et al.*, 1982; Mossner and Lesch, 1998), leukotrienes (Abela and Daniel, 1994), and prostaglandins (Inoue *et al.*, 1984; Ito *et al.*, 1990; Tamaoki *et al.*, 1987). In addition, sympathetic and non-adrenergic non-

cholinergic nerves modulate parasympathetic nerve functions (Hakoda and Ito, 1990; Ito and Hakoda, 1990; Baker *et al.*, 1986; Knight *et al.*, 1981).

3. Sympathetic nerves

Sympathetic nerves are efferent nerves that originate in the spinal cord and synapse onto postganglionic nerves in the cervical thoracic ganglia (Kummer *et al.*, 1992; Belvisi, 2002). In human lungs, sympathetic nerves target mucus glands, blood vessels, and parasympathetic nerves (Richardson and Beland, 1976; Mann, 1971), but do not directly innervate airway smooth muscle nor do they affect smooth muscle tone (Daniel *et al.*, 1986; Richardson and Beland, 1976; Richardson and Ferguson, 1979). However, other species, such as guinea pigs (O'Donnell *et al.*, 1978) dogs (Knight *et al.*, 1981), cats (Dahlstrom *et al.*, 1966), sheep goats, cows and pig have sympathetic innervation of airway smooth muscle (Canning, 2003). Sympathetic innervation in these species allows direct relaxation through neurtotransmitter release.

Stimulation of sympathetic nerves leads to the release of the neurotransmitter, norepinephrine (NE), which binds to adrenoreceptors. Adrenoreceptors are subdivided into α- and β-adrenoreceptors. Human lungs express α_1, β_1, and β_2 receptors (Spina *et al.*, 1989a; Spina *et al.*, 1989b). While humans have little evidence of sympathetic innervation of smooth muscle, they do express β_2-

adrenoreceptors on airway smooth muscle, which can induce relaxation through systemic epinephrine or β_2 agonists (Pack and Richardson, 1984; Kahn; Richardson and Beland, 1976; Billington and Penn, 2003). Smooth muscle relaxation can also be mediated through β_1 receptors on parasympathetic neurons, which decrease neurotransmitter release (Danser *et al.*, 1987).

4. Non-adrenergic non-cholinergic nerves

Non-adrenergic non-cholinergic (NANC) nerves are efferent nerves that innervate airway smooth muscle (Linden, 1996). NANC nerves can be excitatory, mediating smooth muscle contraction through substance P and NKA, or inhibitory, mediating relaxation through nitric oxide (NO) and VIP (Li and Rand, 1991; Belvisi *et al.*, 1992; Matsuzaki *et al.*, 1980; Matsuzaki *et al.*, 1980). These nerves are found in a variety of species, including humans and guinea pigs, where the cell bodies reside in the esophagus and airway parasympathetic ganglia (Li and Rand, 1991; Linden, 1996; Palmer *et al.*, 1986).

FIGURE 1.2. Schematic representations of neuronal synapse and autonomic nerves.

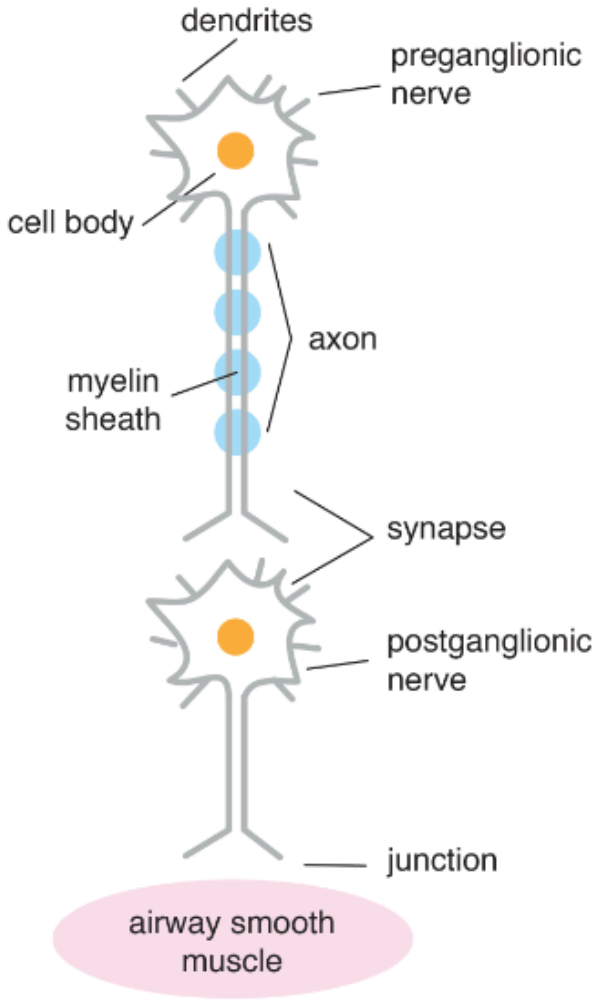

Figure 1.2. Drawing of a basic neuron structure and synapse. The preganglionic nerve has cell bodies that originate in the CNS and has a myelinated axon. The nerve forms a synapse with a postganglionic nerve where neurotransmitters are released. The postganglionic nerve has a cell body outside the CNS with an unmyelinated axon that extends to target tissues and forms a neuronal junction. Based on terminology from (Sherrington, 1897).

FIGURE 1.3 Nerve supply to the airways.

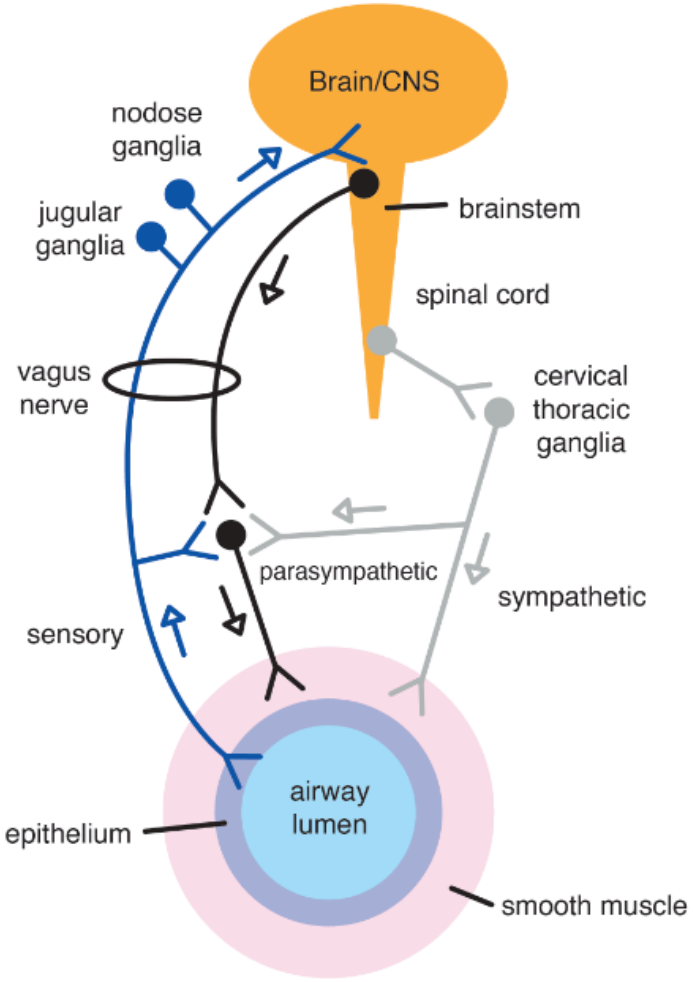

Figure 1.3. Afferent sensory nerves innervate the airways and send information to parasympathetic nerves via a central reflex. Information from sensory nerves (blue) travels up the vagus nerve to the nodose and jugular ganglia and then the CNS. Parasympathetic nerves (black) become activated and send information back down the vagus nerve and innervate the airways. Sympathetic nerves do not innervate airway smooth muscle in humans, but they do in guinea pigs. Figure adapted from (Verhein, 2010).

C. ACETYLCHOLINE AND MUSCARNIC RECEPTORS IN THE LUNG

Acetylcholine (ACh) is a neurotransmitter produced by parasympathetic nerves that mediates bronchoconstriction and mucus secretion. ACh is synthesized by choline acetyltransferase, which catalyzes the formation ACh from choline and acetyl-coenzyme A. Upon release into synapses, ACh is hydrolyzed by acetylcholinesterase, and choline is taken brought back into the neuron through choline transporters. Along with neurons, epithelial, endothelial, smooth muscle, and immune cells are capable of synthesizing and releasing ACh (Proskocil *et al.*, 2004; Reinheimer *et al.*, 1998; Haberberger *et al.*, 2000; Kummer *et al.*, 2008; Wessler *et al.*, 2003). Epithelial cells also express acetylcholinesterase (Proskocil *et al.*, 2004; Koga *et al.*, 1992; Kummer *et al.*, 2008). ACh is normally stored in vesicles in neurons. However, non-neuronal cells lack the ability to store ACh and release it through constitutive secretion (Wessler *et al.*, 2001). The non-neuronal release of ACh is thought to work in an autocrine or paracrine manner to regulate muscosal defense systems and sensory nerve activity (Kummer *et al.*, 2008).

1. Acetylcholine receptors

The functions of ACh was first described and named by Sir Henry Dale as he observed that the ACh has a similar response to actions of muscarine or nicotine (Dale, 1914; Fishman, 1972). ACh can bind to two types of receptors, nicotinic and

muscarinic, both of which are expressed in the lung and contribute to parasympathetic function and control of the airways.

a. Nicotinic receptors in the lungs

Nicotinic receptors are ligand-gated ion channel receptors expressed on postganglionic parasympathetic neurons in the lungs (Racke and Matthiesen, 2004). They are also expressed on macrophages, epithelial cells, eosinophils, neutrophils, and mast cells (Wang *et al.*, 2003a; Blanchet *et al.*, 2007; Iho *et al.*, 2003; Su *et al.*, 2007; Sudheer *et al.*, 2006). Each receptor is a channel made of 5 subunits in the membrane, which can be homomeric or heteromeric combinations of α, β, γ, or δ subunits, depending on the cell type. Stimulation of nicotinic receptor depolarizes the membrane and triggers an action potential mediated by an influx of cations.

b. Muscarinic receptors

Muscarinic receptors are G protein-coupled receptors (GPCR) expressed on a variety of cell types in the lung. There are five identified subtypes (M_1, M_2, M_3, M_4, M_5) (Caulfield and Birdsall, 1998). M_1, M_2, M_3, receptors are expressed on nerves, endothelial cells, smooth muscle, glands, fibroblasts, and immune cells. Some immune cells also express M_4, and M_5 receptors. These subtypes have been identified by RT-PCR, immunohistochemistry, knock-out mice, and selective

antagonist binding studies (Haddad *et al.*, 1996a; Wess, 2004; Mak *et al.*, 1992; Mak and Barnes, 1990; Gies *et al.*, 1989).

i. Signaling

The 5 muscarinic receptor subtypes are coupled to G-proteins, which are heterotrimers of three subunits, which include α, β, and γ subunits. Stimulation of the GPCR induces a conformation change in the receptor that releases the G proteins. This allows the hydrolysis of GTP to GDP and dissociation of the Gα subunit from the βγ dimer. These subunits can then activate or inhibit second messenger systems or associate with ions channels (Peralta *et al.*, 1987).

The muscarinic receptor signaling of the G-proteins are categorized by the Gα subunits and are either $G\alpha_i$, $G\alpha_q$, or $G\alpha_s$ (Caulfield and Birdsall, 1998; Hulme, 1990; Caulfield, 1993). M_1, M_3, M_5 are predominantely coupled to $G\alpha_q$, which stimulates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Exton, 1996) (Figure 1.4). These second messengers can mediate a variety of effects inside the cell. IP3 initiates release of intracellular calcium ions from the endoplasmic reticulum, which can induce smooth muscle contraction (Roffel *et al.*, 1990a; Roffel *et al.*, 1990b). The membrane-bound DAG can activate protein kinase C (PKC), which phosphorylates substrates and also increases intracellular calcium. M_2 and M_4

receptors are coupled to $G\alpha_i$, which inhibits adenylate cyclase (AC), thereby decreasing cyclic adenosine monophosphate (cAMP) production. CAMP normally increases ACh release; therefore, stimulation of M_2 receptors prevents ACh release from parasympathetic nerves (Fryer and Maclagan, 1984; Zhang *et al.*, 1996). Gα can also mediate closing of voltage-gated Ca^{2+} channels, limiting action potentials. The Gβγ subunit can also couple to and activate G-protein-regulated inwardly rectifying K^+ channels (GIRK), preventing excitability. (Ritter and Hall, 2009).

ii. Structure

Muscarinic receptors are GPCRs that are have an extracellular N-terminus, seven transmembrane alpha helices, and an intracellular C-terminus. Three intracellular and three extracellular loops connect the membrane spanning alpha helical domains (Zhang *et al.*, 1996). The G-protein coupling of each subtype is determined by sequence variation in the third intracellular loop (Peralta *et al.*, 1987). The five muscarinic subtypes are similar among different species and are encoded by separate genes lacking introns (Hall *et al.*, 1993; Bonner *et al.*, 1987; Wess, 1993).

iii. Selectivity

The ligand recognition site of muscarinic receptors is in the outer half of the transmembrane portions of the protein. Distinct amino acid sequences in the third intracellular loop of the receptors determine ligand binding, receptor activation, and

G protein coupling (Wess, 1993; Wess *et al.*, 1997; Caulfield and Birdsall, 1998). In addition, an aspartic acid residue in the N-terminal portion of the third extracellular membrane embedded part of the protein is conserved across all muscarinic receptors, and interacts with agonist that have a polar head group, such as ACh (Caulfield and Birdsall, 1998).

Muscarinic receptors have allosteric sites along with the main binding site (Lazareno and Birdsall, 1995). Once an antagonist is bound to the allosteric site, this alters the selectivity for an agonist at the primary site (Caulfield, 1993). For instance, eosinophil major basic protein is an allosteric M_2 receptor antagonist (Jacoby *et al.*, 1993), as is gallamine (Clark and Mitchelson, 1976; Ehlert, 1988).

There are no selective agonists for the different receptor subtypes, but certain antagonists have selectivity for one subtype, such as gallamine for M_2 receptors (Blaber *et al.*, 1985; Fryer and Maclagan, 1984). However, these antagonists only have 10-fold selectivity for one receptor subtype over another (Caulfield and Birdsall, 1998). The main binding site and the allosteric site determine muscarinic receptor antagonist selectivity (Clark and Mitchelson, 1976). Selectivity is determined in the transmembrane and extracellular domains of the receptor, which is demonstrated by chimeric receptor studies (Wess, 1993).

iv. Distribution in the lungs

M_1 receptors are expressed on parasympathetic neurons and regulate neurotransmission (Beck *et al.*, 1987; Bloom *et al.*, 1987; Ashe and Yarosh, 1984; Yang and Biggs, 1991). M_1 receptors are also expressed on epithelial cells; however, these are not innervated by parasympathetic neurons (Casale and Ecklund, 1988; Mak and Barnes, 1990; Gies *et al.*, 1989). Little is known about the receptor function in this cell type, although they may function through ACh release from epithelial cells in an autocrine manner. M_1 receptors are also thought to increase electrolyte release from serous cells in mucus glands (Yang *et al.*, 1988).

M_2 receptors are expressed on parasympathetic ganglia and along neurites (Fryer and Maclagan, 1984; Fryer *et al.*, 1996; van Koppen *et al.*, 1988; van Koppen *et al.*, 1987). Activation of M_2 receptors on postganglionic parasympathetic neurons inhibits the further release of ACh (Fryer and Maclagan, 1984; Fryer, 1995). This autoinhibitory receptor mechanism was first described in guinea pig airways (Fryer and Maclagan, 1984), but has also been demonstrated in humans and several other animal models, including mice, cats, dogs, horses, and rats (Minette *et al.*, 1989; Minette and Barnes, 1988; Larsen *et al.*, 2000; Killingsworth *et al.*, 1992; Aas and Maclagan, 1990; Ito and Yoshitomi, 1988; Brichant *et al.*, 1990; Wang *et al.*, 1995; Belmonte *et al.*, 1997; Blaber *et al.*, 1985). In addition, M_2 receptors are also expressed on airway smooth muscle, where up to 80% of muscarinic receptors on airway smooth muscle are M_2 receptors depending on the species (Haddad *et al.*,

1991; Roffel *et al.*, 1988). These receptors inhibit bronchodilation induced by β agonists by blocking the adenylate cyclase activity in airway sooth muscle (Sankary *et al.*, 1988; Fernandes *et al.*, 1992). However, bronchoconstriction is mediated by M_3 receptors *in vivo*, as M_3 receptor knockout mice do not have induced bronchoconstriction with nerve stimulation or exogenous methacholine (a muscarinic agonist) but M_2 knockout mice do (Fisher *et al.*, 2004).

M_3 muscarinic receptors are expressed on airway smooth muscle, which can range from 20-50% of the muscarinic receptors in the trachea depending on the species (Roffel *et al.*, 1988; Haddad *et al.*, 1991). Activation of M_3 receptors induces contraction of airway smooth muscle and bronchoconstriction (Roffel *et al.*, 1990a). Stimulation of M_3 receptors also induces mucus secretion (Borson *et al.*, 1980; Yang *et al.*, 1988; Baker *et al.*, 1985), increases ciliary beat frequency on epithelial cells (Wong *et al.*, 1988; Klein *et al.*, 2009), and increases vascular dilation (McMahon *et al.*, 1992).

Immune cells also express muscarinic receptors, which can induce or inhibit inflammatory cell mediators depending on the cell type. Human peripheral blood T and B lymphocytes express M_3, M_4, M_5 (Tayebati *et al.*, 2002; Tayebati *et al.*, 1999). M_1 and M_2 mRNA transcripts have also been identified in a human peripheral blood T cells and the Jurkate human T cell line (Fujino *et al*., 1997). However, another studies did not detect M_1 or M_2 transcripts in purified human T cells (Ewa and

Nordberg, 1996). The difference in the findings of these 2 studies may have to do with the experimental conditions of the isolated T cells. In humans and bovine, monocytes and macrophages express M_3 receptors and release leukotriene B_4 (LTB_4) when stimulated with ACh, which is involved in recruitment of blood monocytes, neutrophils, and eosinophils (Sato *et al.*, 1998; Profita *et al.*, 2005). Mast cells express M_1 receptors and ACh stimulation inhibits mast cell histamine release in humans (Fryer, and Jacoby 2008; Reinheimer *et al.*, 1997; Reinheimer *et al.*, 2000), although it should be noted that in other species, such as rats and rabbits, ACh increases histamine release (Masini *et al.*, 1985; Nemmar *et al.*, 1999). Neutrophils and eosinophils have M_3, M_4, M_5 receptor mRNA transcripts (Bany *et al.*, 1999; Verbout *et al.*, 2006), although their functions are unknown.

v. Regulation of bronchoconstriction

Smooth muscle tone and bronchoconstriction is controlled by parasympathetic nerves through the release of ACh onto M_3 receptors on airway muscle (Haddad *et al.*, 1991; Nadel and Barnes, 1984; Roffel *et al.*, 1990a; Boushey *et al.*, 1980). Neuronal M_2 receptors limit ACh release from parasympathetic nerves through a feedback inhibition mechanism, which limits bronchoconstriction (Fryer and Maclagan, 1984; Fryer *et al.*, 1996; Blaber *et al.*, 1985; Minette and Barnes, 1988; Minette *et al.*, 1989). This is demonstrated by knocking out M_2 receptors, which increases vagally induced bronchoconstriction (Fisher *et al.*, 2004). In addition, using the M_2 receptor antagonist, gallamine, vagally induced bronchoconstriction is

potentiated 8-10 fold (Fryer and Maclagan, 1984). Furthermore, ACh release from parasympathetic nerves is increased 5-8 fold when M_2 receptors are blocked with the non-selective muscarinic receptor antagonist, atropine (Baker *et al.*, 1992). Conversely, activating M_2 receptors with agonists inhibits bronchoconstriction in response to electrical stimulation of the vagus nerves up to 80% (Fryer and Maclagan, 1984). This indicates physiological importance of M_2 receptors and ACh release mediating bronchoconstriction.

FIGURE 1.4. Muscarinic receptor signaling via G-proteins

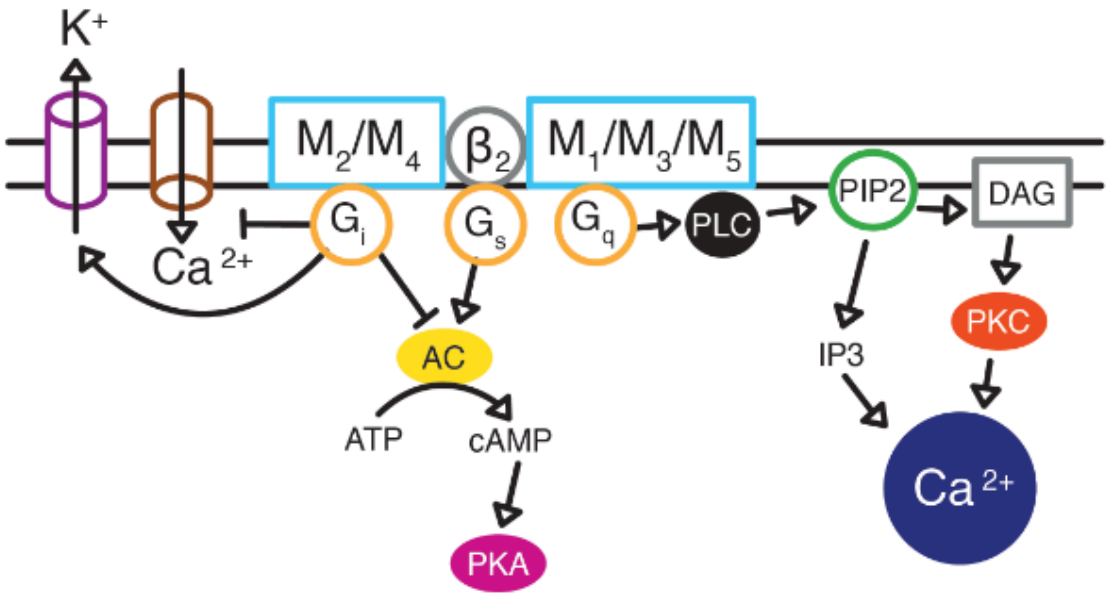

Figure 1.4. This illustration shows the different signaling pathways of the muscarinic G protein-coupled receptors. M_1, M_3, and M_5 are coupled to G_q, which activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 releases Ca2+ from intracellular stores and DAG activates protein kinase C (PKC), which also increases intracellular Ca^{2+} and leads to contraction. M_2 and M_4 are coupled to G_i ,which activates potassium channels and inhibit voltage-gated calcium channels. G_i also inhibits adenylate cyclase (AC), which prevents the conversion of ATP to cAMP and prevents PKA activation. To note, AC is stimulated by β_2 receptor activation coupled to G_s (to which muscarinic receptors are not coupled), which mediate relaxation. Figure adapted from (Verbout, 2008).

FIGURE 1.5. Inhibitory M_2 receptor function on parasympathetic nerves.

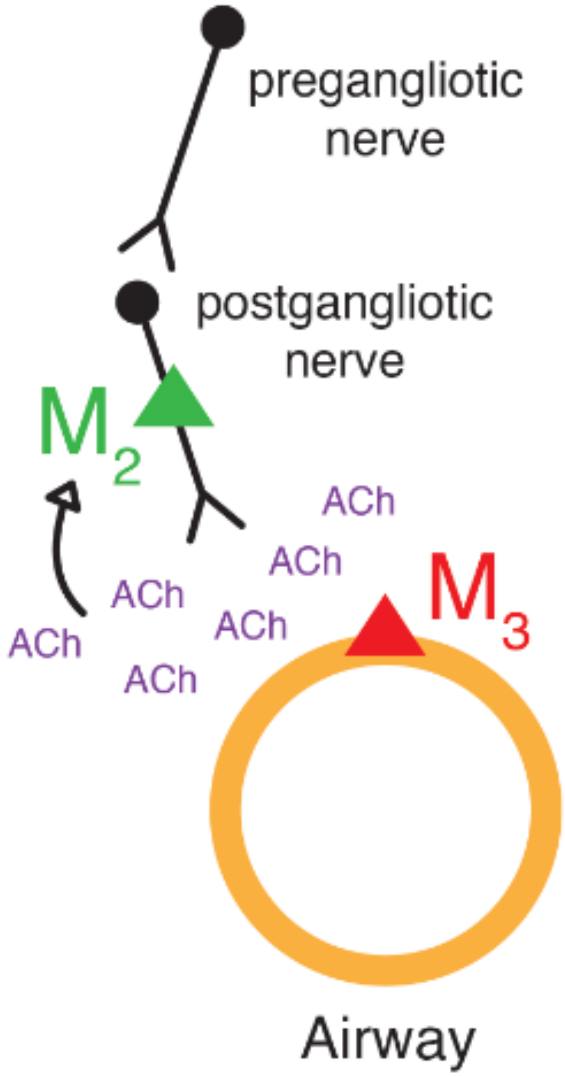

Figure 1.5. Preganglionic nerves activate postganglionic parasympathetic nerves to release acetylcholine onto M_3 muscarinic receptors on airway smooth muscle, inducing contraction. Acetylcholine release can bind to M_2 receptors, which limits further acetylcholine release and bronchoconstriction.

D. ASTHMA AND INFLAMMATION

This thesis focuses on understanding the mechanism that trigger virus-induced asthma exacerbations and possible therapies for virus-induced attacks. Therefore, it is important to discuss the characteristics of asthma and the underlying cellular and inflammatory factors that contribute to obstructed airflow, bronchoconstriction, and hyperreactivity.

1. Definition of asthma

In 2009, approximately 25 million (8%) of the people in the U.S. had asthma, compared to 20 million in 2001 (CDC, 2011b). Asthma is the most common chronic disease in children and affects more than 6 million children in the U.S. (CDC, 2005). In 2007, 185 children and 3,262 adults died from asthma (CDC, 2011b). An estimated 300 million people suffer from asthma worldwide, with approximately 250,000 deaths per year attributed to the disease (WHO, 2007; Bateman *et al.*, 2008; Mallia *et al.*, 2007). Asthma also has a significant burden on health care costs in the U.S., which includes hospital admissions and pharmaceutical costs. Between 2002-2007, asthma related health care costs were $56 billion annually, or approximately $3,300 per person (CDC, 2011b; WHO, 2007). This includes nearly 500,000 hospital admissions and 2 million emergency room visits associated with asthma every year (Mallia *et al.*, 2007).

Asthma is a chronic, heterogeneous condition characterized by airflow obstruction, persistent inflammation, hyperresponsiveness, and reduced lung function (Reddel *et al.*, 2009). The National Heart Lung and Blood Institute has defined asthma as:

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma." (NHLBI, 2007).

As indicated in the NHLBI statement, asthma has clinical features of variable airflow obstruction, reversible bronchoconstriction, and airway hyperreactivity. Symptoms of asthma exacerbations include chest tightness, cough, wheezing, and shortness of breath (Mallia *et al.*, 2007). These symptoms are episodic and often occur at night or early morning and cause disruption of sleep. Symptoms are often triggered by inhaled stimuli, such as smoke or animal dander, or virus infection, exercise, or stress. The obstruction of airflow is often reversible with treatment (NHLBI, 2007).

Asthma symptoms are triggered by an environmental allergen, such as dust mite or pet dander. During first exposure to the inhaled particle, the person becomes sensitized to the allergen and produces antigen specific IgE antibodies that bind to the high-affinity FcεRI receptors on mast cells. Upon re-exposure to the allergen, the allergen binds to IgE antibodies bound to mast cells, which crosslinks the IgE antibodies and releases cell mediators, such as histamine (Holgate, 2008; Bradding *et al*., 2006). Asthma can also be triggered by non-allergic stimuli, including viruses, smoke, exercise, cold air, chemicals, or stress. These are the most common triggers of exacerbations and airway hyperreactivity in asthmatic patients (Smith and McFadden, 1995).

Asthma exacerbations induce airflow obstruction and can be categorized as mild to severe. Mild attacks have decreased airflow that is just outside the normal range of the patient, which usually resolve in hours to days. Moderate exacerbations are attacks that disrupt the daily lives of the patient and need treatment (Reddel *et al.*, 2009). Severe exacerbations are defined by a need for immediate treatment or hospitalization, often to prevent death of the patient, and can last up to 10 days after treatment (Reddel *et al.*, 2009; O'Byrne, 2011). Along with the severity of attacks, frequency can also be categorized as intermittent or persistent. Patients with intermittent asthma have symptoms that are infrequent, usually associated with seasonal allergens or infections. Persistent asthma typically involves frequent or

continuous symptoms that can be mild, moderate, or severe, and may be resistant to β agonist treatment (Shah and Saltoun, 2012; NHLBI, 2007).

2. Asthma characteristics

a. Bronchoconstriction and airway remodeling

Airflow obstruction can be mediated by bronchoconstriction or through structural changes due to persistent inflammation, which is termed airway remodeling. These permanent changes are seen in more severe cases of asthma, which include thickening of the basement membrane under airway epithelium, epithelial fibrosis, smooth muscle hypertrophy, angiogenesis, and gland hyperplasia and mucus plugs (NHLBI, 2007). In general, bronchoconstriction is reversible through pharmacological treatment to relax smooth muscle; however, airflow obstruction can become irreversible after permanent structural and airway remodeling has occurred (NHLBI, 2007).

b. Airway hyperresponsiveness

Airway hyperresponsiveness is a key feature in asthma, and is defined as an increased airway narrowing response to a stimulus that induces little or no bronchoconstriction in a healthy person (Hargreave *et al.*, 1986). This can be determined by assessing contraction or airway narrowing before and after a

constricting agent, such as methacholine, is administered. An asthmatic often has a decreased threshold (increase sensitivity) and thus, potentiated bronchoconstriction to a constricting stimulus compared to a normal subject (Hopp *et al.*, 1985; Tepper, 1987). Several mechanisms contribute to airway hyperresponsiveness, including airway wall thickening, smooth muscle hypertrophy and hyperplasia, inflammation, increased sensory nerve sensitivity, and changes in NANC or parasympathetic nerve function (McParland *et al.*, 2003; Wang *et al.*, 2003b; Black, 2004; James *et al.*, 2012; Coulson *et al.*, 2002; Holgate, 2008). Of particular interest to this thesis is the role of parasympathetic nerves and inflammation in airway hyperresponsiveness.

c. Role of parasympathetic nerves in hyperresponsiveness

Parasympathetic nerves are the dominant controls of airway smooth muscle control and are altered in asthmatic patients (Cropp, 1975). *In vitro* studies using smooth muscle from asthmatic patients demonstrated the contraction response to methacholine was not different between asthmatic and non-asthmatics, indicating hyperresponsiveness is not due to increased sensitivity of airway smooth muscle (Taylor *et al.*, 1985; Armour *et al.*, 1984; Roberts *et al.*, 1985; Whicker *et al.*, 1988). It is more likely that parasympathetic nerves and vagal reflexes play a role in hyperresponsiveness associated with asthma. Electrical stimulation of parasympathetic nerves induces bronchoconstriction, which happens at the areas most innervated by parasympathetic nerves (Nadel, 1971). Further support comes

from an allergic animal model where vagotomy decreased contractions (Gold *et al.*, 1972). In addition, patients with severe asthma who had their vagus nerves severed showed decreased asthma symptoms (Balogh *et al.*, 1957).

Parasympathetic nerves mediate airway hyperreactivity through increased ACh release onto M_3 muscarinic receptors on airway smooth muscle (Drazen *et al.*, 2000; Fryer and Wills-Karp, 1991; Gold *et al.*, 1972). Dysfunctional M_2 receptors on postganglionic nerves lead to increased ACh release and increased bronchoconstriction (Fryer and Maclagan, 1984; Minette and Barnes, 1988; Fryer, 1995). M_2 receptor dysfunction has been demonstrated in several animal models of asthma (Belmonte *et al.*, 1997; Evans *et al.*, 1997; Larsen *et al.*, 1994; Zhang *et al.*, 1999) and humans with asthma (Minette *et al.*, 1989; Ayala and Ahmed, 1989). Loss of inhibitory M_2 receptor function on parasympathetic nerves is induced following ozone exposure (Schultheis *et al.*, 1994) and virus infection (discussed in more detail in section E) (Fryer and Jacoby, 1991; Jacoby *et al.*, 1998; Jacoby, 2004).

d. Inflammation

Inflammation plays a critical role in asthma and is a defining characteristic of the disease. Several immune cell types are involved in the pathophysiology of asthma, including mast cells, eosinophils, neutrophils, macrophages, and T lymphocytes. These cells respond to injury and release pro- and anti-inflammatory cytokines that

affect target tissues and regulate the immune response (Murphy *et al.*, 2008). In addition, all airway cell types, including epithelial, nerve, airway smooth muscle, and inflammatory cells release chemokines (Fryer *et al.*, 2006; Hirst *et al.*, 2002; Dokic and Howarth, 2006). These chemoattractant proteins mediate recruitment of immune cells from the peripheral blood through a concentration gradient. The presence of the inflammatory cells is significantly increased in asthmatic airways, which can be detected in bronchoalveolar lavage (BAL) fluids, peripheral blood, sputum, or tissue biopsies (Jeffery, 1996; Maestrelli *et al.*, 1995; Godard *et al.*, 1982; Broekema *et al.*, 2011; Bousquet *et al.*, 2000). The major inflammatory cells that are involved in respiratory viral infections and asthma are discussed below.

i. Macrophages

Macrophages are the most numerous immune cells in the airways and are one of the first responders to external microbes and environmental insults. They are phagocytic cells that engulf viruses, bacteria, particles, and apoptotic cells. Macrophages can be activated though classical activation, requiring IFN-γ and TNF-α stimulation; alternative activation, mediated through IL-4, IL-13, or glucocorticoid signals; and type II (regulatory) activation, mediated through TLR stimulation and a macrophage stimulating factor, such as IFN-γ (Cohn, 1978; Mosser, 2003; Mosser and Zhang, 2008).

Alveolar macrophages have been widely studied in asthma and they have increased activation in asthmatic BAL (Godard *et al.*, 1982; Joseph *et al.*, 1983; Fuller *et al.*, 1988; Chanez *et al.*, 1994; Chanez *et al.*, 1996; Borish *et al.*, 1992). Activation correlates with disease severity (Kelly *et al.*, 1988; Cluzel *et al.*, 1987). Macrophages are also involved in regulation of airway remodeling by releasing growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), or TGF-β, which are involved in fibrosis (Kovacs and DiPietro, 1994). Macrophages can mediate airways obstruction and regulate airways inflammation through release of prostaglandins (Damon *et al.*, 1989), platelet-activating factor (PAF) (Arnoux *et al.*, 1982), and oxygen free radicals (Majori *et al.*, 1998; Murayama *et al.*, 1990), which can damage tissues. Alveolar macrophages also release cytokines upon activation, such as TNF-α and IL-1β (these cytokines are discussed in more detail later) (Borish *et al.*, 1992; Dinarello, 1996; Chanez *et al.*, 1994; Bousquet *et al.*, 2000). The importance of macrophages in virus-induced exacerbations is revealed in studies where depletion of macrophages prevents airway hyperreactivity and M_2 receptor dysfunction in virus-infected guinea pigs (Lee *et al.*, 2004).

ii. Lymphocytes

Both B and T Lymphocytes are found in the airways and orchestrate immune responses. B cells are important for antigen specific antibody production, including IgA, and IgG, and IgE. In asthma, the production of IgG1 and IgE antibodies are increased (Romagnani, 2002). During respiratory virus infection, the production of

primarily IgG1 and IgG3 are increased in humans (Frasca *et al.*, 2013; Julkunen *et al.*, 1985) or IgG2a in mice (Liang *et al.*, 1999).

T Lymphocytes determine the direction of the immune response depending on the antigen. There are several subclass populations of CD4 T cells, including T helper 1 (Th1), Th2, Th17, T follicular helper (Tfh), and T regulatory cells (Tregs). Th1 cells release cytokines that activate macrophages to aid in the killing of intracellular bacteria. Th2 are important for B cell activation and antigen specific antibody production in response to metazoan parasites and allergens. Th17 stimulate epithelial cells to release chemokines that mediate neutrophil recruitment early in infection (Murphy *et al.*, 2008). Tregs work to dampen the immune response and prevent autoimmunity (Larche, 2007; Murphy *et al.*, 2008).

Evidence suggests there is a skewing of the cytokine profile toward the Th2 CD4+ phenotype and the promotion of eosinoplia in asthma (Cohn *et al.*, 2004). The Th2 cytokines, IL-4, IL-5, IL-9, and IL-13, promote antibody class switching, eosinophilic inflammation, mast cell recruitment, mucus secretion and airway hyperreactivity (Paul and Zhu, 2010; Robinson *et al.*, 1999; Cohn *et al.*, 1998; Mattes *et al.*, 2002; Kim *et al.*, 2010).

Respiratory viruses, including parainfluenza, influenza and respiratory syncytial virus, can also produce a shifted T cell phenotype toward a Th2 response, which may contribute to airway hyperreactivity (Kristjansson *et al.*, 2005; Roman *et al.*, 1997). This idea comes from studies using guinea pigs with allergy resistant/Th1 or allergy prone/Th2 backgrounds (Aida *et al.*, 1997; Sutton *et al.*, 2007). A Th1 background limited the extent of airway hyperreactivity associated with respiratory syncytial virus infection (Sutton *et al.*, 2007). In addition, allergic asthmatic peripheral blood mononuclear cells exposed to rhinovirus produced IL-4 with more IL-10 and less IFN-γ and IL-12 than healthy individuals, indicating a defective Th1 response (Papadopoulos *et al.*, 2002). However, during virus infection, Th1 immunity is typically produced and is important for combating viruses. The Th1 cells release cytokines such as IFN-γ, TNF-α, IL-2 and lymphotoxin-α (Spellberg and Edwards, 2001). In addition, CD8+ T cells are activated and are important in mediating cytotoxic killing of virus-infected cells (Murphy *et al.*, 2008).

iii. Neutrophils

Neutrophils play an important part in the early immune response to viruses and bacteria, where they recognize, ingest, and kill pathogens (Murphy *et al.*, 2008). Neutrophils are generally recruited to the airways early in the inflammatory response to external pathogens or insults through the chemokine IL-8 (Monteseirin, 2009; Roberge *et al.*, 1994). Neutrophils are increased in the sputum during acute asthma exacerbations (Fahy *et al.*, 1995). Respiratory viral infections were reported

in 50% of these asthmatic patients, which correlated with highest neutrophil numbers in their sputum (Fahy *et al.*, 1995). Approximately 50% of severe asthma exacerbations are neutrophilic, while the other 50% are eosinophilic (O'Byrne, 2011). Activated neutrophils release inflammatory cytokines and chemokines, including IL-6, IL-8, TNF-α, IL-1β, and granulocyte-monocyte colony stimulating factor (GM-CSF). They also release matrix metalloproteinases (MMP), which are involved in airway remodeling (Cundall *et al.*, 2003). In addition, neutrophils release neutrophil elastase, which increases goblet cell secretion and forms mucus plugs in asthma (Agusti *et al.*, 1998; Nadel and Takeyama, 1999; Nadel *et al.*, 1999; Takeyama *et al.*, 1998).

iv. Eosinophils

Eosinophils are a granulocytic immune cell that has a major role in asthma and airway hyperreactivity (Hogan *et al.*, 2008; Kay, 2005). When eosinophils become activated they release preformed granules, which contain proteins such as major basic protein (MBP) eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Rosenberg and Domachowske, 1999). While these proteins can be cytotoxic to host tissue, they are also cytotoxic to bacteria and helminth parasites (Paul and Zhu, 2010). ECP and EDN also have ribonuclease activity that helps fight respiratory virus infections (Rothenberg and Hogan, 2006).

In asthma, eosinophil number is inversely correlated with lung function (Horn *et al.*, 1975) and positively correlated with airway hyperreactivity (Bousquet *et al.*, 1990). Eosinophils usually exist in low numbers in healthy individuals. However, BAL fluid and bronchial biopsies from people with chronic asthma show significantly increased eosinophil numbers in the lungs (Bousquet *et al.*, 1990; Lemiere *et al.*, 2006).

Eosinophils have well documented effects on nerve function in the lungs and mediate airway hyperreactivity in allergen-challenged (Fryer *et al.*, 2006; Evans *et al.*, 1997; Evans *et al.*, 2001), ozone (Yost *et al.*, 1999; Yost *et al.*, 2005), and virus-induced (Adamko *et al.*, 1999) animal models of asthma. In humans who have died from asthma, eosinophil numbers are increased in the airways (Gleich *et al.*, 1987) and around airway nerves (Costello *et al.*, 1997). Eosinophils are selectively recruited to airways by eotaxin and IL-5 (Fryer *et al*., 1997; Evans *et al*., 1997; Jose *et al*., 1994; Elbon *et al*., 1995; Yamada *et al.*, 2000). This is demonstrated in studies where blocking the eotaxin receptor, CCR3, prevented eosinophil recruitment and M_2 receptor dysfunction in antigen-challenged guinea pigs (Fryer *et al.*, 2006). Nerves also upregulate ICAM-1 to facilitate attachment of eosinophils to airway nerves (Nie *et al.*, 2007; Sawatzky *et al.*, 2002). When the eosinophils become activated they release preformed granular proteins such as MBP. In humans with fatal asthma, MBP is deposited in the airways, and on airway nerves (Costello *et al.*, 1997). MBP is an M_2 muscarinic receptor antagonist (Jacoby *et al.*, 1993), which

mediates bronchoconstriction through increased ACh release due to the loss of inhibitory M_2 receptor function. In antigen-challenged guinea pigs, M_2 receptor function is inversely correlated with eosinophils around nerves (Costello *et al.*, 1997). In addition, preventing eosinophil recruitment and maturation with an anti-IL-5 antibody prevents nerve dysfunction (Adamko *et al.*, 1999). Furthermore, airway hyperreactivity and M_2 receptor dysfunction can be prevented with heparin, which neutralizes the positive charge on MBP (Fryer and Jacoby, 1992; Jacoby *et al.*, 1993). Eosinophils also produce neurotrophins, such as nerve growth factor (NGF), which is implicated in airway hyperreactivity (Solomon *et al.*, 1998; Wu and Dey, 2006; Verbout *et al.*, 2009; Verhein *et al.*, 2011).

v. Mast cells

Mast cells are primarily associated with allergic asthma and are activated by cross-linking antigen specific immunoglobulin (Ig) E to receptors (FCεRI) on their cell surface (Brightling *et al.*, 2002; Stanworth, 1971; Stanworth, 1972). Mast cell activation mediates bronchoconstriction, mucus secretion, and edema by releasing histamine and other vasoactive mediators, such as PGD_2 and leukotrienes (Boyce, 2003; Robinson, 2004). Histamine can also induce bronchoconstriction by directly contracting airway smooth muscle or through vagal refluxes (Benson and Graf, 1977; Drazen and Schneider, 1978; Ellis and Undem, 1992; Shore *et al.*, 1983).

Mast cells play a major role in acute asthma exacerbations (Broide *et al.*, 1991). Mast cells are found in the bronchi (Pesci *et al.*, 1993; Bradley *et al.*, 1991; Djukanovic *et al.*, 1990; Koshino *et al.*, 1995) and are often degranulated in the airways of asthmatics during stable periods and after allergen challenge (Beasley *et al.*, 1989; Laitinen *et al.*, 1985). Asthmatics also have elevated mast cells in their airway smooth muscle (Brightling *et al.*, 2002). In addition, there are increased levels of tryptase, histamine, and prostaglandins in the BAL fluid of asthmatics (Broide *et al.*, 1991; Laitinen *et al.*, 1985; Tomioka *et al.*, 1984; Casale and Marom, 1983; Jarjour *et al.*, 1991; Wenzel *et al.*, 1988).

3. Current therapies

There are currently a number of therapies that work to limit asthma exacerbations (Reddel *et al.*, 2009). Many target different aspects of the disease and either function to reduce acute exacerbations or are used as continuous treatment to prevent future exacerbations. However, many of these treatments are associated with side effects and do not treat all aspects of the disease. In addition, compliance is often low due to addiction, resistance, or fear of side effects, which contributes to poor asthma control (Horne *et al.*, 2007; Haughney *et al.*, 2008; Mallia *et al.*, 2007). In addition, individuals from lower income often families have poor health and asthma control due to high cost of treatments and inadequate diagnosis (CDC, 2011a). This is also seen in lower income countries that have high prevalence of asthma and mortality

associated with the disease (Mendis *et al.*, 2007; WHO, 2007). This section describes the current treatments and highlights the need for new therapies for asthma.

a. Acute relief

Current guidelines recommend the use of β_2-adrenoreceptor agonists as bronchodilators for immediate rescue (NHLBI, 2007). β_2-adrenoreceptor agonists relax airway smooth muscle through activation of β_2 GPCRs, which are coupled to G_s and stimulate adenylate cyclase and protein kinase A activation (refer to Figure 1.4) (Billington and Penn, 2003). β_2-adrenoreceptor agonists have the side effect of tachycardia, as they bind to adrenoreceptor on the heart and increase heart rate (Mallia *et al.*, 2007). Long-acting β_2-adrenoreceptor agonists are recommended with corticosteroid treatment to enhance bronchodilation (Mallia *et al.*, 2007). This combined therapy has synergistic effects of increasing glucocorticoid sensitivity, increasing β_2-adrenoreceptor expression, and decreasing virus-induced chemokine production from epithelial cells (Edwards *et al.*, 2006).

Muscarinic receptor antagonists effectively dilate airways by binding to M_3 muscarinic receptors on airway smooth muscle and prevent G_q–mediated contraction. While these anticholinergics inhibit acute bronchoconstriction, they also have paradoxical effects by inhibiting muscarinic receptors on other cell types, and potentially worsening symptoms. For instance, muscarinic receptor antagonists

can block both neuronal M_2 receptors and M_3 receptors on airway smooth muscle (Maclagan and Barnes, 1989). Inhibition of M_2 muscarinic receptors on parasympathetic nerves increases ACh release and vagally mediated bronchoconstriction (Fryer and Maclagan, 1984; Blaber *et al.*, 1985). Studies show blocking M_2 receptors can overcome the blockade of M_3 muscarinic receptors on airway smooth muscle and potentiate bronchoconstriction by increasing ACh release through inhibition of M_2 receptors (Fryer and Maclagan, 1987; Groeben and Brown, 1996). In addition, treatment with anticholinergics, such as atropine, can actually increase bronchoconstriction and airway hyperreactivity by inhibiting muscarinic receptors on immune cells, such as eosinophils, and thereby increases activation and MBP release (Verbout *et al.*, 2007; Verbout *et al.*, 2009; Jacoby *et al.*, 2001). Anticholinergics also have unwanted side effects of tachycardia (increased heart rate) by inhibiting M_2 receptors on the heart, and dry mouth and urinary retention by inhibiting M_3 receptors on salivary glands and bladder, respectively (Mallia *et al.*, 2007; Mirakhur, 1978; Nelson *et al.*, 2004; Matsui *et al.*, 2000). However, during acute asthma exacerbation, the anticholinergic ipratropium improves lung function in patients with severe airway obstruction and long duration of symptoms compared to β_2-agonists alone (Rodrigo and Rodrigo, 2000). In addition, a recent study shows the anticholinergic tiotropium increases lung function and decreases asthma symptoms in patients with poorly controlled asthma when added to inhaled glucocorticoid treatment (Peters *et al.*, 2010). While anticholinergics are effective bronchodilators and suggested for patients who do not tolerate β_2 agonists well, they are not currently recommended for long-term

treatment of asthma (NHLBI, 2007). The attempts to use anticholinergics as a treatment in asthma may be hindered in part to the method of administration, dosing, and limited selectivity of the antagonists (Holtzman *et al.*, 1983; Sheppard *et al.*, 1983; Sheppard *et al.*, 1982; Moulton and Fryer, 2011).

b. Chronic prevention

The use of inhaled corticosteroids is the mainstay prophylactic treatment for prevention of future attacks. Corticosteroids improve lung function and prevent exacerbations through decreased airway inflammation, eosinophilia, and airway hyperreactivity (Mallia *et al.*, 2007; O'Byrne, 2011). Inhaled corticosteroids decrease the risk of the first severe asthma exacerbation by 60% and total attacks are reduced by 40% in individuals with mild asthma (O'Byrne *et al.*, 2001). Leukocyte recruitment is decreased with glucocorticoid treatment, which downregulates ICAM-1 expression, thus preventing immune cell adhesion (Caldenhoven *et al.*, 1995). On airway smooth muscle, M_2 and M_3 receptor expression is decreased with glucocorticoid treatment (Emala *et al.*, 1997). This may add to the beneficial effect of glucocorticoid treatment as M_2 receptors normally function to inhibit β_2 agonists and M_3 receptors mediate bronchoconstriction. Glucocorticoids also increase M_2 receptor expression and function on parasympathetic nerves, thus limiting ACh release and airway hyperreactivity (Jacoby *et al.*, 2001). However, this treatment can have serious side effects, including impaired growth in children, osteoporosis, and increased susceptibility to infection (Covar *et al.*, 2000; Abdullah, 2012; Cave *et*

al., 1999). In addition, there are patients with persistent asthma that are resistant to glucocorticoid treatment (Shah and Saltoun, 2012; NHLBI, 2007; Drazen *et al.*, 2000).

Eosinophils and other recruited immune cells also release proinflammatory mediators, such as leukotrienes, which mediate bronchoconstriction (Sawatzky *et al.*, 2002; Cheraim *et al.*, 2008; O'Byrne, 2000). Leukotriene receptor antagonist (LTRA) treatment is effective at improving lung function, bronchodilation, and reducing the risk of asthma exacerbations (Drazen *et al.*, 1999; Barnes and Miller, 2000; Camargo *et al.*, 2003). In addition, exacerbation frequency is reduced and time to first exacerbation is delayed in children with intermittent asthma associated with respiratory virus infection, demonstrating a possible age-related prevention in virus-induced asthma exacerbations (Bisgaard *et al.*, 2005; Mallia *et al.*, 2007).

c. Antiviral therapies

Respiratory viral infection is among most common factor triggering asthma attack in children and adults (Johnston *et al.*, 1995; Atmar *et al.*, 1998). Unfortunately, there are limited therapies for treatment of virus-induced attacks. Vaccination is likely an unrealistic treatment option since a variety of viruses cause similar symptoms. In addition, vaccination is inadequate due to the large number of serotypes of viruses, such as rhinovirus, and ability to develop resistance by the

virus (Mallia *et al.*, 2007). Vaccination has been successful for influenza virus infection (Bridges *et al.*, 2000), which also reduces serious illness and mortality by 50% (Nichol *et al.*, 1994). There are other antiviral therapies currently being investigated and tested in clinical trials; however, none have been approve for treatment of infections. These include capsid-binding inhibitors, which prevent attachment, uncoating, and viral replication; protease 3C inhibitor, which inhibit protein cleavage; and ICAM-1 blocking agents, which prevent rhinovirus entry in to cells (Greve *et al.*, 1989; Mallia *et al.*, 2007).

E. VIRUS INFECTION, AIRWAY HYPERREACTIVITY, AND INFLAMATION

Virus infections cause the majority of asthma attacks in both children and adults (Johnston *et al.*, 1995; Atmar *et al.*, 1998). It is important to understand the mechanisms of airway hyperreactivity that are induced with respiratory viral infections. This section addresses the main respiratory viruses, the causes of airway hyperreactivity, and the potential cytokines involved in exacerbations.

1. Virus infections in asthma

The majority of asthma attacks are associated with viral infection. Original studies using the methods of virus culture and serological testing only showed 10%-50% of acute asthma exacerbations are due to virus infections (Teichtahl *et al.*, 1997; Pattemore *et al.*, 1992; Johnston, 2005). However, after the development of RT-PCR, studies have found that virus infection can be detected in 80% of acute asthma exacerbations in children (Johnston *et al.*, 1995) and 50% in adults (Atmar *et al.*, 1998). However, some studies have shown up to 80% of the exacerbations in adults are attributed to virus infections (Minor *et al.*, 1976; Nicholson *et al.*, 1993). In a study of 9-11 year old children, the viruses that were detected in sputum during acute exacerbations were mostly picornaviruses (mainly rhinovirus), followed by coronavirus, influenza, parainfluenza, and respiratory syncytial virus (Figure 1.6) (Johnston *et al.*, 1995). These are all single-stranded RNA viruses, which are the

most common viruses associated with exacerbations. Typical symptoms of virus-induced exacerbations are wheezing, persistent cough, and bronchial hyperreactivity (Clough *et al.*, 1991; Nicholson *et al.*, 1993; Pattemore *et al.*, 1992).

In children, the seasonal pattern of upper respiratory infections correlates with hospital admissions (Johnston *et al.*, 1996). In adults, viruses were detected in the sputum of 76% of hospital admissions related to asthma exacerbations (Wark *et al.*, 2002). Those with virus infection also had higher levels of lactate dehydrogenase (LDH), a marker for virus-induced lower airway damage, which predicted longer hospital stays and severity of exacerbations. These studies suggest that viruses are the actual causative agent of acute asthma exacerbations and the severity of the infection is the major determining factor of exacerbation severity (Johnston, 2005).

There is much controversy over whether virus infection is protective or predictive in the development of asthma. As a result of increased cleanliness, the argument of the hygiene hypothesis relates the increased prevalence of allergic asthma to the lack of virus infection or exposure to infectious pathogens early in life (Strachan, 1989; Vandenbulcke *et al.*, 2006). However, other studies suggest that virus infections early in life may predispose individuals to develop asthma (Mallia *et al.*, 2007). Specifically, the presence of lower airways infection and severity of infection are demonstrated to be risk factors (Illi *et al.*, 2001; Wark *et al.*, 2002; Mosser *et al.*, 2002; Martinez, 2000). In addition, neonatal rats have permanent changes in lung

function and airway hyperreactivity 16 weeks after infection (Sorkness *et al.*, 1991). However, it should be noted that other environmental or genetic factors may be contributing to asthma development (Mallia *et al.*, 2007; Rodriguez *et al.*, 2002).

2. Virus infections and airway hyperreactivity

Respiratory virus infection is associated with airway hyperreactivity and alterations in the neural control of the airways in human and animals (Papadopoulos *et al.*, 2007; Jacoby, 2002; Jacoby, 2004). Viral infection can cause airway hyperreactivity and increased airway resistance even in a healthy individual. Non-asthmatics have increased airway hyperactivity to exercise, cold air, histamine, citric acid, and carbachol (a muscarinic agonist) (Empey *et al.*, 1976; Aquilina *et al.*, 1980; Johanson *et al.*, 1969; Little *et al.*, 1978). Airway hyperreactivity from inhaled histamine can be inhibited using the nonselective muscarinic receptor antagonist, atropine, to block muscarinic receptors (Empey *et al.*, 1976). This demonstrated that airway hyperreactivity was mediated through a reflex arc, which could be due to either increase in the efferent or afferent arm of the vagal reflux. Later, studies demonstrated bronchoconstriction was mediated through the efferent parasympathetic nerves by showing bronchoconstriction was potentiated in virus-infected animals compared to controls in response to electrical stimulation of the vagus nerves (Buckner *et al.*, 1985). The fact that virus infections increase vagally mediated bronchoconstriction may also explain why anticholinergics are more

effective in acute exacerbations compared to stable asthma (Rodrigo and Rodrigo, 2000; Jacoby, 2002).

3. Virus infections and M_2 receptor dysfunction

Virus infection increases bronchoconstriction and airway hyperreactivity by disrupting the function of parasympathetic nerves that provide autonomic control of the airways. Parasympathetic nerves mediate bronchoconstriction by releasing ACh from their nerve endings onto airway smooth muscle, causing contraction (Nadel and Barnes, 1984; Haddad *et al.*, 1991; Roffel *et al.*, 1990a). ACh also binds to M_2 receptors on the nerves, which inhibits further ACh release and limits bronchoconstriction (Fryer and Maclagan, 1984). Parainfluenza virus causes a decrease in M_2 muscarinic receptor function on parasympathetic nerves, which was evident in studies where pilocarpine, a muscarinic agonist, failed to inhibit vagally mediated bronchoconstriction compared to control guinea pigs (Fryer and Jacoby, 1991). The subsequent loss of inhibitory M_2 receptor feedback causes more ACh to be released onto airway smooth muscle (Fryer and Wills-Karp, 1991).

M_2 receptor dysfunction is found in some asthmatic patients (Minette *et al.*, 1989), while others have normal M_2 receptors that only become dysfunctional during virus infection (Keen *et al.*, 1998). M_2 receptor dysfunction leads to airway hyperreactivity after virus infection or double-stranded RNA produced during viral

replication (Fryer *et al.*, 1994; Bowerfind *et al.*, 2002). Virus-induced M_2 receptor dysfunction is demonstrated in guinea pigs and rats (Fryer and Jacoby, 1991; Sorkness *et al.*, 1994). Dysfunction in M_2 receptors usually reverses in 2-4 weeks after infection (Sorkness *et al.*, 1994). However, the function of M_2 receptors can become changed several weeks after infection, such as becoming linked to cyclooxygenase (COX) II activity (Kahn *et al.*, 1996).

There are several studies investigating the mechanisms of airway hyperreactivity and M_2 receptor dysfunction during virus infection. Direct exposure of M_2 receptors to viruses does not decrease the number of receptors, but does decrease the affinity of agonists for muscarinic receptors (Fryer *et al.*, 1990). This effect is mediated by neuraminidase (NA), which is a surface glycoprotein on parainfluenza and influenza viruses (Scheid *et al.*, 1972; Air and Laver, 1989). NA enzymatically cleaves sialic acid from the M_2 receptor agonist-binding site and reduces agonist affinity (Gies and Landry, 1988). However, other respiratory viruses, such as respiratory syncytial virus and rhinovirus, induce airway hyperreactivity and do not express NA, suggesting other mechanisms are involved (Lemanske *et al.*, 1989; Estripeaut *et al.*, 2008; Pattemore *et al.*, 1992; Flint *et al.*, 2009a). Virus infection decreases neutral endopeptidase activity, an enzyme that degrades tachykinins, such as substance P (Jacoby *et al.*, 1988). Antagonism of the tachykinin NK1 receptor in sensitized, antigen-challenged animals prevents M_2 receptor dysfunction (Costello *et al.*, 1998). Therefore, it is possible M_2 receptor dysfunction is induced by tachykinins in virus

infection, possibly through tachykinin-mediated leukocyte recruitment or adhesion (Baluk *et al.*, 1995; Bertrand *et al.*, 1993; Marasco *et al.*, 1981). Virus infections can increase nitric oxide (NO) production to suppress virus replication (Sanders *et al.*, 1998; Akaike and Maeda, 2000), and NO mediates M_2 receptor dysfunction, which could be another mechanism of airway hyperreactivity (Golkar *et al.*, 2000). Furthermore, direct parainfluenza virus treatment of cultured parasympathetic nerves induces a loss in M_2 receptor function and expression (Jacoby *et al.*, 1998), indicating that if viruses gained access to airway nerves this could be a mechanism for airway hyperreactivity.

Several studies have investigated the possible immune cell mediated causes for the loss of M_2 receptor function during virus infection. Depletion of leukocytes with cyclophosphamide prevents M_2 receptor dysfunction in some virus-infected animals (Fryer *et al.*, 1994). In the animals that lost M_2 receptor function, depleting leukocytes lead to high lung viral titers, which had a significant, positive correlation to the degree of M_2 receptor dysfunction (Fryer *et al.*, 1994). However, in this study, M_2 receptor dysfunction was still present in some guinea pigs with high lung viral titers, despite a lack of immune cells. This indicates another leukocyte-independent mechanism is involved in cases of more severe infections. Selectively depleting macrophages with liposome-encapsulated dichloromethylene-diphosphonate, which induces macrophage apoptosis (Naito *et al.*, 1996), also prevents virus-induced M_2 receptor dysfunction (Lee *et al.*, 2004). The involvement of CD8+ T cells

in virus-induced M_2 receptor dysfunction has also been demonstrated in guinea pigs (Adamko *et al.*, 2003). However, blocking CD8+ T cells only prevented M_2 receptor dysfunction in sensitized animals, but not in non-sensitized animals (Adamko *et al.*, 2003). In sensitized, virus-infected animals, CD8+ T cells induce eosinophil degranulation and subsequent release of the muscarinic antagonist, MBP (Adamko *et al.*, 2003)(Adamko *et al.*, 1999; Jacoby *et al.*, 1993). Sensitization changes the virus-induced loss of M_2 receptor function and inflammatory response to be eosinophil-mediated (Adamko *et al.*, 1999). This is demonstrated by the fact that blocking recruitment of eosinophils in the lungs with an anti-IL-5 antibody or neutralizing MBP with heparin prevents M_2 receptor dysfunction only in sensitized, but not non-sensitized, virus-infected guinea pigs (Adamko *et al.*, 1999). Thus, these findings indicate that eosinophils and CD8+ T cells are not necessary for virus-induced M_2 receptor dysfunction in non-sensitized guinea pigs (Adamko *et al.*, 2003; Adamko *et al.*, 1999).

The immunosuppressive glucocorticoid, dexamethasone, increases M_2 receptor expression and function in cultured parasympathetic nerves (Jacoby *et al.*, 2001). High dose dexamethasone prevents M_2 reception dysfunction in virus-infected guinea pigs (Moreno *et al.*, 2003). Low dose dexamethasone also reduced M_2 receptor dysfunction. However, it did not decrease virus-induced inflammatory cells in the lungs, suggesting an inflammation-independent mechanism of M_2 receptor function at lower doses.

While there is a demonstrated effect of immune cells in virus-induced M_2 receptor dysfunction, little is known about which cellular mediators, such as chemokines or cytokines, are released from these cell types to exaggerate bronchoconstriction. However, some cytokines have been investigated. IFN-γ decreases M_2 receptor expression in cultured guinea pigs parasympathetic nerves. In addition, IFN-γ treatment increases M_2 receptor expression in cultured sympathetic nerves (Grodzki *et al.*, 2011). M_2 receptors on sympathetic nerves limit norepinephrine (NE) neurotransmitter release (Pendry and Maclagan, 1991). NE binds to adrenoreceptors on postganglionic parasympathetic nerves and limits ACh release (Baker *et al.*, 1992; Baker *et al.*, 1983). This means that there would be less NE released from sympathetic nerves due to an increase in M_2 receptor expression. Therefore, increased ACh release from parasympathetic nerves could be indirectly mediated through an IFN-γ-induced decrease in sympathetic neurotransmission. Recent studies have found TNF-α mediates M_2 receptor dysfunction during parainfluenza virus infection in guinea pigs (Nie *et al.*, 2011), and decreases M_2 receptor expression in cultured parasympathetic nerves (Nie *et al.*, 2009). Another study showed M_2 receptor expression decreases in lung fibroblasts with combined TNF-α and IL-1β treatment (Haddad *et al.*, 1996b).

4. Parainfluenza virus infection

Parainfluenza virus belongs to the family paramyxoviridae. There are 4 serotypes of human parainfluenza viruses (type 1, 2, 3, and 4) based on the antigenic

characterization of their viral proteins (Chancock *et al.*, 1958; Enders, 1996). Parainfluenza virus is also subdivided into two genera: respirovirus (type 1 and 3) and rubulavirus (type 2 and 4). Three of the parainfluenza virus subtypes were discovered in the 1950's from children with lower respiratory tract infections and determined to be antigenically different from the similar orthomyxoviruses (influenza). The forth was later discovered in the 1959's (Henrickson, 2003). Parainfluenza type 1 and 2 primarily causes croup (laryngotracheobronchitis), which produces a barking cough and difficulty breathing due to inflammation in the airways. Type 2, 3, and 4 are associated with bronchiolitis and pneumonia; however, type 4 is rare (Peiris, 2005). Children are typically exposed to type 3 by age 2 (Glezen *et al.*, 1984) and type 1 and 2 by age 5 (WHO, 2009a). Except for respiratory syncytial virus, parainfluenza viruses are the most important viral cause of lower respiratory tract illness in children (WHO, 2009a). In addition, parainfluenza type 1 is estimated to cause 5,800- 28,900 hospitalizations every year (WHO, 2009a). Parainfluenza is tied with influenza for the third most prevalent respiratory infection during asthma exacerbations (Johnston *et al.*, 1995).

a. Structure and genome

Parainfluenza virions are spherical, enveloped, and approximately 150-250 nm in diameter (Henrickson, 2003). The viral genome is a linear, non-segmented, and 15 Kb in size. The virus is a single-stranded, negative-sense RNA virus that encodes 6 proteins, including nucleocapsid protein (N), fusion protein (F), hemagglutinin-

neuraminidase (HN), matrix protein (M), large polymerase protein (L), and phosphoprotein (P) (Figure 1.7) (Hall, 2001; Yin *et al.*, 2006; Knipe and Howley, 2007; Flint *et al.*, 2009a). The ribonucleoprotein, which includes N, L, P, and viral RNA, is filamentous and is a fully infectious complex (Zidovec and Mazuran, 1999). The negative strand RNA is not infectious on its own and requires an RNA-dependent RNA polymerase (L) to initiate replication (Marx *et al.*, 1974; Vidal and Kolakofsky, 1989). The P protein also aids synthesis new of viral genome with the L polymerase, and along with the N protein, they coat the coat the viral RNA (Hu and Gupta, 2000; Zidovec and Mazuran, 1999). The HN protein is required for viral attachment and release, and the F protein is needed to fuse the virion membrane with the endocytic vesicle membrane (Fouillot-Coriou and Roux, 2000; Peisajovich *et al.*, 2000). The M protein is important for budding and interacts with the HN glycoproteins, the cytoplasmic tail of the F protein, the lipid bilayer, and the ribonucleoprotein (Ali and Nayak, 2000; Hall, 2001).

b. Virus cycle

The parainfluenza viruses uses hemagglutitin (HA) protein to attach to host cells by binding to sialic acid residues on target cells. The virus enters via clatherin-dependant receptor mediated endocytosis (Flint *et al.*, 2009a). When the endocytic vesicle is acidified, the HA protein is cleaves at site between HA1 and HA2, which induces a conformational change in the protein. The conformational change in HA activates the F protein, which allows the viral membrane to fuse with the endocytic

vesicle membrane (Iorio *et al.*, 2009; Peisajovich *et al.*, 2000; Flint *et al.*, 2009a). The virus is uncoated and the viral ribonucleoprotein enters the cytoplasm. The negative strand viral RNA is transcribed by the L RNA polymerase to make positive strand viral mRNA. The viral mRNA is translated with cellular machinery to make viral proteins. In addition, more genomic viral RNA is generated (Vidal and Kolakofsky, 1989; Zaides *et al.*, 1974). The HN proteins are glycosylated and transported to the cell membrane where they are incorporated. Once a threshold level of F protein accumulates, the virus can assemble (Fouillot-Coriou and Roux, 2000). The M protein associates with the newly synthesized genomic RNA and viral membrane proteins (F, HN), then buds from the plasma membrane (Ali and Nayak, 2000; Flint *et al.*, 2009a). The neuraminidase cleaves the sialic acid, releasing the virus particle and allowing it to spread to neighboring cells (Flint *et al.*, 2009a).

c. Pathogenesis

Parainfluenza virus type 1 and type 2 infections have biannual infection patterns. Type 1 infects on the odd-numbered years and has a sharp increase in the number of cases in the fall. Type 2 infects on the even-numbered years, has a fewer cases than type 1, and appears to be less seasonal. Type 3 infects annually in the spring and summer with less defined outbreaks compared to type 1 and 2 (Hall, 2001). The major symptom of parainfluenza type 1 infection is croup, which is the main cause of hospitalizations with parainfluenza virus infection in children 2-6 years old (WHO, 2009a), and parainfluenza virus is recovered from 65% of croup cases

(Heilman, 1990). Other symptoms, such as runny nose, cough, and fever are also observed. Parainfluenza is transmitted through inhalation of aerosolized virus or contact with contaminated surfaces (Faisca and Desmecht, 2007). The incubation time for parainfluenza is 2-6 days (Vainionpaa and Hyypia, 1994). Virus is shed in the respiratory mucosa and infection induces sloughing of the epithelium, increased mucus production, and is airflow obstruction (Hall, 2001). The virus mainly infects airway epithelium (Faisca and Desmecht, 2007), although infection of macrophages and dendritic cells also occur (Peiris, 2005). During infection, alveolar macrophages participate in phagocytosis of infected cells and release proinflammatory cytokines. Natural killer (NK) cells also become activated in response to interferons and IL-12, which mediate killing of infected cells and contribute to cytokine release. The virus is often cleared through these innate immune responses, but can also be cleared by cytotoxic T cell-mediated killing and neutralization through antibody production. The generated memory T and B cells provide short duration of protection against a future infection (Hall, 2001; Flint *et al.*, 2009a). Reinfection with parainfluenza happens throughout life of an individual; however, it results in a mild respiratory infection and rarely leads to a lower respiratory tract infection (WHO, 2009a; Peiris, 2005).

The parainfluenza type 1 strain used in this thesis is Sendai/52, which was originally determined to be isolated from a fatal case of newborn pneumonitis in Sendai, Japan in 1952 (Kuroya and N, 1953; Kuroya *et al.*, 1953; R *et al.*, 1953;

Jensen *et al.*, 1955). Although the virus was believed to be a human virus when isolated, mice were used to recover virus. Later it was determined that mice in Japan were ubiquitously infected with the virus. In addition, there is also no evidence that humans are the natural host and Sendai virus is now regarded as a rodent virus (Parker and Reynolds, 1968; Faisca and Desmecht, 2007; Brownstein, 2002).

5. Influenza virus infection

Influenza virus is in the family Orthomyxoviridae. There are three types of influenza viruses (A, B, and C), each belonging to their own genera. The subtypes are based on the antigen specificity of the nucleoprotein (NP) and matrix (M) proteins (Palese and Young, 1982; Carr, 2012; Hayashida *et al.*, 1985). Influenza A is also categorized based on the antigenic characterization of the hemagglutinin (HA) and neuraminidase (NA) proteins. Influenza was initially isolated from several animal species, including pigs in 1931 (Shope and Lewis, 1931), and then from humans in 1933 (Smith *et al*., 1933). The influenza A subtype is important as it is the causative agent in of all human influenza pandemic outbreaks, including the "Spanish Flu" in 1918 (H1N1), the "Asian Flu" in 1957 (H2N2), the "Hong Kong Flu" in 1968 (H3N2), and "Bird Flu" in 2004 (H5N1) (Oxford, 2000). The H1N1 serotype is currently the most common seasonal virus in the U.S. (CDC, 2013), and is responsible for the 2009 influenza pandemic outbreak and the Spanish Flu in 1918, which killed an estimated 50 million people worldwide (Taubenberger and Morens, 2006; WHO, 2009b; Oxford, 2000). Every year, up to 15% of the population can be infected with

influenza and approximately 3-5 million severe cases of influenza are reported, with 250,000-500,000 annual deaths (WHO, 2009c). Both young children and people over the age of 65 are at the highest risk for influenza related-complications; however, children have the highest risk of hospitalization and people over 65 have the highest mortality rate associated with infection (Carr, 2012). In regards to asthma exacerbations, influenza is tied with parainfluenza for third as the most detected respiratory virus during acute exacerbations (Johnston *et al.*, 1995).

a. Structure and genome

The influenza virion is spherical, enveloped, and approximately 80 -120 nm in diameter (Stanley, 1944). It has a negative-sense, single-stranded RNA, segmented genome (Figure 1.8). The total genome size is 14 Kb and each of the 8 segments encodes 1-2 proteins, which include the RNA polymerase associated proteins (PB1, PB2, PA), nucleocapsid protein (NP), nuclear export protein (NEP), hemagglutinin (HA), neuraminidase (NA), nonstructural protein (NS1), matrix protein (M1), and ion channel (M2) (Flint *et al.*, 2009a). To accomplish this, the genome undergoes reassortment and variations, which involve RNA splicing and leaky scanning (Marshall *et al.*, 2013; Flint *et al.*, 2009a). The RNA genome is wrapped around a helical core made of NP bound to the RNA, which is determined by the fact the viral RNA is fully accessible to solvent and RNase treatment (Baudin *et al.*, 1994; Duesberg, 1969; Pons *et al.*, 1969; Flint *et al.*, 2009a).

Influenza virus differs from all other RNA viruses, in that RNA synthesis occurs in the nucleus and not the cytoplasm (Flint *et al.*, 2009a). The negative strand RNA is not infectious on its own and requires an RNA-dependent RNA polymerase. The only viral proteins required for transcription are the 4 RNA polymerase proteins, PB1, PB2, PA, and NP (Huang *et al.*, 1990).

The HA glycoprotein mediates cell entry by binding to sialic acid and NA functions to cleave sialic acid to release cell-associated virus progeny and promote spread of the virus (Haywood, 1994). The M2 ion channel functions to import H+ ions into the virion from the acidic vesicle, which mediates fusion and release of viral ribonucleoprotein (RNP) into the cytoplasm. The M1 protein binds to the cell membrane, cytoplasmic regions of HA and NA, and the viral RNP. M1 functions to shut off virus genomic RNA synthesis and export RNP from the nucleus (Flint *et al.*, 2009a). The NS1 protein mainly functions to inhibit polyadenylation and accumulation of cellular mRNA, which interferes with production of host immune responses to infection, such as interferons (Hale *et al.*, 2008). The PB1-F2 is an accessory protein with proapoptotic activity and is made from an overlapping reading frame on the PB1; however, it is not made by all influenza A viruses (Shaw *et al.*, 2008). The virus packages 9 of the 11 proteins into a virion particle, but does not incorporate the NS1 and PB1-F2 proteins (Shaw *et al.*, 2008; Palese and Shaw, 2007).

b. Virus cycle

Influenza HA protein binds to sialic acid containing receptors on host cells and the virus enters the cell via clatherin-dependant receptor mediated endocytosis (Weis *et al.*, 1988; Flint *et al.*, 2009a). Once the endocytic vesicle reaches a pH of approximately 5.0, the HA protein undergoes an acidic-catalyzed conformational change that exposes a fusion peptide (Chen *et al.*, 1998; Carr *et al.*, 1997), which allows fusion of the viral membrane with the late endosome membrane (Carr *et al.*, 1997; Han *et al.*, 2001). The virus is uncoated and M1 is dissociated from the RNP, at which point the viral RNP is imported into the nucleus. The negative strand viral RNA segments are transcribed by the virus RNA polymerase into viral mRNA and exported into the cytoplasm. All viral mRNA are translated by ribosomes in the cytoplasm, except NA, HA, M2, which are translated by ER ribosomes. HA and NA are glycosylated and transported to the cell surface where they are incorporated into the membrane. To assist in making more genomic viral RNA, the 4 RNA polymerase proteins (PB1, PB2, PA, NP) are imported back into the nucleus to make positive stand RNA and then more negative strand genomic RNA. At this point, the M1 protein is transported into the nucleus and associates with the newly synthesized genomic RNA, which shuts down further viral mRNA synthesis and triggers nuclear export (Flint *et al.*, 2009a). The viral nucleocapsid associated with M1 is exported out of the nucleus using NEP, where it is transported to the cell surface to attach to the HA, NA, and M2 proteins in the membrane (Hutchinson and Fodor, 2013). Finally, the virus particle is released from the cell by budding from the cell plasma membrane (Carr, 2012; Flint *et al.*, 2009a).

c. Pathogenesis

Annually, a sharp increase in the number of influenza infections occurs between the months of November through May (Dowell, 2001), with most infections in January or February (Yorke *et al.*, 1979). In fact, influenza is more stable and transmitted more efficiently at low temperatures and humidity (Lowen *et al.*, 2007). Influenza virus also undergoes seasonal antigenic drift through slight mutations in the HA and NA proteins, requiring the host to develop a new antibody response to antigen variations during the next infection cycle (Carr, 2012; Flint *et al.*, 2009b). In general, influenza is transmitted from human to human, although animal to human is also possible. Virus particles are transmitted by respiratory route, often by touching virus-contaminated objects or inhaling respiratory droplets. The virus incubation period is approximately 1-3 days before the first signs of symptoms are observed (Cox and Subbarao, 1999; Carr, 2012), and typical symptoms include, cough, nasal congestion, fever, headache, sore throat body aches, chills and fatigue (Call *et al.*, 2005; Flint *et al.*, 2009b). The peak viral titers are detected 1-3 days after infection and correlate with fever severity. Viral shedding occurs in the respiratory mucosa, initiates 1 day after first symptoms, and lasts approximately 5-10 days (Cox and Subbarao, 1999; Carrat *et al.*, 2008). Influenza virus infects the upper and lower respiratory tracts and primarily targets epithelial cells. Innate immune receptors, such as toll-like receptor (TLR) 3, 7, and 8 recognize the virus and cells release inflammatory mediators, mainly IL-8, RANTES and interferon (Guillot *et al.*, 2005). Like parainfluenza, influenza is often cleared through innate immune mechanism, such as macrophages, cytokines, neutrophils, NK cells, interferons, and fever.

However, if the virus evades these mechanisms, it is cleared through adaptive immune mechanisms involving antigen specific T and B cell effector functions (Carr, 2012; Ada and Jones, 1986; Tamura and Kurata, 2004; Flint *et al.*, 2009b; Murphy *et al.*, 2008). The influenza A virus strain used in this thesis is A/PR/8/34 (H1N1), which was originally isolated from a patient in Puerto Rico in 1934 (Francis, 1935), and was subsequently mouse adapted.

6. Cytokines

a. TNF-α in airway inflammation

TNF-α is a pleiotropic proinflammatory cytokine produced during virus infection by a variety of different cells types and is key regulator of inflammatory responses. Its concentration is increased in the BAL of parainfluenza (Uhl *et al.*, 1998) and influenza virus-infected animals (Vacheron *et al.*, 1990). TNF-α is also implicated in asthma, where TNF-α mRNA and protein levels are elevated in asthmatic patients compared to healthy individuals (Berry *et al.*, 2006; Bradding *et al.*, 1994; Broide *et al.*, 1992; Howarth *et al.*, 2005). In asthma, TNF-α is associated with significant tissue damage, especially with respiratory viral infections (O'Sullivan, 2005). Along with interleukin-1β (discussed below), TNF-α plays a prominent role in asthma pathogenesis by promoting bronchial hyperreactivity and airway remodeling (Kips *et al.*, 1992; Kips and Pauwels, 1996). In addition, TNF-α mediates airway hyperreactivity in antigen-challenged (Nie *et al.*, 2009) and parathion exposed

guinea pigs (Proskocil *et al.*, 2013).

The rationale for investigating TNF-α comes from data demonstrating TNF-α mediates M_2 receptor dysfunction and airway hyperreactivity in sensitized, allergen-challenged (Nie *et al.*, 2009) and virus-infected guinea pigs (Nie *et al.*, 2011). TNF-α also decreases M_2 muscarinic receptor expression in cultured parasympathetic neurons (Nie *et al.*, 2009); however, it is not known if TNF-α decreases M_2 receptor expression *in vivo*.

i. TNF-α

In 1891, William Coley used supernatants from heat-killed bacteria to treat tumors (Bickels *et al.*, 2002). This may be the actual first discovery of tumor necrosis factor-α (TNF-α); however, it was not given a name until 1975 when the endotoxin-like protein was a factor release from host cells with tumor necrosis abilities (Carswell *et al.*, 1975; Terlikowski, 2001). Around the same time, lymphotoxin was discovered as cytotoxic protein released from lymphoid cells (Granger *et al.*, 1969). LT and TNF proteins were cloned in 1984 (Granger *et al.*, 1969; Pennica *et al.*, 1984), and are now well characterized and known to be members of a superfamily of ligand and receptor proteins termed tumor necrosis factor/tumor necrosis factor receptor superfamily proteins (TNF/TNFR SFP). TNF-α is has trimeric symmetry with structural motif called the TNF homology domain (THD). The THD domain is

conserved among all the other TNF proteins and binds to cysteine-rich domains (CRDs) of the TNF receptors (TNFRs) (Bodmer *et al.*, 2002).

TNF-α is synthesized as a transmembrane protein, but can be cleaved by TNF-α converting enzyme to release a soluble form. Both membrane-bound and soluble TNF-α are biologically active (Horiuchi *et al.*, 2010); however, membrane TNF is 1000 times more potent than the soluble form (Schneider *et al.*, 1998). Several different cell types produce TNF-α, including neurons, epithelial, glial, smooth muscle, fibroblasts, and immune cells (Li *et al.*, 2004; Lutfi *et al.*, 2012; Tchelingerian *et al.*, 1993; Heffler *et al.*, 2007; Nie *et al.*, 2011; Murphy *et al.*, 2008). The type of stimulus determines the cell source of TNF-α. T cells produce TNF-α in response to antigen recognition, while macrophages and other cell types produce TNF-α in response to innate immune receptor activation (Keystone and Ware, 2010). TNF-α has several effector functions, such as activation of inflammatory cells, inducing release of inflammatory mediators, upregulation of adhesion molecules, increasing body temperature, leukocyte recruitment, and migration to lymph nodes (Walsh *et al.*, 1991; Nie *et al.*, 2007; Kips *et al.*, 1993; Kips and Pauwels, 1996; Murphy *et al.*, 2008).

TNF-α interacts with nerves, which is determined in studies that demonstrate TNF-α affects nerve function and gene regulation. TNF-α enhances Na^+ currents in primary dorsal root ganglia neurons; however, this response is reduced in TNFR1

knockout mice compared to wild-type (Jin and Gereau, 2006). This indicates TNF-α may induce peripheral hypersensitivity by stimulating TNFR1 in sensory neurons. In addition, studies in knock-out mice have indicated neurotoxic effects associated with TNFR1 and neuroprotective effects associated with TNFR2 (Yang *et al.*, 2002). In addition, TNF-α induces expression of ICAM-1 on parasympathetic nerves, which mediates adhesion of leukocytes (Nie *et al.*, 2007). TNF-α also decreases M_2 muscarinic receptor expression in cultured parasympathetic neurons, and blocking TNF-α decreases the number of eosinophil associated with airway nerves (Nie *et al.*, 2009).

ii. TNF receptors and signaling

TNF-α can bind and activate two tumor necrosis receptors (TNFR), TNFR1 (p55-R) or TNFR2 (p75-R). Both TNF receptors are transmembrane proteins with 3-fold symmetry. TNFR1 is constitutively expressed on almost all nucleated cell types and TNFR2 is inducible and mainly expressed on immune cells (Locksley *et al.*, 2001; Matera *et al.*, 2010). Binding of TNF-α to TNFR1 or TNFR2 initiates signaling cascades that eventually leads to regulation of inflammatory genes; however, TNFR1 is also associated with apoptosis (Kuwano and Hara, 2000; Keystone and Ware, 2010; Matera *et al.*, 2010). TNF-α binding to the TNFR1 receptor recruits TNFR1-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein (RIP) to the cytoplasmic region of the receptor (Figure 1.9). These adaptor proteins recruit and activate the IKK kinase complex, which

phosphorylates the inhibitory protein, IκB, bound to NF-κB. Phosphorylation of IκB leads to its degradation and allows the transcription factor NF-κB to translocate into the nuclease to activate gene transcription (Wajant *et al.*, 2003). A recombinant, dimeric fusion protein that links the TNFR2 to the Fc constant region of IgG, called etanercept, is currently FDA approved treatment for different types arthritis and psoriasis and is used in parts of this thesis (Horiuchi *et al.*, 2010; Hsu *et al.*, 2013).

b. IL-1β in airway inflammation

IL-1β is pleiotropic cytokine that is upregulated during parainfluenza (Yoshizumi *et al.*, 2010) and influenza virus infection (Chiaretti *et al.*, 2013). Overproduction of IL-1β has been associated with the pathology of asthma (Message and Johnston, 2004; Mallia and Johnston, 2006; Proud and Chow, 2006). IL-1β is elevated in both symptomatic and asymptomatic asthmatic bronchoalveolar lavage (BAL) fluid compared to healthy individuals (Broide *et al.*, 1992; Borish *et al.*, 1992; Jarjour and Busse, 1995). IL-1β is produced from asthmatic bronchial epithelium, monocytes, and alveolar macrophages from asthmatic patients (Sousa *et al.*, 1996; Tomita *et al.*, 1995; Borish *et al.*, 1992; Gosset *et al.*, 1991). Furthermore, blocking IL-1β or knocking out IL-1 receptors in mice reduces hyperresponsiveness to inhaled antigens (Johnson *et al.*, 2005; Watson *et al.*, 1993). In addition, asthmatics have increased IL-1β levels and decreased IL-1β receptor antagonist levels after rhinovirus infection compared to non-asthmatics (de Kluijver *et al.*, 2003). These

data indicate an important role for IL-1β in asthma and virus-induced exacerbations.

The importance of determining the role of IL-1β in virus-induced M_2 receptor dysfunction in this thesis is based on data that show IL-1β mediates airway hyperreactivity in ozone (Verhein *et al.*, 2008; Park *et al.*, 2004) and antigen-challenged guinea pigs (Selig and Tocker, 1992; Johnson *et al.*, 2005; Wang *et al.*, 2006). Furthermore, M_2 receptor expression is decreased with combined TNF-α and IL-1β treatment (Haddad *et al.*, 1996b). However, its role in mediating M_2 receptor dysfunction and airway hyperreactivity has not been investigated with an animal model of virus infection.

i. IL-1β

IL-1β was first discovered in 1948 as an endogenous fever-inducing protein from leukocytes (Beeson, 1948). Several studies over the next 4 decades demonstrated multiple immunological properties of the IL-1β protein, such as fever induction, T cell proliferation, and prostaglandin production (Dinarello *et al.*, 1981; Dinarello *et al.*, 1986). Thus, speculation arose as to whether one protein could have such a diverse affect on biological processes (Dinarello, 2005). In 1984, IL-1β was cloned (Auron *et al.*, 1984; Lomedico *et al.*, 1984), and the recombinant protein was used in studies to resolve any confusion from pervious findings (Dinarello, 2005). While the

IL-1 family was originally comprised of 2 members, IL-1α and IL-1β, it has expanded to 11 total proteins including antagonists, coreceptors, decoy receptors, and other binding proteins (Dinarello, 2009). Both IL-1β and IL-1α are agonists and IL-1Ra is a naturally occurring antagonist (Dinarello, 1996). IL-1β and IL-1α are both synthesized from precursor proteins. The production of mature form of IL-1α is mediated by membrane-associated cysteine proteases (Kobayashi *et al.*, 1990), which cleaves pro-IL-1α into IL-1α. IL-1α is rarely seen in systemic circulation, except in cases of severe disease, such as a septic shock-like state, where it can be released from dying cells (Wakabayashi *et al.*, 1991). The mature IL-1β protein is produced by cleavage of the minimally active pro-IL-1β (Jobling *et al.*, 1988, Dinarello, 1996). Pro-1L-1β is cleaved by the IL-1β converting enzyme (ICE), also known as capsase-1 (Black *et al.*, 1988; Dinarello, 1996).

IL-1β is an important cellular mediator of inflammation and there is no evidence of IL-1β being required for homeostasis. This observation is determined from studies of following mice lacking IL-1β over 8 years (Dinarello, 2005). IL-1β production is triggered by IL-1β or TLR ligands, such as viruses or bacteria, which induce the synthesis of the inactive pro-IL-1β protein in monocytes, macrophages, or dendritic cells (Dinarello *et al.*, 1987; Dinarello, 2009).

IL-1β can be released from monocytes, macrophages, dendritic cells, smooth muscle or epithelial cells (Schmitz *et al.*, 2003; Dragon *et al.*, 2007). Once released, IL-1β can

have multiple effects on different cell types. IL-1β increases cellular adhesion molecules and chemokines, which aid in immune cell recruitment to the airways (Dinarello, 1996; Jedrzkiewicz *et al.*, 2000; Birdsall *et al.*, 1992). IL-1β induces eotaxin expression in airway epithelial cells, which mediates eosinophil recruitment (Jeffery, 1995; Ponath *et al.*, 1996; Pease, 2006). In addition, airway smooth muscle hyperresponsiveness induced by rhinovirus is mediated by IL-1β (Hakonarson *et al.*, 1999a).

ii. IL-1 receptors and signaling

IL-1β can bind to multiple receptor types, although only blinding to IL-1 receptor type I initiates a signal transduction (Figure 1.10). IL-1β can also bind to IL-1 receptor type II, which is a decoy receptor that exists as a membrane-bound and soluble form (Dinarello, 2002). IL-1 type II functions as a decoy receptor since it does not have a cytoplasmic domain and does not initiate signaling (Colotta *et al.*, 1993; Heguy *et al.*, 1993). Two new inhibitory receptors have been discovered, single Ig IL-1–related receptor (SIGGRR) (Garlanda *et al.*, 2009) and IL-1 receptor accessory protein (IL-1RAcPb) (Smith *et al.*, 2009). Both these receptor have non-functional Toll/interleukin-1 receptor (TIR), which prevents MyD88 adapter protein recruitment (Dinarello, 2011). The existence of multiple inhibitor receptors, decoy receptors, and antagonists is a way to tightly regulate inflammation and IL-1β mediated effects.

Receptor signaling is initiated when IL-1β binds to the IL-1 type I receptor (IL-1RI) and the co-receptor accessory protein (IL-1RAcP), which induces recruitment of MyD88, IL-1 receptor–associated kinases (IRAKs), and TNF receptor-associated protein factor-6 (TRAF-6) (Figure 1.11) (Dinarello, 2009). Signaling events lead to IKKβ and MAPK activation and downstream activation of the transcription factor, NF-κB. The IL-1β precursor is transcribed and then translated in the cytosol (Fraczek *et al.*, 2008; Dinarello, 2009). The cleavage of IL-1 requires activation and recruitment of the cryopyrin (NALP3) inflammasome complex. The inflammasome is a formation of NALP3 that oligomerizes with other intracellular proteins and functions to convert the inactive procaspase-1 into the active caspase-1 enzyme. However, circulating blood monocytes have constitutively active caspase-1 present (Netea *et al*., 2009). Caspase-1 cleaves pro-IL-1β precursor into an active secreted cytokine (Dinarello, 2009; Thomas *et al.*, 2012). In general, IL-1α participates in local inflammation and regulates intracellular events, while IL-1β is secreted and acts as a systemic regulator of inflammation (Dinarello, 1996).

iii. IL-1 receptor antagonists

A naturally occurring IL-1 receptor antagonist was first described in 1986 as a protein that inhibited IL-1β binding without an agonist activity (Balavoine *et al.*, 1986). The protein was later cloned in 1990 (Eisenberg *et al.*, 1990) and confirmed it binds to the IL-1 receptors without initiating signal transduction (Dripps *et al.*, 1991). IL-1β and IL-1 receptor antagonists share between 26 – 30% sequence

homology; however, there is high structural similarity between the two (Arend, 1993; Arend *et al.*, 1998; Seckinger *et al.*, 1987). In a healthy individual, IL-1 receptor antagonist in the blood ranges from 100-300 ng/ml, while IL-1β levels are often in a range too low to detect (less than 5 pg/ml) (Dinarello, 2011). This concentration ratio is expected as 100-1000-fold excess of this antagonist is needed in order to block biological activity (Dayer-Metroz *et al.*, 1989; Zumsteg *et al.*, 1993; Eizirik *et al.*, 1991; Dinarello and Thompson, 1991). However, in patients with sepsis and burns (Cannon *et al.*, 1992), or healthy people injected with LPS (Cannon *et al.*, 1990; Granowitz *et al.*, 1991), significantly higher levels IL-1β are detected (up to 2.8 ng/ml) (Casey *et al.*, 1993; Dinarello, 2005; Dinarello, 1996). Even a small dose (1 ng/ml) of administered IL-1β is enough to induce fever and increase neutrophils 100% in 4 hours (Tewari *et al.*, 1990). While very small amounts of IL-1β can mediate inflammation, the natural IL-1 receptor antagonist is often sufficient to limit inflammation. However, an imbalance of these proteins can dictate disease severity. For instance, in patients with Lyme arthritis, individuals with higher IL-1β and lower IL-1 antagonist levels had longer recovery times than those with the reverse pattern (Miller *et al.*, 1993). A recombinant human form of the IL-1 receptor antagonist, called anakinra, is currently FDA approved for rheumatoid arthritis treatment and is used in parts of this thesis (Bresnihan *et al.*, 1998; Cohen *et al.*, 2002).

FIGURE 1.6. Viruses detected during respiratory exacerbations.

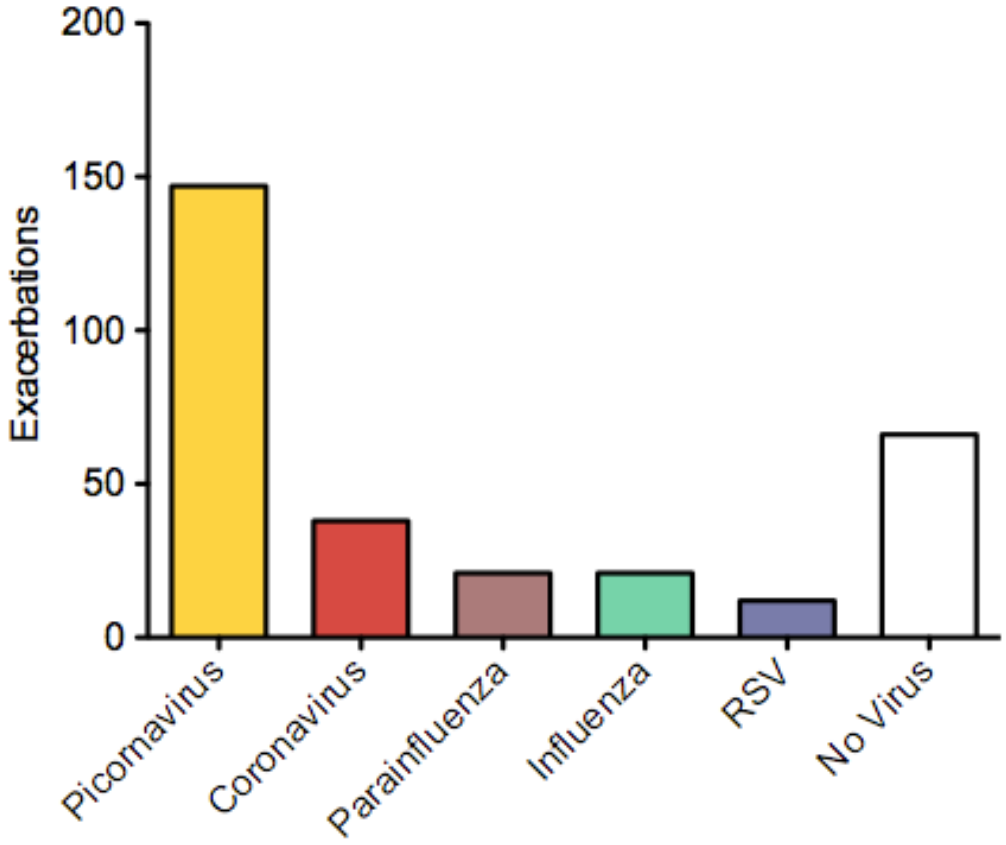

Figure 1.6. Viruses detected in the sputum of patients during asthma exacerbations. Viruses were detected in approximatly 80% of respiratory episodes by PCR, culture, immunofluorescence, or antibody rise by ELISA. ELISA, enzyme-linked immunosorbent assay; RSV, Respiratory syncytial virus. Figure findings from (Johnston *et al.*, 1995).

FIGURE 1.7. Parainfluenza virus structure.

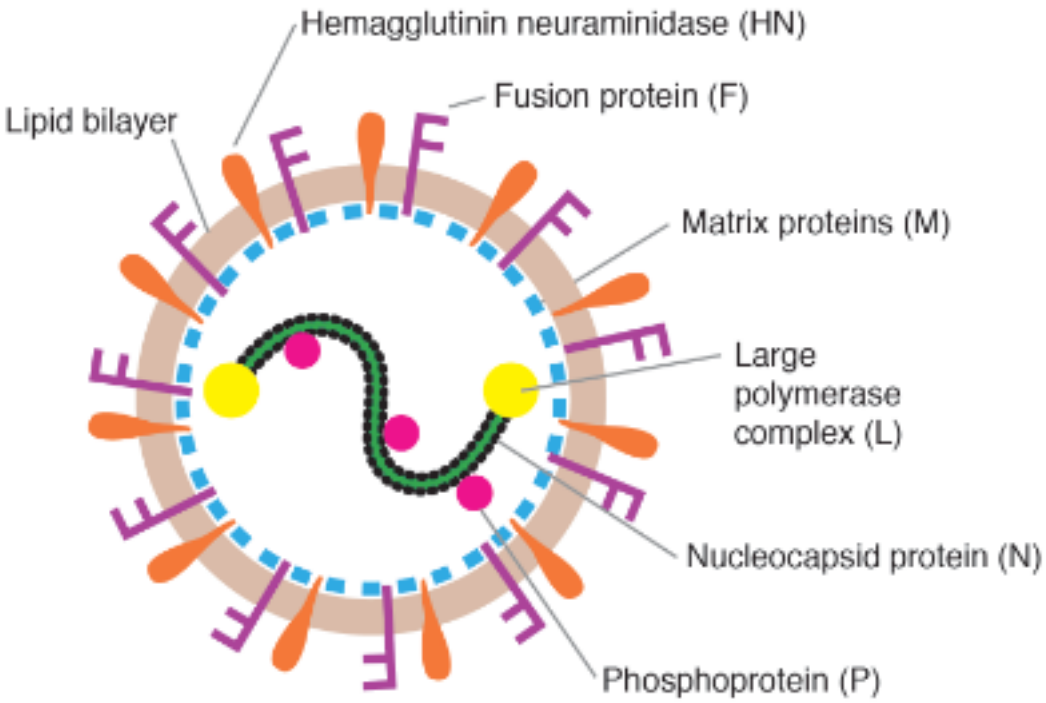

Figure 1.7. The structure of a parainfluenza virion. The components of the virion include nucleocapsid protein (N), fusion protein (F), hemagglutinin-neuraminidase (HN), matrix protein (M), Large polymerase protein (L), and phosphoprotein (P).

FIGURE 1.8. Influenza virus structure.

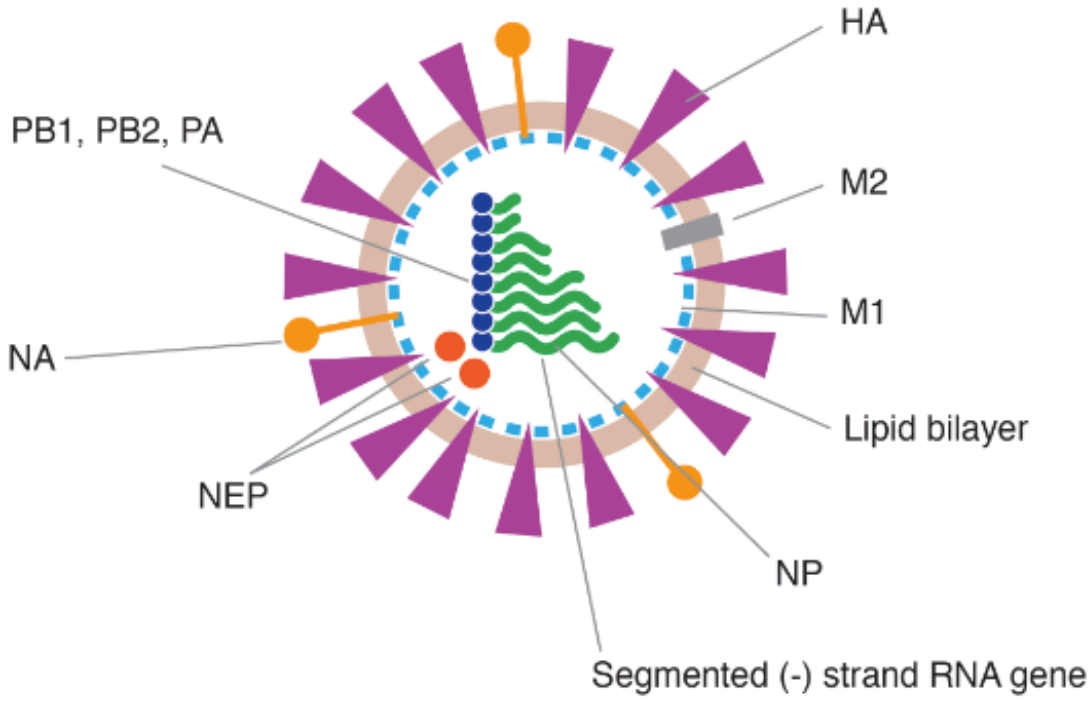

Figure 1.8. The structure of a influenza virion. The components of the influenza virion include 8 segments of genomic RNA, the RNA polymerase associated proteins (PB1, PB2, PA), nucleocapsid protein (NP), nuclear export protein (NEP), hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), and ion channel (M2).

FIGURE 1.9. TNF-α receptor signaling leads to transcription of inflammatory genes.

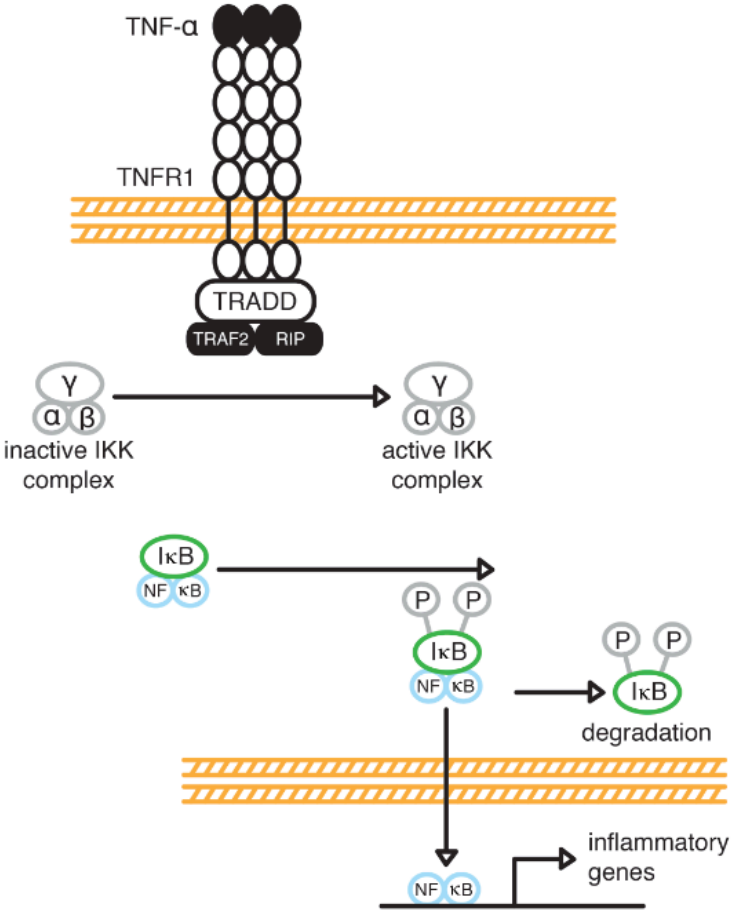

Figure 1.9. TNF-α binding to the TNFR1 receptor recruits the adaptor proteins TNFR1-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein (RIP) to the cytoplasmic domain of the receptor. The IKK kinase complex is then recruited and activated, which phosphorylates IκB, releasing the NF-κB protein from IκB. IκB is degraded and NF-κB translocates into the nuclease to transcribe inflammatory genes. Figure adapted from (Buels, 2011).

FIGURE 1.10. IL-1β receptor types.

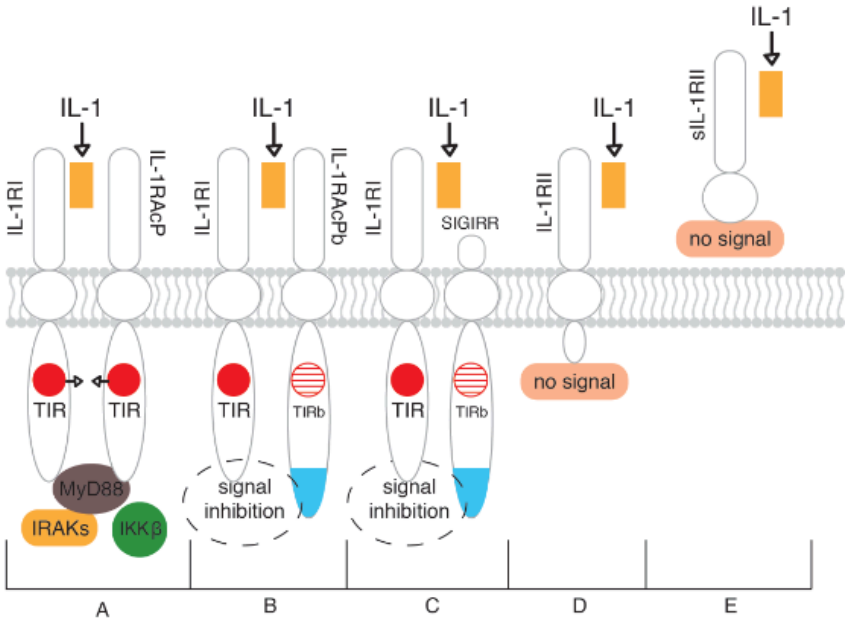

Figure 1.10. IL-1β can bind multiple receptor types. (A) Only IL-1β binding to the IL-1 receptor type I (IL-1RI) and the co-receptor IL-1 receptor accessory protein (IL-1RAcP) will produce downstream signaling. This is due to the functional Toll/interleukin-1 receptor (TIR) domains of the receptor and coreceptor that approximate to facilitate MyD88 binding and kinase activation. IL-1β can bind (B) IL-1 receptor accessory protein (IL-1RAcPb) or (C) single Ig IL-1–related receptor (SIGGRR), but do not produce signals as they do not have functional TIR domains. (D) IL-1β can bind to the decoy IL-1 receptor type II (IL-1RII); however, this does not produce a signal since it has a small cytoplasmic tail and no TIR domain (E) IL-1β can bind soluble IL-1 receptor type II (sIL-1RII) which is a cleaved from of IL-1RII that is not membrane-associated and therefore does not transmit a signal. Figure adapted from (Dinarello, 2011).

FIGURE 1.11. IL-1β activation and receptor types.

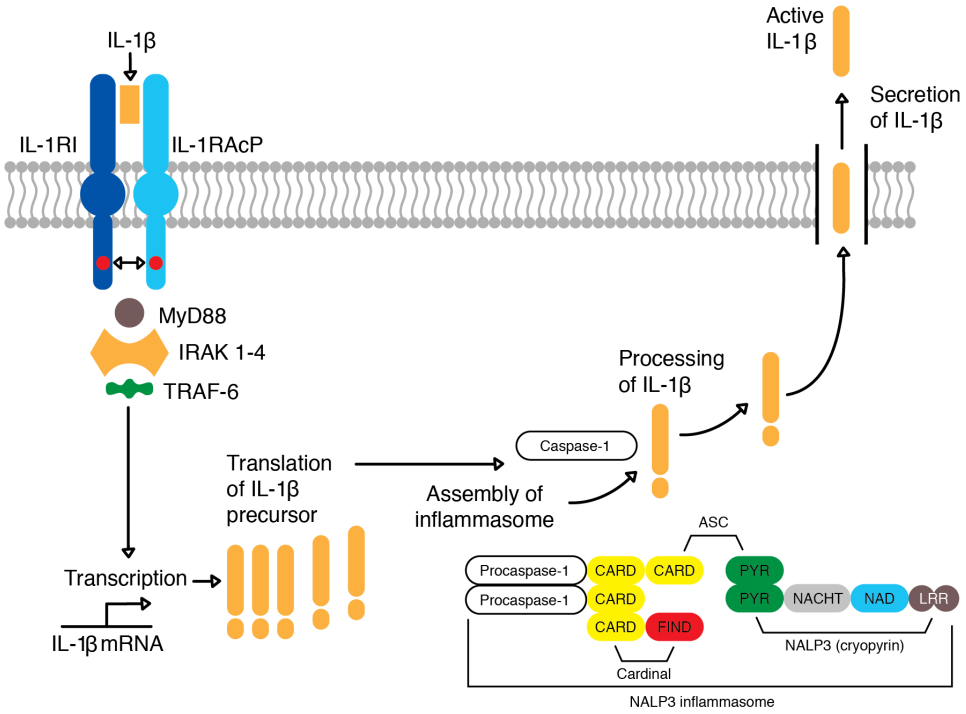

Figure 1.11. (A) IL-1β binds to the IL-1 receptor type I (IL-1RI) and the co-receptor IL-1 receptor accessory protein (IL-1RAcP). The IL-1 receptor heterodimer signaling results in and the recruitment of MyD88, IRAKs, and TRAF-6. The recruited kinases, such as IKKβ and MAPK, leads to NF-κB activation and transcription of pro-IL-1β mRNA. Pro-IL-1β is translated and cleaved by caspase-1 into an active, secreted protein. Activation of caspase-1 is mediated by the NALP3 inflammasome complex, which cleaves inactive procaspase-1 into active caspase-1. Figure adapted from (Dinarello, 2009).

F. HYPOTHESIS AND RESEARCH AIMS

Since respiratory viral infection causes the majority of asthma attacks, it is imperative to understand the molecular mechanism that lead to obstructed airflow, inflammation, and hyperresponsiveness during infection. Virus infection alters control of the airways, but the mechanisms involved in this response are not fully understood.

Certain viruses, such as human metapneumovirus and respiratory syncytial virus, have been shown to directly infect airway nerves *in vivo* or *in vitro*. However, it isn't known if influenza or parainfluenza directly infect airway neurons. Alternatively, there are data indicating a role for inflammatory mediators in virus-induced asthma attacks. The innate immune response is quickly activated during infections, which leads to a release of cytokines and other cell mediators that can alter neuron control of the airways. Specifically, TNF-α and IL-1β are proinflammatory cytokines released during infections that have pathological effects in asthma. Both cytokines mediate hyperresponsiveness *in vitro* and M_2 receptor dysfunction on parasympathetic neurons *in vivo* with either allergen or ozone-challenge. In addition, TNF-α alone or TNF-α combined with IL-1β decreases M_2 receptor expression *in vitro*. This suggests TNF-α and IL-1β may mediate M_2 receptor dysfunction during respiratory virus infection as well. *The overall hypothesis tested is that parainfluenza virus infection does not infect nerves directly, rather that virus infection alters parasympathetic nerve control of the airways indirectly through TNF-*

α and IL-1β production, which mediate neuronal M_2 receptor dysfunction and subsequent airway hyperreactivity.

These studies will determine if parainfluenza or influenza directly infects airway neurons or cell bodies (Figure 1.12; Chapter III). Through the use of a new dissection technique, these studies will test if virus infection decreases M_2 receptor expression *in vivo* and if it is mediated by TNF-α or IL-1β (Chapter IV) and determine whether IL-1β mediates M_2 receptor dysfunction *in vivo* (Chapter V). Finally, they will determine the direct effects of IL-1β *in vitro* and which cytokine is the major factor involved in increased contractile responses *in vitro* (Chapter VI).

FIGURE 1.12. Model of hypothesis tested in this thesis.

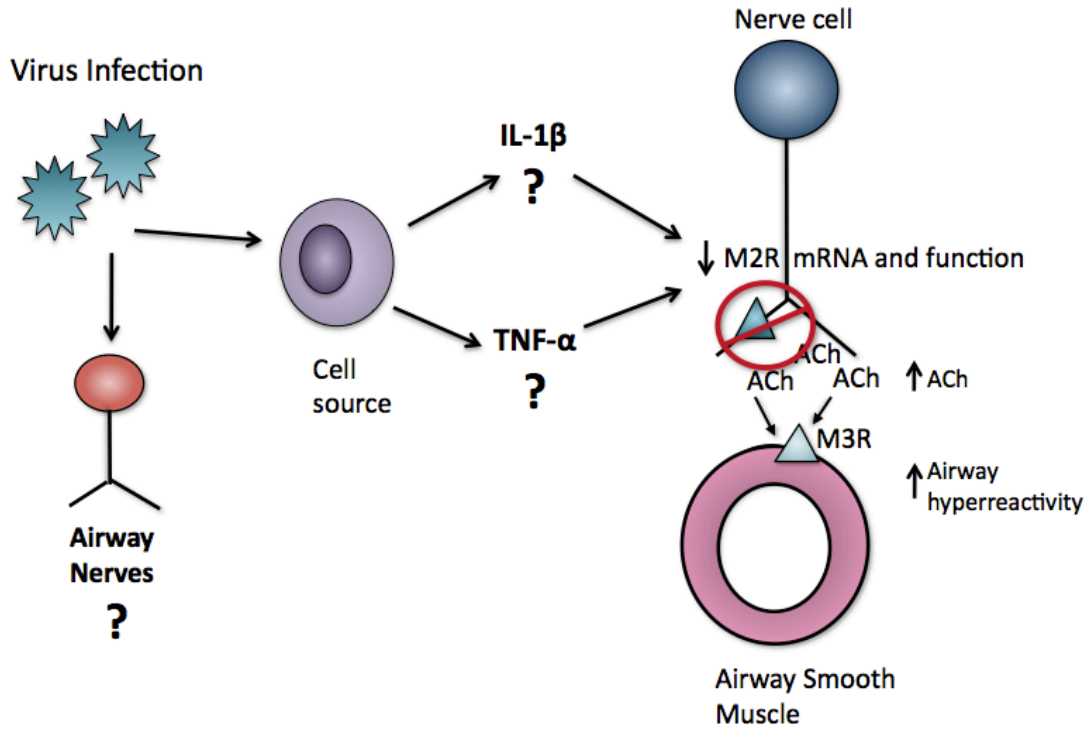

Figure 1.12. Virus infection causes M_2 receptor dysfunction, which increases ACh release from parasympathetic nerves onto M_3 receptors on airway smooth muscle causing airway hyperreactivity. These studies will test if viruses directly infect airway nerves to alter neural control or if the inflammatory cytokines TNF-α and IL-1β mediate M_2 receptor dysfunction. Virus exposure and TNF-α decreases neuronal M_2 receptor expression *in vitro*, but this thesis will test if they also decrease M_2 receptor expression *in vivo*. Finally, this thesis will test if IL-1β decreases M_2 receptor expression and function *in vivo* and *in vitro* and examine possible interactions between IL-1β and TNF-α. The figure illustrates a possible order of events; however, this may not be the exact pathway and does not include all cell sources or mediators in the pathway.

CHAPTER II.

GENERAL METHODS

CHAPTER II: GENERAL METHODS

A. CHOICE OF ANIMAL MODELS AND TISSUE

Mice, guinea pig, and human tissue were used in this thesis. Mice were used to initially study viral infection in airway nerves because of their ease of breeding (10 litters/year and >7 pups/litter) and widespread availability. Both C57BL/6 and BALB/c strains of mice were used since different strains of mice have been shown to have varied levels of susceptibility to parainfluenza virus infection (Faisca *et al.*, 2005; Brownstein *et al.*, 1981). While studies in mice have proved invaluable to the study of allergic inflammation and cell recruitment, this animal model lacks several features of inflammation and airway innervation, making it a less useful model for understanding airway function (Wright and Churg, 2002; Kumar and Foster, 2002; Canning, 2002; Persson *et al.*, 1997). For instance, mice have less inflammation of the airway wall and lack subepithelial fibrosis and epithelial cell proliferation changes seen in airway remodeling (Kumar and Foster, 2002; Holgate *et al.*, 2000). Furthermore, mice do not exhibit the protective cough reflex and lack relaxant innervation in their airway smooth muscle (Canning, 2003; Canning and Fischer, 2001). Mice airway smooth muscle is unresponsive to many of the bronchoconstrictors involved in asthma pathogenesis, including leukotrienes, histamine, neurokinin, bradykinin, and prostanoids (Canning, 2002). Also problematic is that the mediator of bronchospasm in mice is serotonin; whereas human airways are unresponsive to serotonin (Canning, 2003; Bjorck and Dahlen, 1993; Martin *et al.*, 1988). The anatomy of mice is also different from humans in that

they lack subepithelial vasculature (Choi *et al.*, 2000). Also, they rely more on goblet cells for mucus production rather than mucus glands (Choi *et al.*, 2000). For these reasons, mice were not used for the *in vivo* studies as guinea pigs are a better model system for airway physiology.

Guinea pigs have commonly been used to study asthma and were chosen as the animal model for *in vivo* studies of M_2 receptor function after respiratory viral infection and *in vitro* contractile response studies in this thesis. Guinea pigs have also been used to study virus-induced asthma exacerbations and are susceptible to respiratory infections, including respiratory syncytial virus, influenza, parainfluenza, and adenovirus, all of which have been associated with exacerbations (Canning and Chou, 2008). Guinea pigs were also chosen because their airway physiology and receptor pharmacology matches that of humans (Canning, 2003). β_2 agonists, such as epinephrine, induce smooth muscle relaxation in both guinea pigs and humans (Tanaka *et al.*, 2005). Furthermore, smooth muscle contraction is mediated by leukotrienes, methacholine, and histamine in both species (Muccitelli *et al.*, 1987).

The anatomy of the guinea pig also resembles that of a human in that they both have submucosal glands throughout their trachea (Choi *et al.*, 2000; Widdicombe *et al.*, 2001) and pseudo-stratified tracheal epithelium (Dalen, 1983). Guinea pigs also have a significant amount of smooth muscle like humans, as opposed to mice, which

have very little airway smooth muscle (Wenzel and Holgate, 2006; Canning and Chou, 2008). Autonomic nerve innervation of smooth muscle is also similar between humans and guinea pigs, and cholinergic nerves releasing ACh onto postjunctional M_3 receptors controls smooth muscle contraction (Roffel *et al.*, 1990a).

Even though guinea pigs have aspects of pharmacology, physiology, and disease that mimic humans, there are still some drawbacks. For example, there are no transgenic guinea pigs to study the effects of knock-out genes or altered genetics like there are with mice. There are also fewer reagents available for the guinea pig and less known about the genome compared to mice, making it difficult to study novel genes or monitor expression of known genes. In addition, there are some differences between guinea pigs and humans that can limit the ability to compare findings between the species. For instance, the sensory axon reflex that releases local tachykinins (substance P and neurokinin A) is a prominent feature that governs the airways of guinea pigs (Canning and Chou, 2008). However, there is no direct evidence of this axon reflex in human lungs (Verleden, 1996). Even though there are some disadvantages to the guinea pig, there are far more advantages, and thus it was chosen as the major animal model to study airway physiology and receptor function.

When available, donated human tracheas were used to verify findings from guinea pigs to provide evidence that they relate to human disease. The use of human tissue

was important to compare findings in a way most relevant to human disease. Although experimentally administered rhinovirus in asthmatics patients is an approved model for virus-induced asthma exacerbations (Bardin *et al*., 1996; Bardin *et al*., 2000), the cost, access, and availability limited the use of humans for *in vivo* experiments in this thesis. For these reasons, a guinea pig model was used to determine *in vivo* effects of blocking cytokines and M_2 receptor function during virus infection.

1. Mice

Specific pathogen-free female C57BL/6 mice and BALB/c mice (approximately 6 week-old; 25 g) shipped in filtered crates from The Jackson Laboratory (Sacramento, CA), housed in rooms with particulate-filtered air, and fed a normal diet. Animals were handled in accordance with the National Institute of Health guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University.

2. Guinea Pigs

Specific pathogen-free female Dunkin-Hartley guinea pigs (300-350 g and 150-200 g) were shipped in filtered crates from Charles River Laboratories (Kingston, NY), housed in rooms with particulate-filtered air, and fed a normal diet. Animals were

handled in accordance with the National Institute of Health guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University.

3. Human Tracheas

Donated, anonymous human tracheas were provided by the Pacific Northwest Transplant Bank (Portland, OR). Informed consent was obtained from families.

B. VIRUS INFECTION

Both influenza and parainfluenza were used in these studies since combined they represent the second largest group of respiratory viruses detected during asthma exacerbations (Johnston *et al.*, 1995).

1. Growing virus stocks

Influenza A virus (H1N1; A/PR/8/34 strain; VR-1469 from ATCC) and parainfluenza virus (Sendai; Sendai/52 strain; VR-105 from ATCC) were grown in a 175 cm^2 flask of confluent rhesus monkey kidney cells (RMK; ViroMed, Minnetonka, MN). Cultures were incubated in filter sterilized LHC-8 media with 100 units/ml penicillin (Gibco),

100 µg/ml streptomycin (Gibco), and 0.25µm/ml Fungizone (amphotericin B; Gibco, Carlsbad, CA) in a cell culture incubator with 5% CO^2 at 34°C for 7 days. Infectious media was harvested, frozen at -80°C, and thawed. Media was cleared by low-speed centrifugation using an accuSpin 3R centrifuge (Fisher) at 1,500 x g at 4°C for 10 min. Cell-free infectious media was stored at -80°C.

2. Hemadsorption assay and titering

Viral titers were assessed as previously described (Fryer and Jacoby, 1991). Virus stock was diluted 1:10 (10^{-3} – 10^{-9}) in filter-sterilized LHC-8 media with penicillin/streptomycin/fungizone. Confluent RMK cells in 24-well plates were infected with 1ml of diluted virus at 34°C for 7 days. Each dilution was done in triplicate.

Viral stock titers were quantified using tissue culture infective dose ($TCID_{50}$), which is the amount of virus needed to infect 50% of inoculated cell cultures. A hemadsorption assay was used to determine $TCID_{50}$/ml, which takes advantage of the hemagglutinin found in the envelope of both influenza and parainfluenza. Hemagglutinin causes the clumping of red blood cells (agglutination) to the cell surface of virus-infected cells. For the hemadsorption assay, guinea blood (approximately 2 mls) was collected and spun at 1500 x g for 10 min. Red blood cells were rinsed with HBSS with Ca^{2+} and Mg^{2+} (Gibco) and spun at 1500 x g for 10

min for a total of 4 washes. Media was aspirated off and a 0.5% solution of red blood cells was made in HBSS with Ca^{2+} and Mg^{2+}. LHC-8 media was removed from the infected RMK plates and 0.5 mls of 0.5% red blood cell solution was added to each well for at 4°C for 1 hour. Plates were washed 3 times with HBSS with Ca^{2+} and Mg^{2+} and infected wells were visualized using a Nikon Eclipse T5100 inverted microscope. Cells were rated as positively infected based on the adhesion of the red blood cells to the RMK cells (Figure 2.1). The $TCID_{50}$/ml was calculated using the Reed & Muench formula (Reed and Muench, 1938).

3. *In Vivo* infection

a. Guinea pigs

Guinea pigs were anesthetized by intramuscular (i.m.) injection with ketamine (100 mg/kg) and xylazine (5 mg/kg) cocktail. These anesthetics were chosen as they induce surgical anesthesia that the animal can recover from quickly (Dang *et al.*, 2008). Guinea pigs were infected with 250 µl of 1 x 10^6 $TCID_{50}$ parainfluenza or PBS (i.n.). Guinea pigs were monitored until they recovered from anesthesia. Four days later, guinea pigs were used for parasympathetic ganglia removal or *in vivo* experiments. Guinea pigs were euthanized by aortic exsanguination (*in vivo* experiments) or with sodium pentobarbital (150 mg/kg, i.p.) in PBS (parasympathetic ganglia removal). At the end of the experiment, blood, bronchoalveolar lavage (BAL), lungs, and parasympathetic ganglia were removed

(see below). Virus titer from lung tissue was also measured by qRT-PCR to confirm infection. The chosen viral dose was a sublethal doses that produced reliable infection.

b. Mice

Mice were anesthetized by intraperitoneal (i.p.) injection with ketamine (100 mg/kg) and xylazine (5 mg/kg) cocktail. Mice were intranasally (i.n.) infected with 25 µl of 2.8 x 10^4 $TCID_{50}$ parainfluenza, 1x10^3 $TCID_{50}$ influenza, or PBS. Mice were monitored until they recovered from anesthesia. Four days later, mice were euthanized with pentobarbital (120 mg/kg, i.p.). Lungs and nodose and jugular ganglia were removed to assess viral infection of nerves. Virus titer from lung tissue was measured by qRT-PCR to confirm infection (see sections below). The chosen virus doses were sublethal doses that produced reliable infections.

FIGURE 2.1 Infection of rhesus monkey kidney (RMK) cells using hemadsorption assay.

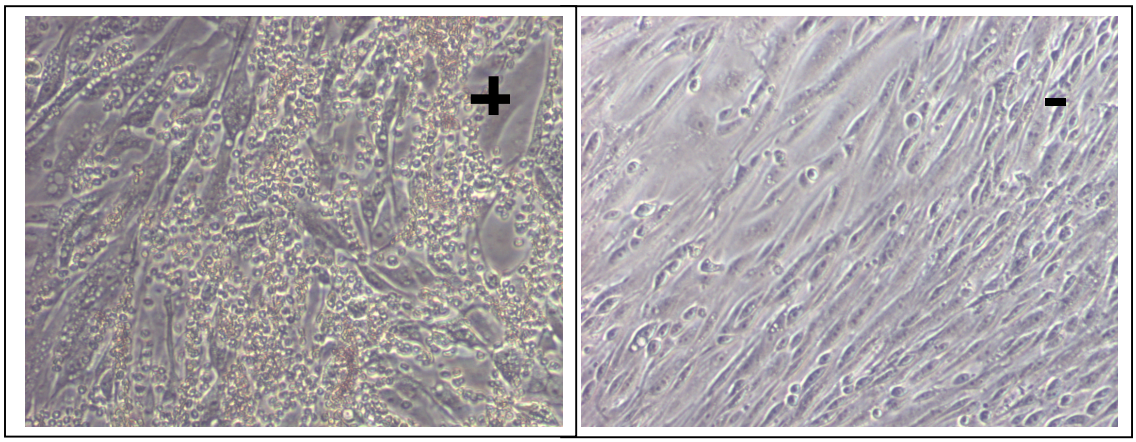

Figure 2.1. RMK cells grown in culture were infected with dilutions of virus to determine viral titers. Cells were rated as positively infected if red blood cells adhered to RMK cells (left panel) and rated as negative if no red cells adhered to RMK cells after washes (right panel). The $TCID_{50}$/ml was calculated using the Reed & Muench formula.

C. *IN VIVO* EXPERIMENTS

1. Pretreatment with etanercept

Guinea pigs were treated with the TNF-α blocker etanercept (3 mg/kg, i.p.) or PBS before infection. Etanercept was administered once 24 hrs before infection (Figure 2.2). Four days after infection, parasympathetic ganglia and lung tissue were harvested after euthanizing the animal.

2. Pretreatment with anakinra

Animals were treated with the IL-1 receptor antagonist anakinra (30 mg/kg, i.p.) or PBS before infection. Anakinra was administered 30 min before infection and once daily until 4 days after infection (Figure 2.3). Four days after infection, *in vivo* airway physiology experiments were conducted, and animals were euthanized through aortic exsanguinations. Brochoalveolar lavage (BAL), blood, parasympathetic ganglia, and lung tissue were harvested after death of the animal.

3. Anesthesia and surgery

Guinea pigs (300-400 g) were anesthetized with urethane (1.9 g/kg, i.p.), which is non-depolarizing central nervous system depressant that causes minimal effects on the heart and lungs (Green, 1982). Urethane causes deep anesthesia for 8-10 hours

(Green, 1982), which was determined by lack of blink and kick response. Typical experiments lasted between 3-4 hours and body temperature was maintained with a 37°C heating pad.

Guinea pigs were paralyzed with succinylcholine (5 μg/min) using a pump 11 plus (Harvard Apparatus, South Natik, MA) to prevent spontaneous breathing (Green, 1982). Due to the use of paralyzing agents, levels of anesthesia were monitored through heart rate and blood pressure. A carotid artery was cannulated to monitor heart rate and blood pressure using a fluid-filled transducer, measured in beats per min (bpm) and mmHg respectively. Heart rate is derived from the blood pressure measurements using a tachograph on the polygraph. Each jugular vein was also cannulated for drug administration (Figure 2.4).

4. Measurement of bronchoconstrictions, heart rate, and blood pressure

Animals were tracheostomized and mechanically ventilated with a positive pressure constant tidal volume with an animal ventilator (Model 683; Harvard Apparatus). A volume of 2.5 ml/100 g and 100 breaths/min was used as these match the average respiratory volumes for guinea pigs (Amdur and Mead, 1958; Guyton, 1947). The Pulmonary inflation pressure (Ppi, mmH_20) pressure required to inflate the lungs

was measured using a pressure transducer (BD DTXplus; Beckton Dickinson, Franklin Lakes, NJ) off the sidearm of the tracheal cannula. Increases in Ppi reflect changes in airflow resistance (Blaber *et al.*, 1985), which can be due to airway narrowing or decreased lung compliance (Nadel *et al.*, 1965). However, studies show acute increases in Ppi correlate to changes in resistance and not lung compliance (Fryer, 1986), so a separate method measuring compliance was not needed.

Ppi, blood pressure, and heart rate was recorded on a Grass polygraph (Model 79WU, Grass Instrument Co., Quincy, MA). Signals from the pressure transducer were fed into a preamplifier (Model 7DAG; Grass) of one channel of the polygraph and total Ppi was measured. The total Ppi output from the driver was also fed into the input of a preamplifier and driver of a second channel of the polygraph. This allowed increased signal sensitivity, providing accurate recordings of Ppi down to 2 mm H_20.

5. Vagal nerve stimulation

Once the guinea pigs were anesthetized, paralyzed, and ventilated, both vagus nerves were crushed with suture thread to avoid vagally mediated reflex constriction (Fryer and Jacoby, 1998; Wagner and Jacoby, 1999). The distal ends of the nerves were hooked to platinum electrodes in mineral oil for vagal nerve

stimulation and both vagus nerves were stimulated (15 Hz, 1-15 V, 0.2 msec duration, 5 sec on, 60 sec off intervals) using an SD9 stimulator (Grass). These parameters were chosen because they represent the normal firing rate of guinea pig vagus nerves (12-15 Hz) and to reduce antidromic impulses from vagal sensory nerves (Mitchell *et al.*, 1987; Undem *et al.*, 1990). The nerve stimulus frequency (15 Hz) was also used since neuronal M_2 muscarinic receptors function best at higher frequencies and the effects of an antagonist are more evident (Blaber *et al.*, 1985). Animals were also chemically sympathectomized with guanethidine (2mg/kg, i.v.) to deplete noradrenaline storages, since guinea pig smooth muscle receives some sympathetic input (Blaber *et al.*, 1985).

Bronchoconstrictions were quantified as the peak increase in pulmonary inflation pressure (mm H_20) above baseline pressure (Dixon, 1903). Bradycardia (fall in heart rate) was measured as a decrease in bpm.

6. Measurement of M_2 receptor function

When functioning properly, inhibitory M_2 receptors on postganglionic nerves limit ACh release from nerve endings (Fryer and Maclagan, 1984). However, virus infection induces M_2 receptor dysfunction, resulting in increased ACh leading to bronchoconstriction (Fryer and Jacoby, 1991). An M_2 receptor antagonist can be

used to test M_2 receptor function by blocking endogenous ACh from activating muscarinic receptors. M_2 receptor function was thereby measured as the ability of gallamine (0.1-10 mg/kg, i.v.), an M_2 receptor antagonist, to potentiate bronchoconstrictions in response to vagal nerve stimulation (Fryer and Maclagan, 1984). If the receptor is not functioning properly, gallamine will be impaired in its ability to potentiate vagally induced bronchoconstrictions.

The vagus nerve was stimulated (15 Hz, 0.2 msec duration, 5 sec on, 60 sec off intervals) and the voltage was adjusted between 1-15 V until it produce repeatable bronchoconstrictions between 5 -20 mm H_20. Once steady bronchoconstrictions were achieved, the voltage remained untouched for the remainder of the experiment. The average of 4 bronchoconstrictions were quantified following each dose of gallamine and divided by the average of 4 bronchoconstrictions before gallamine was given (baseline contractions). An example trace is shown in Figure 2.5.

7. Measurement of postjunctional muscarinic function

Acetylcholine (1-10 μg/kg, i.v.) was given to determine postjunctional M_3 receptor (airway smooth muscle) and M_2 receptor (cardiac muscle) function. Postjunctional M_3 receptor function was measured as an increase in bronchoconstrictions (mm

H_20) and postjunctional M_2 receptor function was measured as a fall in heart rate (bpm). At the end of each experiment, a muscarinic receptor antagonist, atropine (1 mg/kg i.v.), was given to ensure bronchoconstrictions were mediated by cholinergic nerves.

8. Bronchoalveolar Lavage

a. Guinea pigs

At the end of each experiment, guinea pigs were euthanized by aortic exsanguination via the carotid cannula. Lungs were lavaged with 5 x 10 mls of PBS (room temperature) via the tracheal cannula. Cells were spun in an eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germnay) at 300 x g for 10 min and resuspended in 20 mls PBS. Total cell counts were obtained using a hemocytometer. Differential cell counts were obtained by staining cytospun cells with Hemacolor (EMD Chemicals, Philadelphia, PA). Slides were sealed with Cytoseal 60 (Richard-Allan Scientific, Waltham, MA) and a 22 x 60 mm/ #1.5 coverslip (Fisher) weighed down with a 100 g overnight.

b. Mice

Four days after infection, mice were euthanized with an overdose of pentobarbital (120 mg/kg, i.p.). Lungs were lavaged with 3 x 0.5 mls of PBS (room temperature)

via the tracheal cannula. Cells were spun in an eppindorf 5415 tabletop centrifuge (Eppendorf) 500 x rpm for 10 min. Cells were counted and stained the same as guinea pigs.

9. Blood

At the end of the experiment, 2 mls blood was obtained from the carotid artery cannula. Blood smears on slides were obtained from the whole peripheral blood and stained with Hemacolor (EMD Chemicals) to quantify differential cell populations. Slides were sealed with Cytoseal 60 (Richard-Allan Scientific, Waltham, MA) and a 22 x 60 mm/ #1.5 coverslip weighed down by a 100 g overnight. Red blood cells were lysed through the addition of 9.5 mls 0.1N HCL to a 0.5mls blood aliquot. Blood leukocytes were then counted using a hemocytometer.

FIGURE 2.2. Infection and etanercept treatment schedule in guinea pigs.

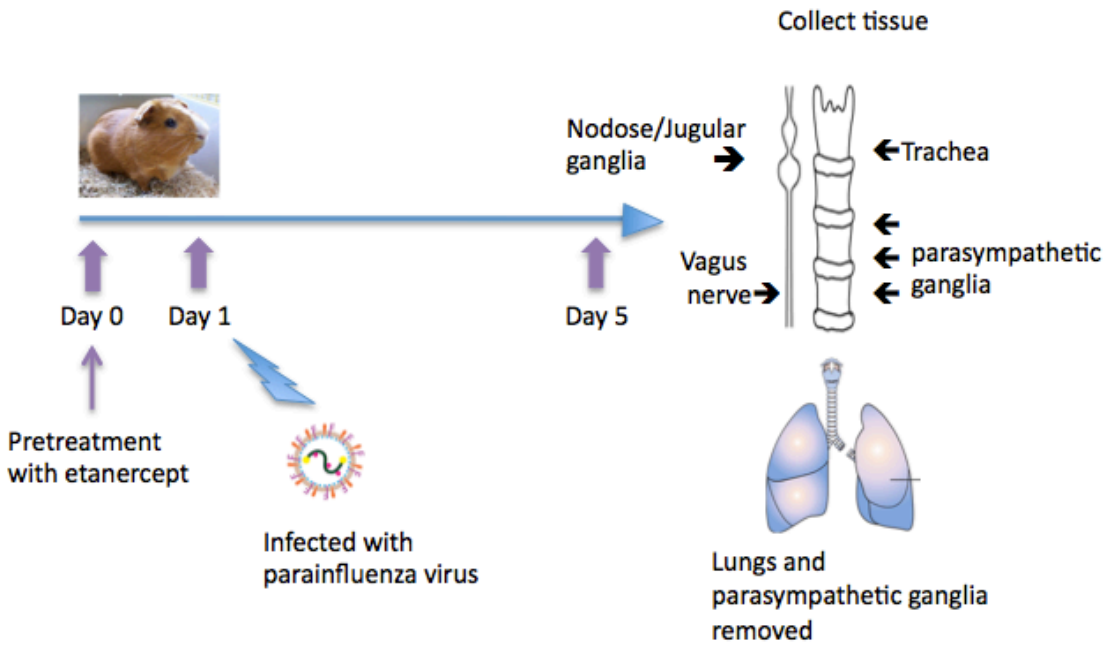

Figure 2.2. Guinea pigs were treated with etanercept (3 mg/kg, i.p.) or PBS once 24 hrs before infection (Day 0). Guinea pigs were intranasally infected with parainfluenza 1 x 10^6 $TCID_{50}$ or PBS (Day 1). Four days after infection (Day 5), parasympathetic ganglia and lung tissue were harvested to measure M_2 receptor expression and viral titers.

FIGURE 2.3. Infection and anakinra treatment schedule in guinea pigs.

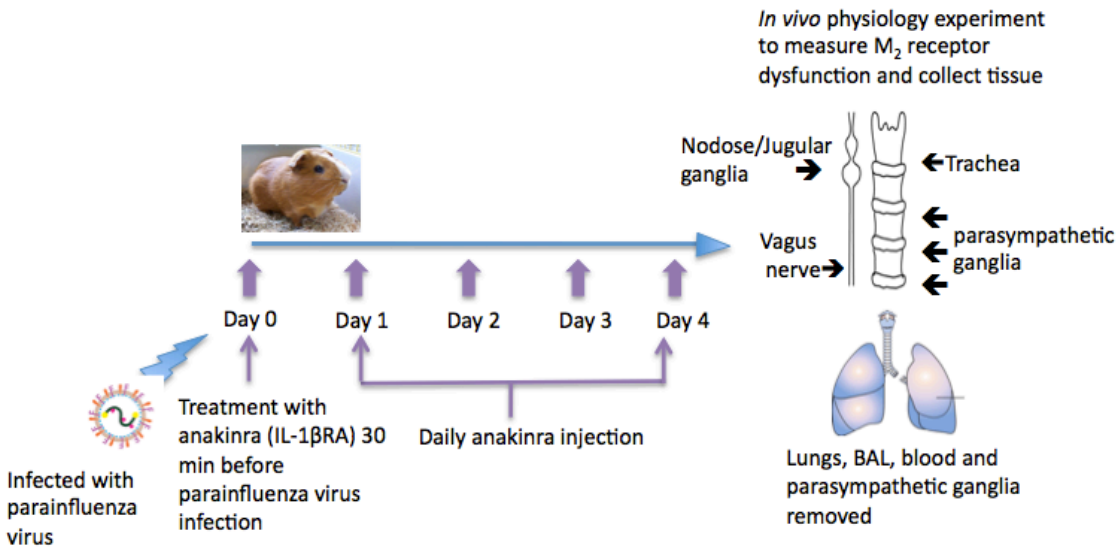

Figure 2.3. Guinea pigs were treated with anakinra (30 mg/kg, i.p.) or PBS once 30 mins before infection (Day 0). Guinea pigs were intranasally infected with parainfluenza 1 x 10^6 $TCID_{50}$ or PBS (Day 0). Four days after infection (Day 4), *in vivo* airway physiology experiments were conducted, and animals were euthanized through aortic exsanguinations. Brochoalveolar lavage (BAL), blood, parasympathetic ganglia, and lung tissue were harvested after death of the animal.

FIGURE 2.4. Surgical preparations for *in vivo* experiments in guinea pigs.

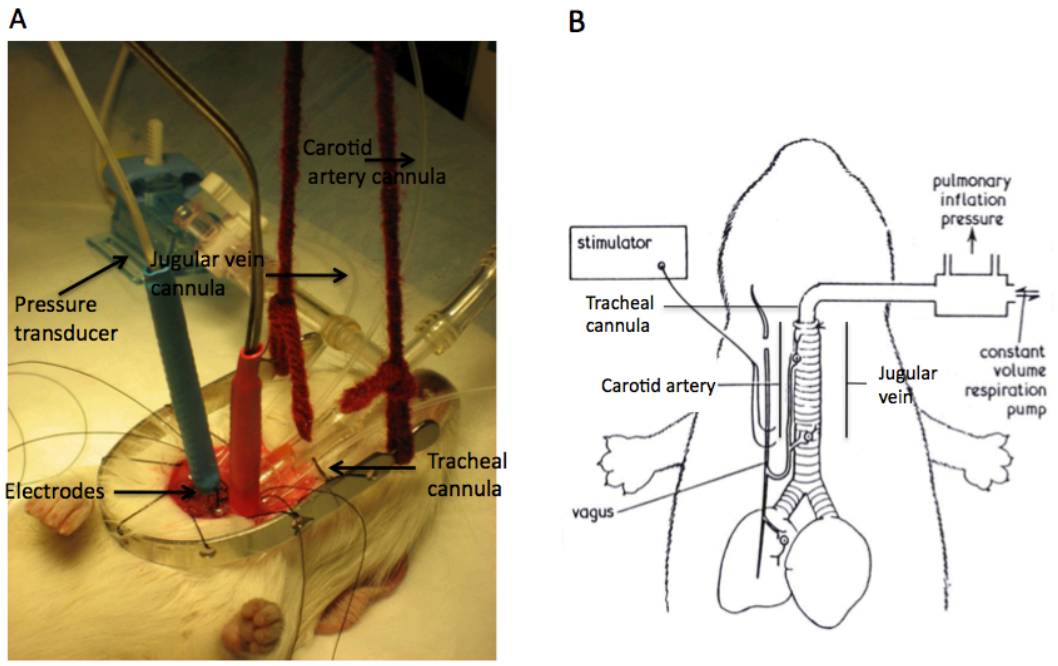

Figure 2.4. Guinea pigs were anaesthetized, paralyzed, and mechanically ventilated. Pulmonary inflation pressure (Ppi) is measured via the sidearm of the tracheal cannula. Vagal nerves are stimulated with electrodes to induce bronchoconstrictions. Drugs are administered through cannulas inserted in jugular veins. Heart rate and blood pressure is measured from a cannula in the carotid artery. (A) Actual set up for *in vivo* measurements. (B) Shows a schematic diagram of *in vivo* preparations. Figure adapted from (Fryer, 1986).

FIGURE 2.5. Trace recordings of pulmonary inflation pressure (Ppi), blood pressure and heart rate.

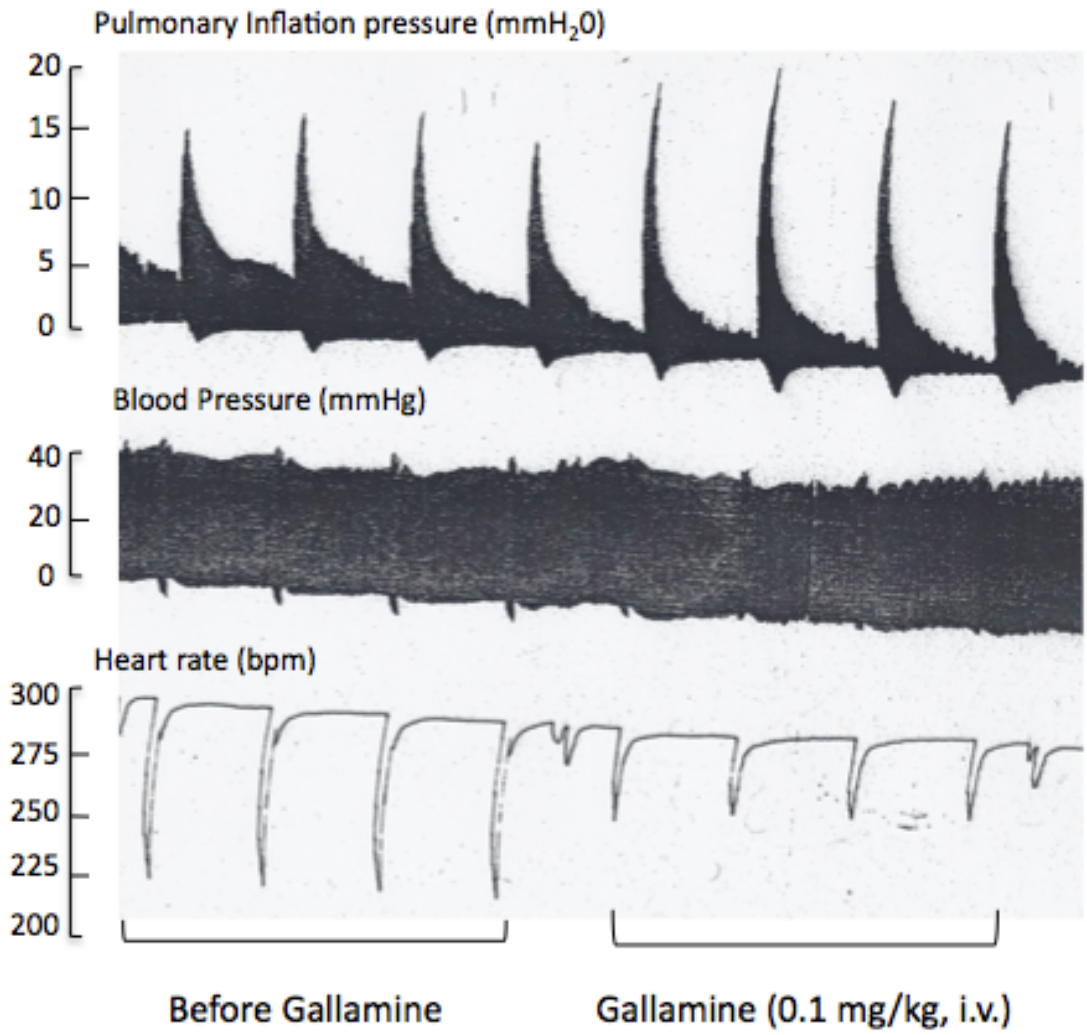

Figure 2.5. An example of pulmonary inflation pressure (Ppi), blood pressure and heart rate in response to vagal nerve stimulation (15 Hz, 1-15 V, 0.2 msec duration, 5 sec on, 60 sec off intervals) before and after administration of gallamine (0.1 – 10 mg/kg, i.v.). Gallamine blocks M_2 muscarinic receptors, which potentiates vagally mediated bronchoconstrictions and prevents a fall in heart rate.

D. TISSUE HARVEST AND GANGLIA DISSECTION

1. Lung Harvest

Guinea pigs were euthanized with sodium pentobarbital (200 mg/kg, i.p.) in PBS and perfused with PBS through the heart. Mice were euthanized with pentobarbital (120 mg/kg, i.p.) in PBS and perfused with PBS through the heart. The right caudal lobe (guinea pig) or the diaphramic lobe (mice) was placed in tinfoil and flash frozen in liquid nitrogen to be used to determine viral titers. The left caudal lobe (guinea pig) or left lobe (mice) was fixed in Zamboni's fixative (American MasterTech Scientific, Lodi, CA) at 4°C overnight. Zamboni's fixative, which contains paraformaldehyde and picric acid, was chosen because it penetrates tissues quickly and optimally preserves nerve morphology and fine structures (Stefanini *et al.*, 1967; Somogyi and Takagi, 1982). Lungs were gently compressed to facilitate diffusion of fixative into alveoli. Tissues were fixed overnight at 4°C and then washed PBS. Lungs were submerged in 1:1 mixture of 18% sucrose (w/v) and Tissue-Tek Optimal Cutting Temperature compound (O.C.T.; Sakura Fintek, The Netherlands) in PBS at 4°C overnight. Transverse sections of lung lobes were cut with a razor blade, embedded in O.C.T in a plastic cryomold (25 x 20 x 5 mm; Tissue-Tek), and stored at -80°C until ready for cryosectioning. Frozen blocks were sectioned into 10 μm sections on ColorFrost Plus slides (Fisher) using a cryostat (Model 0TF5000; Hacker Instruments, Winnsboro, SC). Frozen sections were stored at -80°C until ready for immunostaining.

2. Nodose and jugular ganglia dissection

Mouse nodose and jugular ganglia was removed and stained to determine if parainfluenza or influenza virus infect sensory nerve cell bodies that innervate the lungs. The jugular nerve was exposed by dissecting through the dermis and deeper musculature. The jugular nerve was isolated from the carotid artery and carotid vein and followed up the base of the skull to the jugular foramen where the nodose and jugular could be visualized. The ganglia were harvested together by severing the distal and proximal ends of the nerve and put into Zamboni's Fixative (American MasterTech Scientific, Lodi, CA) at 4°C overnight. The tissue was washed with PBS and submerged in 1:1 mixture of 18% sucrose (w/v) and O.C.T. in PBS at 4°C overnight. The tissue was embedded in O.C.T in a plastic cryomold (15 x 15 x 15 mm) and stored at -80°C until ready for cryosectioning. Frozen blocks were sectioned into 5 μm sections on ColorFrost Plus slides (Fisher) using a cryostat. Frozen sections were stored at -80°C until ready for immunostaining.

3. Parasympathetic ganglia dissection

A new method for guinea parasympathetic ganglia removal was developed to determine the effects of virus infection and blocking IL-1β or TNF-α on M_2 receptor expression *in vivo*. The protocol developed for isolating guinea pig parasympathetic ganglia is discussed in Chapter IV.

E. IMMUNOSTAINING

1. Immunostaining mouse lung tissue

Airway bronchi and bronchioles were imaged in mice from lung sections post-fixed in 4% paraformaldehyde. Sections were blocked and permeabilized with 4% normal goat serum, 5% powdered milk, and 1% Triton X 100 in TBS (pH 7.4). TBS (3 x 5 min) washes were used to minimize non-specific antibody binding. Viral antigen was assessed using chicken anti-Sendai virus antibody (1:250; Abcam, Cambridge, MA) or mouse anti-influenza A virus nucleoprotein (1:500; Abcam). Tissue stained with the influenza A virus nucleoprotein antibody was also blocked with Mouse on Mouse (M.O.M.) blocking reagent (Vector Laboratories, Burlingame, CA) before the regular blocking step to avoid non-specific background from secondary antibody due to endogenous mouse Ig in the tissue. Nerves were assessed using rabbit anti-protein gene product 9.5 (PGP 9.5; 1:1000; Abd Serotec, Raleigh, NC). PGP 9.5 is a ubiquitin hydrolase protein enriched in neurons and a commonly used pan-neuronal nerve marker (Wilkinson *et al.*, 1989; Day and Thompson, 2010). All primary antibodies were incubated overnight at 4°C. Secondary antibodies of goat anti-chicken IgY (H+L) Texas Red (1:250; Abcam), goat anti-mouse IgG (H+L) Alexa 555 (1:1000; Molecular Probes), or goat anti-rabbit IgG (H+L) $F(ab)_2$ Alexa 488 (1:1000; Molecular Probes, Carlsbad, CA) were incubated for 4 hrs at 4°C. No primary antibody and IgG isotypes were used as controls. Sections were mounted with soft set vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and covered with 22 x 60 mm/ #1.5

coverslips. Airway sections were visualized on a Nikon Eclipse E400 microscope and analyzed with MetaMorph imaging software (Version 7.1.2.0; Molecular Devices, Sunnyvale, CA). Data are a mean of 4 animals with photographs of 3 airways per animal.

2. Immunostaining guinea pig lung tissue

The protocol for fixing and staining guinea pig lung tissue was the same for mice, except the M.O.M blocking reagent was not used.

F. NERVE CELL CULTURE EXPERIMENTS

Nerve cell culture experiments were conducted using human SK-N-SH neuroblastoma cells to study the effect of IL-1β and TNF-α on M_2 receptor expression *in vitro*. SK-N-SH were used as they are a neuronal line that maintains their characteristics over many passages, are derived from human neuroblastoma cells, and express the IL-1β and TNF-α receptors (Biedler *et al.*, 1973; Mohan *et al.*, 2010; Nie *et al.*, 2009). However, they are thought to be of sympathetic origin (Biedler *et al.*, 1973; Thiele, 1998), so primary cultures of guinea pig and human parasympathetic neurons were also used to confirm results found in SK-N-SH cells.

1. Human SK-N-SH experiments

Human SK-N-SH neuroblastoma cells from ATCC were thawed and cultured in 75 cm^2 flasks in a cell culture incubator with 5% CO_2 at 37°C. Cells were cultured in minimum essential medium (Cellgro, Manassas, VA) containing 10% FBS (Gibco), 1 mM sodium pyruvate (Gibco), penicillin/streptomycin, and nonessential amino acids (Gibco). When they reached 70-80% confluency, cells were trypsinized with 1 ml trypsin-EDTA for 3 min at 37°C. Cells were reconstituted in 10 mls media and spun at 300 x g for 10 min. Media was aspirated off, and cells were resuspended in 10 mls media and counted on a hemocytometer. Cells were plated in 6 well culture dishes at 1.8 x 10^5 cells/well and grown until 70-80% confluent. Cells were either untreated or treated with 0.01-100 ng/ml human recombinant IL-1β (Sigma) for 6

hrs and RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse transcribed into cDNA and used in qRT-PCR (see below).

2. Guinea pig primary nerve cell culture

Guinea pigs were euthanized with sodium pentobarbital (150 mg/kg, i.p.) and tracheas were removed. Guinea pig parasympathetic nerves were cultured and isolated as previously described (Fryer *et al.*, 1996). Briefly, guinea trachea were rinsed with 5 x penicillin/streptomycin in PBS in a tissue culture hood. The esophagus and connective tissue surrounding the trachealis muscle was removed with sterile scissors. The trachea was opened anteriorly through the cartilage rings and the luminal epithelial layer on the posterior side of the trachea was removed using a sterile cotton swab. The smooth muscle was removed and cut into small pieces and digested in a 25 cm^2 flask in 0.02 μm filtered 0.05% collagenase in HBSS with Ca^{2+} and Mg^{2+} and penicillin/streptomycin for 4 hrs at 37°C. An equal volume of 10% FBS in DMEM and penicillin/streptomycin was added to the cells and spun at 300 x g for 10 min. Media was aspirated off and 10% FBS in 1:1 Ham's F12/DMEM with L-glutamine (Gibco) and penicillin/streptomycin. The cells were plated on a 100 mm culture dish at 37°C overnight. The non-adherent cells were removed and spun at 300 x g for 10 min and resuspended in serum-free media (SFM) containing 1:1 Ham's F12/DMEM with L-glutamine, 0.05% BSA (Fisher), 10 μg/ml insulin (Mediatech; Westwood, MA), 6.7 ng/ml sodium selenite (Mediatech), 20 μg/ml transferrin (Fisher), penicillin/streptomycin, and 100 ng/ml nerve growth

factor (Harlan Bioproducts, Indianapolis, IN). Cells were plated in 6 well plates that were coated with Matrigel (Collaborative Biomedical Products; Bedford, MA) for 1 hr at 37°C. One day later, media was replaced with SFM containing 2 μM cytosine arabinoside (AraC; Sigma) to remove dividing cells and cultured for 3 days. Media was changed every 2-3 days with SFM and experiments were conducted 7 days after plating in 6 well dishes. Cells were then treated for 6 hrs with 0.001-0.1 ng/ml human recombinant IL-1β (Sigma) and 0.01-1 ng/ml recombinant guinea pig TNF-α (R&D) or untreated.

After treatment, parasympathetic neurons were individually isolated in culture by a method developed in our lab (Figure 2.6). Since guinea pig parasympathetic neurons grow in clusters in culture the clusters can be individually removed, which avoids the contamination of non-neuronal cells that still remain after AraC treatment. In this method, a capillary tube attached to a rubber piping and 1 ml syringe is used to suck up the clusters using a in inverted microscope as guidance. The capillary tube is stretched and elongated using a Bunsen burner to create a diameter that is just bigger than the size of a cluster (approximately 200 μm). The end of the tube is used to severe the ends of the neurites of the cluster and to gently dislodge the cluster from the plate. Cell clusters were sucked up using the syringe and put into an microcentrifuge tube. All neuron clusters from one treatment well were pooled together in one tube and stored at -80°C. Once parasympathetic

neurons were removed from culture, RNA was isolated using Power SYBR green Cells-to-C_T kit (Ambion, Carlsbad, CA) described below.

3. Human primary nerve cell culture

Donated human tracheas were cleaned and cultured in the same way as guinea pig tracheas, but due to their size, the luminal epithelial layer was removed using sterile scissors. Media also contained 0.25 μm/ml Fungizone with penicillin/streptomycin and human holo-transferrin (MP Biomedical; Santa Ana, CA) was used in the placement of guinea pig transferring in the SFM media. Human parasympathetic neurons do not grow in clusters in culture, thus isolation contained some contaminating cells.

After cells were seeded in 6 well culture dishes and grown for 7 days, cells were then treated for 6 hrs with 0.001-0.1 ng/ml human recombinant IL-1β (Sigma) and 0.01-1 ng/ml recombinant guinea pig TNF-α (R&D) or untreated. RNA was isolated using the RNeasy mini kit (Qiagen) and reverse transcribed into cDNA to use in qRT-PCR (see below).

FIGURE 2.6. Isolating guinea pig parasympathetic nerve clusters in culture.

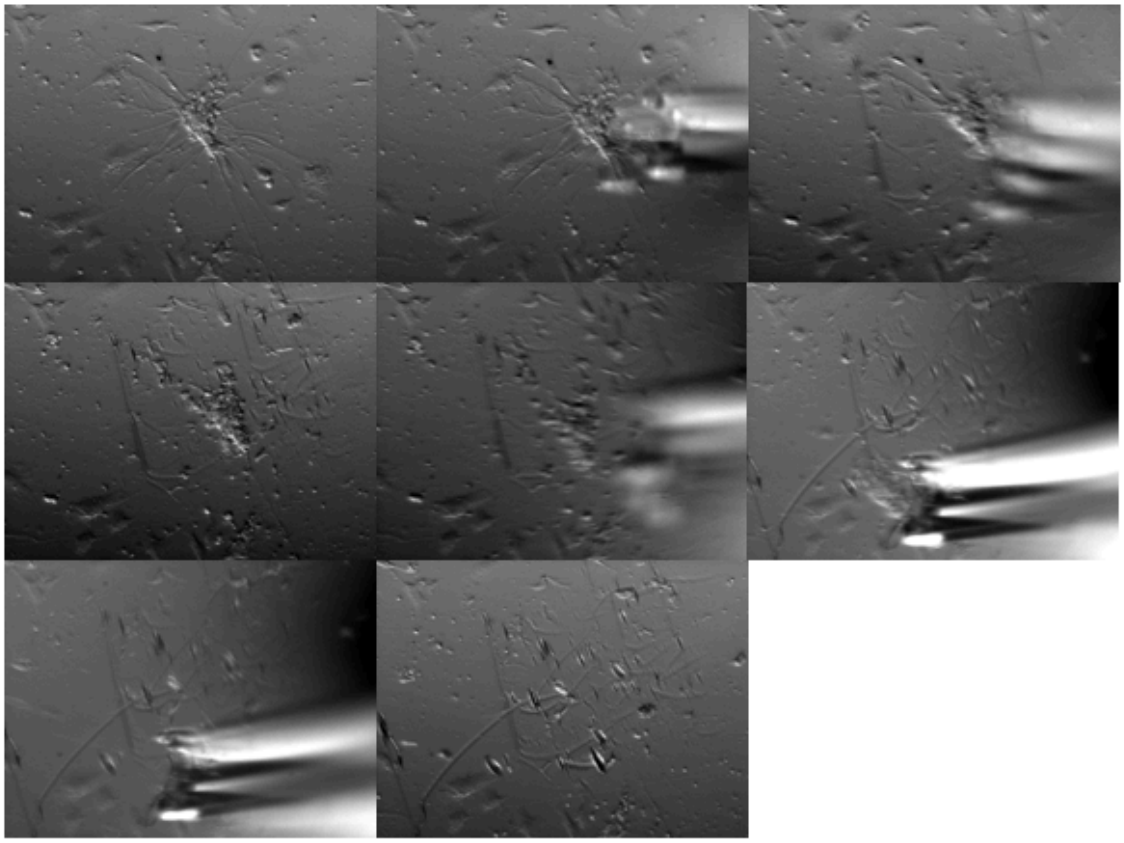

Figure 2.6. Guinea pig primary parasympathetic nerves form clusters when grown in culture. After 7 days, the neurites growing from the cluster severed using an elongated capillary tube. To avoid contaminating cells, only the cluster is sucked up using a syringe and used for RNA isolation.

G. QUANTITATIVE REAL-TIME PCR

1. RNA isolation from lung tissue, SK-N-SH neurons, and human primary airway neurons

RNA was isolated from guinea pig and mice lung homogenates to determine viral titers. RNA was isolated from human SK-N-SH and primary parasympathetic neurons to quantify M_2 receptor expression. Lungs were first weighed (approximately 200 mg) and homogenized in 1 ml RLT buffer/100 g with 10 µl/ml 2-mercaptoethanol (β-ME) using a tissue homogenizer (Polytron Model PT 10-35; Kinematica, Switzerland). Lysates from human neurons and lung homogenates were kept on ice. RNA was extracted from neurons and lung homogenates using RNeasy mini kit (Qiagen) with the additional DNase step. Isolated RNA concentration was determined by measuring the absorbance at 260 nm with a spectrometer (Spectronic BioMate 3; Thermo Electron Corporation, Waltham, MA). RNA (1 µg RNA per 20 µl reaction) was transcribed into cDNA with Superscript III First-Strand Synthesis Kit (Invitrogen) and random hexomer primers using a Veriti 96-well thermal cycler (Applied Biosystems, Carlsbad, CA). See Table 2.1 for cDNA reaction conditions. RNA was stored at -80°C and cDNA was stored at -20°C.

2. RNA isolation from mouse nodose/jugular ganglia, guinea pig parasympathetic ganglia, and guinea pig primary airway neurons

RNA was isolated from mice nodose/jugular ganglia and guinea pig parasympathetic ganglia to determine viral titers and measure M_2 receptor expression. RNA was isolated from guinea pig primary parasympathetic neurons to quantify M_2 receptor expression. Power SYBR green Cells-to-C_T kit (Ambion) was used to isolate RNA, make cDNA, and perform RT-PCR from parasympathetic neurons and ganglia. Briefly, cells or ganglia were spun at 200 x g for 10 min. Excess supernatant was removed, leaving only 5 µl liquid behind in the tube. Cells were lysed with lysis buffer containing DNase I for 7 min at room temperature. During this time, cells were homogenized with an RNase free pestle. The lysis step was terminated with stop solution for 2 min at room temperature and then kept at -80°C. RNA was transcribed into cDNA (10 µl of lysis solution into 50 µl reaction) using a 96-well thermal cycler. See Table 2.2 for PCR conditions.

3. Quantitative real-time PCR

Quantitative real-time PCR was used to measure viral titer as well as changes in gene expression. A standard curve was generated using a non-linear regression to quantify PCR products. For viral products, a parainfluenza and influenza standard curve was used to determine $TCID_{50}$ viral titers using a High-Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) and quantified from rhesus monkey kidney cell titration. Samples were run in duplicate and included a no reverse transcriptase control and a no template control to ensure measurements were not made from genomic contamination. Quantitative RT-PCR was conducted using QuantiTect SYBR

Green PCR Kit (Qiagen) for cDNA made with superscript III or Power SYBR Green for cDNA made with Cells-to-C_T kit. Specific primer sequences were ordered from Invitrogen (Carlsbad, CA) and are listed in Table 2.3. Gene products were analyzed using Applied Biosystems 7500 Fast Real-Time PCR System. See Tables 2.4 – 2.6 for quantitative RT-PCR conditions. Since SYBR green was used, a dissociation curve was added to ensure measurements were not from a non-specific double-stranded PCR products or primers. Influenza, parainfluenza, M_2 receptor, and PGP 9.5 RNA expression were normalized to 18S rRNA. Expression levels were expressed as fold changes relative to controls within each experiment.

TABLE 2.1. Conditions for cDNA reactions using random hexamers.

Temperature	Time	Step
65°C	5 min	denature RNA
4°C	(hold)	add reverse transcriptase
25°C	10 min	primers annealed
50°C	50 min	cDNA synthesis
70°C	15 min	synthesis terminated

TABLE 2.2. Conditions for cDNA reactions using Cells-to C_t kit.

Temperature	Time	Step
37°C	60 min	reverse transcription
45°C	5 min	reverse transcription inactivation
4°C	indefinite	hold

TABLE 2.3. Oligonucleotide primers used in PCR reactions.

Gene	5' Sequence	3' sequence
Virus		
Influenza (H1N1)	CATCCTGTTGTATATGAGGCCCAT	GGACTGCAGCGTAGACGCTT
Parainfluenza (Sendai)	ATGCGGCTGATCTTCTCACT	CTTTGCCACGACATTAGGGT
Human		
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
M_2 receptor	CAAAGGTCACACACCACAGG	TTAAAGTCAACCGCCACC
PGP 9.5	GACTGGAGGACGAGGCTCTG	CACAGGAGTTCCCAATGGTC
Guinea Pig		
M_2 receptor	TTTTCCAATGCTGCTGTCAC	GGCATGTTGTTGTTGTTTGG

TABLE 2.4 Real-Time PCR condition for M_2, PGP 9.5, Sendai, and H1N1 using Quantitect SYBR Green.

Temperature	Time	Step	Repetitions
95°C	15 min	enzyme activation	1
95°C 58°C 72°C	30 sec 1 min 30 sec	denature primers annealed elongation	40
95°C	15 sec	denaturation	1
55- 95°C	1 min each temp	dissociation curve	1
95°C	15 sec	denaturation	1
60°C	15 sec	stabilize products	1

TABLE 2.5. Real-Time PCR condition for 18S using Quantitect SYBR Green.

Temperature	Time	Step	Repetitions
95°C	15 min	enzyme activation	1
95°C 58°C 72°C	20 sec 30 sec 30 sec	denature primers annealed elongation	40
95°C	15 sec	denaturation	1
55- 95°C	1 min each temp	dissociation curve	1
95°C	15 sec	denaturation	1
60°C	15 sec	stabilize products	1

TABLE 2.6. Real-Time PCR condition for M_2, PGP 9.5, Sendai, and H1N1 using Power SYBR Green.

Temperature	Time	Step	Repetitions
95.0 °C	10 min	Enzyme Activation	1
95.0 °C 59 °C	15 sec 1 min	denature primers annealed and elongation	40
95°C	15 sec	denaturation	1
55- 95°C	1 min each temp	dissociation curve	1
95°C	15 sec	denaturation	1
60°C	15 sec	stabilize products	1

H. *IN VITRO* MEASUREMENTS OF CONTRACTION

1. Isolation and culture of guinea pig trachea segments

Segmented guinea pig trachea rings were isolated and cultured as previously described (Makwana *et al.*, 2012). Briefly, guinea pigs were euthanized with sodium pentobarbital (150 mg/kg, i.p.) and tracheas were removed, cut transversely into ring segments with a razor blade. Each segment consisted of 3 cartilage rings, starting 3 segments below the larynx. One guinea pig was able to provide 8-10 segments. Segments were placed in 96-well culture with DMEM (Gibco) substituted with 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). Segments were untreated or treated with guinea pig recombinant TNF-α (100 ng/ml), human recombinant IL-1β (100 ng/ml), human recombinant IL-1RA anakinra (100 ng/ml), or the TNF-α blocker etanercept (100 ng/ml) for 4 days. Some segments were treated with a combination of TNF-α (100 ng/ml) and IL-1RA (100 ng/ml) or IL-1β (100 ng/ml) and etanercept (100 ng/ml) fat 37°C or 4 days. Media and cytokines were replaced daily.

2. *In vitro* measurement of contractions

Contractions were measured *in vitro* as previously described (Murray and Jacoby, 1992). Trachea ring contractions were measured in a 5 ml organ bath (Radnoti Glass Technology, Monrovia, CA) filled with Kreb's-Henseleit solution (117.5 mM NaCL,

5.0 mM KCl, 1.18 mM $MgSO_4$, 1.47 mM NaH_2PO_4, 25 mM $NaHCO_3$, 5.54 mM D-glucose) bubbled with 95% O_2 and 5% CO_2 at 37°C. Each tracheal ring had two loops of suture string threaded through the lumen of the trachea. One loop was tied to an isometric force displacement transducer (Model FT03, Grass Instrument, Quincy, MA) and the other was connected to a stable glass hook in the bottom of the bath (Figure 2.7). Tissues were suspended between platinum electrodes in the bath for electrical field stimulation (EFS). Each segment was allowed to equilibrate to 1.0 g tension for 30 min and washed with Kreb's buffer every 10 min. The tissue's ability to contract was measured by giving KCL (100 mM) and methacholine (MCh; 10 μM) directly to the bath with a 3 x 10 min wash between each drug. KCL was given to depolarize the membrane potential to assess the health of tissue. MCh, a muscarinic receptor agonist, was given to determine if changes in contractile responses were due to M_3 receptors on smooth muscle. Contractile response to EFS (40 V, 1-30 Hz, 0.5 msec duration, 60 sec on, 60 sec off intervals) was measured as the maximum increase above baseline tension. At the end of each experiment TTX, a voltage-gated Na^+ channel blocker (1 μM), was given to confirm contractions were neurally mediated. Contractions were normalized to g tissue weight and recordings were made on a Powerlab/8SP (ADInstruments, Castle Hill, Australia). See Figure 2.7 for a sample recording.

FIGURE 2.7. Measuring *in vitro* tracheal contraction in an organ bath.

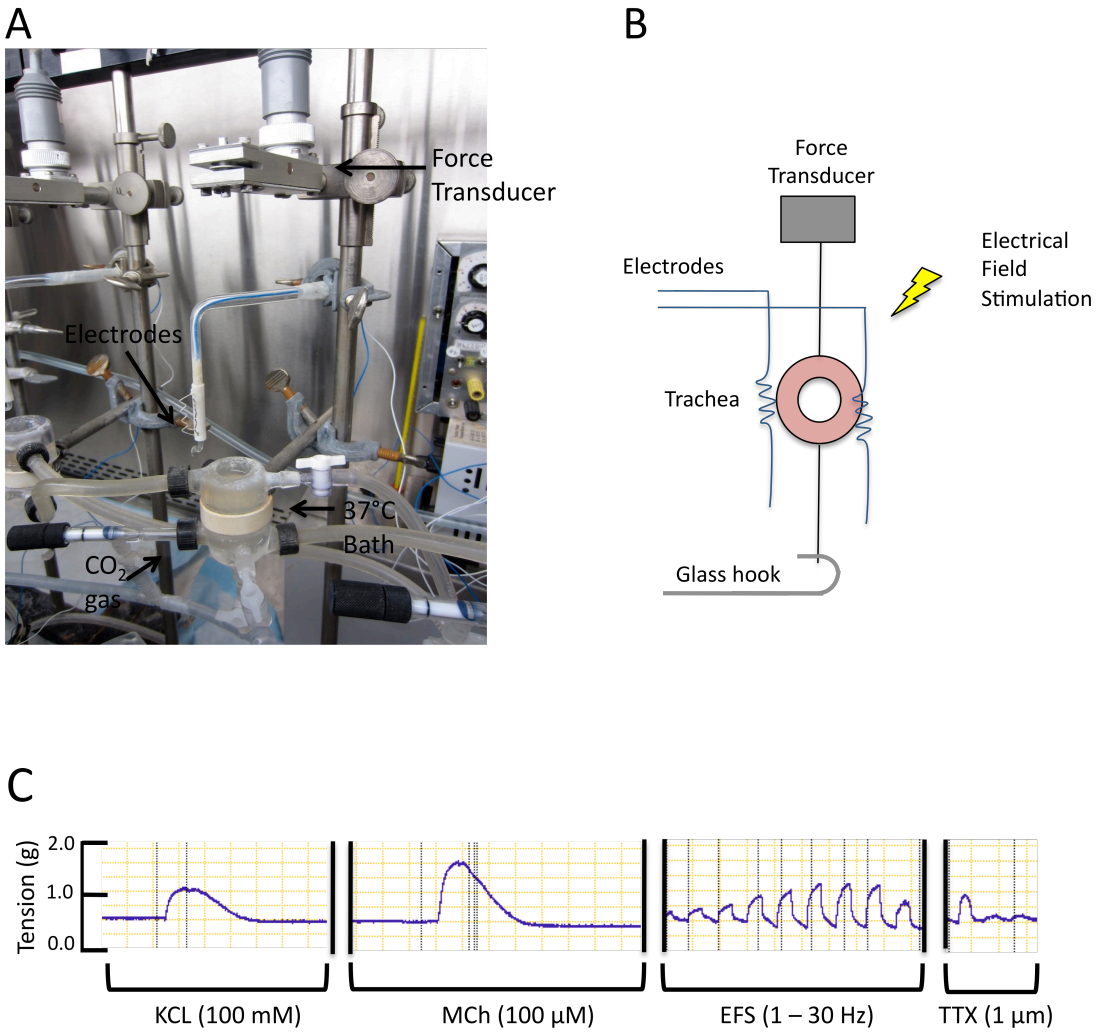

Figure 2.7. (A) Picture of a 5 ml organ bath. The bath contains a 37°C water jacket to keep tissues warm and 95% O_2 and 5% CO_2 is bubbled into the bath. Electrical stimulation is delivered to the tissue through electrodes in the bath and contractions are measured through a force transducer. (B) Schematic of a guinea pig trachea suspended in a 5ml bath by two looped strings attached to a force transducer and glass hook. The trachea piece is positioned in between 2 electrodes. (C) Sample tracing from an *in vitro* contraction experiment. Contraction of tissue

was measure by adding KCL (100 mM) and methacholine (MCh; 10 μM) directly to the bath to measure viability and smooth muscle contractions respectively (with 3 x 10 min washes in between not shown here). Neuronal cholinergic contractions were measured by EFS (electrical field stimulation; 40 V, 1-30 Hz, 0.5 msec duration, 60 sec on, 60 sec off intervals). Tetrodotoxin (TTX; 1 μm) was added at the end of the experiment to confirm contractions were neuronally mediated.

I. MEASUREMENT OF IL-1β IN SUPERNATANT BY ELISA

IL-1β protein levels were measured from supernatants from guinea trachea cultures using a guinea pig DuoSet ELISA kit (R&D systems). A 96-well plate was with capture antibody (0.8 µg/ml) overnight at room temperature. Each well was washed 3 times with 0.05% Tween-20 in PBS. The plate was blocked with 300 µl reagent diluent, which consisted of 0.2 µm filtered 1% BSA in PBS, for 1 hr at room temperature. An IL-1β standard (4000 pg/ml) was diluted in reagent diluent 10 times with 2-fold dilutions. The standard and samples (100 µl) were incubated for 2 hrs at room temperature. The plate was washed 3 times and incubated with detection antibody (200 ng/ml) for 2 hrs at room temperature. Plates were washed 3 times and incubated with streptavidin HRP diluted in reagent diluent (1:200) for 20 min in the dark. Plates were washed and incubated with a 1:1 mixture of reagent A (H_20_2) and reagent B (Tetramethylbenzidine) for 20 min in the dark. The color reaction was stopped using substrate stop solution (2N H_2SO_4). Plates were read at 450 nm and 540 nm on VersaMax microplate reader Molecular Devices, Sunnyvale, CA) using SoftMaxPro 6.1 software (Molecular Devices). IL-1β concentrations were determined from a standard curve created from a four-parameter logistic curve fit.

J. DRUGS AND REAGENTS

Urethane, succinylcholine, gallamine, pentobarbital, methacholine (MCh), KCL, and human recombinant IL-1β were obtained from Sigma-Aldrich (St. Louis, MO).

Acetylcholine (ACh) was purchased from Arco Organics (Fair Lawn, NJ). Anakinra, a recombinant human IL-1 receptor antagonist (IL-1RA), was purchased from Amgen (Thousand Oaks, CA). Guinea pig recombinant TNF-α and a cell culture grade recombinant human IL-1RA was purchased from R&D (Minneapolis, MN). Etanercept, a TNF-α inhibitor, was purchased from Immunex (Thousand Oaks, CA). Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel).

K. STATISTICAL DATA ANALYSIS

Two-way ANOVA with repeated measures test was used for vagally induced bronchoconstrictions and EFS-induced contractions. One-way ANOVA with Bonferroni correction was used for ELISA, BAL leukocyte, blood leukocyte, and M_2 receptor mRNA expression. Student *t* test was used to compare viral titers. Analysis of data was conducted using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Significance is shown as *P* values ≤ *0.05, **0.01, and ***0.001. Data are represented as ± SEM.

CHAPTER III.

INFLUENZA AND PARAINFLUENZA DO NOT INFECT AIRWAY NEURONS IN MICE AND GUINEA PIG

ABSTRACT

Rationale: Viral infection causes 80% of asthma exacerbations in children. Respiratory viral infection alters autonomic neural control of the lungs leading to bronchoconstriction. While viral infection of airway epithelium is well established, it is not known whether viruses directly infect airway nerves or whether changed neural control is secondary to epithelial cell infection. Virus-mediated epithelial cell damage can expose sensory nerve endings to potential viral infection and persistent infection in sensory nerve cell bodies. Therefore, we tested whether influenza and parainfluenza viruses are present in sensory nerves that innervate the lung.

Methods: C57BL/6 and BALB/c mice were infected intranasally with influenza A (H1N1; 1 x 10^3 $TCID_{50}$) or parainfluenza virus (Sendai, 2.8 x 10^4 $TCID_{50}$) virus. Guinea pigs were intranasally infected with parainfluenza (Sendai). Controls received uninfected media. Four days post infection, nerves and virus-infected cells were identified in bronchioles and nodose and jugular ganglia using two-color immunofluorescent staining. A pan-neuronal marker, PGP 9.5, was used to define nerve fibers. Virus infected cells were identified using polyclonal Sendai virus and influenza A nucleoprotein antibodies. Quantitative real-time PCR (qRT-PCR) was preformed on mRNA isolated from lung lobes and from nodose and jugular ganglia, which supply the sensory innervation of the lungs via the vagus nerves.

Results: Immunofluorescence showed strongly positive viral staining of airway epithelium in both mouse strains and guinea pigs infected with either influenza or parainfluenza virus. Nerves were identified in all animals, but no nerves were positive for H1N1 or Sendai infection by immunofluorescence. QRT-PCR of lung mRNA was positive for influenza and parainfluenza virus demonstrating that the lungs were infected, with no difference in viral titers between mouse strains. In addition, mRNA from nodose and jugular ganglia from mice showed no detectable message for either influenza or parainfluenza virus.

Conclusions: These data suggest that both influenza A (H1N1) and parainfluenza (Sendai) virus do not infect airway sensory nerves. Thus, changes in airway neural control seen with viral infection must be due to release of mediators from infected cells other than neurons, or to the inflammatory consequences of infection.

Data from figure 3.2 in this chapter have been submitted as:

Rynko, A., Fryer, A.D., Jacoby, D.B. Interleukin-1beta Mediates Virus-induced M_2 Muscarinic Receptor Dysfunction and Hyperreactivity. *Am J Respir Cell Mol Biol.* (Submitted).

Figures 3.1 and 3.3-3.5 are unpublished and unique to this chapter.

INTRODUCTION

Respiratory virus infections can be detected in 80% of acute exacerbations of asthma in children and 50% in adults (Johnston *et al.*, 1995; Atmar *et al.*, 1998). Virus infections alter the neural control of the airways, which leads to bronchoconstriction during asthma exacerbations.

Infection of the lungs leads to injury and shedding of epithelial cells and disruption of epithelial cell tight junctions (Hashimoto *et al.*, 2008). This increases epithelial barrier permeability leading to exposure of sensory nerves. Most of the sensory nerve fibers that innervate the trachea and airways originate from the nodose and jugular ganglia from the vagus nerve (Springall *et al.*, 1987; Kummer *et al.*, 1992) with a small portion originating from the dorsal root ganglia (Saria *et al.*, 1985; Dalsgaard and Lundberg, 1984). The majority of the vagus nerve fibers are non-myelinated C sensory fibers (Lee *et al.*, 2003). Stimulation and activation of C fibers induces release of neuropeptides, such as tachykinins, which can induce contraction of smooth muscle cells or lead to a parasympathetically induced bronchoconstriction (Papadopoulos *et al.*, 2007; Harrison and Geppetti, 2001; Tripp *et al.*, 2002). Viral infection can increase excitatory and decrease inhibitory responses of sensory nerves (Papadopoulos *et al.*, 2007; Colasurdo *et al.*, 1995; Auais *et al.*, 2003). Studies show parainfluenza and influenza viruses damage epithelium and reduce the activity of the major tachykinin metabolizing enzyme, neutral endopeptidase, which normally decreases the level of tachykinins, thus

potentiating their activity (Jacoby *et al.*, 1988; Dusser *et al.*, 1989; Borson *et al.*, 1989).

While the primary target of respiratory viruses are epithelial cells, studies have shown certain viruses infect airway neurons. Respiratory syncytial virus (RSV) and human metapneumovirus (HMPV) infect airway neurons in mice (Li *et al.*, 2006; Liu *et al.*, 2009). In addition, parainfluenza virus infects parasympathetic neurons *in vitro* (Fryer and Jacoby, 1991) and olfactory neurons *in vivo* (Mori *et al.*, 1995).

To determine if influenza or parainfluenza virus infect airway neurons I tested infection with these viruses directly in mice *in vivo*. I was surprised to find that both viruses do not infect airway neurons in the lungs determined by immunofluorescence. This also was confirmed in the sensory nodose and jugular ganglia, which showed no colocalization or nerve cell bodies with viral antigen by immunofluorescence or detectable viral message by RT-PCR. All of the animals had virus replicating in the lung showing it was not due to lack of infections. The absence of viral infection in airway sensory nerves indicates that the changes in airway control may be due to other mediators released during infection.

EXPERIMENTAL DESIGN

Infection in mice and guinea pigs

C57BL/6 mice and BALB/c mice (approximately 6 week-old; 25 g) were intranasally infected with 25 µl of 2.8 x 10^4 $TCID_{50}$ parainfluenza (in PBS), 1x10^3 $TCID_{50}$ influenza (in PBS), or PBS. Guinea pigs (300 g) were intranasally infected with 250 µl of 1x 10^6 $TCID_{50}$ parainfluenza (in PBS) or PBS. Four days later, mice and guinea pigs were euthanized and lungs and nodose and jugular ganglia were removed to assess viral infection of nerves. See Chapter II general methods for more detailed treatment strategy and growing and titering the virus.

Immunostaining

After 4 days, lungs and nodose and jugular ganglia were removed from animals and fixed, sectioned, and stained to assess viral infection of nerves. See Chapter II general methods for fixing and staining protocols.

qRT-PCR

Viral RNA was isolated from lung and nodose and jugular ganglia tissue using RNeasy kit and viral titer was quantified by qRT-PCR. See Chapter II general methods for qRT-PCR protocol.

RESULTS

Assessing virus infection in mouse and guinea pig airways neurons.

Airway nerves were identified in all animal tissue and were distinguished using a pan-neuronal nerve marker, protein gene product 9.5 (PGP 9.5) (Thompson *et al.*, 1983). Viral antigen was only detected in airways of infected animals using a polyclonal parainfluenza (Sendai) virus and monoclonal influenza A (H1N1) nucleoprotein antibody. Tissue was also labeled with 4',6-diamidino-2-phenylindole (DAPI) for cell nuclei. Parainfluenza virus infection could be identified in the epithelium of bronchioles and bronchi, but did not infect airway nerves of either C57BL/6 or BALB/c mice (Figure 3.1). Guinea pig airway neurons also showed no detectable parainfluenza viral antigen, confirming the results found in mice (Figure 3.2). In addition, influenza A virus antigen could be detected in airway epithelium, but did not colocalize with nerves of BALB/c mice (Figure 3.3). These data illustrate that airway nerves in the lungs of mice and guinea pig are not infected with parainfluenza or influenza A.

Assessing virus infection in sensory nerve cell bodies

To determine if parainfluenza and influenza A infect the nerve cell bodies of mice, the nodose and jugular ganglia was removed and stained for viral antigens and nerves. Nerve cells were identified using PGP 9.5, but no detectable parainfluenza

antigen could be detected in either C57BL/6 or BALB/c strain of mice (Figure 3.4A). Similarly, no influenza A antigen could be detected in the imaged nodose and jugular ganglia of BALB/c mice (Figure 3.4B). These data illustrate that the sensory nerve cell bodies as well as the nerve fibers in the lung are not infected in mice.

Determining viral titers in the lungs and nodose and jugular ganglia of mice

To ensure the animals were infected, viral titers were assessed in the lungs of mice. Viral titers were determined by qRT-PCR from RNA extracted from lung homogenates. Both C57BL/6 and BALB/c of mice proved to be infected with parainfluenza, with no difference in viral titers between strains (C57BL/6 = 3.19 x 10^4 ± 1.17 $TCID_{50}$/mg and BALB/c = 1.95 x 10^4 ± 0.126 $TCID_{50}$/mg; Figure 3.5A). Influenza A infected BALB/c mice also had detectable viral mRNA message (1.79 x 10^5 ± 0.43 $TCID_{50}$/mg; Figure 3.5B). Nodose and jugular ganglia from mice were also assessed for viral message to determine if virus infected the sensory cell bodies that could not be seen from immunostained sections. However, no detectable viral message could be found in nodose and jugular ganglia of mice of infected or mock-infected mice (Figure 3.5A,B). These data indicate influenza A and parainfluenza virus-infected animals had replicating virus in the lungs, but not in the airway sensory neurons.

Effect of virus infection on inflammatory cells in the lungs of mice

The BAL of infected mice was collected and white cells were counted to assess changes in inflammatory cells in the lungs. Parainfluenza-infected mice had a significant increase in total leukocytes compared with mock-infected (parainfluenza-infected BALB/c cells = $7.57 \pm 1.04 \times 10^5$ vs. mock-infected BALB/c = $5.99 \pm 2.69 \times 10^4$ and parainfluenza-infected C57BL/6 = $6.83 \pm 1.48 \times 10^5$ vs. mock-infected C57BL/6 = $6.28 \pm 1.03 \times 10^4$; Figure 3.6A). Similarly, influenza-infected mice had a significant increase in total white cells compared with mock-infected (influenza-infected cells = $6.59 \pm 0.56 \times 10^5$ vs. mock-infected = $4.5 \pm 0.17 \times 10^4$; Figure 3.6B).

FIGURE 3.1. Parainfluenza (Sendai) virus does not infect nerves in lungs of BALB/c or C57BL/6 mice.

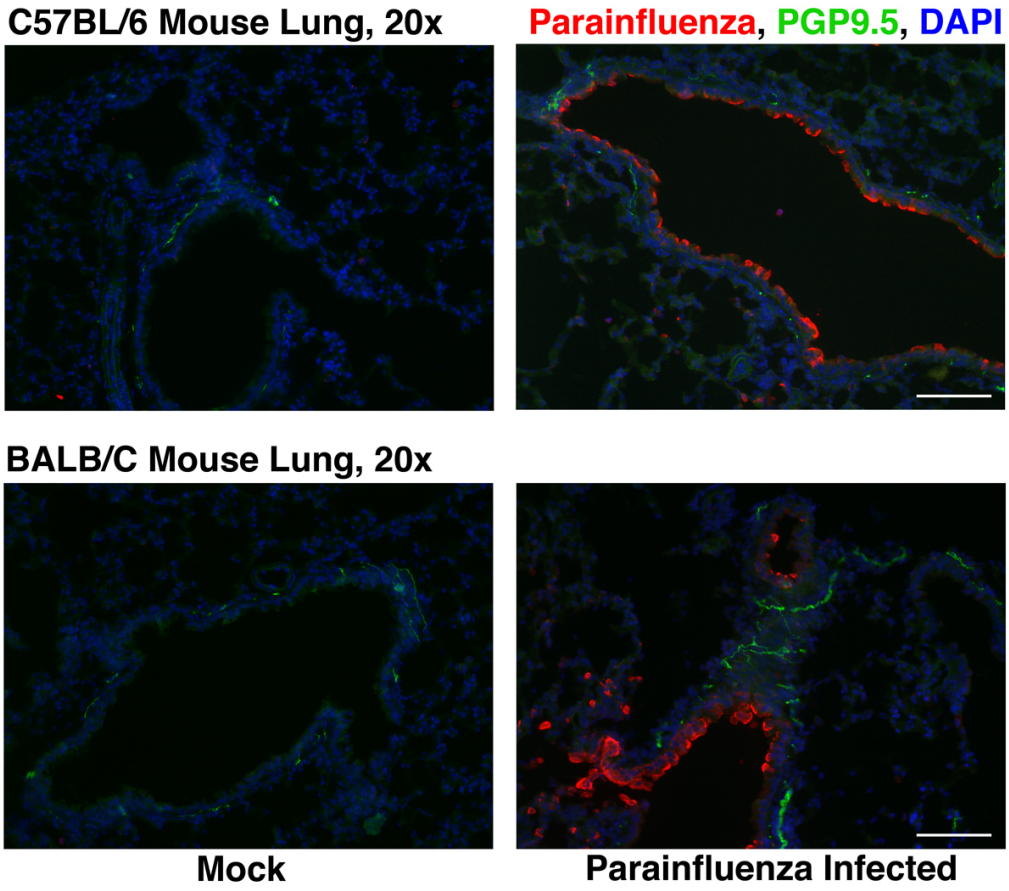

Figure 3.1. Parainfluenza virus antigen does not colocalize airway nerves after infection in the lungs of mice. Mouse lungs infected with 2.8 x 10^4 $TCID_{50}$ parainfluenza for 4 days were fixed, sectioned, and immunostained with a polyclonal antibody to parainfluenza (Sendai) virus antigen (red), a pan-neuronal nerve antibody to PGP 9.5 (green), and DAPI nuclear stain (blue). There was no detectable colocalization with viral antigen and nerves in respiratory bronchi and bronchioles of either strain of mice. (20X magnification, magnification bar = 100 μm, n=4).

FIGURE 3.2. Parainfluenza (Sendai) virus does not infect nerves in lungs of guinea pigs.

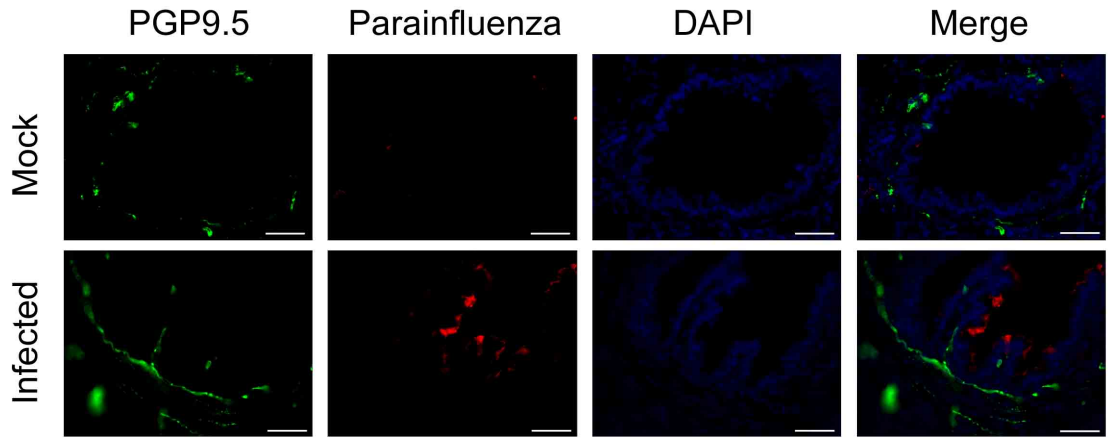

Figure 3.2. Parainfluenza virus antigen does not colocalize airway nerves after infection in the lungs of guinea pigs. Guinea pig lungs infected with 1 x 10^6 $TCID_{50}$ for 4 days were fixed, sectioned, and immunostained with a polyclonal antibody to parainfluenza (Sendai) virus antigen (red), a pan-neuronal nerve antibody to PGP 9.5 (green), and DAPI nuclear stain (blue). Viral antigen (red) is clearly seen in airway epithelial cells, and airway nerves (green) are clearly identified, but there is no colocalization of virus with nerves (40X magnification, magnification bar = 50 μm, n=4).

FIGURE 3.3. Influenza A (H1N1) virus does not infect nerves in lungs of BALB/c mice.

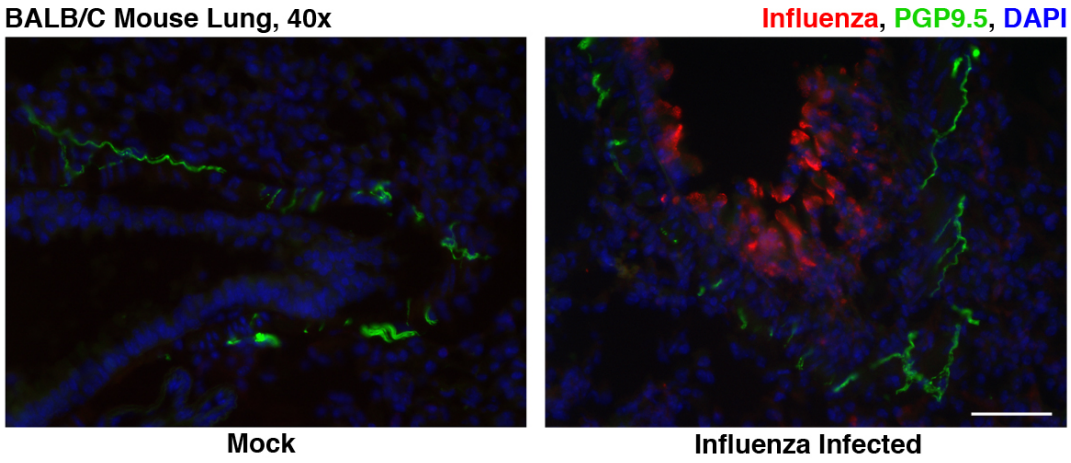

Figure 3.3. Influenza virus antigen does not colocalize airway nerves after infection in the lungs of mice. Mouse lungs infected with 1 x 10^3 $TCID_{50}$ influenza for 4 days were fixed, sectioned, and immunostained with a monclonal antibody to influenza A virus (H1N1) nucleoprotein (red), PGP 9.5 (green), and DAPI nuclear stain (blue). There was no detectable colocalization with viral antigen and nerves in respiratory bronchi and bronchioles. (40X magnification, magnification bar = 50 μm, n=4).

FIGURE 3.4. Parainfluenza (Sendai) and influenza (H1N1) virus do not infect nodose and jugular ganglia of mice.

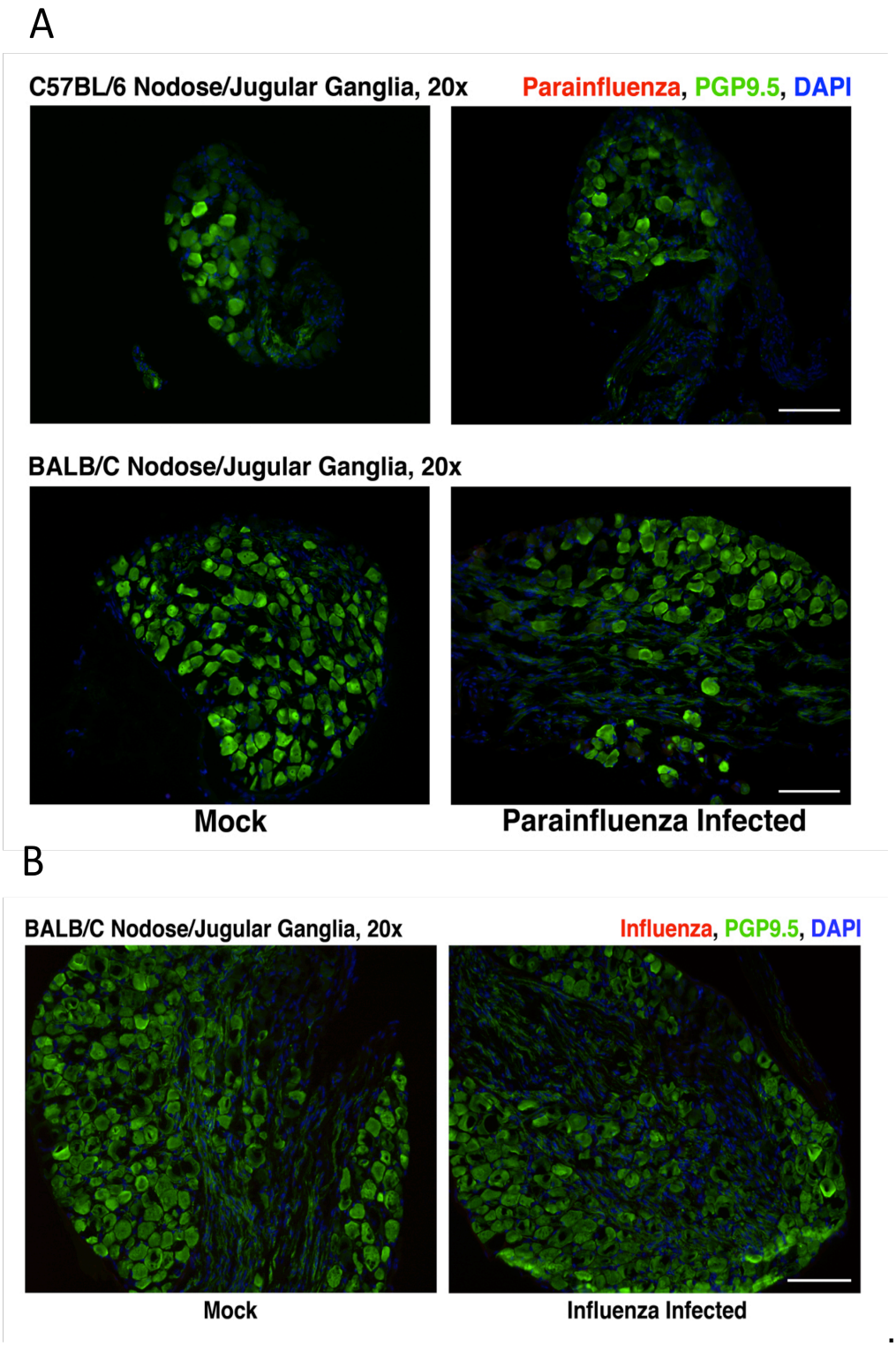

Figure 3.4. Mice were infected with 2.8 x 10^4 $TCID_{50}$ parainfluenza, 1 x 10^3 $TCID_{50}$ influenza virus, or PBS for 4 days. Nodose and Jugular ganglia were removed fixed, sectioned, and immunostained with an antibody to PGP 9.5 (green), DAPI nuclear stain (blue), and either an antibody to influenza A virus (H1N1) nucleoprotein or parainfluenza (Sendai) virus antigen (red). (A) Parainfluenza virus does not infect the nodose and jugular ganglia of BALB/c or C57BL/6 mice (20X magnification, magnification bar = 100 μm, n=6). (B) H1N1 virus does not infect the nodose and jugular ganglia of BALB/c (20X magnification, magnification bar = 100 μm, n=4).

FIGURE 3.5. Mouse lung mRNA was positive for parainfluenza and influenza virus, but mRNA from nodose and jugular ganglia showed no detectable message for either parainfluenza or influenza virus.

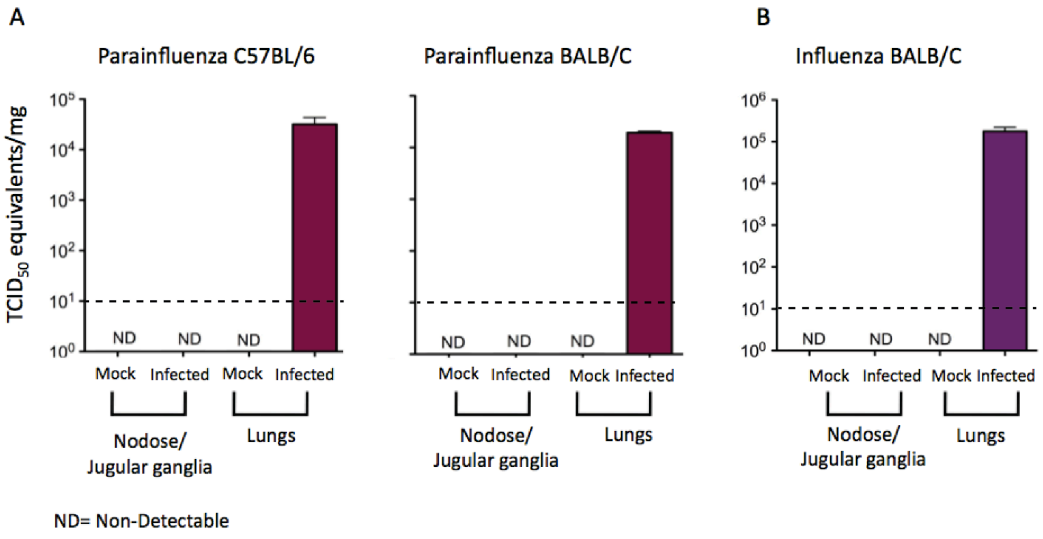

Figure 3.5. Mice were infected with 2.8 x 10^4 $TCID_{50}$ parainfluenza, 1 x 10^3 $TCID_{50}$ influenza virus, or PBS for 4 days. RNA was extracted from nodose and jugular ganglia and lung homogenates and viral message was measured by RT-PCR. Mouse lungs were positive for either parainfluenza or influenza virus infection, but no viral message could be detected in the nodose and jugular ganglia. There was no significant difference in parainfluenza viral message in the lungs four days after infection in either C57BL/6 or BALB/c strains of mice. Viral titers were normalized to 18S rRNA and tissue culture infective dose ($TCID_{50}$) was determined from a parainfluenza standard curve. Dashed lines indicate the limit of detection. Data are shown as ± SEM (n=4).

FIGURE 3.6. Infection with parainfluenza or influenza increased total inflammatory cells in the BAL of mice.

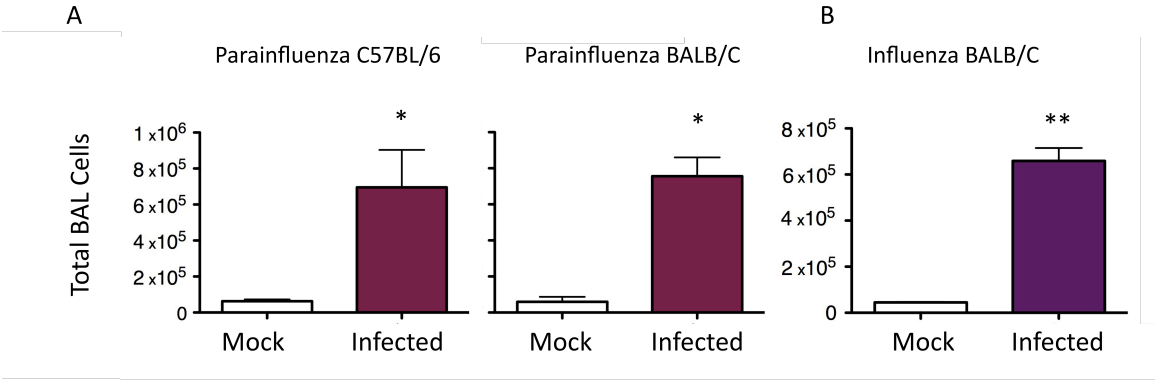

Figure 3.6. Mice were infected with $2.8x10^4$ $TCID_{50}$ parainfluenza, $1x10^3$ $TCID_{50}$ influenza virus, or PBS for 4 days. Lungs were lavaged with PBS at the end of each experiment and total cells were counted. Parainfluenza (A) and influenza A (B) infected mice had a significant increase in total leukocytes in the BAL compared to mock-infected. Data are represented as ± SEM (n=4).

DISCUSSION

As discussed in the introduction, other respiratory viruses can infect airway and olfactory neurons (Li *et al.*, 2006; Liu *et al.*, 2009; Fryer and Jacoby, 1991; Mori *et al.*, 1995). To determine if influenza A and parainfluenza virus infect airway nerves, lungs from infected mice and guinea pigs were removed and immunostained. Both C57BL/6 and BALB/c stains of mice were used since different strains of mice have been shown to have varied levels of susceptibilities to Sendai virus infection (Faisca *et al.*, 2005; Brownstein *et al.*, 1981). Here, I demonstrate parainfluenza and influenza viruses do not infect airway neurons in mice (Figure 3.1 and 3.3). This was surprising since data for other respiratory viruses show viral antigen or mRNA in airway neurons after infection *in vivo* (Li *et al.*, 2006; Liu *et al.*, 2009).

Parainfluenza virus replicates in the nasopharyngeal epithelium and spreads to the trachea, bronchi and bronchioles (Hall, 2001; Brownstein, 2002). However, Mori et al. demonstrated that parainfluenza virus is detected in olfactory bulb neurons after intranasal infection (Mori *et al.*, 1995). In addition, parainfluenza virus infects parasympathetic neurons *in vitro* (Fryer and Jacoby, 1991). The ability of parainfluenza virus to infect neurons *in vivo* and *in vitro* made it seem likely to infect airway neurons, but this was not observed in mice or guinea pigs. Only the airway epithelium of bronchi and bronchioles were identified with viral antigen and this did not colocalize with PGP 9.5 labeled airway nerves. It is also possible that parainfluenza do not infect neurons at all *in vivo* as another study found no invasion

of virus in olfactory bulb neurons after intranasal infection (Lundh *et al.*, 1987). Furthermore, the fact that parainfluenza infects parasympathetic neurons *in vitro* may be due to its uninhibited access to neuronal cells, and appears to be limited to a culture system.

Influenza virus replicates in ciliated epithelium of the conducting airway (Hers, 1966), which consist of several cell types that can become infected throughout the infection process, and infects the trachea down to the alveoli (Jeffery and Li, 1997; Tateno *et al.*, 1966; Janke, 2013; Ebisawa *et al.*, 1969). Influenza infection in airway neurons has not previously been reported; however, CNS impairment with influenza infections has been well described, most commonly influenza-associated encephalopathy, often reported in children (Mizuguchi *et al.*, 2007; Shiomi, 2011; Nicholson, 1998). Other Influenza-associated neurocomplications have been reported, such as influenza-associated myopathy, extrapyramidal based abnormal movements, transverse myelitis, Guillain-Barre syndrome, rhabdomyolysis, and post-infectious acute disseminated encephalomyelitis (Lehmann *et al.*, 2010; Ryan *et al.*, 1999; Studahl, 2003; Bautista *et al.*, 2010; Davis, 2010; Parikh *et al.*, 2010; Yang *et al.*, 2010). In addition, ischemic stroke was reported in a 9 month old infant after infection (Honorat *et al.*, 2012). Furthermore, influenza virus specific RNA fragments were detected in cerebral spinal fluid and brain specimens using RT-PCR and have been reported in cases of encephalitis occurring 24-48 hours after influenza infection (Fujimoto *et al.*, 1998; Ito *et al.*, 1999; Takahashi *et al.*, 2000;

Togashi *et al.*, 2000). Influenza H5N1 has also been shown to be neurotropic in chickens (Ward, 1996). Given the amount of neurocomplications associated with influenza and its ability to induce neuronal changes, it seemed reasonable to hypothesize it could infect airway neurons. However, this hypothesis turned out to be incorrect, as no influenza antigen colocalized with nerves in airways of mice (Figure 3.3).

Sensory nerves have been implicated as possible factors involved in airway hyperreactivity after upper respiratory infections. Viral infections can potentiate neuropeptide release from sensory nerve fibers and cause hyperresponsiveness by increasing leukotriene synthesis (Yang *et al.*, 1997), releasing mast cell mediators (Joos and Pauwels, 1993), modulating cholinergic neurotransmission (Belvisi *et al.*, 1994), and mucus hypersecretion (Coles *et al.*, 1984). Moreover, blocking neuropeptides prior to viral infection can prevent hyperresponsiveness (Ladenius *et al.*, 1995). Sensitivity of sensory nerve receptors has also been suggested as a possible mechanism (Empey *et al.*, 1976), as well as increases in substance P activity from decreased neutral endopeptidase activity, which degrades substance P (Jacoby *et al.*, 1988; Dusser *et al.*, 1989). In addition, influenza and parainfluenza virus infection increases contractile responses to neuropeptides (Saban *et al.*, 1987; Ladenius *et al.*, 1995). Here I showed that both influenza and parainfluenza virus do not infect the sensory nerve fibers or cell bodies of the nodose and jugular ganglia,

as no viral antigen colocalized with nerve cells (Figure 3.4). Moreover, no viral mRNA was detected in the nodose and jugular ganglia.

The limitations of these data are the time point and strain of virus used. While, no virus was detected in the airway nerves at day 4, there may be direct infection of nerves at earlier or later time points. In addition, parainfluenza type 1 (Sendai) or influenza A (H1N1) strain may not infect airway nerves, but other types of parainfluenza or influenza could infect airway nerves. For instance, influenza A (H5N1) infects nerves in birds (Ward, 1996). It is also possible the limit of detection of the RT-PCR could prevent detection of virus mRNA in the ganglia.

All mice were shown to be infected and the infection caused an influx of inflammatory cells to the BAL (Figure 3.5). There was no difference in infectivity between strains for parainfluenza virus as previously reported (Faisca *et al.*, 2005). This is likely due to a difference in the day of viral lung titer measurement (4 days vs. 5-7 days) and the ability of BALB/c mice to almost completely clear the virus by day 7 (Faisca *et al.*, 2005).

These data demonstrate that airway sensory neurons are not infected after influenza or parainfluenza virus infection. The lack of direct viral infection of airway neurons suggest other mechanisms may be involved that change neuronal control

during infections. Possible inflammatory mediators released during infection were investigated in subsequent chapters of this thesis.

CHAPTER IV.

QUANTIFYING M2 RECEPTOR MRNA FROM INFECTED GUINEA PIG PARASYMPATHETIC GANGLIA USING A NOVEL DISSECTION TECHNIQUE

ABSTRACT

Rationale: The majority of asthma exacerbations in children and adults are associated with viral infection. Virus-induced airway hyperreactivity is mediated by loss of neuronal M_2 muscarinic receptors that normally function to limit acetylcholine release and inhibit vagally mediated reflex bronchoconstriction. *In vivo* and in isolated nerve cells, blocking tumor necrosis factor alpha (TNF-α) with etanercept (a TNF-α receptor IgG fusion protein) protects M_2 receptor function and prevents virus-induced hyperreactivity. In cell culture, M_2 receptor gene expression is decreased by direct viral infection of airway nerves or with addition of TNF-α alone. In addition, blocking IL-1β with anakinra, an IL-1β receptor antagonist (IL-1βRA), in antigen-challenged and ozone-challenged guinea pigs prevents airway hyperreactivity *in vivo*. Here we test whether viral infection of airway nerves, TNF-α, or IL-1β is the *in vivo* mechanism of virus-induced decrease in M_2 receptor expression and resulting airway hyperreactivity.

Methods: Guinea pigs were infected with parainfluenza (Sendai) virus. Controls received uninfected media. In some animals, TNF-α was blocked *in vivo* with etanercept 24 hours before virus infection or IL-1β was blocked 30 min before infection. Four days after infection lungs were harvested for viral titers. In addition, guinea pig parasympathetic ganglia were harvested from the airways using a new dissection technique I developed. Viral titers for lung and ganglia were determined

using RNA from tissue homogenates and parasympathetic ganglia by qRT-PCR. M_2 receptor gene expression levels were normalized to 18s and a pan-neuronal marker, PGP 9.5, was used to verify nerve cell content.

Results: Guinea pig parasympathetic ganglia showed a 67% decrease in M_2 receptor mRNA following virus infection. Blocking either IL-1β, with anakinra, or TNF-α, with etanercept, blocked this decrease in M_2 expression. Guinea pig lung viral titers were positive for parainfluenza virus message demonstrating the lungs were infected, and the lung viral titers were not affected by anakinra or etanercept treatment. No virus message could be detected in parasympathetic ganglia by qRT-PCR.

Conclusion: Parainfluenza downregulates M_2 muscarinic mRNA in parasympathetic ganglia *in vivo*. This downregulation is induced by both TNF-α and IL-1β, as it is blocked by either etanercept or anakinra. There is no direct infection of airway parasympathetic nerves. These results show that TNF-α and IL-1β are key mediators of M_2 receptor dysfunction during infection *in vivo*.

Data from this chapter have been submitted as:

Rynko, A., Fryer, A.D., Jacoby, D.B. Interleukin-1beta Mediates Virus-induced M_2 Muscarinic Receptor Dysfunction and Hyperreactivity. *Am J Respir Cell Mol Biol.* (Submitted).

Figures 4.1-4.2 are unpublished and unique to this chapter.

INTRODUCTION

The majority of asthma exacerbations in children and adults are associated with viral infection (Johnston *et al.*, 1995; Atmar *et al.*, 1998). Parainfluenza virus induces airway hyperreactivity by decreasing M_2 muscarinic receptor function on parasympathetic nerves (Fryer and Jacoby, 1991). M_2 receptor dysfunction leads to airway hyperreactivity during virus infection (Fryer *et al.*, 1994).

Previous studies indicate a role for inflammatory cytokines as a mechanism for M_2 receptor dysfunction during virus infection. Tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are two cytokines upregulated during parainfluenza virus infection (Yoshizumi *et al.*, 2010). Overproduction of these and other cytokines has been associated with the pathology of asthma (Message and Johnston, 2004; Mallia and Johnston, 2006; Proud and Chow, 2006). Previous studies showed that TNF-α (Nie *et al.*, 2009) or direct viral infection (Jacoby *et al.*, 1998) decrease M_2 receptor gene expression in cultured neuronal cells. Furthermore, blocking TNF-α *in vivo* with etanercept, a TNF-α receptor (TNF receptor type II) IgG fusion protein, prevents airway hyperreactivity and M_2 receptor dysfunction during virus infection (Nie *et al.*, 2011). In addition, blocking IL-1β with anakinra, a recombinant human IL-1β receptor antagonist (IL-1βRA) selective for IL-1 type I receptors, in antigen-challenged and ozone-challenged guinea pigs prevents airway hyperreactivity *in vivo* (Selig and Tocker, 1992; Verhein *et al.*, 2008).

What is not known is if virus infection decreases M_2 receptor mRNA expression *in vivo*. These studies were conducted to test whether parainfluenza virus infection decreases neuronal M_2 receptor expression and if TNF-α or IL-1β is responsible for virus-induced decrease in M_2 receptor expression and resulting airway hyperreactivity in guinea pigs. These studies used both etanercept and anakinra to demonstrate that virus infection decreases M_2 receptor expression *in vivo* and blocking either TNF-α or IL-1β prevented the decrease in expression.

EXPERIMENTAL DESIGN

Virus infection

Guinea pigs (150-200 g) were intranasally infected with 1 x 10^6 $TCID_{50}$ parainfluenza virus (in PBS). Some animals were treated with guinea pigs were treated with etanercept (3 mg/kg, i.p.), anakinra (30 mg/kg, i.p.), or PBS before infection. Etanercept was administered once 24 hrs before infection and anakinra was administered 30 min before infection and once daily until 4 days after infection. Four days after infection, parasympathetic ganglia (to determine M_2 receptor expression) and lung tissue (to assess viral titers) were harvested after euthanizing the animal. See chapter II for more detailed treatment strategy and methods for isolating, growing, and titering the virus.

Parasympathetic ganglia dissection

To determine the effects of virus infection on M_2 receptor expression *in vivo*, I had to develop a dissection technique to remove parasympathetic ganglia from guinea pig trachea. Isolating ganglia was needed since M_2 receptors are expressed on a variety of cell types in the trachea, such as smooth muscle, epithelial, and other cell types.

Tissue Dissection and Preparation

Guinea pigs (150-200 g) were euthanized with sodium pentobarbital (200 mg/kg, i.p.) and perfused with PBS through heart. Tracheas were removed from the larynx to the bifurcation of the lung. Special attention was taken to remove the esophagus from the posterior side of the trachea since the postganglionic parasympathetic nerve cells are located along adventia and the smooth muscle of the trachea facing the esophagus (Myers, 2001; Zhu and Dey, 2001; Canning and Fischer, 1997; Baker *et al.*, 1986).

Tracheas were cut ventrally through the cartilaginous rings and then pinned with dissecting pins (Fine Science Tools, Foster City, CA) with the lumen side of the trachea down to a ice cold Sylgard dish made from Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) in a glass petri dish (Figure 4.1). All tools used for dissection was rinsed in diethylpyrocarbonate (DEPC, Sigma-Aldrich) treated H_20 for 10 sec, RNase Away (Molecular BioProducts, Waltham, MA) for 30 sec, DEPC treated H_20 for 30 sec, then 70% EtOH made in DEPC treated H_20 for 5 sec.

Neutral Red Staining

Tissue was rinsed with RNase free PBS (ice cold), and then stained for 30 min on ice with 250µl 0.05% neutral red solution (Fisher, Waltham, MA) made in RNase free

PBS (pH 7.4). Neutral red is a lipophilic dye that enters viable cells at neutral pH (Ebner *et al.*, 2009). Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was chosen for its ability to identify and stain neurons in tissue without affecting transmission (Skoogh *et al.*, 1983) and for the characteristic of a non-toxic, vital stain (Grillo and Nadel , 1980; Repetto *et al.*, 2008; Borenfreund and Puerner, 1985). Neutral red is uncharged at neutral pH, but becomes protonated once inside the cell, allowing it to accumulate. Neutral red is a cationic dye that binds to DNA and cytoplasmic RNA that is concentrated on rough endoplasmic reticulum (ER) and ribosomes (Nissl granules) (Scott and Willett, 1966). Because neurons require high protein synthesis, they have a large concentration of rough ER and Nissl granules in their cytoplasm (Kosik and Krichevsky, 2002; Knowles *et al.*, 1996; Nissl, 1894; Fujita *et al.*, 2008). However, since neutral red stains can enter other cell types and stain other organelles, such as lysosomes (Winckler, 1976; Svendsen *et al.*, 2004), the timing of the neutral red staining needed optimization as longer incubation times made it harder to distinguish ganglia.

Tracheas were rinsed with RNase free PBS (ice cold) with the addition of new PBS (ice cold) to keep tissues hydrated. Ganglia were visualized using a Nikon SMZ1000 dissecting stereomicroscope. Some ganglia could be visualized without the addition of neutral red staining; however, the staining significantly enhanced the visibility of ganglia and made smaller and deeper ganglia in the smooth muscle tissue identifiable (Figure 4.2). Ganglia were identified by the light red staining of the

nerve cell bodies that had a swollen appearance. The nerve processes were also stained red and could be visualized projecting from the mass of cells. When determining if a group of cells was a parasympathetic ganglion, the cells were gently nudged in the tissue. Ganglia moved as cluster of cells since they are sheathed by a protective perineurium layer (Chiang and Gabella, 1986; Baluk *et al.*, 1985) and did not break apart as easily compared to other cells that had light red staining. Ganglion clusters removed in these studies were approximately 150-300 μm, which match previous findings (Chiang and Gabella, 1986; Cameron and Coburn, 1984), although smaller ganglion populations of approximately 30-60 μm in size have been reported (Mitchell *et al.*, 1987).

Dissection of parasympathetic ganglia from airway smooth muscle

Airway smooth muscle and adventitia were separated with Dumont no.5 forceps (Fine Science Tools) and the ganglia could be dissected out of tracheal wall. Each process of the ganglia was severed and cleaned in a separated dish with RNase free PBS (ice cold) to remove any non-neuronal tissue (Figure 4.3). All ganglia from each trachea were pooled together in an RNase free microcentrifuge tube. The ganglia were flash frozen in liquid nitrogen and stored at -80°C for RNA isolation using the Cells-to-C_T. In most cases, 8 or more ganglia could be removed and on average 10-12 were removed. The most ganglia I could extract was 16 ganglia from one trachea. However, there are a reported 150-500 total nerve cells in ganglia of 4-20 cells and most (90%) ganglia consist of 12 or less cells in guinea pigs (Baluk and Gabella,

1989; Canning and Fischer, 1997), so it's likely not all parasympathetic ganglia were removed from each animal. See Chapter II general methods for RNA isolation and qRT-PCR protocols.

RNA isolation and qRT-PCR

RNA was isolated from guinea pig parasympathetic ganglia using the Cells-to-C_T kit and quantified by RT-PCR. Tissue was normalized to 18S and not PGP 9.5 since levels of PGP can change throughout development and with injury (Schofield *et al.*, 1995; Olerud *et al.*, 1998). See Chapter II for these general methods.

FIGURE 4.1. Surgical preparations for parasympathetic ganglia dissection guinea pigs.

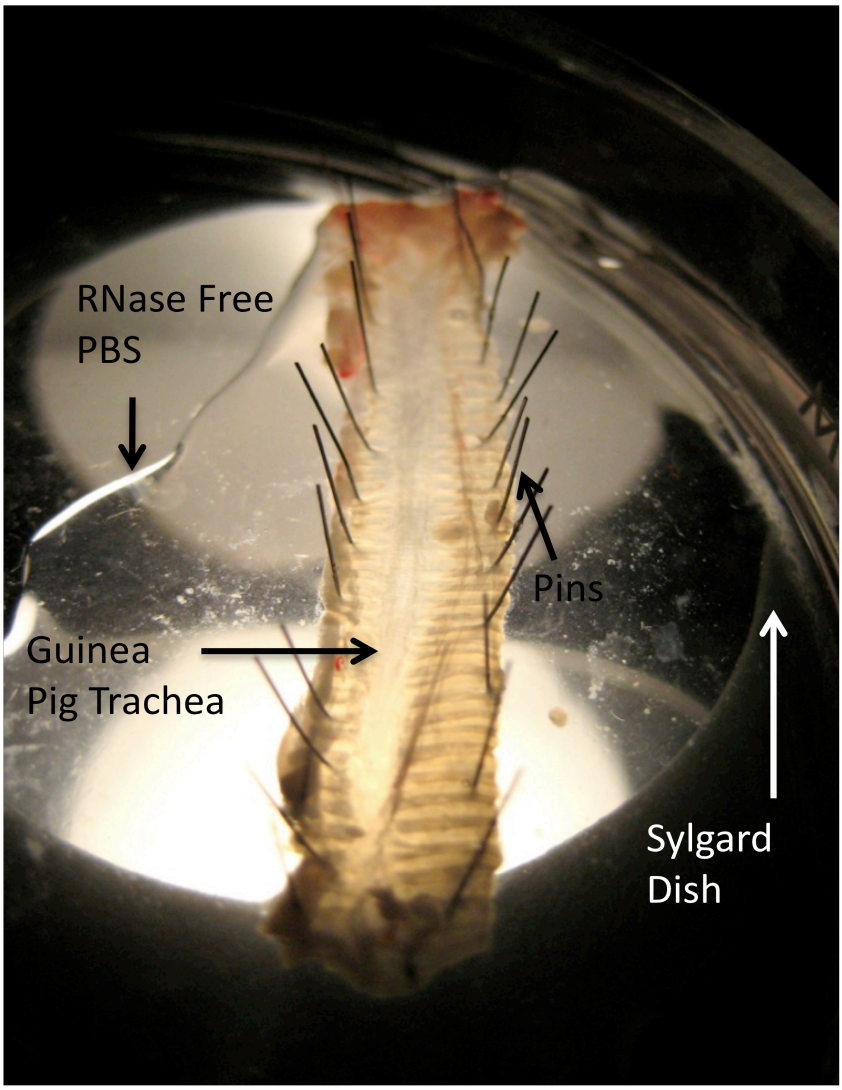

Figure 4.1. Trachea were dissected from guinea pigs and opened up anteriorly through the cartilage rings. Tracheas were flattened and pinned to a sylgard dish with the lumen side down using dissection pins. The dish is kept on ice and ice cold, RNase free PBS is added to the trachea to keep hydrated.

FIGURE 4.2. Parasympathetic ganglia with neutral red staining.

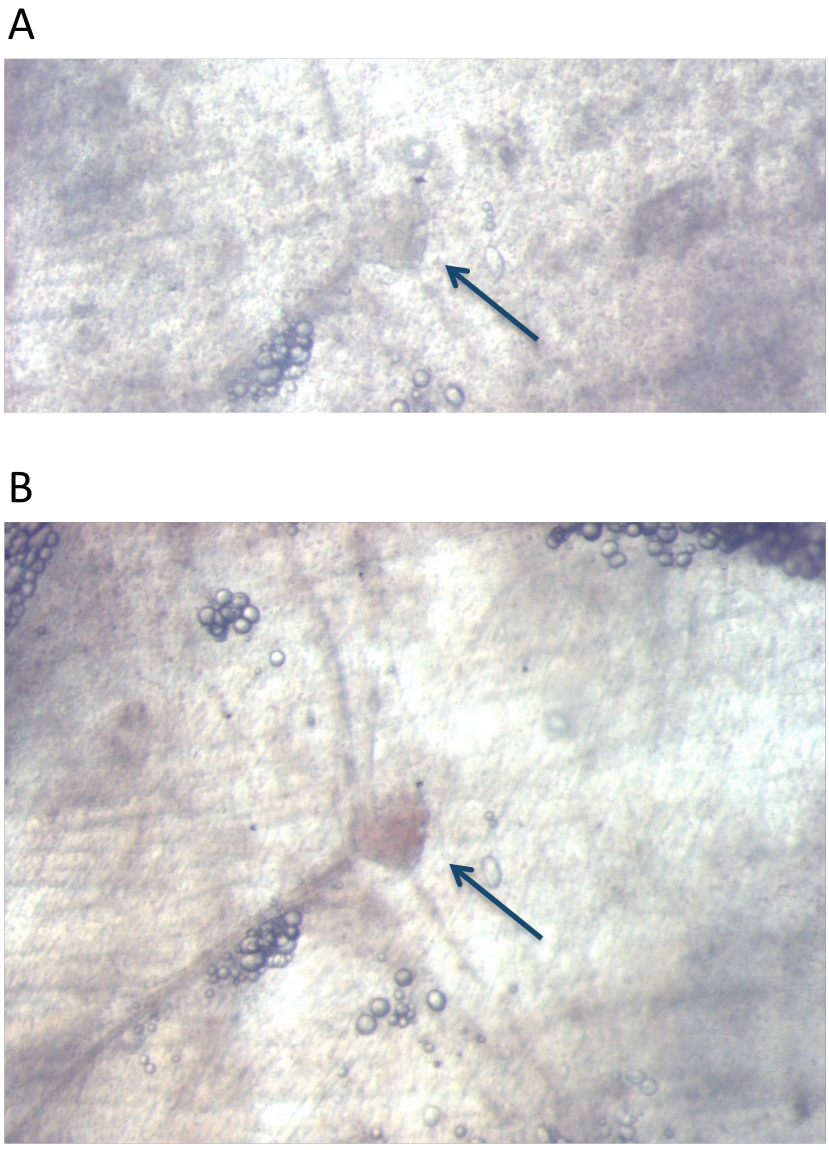

Figure 4.2. Guinea pig tracheas were removed, opened anteriorly, pinned lumen side down. Tracheas were rinsed with cold RNase free PBS and the dish is kept on ice. Ganglia are visualized using a dissecting stereomicroscope. (A) Shows an individual ganglion without staining. (B) Shows the same ganglion after staining with a 0.05% neutral red solution made in RNase free PBS for 30 mins.

FIGURE 4.3. Guinea pig parasympathetic ganglia dissection.

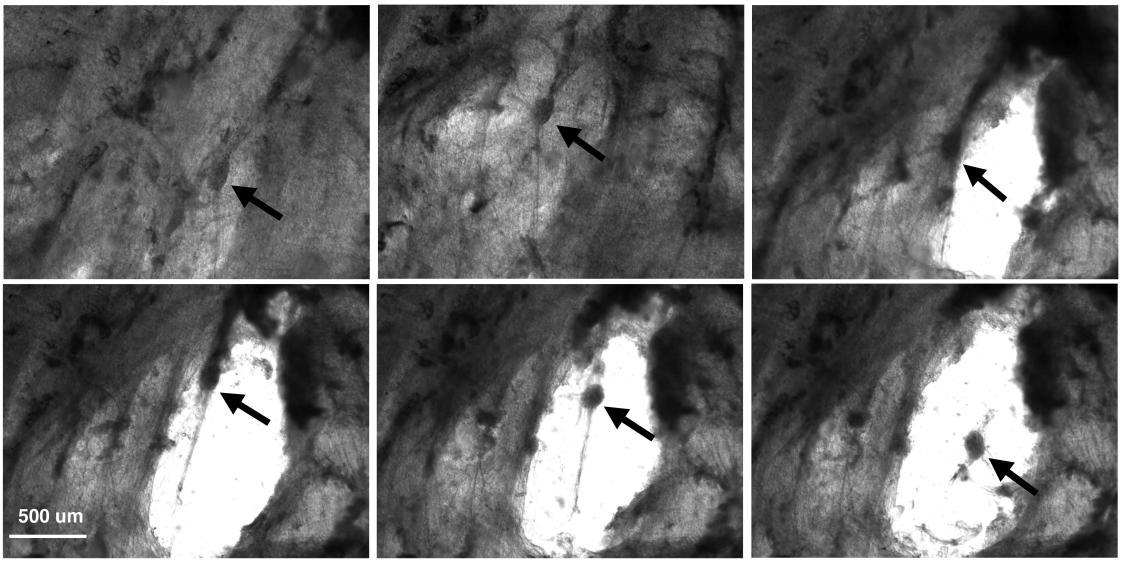

Figure 4.3. Serial images demonstrating the dissection of one parasympathetic ganglion from the tracheal smooth muscle stained with neutral red. Guinea pig tracheas were removed, opened anteriorly, pinned lumen side down, and stained with a 0.05% neutral red solution made in RNase free PBS for 30 mins. Tracheas were rinsed with cold RNase free PBS, and the airway smooth muscle was separated from ganglia with Dumont no.5 forceps using a dissecting stereomicroscope. Each process of the ganglia was severed, and non-neuronal tissue was cleaned away. Ganglia from each trachea were pooled together and stored at -80°C for RNA isolation.

RESULTS

Effects of virus infection and blocking IL-1β and TNF-α on M_2 receptor expression in parasympathetic nerves *in vivo*

Parasympathetic ganglia were individually dissected from guinea pig tracheas to measure M_2 receptor expression. M_2 receptor expression was quantified by RT-PCR from animals treated with either etanercept (3mg/kg, i.p.), to block TNF-α, or anakinra (30mg/kg, i.p.), to block IL-1β. Parainfluenza virus infection caused a 66.92 ± 10.51% decrease in M_2 receptor gene expression compared to mock-infected (Figure 4.4). Virus-infected animals treated with either etanercept or anakinra had M_2 receptor expression similar to controls. Virus-infected animals treated with either etanercept, to block TNF-α, or anakinra, to block IL-1β, had normal M_2 receptor expression.

Effects of blocking IL-1β or TNF-α on viral replication in the lungs and parasympathetic ganglia

Guinea pig lungs were homogenized, and viral titers were assessed by real-time PCR. Infected animals had viral titers less than a log different between all groups (Figure 4.5A). Infected animals had an average titer of 2.86 ± 1.6 x10^3 $TCID_{50}$/mg, which was similar to animals treated with anakinra (4.16 ± 2.7 x10^3 $TCID_{50}$/mg).

Etanercept treated titers (1.9 ±0.6 x 10^4 $TCID_{50}$/mg) also did not change from their matched infected animals without etanercept (3.71 ±1.5 x 10^4 $TCID_{50}$/mg). No viral message could be detected in the parasympathetic ganglia from virus-infected guinea pigs (Figure 4.5B). This further supports the findings of Chapter III that parainfluenza does not infect airway neurons or parasympathetic neurons.

FIGURE 4.4. In vivo parainfluenza virus infection decreases M_2 receptor mRNA expression in airway parasympathetic ganglia. This decrease is prevented by treating with either anakinra or etanercept.

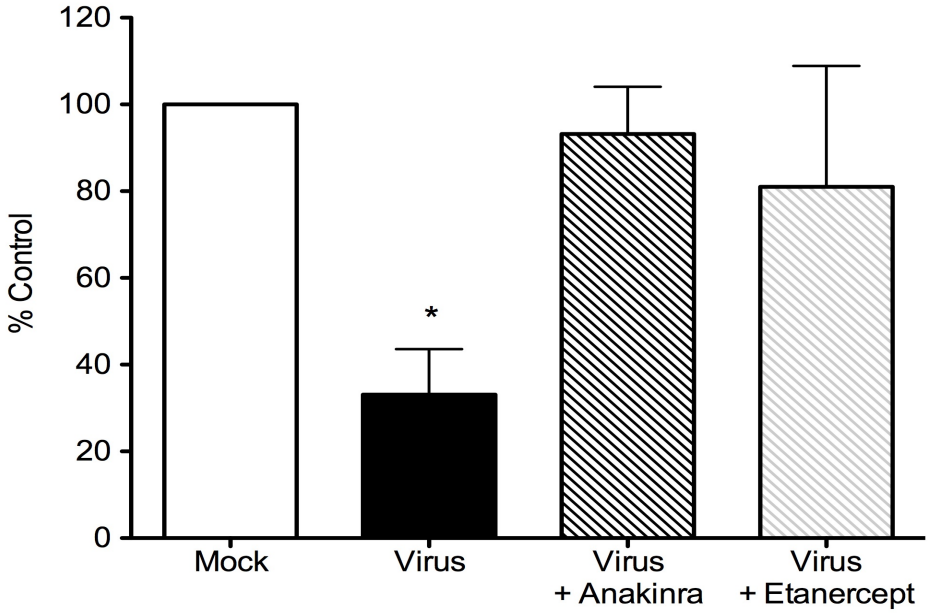

Figure 4.4. Guinea pigs were infected with parainfluenza virus and treated with either etanercept (3 mg/kg, i.p., as a single dose) or anakinra (30 mg/kg, i.p., daily). Four days after infection, tracheas were removed and stained with a 0.05% neutral red solution for 30 mins. Individual parasympathetic ganglia were isolated and pooled together to isolate RNA from each animal. RT-PCR was used to quantify M_2 receptor expression. M_2 receptor mRNA expression was significantly decreased in parainfluenza virus-infected guinea pigs compared to mock-infected animals. Infected animals pre-treated with anakinra or etanercept, to block IL-1β and TNF-α respectively, prevented the decrease in M_2 receptor expression. Data are represented as ± SEM, n=5-9. *$P < 0.05$, compared with mock-infected control group.

FIGURE 4.5 Treatment of parainfluenza virus-infected animals with anakinra or etanercept did not change lung viral titers and no viral message could be detected in parasympathetic ganglia.

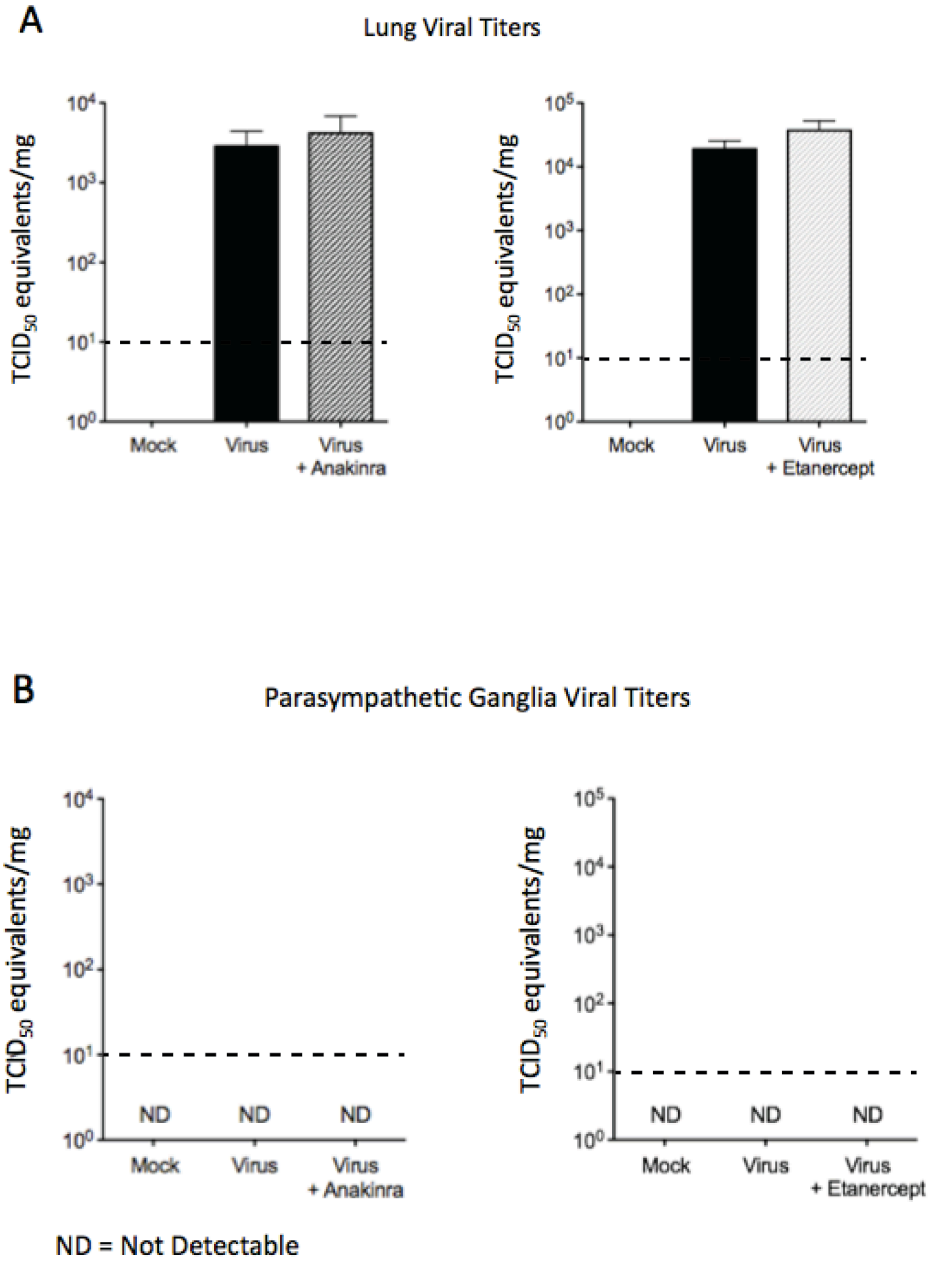

Figure 4.5. Guinea pigs were infected with parainfluenza virus (1 x 10^6 $TCID_{50}$) and pre-treated with either etanercept (3 mg/kg, i.p.) 24 hrs before infection, or anakinra (30 mg/kg, i.p.) 30 min prior to infection with daily injections until 4 days after infection. Four days after infection, RNA was extracted from lung homogenates and viral message was measured by RT-PCR. (A) Anakinra and etanercept pre-treatment did not significantly change viral message in the lungs compared to infected animals with no pre-treatment, n=6-9. Viral titers were normalized to 18S rRNA and tissue culture infective dose ($TCID_{50}$) was determined from a parainfluenza standard curve. Dashed lines indicate the limit of detection. Data are shown as ± SEM. (B) No parainfluenza virus message could be detected in guinea pig parasympathetic neurons. Data are represented as ± SEM, n=6-9.

DISCUSSION

The data in this chapter introduce a novel method for dissecting parasympathetic ganglia and analyzing mRNA expression from guinea pig tracheas. This is demonstrated by the ability to isolate parasympathetic ganglia, extract RNA, and effectively measure gene expression by qRT-PCR. The method presented here shows that parainfluenza decreases M_2 receptor mRNA expression in guinea pigs four days after infection. Blocking TNF-α (with etanercept) or IL-1β (with anakinra) before infection can prevent this decrease(Figure 4.4). This indicates that IL-1β and TNF-α are involved in decreasing M_2 receptor expression in parasympathetic ganglia during infection. This further supports previous findings that TNF-α is a mediator of virus-induced neuronal M_2 receptor dysfunction and airway hyperreactivity *in vivo* (Nie *et al.*, 2011).

The dissection technique was developed to isolate parasympathetic ganglia from the airway wall of guinea pigs (Figure 4.1 – 4.3). Each ganglion consists of 1-60 neurons and are located along the dorsal adventitia surface of the trachea and bronchi of most species (Baker *et al.*, 1986; Baluk and Gabella, 1989; Lees *et al.*, 1997; Yamamoto *et al.*, 1998; Myers, 2000). The parasympathetic ganglia were located in or closely opposed to the trachealis muscle and evenly distributed, which confirms previous findings in guinea pigs (Lees *et al.*, 1997; Fischer *et al.*, 1998; Baluk and Gabella, 1989; Myers, 2000). Each ganglion usually gave 3-4 processes,

which also confirms reports of 3-8 processes projecting from guinea pig tracheal neurons (Lees *et al.*, 1997).

Even though I was able to consistently obtain 10-12 ganglia and reliably get measureable levels of gene expression, I was never able to dissect out every ganglion. Two populations of ganglion cells have been identified in the trachea of cats and ferrets (Mitchell *et al.*, 1987; Cameron and Coburn, 1984; Coburn and Kalia, 1986; Baker *et al.*, 1986). The two types of ganglion cells have slightly different sizes and electrophysiological characteristics (Mitchell *et al.*, 1987). In addition, the larger ganglia of 12 or more nerve cells comprise only 10-20% of the ganglia (Baluk and Gabella, 1989), so the inability to obtain all of the small ganglia could possibly result in a difference in the degree to which M_2 receptor expression is decreased in these studies. A laser capture microdissection technique (Espina *et al.*, 2006) and single cell RNA isolation could be used to extract these remaining ganglia in future studies to further study and verify the effect of virus infection on M_2 receptor expression in parasympathetic ganglia.

The parasympathetic ganglia dissection technique presented here can be used to measure the expression of a variety of genes. While only M_2 receptor, PGP 9.5, and viral RNA expression were looked at in these studies, other expression of genes has been measured, including ICAM-1 and CCL11 (Buels, KS, unpublished data). Once RNA is isolated from the ganglia and transcribed into cDNA, it can be used in other

platforms beyond qRT-PCR, such as microarrays, which can have the added benefit of providing whole genome expression profiles. The method can also be used in different species that have similar or larger tracheal size such as cat, canine, rabbit, or ferret. Due to the size of the mouse trachea, it seems less feasible to do in mice.

Using this dissection and staining technique I demonstrated that M_2 receptor expression is significantly decreased upon parainfluenza virus infection in guinea pigs (Figure 4.4). These data also indicate TNF-α is involved in the virus-induced decrease in expression since blocking TNF-α with etanercept prevents this decrease. Etanercept was also able to block antigen-challenged and virus-induced M_2 receptor dysfunction in guinea pigs (Nie *et al.*, 2009; Nie *et al.*, 2011), and now these data demonstrate that it happens at the level of gene transcription. These studies also support findings *in vitro* that show direct viral infection decreases M_2 receptor gene expression in cultured neuronal cells (Nie *et al.*, 2009; Jacoby *et al.*, 1998). Interestingly, blocking IL-1β with anakinra also fully prevented the decrease in M_2 receptor expression. This suggests IL-1β and TNF-α are involved in the same pathway that effect transcription regulation of M_2 receptor expression in parasympathetic neurons.

Blocking proinflammatory cytokines such as TNF-α and IL-1β can affect the ability to fight off infections. These cytokines prove to be important during infection as several viruses have immune evasion mechanism that target IL-1β or TNF-α to

increase virulence (Alcami and Koszinowski, 2000). Studies in IL-1 receptor knock-out (KO) mice showed increased mortality and persistence of influenza virus infection with only a slight increase in lung viral titers with IL-1R knock-out (KO) mice (Schmitz *et al.*, 2005). However, another study found a significant increase in viral titers in influenza infected IL-1R KO mice (Seki *et al.*, 2010). The data presented here show that that blocking IL-1β does not significantly alter parainfluenza virus lung viral titers (Figure 4.5). Previous studies also show TNF-α significantly inhibits viral replication and production of infectious particles for influenza (Van Campen, 1994) but not RSV (Hussell *et al.*, 2001) or parainfluenza virus (Nie *et al.*, 2011). However, one study did report increased viral replication when blocking TNF-α during parainfluenza virus infection in rats (Uhl *et al.*, 1998). I have also shown no difference in viral titer in the lungs after blocking TNF-α, which confirms findings for RSV and parainfluenza (Hussell *et al.*, 2001; Nie *et al.*, 2011). Its should be noted that these experiments only measured viral RNA in the lungs, so this was only a determination of replicating virus and not a direct correlation to the amount of infectious particles, which was not measured in these studies. However, previous studies show qRT-PCR detection correlates well with $TCID_{50}$ measurements for influenza (Quinlivan *et al.*, 2005) and parainfluenza virus (Aguilar *et al*., 2000).

In summary, the data presented here demonstrate that parainfluenza virus induces a decrease in M_2 receptor expression in parasympathetic ganglia. Pre-treating

guinea pigs with either anakinra, to block IL-1β, or etanercept, to block to TNF-α, resorted M_2 receptor expression in parasympathetic neurons. In addition, blocking either TNF-α or IL-1β did not alter viral content of the lung. The interaction between TNF-α and IL-1β and their effect on M_2 receptor expression and contractile responses is further investigated in Chapter VI.

CHAPTER V.

IL-1β MEDIATES M2 RECEPTOR DYSFUNCTION IN PARAINFLUENZA VIRUS-INFECTED GUINEA PIGS

ABSTRACT

Rationale: Viral infections cause asthma attacks. Viruses cause loss of function of inhibitory M_2 receptors on parasympathetic nerves. Blocking IL-1β prevents M_2 receptor dysfunction in antigen-challenged guinea pigs. Here I have investigated whether blocking IL-1β would prevent M_2 receptor dysfunction in virus-infected guinea pigs.

Methods: Guinea pigs were infected with parainfluenza. In some animals, IL-1β was blocked with anakinra (30 mg/kg, i.p.) 30min before infection and every 24 hours after infection. Four days after infection, guinea pigs were mechanically ventilated and M_2 receptor function was measured as the ability of gallamine to increase vagally stimulated bronchoconstriction. Viral titers were determined by measuring viral RNA in the lungs. White cells in blood and lung lavage were counted.

Results: Virus infection caused M_2 receptor dysfunction, seen as a loss of the ability of gallamine to increase vagally mediated bronchoconstriction. Blocking IL-1β with anakinra prevented M_2 receptor dysfunction in infected animals. White cell counts were increased in lavage and decreased in the blood of infected animals. Anakinra did not affect viral titers or white cell counts.

Conclusions: Treatment with an IL-1β receptor antagonist prevents M_2 receptor dysfunction in parainfluenza infected guinea pigs.

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INTRODUCTION

The majority of asthma attacks are associated with viral infection, which can be detected in 80% of acute asthma exacerbations in children (Johnston *et al.*, 1995) and 50% in adults (Atmar *et al.*, 1998). Viral infection can cause airway hyperreactivity and increased airway resistance even in a healthy individual (Empey *et al.*, 1976; Aquilina *et al.*, 1980; Johanson *et al.*, 1969). Virus infection can cause increased bronchoconstriction and airway hyperreactivity by disrupting the function of parasympathetic nerves that provide autonomic control of the airways (Fryer and Jacoby, 1991; Nie *et al.*, 2011; Lee *et al.*, 2004; Adamko *et al.*, 2003; Jacoby and Fryer, 1991).

Parasympathetic nerves mediate bronchoconstriction by releasing acetylcholine (ACh) from their nerve endings onto airway smooth muscle, causing contraction (Nadel and Barnes, 1984; Haddad *et al.*, 1991; Roffel *et al.*, 1990a). ACh also binds to M_2 receptors on the nerves, which inhibits further ACh release and limits bronchoconstriction (Fryer and Maclagan, 1984). Parainfluenza virus induces airway hyperreactivity by decreasing M_2 muscarinic receptor function on parasympathetic nerves (Fryer and Jacoby, 1991), and the loss of M_2 receptor negative feedback causes more ACh to be released onto airway smooth muscle (Fryer and Wills-Karp, 1991). M_2 receptor dysfunction is found in some asthmatic patients (Minette *et al.*, 1989), and leads to airway hyperreactivity after virus

infection or double-stranded RNA production (Fryer *et al.*, 1994; Bowerfind *et al.*, 2002).

IL-1β is a proinflammatory cytokine induced during virus infection in response to double-stranded RNA production during viral replication. IL-1β is elevated in both symptomatic and asymptomatic asthmatic bronchoalveolar lavage (BAL) fluid (Broide *et al.*, 1992; Borish *et al.*, 1992; Jarjour and Busse, 1995), asthmatic bronchial epithelium (Sousa *et al.*, 1996), monocytes (Tomita *et al.*, 1995), and alveolar macrophages from asthmatic patients (Sousa *et al.*, 1996; Borish *et al.*, 1992; Gosset *et al.*, 1991). Moreover, asthmatics have increased IL-1β levels and decreased IL-1β receptor antagonist (IL-1βRA) levels after rhinovirus infection (de Kluijver *et al.*, 2003). IL-1β gene polymorphisms has been associated in men with asthma (Karjalainen *et al.*, 2002) and IL-1RA gene polymorphisms is increased in German, Italian, and Turkish families with asthma (Pattaro *et al.*, 2006; Gohlke *et al.*, 2004; Zeyrek *et al.*, 2008). In addition, IL-1β-mediated activation of TH_2 cells is necessary for airway hyperreactivity (Nakae *et al.*, 2003; Schmitz *et al.*, 2003). Blocking IL-1β or IL-1 receptors prevents ozone-induced and antigen-challenged airway hyperreactivity (Verhein *et al.*, 2008; Selig and Tocker, 1992; Watson *et al.*, 1993). Furthermore, parainfluenza virus-treated cells increase production of IL-1β (Yoshizumi *et al.*, 2010; Hua *et al.*, 1996) and blocking IL-1β prevents bronchial hyperresponsiveness in isolated rhinovirus-infected tissue (Hakonarson *et al.*,

1999a); however, the role of IL-1β has not been investigated in a parainfluenza virus infection animal model.

To determine the effects of IL-1β on M_2 receptor function, these studies tested the effects of blocking IL-1β with anakinra during parainfluenza virus infection in guinea pigs. These data demonstrate that blocking IL-1β prevented M_2 receptor dysfunction and blocking IL-1β prevented a decrease in M_2 receptor expression after infection in parasympathetic nerves *in vivo*.

EXPERIMENTAL DESIGN

Virus Infection

Guinea pigs were infected with 250 µl 1 x 10^6 $TCID_{50}$ parainfluenza virus (in PBS) or PBS. See chapter II methods for more detailed infection methods and for growing and tittering of virus.

Treatments

Some infected guinea pigs (300 g) were treated with an IL-1β receptor antagonist, anakinra (30 mg/kg, i.p.), or vehicle (6.5 mM sodium citrate, 140 mM NaCl, 48 mM EDTA, 1 mg/ml polysorbate 80) before infection. Anakinra was administered 30 min before infection and once daily until 4 days after infection. See Chapter II methods for schematic of treatment schedule.

Measurements

Four days after infection, *in vivo* airway physiology experiments to measure pulmonary inflation pressure and M_2 receptor expression were conducted, and animals were euthanized through aortic exsanguinations. Bronchoalveolar lavage (BAL), blood, and lung tissue were harvested after death of the animal. Total and differential leukocytes were counted from collected blood and BAL. Viral RNA was isolated from homogenized lung tissue using cells RNeasy kit and quantified by qRT-PCR. See Chapter II for detailed protocols of these methods.

RESULTS

Effects of blocking IL-1β on M_2 muscarinic receptor function

Guinea pigs were matched in age before experiments. There were no differences in heart rate, blood pressure, or weights among the groups of animals (Table 5.1).

Electrical stimulation of the vagus nerves produced repeatable, frequency-dependent bronchoconstrictions. M_2 receptor function was measured as the ability of gallamine (0.1-10 mg/kg i.v.), an M_2 receptor antagonist, to potentiate bronchoconstrictions, measured as an increase in pulmonary inflation pressure above baseline constrictions. This increase is due to a blockade of the inhibitory M_2 receptor of the on postganglionic nerves resulting in an increase in ACh release from nerves. Parainfluenza virus infected animals had a decreased response to gallamine compared to mock-infected animals, indicating M_2 receptor dysfunction (Fig. 5.1A). Animals pre-treated with anakinra to block IL-1β had normal M_2 receptor function after infection.

M_2 receptors on cardiac muscle mediate ACh-induced bradycardia. Gallamine (0.1-10 mg/kg i.v.) blocked M_2 receptors on cardiac muscle and prevented vagally mediated decreases in heart rate in all groups (Fig. 5.1B). Thus neither virus infection nor anakinra treatment affected the M_2 receptors in the heart.

Effects of blocking IL-1β on airway smooth muscle and cardiac muscle

ACh (1-10 μg/kg, i.v.) produced dose-dependant bronchoconstriction by binding to M_3 muscarinic receptors on airway smooth muscle (Fig. 5.2A) and bradycardia by binding to M_2 muscarinic receptor on cardiac muscle (Fig. 5.2B). Neither virus nor anakinra affected ACh-induced bronchoconstriction or bradycardia compared to controls, indicating that cardiac and airway smooth muscle responsiveness was not altered by infection or drug treatment.

Effect of virus infection and blocking IL-1β on inflammatory cells

Virus infection decreased total inflammatory cells in the blood (Fig. 5.3A). The leukopenia was largely due to a decrease in neutrophils and lymphocytes. This change was not changed by anakinra. In the BAL fluid of the lungs, virus infection increased total inflammatory cells compared to mock-infected, and again this was not changed by anakinra (Fig 5.3B). The increase was mostly due to an influx of macrophages and neutrophils during infection. The changes in cell populations in the blood and BAL were not significantly different between virus-infected and anakinra-treated animals.

Effects of blocking IL-1β or TNF-α on viral replication in the lungs

Guinea pig lungs were homogenized, and viral titers were assessed by real time PCR. Infected animals had viral titers less than a log different treated groups (Fig. 5.4A,B). Infected animals had an average titer of 2.86 ±1.6 x10^3 $TCID_{50}$/mg, which was similar to animals treated with anakinra (4.16 ±2.7 x10^3 $TCID_{50}$/mg).

TABLE 5.1. Baseline physiology measurements.

	Weight (g)	**Ppi, mmH_20**	**Heart Rate, bpm**	**Systolic BP, mmHg**	**Diastolic BP, mmHg**
Mock	401.6 ± 26.3	80 ± 9.5	235 ± 12.5	33.6 ± 3.2	13.6 ± 2.2
Virus	368.8 ± 21.5	76 ± 5.8	263 ± 10.9	40 ± 3.4	14.4 ± 1.4
Virus + Vehicle	363.8 ± 2.0	99 ± 8.1	267 ± 21.7	35.6 ± 7.5	14.2 ± 2.1
Virus + Anakinra	360.8 ± 14.0	97.5 ± 6.3	250 ± 4.1	35.5 ± 6.3	12.3 ± 0.6

Abbreviations: Ppi, pulmonary inflation pressure; bpm, beats per minute; BP, blood pressure. Data is represented as ± SEM.

FIGURE 5.1. Parainfluenza virus infection induces pulmonary M_2 receptor dysfunction, which is prevented by blocking IL-1β.

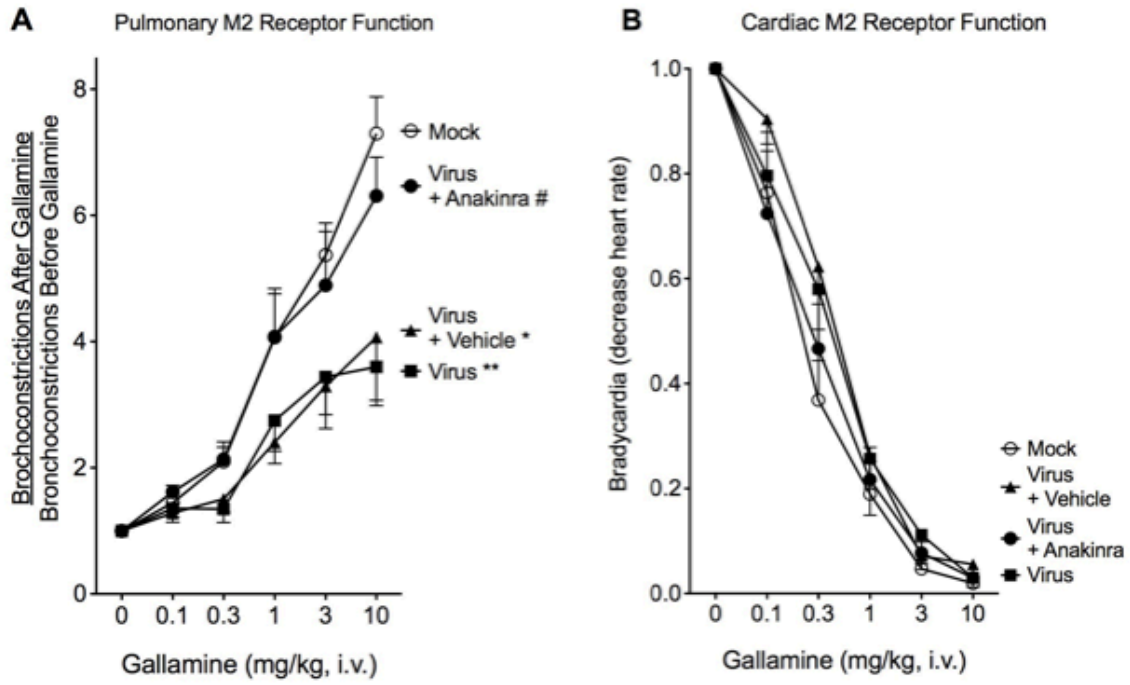

Figure 5.1. Guinea pigs were treated with an IL-1β receptor antagonist, anakinra (30 mg/kg/day, i.p.), 30 min before parainfluenza virus infection (1 x 10^6 $TCID_{50}$) or PBS mock infection. Bronchoconstriction were induced by electrical stimulation of both the vagus nerves four days after infection (15Hz, 10V, 0.2ms duration, 5sec on, 60sec off intervals). M_2 receptor function was measured as the potentiation in bronchoconstrictions after increasing doses of gallamine (0.1-10 mg/kg i.v.), an M_2 receptor antagonist, was administered. (A) Gallamine potentiated bronchoconstrictions in mock-infected animals (A,B white circles). The parainfluenza virus infected animals had significantly reduced responses to gallamine indicating M_2 receptor dysfunction (A,B black squares). Pre-treatment

with anakinra to block IL-1β prevented M_2 receptor dysfunction in virus-infected animals (A,B black circles). Vehicle treatment did not prevent M_2 receptor dysfunction in infected animals (A,B black triangles). (B) Gallamine blocked cardiac M_2 receptor function and prevented the fall in heart rate (bradycardia) in mock-infected animals, which was unaffected by parainfluenza virus infection, anakinra, or vehicle treatment. Data are expressed as ±SEM, n=4-5. *$P < 0.05$ and **$P < 0.005$, compared with mock-infected control group. #$P < 0.05$, compared with infected group.

FIGURE 5.2. Neither virus infection nor blocking IL-1β affected acetylcholine-induced bronchoconstrictions or bradycardia.

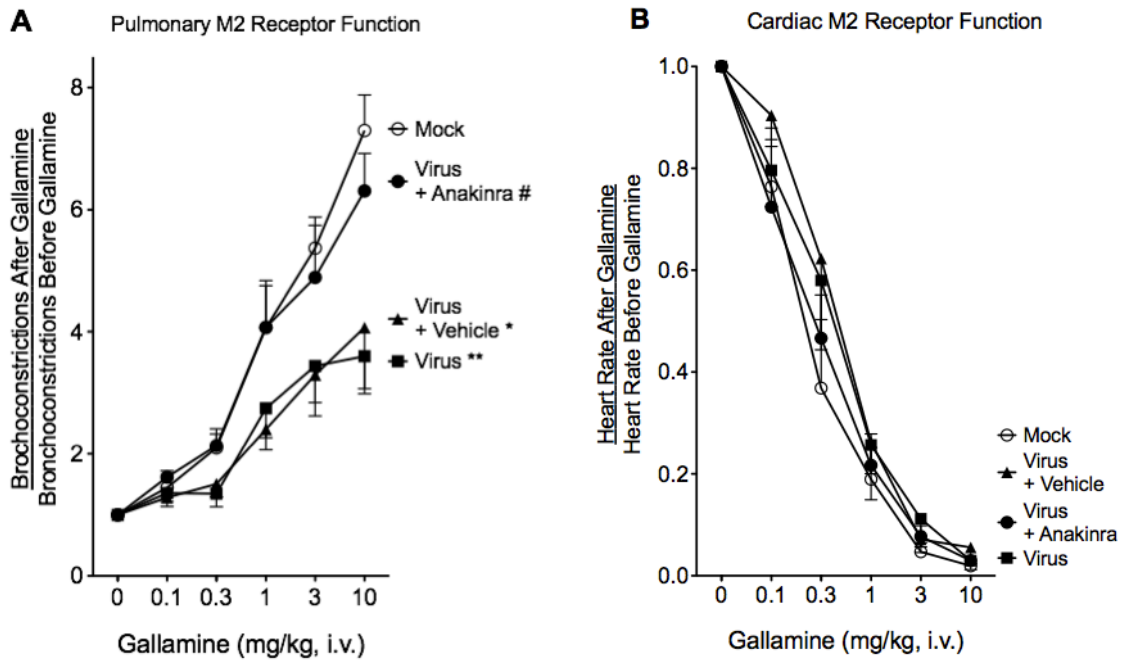

Figure 5.2. Guinea pigs were treated with an IL-1β receptor antagonist, anakinra (30 mg/kg/day, i.p.), 30 min before parainfluenza virus infection (1 x 10^6 $TCID_{50}$) or PBS mock infection. ACh-induced (1-10μg/kg, i.v.) bronchoconstrictions (B) and bradycardia (B) were not changed with virus infection or anakinra treatment. Vehicle treatment did not change bronchoconstrictions or bradycardia in response to ACh. Data is expressed as ±SEM, n=4-5.

FIGURE 5.3. Parainfluenza infection alters leukocytes in the blood and bronchoalveolar lavage (BAL) of guinea pigs. Neither is significantly affected by blocking IL-1β.

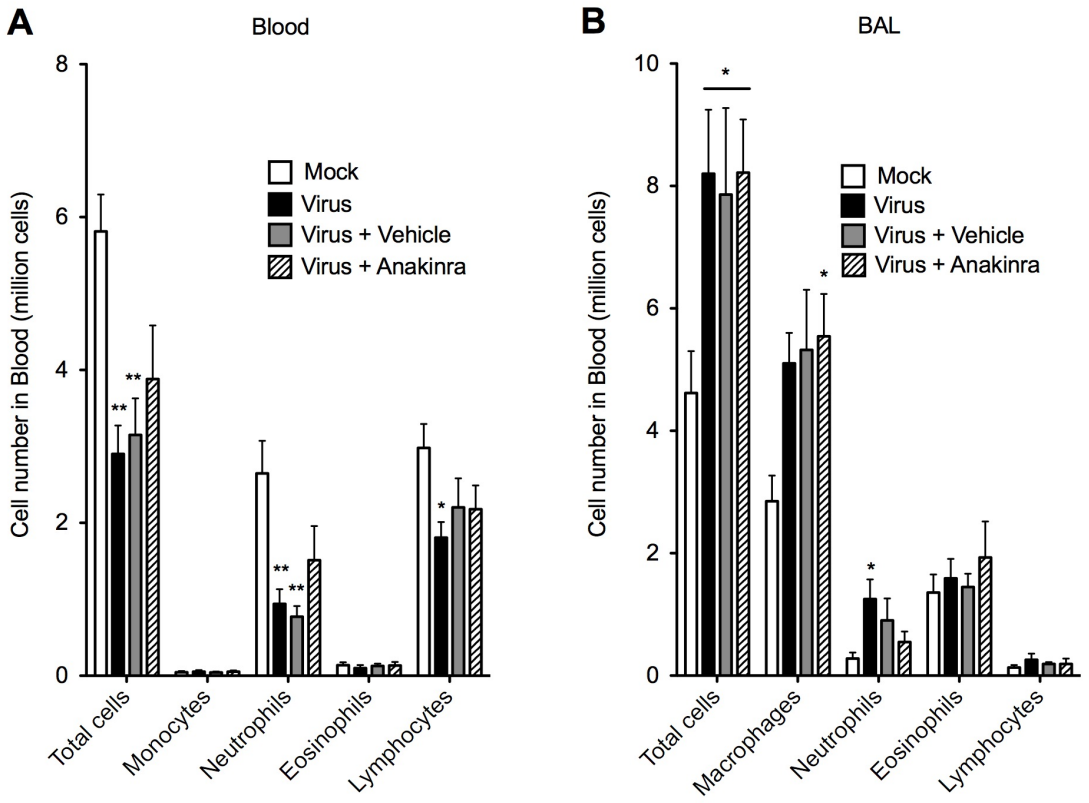

Figure 5.3. Blood was collected at the end of each experiment and red blood cells were hydrolysed. Lungs were lavaged with PBS. Total cells were counted and stained differential cell populations were counted. (A) Infection decreased total blood leukocytes, which was mainly neutrophils and lymphocytes. This was not significantly different with anakinra treatment, n=8-9 (B) Infection increased total BAL leukocytes, mainly neutrophils and macrophages. Again, this was not significantly affected by anakinra treatment, n=7-9. Data are represented as ± SEM

*P < 0.05 significance compared with control group. **P < 0.01 significance compared with control group.

FIGURE 5.4. Treatment of parainfluenza virus-infected animals with anakinra did not change lung viral tite

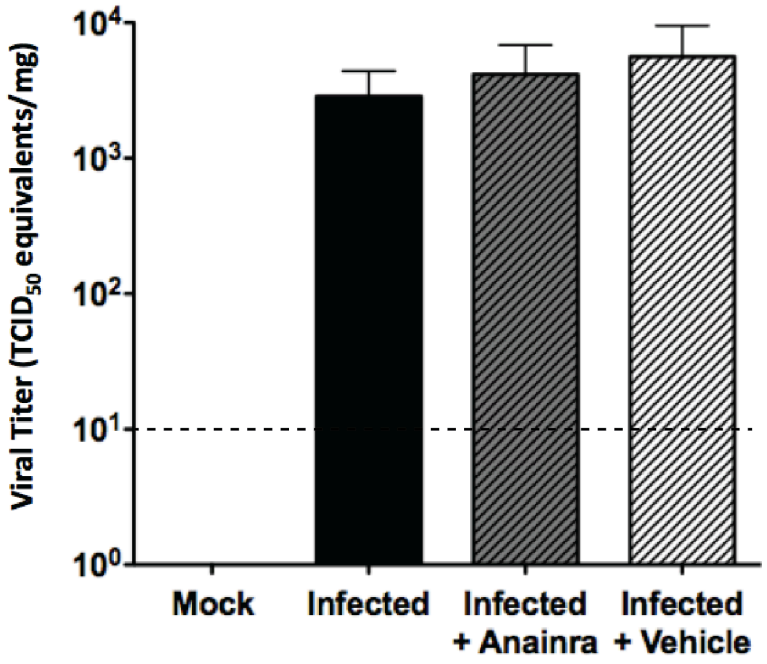

Figure 5.4. Guinea pigs were pre-treated with anakinra (30 mg/kg/day, i.p.) before parainfluenza virus infection (1 x 10^6 $TCID_{50}$). RNA was extracted from lung homogenates and viral message was measured by RT-PCR. Anakinra or vehicle pre-treatment did not significantly change viral message in the lungs four days after infection compared to infected animals with no pre-treatment, n=6-9. Viral titers were normalized to 18S rRNA and tissue culture infective dose ($TCID_{50}$) was determined from a parainfluenza standard curve. Dashed lines indicate the limit of detection. Data are shown as ± SEM.

DISCUSSION

Virus infection causes M_2 receptor dysfunction and airway hyperreactivity (Fryer and Jacoby, 1991; Lee *et al.*, 2004) and is associated with the majority of asthma exacerbations (Johnston *et al.*, 1995; Atmar *et al.*, 1998). Here I showed that IL-1β mediates neuronal M_2 receptor dysfunction during parainfluenza virus infection *in vivo* (Figure 5.1). I also demonstrated blocking IL-1β did not affect lung viral titers or white cell populations in the blood or BAL (Figure 5.3 and 5.4).

IL-1β is increased in parainfluenza virus-treated cells (Yoshizumi *et al.*, 2010; Hua *et al.*, 1996) and blocking IL-1β prevents airway hyperresponsiveness in virus-infected tissue (Hakonarson *et al.*, 1999a). To determine if IL-1β mediates M_2 receptor dysfunction *in vivo*, I infected guinea pigs with parainfluenza and measured M_2 receptor function. Parainfluenza virus infection decreased M_2 receptor function on postganglionic nerves, confirming previous findings (Fryer and Jacoby, 1991; Lee *et al.*, 2004; Nie *et al.*, 2009; Fryer *et al.*, 1994; Adamko *et al.*, 2003), and I now showed blocking IL-1β with anakinra restored M_2 receptor function (Figure 5.1). This demonstrates that IL-1β mediates M_2 receptor dysfunction during respiratory viral infection. There was no difference in M_2 receptor function between virus alone and vehicle-treated animals.

In addition, ACh-induced bronchoconstrictions were not changed in virus-infected animals compared to controls, which matches other reports with parainfluenza virus infection (Nie *et al.*, 2011; Drake *et al.*, 2013), and there was no difference between any virus treated groups (Figure 5.2). Since there was no increase in bronchoconstrictions with intravenous ACh shown in these studies, and thus no changes in M_3 receptor function on airway smooth muscle or possible change in acetylcholinesterase activity (the enzyme that hydrolyzes ACh), it seems likely that airway hyperreactivity is mediated by virus-induced M_2 receptor dysfunction and prevented by blocking IL-1β.

Virus infection alters inflammatory cell populations in the blood and the lungs (Figure 5.3). I demonstrated a decrease in total white blood cells in the periphery, which has been previously documented with parainfluenza virus (Fryer *et al.*, 1994; Drake *et al.*, 2013). This leukopenia was mainly due to a decrease in neutrophils and lymphocytes. An increase in total cells in the BAL was also shown, which demonstrated there was an increase in leukocyte infiltrates into the lungs that is consistent with previous findings with parainfluenza (Fryer *et al.*, 1994; Adamko *et al.*, 2003; Drake *et al.*, 2013). This was due to increased macrophages and neutrophils, similar to prior data (Fryer *et al.*, 1994; Drake *et al.*, 2013). The change in cell populations in the lung lavage and blood was not significantly changed with anakinra treatment compared to virus alone.

There has been a documented role of several immune cells in M_2 receptor function with parainfluenza virus infection. Eosinophils have been shown to be important in sensitized, virus-infected, but not in non-sensitized, virus-infected guinea pigs (Adamko *et al.*, 2003). Macrophages (Lee *et al.*, 2004), CD8+ T cells (Adamko *et al.*, 2003), and total leukocyte (Fryer *et al.*, 1994) depletion also protects M_2 receptor dysfunction after infection. In my studies, there was not a significant change in differential leukocyte populations in the blood or BAL with infection with anakinra treatment compared to virus alone. There was a slight, non-significant increase in neutrophil numbers in the blood with anakinra treatment (Figure 5.3A). RSV-infected (Wang *et al.*, 1998) and influenza-infected (Bordon *et al.*, 2013b) humans demonstrate increased neutrophil apoptosis upon infection as a way to limit inflammation and leakage of intracellular contents (Savill *et al.*, 1989; Bordon *et al.*, 2013a), and blocking IL-1β may modestly prevent apoptosis, possibly through TNF-α or prostaglandin E2 (PGE_2) (Cross *et al.*, 2008; Ottonello *et al.*, 1998; Molina-Holgado *et al.*, 2000; Akgul and Edwards, 2003).

There was also a slight non-significant decrease in neutrophils in the BAL with anakinra treatment, which indicates IL-1β is important for neutrophil recruitment to the lungs during parainfluenza infection (Figure 5.3B). This may be mediated through up-regulation of ICAM-1 or other adhesion molecules (Ley *et al.*, 2007; Sadik *et al.*, 2011), as expression levels are increased in tracheal epithelial cells during rhinovirus infection and this is mediated by IL-1β (Terajima *et al.*, 1997).

Data also show IL-1 receptor KO mice have decreased neutrophil recruitment involving a decrease in expression of the chemotactic protein, macrophage inflammatory protein-2 (Kato *et al.*, 2002). Neutrophil recruitment can also be initiated through leukotriene B4 (LTB_4) production (Lammermann *et al.*, 2013; Sadik *et al.*, 2011), and IL-1β has been shown to increase neutrophil migration through LTB_4 release (Oliveira *et al.*, 2008). This indicates blocking virus-induced IL-1β production could be leading to decreased neutrophil inflammation in the lungs during parainfluenza virus infection through reduced adhesion molecule or chemoattractant proteins. Lastly, even though the cell number of each population didn't change with treatment, it is possible some of the cells types may have become primed and activated, which can induce adhesion molecules, chemokine, cytokine, and superoxide production that may contribute to M_2 receptor dysfunction with virus infection (Bermejo-Martin *et al.*, 2007; Terajima *et al.*, 1997; Busse *et al.*, 1991; Folkerts and Nijkamp, 1995; Bordon *et al.*, 2013a; Ryan and Majno, 1977; Hussell and Goulding, 2010).

These data demonstrate IL-1β-mediated loss of M_2 receptor function on parasympathetic nerves. Moreover, the studies show that targeting IL-1β may be beneficial in preventing the loss of inhibitory M_2 receptors and restoring normal nerve function during infection. These data suggest that blocking IL-1β can be a potentially effective treatment therapy for virus-induced asthma exacerbations.

CHAPTER VI.

IN HUMANS, IL-1β INDUCES DOWNREGULATION OF M2 RECEPTOR MRNA. TNF-α INCREASES CONTRACTILE RESPONSES, WHICH INVOLVES IL-1β PRODUCTION IN GUINEA PIG TRACHEAS *IN VITRO*.

ABSTRACT

Rationale: Viral infections are associated with the majority of asthma attacks in children and adults. Inhibitory M_2 receptors on parasympathetic nerves, which regulate acetylcholine (ACh) release, are dysfunctional after viral infection. Because IL-1β and TNF-α are upregulated during respiratory viral infections, and blocking either TNF-α or IL-1β prevents M_2 receptor dysfunction after infection, I investigated whether the cytokines work synergistically together or if one cytokine induces production of the other to cause M_2 receptor dysfunction during parainfluenza virus infection.

Methods: Human SK-N-SH neuroblastoma cells and cultured guinea pig parasympathetic neurons were treated with IL-1β for 6 hrs (0.01-100 ng/ml). Human parasympathetic neurons were treated IL-1β (0.01-10 ng/ml) for 6 hrs and M_2 receptor expression was measured by RT-PCR. Cultured guinea pig tracheal ring segments were treated with TNF-α (100 ng/ml) or IL-1β (100 ng/ml) for 4 days. Some TNF-α treated rings were also treated with an IL-1 receptor antagonist (100 ng/ml) and some IL-1β treated rings were treated with etanercept (100 ng/ml). Trachea rings were immersed in an organ bath and contractions were induced by electrical field stimulation (EFS) and MCh (10 μM). IL-1β protein in the supernatants from cultured trachea rings was measured by ELISA.

Results: Treatment of SK-N-SH neuroblastoma cells, primary cultures of guinea pig, and human parasympathetic neurons with IL-1β decreased M_2 receptor mRNA. The decrease in M_2 receptor expression by IL-1β was not synergistic with TNF-α treatment in guinea pig parasympathetic neurons. Cultured segments of guinea pig tracheas treated with either TNF-α or IL-1β, increased trachea contractions in response to activation of airway nerves by electrical field stimulation. IL-1β was produced from the cultured trachea segments.

Conclusions: These data show that virus-induced hyperreactivity and M_2 dysfunction involves both IL-1β and TNF-α, likely in sequence with virus-induced TNF-α increasing production of IL-1β.

Data from figures 6.2-6.5 in this chapter have been submitted as:

Rynko, A., Fryer, A.D., Jacoby, D.B. Interleukin-1beta Mediates Virus-induced M_2 Muscarinic Receptor Dysfunction and Hyperreactivity. *Am J Respir Cell Mol Biol.* (Submitted).

Figures 6.1, 6.2B, and 6.6 are unpublished and unique to this chapter.

INTRODUCTION

Both IL-1β and TNF-α are pleiotropic cytokines that have been implicated in asthma pathogenesis and are upregulated during parainfluenza virus infection (Hallsworth *et al.*, 1994; Yoshizumi *et al.*, 2010; Holgate *et al.*, 2010). Both cytokines are released from macrophages or monocytes, T-cells, or infected epithelial cells (Gosset *et al.*, 1991; Lloyd and Hessel, 2010; Takeuchi and Akira, 2009; Message and Johnston, 2004; Nie *et al.*, 2011). In addition, both cytokines are crucial for the initiation of cytokine cascades that induce adhesion molecule expression and chemokine production that lead to leukocyte recruitment (Dinarello, 1997; Dinarello, 1989; Wajant *et al.*, 2003; Herbein and O'Brien, 2000).

Previous data demonstrate blocking TNF-α prevents M_2 receptor dysfunction and subsequent airway hyperreactivity in antigen-challenged (Nie *et al.*, 2009) and virus-infected (Nie *et al.*, 2011) guinea pigs. However, blocking IL-1β also prevents M_2 receptor dysfunction after infection (Chapter V). Given that blocking either cytokine can prevent M_2 receptor dysfunction, it seemed TNF-α and IL-1β might be working synergistically together, or that one cytokine is stimulating production of the other. Since previous findings show that IL-1β and TNF-α work synergistically to decrease M_2 receptor expression in human lung fibroblasts (Haddad *et al.*, 1996b), I investigated if the cytokines work synergistically in parasympathetic neurons. Furthermore, since IL-1β and TNF-α are each capable of regulating the other (Nawroth *et al.*, 1986; Tillie-Leblond *et al.*, 1999; Zhang and Rom, 1993; Zwerina *et*

al., 2007; Winzen *et al.*, 1993; Hultner *et al.*, 2000; Le and Vilcek, 1987), I also tested whether one cytokine is upstream of the other mediating an increased hyperresponsiveness in guinea pig tracheas.

In addition, blocking either cytokine prevented the virus-induced decrease in M_2 receptor expression *in vivo* (Chapter IV), and direct treatment of neurons with TNF-α alone decreases neuronal M_2 receptor expression *in vitro* (Nie *et al.*, 2009). However, it is not known if direct treatment of airway neurons with IL-1β decreases M_2 receptor mRNA, which I also investigated. In this chapter, I determined IL-1β directly downregulates M_2 receptor expression in cultured parasympathetic neurons and TNF-α is a major contributor to IL-1β production mediating increased contractile responses in guinea pigs.

EXPERIMENTAL DESIGN

Nerve culture experiments

SK-N-SH

Human SK-N-SH neuroblastoma cells were plated in 6 well culture dishes at 1.8 x 10^5 cells/well and grown until 70-80% confluent. Cells were either untreated or treated with 0.01-100 ng/ml human recombinant IL-1β for 6 hrs and 24 hrs. RNA was isolated using the RNeasy mini kit. RNA was reverse transcribed into cDNA and used in qRT-PCR. See Chapter II general methods for detailed culturing protocol.

Guinea pig primary parasympathetic neurons

Guinea pig parasympathetic nerves were cultured and isolated as previously described (Fryer *et al.*, 1996). Cells were then treated for 6 hrs with 0.001-0.1 ng/ml human recombinant IL-1β and 0.01-1 ng/ml recombinant guinea pig TNF-α or untreated. See Chapter II general methods for detailed culturing and nerve cluster removal protocol.

Human primary parasympathetic neurons

Human parasympathetic nerves from donated human tracheas were cultured as previously described (Fryer *et al.*, 2006; Nie *et al.*, 2007). Cells were then treated for 6 hrs with 0.01-0.10 ng/ml human recombinant IL-1β or untreated. RNA was

isolated using the RNeasy mini kit (Qiagen) and reverse transcribed into cDNA to use in qRT-PCR. See Chapter II general methods for detailed culturing protocol.

qRT-PCR

See Chapter II general methods for qRT-PCR method.

Isolation and culture of guinea pig trachea segments

Segmented guinea pig trachea rings were isolated and cultured as previously described (Makwana *et al.*, 2012). Briefly, guinea pigs trachea ring segments were untreated or treated with guinea pig recombinant TNF-α (100 ng/ml), human recombinant IL-1β (100 ng/ml), human recombinant IL-1 receptor antagonist (IL-1RA; 100 ng/ml), or etanercept (100 ng/ml) for 4 days. Some segments were treated with a combination of TNF-α (100 ng/ml) and IL-1RA (100 ng/ml) or IL-1β (100 ng/ml) and etanercept (100 ng/ml) at 37°C or 4 days. Media and cytokines were replaced daily. See Chapter II general methods for isolation and culturing protocol.

In vitro measurement of contractions

Contractions were measured *in vitro* as previously described (Murray and Jacoby, 1992). Trachea ring contractions were measured in a 5 ml organ bath. Tissues were suspended between platinum electrodes in the bath for electrical field stimulation (EFS). The tissue's ability to contract was measured by giving KCL (100 mM) and methacholine (MCh; 10 μM) directly to the bath with a 3 x 10 min wash between each drug. Contractile responses to EFS (40 V, 1-30 Hz, 0.5 msec duration, 60 sec on, 60 sec off intervals) were measured as the maximum increase above baseline tension. At the end of each experiment TTX, a voltage-gated Na channel blocker (1 μM), was given to confirm contractions were neurally mediated. See Chapter II general methods for detailed protocol.

Measurement of IL-1β In supernatant by ELISA

IL-1β protein levels were measured in supernatants from guinea trachea cultures using a guinea pig DuoSet ELISA kit (R&D). See Chapter II general methods for ELISA method.

RESULTS

Effect of IL-1β on M_2 receptor expression in human neurons in vitro.

To initially test the direct effects of IL-1β on neurons, I cultured human SK-N-SH neuroblastoma cells with IL-1β (1-500 ng/ml) for either 6 or 24 hours. RNA was isolated from cells and M_2 receptor expression was measured by qRT-PCR. The preliminary data show IL-1β induced a decrease in SK-N-SH cells at both 6 and 24 hours (Figure 6.1). IL-1β approximately decreased M_2 receptor expression 97.59% at the 6 hours and 87.35% at the 24 hours, which amounted to a 5.25-fold greater decrease at 6 hours compared to 24 hours. An approximate 10% greater decrease in receptor expression was seen at 6 hours compared to 24 hours at each dose tested.

Experiments were continued at the 6 hour time point to determine the effect of IL-1β on human cultured neurons. M_2 receptor expression was determined by real time qRT-PCR from human SK-N-SH neuroblastoma cells and from primary cultures of guinea pig airway parasympathetic neurons treated with IL-1β (0.01-100 ng/ml). IL-1β treatment produced a 92.61 ±0.86% decrease in M_2 mRNA at 100ng/ml and had an IC_{50} of 0.0138ng/ml in human SK-N-SH cells. (Figure 6.2A). IL-1β treatment decreased M_2 receptor mRNA expression in a dose dependant manner in human cultered parasympathetic neurons (Figure 6.2B).

Effects of combined IL-1β and TNF-α treatment on guinea pig parasympathetic neurons.

To test if the IL-1β and TNF-α were synergistic, guinea pig parasympathetic neurons were treated with combined doses of IL-1β (0.001-0.01ng/ml) and TNF-α (0.01-0.1ng/ml) for 6 hrs. While both cytokines alone decreased M_2 receptor expression, there was no synergistic decrease when the cytokines were combined (Figure 6.3).

Effects of TNF-α and IL-1β treatment on EFS- and MCh-induced contractions of isolated guinea pig tracheas.

To determine if the potentiated contractile response was due to one cytokine (TNF-α or IL-1β) inducing the production of the other, guinea pig trachea were cut into rings, and cultured for 4 days. The TNF-α blocker etanercept (100 ng/ml) added to some tracheas with or without IL-1β (100 ng/ml) and an IL-1 receptor antagonist IL-1RA (100 ng/ml) was added to some tracheas with TNF-α (100 ng/ml). After four days, tracheal contractions were measured in an organ bath. Tracheal segments gave reproducible contractions in response to electrical field stimulation (EFS) of airway nerves (40 V, 1-30 Hz, 0.5 ms duration, 60 sec on, 60 sec off intervals) and to KCL (100 mM), which depolarizes the smooth muscle membrane to induce contractions. Contractile responses to MCh (10 μM), added directly to the bath, were not changed between treatment groups (Fig 6.4C), indicating that the tissue hyperresponsiveness was not mediated by increased smooth muscle sensitivity. In

contrast, tracheal segments cultured with either TNF-α (100 ng/ml) or IL-1β (100 ng/ml) alone had increased contractile responses to EFS compared to untreated (Figure 6.4 A,B). Blocking IL-1β during TNF-α treatment prevented the potentiated contractions (Figure 6.4A). However, blocking TNF-α during IL-1β treatment did not (Figure 6.4B). This suggests that TNF-α is mediating increased contractile responses through increasing the production of IL-1β.

IL-β production from the supernatants of the cultured trachea rings was confirmed by ELISA (Figure 6.5). IL-1β was increased 5.91 ± 0.7-fold after 24 hrs when treated with TNF-α compared to untreated controls. Trachea segments treated with TNF-α and IL-1βRA also had a significant increase above controls (5.18 ± 0.31-fold) at 24 hours.

FIGURE 6.1. Direct treatment of human SK-N-SH neuroblastoma cells with IL-1β decreases M_2 receptor expression at 6 hrs and 24 hrs.

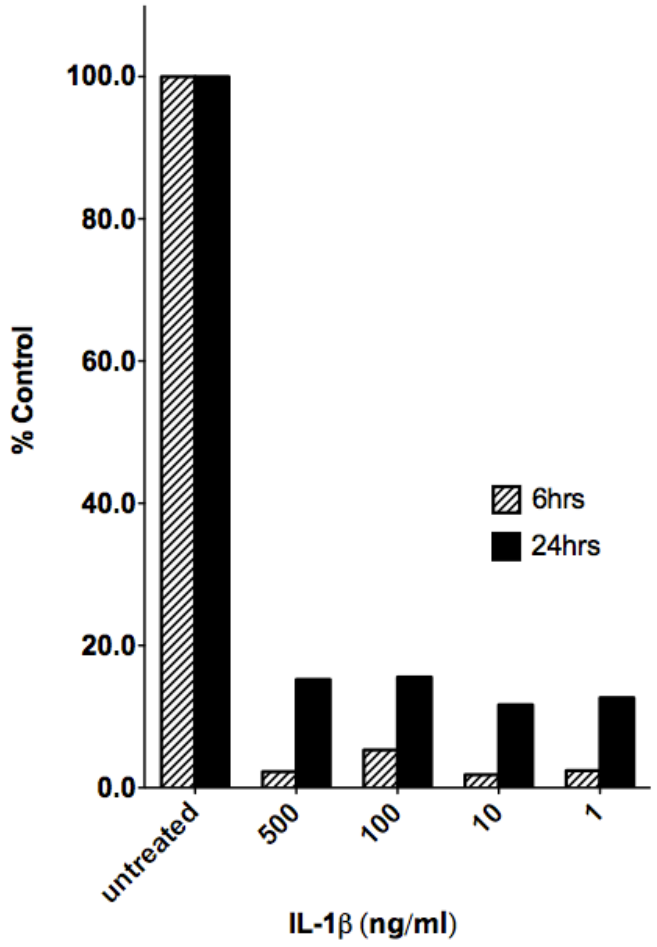

Figure 6.1. Human SK-N-SH neuroblastoma cells were treated for 6 hrs or 24 hrs with IL-1β (1-500 ng/ml). RNA was extracted from cells and M_2 receptor expression was quantified by RT-PCR and normalized to 18S rRNA. Preliminary data show IL-1β decreased M_2 receptor mRNA expression in SK-N-SH cells at 6 hrs and 24 hrs. n=1.

FIGURE 6.2. Direct treatment of human neuronal cells with IL-1β decreases M_2 receptor expression.

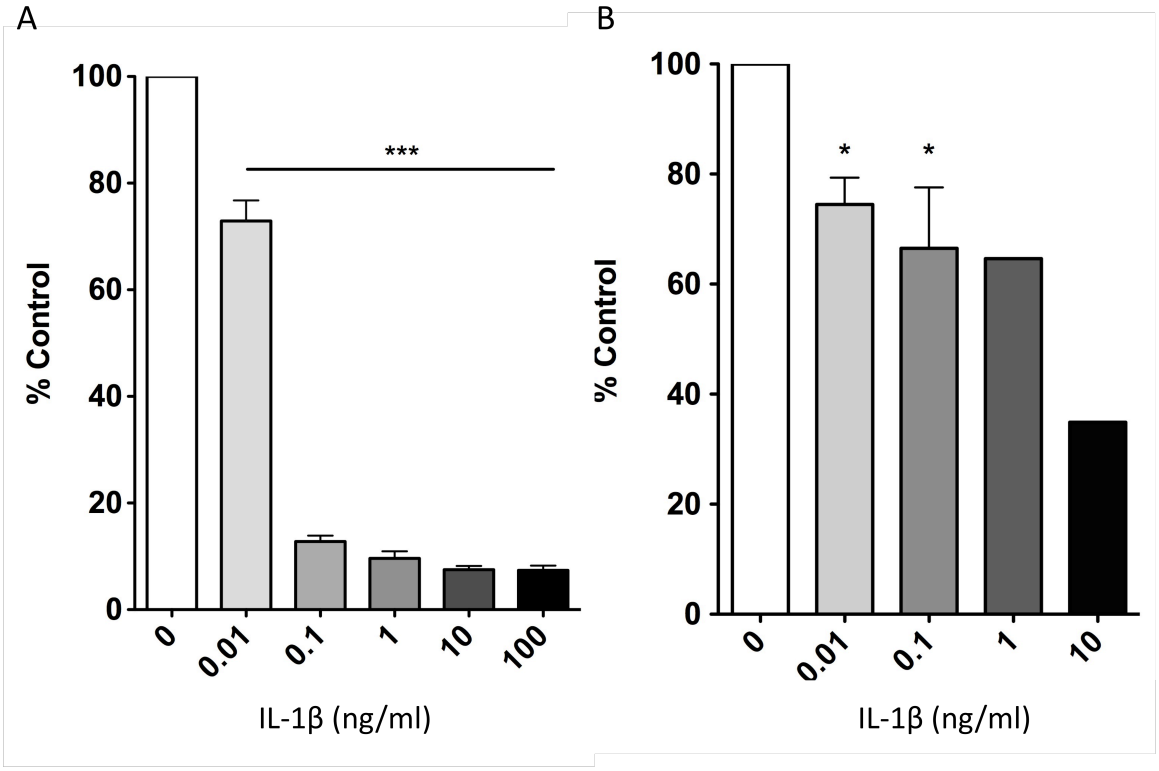

Figure 6.2. Human SK-N-SH neuroblastoma cells were treated for 6 hrs with IL-1β (0.01-100 ng/ml). Human parasympathetic neurons were treated for 6 hrs with IL-1β (0.01-10 ng/ml). RNA was extracted from cells and M_2 receptor expression was quantified by RT-PCR and normalized to 18S rRNA. (A) IL-1β caused a significant, dose-dependant decrease in M_2 receptor mRNA expression in SK-N-SH cells. n=3. ***P < 0.0001 significance compared with control group. (B) IL-1β significantly decreased M_2 receptor mRNA expression in human parasympathetic neurons at 0.1 and 0.001 ng/ml IL-1β, n=3. Preliminary data show M_2 receptor expression is decreased at 1 and 10 ng/ml doses of IL-1β, n=1. Data are represented as ± SEM.

FIGURE 6.3. Direct treatment of guinea pig parasympathetic neurons with IL-1β decreases M_2 receptor expression that is not synergistic with TNF-α treatment.

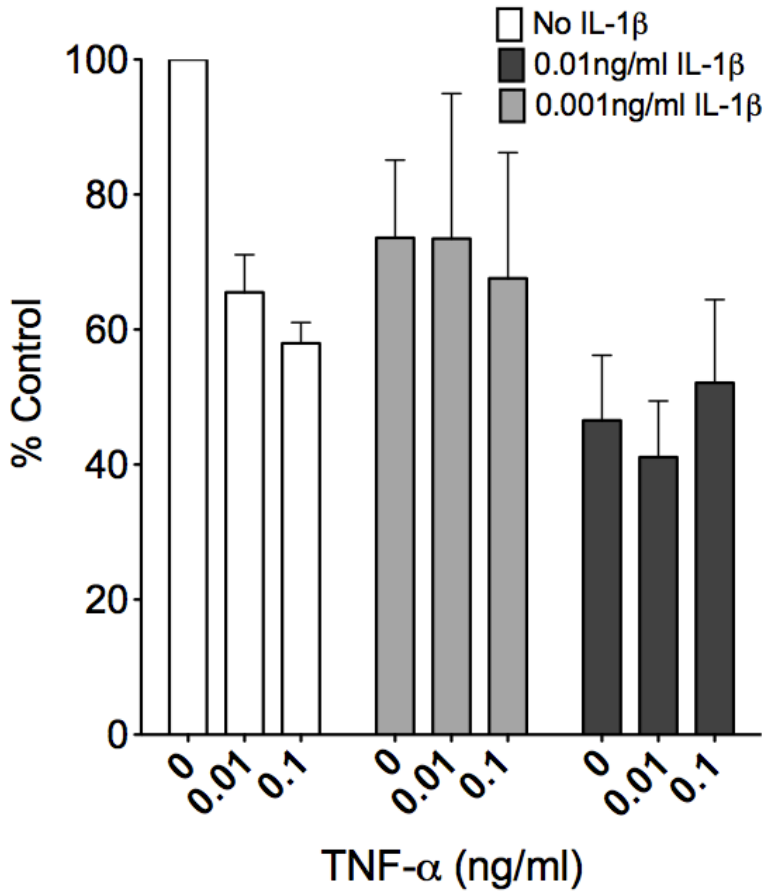

Figure 6.3. Parasympathetic neurons from guinea pig tracheas were cultured for 7 days and treated with combined doses of IL-1β (0.001-0.01 ng/ml) and TNF-α (0.01-0.1 ng/ml) for 6 hrs. RNA was extracted from cells and M_2 receptor expression was quantified by RT-PCR and normalized to 18S rRNA. IL-1β and TNF-α are not synergistic in decreasing M_2 receptor expression in cultured guinea pig airway parasympathetic neurons. Data are represented as ± SEM, n=3.

FIGURE 6.4. IL-1β and TNF-α increase guinea pig contractions *in vitro*; blocking IL-1β prevents the effect of TNF-α, but blocking TNF-α does not prevent the effect of IL-1β.

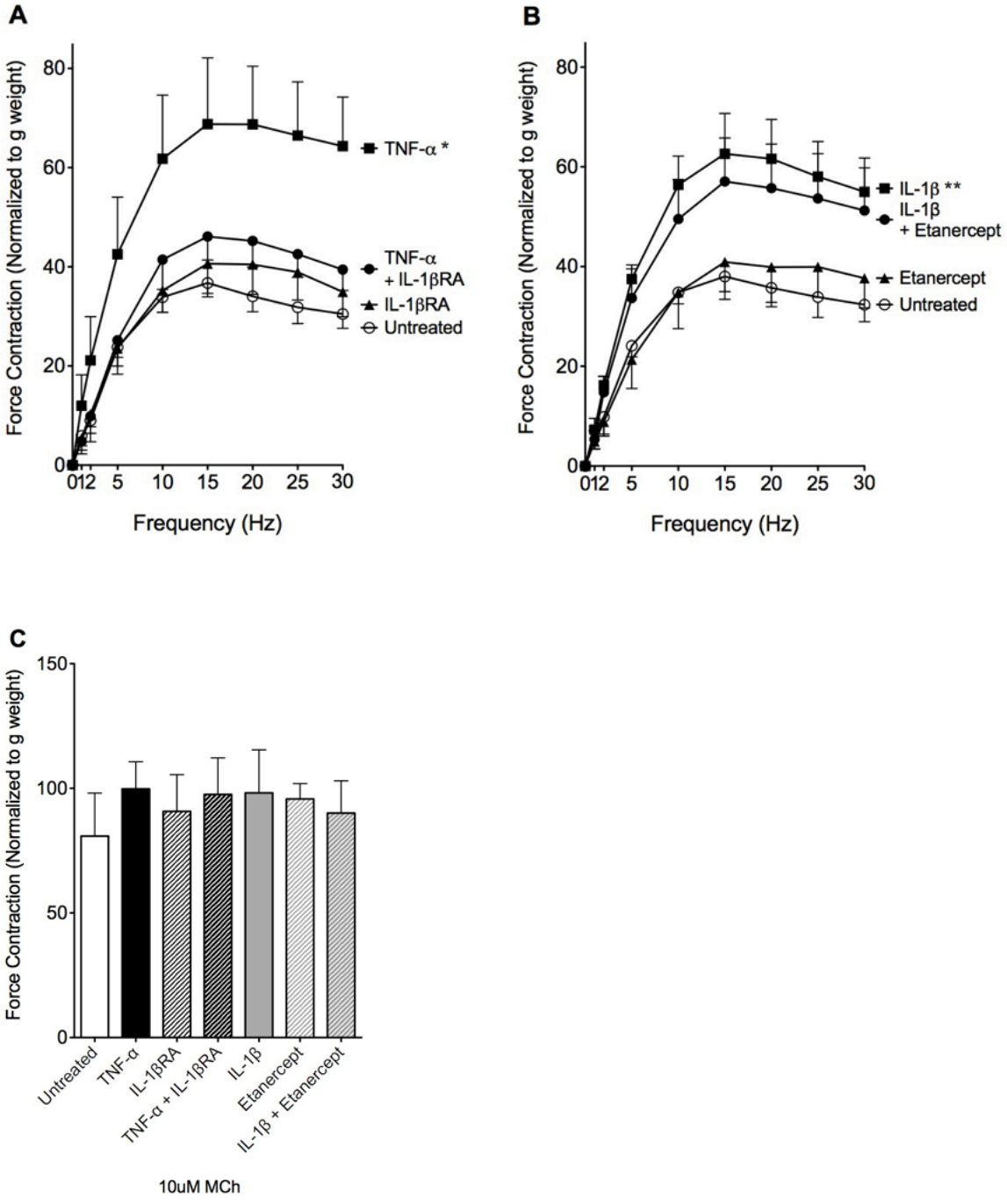

Figure 6.4. Guinea trachea rings were treated for 4 days with TNF-α (100 ng/ml) or IL-1β (100 ng/ml). Some TNF-α treated rings were also treated with an IL-1 receptor antagonist (100 ng/ml) and some IL-1β treated rings were treated with etanercept (100 ng/ml). Trachea rings were immersed in an organ bath and contractions were induced by electrical field stimulation (EFS; 40 V, 1-30 Hz, 0.5 msec duration, 60 sec on, 60 sec off intervals) and MCh (10 μM). Contractions were normalized to g tissue weight. (A) TNF-α potentiated EFS induced contractions (black squares) compared to untreated (white circles), which was prevented by blocking IL-1β during TNF-α treatment (black circles). The IL-1 receptor antagonist alone did not potentiate responses (black triangles). (B) IL-1β treatment also potentiated contractions (black squares) compared to untreated (white circles). In contrast, blocking TNF-α, with etanercept, in IL-1β treated groups did not prevent EFS-induced contractions (black circles). Blocking TNF-α alone did not potentiate responses (black triangles). (C) MCh-induced contractions were not different between treatments groups. Data are represented as ± SEM, n=4-9. *$P < 0.05$ significantly different from control group. **$P < 0.01$ significantly different from control group.

FIGURE 6.5. IL-1β is produced from guinea pig tracheas treated with TNF-α.

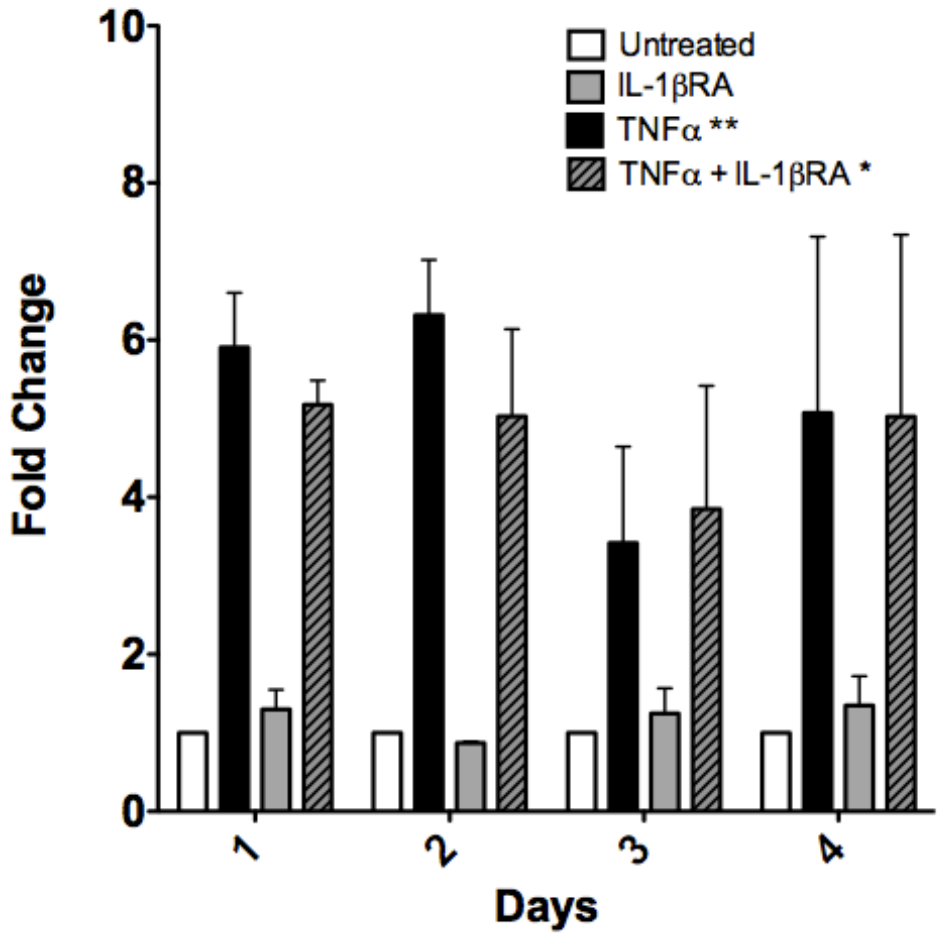

Figure 6.5. Guinea trachea rings were treated for 4 days with TNF-α (100 ng/ml). Some TNF-α treated rings were also treated with an IL-1 receptor antagonist (IL-1RA; 100 ng/ml). Media was changed daily and supernatants were used to determine IL-1β production by ELISA. IL-1β was produced in airway segments treated with TNF-α alone or with TNF-α and IL-1βRA. Data are represented as ± SEM, n=3. *P < 0.05 significantly different than control group. **P < 0.01 significantly different than control group.

DISCUSSION

Here I showed IL-1β decreased M_2 receptor mRNA expression in cultured neurons. These data also strongly suggest that viral infection of the airways leads to production of TNF-α, which induces production of IL-1β, and that it is IL-1β that ultimately mediates loss of neuronal expression and function of M_2 receptors, causing airway hyperreactivity (Chapter IV and V).

I also demonstrated that TNF-α and IL-1β did not work synergistically in the downregulation of M_2 receptor mRNA in neurons (Figure 6.3). TNF-α and IL-1β have been shown to work synergistically in fetal brain neurons to increase production of nitric oxide (Chao *et al.*, 1995) and human lung embryonic lung fibroblasts to decrease M_2 receptor expression (Haddad *et al.*, 1996b). Haddad *et al.* show M_2 receptor expression decreases only with combined IL-1β and TNF-α treatment and that neither IL-1β nor TNF-α alone could decrease receptor expression in fibroblasts (Haddad *et al.*, 1996b). In contrast, in more relevant cells, I demonstrated TNF-α (Nie *et al.*, 2009) or IL-1β alone decreases M_2 receptor expression in human SK-N-SH (Figure 6.2A) and primary cultures of human (Figure 6.2B) and guinea pig airway parasympathetic neurons (Figure 6.3). Thus, this demonstrated a mechanism for M_2 receptor down-regulation involving IL-1β and TNF-α that is different between fibroblasts and neurons. The decrease in M_2 receptor expression could be due to de novo protein seen with TNF-α and IL-1β treatment in fibroblasts (Haddad and Rousell, 1998; Haddad *et al.*, 1996b) or possibly by destabilization of

mRNA seen with TNF-α treatment in parasympathetic neurons (Nie *et al.*, 2009). The IC_{50} of IL-1β on M_2 receptor expression reported here is similar to findings of the IC_{50} of IL-1β on opioid receptor expression at the same time point and in the same cell line (Mohan *et al.*, 2010).

While the data presented here show a direct effect of IL-1β on M_2 receptor expression *in vitro*, it is possible other mechanisms involving IL-1β are happening *in vivo*. The M_2 receptor expression changes may also be due to regulation of transcriptional proteins, such as NF-κB. Both IL-1β and TNF-α can activate the transcription factor NF-κB (Beg *et al.*, 1993; Edwards *et al.*, 2005; Newton *et al.*, 1997), which has been shown to regulate the expression of several genes involved in inflammation responses, including the induction of interleukin-8 (IL-8) and intracellular adhesion molecule-1 (ICAM-1) (Stein *et al.*, 1993a; Baeuerle, 1991; Lenardo and Baltimore, 1989; Stein *et al.*, 1993b; Grilli *et al.*, 1993). For instance, stimulated monocytes and macrophages from asthma patients have increased IL-1β and TNF-α production with a 20-fold increase in IL-8 production compared to controls (Hallsworth *et al.*, 1994). IL-8 is increased in virus-infected bronchial epithelial cells *in vitro* and *in vivo* through NF-κB activation (Griego *et al.*, 2000; Zhu *et al.*, 1996; Zhu *et al.*, 1997). In addition, ICAM-1 is upregulated in the respiratory epithelium during virus infection through IL-1β production and NF-κB activation (Terajima *et al.*, 1997; Papi and Johnston, 1999). ICAM-1 is also increased on parasympathetic nerves following antigen challenge *in vivo* or with direct TNF-α and

IFN-γ treatment *in vitro*, which also involves NF-κB (Nie *et al.*, 2007). Thus, IL-1β could be activating NF-κB to regulate chemokines, adhesion molecules, or other proteins that may affect inflammation and contribute to M_2 receptor dysfunction *in vivo*.

Our lab's previous data show blocking TNF-α can prevent M_2 receptor dysfunction during infection (Nie *et al.*, 2011), and now I have shown TNF-α is a major contributor to IL-1β production mediating M_2 receptor dysfunction (Figue 6.4). There was potentiated contractile response to EFS with IL-1β and TNF-α, which was blocked with tetrodotoxin (TTX) demonstrating the contractions were neuron-mediated. In addition, smooth muscle contractility was not increased. IL-1β can increase expression of TNF-α in a variety of cell types, including fibroblasts, mast cells, monocytes, macrophages, and T cells (Winzen *et al.*, 1993; Hultner *et al.*, 2000; Burger and Dayer, 2002; Bethea *et al.*, 1992; Le and Vilcek, 1987). However, since potentiated contractile responses were only prevented when the TNF-α treated segments were treated with an IL-1 receptor antagonist, this suggests that TNF-α is upstream of IL-1β in the signaling cascade. Although, IL-1β could still be increasing TNF-α production, this is not the major pathway causing increased contractile responses.

Increased contractile responses may also be mediated through autocrine action of IL-1β, which has been demonstrated in sensitized tracheal tissue (Hakonarson *et al.*,

1997) and rhinovirus-infected airway smooth muscle (Hakonarson *et al.*, 1999a) tracheal tissue. The data in these studies also do not rule out the idea that other cytokines are involved in the cascade of induction, and that it may not just be IL-1β and TNF-α in the pathway. For instance, interferon-γ (IFN-γ) could be involved, as M_2 receptor expression decreases on parasympathetic (Jacoby *et al.*, 1998) and increases on sympathetic (Grodzki *et al.*, 2011) cultured neurons after IFN-γ treatment. This data also doesn't rule out induction of transcription factors, intracellular adhesion molecules, chemokines, or other signaling molecules that have been shown to be upregulated with IL-1β and TNF-α treatment in airway tracheal tissue (Hakonarson *et al.*, 2001).

Previous studies have shown TNF-α and IL-1β can induce increased contractility through increased airway smooth muscle sensitivity in cultured guinea pig tracheas (Parris *et al.*, 1999; Pennings *et al.*, 1998; Wills-Karp *et al.*, 1993; Wu *et al.*, 2002) or mediated by TNF-α-induced loss of bronchodilating mechanisms of β-adrenergic receptors (Wills-Karp *et al.*, 1993; Amrani, 2006). My data show TNF-α and IL-1β increased contractile responses compared to controls, which confirms previous findings (Makwana *et al.*, 2012; Wu *et al.*, 2002). However, the cytokine treatments (or antagonist treatments) in my studies did not augment MCh-induced contractile responses *in vitro*. This indicates that airway smooth muscle responsiveness is not induced through a change in M_3 muscarinic receptors. It is possible that the initial findings of TNF-α potentiation (Parris *et al.*, 1999; Pennings *et al.*, 1998) on smooth

muscle was possibly due to the differences in incubation times (30 min-24hrs vs. 4 days).

The sources of IL-1β and TNF-α production are not clear from these experiments. IL-1β is mainly produced by alveolar macrophages (Borish *et al.*, 1992), however other cell types produce IL-1β as well (Blasi *et al.*, 1999; Vitkovic *et al.*, 2000). For instance, rhinovirus infection has been shown to induce production of IL-1β from bronchial epithelial cells (Griego *et al.*, 2000) and respiratory submucosal glands (Yamaya *et al.*, 1999). TNF-α is released from a variety of cell types as well including, macrophages, neutrophils, and T cells (Sedgwick *et al.*, 2000; Grivennikov *et al.*, 2005; Fleischmann *et al.*, 2003; Vitkovic *et al.*, 2000). Moreover, *in vitro* data show respiratory epithelial cells are a major contributor of TNF-α when exposed to parainfluenza virus (Nie *et al.*, 2011). It's also possible airway neurons themselves are contributing to the production of these cytokines, as studies show TNF-α and IL-1β are produced from neurons in the brain (Breder *et al.*, 1988; Ignatowski *et al.*, 1997; Vitkovic *et al.*, 2000).

In summary, these data illustrate the role of TNF-α and IL-1β in M_2 function during infection. Since M_2 receptor dysfunction is a common mechanism of airway hyperreactivity, IL-1β may be involved in the observed airway hyperreactivity seen with virus infection *in vivo* (Empey *et al.*, 1976; Aquilina *et al.*, 1980; Gregg, 1972; Schwarze *et al.*, 1997; Ikemura *et al.*, 2000; Adamko *et al.*, 2003). These data provide

a better understanding of the mechanism involved in virus-induced asthma, which are imperative in determining better treatment strategies for acute virus-induced attacks. I have demonstrated IL-1β-mediated loss of M_2 receptor expression on parasympathetic nerves *in vivo* and *in vitro,* and a TNF-α-mediated loss *in vivo* as well. Moreover, targeting IL-1β (Chapter V) or TNF-α (Nie *et al.*, 2011) may be beneficial in preventing the loss of inhibitory M_2 receptors and restoring normal nerve function during infection. Furthermore, I have illustrated that it is TNF-α is the major factor mediating the IL-1β-induced increase in contractility. Collectively, these data suggest that blocking IL-1β can be a potentially effective treatment therapy for virus-induced asthma exacerbations.

CHAPTER VII.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The data presented in this thesis provide novel findings in the field of virus-induced M_2 receptor function and airway hyperreactivity. I used mice to demonstrate that influenza and parainfluenza infect airway epithelium and do not infect airway neurons. This was also confirmed with parainfluenza virus in guinea pigs (Chapter III). Given that virus-infection has a documented physiological effect on M_2 receptor function on parasympathetic nerves (Jacoby, 2004), I developed a novel dissection technique in guinea pigs to show that parainfluenza virus infection decreased M_2 receptor expression in parasympathetic neurons *in vivo*, and that this can be prevented by blocking either TNF-α or IL-1β (Chapter IV). Using a guinea pig model, I also demonstrated blocking IL-1β, with anakinra, during parainfluenza virus infection prevented M_2 receptor dysfunction (Chapter V). *In vitro*, I determined IL-1β directly decreases M_2 receptor expression in human and guinea pig parasympathetic neurons and that TNF-α is the major contributing factor of IL-1β production (Chapter VI). I have updated figure 1.17 to include the findings presented here into figure 7.1. This figure illustrates that airway nerves are not infected during infection and that TNF-α is upstream of IL-1β production resulting in M_2 receptor dysfunction, increased ACh release, and airway hyperreactivity.

It is known that viruses generally infect the airway epithelium, which can damage cells and tight junctions and can leave sensory nerve endings exposed (Hashimoto *et al.*, 2008; Papadopoulos *et al.*, 2007). Previous data have demonstrated viral

infection can increase excitatory and decrease inhibitory responses of sensory nerves (Papadopoulos *et al.*, 2007; Colasurdo *et al.*, 1995; Auais *et al.*, 2003). It has also been shown that certain respiratory viruses, such as respiratory syncytial virus and human metapneumovirus, can infect airway nerves (Li *et al.*, 2006; Liu *et al.*, 2009). My data definitively shows that influenza A (H1N1) and parainfluenza (Sendai) virus do not infect airway sensory neurons in mice four days after infection (Chapter III). In addition, parainfluenza does not infect airway neurons or parasympathetic ganglia in guinea pigs (Chapter III and IV). This suggests that alteration in neural control seen with these respiratory viruses involve other mediators released during infection that indirectly change nerve function. I have now shown that TNF-α and IL-1β are the inflammatory mediators inducing these changes in neuronal control of the airways.

Previous research has demonstrated that virus-induced airway hyperreactivity is mediated through a loss in inhibitory M_2 receptors on parasympathetic nerves (Fryer and Jacoby, 1991; Fryer *et al.*, 1994). Work from our lab has shown TNF-α treatment (Nie *et al.*, 2009) or direct viral infection (Jacoby *et al.*, 1998) decreases M_2 receptor gene expression in cultured neurons. In addition, blocking TNF-α *in vivo* prevents airway hyperreactivity and M_2 receptor dysfunction during allergen-challenged and virus infected guinea pigs (Nie *et al.*, 2009; Nie *et al.*, 2011). IL-1β has also been shown to mediate airway hyperreactivity in antigen-challenged, toluene diisocyanate-challenged, and ozone-challenged animals (Selig and Tocker,

1992; Johnson *et al.*, 2005; Verhein *et al.*, 2008; Park *et al.*, 2004; Townley and Horiba, 2003). Furthermore, previous findings indicate a role for IL-1β and TNF-α in the pathology of asthma (Message and Johnston, 2004; Mallia and Johnston, 2006; Proud and Chow, 2006). Given the effects of virus infection, TNF-α, and IL-1β on M_2 receptor function *in vivo*, I wanted to determine if virus infection would decrease M_2 receptor expression *in vivo*. Therefore, I developed a dissection technique to remove parasympathetic ganglia and determined parainfluenza virus infection does decrease M_2 receptor expression (Chapter IV). I also demonstrated that blocking either TNF-α or IL-1β could prevent the decrease in M_2 receptor expression in parasympathetic neurons. This indicated IL-1β and TNF-α are important in mediating M_2 receptor regulation during virus infection and further confirmed results showing TNF-α mediates M_2 receptor down-regulation (Nie *et al.*, 2011; Nie *et al.*, 2009).

While I showed no airway neurons or cell bodies from mouse sensory nodose and jugular (Chapter III) and guinea pig parasympathetic ganglia (Chapter IV) were infected, it is still possible other nerve fibers can be infected. Esophageal postganglionic parasympathetic neurons have shown to innervate the tracheal ganglia (Fischer *et al.*, 1998; Zhu and Dey, 2001; Mazzone and McGovern, 2010), evident by nitric oxide synthase (NOS) localizing to parasympathetic ganglia (Dey *et al.*, 1993; Fischer and Hoffmann, 1996), and release of nitric oxide (NO), a free radical known to have various physiological effects, including smooth muscle

relaxation and antimicrobial actions (Beckman and Koppenol, 1996; Nathan and Hibbs, 1991). Moreover, virus infection can alter NOS, which catalyzes NO from L-arganine, and NO release (Akaike and Maeda, 2000). Inducible NOS (iNOS) has shown to be regulated by direct infection, as in the cases of RSV or human immunodeficiency virus (HIV) (Tsutsumi *et al.*, 1999; Akaike and Maeda, 2000), but can also be indirectly affected via IFN-γ induction as reported with influenza infection and other viruses (Akaike *et al.*, 1998; Akaike and Maeda, 1999). Furthermore, influenza virus infection has been shown to increase neuronal NOS (nNOS) expression (Fatemi *et al.*, 1998). Alterations in NO production can affect M_2 receptors, as data show NO can induce neuronal M_2 receptor dysfunction on parasympathetic nerves (Golkar *et al.*, 2000). Thus, it is conceivable esophageal neurons can be exposed and infected during influenza or parainfluenza virus infection and may be altering NOS expression or NO release locally on tracheal parasympathetic ganglia. Future studies could be done to assess viral infection in esophageal neurons and isolate the esophageal ganglia, possibly with same tracheal parasympathetic ganglia removal technique, and determine if viruses infect the cell bodies.

Parainfluenza virus treated cells increase IL-1β production in culture (Yoshizumi *et al.*, 2010; Hua *et al.*, 1996) and blocking IL-1β prevents airway hyperresponsiveness in virus-infected tissue (Hakonarson *et al.*, 1999a). My studies have added that IL-1β mediates virus-induced M_2 receptor dysfunction *in vivo,* which I demonstrated

through the use of an IL-1 receptor antagonist in guinea pigs (Chapter V). Furthermore, blocking IL-1β did not significantly affect leukocytes in the BAL or blood or viral lung titers compared to untreated infected animals.

I demonstrated blocking either TNF-α or IL-1β prevented a virus-induced decrease in M_2 receptor expression *in vivo* (Chapter VI), and previous work show direct treatment of cultured neurons with TNF-α can induce this decrease *in vitro* (Nie *et al.*, 2009). I have now shown that IL-Iβ directly decreased M_2 receptor expression in cultured parasympathetic neurons from guinea pigs and humans as well as SK-N-SH cells.

IL-1β and TNF-α have been shown to work synergistically to decrease M_2 receptor expression in human lung fibroblasts (Haddad *et al.*, 1996b). However, I have demonstrated IL-1β and TNF-α do not work synergistically in cultured guinea pig parasympathetic neurons (Chapter VI), which suggest the receptor regulation is mediated differently in the two cell types. Data show that M_2 regulation can be mediated through a variety of cellular kinases (Haddad and Rousell, 1998). In human lung embryonic (HEL) fibroblasts cells, M_2 receptor regulation was mediated by protein kinase C (PKC) and involved synthesis of new protein (Rousell *et al.*, 1995). Haddad *et al.* demonstrated that IL-1β and TNF-α synergistic M_2 receptor regulation in HEL cells was mediated through cAMP-dependant protein kinase (PKA), and demonstrated blocking PKC had no effect on expression (Haddad *et al.*,

1996b). In addition, platelet-derived growth factor (PDGF) downregulation of M_2 receptors involved ERK1 and 2, but not PKC or PKA (Rousell *et al.*, 1997). Therefore, given the different kinases involved in M_2 receptor regulation in different cell types, the signaling mechanisms and kinases involved in M_2 receptor regulation should also be investigated in parasympathetic neurons. Inhibiting various kinases downstream of the IL-1 receptor, such as IKK or MKKs, during IL-1β treatment could be done to determine the signaling pathways. Future experiments could also be done to determine if the decrease in M_2 receptor expression in parasympathetic neurons is due to de novo protein synthesis or destabilization of mRNA.

Given that IL-1β and TNF-α do not work synergistically, and since IL-1β and TNF-α can regulate regulating each other (Nawroth *et al.*, 1986; Tillie-Leblond *et al.*, 1999; Zhang and Rom, 1993; Zwerina *et al.*, 2007; Winzen *et al.*, 1993; Hultner *et al.*, 2000; Le and Vilcek, 1987), I wanted to determine which cytokine might be upstream of the other in mediating increased hyperresponsiveness. I demonstrated that TNF-α is the main contributor of IL-1β production leading to increased contractility, as blocking IL-1β during TNF-α treatment prevented TNF-α-induced increased contractions in isolated guinea pig tracheas (Chapter VI). While TNF-α may be produced with IL-1β treatment in the cultured tracheal segments, it may not be at levels high enough to induce increased contractility since blocking TNF-α did not prevent IL-1β-induced increased contractility. The increased contractility with TNF-α or IL-1β was neurally mediated, and did not enhance cholinergic airway smooth

muscle contractile responses since the response to MCh was similar across all the groups.

IL-1β induction by TNF-α is orchestrated by multiple events. IL-1β is produced from monocytes, macrophages or dendritic cells and is mediated by TNF-α, IL-1β, or TLR stimulation from pathogens (Netea *et al*., 2009). TNF-α signaling activates NF-κB and transcription of IL-1β mRNA that is translated into inactive pro-IL-1β (Beg *et al.*, 1993; Dinarello, 2011). The induction of IL-1β by TNF-α most likely requires de novo protein synthesis as cyclohexamide treatment prevents IL-1β production in other TNF-α treated cells (Naworth *et al.*, 1986). As discussed in the introduction (Chapter I), pro-IL-1β is cleaved into active IL-1β by caspase-1, which also needs to be activated through autoproteolysis (Broz *et al*., 2010). It should be noted that active caspase-1 is present in blood monocytes from healthy individuals (Netea *et al*., 2009). During infection, activation of caspase-1 is mediated by inflammasome activation. The inflammasome oligomerization is initiated by a fall in intracellular K+ resulting from a build up of extracellular ATP released from activated monocytes or macrophages (Agostini *et al*., 2004, Dinaerllo, 2011). Excess ATP activates the P2X7 receptor on the cell surface, which leads to a rapid efflux of K+ out of the cell, resulting in decreased intracellular K+ (Perregaux *et al*., 2000; Solle *et al*., 2001, Dinarello, 2011). Once caspase-1 is activated by the inflammasome, active IL-1β is produced and released from the cell though the loss in membrane integrity,

phospholipase C and calcium-dependent phospholipase A activation mediated by an increase in intracellular calcium levels (Andrei *et al*., 2004; Dinarello, 2011).

Even though I have demonstrated *in vitro* that IL-1β can directly decrease M_2 receptor expression, its possible other or additional mechanisms affect receptor function *in vivo*. IL-1β increases the production of chemokines from airway smooth muscle (Oltmanns *et al.*, 2003; Wuyts *et al.*, 2003) and can induce IL-8, RANTES, and granulocyte-macrophage colony stimulating factor (GM-CSF) from bronchial epithelial cells (Manni *et al.*, 1996; Marini *et al.*, 1991; Hashimoto *et al.*, 2000; Matsumoto *et al.*, 1998). These can all lead to stimulation and recruitment of leukocytes, including macrophages and CD8+ T cells, which have been shown to be involved in virus-induced M_2 receptor dysfunction (Lee *et al.*, 2004; Adamko *et al.*, 2003; Fryer *et al.*, 1994; Fryer *et al.*, 1999). These immune cells can release other cell mediators and cytokines, including IFN-γ, TNF-α, NO, transforming growth factor-β1, fibroblast growth factor, and platelet-derived growth factor, which alter M_2 receptor mRNA, protein, or function *in vitro* (Jacoby *et al.*, 1998; Grodzki *et al.*, 2011; Nie *et al.*, 2009; Hua *et al.*, 1996; Haddad *et al.*, 1996c; Golkar *et al.*, 2000; Smith and Kessler, 1988; Jackson and Nathanson, 1997; Rousell *et al.*, 1997; Young and Hardy, 1990).

Recent reports are shedding light on the reasons why people with asthma have severe symptoms associated with virus infections, which may involve genetic

polymorphisms. Polymorphisms of TNF-α, IL-1β, and the IL-1RA gene are associated with asthmatic patients (Jimenez-Morales *et al.*, 2009; Karjalainen *et al.*, 2002; Pattaro *et al.*, 2006; Gohlke *et al.*, 2004; Zeyrek *et al.*, 2008; Malerba and Pignatti, 2005). The polymorphisms observed with IL-1β and IL-1RA gene may contribute to virus-induced asthma exacerbations. For instance, IL-1RA has three isoforms, one of which has an attenuated inhibitory effect on the IL-1 receptor than the other two isoforms (Malyak *et al.*, 1998). Its possible the IL-1RA gene polymorphism affects the ability of IL-1RA to bind to or inhibit the IL-1 receptor since significant differences in serum levels of IL-1RA are not detected between asthmatic and normal subjects (Pattaro *et al.*, 2006; Gohlke *et al.*, 2004). Conversely, polymorphisms in the IL-1β gene may result in stronger interaction with the IL-1 receptor.

In addition, a polymorphism in the IL-1 receptor associated kinase (IRAK) gene has also been identified and associated with early onset asthma (Balaci *et al.*, 2007; Bartlett *et al.*, 2009). IRAK regulates the toll-like receptor (TLR) and IL-1 receptor pathways (Wesche *et al.*, 1999; Kobayashi *et al.*, 2002). Thus, if this polymorphism affects the ability of the IRAK to regulate IL-1 receptor signaling, this could contribute to virus-induced asthma exacerbations. It could also possibly lead to dysregulation of TLR signaling, which could prevent the bronchodilator effects of the single-stranded RNA-activated TLR7 (Kaufman *et al.*, 2011; Boehme and Compton, 2004; Kawai and Akira, 2006). Dysregulation of TLR stimulation could

also result in TH2 immunity instead of TH1 responses, which has shown to induce TH2 cytokines and asthma development (Redecke *et al.*, 2004; Piggott *et al.*, 2005; Hakonarson *et al.*, 1999b; Gangloff and Guenounou, 2004; Finberg and Kurt-Jones, 2004). Indeed, respiratory syncytial virus has shown to promote skewed TH2 responses and airway hyperresponsiveness with a strong association of asthma development (Sutton *et al.*, 2007; Kristjansson *et al.*, 2005; Perez-Yarza *et al.*, 2007). Thus, IRAK polymorphisms might be contributing to increased virus-induced airway hyperreactivity in asthmatics compared to healthy individuals (Johnston, 2005; Atmar *et al.*, 1998; Aquilina *et al.*, 1980).

In addition, TNF-α polymorphisms in the promoter region and flanking regions have been associated with asthma severity and susceptibility (Jimenez-Morales *et al.*, 2009; Gao *et al.*, 2006; Lin *et al.*, 2002; Seki *et al.*, 1999; Witte *et al.*, 2002; Albuquerque *et al.*, 1998; Zhu *et al.*, 2000; Winchester *et al.*, 2000; Kamali-Sarvestani *et al.*, 2007; Aoki *et al.*, 2006; Shin *et al.*, 2004; Moffatt and Cookson, 1997). The polymorphisms in the promoter may affect binding of transcription factors and alter regulation of the TNF-α gene, leading to increased expression of TNF-α and subsequent IL-1β (Capter VI) that could cause virus-induced asthma attacks. Given both the polymorphisms TNF-α and IL-1, it would be interesting to express them in neurons and determine if they affect signaling and subsequent M_2 receptor regulation. It would also be beneficial to study asthmatic children and

adults with these polymorphisms during respiratory infections and determine if they contribute to increased severity of exacerbations.

There is a large population of severe refractory asthmatic patients that are difficult to treat and are in need of novel therapies (Dolan *et al.*, 2004; Holgate *et al.*, 2006; Chanez *et al.*, 2007). Blocking TNF-α and IL-1β are common for the treatment of rheumatoid arthritis and other inflammatory diseases (Singh *et al.*, 2012; Silva *et al.*, 2010; Dinarello *et al.*, 2012). Anakinra, or other treatments to block IL-1β, may also be helpful in severe asthmatic patients on inhaled corticosteroid that are still symptomatic (Wenzel and Szefler, 2006; Ito *et al.*, 2006). Furthermore, some patients with severe asthma who are resistant to traditional treatments have benefited from anti-TNF-α treatment (Howarth *et al.*, 2005; Cazzola and Polosa, 2006; Berry *et al.*, 2006). These patients may have further relief with an IL-1 receptor antagonist treatment, either alone or possibly with etanercept, as I have shown TNF-α is a major contributor to IL-1β production inducing airway hyperresponsiveness.

It should be noted however, that a slight increase in incidence of infections when blocking IL-1β with anakinra has been reported, which was not different with high-risk patients with 1 or more comorbidities (Schiff *et al.*, 2004; Fleischmann *et al.*, 2006; Fleischmann *et al.*, 2003). However, one meta-analysis found higher doses of anakinra (100 mg/day) increased the risk of serious infections in individuals with

comorbidities (Salliot *et al.*, 2009). Blocking IL-1β with anakinra does not increase the incidence of opportunistic infections, though (Fleischmann *et al.*, 2003; Salliot *et al.*, 2009). Conversely, blocking TNF-α has demonstrated to increase the risk of opportunistic infections, bacterial and fungal (Slifman *et al.*, 2003; Bergstrom *et al.*, 2004; Smith and Kauffman, 2009), as well instances of reactivation of latent tuberculosis (Lin *et al.*, 2010; Mohan *et al.*, 2004; Keane, 2005; Keane *et al.*, 2001; Fallahi-Sichani *et al.*, 2012; Dogra and Khullar, 2013; Furst, 2010; Crawford and Curtis, 2008; Harris and Keane, 2010). In addition, an increased risk of other viral infections such as varicella zoster virus, Epstein-Barr virus, cytomegalovirus, and human papillomavirus have been reported while taking TNF-α inhibitors (Strangfeld *et al.*, 2009; Petersen and Lorentzen, 2008; Losco *et al.*, 2004; Antoniou *et al.*, 2008; Adams *et al.*, 2004; Kim and Solomon, 2010; Wallis, 2011). Furthermore, blocking TNF-α has been reported to reactivate hepatitis B virus (HBV) and hepatitis C virus (HCV) in patients taking TNF-α inhibitors for arthritis (Vassilopoulos and Calabrese, 2013; Vassilopoulos and Calabrese, 2012), and those with a current or previous history of HBV or HCV are often treated with anti-viral therapy such as tenofovir or entecavir (Vassilopoulos *et al.*, 2010; Woo *et al.*, 2010). Lastly, influenza and parainfluenza infection are also associated with an increased risk of secondary infections (LeVine *et al.*, 2001; Pittet *et al.*, 2010), with the majority of deaths resulting from secondary bacterial pneumonia (Morens *et al.*, 2008; Louria *et al.*, 1959; Hers *et al.*, 1958). Given the increased risk of infections with blocking IL-1β or TNF-α as well as with respiratory infection, precaution would have to be taken if one were blocking these cytokines to prevent virus-induced

asthma exacerbations. Since blocking IL-1β in my studies prevented M_2 receptor dysfunction and increased tracheal contractility, it may be a more effective treatment for virus-induced asthma attacks than blocking TNF-α, as anti-IL-1β studies show limited risk of other infections compared to anti-TNF-α studies.

While blocking IL-1β with anakinra alone is suggested to be effective in my studies (Chapter V and VI), it is also possible that a combined treatment of etanercept and anakinra could be an alternative in patients with severe refractory asthma. However, the dosing and effectiveness would still need to be investigated during virus infections. Studies have also found that taking etanercept and anakinra together for rheumatoid arthritis treatment increased the risk of infections versus etanercept alone treatment (Genovese *et al.*, 2004; Weinblatt *et al.*, 2006). Therefore, the benefit-to-risk of combined treatment would also have to be considered given the possibility of increased incidence of other infections as discussed earlier.

It would also be beneficial to study the effects of blocking IL-1β alone or combined with a TNF-α blocker in an asthma model of virus-infection. Blocking TNF-α has previously shown to be effective at preventing M_2 receptor dysfunction and airway hyperreactivity in sensitized, allergen-challenged guinea pigs (Nie *et al.*, 2009). Anti-TNF-α antagonism has previously been investigated for the treatment of asthma in humans as well. These studies show blocking TNF-α in patients with severe asthma

can improve lung function, airway inflammation, tissue remodeling, and bronchial hyperresponsiveness (Berry *et al.*, 2006; Erin *et al.*, 2006; Morjaria *et al.*, 2008; Antoniu *et al.*, 2008), but is less effective in people with mild asthma (Rouhani *et al.*, 2005; Taille *et al.*, 2013; Antoniu, 2009). As TNF-α is shown to be a primary mediator of cell injury during infection and is relatively dispensable for viral clearance (Damjanovic *et al.*, 2011; Bruder *et al.*, 2006), blocking TNF-α could be a valuable treatment specifically for acute virus-induced asthma exacerbations in preventing immunopathology and severe lung injury without greatly affecting clearance of the virus. There are, however, no published data investigating the effectiveness of blocking IL-1β as treatment for asthma or virus-induced asthma exacerbation in humans. I also think it would be beneficial to investigate if blocking IL-1β would be effective in a sensitized, virus-infected animal model as well, since data show mechanisms of virus-induced M_2 receptor dysfunction and airway hyperreactivity can differ in non-sensitized vs. sensitized infected animals, which have more eosinophil-mediated effects on M_2 receptor function (Adamko *et al.*, 2003).

In conclusion, my thesis research has provided a new understanding of virus-induced M_2 receptor dysfunction and airway hyperreactivity. I have demonstrated that influenza and parainfluenza virus do not infect sensory neurons or parasympathetic ganglia. I also developed a new dissection technique and showed parainfluenza virus infection indirectly decreased M_2 receptor expression in

parasympathetic neurons, which is mediated by TNF-α and IL-1β. Moreover, the IL-1β-mediated loss of M_2 receptor expression happens *in vivo* and *in vitro*. Furthermore, I demonstrated that blocking IL-1β *in vivo* prevented the loss of inhibitory M_2 receptors during parainfluenza virus infection. These studies suggest that blocking IL-1β can be an effective treatment for virus-induced asthma exacerbationsm, providing a potential new drug target for future therapies.

FIGURE 7.1. Summary of findings of IL-1β and TNF-α relevant to virus-induced asthma exacerbations.

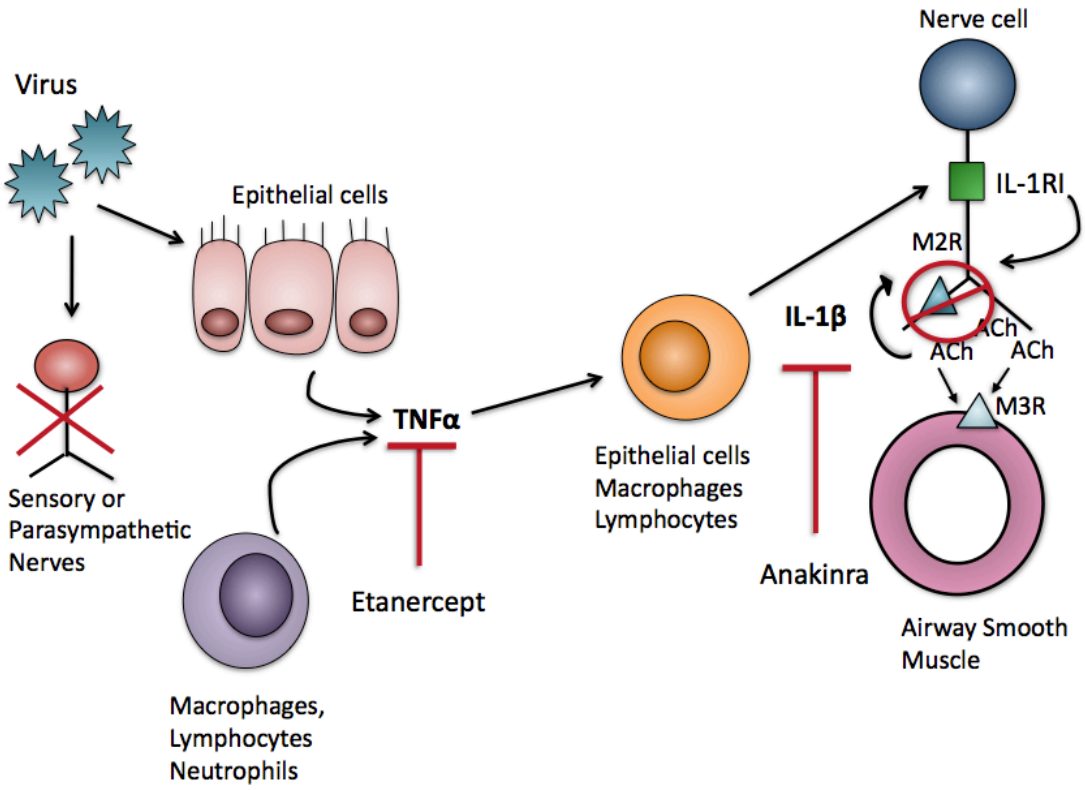

Figure 7.1. I have demonstrated the respiratory viruses influenza and parainfluenza primarily infect airway epithelium in mice (and guinea pigs for parainfluenza) and do not infect airway neurons (Chapter III). During infection, TNF-α is upregulated from infected epithelial cells and immune cells, presumably macrophages, lymphocytes, and neutrophils. TNF-α induces IL-1β production from several cell types such as epithelial, macrophges, lymphocytes, and others. Through a novel dissection technique, I showed that infection decreases M_2 receptor expression in parasympathetic neurons *in vivo*, which can be prevented by blocking either TNF-α, with etanercept, or IL-1β, with anakinra (Chapter IV). Blocking IL-1β, with anakinra,

during parainfluenza virus infection prevented M_2 receptor dysfunction, thus preventing the loss of negative feedback inhibition and increased ACh release onto M_3 muscarinic receptors on airway smooth muscle (Chapter V). IL-1β directly decreases M_2 receptor expression in human and guinea pig parasympathetic neurons and TNF-α is the major contributing factor of IL-1β production (Chapter VI).

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