ACUTE BRONCHODILATOR EFFECTS OF TOLL-LIKE RECEPTOR 7 AGONISTS

by

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ABSTRACT

Asthma exacerbations are caused by respiratory virus infections. Toll-like receptor 7 (TLR7) of the innate immune system recognizes single-stranded RNA viruses and mounts an immune response to clear the infection. TLR7 polymorphisms are associated with asthma. While its role in innate immune defense in the lung is appreciated, little is known about the acute effects of TLR7 signaling on airway physiology.

The results in this thesis demonstrate that TLR7 agonists are rapid bronchodilators in guinea pigs, mice, and humans, abolishing airway smooth muscle contraction within minutes of administration (Chapter III). Depending on species and ligand, the bronchodilating effect is mediated in part by TLR7, and in part by another target, most likely TLR8 (Chapter IV). TLR7-dependent bronchodilation is mediated by nitric oxide, and TLR7-independent bronchodilation is mediated by prostaglandins and large conductance calcium-gated potassium channels (Chapter IV). There is dysfunction in the bronchodilating effect with reduced ligand potency in allergen-sensitized and virus-infected guinea pigs *in vivo*, which may account in part for the more severe response to respiratory virus infections in humans with asthma (Chapter V). It may also explain why TLR7 polymorphisms are closely associated with asthma. Though the bronchodilating potential is reduced in models of asthma, TLR7 agonists are still bronchodilators, suggesting that TLR7 may serve as a valuable therapeutic target in asthma.

This novel finding represents an endogenous protective mechanism to maintain breathing during the inflammation necessary to clear respiratory virus infections. It also suggests that TLR7 agonism may be a potent rescue therapy during active bronchoconstriction associated with asthma attacks. Others have proposed TLR7 agonists as prophylactic therapy for asthma by harnessing the immunomodulatory capabilities of this receptor in skewing the immune system in the airways away from T_h2-type and towards T_h1-type immunity (a form of anti-allergic inflammation). Combined with their prophylactic capabilities, the acute bronchodilation mediated by TLR7 agonists demonstrated here suggests a novel and unique combined prophylactic and rescue therapy for asthma in one medication. Current standard of therapy for asthma uses one medication to prevent airway inflammation, and a second medication to limit bronchoconstriction. As these are often combined into one formulation, it is not uncommon for these patients to have a third rescue medication for acute bronchoconstriction during asthma attacks. As individual medications are all associated with some side effects, reducing the number of medications administered may potentially reduce side effects.

CHAPTER I.

INTRODUCTION

INTRODUCTION

This thesis addresses the association of respiratory virus infections with asthma attacks, by investigating the involvement of Toll-like receptor 7 function in airway physiology, pharmacology, and pathology.

A. AIRWAY ANATOMY

Because this thesis addresses airway dysfunction, this section describes the anatomy and normal function of the lung from its overall architecture to the individual cell types that contribute to its overall function. As will be discussed further in this section, the lung functions to oxygenate the blood, and the unobstructed passage of air through the airways within the lung. Section C describes the asthmatic condition in which the passage of air is obstructed, limiting breathing essential for life.

1. Lung architecture and function

Beginning at the trachea, the airways undergo multiple divisions into progressively smaller generations of bronchi and bronchioles, terminating at alveolar sacs. Alveoli are small air sacs at the distal end of the branching airways in the lungs. Oxygen and carbon dioxide are exchanged between inhaled air and blood in capillaries in the alveolar walls. The millions of alveoli provide a large surface area for gas exchange. Deoxygenated blood is pumped by the right heart into pulmonary circulation

through the pulmonary artery into the capillaries, where it is oxygenated. Oxygenated blood moves from the capillaries through the pulmonary veins back to the left heart, where it is pumped into systemic circulation providing oxygen to the whole body (West, 2000). The pulmonary circulation is distinct from bronchial circulation, arising from the systemic circulation and providing oxygenated blood to the airway tissues.

2. Airway nerves

The autonomic nervous system controls functions in the lung including airway smooth muscle tone and contraction, cough, mucus secretion, vascular permeability, and blood flow.

Sensory afferent nerves

Sensory afferent nerve fibers branch and spread throughout the subepithelium, between the epithelial cells, and reach the airway lumen (Jeffery, 1995). Sensory nerves relay sensation to stimuli including mechanical, pH, temperature, allergen, cigarette smoke, and pollutants (Lee et al., 1989; Coleridge et al., 1993; Giesbrecht et al., 1993; Coleridge and Coleridge, 1994). The vast majority of these sensory afferents have cell bodies in the nodose or jugular ganglia, while the others have cell bodies in thoracic and cervical dorsal-root ganglia (Jeffery, 1995). They project to the central nervous system and, after central processing of information, send information to efferent parasympathetic, sympathetic, and non-adrenergic non-

cholinergic nerves that innervate the airways (Kalia, 1987). Reflex responses include changes in airway caliber, cough, and mucus secretion (Coleridge and Coleridge, 1994). Importantly, airway smooth muscle tone at rest is also mediated by a reflex arc from tonic sensory afferent nerve activity to the central nervous system (O'Donnell et al., 1978; Kesler and Canning, 1999).

In addition neuropeptides released from sensory nerves in the airways can mediate local reflexes without relay to the central nervous system. Airway smooth muscle contraction, mucus secretion, and vasodilation can be regulated locally by neuropeptides, such as substance P, neurokinin A, and calcitonin gene-related peptide (Lundberg et al., 1983; Maggi et al., 1991; Ollerenshaw et al., 1991; Lou et al., 1993). These sensory neuropeptides can also induce inflammation that can affect airway function (Barnes, 2001). The function of local mediator release by sensory nerves in the airway is discussed further below in the section on non-adrenergic non-cholinergic innervation.

Parasympathetic efferent nerves

The myelinated preganglionic parasympathetic nerves project from the brain stem, travelling in the vagus nerve to the airway tissue, where they synapse with unmeylinated postganglionic parasympathetic nerves in various airway effector structures. For control of the trachea and bronchi, the preganglionic nerves synapse with clusters of postganglionic cell bodies arranged in chains along the smooth muscle. In all species, parasympathetic nerves provide the dominant autonomic

control of the airways, mediated by the neurotransmitter acetylcholine. Stimulation of airway parasympathetic nerves causes release of the neurotransmitter acetylcholine, which results in bronchoconstriction, mucus secretion, and vasodilation by activating post-junctional M_3 muscarinic receptors (Belvisi, 2002).

Sympathetic efferent nerves

The preganglionic sympathetic nerves are shorter than the parasympathetic preganglionic nerve fibers. They project from the first four or five thoracic vertebrae and synapse onto paravertebral postganglionic nerves that travel to the airway along, but separate from, the vagus nerve. The postganglionic sympathetic nerve fibers innervate submucosal glands and bronchial blood vessels (Belvisi, 2002).

Human airway smooth muscle has little or no sympathetic innervation (Richardson and Beland, 1976), in contrast to other species, such as guinea pig (O'Donnell et al., 1978; Oh et al., 2006). Sympathetic innervation exerts a relaxant effect on airway smooth muscle mediated by the neurotransmitter norepinephrine.

Non-adrenergic non-cholinergic efferent nerves

The non-adrenergic non-cholinergic innervation of the airway smooth muscle is the only relaxant innervation in human airways, which do not have significant sympathetic relaxant innervation (Richardson and Beland, 1976). The non-adrenergic non-cholinergic nerves were identified when blockade of sympathetic

and parasympathetic innervation in airway smooth muscle strips unveiled a neural component (tetrodotoxin-sensitive) of relaxation upon electrical field stimulation (Coburn and Tomita, 1973; Richardson and Beland, 1976). The neurotransmitters mediating this effect were identified to include stimulatory transmitters, such as substance P, as well as inhibitory transmitters, such as nitric oxide or vasoactive intestinal peptide (Matsuzaki et al., 1980; Stewart and Fennessy, 1986; Belvisi et al., 1992). This non-adrenergic non-cholinergic innervation is provided by the efferent arm of the sensory nerves in the airway.

3. Airway smooth muscle

Airway smooth muscle controls airway diameter, which affects the resistance to airflow and ultimately gas exchange. In the trachea and extrapulmonary bronchi, incomplete rings of supportive cartilage are connected dorsally by transverse bands of airway smooth muscle. The intrapulmonary bronchi are encircled by opposing spirals of airway smooth muscle internal to the cartilage, so that when the smooth muscle contracts, the airways both shorten and constrict (Jeffery, 1995). This is accomplished by bundles of individual long and narrow smooth muscle cells arranged in a parallel orientation.

Airway smooth muscle is neither of the single unit classification, in which cells have little or no innervation with spontaneous myogenic contraction, nor is it purely multiunit, where every cell is individually innervated. Rather airway smooth muscle is of an intermediate classification, with sparse innervation of the cells in the

outermost layer that controls contraction of multiple airway smooth muscle cells (Stephens, 2001). Parasympathetic nerves, which provide the major motor control of the airway smooth muscle, run in the connective tissue between airway smooth muscle bundles. Gap junctions between the smooth muscle cells within bundles allow for coordinated contraction (Daniel et al., 1986).

Airway smooth muscle function and signaling mechanisms regulating contraction and relaxation will be discussed in section B of this introduction.

4. Airway epithelium

Epithelial cells line the bronchial and alveolar lumen. In the alveoli, attractive forces between the water molecules in the liquid lining increase surface tension and decrease surface area of the air-liquid interface. This surface tension would collapse the alveoli, but type II pneumocytes secrete pulmonary surfactant that disrupts attractive forces between water molecules, decreasing surface tension and preventing alveolar collapse (West, 2000).

The enormous surface area of the lung is directly exposed to inhaled air from the environment. To protect the lung from inhaled particles, mucus secreted from submucosal glands and goblet cells in the airway walls traps the potential irritants and together they are propelled upwards out of the lung by the rhythmic movement of cilia on bronchial epithelial cells. Because the alveolar epithelial cells do not have cilia, cells of the immune system, particularly alveolar macrophages, are required

for protection of the alveoli (West, 2000). Alveolar macrophages engulf the particles for destruction and trafficking away from the lung.

The airway epithelium also mediate sodium secretion and chloride absorption to control the movement of water through paracellular pathways down the hydrostatic pressure gradient (Jeffery, 1995). Mucus function depends on proper hydration from the movement of water to the airway lumen.

The airway epithelial cells are directly exposed to pathogens and hazardous particles from the environment, so they also serve an important barrier function to prevent systemic exposure and disease. Furthermore, epithelial cells express many receptors of the innate immune system, including Toll-like receptors, to detect these pathogens and respond accordingly to prevent or clear infections (Bals and Hiemstra, 2004). In addition to inflammatory cytokines and chemokines, airway epithelial cells produce a number of antimicrobial substances, such as defensins, secretion of which is upregulated in response to infection (Hiratsuka et al., 1998; Hiemstra, 2001; Ashitani et al., 2002). Because of its important function in host defense, the airway epithelium itself can be considered an immune organ. Damage to the airway epithelium from virus infections, toxins, or environmental oxidants reduces its very important barrier function and can lead to disease. The innate immune system and Toll-like receptors will be discussed in more detail in section D of this introduction.

5. Submucosal glands

Throughout the human airways there are submucosal glands. These glands secrete neutral and acidic mucus and serous fluid, to maintain fluid dynamics and remove foreign particles and pathogens from the airway. There are also mucus secreting goblet cells interspersed with the epithelium (Jeffery, 1995).

6. Immune cells and structures

Macrophages, dendritic cells, T and B lymphocytes, mast cells, eosinophils, and neutrophils are all resident in the airway tissues. Encapsulated lymph nodes are present in peribronchial tissue of the large bronchi (Jeffery, 1995). In addition, lymphoepithelial nodules termed bronchus-associated lymphoid tissue, resembling Peyer's patches, are present in the bronchial mucosa (Bienenstock et al., 1973).

B. AIRWAY SMOOTH MUSCLE

The airway smooth muscle surrounding the lower airways and on the dorsal side of the trachea control airway caliber, the diameter of the airway lumen. Thus, airway smooth muscle regulates the passage of air into the airways for gas exchange in the alveoli. Furthermore, airway smooth muscle can secrete inflammatory mediators that contribute to host defense in the airways. This section describes what is known about how contraction and relaxation are regulated within the airway smooth

muscle, as well as its inflammatory functions. An understanding of these mechanisms is particularly important when considering pathology and therapy of obstructive pulmonary diseases, such as asthma.

1. Contractile mechanism

The crux of the contractile mechanism is the adenosine triphosphate (ATP)-dependent sliding of smooth muscle myosin filaments on actin filaments, by the cross bridging and stepping of globular heads of myosin filaments (Gabella, 1984). Contraction is triggered by phosphorylation of myosin-light chain by myosin light-chain kinase, and is terminated by dephosphorylation by myosin light-chain phosphatase (Kamm and Stull, 1985, 1986; Word et al., 1994). Myosin light chain kinase is activated by calcium-calmodulin complexes, therefore, calcium is a central trigger of airway smooth muscle contraction in response to a wide range of stimuli (Figure 1.1A).

2. Excitation-contraction coupling

Contraction of airway smooth muscle can occur from electromechanical mechanisms, as there are a number of voltage-gated calcium channels in the smooth muscle membrane that can respond to slight changes in membrane potential. Airway smooth muscle contraction can also occur from direct pharmacological agents that trigger signal cascades with little or no dependence on membrane depolarization. Acetylcholine-induced contraction of smooth muscle evokes a slight

depolarization, but repolarizing currents and blockade of voltage-gated calcium channels do not reverse contraction (Coburn and Yamaguchi, 1977). Contractile agonists induce slight depolarization of airway smooth muscle cells, probably through the action of calcium-gated chloride channels, but an action potential is not detected due to rectification from increased potassium conductance through voltage-gated potassium channels. This can be mimicked experimentally in the absence of contractile agonists by direct chemical depolarization of the airway smooth muscle membrane using KCl (Stephens, 2001). The trigger of signal cascades in response to depolarization of the cell membrane, and the induction of membrane depolarization in response to contractile agonists, suggests an interdependence of the electromechanical and pharmacologically evoked signal cascades. These mechanisms might cross regulate each other to generate sustained muscle contraction, particularly due to the transient and oscillatory nature of intracellular calcium signals.

3. Calcium signaling and calcium sensitivity

Calcium signaling is an important component of contractile mechanisms. Though electromechanical coupling requires extracellular calcium for influx through voltage-gated calcium channels, pharmacomechanical coupling operates even in the absence of extracellular calcium. Rather, their action results in calcium release from intracellular stores (Stephens, 2001). Contraction evoked from electromechanical coupling only occurs independently in experimental conditions by direct electrical stimulation of the smooth muscle (as opposed to the nerves) or chemical

depolarization with KCl. Physiologically, changes in electrical characteristics of the smooth muscle membrane are induced by the pharmacological action of neurotransmitters like acetylcholine or serotonin released by the prejunctional nerve on receptors in the airway smooth muscle membrane.

Agonist-induced contraction of airway smooth muscle most-frequently occurs through G-protein coupled receptors that couple to G_q, activating phospholipase C. The action of phospholipase C generates the second messenger inositol trisphosphate (IP_3) from the membrane phospholipid phosphatidylinositol 4,5,-bisphosphate (PIP_2), which initiates calcium release from the sarcoplasmic reticulum via IP_3-gated calcium channels (Baba et al., 1989; Baron et al., 1989). Calcium-release triggers further calcium release from the opening of calcium-gated calcium channels on the sarcoplasmic reticulum, such as ryanodine receptors, propagating a calcium wave across the cell (Collier et al., 2000). There is an initial rise in calcium within 10 to 15 seconds that rapidly declines to a lower plateau or oscillations of increasing and decreasing intracellular calcium (Murray and Kotlikoff, 1991; Murray et al., 1993). The sustained calcium signal is required for sustained contractions. It is clear that the initial rise in calcium comes from IP_3-gated intracellular stores, but the sustained calcium plateau or oscillations are maintained by influx of extracellular calcium (Murray and Kotlikoff, 1991). It is unclear whether extracellular calcium influx is through store-operated calcium channels that open in response to elemental calcium, ligand-gated calcium channels activated by

diacylglycerol another product of phospholipase C activity, or voltage-gated calcium channels activated in response to slight depolarization.

Sustained airway smooth muscle contraction is probably achieved by a combination of all of these mechanisms, where agonist-induced intracellular calcium release induces membrane depolarization through calcium-gated chloride efflux and calcium-gated calcium influx, which in turn amplifies calcium influx through voltage-gated calcium channels. Calcium then repolarizes the smooth muscle membrane by potassium efflux through calcium-gated potassium channels, allowing the reuptake of intracellular calcium into the sarcoplasmic reticulum through the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), to restart the whole contractile cycle (Berridge, 2008) (Figure 1.1B). Because not all of the airway smooth muscle cells are innervated to receive the contractile neurotransmitter agonist signal, changes in intracellular ion concentrations are propagated through gap junctions to synchronize the airway smooth muscle cells.

Calcium sensitivity describes the difference in contractile responses to the same levels of intracellular calcium (Sanderson et al., 2008). Though the initiation of contraction of airway smooth muscle is dependent on intracellular calcium, the degree of tension does not always correlate with intracellular calcium levels (Perez and Sanderson, 2005). This is due to a number of mechanisms that change the sensitivity of the contractile mechanism to intracellular calcium levels. A striking example of this phenomenon was demonstrated when airway smooth muscle was able to relax although the intracellular calcium concentration was held at contractile

levels, and addition of contractile agonists induced further contraction without changing intracellular calcium concentration (Bai and Sanderson, 2006). Differences in calcium sensitivity most often reflect changes in the activity of myosin light chain phosphatase, which exerts an inhibitory effect on contraction (Somlyo and Somlyo, 2003). For example, increased myosin light chain phosphatase activity at equivalent intracellular calcium levels will decrease contractility. Changes in myosin light chain phosphatase activity are frequently regulated by Rho kinase and protein kinase C, both of which can be activated by G protein coupled receptor agonists (Chiba and Misawa, 2004). In this way, different G protein coupled receptor agonists can induce different levels of contraction to equivalent calcium signals. While a number of mechanisms are proposed to explain airway hyperreactivity characteristic of asthma, some propose a model in which increased calcium-sensitivity in airway smooth muscle of asthmatics accounts for the increased responsiveness to equivalent stimuli (Sanderson et al., 2008).

4. Signaling pathways regulating airway smooth muscle contractility

The number of signaling entities that regulate airway smooth muscle contraction is ever increasing. This section focuses on some of the more well-characterized pathways.

G-protein coupled receptors

Parasympathetic innervation provides the dominant autonomic control of the airway smooth muscle, and specifically acetylcholine released from nerve terminals activates the M_3 muscarinic receptors on smooth muscle. These receptors are G_q-coupled, and trigger a signal cascade resulting in intracellular calcium release from the sarcoplasmic reticulum as described above. Histamine, a well-characterized transmitter of allergic airway contraction, is released by mast cells onto histamine H_1 receptors expressed on the smooth muscle, which are also coupled to IP_3-mediated calcium release. Other bronchoconstrictive agonists that signal through G_q include bradykinin, substance P, leukotriene D_4, and serotonin (Panettieri et al., 1989; Farmer et al., 1991; Abela and Daniel, 1994; Yang et al., 1994) (Figure 1.2A).

Interestingly, not all G_q-coupled receptors induce contraction. It was recently shown that bitter taste receptors expressed on airway smooth muscle can mediate airway relaxation in response to bitter tastants (Deshpande et al., 2010). This is due to a localized calcium response near the smooth muscle cell membrane that activates large-conductance calcium-gated potassium channels resulting in membrane hyperpolarization. This surprising finding suggests that not all calcium signaling is the same even within the same cell type, and subcellular localization of signaling components determined by organelles and scaffolding structures may be critical to the function of the calcium signal.

In general, G_s-coupled receptors in airway smooth muscle mediate relaxation (Figure 1.2A). Beta agonists, which bind to G_s-coupled β_2-adrenergic receptors on airway smooth muscle, are used as rescue therapy for bronchoconstriction in

asthma. Other G_s-coupled relaxant receptors in the airways include the prostaglandin E_2 receptors, vasoactive intestinal peptide receptors, and adenosine A_{2B} receptors (Hall et al., 1992; Maruno et al., 1995; Mundell et al., 2001). Agonists at G_s-coupled receptors induce generation of cyclic adenosine monophosphate (cAMP) via activation of adenylyl cyclase. cAMP in turn activates protein kinase A, which regulates many downstream proteins by phosphorylation. Some of these downstream targets have relaxant effects. IP_3 production can be inhibited by protein kinase A, limiting calcium release from the sarcoplasmic reticulum and subsequent contraction (Hall and Hill, 1988). Calcium release or reuptake through channels can be inhibited by protein kinase A, again limiting the propagation of calcium oscillations and contraction (Volpe and Alderson-Lang, 1990). The large-conductance calcium-activated potassium channels described above are activated by protein kinase A, which hyperpolarizes the membrane and limits contraction (Kume et al., 1994). Myosin light-chain kinase can be inactivated by phosphorylation at a protein kinase A regulatory domain, limiting myosin light-chain activation and contraction (Anderson, 2006). These protein kinase A-mediated mechanisms explain the relaxant effects of agonists at G_s-coupled receptors (Figure 1.2A).

G_i-coupled receptors generally mediate contraction in airway smooth muscle (Figure 1.2A). Activation of these receptors inhibits adenylyl cyclase and counteracts relaxant effects of G_s-coupled receptors. G_i can also directly inhibit the large-conductance calcium-activated potassium channels, inhibiting hyperpolarization and relaxation (Kume et al., 1992). G_i can also promote

contraction via activation of RhoGTPase, which mediates actin polymerization and contractility (Hirshman and Emala, 1999) (Figure 1.2A). The M_2 muscarinic receptors and A1 adenosine receptors are G_i-coupled receptors that contribute to contraction (Widdop et al., 1993; Mundell et al., 2001).

Nitric oxide

The mechanisms listed above are all mediated by G-protein coupled receptors, but the membrane permeant gas nitric oxide mediates airway smooth muscle relaxation independent of surface receptors. Nitric oxide is generated in the airway epithelium, as well as in parasympathetic and non-adrenergic non-cholinergic neurons, from L-arginine by the enzyme nitric oxide synthase (Fischer et al., 1993; Yoshihara et al., 1998; Kesler et al., 2002). Nitric oxide diffuses into the smooth muscle where it activates guanylyl cyclase, in turn generating cyclic GMP (cGMP). Similar to protein kinase A activation by cAMP, cGMP activates protein kinase G which phosphorylates a number of downstream targets to induce smooth muscle relaxation (Hamad et al., 2003). Furthermore, myosin light chain phosphatase activity is potentiated, also exerting a relaxant effect (Figure 1.2B). Nitric oxide synthase activity can be inhibited with N-methyl-L-arginine (L-NMMA) (Salerno et al., 2002).

Potassium channels

As stated above, other than small fluctuations, the airway smooth muscle membrane potential is relatively stable. Small changes in membrane potential are due to the

activity of ion channels, including calcium, chloride, and potassium channels. Delayed rectifier potassium channels contribute to the membrane potential stability (Boyle et al., 1992). In general, potassium channel activity repolarizes or hyperpolarizes the airway smooth muscle membrane by outward potassium currents, exerting an inhibitory or relaxant effect (Figure 1.2C). The large-conductance calcium-activated potassium channel (MaxiK or BK) mediates a part of the relaxant effects of the G_s-coupled receptors described above (Kume et al., 1989). This channel is activated both by membrane depolarization and intracellular calcium (Ghatta et al., 2006), in effect only opening under contractile conditions to induce relaxation. Further regulation of MaxiK is contributed by protein kinase A activity in response to Gs-coupled receptor agonists (Kume et al., 1992; Kume et al., 1994). MaxiK potassium channels can be blocked with paxilline (Knaus et al., 1994) (Figure 1.2C).

Prostaglandins

Prostaglandins are eicosanoid lipid mediators with potent effects on airway physiology and inflammation. They are metabolites of the essential fatty acid arachidonic acid (Bergstroem et al., 1964). Arachidonic acid is released from the cell membrane by phospholipase A_2, and in turn is converted into prostaglandin H_2 (PGH_2) by the cyclooxyegenase-1 and -2 enzymes. PGH_2 is further converted by a family of metabolic enzymes into the other prostaglandin subtypes. The prostaglandins are an autocrine or paracrine molecule, signaling at or close to their location of synthesis respectively, through G-protein coupled receptors (Funk,

2001). Multiple cell types in the airways can synthesize prostaglandins, including epithelium, smooth muscle, neurons, and inflammatory cells. Additionally, these cell types also contain specific prostaglandin receptors to respond to these molecules (Coleman et al., 1994). As described above, the downstream physiological effects of prostaglandins depend on the G-protein to which the prostaglandin receptor couples. In general, airway smooth muscle relaxation results from the G_s-coupled prostaglandin receptors (Figure 1.2D), while contraction results from those coupled to G_q or G_i (Clarke et al., 2009). Prostaglandin signaling results in constrictive and relaxant effects on airway vasculature and airway smooth muscle, but they are also involved in fever, edema, and chemotaxis of various immune cells in the inflammatory response, which in turn can affect airway smooth muscle function (Funk, 2001). Prostaglandin E_2 (PGE_2) is the predominant prostaglandin generated in airway smooth muscle, and the EP_2 prostaglandin receptor coupled to G_s is the predominant receptor expressed by airway smooth muscle, exerting a relaxant effect on contracted airway smooth muscle (Delamere et al., 1994; Belvisi et al., 1997; Penn et al., 2001). Prostaglandin synthesis can be inhibited by the cyclooxygenase-1 and -2 inhibitor indomethacin (Sharma and Sharma, 1997) (Figure 1.2D). There are a number of other eicosanoids, such as leukotrienes, generated from arachidonic acid with potent inflammatory and physiological effects that are not discussed further here.

Of particular importance to this thesis is that prostaglandin signaling can regulate the activity of the large-conductance calcium-activated (MaxiK) potassium channels

described above (Moreau et al., 1996; Lee et al., 1999). Some prostaglandins, such as prostaglandin E_2 (PGE_2) and prostaglandin I_2, relax airway smooth muscle (Tamaoki et al., 1993; Hartney et al., 2006), as does opening of the MaxiK potassium channel. In human corporal smooth muscle, PGE_1 induces relaxation mediated by MaxiK, accounting for the increased blood flow and pro-erectile effects of PGE_1 (Lee et al., 1999). In human osteoblast bone cells, PGE_2 activates MaxiK (Moreau et al., 1996). Though not directly shown, it is possible that MaxiK mediates some of the relaxant effects of prostaglandins on airway smooth muscle (Figure 1.2E).

Nucleotides

Nucleotides also modulate airway smooth muscle function. Adenosine acts through a number of adenosine receptor subtypes, G-protein coupled receptors coupled to G_s, G_i, and G_q (Wilson, 2008). Depending on the tone of the airway smooth muscle, adenosine can exert either contractile or relaxant effects. Adenosine A_1 receptors are expressed on airway smooth muscle cells, and mediate contraction through G_i-coupling (Ethier and Madison, 2006). Adenosine A_{2a} receptors are also expressed on airway smooth muscle, but mediate relaxation through Gs-coupling (Wilson, 2008). The resulting airway tone is probably due to complex regulation by multiple receptor subtypes with opposing effects, and the role of individual subtypes might change with airway tone. For example adenosine has been shown to contract airway smooth muscle at rest, but relax precontracted airway smooth muscle (Caparrotta et al., 1984; Krzanowski et al., 1987; Farmer et al., 1988). Another level of complexity arises when considering all of the cell types in the lung that express adenosine

receptors, including immune cells, which may mediate indirect effects of adenosine on airway smooth muscle function. Additionally, the purine ATP can bind to P2X and P2Y receptors in the airway to further affect airway function (Bergner and Sanderson, 2002; Mounkaila et al., 2005).

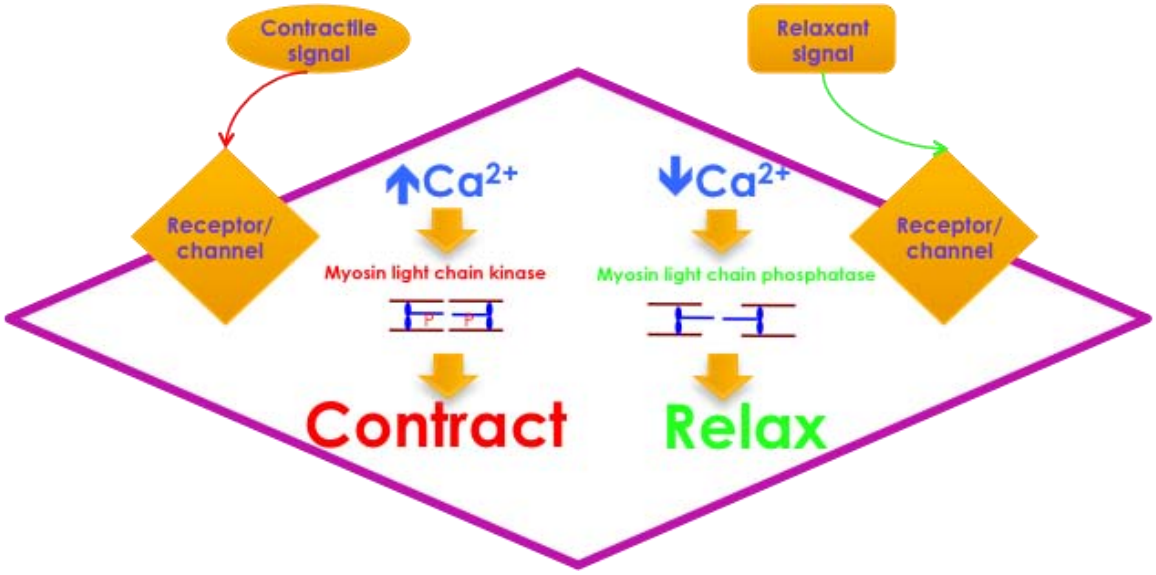
5. Inflammation

While the primary function of the airway smooth muscle is to regulate airway caliber, the airway smooth muscle cells are capable of generating and responding to inflammatory signals in response to infection (Halayko and Amrani, 2003; Hirst, 2003; Chung, 2005). Inflammatory cytokines, such as interferon-γ and tumor necrosis factor-α, act directly on receptors expressed by airway smooth muscle, to stimulate the release of more inflammatory cytokines, chemokines, growth factors, and matrix metalloproteinases, as well as to stimulate increased intercellular adhesion molecule expression (Amrani et al., 1999; Chung, 2005; Tliba and Amrani, 2008). These mediators induce recruitment of immune cells and remodeling of the airway wall (Hirst, 2003). These can have both beneficial effects, such as protection from infection, but also deleterious effects, such as asthma. Airway smooth muscle cells also express IgE receptors to respond to IgE released from B cells during allergic inflammation (Gounni et al., 2005).

In the next sections, inflammation in the airways will be discussed in greater detail with regards to both pathology, such as in asthma, as well as innate host defense.

FIGURE 1.1. Schematics of airway smooth muscle contraction and relaxation mediated by intracellular calcium and membrane potential.

A



B

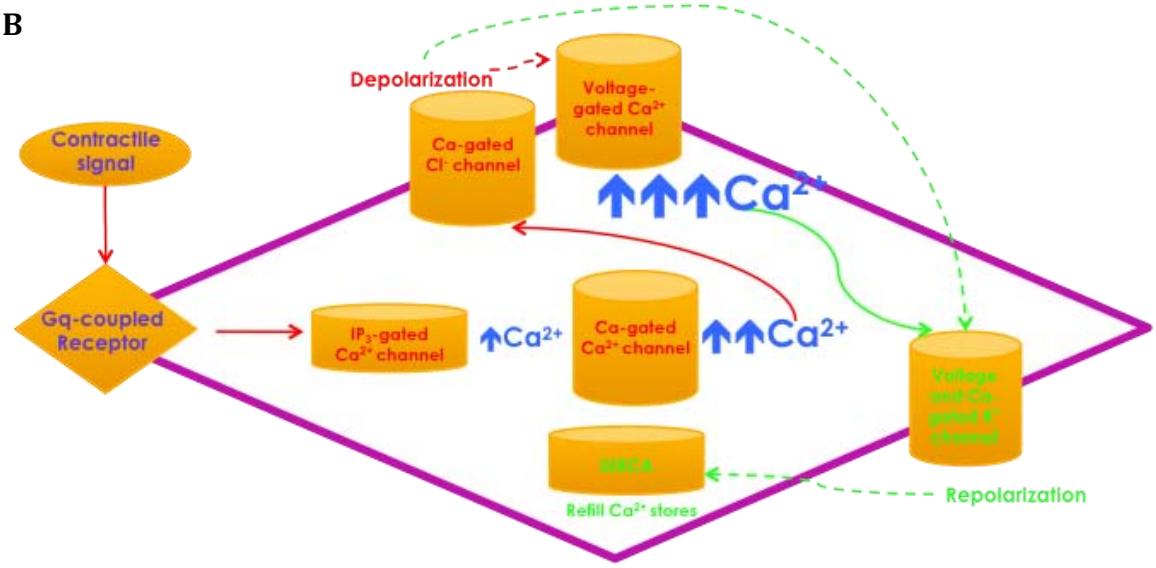
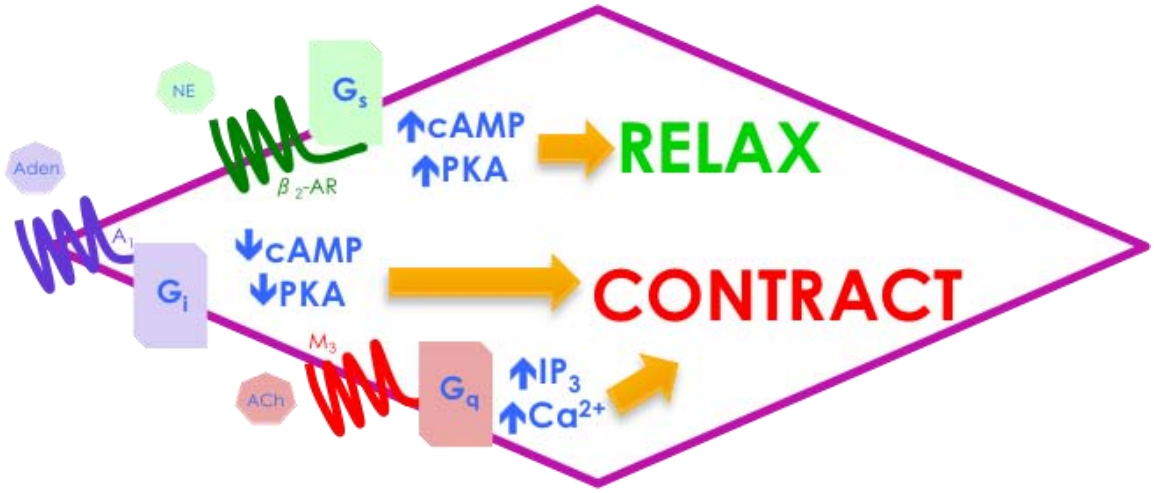


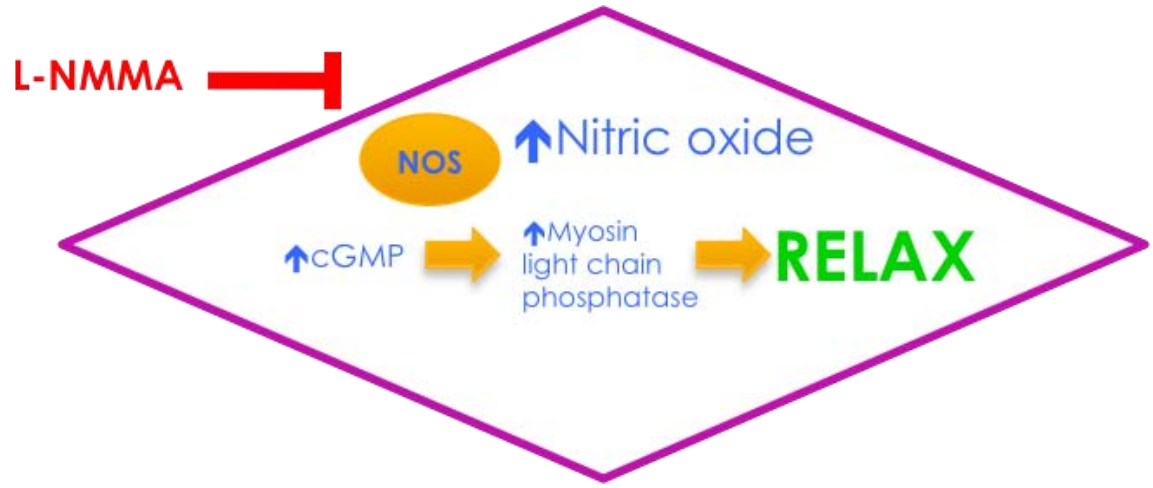
Figure 1.1. (A) In general, airway smooth muscle cells contract in response to global increases in intracellular calcium, and relax in response to decreases in intracellular calcium. The phosphorylation state of myosin light chain in response to intracellular calcium concentration is the primary control for airway smooth muscle contraction and relaxation. (B) In addition to calcium mechanisms, small changes in the airway smooth muscle membrane potential contribute to contraction and relaxation. These changes in membrane potential are controlled by ligand-gated (IP3) and voltage-gated ion channels, but are also regulated by intracellular calcium released from and taken back up in intracellular stores. Red arrows denote contractile contributions and green arrows denote reset contributions of an oscillatory response. Dashed arrows denote membrane potential contributions. SERCA is the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase for calcium reuptake and replenishing of the intracellular calcium storage.

FIGURE 1.2. Signaling mechanisms regulating airway smooth muscle contraction and relaxation.

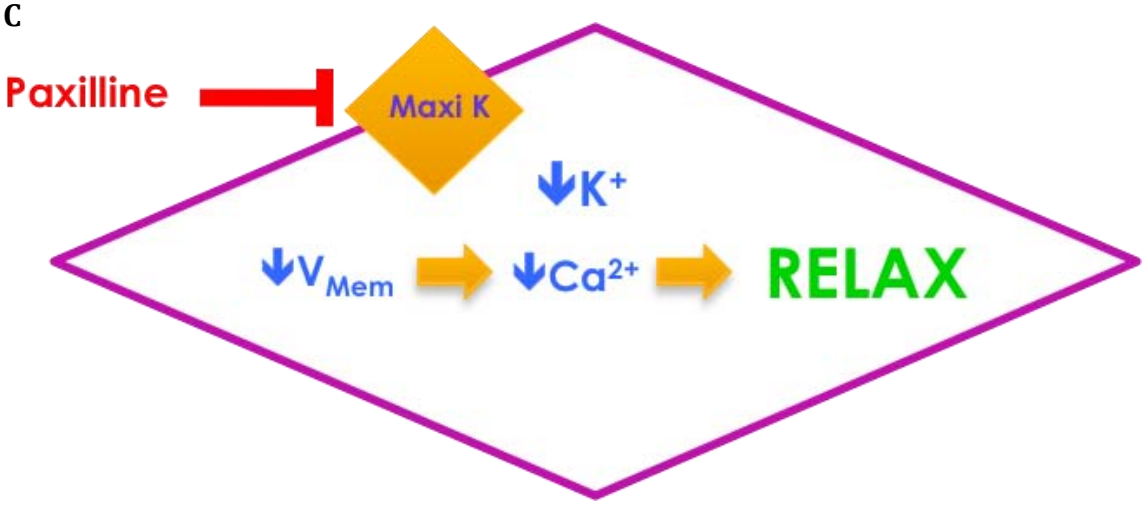
A



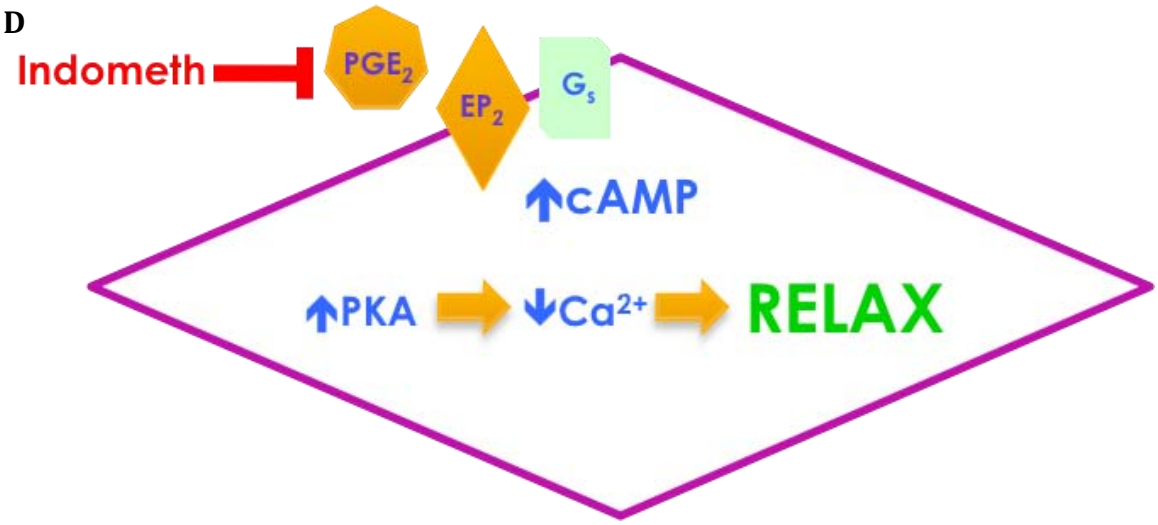
B



C


Paxilline
Maxi K
↓K+
↓VMem
↓Ca2+
RELAX

D


Indometh
PGE2
EP2
Gs
↑cAMP
↑PKA
↓Ca2+
RELAX

E


PGE2
EP2
Gs
Maxi K
↑cAMP
PKA
↓K+
RELAX
↓VMem

Figure 1.2. (A) Schematic of the main G-protein coupled receptor-mediated mechanisms through G_s, G_i, and G_q of airway smooth muscle contraction and relaxation. Acetylcholine (ACh) induces contraction through G_i-coupled M_3 muscarinic receptors. Norepinephrine (NE) induces relaxation through G_s-coupled β_2 adrenergic receptors (β_2-AR). Adenosine (Aden) induces contraction through A_1 adenosine receptors. Cyclic adenosine monophosphate (cAMP); protein kinase A (PKA); inositol trisphosphate (IP_3). (B) Schematic of nitric oxide mediated relaxation of airway smooth muscle. L-NMMA is a NOS inhibitor. Nitric oxide synthase (NOS); Cyclic guanine triphosphate (GMP). (C) Schematic of the large conductance calcium-activated potassium channel (MaxiK) mediated relaxation of airway smooth muscle. Activation results in potassium efflux and repolarization or hyperpolarization of the membrane. Paxilline is a MaxiK blocker. Membrane potential (V_{mem}). (D) Schematic of prostaglandin E_2 (PGE_2) mediated relaxation of airway smooth mucle. Indomethacin (indometh) is a cyclooxygenase inhibitor that inhibits prostaglandin synthesis. Prostaglandin E_2 receptor (EP_2). (E) Schematic of potential relaxant mechanism of prostaglandin E_2 regulating MaxiK function.

C. ASTHMA

The work in this thesis contributes to the understanding of asthma and potential therapeutics. The previous sections detailed the function and cellular components of the normal lungs with particular emphasis on the function and signaling mechanisms in airway smooth muscle. It is important to understand the dysfunction in the lung contributing to asthma pathology. This section describes what is known about the characteristics, causes, existing therapies, and animal models used to investigate asthma.

1. Characteristics

As of 2008, the Global Initiative for Asthma (GINA), a collaboration of the National Heart, Lung, and Blood Institute, National Institutes of Health, USA, and the World Health Organization with asthma experts from around the world, reported 300 million cases of asthma worldwide (Bateman et al., 2008). By estimating disability-adjusted life years, 1% of the global disease burden is attributed to asthma (Masoli et al., 2004). The majority of asthma starts early in childhood and may remit later in life, though relapses are frequent (Sears et al., 2003; Sly et al., 2008). GINA provides an operational description of asthma as follows:

> *Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to 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 within the lung that is often reversible either spontaneously or with treatment.

From the operational description above, the hallmarks of asthma can be broken down to two main interdependent components, airway inflammation and airway hyperreactivity, defined as excessive airway narrowing in response to stimuli that have little effect in healthy individuals. The end result is airway obstruction that makes breathing difficult (Figure 1.3). This airway obstruction can be uncomfortable or limit the range of physical activity, but it can also be lethal, with approximately 250,000 asthma-associated deaths yearly worldwide. The burden of the disease extends to school and work absences, hospital admissions, and health care costs. There are 2 million asthma-related emergency room visits and 5 hundred-thousand hospital admissions in the United States each year (Mallia et al., 2007). The following subsections describe the airway inflammation and hyperreactivity components of the asthmatic condition.

Inflammation

Many immune cells coordinate the inflammatory response including eosinophils, mast cells, and CD4+ T lymphocytes. The CD4+ T lymphocytes are usually of the T_h2 phenotype associated with allergic inflammation (Anderson, 2002). In response to crosslinking of their high affinity IgE receptors, mast cells release airway smooth muscle contractile agonists, such as histamine and leukotrienes, as well as mediators of airway remodeling, such as tryptase and matrix metalloproteinases

(Bradding et al., 2006). Upon activation, eosinophils release a number of cytokines, chemokines, and preformed granule proteins and tissue-damaging superoxides that mediate inflammation and combat helminth worm infections (Kariyawasam and Robinson, 2006). Eosinophils play a key role in airway hyperreactivity that will be discussed further below in the section on airway hyperreactivity. Consequences of the asthmatic inflammatory response include excessive mucus secretion and edema, epithelial cell damage, remodeling and thickening of the airway wall, and immune cell infiltration (Holgate, 2008). These are all factors causing airway obstruction, resulting in difficulty breathing.

T_h2–type immunity describes a specific immune environment characterized in large part by a subtype of CD4+ T helper lymphocytes and the specific cytokines they produce. This immune environment is well suited for host immunity to extracellular parasites, such as helminths (Paul and Zhu, 2010). The T_h2 phenotype of T helper lymphocytes is characterized by T cells that express the CCR4 chemokine receptor and release the cytokines interleukin (IL)-4, IL-5, IL-9, and IL-13. Among other effects, IL-4 and IL-13 facilitate class switching of B cell antibodies to IgE (Larche et al., 2003). IL-5 recruits eosinophils to the lungs (Robinson et al., 1999), and IL-9 is involved in mast cell recruitment to the lungs (Paul and Zhu, 2010). Though the environment is categorized by T helper cell subtype, other immune cells, epithelial cells, nerves, and smooth muscle cells in the airways can release cytokines contributing to the T_h2 immune environment. Allergy and asthma are often correlated and both are categorized as T_h2 diseases. It should be noted that the distinction of asthma as a purely T_h2 disease is an oversimplification, as the immune

system is a complex network of regulatory systems. In fact, T lymphocytes of the T_h1 subtype that secrete TNF-α and interferon-γ contribute significantly to tissue damage associated with severe asthma, particularly in response to virus infection (O'Sullivan, 2005). Overall, however, airway environments with T_h2-polarized immune responses are characterized by allergy, eosinophilia, mucus secretion, goblet cell hyperplasia, airway hyperreactivity, and airway remodeling, characteristics also attributed to asthma (Kim et al., 2010).

The T_h1 immune environment is characterized by cytokines secreted by T helper type 1 lymphocytes, such as type I interferons, TNF-α, and IL-10. The T_h1 immune environment is particularly suited for combating virus or intracellular microbe infections, as opposed to the extracellular parasites for which the T_h2 immune environment is suited. By the action of cytotoxic CD8+ T cells and interferon-γ and TNF-α secreting T_h1-type CD4+ T helper lymphocytes, virus infected cells in the airways are recognized and killed (Papadopoulos et al., 2007). Some consider asthma and allergic disease to be a problem in the balance in the T_h2 and T_h1 immune environments, with skewing towards a more T_h2-dominated environment (Vandenbulcke et al., 2006). In fact proponents of the 'hygiene hypothesis' attribute the increase in allergic diseases and asthma with increased cleanliness and sterilization due to a lack of exposure to infectious pathogens (Hertzen, 1998). The result is skewing away from a T_h1-type and towards a T_h2-type immune environment. This is in some contradiction to others who believe that early-life virus infections predispose individuals to asthma development discussed later in

this section (Mallia and Johnston, 2002). However, these theories can be unified by contributions from other genetic and environmental factors.

In response to tissue damage from a wide range of insults from microbial to environmental, the airway tissue needs to repair itself. Tissue repair is a beneficial result of normal inflammation in response to tissue damage. There is excessive damage and shedding of the airway epithelium in the asthmatic condition (Carroll et al., 1993). The subsequent airway repair, however, is dysfunctional, and aberrant repair leads to airway remodeling, resulting in a different airway composition compared to prior to the damage (Holgate, 2008). T_h2 cytokines are associated with aberrant tissue repair. As these cytokines are often upregulated in asthmatic airways, they are an explanation for the excessive airway remodeling. Persistent inflammation in response to these cytokines leads to the release of a number of growth factors leading to airway remodeling (Holgate et al., 2001). As the name "airway remodeling" implies, the lung was originally modeled in development, and many growth factors that are over- or aberrantly expressed are the same growth factors that guide development of the fetal lung (Jeffery, 2001). They are, furthermore, characteristic of wound healing that is necessary in response to airway tissue injury, but it is the persistence of the remodeling components that is pathogenic in asthma (Jeffery, 2001).

In asthmatics, aberrant airway remodeling results in mucus gland, airway smooth muscle, goblet cell, and epithelial hypertrophy and hyperplasia, as well as increased deposition of extracellular matrix components, resulting in thickening of the airway

wall and thinning of the airway lumen, contributing to the severity of the disease (Bumbacea et al., 2004). Subepithelial fibrosis from the increased deposition of extracellular matrix components alters the integrity and function of the overlying epithelium (Jeffery, 2001). Goblet cell metaplasia from dedifferentiated preexisting epithelial cells increases the number of goblet cells and subsequently increases mucus secretion (Curran and Cohn, 2009). Airway smooth muscle mass is increased in asthmatic airways due to hyperplasia and hypertrophy (Ebina et al., 1993), but also potentially due to migration of dedifferentiated fibromyocytes and reduced apoptosis of smooth muscle cells (Gizycki et al., 1997; Martin and Ramos-Barbon, 2003). Neuroplastic changes are also observed with potential physical increases in sensory nerve innervation as well as changes in the phenotype and function of the efferent nerves (Fryer and Jacoby, 2002; Nassenstein et al., 2006). An increase in sensory nerve innervation may result in excessive mucus secretion and bronchoconstriction.

As stated above, inflammation and hyperreactivity are interdependent components of asthma, and the persistent influx of immune cells and constant exposure to inflammatory mediators contribute to the physical and functional remodeling of the lungs that result in airway hyperreactivity and airflow obstruction.

Airway hyperreactivity

I humans with asthma, a number of mechanisms contribute to airway hyperreactivity, defined by airway narrowing in response to stimuli that evoke little or no airway narrowing in healthy subjects. These include thickening of the airway

wall, which changes the contractile properties of the airway; smooth muscle hypertrophy or increased calcium sensitivity, which increase smooth muscle contraction to equivalent stimuli; increased sensitivity of sensory nerves, which subsequently increases the activity of the efferent reflex parasympathetic contractile innervation; or direct changes in efferent parasympathetic or non-adrenergic non-cholinergic nerve function (Fryer and Jacoby, 2002; McParland et al., 2003; Wang et al., 2003; Black, 2004). Symptoms of acute exacerbations of airway hyperreactivity include shortness of breath, cough, wheezing, and chest tightness (Mallia et al., 2007).

Eosinophils and airway hyperreactivity

Because of its direct application to work in this thesis, the rest of this section will focus on eosinophil-mediated changes in efferent parasympathetic nerve function as a mechanism that results in airway hyperreactivity. The work in this thesis will add a new proposed mechanism of airway hyperreactivity in asthmatic airways, while contributing to what is already known about normal airway physiology and maintaining a role for eosinophil-mediated airway hyperreactivity. It should be stated, however, that a large number of asthmatics are refractory to anti-inflammatory therapies, suggesting mechanisms of airway hyperreactivity inherent to non-immune resident cells in the lung (Brusasco et al., 1998; Crimi et al., 1998).

Airway parasympathetic nerves induce contraction of airway smooth muscle by the release of acetylcholine onto M3 muscarinic receptors on the airway smooth muscle. In a self-regulatory system of autoinhibition, acetylcholine also acts through

inhibitory M2 muscarinic receptors on the airway parasympathetic neurons, limiting further acetylcholine release from the nerve terminal, in effect limiting contraction of the airway smooth muscle (Fryer and Maclagan, 1984, 1987a, b) (Figure 1.4A). Airway eosinophilia is a hallmark of asthma. Increased eosinophil numbers are found in the sputum or biopsies of the majority of asthma cases, and around the airway nerves of humans who died of asthma (Costello et al., 1997; Kay, 2005; Lemiere et al., 2006). The eosinophils chemotax to the airway nerves in response to chemokines that bind CCR3 on the eosinophils (Fryer et al., 2006). Upon reaching the nerve and binding to ICAM, the airway eosinophil is activated and releases preformed granule proteins, such as major basic protein (Sawatzky et al., 2002; Nie et al., 2007). Major basic protein is found deposited throughout the airways of humans who died of asthma (Costello et al., 1997). Eosinophil activation at the nerve can be triggered by virus infection, antigen inhalation in pre-sensitized airways, or ozone exposure, all known causes of asthma exacerbations discussed in the next section (Evans et al., 1997; Adamko et al., 1999; Yost et al., 2005; Proskocil et al., 2008). Major basic protein released by the activated eosinophils is an allosteric M2 muscarinic receptor antagonist. Its action at airway parasympathetic nerves results in more acetylcholine release and more bronchoconstriction (Jacoby et al., 1993). Eosinophil-mediated airway dysfunction can be inhibited with an antibody against IL-5, a cytokine for eosinophil recruitment and maturation, as well as by antibody or heparin-mediated charge neutralization of major basic protein (Fryer and Jacoby, 1992; Jacoby et al., 1993; Adamko et al., 1999) (Figure 1.4B). IL-5

is a T_h2 cytokine, again emphasizing the role of this type of immune environment in asthma pathology.

2. Causes of disease development and exacerbations

Factors contributing to the pathogenesis of asthma include genetic factors, such as predisposition to allergy or obesity, as well as environmental factors, such as allergens, infections, pollutants, cigarette smoke, occupational exposures, and diet. These factors probably operate in distinct mechanisms but converge on final common pathways that result in the airway inflammation, bronchoconstriction, and airflow obstruction of asthma. For example, some pathogens might trigger a more neutrophilic inflammation and allergens a more eosinophilic inflammation, while oxidant pollution directly inflames airway epithelium. All of these pathways can converge on asthmatic airway inflammation and hyperreactivity. While this is an oversimplification, it is in this way that asthma is considered a very heterogeneous disease. This section will briefly discuss some of the known triggers of asthma exacerbations, with particular attention given to respiratory virus infections.

Allergy

Allergy plays a central role in asthma. More than 80% of children with asthma are sensitized to environmental allergens (Singh and Busse, 2006). Again in support of an association of the T_h2-type immune environment in the lungs with asthma, allergy is categorized as a T_h2 disease, and prolonged exposure to aerosolized allergens results in T_h2-driven allergic inflammation of the airways (Peden, 2001). Common allergens include pollen, fungi, mites, dust, and pets. Following an allergen

challenge, the asthmatic airway is characterized by eosinophilic inflammation and elevated levels of the T_h2 cytokines IL-5 and IL-13 (Singh and Busse, 2006). The role of eosinophils in the exacerbation of asthma was described above. The role of allergy in asthma might be to set the immune environment that amplifies the effects of other asthma exacerbating insults, so that the inflammatory effects are more severe. The subsequent insult could be exposure to the sensitizing allergen, virus infection, or pollutants.

Respiratory viruses

In a study of asthma in 9 to 11 year old children, up to 85% of exacerbations defined by excessive wheezing, cough, and fall in peak expiratory airflow, had upper respiratory virus infections as determined by polymerase chain reaction (Johnston et al., 1995). These infections were predominantly attributed to rhinovirus, a picornavirus, which is associated with the common cold. In studies in adults that experienced an asthma exacerbation, as defined as a decrease in peak expiratory flow rates, 57% of the patients had virus upper respiratory infections, mostly attributed to rhinovirus (Nicholson et al., 1993). Furthermore, the rate of hospital admission for asthma attacks closely correlates with the seasonal pattern of rhinovirus infections (Johnston et al., 1996). Other studies suggest that as many as 80% of adult asthma exacerbations are likely associated with respiratory virus infections (Minor et al., 1976). Though these studies focused on respiratory virus infections of the upper airways, rhinovirus infection can spread to the lower airways, and it is proposed that this might account for more sever asthma

symptoms (Mosser et al., 2002; Singh and Busse, 2006). Other respiratory viruses associated with asthma exacerbations include coronavirus, influenza, parainfluenza, and respiratory syncytial virus (Johnston et al., 1995). These are all viruses that have a single stranded RNA (ssRNA) genome.

The airway epithelium is the primary site of entry for respiratory viruses (Papadopoulos et al., 2000). The viruses continue to replicate with virus shedding and infection of neighboring cells. The epithelial cells release cytokines and signal the presence of the virus infection, triggering an immune response to combat and clear the infection (Jacoby, 2002). Respiratory viruses can also enter and replicate in alveolar macrophages contributing further immune surveillance and releasing cytokines to signal the presence of a virus infection (Cirino et al., 1993).

Both healthy humans and humans with asthma are infected by and clear respiratory virus infections, but the symptoms are much more severe in humans with asthma. In support of the skewing of the airway immune environment towards a T_h2 allergic environment as a factor in asthma severity, it was found that rhinovirus shedding and symptom severity were directly correlated with the T_h2-type cytokine IL-5, but inversely correlated with the T_h1-type cytokine interferon-γ (Gern et al., 2000; Parry et al., 2000). This supports the idea that while both allergy and viruses exacerbate asthma, exacerbation by a virus infection might be more potent in an already allergic environment. Conversely, the virus infection might potentiate subsequent allergic responses (Lemanske et al., 1989). This tells us the importance of the cytokines and cell types in the immune environment, but tells us little about

the direct mechanisms mediating the asthma symptoms. Answering the question of why the symptoms are so much more severe and result in exacerbations in the asthmatic conditions is a focus of the work in this thesis.

Some of the proposed mechanisms of virus-mediated airway hyperreactivity in asthma are described here. Neutral endopeptidase activity in the epithelium is reduced during respiratory virus infection. Neutral endopeptidase in the airways inactivates tachykinins, such as substance P, which are non-adrenergic non-cholinergic peptide transmitters released from sensory nerves that induce airway smooth muscle contraction, recruit lymphocytes, and stimulate mucus secretion through neurokinin receptors. As activity of this enzyme is reduced during respiratory virus infection, there is reduced inactivation of contractile tachykinins, allowing their effects to persist (Jacoby et al., 1988; Dusser et al., 1989). Histamine also induces reflex bronchoconstriction. Virus infections can reduce the activity of histamine N-methyltransferase, which inactivates histamine (Nakazawa et al., 1994). The result is increased histamine persistence in the airway and increased bronchoconstriction. Viruses can potentiate IgE–mediated histamine release from basophils and mast cells in the airways (Ogunbiyi et al., 1988; Clementsen et al., 1991). Virus-mediated potentiation of 5-lipoxygenase increases the production of contractile leukotrienes lipid mediators described earlier (Seymour et al., 2002). Decreased non-adrenergic non-cholinergic nerve function in response to virus infection can reduce the bronchodilating effects of nitric oxide and vasoactive intestinal peptide (Folkerts et al., 1998). Epithelial damage in response to virus infection can occlude the airways with cellular debris, as well as expose sensory

nerve endings, increasing the sensory-mediated reflex bronchoconstriction described earlier (Papadopoulos et al., 2007).

As previously described, asthma is associated with a pathological skewing of the immune environment away from an antiviral T_h1-type towards a T_h2-type. Regardless of other T_h2-type allergic symptoms, the reduced ability to combat virus infections in the T_h2-type environment is also proposed for the more severe and longer sustained symptoms of respiratory virus infections (Wark et al., 2005). For example, viruses could persist due to reduction of interferons characteristic of the T_h1-type immune response (Graham et al., 1994). Interferons are particularly well suited to combat virus infection by priming cells to inhibit infection and replication of the virus (Contoli et al., 2006).

In a previous section, a mechanism of eosinophil-mediated airway hyperactivity was described. A virus-induced increase in intercellular adhesion molecule expression on airway effector structures can increase eosinophil recruitment (Papi and Johnston, 1999; Grunberg et al., 2000). Virus-induced eosinophil activation can trigger airway hyperreactivity by causing dysfunction of prejunctional M2 muscarinic receptor on parasympathetic nerve terminals (Adamko et al., 1999). Reduced M2 muscarinic receptor function might also occur by reduced M2 receptor expression in response to virus infection or interferon signaling, or direct modification of the receptor by the activity of viral neuraminidase (Fryer et al., 1990; Fryer and Jacoby, 1991; Jacoby et al., 1998).

Although eosinophils can be activated by virus to mediate airway hyperreactivity, it is important to note that eosinophils also exert potent antiviral properties (Adamko et al., 1999). This presents an interesting conundrum that emphasizes the complexity of the immune system and the limitations of oversimplified interpretations. Eosinophilia and asthma are associated with T_h2-type immune responses, but eosinophils themselves are potent antiviral effector cells, which is a property of a T_h1-type immune response. Furthermore, allergic eosinophil activation, responsible for M2 muscarinic receptor dysfunction, is associated with T_h2 responses, but virus infections induce interferon production associated with T_h1 responses that can also reduce M2 muscarinic receptor function (Fryer et al., 1990; Jacoby et al., 1998). This emphasizes the need to consider a more complex spectrum of immune phenotypes rather than absolute definitions of immune environments. It further emphasizes the need to consider functional changes in immune cells, such as the eosinophils, under different conditions, rather than attributing the presence of a particular cell type to an absolute type of immune response, suggesting that eosinophil subtypes may mediate different responses.

Bacterial infections of the respiratory tract, such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, are also associated with asthma exacerbations, but are not the focus of this work (Johnston and Martin, 2005).

Pollution

Pollutants associated with asthma exacerbations include tobacco smoke, ozone, and organophosphorus pesticides (Bernstein et al., 2004; Brims and Chauhan, 2005).

Occupational exposures to a number of chemical and animal products add to the growing list of agents that exacerbate asthma (Mapp et al., 2005). As they are not the focus of the work in this thesis, they will not be discussed further. However, it should be stated again that asthma is a heterogeneous disease with common symptoms in part driven by ubiquitous mechanisms of airway physiology, so proposed therapies that arise from the work in this thesis, though discovered in a context of virus-induced asthma, might extend to multiple types of asthmatic airway dysfunction. Furthermore, as the effects of virus infection might be amplified in an allergic immune environment, the effects of virus infections can be amplified in airways also exposed to pollutants, and the effect of pollutants can be more severe in an allergic airway environment (Chauhan et al., 2003; Proskocil et al., 2008).

3. Therapies

A large and ever growing number of asthma therapeutics are marketed or in preclinical and clinical testing phases. Because asthma is a heterogeneous disease, there are numerous signaling pathways and cell types that therapeutic interventions can target. None of these completely treat all the symptoms of asthma, and all are associated with side effects. Furthermore, due to the frequency of administration or fear of side effects, resistance, or addiction, compliance with treatment regiments are low (Horne et al., 2007; Haughney et al., 2008). Despite numerous therapies, prohibitive costs and inadequate diagnosis in lower income countries results in higher morbidity and mortality of the disease (Mendis et al., 2007). For virus-induced asthma specifically, vaccination remains insufficient due

to the large number of viruses and high rate of mutation conferring resistance (Mallia et al., 2007). Asthma medications fall into two main categories: those for rescue from acute exacerbations and those for chronic prevention against future attacks. This section describes some of the available asthma therapies, future proposed therapies, and highlights the need for new and improved medicines.

Chronic prevention

The standard chronic prophylactic treatment for asthma is inhaled corticosteroid (fluticasone, budesonide) treatment. Among the many benefits of corticosteroid steroid therapy are reduced inflammation, eosinophilia, and airway hyperreactivity (Pauwels et al., 2003; Sin et al., 2004). These effects are mediated by glucocorticoid nuclear receptors that both up- and downregulate the expression of a number of genes. ICAM-1 expression is reduced, preventing the adhesion of immune cells in the airways. M2 muscarinic receptor expression and function is increased on parasympathetic nerves, limiting acetylcholine release and limiting bronchoconstriction (Jacoby et al., 2001). Because of the immunosupressive effects of corticosteroids, a side effect is an increased susceptibility to infection. Furthermore, a subpopulation of asthmatic patients are resistant to glucocorticoid therapy (Drazen et al., 2000). A less standard anti-inflammatory treatment are leukotriene receptor antagonists (montelukast), and these have the added benefit of being acute bronchodilators for rescue during ongoing attacks (Drazen et al., 1999; Camargo et al., 2003).

Acute rescue

Acute and severe asthma attacks are treated with rescue medications that relax the airway smooth muscle almost immediately upon administration. The most widely used rescue therapies are β_2-adrenergic receptor agonists and muscarinic receptor antagonists. β_2-adrenergic receptor agonists (salmeterol, fomoterol, albuterol) activate protein kinase A-mediated airway smooth muscle relaxant mechanisms discussed previously. Side effects include tachycardia due to activity at adrenergic receptors in cardiac muscle (Mallia et al., 2007). Although β_2-adrenergic receptor agonists have anti-inflammatory effects during chronic therapy, they are recommended for use in combination therapy with an inhaled corticosteroid for its anti-inflammatory properties (Mallia et al., 2007). Furthermore, corticosteroid treatment upregulates β_2-adrenergic receptor expression, increasing airway responsiveness to combined agonists, and β_2-adrenergic receptor agonists increase sensitivity to glucocorticoids, providing synergistic effects from combined therapy (Edwards et al., 2006). At the very least, combination therapies allow a multipronged approach to treat the two main components of asthma, bronchoconstriction due to airway hyperreactivity and inflammation.

Muscarinic receptor antagonists (ipratropium, tiotropium) acutely antagonize M3 muscarinic receptor on airway smooth muscle, inhibiting G_q-mediated intracellular calcium signaling, resulting in decreased airway smooth muscle contraction. The administration of these rescue medications reduces hospital admissions and duration of hospitalization (Brophy et al., 1998; Rodrigo and Rodrigo, 2000).

Though acute effects of muscarinic receptor antagonists are beneficial, chronic use of these medicines has mixed results, and can potentially worsen asthma symptoms. This is in part due to the blockade of inhibitory M2 muscarinic receptors on parasympathetic nerves, which increases bronchoconstriction, and in part due to blockade of inhibitory muscarinic receptors on immune cells, altering their function (Jacoby and Fryer, 2001; Verbout et al., 2007; Verbout et al., 2009). Other side effects include dry mouth due to blockade of M3 muscarinic receptors on salivary glands and tachycardia due to blockade of M2 muscarinic receptors on cardiac muscle.

Antiviral

Virus-specific treatments for asthma are few. Only influenza is successfully managed with vaccination (Bridges et al., 2001). Other antiviral approaches that have undergone clinical trials include capsid-binding inhibitors that prevent the attachment of the virus to it receptor, viral protease 3C inhibitors that inhibit the processing of viral proteins, and ICAM-1 blockers that block the rhinovirus receptor for cellular entry (Mallia et al., 2007).

Other therapies

The association of asthma and its propensity for skewing towards T_h2-type immunity makes targeting the mechanisms determining the immune environment a novel and intriguing therapy. As cytokines released from multiple cell types define the architecture and composition of the airway immune environment, cytokine-based therapies are one approach at attempting to counteract the effects of T_h2-type

immunity. A humanized monoclonal antibody to IL-5 (mepolizumab), a T_h2 cytokine for eosinophil recruitment and maturation, reduced sputum eosinophil numbers, but was of limited benefit in the allergic asthmatic response, though in a separate trial there was a prednisone sparing effect of this therapy, suggesting that it had beneficial anti-inflammatory effects (Leckie et al., 2000; Flood-Page et al., 2007; Nair et al., 2009). The beneficial effects of this therapy are more pronounced when trials are designed in patients with refractory eosinophilic asthma (Haldar et al., 2009). Anti-IgE therapy with a human monoclonal antibody (omalizumab) reduces the effects of IgE-mediated asthma symptoms in severe allergic asthma (Scheinfeld, 2005). Though it has few side effects, this therapy is cost prohibitive and only used in severe cases of asthma. Anti-TNF-α therapy (etanercept) with a soluble receptor to deplete TNF-α and inhibit inflammation has shown promise in clinical trials, but increased susceptibility to infection is a serious side effect (Palladino et al., 2003). An opposite approach is to stimulate T_h1 immunity by the administration of T_h1 cytokines. In clinical trials, administering a human recombinant T_h1 promoting cytokine IL-12 reduced sputum eosinophil counts, but did not improve airway hyperreactivity (Mallia et al., 2007). The success of any individual therapy can be masked by the heterogeneous nature of asthma. Though all patients are suffering from airway obstruction, the causes of this obstruction can be different. When asthma is the only prerequisite for patient entry into a clinical trial, the beneficial effects in subpopulations of asthmatics may be lost in the overall population tested. It is therefore important to properly design trials to focus on the types of asthmatic subpopulations for whom that therapy could be beneficial. Along those lines, it is

also important not to over interpret failures of clinical trials, as they might not have been designed with the power necessary to unveil beneficial effects.

The newest therapeutic approach to skewing the immune environment in the airways away from T_h2 and towards T_h1 is the use of immunomodulatory drugs that stimulate Toll-like receptors of the innate immune system and induce the production of interferons and T_h1 cytokines (Agrawal and Kandimalla, 2002). The work in this thesis contributes to the body of knowledge of the therapeutic benefit of targeting Toll-like receptors in asthma. Toll-like receptors will be described in greater detail in the next section.

Other existent and proposed therapies target all aspects of signaling associated with both normal and pathological airway physiology. These include phosphodiesterase inhibitors that inhibit cAMP breakdown and allow PKA-mediated relaxant effects to persist, blocking of chemokine receptors to block the recruitment of immune cells, transcription factor inhibitors to inhibit inflammatory transcription, cell proliferation inhibitors to inhibit airway remodeling, and ion channel modulators that increase relaxant repolarization of airway smooth muscle (Barnes, 2004). Even with many available medications, due to resistant subpopulations of asthmatic patients, numerous side effects, and unsatisfactory maintenance of symptoms, the need for new medications is clear.

4. Animal models

Human asthma is predominantly investigated by histological analysis of postmortem or biopsied tissue and minimally invasive techniques, such as

spirometry and whole body plethysmography, in asthmatic patients. Measurements provided by these types of methods include forced vital capacity (VC), which is the total volume of air that can be exhaled or inhaled during a respiratory maneuver, the forced expiratory volume in one second (FEV_1), a measure of how quickly the lungs can be emptied, and peak expiratory flow (PEF), a measure of flow rate (Mallia et al., 2007). These measurements are used to diagnose and study asthma. Rhinovirus infection of the airways of mild asthmatic humans is an approved model and recapitulates many of the features of virus-induced asthma exacerbations (Bardin et al., 1996; Bardin et al., 2000). This is an important model, because it allows for measurements at baseline before virus infection, and for separate measurements following virus infection. Because of the ethical, cost prohibitive, access, and availability issues surrounding human research, a number of animal models of airway physiology and asthma pathophysiology are used.

The quality of an animal model depends on which aspect of disease the research is investigating, be it acute exacerbations, chronic inflammation, pathogenesis, or lung development. The main disease characteristics for asthma that need to be addressed by the model are airway inflammation and airway hyperreactivity. However, an animal model in which airway inflammation is recapitulated, but airway smooth muscle physiology and innervation are completely irrelevant to the human condition is of limited utility. In the same way, an animal model in which the airway physiology is identical to humans, but the allergic response is dominated by a different cytokine or immune effector cell is also of limited utility. Developmental differences must also be considered when studying asthma in different age

populations or in pathogenesis. Asthma has been modeled in large animals, such as cats and pigs, but pertaining to work in this thesis, this section will focus on guinea pig and mouse models of airway physiology and asthma.

Guinea pig

Guinea pigs are commonly used as animal models for studying asthma. This is because guinea pig airway physiology is dominated by receptors whose pharmacology is the closest to that of humans (Canning and Chou, 2008). Furthermore, guinea pig airway anatomy is similar to humans, as opposed to mice. For example, mice lack subepithelial vasculature and have very few mucus glands, rather they depend more on goblet cells for mucus production (Choi et al., 2000). Guinea pigs also have similar airway smooth muscle anatomy and physiology to humans, as opposed to mice which have very little airway smooth muscle (Canning and Chou, 2008). Guinea pig airway smooth muscle has spontaneous tone controlled by autocrine and paracrine signaling very similar to humans, and it also secretes the chemokine eotaxin for eosinophil chemotaxis as in humans (Lambley and Smith, 1975; Ellis and Undem, 1994; Li et al., 1997; Ghaffar et al., 1999). Similar to humans, the autonomic innervation of airway smooth muscle in guinea pig is dominated by cholinergic nerves releasing acetylcholine onto postjunctional contractile M3 muscarinic receptors, with sparse adrenergic relaxant innervation of the intrapulmonary airways (Roffel et al., 1990; Preuss and Goldie, 1999). Non-adrenergic non-cholinergic relaxant innervation, as opposed to adrenergic relaxant innervation, is mediated by nitric oxide and vasoactive intestinal peptide and is the

more dominant form of relaxation of airway smooth muscle in both guinea pigs and humans (Li and Rand, 1991; Ward et al., 1995). The reflex responses in the airway triggered by sensory afferents are also similar in guinea pigs and humans, particularly the cough reflex, which some argue is absent in mice (Canning, 2006; Canning et al., 2006). Finally, histamine is the allergic mediator released by mast cells in response to cross linking of their high affinity IgE receptors in humans and guinea pigs, but in mice it is serotonin (Martin et al., 1988; Bjorck and Dahlen, 1993). This makes the guinea pig a more relevant model of allergic sensitization. While the guinea pig airway models many of the important features of human airway physiology, pharmacology, and disease, there are some differences from humans. For example, the sensory axon reflex of local tachykinin release without relay to the central nervous system is a hallmark of guinea pig responses, but is largely absent in humans (Canning and Chou, 2008). Beyond the similarities in the guinea pig and human airways, the larger size of the guinea pig relative to the mouse makes both *in vivo* and *in vitro* measurements of airway physiology more feasible. Models of asthma in the guinea pig used in the work presented in this thesis include allergen sensitization and respiratory virus infection.

Mouse

The mouse is a widely used laboratory animal model. Its advantages are numerous from size to rapid breeding, as well as widespread availability of reagents for research. The particular advantage that makes mouse indispensible is the widespread generation of transgenic strains with genes knocked out or

overexpressed to study the function of all aspects of biological functions, by the presence, absence, or overexpression of a gene product. For this reason, despite differences in airway physiology, the mouse model is still an important tool for understanding the airways. The development of tools for the generation of more transgenic species holds promise that similar studies will one day be carried out in a more pertinent animal model for airway physiology. The mouse has shortcomings beyond those described in the previous section, particularly with regards to modeling allergic asthma. Accumulation of eosinophils in the epithelium of allergen sensitized and acutely challenged mice is lacking, and the eosinophils present are not readily degranulated upon activation (Korsgren et al., 1997; Malm-Erjefalt et al., 2001). The consequences of inflammation are also very different, with less inflammation of the airway wall, subepithelial fibrosis, and epithelial cell proliferation (Kumar and Foster, 2002). Nonetheless, the positive contributions from transgenic strains outweigh the negative, and work in this thesis is also carried out in allergen sensitization and challenge, virus infection, and transgenic mouse models.

FIGURE 1.3. The asthmatic human airway is obstructed by inflammation and hyperreactive airway smooth muscle contraction, making breathing difficult.

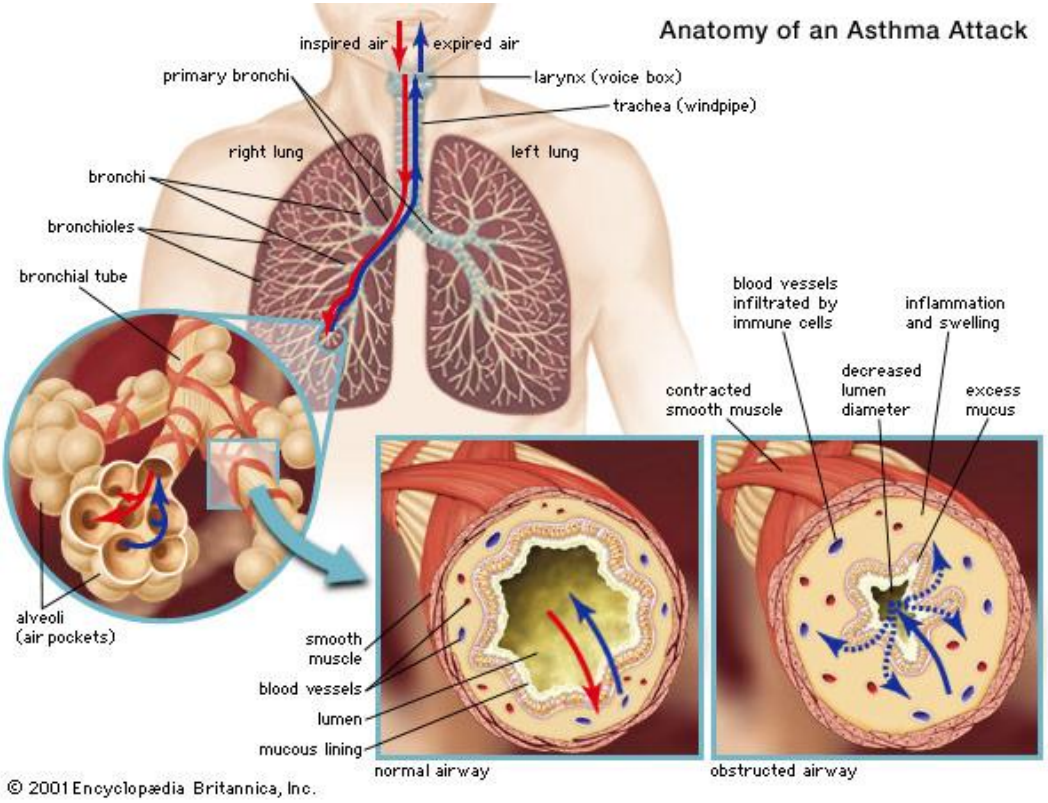
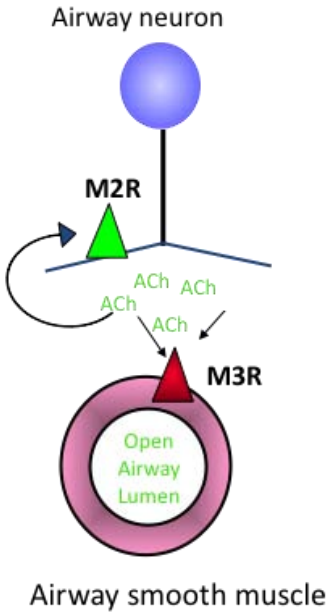


Figure 1.1. Inflammation of the airways in the human lungs combined with hyperreactive contraction of the airway smooth muscle, results in narrowing of the airway lumen and airway obstruction. In the lower left is a normal airway and on the right, an obstructed airway. The increased swelling, mucus secretion, airway smooth muscle contraction, and immune cell infiltration indicated in the schematic all contribute to narrowing the airway diameter. By courtesy of Encyclopaedia Britannica, Inc., copyright 2001; used with permission.

FIGURE 1.4. Inhibitory M2 muscarinic receptors and airway hyperreactivity mediated by M2 dysfunction following virus-induced eosinophil activation.

A



B

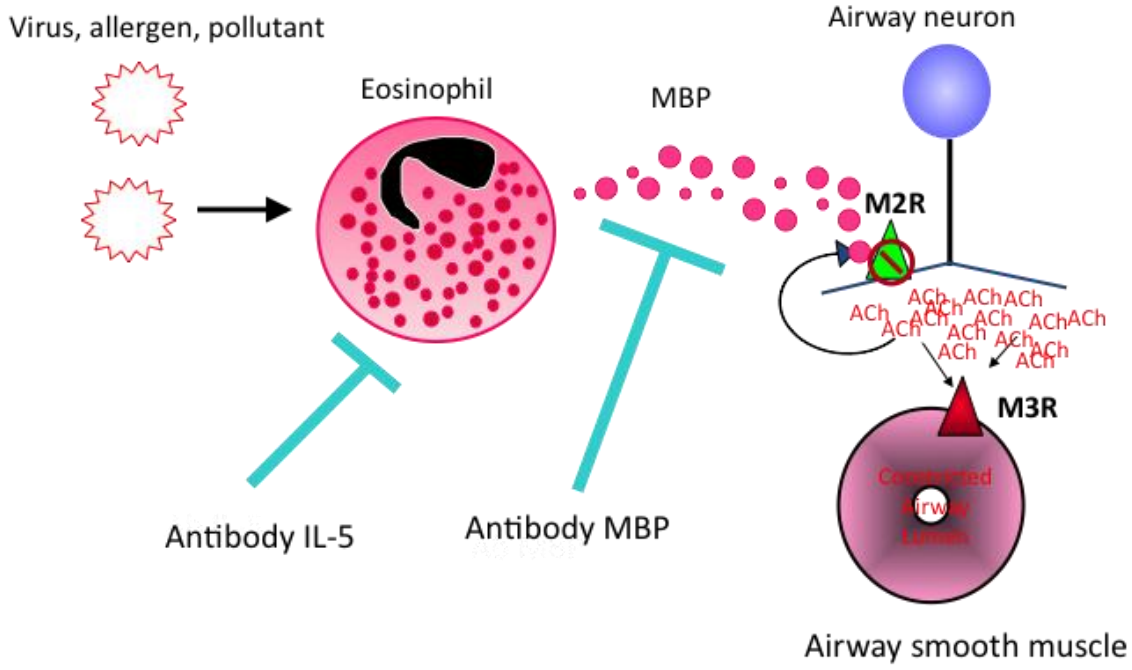


Figure 1.4. (A) This schematic shows the parasympathetic innervation of airway smooth muscle around the airway lumen of a normal airway. Acetylcholine released from the nerve terminals induces contraction via M3 muscarinic receptors on the airway smooth muscle. Acetylcholine also feeds back onto inhibitory M2 muscarinic receptors limiting further acetylcholine release and limiting bronchoconstriction. (B) This schematic shows the effects of virus, allergen, or pollutant induced eosinophil activation around parasympathetic nerves in an asthmatic airway. Major basic protein (MBP) released from the eosinophils antagonizes the inhibitory M2 muscarinic receptors. The result is increased acetylcholine release and increased bronchoconstriction. This effect can be inhibited by depleting eosinophils with an antibody to IL-5 or neutralizing major basic protein with an antibody.

D. INNATE IMMUNITY AND TOLL-LIKE RECEPTOR 7

This thesis focuses on virus-induced exacerbations of asthma, described in the previous section. Asthma and allergy may be part of a defective immune response. Furthermore, some of the deleterious effects of asthma, such as airway remodeling, arise from aberrant tissue damage due to overactivity or autoactivity of the immune system. This section describes mechanisms by which the lungs detect respiratory virus infections and the subsequent immune response to clear the infections. Because the experimental body of this work addresses the role of Toll-like receptor 7, particular attention is given to the detection of viruses by this receptor of the innate immune system.

1. The innate immune system

The adaptive immune system provides protection from invading pathogens in the form of memory from the first encounter, with specificity towards that pathogen to mount the appropriate response to combat subsequent encounters. The adaptive immune response, even after the first encounter, requires several days of clonal expansion of the specific lymphocytes involved in the response. Before the recruitment of T and B lymphocytes to the site of infection for cytotoxic or antibody-mediated protection, pathogens are first recognized by receptors of the innate immune system which mount an early immune response to fight the pathogen and recruit the adaptive immune system (Janeway and Medzhitov, 2002). These receptors recognize conserved molecular patterns against whole classes of pathogens. Their ability to recognize these patterns is genetically encoded and not

the result of somatic recombination, as is the case for antibodies. For example, a molecular structure from a highly conserved component of a virus genome will trigger a general antiviral response, in spite of not recognizing which specific virus subtype it is. In spite of its lack of specificity, the innate immune system has the ability to distinguish between pathogens and self on resident cells and structures.

Allergy arises from the triggering of a T_h2-type immune response to allergens. The innate immune system is responsible for initially recognizing the allergen and mounting this defense. This comes in the form of an IgE dominated antibody response, and as discussed previously, crosslinking of high affinity IgE receptors on mast cells resulting in their degranulation and release of allergic mediators. These are the same mediators required to fight extracellular parasites like helminth worm infections, so there is an evolutionary basis for maintaining this seemingly pathological immune response (Medzhitov, 2010). Some allergens contain proteases that mimic proteases found in parasites that trigger T_h2 responses, so the recognition of these allergens might come from the misinterpretation of enzymatic activity as that of a parasite (Sokol et al., 2008). Allergens may have structures that resemble parasitic structures, or alternatively adjuvant properties of microbial pathogens encountered at the same time as sensitizing allergens could mount a response against those allergens (Akira et al., 2001). Regardless how the response is initiated, the immune environment in the lung is set by the cytokines released by various resident and immune cells in response to the antigens breathed into the airways and by the signaling cascades triggered by pattern recognition receptors of the innate immune system. Pathogenic imbalance of the immune system skewing it

towards T_h2 away from T_h1 is the result of these signal cascades triggering the production of T_h2-type cytokines, such as IL-5 and IL-13, or the absence of signal cascades that trigger the production of T_h1-type cytokines, such as IL-10 and interferon-γ. The pattern recognition receptors that trigger these signal cascades include protein kinase R, 2'-5'-oligoadenylate synthase, Nod-like receptors, RIG-1, and Toll-like receptors (Janeway and Medzhitov, 2002). Interferon production in response to innate recognition of a virus infection results in many antiviral responses including development of a stronger more specific adaptive immune response, upregulation of major histocompatibility complex type I for presentation of intracellular virus antigens, induction of CD8+ cytotoxic T cells to kill virus infected cells, and the inhibition of virus RNA replication and protein synthesis (Iwasaki and Medzhitov, 2004; Theofilopoulos et al., 2005).

2. Toll-like receptors (TLRs)

Toll-like receptors were first identified for their role in dorso-ventral axis formation in *Drosophila* development (Hashimoto et al., 1988). It was recognized that the *Drosophila* TLR and the human IL-1 receptor have homologous cytoplasmic adaptor domains now termed Toll-interleukin 1 receptor (TIR domains), and both receptors induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (Belvin and Anderson, 1996). It was later found that *Drosophila* Toll mutants were susceptible to fungal infection (Lemaitre et al., 1996), and the association of mammalian TLRs and immunity became increasingly clear.

Structurally, TLRs are single pass type I transmembrane proteins with extracellular leucine-rich repeats that participate in ligand recognition, and an intracellular TIR domain for signal transduction (Kawai and Akira, 2010).

TLRs recognize pathogen associated molecular patterns including lipids, proteins and nucleic acids from bacteria, funguses, parasites, and viruses (Janeway and Medzhitov, 2002) (Figure 1.5). Though differences exist based on the specific cell type and TLR, canonical TLR signaling proceeds through the TIR domain containing adaptor molecule MyD88, and the assembly of a signal scaffold that triggers the downstream activation of transcription factors (Figure 1.5). These transcription factors, including NF-κB, interferon regulatory factors, and AP-1 from the MAPK pathway, regulate the expression of a wide range of inflammatory genes, including those for cytokines, chemokines, antigen presentation, antimicrobial peptides, and costimulatory molecules . These products are involved in mounting an immediate host defense and the induction of an adaptive immune response against the invading pathogen (Janeway and Medzhitov, 2002). Of note, TLR3 for double stranded (ds) virus RNA, does not signal through MyD88, but instead the TIR domain containing TRIF (TIR domain containing adaptor inducing interferon-β), and TLR4, for bacterial lipopolysaccharide, can signal through both MyD88 and TRIF (Diebold et al., 2003; Yamamoto et al., 2003).

The TLRs that recognize viral products predominantly recognize virus nucleic acids, though other TLRs might recognize molecular patterns of the virus coat. TLR7 and TLR8 recognize virus ssRNA, TLR3 recognizes virus dsRNA, and TLR9 recognizes

virus or bacterial DNA. Because viruses are intracellular pathogens, TLR3, TLR7, TLR8, and TLR9 predominantly signal intracellularly in acidic endosomes (Ahmad-Nejad et al., 2002; Heil et al., 2003; Matsumoto et al., 2003; Funami et al., 2004). They translocate from the endoplasmic reticulum to the endosome upon ligand stimulation (Kim et al., 2008). The current thought is that this localization prevents the recognition of self-RNA and DNA to prevent autoimmunity (Barton et al., 2006; Diebold et al., 2006). This subcellular localization and translocation, however, has been characterized in immune cells, and other subcellular or cell surface localization cannot be ruled out at this time. Importantly, the subcellular localization is controlled by coordinated signaling of a number of components (Figure 1.5), so defects in any of these components could result in TLR dysfunction.

TLR dysfunction is associated with a number of human diseases, as it perturbs the delicate balance of the immune system's protective and deleterious functions. TLR4 polymorphisms in humans are associated with reduced responsiveness to gram-negative bacteria and respiratory syncytial virus, both ligands for TLR4 (Kadowaki et al., 2001; Agnese et al., 2002; Lorenz et al., 2002; Zarember and Godowski, 2002; Child et al., 2003; Tal et al., 2004). These infections result in severe health problems including bacterial sepsis and bronchiolitis. TLR2 mutations in humans are associated with increased susceptibility to tuberculosis and leprosy (Kang and Chae, 2001; Ben-Ali et al., 2004).

Exposure to lipopolysaccharide early in life is associated with decreases in asthma and allergy (Michel et al., 1996; Braun-Fahrlander et al., 2002). TLR4 activity

induces T_h1-type cytokine signaling skewing the airway towards this immune environment. The early exposure in life to lipopolysaccharide might result in strong induction of T_h1 cytokine production, reducing the occurrence of asthma and allergy.

Because of their immunomodulatory capability, TLRs are a therapeutic target of growing interest. Tumors have been targeted with TLR ligands to induce the production of cytokines to initiate an immune response that will kill as well as to induce apoptosis in the tumor cells and protect against future recurrence (Carpentier et al., 2006; Quirk et al., 2010). TLR agonists have also been studied for their potential neuroprotective effects from stroke and cerebral ischemia (Bsibsi et al., 2006; Marsh and Stenzel-Poore, 2008). TLR ligands have been proposed for protection against lupus, rheumatoid arthritis, Chrohn's disease, and atherosclerosis, to reduce the autoinflammation associated with the diseases (Andreakos et al., 2004; Barrat et al., 2005).

3. Toll-like receptor 7 and Toll-like receptor 8

The work in this thesis focuses on TLR7 signaling. TLR7 recognizes virus ssRNA as well as synthetic molecules of the imidazoquinoline and guanosine analog structural classes (Hemmi et al., 2002; Heil et al., 2003; Lee et al., 2003). One of the imidazoquinoline compounds, R837 (Imiquimod), is marketed as Aldara for the treatment of genital warts and other virus induced lesions, including cancers (Chang et al., 2005). The mechanism of action is the induction of interferons and the infiltration of immune cells to destroy the virus infection. The ability of the innate

immune system to recruit the adaptive immune system provides future protection and limits recurrence of these virus-induced lesions. Because TLR7 recognizes virus ssRNA, synthetic ssRNA ligands have been developed based off virus nucleotide sequences derived from human immunodeficiency virus, vesicular stomatitis virus, and influenza virus (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). There is, however, much species-dependence on which synthetic ligands and virus sequences are recognized by TLR7, TLR8, or both. Though it will not be discussed further here, TLR9 recognizes viral CpG DNA from Herpes simplex viruses, and induces interferon production to combat the virus infection (Lund et al., 2003; Krug et al., 2004).

TLR7 and TLR8 both recognize virus ssRNA and induce interferon production for antiviral immunity (Hoshino et al., 2002; Hemmi et al., 2003). This overlapping or redundant function probably exists for a reason. Perhaps the difference lies in the specific virus ssRNA sequences they recognize. Some propose that the guanine and uridine (G/U) content of the virus ssRNA sequence confers specificity to TLR8, but there are contradictions in this claim across groups (Heil et al., 2004; Hornung et al., 2005; Meier et al., 2007). Another possibility is the differential expression allows specific responses from different cell types. For example, human plasmacytoid dendritic cells express TLR7 and myeloid dendritic cells express TLR8 in humans (Diebold, 2008). With the recent development of a TLR8-deficient mouse, it was shown that TLR8 serves a role in regulating TLR7 function, with hyperreactive TLR7 responses in dendritic cells deficient in TLR8 (Demaria et al., 2010). However, a compensatory TLR7 upregulation cannot be ruled out as the reason for this

observation. TLRs dimerize in response to ligand binding, and TLR2 has been shown to heterodimerize with TLR1 and TLR6 to differentiate between diacyl and triacyl bacterial lipopeptides respectively (Takeuchi et al., 2001; Takeuchi et al., 2002). Potentially, TLR7 and TLR8 heterodimerize to make a unique signaling species from TLR7 or TLR8 homodimers. When cotransfected into HEK293 cells physical interactions cross regulating function were shown between TLR7, 8, and 9 (Wang et al., 2006a). Many of the problems differentiating the roles for these receptors might arise from their expression alone in recombinant cell lines or their individual knockout in deficient mouse strains. The absence of a response to specific ligands might not be due to the absence of a single receptor, rather the absence of the ability to form heterodimers. For example the basis for the redundant recognition of the imidazoquinoline compound R848 by both TLR7 and TLR8 comes from the lone recombinant expression of either TLR7 or TLR8 in HEK293 cells (Jurk et al., 2002). Furthermore, the presence of thymidine homopolymer oligodeoxynucleotides induced plasticity in ligand recognition between human TLR7 and TLR8, suppressing TLR7 function and promoting TLR8 function, again demonstrating a lack of a simple distinction as redundant receptors (Jurk et al., 2006).

Another example of the poor characterization of ligand recognition by TLR7 and TLR8 is that TLR8 was identified as nonfunctional in mice (Heil et al., 2004). This is a dubious claim due to the evolutionary pressure necessary in the maintenance of a vestigial receptor. This citation is frequently referenced to demonstrate that TLR8 is nonfunctional in mice, but is based on unpublished results claiming a lack of response to ssRNA in murine TLR8-deficient dendritic cells. This TLR8-deficient

mouse is not available in strain banks and no other publications exist from this mouse. To put to rest the concept of nonfunctional mouse TLR8, it was shown that coincubating TLR7 ligands with thymidine homopolymer oligodeoxynucleotides potentiates the function of mouse TLR8 and abrogates the function of mouse TLR7 expressed in HEK293 cells (Gorden et al., 2006). More recently it was shown that TLR8 mediates the activation of mouse plasmacytoid dendritic cells by vaccinia viral DNA, a non-ssRNA pathogen molecule (Martinez et al., 2010). As of recently, a new TLR8-deficient mouse strain was generated (Demaria et al., 2010). Responses to TLR7 and TLR8 ligands in these mice compared to TLR7-deficient and TLR7/8 double-deficient mice will be important for understanding the differences or similarities between these receptors and their potential interactions.

When considering how viruses interact with the airways, it is important to determine in which organs and cells TLR7 and TLR8 are expressed. Based on mRNA pooled from whole immune organs, it was determined that the highest TLR7 and TLR8 expression levels are in the lung and spleen (Chuang and Ulevitch, 2000; Nishimura and Naito, 2007). Because its original mammalian functional identification was in the innate immune system, TLR7 expression has been highly characterized in immune cells. TLR7 is expressed in eosinophils and mast cells, and functions in the activation of these allergy-related immune cells (Nagase et al., 2003; Kulka et al., 2004; Heib et al., 2007; Phipps et al., 2007; Wong et al., 2007). This activation is rapid, and is an example of a non-canonical TLR7 signaling mechanism probably not involving gene regulation to be discussed later with regards to the work in this thesis. Human eosinophils from atopic donors are hyperreactive in

response to TLR7 ligands (Mansson and Cardell, 2009). There are conflicting reports in the literature about whether TLR7 is expressed in neutrophils, but consensus exists that they express TLR8 (Francois et al., 2005; Wang et al., 2008; Janke et al., 2009). Consistent with their primary function as antigen presenting cells, pulmonary plasmacytoid dendritic cells express TLR7 and pulmonary myeloid dendritic cells express TLR8 (Demedts et al., 2006). TLR7 expression in B cells is critical for driving the appropriate antibody isotype switching in response to influenza virus infection (Heer et al., 2007).

TLR7 is also expressed in a wide range of nonimmune cell types. TLR7 expression was identified in cultured airway smooth muscle, which was upregulated by incubation with IL-1β and TNF-α (Sukkar et al., 2006). Though they are not airway cells, the expression of TLR7 and TLR8 in cardiac muscle further supports the ability of contractile muscle cells to express these TLRs (Triantafilou et al., 2005). As airway epithelial cells are among the first exposed to inspirated respiratory viruses, they also express TLR7. TLR7 is expressed in endothelial cells affecting immune cell extravasation into the tissue (Gunzer et al., 2005). Though it has yet to be shown in airway neurons, the precedence of TLR7 and TLR8 expression and function in peripheral and central neurons and microglial cells has been established (Olson and Miller, 2004; Su et al., 2005; Ma et al., 2006; Butchi et al., 2010; Goethals et al., 2010; Liu et al., 2010). It is clear that TLR7 and TLR8 are expressed in a wide range of cell types, suggesting their importance in mediating antiviral protection, particularly in the lung.

Almost all of the effects of TLR7 described above arise from canonical signaling via modulation of gene expression. The work in this thesis focuses on non-canonical signaling mechanisms of TLR7 that can result in faster effects on airway physiology. Non-canonical TLR7 signaling remains largely uncharacterized. Some of the signaling pathways described in an earlier section on airway smooth muscle have been implicated at some level with TLR signaling and will be discussed here.

The TLR4 ligand lipopolysaccharide can induce prostaglandin E_2-dependent relaxation of isolated mouse trachea *in vitro* (Balzary and Cocks, 2006). Though the maximum relaxant effect of lipopolysaccharide was achieved in 60 minutes, acute relaxation was measured within 15 minutes of administration to the organ bath. In human neutrophils, the TLR7 and TLR8 agonist R848 primes the cells for release of prostaglandin E_2 in response to N-Formylmethionyl-leucyl-phenylalanine (fMLP) stimulation within 10 minutes of administration (Hattermann et al., 2007). These data suggest that TLR7 signaling can induce prostaglandin E_2 release, a mediator with fast signaling effects through G-protein coupled receptors as described earlier.

TLR4 and TLR2 ligands can amplify MaxiK potassium channel activity to activate human macrophages within minutes of administration (Scheel et al., 2006). If this mechanism is conserved in the context of airway smooth muscle membrane repolarization and relaxation, it demonstrates the ability of TLRs to signal through this critical regulator of smooth muscle physiology.

The relaxant effect of lipopolysaccharide mentioned above also occurs in isolated guinea pig tracheas, and is partially dependent on nitric oxide (Fedan et al., 1995).

The TLR7 agonist R837 induces nitric oxide production in B cells (Tumurkhuu et al., 2009). These data suggest that nitric oxide signaling is functionally coupled to TLR signaling in smooth muscle and that a TLR7 agonist can also induce nitric oxide production. Therefore, the possibility exists that TLR7 signaling in airway smooth muscle cells can result in the production of nitric oxide, a rapid regulator of airway smooth muscle physiology. Phosphoinositide 3-kinase, which can be activated by TLR7 agonists, is involved in the activation of the nitric oxide synthase enzyme for the production of nitric oxide (Francois et al., 2005; Wang et al., 2006b; Hazeki et al., 2007). Perhaps the regulation of nitric oxide production is accomplished through phosphoinositide 3-kinase, which is involved in the recruitment of a number of signal scaffolding molecules to the cell membrane, affecting a number of pathways regulating airway smooth muscle physiology.

Mitogen activated protein kinase (MAPK) pathways, though primarily involved in the regulation of gene expression, also have targets along their signal cascades that can mediate acute changes in airway physiology. MAPK is also a pathway involved in canonical TLR7 signaling. Focal adhesion kinase is involved in MAPK activation of eosinophil migration in response to microbial infection, and the TLR7 agonist R837 activates eosinophils through focal adhesion kinase (Cheung et al., 2008). Focal adhesion kinase regulates actin polymerization and depolymerization during contraction and relaxation in airway smooth muscle, as well as rapid changes in the cytoskeleton of the smooth muscle in response to changes in airway inflation (Smith et al., 1997; Gunst et al., 2003). If the focal adhesion kinase signaling pathway is

functionally coupled to TLR7 signaling in airway smooth muscle cells, it too could have rapid effects on airway smooth muscle function.

Adenosine receptors are G-protein coupled receptors whose signaling can have bronchoconstrictive or bronchodilatory affects on airway smooth muscle, as described earlier (Smith and Broadley, 2008). The TLR7 agonist R837 has been shown to interact with adenosine receptors as an off target effect, but synergy between adenosine A2a receptors and TLR7 has also been reported (Pinhal-Enfield et al., 2003; Schon et al., 2006).

Canonical TLR7 signaling involving regulation of gene expression is not the only effect of TLR7 stimulation. Non-genomic fast signaling mechanisms implicated in airway smooth muscle physiology might also be involved.

TLR7 and TLR8 polymorphisms are associated with asthma. This was determined by linkage analysis in families with high asthma prevalence, as well as reduced TLR7 responsiveness to R837 in blood mononuclear cells from adolescents with asthma (Moller-Larsen et al., 2008; Roponen et al., 2010). The reduced responsiveness might result in defective immunity against viruses, or it might result in skewing towards T_h2 type immunity due to the reduced production of T_h1 cytokines.

Asthma and allergy are associated with T_h2-type immunity, which results in reduced resistance to virus infections and increased inflammation and airway remodeling mediated by T_h2 cytokines. TLR7 signaling is associated with upregulation of cytokines for T_h1-type immunity skewing away from T_h2-type immunity. Therefore, many groups have shown that administration of TLR7 ligands in animal models of

allergic inflammation results in reduced airway remodeling, IgE production, airway hyperreactivity, and inflammation (Stokes et al., 1998; Quarcoo et al., 2004; Napolitani et al., 2005; Moisan et al., 2006; Camateros et al., 2007; Sel et al., 2007; Xirakia et al., 2010). These characteristics have led to the proposal of TLR7 agonists as prophylactic therapeutics for humans with asthma, providing chronic protection from T_h2-type inflammation, virus infection, and asthma. Little is known about the acute effects of these ligands on airway physiology in short time frames irrelevant to most inflammatory processes. The work in this thesis addresses the rapid effects of TLR7 signaling in airway physiology, and proposes a further therapeutic use of TLR7 agonists as rescue medications from acute bronchoconstriction during an active asthma attack.

FIGURE 1.5. Canonical Toll-like receptor ligands and signaling.

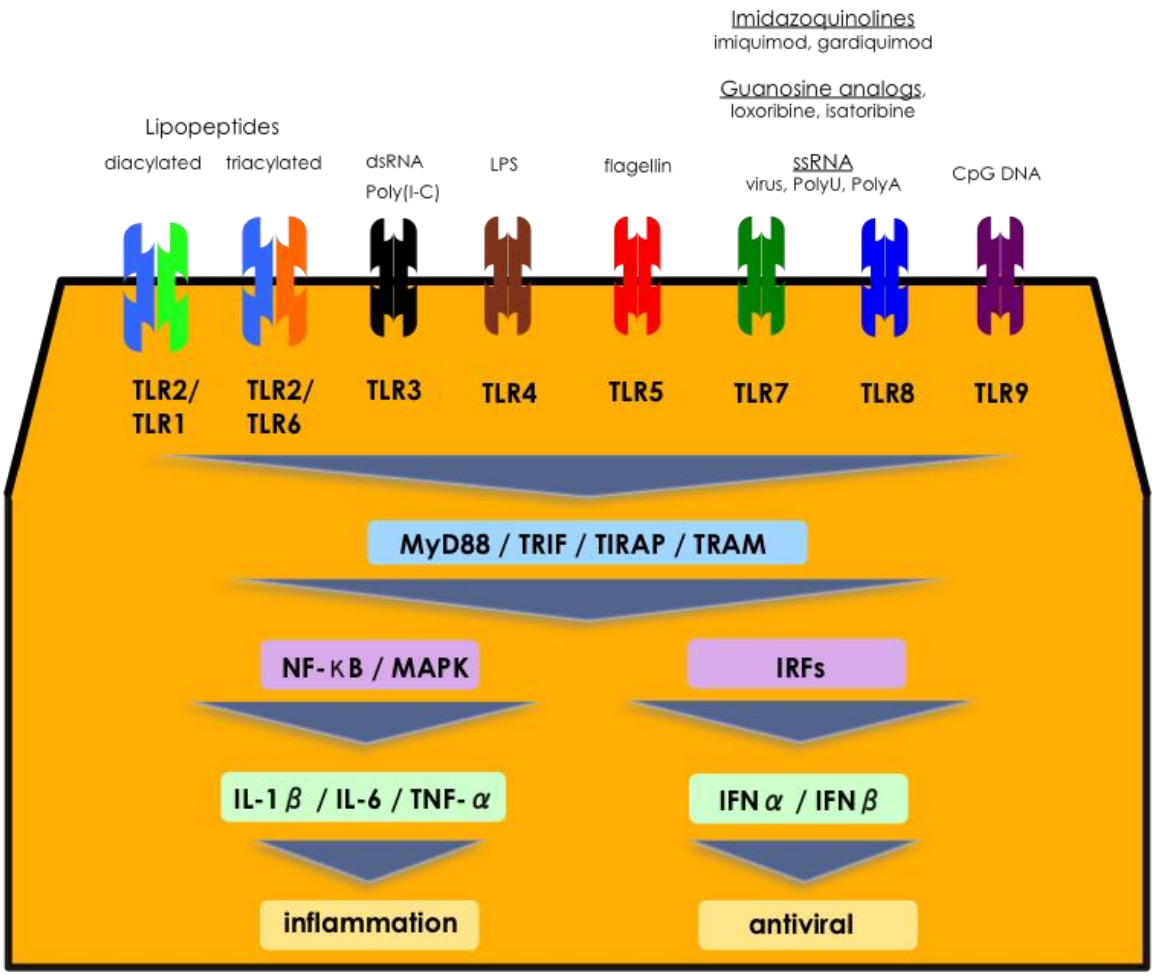


Figure 1.5. Upon recognition of their cognate pathogen associated molecular pattern or synthetic agonist, TLR signaling propagates through a scaffold of TIR-domain containing proteins for the downstream activation of transcription factors. The end result is upregulation of genes involved in inflammation and those involved in a direct antiviral response for the clearance of the infection. TLR is Toll-like receptor; LPS is lipopolysaccharide; MyD88 is myeloid differentiation factor 88; TIR

is Toll interleukin 1-β receptor homology domain; TRIF is TIR domain-containing adaptor inducing interferon-β; TIRAP is TIR domain-containing adaptor protein; TRAM is TRIF-related adaptor molecule.

E. HYPOTHESIS AND RESEARCH AIMS

Because of the close association of respiratory viruses and asthma attacks, it is important to understand why the symptoms of respiratory viruses in subjects with asthma are more severe and longer lasting than in healthy humans who are also subject to the same respiratory virus infections. When considering how viruses can mediate or trigger the asthmatic response, the mechanisms through which viruses can be recognized must be considered. The innate immune system is involved in early detection of virus infection to mount an acute antiviral inflammatory response in the lungs. The main symptoms of asthma, airway hyperreactivity and inflammation, are interdependent. Polymorphisms of Toll-like receptor 7 (TLR7), a receptor of the innate immune system for virus ssRNA that is expressed at the highest levels in the lungs, are associated with human asthma. Perhaps the innate immune response to the virus infection is defective in humans with asthma, contributing to a hyperreactive bronchoconstrictor response.

Eosinophils found in the airways of many humans with asthma and allergen sensitized guinea pigs express TLR7 and can be activated by synthetic TLR7 ligands and ssRNA viruses *in vitro*. Eosinophil activation by respiratory viruses is associated with airway hyperreactivity mediated by postganglionic prejunctional M2 muscarinic receptor dysfunction *in vivo*. My original hypothesis, therefore, was that TLR7 agonist-mediated activation of eosinophils in the airways of allergen-sensitized guinea pigs is responsible for virus-induced airway hyperreactivity. My preliminary data, however, suggested otherwise. I was surprised to find that

intravenous (i.v.) administration of a TLR7 agonist, R837, abolished vagal and i.v. acetylcholine-induced bronchoconstriction in guinea pigs *in vivo* within minutes of administration. This finding was surprising not only because the TLR7 agonist was a bronchodilator instead of a bronchoconstrictor. It was also surprising because the effect was rapid occurring in seconds and minutes, while canonical TLR7 signaling is associated with the regulation of expression of genes whose products contribute to the appropriate antiviral inflammatory response, effects that take place over hours and days.

An acute bronchodilator effect mediated by TLR7 is interesting both because of the novel non-canonical signaling pathways mediated by TLR7, as well as the potential therapeutic benefit of acute bronchodilators as rescue medications for asthma attacks. Furthermore, the bronchodilatory effect of a TLR7 agonist might represent a bronchoprotective mechanism to maintain unobstructed breathing during a respiratory virus infection. An inflammatory response is beneficial for clearing the respiratory virus infection, but symptoms of disease are often due to the deleterious effects of this inflammatory response. The mucus secretion, sloughing off epithelium, edema, swelling, and immune cell infiltration contribute to clearance of the virus infection, but if these effects remain unopposed, the resulting airway obstruction limits breathing essential for the host to live. While much is known about how the immune system contributes to inflammation, less is known about how the deleterious effects of the immune system are controlled. Perhaps a mechanism has evolved so that the airways dilate in response to respiratory viruses

to maintain airflow and breathing during the inflammation associated with the clearance of the infection. *The overall hypothesis tested in this thesis is that noncanonical TLR7 signaling mediates rapid bronchodilation within minutes, an effect that is defective in asthma.* Specifically, these studies will determine if TLR7 mediates the bronchodilatory effects of TLR7 agonists, at which cell types in the airway this effect is mediated, the signaling mechanisms involved, if the effect translates to humans for therapeutic potential, and if this relaxant effect is intact in animal models of asthma.

CHAPTER II.

GENERAL METHODS

A. ANIMALS AND TISSUES

1. Choice of animals versus humans

As described in the introduction, the guinea pig airway is a good model for the human airway with high similarity in innervation, pharmacology, and inflammation. Though the mouse has many differences in airway physiology than humans, the availability of TLR7-deficient mice made it necessary to characterize effects in mouse including wildtype, TLR7-deficient, and models of asthma.

1. Guinea pigs

Specific pathogen-free female Hartley guinea pigs (300 – 350 g; Elm Hill Breeding Labs Chelmsford, MA) were shipped in filtered crates, kept in high-efficiency particulate-filtered air, and fed a normal diet.

Animals were handled in accordance with NIH guidelines. Protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

2. Mice

C57BL/6 mice (approximately 6 week-old; The Jackson Laboratory Sacramento, CA) were shipped in filtered crates, kept in high-efficiency particulate-filtered air, and a

fed a normal diet. Mice used for virus infections and corresponding uninfected controls were kept in a BSL-2 facility prior to infection.

TLR7-deficient mice (B6.129S1-Tlr7tm1Flv/J; The Jackson Laboratory Sacramento, CA) were generated as previously described, with a LacZ cassette disrupting TLR7 from upstream nucleotide (291 bp) corresponding to the promoter of the TLR7 gene to the downstream nucleotides corresponding with amino acid 803 of the TLR7 gene (Lund et al., 2004). Homozygotes for the knockout construct were used in experiments, however they were not successful breeders. Therefore heterozygous females were mated to hemizygous males (TLR7 is on the X chromosome, so males only have one copy).

NJ1726 mice were a generous gift from Jamie and Nancy Lee (Mayo Clinic, Scottsdale, AZ) and generated as previously described (Lee et al., 1997). Briefly, these mice have IL-5 expressed under a Clara cell-specific promoter resulting in IL-5 overexpression in the lungs and increased eosinophil numbers in the lungs.

PHIL mice were a generous gift from Jamie and Nancy Lee (Mayo Clinic, Scottsdale, AZ) and generated as previously described (Lee et al., 2004). Briefly, these mice have diptheria toxin expressed under an eosinophil-specific promoter, depleting eosinophils.

NJ1726/PHIL mice were generated by mating NJ1726 with PHIL mice.

Animals were handled in accordance with NIH guidelines. Protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

3. Human trachea

Human tracheas were obtained from the Pacific Northwest Transplant Bank (Portland, OR).

B. MEASUREMENTS OF AIRWAY PHYSIOLOGY

1. *In vivo* guinea pig bronchoconstriction

Guinea pigs were anaesthetized with urethane (1.9 mg kg^{-1} i.p.), a non-depolarizing drug with little effect on respiratory and cardiovascular systems. They were chemically sympathectomized with guanethidine (5 mg kg^{-1} i.v.), to deplete norepinephrine and limit adrenergic input. They were paralyzed with a constant infusion of succinylcholine (10 μg kg^{-1} min^{-1} i.v.), to prevent breathing patterns controlled by the animal. Animals were tracheostomized and mechanically ventilated (tidal volume 2.5 ml, 100 breaths per minute). Both vagi were cut and distal portions attached to platinum electrodes submerged in liquid paraffin. Drugs were administered through cannulas in both jugular veins. Heart rate and blood pressure were measured by a transducer connected to a cannula in one carotid artery. Pulmonary inflation pressure was measured by a transducer connected to a

side arm of the tracheal cannula. Bronchoconstriction was induced by electrical stimulation of the vagi (10 V, 10 Hz, 0.2 msec pulse duration, 5 sec on, 60 sec off) or administration of acetylcholine (2ug kg^{-1}). See Figure 2.1 for schematic representation.

2. *In vitro* contraction of isolated guinea pig trachea

Contractions of isolated tracheas were measured *in vitro* as previously reported (Murray and Jacoby, 1992). Guinea pigs were killed with an overdose of pentobarbital (150mg/kg). The trachea was removed and cut between tracheal rings into segments. 0.5 cm rings of trachea were suspended in a 5 ml organ bath (Radnoti Glass Technology Inc., Monrovia, CA) that contained Kreb's 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 a 95%O_2-5%CO_2 gas mixture). The segments were supported by loops of silk through the tracheal lumen, with the lower thread tied to a hook at the bottom of the bath and the upper thread tied to a Grass FT03 isometric force transducer (Grass Instrument Co, Quincy, MA). Except for studies involving isoproterenol, propranalol (1 μM) was added to the Kreb's solution. Segments were equilibrated in the bath at 1 g tension for 60 min, and washed with Krebs solution every 15 min. Tracheal contractions were induced with methacholine (10 μM), histamine (10 μM), KCl (20 mM or 100 mM), or electrical field stimulation (EFS; 100 V, 20 Hz, 0.2 msec pulse duration, 15 sec on, 150 sec off). Tension recordings were made on a Powerlab/8SP (ADinstruments, Castle Hill, Australia). Except for EFS experiments, drugs were administered cumulatively to the bath following induction

of sustained contractions. For EFS drugs were administered between contractions, as it induces transient contractions. 100% maximum contraction is defined as the maximum contraction induced by the indicated contracting stimulus after administration of vehicle and before the administration of bronchodilating drugs. Values are normalized to a time-control corresponding to each dose. For guinea pig trachea, a single animal can provide six to eight tracheal segments, each in its own bath. See Figure 2.2 for picture and schematic.

3. *In vitro* contraction of isolated human trachea.

The procedure is the same as in guinea pig, but instead of the trachea being cut into rings, the trachea segments are suspended as strips due to the large size of the trachea and inability for rings to fit in 5 ml organ baths. Briefly, the human trachea is cut ventrally through the cartilage to open it. Excess cartilage is removed leaving only 0.5 cm on both sides of the trachealis muscle on the dorsal side of the trachea. Strips are cut perpendicular to the trachealis muscle, and strung up in the organ bath with a loop of silk thread going through the cartilage on both sides of the trahchealis muscle. Depending on the size of trachea obtained, four to eight 1 cm wide tracheal strips can be obtained from a single donor, each strip in its own bath.

4. *In vitro* contraction of isolated mouse trachea.

The procedure is the same as in guinea pig, though due to the small size of the trachea it is not cut into segments. Instead, a whole trachea removed from a single

mouse is cut just above the bifurcation into the main bronchi, and below the 4th tracheal ring after the larynx. Therefore, a single mouse provides tissue for a single organ bath.

FIGURE 2.1. *In vivo* measurement of bronchoconstriction.

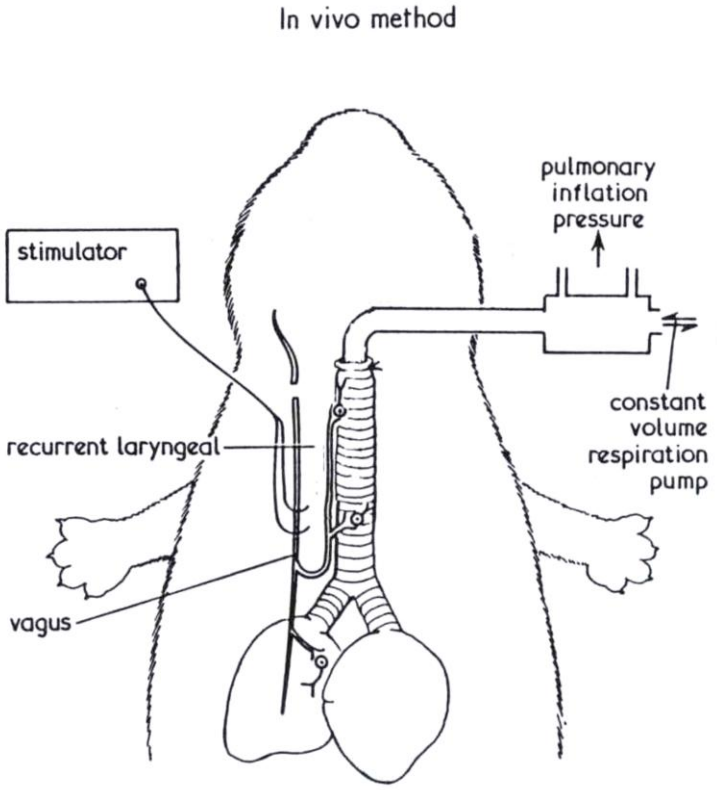
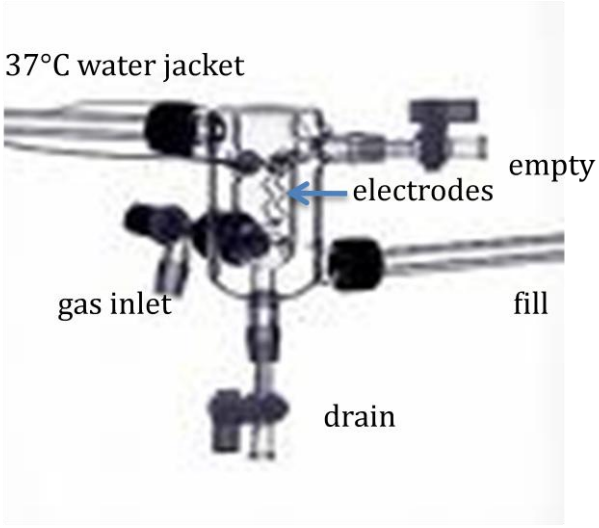


Figure 2.1. Schematic representation of an anaesthetized and paralyzed guinea pig on its back. Shown in the schematic are the canulated trachea through which the animal is mechanically ventilated and pulmonary inflation pressure is measured. Also shown is the electrical stimulation of the vagus nerve to induce bronchoconstriction. Drugs are administered i.v. through a canula in the jugular vein, not shown here. Figure adapted from (Fryer, 1986).

FIGURE 2.2. *In vitro* measurement of tracheal contraction.

A

B



C

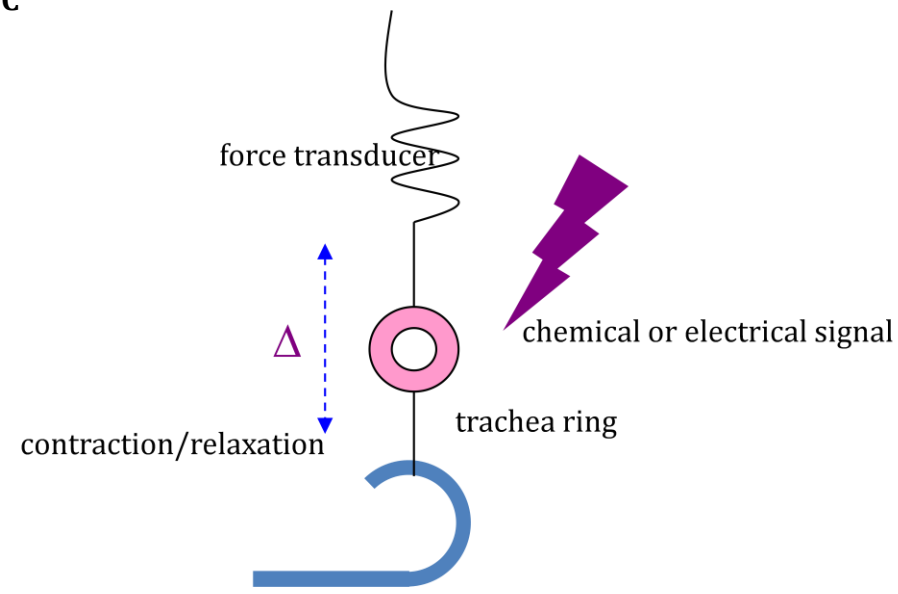


Figure 2.2 (A) Picture of a full organ bath system with four baths and one buffer reservoir feeding the individual baths. (B) Picture of an individual bath, in which a tracheal segment is suspended. Buffer enters through the tube on the bottom right, and leaves through the top right tube during washout. A mixture of 95% O_2 and 5%CO_2 is bubbled into the bath through the back tube. A 37°C water jacket circulates around the bath through the upper left tube. Electrical stimulation is delivered through the electrodes visible in the bath. (C) Schematic of the setup of the trachea segment within an individual bath and how changes in tension are measured.

C. MATERIALS

TLR agonists R837, R848, CLO97, gardiquimod, peptidoglycan, and flagellin, and the MyD88 inhibitor peptide were obtained from Invivogen (San Diego, CA). The previously described TLR7 antagonist IRS661 (Barrat et al., 2005), and the 21-mer phosphorothioated ssRNA oligonucleotides, PolyUs and PolyAs, were custom synthesized by Invitrogen (Carlsbad, CA). Sendai virus stock was obtained from ATCC. All other drugs and chemicals were obtained from Sigma Aldrich (St. Louis, MO).

D. STATISTICS

Two-way ANOVA analysis with repeated measures and Bonferroni's multiple comparison post-test was used to compare multiple means across groups. One-way ANOVA analysis with repeated measures and Bonferroni's multiple comparison post-test was used for the effect of dose within a single group. Analysis was done using GraphPad Prism software. Significance is indicated as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All error bars represent the s.e.m.

CHAPTER III.

TOLL-LIKE RECEPTOR 7 AGONISTS INDUCE ACUTE BRONCHODILATION WITHIN MINUTES OF ADMINISTRATION IN GUINEA PIGS, MICE, AND HUMANS

ABSTRACT

Respiratory virus infections result in asthma exacerbations. TLR7 is a receptor for virus single-stranded RNA and is expressed at high levels in the lungs. Because TLR7 polymorphisms are associated with asthma, I examined effects of TLR7 agonists in guinea pig airways *in vivo*, as well as in isolated guinea pig, mouse, and human tracheas *in vitro.* I induced bronchoconstriction in guinea pigs *in vivo* by electrical stimulation of the vagus nerve or by i.v. administration of acetylcholine and measured the effect of a TLR7 agonist administered i.v. I induced contraction of airway smooth muscle in segments of isolated guinea pig, mouse, and human trachea *in vitro* with electrical field stimulation, methacholine, histamine, and potassium chloride, and measured the effect of TLR7 agonists administered directly to the bath. TLR7 agonists dose-dependently and acutely inhibited bronchoconstriction *in vivo* and relaxed contraction of airway smooth muscle *in vitro* within minutes of administration. These data demonstrate a novel protective mechanism to limit bronchoconstriction and maintain airflow during respiratory virus infections. The fast time frame is inconsistent with canonical TLR7 signaling, suggesting a previously undescribed nontranscriptional component to TLR7 signaling. TLR7 is an attractive therapeutic target for its ability to reverse bronchoconstriction within minutes.

INTRODUCTION

Viruses have been detected in 80% of acute exacerbations of asthma in children and 50% of acute exacerbations in adults (Johnston et al., 1995; Atmar et al., 1998). These airway viruses induce inflammatory responses in lungs via multiple signaling pathways, including protein kinase R, retinoid inducible gene-I (and related helicases), and multiple toll-like receptors.

Toll-like receptors (TLRs) respond to multiple viruses as an early and rapid defense. TLR signaling results in an immediate inflammatory response and induction of an adaptive immune response to clear viral infections (Kawai and Akira, 2006). Most respiratory viruses have a single-stranded RNA (ssRNA) genome. Upon recognition of viral ssRNA (Diebold et al., 2004), TLR7 signals through the adaptor molecule MyD88, activating transcription factors that induce production of antiviral cytokines, such as interferons, and inflammatory cytokines, such as TNF-α (Kawai and Akira, 2006). While necessary to clear infection, the resulting inflammation may also impair airway function and participate in virus induced asthma attacks. The highest TLR7 expression level is found in the lungs (Chuang and Ulevitch, 2000), most likely in multiple cell types including inflammatory cells (Demedts et al., 2006), epithelial cells(Uehara et al., 2007), and human airway smooth muscle cells (Sukkar et al., 2006). Furthermore, expression in human smooth muscle cells is potentiated by IL-1β and TNFα, cytokines commonly found in lungs in response to infection (Sukkar et al., 2006). TLR7 polymorphisms have been associated with asthma, although the functional effects of these polymorphisms are not known

(Roponen et al.; Moller-Larsen et al., 2008), so it is important to understand contributions of TLR7 signaling to airway physiology.

To address whether TLR7 directly affects respiratory physiology, I tested the effects of a synthetic TLR7 agonist, R837 (imiquimod), in guinea pigs *in vivo.* I was surprised to find that R837 rapidly and completely abolished bronchoconstriction induced by electrical stimulation of the vagus nerve or by intravenous administration of acetylcholine. The inhibition of bronchoconstriction was rapid, occurring within minutes of administration, a time frame inconsistent with canonical TLR7 signaling. This was reproduced in isolated guinea pig tracheas, mouse tracheas, and human tracheas *in vitro*. R837 inhibited contractions of guinea pig tracheas induced by electrical field stimulation to stimulate the nerves in the tissue, as well as contractions induced by administration of methacholine, histamine, or potassium chloride. This indicates that the bronchodilating effect of TLR7 agonists is not limited to neuronally evoked or cholinergic contractions, nor is it limited to contractions induced by G-protein coupled receptors on the smooth muscle membrane. Several structurally related imidazoquinoline ligands, as well as structurally unrelated single stranded RNA ligands, which are known TLR7 agonists, all dose-dependently inhibited methacholine-induced contractions of guinea pig trachea. That multiple TLR7 ligands of different structural classes all showed similar relaxant effects, suggests that TLR7 may be mediating these effects. This may represent a potent, physiological mechanisms of bronchodilation in the presence of respiratory virus RNA by which airflow may be maintained during virus-induced

inflammation and counteract virus induced airway occlusion. TLR7 polymorphisms, if associated with loss of expression or function, might predispose to asthma by eliminating this compensatory bronchodilation during viral infections. The rapid bronchodilation observed within minutes makes TLR7 an attractive, novel target for the treatment of asthma.

EXPERIMENTAL DESIGN

In vivo measurements of bronchoconstriction

See Chapter II General Methods.

In vitro measurements of tracheal contraction

See Chapter II General Methods.

Epithelial removal

Airway epithelium was removed from guinea pig tracheas by gentle abrasion with a cotton swab.

RESULTS

A TLR7 agonist, R837, inhibits bronchoconstriction in guinea pigs *in vivo*.

Electrical stimulation of the vagus nerves (10 V, 10 Hz, 0.2 msec pulse duration, 5 sec on, 60 sec off) or i.v. administration of acetylcholine (2 μg kg^{-1}) caused reproducible bronchoconstriction in anesthetized guinea pigs *in vivo.* R837 (0.003 – 10 mg kg^{-1}) dose-dependently inhibited subsequent bronchoconstriction induced by both vagal stimulation and by i.v. acetylcholine (Figure 3.1; vagal stimulation IC_{50} = 0.19 mg kg^{-1}; acetylcholine IC_{50} = 0.49 mg kg^{-1}). The effect occurred within 1 minute of administration of R837, and at higher doses bronchoconstriction was completely inhibited. The effect of R837 was rapidly reversed, with recovery of bronchoconstriction within 20 minutes.

A TLR7 agonist, R837, reverses contraction of isolated guinea pig trachea *in vitro*.

Electrical field stimulation of isolated guinea pig tracheas in organ baths. (EFS; 100 V, 20 Hz, 0.2 msec pulse duration, 15 sec on, 150 sec off) caused reproducible contractions that are blocked by atropine indicating that they are mediated via release of acetylcholine. R837 (3 – 1000 μM) acutely reduced subsequent contractions induced by EFS (IC_{50} = 40 μM; Figure 3.2A), confirming *in vivo* inhibition of bronchoconstriction induced by electrical stimulation of the vagus nerves (Figure 3.1).

To determine whether R837 also relaxed contractions induced directly at airway smooth muscle, reproducible contractions were induced by methacholine (3μM) or histamine (10μM), both of which signal through G-protein coupled receptors. R837 relaxed established and sustained contractions induced by methacholine and histamine in a concentration-dependent manner (IC_{50} = 32 μM for tissues contracted with methacholine and 7.2 μM for tissues contracted with histamine; Figure 3.2A).

To bypass surface receptors on smooth muscle, I used KCl (20 mM and 100 mM) to directly depolarize the membrane and contract tracheal smooth muscle. R837 also relaxed KCl-induced contraction (Figure 3.2B). The R837-mediated relaxation of contractions induced by 20 mM KCl (IC_{50} = 113 μM) was more potent and complete than the relaxation of contractions induced by 100 mM KCl (IC_{50} = 779 μM), suggesting a mechanism that may involve K^+ channels.

As seen *in vivo*, relaxation of airway smooth muscle by R837 *in vitro* was reversible and the full contractile response recovered within 15 minutes of washing R837 from the bath, demonstrating that relaxation of contracted airways is not due to toxic effects at airway smooth muscle.

Because I was able to replicate the *in vivo* bronchodilatory effect of R837 *in vitro*, all of the subsequent experiments were carried out *in vitro*.

R837-mediated reversal of guinea pig airway smooth muscle contraction is not dependent on airway epithelium.

To determine whether R837 relaxes smooth muscle contraction by bronchodilators released from the epithelium (Freed et al., 1987), I removed the epithelium from guinea pig tracheal segments with a cotton swab. Epithelial removal was confirmed by microscopic evaluation of tracheal segments used in the bath (Figure 3.3A). Removal of airway epithelium had no effect on the ability of R837 to relax airway smooth muscle precontracted with methacholine (3 μM) *in vitro*, indicating that R837 does not act through the epithelium to induce relaxation (Figure 3.3B).

Other TLR7 agonists also relax guinea pig airway smooth muscle *in vitro,* but not TLR2 or TLR5 agonists.

Other TLR7 agonists, CL097, R848, and gardiquimod also relaxed isolated guinea pig tracheal smooth muscle contracted with methacholine (3 μM) in a concentration-dependent manner (Figure 3.4A). Since R837 and these three other agonists are structurally related imidazoquinolines, I also tested a reported TLR7 ligand of a different structural class, a 21mer polyU ssRNA oligonucleotide on a phosphorothioate backbone (PolyUs) (Diebold et al., 2006). PolyUs partially relaxed contraction induced by methacholine (3 μM) in a concentration-dependent manner (Figure 3.4B). Mixed polyU oligonucleotides of various lengths on a phosphodiester backbone (PolyUo), did not induce relaxation, suggesting the relaxant effect is specific to TLR7 ligands. Another related compound, 21mer polyA ssRNA oligonucleotide on a phosphorothioate backbone (PolyAs) also partially relaxed methacholine-contracted trachea (Figure 3.4B). The TLR2 agonist, peptidoglycan,

and the TLR5 agonist, flagellin, did not relax contraction induced by methacholine in the dose ranges tested (Figure 3.4C).

TLR7 agonists reverses contraction of isolated human and mouse trachea *in vitro*.

If TLR7 agonists were to be used for therapeutic purposes, the bronchodilating effect must translate to human tissue as well. To address whether TLR7 agonists have a relaxant effect in human airway smooth muscle, I measured the effect of R837 and PolyUs on contraction of isolated human trachea *in vitro*. Reproducible sustained contractions were induced by methacholine (3 μM) and cumulative increasing doses of R837 or PolyUs were added directly to the bath. R837 (0.1 – 100 μM) and PolyUs (0.3 – 300 μg/ml) dose-dependently inhibited contraction (IC_{50} = 15 μM for R837, and IC_{50} = 1.3 μg/ml for PolyUs; Figure 3.5A). The inhibition of contraction by R837 was more complete than that by PolyUs.

For future genetic studies and disease models in mice, it is necessary to replicate the relaxant effect of TLR7 agonists in mice as well. To address whether TLR7 stimulation has a relaxant effect in mouse airway smooth muscle, I measured the effect of R837 and PolyUs on contraction of isolated mouse trachea *in vitro*. Reproducible sustained contractions were induced by methacholine (3 μM) and cumulative increasing doses of R837 or PolyUs were added directly to the bath. R837 (0.1 – 100 μM) and PolyUs (10 – 300 μg/ml) dose-dependently inhibited contraction (IC_{50} = 3.4 μM for R837, and IC_{50} not determined for PolyUs; Figure 3.5B).

FIGURE 3.1. A TLR7 agonist, R837, inhibits bronchoconstriction in guinea pigs *in vivo*.

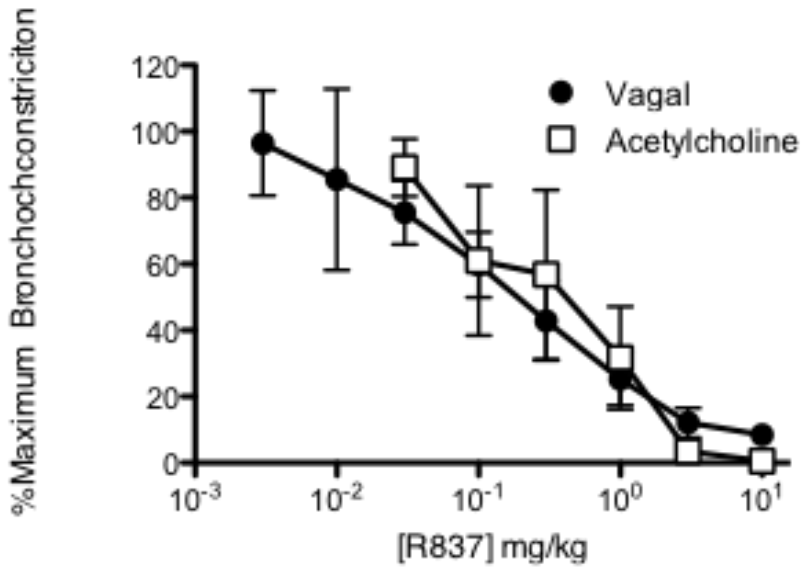


Figure 3.1. Bronchoconstrictions were induced by electrical stimulation of the vagus nerves (circles) or by i.v. administration of acetylcholine (squares), and the effect of cumulative increasing doses of R837 administered i.v. was measured. Data are expressed as the mean ± SEM. (*n*=9 for vagal stimulation, maximum=93.0±21.5 mm H_20; *n*=4 for acetylcholine, maximum=70.5±32.2 mm H_20; p≤0.001 for effect of dose).

FIGURE 3.2. A TLR7 agonist, R837, relaxes isolated guinea pig trachea *in vitro*.

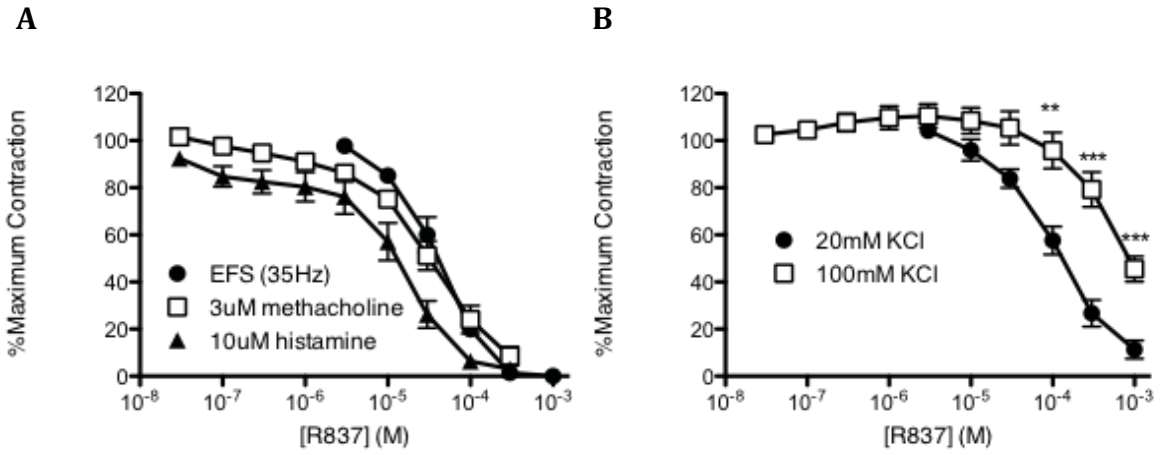


Figure 3.2. (A) Contraction of tracheal segments was induced by electrical field stimulation (EFS; circles), methacholine (squares), and histamine (triangles), and the effect of cumulative increasing doses of R837 was measured. Data are expressed as the mean ± SEM. (*n*=3 for EFS, maximum=0.53±0.08 g; *n*=6 for methacholine, maximum=1.98±0.31 g; *n*=6 for histamine, maximum=1.04±0.13 g; p≤0.001 for effect of dose). (B) Contraction of tracheal segments was induced by KCl (20 mM - circles; 100 mM - squares), and the effect of cumulative increasing doses of R837 was measured. Data are expressed as the mean ± SEM. (*n*=3 for 20 mM KCl, maximum=0.78±0.23 g; *n*=6 for 100 mM KCl, maximum=1.47±0.30 g; 20 mM KCl vs. 100 mM KCl p≤0.01 at 100 μM R837 and p≤0.001 at 300 μM and 1000 μM R837; p≤0.001 for effect of dose).

FIGURE 3.3. R837-mediated inhibition of guinea pig airway smooth muscle contraction is not dependent on airway epithelium.

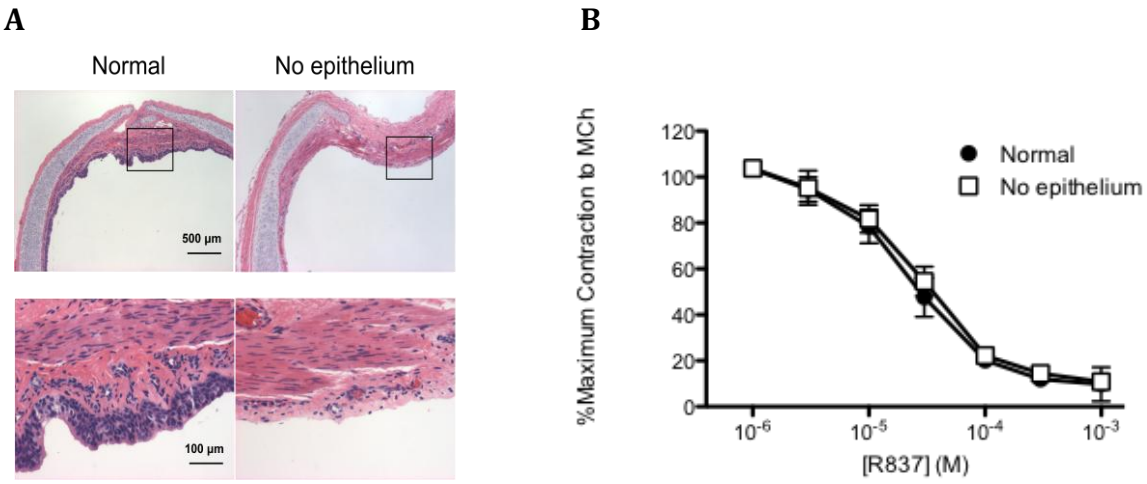


Figure 3.3. (A) 4X magnification of hematoxylin and eosin stain stained sections of trachea segments with and without the epithelium, with a 20X magnification of the region in the black box. The removal of epithelium can be viewed as the absence of purple nuclei lining the airway lumen. (B) The effect of cumulative increasing doses of R837 on contraction was measured in intact tracheal segments (circles) or tracheal segments with the epithelium removed (squares). Data are expressed as the mean ± SEM. (n=2; maximum with epithelium=1.48±0.1 g; maximum without epithelium=0.76±0.28g; $p \leq 0.001$ for effect of dose).

FIGURE 3.4. Other TLR7 agonists also relax isolated guinea pig trachea *in vitro*, but not TLR2 or TLR5 agonists.

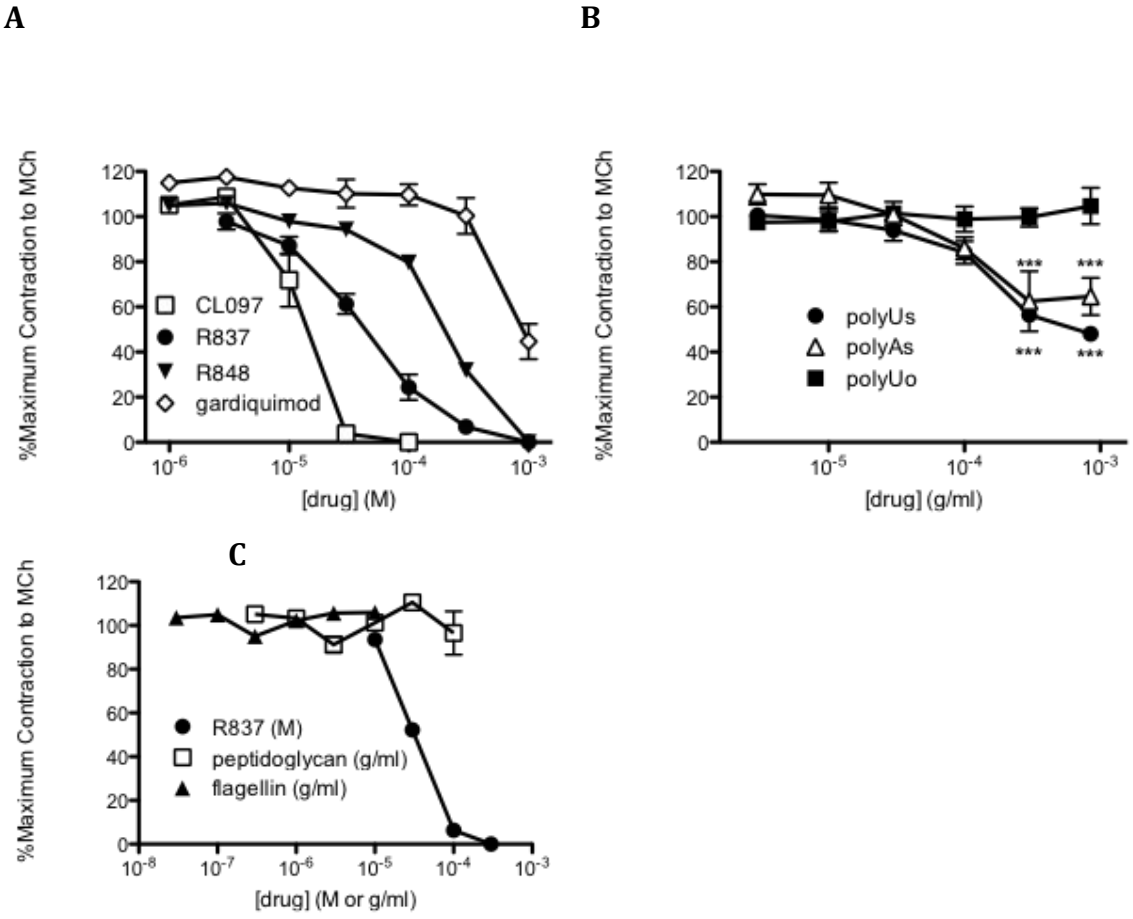


Figure 3.4. (A) Contraction of tracheal segments was induced by methacholine (3 μM), and the effect of cumulative increasing doses of R837 (circles), CL097 (squares), R848 (triangles), and gardiquimod (diamonds) was measured. Data are expressed as the mean ± SEM. (R837 *n*=8; R848 *n*=4; gardiquimod *n*=3; CL097 *n*=2; maximum=1.64±0.17 g; p≤0.001 for effect of dose). (B) Contraction of tracheal segments was induced by methacholine (3 μM), and the effect of cumulative increasing doses of 21mer PolyU on a phosphorothioate backbone (PolyUs; circles)

mixed lengths of PolyU on a phosphodiester backbone (PolyUo; squares), and 21mer PolyA on a phosphorothioate backbone (PolyAs; triangles) was measured. Data are expressed as the mean ± SEM. (n=3; maximum=1.27±0.14 g; 300 μg ml^{-1} and 1000 μg ml^{-1} PolyUo vs. PolyUs and PolyUo vs. PolyAs $P \leq 0.001$; for effect of dose of PolyUs $P \leq 0.001$, PolyAs $P \leq 0.01$). (C) Contraction of tracheal segments was induced by methacholine (3 μM), and the effect of cumulative increasing doses of R837 (circles), peptidoglycan (squares), or flagellin (triangles) was measured. (R837 n=1; peptidoglycan and flagellin n=3).

FIGURE 3.5. TLR7 agonists inhibit contraction of isolated human and mouse trachea *in vitro*.

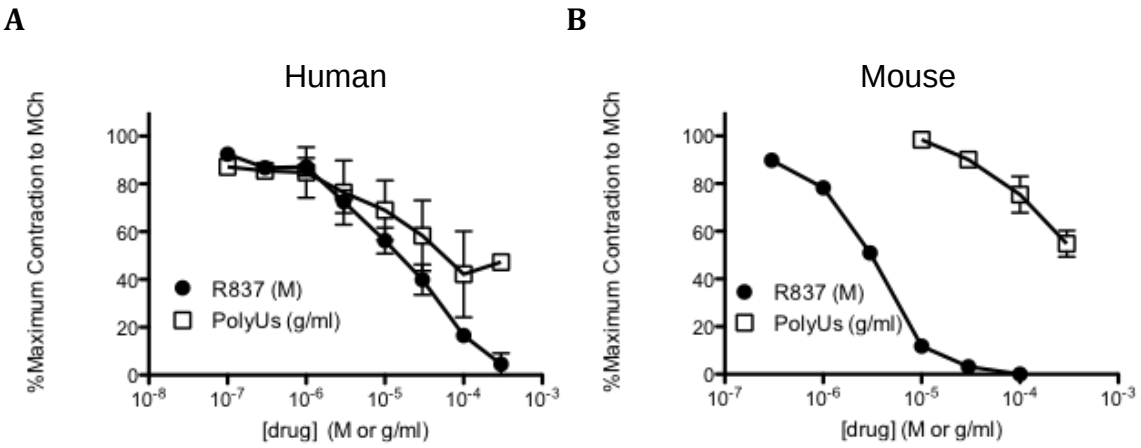


Figure 3.5 (A) Contraction of human tracheal segments was induced by methacholine (3 μM), and the effect of cumulative increasing doses of R837 (circles) or PolyUs (squares) was measured. Data are expressed as the mean ± SEM. (R837 *n*=3; PolyUs *n*=2; maximum=± g). (B) Contraction of mouse tracheal segments was induced by methacholine (3 μM), and the effect of cumulative increasing doses of R837 (circles) or PolyUs (squares) was measured. Data are expressed as the mean ± SEM. (R837 *n*=3; PolyUs *n*=3; maximum=0.52±0.27 g).

DISCUSSION

Because of the association of ssRNA respiratory viruses with asthma attacks (Johnston et al., 1995; Johnston et al., 1996; Atmar et al., 1998), the relatively high expression of TLR7 in the lungs (Chuang and Ulevitch, 2000), and the association of TLR7 polymorphisms with asthma (Roponen et al.; Moller-Larsen et al., 2008), I investigated the effects of acute TLR7 signaling on airway physiology. I was surprised to find that the TLR7 agonist, R837, completely and potently abolished bronchoconstriction *in vivo* and trachealis muscle contraction *in vitro*, both effects occurring within minutes. This drug clearly has direct potent effects on airway smooth muscle, as seen by its ability to relax contractions induced by methacholine and histamine, although additional effects on airway nerves cannot be excluded. It is also possible that other cells, including leukocytes, which are known to express TLR7, may be contributing bronchodilating mediators.

The effect is not specific to GPCR-mediated contractions, as R837 relaxed contraction induced by chemical depolarization of the smooth muscle membrane with KCl. The mechanism of R837 induced relaxation is independent of epithelium, as removal of the epithelium did not change the relaxant response to R837 *in vitro.*

Other structurally related TLR7 agonists, R848, CL097, and gardiquimod, as well as structurally unrelated TLR7 agonists PolyUs and PolyAs, also relaxed guinea pig trachea *in vitro.* Unlike R837, these latter two compounds only partially relaxed contraction. PolyAs was originally selected as a negative control for PolyUs, based

on reports that PolyAs is not a TLR7 ligand. The bronchodilation induced by the negative control was inconsistent with claims that the effect is TLR7-mediated. In the interim, however, it was shown in a new TLR8-deficient mouse, that PolyAs is a TLR8 agonist (Demaria et al.; Martinez et al.). This leaves open the possibility that both TLR7 and TLR8 can mediate rapid relaxation of airway smooth muscle.

Bacterial TLR ligands peptidoglycan from *Bacillus subtilis*, a TLR2 ligand, and flagellin from *Bacillus subtilis*, a TLR5 ligand, did not relax the contracted trachea. This might be an interesting distinction that bacterial TLR ligands do not induce bronchodilation, but viral ligands do. Seemingly contradictory evidence exists in the literature that the bacterial TLR4 ligand lipopolysaccharide induces relaxation of airway smooth muscle (Fedan et al., 1995; Balzary and Cocks, 2006). This might not be inconsistent with the distinction of the relaxant effect to viral TLR ligands, as TLR4 is the primary detector of respiratory syncytial virus, a virus highly associated with asthma (Haynes et al., 2001; Tal et al., 2004; Cyr et al., 2009). It will be interesting to test a whole panel of bacterial and viral TLR ligands to determine if there is a distinction in this acute bronchodilatory property to viral TLR ligands.

TLR7 agonist-mediated relaxation occurred in minutes *in vivo* and *in vitro*. The speed of this effect is important, but is not consistent with typical TLR signaling via induction of gene expression, which requires new synthesis, processing, and trafficking of mRNA and protein.

Because of the rapid bronchodilation in response to TLR7 agonists observed in guinea pig both *in vivo* and *in vitro*, these drugs are attractive for their therapeutic potential in diseases marked by airway contraction, particularly asthma. For therapeutic value, however, the same bronchodilatory effect must be replicated in human tissue. Because R837 and PolyUs were able to rapidly relax isolated human trachea *in vitro*, these ligands remain an interesting rescue therapy for acute bronchoconstriction. PolyUs is reported as a TLR8 agonist in humans, again supporting a bronchodilating role for TLR8 in addition to TLR7 (Heil et al., 2004).

Others have explored TLR7 as a therapeutic target for asthma (Stokes et al., 1998; Moisan et al., 2006; Camateros et al., 2007; Camateros et al., 2009; Xirakia et al., 2010), but in a context of chronic protection from airway inflammation rather than rescue from an acute asthma attack. This is shown not by the inhibition of bronchoconstriction as I show here, but by the inhibition of airway hyperreactivity and allergic inflammation characteristic of asthma. This is thought to be a consequence of canonical TLR7 signaling, resulting in increased expression of T_h1 cytokines, such as IL-12 and interferon-γ (Tomai et al., 1995; Fogel et al., 2002), skewing the airway response away from a T_h2 immune environment (Holgate, 2008). My data show there is also a fast bronchodilating action of these drugs that is too rapid in onset to occur via modulation of the immune environment via changes in gene expression. In sum, this class of compounds may provide both chronic protection against airway hyperreactivity, and rapid bronchodilation in the event of an exacerbation, indicating that they might be successful as both prophylactic and

rescue medications. Furthermore, the data emphasize the need to test shorter incubation times when conducting studies with TLR7 ligands, and to consider noncanonical TLR7 signaling as well as off-target effects of these ligands.

Because the mouse provides opportunities for genetic manipulation of the TLR7 pathway, and because of available mouse models of allergy and asthma, I wanted to determine if there was also a relaxant effect of TLR7 agonists in mouse. R837 and PolyUs both reversed contraction of isolated mouse trachea *in vitro*. Because mouse airway physiology is very different from that in human and guinea pig, this was not a trivial finding and indicates a highly conserved mechanism across three mammalian species.

The TLR7 agonist-mediated bronchodilation suggests this conserved mechanism might limit airway obstruction resulting from inflammation, edema, and sloughing of dead cells associated with clearance of respiratory virus infections. TLR7 polymorphisms are associated with asthma (Roponen et al.; Moller-Larsen et al., 2008), and it is tempting to speculate that these may result in a defective bronchodilator response to respiratory viruses, leaving bronchoconstrictive effects of viral infection unopposed in asthma.

In summary, TLR7 agonists are potent bronchodilators *in vivo* and *in vitro*, a rapid effect inconsistent with the time frame of canonical TLR signaling. The rapid and potent bronchodilatory effects of TLR7 agonists, along with their longer-term effects of biasing the immune response away from T_h2 and towards T_h1, make these

compounds attractive candidates for treatment of asthma. Regardless of the target mediating the bronchodilating effects of these molecules, their therapeutic potential is clear. Interestingly, R837 (Imiquimod), was approved for topical treatment of genital warts solely based on its efficacy and safety profile before the target mediating the effects was known (Medzhitov et al., 1997; Beutner et al., 1998; Gupta et al., 2002). It was only known to be an immune-response modifier for its ability to upregulate alpha-interferon, tumor necrosis factor alpha, and interleukin-6 (Kono et al., 1994; Reiter et al., 1994). However, for further development and improvement of these drugs, it is important to know the target and mechanism mediating the bronchodilating effect. Furthermore, if the bronchodilating effect is TLR7-mediated, the fast time-frame suggests a new mode of TLR7 signaling, which would be of great interest to the field. In Chapter IV, I address the target and mechanism mediating the bronchodilating effects of TLR7 agonists.

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CHAPTER IV.

THE MECHANISM OF TOLL-LIKE RECEPTOR 7 AGONIST-MEDIATED BRONCHODILATION INVOLVES PROSTAGLANDINS, A LARGE CONDUCTANCE CALCIUM-GATED POTASSIUM CHANNEL, AND NITRIC OXIDE

ABSTRACT

Because of the association of respiratory viruses and asthma, I examined the effects of TLR7 agonists on airway physiology. Canonical TLR7 signaling is associated with changes in gene expression, involving new synthesis, processing, and trafficking of mRNA and protein in a time frame of hours and days. I previously showed that TLR7 agonists cause rapid bronchodilation within minutes of administration, a time frame inconsistent with the known effects of TLR7 signaling. Here, I have taken several approaches to determine whether TLR7 is mediating the rapid relaxing effects of TLR7 agonists. These include pharmacological approaches with a TLR7 antagonist, as well as genetic approaches using TLR7-deficient mice. In guinea pigs, airway relaxation induced by the TLR7 agonist, R837, was partially blocked with a TLR7 antagonist. Another TLR7 agonist, PolyUs, mediated relaxation that was completely blocked by a TLR7 antagonist. This suggests that R837-mediated bronchodilation is partially TLR7-dependent, while PolyUs-mediated relaxation is completely TLR7-dependent. In mice, I found that R837 and PolyUs inhibited contraction of airway smooth muscle from both wildtype and TLR7-deficient mice, suggesting a non-TLR7 mediated mechanism of relaxation. However, in the presence of a TLR7 antagonist, the relaxant effect of R837 in the wildtype, but not the TLR7-deficient mice was significantly reversed. This suggests there are TLR7-dependent and -independent relaxant effects of R837 in the mouse, but that the effective dose range of both targets are very close together. In human tissue, I found that R837 and PolyUs inhibited contraction of airway smooth muscle, but only the R837-mediated effect

was reversed by a TLR7 antagonist. I also undertook studies to understand the mechanism of both TLR7-dependent and –independent R837-mediated bronchodilation. Candidate targets for mechanistic studies were selected from Toll-like receptor associated signaling pathways that also serve a role in rapid bronchodilation. These include prostaglandins, the large conductance calcium-gated potassium channels (MaxiK), and nitric oxide. Using combinations of inhibitors of these pathways along with the TLR7 antagonist in guinea pig trachea *in vitro*, I have determined that TLR7-independent bronchodilation is mediated by prostaglandins and MaxiK, while TLR7-dependent bronchodilation is partially mediated by nitric oxide. Finally, adenosine receptors, known to bind R837, are ruled out as mediating the majority of relaxant effects of R837.

INTRODUCTION

Respiratory virus infections are closely associated with asthma exacerbations. A receptor of the innate immune system, TLR7, signals in response to virus ssRNA (Diebold et al., 2004; Heil et al., 2004). Furthermore, TLR7 polymorphisms are associated with asthma (Roponen et al.; Moller-Larsen et al., 2008). To understand how viruses interact with and affect airway physiology, I previously tested the effects of TLR7 agonists on bronchoconstriction in guinea pigs *in vivo*, and on contraction of isolated trachea from guinea pig, mouse, and human *in vitro*. I was surprised to find that a number of TLR7 agonists of two structural classes all inhibited bronchoconstriction or reversed contraction of airway smooth muscle. This was surprising both because it has never been shown and because of the fast time-frame in which the effect took place. TLR7 signaling is known to upregulate various proinflammatory and antiviral cytokines over hours and days for the clearance of virus infections. Little is known, however, about fast effects of TLR7 signaling within minutes. That TLR7 agonists act as bronchodilators is a novel finding, and, if it is mediated by TLR7, is one of few examples of fast TLR signaling. If TLR7 mediates bronchodilation, it may represent a protective mechanism to maintain airflow during the inflammation and airway occlusion associated with respiratory virus infections. TLR7 would effectively adjust airway physiology to compensate for TLR7-mediated inflammation. The rapid bronchodilation is an important characteristic of a rescue medication for an ongoing asthma attack. Both claims of an endogenous protective mechanism, as well as therapeutic value require

an understanding of the receptor mediating the relaxant effects of TLR7 agonists and the mechanism by which they relax airway smooth muscle. That multiple TLR7 agonists of different structural classes all show a bronchodilatory effect supports TLR7 as the receptor mediating the bronchodilation, but other receptors cannot be ruled out, particularly as this is a finding with little precedent.

To determine if TLR7 mediates the relaxant effects of TLR7 agonists in guinea pig, humans, and mice, I took both pharmacological and genetic approaches. Pharmacologically, I measured the effect of two structurally distinct TLR7 agonists in the presence of a TLR7 antagonist *in vitro*. If TLR7 mediates the relaxant effects of the agonists, then it would be expected that an antagonist would block the receptor and reverse these relaxant effects. For the genetic approach, the availability of a TLR7-deficient strain of mice allowed me to measure the effect of TLR7 agonists on airway smooth muscle contraction *in vitro* in the absence of TLR7. If TLR7 exclusively mediates the relaxant effects of TLR7 agonists in mice, then these effects should be abrogated when there is no available receptor. I was also able to combine these approaches using the TLR7 antagonist in TLR7-deficient mice. Even if there are multiple targets mediating the relaxant effects in addition to TLR7, the antagonist should reverse any TLR7-mediated effects only when the receptor is present in wildtype, but not in TLR7-deficient mice.

I show here that in the presence of a TLR7 antagonist the relaxant effects were partially or completely reversed depending on the agonist and species tested. Together, that multiple TLR7 agonists caused bronchodilation and that a TLR7

antagonist could reverse the bronchodilating effect, suggests the effect is at least partially mediated by TLR7 in guinea pigs and mice, but that TLR7-independent relaxant effects also exist. The almost complete reversal of R837-mediated relaxation in human tracheas, suggests the relaxant effects R837 are completely TLR7-mediated in humans. The relaxant effect of PolyUs was not reversed by a TLR7 antagonist in human tracheas, so this might not be mediated by TLR7.

I also conducted studies to determine the mechanism of TLR7-agonist mediated bronchodilation. Candidates were selected from pathways known to have fast bronchodilating effects and that are somehow associated with TLR signaling, including prostaglandins, the large conductance calcium-gated potassium channel (MaxiK), and nitric oxide. Using inhibitors to block these pathways I tested the relaxant effect of R837 *in vitro*. I also measured the effects of R837 in the presence of these inhibitors combined with a TLR7 antagonist, to determine if they were part of the TLR7-dependent pathway or the TLR7-independent pathway.

I found that the mechanism of TLR7-dependent bronchodilation in guinea pigs involves nitric oxide, while the TLR7-independent bronchodilation involves prostaglandins and MaxiK potassium channels.

R837 has been reported as an antagonist at adenosine A_1 and A_{2a} receptors (Schon et al., 2006), thus it is important to determine whether R837 elicits its relaxant effects by adenosine receptor signaling. Adenosine receptors can modulate airway physiology with both contractile and relaxant effects depending on conditions and

concentrations used (Farmer et al., 1988; Ali et al., 1992; Ethier and Madison, 2006). To address the role of adenosine receptors in R837-mediated relaxation of airway smooth muscle, I measured the effect of adenosine ligands alone or when coincubated with R837 or a TLR7 antagonist in isolated guinea pig trachea *in vitro*. I found that R837 could not be acting as an adenosine receptor antagonist to cause bronchodilation. Furthermore, it does not elicit the majority of its effects by acting as an adenosine receptor agonist or partial agonist.

Others are also testing the effects of imidazoqinoline TLR7 agonists in chronic models of allergic asthma through canonical TLR7 signaling and upregulation of T_h1 cytokines, skewing away from an allergic immune environment (Stokes et al., 1998; Quarcoo et al., 2004; Napolitani et al., 2005; Moisan et al., 2006; Camateros et al., 2007; Sel et al., 2007; Xirakia et al., 2010). Together with the data presented here, this suggests that these molecules may be useful as both prophylactic therapies against future asthma attacks and as a rescue medication for ongoing attacks.

EXPERIMENTAL DESIGN

In vitro measurements of tracheal contraction

See Chapter II General Methods.

Treatments

TLR7 agonists (R837, R848, CL097, gardiquimod, PolyUs, and PolyAs), the β_2-adrenergic receptor agonist, isoproterenol, and the adenosine receptor agonist, NECA, were added cumulatively in increasing concentrations to tracheas precontracted with methacholine (MCh; 3 μM). Before the agonist dose responses *in vitro*, some tracheal segments were treated for one hour with the TLR7 antagonist IRS661 (100 μM), the MyD88 inhibitor peptide (64.4 μM), or the β_2-adrenergic receptor antagonist propranolol (1 μM), for 15 minutes with the cyclooxygenase inhibitor indomethacin (1 μM), the MaxiK K^+ channel blocker paxilline (20 μM), the general K^+ channel blocker TEA (20 mM), the adenosine A_1 receptor antagonist DPCPX (2 μM), the adenosine A_{2A} receptor antagonist SCH58261 (2 μM), or for 30 minutes with the nitric oxide synthase inhibitor L-NMMA (100 μM).

RESULTS

The TLR7 antagonist, IRS661, partially blocks R837-mediated relaxation of guinea pig airway smooth muscle and completely blocks PolyUs- and PolyAs-mediated relaxation.

While multiple TLR7 agonists had bronchodilatory effects, whether these effects are TLR7-mediated or mediated by another target remains unknown. If the relaxant effects are TLR7-mediated, they should be reversed by a specific TLR7 antagonist. The TLR7 antagonist, IRS661 (100 μM), partially and significantly blocked smooth muscle relaxation by R837 (100 μM) (Figure 4.1). IRS661 (100 μM) was able to completely block the relaxation by PolyUs and PolyAs (300 μg ml^{-1}) (Figure 4.1). The ability of IRS661 to block relaxation was specific to TLR7 agonists since it did not block relaxations induced by the β_2-adrenergic receptor agonist, isoproterenol (right bars; Figure 4.1). The β_2-adrenergic receptor antagonist, propranolol, blocked relaxation by isoproterenol, but did not block R837-mediated relaxation of airway smooth muscle (Figure 4.1), demonstrating that R837 is not mediating relaxation through β_2-adrenergic receptors. Together, these data indicate that R837-induced relaxation is partially mediated by TLR7, while relaxation by PolyUs and PolyAs is completely mediated by TLR7.

The MyD88 inhibitor peptide does not block R837-mediated relaxation of guinea pig trachea *in vitro*, but also does not block R837-mediated NF-κB signaling *in vitro.*

Because the mechanism of canonical TLR7 signaling proceeds directly downstream through the adaptor molecule MyD88, I measured the effect of a MyD88 inhibitor peptide to determine if R837-mediated relaxation proceeds through the canonical TLR7 signaling pathway. Neither a MyD88 inhibitor peptide (64 μM) nor a MyD88 control peptide (64 μM) had any effect on R837-mediated relaxation of isolated guinea pig trachea *in vitro* (Figure 4.2A). To test whether the MyD88 inhibitor peptide worked, I removed the tracheas from the organ bath experiments and measured NF-κB activation by ELISA. NF-κB activation serves as a readout for the canonical TLR7 signaling pathway involving regulation of gene expression. R837 induced NF-κB activation (Figure 4.2B), as expected by canonical TLR7 signaling. The MyD88 inhibitor did not inhibit R837-induced NF-κB activation (Figure 4.2B), suggesting that the MyD88 inhibitor does not work in guinea pig tracheas under the conditions used. To confirm that I could detect inhibition of canonical TLR7 signaling by this method, I also included trachea pretreated with the TLR7 antagonist, IRS661. IRS661 (300 μM) blocked R837-induced NF-κB activation to unstimulated levels (Figure 4.2B), confirming the ability to measure blockade of this pathway.

R837-mediated relaxation of guinea pig airway smooth muscle involves prostaglandins, large conductance Ca^{2+}-activated potassium (MaxiK) channel, and nitric oxide synthase.

Because the known effects of TLR7 signaling involve changes in gene expression, the TLR7-mediated relaxation is too fast to occur by these signaling mechanisms. To

address the unexpected rapid nature of TLR7 signaling, I investigated the involvement of pathways implicated in TLR signaling that are also able to rapidly modify airway physiology,. These include prostaglandins(Balzary and Cocks, 2006; Hattermann et al., 2007), MaxiK potassium channels(Scheel et al., 2006), and nitric oxide (Hammadi et al., 2008; Tumurkhuu et al., 2009).

Cyclooxygenase is an enzyme involved in the synthesis of prostaglandins. The cyclooxygenase inhibitor, indomethacin (1 μM), partially blocked R837-mediated relaxation of contractions induced by methacholine (3 μM; Figure 4.3), indicating that prostaglandins are partially responsible for R837-mediated relaxation.

To determine if MaxiK potassium channels are involved in the relaxing mechanism, a specific MaxiK channel blocker was used. To determine if other potassium channels are involved, a general potassium channel blocker was used. Both TEA (Nakajima, 1966) (a general potassium channel blocker; 20 mM) and paxilline(Knaus et al., 1994) (a selective MaxiK channel blocker; 20 μM) partially and equivalently blocked relaxation induced by either 30 μM or 100 μM R837 (Figure 4.4). This indicates that MaxiK channels are involved in the relaxation, as there is no additional blockade when all K^+ channels are blocked by TEA.

Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase. The nitric oxide synthase inhibitor N-monomethyl-L-arginine (L-NMMA) (100 μM) partially blocked relaxation induced by either 30 μM or 100 μM R837 (Figure 4.5A), indicating that nitric oxide is involved in R837-mediated relaxation. However, L-NMMA had no effect on PolyUs-mediated relaxations (Figure 4.5B), suggesting that a

different mechanism and possibly receptor mediate the effect of PolyUs. The complete reversal of PolyUs-mediated relaxation by the TLR7 antagonist is inconsistent with a different receptor, but it is possible that the specificity of IRS661 is not limited to TLR7 in the guinea pig.

To test whether TLR7 receptors, prostaglandins, MaxiK channels, and nitric oxide synthase are part of the same or parallel pathways of R837 mediated relaxation, I used combinations of inhibitors *in vitro*. Indomethacin, paxilline, L-NMMA, and IRS661 (TRL7 antagonist) each blocked R837 induced relaxation to the same extent. Blockade of R837-mediated relaxation by IRS661 was significantly greater in the presence of either indomethacin or paxilline compared to IRS661, indomethacin, or paxilline alone. However, blockade by IRS661 was not significantly greater in the presence of L-NMMA (Figure 4.6). There was no additive effect of indomethacin with paxilline (Figure 4.6). Together, these data indicates that R837 induced relaxation through two different pathways, one that is mediated through TLR7 and nitric oxide synthase, and a separate bronchodilator pathway that signals through prostaglandins and the MaxiK channels.

Adenosine antagonists do not relax guinea pig airway smooth muscle, and a TLR7 antagonist does not block adenosine receptors *in vitro*.

I tested whether relaxing effects of R837 are via adenosine receptors since it is a reported antagonist at A_1 and A_{2A} adenosine receptors (Schon et al., 2006). Neither the A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) nor the A_{2a} receptor antagonist SCH58261 relaxed contractions induced by methacholine

(Figure 4.7A). Therefore, R837 does not relax airway smooth muscle by acting as an antagonist at A_1 or A_{2A} receptors.

To address whether the adenosine antagonists bind and compete at the same receptor as R837, but fail to relax contraction, I measured the ability of R837 to relax methacholine-induced contractions when co-incubated with adenosine receptor antagonists. When coincubated with DPCPX, R837-mediated relaxation was partially reversed (Figure 4.7B left). Due to the minimal reversal, it is unlikely that these receptors are mediating the majority of the R837-mediated relaxation. I also tested whether PolyUs induced relaxation through adenosine receptors, and found no effect of coincubation with both the adenosine receptor antagonists, suggesting PolyUs does not relax airway smooth muscle through adenosine receptors (Figure 4.7B right).

To test whether R837 could be acting as a partial adenosine receptor agonist rather than an antagonist, I measured the effect of the general adenosine receptor agonist NECA in guinea pig trachea *in vitro*. NECA caused a dose-dependent partial relaxation (Figure 4.7C). However, the TLR7 antagonist IRS661 (100 μM) did not reverse the relaxation of tracheal segments induced by 30 μM or 100 μM NECA (a general adenosine receptor agonist; Figure 4.7D), indicating that IRS661 is not blocking adenosine receptors and specifically reverses the relaxant effects of TLR7 agonists. The relaxant effect of NECA was completely reversed by coincubation with the adenosine receptor antagonists DPCPX and SCH58261 (10 μM each; Figure

4.7D) confirming both that NECA-induced relaxation can be reversed and that it is mediated by adenosine receptors.

The TLR7 antagonist, IRS661, and the nitric oxide synthase inhibitor, L-NMMA, reverse R837-, but not PolyUs-mediated relaxation of isolated human tracheas *in vitro*.

As previously stated, for therapeutic purposes it is important to determine if the relaxant effects of TLR7 agonists translate to humans. While these drugs are bronchodilators regardless of the target, the further development of more efficacious or longer lasting drugs would benefit from understanding of structure-activity relationships, and this depends on the specific receptor mediating the drugs' effects. To address whether the relaxant effect of TLR7 agonists is TLR7-dependent in humans, I used the TLR7 antagonist, IRS661, to determine if it reverses the effects of TLR7 agonists. Methacholine (3 μM)-induced contraction was dose-dependently inhibited by R837 and PolyUs (Figure 4.8A). IRS661 (40 μM) almost completely reversed R837-mediated bronchodilation (Figure 4.8A), but did not reverse PolyUs-mediated bronchodilation. This suggests that in humans R837-mediated bronchodilation is completely TLR7-dependent, while PolyUs-mediated bronchodilation is TLR7-independent.

Because of the complete TLR7-dependence of R837 and apparent TLR7-independence of PolyUs in relaxation of human trachea, and our hypothesis that the TLR7-dependent pathway involves nitric oxide synthase, I measured the effect of the nitric oxide synthase inhibitor, L-NMMA (100 μM) on R837 and PolyUs-

mediated relaxation of human trachea. Consistent with the TLR7 antagonist, L-NMMA almost completely reversed the relaxant effect of R837, but had no effect on PolyUs-mediated relaxation (Figure 4.8B). Together, this suggests that the TLR7-dependent relaxant pathway involves nitric oxide as seen with R837, while a TLR7-independent relaxant pathway does not involve nitric oxide as seen with PolyUs. PolyUs has been reported to be a TLR8 agonist in humans (Heil et al., 2004), and we have shown that PolyUs fails to activate the canonical TLR7 pathway in a human TLR7 expressing cell line with a secretable alkaline phosphatase reporter under a NF-κB sensitive promoter (data not shown).

The TLR7 antagonist, IRS661, partially reverses R837 and PolyUs-mediated relaxation of isolated wildtype mouse tracheas *in vitro*, but not in tracheas from TLR7-deficient mice.

Using a pharmacological approach, I have shown that a TLR7 antagonist can partially reverse TLR7 agonist-mediated relaxation of both guinea pig and human tracheas. Though multiple TLR7 agonists showed the same relaxant effect and an antagonist reversed that effect in guinea pigs and humans, there was only a partial reversal in guinea pigs, leaving open the possibility that another target mediates the relaxant effects. To address whether there is TLR7-independent relaxation to TLR7 agonists, I used a genetic approach with a TLR7-deficient strain of mice. Methacholine (3 μM)-induced contraction was dose-dependently inhibited by R837 and PolyUs in both wildtype and TLR7-deficient mice (Figure 4.9A and B). This suggests that in mouse the relaxant effects of TLR7 agonists are not mediated by

TLR7, but by another target. However, in the presence of the TLR7 antagonist IRS661 (100 μM), the relaxant effect of R837 (10 μM) and PolyUs (300 μg/ml) was partially and significantly reversed only in the wildtype mouse tracheas and not in the TLR7-deficient mouse tracheas (Figure 4.9C and D). This suggests a TLR7-dependent and -independent pathway in mice, but the effective dose ranges are so close that it is not possible to see the difference between wildtype and TLR7-deficient mice. The only difference that can be detected is when the TLR7 component in the wildtype is blocked, whereas in the TLR7-deficient mice there is no difference with the TLR7 antagonist, because there is no TLR7.

The nitric oxide synthase inhibitor L-NMMA reverses R837-mediated relaxations in both wildtype and TLR7-deficient mouse tracheas *in vitro*.

Though R837 appears to exert relaxant effects through both TLR7-dependent and –independent pathways, as it relaxes tracheas from both wildtype and TLR7-deficient mice, I wanted to determine if the TLR7-dependent component is also nitric oxide-dependent. I, therefore, measured the effect of the nitric oxide synthase inhibitor L-NMMA (100 μM) on R837-mediated relaxation of tracheas from wildtype and TLR7-deficient mice. L-NMMA partially reversed R837-mediated relaxation of tracheas from both wildtype and TLR7-deficient mice to a similar extent (Figure 4.10). This suggests that unlike in guinea pig and human, both TLR7-dependent and –independent relaxant effects of R837 are mediated by nitric oxide in the mouse.

FIGURE 4.1. The TLR7 antagonist, IRS661, partially reverses R837-mediated relaxation and completely reverses PolyUs- and PolyAs-mediated relaxations, but does not reverse β_2-AR mediated relaxation of isolated guinea pig trachea *in vitro*.

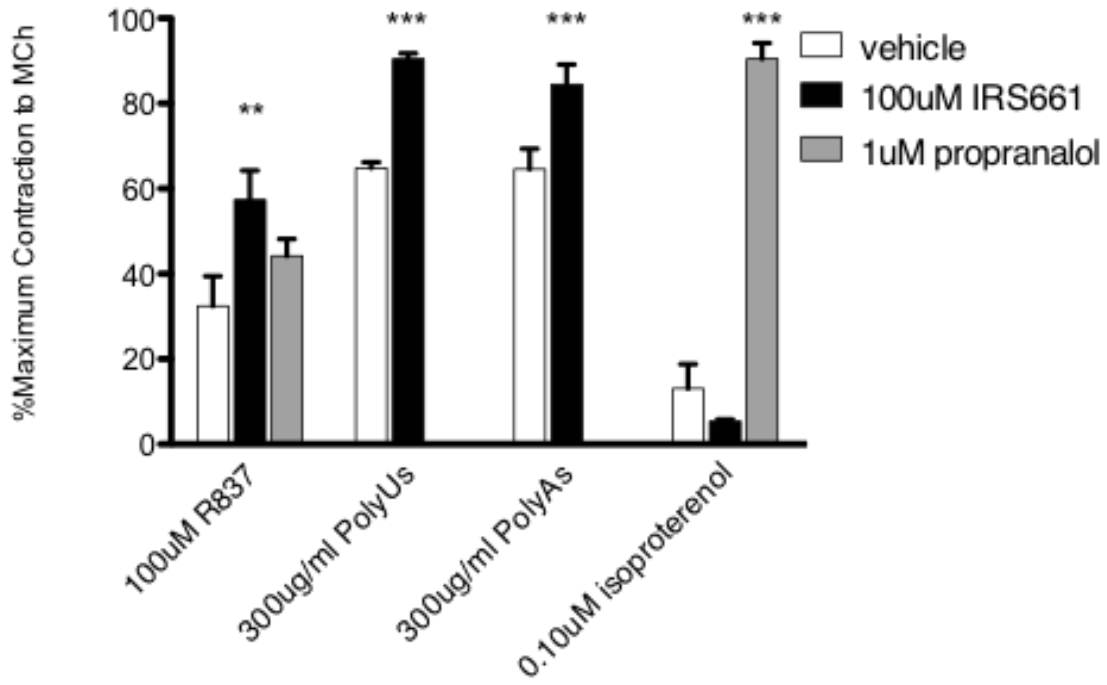


Figure 4.1. Trachea segments were preincubated with vehicle (open bars), IRS661 (black bars), or propranolol (grey bars). Contraction of tracheal segments was induced by methacholine and the effect of R837, PolyUs/As, or isoproterenol was measured. Data are expressed as the mean ± SEM. (n=3; maximum=1.49±0.10 g; R837 ± IRS661 $P \leq 0.01$; PolyUs/As ± IRS661 $P \leq 0.001$; isoproterenol ± propranolol $P \leq 0.001$).

FIGURE 4.2. The MyD88 inhibitor peptide does not block R837-mediated relaxation of guinea pig trachea *in vitro*, but it also does not block R837-mediated NF-κB signaling *in vitro.*

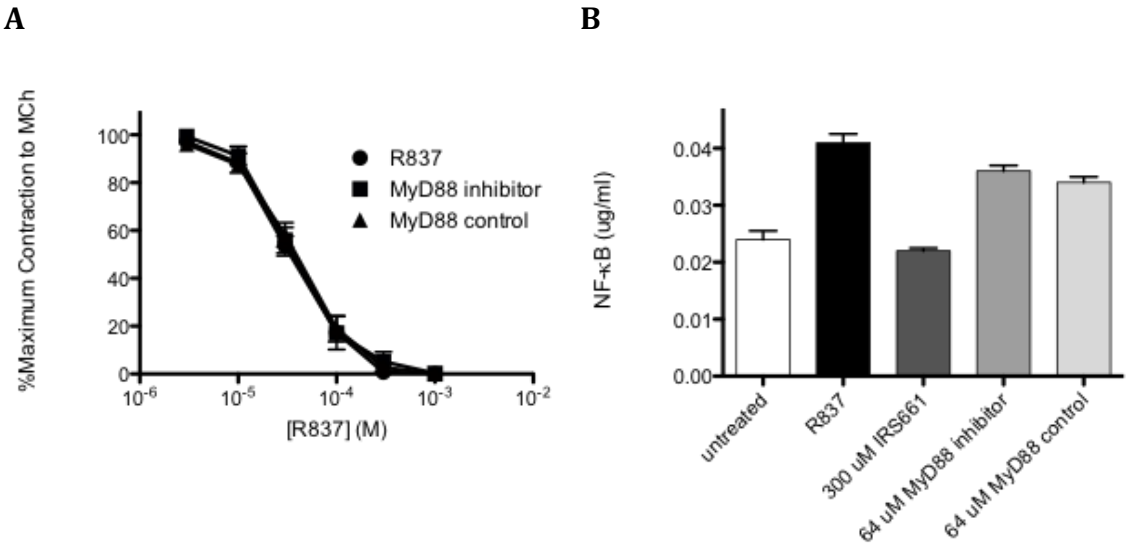


Figure 4.2 (A) Trachea segments were incubated with a MyD88 inhibitor or control peptide, contraction was induced by methacholine, and the effect of R837 was measured. Data are expressed as the mean ± SEM. (*n*=4; maximum=1.21±0.41 g). (B) Protein was extracted from trachea segments at end of organ bath experiment and NF-κB activation was measured by ELISA. Also included measurements from tissue pretreated with IRS661. Data are expressed as the mean ± SEM. (*n*=4).

FIGURE 4.3. R837-mediated relaxation of isolated guinea pig trachea *in vitro* is partially reversed by indomethacin, an inhibitor of prostaglandin synthesis.

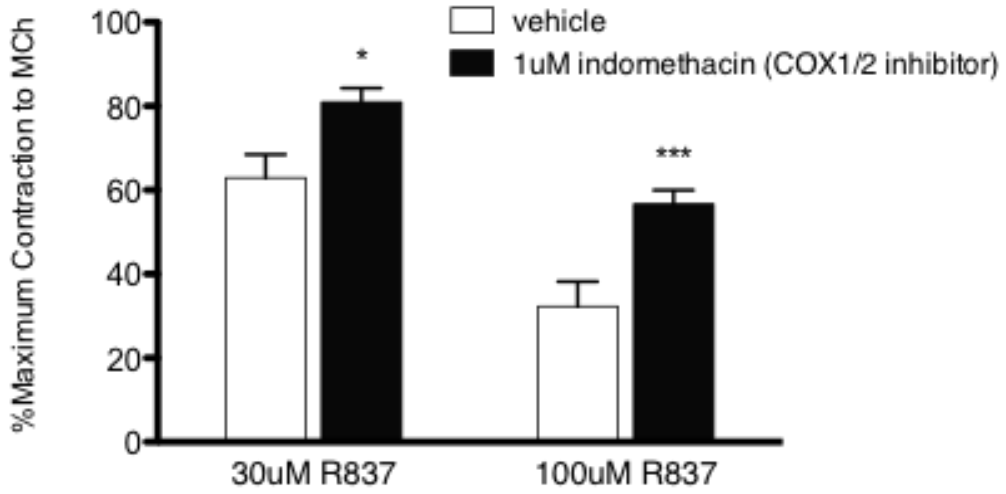


Figure 4.3 Trachea segments were preincubated with vehicle or the cyclooxygenase inhibitor indomethacin, contraction was induced by methacholine, and the effect of R837 was measured. Data are expressed as the mean ± SEM. (n=3; maximum=1.53±0.18 g; 30 μM R837 $P \leq 0.05$; 100 μM R837 $P \leq 0.001$).

FIGURE 4.4. R837-mediated relaxation of isolated guinea pig trachea *in vitro* is partially reversed by paxilline, a MaxiK potassium channel blocker.

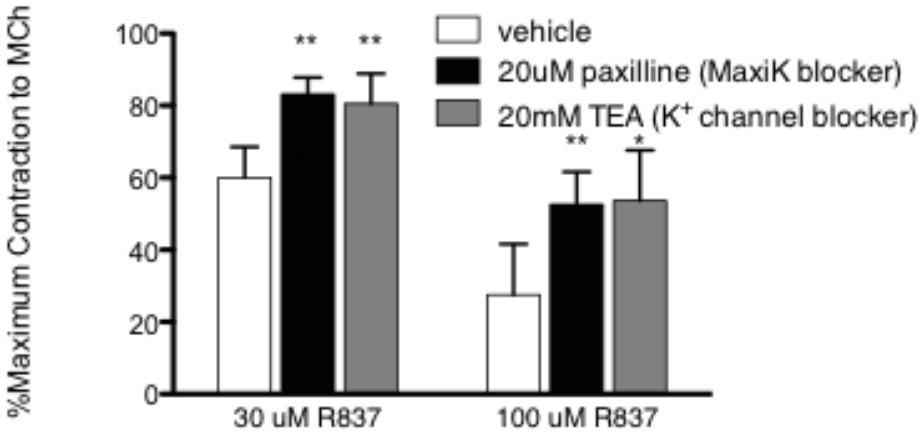


Figure 4.4 Trachea segments were preincubated with vehicle, paxilline, or TEA, contraction was induced by methacholine, and the effect of R837 was measured. Data are expressed as the mean ± SEM. (n=3; maximum=1.22±0.09 g; 30 μM R837 vs. paxilline $P \leq 0.01$, 30 μM R837 vs. TEA $P \leq 0.01$; 100 μM R837 vs. paxilline $P \leq 0.01$, 100 μM R837 vs. TEA $P \leq 0.05$).

FIGURE 4.5. R837- but not PolyUs-mediated relaxation of isolated guinea pig trachea *in vitro* is partially reversed by L-NMMA, a nitric oxide synthase inhibitor.

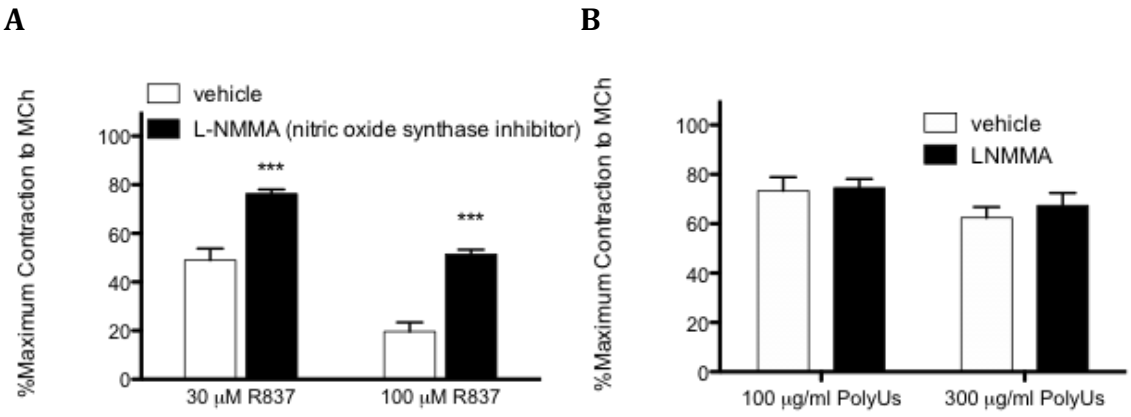


Figure 4.5 (A) Trachea segments were preincubated with vehicle or L-NMMA, contraction was induced with methacholine, and the effect of R837 measured. Data are expressed as the mean ± SEM. (n=3; maximum=2.14±0.17g; P≤0.001). (B) Trachea segments were preincubated with vehicle or L-NMMA, contraction was induced with methacholine, and the effect of PolyUs measured. Data are expressed as the mean ± SEM. (n=3; maximum=1.16±0.14g).

FIGURE 4.6. The effect of inhibition of prostaglandin synthesis or the MaxiK potassium channel, but not inhibition of nitric oxide synthesis, is additive with TLR7 blockade.

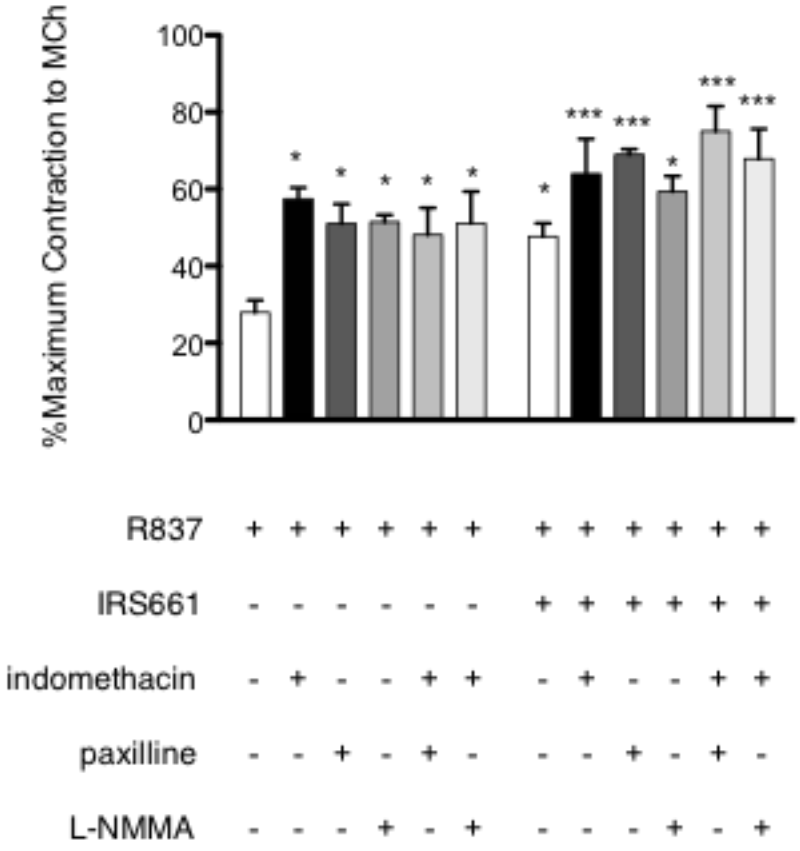
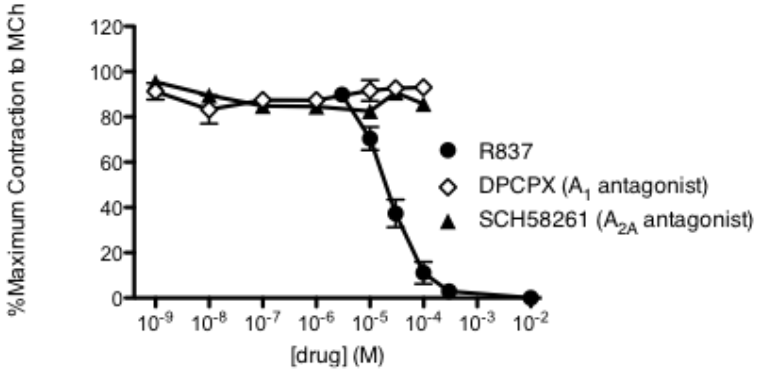


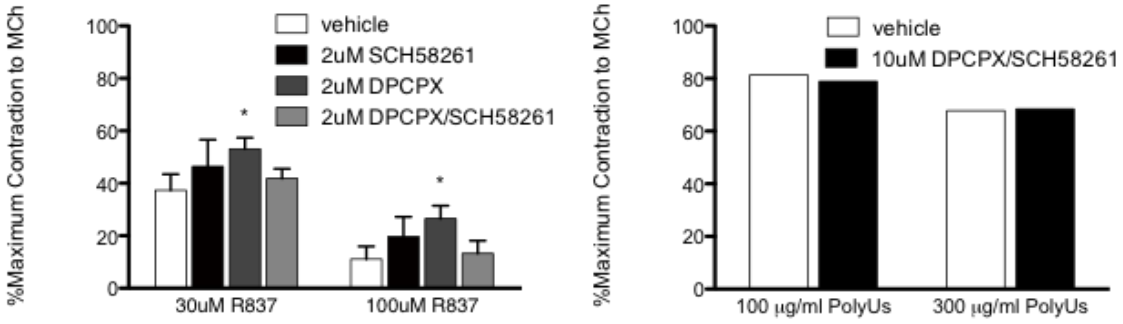
Figure 4.6 Trachea were preincubated with vehicle control or IRS661 in combination with vehicle control, indomethacin, paxilline, L-NMMA, indomethacin and paxilline together, or indomethacin and L-NMMA together. Contraction was induced by methacholine and the effect of R837 was measured. Data are expressed as the mean ± SEM. (*n*=3; maximum=1.93±0.15 g; *significantly different than R837 alone; ***significantly different than both R837 alone and R837 + IRS661).

FIGURE 4.7. Adenosine receptor antagonists do not induce relaxation and partially reverse the effect of R837-mediated relaxation of isolated guinea pig trachea *in vitro*, and adenosine agonist-mediated bronchodilation is not reversed by a TLR7 antagonist.

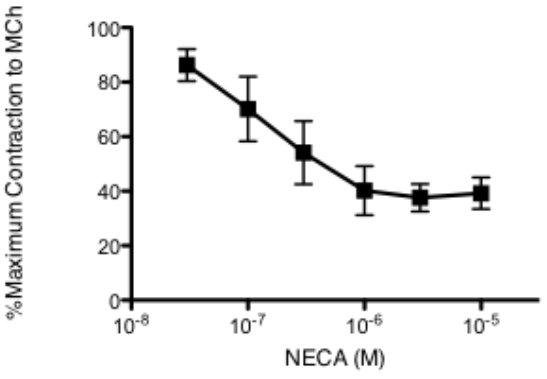
A



B



C



D

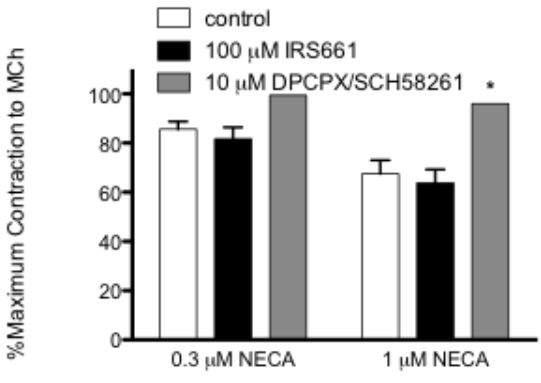


Figure 4.7 (A) Contraction of tracheal segments was induced by methacholine and the effect of cumulative increasing doses of R837 (circles), the adenosine A_1 receptor antagonist DPCPX (diamonds), or the adenosine A_{2A} receptor antagonist SCH58261 (triangles) was measured. Data are expressed as the mean ± SEM. (*n*=3; maximum=1.29±0.10 g; effect of dose of R837 $P \leq 0.001$). (B) Contraction of tracheal segments was induced by methacholine, incubated in the absence or presence of SCH58261, DPCPX, or SCH58261 and DPCPX together, and the effect of R837 (left) or PolyUs (right) was measured. Data are expressed as the mean ± SEM. (R837 *n*=3, PolyUs *n*=1; maximum=1.3±0.16 g; R837 vs. 2 μM DPCPX $P \leq 0.05$). (C) Contraction of tracheal segments was induced by methacholine and the effect of cumulative increasing doses of the adenosine agonist NECA was measured. Data are expressed as the mean ± SEM. (*n*=3; maximum=0.95±0.31 g; effect of dose of NECA $P \leq 0.01$). (D) Trachea segments were preincubated with vehicle (open bars), IRS661 (black bars), or DPCPX and SCH58261 (grey bars). Contraction of trachea segments was induced by methacholine, and the effect of NECA was measured. Data are expressed as the mean ± SEM. (control *n*=3, IRS661 *n*=2, DPCPX/SCH58261 *n*=1; maximum=1.78±0.30 g; 1 μM NECA vs. 10 μM DPCPX/SCH58261 $P \leq 0.05$).

FIGURE 4.8. The TLR7 antagonist, IRS661, and the nitric oxide synthase inhibitor, L-NMMA, almost completely reverse R837-mediated relaxation of human trachea *in vitro*, but not PolyUs-mediated relaxation of human trachea.

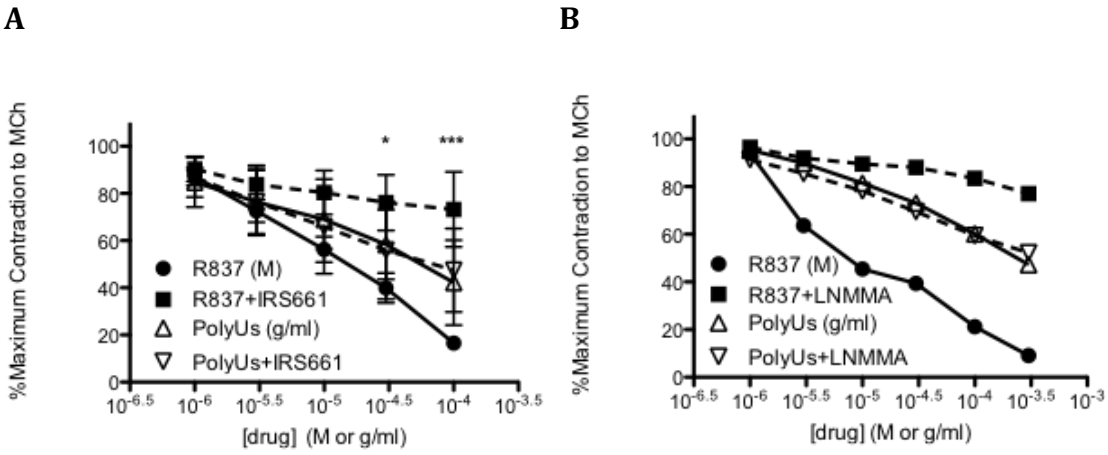


Figure 4.8. (A) Human trachea segments were preincubated with vehicle (solid lines) or IRS661 (dashed lines). Contraction of tracheal segments was induced by methacholine and the effect of R837 (circles, squares) or PolyUs (triangles) was measured. Data are expressed as the mean ± SEM. (R837 *n*=3. PolyUs *n*=2; maximum=0.93±0.67g; R837 vs. R837 ± IRS661 30 μM $P \leq 0.05$, 100 μM $P \leq 0.001$) (B) Human trachea segments were preincubated with vehicle (solid lines) or L-NMMA (dashed lines). Contraction of tracheal segments was induced by methacholine and the effect of R837 (circles, squares) or PolyUs (triangles) was measured. Data are expressed as the mean ± SEM. (*n*=1; maximum=1.02±0.72 g)

FIGURE 4.9. R837 and PolyUs relax contraction of tracheas from wildtype and TLR7-deficient mice, but the TLR7 antagonist, IRS661, only reverses relaxation of wildtype mouse tracheas *in vitro*.

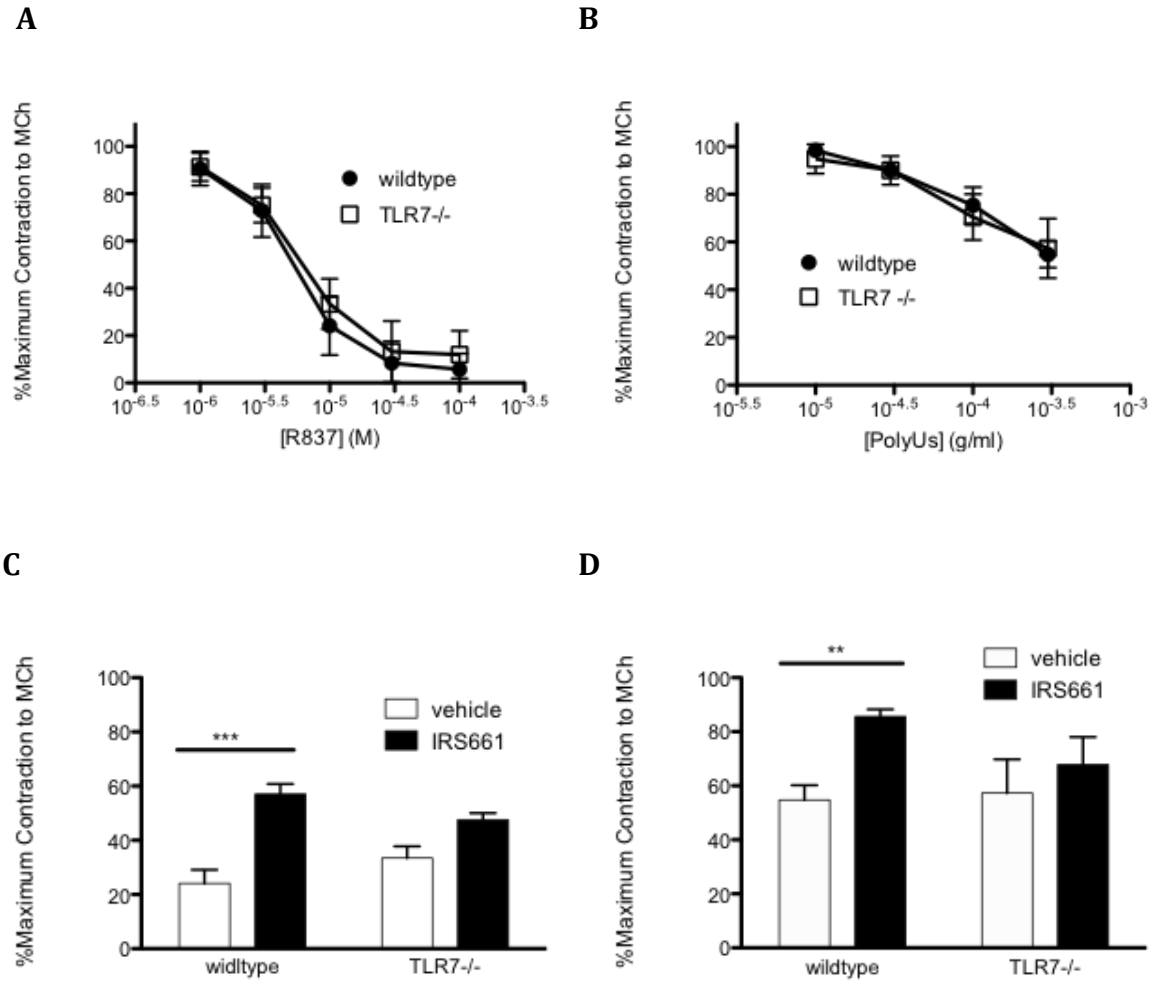


Figure 4.9 (A) Contraction of trachea segments from wildtype (circles) and TLR7-deficient (squares) mice was induced by methacholine and the effect of cumulative increasing doses of R837 was measured. Data are expressed as the mean ± SEM. (*n*=6; maximum wildtype=0.321±0.076 g; maximum TLR7-/-=0.304±0.105 g). (B) Contraction of trachea segments from wildtype (circles) and TLR7-deficient

(squares) mice was induced by methacholine and the effect of cumulative increasing doses of PolyUs was measured. Data are expressed as the mean ± SEM. (*n*=3; maximum wildtype=0.523±0.275 g; maximum TLR7-/-=0.280±0.045 g). (C) Trachea segments were preincubated with vehicle (open bars) or IRS661 (closed bars) and the effect of R837 (10 μM) was measured. Data are expressed as the mean ± SEM. (*n*=6; ***$P \leq 0.001$). (D) Trachea segments were preincubated with vehicle (open bars) or IRS661 (closed bars) and the effect of PolyUs (300 μg/ml) was measured. Data are expressed as the mean ± SEM. (*n*=3; **$P \leq 0.01$).

Figure 4.10. The nitric oxide synthase inhibitor L-NMMA reverses R837-mediated relaxations in both wildtype and TLR7-deficient mouse tracheas *in vitro*.

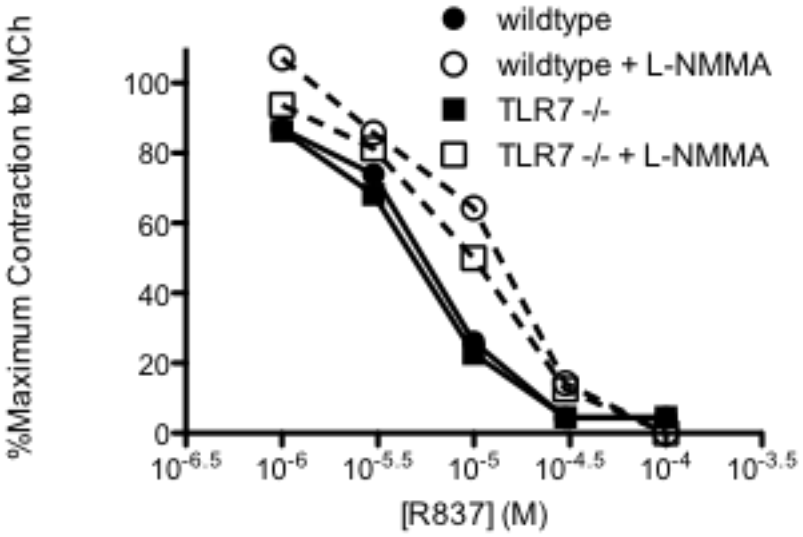


Figure 4.10 Contraction of trachea segments from wildtype (circles) and TLR7-deficient (squares) mice was induced by methacholine and the effect of cumulative increasing doses of R837 was measured before (solid lines) and after (dashed lines) incubation with L-NMMA. (*n*=1; maximum wildtype=0.19±0.04g, maximum TLR7-/-=0.19±0.06g).

DISCUSSION

My surprising finding that TLR7 agonists caused rapid bronchodilation within minutes of administration in guinea pigs *in vivo* and in isolated guinea pig, human, and mouse tracheas *in vitro*, demonstrated that these ligands could potentially be used as therapies for disorders marked by bronchoconstriction. Their therapeutic potential lies in their bronchodilatory ability regardless of the receptor mediating the effect. Though one of these ligands, R837 (Imiquimod), is a commercially available therapeutic for virus induced lesions and tumors, it is currently approved for topical use with limited systemic exposure (Beutner et al., 1998; Harrison et al., 2004). It is unlikely that it would have access to the airways in sufficient concentrations to be efficacious, however experiments using oral administration of R837 for cancer therapy show promise for more extensive distribution of the drug (Dharmapuri et al., 2009). To further develop and improve drugs of this class for asthma therapies with an intelligent approach, more information is needed about the receptor mediating the bronchodilating effects as well as the mechanism.

Because multiple TLR7 ligands of two structural classes all exhibited a bronchodilatory effect, TLR7 remained a viable candidate as the receptor mediating that effect. It is still possible that all of these ligands share a common off target effect of bronchodilation not mediated by TLR7. I, therefore, undertook studies to determine the extent of the involvement of TLR7 in the observed relaxant effect. This was accomplished using multiple approaches, including pharmacological receptor blockade and genetic abrogation of TLR7 function.

A TLR7 antagonist, IRS661, partially reversed R837-mediated relaxation and completely reversed relaxation induced by PolyUs and PolyAs in guinea pigs. Though PolyAs is not reported to be a TLR7 agonist, the reversal of the relaxant effect by a TLR7 antagonist, suggests that this might depend on the system in which the ligand was tested. For example, maybe PolyAs fails to activate NF-κB, but succeeds at rapidly stimulating TLR7 for relaxation of airway smooth muscle. This might depend on the length of time of receptor occupancy by the ligand or how efficient the ligand is at inducing conformational changes in the receptor. More recently it was shown that PolyAs is a TLR8 agonist, so perhaps IRS661 acts as both a TLR7 and TLR8 antagonist in guinea pig (Demaria et al.; Martinez et al.).

The effect of R837 was almost completely reversed by a TLR7 antagonist in human tracheas, suggesting that the degree of TLR7-dependence is species-dependent. Another possibility is that IRS661 is a more efficient antagonist in humans than in guinea pigs. PolyUs-mediated relaxation, however, was not reversed by a TLR7 antagonist, suggesting that in humans PolyUs does not mediate bronchodilation through TLR7. It is reported that PolyUs does not activate human TLR7, but instead activates human TLR8 (Heil et al., 2004). It is interesting to consider TLR8 as the target mediating TLR7-independent effects, but TLR8-specific agonists, antagonists, as well as a TLR8-deficient strain of mice must be further developed to address this question. Together, that multiple TLR7 agonists are bronchodilators, and an antagonist reverses some or all of this bronchodilation, strongly suggests a role for TLR7 in mediating the bronchodilating effects. The fact that in humans a TLR8-

specific agonist induces bronchodilation that cannot be reversed by a TLR7 antagonist, suggests that perhaps TLR8 mediates the TLR7-independent relaxant effects.

The possibility of TLR7-dependent and -independent effects of R837 depending on the species as opposed to the complete TLR7-dependence or TLR8-dependence of PolyUs is an important distinction. Many groups currently use imidazoquinolines, rather than PolyUs, to study TLR7 and may be seeing off target effects with the R837-related imidazoquinolines. This could result in incorrectly attributing observed effects to TLR7 activation.

I also used a genetic approach to determine the involvement of TLR7 in TLR7 agonist-mediated bronchodilation using a TLR7-deficient strain of mice. I observed almost identical bronchodilation in wildtype compared to TLR7-deficient mice in response to both R837 and PolyUs. At first glance, this would seem to suggest that TLR7 plays no role in R837- or PolyUs-mediated bronchodilation in the mouse. Another possibility is the upregulation or already sufficient existence of a compensatory bronchodilating mechanism in response to TRL7 agonists. I next used a TLR7 antagonist and measured its ability to reverse TLR7 agonist-mediated relaxation in both wildtype and TLR7-deficient mice. The TLR7 antagonist could partially and significantly reverse both R837- and PolyUs-mediated relaxation in wildtype, but not in TLR7-deficient mice. This supports a TLR7-dependent and –independent bronchodilatory effect of R837 and PolyUs in mice, and further suggests that the two pathways have a very close effective dose range. When TLR7

is present in wildtype mice, it contributes to the bronchodilatory effect of TLR7 agonists as seen by partial reversal by the TLR7 antagonist. In the absence of TLR7 function in the TLR7-deficient mice, TLR7 agonists still bronchodilate through the TLR7-independent pathway, but the TLR7 antagonist has no effect due to the absence of a TLR7-dependent contribution. It is possible that TLR8 mediates a large portion of the bronchodilating effects of these ligands in mouse, or that TLR8 compensates for the loss of TLR7 function in the TLR7-deficient mice.

MyD88 is an adaptor molecule through which TLR7 signals to regulate gene expression (Hemmi et al., 2002). This canonical signaling results in the downstream activation of a number of transcription factors through NF-κB, MAPK, and interferon regulatory factor signaling (Diebold et al., 2004). To determine if the relaxant effect of TLR7 agonists is also MyD88-dependent, I measured the effect of a MyD88 inhibitor peptide. Blockade of MyD88 signaling with the inhibitor peptide had no effect on the relaxant effect of R837 in guinea pig trachea *in vitro*, initially suggesting that the rapid relaxation is not MyD88-dependent in guinea pig. However, by measuring NF-κB activation as a marker of canonical TLR7 signaling, I also found that the MyD88 inhibitor did not block R837-induced NF-κB activation. R837-induced NF-κB activation was blocked by the TLR7 antagonist, so the lack of effect of the MyD88 inhibitor is not because I cannot measure inhibition of R837-induced NF-κB activation. This suggests either that the MyD88 inhibitor does not work in our system or that TLR7-signaling is not MyD88-dependent in the guinea pig. Even if the transcriptional components of TLR7-signaling are MyD88-dependent in guinea

pig, the fast relaxant effects of TLR7 ligands might be MyD88-independent. As of now, I have not been able to determine the role of MyD88 in the observed relaxant effect of TLR7 agonists.

To further understand the mechanism of TLR7-dependent and –independent bronchodilation in the guinea pig, I conducted mechanistic studies using pharmacological inhibitors of bronchodilating pathways. These signaling pathways were selected from those which have some reported association with TLR signaling, including prostaglandins, the large conductance calcium-activated potassium channel (MaxiK), and nitric oxide (Fedan et al., 1995; Balzary and Cocks, 2006; Scheel et al., 2006; Hattermann et al., 2007; Tumurkhuu et al., 2009). Though a specific connection between TLR7 ligands and MaxiK has not been directly shown, it is known that influenza activates the inflammasome through TLR7, and inflammasome activity is dependent on potassium efflux from the cell (Petrilli et al., 2007; Ichinohe et al., 2010). While this has been widely considered to be due to the activity of ATP at purinergic channels, it is interesting to consider a possible association with other potassium channels, such as MaxiK.

Inhibition of prostaglandin synthesis by indomethacin, MaxiK potassium channel blockade by paxilene, and inhibition of nitric oxide synthesis by L-NMMA, all partially and significantly reversed the relaxant effect of R837 in guinea pig trachea. To address whether any or all of these mechanisms are part of the TLR7-dependent or –independent pathways, I used various combinations of the inhibitors with a TLR7 antagonist. Both inhibition of prostaglandin synthesis and MaxiK blockade had

an additive reversal of R837-mediated relaxation in combination with the TLR7 antagonist, suggesting that they are part of separate TLR7-independent pathways. Combining inhibition of prostaglandin synthesis with MaxiK blockade did not have an additive reversal, suggesting they are part of a common TLR7-independent pathway. This is not surprising as prostanoid signaling is known to feed into MaxiK function (Yamaki et al., 2001). Inhibition of nitric oxide synthesis did not have a clear and significant additive reversal in combination with the TLR7 antagonist, suggesting that nitric oxide synthesis may be the mechanism of the TLR7-dependent bronchodilation.

Interestingly, in human tracheas, the nitric oxide synthase inhibitor completely reversed the relaxant effect of R837, but not that of PolyUs. This resembles the differential sensitivity to the TLR7 antagonist of these ligands in humans. If TLR7-dependent relaxation involves nitric oxide, as determined in the guinea pig, then only the TLR7 ligand should be reversed both by a TLR7 antagonist or a nitric oxide synthase inhibitor, as is R837. As stated above, it has been shown that R837 activates human TLR7, but PolyUs does not. This further confirms that TLR7-independent relaxation does not involve nitric oxide, as PolyUs is reversed neither by the TLR7 antagonist nor the nitric oxide synthase inhibitor.

If the TLR7-dependent component of R837-mediated bronchodilation is mediated by nitric oxide, then it would be expected that R837-mediated bronchodilation should be reversed in the wildtype, but not the knockout mouse. This, however, was not the case, as L-NMMA reversed R837-mediated relaxation of tracheas from both

wildtype and TKR7-deficient mice. This might suggest that in the mouse, both TLR7-dependent and TLR7-independent relaxant effects of R837 are mediated by nitric oxide. Another possibility is that compensatory pathways, such as TLR8, might utilize the nitric oxide-mediated relaxant effects in TLR7-deficient animals, even if they do not normally do so in wildtype animals. More experiments with TLR7- and TLR8-specific ligands, as well as TLR8-deficient and TLR7/8-deficient mice are needed to further address the nitric oxide dependency of TLR7-mediated bronchodilation in the mouse.

R837 is reported as an antagonist at adenosine A_1 and A_{2a} receptors, so I tested the possibility that antagonism of adenosine receptors account for TLR7-independent bronchodilation in guinea pig tracheas *in vitro*. If R837 was acting as an adenosine A_1 or A_{2a} receptor antagonist to cause relaxation of airway smooth muscle, then it would be expected that other known antagonists would have a similar effect. Neither the adenosine A_1 receptor antagonist (DPCPX) nor the adenosine A_{2a} receptor antagonist (SCH58261) caused any relaxation of guinea pig trachea *in vitro*. Therefore, R837 does not act as an adenosine receptor antagonist to relax guinea pig tracheas. To further address this question, I coincubated R837 with the adenosine antagonists to determine if there is any competition at a common receptor. There was minimal though significant reversal of R837-mediated relaxation by the adenosine A_1 receptor antagonist DPCPX. It seems unlikely that this minimal reversal could account for the TLR7-independent pathway. The

adenosine antagonists did not reverse the relaxant effect of PolyUs, confirming that these relaxations are also not mediated by adenosine receptors.

A common problem in pharmacology is the misclassification of partial receptor agonists as antagonists, because they will inhibit the effect of a full agonist when coincubated. To address whether R837 acts as an adenosine receptor agonist, I measured the effect of a full adenosine receptor agonist (NECA). NECA dose-dependently induced partial relaxation of guinea pig tracheas, leaving the possibility that R837 acts as an adenosine receptor agonist to account for the TLR7-independent pathway. The effect of NECA was completely adenosine receptor mediated and blocked by the adenosine receptor antagonists. I once again tested the specificity of the TLR7 antagonist by measuring its effects on adenosine receptor agonist mediated relaxation. The TLR7 antagonist did not reverse the relaxant effect of the adenosine receptor agonist, showing again that the TLR7 antagonist is specific to and only reverses the effects of TLR7 agonists.

The question of what receptor is mediating the TLR7-independent effects in guinea pig and mouse remains unanswered. There are conflicting reports based on species and organ system as to whether existing TLR7 agonists also stimulate TLR8(Jurk et al., 2002; Heil et al., 2004; Spaner et al., 2005; Gorden et al., 2006; Lan et al., 2007; Zhu et al., 2008), leaving open the possibility that some bronchodilator effect is TLR8-mediated in guinea pig and mice. The lack of TLR8 antagonists or a TLR8-deficient strain of mice makes this determination difficult. Recently, a TLR8-deficient mouse was reported (Demaria et al.). It will be of great utility to test the

relaxant effect of TLR7/8 ligands in TLR8-deficient mice, as well as mice deficient in both TLR7 and TLR8.

The data suggest that in humans the relaxant effect of R837 is completely TLR7-dependent and completely nitric oxide-dependent, whereas that of PolyUs is completely TLR7- and nitric oxide-independent. Because PolyUs is a TLR8 agonist in humans, I propose that the non-TLR7 mediated relaxant effects of TLR7/8 ligands are mediated by TLR8. Though, more experiments are needed in human tissue, based on the TLR7-independent effects in guinea pig, I propose that the TLR7-independent TLR8-dependent relaxant effects are mediated by prostaglandins and the MaxiK potassium channel. A proposed model of TLR7-dependent and TLR8-dependent effects of TLR7/8 ligands is shown in Figure 4.11. Also shown in the model is R848, which is reported to signal through both human TLR7 and TLR8, and therefore would be expected to have relaxant effects mediated by both pathways.

I have now shown that TLR7 agonists are potent bronchodilators and, depending on the drug and species tested, the effect is at least partially TLR7-mediated. This might be a protective mechanism to maintain normal breathing during the inflammation associated with a respiratory virus infection. The question remains of why humans with asthma suffer more severe effects of virus infections. One possibility is that this is a functional consequence of the reported TLR7 polymorphisms associated with asthma. Perhaps these patients do not have the bronchodilatory protection provided by TLR7. Beyond polymorphisms in TLR7, the allergic immune environment often associated with asthma or virus infection itself might change the

bronchodilatory potential of TLR7. The effect of TLR7 signaling on airway physiology in the asthmatic condition will be addressed in Chapter V, in which I test the effect of TLR7 ligands in animal models of asthma.

Data in this chapter have been published as:

Kaufman EH, Fryer AD, Jacoby DB. Toll-like receptor 7 agonists are potent and rapid bronchodilators in guinea pigs. *J. Allergy Clin Immunol*, 2010. Article in press.

FIGURE 4.11. Proposed model of TLR7-dependent and TLR8-dependent airway smooth muscle relaxant effects of TLR7/8 agonists in humans.

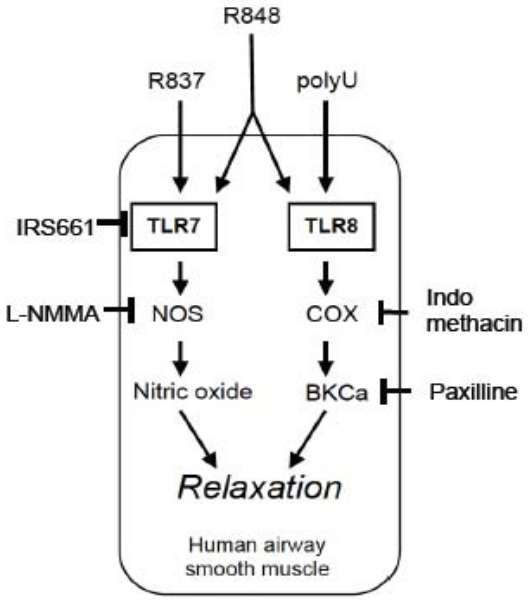


Figure 4.11 My data suggest the pathway on the left for TLR7-dependent airway smooth muscle relaxation, and the pathway on the right for TLR8-dependent airway smooth muscle relaxation. R837 relaxes the airway smooth muscle through TLR7 signaling and nitric oxide production. PolyU relaxes the airway through TLR8, independently of nitric oxide. The TLR8 pathway may be responsible for non-TLR7 effects of R837 at high concentrations in other species, which are mediated via prostanoid production and opening of the MaxiK channel. The dual TLR7/8 agonist R848 would be expected to work through both relaxant pathways.

CHAPTER V.

THE EFFECT OF TOLL-LIKE RECEPTOR 7 AGONISTS ON BRONCHODILATION IN ANIMAL MODELS OF ASTHMA

ABSTRACT

Because of the association of asthma attacks with virus infections, I explored the effects of signaling through a virus-detecting receptor of the innate immune system on airway physiology. I showed that TLR7 agonists are rapid and potent bronchodilators *in vivo* and *in vitro*, by a mechanism involving prostaglandins, the MaxiK potassium channel, and nitric oxide. This bronchodilating pathway might maintain breathing during the airway occlusion brought on by inflammation associated with clearing a virus infection. Both healthy humans and those with asthma get respiratory virus infections, but the symptoms are more severe in humans with asthma. Because of TLR7 polymorphisms associated with asthma, as well as a T_h2-dominated allergic immune environment in the lungs that may result in reduced TLR7 expression, perhaps humans with asthma have a deficiency in the described TLR7-mediated bronchodilating protective mechanism. To address the effects of TLR7 signaling on airway physiology in the asthmatic condition, I measured the effect of the TLR7 agonist, R837, in several animal models of asthma. These include allergen sensitization or virus infection in both guinea pig and mouse, as well as transgenic mouse models that have increased or decreased numbers of eosninophils in the lungs. I found that both allergen sensitization and Sendai virus-infection reduced the bronchodilating potency of R837 in guinea pigs *in vivo*. To determine if TLR7-mediated eosinophil activation is responsible for the reduced R837 potency, I depleted eosinophils with an antibody to IL-5, but was not able to rescue the potency. There was no difference in the relaxant potency of R837 in

tracheas from allergen sensitized, sensitized and challenged, and Sendai virus-infected mice *in vitro.* To determine whether these asthma models were insufficient to induce TLR7 dysfunction in mice, I also measured the effect of R837 in transgenic mice with and without eosinophils in the lung, but found no differences in the relaxant potency of R837. The data suggest that guinea pig models of asthma are better for measuring TLR7 dysfunction, and that eosinophils that can be depleted by an antibody to IL-5 are not responsible for dysfunction in the relaxant potency of TLR7.

INTRODUCTION

In the previous chapters I demonstrated that TLR7 agonists are potent and rapid bronchodilators within minutes of administration in guinea pigs, mice, and humans. Because of the inconsistency of the fast timing of the effect with known effects of TLR7 signaling, I undertook experiments to show that TLR7 does mediate some or all of the effects of TLR7 agonists depending on the species. The mechanism involves effectors known to have fast effects on airway smooth muscle physiology including prostaglandins, the MaxiK potassium channel, and nitric oxide.

This highly conserved mechanism might serve to maintain breathing by bronchodilating the airways during the obstruction associated with the clearance of the virus infection. While inflammation is beneficial for protection against pathogenic infections, there are numerous deleterious effects, and mechanisms evolved to limit these effects are very important. Both healthy humans and humans with asthma are subject to respiratory virus infections. While healthy humans experience symptoms consistent with inflammation to clear the infection, humans with asthma suffer more severe symptoms that present as asthma attacks that can result in hospital admissions or death. As TLR7 polymorphisms are associated with asthma, perhaps TLR7 dysfunction limits the bronchodilating effect of this protective mechanism. The result is more constriction and obstruction during the virus infection, hallmarks of the asthmatic condition. Because the asthmatic condition is often associated with allergy and a T_h2-skewed immune environment in the lung, another possibility could be reduced TLR7 expression or function. TLR7

signaling is associated with skewing towards a T_h1 immune environment characterized by antiviral cytokines like interferon-γ. Here I show, as others have (Nagase et al., 2003), that interferon-γ can potentiate TLR7 expression and function, so reduced interferon-γ production in a T_h2 environment might result in reduced function of a TLR7-mediated bronchodilating mechanism.

To address whether the TLR7-mediated bronchodilating protective mechanism is dysfunctional in the asthmatic condition, I measured the effect of the TLR7 agonist, R837, in several animal models of asthma. These include allergen sensitization models of allergic asthma and Sendai virus infection models in guinea pig and mouse. I found that allergen sensitization and Sendai virus-infection reduced the bronchodilating potency of R837 in guinea pigs *in vivo*. The reduced potency is the result of the loss of a high potency phase from a biphasic dose-response in control animals, leaving only the lower potency phase remaining. The results suggest that a reduction in TLR7 function might account for reduced bronchodilation in humans with asthma during a respiratory virus infection.

A hallmark of asthma is higher numbers of eosinophils in the airways, a characteristic shared by allergen-sensitized guinea pigs (Costello et al., 1997; Kay, 2005; Lemiere et al., 2006). Activation of these eosinophils by allergen challenge or virus infection results in the release of various inflammatory mediators, including major basic protein. Major basic protein is an allosteric antagonist of M2 muscarinic receptors. Prejunctional M2 muscarinic receptors on airway parasympathetic nerves limit acetylcholine release from the nerve terminal, a negative feedback

mechanism limiting bronchoconstriction. Antagonism of these M2 muscarinic receptors by major basic protein eliminates the negative feedback, increasing bronchoconstriction.

In this chapter, I show that human eosinophils express TLR7 and can be activated by TLR7 agonists. In allergen-sensitized guinea pigs, perhaps TLR7-mediated activation of eosinophils exerts a bronchoconstricting effects that opposes TLR7-mediated bronchodilation, accounting for the reduced potency of R837. To test this hypothesis, I depleted eosinophils from allergen-sensitized animals using a monoclonal antibody to IL-5, a cytokine that promotes eosinophil survival and maturation. This treatment did not rescue the potency of R837, suggesting that eosinophils are not responsible for the reduced potency in allergen-sensitized guinea pigs.

In mice, there was no effect of allergen sensitization, allergen challenge, or virus infection on the relaxant potency of R837 *in vitro*. Because mouse airway physiology is so different from guinea pigs and humans and not all characteristics of asthma are recapitulated in the allergic and virus-infected mouse models, I also used transgenic mouse lines. NJ1726 mice have IL-5 expressed under a Clara cell-specific promoter resulting in IL-5 overexpression in the lungs and increased eosinophil numbers in the lungs (Lee et al., 1997). Because increased eosinophil numbers have long been associated with asthma, this model allowed me to measure the effect of bronchodilating TLR7 signaling at the same time as that from TLR7 in eosinophils. The PHIL strain of mice, have the diphtheria toxin A chain expressed under the

control of the eosinophil-specific eosinophil peroxidase promoter, depleting eosinophils from these mice. The PHIL strain allows me to determine if the absence of eosinophils shows a different bronchodilator response to TLR7 signaling, suggesting a role for eosinophil activation. A third strain, NJ1726/PHIL, has IL-5 overexpression in the lungs, but no eosinophils, allowing me to determine whether IL-5 overexpression in the lungs itself has an effect regardless of the presence of eosinophils. A T_h2 allergic immune environment is characterized by increased IL-5 expression, so non-eosinophilic IL-5 effects cannot be discounted. I observed no significant difference in the relaxant effect of R837 in any of these strains of mice from wildtype controls.

The data suggest that allergy and virus infection, often associated with asthma attacks, reduce the bronchodilating potency of TLR7 agonists in guinea pig. This might reflect a defect reducing bronchodilation in response to respiratory viruses in humans with asthma. The result could be more bronchoconstriction to equivalent stimuli during the respiratory virus infection, which presents as the airway hyperreactivity characteristic of the asthmatic condition. This might explain why humans with asthma suffer more severe obstructive respiratory symptoms, more hospitalizations, and more fatalities than healthy humans in response to respiratory virus infections. It also suggests that therapies that restore the bronchodilating effects of TLR7 signaling could be beneficial for asthma. The data also suggest that guinea pigs remain a better model of human airway physiology and pharmacology than mice.

EXPERIMENTAL DESIGN

In vivo measurements of bronchoconstriction

See Chapter II General Methods.

In vitro measurements of tracheal contraction

See Chapter II General Methods.

Guinea pig ovalbumin sensitization

Guinea pigs were sensitized by 0.5 ml intraperitoneal injections of Grade II chicken egg ovalbumin (6 mg/ml in PBS) every other day for 3 total injections. 3 weeks later guinea pigs were used for *in vivo* measurements of respiratory physiology. Some animals were administered monoclonal rat anti-mouse/human IL-5 (240 μg/kg; BD Biosciences, San Diego, CA) 5 days before *in vivo* measurements.

Lungs were removed at the end of the experiment, fixed overnight at 4°C in zinc-buffered formalin, and embedded in paraffin for transverse sectioning. Sections were dewaxed and stained for 60 seconds with Chromotrope 2R (Sigma Aldrich, St Louis, MO) to identify eosinophils in the tissue. Sections were rinsed in tap water and then counterstained for 30 seconds with methyl green (Vector Laboratories, Burlingame, CA) to identify nuclei of cells in the tissue for orientation. 5 airways per animal were photographed by a digital camera attached to a Nikon upright microscope, and eosinophils in the epithelium and in the smooth muscle were

counted and normalized to the area of the airway epithelium and smooth muscle using Metamorph imaging software.

Guinea pig Sendai virus infection

Guinea pigs were anaesthetized with ketamine (30mg/kg intramuscular; JHP Pharmaceuticals LLC, Rochester MI) and xylazine (5 mg/kg intramuscular; Vedco Inc., St Joseph, MO) and infected by intranasal administration of 1 x 10^6 $TCID_{50}$ units (determined from dosage at which 50% of rhesus monkey kidney cells are infected by Sendai virus) in 250 μL PBS. 4 days later guinea pigs were used for *in vivo* measurements of respiratory physiology. Lungs were removed at the end of the experiment and virus titer was measured by PCR to confirm infection.

Mouse ovalbumin sensitization and challenge

Mice were sensitized by a single intraperitoneal injection of 100 μL. 3 weeks later the mice were anaesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) and challenged by intratracheal administration of 100 μL of 2% ovalbumin (chicken egg ovalbumin (Grade II, Sigma Aldrich, St. Louis, MO) dissolved in PBS and emulsified in aluminum hydroxide/magnesium hydroxide (Pierce, Rockford, IL)). The challenge was repeated 3 times on alternating days. 24 hours later mice were used in *in vitro* experiments.

Mouse Sendai virus infection

Mice were anaesthetized with ketamine (100mg/kg) and xylazine (5mg/kg) and infected intranasally with Sendai virus (2.8×10^4 $TCID_{50}$ units). 4 days later mice were used in *in vitro* experiments. Lungs were removed at the end of the experiment and virus titer was measured by PCR to confirm infection.

Eosinophil isolation

Human eosinophils were isolated from human blood using a modified protocol from a commercially available kit (MACS Eosinophil Isolation Kit, Miltenyi Biotec, Auburn, CA). 180 ml of blood was collected in a syringe containing 0.5 M EDTA to prevent clotting and stored on ice. The blood was then diluted 1:1 with ice cold PBS. 20 ml of blood was layered over 20 ml cold sterile Ficoll in a 50 ml conical vial, and centrifuged in a tabletop centrifuge (600 x g for 30 minutes). This centrifugation separates the granulocytes and red blood cells from the mononuclear cells. Red blood cells were then hyptonically lysed in water. The cells were centrifuged again (300 x g for 30 minutes) and supernatant removed by aspiration. The cells were resuspended in dilution buffer (PBS, 2.5% BSA, 2mM EDTA; 40 μL per 10^7 cells) and incubated with a biotin-conjugated antibody cocktail for 10 minutes on ice. This antibody cocktail is specific for markers on non-eosinophil cells, including neutrophils, T cells, B cells, NK cells, dendritic cells, and monocytes. After washing, the cells were incubated with a secondary antibody against biotin conjugated to magnetic microbeads for 15 minutes. The cells were washed and resuspended in

dilution buffer (500 μL per 10^8 cells). The suspension was run over a column filled with magnetic beads and held in a magnetic block, so that antibody labeled cells were retained in the column and unlabeled cells flowed through. The column was rinsed three times, and the eosinophil purity and viability was assessed by differential staining and Trypan blue exclusion of a sample of the effluent.

Eosinophil immunostaining

Eosinophils were treated overnight with or without interferon-γ (300 U/ml). The following day, eosinophils were cytofuged onto a glass slide (10K to 30K cells) and fixed in paraformaldehyde (4% for 10 minutes). Slides were washed 3 times for 5 minutes in 0.5% Triton-PBS for permeabilization, or just PBS for nonpermeabilized cells. The slides were then incubated in 0.1% trypsin for 10 minutes at 37°C for antigen unmasking. Hydrophobic barriers were drawn around cells. Slides were blocked in 10% normal goat serum and 10% fetal bovine serum for 60 minutes at 37°C in a humid chamber. Slides were incubated with rabbit anti-TLR7 primary antibody (0.125 μg/ml; Invitrogen, Carlsbad, CA) overnight at 4°C in a humid chamber. The following day slides were rinsed 5 times in PBS, then incubated with a fluorescent secondary goat anti-rabbit IgG antibody (1:1000; Alexafluor488; Invitrogen, Calrsbad, CA) at 37°C for 60 minutes in a humid chamber. Slides were washed 5 times in PBS and mounted with 4', 6-diamidino-2-phenylindole (DAPI) softset media (Vector Laboratories, Burlingame, CA) to label the nuclei. Images were captured with a digital camera on a Nikon upright microscope, and analyzed with Metamorph imaging software.

Transgenic mice

See Chapter II General Methods.

RESULTS

R837 is less potent in allergen-sensitized guinea pigs *in vivo* due to the absence of a high-potency relaxant phase of the R837 dose-response.

Allergies are a common trigger of asthma, and ovalbumin sensitization of guinea pigs is a commonly used model of allergic asthma. To address whether allergy changes the acute bronchodilatory response to TLR7 agonists to explain the association of respiratory viruses with asthma, I measured the effect of R837 on bronchoconstriction in ovalbumin-sensitized guinea pigs *in vivo.* Because allergen-sensitization and challenge is associated with airway hyperreactivity mediated by M2 muscarinic receptor dysfunction on the prejunctional parasympathetic nerves (Fryer and Wills-Karp, 1991), bronchoconstriction was induced by electrical stimulation of the vagus nerve (10 V, 10 Hz, 0.2 msec pulse duration, 5 sec on, 60 sec off). This is opposed to direct stimulation of M3 muscarinic receptors on the smooth muscle by the exogenous administration of acetylcholine. R837 rapidly and dose-dependently abolished bronchoconstriction in both control nonsensitized and ovalbumin-sensitized guinea pigs *in vivo*, however the bronchodilatory effect was significantly less potent in ovalbumin-sensitized guinea pigs (Figure 5.1A). Further analysis by curve fitting reveals a shallow Hill slope for the dose-response to R837 in control nonsensitized compared to that in sensitized guinea pigs (Figure 5.1B left; Nonsensitized Hill Slope=-0.52, Sensitized Hill Slope=-0.99), indicating a multiphasic fit (Figure 5.1B right). A comparison of monophasic and biphasic fits by hypothesis testing with a monophasic fit as the null hypothesis (Figure 5.1B bottom) concludes

that a biphasic fit is preferred for the R837 dose-response in nonsensitized controls and a monophasic fit for the R837 dose-response in ovalbumin-sensitized guinea pigs. There is a high potency phase in control animals (IC_{50}=0.005 mg/kg) that is lost in the sensitized animals. The lower potency phase in control animals (IC_{50}=0.48 mg/kg) is approximately equivalent to the potency of the monophasic dose-response in sensitized animals (IC_{50}=0.41 mg/kg), and 100-fold less potent than the high potency phase.

Isolated human eosinophils express TLR7, and expression is potentiated by pretreatment with interferon-γ.

Among other symptoms, ovalbumin sensitization of guinea pigs results in eosinophil recruitment to the lungs . Activation of these eosinophils mediates airway hyperreactivity induced by allergen challenge, as well as virus infection of allergen sensitized animals (Fryer and Wills-Karp, 1991; Adamko et al., 1999). To address whether eosinophils could be activated by TLR7 ligands, I first stained isolated human eosinophils to determine if they expressed TLR7. By immunofluorescence, I found that isolated human eosinophils do express TLR7 (Figure 5.2A). The staining was more pronounced in permeabilized (Figure 5.2A left) compared to nonpermeabilized (Figure 5.2A right) eosinophils, possibly suggesting a predominantly intracellular localization. Because interferon-γ, a T_h1 cytokine, can upregulate TLR7 expression in some systems (Mohty et al., 2003; Nagase et al., 2003), I tested the effect of overnight pretreatment with interferon-γ (300 U/ml), on TLR7 expression in human eosinophils. Pretreatment with interferon-γ resulted in

an increase of TLR7 staining (Figure 5.2A bottom). This could be due to an increase in TLR7 expression, or a potential redistribution of TLR7 localization to the cell surface, as nonpermeabilized eosinophils have much higher staining with interferon-γ pretreatment (Figure 5.2A bottom right). A 55-fold increase in TLR7 mRNA expression in interferon-γ pretreated eosinophils was also detected by RT-PCR (Figure 5.2B). The increase in TLR7 expression following interferon-γ pretreatment corresponded functionally, with increased eosinophils activation in response to virus and synthetic TLR7 agonists.

Depletion of eosinophils with an antibody to IL-5 does not rescue the potency of R837-mediated bronchodilation in allergen-sensitized guinea pigs *in vivo*.

To address whether it is the eosinophils in the airways of ovalbumin-sensitized guinea pigs that cause the reduced bronchodilatory potency of R837, I tested the effect of R837 in allergen-sensitized animals *in vivo* in which the eosinophils have been depleted. To deplete eosinophils, I treated ovalbumin-sensitized animals with a monoclonal antibody to IL-5, a cytokine for eosinophil maturation and survival. Eosinophil depletion did not rescue the potency of R837 to control levels (Figure 5.3A), suggesting that eosinophils do not mediate the reduced bronchodilator potency of R837 in allergen-sensitized guinea pigs *in vivo.* To confirm that the eosinophils were depleted by the monoclonal antibody to IL-5, eosinophils around medium sized airways were counted in sections of guinea pig lungs. Though not statistically significant, there appears to be a reduction in eosinophil number around the airways of sensitized animals treated with the antibody to IL-5 (Figure

5.3B). This is not a complete depletion of eosinophils, however, so eosinophil activation could still be an explanation for the reduced potency of R837.

There is no difference in the relaxant potency of R837 in mouse tracheas *in vitro* from allergen sensitized, allergen sensitized and challenged, and transgenic models with overexpression of IL-5 or eosinophils in the lungs.

Because of the availability of TLR7-deficient mice, the mouse still remains an intriguing model for the possibility of complete abrogation of TLR7 function in a non-pharmacological manner, particularly in disease states in which TLR7 function might be changed. I described a reduction of TLR7-mediated bronchodilation in allergen-sensitized guinea pigs, and I wanted to determine whether this effect could be replicated in mouse to determine if the mouse model remains viable for detecting changes or deficiencies in a TLR7-mediated bronchodilatory response. There was no difference in the relaxant potency of R837 in tracheas from ovalbumin-sensitized and control nonsensitized mice (Figure 5.4A). As in the guinea pig, these mice were allergen sensitized, but not challenged. To determine if the mouse needs an additional hit from an allergen challenge following sensitization to detect changes in the relaxant potency of R837, I also tested the effect of R837 in allergen sensitized and challenged mouse tracheas *in vitro*, but again saw no difference from control nonsensitized mice (Figure 5.4A). Thus, unlike guinea pig, the allergen-sensitized mouse is not a suitable model for measuring changes in the relaxant ability of TLR7 signaling. Another possibility is that differences were not observed, because these

mouse experiments were done in an *in vitro* organ bath setup, while the guinea pig experiments were done *in vivo*.

Another mouse model of allergic or eosinophilic asthma that is available is a transgenic mouse model. In this model, NJ1726 mice express IL-5 under the control of the airway epithelium specific Clara cell promoter for increased numbers of eosinophils in the lungs (Lee et al., 1997). There is no need for allergen sensitization or challenge to recruit these eosinophils, as IL-5 is constitutively elevated in the lungs of these animals. If eosinophils play any role in changing the relaxant response to TLR7 agonists, then this model allows me to detect those changes while bypassing any problems with allergen sensitization and challenge in the mouse. There was no difference in the relaxant potency of R837 in tracheas from NJ1726 mice compared to control wildtype mice *in vitro* (Figure 5.4B). The PHIL strain of mice express the diptheria toxin A chain under the control of the eosinophil specific promoter for eosinophil peroxidase to kill all eosinophils. Perhaps the effects of eosinophils are already saturated between wildtype and NJ1726 mice, but the PHIL mice allow me to detect any changes in the relaxant potency of R837 when eosinophils are completely absent, if there is a contribution from eosinophils in wildtype control or NJ1726 mice. There was no difference in the relaxant potency of R837 in tracheas from PHIL mice compared to control wildtype mice *in vitro* (Figure 5.4B). I also tested the effect of R837 in tracheas from NJ1726/PHIL mice that have overexpression of IL-5 in the lungs but no eosinophils, in case IL-5 itself causes changes in the relaxant potency of R837. As expected from the single transgenic NJ1726 or PHIL mice, there was no difference in the relaxant potency of R837 in

tracheas from NJ1726/PHIL mice compared to control wildtype mice *in vitro* (Figure 5.4B). The data suggest that transgenic mouse models of asthma are not sufficient to detect changes in the relaxant potency of R837 *in vitro*. Again, another possibility is the failure of the *in vitro* system to detect the changes that were detected in the guinea pig *in vivo.*

The relaxant potency of R837 is reduced in Sendai virus-infected guinea pigs *in vivo*, but not Sendai virus-infected mouse tracheas *in vitro*.

Respiratory virus infections are associated with asthma attacks, regardless of the atopic status of the patient. To determine whether virus infection changes the bronchodilatory potency of TLR7 signaling, I measured the effect of R837 on bronchoconstriction *in vivo* 4 days after Sendai virus infection of guinea pigs. Virus-infection was confirmed by real time PCR on lung tissue after physiological measurements were taken (data not shown). The bronchodilatory potency of R837 was reduced in Sendai virus-infected guinea pigs compared to control uninfected guinea pigs (Figure 5.5A). The reduced potency was very close to the reduced potency observed upon allergen sensitization, perhaps also due to the loss of a high potency phase of a biphasic dose response. The data suggest that inflammation induced by virus infection, similar to allergic inflammation, changes the ability of TLR7 agonists to relax guinea pig airways. Another possibility is that the Sendai virus ssRNA genome is occupying TLR7 receptors, so the starting bronchoconstriction is already partially bronchodilated by TLR7 occupancy, the hypothesized endogenous protective mechanism against these respiratory virus

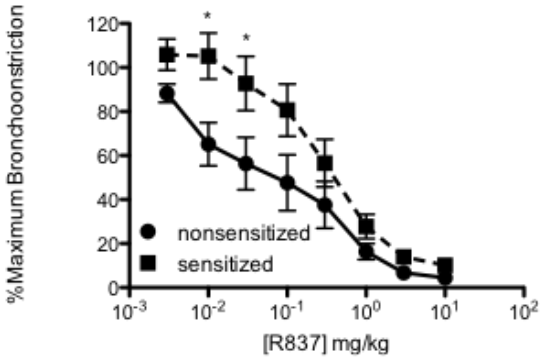
infections. Low doses of R837 will occupy only the fraction of receptors dictated by the inherent affinity of R837, but if these are already occupied by virus ssRNA, then no further bronchodilation will be observed. Further bronchodilation will only be observed at higher concentrations of R837, as R837 begins to occupy more receptors left available. Consistent with this hypothesis, the average baseline bronchoconstriction induced by electrical stimulation of the vagus nerve (10 V, 10 Hz, 0.2 msec pulse duration, 5 sec on, 60 sec off) was 97 mm H_2O for control guinea pigs compared to 53 mm H_2O for virus-infected guinea pigs, suggesting that the virus-infected guinea pigs start out partially bronchodilated by the virus.

Though neither allergic inflammation nor transgenic models of eosinophil presence and absence changed the relaxant potency of R837 in mouse tracheas *in vitro*, I wanted to determine whether differences could be detected upon respiratory virus infection in the mouse. I measured the relaxant effect of R837 *in vitro* in tracheas from uninfected control mice and mice 4 days after Sendai virus infection. Virus infection was confirmed by real time PCR on lung tissue (data not shown). I observed no differences in the relaxant potency of R837 in tracheas from uninfected control and Sendai virus-infected mice (Figure 5.5B). This suggests that the mouse airway is not a good model for virus-induced changes in rapid TLR7 signaling. The expression of TLR7 was measured in these mice by quantitative real time PCR, and I observed a 5-fold increase in TLR7 mRNA in Sendai-virus infected mice (Figure 5.4C). Another explanation is that any changes in TLR7 signaling are overcome by the increased TLR7 expression, so that overall no differences are detected. Yet

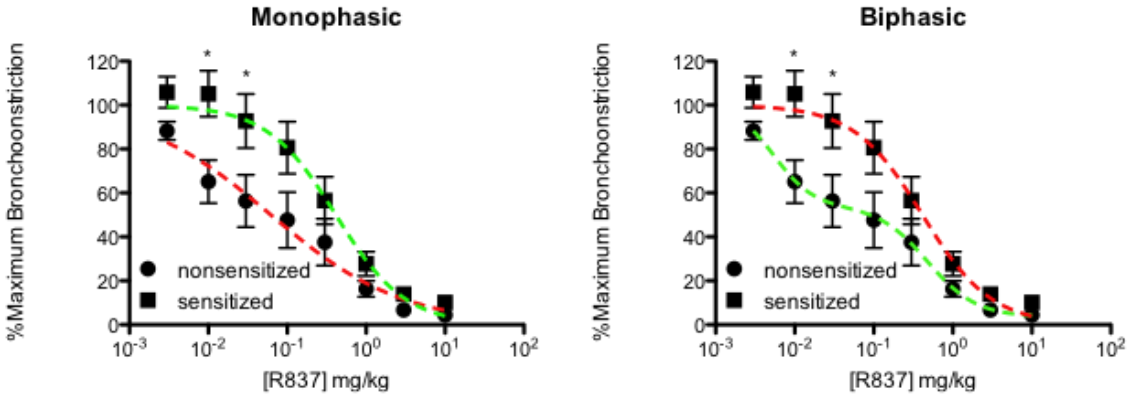
another possibility is that changes observed in the guinea pig *in vivo* are not detectable in the *in vitro* organ bath experimental system.

FIGURE 5.1. R837 is less potent in allergen-sensitized guinea pigs *in vivo* due to the absence of a high-potency relaxant phase of the R837 dose-response.

A



B



	nonsensitized	sensitized
HillSlope	-0.52	-0.99
IC50 (mg/kg)	0.06	0.41

	nonsensitized	sensitized
IC50 phase 1	0.005	0.03
IC50 phase 2	0.48	0.40

Comparison of Fits	Nonsensitized	Sensitized
Null hypothesis	Monophasic	Monophasic
Alternative hypothesis	Biphasic	Biphasic
P value	0.0066	
Conclusion (alpha = 0.05)	Reject null hypothesis	
Preferred model	Biphasic	Monophasic

Figure 5.1 (A) Bronchoconstrictions were induced by electrical stimulation of the vagus nerves in nonsensitized control (circle) and ovalbumin-sensitized (square) guinea pigs *in vivo*, and the effect of cumulative increasing doses of R837 administered i.v. was measured. Data are expressed as the mean ± SEM. (n=5 for vagal stimulation, maximum nonsensitized=93.0±21.5 mm H_2O, maximum sensitized=68.5±11.8 mm H_2O; nonsensitized vs. sensitized $p \leq 0.05$ at 0.01 and 0.03 mg/kg). (B) Monophasic (left) and biphasic (right) nonlinear regression analysis were used to fit the R837 dose-response curves. The Hill slope and monophasic IC50 is shown below the graph on the left, while the IC50 for each phase of the biphasic fit is shown below the graph on the right. A comparison of fits is reported, and the preferred (green) and rejected (red) model is indicated on the graph.

FIGURE 5.2. Isolated human eosinophils express TLR7, and expression is potentiated by pretreatment with interferon-γ.

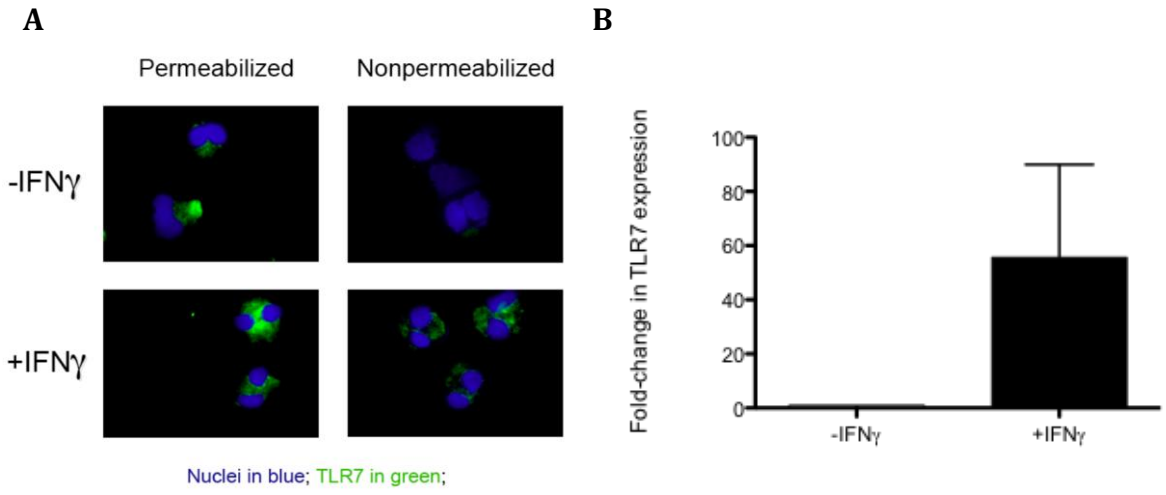


Figure 5.2 (A) Permeabilized or nonpermeabilized isolated human eosinophils were pretreated overnight with vehicle or interferon-γ, stained for TLR7 (green), and mounted in DAPI containing media to label nuclei (blue). (B) TLR7 mRNA expression in human eosinophils pretreated overnight with vehicle or interferon-γ was measured by quantitative real time PCR and normalized to expression in vehicle treated eosinophils (*n*=3).

FIGURE 5.3. Depletion of eosinophils with an antibody to IL-5 does not rescue the potency of R837-mediated bronchodilation in allergen-sensitized guinea pigs *in vivo*.

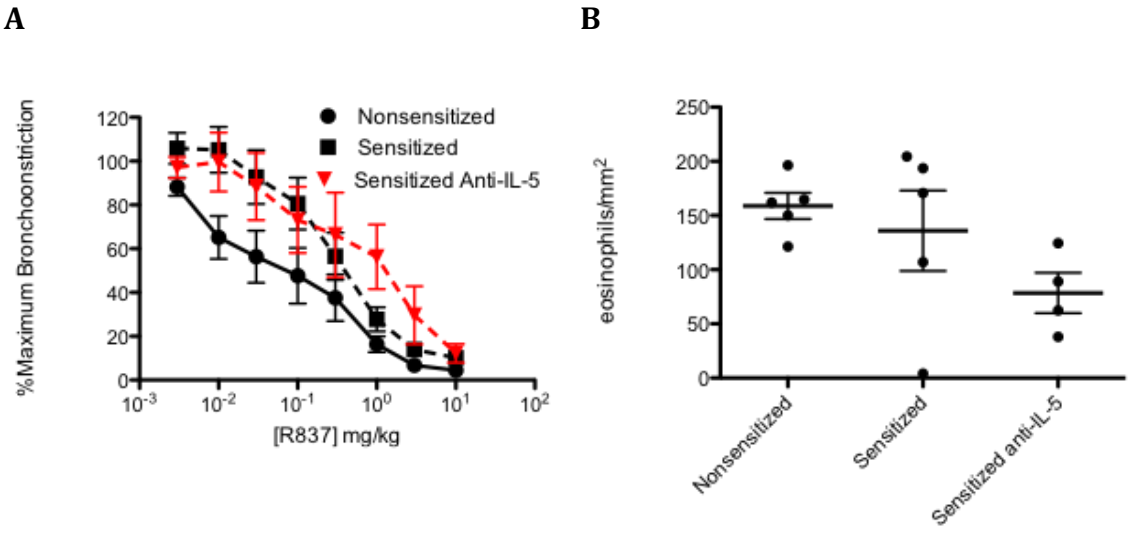


Figure 5.3 (A) Bronchoconstrictions were induced by electrical stimulation of the vagus nerves in nonsensitized control (circles), ovalbumin-sensitized (squares), and ovalbumin-sensitized with anti-IL-5 treatment (red triangles) guinea pigs *in vivo*, and the effect of cumulative increasing doses of R837 administered i.v. was measured. Data are expressed as the mean ± SEM. Data previously shown in Figure 5.1 are redisplayed in black with new data in red. (*n*=5; maximum nonsensitized=93.0±21.5 mm H_2O, maximum sensitized=68.5±11.8 mm H_2O, maximum Anti-IL-5=94.0±55.9 mm H_2O). (B) Average number of eosinophils per area of 4 individual airways per section of guinea pig lung (*n*=5).

FIGURE 5.4. There is no difference in the relaxant potency of R837 in mouse tracheas *in vitro* from allergen sensitized, allergen sensitized and challenged, and transgenic models with overexpression of IL-5 or eosinophils in the lungs.

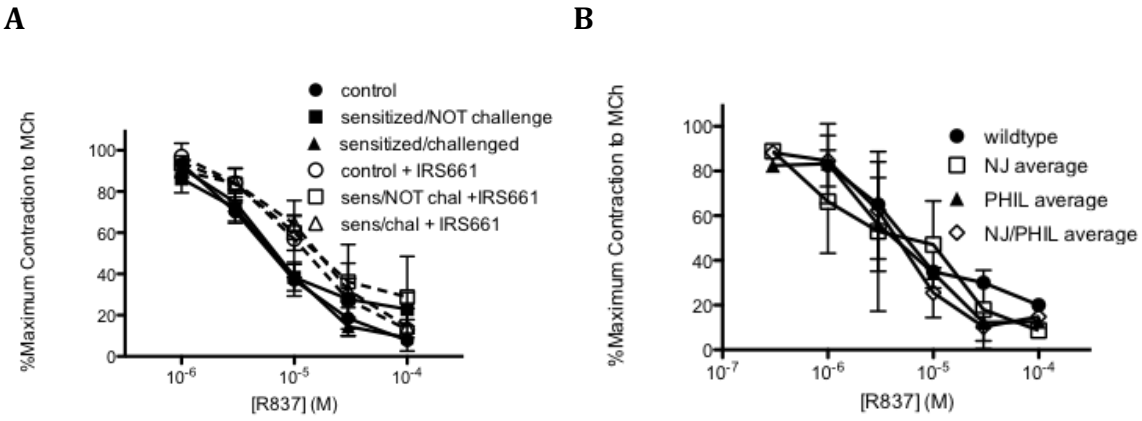


Figure 5.4 (A) Trachea segments were preincubated with vehicle (solid lines) or IRS661 (dashed lines). Contraction was induced by methacholine and the effect of cumulative increasing doses of R837 was measured in tracheas from nonsensitized control (circles), ovalbumin-sensitized but not challenged (squares), and ovalbumin-sensitized and challenged (triangles) mice. Data are expressed as the mean ± SEM. (nonsensitized control *n*=3, sensitized *n*=2, sensitized and challenged *n*=6; maximum nonsensitized control=0.32±0.06 g, maximum sensitized=0.33±0.03 g, maximum sensitized and challenged=0.28±0.06 g). (B) Contraction was induced by methacholine and the effect of cumulative increasing doses of R837 was measured in tracheas from wildtype (circles), NJ1726 (squares), PHIL (triangles), and NJ1726/PHIL (diamonds) mice. Data are expressed as the mean ± SEM. (wildtype *n*=1, NJ1726 *n*=2, PHIL *n*=2, NJ1726/PHIL *n*=2).

FIGURE 5.5. The relaxant potency of R837 is reduced in Sendai virus-infected guinea pigs *in vivo*, but not Sendai virus-infected mouse tracheas *in vitro*.

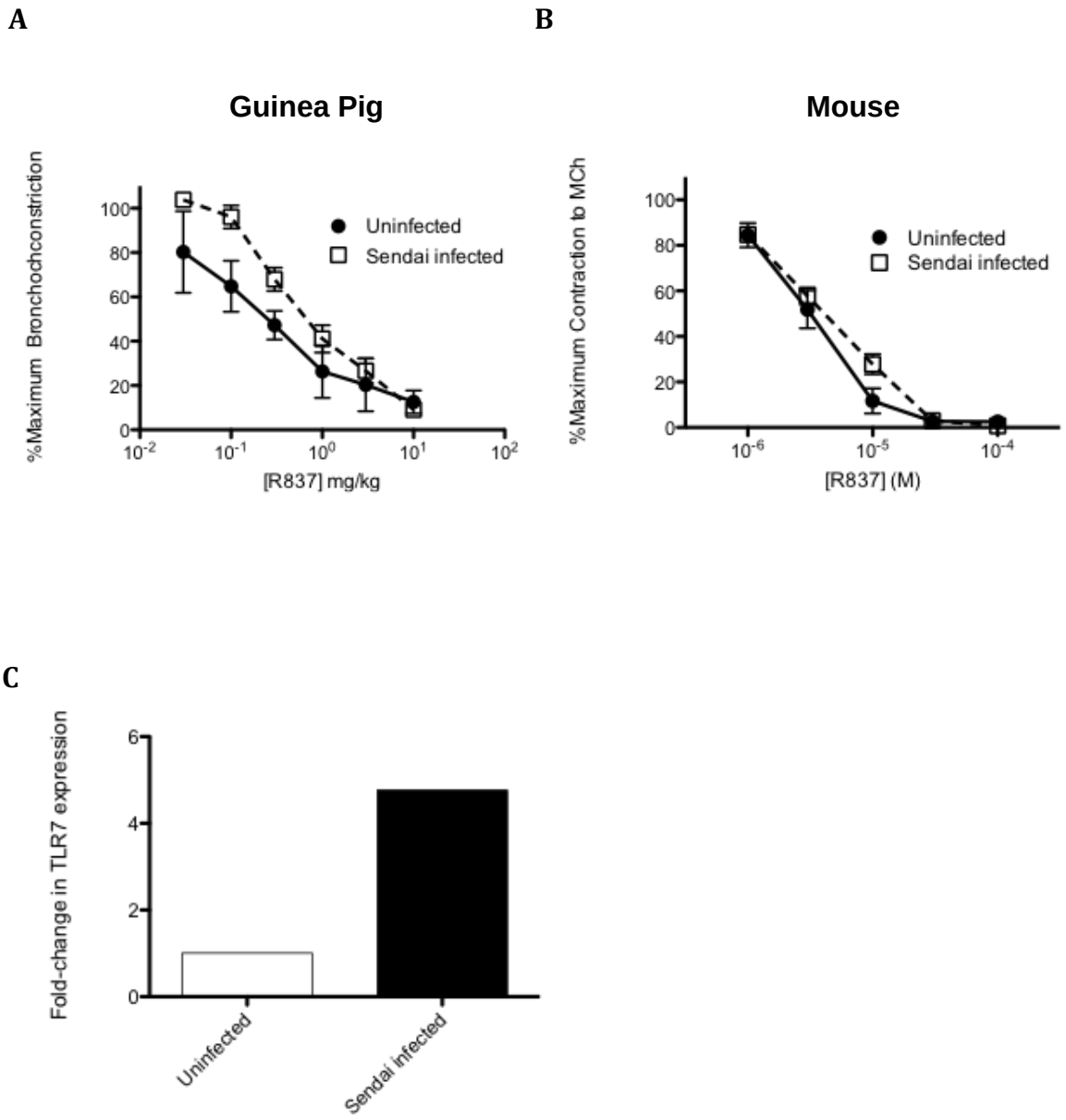


Figure 5.5 (A) Bronchoconstrictions were induced by electrical stimulation of the vagus nerves in uninfected control (circles) and Sendai virus-infected (squares)

guinea pigs *in vivo*, and the effect of cumulative increasing doses of R837 administered i.v. was measured. Data are expressed as the mean ± SEM. (uninfected control n=2, Sendai virus-infected n=3; maximum uninfected control=97±36 mm H_2O, maximum Sendai virus-infected=53±10 mm H_2O; effect of Sendai virus infection on R837-dose response $p \leq 0.05$). (B) Contraction was induced by methacholine and the effect of cumulative increasing doses of R837 was measured in tracheas from uninfected controls (circles) and Sendai virus-infected (squares) mice. Data are expressed as the mean ± SEM. (n=4; maximum uninfected control=0.42±0.07 g, maximum virus-infected=0.36±0.09 g). (C) TLR7 mRNA expression was measured by quantitative real time PCR and normalized to expression in uninfected control mice (n=5).

DISCUSSION

In the previous 2 chapters I described my initial observation of a bronchodilatory effect of TLR7 agonists *in vivo* and *in vitro.* The bronchodilatory effect is conserved across three mammalian species, suggesting an important mechanism that has been maintained with evolution. The reason for its importance might be to relax and open the airways to maintain breathing during a respiratory virus infection, the clearance of which involves inflammation that is obstructive and constrictive to the airways.

TLR7 was initially selected as a target that might affect airway physiology, because of the close association of respiratory ssRNA viruses with asthma attacks, and because TLR7 polymorphisms are associated with asthma. I was expecting to measure differences in TLR7 signaling in animal models of asthma to account for the more severe symptoms of respiratory virus infections in humans with asthma. The initial bronchodilatory observations were made in healthy animals, but were unexpected. The question remains why are the symptoms of respiratory virus infections more severe in humans with asthma?

One possibility is that described TLR7 polymorphisms associated with asthma result in a defective bronchodilator response in the presence of respiratory viruses. This could manifest as more bronchoconstriction to equivalent stimuli during the virus infection, the definition of airway hyperreactivity. In the absence of TLR7 polymorphisms, perhaps some other aspect of the asthmatic condition affects TLR7 signaling.

As allergy is also associated with asthma, the allergic immune environment of the lung could also contribute to a defective TLR7 bronchodilator response. The allergic immune environment in the lung is characterized by T_h2 cytokines like IL-4, IL-5, and IL-13. TLR7 signaling promotes a T_h1 immune environment characterized by cytokines like IL-10 and interferon-γ. T_h1 cytokines like interferon-γ have been shown to promote TLR7 signaling. Here, I show corroborating data that interferon-γ potentiates TLR7 expression and function in isolated human eosinophils. In the absence of T_h1 cytokines, as in allergic airways, TLR7 expression and bronchodilating function might be decreased, accounting for the more severe effects of respiratory virus infection.

To address whether there is dysfunction in the TLR7-mediated bronchodilating mechanism in a model of allergic asthma, I tested the effect of R837 on bronchoconstriction in ovalbumin-sensitized guinea pigs *in vivo*. I found in these animals that the bronchodilating potency of R837 is significantly reduced. By curve-fitting analysis, it seems that the loss of potency is due specifically to the loss of a high-potency bronchodilating phase of the dose-response that is present in control nonsensitized animals, leaving a lower potency bronchodilating phase intact. These results confirm that allergic inflammation reduces the protective effect of TLR7 signaling in the airways, possibly explaining the more severe symptoms of respiratory virus infections in humans with asthma.

A hallmark of lung inflammation is the influx of immune cells. Multiple immune cells are known to express TLR7 (Nagase et al., 2003; Kulka et al., 2004; Demedts et al.,

2006), and perhaps TLR7 signaling in these cells can modulate the bronchodilating effects of TLR7 signaling in the airways. Here, I show that isolated human eosinophils express TLR7 and are activated in response to TLR7 agonists, both virus ssRNA and synthetic. Eosinophils have long been associated with asthma. The activation of eosinophils around airway parasympathetic nerves results in the release of major basic protein, an allosteric M2 muscarinic antagonist (Jacoby et al., 1993). Prejunctional inhibitory M2 muscarinic receptors on parasympathetic nerve terminals limit acetylcholine release onto smooth muscle, limiting bronchoconstriction under healthy conditions (Figure 1.4A). The functional consequence of major basic protein release from activated eosinophils is that blockade of prejunctional inhibitory M2 muscarinic receptors results in more acetylcholine release from the nerve terminals and more bronchoconstriction mediated by M3 muscarinic receptors on the airway smooth muscle (Figure 1.4B). This airway hyperreactivity can be reversed by depleting eosinophils with an antibody to IL-5 or with a neutralizing antibody against major basic protein.

In allergen-sensitized guinea pigs, eosinophil activation can be accomplished by allergen challenge or by respiratory virus infection (Fryer and Wills-Karp, 1991; Adamko et al., 1999), both resulting in airway hyperreactivity. As I show here that eosinophils can be activated by respiratory viruses or R837, perhaps TLR7 mediates this eosinophil activation and airway hyperreactivity in allergen sensitized guinea pigs. Even if there is a bronchodilating effect of TLR7 signaling in the airways as I have described in healthy animals, an opposing bronchoconstrictive effect from

TLR7-mediated eosinophil activation would limit the overall bronchodilating effect (Figure 5.6). In this way, eosinophils or other TLR7-expressing immune cells might limit the bronchodilating protective mechanism of TLR7 signaling during allergic inflammation.

To address whether eosinophils mediate the dysfunction in the TLR7-mediated bronchodilating mechanism in allergen-sensitized guinea pigs *in vivo*, I attempted to deplete eosinophils in allergen-sensitized guinea pigs using a monoclonal antibody to IL-5, a cytokine for eosinophil maturation and survival. Complete eosinophil depletion was not accomplished, but I did observe a statistically insignificant reduction in eosinophils around airways in guinea pig lung sections. Treatment with the antibody against IL-5 did not rescue the bronchodilating potency of R837 *in vivo*, This might suggest that eosinophils do not mediate the reduced potency of R837, but the data are inconclusive as complete depletion of eosinophils was not accomplished. Furthermore, the changes in potency could be due to IL-5 insensitive eosinophils. There are many changes from allergic inflammation, beyond influx of eosinophils, and other immune cell types as well as tissue remodeling can not be ruled out either. It has been shown that ovalbumin sensitization and challenge of guinea pigs results in decreased nitric oxide synthase activity in tracheal smooth muscle (Samb et al., 2001). As I have determined that nitric oxide synthase mediates some of the relaxant effects of TLR7 agonists *in vitro*, perhaps a reduction in nitric oxide production results in the decreased sensitivity to R837 in ovalbumin sensitized guinea pigs *in vivo*. This is one of many possible changes that could

explain changes in the relaxant potency of R837 upon allergen sensitization.

Figure 5.6. Hypothesized effect of TLR7-mediated bronchodilation in the presence of TLR7-mediated eosinophil activation by the agonist R837.

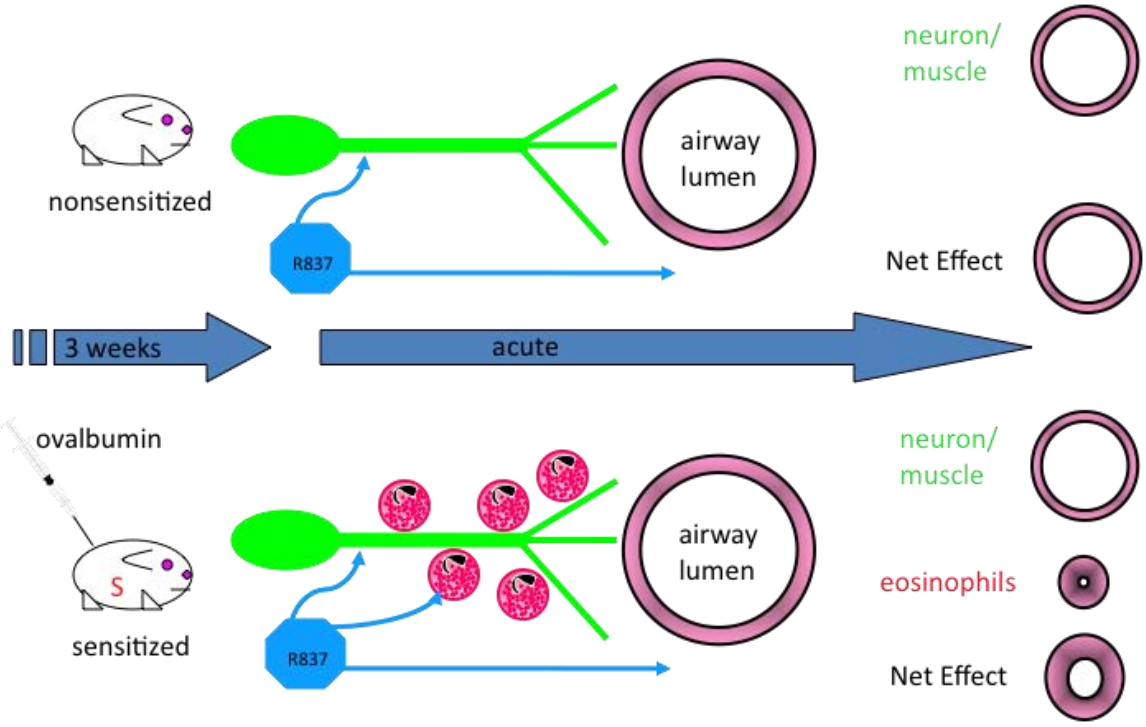


Figure5.6. This schematic shows the activation of bronchodilatory mechanisms in nonsensitized guinea pigs (top) and the activation of both bronchodilatory mechanisms and bronchoconstrictive eosinophil-mediated mechanisms in ovalbumin-sensitized guinea pigs (bottom). This is a possible explanation for the reduced relaxant potency of R837 in ovalbumin-sensitized guinea pigs *in vivo.*

CHAPTER VI.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Exacerbations of asthma, a disease characterized by airway inflammation and hyperreactivity, are associated with respiratory virus infections (Nicholson et al., 1993; Johnston et al., 1995). While healthy individuals and patients with asthma are susceptible to the same respiratory virus infections, the symptoms are more severe and last longer in humans with asthma. As respiratory viruses induce an inflammatory response to clear the infection and protect the host, perhaps it is this inflammatory response that is defective in humans with asthma. Indeed, asthma and allergy have been associated with a defective adaptive immune response described by the predominance of helper T lymphocytes of the T_h2 subtype, which produce cytokines such as IL4, IL-5, and IL-13, and results in IgE antibody class switching, mucus hypersecretion, airway remodeling, and airway hyperreactivity (Anderson, 2002; Larche et al., 2003; Bradding et al., 2006; Holgate, 2008). This immune response is not purely pathological, and is appropriate for the clearance of extracellular parasitic infections, such as helminth worm infections (Kariyawasam and Robinson, 2006; Paul and Zhu, 2010). For the clearance of respiratory virus infections, however, the healthy human airways mount an adaptive immune response characterized by helper T lymphocytes of the T_h1 subtype, which produce cytokines such as IL-10 and interferon-γ, effectively clearing the virus infection while limiting the deleterious effects of the immune system on the airway environment (Papadopoulos et al., 2007). While a misdirected T_h2 adaptive immune response is associated with asthma, the adaptive immune response is determined

by the initial recognition of the respiratory virus by the innate immune system. It might be the innate recognition of the virus that is defective, resulting in the mounting of an inappropriate adaptive immune response. Viruses are recognized by receptors of the innate immune system that recognize conserved molecular patterns characteristic of viruses, particularly their genomes. Most respiratory viruses have a ssRNA genome that can be recognized by TLR7 and TLR8 (Diebold, 2008). TLR7 and TLR8 are expressed at the highest levels in the lung, and polymorphisms in these receptors are associated with asthma (Roponen et al.; Chuang and Ulevitch, 2000; Nishimura and Naito, 2005; Moller-Larsen et al., 2008). It could be inferred, therefore, that a defective innate immune response to respiratory viruses mediated by TLR7 or TLR8 could result in an inappropriate immune environment that is inefficient at clearing virus infections and is characterized by the inflammation associated with asthma. Drugs that can skew the lung immune environment towards T_h1 away from T_h2 have been proposed as a prophylactic therapy for asthma. In particular, TLR7 agonists have been proposed for this role, as their recognition mounts an antiviral immune response, which itself skews the environment away from T_h2 and towards T_h1 inflammation (Stokes et al., 1998; Quarcoo et al., 2004; Napolitani et al., 2005; Moisan et al., 2006; Camateros et al., 2007; Sel et al., 2007; Xirakia et al., 2010).

Eosinophils, immune effector cells associated with T_h2-type immunity and asthma, can be activated in the lungs of humans with asthma to mediate airway hyperreactivity characteristic of asthma (Costello et al., 1997). These cells can be

activated by allergen exposure, pollutants, and respiratory viruses (Evans et al., 1997; Adamko et al., 1999; Yost et al., 2005; Proskocil et al., 2008). Eosinophils express TLR7 that can mediate eosinophil activation in response to both virus ssRNA and synthetic TLR7 agonists (Nagase et al., 2003; Mansson and Cardell, 2009). The early goal of my research was to reproduce the airway hyperreactivity in response to a respiratory virus infection by activating eosinophils in allergen sensitized guinea pigs using a synthetic TLR7 agonist. Upon administration of the TLR7 agonist R837 *in vivo,* I instead found the drug rapidly and completely abolished bronchoconstriction within minutes of administration. Not only could I not replicate the airway hyperreactivity characterized by increased bronchoconstriction, but I found a bronchodilatory role for this drug. Furthermore, this bronchodilation occurred in minutes, which is inconsistent with the timeframe of transcriptional regulation associated with canonical TLR signaling. This rapid bronchodilation is the primary characteristic of a rescue therapy for the ongoing bronchoconstriction of an asthma attack. Combined with the T_h1-skewing potential of TLR7 agonists, this single drug holds the unique potential for both chronic prophylactic therapy and acute rescue therapy. Currently standard asthma therapy consists of anti-inflammatory steroids for prophylaxis in combination with beta-agonists for acute bronchodilatory rescue. Both of these drugs are associated with a number of side effects. A TLR7 agonist has the potential to do both.

If the relaxant effect of R837 is indeed mediated by TLR7 itself, it suggests a mechanism that evolved to bronchodilate the airways during a respiratory virus

infection. Respiratory virus infections are associated with inflammation, which clears the infection but can also obstruct the airways and breathing. It is reasonable that the airways may have also developed a mechanism to bronchodilate in response to respiratory virus infections, to increase the diameter of the airway lumen, and maintain the passage of air and effective breathing. Defects in this bronchoprotective mechanism, perhaps associated with TLR7 polymorphisms or T_h2 cytokine-mediated downregulation of TLR7 expression or function, could explain the more severe effects of respiratory virus infections in humans with asthma. While they experience inflammation that is necessary to clear the virus infection, they may not experience the beneficial bronchodilation to maintain breathing.

Because of interest in both the therapeutic potential and a possible endogenous protective mechanism, I shifted the focus of my research to the bronchodilatory effects of TLR7 agonists. As this was a novel finding, it required extensive characterization. I was interested in determining whether TLR7 itself mediated the bronchodilating effects of the drug or whether off target effects were responsible. Because of the fast timeframe inconsistent with canonical TLR7 signaling, I wanted to determine what intracellular signaling mechanisms could mediate this effect. Because of its obvious therapeutic potential, I wanted to determine if the effect was conserved in human airway tissue. Finally, I wanted to determine whether the bronchodilating effect was defective in animal models of asthma, in part to explain

some of the more severe effects of respiratory virus infections in asthma and in part to determine if the therapeutic potential carries over to asthma.

In Chapter III, I demonstrate that the TLR7 agonist relaxed bronchoconstriction in guinea pigs *in vivo* and *in vitro*. The mechanism used to induce contraction *in vitro* gave me information about the location of action of the relaxant effects of the drug. R837 reversed contractions induced by electrical field stimulation of the nerves in the trachea as well as by administering acetylcholine directly to the bath, with identical dose responses. As electrical field stimulation induces acetylcholine release from nerve terminals, these are both cholinergic mechanisms of contraction. It seems unlikely then that R837 would act at the nerve upstream from the muscle, when R837 had an identical dose response of relaxation when stimulating the downstream muscle directly with acetylcholine. Reversal of histamine-induced contractions demonstrated that the relaxant effect of R837 was not specific to cholinergic contractions. It suggests that R837 is not disrupting muscarinic receptor function or signaling specifically. Histamine receptors, like muscarinic receptors, are G_q-coupled receptors, so the possibility existed that R837 interfered specifically with G_q-mediated calcium signaling. The reversal of KCl-induced contractions by R837, suggests that this is not the case. KCl induces contraction of airway smooth muscle by depolarizing the smooth muscle membrane and opening cell-surface voltage-gated calcium channels (Mitchell, 1987). Though, this calcium also causes contraction of the smooth muscle, it is a global increase in calcium from the extracellular environment, rather than smaller release of calcium from the

sarcoplasmic reticulum. This suggests that the effect is not specific to G_q-mediated increases in intracellular calcium, and rather acts further downstream to general mechanisms of smooth muscle contraction beyond the specific stimulus. The increased potency of R837-mediated relaxation of contractions induced by 20 mM KCl compared to 100 mM KCl, was an early indication of sensitivity of the mechanism to potassium concentration and that potassium channels might be involved in the relaxant mechanism. When I removed the epithelium from the guinea pig trachea segments, there was no difference in the relaxant potential of R837, so I was also able to rule out the epithelium as a site of action. With the nerves and epithelium ruled out, it seems most likely that the relaxant effects of R837 occur directly at the airway smooth muscle, but other immune cells and endothelial cells resident in the lungs cannot be ruled out at this time. In preliminary experiments not shown here, I measured the effect of R837 on intracellular calcium release in cultured airway smooth muscle cells and saw both inhibition of calcium transients as well as induction of calcium transients. Though the effect with regards to increasing or decreasing calcium transients is unclear, the ability to modulate calcium in airway smooth muscle cells, suggests that the relaxant mechanism of R837 may occur in these cells.

Using multiple TLR7 agonists of two structural classes, I showed that all of these agonists had a bronchodilating effect *in vitro.* While it remains possible that all of the TLR7 agonists relax airway smooth muscle through a non-TLR7 target, the fact

that this property is shared by TLR7 agonists of different structural classes supports TLR7 as the target.

An important finding in Chapter III is that TLR7 agonists could relax trachea from guinea pigs, mouse, and human. The conservation of the relaxant mechanism across all species through evolution, suggests an important mechanism that is necessary to protect the airways during respiratory virus infection.

I also showed that the bronchodilating effect of TLR7 agonists is not replicated by the TLR2 agonist, peptidoglycan, or the TLR5 agonist, flagellin. One possible interpretation is that the bronchodilator effect is specific to virus pattern recognition receptors and not bacterial pattern receptors. At first glance, this is not consistent with reports of acute relaxation of airway smooth muscle induced by lipopolysaccharide, a TLR4 ligand (Fedan et al., 1995; Balzary and Cocks, 2006). Although more appreciated for its role in detecting bacterial lipopolysaccharide, TLR4 mediates recognition of respiratory syncytial virus, a ssRNA virus highly associated with asthma (Haynes et al., 2001; Tal et al., 2004; Cyr et al., 2009). It is possible, therefore, that TLR-mediated bronchodilation is a bronchoprotective mechanism in response to respiratory viruses and not bacteria. Consistent with this interpretation, bitter taste receptors that can respond to bacterial components also induce acute bronchodilation, and therefore TLR contribution may not be necessary (Deshpande et al., 2010). Future experiments should include a comprehensive measurement of the effects of a wide range of TLR ligands, spanning all mammalian

TLRs with known ligands (TLR1 – TLR9), on airway smooth muscle contraction to determine if this effect is specific to TLRs responsible for virus detection.

An important finding in Chapter III is that TLR7 agonists relax trachea from guinea pigs, mice, and humans. Though these are all mammalian species, the conservation of the relaxant mechanism across three distantly related species suggests it is of great importance and has been maintained throughout evolution. If this represents a bronchoprotective mechanism, it makes sense that it would be conserved across species that are susceptible to respiratory virus infections.

In Chapter IV, I showed that the relaxant effects of TLR7 agonists are partially or completely reversed by a TLR7 antagonist *in vitro*, lending further support to TLR7 as the target mediating the relaxant effects of the agonists. A Schild analysis would be useful to determine whether there is competition at the same binding site on the same receptor for the TLR7 antagonist and agonists. The original compounds I tested in guinea pig tracheas *in vitro* were the imidazoquinolines, which seem to have TLR7-dependent as well as TLR7-independent effects. Because of the lack of competition at a single site, the assumptions of the Schild analysis were violated and could not be completed. The Schild analysis assumes perfect competition such that no matter what concentration of agonist is used, it should be overcome by a high enough concentration of antagonist, and no matter what antagonist concentration is used it should be overcome by a high enough concentration of agonist. The TLR7 antagonist could only reverse the TLR7-dependent effects at lower doses of the agonist, but at higher concentrations of agonist, the antagonist could no longer

reverse the effects as they are TLR7-independent, no matter how high of the concentration of the antagonist. I was able to completely reverse the relaxant effects of PolyUs *in vitro,* but this compound was available in limited quantities, due to custom synthesis, and a Schild analysis fell to lower priority amongst the other experiments I wanted to perform. It would be useful in the future to complete a Schild analysis with PolyUs to demonstrate competition with the TLR7 antagonist at the same ligand binding site and to determine if TLR7 mediates the effects. When I conducted the experiments in human trachea for translational purposes, I was surprised to find that R837 was completely reversed by the TLR7 antagonist, suggesting complete TLR7-dependence in humans. Furthermore, PolyUs, which is reported as a TLR8 agonist in humans, was not reversed at all by the TLR7 antagonist (Diebold et al., 2006). This gives support to the specificity of the TLR7 antagonist. It also suggests that the closely related TLR8 mediates the TLR7-independent relaxant effects of ligands with mixed reports of TLR7 versus TLR8 selectivity. In this case, even though there is complete reversal of the relaxant effects of R837, a Schild analysis was not carried out due to limitations in the availability of human tracheas. Again, this would be a useful experiment to complete in the future as definitive proof of the TLR7-dependence of the relaxant effects of TLR7 agonists in human tissue.

Because the fast timeframe of TLR7-mediated bronchodilation, it seems unlikely that the relaxation occurs through canonical TLR7 signaling, which involves changes in gene expression. I, therefore, conducted mechanistic studies to determine how

TLR7 could mediate fast effects on airway smooth muscle in minutes, as opposed to long-term effects of TLR7 signaling. As opposed to a fishing expedition, I narrowed my search to signaling pathways associated with some form of TLR signaling and pathways that can have rapid effects on airway smooth muscle physiology. These pathways include nitric oxide, prostaglandins, and the large-conductance calcium-gated potassium channel (MaxiK) (Fedan et al., 1995; Balzary and Cocks, 2006; Scheel et al., 2006; Hattermann et al., 2007; Tumurkhuu et al., 2009). Of these pathways it appears as though TLR7-mediated mechanisms involve nitric oxide, whereas TLR7-independent relaxant mechanisms involve prostaglandins and MaxiK. This was most clearly demonstrated in human tracheas in which R837 induced relaxation is both completely TLR7-dependent and completely nitric oxide-dependent, whereas PolyUs-induced relaxation is both completely TLR7-independent and nitric oxide-independent. This is consistent with the reported selectivity of human TLR7 for these ligands, as R837 is reported as TLR7-selective, whereas PolyUs is reported as TLR8 selective in humans (Heil et al., 2004).

Questions of ligand-selectivity make mechanistic studies in the guinea pig difficult to interpret. For example, the TLR7 antagonist almost completely blocked the relaxant effects of PolyUs, but the nitric oxide synthase inhibitor had no effect. One interpretation is that the TLR7 antagonist blocks TLR8-mediated effects in guinea pigs rather than TLR7. Therefore, the TLR7 antagonist partially blocks the relaxant effects of R837 and completely blocks the relaxant effects of PolyUs, both mediated by TLR8. This is not consistent with the absence of an additive effect in blocking

R837-mediated relaxations when using the TLR7 antagonist and the nitric oxide synthase inhibitor together.

It is tempting to speculate about heterodimerization when observing strange selectivity effects as described above. Fore example TLR2 herterodimerizes with TLR1 and TLR6 to distinguish between diacyl and triacyl bacterial lipopeptides respectively (Takeuchi et al., 2001; Takeuchi et al., 2002). Furthermore, physical interactions cross-regulating function have been shown amongst TLR7, TLR8, and TLR9 (Wang et al., 2006a). Perhaps PolyUs is recognized by a TLR7/TLR8 heterodimer that is sensitive to the TLR7 antagonist but does not signal through nitric oxide. Therefore, PolyUs should be sensitive to inhibitors of prostaglandin synthesis or MaxiK. The next experiments should measure the relaxant effects of PolyUs in combination with inhibitors of prostaglandin synthesis and MaxiK to determine if this is a common or independent pathway with a TLR7/TLR8 heterodimer. Though the guinea pig is an excellent model for human airway physiology, the lack of anecdotal evidence of ligand selectivity in the literature, let alone a binding assay, highlights one of the difficulties in using this species.

The mouse provided me with a different airway physiology from human and the availability of abrogation of TLR7 function without pharmacological intervention in a TLR7-deficient mouse strain. R837 and PolyUs induced relaxation of both the wildtype and TLR7-deficient mouse tracheas *in vitro*. The TLR7 antagonist partially reversed the relaxant effect of R837 and almost completely reversed the effect of PolyUs in the wildtype, but not the TLR7-deficient mouse tracheas. These data

indicate that relaxation induced by PolyUs is more TLR7-dependent than R837 in mouse. The near equivalent relaxation in TLR7-deficient and wildtype mice suggests that, when TLR7 is available in the mouse, it is the dominant mediator of relaxations induced by PolyUs, but in the absence of TLR7, TLR8 can mediate relaxant effects of PolyUs as a form of compensation. The reversal of R837-mediated relaxation by the nitric oxide synthase inhibitor in both the TLR7-deficient and wildtype mouse strains suggests that the TLR8-dependent relaxations are mediated by nitric oxide in mouse. The next experiments need to measure the relaxant effect of PolyUs in the presence of a nitric oxide synthase inhibitor in the wildtype mouse to determine if a ligand that is predominantly TLR7- and minimally TLR8-dependent is only minimally nitric oxide-dependent. Again, confounding data due to lack of heterodimerization in a TLR7-deficient strain make results difficult to interpret. Experiments in TLR8-deficient mice and TLR7/TLR8 double-deficient mice are needed to determine if these are the receptors mediating effects in mouse.

Having identified a bronchoprotective mechanism of relaxation in the presence of respiratory virus infection, I next wanted to determine if these pathways function in models of asthma. The purpose of this approach is two-fold. First, for therapeutic potential, the TLR7 agonists must be bronchodilators in asthmatic as well as healthy airways. Second, I wanted to determine if this bronchoprotective pathway is defective in models of asthma to explain the more severe effects of respiratory viruses in humans with asthma. In Chapter V, I used respiratory virus infection and allergen-sensitization models of asthma in both guinea pigs and mice. In mice, in

which conventional models of asthma are of limited utility, I also used a transgenic strain with eosinophils localized in the lungs.

In ovalbumin-sensitized guinea pigs that were not challenged with ovalbumin, eosinophils are recruited to the lungs. I measured the bronchodilating effect of R837 in these animals *in vivo*. R837 was still able to completely and rapidly abolish bronchoconstriction induced by electrical stimulation of the vagus nerve. This observation is important because it suggests that R837 could have therapeutic benefit even in a dysfunctional airway representative of the asthmatic condition. Compared to control nonsensitized animals, however, the bronchodilating potency of R837 was reduced. In addition, the R837 dose-response curve had a biphasic fit in control nonsensitized guinea pigs, suggesting activity at multiple sites or receptors. In allergen-sensitized guinea pigs, there is a monophasic dose response curve with a loss of the higher potency phase, and correlating with the lower potency phase of the control biphasic curve. One proposed mechanism is that TLR7-mediated eosinophil activation exerts an opposing bronchoconstrictive effect on TLR7-mediated bronchodilation. Eosinophil depletion with an antibody to IL-5 did not rescue the potency of R837 *in vivo*. The eosinophil depletion may not have been successful, as even though there are a reduced number of eosinophils in the airway tissue, there was not a complete depletion. Another possibility is that the antibody to IL-5 mediates other effects that also reduce the bronchodilating potency of R837, so that contributions from eosinophil-depletion are masked by the anti-IL-5

contributions. Yet another possibility is different subpopulations of eosinophils with differential sensitivities to the effects of IL-5.

If eosinophils are not responsible for the reduce potency of R837 in allergen-sensitized guinea pigs, other explanations are still available. Reduction in TLR7 or TLR8 expression in a T_h2 cytokine dominated allergic environment, might reduce the bronchodilating contribution of either of these receptors. Changes in any of the components of the TLR7 signaling pathway might change the potency of the response. Other possibilities include changes in calcium sensitivity, so that the same intracellular calcium levels in control and allergen-sensitized guinea pigs correspond to different amounts of airway smooth muscle contraction. Focusing on the nitric oxide-dependent component of the relaxant mechanism, dysfunction could occur at multiple points relevant to nitric oxide signaling. Upstream of nitric oxide production, loss of nitric oxide synthase, the enzyme responsible for generating nitric oxide from L-arginine, could reduce relaxant nitric oxide production (Samb et al., 2001). Instead of affecting enzyme function, reduced availability of the substrate arginine by increased arginase activity is an alternative explanation for reduced nitric oxide production (Meurs et al., 2002; Zimmermann et al., 2003). Downstream of nitric oxide production, nitric oxide activates guanylyl cyclase for the production of cGMP. cGMP is a relaxant second messenger that is broken down by phosphodiesterase 5 (PDE5). The relaxant effect of nitric oxide could be reduced by increased PDE5 activity, limiting cGMP (Mullershausen et al., 2006).

The bronchodilating potency was also reduced in Sendai virus-infected guinea pigs *in vivo*. These data suggest that the reduced potency is not a characteristic of allergic inflammation, but rather of general inflammation. Another possible interpretation, however, involves receptor occupancy. If TLR7 is already occupied by virus ssRNA in the virus-infected animals, then the baseline bronchoconstriction measured might be one with TLR7-mediated bronchodilation already integrated into the overall response. Further bronchodilating effects of R837 would not be observed until higher doses, when TLR7 occupancy by R837 can overcome that already accomplished by the virus ssRNA. This would be consistent with the endogenous bronchoprotective mechanism I have hypothesized, that respiratory viruses relax the airways. If the airways are already partially relaxed relative to the intrinsic maximum bronchoconstriction, relaxation at sub-maximal occupancy doses of R837 will be masked.

The next experiments should include the effects of PolyUs in control, sensitized, and virus-infected guinea pigs *in vivo*. I have proposed that R837 has multiple targets, as evidenced by the biphasic dose-response curve, the incomplete reversal by the TLR7 antagonist in guinea pigs, and the fact that PolyUs seems to have one target, as it is almost completely reversed by the TLR7 antagonist. If the loss of the high potency phase of R837-mediated bronchodilation in ovalbumin-sensitized guinea pigs reflects the loss of either TLR7 or TLR8 function, the relaxant effect of PolyUs, which is specific to only one of these targets, should be either unaffected or nonexistent, depending on which target is being affected by the sensitization protocol. It will also

be interesting to test these ligands in an allergen sensitization and challenge model of asthma, as well as a virus infection in an allergen sensitization model. This would determine if hyperreactive bronchoconstriction induced by eosinophil activation can also be reversed by TLR7 agonists.

Differences in the bronchodilatory potency of R837 were not observed in any of the mouse models of asthma, including allergen sensitization with and without challenge, Sendai virus infection, and transgenic over-expression of IL-5 in the lungs. The mouse airway is different from those of the guinea pig and human airways, in terms of both airway smooth muscle physiology and airway inflammation. Though the guinea pig does not necessarily reflect changes in the human, the mouse is historically less predictive. Another difference is that the asthma model mouse experiments were done *in vitro* whereas those in guinea pig were done *in vivo*. The guinea pig experiments should be done *in vitro* to confirm whether the reduced potency of R837 is maintained. Alternately, we now have the capability of performing *in vivo* measurements of airway pressure, so measuring the effect of R837 in mouse models of asthma *in vivo* are among the next experiments to be conducted.

Currently, I have not conducted any asthma model studies in human tissue. Clearly, the long-term goal is to test TLR7 agonists in humans with asthma in clinical trials. The human tissue I used in the study was donated from Pacific Northwest Transplant bank and was not selected for an asthma phenotype. If possible, a very useful experiment would be to compare the response to R837 in both healthy and

asthmatic human airway tissue. If this type of segregation is not possible, a useful experiment would be to culture human tracheas in allergic or asthmatic serum or with T_h2 cytokines. Another possibility would be to express the various identified human polymorphisms in cell lines and measure their function in the canonical TLR7 signaling pathway, as well as faster signaling mechanisms like intracellular calcium or nitric oxide production. It will be interesting to determine if these polymorphisms affect the TLR7 response so that the bronchoprotective mechanism is defective.

Regardless of the effects in models of asthma, the bronchodilating effect of TLR7 agonists is maintained, and the therapeutic potential is clear for this class of drugs as acute rescue medications for asthma attacks. Along with the acute bronchodilating effect, these drugs may provide chronic anti-inflammatory and anti-hyperreactivity properties in the airways. The combination of prophylaxis and rescue in one medication holds potential as an alternative to the current standard of a steroid for anti-inflammation combined with a beta-agonist for bronchodilation. Side effects could include fever and acute inflammation due to the induction of interferon and tumor necrosis factor-α production. Another side effect could be increased susceptibility to virus infection due to desensitization of TLR7 by persistent stimulation. Further animal studies are needed for this determination. That being said, TLR7 agonists have been well tolerated in clinical trials, with minimal side effects (Witt et al., 1993; Savage et al., 1996). Furthermore masked prodrugs are being developed to limit systemic toxicity. For example by the

generation of TLR7 agonist esters that are inactivated by esterases in the blood, these immunomodulators can be inactivated systemically, while remaining active at specific locations (Fletcher et al., 2006).

The work in this thesis contributes a novel bronchodilatory mechanism to the field of airway physiology. It also contributes a possible explanation for virus-related airway pathophysiology, such as in asthma. It will be interesting to see if TLR7 remains a viable drug target for asthma therapy in the coming years with a rapidly expanding body of TLR research. As the TLR field grows, it is my opinion that research needs to consider effects beyond those of canonical pathways involving upregulation of inflammatory cytokines. Canonical functions of a receptor are generally assigned based on the initial discovery. The pitfalls are that many important effects may be missed. More kinetic studies with shorter time points are needed in all experimental systems considering TLRs, rather than relying strictly on assays measuring gene effects 24 hours later. The rapid protective mechanism I describe in this thesis suggests the possibility that other TLRs can affect physiological systems in a rapid time frame. These effects might reflect potent homeostatic mechanisms needed during the many changes associated with inflammation. While they might not be considered traditional effects of the immune system, these effects would suggest that the immune system plays a role beyond protection of infection to maintain physiological functions. The need to consider non-canonical effects of these receptors extends to determination of ligand selectivity. Claims of ligand recognition by a receptor need to be tempered based on

the experimental system used. If only measuring the effect of a ligand on canonical TLR-mediated gene regulation, it is not possible to claim that a ligand is not recognized by the receptor if this has not been determined in other functional assays or shorter time frames. It further emphasizes the need for more binding assays to conclusively determine whether ligands bind to TLRs regardless of functional effects. It is exciting to consider the rapid effects of TLR signaling that might be discovered with further research in the coming years.

In summary, the TLR7 agonist R837 has TLR7-dependent and TLR7-independent bronchodilatory effects in guinea pig *in vivo* and *in vitro* and in mouse *in vitro*. R837 has only TLR7-dependent bronchodilatory effects in human trachea *in vitro*. PolyUs has almost completely TLR7-dependent bronchodilatory effects in guinea pig *in vitro*, but is of mixed TLR7-dependence and TLR7-independence in the mouse *in vitro*. Consistent with reports that PolyUs is a TLR8 agonist in human, PolyUs has completely TLR7-independent bronchodilatory effects. Based on the possible TLR8-dependence of PolyUs in the humans, and the strong sequence conservation across species, I suggest that TLR8 mediates the TLR7-independent relaxant contributions of these ligands in all three species tested. Depending on species, the TLR7-dependent and –independent relaxant mechanisms involve prostaglandins, MaxiK, and nitric oxide. In guinea pig models of asthma including allergen sensitization and virus infection, the bronchodilatory potency of R837 is reduced *in vivo*, whereas there is no effect *in vitro* in similar mouse models of asthma. The TLR7/8-mediated bronchodilatory mechanism is highly conserved across three species suggesting

importance in protecting the airways from excessive obstruction during virus infection. The translation of the bronchodilatory effect to human tissue combined with the T_h1-skewing properties suggests TLR7/8 agonists represent a novel class of both acute rescue and chronic prophylactic asthma therapeutics.

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