REGULATION OF AIRWAY REACTIVITY: INTERACTIONS BETWEEN NEURONAL AND IMMUNE PATHWAYS IN THE LUNG

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The prevalence of asthma has been on the rise since the 1970s, affecting more than 300 million people worldwide. In recent years, approximately 250,000 deaths per year have been attributed to the condition. The majority of these deaths are likely a direct result of airway obstruction, of which constriction of airway smooth muscle plays a significant role. Although several bronchodilators are currently on the market for alleviation of airway constriction, not all patients are responsive to these treatments. This underlies the importance of understanding the mechanisms triggering smooth muscle constriction so that additional drug targets can be discovered to help prevent or reverse airway constriction during asthmatic exacerbations.

In this dissertation I utilize two approaches to analyze immune-related airway constriction in the mouse in an attempt to further understand the mechanism behind the human condition. The majority of asthmatics also have allergies, which can trigger both enhanced inflammation of the airways and airway constriction. Therefore, the first approach involved modeling allergic airway constriction in both the naïve and inflamed mouse lung to examine the mechanisms of IgE-mediated bronchoconstriction. Genetic, pharmacological, and surgical methods were then used to explore the cell types, mediators, and receptors involved in this response.
The second approach involved the triggering of airway constriction through a non-allergic mechanism. In these studies, the thromboxane analog U46619 was used to elicit dose-dependent airway constriction. As the thromboxane receptor, Tp, is expressed on multiple cell types, mice carrying a tissue-specific deletion of this receptor were examined to define the cell types involved in U46619-mediated bronchoconstriction in both the naïve and inflamed lung.

Asthma is a highly complex disease involving the combined effects of both genetic and environmental effects and therefore it is unlikely to be able to model the disease itself accurately in a laboratory animal that does not spontaneously develop the condition. However, it is possible to closely approximate several pathophysiological symptoms of asthma in the mouse, and using these models, the many mechanisms underlying the conditions of asthma can be teased away from the complexity of the condition as a whole.
ACKNOWLEDGEMENTS

I would like to offer my heartfelt thanks to several individuals which have made the work described in this dissertation possible. First and foremost, I would like to thank my advisor, Bev Koller, not only for her training and advice but also for her willingness to believe in my abilities and my work. Under her guidance I have grown as a scientist, and have learned to think critically about my experiments and to be able to evaluate the results not only as to how they fit into the scope of my project, but to how they fit into a broader context. Her belief and support has helped me to develop skills that I would previously have thought impossible, and has enabled me to challenge myself both technically and mentally. Bev, I would like to express my sincere gratitude for all of the time, patience, and advice that you have given me over the years. I can only hope that my accomplishments in the future will make you proud.

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<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>5-LO</td>
<td>5-lipoxygenase</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>ANS</td>
<td>autonomic nervous system</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BMMCs</td>
<td>bone marrow-derived mast cells</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>c-KIT</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>cysLT</td>
<td>cysteinyl leukotriene</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>E</td>
<td>elastance</td>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophilic cationic protein</td>
</tr>
<tr>
<td>ESF</td>
<td>electrical field stimulation</td>
</tr>
<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>ESMC</td>
<td>embryonic stem cell-derived mast cell</td>
</tr>
<tr>
<td>FEV₁</td>
<td>forced expiratory volume in the first minute</td>
</tr>
<tr>
<td>FLAP</td>
<td>5-lipoxygenase activating protein</td>
</tr>
<tr>
<td>FOT</td>
<td>forced oscillatory technique</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>G</td>
<td>tissue damping</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>H</td>
<td>tissue elastance</td>
</tr>
<tr>
<td>HBSS</td>
<td>hanks buffered saline solution</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>prostacyclin receptor</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>mAChR</td>
<td>muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MCh</td>
<td>methylcholine</td>
</tr>
<tr>
<td>MBP</td>
<td>major basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMEF</td>
<td>maximal midexpiratory flow</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
</tbody>
</table>
NKA  neurokinin A
NO   nitric oxide
NOS  nitric oxide synthase
NPY  neuropeptide Y
OVA  ovalbumin
PAF  platelet activating factor
PAR  protease-activated receptor
PCLS precision cut lung slices
PCPA 4-chloro-DL-phenylalanine
PEF  peak expiratory flow
Penh enhanced pause
PGHS prostaglandin H synthase
PKA  protein kinase A
PLA₂ phospholipase A2
PLC  phospholipase C
PNS  peripheral nervous system
PSA  passive systemic anaphylaxis
R   pulmonary resistance
RARs rapidly adapting receptors
Rₘₐₜ airway resistance
Rₐ lung resistance
SARs slowly adapting receptors
SCF  stem cell factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCG</td>
<td>superior cervical ganglia</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SNS</td>
<td>somatic nervous system</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>Tgln</td>
<td>transgelin</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane</td>
</tr>
<tr>
<td>TXAS</td>
<td>thromboxane synthase</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
</tr>
<tr>
<td>WBM</td>
<td>whole bone marrow</td>
</tr>
<tr>
<td>WBP</td>
<td>whole body plethysmography</td>
</tr>
<tr>
<td>Wsh</td>
<td>C57BL/6 Kit&lt;sup&gt;Wsh&lt;/sup&gt;/Kit&lt;sup&gt;Wsh&lt;/sup&gt;</td>
</tr>
<tr>
<td>Z</td>
<td>input impedance</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
STRUCTURE, FUNCTION AND REGULATION OF THE HEALTHY LUNG

The lung is the essential respiration organ in air-breathing animals. Air passes through the nose or mouth and travels through the oropharynx, nasopharynx, larynx, and trachea into the main conducting airways of the lung until it finally reaches the alveoli where the gas exchange of carbon dioxide and oxygen takes place. The structure of the human lung, although similar in many ways, is relatively complex when compared to that of experimental animals, especially the mouse (Figure 1.1). These differences are reviewed extensively by Hyde et al. (1). Briefly, the tracheobronchial conducting airways form a complex branching pattern that extends to the gas exchange peripheral airways. The bronchi, the largest branches, have abundant cartilage while distally the smaller bronchioles have thinner walls with little to no cartilage and a greater proportion of smooth muscle. Conversely, in the mouse cartilage is found predominantly in the trachea. The average number of airway generations from the trachea to the alveolar spaces is roughly the same for most mammals; however, branching is unique in primates (including humans). Furthermore, the organization of the transition zone between the conducting airways and the gas exchange zone differs significantly between the species. Primates have many respiratory bronchioles, while mice have few. Finally, the cellular make-up of the airways also differs (Table 1.1).

The lungs are primarily responsible for the exchange of oxygen and carbon dioxide between the air we breathe and the blood, supplying a source of energy to the body. On average, humans inhale and exhale 22,000 times per day and process around 300 cubic feet (8.5 cubic meters) of air, which brings not only nourishing oxygen but also a multitude of pollutants and noxious particles from which the lung needs to protect.
Figure 1.1 Structure of human and mouse lung.*  Humans have an extensive branching of the lung (including the intrapulmonary bronchus), as well as a greater area of transition between the most distal conducting airway, the terminal bronchiole, and the alveolar ducts. The mouse airway has less branching; primary bronchus leading directly to terminal bronchioles. The distal airway of mice lacks the extensive respiratory bronchioles and is usually characterized by the terminal bronchiole branching directly into the alveolar ducts. *Adapted from (1).
itself. The airway wall is made up of several “compartments” including, 1) the epithelial compartment, comprised of surface epithelium and submucosal glands; 2) the interstitial compartment, including the basement membrane, fibroblasts, smooth muscle, cartilage, and vasculature; 3) the nervous compartment, both afferent and efferent branches; 4) the vascular compartment which encompasses capillaries, arterioles and venules from the bronchial circulation, and lymphatic vessels; and 5) the immunological compartment, including both migratory and resident inflammatory immune cells. Together these compartments help to coordinate proper function, as well as defense mechanisms to protect the airways from environmental irritants, pollutants, and allergens inhaled on a regular basis.

**Epithelium**

The bronchial epithelium is a stratified structure consisting of a columnar layer comprising ciliated and secretory cells that are supported by basal cells. This represents the primary physical barrier that protects the internal milieu of the lungs and intra-epithelial sensory nerves from inhaled irritants, pollutants, infectious agents, and other particulate matter. Under normal conditions, the epithelium secretes mucus, as well as cytoprotective molecules in order to trap and inactivate inhaled components. The epithelium can also work in conjunction with the immune system by secreting cytokines and chemokines, as well as through expression of adhesion molecules; thereby providing a mechanism of tissue repair and debris removal in the event of epithelial compromise.
Table 1.1 Comparison of species differences in airway organization.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tracheobronchial airway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage in wall</td>
<td>trachea to distal bronchiole</td>
<td>trachea to distal bronchiole</td>
</tr>
<tr>
<td>Nonrespiratory bronchioles</td>
<td>several generations</td>
<td>several generations</td>
</tr>
<tr>
<td>Respiratory bronchioles</td>
<td>several generations</td>
<td>several generations</td>
</tr>
<tr>
<td>Generations to alveolarised bronchiole</td>
<td>17-21</td>
<td>13-17</td>
</tr>
<tr>
<td>Branching pattern</td>
<td>dichotomous</td>
<td>monopodial</td>
</tr>
<tr>
<td><strong>Trachea</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Cartilage</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Submucosal glands</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><strong>Epithelium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (µm)</td>
<td>50-100</td>
<td>11-14</td>
</tr>
<tr>
<td>Cells/mm basement membrane</td>
<td>303±20</td>
<td>215</td>
</tr>
<tr>
<td>% goblet cells</td>
<td>9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>% serous cells</td>
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</tr>
<tr>
<td>% clara cells</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>% ciliated cells</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>% basal cells</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>% other cells</td>
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<td><strong>Intrapulmonary airways</strong></td>
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<td>Smooth muscle</td>
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<td>Cartilage</td>
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<td>absent</td>
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<td>Submucosal glands</td>
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<tr>
<td><strong>Epithelium</strong></td>
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<tr>
<td>Thickness (µm)</td>
<td>40-50</td>
<td>8-16</td>
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<td>Cells/mm basement membrane</td>
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<tr>
<td>% goblet cells</td>
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<tr>
<td>% serous cells</td>
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<td>&lt;1</td>
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<tr>
<td>% clara cells</td>
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<td>61</td>
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<td>37</td>
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<td>% basal cells</td>
<td>32</td>
<td>&lt;1</td>
</tr>
<tr>
<td>% other cells</td>
<td>18</td>
<td>2</td>
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<tr>
<td><strong>Terminal bronchioles</strong></td>
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<td>Wall</td>
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<tr>
<td><strong>Epithelium</strong></td>
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<tr>
<td>Thickness (µm)</td>
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<td>7-8</td>
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<tr>
<td>Cells/mm basement membrane</td>
<td>?</td>
<td>?</td>
</tr>
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<td>% goblet cells</td>
<td>35</td>
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<td>0</td>
</tr>
<tr>
<td>% clara cells</td>
<td>60-80</td>
<td>60-80</td>
</tr>
<tr>
<td>% ciliated cells</td>
<td>52</td>
<td>40-20</td>
</tr>
<tr>
<td>% basal cells</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>% other cells</td>
<td>13</td>
<td>0</td>
</tr>
</tbody>
</table>

*Modified from (1).
**Airway Smooth Muscle**

In normal airways, the role of airway smooth muscle (ASM) is controversial. ASM may provide structural support, promote gas exchange, aid in mucus clearance, protect against particulate infiltration through narrowing of the airways, and/or contribute to the cough reflex. On the other hand, it is possible that ASM does not contribute significantly to the healthy lung. In human and animal airways, there is a small degree of resting tone maintained by constitutive release of acetylcholine (ACh) from parasympathetic nerves within the airway. However, this resting tone does not appear to be critical to healthy lung function, as treatment with muscarinic antagonists does not appear to have adverse effects on airway function. It is unclear if proximal and distal ASM behaves in the same way, as innervation of the distal airways is sparse and receptor distribution varies within different degrees of the airways.

**Resident Immune Cells**

*Dendritic Cells*

Dendritic cells (DCs) are bone marrow-derived cells. Their primary function is to act as sentinels in the mucosa and internal organs of the body in search of foreign antigens, viruses, and bacteria (2). DCs bind, internalize, and process antigens which are then displayed on their surface and presented to lymphocytes. This process drives the adaptive immune response. In addition to their role in the adaptive immune response, DCs also tolerize T cells to antigens that are innate to the body (self antigens), minimizing autoimmune reactions and thereby linking adaptive and innate immune responses.
Mast cells

Mast cells, which arise from CD34+ pluripotent hematopoietic cells in the bone marrow, are present in most tissues in the vicinity of blood vessels and are especially predominant near external boundaries such as the lung mucosa, as well as the nose and mouth. Unlike other inflammatory cells which mature before circulation throughout the body, mast cells circulate in an immature form and finally mature after they have been established at a tissue site (reviewed in (3)). Since mast cells mature after reaching different tissue types, they can develop different properties depending on the tissue that they occupy. There are two types of mast cells recognized to date, 1) connective tissue; and 2) mucosal mast cells. Typically, the connective tissue mast cell is found in the serosal tissue and in skin, while the mucosal type is located in the mucosa of both the lung and gut (4). Although best known for their role in allergy and anaphylaxis, mast cells are also involved in functions such as blood flow and coagulation, mucosal secretion, wound healing, regulation of innate and adaptive immune responses, as well as peripheral tolerance.

The primary activating mechanism of mast cells is through an FcεRI dependent mechanism. This involves interaction of a multivalent antigen (allergen) with its specific IgE antibody attached to the cell membrane via the high affinity FcεRI receptor. Cross-linkage of IgE by the interaction of allergen initiates mast cell activation and mediator generation and release. Mast cells also express low affinity IgG receptors, these low affinity receptors may regulate high-affinity IgE receptor-mediated activation. Mast cells can also be activated by additional stimuli including neuropeptides, complement...
components, and certain drugs such as opiates. Morphologically, degranulation resulting from different modes of activation is similar (reviewed in (3)).

**Macrophages**

Macrophages are present within interstitial tissues, alveolar spaces, and on mucosal surfaces throughout the body, including the lung. They are derived from myloid precursors, or monocytes, in bone marrow, spleen, and fetal liver that migrate to the lung. Similar to mast cells, the environment of their final destination influences the function of the developing macrophage. Generally, macrophages function as a link between the innate and adaptive immune system. Their primary role is phagocytosis of microbial pathogens, particulate matter, or debris in response to infection or injury; however, they are also capable of secreting an assortment of proinflammatory cytokines and chemokines.

**Neural Control**

The nervous system is divided into the somatic nervous system (SNS) and the autonomic nervous system (ANS). A generalized diagram can be seen in Figure 1.2. Organs under the control of the SNS are considered to be under voluntary control, while the ANS regulates involuntary organ function and maintains homeostasis. The ANS primarily functions as an efferent system transmitting signals from the central nervous system (CNS) to the peripheral nervous system (PNS); however some afferent autonomic fibers are carried to the CNS by major autonomic nerves such as the vagus nerve, which are involved in the mediation of visceral sensation and regulation of vasomotor and respiratory reflexes. The ANS is primarily involved in reflex arcs, transmitting impulses
from the CNS to peripheral organ systems. For example, afferent fibers convey stimuli
from peripheral pain, mechano-, or chemo-receptors to the CNS to activate efferent
sympathetic or parasympathetic transmission to the peripheral organ systems. A
generalized schematic of airway vagal innervation can be seen in **Figure 1.3**.

*Afferent Nerves*

Sensory nerves in the respiratory tract are adapted to detect various elements of
the physical and chemical environment of the airways and transmit this information to the
CNS in the form of action potentials. An action potential is initiated by depolarization of
the nerve membrane, which when great enough, leads to the formation of the action
potential via the activation of voltage-gated sodium channels. The action potential is
conducted along the axon until it reaches a central terminal, causing neurotransmitter
release into the synapse of secondary neurons ultimately leading to reflex reactions
(reviewed in (5)).

The vagus nerve contains the majority of airway sensory afferents (reviewed in
(6)). Sensory nerves are divided into two general categories (**Table 1.2**), stretch
receptors (which respond to mechanical forces caused by inflation and deflation during
respiration) and nociceptors (which respond to the threat of tissue damage). The stretch
receptors are classified as “A fibers” because their action potentials are conducted at a
fast rate along their axons (~10-50 m/s), and are located from the nose to the
cartilaginous bronchi occurring with greater frequency in the large airways (7). These
receptors can be further subdivided into slowly adapting receptors (SARs) and rapidly
adapting receptors (RARs) based on their adaptation properties to a sustained inflation.
Most nociceptors in the lung are classified as “C fibers” due to their slow velocity action
Figure 1.2 Schematic of the different divisions of the nervous system. Sensory nerves receive signals from receptor tissues and send a signal through the peripheral nervous system (PNS) to the central nervous system (CNS). The CNS then sends the signal down through the PNS to either the somatic nervous system or the autonomic nervous system, where the nerves release neuropeptides that act on specific effectors.
Figure 1.3 Airway innervation*. Afferent sensory nerve endings in the airway epithelium are activated by environmental stimuli which results in neuropeptide release from sensory “efferent” fibers, as well, as perpetuation of the signal to the CNS. The signal in the CNS then results in a cholinergic reflex and subsequent release of ACh from preganglionic parasympathetic nerves. The release of ACh then activates the parasympathetic ganglion neurons and facilitates the release of ACh from post-ganglionic fibers. *Adapted from (8)
# Table 1.2 Pulmonary receptors and their reflex effects*

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Receptor Subtype</th>
<th>Reflex Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stretch Receptors</td>
<td>SAR</td>
<td>Inspiratory termination, expiratory facilitation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enhancement of inspiratory effort</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bronchodilation</td>
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<tr>
<td></td>
<td></td>
<td>Tachycardia</td>
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<tr>
<td></td>
<td>RAR</td>
<td>Cough</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Broncho- and laryngoconstriction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Augmented breath/gasp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Irregular inspiration, shortened expiration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Airway mucus secretion</td>
</tr>
<tr>
<td>Nociceptors</td>
<td>Bronchopulmonary C fibers</td>
<td>Rapid, shallow breathing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apnea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Broncho- and laryngoconstriction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Airway mucus secretion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vasodilation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bradycardia</td>
</tr>
</tbody>
</table>

*Adapted from (9)
potentials (~0.3-2 m/s). These C fibers can also be subdivided based on their ability to respond to the chemical capsaicin, a component of hot peppers. Capsaicin-sensitive C fibers exhibit slower action potentials than those of capsaicin-insensitive C fibers (0.3-0.7 m/s and 0.7-2 m/s, respectively) (reviewed in (10)). The nerve cell bodies of sensory nerves in the airways are localized to both the vagal sensory ganglia (subdivided into the nodose and jugular ganglions) and the dorsal root ganglia. RARs and SARs are derived from nodose neurons (11, 12), whereas bronchopulmonary C fibers are derived from both nodose, jugular, and dorsal root ganglia (13, 14).

SARs respond to changes in tension across the airway wall and are thought to regulate changes in bronchial tone with inspiration. There is also evidence in canines that SARs and CO₂-sensitive chemoreceptors interact at the reflex level; lengthening of expiratory duration is attenuated by increased levels of CO₂, or hypercapnia (15). Activation of these receptors leads to inhibition of inspiration and inhibition of parasympathetic activity, consequently resulting in relaxation of ASM (16). RARs, also known as irritant receptors, are thin, myelinated nerve terminals stimulated by mechanical, chemical, and inflammatory stimuli. Activation of these receptors leads to increased respiratory rate, increased inspiratory effort, and increased parasympathetic output resulting in contraction of ASM and mucus secretion (16). RARs are also responsible for the cough reflex, which is primarily used as a means to rid the airway of inhaled pollutants or irritants. These particles typically get trapped in the protective mucus layer and then expelled upon activation of RARs and initiation of cough. It was once thought that mice lack RARs, due to the absence of cough reflex. Although RARs have been found to be located in neurites of cultured adult mouse neurons and in adult
mouse skin samples (17, 18), initially no RARs were detected in either the tracheal or bronchial epithelium of this species (19). However, more recent methods have made it possible to record RAR activity in vivo in the mouse lung (20). These studies concluded that lung sensory behavior in the mouse is similar to large animals (such as dogs, cats, and rabbits) and most closely mimics that of the rat.

Morphological studies in the cat have shown that approximately 75% of afferent fibers in the lung are slow conducting, unmyelinated C-fibers, which span the entire respiratory tract from trachea to the parenchyma to the central airways (21). While typically quiescent during tidal breathing, C fiber activation provides excitatory input to neuronal pathways driving autonomic output to the airways, resulting in bronchoconstriction, mucus secretion, and vasodilation (22, 23). These reflexes can be prevented by treatment with centrally acting tachykinin receptor antagonists, suggesting that C-fibers use Substance P (SP) and neurokinin A (NKA) as their primary neurotransmitters (23). In the CNS, neurokinins augment synaptic transmission through the action of G-protein coupled neurokinin receptors (NK₁, NK₂, and NK₃) located on secondary neurons (24). In addition, some C fibers may branch to synapse on local parasympathetic ganglia where release of tachykinins can elicit excitatory postsynaptic potentials (25). Subsets of afferent nerves in the airways can contain neurotransmitters other than tachykinins, such as calcitonin gene related peptide (CGRP), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY).

Efferent nerves

The efferent portion of the ANS is further divided into the parasympathetic nervous system and the sympathetic nervous system. Both of these systems consist of
myelinated preganglionic fibers which make synaptic connections with unmyelinated postganglionic fibers which then innervate the effector organ. These synapses typically occur in clusters, or ganglia. Most organs are innervated by fibers from both divisions of the ANS, which tend to have opposing influences.

The predominant control of human and animal airways is exerted by parasympathetic (or cholinergic) nerves, which travel in the vagus nerve. The parasympathetic division primarily releases ACh from both its pre- and postganglionic terminals and is involved in responses such as pupil constriction, digestion, smooth muscle contraction, reduction of heart rate, and various organ and gland secretions. Parasympathetic nerves are the major neural bronchoconstrictor mechanism within human airways. Specifically, the vagus nerve carries efferent cholinergic fibers that synapse in small ganglia within the airway wall, from which short postganglionic fibers innervate airway smooth muscle and submucosal glands (26). In humans, cholinergic innervation is abundant in the trachea and in the large central airways, but diminishes peripherally (27). In addition, human airways have a resting tone that is maintained by a constitutive low-level release of ACh from these cholinergic nerves. This resting tone is also evident in mouse airways and also appears to be mediated by cholinergic nerves as baseline lung resistance is reduced in animals after vagotomy (28).

Stimulation and activation of cholinergic fibers results in the release of ACh, which can act directly on either nicotinic acetylcholine receptors (nAChRs) or muscarinic acetylcholine receptors (mAChRs). nAChRs mediate ganglionic transmission in airways and are located directly on parasympathetic ganglia (excitatory), as well as on preganglionic junctions (inhibitory). These receptors are ligand-gated ion channels
formed by 5 homologous or identical subunits, arranged to build a central ion channel. nAChRs directly on parasympathetic ganglia are required for activation of post ganglionic parasympathetic fibers that innervate the airways, which can be blocked pharmacologically by treatment with nicotinic antagonists such as hexamethonium and mecamylamine.

In mammals, 5 distinct mAChRs have been identified (M1-M5), and in the lung, only the M1, M2, and M3 mAChRs have a defined physiological role (29). In mice and humans, the location of the muscarinic receptors has been well established. M1 mAChRs are primarily localized to the parasympathetic ganglia, where they facilitate neurotransmission, but are also located postjunctionally, directly on vascular smooth muscle (VSM) and submucosal glands (30). M2 mAChRs are located postjunctionally, directly on ASM and prejunctionally, along postganglionic cholinergic nerve terminals (31), where they act as feedback regulators of ACh release. M3 mAChRs are located postjunctionally, directly on ASM, VSM, and along submucosal glands (32), where they facilitate airway and vascular constriction and enhance mucus production, respectively. The M1 and M3 mAChRs preferentially couple to G-proteins of the Gq family and upon stimulation exert their primary physiological effects through the activation of protein kinase A (PKA), phospholipase C (PLC), and increases in intracellular calcium [Ca^{2+}]_i (33); which, in the case of M3 receptor activation, results in ASM contraction (Figure 1.4 A). The M2 mAChR is selectively linked to G-proteins of the Gi/o family and exerts its effects through the inhibition of adenylate cyclase activity and the subsequent decrease in intracellular cyclic AMP (cAMP) levels (33).
The sympathetic division is typically involved in “fight or flight” responses such as pupil dilation, vascular dilation, smooth muscle relaxation, and increased heart rate. Sympathetic nerves emerge from the spinal cord and release ACh onto the sympathetic trunk on either side of the spinal cord. The postganglionic fibers extend to the lung where they release norepinephrine, which elicits its effects via activation of adrenergic receptors. The extent of sympathetic innervation in the lungs appears to be species specific. Although sympathetic nerves extend into the ASM of both cat and guinea pig airways, in humans these nerve fibers innervate submucosal glands, blood vessels, and parasympathetic ganglia but do not directly innervate ASM (reviewed in (34)). Despite the species-dependent innervation patterns, adrenergic receptors appear to be present throughout the lung even in humans (35).

The adrenergic receptors are G-protein coupled receptors that can be grouped into two families. The \( \alpha \)-adrenergic receptors, \( \alpha_1 \) and \( \alpha_2 \) are coupled to \( G_q \) and \( G_{i/o} \), respectively, which function as described above. Conversely, \( \beta \)-adrenergic receptors are coupled to \( G_s \), where stimulation activates adenylate cyclase to increase cAMP. cAMP then increases PKA activity, which in turn phosphorylates downstream protein modulators (36). This reaction ultimately leads to a fall in intracellular \( \text{Ca}^{2+} \) levels, and activation of large conductance potassium channels to initiate the bronchodilation response (Figure 1.4 B).

In addition to their actions on smooth muscle, adrenergic and muscarinic receptors also modulate norepinephrine and ACh release from sympathetic nerves and parasympathetic nerves, respectively. On sympathetic nerves, \( M_1 \) mAChRs (37) and \( \beta_2 \)-adrenergic (38) receptors enhance the release of norepinephrine, whereas \( M_2 \) mAChRs...
Figure 1.4 Gq and Gs regulation of airway smooth muscle. A) Release of ACh from parasympathetic nerves mediates airway smooth muscle constriction through the M₃ receptor, a Gq-protein coupled receptor. Activation of M₃ initiates dissociation of the Gq+GTP complex and activation of phospholipase C (PLC). Activated PLC catalyzes the hydrolysis of PIP2 into DAG and IP3. IP3 initiates the release of calcium from the sarcoplasmic reticulum into the cytosol. The resulting increase in calcium promotes the binding of calmodulin (CaM). The calcium/calmodulin complex activates myosin light chain kinase (MLCK), which phosphorylates myosin light chains and promotes the ATPase activity of myosin, thereby promoting cross-bridge cycling and contraction. B) Release of norepinephrine (NE) from sympathetic nerves initiates smooth muscle relaxation by activation of protein kinase A (PKA) via a Gs-protein coupled receptor. Activated PKA can phosphorylate Gq coupled receptors, PLC, IP3 receptors, as well as MLCK. This phosphorylation results in reduced generation of IP3, attenuated ability of IP3 to activate IP3 receptors and increase intracellular calcium levels, and decreased affinity of MLCK for the calcium/calmodulin complex- all of which reduces the contractility of airway smooth muscle.
(39) and α₂-adrenergic (40) receptors inhibit its release. On parasympathetic ganglia, β₂-adrenergic receptors have been shown to enhance the release of ACh from guinea pig postganglionic nerves in vivo (38), but have also been shown to inhibit this release in human trachea and bronchi in vitro (41, 42). Norepinepherine is colocalized with NPY in sympathetic nerves (43). NPY, although a potent vasoconstrictor, has little if any direct effects on ASM, but may also modulate sympathetic reflexes by inhibiting the release of norepinepherine via prejunctional activation of Y₂ receptors.

**Lung function measurements**

In humans, lung function is most commonly measured using parameters derived from forced expiration measurements, in which patients inhale a maximum volume and exhale as rapidly as possible into a device that records expired volume versus time. Using this method, peak expiratory flow (PEF), forced expiratory volume in the first second (FEV₁), and maximal midexpiratory flow (MMEF) can be assessed easily and noninvasively in patients without instrumentation. During this forced expiration, the rate at which air is expelled from the lungs can determine the mechanical properties of the airway using the wave-speed equation for flow through collapsible tubes (44).

Due to several advantages that the mouse provides, including a well-characterized genome, ease of genetic manipulation, and short reproductive cycle, mice are commonly used for pulmonary research. Several techniques are available for the evaluation of airway mechanics in the mouse (Table 1.3), including those that require removal of lungs from the host, noninvasive in vivo measurements, and invasive in vivo measurements that require instrumentation of the airway (reviewed in (45, 46)). In vitro, bronchial and/or
tracheal strips and rings can be used to study smooth muscle constriction and hyperresponsiveness to various agents and stimuli by measuring isometric or isotonic responses of the tissues attached to a force transducer (47-49). In addition, precision cut lung slices (PCLS), introduced by Krumdieck et al. (50) in 1980, have been a valuable tool for studying airway constriction. Using the PCLS technique, preparations of viable tissue slices can be taken into culture where constriction of airways of different diameter can be directly visualized and analyzed using videomicroscopy.

Whereas these in vitro techniques offer several advantages, such as the study of several replicate samples per animal and ease of data interpretation due to the collection of direct measurements, in vivo obstruction of the airways results not only as a result of smooth muscle contraction, but also from release of neurotransmitters, release of secretions into the lumen, and inflammatory cell infiltration of the airways, which is not adequately represented in isolated airway assays. Several methods for measuring lung function in vivo have been developed; however, the most frequently used are the noninvasive measurement of enhanced pause (Penh) using barometric whole body plethysmography (WBP) and the invasive measurement of airway resistance using either the equation of motion or the forced oscillatory technique (FOT).

WBP involves the use of unrestrained, conscious, spontaneously breathing mice to evaluate Penh, a dimensionless measurement that reflects a combination of the pressure signal from inspiration and expiration and the timing of expiration (51). Although there are several advantages to this approach; such as ease of use, high throughput capability, and the ability to assess responses from the same animal over time, there has consistently been controversy as to the ability of Penh to accurately reflect
Table 1.3 Lung function assessments in the mouse.

<table>
<thead>
<tr>
<th><strong>In vitro</strong></th>
<th>Method</th>
<th>Parameter(s)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated airway segments</td>
<td>Tension</td>
<td>1) Several replicates per animal 2) Direct measurement of contractions</td>
<td>Circulatory and nervous system interrupted</td>
<td></td>
</tr>
<tr>
<td>Precision cut lung slices (PCLS)</td>
<td>Airway area</td>
<td>1) Viable lung tissue 2) Several replicates per animal 3) Direct measurements 4) Visualization of airways</td>
<td>Circulatory and nervous system interrupted</td>
<td></td>
</tr>
<tr>
<td>Isolated, perfused lungs</td>
<td>Airway resistance (R) Compliance (Cdyn)</td>
<td>1) Intact airways 2) Separation of pulmonary and systemic responses</td>
<td>1) Indirect measurement of mechanics 2) Only one sample per animal 3) Does not reflect metabolic and humoral input from other organs</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>In vivo</strong></th>
<th>Method</th>
<th>Parameter(s)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Body Plethysmography (WBP)</td>
<td>Penh</td>
<td>1) High throughput and ease of measurement 2) Evaluation of mice during normal physiological conditions 3) Ability to measure serial changes over time in the same animal</td>
<td>1) Lack of specificity of the Penh parameter 2) Inability to bypass the upper airway (nose and trachea) 3) Inability to distinguish between central, peripheral, and tissue contributions</td>
<td></td>
</tr>
<tr>
<td>Airway Pressure Time Index (APTI)</td>
<td>Airway resistance</td>
<td>Only requires measures of respiratory pressures, not volumes and flows</td>
<td>Inability to distinguish between central, peripheral, and tissue contributions</td>
<td></td>
</tr>
<tr>
<td>Invasive ventilation: Equation of motion - single compartment model</td>
<td>Resistance (R₁) Elastance (E) Compliance (Cdyn)</td>
<td>Can simultaneously assess airway resistance, the ease at which the lungs can be expanded, and rigidity</td>
<td>1) Over simplified model of the lung 2) Lacks the ability to differentiate between changes occurring in various parts of the respiratory tree 3) Airway modulation by neural components may be suppressed due to anesthesia</td>
<td></td>
</tr>
<tr>
<td>Invasive ventilation: Forced Oscillation Technique (FOT) – constant phase model</td>
<td>Airway resistance (Rₚ₀₉) Tissue damping (G) Tissue elastance (H)</td>
<td>Capable of distinguishing between central and peripheral mechanics</td>
<td>1) Difficulty of data interpretation 2) Airway modulation by neural components may be suppressed due to anesthesia</td>
<td></td>
</tr>
</tbody>
</table>
pulmonary resistance (52-55). Due in part to these discrepancies, invasive measurement of pulmonary resistance in anesthetized, paralyzed, mechanically ventilated animals is still considered the ‘gold standard’ of evaluating lung function in the mouse [reviewed in (56)]. Traditionally, pulmonary resistance (R) and elastance (E) are obtained from fitting fixed pressure, volume, and flow data to the equation of motion using a single-compartment model of the lung. However, the values R and E using this simplified model of the lung vary with the frequency at which the respiratory system is oscillated, which has been noted to be significant over the range of normal breathing (57). Therefore, determining R and E at a single frequency may not accurately reflect a complete characterization of respiratory mechanics. Measurement of respiratory input impedance (Z), perturbing the respiratory system with a broad-band flow waveform that simultaneously contains a range of frequencies (i.e. FOT), can be used to ameliorate this limitation. Furthermore, by fixing Z to the equation of motion using the constant-phase model of the lung several more parameters can be calculated to evaluate more precise lung function (58) and provide a clearer distinction between central and peripheral events in the lung.

REGULATION OF THE DISEASED LUNG

Asthma is one of the most common respiratory diseases, affecting approximately 300 million adults and children worldwide (59). Phenotypically, asthma is a heterogenous disease manifesting in many subtypes and affected by interactions of both environmental and genetic factors. Initially asthma was defined as “spasmodic afflictions of the bronchial tubes” (reviewed in (60)); however, as understanding of the asthmatic condition has developed, so has the characterization of the disease. Currently asthma is
characterized by 1) partially reversible airway obstruction brought on by airway narrowing and increased mucus production and plugging; 2) chronic inflammation represented by eosinophilia and increased number of mast cells; 3) airway hyperresponsiveness (AHR) to otherwise mildly provoking stimuli; and 4) airway remodeling manifesting in ASM hypertrophy, collagen deposition, and basement membrane thickening. The presence of these features distinguishes asthma from other obstructive airway diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (61).

Allergy is acknowledged as a major risk factor for asthma. Atopic (allergic) asthmatics can have extreme sensitivity to common allergens, which may cause a severe allergic reaction, or anaphylaxis. The acute phase of allergy is represented by an immediate-type hypersensitivity response initiated by allergen-induced crosslinking of specific IgE antibody bound to mast cells. Bronchoconstriction is the immediate symptom of the acute allergic response, while the late response is characterized by airway perivascular edema, mucus plugging, and inflammatory cell recruitment. Although the mast cell is the primary effector cell in the allergic response, a variety of airway, immune, and neural cells function together to contribute to the symptoms and development of asthma. The contribution of allergy to asthma however is particularly complex: although most asthmatics are allergic to at least one allergen, the majority of allergic subjects do not develop asthma.
Epithelium

In asthma, the epithelium shows evidence of stress and injury. Damage to the epithelial layer can affect airway responsiveness and induce airway remodeling. After epithelial damage, inhaled particles can access the underlying nerves and smooth muscle more easily. Sensory nerves involved in neuropeptide release can be stimulated, resulting in reflex bronchoconstriction. Furthermore, dysfunction of the epithelial layer could result in an increase in the concentration of several contractile agents. For example, histamine is metabolized by diamine oxidase and neuropeptides are metabolized by neutral endopeptidases within this layer (62).

Under normal conditions epithelial cells release factors that suppress mesenchymal cells. Upon injury or damage, repair responses promote airway remodeling by activating fibroblasts that lie under the epithelial layer; this signaling supports the growth and survival of mesenchymal cells which can lead to differentiation into connective tissue and/or additional smooth muscle cells. PGE₂, the predominant ecosaniod product of epithelial cells, is a potent inhibitor of mesenchymal cell chemotaxis (63), mitogenesis (64), and collagen synthesis (65), as well as a bronchial relaxing factor (66). In some asthmatics, the respiratory epithelium has a diminished capacity to synthesize PGE₂ (67), which can lead to increased airway remodeling and bronchoconstriction. In vivo (68) and in vitro (69) studies have shown that injury to the epithelium causes a release of fibroproliferative and profibrogenic growth factors which can promote the proliferation of underlying fibroblasts and promote differentiation of these fibroblasts into myofibroblasts that can secrete collagen. This process can lead to fibrosis of the basement membrane and overall stiffening of the airways.
Mucus production and edema

Normally, airway mucus is a protective film that prevents inhaled particles from damaging the airway epithelium. Mucus hypersecretion is a pathological feature of asthma that contributes significantly to airway obstruction. Mucins are secreted by submucosal glands and unique epithelial cells known as goblet cells. Goblet cells produce mucins in response to several endogenous factors including leukotrienes (LT) and T-helper-type 2 (Th2) cytokines, such as interleukin (IL)-13, that are secreted in response to allergens. LTD₄ released after antigen challenge enhances mucus secretion, which can be prevented via administration of cysteinyl leukotriene (cysLT) antagonists such as pranlukast (70). Further study has revealed that cysLTs are mediators for both the early and late phase mucus synthesis and secretion from goblet cells (71).

Interestingly, goblet cell hyperplasia and mucus overproduction in an asthma model also appear to be regulated by the neurotransmitter GABA (72), linking the immune and neural systems in asthma pathology. Along these lines, the production of airway mucus in the central airways is under cholinergic control, and ACh has been shown to be the primary neurotransmitter involved in mucus secretion in these airways (73) specifically via submucosal glands that express functional M₁ and M₃ mAChRs (32).

In addition to excess mucus production, a major aspect of airway inflammation is the extravasation of plasma (or vascular leakage), which can bring with it plasma-derived proteins and/or peptides that have potent adhesive and leukocyte-activating properties (74). Microvascular exudation of plasma occurs both in human asthma (75), as well as animal models of asthma (76-78). Specifically in a mouse model of asthma, allergen challenge results in plasma exudation in the trachea and bronchi but not in the lung
parenchyma (78). Many mediators of asthma are capable of inducing vascular leakage. Platelet activating factor (PAF), a mediator released by mast cells, is involved in both early and late-phase plasma exudation in guinea pig tracheobronchial airways (79). The thromboxane A$_2$ (TXA$_2$) analog U46619, despite being a potent vasoconstrictor, has also been shown to induce vascular leakage, as well as airway constriction, in guinea pigs (80). After allergen challenge, prolonged vascular leakage may be due to sequential effects of these and several other mediators such as histamine, bradykinin, tachykinins, and leukotrienes.

**Airway Smooth Muscle**

ASM plays a central role in regulating bronchomotor tone. In the asthmatic airway, ASM mediates acute bronchoconstriction and participates in AHR. Contraction of ASM requires an increase in intracellular calcium concentration, which is maintained at a low level during rest. Activation of surface receptors by contractile agonists results in an increased calcium concentration and consequent contraction of the cell. ASM can constrict in response to many agents, either directly via receptors on the muscle, or indirectly through activation of immune cells or nerves which then release mediators capable of contracting the muscle. Although there is little evidence to suggest that ASM constriction is abnormal in asthma, there is evidence of dysfunction of relaxant mechanisms. For example, ASM relaxation *in vitro* to β$_2$-adrenoreceptor agonists is impaired in patients that died of asthma exacerbations (81). This impairment was not found to be a result of decreased receptor density or affinity (82, 83). Animal models
have suggested that this impairment is mediated in part by the inflammatory cytokine IL-1β (84, 85).

During chronic asthma, ASM undergoes phenotypic alterations including hypertrophy (increase in size) and hyperplasia (increase in number) (86). Post mortem studies of asthmatics have suggested that the number of smooth muscle cells is increased 2 to 3 fold that of normal airways (86, 87), and in vitro studies have shown that proliferation of ASM cells is increased in cultures from asthmatic patients (88). Increased thickness of ASM may have an important effect on the contractile response of the airways and may contribute to asthmatic AHR (89). In addition to increased ASM, thickening of the airway wall and changes within the wall itself can increase airway narrowing due to purely structural reasons.

ASM can also perpetuate airway inflammation through the secretion of cytokines and chemokines. Human ASM is capable of producing eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), RANTES, CXCL10, IL-8, and IL-6 (90-94). Eotaxin, RANTES and GM-CSF promote recruitment and survival of eosinophils, while CXCL10 is a potent chemokine for activated T cells and mast cells (94, 95).

**Allergic Inflammation**

*Dendritic cells*

The recognition of allergen by antigen presenting cells is the first step of immune sensitization that eventually leads to the formation of Th2 immunity to inhaled allergen, as well as to the development of allergic asthma. DCs are present in the airways and are able of capturing and processing antigen for presentation to T cells, and have been shown
to be crucial for the induction of primary immune responses to inhaled allergen that ultimately leads to sensitization (96). Upon recognition of foreign antigen, DCs migrate to the draining lymph nodes where they present the antigen to T cells.

In addition to their role in sensitization, it is likely that DCs contribute to the chronic secondary Th2 response in asthmatic airways. Increased numbers of DCs are present in the airways of asthmatics (97), as well as in a rat model of eosinophilic airway inflammation (98). Selective depletion of DCs in sensitized animals resulted in complete disappearance of eosinophilia and goblet cell hyperplasia in the airways (99). Furthermore, chronic inflammation and airway structural changes may lead to local maturation of DCs. Cytokines such as GM-CSF, tumor necrosis factor alpha (TNF-α), and IL-4 expression in the asthmatic epithelium can potentially up-regulate the costimulatory function of DCs (2). Also, mast cells within the airways can express cytokines that can activate and prolong survival of DCs (100). In addition to activating T cells, DCs produce chemokines that attract recently activated Th2 cells (101), ensuring that recently activated effector cells remain in the airways.

**Lymphocytes**

T helper cells, specifically CD4+ Th2 cells, play a critical role in asthma and allergy. Th2 cells secrete a specific pattern of cytokines including IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, and TNF-α (102). Through release of these cytokines, Th2 cells can influence differentiation and proliferation of B cells, IgE class switching, eosinophil and mast cell maturation and recruitment, and regulation of adhesion molecules involved in cell migration to the epithelium. Indeed, these cytokines, as well as activated CD4+ Th2 cells, have been identified in biopsies of both atopic and non-atopic asthmatics.
Selective depletion of CD4+ T cells by injection of anti-CD4 monoclonal antibodies or use of major histocompatibility complex (MHC) class II–/– mice abolishes eosinophilic inflammation, AHR, and goblet cell hyperplasia following ovalbumin (OVA) exposure (104, 105).

Th2 cytokines play various roles in the asthmatic response. IL-4 is most important for inducing the Th2 response; therefore, IL-4 released by Th2 cells acts as a feedback loop to perpetuate the response. Studies in IL-4 deficient mice have demonstrated that IL-4 is also essential for the development of allergic airway inflammation (104, 106). Antibody neutralization of IL-4 prior to allergen sensitization prevents allergic inflammation; however, abolishment of IL-4 during airway challenge does not affect eosinophil recruitment or AHR (107, 108), suggesting that IL-4 is involved in the sensitization process and not in the recruitment of inflammatory cells. On the other hand, the importance of IL-5 in eosinophilia and AHR is apparent in IL-5–/– mice, which exhibit significant reduction of blood and tissue eosinophils after antigen challenge (109). IL-6 effects B cell immunoglobulin production, however IL-6–/– mice show normal eosinophil recruitment and AHR in an asthma model (110). IL-13 is a major cytokine regulating AHR and mucus hypersecretion independently of tissue eosinophilia (111). Studies in IL-13 deficient mice demonstrated that sensitization and challenge with allergen failed to elicit AHR or mucus hypersecretion, despite having increased levels of both IL-4 and IL-5. Furthermore, reconstitution of the IL-13 deficient mice with recombinant IL-13 recapitulated the AHR and mucus hypersecretion seen in wild-type animals (112). IL-10 has been shown to be important for B cell differentiation; however its role in eosinophilia and AHR is a matter of debate.
B-lymphocytes are the primary effector cells targeted by IL-4 and IL-13 released by Th2 cells. B-cells produce antigen specific IgE following isotype switching from IgM to IgE in response to cytokines released from Th2 cells. IgE antibodies bind to high-affinity FceRI receptors on mast cells. Following subsequent allergen exposure, IgE bound to FceRI receptors triggers mast cell activation after cross-linking with specific allergen, resulting in the synthesis and release of a variety of proinflammatory mediators (Figure 1.5).

**Eosinophils**

In the airways, eosinophils are the primary effector leukocytes recruited to sites of inflammation (Figure 1.6) and may contribute to allergic lung disease by enhancing goblet cell metaplasia, matrix deposition, ASM hypertrophy, and AHR (113). Many factors mediate eosinophil recruitment to the inflamed lungs; however, the mechanisms underlying airway eosinophilia and allergic lung disease are quite complex and not completely understood.

Many receptor-ligand interactions are necessary for eosinophil recruitment to the lungs following inhalation of allergen. G protein-coupled receptors are capable of responding to various stimuli, including gradients of chemoattractants needed for tissue-specific recruitment of proinflammatory leukocytes (114). Specifically, Gq-coupled receptors are expressed in many tissues/cells associated with allergic reactions, and significantly increased levels of Gαq protein has been detected following OVA challenge in the lungs of both rats and guinea pigs (115, 116). The most compelling data regarding the significance of the Gαq protein in the development of allergic airway inflammation has been produced from studies utilizing Gαq deficient mouse lines. These
Figure 1.5 IgE-dependent mast cell activation*. Uptake of allergen by dendritic cells results in the expansion of Th2-type cells resulting in the stimulation of B cells and production of antigen-specific IgE. The IgE binds to high affinity FcεRI receptors on mast cells. Allergen then binds to and causes cross-linking of IgE to the receptor, which results in mast cell activation. Finally the activated mast cell releases preformed and newly synthesized mediators. *Modified from (117)
Figure 1.6 Th2 mediated inflammation*. Presentation of allergens at the airway surface by dendritic cell results in Th2 cell differentiation and cytokine production (IL-4, 5 and 13) in atopic asthmatics. CD4+ T cells orchestrate the ensuing inflammatory response characterized by an influx of eosinophils into the airway spaces in response to cytokines. Eosinophils are recruited into the airway from bone marrow through several steps which are coordinated by Th2 cytokines. Eosinophils develop in the bone marrow from CD34+ progenitor cells (mediated by IL-5, IL-13, and GM-CSF) which are produced by CD4+ T cells. Once cells are committed to the eosinophil lineage, they move from the bone marrow into the vasculature under the guidance of IL-5. Their movement through the blood vessel walls into sites of Th2 mediated inflammation is controlled by IL-4 and 13. Once activated by IL-5 or eotaxin, eosinophils may contribute to the pathogenesis of allergic asthma by acting on various cells in the airway walls (including fibroblasts, smooth muscle cells, and epithelial cells) or secrete further Th2 cytokines. *Modified from (113).
animals clearly demonstrate significantly attenuated airway eosinophilia, as determined by both bronchoalveolar lavage fluid (BALF), cellularity, and histological examination of the lungs (118). A significant decrease in local (BALF) GM-CSF production was observed in the \( \Gamma_\alpha_q \) deficient animals, which was suggested to be the underlying mechanism behind the attenuated airway eosinophilia (118).

Once recruited to the inflamed tissue, eosinophils contribute to allergic symptoms through the release of granule proteins and pro-inflammatory mediators which act to increase vascular permeability, stimulate mucus production, and facilitate AHR. Eosinophils release granule proteins such as eosinophilic cationic protein (ECP) and eosinophil peroxidase (EPO), which are toxic to the respiratory epithelium (119), as well as major basic protein (MBP), which can induce mast cell activation and dysregulate vagal muscarinic receptor function (120). Inhibition of eosinophilia in a rat model of allergy using anti-PMN treatment indicates that eosinophils are important for the late-phase reaction of mucus secretion from epithelial goblet cells (71). The development of eosinophil-deficient mice has further defined the role of this leukocyte population in asthma models. Antigen challenge in eosinophil-deficient mice resulted in significantly reduced epithelial hypertrophy, goblet cell metaplasia/mucus accumulation, and AHR to the ACh analog methacholine (MCh) when compared to WT mice (121), linking eosinophils to these symptoms associated with asthma.

*Mast Cells*

Mast cells are the primary effector cells of acute allergic reactions. Mast cells play a critical role in the early phase of allergic airway disease, including airway obstruction and recruitment/activation of additional inflammatory cells. Many mediators
released from mast cells are capable of stimulating events that may contribute to asthmatic symptoms (Table 1.4). Release of preformed granules containing mediators such as histamine, serotonin, chymase, and tryptase, as well as synthesis and secretion of various lipid mediators such as prostaglandins and leukotrienes can generate airway obstruction by inducing smooth muscle constriction, vascular leakage, and mucus hypersecretion. Furthermore, mast cell-derived cytokines and chemokines, such as IL-4, IL-5, IL-6, LTB₄, PAF, TNF-α and GM-CSF can further perpetuate the inflammatory cascade through local recruitment and activation of eosinophils and lymphocytes.

Histamine has long been thought to be a mediator of acute reactions in humans. Histamine effects mediated through the H1 receptor include smooth muscle contraction, increased vascular permeability, prostaglandin generation, and activation of vagal reflexes. H2-mediated effects include increased lower airway mucus secretion, esophageal contraction, and inhibition of neutrophil activation (reviewed in (122). Furthermore, histamine has been found in the airways of asthmatic patients, along with increased number of degranulated mast cells. The level of histamine in BALF was found to correlate with the severity of asthma and AHR (123). Aside from humans, studies in guinea pigs suggest that mast cell degranulation enhances the excitability of sensory C-fibers through the activation of histamine H1 receptors (124). In the mouse, blockade of the H2 receptor was shown to attenuate AHR to MCh after OVA challenge (125). This differs in the common view that histamine induced bronchoconstriction is mediated exclusively through H1 receptors, but still points to a role of histamine in allergic airway constriction in the mouse. There has also been some contradiction as to whether
Table 1.4 Mast cell mediators, receptors, and function in allergy and asthma.*

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Receptor(s)</th>
<th>Receptor Location</th>
<th>Effect</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>H1</td>
<td>Epithelium, ASM, sensory nerves</td>
<td>ASM constriction, ↑ neuropeptide release</td>
<td>(6, 126)</td>
</tr>
<tr>
<td></td>
<td>H2</td>
<td>Epithelium, ASM, submucosal glands, immune cells</td>
<td>Inhibition of chemotaxis, elevation of cAMP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H3</td>
<td>Sensory nerves</td>
<td>↓ neuropeptide release</td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1</td>
<td>ASM, sensory nerves</td>
<td>Pulmonary vasoconstriction</td>
<td>(127-129)</td>
</tr>
<tr>
<td></td>
<td>5-HT2</td>
<td>Epithelium, ASM</td>
<td>ACh release, ASM constriction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HT3</td>
<td>Sensory nerves</td>
<td>Neural excitation</td>
<td></td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>DP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Epithelium, ASM</td>
<td>Bronchoconstriction, bronchodilation, vasodilation, eosinophil activation</td>
<td>(130-132)</td>
</tr>
<tr>
<td></td>
<td>DP&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>Inflammatory cell recruitment</td>
<td></td>
</tr>
<tr>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>B-LT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Leukocytes</td>
<td>Chemotactic activity for neutrophils and eosinophils</td>
<td>(131)</td>
</tr>
<tr>
<td>LTC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>CysLT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>ASM</td>
<td>Bronchoconstriction</td>
<td></td>
</tr>
<tr>
<td>Tryptase</td>
<td>PAR2</td>
<td>Epithelium, ASM, sensory nerves, mast cells</td>
<td>Triggers release of PGE&lt;sub&gt;2&lt;/sub&gt;, degrades VIP, reflex bronchoconstriction, sensitizes C-fibers</td>
<td>(133)</td>
</tr>
<tr>
<td>IL-4</td>
<td>IL-4R</td>
<td>Hematopoietic, endothelial, epithelial, and muscle cells</td>
<td>B-cell class switching; stimulating and maintaining Th2 cell proliferation; promotes mast cell aggregation</td>
<td>(134-136)</td>
</tr>
<tr>
<td>IL-5</td>
<td>IL-5R</td>
<td>Eosinophils</td>
<td>Activation and recruitment of eosinophils</td>
<td>(137)</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6R</td>
<td>T cells, B cells, nerve cells, epithelial cells</td>
<td>Promotes mucus production, sensitizes sensory nerves, tissue maintenance and remodeling, enhances effects of IL-4</td>
<td>(138-141)</td>
</tr>
<tr>
<td>PAF</td>
<td>PAF-R</td>
<td>platelets, eosinophils, neutrophils,</td>
<td>activates/recruits eosinophils, induces microvascular leakage and mucus secretion</td>
<td>(142-146)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNFR1</td>
<td>ASM, nerves, inflammatory cells</td>
<td>increase G&lt;sub&gt;i&lt;/sub&gt; and G&lt;sub&gt;q&lt;/sub&gt; alpha protein expression in ASM, sensitizes sensory nerves, induces histamine release from mast cells, recruits neutrophils and eosinophils</td>
<td>(138, 147-150)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNFR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>GM-CSFr</td>
<td>epithelium, monocytes, granulocytes, macrophages</td>
<td>Activates dendritic cells, eosinophil recruitment</td>
<td>(118, 151, 152)</td>
</tr>
</tbody>
</table>

*Mast cell mediators reviewed in (3).
exogenous histamine can cause airway constriction in the mouse. Using tracheostomized mechanically ventilated mice, Martin et al. (1988) could not detect airway constriction following iv histamine administration (153). However, aerosolized histamine was able to induce only peripheral airway resistance (measured as a decrease in dynamic compliance, Cdyn), with no central airway constriction using barometric plethysmography (154).

Serotonin has been shown to cause bronchoconstriction in many mammalian species (155). Levels of free serotonin in plasma have been shown to be increased in patients with asthma, and these levels correlated positively with clinical status and negatively with pulmonary function (156). Although exogenous serotonin fails to elicit bronchoconstriction in healthy or asthmatic human subjects (157), both tryptophan hydroxylase and serotonin have been measured in human mast cells (158). Additionally, serotonin has been reported to activate human airway smooth muscle ex vivo (159) and 5-HT receptor activation has been shown to facilitate cholinergic bronchoconstriction in human airways (160), which points to the possible involvement of serotonin in anaphylactic bronchoconstriction in man. Additionally, in mice, serotonin has been shown to contribute to nociceptive processing via a subset of afferent fibers that do not express SP, most likely RARs, through the 5-HT3 receptor (129). Furthermore, in human and guinea pig airways, 5-HT receptor activation causes depolarization of airway cholinergic nerve terminals via 5-HT3 receptors (160). Serotonin is also able to elicit a dose dependent increase of pulmonary RAR activity in the rabbit (161).

PGD2 is a bronchoconstricting agent in humans. When inhaled, PGD2 is 25-fold more potent as a bronchoconstrictor than histamine in non-asthmatic human subjects (162). In contrast, exogenously administered PGD2 fails to cause airway constriction in
mice. Highly purified human lung mast cells, as well as, human BALF mast cells produce PGD2. PGD2 has also been shown to be synthesized and released during IgE-dependent activation of mouse bone marrow derived mast cells (BMMCs) (163). However, the lack of significant benefit of nonselective cyclooxygenase inhibitors to treat asthmatic exacerbations (164) have led to the mast-cell derived prostanoids being largely discounted as significant mediators of bronchoconstriction in asthma.

Despite the disfavor of PGD2 as an asthmatic bronchoconstrictor, there has been a significant amount of evidence that points to the involvement of PGD2, through the DP2 receptor, in allergic inflammation, specifically in inflammatory cell recruitment (reviewed in (130). Administration of exogenous PGD2 into the airways of dogs results in increased inflammatory recruitment (165). In mice, administration of PGD2 increased OVA-mediated recruitment of eosinophils and CD4+ T cells into the lung (166), while inhibition of DP2 with the selective antagonist TM30089 reduced OVA-induced airway eosinophilia and mucus production (167). Furthermore, DP-deficient mice have reduced eosinophil and lymphocyte recruitment, as well as reduced cytokine expression compared to WT controls in an OVA-induced model of asthma (168), indicating that PGD2 is important for such responses.

Leukotrienes are potent constrictors of both human bronchi (169) and guinea pig lung (170). In humans, the leukotrienes appear to play a substantial role in asthmatic airway obstruction. Inhaled LTC4 and LTD4 are potent constrictors of human airways (171), and indirect evidence supports a model in which the release of this mediator by mast cells contributes to airway constriction during anaphylaxis. For example, in human PCLS, antigen induced airway constriction was attenuated after treatment with the
leukotriene receptor antagonist montelukast (172). Increased urinary cysLT metabolites are detected after challenge of asthmatics with inhaled allergen (173). Also, allergic asthmatics exhibit increased LTC4 in their BALF after antigen challenge (174). In addition, a multitude of pharmacological studies have suggested the involvement of leukotrienes in several features of human asthma, including airway obstruction (175-178).

Although there appears to be an important role for leukotrienes in asthma, it has been difficult to demonstrate the importance of this class of constricting agents in the mouse, specifically in constriction of ASM, because pulmonary responses following leukotriene administration in mice are weak at best. Reports regarding the ability of this mediator to induce changes in airway mechanics have been conflicting. Early studies by Martin et al. report no changes in airway mechanics after i.v. delivery of LTC4 or LTD4 (153). In contrast, Yang et al. reported changes in Penh and a small increase in lung resistance (Rl) after exposure of mice to LTD4, either inhaled or after i.v. administration, respectively (179). In addition LTD4 was capable of constricting mouse tracheal rings in a dose dependant manner. These changes were somewhat increased in mice over-expressing a human cysLT1R transgene.

Interestingly, when analyzing separate components of this pathway in mice, it appears to be fully intact and comparable to humans. Mouse mast cells are fully capable of leukotriene synthesis; LTC4 has been reported to be synthesized in mouse BMMCs and was shown to be released upon mast cell activation (180). Increased levels of LTC4 in BALF have been reported in both allergic asthmatics (174) and sensitized mice (181) after antigen challenge. Furthermore, characterization of the cysLT1 receptor in mice has
shown comparable binding affinities and mRNA expression in the lung to that of the human cysLT₁ receptor (182-184). Immunocytochemistry and *in situ* hybridization for the cysLT₁ receptor have confirmed its expression in both mouse (185) and human (186) airway smooth muscle, respectively. A second cysteinyl leukotriene receptor, cysLT₂, has also been characterized. Although cysLT₂ has proven to be involved in tracheal constriction in sheep (187) little to no expression of this receptor was detected in either the mouse (188) or human (189) lung. Finally, addition of LTD₄ or LTC₄ to mouse tracheal smooth muscle cells is able to elicit dose-dependent Ca²⁺ signaling (179), indicating that the mouse receptor is functional and able to respond to leukotrienes. In light of these studies, it is unclear why mice have such a minimal response to leukotrienes, as they appear to have the necessary components to induce leukotriene-dependent bronchoconstriction.

In addition to airway constriction, cysLTs may contribute to airway remodeling through their roles in inflammation and their direct effects on mesenchymal cells. In a model of asthma, repeated exposure to OVA in OVA-sensitized rats increased ASM mass, which was partially inhibited by the cysLT receptor antagonist MK-571 (190). Mice with allergen-induced chronic lung inflammation develop eosinophilia, goblet cell hyperplasia, increased thickness of ASM, and deposition of collagen beneath the epithelial cell layer. Pretreatment with the cysLT receptor antagonist montelukast significantly inhibited airway eosinophilia, hyperplasia of both goblet and smooth muscle cells, and subepithelial fibrosis (191), implicating leukotrienes in these aspects of the asthmatic condition.
The most abundant mediator found in human mucosal mast cells is tryptase, which can influence several airway processes including: airway fibrosis and extracellular matrix turnover, ASM and epithelial cell hyperplasia, airway inflammation, and alterations in bronchial tone. Tryptase can stimulate the release or IL-8, a granulocyte chemoattractant (192) and induce expression of mRNA for IL-1β, which may also be involved in recruitment of inflammatory cells (193). In addition, stimulation of human ASM cells with tryptase induced cytokine release and subsequently enhanced chemotaxis of mast cells to ASM (194). Tryptase contributes to airway remodeling through its ability to stimulate fibroblast proliferation and chemotaxis (195, 196), increase the synthesis and secretion of type 1 collagen (197), and increase the amount of collagenolytic activity in fibroblasts (197). Tryptase is also a known mitogen for dog tracheal smooth muscle cells (198, 199), as well as human ASM (200) and epithelial cells (192).

Although tryptase is typically involved in airway remodeling and inflammatory recruitment, it may also be involved in mast cell-mediated airway constriction through activation of protease-activated receptor (PAR) receptors. PAR2 receptors are found on the airway epithelium, ASM, mast cells, as well as on sensory neurons. Tryptase causes excitation of sensory neurons, which can lead to the release of neurotransmitters which can act to augment lung function (reviewed in (201)). While some of the peptides released, notably the bronchodilating peptide VIP and the vasodilator CGRP, may subsequently be rapidly cleaved by tryptase; bronchoconstricting tachykinins are resistant to inactivation by tryptase, shifting the overall response towards bronchoconstriction. In the mouse, exogenous administration of PAR2-activation peptide (PAR2-AP) results in
increases in airway pressure and lung water that is mediated through a reflex effect involving NK2 receptors (202), presumably through the release of NKA from sensory nerves.

**Lipid Mediators**

*Arachidonic acid pathway*

Eicosanoids are a family of lipid mediators that includes prostanoids (prostaglandins and thromboxane), leukotrienes, and HETES which are derived from phospholipase-released arachidonic acid (AA). The first step in the production of all eicosanoids is the phospholipase A2 (PLA2)-induced hydrolysis of the sn-2 position of membrane glycerophospholipids to release AA (203). PLA2 activity can be stimulated by a variety of components including: Fc-receptor activation, complement, immune complexes, and microorganisms. The expression and activity of various enzyme pathways within a given cell determines the eicosanoid produced from the released AA (Figure 1.7).

Prostaglandins are not stored, but synthesized de novo from membrane-released AA when cells are activated by cytokines, mechanical trauma, or other stimuli (reviewed in (131)). Briefly, released AA is metabolized into the intermediate prostaglandin PGH2 through the action of prostaglandin H synthase (PGHS; cyclooxygenase; COX), which exists in the two isoforms COX-1 and COX-2. Simplistically, COX-1 is responsible for constitutive production of prostaglandins, while COX-2 is involved in various inflammatory and induced productions. PGH2 is then further metabolized by downstream enzymes such as thromboxane synthase, PGF synthase, PGD synthase, and PGE synthase.
to generate thromboxane and various other prostaglandins. Leukotrienes are predominantly synthesized by inflammatory cells such as macrophages and mast cells. Activation of these cells initiates translocation of PLA2 to the nuclear envelope. Released AA is then transformed to LTA4 through the actions of 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP). LTA4 then transforms through either hydrolysis, conjugation with glutathione, or transcellular metabolism to generate LTB4, LTC4, LTD4, and LTE4 (reviewed in (131)).

**Thromboxane**

Thromboxane is well known for its role in platelet aggregation, vasoconstriction, and vascular smooth muscle proliferation; however, it is also capable of initiating ASM contraction (204-206). TXA2 has been implicated in inducing AHR in models of allergic inflammation (207), endotoxin models (208), and IL-8-mediated airway neutrophil accumulation (209). Urinary excretion of TXA2-derived products are shown to be markedly increased in subjects with severe acute asthma when compared to nonsmoking controls; however, antigen challenge in atopic volunteers did not significantly increase these products (210). This data implies that TXA2 may have biological actions in asthma in general, but may not contribute significantly to the allergic component of the disease.

All *in vivo* actions of TXA2 are mediated through the cell surface G protein-coupled thromboxane/prostanoid (Tp) receptor, and many of its physiological actions have been attributed to Gq-mediated activation of PLC and increases in intracellular calcium concentration. The Tp receptor is maintained at high levels in a variety of tissues including the thymus, lung, brain, and kidney (211).
Figure 1.7 Generation and metabolism of arachidonic acid.
Tp receptor expression has been reported on several cell types including ASM cells (212) and nodose ganglion neurons (213), where activation may lead to ASM constriction through either direct or indirect mechanisms, respectively. Considerable evidence has suggested that TXA2 mediates ASM constriction through neural mechanisms affecting ACh release (214-216). Recent studies in the mouse have demonstrated that the constrictor effect of TXA2 in both the naïve and inflamed airway is dependent on the activity of mAChR-mediated cholinergic pathways (28). A possible model suggested by these findings describes a situation whereby the release of ACh from the postsynaptic neurons is enhanced by TXA2 binding to presynaptic Tp receptors on the vagus nerve. Activation of these Tp receptors on the nerve or nerve terminals results in a substantial increase in ACh release and the subsequent increase in M3 mAChR-mediated ASM constriction. However, alternative scenarios can be envisioned, and further investigation is required to elucidate the mechanism of TXA2-mediated ASM constriction.

**Neural regulation**

*Sensory “efferent” neurotransmitters*

The release of neuromediators (**Table 1.5**) from airway nerves can elicit airway responses including bronchoconstriction/bronchodilation (**Figure 1.8**), increased neurotransmission, and neurogenic inflammation. Whereas immunologic inflammation results from the binding of antigen to antibody, neurogenic inflammation occurs when neuropeptides released from sensory nerves produce an inflammatory response characterized by microvascular leakage, vasodilation, mucus secretion, and
recruitment/activation of inflammatory cells. Other neuropeptides released from sensory nerves act as bronchodilators, and dysregulation or inhibition of their release may contribute to symptoms of asthma. Sensory nerve fibers also project into local airway ganglia, where release of neurotransmitters can affect neurotransmission of these nerves (217).

The tachykinins NKA and SP, which are localized to capsaicin-sensitive sensory nerves, can exert numerous effects on respiratory target cells. NKA potently constricts human ASM in vitro through NK₂ receptors, specifically in the small airways (218), suggesting that nerve-derived tachykinins may have a role in peripheral airway constriction whereas cholinergic effects dominate the central airways. Tachykinins released from sensory “efferent” nerves can also enhance neurotransmission through parasympathetic ganglia resulting in an increased release of ACh from the nerve ending (219). NK₁ and NK₂ receptors have been shown to be involved in the facilitation of AHR in a guinea pig model of allergic airway disease (220); although, using the same model, were found to not be involved in inflammatory cell recruitment (221). The NK₁/NK₂ selective antagonist AVE5883 also failed to reduce allergen-induced airway inflammation (222). However, SP, through NK₁ receptors, is involved in other aspects of neurogenic inflammation including plasma vasodilation and exudation, as well as submucosal secretion (219). Additional studies have shown that the NK₃ receptor is involved in inflammatory cell recruitment in OVA sensitized and challenged mice (223).

CGRP is expressed and co-localized with tachykinins in sensory nerve fibers. CGRP is involved in vasodilation (224) and causes dose-dependent relaxation of human pulmonary arteries (225). In addition, this protein also enhances protein extravasation
<table>
<thead>
<tr>
<th>Transmitter</th>
<th>Receptor(s)</th>
<th>Location</th>
<th>Effect</th>
<th>Reference(s)</th>
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</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Nicotinic (N)</td>
<td>Epithelium, PNS ganglion, PNS preganglionic synapse, sensory nerves</td>
<td>Activation of postganglionic nerve, feedback inhibition, ↑ neuropeptide release</td>
<td>(30-32, 226, 227)</td>
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<tr>
<td></td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>PNS ganglion, submucosal glands</td>
<td>Facilitates ACh release, ↑ mucus production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ASM, PNS post ganglionic synapse</td>
<td>Feedback inhibition of ACh release</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M&lt;sub&gt;3&lt;/sub&gt;</td>
<td>ASM, submucosal glands</td>
<td>ASM constriction, ↑ mucus production</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>α&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Throughout lung, ASM, sympathetic nerves, parasympathetic nerves</td>
<td>Bronchoconstriction, inhibit release of NE</td>
<td>(36, 38, 40)</td>
</tr>
<tr>
<td></td>
<td>β&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
<td>Bronchodilation, enhance release of NE and ACh</td>
<td></td>
</tr>
<tr>
<td>NKA</td>
<td>NK&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prejunctional, ASM</td>
<td>Cholinergic reflexes, bronchoconstriction</td>
<td>(24, 25, 218)</td>
</tr>
<tr>
<td>CGRP</td>
<td>CRLR+RAMP1</td>
<td>Lung, dendritic cells</td>
<td>Vasodilation</td>
<td>(228)</td>
</tr>
<tr>
<td>VIP</td>
<td>VPAC1</td>
<td>CNS, lung, T-lymphocytes</td>
<td>↓ neuropeptide release, inhibits release of ACh from cholinergic nerves, anti-inflammatory</td>
<td>(226)</td>
</tr>
<tr>
<td></td>
<td>VPAC2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAC1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO (nNOS)</td>
<td>sGC</td>
<td>ASM</td>
<td>ASM constriction, ASM relaxation, Inhibition of ASM proliferation</td>
<td>(229-231)</td>
</tr>
<tr>
<td>NPY</td>
<td>Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Sensory nerves, postganglionic cholinergic nerves, prejunctional sympathetic nerves</td>
<td>↓ neuropeptide release, inhibit adenylyl cyclase</td>
<td>(226)</td>
</tr>
<tr>
<td>SP</td>
<td>NK&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Mast cells, parasympathetic ganglion, blood vessels, airway epithelium, submucosal glands</td>
<td>Membrane ruffling/activation, facilitatory effects on cholinergic neurotransmission, vasodilation, epithelium goblet cell secretion</td>
<td>(24, 219, 232, 233)</td>
</tr>
</tbody>
</table>
Figure 1.8 Neural control of ASM. Release of neurotransmitters from airway nerves augments bronchial tone.
induced by tachykinins (234). Although CGRP appears to have no direct effect on ASM tone, it has been shown to protect against SP-induced airway constriction in human and guinea pig airways. However, this bronchoprotective property was impaired during OVA-induced inflammation (235), suggesting that the ability of CGRP to regulate ASM tone may be altered in inflammatory diseases. In support of this theory, CGRP levels are depleted in the airways of mice with allergic airway inflammation despite having no difference in the overall density of nerve fibers between the control and sensitized/challenged groups (236). Furthermore, treatment of OVA-sensitized mice with exogenous CGRP prevented the development of OVA-induced AHR to MCh, indicating the possibility of CGRP therapy in the treatment/prevention of AHR.

VIP is a cotransmitter, together with nitric oxide (NO), of airway relaxation, as well as a modulator of various immune and inflammatory responses. VIP and NO work synergistically to enhance ASM relaxation through the combined activation of adenylate cyclase and guanylyl cyclase, respectively (237). Mice deficient in VIP exhibit spontaneous AHR to MCh challenge, as well as increased airway inflammation (eosinophils) and levels of inflammatory cytokines in BALF. Theses phenotypes are partially reversible by exogenous VIP treatment (237), suggesting that endogenous VIP helps protect the airways and altered expression or dysregulation of this peptide may contribute to asthmatic conditions. VIP has also been shown to suppress ASM proliferation (238), a key component to airway remodeling seen in chronic asthma. In addition, inflammation and mast cell activation and degranulation in allergic asthma can influence the activity of neuropeptides. For example, tryptase released by mast cells actively degrades VIP, effectively inhibiting its dilation effect (reviewed in (226)).
addition to being released from sensory nerves, VIP can also be released by immune
cells, where it’s involved in many biological functions, namely immunomodulation,
predominantly acting as a potent anti-inflammatory factor and suppressive agent for Th1
responses (239).

NO is a short-lived molecule that is produced by 3 nitric oxide synthases (NOSs).
Endothelial NOS (eNOS, NOS3) and neural NOS (nNOS, NOS1) are constitutively
expressed, whereas NOS found in other cell types like macrophages, epithelial cells, and
neutrophils is inducible (iNOS, NOS2). The role of NO in asthma is controversial.
Although NO concentrations appear to be elevated in exhaled air from asthmatics (240),
there are contradicting results reported on the ability of NO to act as a bronchodilator
(241, 242). Studies in mice with targeted deletions in the genes for these NOS isoforms
have shown a link between nNOS and airway responsiveness in a murine model of
allergic asthma; mice deficient in nNOS exhibited decreased AHR and exhaled NO
concentrations with no difference in inflammatory recruitment or IgE production (229,
243). These studies imply that NO (produced through nNOS expression) increases or
positively regulates AHR. However, another study in mice has shown that increased
NOS2 (iNOS) expression helped to protect against AHR (244), suggesting that NO acts
to relax the airways. Finally, yet another study in mice revealed that NO derived from
eNOS is involved in regulating bronchial reactivity; mice lacking eNOS were
hyperresponsive to MCh challenge while inhibition of iNOS had no effect (245). Similar
to the conflicting results found in human studies, it is clear that the role of NO and its
specific synthases in the mouse is still controversial.
Additionally, it has also been proposed that the NO “receptor” may be dysregulated in asthma. NO activates soluble guanylyl cyclase (sGC), which catalyzes the conversion of GTP to cGMP. cGMP is able to induce smooth muscle relaxation by lowering intracellular Ca\(^{2+}\) concentration. Despite the high levels of NO present in the asthmatic airway (240) capable of activating sGC and relaxing ASM, airway tone is significantly elevated in asthmatics. In an animal model of asthma, mice sensitized and challenged with OVA, while exhibiting many characteristics of asthma, also displayed reduced mRNA and protein levels of the sGC subunits (230). This study also showed that treatment of naïve mice with a sGC inhibitor induced spontaneous AHR to MCh, suggesting that inhibition of sGC could contribute to AHR seen in asthma.

**Dysregulation of neural activity**

Sensory and parasympathetic nerves extending into the lungs may contribute significantly to airway obstruction and hyperreactivity in the diseased lung. As discussed above, neuropeptides released from nerves can elicit effects on further neurotransmission, mucus secretion, ASM, epithelial cells, and inflammatory cells. Furthermore, dysregulation of these neurotransmitters, airway receptors (Figure 1.9), or the response of nerves during a disease state may lead to an alteration of normal airway tone or an increase/decrease in nerve excitability and signal transduction. One hypothesis is that asthma represents an imbalance or dysregulation of the autonomic nervous system.

There is evidence that suggests abnormalities in β-adrenergic and/or muscarinic receptor function occur in asthma (26), resulting in AHR. Probably the best example of this is shown in studies conducted by Fryer and colleagues on dysfunctional M\(_2\) mAChRs in guinea pig models of asthma (reviewed in (246)). Release of ACh from
Figure 1.9 Receptor localization within the airway. Cartoon representation of mast cell mediator and neurotransmitter receptor localization within the lung.
parasympathetic nerves is normally regulated by prejunctional M₂ mAChRs, providing a negative feedback mechanism which inhibits further release of ACh (247). They showed that guinea pigs sensitized and challenged with antigen exhibited enhanced release of ACh from vagus nerves (248), and this release ultimately lead to hyperresponsiveness. Furthermore, M₂ receptors were shown to be dysfunctional in this model, as the selective agonist pilocarpine no longer inhibited vagally induced bronchoconstriction (248), providing evidence that loss of neuronal M₂ function may be a mechanism for airway hyperresponsiveness. These findings were also reported in studies of *in vitro* mouse tracheas (249). As in animal models of asthma, there is evidence of M₂ dysfunction in humans with asthma. Human parasympathetic nerves from patients with asthma show an enhanced release of ACh in response to electrical field stimulation *in vitro* compared to controls (250). Furthermore, M₂ agonists failed to inhibit bronchoconstriction in patients with mild asthma (251).

In addition to hyperresponsiveness, eosinophils are also present in the lung after antigen challenge in sensitized animals (252-255), as well as in patients with asthma, where they appear to be selectively recruited to airway nerves (256). Eosinophils release the charged protein MBP, which can act as an allosteric antagonist to the M₂ receptor (120), which is sensitive to inhibition by positively charged proteins. Inhibition of eosinophil migration into the lungs also appears to inhibit hyperresponsiveness (257). Furthermore, inhibition of MBP successfully inhibited AHR in antigen challenged guinea pigs (252) despite the continued presence of eosinophils in the airways and fully functional M₃ mAChRs. These data effectively link the loss of M₂ mAChR function via
release of eosinophil major basic protein as a mechanism for AHR in antigen challenged animals.

Inflammatory cell and neural interactions

Inflammatory cells present in the diseased lung have been shown to interact with nerves. Direct contact between DCs and peripheral sensory nerves has been documented in the skin, liver, and lung (258-260). In the airways, DCs are contacted by SP-containing fibers, and their recruitment to the airways after antigen challenge is dependent on SP (259). Quantitative mapping of airway nerves and mucosal DCs has shown many contact points between DCs and sensory fibers containing both CGRP and SP, including neuroepithelial bodies and airway ganglia, and the percentage of co-localization showed an increase in inflamed airways (261).

Some airway nerves are capable of recruiting and binding eosinophils. Primary cultures of airway parasympathetic nerves express eotaxin (262), as well as adhesion molecules capable of binding eosinophils (263). As detailed above; eosinophils are selectively recruited to airway nerves in both a guinea pig model of asthma, as well as in human patients with asthma (256). Eosinophil-nerve interactions were shown to influence the function of prejunctional M₂ mAChRs, resulting in a loss of feedback inhibition through this receptor. M₂ receptor dysfunction results in an increased release of ACh from parasympathetic nerves and subsequent enhanced activation of M₃ mAChRs on ASM, resulting in AHR.

In addition to DCs and eosinophils, mast cells have been shown to be closely associated, or even attached to neurons *in vitro*. For example, nerve-mast cell interaction has been reported in co-cultures of murine BMMCs and superior cervical ganglion
neurons (SCGs). In this study, it was shown that mast cells can attach to neurites through the homophilic binding of synaptic cell adhesion molecule located on both the neurite and the mast cell (264). Furthermore, mast cells have been shown to be located near the parasympathetic ganglia neurons in *ex vivo* guinea pig lungs (265).

It is known that cross linking of high affinity IgE receptors on mast cells by allergen causes degranulation of the mast cells and the subsequent release of proinflammatory mediators that can induce smooth muscle constriction (3, 266, 267). However, these inflammatory mediators may also influence the release of neurotransmitters via activation of sensory nerves or may increase the release of ACh from cholinergic nerve terminals (226). For example, degranulation of purified human lung mast cells enhanced the excitability of rabbit visceral sensory C-fibers *in vitro* (268). Furthermore, antigenic stimulation of sensitized guinea pig bronchi caused increased sensitivity of sensory nerve endings and parasympathetic ganglion neurons in the airway wall, as well as degranulation of mast cells surrounding and within the bronchial parasympathetic ganglia (269).

Receptors for the mast cell mediators histamine (126), serotonin (127-129), PGD$_2$ (132) and tryptase (133) have been functionally shown to be located on nerves (*Figure 1.9*). Therefore, mast cell derived mediators may modulate neural activity and/or excitability during the immune response. One of the most dramatic neural changes that occur in response to inflammation is the allergen-induced synthesis of SP in large-diameter A-fibers. Although SP is seldom expressed in A-fiber vagal nerves, OVA inhalation in sensitized guinea pigs results in a significant increase in SP expression in large-diameter nodose ganglion neurons that innervate the lungs (270). Mast cell
mediators may also have an effect on neurotransmitters released from sensory nerves. For example, VIP released by airway nerves typically has a bronchodilator effect on human and animal airways, possibly through its ability to inhibit ACh release from post ganglionic parasympathetic nerves. It is highly abundant in the upper and lower respiratory tract of humans (271, 272). However, tryptase released by mast cells actively degrades VIP, effectively inhibiting its dilation effect (reviewed in (226)).

**Summary**

The overall aim of this dissertation was to assess the mechanisms through which inflammatory cell mediators interact with different cell types within the lung to induce airway constriction. The first aim of this work focuses on assessing the mechanism through which mast cell activation contributes to anaphylactic airway constriction in the mouse. Although it is known that mast cells are intimately involved in the anaphylactic response, the mechanism through which airway constriction is initiated is poorly defined. This question is addressed using genetic, pharmacological, and surgical approaches in conjunction with *in vivo* assessment of airway mechanics in the mouse. The second aim of this work directly analyzes C57BL/6 Kit<sup>Wsh/Wsh</sup> (Wsh) mast-cell deficient mice as a tool for studying mast cell function within the lung. Due to their fertility, lack of anemia, and C57BL/6 background, Wsh mice have become a common research tool in the mast cell field. However, the usefulness of these mice as a model of mast cell function in the lung, specifically in the context of airway constriction, is not well defined. To address this aim, we utilized different methods of mast cell reconstitution in these mice and analyzed the ability of each method to repopulate the lung with mast cells, as well as
recapitulate anaphylactic responses including antigen-mediated bronchoconstriction. The third research aim utilizes a genetic approach to define the mechanism through which the lipid-mediator TXA$_2$ contributes to airway reactivity. To address this aim, we generated mice carrying a Tp receptor locus that is sensitive to disruption by cre recombinase. By breeding these mice to animals that express cre driven by either the nestin or SM22 promoter, mice were generated which lack Tp in neurons or smooth muscle cells, respectively. The in vivo airway mechanics of the resultant tissue-specific Tp-deficient mice were then characterized.

**Organization of the dissertation**

Chapter 2 of this dissertation introduces a mechanism through which mast cell-derived serotonin initiates anaphylactic bronchoconstriction through the cooperation of a neural pathway. Chapter 3 introduces mast cell reconstitution of Wsh mast-cell deficient mice, and describes a detailed analysis of the ability of different reconstitution methods to introduce mast cells into the lung and to recapitulate antigen-mediated bronchoconstriction. Chapter 4 further defines M$_3$ mAChR-dependent TXA$_2$-mediated ASM constriction using tissue-specific Tp receptor deficient lines which lack functional Tp receptors throughout the nervous system or on smooth muscle cells. Together, the work presented in these chapters describes the interactions of neuronal and smooth muscle cells together with inflammatory cell mediators in orchestrating airway reactivity in mouse models of allergy and asthma. Finally, Chapter 5 provides a cumulative discussion on mechanisms of airway constriction in the mouse, as well as the use of the mouse as a model of human disease.
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CHAPTER 2

Co-operation between mast cells and neurons is essential for antigen-mediated bronchoconstriction

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Abstract

Mast cells are important sentinels guarding the interface between the environment and the body: a breach in the integrity of this interface can lead to the release of a plethora of mediators which engage the foreign agent, recruit leukocytes, and initiate adaptive physiological changes in the organism. While these capabilities make mast cells critical players in immune defense, it also makes them important contributors to the pathogenesis of diseases such as asthma. Mast cell mediators induce dramatic changes in smooth muscle physiology, and the expression of receptors for these factors by smooth muscle suggests that they act directly to initiate constriction. Contrary to this view, we show here that mast cell-mediated bronchoconstriction is observed only in animals with intact innervation of the lung and that serotonin release alone is required for this action. While ablation of sensory neurons does not limit bronchoconstriction, constriction after antigen challenge is absent in mice in which the cholinergic pathways are compromised. Linking mast cell function to the cholinergic system likely provides an important means of modulating the function of these resident immune cells to physiology of the lung, but may also provide a safeguard against life-threatening anaphylaxis during mast cell degranulation.
Introduction

IgE-dependent mast cell activation is the central mechanism underlying immediate allergic reaction (1). Cross-linking of IgE bound by Fc receptors on mast cells leads to release of preformed mediators stored in the mast cell granules, as well as, production of lipid mediators and up-regulation of cytokine synthesis (1). These mediators can profoundly influence the physiology of the organism and thus systemic release of mast cell mediators can result in life threatening anaphylaxis. Mast cells can also contribute to chronic inflammatory diseases, including asthma, where activation by environmental allergens contributes to inflammation and reversible airway constriction, a hallmark of this disease. Consistent with a role for mast cells in this aspect of asthma, an increase in airway mast cells has been reported in asthmatic patients compared to healthy individuals (2).

The mechanism(s) through which mast cell mediators contribute to the airway obstruction is not yet completely understood. However, airway smooth muscle (ASM) expresses many receptors for mediators produced by mast cells (3). Thus, in the simplest model, mast cell mediators, including cysteinyl leukotrienes (cysLTs), histamine and serotonin (5-HT) act directly on smooth muscle, triggering increases in intracellular Ca\textsuperscript{2+} followed by assembly of the contractile apparatus. Direct ability of several mast cell mediators to elicit Ca\textsuperscript{2+} flux in muscle cells and constriction of tracheal rings further supports this model (4-6).

In a number of organ systems, close interaction of mast cells with both sensory and parasympathetic neurons has been demonstrated, suggesting that mast cells may induce bronchoconstriction by altering the activity of the neuronal pathways which
function to determine ASM tone. For example, histamine released by mast cells is reported to stimulate parasympathetic neurons in the guinea pig heart (7), while antigen challenge in sensitized rats results in the activation of afferent nerve fibers in the gut mediated by both histamine and serotonin (8). Consistent with this, mucosal mast cell mediators from patients with irritable bowl syndrome were reported to excite rat nociceptive visceral sensory nerves (9). A number of studies suggest that this relationship is also present in other organs including the lung. For example it has been reported that substance P containing nerves in the rat trachea interact with mast cells to cause antigen-specific, and dependent, changes in lung solute clearance and epithelial chloride ion secretion (10-12).

In humans, the cholinergic parasympathetic nervous system represents the predominate bronchoconstrictor pathway in the airways. Efferent cholinergic nerve fibers run through the vagus nerve and synapse in small ganglia within the airway wall. These release acetylcholine (ACh), which binds nicotinic acetylcholine receptors (nAChRs) on the parasympathetic ganglion, which activates postganglionic fibers innervating ASM and submucosal glands. These postganglionic fibers release ACh, which binds to muscarinic acetylcholine receptors (mAChRs) directly on ASM. The activity of the parasympathetic pathway is modulated by sensory neurons present throughout the airway. Supporting a role for the mast cell interaction with the autonomic nervous system, mast cells have been shown to be closely associated with airway nerves (13), and receptors for many mast cells mediators, including histamine and serotonin, are expressed by neurons (14-16). In the studies detailed here we demonstrate that in allergic
mice, antigen challenge induces constriction of the central airways, and this constriction is dependant not only on mast cells but also on an intact parasympathetic system.

Methods

Experimental animals. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of North Carolina at Chapel Hill. Mast cell deficient C57BL/6 KitW-/-, PAR2-/- (17), C57BL/6J, and BALB/c mice were purchased from Jackson Laboratory. Mice deficient in 5-lipoxygenase (5LO-/-) were generated as previously described (18). M1 and M3 mAChR-deficient mice and their controls were bred as previously described (19, 20). The generation of congenic 5HTT-deficient mice has been previously described (21). All experiments were carried out using 8-12 week old mice, with the exception of the 5-HTT mice. Both control and experimental animals were 6 months of age.

Airway measurements in intubated mice. Mechanical ventilation and airway measurements were carried out as previously described (22). After baseline measurements, serotonin (6.25 µg/ml, Sigma Chemical) was administered i.v. through a jugular catheter in increasing doses (0.3, 0.6, and 1.2 pg/ml). Single doses of DNP (250 µl, 5 mg/ml, Sigma Chemical) or OVA (50 µl, 10 mg/ml, Grade V, Sigma Chemical) were also delivered i.v. through a jugular catheter. After administration, airway mechanics were determined using the Forced Oscillatory Technique (FOT) every 10 s for 3 min. The resultant pressure and flow data were fit to a constant phase model as
previously described (23). Similar to other studies assessing FOT, we confined our
analysis to $R_{aw}$ ($R_n$; Newtonian resistance), which assesses the flow resistance of the
conducting airways, and $G$ (tissue damping), which reflects tissue resistance in the
peripheral airways (24). Total lung resistance ($R_L$) was calculated from the FOT data by
adding tissue resistance ($R_{ti}$) to $R_{aw}$ as previously described (24, 25). Where $R_{ti}$ is given
by:

$$R_{ti} (f) = \frac{G}{(2\pi f)^\alpha}$$

where $f$ is the frequency in Hz and $\alpha = \frac{2}{\pi} \tan^{-1}(H/G)$. All data are represented as
percent baseline $R_L$, $R_{aw}$ or $G$.

To determine the contribution of various mediators and/or receptors in airway
responses, mice received a pre-treatment i.p. injection of pharmacological antagonist
before exogenous administration of mediators or antigen. To assess the role of mACHRs
and nACHRs to airway responses, mice received a pre-treatment i.p. injection of atropine
sulfate (10µM/kg, American Pharmaceutical Partners) or mecamylamine (4mg/kg, Sigma
Chemical), respectively. To assess the role of prostaglandins in IgE-dependent
anaphylactic airway constriction, mice were pretreated with 10 mg/kg indomethacin (1
mg/ml, i.p., Sigma Chemical) 1 hour prior to airway measurements. To determine the
efficacy of prostaglandin depletion, levels of PGD$_2$ were measured by EIA (Assay
designs) from harvested gut samples. To determine the contribution of HR1 and HR2 to
airway responses, passively sensitized mice received a pre-treatment i.p. injection of
pyrilamine (40mg/kg, Sigma Chemical) or cimetidine (25mg/kg, Sigma Chemical),
respectively. Methiothepin (2mg/kg, i.p.), ketanserin (12 mg/kg, i.p.), and ondansetron (1
mg/kg, i.p.) were administered to block serotonin receptors. Methiothepin is a
nonspecific antagonist, whereas ketanserin and ondansetron are specific antagonists for 5-HT$_{2A}$ and 5-HT$_3$ receptors, respectively.

**Anaphylaxis protocols.** *IgE-dependent passive anaphylaxis:* Mice were sensitized with 20 µg mouse monoclonal anti-DNP IgE (1.0 mg/ml, Sigma Chemical; 200 µl total volume in saline) or an equal volume PBS i.v. through the tail vein. Approximately 19-24 hours after anti-DNP IgE injection, mice were anesthetized, catheterized, tracheostomized and mechanically ventilated as described above. Immediately following the baseline measurements, 0.25 ml of 5 mg/ml DNP (or an equal volume of PBS) was injected into the jugular and airway parameters were measured every 10 s for 3 min immediately following anaphylaxis induction. Bar graphs represent the area under the curve.

*Active systemic anaphylaxis to OVA:* Mice received a single i.p. injection of 0.2 ml sterile 0.9% NaCl containing 20 µg OVA emulsified in 2.25 mg aluminum hydroxide gel (Sigma Chemical). 18-21 days after OVA immunization, mice were anesthetized, placed on a ventilator, challenged with a single i.v. infusion (via a jugular catheter) of 500 µg of OVA in 50 µl of sterile 0.9% NaCl and airway measurements were taken every 10 s for 3 min (26). Control mice were challenged with an equal volume of sterile 0.9% NaCl. Bar graphs represent the area under the curve.

*Acute anaphylaxis to OVA following induction of allergic airway disease:* Mice were sensitized by i.p. injection of 20 µg OVA emulsified in 2.25 mg aluminum hydroxide gel in a total volume of 200 µl on days 1 and 14. Mice were challenged (45 min) via the airways with OVA (1% in saline) for 5 days (days 21 – 25) using an ultrasonic nebulizer (DeVillbiss Health Care, Somerset, PA). On day 26 mice were
anesthetized, placed on a ventilator, challenged with a single iv infusion (via a jugular catheter) of 500 µg of OVA in 50 µl of sterile 0.9 % NaCl. Airway measurements were taken every 10 s for 3 min. Bar graphs represent the area under the curve.

After airway measurements, mice were sacrificed and 0.5-1.0 ml of blood was collected by cardiac puncture. Following coagulation, serum was collected. Total IgE levels in the serum were measured by ELISA (R&D systems). BAL was performed five times with 1.0 ml of sterile HBSS each time. Cells present in the BAL were determined using a hemocytometer. A differential cell count was conducted on a cytospin prepared from 150 µl of BAL fluid and stained with Diff-Quik solution (Sigma Chemical). The remaining BAL fluid was centrifuged to remove cells. Histamine levels were measured in the cell-free supernatant by ELISA (Beckman Coulter).

**Histology.** For histopathologic examination, lungs were fixed by inflation (20 cm pressure) and immersion in 10% formalin. To evaluate airway eosinophilia, fixed lung slices were subjected to hematoxylin and eosin (H & E) staining. H&E stained lung slices were digitally imaged midway along the length of the main axial airway, as well as, at both ends of the same airway. Each of the three images was then divided into 4 quadrants and the levels of inflammation in each quadrant were semi quantitatively assessed based on a score of 0 (no inflammation) to 3 (severe inflammation). Scores from all quadrants for each of the 3 pictures representing 1 airway were then averaged together to represent the average H&E score of that airway (27). To assess goblet cell hyperplasia, serial sections of the left lobes of the lungs that yield maximum longitudinal visualization of the intrapulmonary main axial airway were analyzed following Alcian-blue/periodic acid-schiff reaction (PAS) staining. To avoid bias for a certain region, and
to consistently view the identical region in all slides, a 2-mm length of airway, located midway along the length of the main axial airway, was digitally imaged. Using ImageJ software (NIH, National Technical Information Service, Springfield, VA), the area and length of the PAS/AB-stained region in the sections were measured and the data expressed as the mean volume density \( V_s = n l / m m^2 \) basal lamina + SEM of PAS/Abstained material within the epithelium) as previously described (28).

**Inhibition of serotonin with 4-Chloro-DL-phenylalanine (PCPA).** Mice received a single i.p. injection of 0.2 ml sterile 0.9% NaCl containing 20 µg OVA emulsified in 2.25 mg aluminum hydroxide gel. 18-21 days after OVA immunization, mice were treated with PCPA (150 mg/kg, i.p.; 25 mg/ml in 1N HCl) and immediately challenged with 250 µg of OVA in 50 µl of sterile 0.9% NaCl (i.v.) to deplete serotonin stored in mast cells. 24 hours later, mice received a second treatment of PCPA (150 mg/kg, i.p.) and were then anesthetized, placed on a ventilator, and challenged with a single i.v. infusion (via a jugular catheter) of 500 µg of OVA in 50 µl of sterile 0.9% NaCl and airway measurements taken every 10 s for 3 min. Control mice were challenged with an equal volume of sterile 0.9% NaCl.

After airway measurements, mice were sacrificed and 0.5-1.0 ml of blood collected by cardiac puncture with a syringe containing 20 µl 100 U/ml heparin. The blood was then centrifuged at 12000 rpm for 5 min to collect plasma. Serotonin levels were measured in the plasma by ELISA (Beckman Coulter).

**Sensory ablation.** *Adults:* Capsaicin was administered to ablate sensory neurons as previously described (29, 30). For pretreatment, mice received a s.c. dose of 25 mg/kg capsaicin at 0 hrs and a second s.c. dose of 75mg/kg capsaicin at 24 hrs (5 and 15 mg/ml
capsaicin dissolved in 1:1:8 ethanol:Tween80:saline, respectively). To minimize the respiratory effects associated with capsaicin injection, animals were first anesthetized with avertin (250 mg/kg, i.p.) and then treated with 10 mg/kg theophylline (s.c., 5 mg/ml in distilled water, Sigma Chemical) and 0.1 mg/kg terbutaline (i.p., 0.05 mg/ml in saline, Sigma Chemical). Airway assessment was conducted 10-12 days after the final capsaicin treatment.

**Neonates:** Pups were injected with 50 mg/kg capsaicin (15 mg/ml, s.c.) at day 2 to 3 after birth to degrade sensory neurons. Animals were aged to 8 weeks of age before conducting studies.

To check for effectiveness of the treatment, a drop of 0.1 mg/ml capsaicin was instilled into one eye of each mouse and wiping movements were counted as previously described (31, 32). For substance P measurements, lungs were lavaged with 0.4 ml chilled PBS containing 500 KIU/ml aprotinin (Sigma Chemical). Substance P was measured from BAL by ELISA (R&D systems).

**Vagotomy.** A surgical vagotomy was conducted to assess the contribution of intact parasympathetic innervation as previously described (33). Briefly, the right and left vagus nerves running parallel to the trachea were isolated and a piece of surgical string was passed underneath to facilitate cutting during mechanical ventilation. In experimental animals, basal lung mechanics were established before severing of both vagus nerves. Post vagotomy baseline was then reassessed prior to i.v. challenge. Control animals received a sham operation which involved simply lifting and releasing of the surgical string along with the nerve fibers.
**MCh treatment in vagotomized mice.** Anesthetized, paralyzed, vagotomized, mechanically ventilated mice were treated with i.v. MCh (20 µg/ml, Sigma Chemical) administered through a jugular catheter in increasing doses (50 µl, 100 µl, and 200 µl) or an aerosolized dose response of MCh (12.5-50 mg/ml) delivered via a nebulizer through a side port in the ventilator circuit for 30 s at a rate of 200 breaths/min with a tidal volume of 0.15 ml. Immediately following the aerosol challenge, the nebulizer was isolated from the inspiratory circuit and the original mechanical ventilation was resumed.

**Statistical analysis.** Data are represented as means ± SEM. Analysis Of Variance (ANOVA) followed by Tukey-Kramer Honestly Significant Difference for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by Student’s two-tailed t-test. A $P$ value of <0.05 was considered statistically significant.

**Results**

**Change in airway resistance during anaphylaxis is dependant on mast cells, primarily those located in the central airways.**

We first determined the contribution of mast cells to changes in airway mechanics. Previous studies have shown that in the mouse, antigen treatment following sensitization results in rapid decrease in lung conductance ($G_L$) (26), which is the inverse of lung resistance ($R_L$). Similarly, we found that sensitization of wild-type C57BL/6 (B6) mice with OVA plus adjuvant, followed by exposure to antigen, resulted in a rapid and dramatic increase in $R_L$ (Figure 2.1A). Similar immunization and challenge of C57BL/6 $Kit^{W-sh}/Kit^{W-sh}$ (Wsh/Wsh) mice, which lack mast cells, failed to induce changes in lung
mechanics, indicating that this response was dependent on the normal development of this cell population (Figure 2.1B). To verify that the difference in R_L was not due to a reduction in overall IgE production in the mast cell deficient animals, we compared total serum IgE levels in the B6 and Wsh/Wsh congenic pairs (Figure 2.1C). There was no difference in IgE production between the two groups.

Mast cells are located throughout the airways including the trachea, bronchus, and sporadically in the parenchyma (34), yet it is unclear whether all of these cells contribute to changes in lung physiology during anaphylaxis. To examine this point more closely, we monitored changes in airway mechanics after sensitization and exposure to OVA using FOT. Interestingly, using this method we found that the changes in lung mechanics were largely limited to a parameter termed ‘airway resistance’ or R_aw, which a number of theoretical and experimental studies suggest is sensitive primarily to changes in the central airways (35). As can be seen in figure 2.1D, antigen induced a significant and robust change in R_aw in B6 mice previously sensitized to OVA plus adjuvant and little to no change in G, a parameter termed ‘tissue damping’ which is associated with changes in the distal lung. In contrast, no change in R_aw was observed in mast cell deficient Wsh/Wsh mice after antigen challenge or in mice treated with vehicle (Figure 2.1E). To further verify these findings, we examined changes in airway mechanics in a mouse model of passive anaphylaxis. Mice received i.v. monoclonal anti-DNP IgE, and the following day, after establishment of baseline lung mechanics, mast cell degranulation was induced by i.v. delivery of antigen (DNP). DNP induced similar increases in R_aw as those seen during active anaphylaxis to OVA (data not shown). Unlike antigen induced constriction, airway constriction induced with methacholine (MCh), a stable
acetylcholine analog, resulted in both changes in $R_{\text{aw}}$ and $G$ consistent with both changes in the central and distal airways in response to this neurotransmitter (Figure 2.1F). Similar responses to both antigen and MCh challenge were also seen in the BALB/c and 129/SvEv strains (data not shown). This suggests that although the ability of methacholine to induce airflow obstruction is distributed throughout the lung, mast cell degranulation results in only central airway constriction, despite the presence of these cells in peripheral airways.

We next asked whether this is also the case in mice with inflamed airways, particularly in those with allergic lung disease. To do this we induced allergic lung disease in B6 and Wsh/Wsh mice by sensitization and challenge with OVA using an immunization protocol previously shown to induce similar levels of inflammation in both lines (36). As expected, the absence of mast cells in the Wsh/Wsh mice did not significantly alter any of the disease parameters including IgE production, cellularity of the BAL fluid, mucus cell hyperplasia or the induction of the Th2 pathway (Figure 2.2A-E). Twenty-four hours after the last OVA challenge, changes in airway resistance in response to antigen were measured using FOT, monitoring both $R_{\text{aw}}$ and $G$ changes in lung mechanics in the OVA treated mice. Despite robust inflammation in the central and distal lung, changes in airway mechanics were limited to parameters associated with the central airways. A significant increase in $R_{\text{aw}}$ was observed; however, no significant increase in $G$ was observed after infusion of antigen into mice with inflamed lungs (Figure 2.3A). Similar to actively sensitized animals, the increase in $R_{\text{aw}}$ after OVA challenge in mice with allergic airway disease was dependent on mast cells; Wsh/Wsh mice failed to respond to antigen challenge (Figure 2.3B). As would be expected given
the absence of mast cells in the Wsh/Wsh mice, no histamine was detected in the Wsh/Wsh animals, while this mediator could easily be detected in the BAL from B6 mice following antigen challenge (Figure 2.3C). Similar to the naïve airway, antigen induced constriction appears to be located within the central airways.

**Mast cell release of serotonin alone mediates airway constriction during anaphylaxis.**

To address the contribution of the various mast cell mediators to allergen-induced bronchoconstriction we took advantage of a number of pharmacological and genetic tools, examining the impact of loss of various pathways on the changes in $R_{aw}$ during anaphylaxis. Anaphylaxis results in a measurable increase of leukotriene (LT) C$_4$ in the BAL (37). To examine the contribution of LTC$_4$ to anaphylactic bronchoconstriction, we utilized animals lacking the enzyme 5-lipoxygenase (5LO), an enzyme required for the synthesis of all leukotrienes. The inability of the mast cells to produce this lipid mediator did not alter bronchoconstriction after induction of passive anaphylaxis in 5LO$^{-/-}$ mice (Figure 2.4A). Although prostaglandin (PG) D$_2$ has been shown to be synthesized and released during IgE-dependent activation of mouse bone marrow-derived mast cells (38), no attenuation of airway resistance was noted in mice in which the production of prostaglandins was inhibited by treatment with the NSAID indomethacin (Figure 2.4B). Similarly, antagonists which block the H1 and H2 histamine receptors did not significantly alter anaphylactic bronchoconstriction (Figure 2.4C). The most abundant mediator found in human mucosal mast cells, tryptase, has the ability to activate protease activated receptor 2 (PAR2) receptors, which can mediate airway constriction under some
experimental conditions (39). To determine whether this pathway was involved in bronchoconstriction associated with anaphylaxis, we examined this response in PAR2−/− mice (17); no difference in R_{aw} was noted between the mutant and control animals (Figure 2.4D).

Mast cells express the enzyme required for the production of serotonin (40), and previous studies have indicated that serotonin can mediate changes in lung resistance (41). We therefore first asked whether the change in airway mechanics mediated by serotonin were also limited to the central airways. As can be seen in figure 2.5A, exogenous serotonin induced a dose-dependent increase in R_{aw} with little change in G, a pattern similar to what is observed after mast cell degranulation. To address the role of serotonin in mast cell mediated bronchoconstriction more directly, we examined the impact of PCPA on airway resistance. PCPA inhibits the activity of tryptophan hydroxylase, an enzyme which carries out the essential step in the synthesis of serotonin. Mice were sensitized with OVA and three weeks later treated with either vehicle or PCPA prior to the induction of anaphylaxis by injection of antigen. Antigen exposure resulted in a robust increase in airway resistance in the vehicle treated animals. In contrast, airway resistance was abolished in mice treated with PCPA, despite the normal degranulation of mast cells in these animals; histamine levels in the BAL collected immediately after induction of anaphylaxis were not significantly different between the two groups (Figure 2.5B). Furthermore, no difference was observed between the PCPA and control animals in the ability of their airways to respond to MCh (Figure 2.5C). As expected, anaphylaxis resulted in a measurable increase in plasma serotonin levels, but this increase was not observed in the PCPA treated mice (Figure 2.5D). Thus, the
protection of the PCPA treated mice from bronchoconstriction was due to attenuated serotonin release and not to alterations in either smooth muscle function or the responsiveness of mast cells to antigen.

To rule out a possible role for platelet-derived serotonin in this model, we examined the response to IgE and antigen in mice lacking the serotonin transporter. While mast cells express tryptophan hydroxylase and thus can produce serotonin, platelets acquire this mediator via the serotonin transporter as they pass through the intestinal circulation. Thus platelets from mice and rats lacking the transporter also lack serotonin (42, 43). 5HTT-deficient mice demonstrate wild-type levels of constriction after antigen challenge (Figure 2.5E) indicating that platelet-derived serotonin is not critical for this response. Serotonin-dependent constriction was also observed in BALB/c mice (Figure 2.6), which are commonly thought of as prototypical Th2 responders.

If bronchoconstriction is mediated by mast cell-derived serotonin, it would follow that agents that block serotonin receptors would also attenuate changes in $R_{aw}$. Serotonin-induced increases in $R_{aw}$ were abolished by both the non-specific antagonist methiothepin and the 5-HT$_{2A}$-specific antagonist ketanserin, whereas the 5-HT$_{3}$-specific antagonist ondansetron failed to attenuate this increase in airway mechanics (data not shown). Both methiothepin and ketanserin abolished allergic airway constriction in passively sensitized mice (Figure 2.5F). Together these studies indicate that while mast cells release many mediators that may modulate the inflammatory response, serotonin alone mediates the changes in airway resistance during anaphylaxis through its action on 5-HT$_{2A}$ receptors.
Intact sensory innervation is not required for mast cell mediated bronchoconstriction

Our observation that the ability of mast cells to mediate bronchoconstriction was sensitive to the location of these cells in the lung, and our observation that serotonin alone mediated this response, suggested to us that the mast cell mediated constriction may result from indirect stimulation of sensory neurons, triggering a bronchial reflex. To test this hypothesis we examined the ability of IgE and antigen to induce constriction in mice in which sensory neurons had been ablated by treatment with capsaicin. Ablation of sensory C-fibers with capsaicin in both adults and neonates failed to significantly decrease $R_{aw}$ after antigen challenge in passively sensitized mice (Figure 2.7A-B), suggesting that capsaicin-sensitive C-fibers do not play a primary role in allergic airway constriction. Substance P, the primary neurotransmitter stored in C-fibers, was measured to evaluate the efficacy of capsaicin treatment. Antigen challenge in passively sensitized mice increased substance P levels in the BAL, but was attenuated in mice pretreated with capsaicin (Figure 2.7C), verifying that the capsaicin treatment ablated substance P containing C-fibers in these animals.

Vagotomy abolishes anaphylactic airway constriction in both the naïve and allergic airway

The tone of ASM is largely dependent on parasympathetic innervation, which travels in the vagus nerve. Mast cells have been observed close to the vagus nerve and parasympathetic ganglia in other organisms and tissues (7, 13, 44), raising the possibility of cooperation between mast cells and these cholinergic fibers in bronchoconstriction.
To test this hypothesis, we examined mast cell-mediated bronchoconstriction in mice in which the cholinergic pathways were disabled using a number of approaches.

Blockade of nAChRs with the nonspecific nicotinic antagonist mecamylamine, prior to antigen challenge, abolished antigen-induced increases in $R_{aw}$ in passively sensitized mice (Figure 2.8A). Post ganglionic fibers release ACh which binds mAChRs expressed by smooth muscle cells to initiate contraction. Blockade of mAChRs with the nonspecific muscarinic antagonist atropine resulted in complete absence of airway constriction after antigen challenge in B6 mice (Figure 2.8B). To further define the muscarinic receptors required for mast cell mediated bronchoconstriction, we examined a series of mouse lines deficient in M1 or M3 mAChRs. Complete attenuation of $R_{aw}$ was only observed in the mice lacking the M3 mAChR (Figure 2.8C), which has been shown to be expressed by airway smooth muscle (3).

The observation that disruption of both nicotinic and muscarinic neuronal transmission inhibits anaphylactic changes in airway mechanics suggests that an intact parasympathetic pathway is required for this mast cell function. If this is the case, we would expect that severing the incoming neural fibers would eliminate IgE/antigen mediated bronchoconstriction. To test this hypothesis, mice were treated with either IgE or saline and the following day the vagus nerve was severed prior to treatment with antigen. Control groups in which the nerve was isolated but not severed were also prepared. Vagotomy abolished antigen-mediated airway constriction in B6 mice (Figure 2.9A). We next determined whether severing the vagus nerve altered the ability or extent of mast cell degranulation in the airways by measuring histamine levels in the BAL
immediately after measurement of airway parameters. No difference in histamine levels was seen following vagotomy (Figure 2.9B).

Inflammation results in the recruitment of cells to the lung. This raises the possibility that, in such an environment, mast cells may bring about changes in airway caliber in response to antigen independent of parasympathetic pathways. To test this, we induced allergic lung disease in mice and examined the impact of vagotomy on these animals. Again, vagotomy abolished increases in $R_{aw}$ following antigen challenge (Figure 2.9C).

Allergic airway constriction in BALB/c mice was also sensitive to atropine and vagotomy (Figure 2.10). To verify that the vagotomy did not alter fundamental properties of the ASM, we confirmed that this surgical procedure did not alter the response of the mice to MCh (Figure 2.11).

If serotonin released by mast cells collaborates with signals derived from the parasympathetic pathways, one would expect that constriction of the airways to exogenous serotonin to show similar sensitivity to changes in the activity of this system. Supporting the role of serotonin in neural mediated anaphylaxis, administration of exogenous serotonin exhibited dose dependent central airway constriction that was sensitive to both vagotomy and atropine (Figure 2.9D). Additionally, pretreatment with a non-provoking dose of serotonin resulted in an additive effect on bronchoconstriction to a non-provoking dose of MCh (Figure 2.12). Together these studies support a model in which mast cell derived serotonin collaborates with cholinergic pathways of the lung to bring about changes in airway patency during anaphylaxis.
Discussion

We show here that IgE-mediated degranulation of mast cells leads to an increase in the release of mast cell specific mediators in the mouse lung accompanied by changes in airway mechanics. These changes in airway mechanics are dependent on both mast cell release of serotonin and intact cholinergic innervation of the lung. Similar to previous studies (45) we show that there was a dramatic increase in total $R_L$ following antigen challenge, similar in magnitude to that generally achieved with 2 µg i.v. MCh (data not shown). More recently it has become possible to evaluate changes in airway mechanics using FOT. Using the input impedance of prime waves of various amplitudes, fit to the constant phase model of the lung, airflow obstruction following anaphylaxis can be distinguished between the central and peripheral airways (24, 35). Using this method we found that while MCh affects parameters associated with changes in mechanics of both the central and peripheral airways, changes observed after mast cell degranulation were surprisingly limited to the central airways despite the fact that these cells can be found throughout the lung, including within the distal airways and lung parenchyma (34). A number of different explanations are possible for this observation. First while mast cells are found throughout the lung in B6 mice, a larger concentration are localized within close proximity of bronchial smooth muscle while few are found in the alveolar spaces and within the lung parenchyma (34). While antigen exposure may lead to degranulation of cells in the distal lung, the distribution of the receptor(s) sensitive to mast cell mediators may also be biased towards smooth muscle of the central airways. Alternate explanations are also possible. For example, it is possible that cholinergic innervation is more abundant in the central airways, or that the mast cell localization in
respect to these neurons differs at different levels of the airways. However, our observation that inhaled serotonin leads to changes in lung mechanics similar to that observed upon mast cell degranulation suggests that the distribution of serotonin receptors plays a role in limiting the response to the larger airways. This finding differs from studies carried out on precision cut lung slices prepared from rats in which constriction of airways was observed in both the distal and proximal airways (46). In this study antigen challenge of passively sensitized lung slices resulted in both stronger and faster constriction of the small airways when compared to the large conducting airways. Similarly, serotonin-provoked responses were also greater in the peripheral airways. In addition, passively sensitized lung slices from humans have also been reported to have an increased response to antigen with decreasing airway size (47). The difference in these findings underscores the continuing importance of verification of ex vivo findings in an animal model. Alternately, this may reflect differences between species in the anatomical distribution of mast cells, ASM, airway receptors, or a combination thereof; and may also indicate that while bronchoconstriction in mice is restricted to central airways this may not be representative of all species.

Mast cells produce a number of mediators that have been shown, both in vitro and in vivo, to mediate airway constriction, including LTC₄/LTD₄, PGD₂, tryptase and histamine. Direct testing using mice lacking 5LO indicated that leukotrienes are not required for IgE/antigen mediated airway constriction, at least in this species. Likewise, inhibition of endogenous prostaglandin production using the NSAID indomethacin failed to inhibit antigen-induced increases in Rₐw, ruling out the involvement of PGD₂ in this response. Furthermore, mice lacking PAR2 responded similarly to wild-type animals,
indicating that, in the mouse, activation of PAR2 receptors by tryptase is not linked to allergic airway constriction. Nor did we find evidence supporting a role for mast cell derived histamine in this response, despite the ability of inhaled histamine to induce an increase in airway resistance in humans (48) and to modestly increase $R_{aw}$ in some mouse strains (data not shown). In contrast, both inhibition of serotonin synthesis and blockage of the serotonin 5-HT$_{2A}$ receptor completely abolished the change in airway mechanics after antigen challenge. This finding is consistent with a number of previous studies using mice, in which pharmacological agents were shown to attenuate changes in airway mechanics. For example, Eum et al. reported that pretreatment of antigen challenged mice with the non-specific serotonin receptor antagonist methysergide significantly attenuated anaphylactic bronchoconstriction (45). 5-HT$_{2}$ receptor specific antagonists have also been shown to attenuate serotonin-induced increases in pulmonary mechanics (49).

Serotonin has been reported to activate human airway smooth muscle \textit{ex vivo} (50). However, unlike LTC$_{4}$ and histamine, serotonin inhalation does not lead to increased airway constriction, even in asthmatics (51). Furthermore, until recently, it was believed that human mast cells, unlike the rodent counterpart, could not produce serotonin. Careful study of these cells has revealed both the expression of tryptophan hydroxylase and the localization of serotonin (40); however, these findings are limited to cells derived \textit{ex vivo} and levels were quite small relative to the amounts stored in rodent mast cell granules. Despite the differing expression between human and mouse mast cells, a re-evaluation of potential roles for this pathway in human mast cells is warranted. For example it is possible that localized release of this mediator in close apposition to
nerves or ASM can produce a significant change in the activity of the parasympathetic pathway, and that this activity is not easily mimicked by delivery to the epithelial surface.

The vagus nerve contains both the incoming parasympathetic efferents and the sensory nerves originating from the lung, and thus the loss of response in mice after vagotomy is consistent with a model in which mast cells release mediators that stimulate sensory neurons. Sensory neuron activation can lead to both local release of mediators and a bronchial reflex resulting in increased activity of the parasympathetic pathway, a response lost in the vagotomized mice. The serotonin 5-HT₃ receptor is known to be involved in activation and depolarization of sensory neurons in several species including the mouse (15). It is possible that serotonin released by mast cells activates sensory neurons to elicit a bronchial reflex. Past studies in various species have suggested the involvement of sensory neurons in the allergic response. For example, degranulation of purified human lung mast cells enhanced the excitability of rabbit visceral sensory C-fibers in vitro (52). Furthermore, antigenic stimulation of sensitized guinea pig bronchi caused increased sensitivity of sensory nerve endings (53). Consistent with this, Yu et al. showed that mast cell degranulation enhanced the excitability of guinea pig esophageal C-fibers to mechanical and chemical stimulation (54). In the rat, capsaicin-sensitive neurons interact with mast-cells to influence lung solute clearance and chloride ion secretion in the trachea (10-12). Our observation however does not support this mechanism, as pretreatment of the mice with capsaicin had little impact on mast cell mediated airway constriction while, as expected, release of substance P by these neurons was all but eliminated. Consistent with this, pretreatment of mice with the 5-HT₃ receptor specific antagonist ondansetron did not attenuate serotonin-induced increases in
airway mechanics (data not shown). While it is unlikely that substance P is involved in this reaction, as animals were treated both as adults and as neonates, the involvement of this neurotransmitter cannot be completely discounted as effects of specific NK receptor antagonists were not evaluated.

A number of lines of evidence presented here support a critical role for parasympathetic neurons in mast mediated bronchoconstriction. This includes the loss of the antigen-induced contractile response in vagotomized mice, the ability of antagonists of both nAChRs and mAChRs to block the response, and the inability of IgE and antigen to trigger bronchoconstriction in mice lacking the M3 receptor. In recent years, studies have suggested that the cholinergic contractile response to serotonin depends on a non-neuronal source of ACh, specifically, serotonin released from the epithelium (49). This hypothesis was based largely on in vitro studies of mouse isolated trachea challenged with exogenous serotonin. They showed that serotonin induced contractions of tracheal smooth muscle was sensitive to epithelial removal but not to inhibition of neural pathways, and that these contractions were dependent on the release of ACh. Our studies do not support this hypothesis, as it would predict that severing the vagus nerve would have no effect on the ability of serotonin to induce bronchoconstriction. Our data show that in vivo, airway constriction from both exogenous serotonin and mast cell-derived serotonin is sensitive to vagotomy.

A number of models for mast cell interaction with the cholinergic neural pathway are possible. First it is possible that mast cells increase transmission at the preganglionic terminal, either by increasing ACh release or augmenting the activity of nAChRs. Second, mast cell mediators could amplify the release of ACh from postganglionic nerve
terminals. Finally, activation of serotonin receptors on ASM may lead to smooth muscle contraction only when ACh, which is released at low levels constitutively by the parasympathetic fibers, occupies M₃ receptors present on ASM. A number of studies support the models in which antigen challenge induces an increase in release of ACh. *Ex vivo* studies have measured an increase in ACh levels after stimulation with antigen in both mouse and canine tracheal rings (55, 56). Drugs which decrease ACh metabolism increased bronchoconstriction after either antigen or 5-HT challenge (45). However, our finding that only the 5-HT₂A receptor antagonist attenuates this response, coupled with evidence that supports the localization of this receptor to ASM (57) and not cholinergic fibers, supports the later model in which the impact of engagement of the 5-HT₂A receptors is dependent on M₃ receptors present on ASM. The need for this “second signal” is not clear. Serotonin alone can shorten smooth muscle *in vitro* and in *ex vivo* lung slices (57, 58), and therefore the observation that in the mouse its ability to alter smooth muscle activity *in vivo* is regulated by parasympathetic pathways is somewhat surprising. However, it is interesting to speculate that this relationship represents one of a number of “checks and balances” designed to safeguard the patency of the airways, limiting the activity of smooth muscle modulators stored by mast cells, while at the same time allowing mast cells to safeguard the airway against infectious agents by rapid deployment of their arsenals of inflammatory mediators.
Figure 2.1 Changes in airway physiology of wild-type and mast cell deficient mice in response to OVA and MCh. (A) OVA challenge in sensitized mice \((n=5)\) induced an immediate increase in \(R_L\) that gradually returned to baseline. Control animals \((n=4)\) sensitized with OVA but challenged with saline had no change in \(R_L\) over baseline \((^*P<0.05\) compared to OVA/saline). (B) Increases in \(R_L\) are dependent on mast cells, as there was no response in mast cell deficient Wsh/Wsh mice \((n=8)\) after antigen challenge \((**P<0.001\) compared to all other groups). (C) IgE levels were comparable between wild-type \((n=7)\) and mast cell deficient \((n=7)\) mice sensitized and challenged with OVA. (D) FOT measurement of airway mechanics after antigen challenge. OVA challenge in sensitized mice \((n=5)\) resulted in a significant increase in \(R_{aw}\) but no change in \(G\) compared to controls \((n=4)\) \((^*P<0.05\) compared to saline). (E) Increases in \(R_{aw}\) in sensitized mice are dependent on mast cells. Sensitized Wsh/Wsh mice \((n=8)\) had no response to antigen challenge \((**P<0.001\) compared to all other groups). (F) Aerosol methacholine (MCh) challenge in B6 mice \((n=13)\) results in a dose dependent increase in both \(R_{aw}\) and \(G\).
Figure 2.2  Airway inflammation in B6 and Wsh/Wsh mice with allergic lung disease.  (A) There was no significant difference in serum IgE levels in wild-type (n=8) and mast cell deficient mice (n=6).  (B) The resulting cell profile was the same for the wild-type (n=6) and mast cell deficient mice (n=8), with the majority of the cells (>80%) being eosinophils.  (C) IL-13 levels were used to represent the amount of inflammation present in the airways.  There was no significant difference in IL-13 between the wild-type (n=8) and mast cell deficient mice (n=9).  (D) The average H&E score was semi quantitatively measured for each main axial airway, and did not differ between the C57BL/6 (n=9) and C57BL/6 KitW-sh/KitW-sh (n=9) mice.  (E) There was no significant difference in mucus levels in the wild-type (n=8) and mast cell-deficient (n=8) mice.
Figure 2.3  Anaphylactic bronchoconstriction in wild-type and mast cell deficient mice with allergic lung disease.  (A) Allergic lung disease was induced by sensitization and repeated aerosol with OVA. B6 mice with inflamed airways (n=5) exhibited a significant increase in $R_{aw}$ after antigen challenge but no change in $G$ (*$P<0.05$ compared to saline). Saline challenged controls (n=3) had no response.  (B) Mast cell deficient Wsh/Wsh mice with an inflamed airway (n=7) failed to respond to antigen challenge (***$P<0.001$ compared to all other groups). (C) Histamine levels in B6 mice with inflamed airways were significantly increased after antigen injection (n=6) compared to levels after saline injection (n=3) (***$P<0.001$ compared to saline). Histamine was undetectable in Wsh/Wsh mice (n=7).
Figure 2.4 Pharmacological and genetic inhibition of mast cell mediator pathways during passive anaphylaxis. (A) Passively sensitized leukotriene deficient (5LO⁻/⁻) mice and their wild-type C57BL/6 controls showed no difference in $R_{aw}$ after antigen challenge. 5LO⁺/⁺ - controls, $n=3$; IgE+DNP, $n=6$. 5LO⁻/⁻ - controls, $n=3$; IgE+DNP, $n=6$ (*$P<0.001$, #$P<0.001$). (B) Pretreatment of passively sensitized C57BL/6 mice with indomethacin (10 mg/kg), to deplete endogenous prostaglandin production, had no effect on $R_{aw}$ after antigen challenge. Saline- controls, $n=3$; IgE+DNP+saline, $n=6$. Indomethacin- controls, $n=3$; IgE+DNP+indomethacin, $n=7$ (*$P<0.001$, #$P<0.001$). (C) C57BL/6 mice were pretreated with either pyrilamine (40 mg/kg) or cimetidine (25 mg/kg) to block histamine H1 and H2 receptors, respectively. Antagonism of histamine receptors had no effect on anaphylactic airway constriction in passively sensitized mice. Saline- controls, $n=3$; IgE+DNP, $n=13$. Pyrilamine- controls, $n=3$; IgE+DNP, $n=10$. Cimetidine- controls, $n=3$; IgE+DNP, $n=13$ (*$P<0.001$, #$P<0.01$, †$P<0.001$). (D) Antigen induced airway constriction was equivalent in wild-type and PAR2⁻/⁻ passively sensitized mice. PAR2⁺/⁺ - Controls, $n=4$; IgE+DNP, $n=6$. PAR2⁻/⁻ - Controls, $n=5$; IgE+DNP, $n=4$ (*$P<0.001$; #$P<0.001$).
Figure 2.5 Changes in airway physiology in response to serotonin and after inhibition of serotonergic pathways. (A) Similar to antigen exposure, exogenous i.v. serotonin induces dose dependent increases in $R_{aw}$, but no change in $G$ ($n=13$). (B) Vehicle treated mice ($n=10$) sensitized and challenged with OVA had a significant increase in $R_{aw}$. Pretreatment with PCPA (150 mg/kg, $n=15$) abolished anaphylactic airway constriction in actively sensitized mice challenged with OVA ($**P<0.001$ compared to all other groups). PCPA treatment had no effect on the ability of mast cells to degranulate; histamine levels were the same in both groups of OVA treated animals ($n=10-15$ per group). (C) PCPA treatment had no effect on the ability of the airway smooth muscle to constrict to aerosolized MCh administered after antigen challenge ($n=3$). (D) PCPA treatment significantly attenuated plasma serotonin levels in OVA challenged animals ($n=4-6$ per group) (*$P<0.05$ compared to all other groups). (E) Passively sensitized serotonin transporter (5HTT)-deficient ($n=4$) and hererozygous mice ($n=5$) challenged with DNP had a significant increase in $R_{aw}$ compared to controls ($n=2$ per group). This response was similar to that of WT animals ($n=5$). (*$P<0.001$; #$P<0.001$; $\#P<0.001$; $\#P<0.001$). (F) Blockade of serotonin receptors with methiothepin (2 mg/kg, $n=5$) or ketanserin (12 mg/kg, $n=5$) abolished airway constriction after antigen challenge in passively sensitized animals ($**P<0.001$ compared to all other groups).
Figure 2.6 Serotonin mediates antigen-induced central airway constriction in BALB/c mice. Vehicle treated mice sensitized with OVA and challenged with either PBS (Controls, \(n=2\)) or OVA (OVA, \(n=5\)). OVA challenged mice had a significant increase in \(R_{aw}\). Pretreatment with 150 mg/kg PCPA (OVA+PCPA, \(n=4\)) abolished anaphylactic airway constriction in actively sensitized mice challenged with OVA (**\(P<0.001\) compared to all other groups).
Figure 2.7 Anaphylactic bronchoconstriction following ablation of sensory C-fibers with capsaicin. (A) Sensory ablation with capsaicin (25-75 mg/kg) in adults (n=6) had no significant effect on airway constriction after antigen challenge in passively sensitized mice (*P<0.001, #P<0.01). (B) Degradation of C-fibers with capsaicin (50 mg/kg) in newborn mice (n=7) had no effect on airway parameters after antigen challenge in passively sensitized mice (*P<0.001, #P<0.001). (C) Antigen challenge in passively sensitized mice (n=10) increased substance P content in the BAL. Neonatal treatment of capsaicin (n=5) reduced BAL substance P after antigen challenge to levels seen in control animals (n=3) (**P<0.05, compared to all other groups).
Figure 2.8 Antigen-induced bronchoconstriction following cholinergic inhibition. (A) Blockade of cholinergic nAChRs with mecamylamine (4 mg/kg, $n=8$) abolished airway constriction following antigen challenge in passively sensitized wild-type mice ($**P<0.001$ compared to all other groups). (B) Blockade of mAChRs with atropine (10 µM/kg, $n=6$) resulted in complete loss of airway constriction after antigen challenge in passively sensitized mice ($**P<0.01$ compared to all other groups). (C) In passively sensitized animals, loss of the M$_3$ receptor ($n=6$) resulted in abolishment of anaphylactic airway constriction, whereas M$_1$ receptor deficiency ($n=6$) resulted in a significant attenuation, but not complete loss of the response to antigen ($*P<0.001$, $**P<0.01$, $^#P<0.001$, $^##P<0.001$, $§P<0.01$).
Figure 2.9 Vagotomy attenuates antigen and serotonin-induced changes in airway resistance. (A) Passively sensitized and challenged wild-type mice after dissection of the vagus nerve ($n=18$) or a surgical sham ($n=28$). Antigen challenged mice showed a significant increase in $R_{aw}$ that was abolished in vagotomized animals ($**P<0.001$ compared to all other groups). (B) Histamine levels in the BAL fluid were measured after induction of passive anaphylaxis. Vagotomy did not affect the levels of histamine in the BAL fluid ($n=5$ per group). (C) Changes in $R_{aw}$ were abolished after vagotomy in OVA challenged sensitized mice ($n=7-14$ per group) with allergic lung disease ($**P<0.001$ compared to all other groups). (D) Serotonin was delivered i.v. following either vagal dissection or pretreatment with atropine. Exogenous serotonin ($n=11$) resulted in a dose dependent increase in $R_{aw}$ that was sensitive to both vagotomy ($n=5$) and atropine ($n=6$) ($**P<0.01$).
Figure 2.10 Antigen-induced bronchoconstriction following vagotomy and atropine in BALB/c mice. Passively sensitized and challenged wild-type BALB/c mice after dissection of the vagus nerve or pretreatment with atropine. Antigen challenged mice ($n=6$) showed a significant increase in $R_{aw}$ compared to controls ($n=3$). Airway constriction was abolished in vagotomized animals ($n=5$). Blockade of mAChRs with atropine (10 µM/kg, $n=6$) resulted in complete loss of central airway constriction after antigen challenge in passively sensitized mice. No significant change in $G$ was observed. (**$P<0.001$ compared to all other groups)
Figure 2.11  Airway mechanics in response to MCh challenge after vagotomy.  (A) Vagotomy also did not significantly affect the response to iv MCh.  MCh, n=3; MCh+vagotomy, n=3.  (B) Constriction of airway smooth muscle following aerosolized MCh was not altered following dissection of the vagus nerve.  MCh, n=7; MCh+vagotomy, n=7.
Figure 2.12  Increased airway resistance in mice treated with non-provoking doses of both serotonin and MCh. B6 mice exhibited increased sensitivity to serotonin when coupled with exposure to a non-provoking dose of MCh. Low levels of either i.v. serotonin (0.075 pg, $n=3$) or aerosolized MCh (6 mg/ml, $n=3$) does not result in a significant increase in $R_{aw}$ compared to saline challenge. A significant increase in $R_{aw}$ was detected after non-provoking doses of serotonin and MCh were delivered together ($n=3$) (**$P<0.001$ compared to all other groups).


mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. J Neurosci 21:6348-6361.


CHAPTER 3

Unique populations of lung mast cells are required for antigen-mediated bronchoconstriction

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Abstract

Background Studies in both human and mouse indicate that mediators released by mast cells can lead to bronchoconstriction, and thus these are important effector cells in life threatening anaphylaxis. Much of our understanding of the various functions of mast cells emanates from the study of mice lacking theses cells, particularly mice carrying mutations in the tyrosine kinase gene Kit. Definitive evidence for the role of mast cells in the altered immune response requires the demonstration that this response can be normalized by reconstitution of the mice with cultured bone marrow-derived mast cells (BMMCs). While many mast cell niches can be restored with BMMCs, this has not been demonstrated for mast cells present in the airways of the lung, cells poised to mediate bronchoconstriction during allergic responses.

Objective To determine if mast cell deficient Kit$^{Wsh/Wsh}$ reconstituted lines are an appropriate model for the study of the role of these cells in bronchoconstriction associated with allergic responses.

Methods Kit$^{Wsh/Wsh}$ mice were reconstituted with either whole bone marrow (WBM) or BMMCs and responses to IgE-mediated mast cell activation determined; including systemic hypothermia, mediator release, and bronchoconstriction in anesthetized, mechanically ventilated animals.

Results Engraftment of Kit$^{Wsh/Wsh}$ mice with WBM and BMMCs results in reconstitution of the central airways with mast cells. While the treatment of the two groups of animals resulted in similar systemic changes when challenged with IgE/Ag in a model of passive anaphylaxis, bronchoconstriction was observed only in Kit$^{Wsh/Wsh}$ animal which had received a bone marrow transplant.
Conclusions While BMMCs can populate the lung, they cannot restore IgE/Ag-mediated bronchoconstriction to mast cell-deficient animals. This suggests that the mast cell population which mediates this function may be unique, and to fill this niche in the lung cells must undergo a specific developmental program, one that is no longer available to cultured mast cells.
Introduction

Mast cells arise as precursors in the bone marrow. This precursor cell is released into the blood stream and then migrates to various tissues where it undergoes its final differentiation, a process which is believed to be influenced by the local environment. At all stages of maturation mast cells express the receptor tyrosine kinase (CD117, c-KIT) and require the Kit ligand, stem cell factor (SCF), for survival. In the mouse, c-KIT and SCF are encoded at the white spotting (W) and steel (Sl) loci, respectively. Mutations in the W and/or Sl loci results in deficiencies in the production of melanocytes, germ cells, and hematopoietic cells (reviewed in (1)). While several mutations at this locus have been described, the most common mutations used for studying mast cells in mice are Sl/St^d, W/W^v, and W^sh/W^sh. The Kit^W/W^v (Wv) mouse, is a compound heterozygous animal, with the mutation deleting segments of the Kit coding region. In contrast, analysis of Kit in Kit^Wsh/Wsh (Wsh) mice revealed no alteration in either the sequence or organization of the gene, but rather an inversion in the regulatory region (2, 3). While all these mutations lead to profound mast cell deficiencies, as well as absence of coat pigment, Sl/St^d and W/W^v mutant mice are also anemic and sterile, phenotypes which are not seen in W^sh/W^sh mice. Therefore, the Wsh mouse has increasingly been used for study of mast cell function: the fertility of these mice simplifies the generation of these animals and their intercross with lines carrying other mutations.

Due to the lack of mast cells in the Wsh and Wv mice, alterations in the response of these lines to various pathogens and in models of autoimmune disease has been broadly used to support a role for the mast cells in these immune responses (4-7). However, because the phenotype of lines lacking mast cells is not limited to this cell
type, confirmation of mast cell function is dependant on the demonstration that the deficit in these mice can be corrected by restoration of the mast cell population. This can be done in one of two ways. The mice can be reconstituted with whole bone marrow (WBM) isolated from a wild type congenic animal. Alternatively, mast cell cultures can be established from bone marrow of wild type animals. Once the purity of these cultures is verified, these cells can be introduced into the Wsh or Wv mouse. The primary advantage of this later approach is that only the mast cell compartment is of donor origin: when total bone marrow is used, other hematopoetic compartments are also restored. The use of Wsh and Wv mice reconstituted with BMMCs has become increasingly common with the availability of mast cell cultures derived from mice carrying mutations generated by homologous recombination. This has allowed the identification of the role of specific pathways within mast cells, as well as assignment of mast cell mediators to specific pathophysiological changes during the immune response.

Mucosal type mast cells in the lung are intimately involved in allergic immune responses and are known to contribute to airway reactivity and hyperresponsiveness in models of anaphylaxis and asthma. In normal mice, mast cells are located throughout the main airways including within the trachea and bronchus, with few mast cells being found within the parenchyma (8). Histological analysis has demonstrated the presence of mast cells in the lung of Wsh mice reconstituted with both WBM and BMMCs (9-11). In the latter case, however, there has been disagreement on the extent to which the BMMCs can reconstitute various regions of the lung. Although it is generally accepted that BMMCs cannot reconstitute the trachea, histological studies by Wolters and colleagues (11) showed mast cells close to or within the smooth muscle layer of the airways of
reconstituted Wsh mice. Conversely, studies by Grimbaldeston et al. (10) only noted reconstitution of mast cells in the lung parenchyma of Wsh mice engrafted with BMMCs.

Immunological studies also indicate that BMMC reconstitution can restore the immune response of Wsh mice, including their response in ovalbumin-induced models of asthma (12). Some studies have also evaluated airway hyperresponsiveness (AHR) in the reconstituted mice (12-14). In these instances the response of the mice to inhaled methacholine, a stable derivative of Ach which acts directly on the smooth muscle wall to elicit airway constriction, was evaluated and shown to be restored in the reconstituted animals. However, it is not unlikely that restoration of AHR in the Wsh mice that received BMMCs was the indirect consequence of normalization of the immune response. The possibility remains that, while some mast cell functions are restored in the Wsh mice, the mechanism(s) by which these cells contribute to pathophysiology during inflammation may not completely mimic that of a normal animal.

Recently we have shown that mast cells interacted with the parasympathetic pathways in the lung to mediate bronchoconstriction during anaphylaxis: the response was absent in Wsh mice but also absent in mice in which the parasympathetic pathways are inhibited (15). This suggests that interactions between mast cells and neurons regulate airway caliber during inflammatory responses and raises the possibility that a unique subpopulation of mast cells may be required for this function. To begin to address this question we evaluated the ability of culture bone marrow derived mast cells to restore this response in the Wsh mice.
Methods

Experimental animals

Mast cell deficient C57BL/6 KitW-sh/W-sh mice and their wild-type C57BL/6J controls were purchased from Jackson Laboratory. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of North Carolina at Chapel Hill.

Reconstitution of mast cell-deficient mice

Whole bone marrow: Bone marrow cells were collected from femurs of wild type C57BL/6J or C57BL/6 KitW-sh/W-sh mice. Whole bone marrow (WBM) cells were centrifuged at 1200 rpm for 5 min and resuspended in PBS (200 µl per animal of bone marrow). Recipient mice were irradiated before WBM injection with a split dose of 10 Gy (two 5-Gy doses 3 h apart). WBM was injected i.v. via the tail vein into 4-8 weeks old C57BL/6 KitW-sh/W-sh recipient mice. Animals were analyzed after 12 weeks. In order to distinguish the effect of mast cells in bone marrow reconstituted animals, we compared C57BL/6 KitW-sh/W-sh animals reconstituted with WBM from C57BL/6J mice with C57BL/6 KitW-sh/W-sh animals reconstituted with WBM from C57BL/6 KitW-sh/W-sh mice.

BMMCs: Bone marrow was harvested from C57BL/6J mice and cultured in the presence of 5 ng/ml recombinant IL-3 and 10 ng/ml recombinant stem cell factor (SCF). Cells were cultured for 5 weeks, when cell populations consisted of >95% mast cells as assessed by expression of c-Kit and FceRI on cell surface by fluorescence activated cell sort analysis (FACS). Ten million BMMC in 200 µl of PBS were injected i.v. via the tail
vein into 3-9 week old C57BL/6 $Kit^{W-sh/W-sh}$ mice. Mice were analyzed 12-16 weeks after injection.

**Anaphylaxis protocols**

*Passive systemic anaphylaxis (PSA):* Mice were sensitized with 20 µg mouse monoclonal anti-DNP IgE (1.0 mg/ml, Sigma Chemical; 200 µl total volume in PBS) or an equal volume of PBS i.v. through the tail vein. Approximately 19-24 hours after anti-DNP IgE injection, mice were injected with 250 µl of 5 mg/ml DNP and rectal temperature monitored every 10 min for 40 min.

*IgE-dependent passive anaphylaxis:* Mice were sensitized with 20 µg mouse monoclonal anti-DNP IgE or an equal volume PBS i.v. through the tail vein, as described above. Approximately 19-24 hours after anti-DNP IgE injection, mice were anesthetized, catheterized, tracheostomized and mechanically ventilated as previously described (16). Immediately following the baseline measurements, 250 µl of 5 mg/ml DNP (or an equal volume of PBS) was injected into the jugular vein. After administration, airway mechanics were determined using the Forced Oscillatory Technique (FOT) every 10 s for 3 min. Bar graphs represent the area under the curve.

**Histology**

For histopathologic examination, lungs were fixed by inflation (20 cm pressure) and immersion in 10% formalin. After paraffin embedding, 5-µm sections were cut and stained with toluidine blue. Positively stained mast cells were counted in longitudinal sections through one mainstream bronchus. Mast cell numbers for each mouse were the average of the counts in 10 randomly selected 10× high-power fields on each slide.
**Receptor expression analysis of BMMCs**

RNA was collected from 1 million BMMCs using RNAbee reagent (Qiagen). RNA was then converted to cDNA using a High Capacity cDNA RT kit (Applied Biosystems) and FcεRI-α, FcεRI-β, and tryptophan hydroxylase (Tph1) expression was assessed by real-time PCR using commercially available probes (Applied Biosystems). Results were standardized to GAPDH expression.

**Analysis of histamine release from mast cells**

Mice were sensitized with 20 µg mouse monoclonal anti-DNP IgE or an equal volume PBS i.v. through the tail vein, as described above. Approximately 19-24 hours after anti-DNP IgE injection, mice were injected with 250 µl of 5 mg/ml DNP i.v. through the tail vein. 2 min following injection of antigen, mice were sacrificed by inhalation of CO₂ and 0.5-1.0 ml of blood collected by cardiac puncture with a syringe containing 20 µl 100 U/ml heparin. The blood was then centrifuged at 12000 rpm for 5 min to collect plasma. Bronchoalveolar lavage was performed five times with 1.0 ml of sterile HBSS each time. The recovered fluid (BALF) was centrifuged to remove cells. Histamine levels were measured in the plasma and BALF cell-free supernatant by ELISA (Beckman Coulter).

**Statistical analysis**

Data are represented as means ± SEM. Analysis Of Variance (ANOVA) followed by Tukey-Kramer Honestly Significant Difference for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by Student’s two-tailed t-test. A P value of <0.05 was considered statistically significant.
Results

*Engraftment with WBM and BMMCs can reconstitute peripheral responses to passive systemic anaphylaxis (PSA)*

Cohorts of Wsh mice were reconstituted with either cultured isogenic C57BL/6 BMMCs or with WBM cells isolated from C57BL/6 mice. Control groups received bone marrow cells from the Wsh mice. This provides a control for the repopulation of cell linages in addition to mast cells that may be absent or compromised in the Wsh mice. Wsh mice receiving bone marrow were exposed to low doses of irradiation. Eight weeks after reconstitution we examined the response of the groups in a model of IgE mediated anaphylaxis which previously has been shown to be mast cells dependant. Mice received i.v. monoclonal IgE and after 24 hours were exposed to antigen. This treatment results in systemic anaphylaxis, characterized by lethargy, hypotension, airway obstruction, and hypothermia (17, 18). To quantitate these changes, we monitored the drop in body temperature associated with this response. No change in temp was observed in the Wsh mice, consistent with the lack of mast cells in these animals. A rapid and sustained drop in body temperature was observed in the mice reconstituted with both the cultured BMMC and with the bone marrow cells. This response was similar to that observed in wild type mice (*Figure 3.1*).

*WBM and BMMCs transfer results in reconstitution of mast cell populations in the central airways*

The systemic shock leading to drop in body temperature in response to IgE and antigen is mediated by the release of mast cell histamine in the blood stream (19), which
is thought to act in the hypothalamus to trigger this response (20), and therefore would not be dependent on reconstitution of various mast cell populations in the lung. To address this, we therefore first carried out histological examination of the lungs, comparing the number and distribution of mast cells in wild type mice, mice reconstituted with BMMCs and mice reconstituted with WBM. Consistent with previous studies of the W\textsuperscript{v} mast cell deficient line (9), no mast cells were detected along the main bronchi of Wsh mice (Figure 3.2A), locations at which these cells were easily observed in the C57BL/6 mice. No mast cells were detected in the parenchyma of C57BL/6 mice or the Wsh animals (Figure 3.2B). Reconstitution of Wsh mice with WBM or BMMCs resulted in remarkably increased numbers of mast cells along the central airways, as well as some mast cells present in the lung parenchyma (data not shown). This increase in mast cell number was even more pronounced in the mice reconstituted with WBM (Figure 3.2C-E). The increase in total mast cell numbers in the reconstituted animals was in part due to mast cells present in the parenchyma of the lung, a region in which few mast cells are observed in wild type mice. No apparent difference was noted in the location of the mast cells within the airways of the wild type, BMMC, and WBM reconstituted animals.

\textit{Allergic bronchoconstriction is recapitulated in Wsh mice after reconstitution with WBM but not with BMMCs}

As both BMMC and WBM reconstitution of Wsh mice resulted in mast cells present in elevated numbers in the airways and parenchyma, we next asked whether the reconstituted mice would respond to allergen and IgE with a comparable increase in the
level of bronchoconstriction. To address this question we again treated mice with monoclonal IgE. After 24 hour mice were anesthetized, tracheotomized and mechanically ventilated. Antigen was then delivered via a jugular catheter and the changes in airway resistance determined using the Forced Oscillation Technique to measure respiratory system input impedance and evaluate the Constant Phase Model. This technique allows for unique distinction between central and peripheral lung mechanics. As expected, treatment of the B6 mice that had received anti-DNP IgE showed a significant central airway constriction ($R_{aw}$) after challenge with DNP when compared with sensitized animals that received a PBS challenge. This response was absent in antigen challenged Wsh mice: no bronchoconstriction was observed and their airway mechanics did not differ from the PBS-challenged controls.

As expected, this response was observed in the Wsh mice in which the mast cell population had been reconstituted by a bone marrow transplant: the increase in airway resistance in the antigen treated animals did not differ significantly from the wild type B6 animals (Figure 3.3A). To verify that the response observed in these animals was in fact due to reconstitution of mast cells and not the indirect consequences of the bone marrow transplant we examined cohorts of Wsh reconstituted with Wsh marrow. As expected, the airways of Wsh mice reconstituted with Wsh WBM did not constrict following antigen challenge.

We next evaluated the response of Wsh mice in which the mast cell population had been reconstituted with BMMCs. Again mice were treated with IgE, ventilated, and changes in airway resistance monitored during and after iv delivery of antigen. No change in $R_{aw}$ was observed in these animals (Figure 3.3B). This was surprising given
the high number of mast cells in the lungs of these animals and the hypothermia secondary to PSA observed on treatment of similar groups with IgE and antigen.

*Release of mast cell mediators in Wsh reconstituted populations*

It is possible that lack of airway constriction in BMMC engrafted Wsh mice was due to improper receptor and/or mediator expression; therefore we characterized the BMMCs *in vitro* and analyzed their receptor expression and ability to degranulate. FACs analysis revealed that BMMCs expressed both c-kit and the FcεRI receptor (*Figure 3.4A*), two markers synonymous with mast cells. Real-time PCR analysis of BMMCs also indicated the presence of both α and β chains of FcεRI (*Figure 3.4B*), and IgE-dependent activation of BMMCs *in vitro* was confirmed by measuring hexosaminidase release after antigen challenge (data not shown). It has been shown that mast cell-derived serotonin is responsible for antigen-mediated central airway constriction (15). Tryptophan hydroxylase, the enzyme required for serotonin synthesis, was also abundantly expressed in BMMCs (*Figure 3.4B*).

One possible explanation for the differences between the phenotype of the Wsh reconstituted by WBM versus BMMCs is that the BMMC-derived cells do not produce or release mast cell mediators necessary for bronchoconstriction in quantities similar to those released by mast cells differentiated from hematopoetic precursors. To begin to address this we used histamine as a surrogate marker for mast cell degranulation, comparing histamine release in both the bloodstream and lungs of WBM and BMMC reconstituted Wsh mice after sensitization and challenge with antigen. Antigen challenge of wild-type B6 mice resulted in elevated levels of histamine in both BALF and blood,
while histamine was undetected in both PBS challenged controls and mast cell-deficient Wsh mice (Figure 3.5A-B). Following antigen challenge, histamine levels in both BALF and blood of WBM reconstituted Wsh mice were comparable to similarly challenged B6 mice (Figure 3.5A-B). Wsh mice reconstituted with BMMCs had significantly attenuated levels of histamine in their BALF after antigen challenge (Figure 3.5A). However, this deficit was limited to the airways as circulating levels of histamine were comparable to both B6 and WBM engrafted mice (Figure 3.5B). Despite this difference, quantity of granules in lung mast cells of WT and reconstituted Wsh mice appeared equivalent after histological examination (Figure 3.6A-C).

Discussion

Antigen mediated degranulation of mast cells in the airways can lead to life threatening bronchoconstriction, presumably through the actions of chemical mediators stored in mast cell granules on airway smooth muscle. Similarly in mice, an increase in airway resistance can be measured in response to treatment with IgE and antigen. The dependence of this response on mast cells is supported by the failure to observe bronchoconstriction in Wsh mice (15). The lack of mast cells in Wsh mice and the ability to restore many mast cell functions by reconstitution of this mouse line with cultured bone marrow derived mast cells suggests that this strategy could also be used to investigate the mechanism(s) by which mast cells alter smooth muscle physiology in the lung and mediate bronchoconstriction in response to antigen challenge. However, we report here that, while mast cells are observed in Wsh mice reconstituted with BMMCs there are important qualitative differences between these populations and those in both
wild type mice and mice reconstituted with hematopoietic stem cells from whole bone marrow. A critical mast cell function in the lung, the ability to alter smooth muscle tone, is not restored in these animals.

There are a number of possible reasons for the lack of response in reconstituted Wsh mice. Wsh mice carry a mutation in the regulatory region upstream of the ckit promoter which negatively effects the expression of ckit in a tissue-specific manner. This limits the development of mast cells. However, altered expression of ckit has additional consequences including cardiac hypertrophy, splenomegaly with expanded myeloid and megakaryocyte populations, and bone marrow abnormalities such as neutrophilia and thrombocytosis (21). In addition, the Wsh mutation also results in alteration in the development of interstitial cells of Cajal (ICC). ICCs in the gut serve as “pacemaker” cells that trigger gut contraction and have also been shown to modulate neurotransmission (22, 23). Other smooth muscle containing tissues are also known to contain these cells, although their function in other tissues is still being investigated. Therefore it is possible that the lack of ability of BMMCs to restore function may reflect the fact that attenuated bronchoconstriction is not due to the loss of mast cell function, but rather other physiological consequences of altered ckit expression, such as alteration of the smooth muscle physiology or innervation of the airways. This is not supported by our demonstration that the response can be restored by treatment of mice with total bone marrow. However, we cannot formally rule out the possibility that a population of cells of hematopoietic origin, in addition to mast cells, is restored under these experimental conditions.
It is also possible that the cultured mast cells cannot efficiently reconstitute the lung or migrate to specific compartment of lung. However, similar to previous studies (10, 11), we report that resident mast cells are observed in Wsh mice reconstituted with either whole bone marrow or cultures of bone marrow derived mast cells. In fact, in our study the number of mast cells was dramatically increased in the reconstituted animals compared to wild type B6 mice. Because mast cell-mediated airway constriction occurs exclusively within the central airways of wild-type B6 mice (15), mast cell location after reconstitution may be of particular importance when studying this reaction. Mast cells in wild-type B6 mice are extremely rare in the lung parenchyma but are commonly found within the trachea and around the bronchi, specifically within close proximity to bronchial smooth muscle (8). Wolters et al. (11) reported that 12 weeks after i.v. injection of $10^7$ BMMCs, the lungs, but not trachea, of reconstituted Wsh mice contained mast cells, and histologically the majority of these mast cells were seen in close proximity of the large airways. Conversely, the study by Grimbaldeston et al. (10), while supporting the inability of the trachea to reconstitute mast cells, reported that in the reconstituted animals the majority of the mast cells were localized to the parenchyma. We observed higher total number of mast cells in the animals reconstituted with both WBM and BMMCs. Mast cells were observed in the submucosa of the conducting airways of the BMMC reconstituted mice in locations that appeared similar to those observed in both WBM reconstituted animals and wild type B6 animals. However, we cannot rule out the possibility that there are subtle but important differences, not apparent using the methods employed in this study, that distinguish the location of the mast cells in BMMC and WBM reconstituted animals. For example, it is possible that the location of
the mast cells vis a vie the neurons present in the airways is not equivalent, and we have recently reported interaction between these cell types is essential for bronchoconstriction (15).

A second possibility is that limited differentiation pathways are available to cultured BMMCs, and these alter the spectrum of physiological activities in which these mast cells can participate. We did however verify that the BMMCs used for the reconstitution experiments expressed c-kit and the FcεRI receptor, as well as tryptophan hydroxylase for serotonin synthesis, which is the mediator required for antigen-mediated bronchoconstriction (15). In addition, BMMCs are able to degranulate following IgE-mediated activation in vitro, as measured by hexosiminidase release (data not shown). The normal differentiation of the BMMC after reconstitution of Wsh mice is supported by our demonstration that these animals displayed other pathophysiological changes characteristic of passive anaphylaxis. Treatment of these animals with monoclonal IgE and antigen resulted in a rapid drop in body temperature. This response is attributed to the action of mast cell histamine in the brain, specifically through binding to histamine receptors on the hypothalamus (20). Blood histamine levels following antigen challenge of sensitized mice were comparable to both WBM reconstituted and wild-type mice, consistent with the re-establishment of the PSA response in these animals. However, we cannot rule out the possibility that this maturation is attenuated in the lung mast cells and/or that the production of other important mediators is compromised in the BMMCs, as the histamine release in BALF was attenuated in animals reconstituted with these cells.

Several studies have used Wsh mice to analyze mast cell function in allergic inflammation and AHR to methacholine (13, 14). In many of these studies the
attenuation of AHR in the Wsh mice was restored in mice constituted with wild type BMMCs. However, in these studies antigen-mediated airway constriction was not assessed. It is quite likely that the ability of the BMMCs to restore AHR to methacholine is the result of the production of inflammatory mediators by lung mast cells and the impact of these mediators and/or recruited inflammatory cells on smooth muscle physiology. This likely reflects a different and additional role for the mast cell in airway physiology, one that is independent from the ability of preformed mediators in mast cell granules to initiate rapid constriction of smooth muscle upon mast cell degranulation by IgE/antigen.

In summary, our study suggest that while functions of mast cells can be studied in vivo using the Wv/Wsh reconstitution model, this does not currently include the study of antigen/IgE mediated bronchoconstriction. A further study of this model, however, may lead to the identification of the mechanism(s) by which maturing mast cells or their precursor cells develop the ability to populate very specific niches in the lung and in other organs. It is interesting to speculate that differences in the propensity of the mast cell to populate these specific sites may profoundly alter the susceptibility of individuals to allergic disease and or the manifestations of various symptoms of these diseases once they are acquired.
Figure 3.1  Body temperature after induction of passive systemic anaphylaxis. Mice were sensitized with anti-DNP IgE. Following antigen challenge, rectal temperature was recorded every 10 min for up to 40 min. Wild-type (B6, $n=2$) mice exhibited significant reduction in rectal temperature after antigen challenge, whereas the temperature of mast cell-deficient (Wsh, $n=2$) mice increased slightly. Reconstitution of Wsh mice with either WBM (WBM$\rightarrow$Wsh, $n=2$) or BMMCs (BMMC$\rightarrow$Wsh, $n=2$) resulted in temperature drop after antigen challenge.
Mast Cells

E

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Figure 3.2 Airway mast cells. Lungs of mast cell-deficient Wsh (A), B6 (B), WBM reconstituted Wsh (C), and BMMC reconstituted Wsh (D) were fixed in 10% formalin and stained with toluidine blue for mast cell identification and viewed at 10-20x magnification. Mast cells were undetected in Wsh airways (A). Mast cells were visualized in close proximity to the main central airway in B6 and in both WBM and BMMC reconstituted animals (B-D). The average number of mast cells in the lung was quantitated by counting stained cells in 10 random fields along the mainstream bronchus (E). Mast cells were undetected in Wsh mice. Few mast cells were detected in wild-type B6 airways, while significantly larger quantities of mast cells were detected in both WBM and BMMC reconstituted animals. *P<0.001, #P<0.001, ψP<0.01.
Figure 3.3  Anaphylactic bronchoconstriction after mast cell reconstitution of Wsh mice.  A) WBM was harvested from either wild-type B6 or mast cell-deficient Wsh mice and used to reconstitute Wsh animals. Non-reconstituted B6 and Wsh mice were evaluated for comparison. Mice were sensitized with anti-DNP IgE, anesthetized, paralyzed, and mechanically ventilated. Experimental mice were then challenged with antigen (DNP) and airway mechanics measured. Control animals (B6, \( n=2 \)) were challenged with PBS. Significant airway constriction was seen in B6 mice (\( n=3 \)) and in Wsh animals reconstituted with B6 WBM (B6→Wsh, \( n=3 \)) after antigen challenge. The airways of Wsh and Wsh animals reconstituted with Wsh WBM (Wsh→Wsh, \( n=2 \)) did not differ from control animals treated with saline.  B) Mast cells were cultured from B6 bone marrow and used to reconstitute mast cell-deficient Wsh animals. Mice were sensitized with anti-DNP IgE, anesthetized, paralyzed, and mechanically ventilated. Mice were then challenged with DNP and airway mechanics measured. The central airways of B6 mice (\( n=5 \)) constricted significantly following antigen challenge. There was no response to antigen in either the Wsh (\( n=5 \)) or the reconstituted animals (BMMC→Wsh; \( n=6 \)).
Figure 3.4 Characterization of BMMCs. A) Cell surface expression of FcεRI and c-Kit was verified by fluorescence activated cell sort analysis (FACS). Mast cells were exposed to IgE for 2 h and stained with FITC-labeled anti-IgE antibody (right panel gray), PE-labeled anti-c-Kit antibody (left panel gray) or rat IgG1-FITC (right panel transparent) and rat IgG2b-PE (left panel transparent) as isotype-matched controls. B) Expression of the FcεRI receptor and tryptophan hydroxylase was verified using real-time RT-PCR from RNA collected from 1 million BMMCs. Relative expression was standardized using GAPDH as a control.
Figure 3.5 Histamine release following IgE-dependent passive anaphylaxis. Mice sensitized with anti-DNP IgE. 24 hours following sensitization, mice were challenged with either DNP or PBS. 2 min after challenge, animals were sacrificed and BALF (A) and plasma (B) collected for analysis of histamine release. Significant levels of histamine was measured in the BALF of wild-type (\(n=2\)) and WBM reconstituted Wsh (\(n=3\)) mice. Histamine was detected in the BALF of BMMC reconstituted Wsh (\(n=3\)) mice, although the level was significantly attenuated compared to both B6 and WBM reconstituted animals. Large quantities of histamine were present in the plasma of wild-type mice and both groups of reconstituted animals. There was no significant difference in plasma histamine levels between those 3 groups. No histamine was detected in the BALF or plasma of either Wsh (\(n=2\)) mice or B6 mice challenged with PBS (controls, \(n=2\)). *\(P<0.05\), #\(P<0.05\).
**Figure 3.6 Mast cell granules.** Lungs of B6 (A), WBM reconstituted Wsh (B), and BMMC reconstituted Wsh (C) mice were fixed in 10% formalin and stained with toluidine blue for mast cell identification and viewed at 100x magnification. Histologically, quantity of granules in lung mast cells is similar in all groups.
References


CHAPTER 4

Mechanistic differences in thromboxane-mediated airway constriction in the naive and allergic lung

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Abstract

Thromboxane A₂ (TXA₂) is a potent lipid mediator released by platelets and inflammatory cells and is capable of inducing vasoconstriction and bronchoconstriction. In the airways, recent studies have demonstrated that TXA₂ mediated increases in airway constriction is dependent on vagal innervation and that Tp receptor-mediated changes in airway constriction requires the expression of the M₃ muscarinic acetylcholine receptor (mAChR). To further define the mechanism underlying TXA₂-mediated airway constriction, we generated mice carrying a Tp receptor locus that is sensitive to disruption by cre recombinase. These mice were crossed with both nestin-cre transgenic mice, which express cre recombinase throughout both the central and peripheral nervous systems, and with SM22-cre transgenic mice, which express cre recombinase in smooth muscle cells. Here we demonstrate that loss of the Tp receptor throughout the nervous system does not significantly affect naïve airway reactivity induced by the stable TXA₂ analog, U46619. However, the resultant smooth muscle Tp-deficient animals demonstrate attenuated airway responses following aerosol challenges with U46619 and also exhibit attenuated TXA₂-mediated airway hyperreactivity (AHR) to cholinergic stimuli. Conversely, in the inflamed airway, receptor contribution switches to dependence on neural Tp receptors. Following OVA-induced inflammation, smooth muscle Tp-deficient mice regain responsiveness to U46619, while the response in neural Tp-deficient mice is lost. These findings suggest that TXA₂ mediates airway reactivity in the naïve lung through collaborations involving smooth muscle Tp receptors but through neural Tp receptors in the inflamed lung.
Introduction

Since its discovery and description as the active extract from human platelets (1), thromboxane A$_2$ (TXA$_2$) has been the focus of intense research describing the interactions of this prostanoid with components of the cardiovascular system. Production of TXA$_2$ results in the promotion of platelet aggregation, increased cholesterol loading, and vascular smooth muscle proliferation (2). However, in addition to balancing proper cardiovascular health, TXA$_2$ is also capable of inducing potent smooth muscle constriction, which can influence diverse physiological processes including vasoconstriction, bronchoconstriction, uterine contractions, and intestinal contractions. Similar to other prostanoids, TXA$_2$ synthesis is initiated by the oxidation of arachidonic acid (AA) by either cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) into prostaglandin H$_2$ (PGH$_2$). The thromboxane synthase enzyme (TXAS) catalyzes the isomerization of PGH$_2$ to generate TXA$_2$, which has a very short half-life in aqueous solution and is rapidly hydrolyzed to the stable inactive metabolite thromboxane B$_2$ (TXB$_2$). Because of this instability, most experimental studies of TXA$_2$ biology have utilized stable TXA$_2$ mimetics such as U46619 (3-5). TXA$_2$ exerts its biological functions through interactions with the heterotrimeric G protein-coupled thromboxane prostanoid (Tp) receptor (6-9). All of the known physiologic functions of TXA$_2$ are dependent upon the expression of this receptor, which associates with the Gq family of proteins and asserts most of its physiological actions through the activation of phospholipase C (PLC) and increases in intracellular calcium concentrations ([Ca$^{2+}$]).

Pharmacological assessments with selective TXA$_2$ antagonists and conventional Tp receptor deficient mouse lines have been crucial in implicating TXA$_2$ in a number of
pathophysiological conditions including cardiovascular disease (10), intrauterine growth retardation (11), various kidney diseases (12-14), and angiogenesis (15). In addition to these disorders, pharmacological and genetic approaches have been essential in establishing TXA₂ as a potent mediator of human and mouse airway constriction, where it has been shown to be capable of influencing a variety of mechanisms associated with lung diseases such as asthma and chronic obstructive pulmonary disease (COPD)(16-19). However, the intrinsic limitations of pharmacological assessments (potency, specificity, and dosage) have hampered efforts to explore many of the underlying mechanisms affecting these pathophysiological processes. Likewise, conventional Tp receptor-deficient mice, which lack functional Tp receptors in all cell types, lack the resolution to discern which Tp receptors are responsible for specific physiological outcomes. This is especially troublesome in tissues where multiple cell types express Tp receptors.

Bronchoconstriction is a dynamic process that involves airway smooth muscle (ASM) and parasympathetic innervation, and can also be influenced by bronchial epithelial cells and leukocytes. Each of these cell types has been suggested to express Tp receptors (20-25), thus the classical pharmaceutical and genetic techniques are inadequate to further define the in vivo mechanism underlying TXA₂ induced ASM constriction. Considerable evidence has suggested that TXA₂ mediates ASM constriction through neural mechanisms affecting acetylcholine release (19, 26, 27). Indeed, the Tp receptor has been found expressed on neurons and in discrete regions of the brain, where TXA₂ has been implicated in the proliferation and survival of oligodendrocytes (28, 29). Direct evidence has also demonstrated that these receptors are present on peripheral and/or sensory nerves of the mouse, pig, and rabbit (30-32). Here we directly assess the
contribution of neurally expressed Tp receptors in mediating airway reactivity by generating mice carrying a Tp locus that is sensitive to disruption by cre recombinase.

In addition to neural expression, Tp receptors are also abundantly expressed on ASM cells. To evaluate the role of these receptors, mice carrying the floxed Tp locus were crossed with a mouse line in which cre expression is under the control of the smooth muscle specific SM-22 promoter. Here we demonstrate that, in vivo, smooth muscle-specific Tp receptor-deficient mice exhibit attenuated increases in airway reactivity in response to U46619, as well as, attenuated U46619-mediated AHR to cholinergic stimuli in the naïve lung. These findings suggest that TXA2-induced ASM constriction and AHR in the naïve airway are predominately mediated by Tp receptors expressed by the smooth muscle. Conversely, in the inflamed airway, smooth muscle Tp receptor-deficient mice regain reactivity to U46619 following OVA-induced inflammation while neural Tp receptor-deficient mice are unresponsive. This suggests differing mechanisms mediating TXA2-induced airway reactivity in naïve and inflamed airways.

Methods

Generation of mice carrying a Tp locus sensitive to Cre-mediated disruption.
Segments of the Tp receptor gene were amplified by PCR. The resultant PCR products were utilized to create a plasmid capable of undergoing homologous recombination with the endogenous locus and in doing so, introduce loxP sites flanking the major coding exon of the Tp receptor (Figure 4.1). Three fragments of the Tp receptor gene were amplified using the following primer sets: 5’-ATAAGCTTTGCGGCCGCAGTTTCCCTGGTGGTACGTG-3’ and 5’-ATATCGATTAGCCCTAGCTGTCCTGGAA-3’ (to amplify a 3073 bp region of homology in intron 1), 5’-GCTGCCTCAAAG

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AAAGGGTA-3’ and 5’-GTGCGG CCGCTCCTAAAGCCCCAAAGACCT-3’ (to amplify a 1032 bp region containing exon 2), and 5’-
GTGGATCCCTGCAGGATGTAACAGGAAAGA-3’ and 5’-GTGGTACCAGA
ACCCATCCCAGTTCTGA-3’ (to amplify a 4424 bp region of homology in intron 2).
These fragments were sub-cloned into the pCR 2.1 vector (Invitrogen) and sequenced to verify that the PCR amplification did not introduce mutations into essential sequences.
The fragment corresponding to intron 2 was directly cloned into the pXenaLF² vector, 3’ to the neomycin cassette and 5’ to the pgk-tk cassette (Figure 4.1). The pXenaLF² vector contains the selectable marker gene for neomycin resistance (pgk-neo) flanked on both 5’ and 3’ sides by a loxP and frt site. The fragments corresponding to intron 1 and exon 2 of the Tp receptor were subcloned 5’ and 3’ of the loxP cassette, respectively, in the pNebLox vector. This generated a fragment of Tp receptor DNA with a loxP site placed in intron 1. This newly generated 5’arm-loxP-exon 2 cassette was subsequently removed from pNebLox and cloned into the pXenaLF² vector, 5’ to its loxP cassette (Figure 4.1).

The targeting plasmid was linearized with PvuI and introduced into embryonic stem (ES) cells derived from 129/SvEv mice and transformants were isolated using standard methodologies (33). A DNA probe corresponding to the region immediately downstream of the targeted region (3’ probe) was generated as previously described (9) and a probe corresponding to exon 2 of the Tp receptor (internal probe) was generated using the following primer sets: 5’-GCTGCCTCAAAAGAAAGGTA-3’ and 5’-
GTGCGGCGG CTCCTAAAGCCCCAAAGACCT-3’. These probes were used to identify targeted ES cells by Southern blot and were used to confirm the incorporation of loxP sites in successfully targeted ES cells. These probes were also used to genotype the
resultant mice. ES cells in which the plasmid integrated by homologous recombination and contained the loxP-exon2-loxP-flp-neo-flp cassette were used to generate chimeric animals, which in turn were bred to generate animals heterozygous for the floxed allele. The neomycin gene was then removed by breeding the heterozygous mice with C57BL/6J mice that express flp recombinase in the germline (B6;SJL-Tg(ACTFLPe)9205Dym/J, Jackson Laboratories). Following digestion with BamHI, the exon 2 probe discussed above was utilized to confirm the removal of the neomycin cassette.

Mice expressing cre recombinase under the control of the SM22 promoter are commercially available (STOCK Tg(Tagln-cre)1Her/J, Jackson Laboratory) and maintained on a mixed genetic background of 129S5/SvEvBrd, C57BL6, and SJL. These animals have been shown to express cre throughout the smooth muscle from all tissues examined (34-36). C57BL/6J mice expressing cre recombinase under the control of the nestin promoter are also commercially available (B6.Cg-Tg(Nes-cre)1Kln/J, Jackson Laboratory). These mice express cre early in embryonic development in cell lineages that give rise to all neurons including sensory neurons, neurons of the CNS, and parasympathetic and sympathetic pathways (37). The introduction of these transgenes onto the 129/SvEv background was initiated and animals were backcrossed for 7 generations. A DNA probe corresponding to the region immediately 5’ of the targeted region was generated by PCR (5’-AACCTGAGTCTGTGGG GTTG-3’ and 5’-ACAAGCATCA AGGAGGGATG-3’) and was used, in conjunction with a BamHI digest, to assess Tp exon 2 removal by cre/lox in selected tissues. 129/SvEv Tp+/− mice were generated as previously described (9). All animal studies were conducted in
acCORDANCE WITH THE NATIONAL INSTITUTES OF HEALTH GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS AS WELL AS THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) GUIDELINES OF THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL.

**Measurement of Tp expression in cultured airway smooth muscle cells and peripheral nerves.** Airway smooth muscle was isolated from tracheal explants. Briefly, the trachea between the larynx and the mainstem bronchi was removed and placed in a sterile Petri dish containing HBSS solution with a 2X concentration of antibiotic/antimycotic (penicillin/streptomycin/genomycin). The trachea was dissected free of surrounding tissues and epithelium was removed by rubbing the luminal surface with a plastic blade. The trachea was then dissected into 2-3 mm segments and placed intima side down in a sterile culture dish. Explants were allowed to adhere to dish and then covered with 3ml of DMEM+20%FCS+2X antibiotic/antimycotic and incubated at 37°C in a humidified chamber. After 3 days of cell growth, FCS was reduced to 10% and antibiotic/antimycotic to 1X. At confluent density, cells were harvested for RNA collection.

Nodose and superior cervical ganglia (SCG) were collected from 5-6 mice and snap frozen in liquid nitrogen. Pools of ganglia were homogenized using a glass pestle prior to RNA collection. RNA was collected using RNAbee reagent (Qiagen) and was converted to cDNA using a High Capacity cDNA RT kit (Applied Biosystems) and Tp receptor expression was assessed by real-time PCR using a commercially available Tp probe and 18S probe as an internal control (Applied Biosystems). Data is shown relative to 129Sv/Ev Tp⁺/⁺.
Measurement of Airway Reactivity in Intubated Mice. Mice were anesthetized with 70-90 mg/kg pentobarbital sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated at a rate of 300 breaths/min, tidal volume of 6 cc/kg, and positive end-expiratory pressure of 3-4 cm H2O with a computer controlled small-animal ventilator (Scireq, Montreal, Canada). Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide. Following baseline assessments, mice were exposed to aerosol challenges via an ultrasonic AeroNeb nebulizer (Scireq) connected through inspiratory line of the ventilator circuit. Animals were ventilated at a rate of 200 breaths/min for 30 seconds with a tidal volume of 0.15 mls. Airway mechanics using the Forced Oscillatory Technique (FOT) were determined every 10 seconds for the following 3 minutes, as previously described (38). Briefly, following passive expiration, a broadband (1-19.625 Hz) volume perturbation was applied to the lungs while the pressure required to generate the perturbations was assessed (38-40). The resultant pressure and flow data were fit into a constant phase model as previously described (38, 41). Similar to other studies assessing forced oscillatory mechanics, we confined our analysis to $R_{aw}$ (Rn; Newtonian resistance), which assesses the flow resistance of the conducting airways and G (tissue damping), which reflects tissue resistance (38, 39). To assess TXA2-mediated airway reactivity, mice were exposed to aerosol challenges of the stable thromboxane analog U46619 (Caymen Chemical, Ann Arbor, MI) as previously described (17). Briefly, mice were exposed to a 30-second aerosol dose response challenge of $10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M. Following each aerosol challenge, airway responses were assessed for 3 minutes as previously described.
Because thromboxane has also been suggested to induce airway hyperresponsiveness, we also assessed the increases in airway mechanics to methacholine (MCh), an acetylcholine analog, following pretreatment with a mildly provoking dose of U46619 in naïve mice. Airways were exposed to a 30-second aerosol dose of U46619 (10^{-5} M) or vehicle (10% EtOH) and changes in airway mechanics were assessed for 3 minutes. Following this exposure, mice were challenged with a 30-second aerosol dose MCh (3 mg/ml; Sigma Chemical, St. Louis, MO).

**Induction of allergic airway disease.** To evaluate the role of tissue specific Tp receptors in inflammatory cell recruitment and airway hyperresponsiveness mice were sensitized by i.p. injection of 20 µg OVA emulsified in 2.25 mg aluminum hydroxide gel in a total volume of 200 µl on day 1. Mice were challenged (1 hour) via the airways with OVA (1% in saline) for 3 days (days 14-16) using an ultrasonic nebulizer (DeVillbiss Health Care, Somerset, PA). On day 17 mice were anesthetized, placed on a ventilator and challenged with increasing doses of U46619 (10^{-5} M-10^{-3} M). Airway measurements were taken every 10 s for 3 min.

After airway measurements, mice were sacrificed and 0.5-1.0 ml of blood was collected by cardiac puncture. Following coagulation, serum was collected. Total IgE levels in the serum were measured by ELISA (R&D systems). Bronchoalveolar lavage was performed five times with 1.0 ml of sterile HBSS each time. Cells present in the bronchoalveolar lavage fluid (BALF) were determined using a hemocytometer. A differential cell count was conducted on a cytospin prepared from 150 µl of BALF and stained with Diff-Quik solution (Sigma Chemical). The remaining BALF was centrifuged to remove cells. IL-13 levels were measured in the cell-free supernatant by
ELISA (R&D systems). In some animals, the right lobes of the lung were snap frozen in liquid nitrogen and homogenized for RNA collection. RNA was then converted to cDNA using a High Capacity cDNA RT kit and a surrogate marker of mucus production was assessed by real-time PCR using a commercially available Muc5a probe and an HPRT probe as an internal control (Applied Biosystems). Data is shown relative to naïve lung.

**Histology.** For histopathologic examination, lungs were fixed by inflation (20 cm pressure) and immersion in 10% formalin. To evaluate airway eosinophilia, fixed lung slices were subjected to hematoxylin and eosin (H & E) staining. H&E stained lung slices were digitally imaged midway along the length of the main axial airway, as well as, at both ends of the same airway. Each of the three images was then divided into 4 quadrants and the levels of inflammation in each quadrant were semi quantitatively assessed based on a score of 0 (no inflammation) to 3 (severe inflammation). Scores from all quadrants for each of the 3 pictures representing 1 airway were then averaged together to represent the average H&E score of that airway (42). To assess goblet cell hyperplasia, serial sections of the left lobes of the lungs that yield maximum longitudinal visualization of the intrapulmonary main axial airway were analyzed following Alcian-blue/periodic acid-schiff reaction (AB-PAS) staining. To avoid bias for a certain region, and to consistently view the identical region in all slides, a 2-mm length of airway, located midway along the length of the main axial airway, was digitally imaged. Using ImageJ software (NIH, National Technical Information Service, Springfield, VA), the area and length of the AB-PAS-stained region in the sections were measured and the data expressed as the mean volume density ($V_s = nl/mm^2$ basal lamina + SEM of AB-PAS stained material within the epithelium) as previously described (43).
Statistical Analysis. Data are presented as the means ± standard error of the mean (SEM). Analysis Of Variance (ANOVA) followed by Tukey-Kramer HSD for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student’s two-tailed t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Generation of mice with tissue specific loss of the Tp receptor

The development of mouse lines in which loss of gene expression is restricted to specific populations of cells provides a powerful means with which to identify the effector population(s) through which a particular gene product contributes to complex physiological responses. This experimental system requires the placement of loxP sites around a critical exon of the gene under study. To obtain a Cre recombinase-mediated deletion of the Tp receptor, a targeting vector was designed which would introduce a Neo cassette flanked by loxP-frt sites downstream from exon 2. A third loxP site was introduced into the first intron of the murine Tp receptor gene, thereby flanking exon 2, with loxP sites (Figure 4.1A). Exon 2 contains the ATG start codon and encodes 6 of the 7 transmembrane domains of the Tp receptor. Following electroporation of the targeting plasmid, Southern blot analysis of the ES cell clones was used to identify those carrying a floxed TP allele.

For the initial screen, ES clones were digested with BamHI and probed with a 3’ probe that lies outside of the targeting plasmid. Digestion of the DNA from eight neomycin resistant clones produced the 7.4 kb fragment that is indicative of successful
integration of the targeting plasmid via homologous recombination (Figure 4.1B). These clones were then probed with an internal, exon 2 specific probe to identify those producing a 6.5 kb fragment, which suggests that the floxed Tp exon 2 and loxP-frt-neo-loxP-frt cassette were successfully integrated (Figure 4.1C). The targeted ES cell clones were further analyzed to identify those in which the crossover event during homologous recombination resulted in the introduction of the 5’ loxP site in addition to the neomycin gene at the Tp locus. DNA was digested with BamHI and EcoRI and analyzed by Southern blot using the exon 2 probe, which recognizes a 7.3 kb wild type allele and a 5.5 kb fragment in those clones in which the crossover event failed to introduce the 5' loxP site. The probe is expected to hybridize to a 3.3 kb fragment both in ES cells in which the targeting plasmid integrated randomly and in those clones which underwent homologous recombination with the desired crossover event. Clone number 7, was the only ES cell clone identified as clearly carrying a floxed Tp receptor exon 2 allele (Figure 4.1D). Subsequent mouse lines were generated from this ES cell line.

Removal of the neo gene from the Tp locus of the 129 ES cell-derived mice required crossing the mice with the germline-flp transgenic line, which is on a C57BL/6 genetic background. In addition, the nestin transgene was obtained on a C57BL/6 genetic background, while the SM22 transgene was on a mixed genetic background (129S5/SvEvBrd, C57BL6, and SJL). All resulting mice were then backcrossed onto the 129/SvEv background for 7 generations.

Because of the difficulty of obtaining sufficient numbers of experimental animals by simply intercrossing the Tp^{loxTp/+} and cre-transgene positive Tp^{loxTp/+} mice (only 1 in 8 mice would be expected to be useful), we first crossed the transgenic mice with Tp^{−/−}.
animals on a 129/SvEv genetic background. The cre-transgene positive female Tp<sup>+/−</sup>
mice were then crossed to mice homozygous for the Tp<sup>loxEV</sup> allele. Four different
genotypes of mice were generated from this cross at approximately equal frequencies.
The Tp<sup>loxEV/+</sup> mice should behave essentially the same as wild type mice, since the
presence of the loxP sites in the introns of the Tp receptor is not expected to affect gene
expression. The Tp<sup>loxEV/−</sup> mice generated would be expected to have a slight attenuation
of response to U46619 based on our previous studies showing a decrease in this response
in heterozygous animals. The response of these mice should be indistinguishable from
that of the cre-transgene positive Tp<sup>loxEV/+</sup> animals, if Tp expression is critical in the cre
expressing tissues, as either the smooth muscle (Tp<sup>loxEV/+ Tgln+ve</sup>) or neural (Tp<sup>loxEV/+ nestin+ve</sup>)
tissues will essentially be +/- for the Tp receptor. If this is not the critical
tissue type, then these mice should behave similar to a wild type mouse. Finally, the cre-
transgene positive Tp<sup>loxEV/−</sup> mice are expected to have no expression of Tp in the smooth
muscle (Tp<sup>loxEV/− Tgln+ve</sup>) or neural (Tp<sup>loxEV/− nestin+ve</sup>) tissues and are heterozygous for
Tp in all other tissues. Thus the appropriate comparisons should be made between the
cre-transgene positive Tp<sup>loxEV/−</sup> mice and their Tp<sup>loxEV/−</sup> littermates. In summary, we would
expect if a particular tissue is critical for a response, the following rank order of
responses is expected (from lowest to highest): cre-transgene positive Tp<sup>loxEV/−</sup> < Tp<sup>loxEV/−</sup>
= cre-transgene positive Tp<sup>loxEV/+</sup>.

Southern blot analysis was utilized on select tissues to confirm successful, tissue
specific, disruption of the Tp receptor. Tissues were harvested from Tp<sup>loxEV/+</sup> mice, which
either carried the cre transgene under the control of the SM22 (transgelin; Tgln) promoter
(Tgln+) or were negative for the cre transgene (Tgln−) (Figure 4.2A). Tissues were
harvested that demonstrate high concentrations of smooth muscle (trachea, intestine, and uterus), cardiac muscle (heart), and skeletal muscle (thigh muscles), as well as, high Tp receptor expression (kidney). DNA was extracted from each of these tissues and digested with BamHI for Southern blot analysis with probes specific for cre (data not shown), exon 2 (data not shown), and for a region 5’ of exon 2. All tissues demonstrate the endogenous 11.2 kb fragment indicative of the wild type allele and a 4.5 kb fragment indicative of the floxed Tp allele. However, an additional 3.3 kb fragment is present in smooth muscle containing tissues, which suggests successful disruption of the Tp receptor, at least in some cells (Figure 4.2A). To verify Tp receptor loss in the ASM, 3 denuded tracheas were pooled together from Tp\textsuperscript{loxTP+} and Tp\textsuperscript{loxTP-} mice, which either carried the SM22-cre transgene or were transgene negative (Figure 4.2B). As with the SM22 mice, tissues were also harvested from Tp\textsuperscript{loxTP+} and Tp\textsuperscript{loxTP-}, which carried the cre transgene under the control of the nestin promoter. The brain, intestine, and kidney were harvested and Southern blot analysis was performed as described above. Animals that are wild type for the Tp receptor (Tp\textsuperscript{+/+} and the Tp\textsuperscript{loxTP+} mice) demonstrate an 11.2 kb fragment, while mice that carry the Tp receptor disrupted by insertion of a neomycin cassette (Tp\textsuperscript{loxTP-} nestin+, and Tp\textsuperscript{loxTP-} mice) demonstrate a 4.1 kb fragment (Figure 4.2C). In non-neuronal tissues (kidney and intestine) a 4.5 kb fragment is present that represents the intact floxed Tp receptor. However, in the brain, a 3.3 kb fragment is generated in the nestin+ mice, which is indicative of successful neural disruption of the Tp receptor by cre recombinase (Figure 4.2C).

To verify loss of Tp receptors specifically in ASM and peripheral neurons in cre-transgene expressing mice, Tp expression in tracheal smooth muscle cultures and
nodose/SCG ganglia pools was verified by real-time PCR (Figure 4.3A-B). Relative to wild-type 129/SvEv, expression of Tp receptors in ASM and peripheral ganglia in Tp$^{\text{loxTpr/-}}$-Tgln+ve mice (Figure 4.3A) and Tp$^{\text{loxTpr/-}}$-Nestin+ve mice (Figure 4.3B), respectively, did not differ from that seen in Tp$^{-/-}$ mice, verifying the loss of this receptor in those tissues. In addition, heterozygous mice exhibited approximately half the expression compared to wild-type animals.

**Neurally deficient Tp receptor mice demonstrate increased airway resistance ($R_{aw}$) and tissue damping (G) following stimulation with U46619**

Initial reports suggest that TXA$_2$ contributes to ASM constriction through the potentiation of vagal nerve neuro-effector transmission (18). Inflammatory mediators such as TXA$_2$ could affect neural activity at a variety of levels, including the primary afferent sensory nerve, autonomic ganglia, and autonomic neuroeffector junction. If this hypothesis were true, we would expect that U46619 induced increases in $R_{aw}$ and G would be severely attenuated in mice with reduced neural expression of Tp receptors. U46619 induced a significant, dose dependent increase in both $R_{aw}$ and G in 129/SvEv wild type mice (Tp$^{+/+}$), while Tp receptor deficient animals (Tp$^{-/-}$) were unresponsive (Figure 4.4A-B). No significant differences in $R_{aw}$ or G were observed between the Tp$^{\text{loxTP/+}}$ wild type mice, the Tp$^{\text{loxTpr/-}}$ nestin+ve neurally deficient Tp receptor mice, the Tp$^{\text{loxTpr/-}}$ heterozygous mice, or the Tp$^{\text{loxTpr/+}}$ nestin+ve neurally heterozygous Tp receptor mice (Figure 4.5A-B).
Airway reactivity is attenuated in smooth muscle Tp receptor deficient mice after exposure to U46619

Neural disruption of the Tp receptor failed to significantly attenuate airway reactivity to U46619, suggesting that TXA₂ mediated airway constriction may be mediated by other cell types known to express the Tp receptor. TXA₂ receptor expression by smooth muscle cells is well documented, as is the ability of Tp receptor activation to mediate increases in [Ca²⁺], in cultured human primary ASM cells (44). Here, we sought to examine the possibility that TXA₂-mediated increases in airway reactivity could be mediated by Tp receptors located directly on ASM cells. If this hypothesis were true, we would expect that U46619-induced increases in R_{sw} and G would be severely attenuated in mice with reduced expression of TP receptors on smooth muscle. As mentioned above, U46619 induced a significant, dose dependent increase in both R_{sw} and G in wild type mice (Tp⁺/⁺), while Tp receptor deficient animals (Tp⁻/⁻) were unresponsive. Like the Tp⁻/⁻ animals, the Tp^{loxTp⁻/⁻tgln+}ve smooth muscle receptor deficient mice also demonstrated a significantly attenuated R_{sw} response compared to both Tp^{loxTp⁻/⁻} heterozygous and Tp^{loxTp⁺/⁺} wild type mice. The heterozygous Tp^{loxTp⁻/⁻} animals demonstrated an intermediate phenotype with a significantly enhanced R_{sw} compared with the Tp^{loxTp⁻/⁻tgln+ve} smooth muscle receptor deficient mice and significantly attenuated compared with the Tp^{loxTp⁺/⁺} wild type animals (Figure 4.6A). Similar to Tp⁻/⁻ animals, no G response was observed in animals that were deficient of Tp receptors in their smooth muscle (Figures 4.6B). Although a mild dose-dependent increase in G was seen following U46619 challenge, this response did not differ between the Tp^{loxTp⁻/⁻}, Tp^{loxTp⁺/⁺tgln+ve}, and the Tp^{loxTp⁺/⁺} groups.
Exposure to U46619 increases airway sensitivity to cholinergic stimuli in the naïve lung

The ability of U46619 to induce AHR to muscarinic receptor agonists has been characterized in a variety of model organisms (45-47). However, while this phenomenon has been identified in canine, guinea pig, and human studies, the mechanism behind this increased sensitivity is not well defined. Therefore, we sought to assess and characterize this TXA2-mediated AHR in mice. Both Tp+/+ and Tp-/- mice were exposed to a mildly provoking dose of U46619 (10⁻⁵ M). The initial treatment with U46619 was followed, 3 minutes later, by exposure to a weakly provoking dose of MCh (3 mg/ml). Pretreatment with U46619 induced a robust MCh response in wild-type animals, but not in Tp-deficient animals (Figure 4.7A-B). Increased responsiveness to MCh after U46619 challenge was evident in both the Rsw (Figure 4.7A) and G (Figure 4.7B) parameter of Tp+/+ mice, signifying AHR in both the central and peripheral airways. In contrast, U46619 pretreatment did not increase responsiveness to non-cholinergic stimulation with serotonin (5-HT), adenosine, PGD₂, or PGF₂α (Figure 4.8).

Following the identification of a primary role for smooth muscle Tp receptors in mediating U46619-induced airway reactivity in the naïve model described above, we next wanted to assess whether induction of AHR is dependent on smooth muscle expression of Tp receptors, specifically the ability of U46619 to enhance the response to low doses of MCh in the smooth muscle Tp receptor-deficient mice and their controls (TploxTp/- heterozygotes). TploxTp/- heterozygous mice had a significantly increased response to MCh in the central airways (Rsw) following pretreatment with U46619 (Figure 4.7C). This AHR was not seen in the smooth muscle Tp-deficient animals. Although the
heterozygous mice also trended towards AHR to MCh after U46619 challenge in the peripheral airways, this response was not statistically significant (Figure 4.7D).

Pretreatment of smooth muscle Tp-deficient mice with U46619 also failed to increase responsiveness to MCh in the G parameter.

**Airway reactivity in the inflamed airway**

Allergic lung disease was induced by sensitization and repeated aerosol challenges with OVA. Control animals were sensitized to OVA but challenged with saline. Aerosol challenge of U46619 induced smooth muscle Tp receptor-dependent central (Figure 4.9A) and peripheral (Figure 4.9B) airway constriction in saline challenged animals. Saline challenged Tp\(^{/-}\) and smooth muscle-deficient Tp\(^{loxp/-}\) Tgln+ve had no response to U46619 at any dose. Surprisingly, OVA challenged Tp\(^{loxp/-}\) Tgln+ve mice showed significant airway reactivity when compared to saline challenged animals, which implies the loss of smooth muscle Tp-receptor dependence for airway constriction in the inflamed airway. General airway inflammation was the same in both Tp\(^{loxp/-}\) and Tp\(^{loxp/-}\) Tgln+ve OVA challenged mice; there was no difference in the average H&E score (Figure 4.10A), total BALF cell count (Figure 4.10B), cellular profile (Figure 4.10C), BALF IL-13 levels (Figure 4.10D), serum IgE (Figure 4.10E) or mucus production (Figure 4.10F-G) between the two groups. As expected, these parameters were all elevated compared to the saline treated animals (some data not shown).

Aerosol challenge of U46619 induced dose-dependent central and peripheral airway constriction in saline challenged animals that was independent of neural Tp
expression (Figure 4.11A-B). Surprisingly, while OVA challenge increased the responsiveness of $T_p^{\text{loxT}_{p/-}}$ mice to U46619, OVA challenged neural deficient $T_p^{\text{loxT}_{p/-}}$ Nestin+ve mice failed to respond to U46619 when compared to saline challenged animals, suggesting a dependence on neural Tp receptors for this response. This change in airway response was not a result of altered inflammatory conditions in the airways, as there was no difference in airway inflammation, BALF cellularity, BALF IL-13, serum IgE, or mucus production between $T_p^{\text{loxT}_{p/-}}$ Nestin+ve and $T_p^{\text{loxT}_{p/-}}$ mice (Figure 4.12A-G).

**Discussion**

Extensive characterization of TXA$_2$-mediated airway reactivity has suggested that this potent lipid mediator indirectly induces ASM constriction by potentiating the neuro-effector transmission of cholinergic stimuli. The basis for this hypothesis stems from microelectrode and tension recording assessments of smooth muscle cells and nerve preparations isolated from the canine trachea. Under these *ex vivo* conditions, sub-threshold levels of TXA$_2$ ($10^{-10}$-10$^{-7}$ M, depending on study) do not affect the membrane potential, input membrane resistance, or the sensitivity to acetylcholine of canine trachea smooth muscle cells; however, this concentration is sufficient to significantly increase the amplitude of contractions evoked by electrical field stimulation (EFS) (18, 48). This amplification has been shown to be sensitive to atropine, tetrodotoxin, and selective TXA$_2$ antagonists. Expanding upon these findings, the ability of TXA$_2$ to augment the *in situ* central airway contraction of the canine trachea following efferent nerve stimulation was also assessed (49). These studies monitored airway responses to intravenous drug
administration and vagal stimulation by in situ isometric measurements of tracheal smooth muscle tension. These data demonstrate that U46619 substantially augments the tracheal contraction induced by vagal stimulation. However, when acetylcholine was given instead of vagal stimulation, U46619 did not enhance the tracheal contractions (49). It was therefore reasoned that U46619 potentiation is caused by a pre-junctional action rather than by nonspecific pre-contraction of ASM with another agonist. Based on previous work demonstrating that U46619 did not enhance afferent vagal stimulation of canine ASM (50) and the observation that canine airways lack non-adrenergic innervation (51), the authors presumed that TXA2 augmentation depended on efferent pre-junctional vagal mechanisms (49).

In addition to data generated with canine tracheal preparations, data that supports the enhancement of neural signaling via TXA2 has also been generated by assessments of canine bronchial preparations. As with tracheal preparations, U46619 has also been demonstrated to constrict bronchial smooth muscle. To further characterize this constriction, microelectrode studies demonstrated that sub-threshold doses of U46619 (10-9 M) greatly potentiated the amplitude (252%) and the duration (over 2-fold) of the EFS excitatory junction potential without significantly altering the membrane potential or tonic contractions of the ASM (21, 52). This pre-junctional effect was shown to be sensitive to TXA2 receptor antagonists and therefore suggests that U46619 is capable of pre-junctionally stimulating nerves, via the Tp receptor, to enhance the release of ACh (21).

Based on the extensive data suggesting that TXA2-mediated ASM constriction is dependent on a neural mechanism, it was surprising that we did not observe a more
dramatic phenotype in naïve mice lacking neural expression of Tp receptors, despite verified disruption of the receptor in both central and peripheral nerves by southern blot analysis and real-time PCR, respectively. However, it is also apparent that this potent lipid mediator can also mediate airway constriction though post-junctional interactions with the Tp receptor. Indeed, many of the studies discussed above that effectively demonstrated the presence of a neurally mediated mechanism underlying TXA2-induced ASM constriction, also characterized post-junctional activities. For example, as previously discussed, Janssen and Daniel demonstrated that U46619 was capable of pre-junctional stimulation of nerves. However, they also observed that U46619-induced membrane depolarization and powerful ASM constrictions through a mechanism that utilized intracellular Ca\(^{2+}\) rather than extracellular Ca\(^{2+}\) in canine bronchial smooth muscle preparations (21, 48). These contractions were sensitive to a Tp receptor antagonist (21) and were insensitive to TTX and atropine (52). Together, these data suggest that ASM Tp receptors may also have some role in influencing airway constriction. Extensive data clearly demonstrates that Tp receptors are expressed on smooth muscle (53-55) and as discussed above, several studies have suggested that these receptors may functionally contribute to ASM constriction. Consistent with this, we have shown that U46619-induced constriction in the naïve mouse lung is dependent on Tp receptors specifically located on smooth muscle.

A number of models could be proposed to describe possible mechanisms involved in the modulation of smooth muscle Tp receptor signaling in the naïve lung that are consistent with previous work showing a dependence on the M3 mAChR for TXA2-mediated ASM constriction (17). Because Tp receptors and M3 mAChRs are
both present on smooth muscle, one potential model suggests that stimulation of the Tp receptor fails to increase intracellular Ca\textsuperscript{2+} to levels sufficient to mediate constriction. However, binding of TXA\textsubscript{2} to its receptor potentates the activity of the M\textsubscript{3} mAChR receptor, thereby increasing the sensitivity of this receptor to basal release of acetylcholine. While this is the simplest model, other possible models can be envisioned, which reflect a possible modulation of M\textsubscript{3} mAChR signaling by the Tp receptor. It is well established that G-protein coupled receptors (GPCR) function via distinct signal transduction pathways; however, it is also accepted that “cross-talk” or synergy can also occur. This synergism can occur through mechanisms such as heterodimerization, cross-reactions among GPCR downstream effectors, and G-protein redistribution (56). While downstream effector cross-signaling has not been defined for Tp receptors, some evidence does suggest the Tp receptors are capable of both heterodimerization and cross-talk with other GPCRs via redistribution of G-proteins. Tp receptors have been shown to form heterodimers with the prostacyclin receptor (IP) in vascular smooth muscle cells (57, 58), suggesting the possibility of heterodimerization with M\textsubscript{3} receptors on ASM. Evidence for cross-talk stems from data suggesting synergism between platelet PAR1 receptors and Tp receptors, as well as, anecdotal evidence suggesting a similar synergism between platelet-activating factor (PAF) and Tp receptors (59). The PAR1/Tp synergism model describes a dynamic equilibrium between the Tp receptor and other GPCRs that couple to the same G\alpha-subunits as Tp (56). Following this model, a G\alpha driven competition may exist between Tp and other GPCRs. Upon activation, the disassociation of the G\alpha-subunit from these GPCRs, and the subsequent G\alpha reassociation to Tp is capable of shifting Tp to a higher affinity state.
and thereby enhancing Tp receptor mediated signaling events (59, 60). It is tempting to speculate a similar synergism may exist between the Tp receptor and the M₃ mAChR.

In addition to being capable of inducing direct airway constriction, TXA₂ can also contribute to AHR. While the contribution of TXA₂ to cholinergic AHR has been identified, little progress has been made at addressing the underlying mechanism. Previous reports have speculated that inflammation is the underlying mediator of this TXA₂ induced AHR (16). However, the notion that inflammation is the only factor mediating TXA₂-mediated AHR is not possible as previous studies have demonstrated that the U46619-mediated enhanced MCh response has been observed in *ex vivo*, blood leukocyte free models (47), as well as, our data presented here showing U46619-mediated cholinergic AHR in naïve animals. Here we show that smooth muscle Tp receptor activation appears to be the primary contributing factor in TXA₂-mediated AHR to MCh in the naïve lung. However, OVA-induced inflammation has been shown to induce AHR to U46619 which was mediated by both the Tp receptor and M₃ mAChR (17). In the current study, we show that although smooth muscle Tp-deficient mice are unresponsive to U46619 in the naïve lung, they regain responsiveness similar to heterozygous animals in the inflamed airway. In addition to this, following OVA treatment, neural Tp-deficient mice failed to respond to U46619 challenge. This would imply a mechanistic switch from smooth muscle Tp receptors to neural Tp receptors in the inflamed airway. From the data presented, it can be envisioned that in the allergic airway smooth muscle Tp receptors are responsible for initial “baseline” constriction to U46619 while neural Tp receptors mediate enhanced responsiveness to the agonist in the presence of inflammation.
Our data extends the findings that TXA$_2$ contributes to the development of allergic airway disease, and in particular to changes in airway reactivity, through its ability to amplify the actions of ACh. Based on the data generated here, it is likely that the primary mechanism by which TXA$_2$ enhances subsequent responses to ACh in the naïve lung is via the same pathway by which Tp itself brings about changes in airway reactivity: through Tp receptors directly on ASM. However, upon induction of allergic inflammation, neural Tp receptors activation mediates airway constriction. It is still unclear whether or not TXA$_2$ contributes to the nonspecific AHR observed in asthmatic individuals; however, the improvement in subpopulations of asthma patients following TXA$_2$ antagonist therapy suggests that this mechanism may play an important, yet limited, role in asthma pathophysiology.
A

Murine Tp Receptor
Endogenous Locus

1 kb

7.3 kb

3' Probe

7.4 kb Cre Recombinase Mediated

3' Probe

7.4 kb

BamHI Digest

4 7 11 32

11.2

7.4

3' Probe

BamHI + EcoRI Digest

Exon Probe

11 7 32

7.3

5.5

3.3

Exon Probe

III.

Targeting Plasmid

pgk-neo

loxP-loxP-frt-neo-loxP-frt

Non Probe

Exon 2 Probe

IV.

Removal of pgk-neo

lop-rec

Exon 2 Probe

Removal of Exon 2

V.

removal of pgk-neo

lop-rec

Exon 2 Probe

removal of Exon 2

B

C

D

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Figure 4.1 Schematic depicting the generation of a floxed TP allele in ES cells. A) The organization of the endogenous locus, the targeting plasmid, and the Tp allele generated after homologous recombination of the plasmid with the wild type Tp allele are shown. The DNA fragments required for assembly of the targeting plasmid were prepared from genomic DNA isolated from 129 derived ES cells and primers designed using the sequence for the TP gene available in the Celera mouse genome. Fragments were cloned into the vector pXenaLF2, which was constructed specifically for the rapid assembly of plasmids capable of generating floxed alleles. LoxP sites are denoted with triangles, frt sites by diamonds, and the neo gene is represented by the shaded arrow. Relevant restriction sites are abbreviated as follows: B, BamHI; A, Apal; E, EcoRI. Greyed boxes represent coding Tp receptor exons. B-D) Southern blot analysis of the ES cell clones to identify those carrying a floxed TP allele. DNA was prepared from neomycin resistant clones obtained after electroporation of the targeting plasmid into ES cells. A 3' probe not included in the targeting plasmid was used as a first screen to identify those ES cell clones in which the plasmid was integrated by homologous recombination. Eight such clones were identified and further analyzed using a TP exon 2 specific probe. Some of the targeted ES cell clones were further analyzed to identify those in which the crossover event during homologous recombination resulted in introduction of the 5' lox site in addition to the neomycin gene at the Tp locus. To do this, DNA was digested with BamHI and EcoRI and analyzed by Southern blot using the exon 2 probe. This probe recognizes a 7.4 kb wild type allele and a 5.5 kb fragment in those clones in which the crossover event failed to introduce the 5'lox P site. The probe is expected to hybridize to a 3.3 kb fragment both in ES cells in which the targeting plasmid integrated randomly and in those clones which underwent homologous recombination with the desired crossover event. Clone number seven clearly represents such an ES cell clone.
Figure 4.2  Southern Blot characterization of floxed Tp mice.  A) Southern blot analysis of organs and tissues from Tp receptor wild type mice carrying the floxed Tp allele (loxTp+/+) either with (Tgln+) or without (Tgln-) the cre transgene under the control of the SM22 (transgelin; Tgln) promoter. Smooth muscle containing tissues (Intestine and Uterus) demonstrate smooth muscle specific disruption of the Tp receptor.

B) Southern blot analysis of tracheas harvested and pooled from Tp receptor wild type (loxTp/+) or Tp receptor deficient (loxTp/-) mice carrying the floxed Tp allele either with (Tgln+/+) or without (Tgln/-) the cre transgene under the control of the SM22 promoter. Airway smooth muscle specific disruption of the Tp receptor was observed in the tgln/+ preparations.

C) Southern blot analysis of organs and tissues from Tp receptor wild type (loxTp/+) or Tp receptor deficient (loxTp/-) mice carrying the floxed Tp allele either with (Nestin+) or without (Nestin-) the cre transgene under the control of the Nestin promoter. Neural specific disruption of the Tp receptor was observed in brain preparations.
Figure 4.3 Expression of Tp receptors in airway smooth muscle and peripheral ganglia. Expression of Tp receptors in airway smooth muscle and peripheral nerves was confirmed by real-time PCR on smooth muscle cells cultured from tracheal explants (A) and pools of nodose and SCG neurons (B). Relative to wild type mice, heterozygous mice exhibited approximately 50% Tp expression, whereas Tp<sup>−/−</sup> mice had no Tp expression. Tp receptor expression in Tp<sup>loxTp<sup>−/−</sup>Tgln+ve</sup> and Tp<sup>loxTp<sup>−/−</sup>Nestin+ve</sup> mice did not differ from Tp<sup>−/−</sup> mice, indicating effective disruption of Tp receptors in airway smooth muscle and peripheral nerves, respectively.
Figure 4.4 Mice heterozygous for the Tp receptor (Tp⁺/⁻) demonstrate an intermediate response to a U46619 dose response challenge. Increases in both A) airway reactivity (Rₐw) and B) tissue damping (G) were observed in the Tp receptor wildtype mice (Tp⁺/+), while no increases were observed for the Tp receptor deficient mice (Tp⁻/⁻). Mice that were heterozygous for the Tp receptor (Tp⁺/⁻) demonstrated intermediate phenotypes, which were neither significantly increased compared to the Tp⁻/⁻ mice (with the exception of the 10⁻⁴M U46619 dose under the Raw parameter) nor significantly reduced versus the Tp⁺/+ animals. Tp⁻/⁻, n = 12; Tp⁺/⁻, n = 6; Tp⁺/+, n = 18. *P < 0.05; †P < 0.05.
Figure 4.5 Airway reactivity of mice deficient in neural Tp receptors to U46619.

Neural specific deletion of Tp receptors does not significantly affect airway resistance (A) and tissue damping (B) induced by U46619 aerosol challenge. Tp^{-/-}, n=4; Tp^{loxTp^{-/-}} nestin+ve, n = 8; Tp^{loxTp^{-/-}}, n = 4; Tp^{loxTp^{+/loxp}}, n = 4; Tp^{loxTp^{+/loxp}} nestin+ve, n = 5. *P<0.05.
Figure 4.6 Airway reactivity of mice deficient in smooth muscle Tp receptors to
U46619. Smooth muscle specific deletion of Tp receptors significantly attenuates airway
resistance and tissue damping induced by U46619 aerosol challenge. A) Airway
resistance ($R_{aw}$) and B) tissue damping ($G$) are significantly reduced in Tp receptor
deficient mice carrying the floxed Tp allele and the cre transgene under the control of the
SM22 promoter ($Tp^{loxp/-}\text{tgln+ve}$). The wild type mice carrying the floxed Tp allele
($Tp^{loxp/-}$) demonstrated a significant increase in the U46619 response. The heterozygous
wild type mice carrying the floxed Tp allele and the cre transgene ($Tp^{loxp/+}\text{tgln+ve}$)
(thus heterozygous for the Tp receptor on the smooth muscle) and Tp receptor-deficient
mice carrying the floxed Tp allele but no cre transgene ($Tp^{loxp/-}$) (thus heterozygous in
all tissues) demonstrated intermediate phenotypes in response to U46619 challenge. $Tp^{-/-}$,
$n=5$; $Tp^{loxp/-}\text{tgln+ve}$, $n = 6$; $Tp^{loxp/+}$, $n = 4$; $Tp^{loxp/+}\text{tgln+ve}$, $n = 5$; $Tp^{loxp/+}\text{tgln+ve}$, $n = 3$.

* $P<0.05$, ** $P<0.01$, # $P<0.05$. 
Figure 4.7 Non-provoking dose of U46619 increases airway sensitivity to cholinergic stimuli. Airways were exposed to low levels of U46619 (10^{-5} \text{ M}) followed by subsequent cholinergic stimulation with low levels of MCh (3 mg/ml). Within 3 minutes of vehicle or U46619 treatment, mice were exposed to an aerosol produced from a 3 mg/ml solution of MCh, which has been demonstrated to be only mildly provoking. Tp^{+/+} mice exhibited increased sensitivity to cholinergic stimuli after challenge with a non-provoking dose of U46619 in both the R_{aw} (A) and G (B) parameters. Sensitivity of Tp^{-/-} mice to MCh did not change following U46619 challenge. Control Tp^{loxTp/-} heterozygous mice behaved similarly to wildtype animals; sensitivity of the central airways (R_{aw}) to MCh was significantly increased following U46619 challenge (C). While the increased sensitivity of the peripheral airways (G) to MCh was not statistically significant, it trended in the same manner as wildtype mice (D). Mice lacking Tp receptors in smooth muscle (Tp^{loxTp/-} Tgln+ve) failed to exhibit increased sensitivity to cholinergic stimuli following U46619 challenge. MCh only: Tp^{+/+}, n=4; Tp^{-/-}, n=3; Tp^{loxTp/-} Tgln+ve, n = 3; Tp^{loxTp/-}, n = 4. U46619+MCh: Tp^{+/+}, n=7; Tp^{-/-}, n=6; Tp^{loxTp/-} Tgln+ve, n = 7; Tp^{loxTp/-}, n = 8. *P<0.05, **P<0.01, #P<0.05.
Figure 4.8 Airway reactivity to non-cholinergic stimulation following exposure to non-provoking dose of U46619. Airways were exposed to low levels of U46619 (10⁻⁴ M) followed by subsequent stimulation with non-cholinergic agents. Two groups of wild type 129/SvEv mice were intubated and baseline lung mechanics established. The first cohort of wild type mice were then exposed to vehicle followed by an aerosol produced from a 1x10⁻⁴ M solution of U46619. The second group of wild type mice was only treated with vehicle. Within 3 minutes of vehicle or U46619 treatment, all groups of mice were exposed to an aerosol produced from non-provoking doses of non-cholinergic agents. Data are shown as the % change in $R_{aw}$ or $G$ observed between challenge 1 and challenge 2. A-B) The effects of exposing mice to a mildly provoking dose of U46619 (10⁻⁴ M) followed by exposure to serotonin (5-HT; 5 mg/ml), adenosine (6 mg/ml), prostaglandin D$_2$ (PGD$_2$; 10⁻⁴ M), and prostaglandin PGF$_{2\alpha}$ (PGF$_2$; 10⁻⁴ M) were also assessed. No increases in AHR were observed for any of these mediators. PBS/5-HT, n = 4; U46619/5-HT, n = 4; PBS/adenosine, n = 3; U46619/adenosine, n = 3; PBS/PGD$_2$, n = 7; U46619/PGD$_2$, n = 3; PBS/PGF$_2$, n = 12; U46619/PGF$_2$, n = 3.
Figure 4.9 Airway reactivity of smooth muscle-deficient Tp mice to U46619 in an inflamed airway. Allergic lung disease was induced by sensitization and repeated aerosol with OVA. Control animals were sensitized to OVA but challenged with saline. A dose dependent increase in both airway reactivity ($R_{aw}$) ($A$) and tissue damping ($G$) ($B$) were observed in the saline challenged $\text{Tp}^{lox\text{Tp}/-}$ heterozygous mice in response to U46619. Saline challenged smooth muscle-deficient $\text{Tp}^{lox\text{Tp}/-}$ $\text{Tgln}^{+ve}$ had no response to U46619 at any dose. OVA challenged $\text{Tp}^{lox\text{Tp}/-}$ $\text{Tgln}^{+ve}$ mice showed significantly increased airway reactivity when compared to saline challenged animals, similar to that observed in $\text{Tp}^{lox\text{Tp}/-}$ heterozygous mice. Saline: $\text{Tp}^{+/}$, $n=3$; $\text{Tp}^{lox\text{Tp}/-}$ $\text{Tgln}^{+ve}$, $n=5$; $\text{Tp}^{lox\text{Tp}/-}$, $n=7$. OVA: $\text{Tp}^{+/}$, $n=2$; $\text{Tp}^{lox\text{Tp}/-}$ $\text{Tgln}^{+ve}$, $n=5$; $\text{Tp}^{lox\text{Tp}/-}$, $n=6$. *$P<0.05$. 
Figure 4.10 OVA-induced airway inflammation in smooth muscle-deficient Tp mice.

Allergic lung disease was induced by sensitization and repeated aerosol with OVA. Control animals were sensitized to OVA but challenged with saline. Airway inflammation (A), total cell recruitment (B), cellular profile (C), BALF IL-13 levels (D), serum IgE levels (E), and mucus production, as measured by AB-PAS staining (F) and MUC5a expression (G), were equal in OVA challenged TploxTp/- and TploxTp/- Tgln+ve mice. Saline: TploxTp/- Tgln+ve, n = 8; TploxTp/-, n = 6. OVA: TploxTp/- Tgln+ve, n = 8; TploxTp/-, n = 7. **P<0.01.
Figure 4.11 Airway reactivity of neural-deficient Tp mice to U46619 in an inflamed airway. Allergic lung disease was induced by sensitization and repeated aerosol with OVA. Control animals were sensitized to OVA but challenged with saline. A dose dependent increase in airway reactivity ($R_{aw}$) (A) and a slight increase in tissue damping ($G$) (B) was observed in the saline challenged $T_{p}$lox$T_{p}$/− and $T_{p}$lox$T_{p}$/− Nestin+ve mice in response to U46619. An increase in airway reactivity following U46619 challenge was seen in $T_{p}$lox$T_{p}$/− OVA-treated mice but not in OVA-treated $T_{p}$lox$T_{p}$/− Nestin+ve mice. Saline: $T_{p}$−/−, n=3; $T_{p}$lox$T_{p}$/− Nestin+ve, n=4; $T_{p}$lox$T_{p}$/−, n=4. OVA: $T_{p}$−/−, n=3; $T_{p}$lox$T_{p}$/− Nestin+ve, n=4; $T_{p}$lox$T_{p}$/−, n=6. *$P$<0.05, #$P$<0.01.
Figure 4.12 OVA-induced airway inflammation in neural-deficient Tp mice.

Allergic lung disease was induced by sensitization and repeated aerosol with OVA. Control animals were sensitized to OVA but challenged with saline. Airway inflammation (A), total cell recruitment (B), cellular profile (C), BALF IL-13 levels (D), serum IgE levels (E), and mucus production, as measured by AB-PAS staining (F) and MUC5a expression (G), were equal in OVA challenged Tp^{loxTP/-} and Tp^{loxTP/-} Nestin+ve mice. Saline: Tp^{+/+}, n=3; Tp^{loxTP/-} Nestin+ve, n = 4; Tp^{loxTP/-}, n = 5. OVA: Tp^{loxTP/-} Nestin+ve, n = 5; Tp^{loxTP/-}, n = 7. *P<0.05, **P<0.01.
References


CHAPTER 5

CONCLUDING REMARKS
Mechanisms of airway reactivity in the mouse

Chapters 2 and 4 of this dissertation detail allergic and non-allergic constriction of the mouse airways in response to allergen and thromboxane (TXA₂), respectively. Briefly, allergic airway constriction in the mouse is a concerted effort between mast cell-derived serotonin (5-HT) and neurally released acetylcholine (ACh), specifically requiring both the 5-HT₂A receptor and M₃ muscarinic acetylcholine receptor (mAChR). TXA₂-mediated constriction in the naïve airway also requires some neural input (1), as well as cooperation between the M₃ mAChR (1) and smooth muscle Tp receptors. In these examples, the method of bronchoconstriction following different stimuli is surprisingly similar. However, to further define the exact mechanism being employed, additional experimentation is needed in both cases.

Identifying the 5-HT₂A population involved in allergic constriction

Like the Tp receptor, the 5-HT₂A receptor is found on several cell types in addition to airway smooth muscle (ASM) including: neurons, vascular smooth muscle, epithelium, and platelets. Although pharmacological inhibition with receptor antagonists is a standard method in determining receptor involvement in a given response, there are certain pitfalls to this approach. The specificity of the drug, as well as the dose delivered, is always of primary concern when using pharmacological agents. When possible, using animals with a genetic deficiency for the gene of interest is a more reliable method. Using homologous recombination in mice, not only can gene deletions be developed, but also tissue- and temporal- specific deletions. The next step in elucidating the mechanism of antigen-mediated bronchoconstriction would be to determine which subset of 5-HT₂A receptors is involved in this response. This could be accomplished through the
generation of mice carrying a 5-HT$_{2A}$ receptor locus that is sensitive to disruption by cre recombinase. These mice can then be bred to various lines of mice expressing Cre in specific tissues, such as neurons (Nestin-cre), smooth muscle (SM22-cre), or pulmonary epithelial cells (FOXJ1-cre) (2-4), to create mice with tissue-specific deletion of the 5-HT$_{2A}$ receptor. Determining the location of receptor activation would help to define the tissue(s) involved and lend some insight into the role it plays in the response.

Mechanisms of 5-HT$_{2A}$ /Tp receptor interactions with the M$_3$ receptor

For both the 5-HT$_{2A}$ receptor and the Tp receptor, the simplest mechanism involving M$_3$ mAChR cooperation would be one in which additive effects from concurrent receptor activation is needed to elicit downstream Ca$^{2+}$ signaling to initiate contraction of ASM (Figure 5.1A). In this case, the neural component is simply providing basal levels of ACh which activates M$_3$ receptors, causing a slight increase in intracellular Ca$^{2+}$ and giving the lung a baseline tone. 5-HT$_{2A}$ /Tp receptor stimulation also increases intracellular Ca$^{2+}$, although levels are insufficient to cause contraction of ASM. However, activation of both receptors increases Ca$^{2+}$ levels sufficiently to activate the contractile apparatus.

In vivo experiments in both Chapter 2 and Chapter 4 involving non-provoking doses of 5-HT or TXA$_2$ in combination with a non-provoking dose of methacholine (MCh), an ACh analog and M$_3$ agonist, provides suggestive evidence for this Additive Effects model. For both 5-HT and TXA$_2$, low doses that were insufficient to cause ASM constriction were able to induce moderate constriction when coupled with a non-provoking dose of MCh. Separately, these doses of 5-HT, TXA$_2$, and MCh were
A) Additive Effects Model

Naïve airways with cholinergic tone

Elevated 5HT/TXA2 and activation of 5HT2A/Tp receptors

↑Ca++

B) Dimer Formation Model

I) and II) Agonist binding to a GPCR monomer drives heterodimer formation or III) the agonist could bind to a preexisting dimer. Loading the GPCR with agonist induces a conformational shift that allows the accommodation of the heterotrimeric G protein binding. GPCR activation results in the dissociation of the G protein subunits ultimately resulting in increased intracellular Ca^{2+} and increased smooth muscle constriction.

Figure 4.9 Schematic depicting possible models of 5-HT_{2A}/Tp receptor and M3 mAChR-mediated naïve airway smooth muscle constriction. A) Additive Effects Model: 5-HT_{2A}/Tp receptor stimulation fails to increase intracellular Ca^{2+} to levels sufficient to mediate smooth muscle constriction. Acetylcholine acts on M3 mAChRs to maintain cholinergic tone. However, binding of 5-HT/TXA_{2} to its receptor potentiates the activity of the M3 mAChR, thereby increasing the sensitivity of this receptor to basal release of acetylcholine and leading to increased intracellular Ca^{2+} and smooth muscle constriction. B) Dimer Formation Model: I) and II) Agonist binding to a GPCR monomer drives heterodimer formation or III) the agonist could bind to a preexisting dimer. Loading the GPCR with agonist induces a conformational shift that allows the accommodation of the heterotrimeric G protein binding. GPCR activation results in the dissociation of the G protein subunits ultimately resulting in increased intracellular Ca^{2+} and increased smooth muscle constriction.
sufficient to elicit a response, but in combination were able to provoke airway constriction. Interestingly, low doses of 5-HT or TXA$_2$ specifically required an addition of MCh to induce contraction, but low doses of 5-HT in combination with TXA$_2$ was insufficient to induce airway reactivity.

One step towards confirmation of this model would be to stimulate cultured ASM cells in vitro with combinations of 5-HT, TXA$_2$, and MCh and measure the resulting increase in intracellular Ca$^{2+}$. Although this appears to be a straightforward approach, cultured ASM cells undergo a degree of differentiation that rapidly decreases M$_3$ receptor expression (5). However, several studies in canine primary ASM cells have shown that serum starvation can give rise to a subpopulation of cells that require high M$_3$ expression (6, 7). If this system were shown to function the same way in primary cultures of mouse ASM, these cells could be used to elucidate the interaction between the 5-HT$_{2A}$/Tp receptor and the M$_3$ receptor.

It has been well established that G protein-coupled receptors (GPCRs) can function as homo- or heterodimers, and specifically the M$_3$ receptor has been shown to not only exist as a monomer but to also form heterodimers with other GPCRs (reviewed in (8)). Furthermore, 5-HT$_{2A}$ receptor heterodimerization with mGluR$_2$ receptors in cortical networks of the central nervous system have been identified (reviewed in (9)), and Tp receptors are known to form heterodimers with prostacyclin receptors (IP) in vascular smooth muscle (9-11). In addition, 5-HT$_{2A}$, Tp, and M$_3$ are all G$_q$-protein coupled, a protein commonly associated with its role in promoting ASM contraction. Briefly, activation of these receptors initiates dissociation of the G$_q$+GTP complex and activation of phospholipase C (PLC), which then catalyzes the hydrolysis of PIP$_2$ into
DAG and IP3. IP3 initiates the release of Ca\(^{2+}\) from the sarcoplasmic reticulum into the cytosol, resulting in increased Ca\(^{2+}\) concentration, which promotes the binding of calmodulin (CaM). The Ca\(^{2+}/CaM\) complex activates myosin light chain kinase, which phosphorylates myosin light chains and promotes the ATPase activity of myosin, thereby promoting cross-bridge cycling and contraction.

Knowing that these receptors are all coupled to the same G protein and are capable of forming heterodimers with unrelated GPCRs suggests coupling of M3-5-HT\(_{2A}\) and/or M3-Tp receptors on ASM as a possible mechanism regulating antigen- and TXA\(_2\)-mediated airway constriction, respectively (Figure 5.1B). This Dimer Formation Model could function in one of two ways. Binding of ligand on either receptor could drive the formation of dimmers, which are needed for association of the G protein, which dissociates upon binding of the second ligand. Alternatively, and more consistent with current knowledge of GPCR dimers, heterodimers preexist in the tissue and binding of both ligands is needed for dissociation of the G protein and consequent downstream signaling.

Several experiments could be done to verify/refute the ability of these GPCRs to form heterodimers. Suggestive evidence can be obtained using ASM cultures treated with 5-HT or TXA\(_2\), activating 5-HT\(_{2A}\) or Tp receptors, respectively. This activation should trigger release of intracellular Ca\(^{2+}\). However, if these receptors exist as heterodimers with M\(_3\) receptors, pretreatment with atropine should be able to block rises in Ca\(^{2+}\) resulting from 5-HT or TXA\(_2\) stimulation. Alternatively, immunoprecipitation assays could be carried out on either primary ASM cells or cells co-transfected with 5-HT\(_{2A}\) or Tp receptors and M\(_3\) receptors. If the GPCRs exist as dimers,
immunoprecipitation of one receptor would result in precipitation of the other receptor. Finally, cells can be co-transfected with 5-HT$_{2A}$ or Tp receptors labeled with a donor fluorophore and M$_3$ receptors labeled with an acceptor fluorophore. Analysis of the molecular interaction of the two co-transfected receptors can then be determined using Fluorescence Resonance Energy Transfer (FRET).

**Representation of human disease in a mouse model**

Determination of mechanisms involved in airway reactivity in experimental animals such as the mouse can lead to a better understanding of bronchoconstriction in humans with asthma. It’s well understood that asthma is a complex disease involving both environmental and genetic factors that cannot be fully modeled in an animal that does not spontaneously develop the disease; however, modeling specific symptoms of asthma in the mouse can hopefully provide insight into the human condition. In addition, *in vitro* data often does not completely correlate with *in vivo* data even from the same species, and most “*in vivo*” human studies are suggestive measurements of byproducts that are either elevated/decreased in asthmatic patients. Although no animal model is going to perfectly portray the human condition, the inability to perform substantial *in vivo* experimentation on human subjects highlights the importance of using an animal model.

Small size, simple housing requirements, short reproductive cycle, and ease of genome manipulation have made the mouse a desirable and common animal model for studying human disease. The mouse has been the most common animal for modeling the classic Th2 asthmatic phenotype and for studying allergic responses in the airways (reviewed in (12)). In addition, airway reactivity is easily measured in both conscious,
spontaneously breathing, as well as anesthetized, artificially ventilated animals. This begs the question ‘how good is the mouse as a model of airway reactivity’?

**Neuronal involvement in asthma**

A common theme in the research presented in this dissertation is the reliance of a neural component for allergen- and TXA$_2$-induced airway reactivity. In the mouse, we have seen that surgical vagotomy attenuates bronchoconstriction following challenge with either allergen or the TXA$_2$ analog U46619; furthermore, pretreatment with atropine and the use of mAChR-deficient mice has shown the requirement of the M$_3$ receptor in both models of airway reactivity (1, 13). This data emphasizes the absolute requirement of an intact neural circuit for bronchoconstriction in the mouse, but can the same be said for humans?

Neural involvement in asthma is not a new idea. As far back as the 1920s medical researchers have experimented with the ability of neural resection and lung denervation to cure the asthmatic condition (reviewed in (14)). Although results generally varied, many patients improved and a few were even thought to be cured. Since then, anticholinergic treatment of asthmatics has also shown varied results. Anticholinergic treatment in combination with $\beta_2$-agonists (bronchodilators) has been shown to improve lung function in children and adults with severe acute asthma; however addition of anticholinergics made no difference in patients with mild to moderate asthma (15). Some studies have suggested that asthma is a result of neural dysregulation. Human parasympathetic nerves from patients with asthma have increased release of acetylcholine (ACh) in response to electrical field stimulation (16). In addition M$_2$ agonists, which should prevent further release of ACh from parasympathetic nerves, fail
to inhibit bronchoconstriction in some patients with asthma (17), suggesting dysfunction
of muscarinic receptors in asthma. Like many other complex diseases, asthma can be
divided into many different subsets, some of which appear to be at least partially
dependent on cholinergic signaling.

Serotonin and asthma

We have shown that allergen-induced airway reactivity in the mouse is mediated
by mast cell-derived 5-HT (13). Although 5-HT has been shown to activate human ASM
ex vivo (18), as well as increase Ca\(^{2+}\) signaling in cultured human ASM (19), it is
generally accepted that inhalation of 5-HT does not lead to increased airway constriction,
even in asthmatics (20). However, some evidence for the involvement of 5-HT in asthma
has been presented. Levels of free 5-HT in plasma have been shown to be increased in
patients with asthma, and these levels correlated positively with clinical status and
negatively with pulmonary function (21). Additionally, tryptophan hydroxylase and
serotonin have been measured in human mast cells (22), albeit in much smaller amounts
than seen in their mouse counterparts. Although inhalation of 5-HT does not effect lung
function in asthmatics, it may increase airway reactivity given that 5-HT receptor
activation has been shown to facilitate cholinergic bronchoconstriction in human airways
(23).

A few clinical studies have suggested the possible involvement of serotonin in
anaphylactic bronchoconstriction in man. Inhibition of 5-HT\(_{2A}\) receptors with droperidol
successfully treated two asthmatic patients who were unresponsive to conventional
therapy (24). Another study by Lechin et al. (25) reported the use of placebo and
tianeptine, an antidepressant that induces uptake of 5-HT by platelets, in a double-blind
cross-over study to reduce the levels of free serotonin in patients with asthma. Tianeptine treatment provoked a dramatic decrease in clinical rating and increase in pulmonary function. These results suggested the potential for 5-HT modifiers in the treatment of asthma; however, no 5-HT modifiers are currently used for the treatment of asthma.

*Thromboxane and asthma*

Human allergic airway constriction modeled in precision cut lung slices (PCLS) was shown to be mediated by a combination if leukotrienes and thromboxane (26), although no *in vivo* studies have been done to link thromboxane to allergen-induced bronchoconstriction in asthmatics. Clinical studies have been done to asses the role of TXA₂ on airway hyperresponsiveness (AHR) to cholinergic agents, and have shown that inhibition of either thromboxane synthetase or Tp receptors improves AHR in asthmatic patients (27, 28).

Inconsistent with the human PCLS studies, leukotriene- and Tp-deficient mice have no defect in allergen-mediated bronchoconstriction. However, the TXA₂ analog U46619 has significant dose-dependent effects on airway reactivity in the mouse. Chapter 4 details the involvement of specific subsets of Tp receptor in airway reactivity in both the naïve and the allergic. Briefly, smooth muscle Tp receptors facilitate U46619-mediated airway constriction in the naïve lung, while both smooth muscle and neural Tp receptors are involved in constriction and AHR in allergic airways. Because little is known about specific receptor subset involvement in airway reactivity in human asthma, it is tempting to speculate a similar mechanism to that seen in the mouse, although studies to confirm this would be difficult at best.
Making the mouse more like us

Given the differences between humans and the mouse, attempts have been made to “humanize” the mouse. For example, transgenic mice have been made that express the human leukotriene receptor, cycLT₁R (29). In these animals, the inability of the mouse airways to respond to leukotriene challenge is partially corrected, making their airways more representative of human airways. Using advanced homologous recombination techniques it is now possible to completely replace specific mouse genes with their human counterparts, including regulatory regions. Once mechanisms are elucidated in the mouse using both in vivo and in vitro methods, these mechanisms can be compared to what is known about the human condition and steps to “humanize” the mouse through replacement with human genes can initiated. This method has the potential to provide a more reliable mouse model for studying human disease.
References


