The role of pulmonary mast cells in neurotrophin 4 mediated cholinergic neuroplasticity in neonatal asthma
THE ROLE OF PULMONARY MAST CELLS IN NEUROTROPHIN 4
MEDIATED CHOLINERGIC NEUROPLASTICITY IN NEONATAL ASTHMA

by

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DEDICATION

I would like to dedicate this thesis to my beloved husband Sagar Naik and my adorable nephew Sahil
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THE ROLE OF PULMONARY MAST CELLS IN NEUROTROPHIN 4 MEDIATED CHOLINERGIC NEUROPLASTICITY IN NEONATAL ASTHMA

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ABSTRACT

Asthma is a chronic inflammatory lung disease characterized by recurrent wheezing, coughing and difficulties in breathing. Asthma affects 25.7 million people in the USA including 8 million children. Asthma is often associated with early-life exposure to environmental insults. However, mechanisms that link early-life insults to persistent airway dysfunction are unknown. Our previous studies in mice showed that early-life allergen exposure increases the levels of neurotrophin 4 (NT4) causing airway smooth muscle (ASM) hyperinnervation and persistent airway hyper reactivity (AHR). I show that early-life allergen exposure selectively increases cholinergic innervation. Notably, cholinergic nerves release acetylcholine, a potent airway constrictor that signals through the M3 receptor in ASM. Building upon these findings, my thesis encompasses two components. Firstly, how is NT4 expression aberrantly up regulated following early-life allergen exposure? Secondly, what is the effect of enhanced cholinergic innervation on the neonatal ASM?
I find that NT4 is selectively expressed by ASM and mast cells in mice, nonhuman primates and humans. We show in mice that while NT4 expression in ASM remains unchanged upon allergen exposure, mast cells expand in number and degranulate to release NT4 thereby increasing NT4 levels in the lung. Adoptive transfer of wild-type mast cells, but not $NT4^{-/-}$ mast cells restores ASM innervation and AHR in $Kit^{W+/-}sh/-$ mice following early-life insults. In an infant primate model of asthma, the increased ASM innervation is also associated with the expansion and degranulation of mast cells. Therefore, pulmonary mast cells are a key source of aberrant NT4 expression following early-life insults in both mice and possibly primates.

Next, I speculated that an increased cholinergic output in the neonatal lung might lead to persistent AHR. Using recurrent methacholine exposure and M3 receptor blocker, 4-DAMP, I show that enhanced cholinergic signaling in neonatal mice leads to persistent AHR without inflammation. In contrast, methacholine exposure in adult mice has no prolonged effects on airway reactivity. Together, my findings support a model in which deregulated neural activities following early-life insults cause persistent ASM hypercontractility. Thus, early-life interventions to block mast cell degranulation and the cholinergic pathway may benefit children with recurrent wheezing.
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<tbody>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyper-reactivity</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchial alveolar lavage fluid</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>ChAT</td>
<td>cholinergic acetyltransferase</td>
</tr>
<tr>
<td>COLIA</td>
<td>collagen IA</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disorder</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FV</td>
<td>Flexivent</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association studies</td>
</tr>
<tr>
<td>HDMA</td>
<td>house dust mite antigen</td>
</tr>
<tr>
<td>ICS</td>
<td>inhaled corticosteroids</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>M2</td>
<td>muscarinic receptor 2</td>
</tr>
<tr>
<td>M3</td>
<td>muscarinic receptor 3</td>
</tr>
<tr>
<td>Mch</td>
<td>methacholine</td>
</tr>
<tr>
<td>MCp</td>
<td>mast cell progenitor</td>
</tr>
</tbody>
</table>
NEB………………………………………………………………...neuroendocrine bodies
NF………………………………………………………………...neurofilament
NGF………………………………………………………………...nerve growth factor
NT………………………………………………………………...neurotrophin
NT4………………………………………………………………...neurotrophin 4
O₃………………………………………………………………...ozone
OVA………………………………………………………………...ovalbumin
P………………………………………………………………...postnatal day
PBS………………………………………………………………...phospho-buffered saline
PFA………………………………………………………………...paraformaldehyde
P75………………………………………………………………...low-affinity neurotrophin receptor
RSV………………………………………………………………...respiratory syncytial virus
SABA………………………………………………………………...short acting beta agonists
SNP………………………………………………………………...single nucleotide polymorphisms
Th………………………………………………………………...T- helper cell
TH………………………………………………………………...tyrosine hydroxylase
Trk………………………………………………………………...tyrosine kinase receptor
TRPA1……………………………………………………………transient receptor potential cation channel, member A1
VACHT…………………………………………………………...vesicular acetylcholine transporter
WT………………………………………………………………...wild type
CHAPTER ONE

Childhood Asthma

1.1 Summary

Asthma is an inflammatory disease of the airways characterized by increased airway constriction and mucus hyper secretion. The risk factors for asthma include genetic predisposition, early-life exposures to allergen, cigarette smoke, and respiratory syncytial virus (RSV) infections. Asthma is known to manifest itself in childhood as recurrent wheezing and often persists into adulthood. Several studies revealed that children with severe and persistent wheeze continue to have lower lung function during adulthood \(^{(1),(2)}\). Early-life insults are speculated to alter the neonatal lung structure leading to a prolonged reduction in pulmonary function and increased airway hyper reactivity (AHR). Although an initial immune response is required for the manifestation of childhood asthma, we find that the progression of asthma in adulthood rely on factors and pathways independent of the inflammatory response.

Our study in the neonatal mice has shown an association of increased lung innervation during development with asthma progression into adulthood \(^{(3)}\). The innervation of the lung occurs through neurotrophic factors expressed by the ASM \(^{(4)}\). Studies have identified that increased levels of neurotrophins in the BALF/serum is associated with asthma severity in children \(^{(5)}\). Our previous work in a neonatal mouse model of asthma showed that early-life allergen exposure increases NT4 levels thus triggering hyper
innervation of ASM leading to persistent AHR (6). In this chapter, I discuss the risk factors, innate immune responses and neuronal alterations involved in childhood asthma.

1.2 Introduction

Asthma is a complex disease of the airways that includes inflammation and reversible airway hyper contraction leading to difficulty in breathing. The incidence of asthma has been increasing worldwide. According to the center of disease control, in the USA alone around 9 million children suffer from asthma. Asthma in children is linked with exposure to pollutants, cigarette smoke, allergens or viral respiratory infection (7),(8),(9). All of these factors may differentially determine the degree of atopy and wheeze later in life (10).

The lung continues to develop post-birth. In mice, the respiratory tract reaches maturity around 3-4 weeks postnatally (11, 12). The non-human primate lung shows rapid growth through age 2 with slower growth until 7 years of age (13). The human lung shows rapid growth through age 3 with slower growth until age 8 (14) but is not considered fully mature until early adulthood. Childhood asthma often persists into adulthood. Data from longitudinal birth cohort studies show that children who presented with recurrent wheeze in pre-school years continued to have reduced lung function even as adults with or without asthma symptoms (15),(16). Adults who had early-onset (before three years of age) persistent wheezing as compared to late-onset wheezing are more likely to have significantly lower forced expiratory flows. This indicates that insults to a developing lung may cause permanent alterations in the lung thereby lowering lung function.
Although childhood asthma is inflammation driven, structural changes occurring in the postnatal developing lung may contribute to the disease progression independent of inflammation. However, the exact mechanism involved in early-life airway insults that lead to development of persistent asthma remain unknown. Although there are several oral and inhaled corticosteroid treatment options available for asthmatics, they are not effective in altering the progression of the disease. Thus, it is crucial to understand the underlying mechanisms involved in the pathophysiology of neonatal asthma for effective therapeutics.

Using an OVA allergen neonatal mouse model of asthma, we showed that early-life insults lead to increases in ASM innervation associated with persistent AHR \(^{(6)}\). Our studies in infant primates show that early-life exposure to O\(_3\)+house dust mite allergen (HDMA) leads to increased ASM innervation (Figure 3.4.1). In contrast, insults to mature adult lungs in animal models have no effect on airway innervation and elicit transient airway dysfunction \(^{(6)}\). These findings suggest that aberrant ASM innervation upon early-life insults is central to asthma progression later in life.

The lung is innervated by a combination of intrinsic neurons \(^{(17)}\) and extrinsic neurons. Intrinsic neurons have their cell bodies within the lung tissue while the cell bodies of extrinsic neurons are located in the central and peripheral nervous system. The axons of extrinsic neurons innervate ASM and neuroendocrine cells. The neuronal outgrowth
factors called neurotrophins that provide signals required for the survival, development and functions of the neurons\textsuperscript{(18)}. A mixed innervation of cholinergic neurons and non-adrenergic non-cholinergic sensory neurons regulate the airway tone\textsuperscript{(19)}. The cholinergic neurons release acetylcholine (Ach) from post-ganglionic nerve fibers. A post-ganglionic nerve fiber relays messages from the ganglion to the effector organ. The released Ach signals through the muscarinic receptors on the ASM leading to bronchoconstriction.

The objective of my thesis is to unravel the mechanism involved in the immune-nerve interaction in a neonatal mouse model of asthma. My study shows a unique role of the mast cells as a source of neurotrophin 4 (NT4) mediated neuroplasticity in the neonatal lung upon allergen exposure. Furthermore, my research shows that upon allergen exposure in early-life, mast cell-derived NT4 increases cholinergic innervation in the neonatal lung leading to persistent AHR. These data suggest a novel pathway driven by enhanced cholinergic activity in early-life, which in part, leads to lower lung function in adults.

1.3 Childhood asthma risk factors, predictors and medication
Development of childhood asthma has several risk factors that include underlying genetic predisposition, viral infections, early-life allergen exposures, epigenetic and environmental risk factors (and their interactions), natural history and gender. Asthma susceptibility is influenced by the interaction of genes and environment. Genome-wide association studies (GWAS) showed an association of single nucleotide polymorphisms
(SNPs) in IL33, IL13R, IL13, STAT6 and IL18R1 genes, which are involved in Th2 inflammation pathway with asthma and asthma-related phenotypes. Additional SNPs in ORMDL3 and TSLP genes are also associated with asthma. Based on GWAS studies a strong association is seen between a SNP in the ORMDL3 locus with childhood asthma \(^{(20)}\). However, the exact function of ORMDL3 in childhood asthma remains unknown. Another study showed an association of SNPs in thymic stromal lymphopoietin (TSLP) with IgE (in girls) and asthma \(^{(21)}\). Gender and age also act as important risk factors for asthma. Childhood asthma has a higher prevalence in boys than girls whereas adult-onset asthma is more prevalent in women than men. Children with recurrent wheeze and reduced lung function in the ages between 3-6 years are at a higher risk to develop asthma at school age and continue to have asthma in adolescent life \(^{(22)},(23)\). Based on our studies, early-life OVA exposure-induced asthma in neonatal mice persists into adulthood. Studies also show epigenetic predictors such as DNA methylation signatures as risk factors for asthma in children \(^{(24)}\). Though several risk factors and predictors for childhood asthma are well known, the pathophysiology underlying the disease and its progression remains to be understood.

Asthma in children is managed by alleviating symptoms and risk factors. Most of the children suffering from asthma receive a low dose of inhaled corticosteroids (ICS) and short-acting beta agonists (SABA) as the first-line of therapy. Though both these treatments reduce asthma symptoms and improve the quality of life, they have several side effects in children. Children using inhaled corticosteroids at moderate to high doses
are prone to oral candidiasis. Also, these medications are associated with reduction in their growth probably due to poor bone metabolism and reduced osteoblast proliferation (25). Several other treatment options include leukotriene receptor modifiers, systemic corticosteroids and combination therapy. These treatment options alleviate asthma symptoms but do not alter the disease progression in children. It has been difficult to control asthma in children due to lack of thorough knowledge of disease onset and progression. To understand the mechanism of early-life insults to a neonatal lung, we established the neonatal mouse model of asthma using OVA-alum sensitization and challenge. Based on this model, our laboratory showed that early-life insults alter ASM phenotype. My thesis work aims to provide novel drug targets in the neonatal age to alter significantly asthma progression into adulthood.

1.4 Early-life insults, immune modulation and development of childhood asthma

The neonatal immune system is different from the adult mature immune system in its response to bacterial and viral infections (26). Neonatal immunity is immature due to lack of memory cells and impaired function of effector B and T cells. It is mainly dependent on innate immune responses and the passive antibodies acquired from the mother. The neonatal immune system is capable of mounting a slow adaptive CD4+ T cell response (27). Innate immune responses are the first line of defense and occur mainly after primary sensitization. The Toll-like receptors (TLRs) are present on the surface of antigen-presenting cells and recognize pathogen/allergen-specific patterns. This triggers release of specific cytokines from innate immune cells that help in Th1/Th2 polarization. Studies
have shown that activation of TLR in neonatal monocytes and dendritic cells induces a reduction in expression of Th1 polarizing cytokines while increasing the expression of Th2 polarizing cytokines IL-6 and IL-10 (28),(29). In the absence of a strong Th1 polarizing signal, the neonatal immunity is thus skewed towards a deregulated Th2 response. Severe RSV infection in early-life can lead to asthma in children by altering the Th1/Th2 balance. RSV activates TLR-4 on the lung epithelium, through a mechanism similar to house dust mite allergen, which is a common cause of asthma in children (30),(31),(32),(33). Thus, the neonatal immunity is prone to allergy development owing to a highly polarized Th2 environment.

The change in lifestyle and urbanization in western countries led to decrease in incidence of infectious diseases thus causing an imbalance in the Th1/Th2 immune response in neonatal immunity. This led to the development of the “hygiene hypothesis”. The hygiene hypothesis states that early-life bacterial infections can alter the tendency to develop allergic diseases by augmenting a Th1 immune response. Studies show that neonates subjected to repeated high-level exposures of LPS, an endotoxin found in the cell wall of gram-negative bacteria, activates the Th1 immune response which protects against house dust mite-mediated allergy development (34). The repeated exposure of LPS activates the TLRs on the antigen presenting cells which causes an increase in the Th1 immune response. Although some studies support the hygiene hypothesis (35), the timing, microbiome, dose, site of activation involved in establishing the immune modulation and causing allergy development in children warrants further study.
1.5 Innervation changes in childhood asthma

During embryogenesis, the outgrowth of axons into the distal lung is closely associated with the formation of ASM. The immature developing lung demonstrates increased levels of axonal growth referred to as neuroplasticity. We showed that during normal postnatal lung development innervation peaks at P14 and stabilizes around P21 coinciding with the maturation of the airway smooth muscle (6). Based on our neonatal mouse model of ovalbumin (OVA) and cockroach allergen exposure, we discovered that early-life insults have long-term effects on the developing ASM due to aberrant innervation changes. The ASM is innervated by sympathetic, parasympathetic and sensory neurons. The sympathetic nerves release catecholamines which signal through adrenergic receptors causing relaxation of the airway. The parasympathetic nerves release acetylcholine that causes constriction of airways. The sensory afferent nerves release mediators such as substance P, neurokinin A also called as tachykinins and calcitonin gene-related peptide (CGRP). Tachykinins constrict airways through NK-1 (activated by Substance P) and NK-2 receptors (activated by Neurokinin A).

Studies in different animal models suggest an association of aberrant nerve changes with asthma in children. RSV infection, a major risk factor for childhood asthma causes increases in peptidergic sensory nerves in the lower airway of neonatal guinea pigs (36). Early-life exposure to ozone (O3) or cigarette smoke in rats and mice similarly increases sensory and sympathetic innervation of the airway (37). Studies in neonatal rats showed
that early-life ozone exposure leads to an increase in substance P-containing nerve fibers (37).

Furthermore, we show that early-life OVA exposure leads to changes in ASM innervation, when adult mice are challenged with OVA allergen there is no measurable change in levels of innervation (6). In support of these findings, Wu Z.X et al. has shown that early postnatal exposure to tobacco in mice lead to long-term increases in lung innervation while late postnatal tobacco exposure does not cause any changes in innervation or airway hyper responsiveness (3). These studies show that the postnatal developing lung is vulnerable to changes in innervation upon allergen exposure leading to persistent airway hyper reactivity (38),(6),(3). However, the effects of increasing innervation are limited to the early postnatal period. Therefore, it is important to understand how these specific innervation changes are brought about in childhood and how are they associated with a sustained decline in lung function into adulthood.

1.6 Neurotrophins and childhood asthma

Neurotrophins are growth factors for nerves that provide survival, proliferation and guidance cues to axons. There are four different kinds of neurotrophins: NGF, NT3, BDNF and NT4. They signal through low-affinity P75NTR and high-affinity tropomyosin kinase receptors; Trk A, Trk C and Trk B. BDNF and NT4 signal through Trk B receptors. Neurotrophins are synthesized as preproprotein precursors (~27 kDa) and are intracellularly cleaved to a pro-neurotrophin that is further processed by
proteinases such as matrix metalloproteinase (MMP) to generate mature NTs (≈14 kDa). During development, NT4 and BDNF derived from the ASM act as essential signals for establishing innervation (4).

Studies in animal and human models with early-life RSV infections showed increases in NGF levels in the BALF/serum (39). Similarly, our study in the neonatal mice shows that early-life exposure to OVA increases NT4 levels in the lung causing increased ASM innervation (6). Though it is known that deregulated neurotrophin levels are associated with asthma severity in children (40), it is unclear which cells contribute to the deregulated release. During development neurotrophins derived from target tissues establish lung innervation. However, there may be additional cell sources that express NTs. During allergen exposure, neurotrophins are speculated to be secreted by activated immune cells such as mast cells, dendritic cells and T cells (41),(42). Mast cells play an important role in allergic inflammation (43).

Mast cells are located adjacent to blood and lymphatic vessels, beneath epithelial surfaces and in proximity to ASM. Mast cells are known to produce NT3, NT4, BDNF and NGF (44). Human mast cells express NGF and functional Trk A (45). Mast cells directly activate both the neurons and ASM. Dendritic cells release NGF and BDNF by LPS and allergen stimulation (46). Data show expression of Trk A and p75NTR receptors on dendritic cells (47). Adult human T cells are shown to express Trk A receptors on the cell surface (48),(49). In the neonatal mouse lung, Trk B receptors are present on the endothelial
cells and nerves, but there is no expression of Trk B on immune cells\(^6\). After allergen exposure, NT4 signals through Trk B receptors expressed on the nerves to induce neuroplasticity. In our neonatal mouse model of asthma, the cellular source responsible for elevated NT4 levels in the lung remains unclear. My research focuses on determining the precise cellular source of NT4 upon allergen exposure as well as understanding the mechanism of NT4 secretion and release\(^{50}\).

1.7 Conclusion

Data from clinical and animal studies show that early-life allergen exposure leads to altered lung function, which persists into adulthood. The significance of these findings is four-fold. Firstly, the developing immature lung seems more prone to environmental insults than the mature adult lung. Secondly, risk factors for childhood asthma are complex ranging from environmental exposures to viral infections to age on-set. Thirdly, early-life insults in a neonatal mouse model of asthma causes increases in neurotrophin levels, particularly NT4 leading to hyper innervation of the ASM. Lastly, the increase in innervation in early-life is functionally linked to persistent changes in the ASM contraction rendering the ASM hyper-responsive through adulthood. Our studies show that an enhanced neural activity leads to persistent changes in neonatal ASM phenotype (Figure 4.6). Thus, children with asthma may show continued decline in lung function due to deregulated neural signaling during early life. These findings indicate that there may be a therapeutic time-window in early childhood when the lung is still developing to prevent asthma outcome as adults. Thus, it becomes essential to understand the allergen-
nerve-ASM crosstalk to provide specific drug targets. The following chapters will focus in depth on the mast cell regulation of NT4 secretion and effect of enhanced cholinergic activity in early-life on persistent AHR.
CHAPTER TWO

Mast cells

2.1 Summary

Mast cells are hematopoietic cells that originate in the bone marrow and circulate in the blood as precursors i.e. mast cell progenitors (MCps). Upon degranulation via IgE-dependent or independent mechanisms mast cells release several mediators such as cytokines, histamine, leukotriene and neuropeptides. Mast cells are major effector cells in allergic asthma and contribute to chronic airway inflammation, mucous metaplasia and airway constriction. Mast cells and nerves communicate due to their spatial proximity in tissues such as skin, GI tract and lung. In the skin, mast cells and nerves crosstalk in response to infections, injury and wound healing. In the GI tract, during normal conditions, the communication between mast cells and enteric nerves is required for maintenance of mucosal homeostasis. During pathological conditions such as inflammatory bowel disease, the increase in mast cell number is associated with increase in neuropeptide release and enteric nerve activation. In the lung, mucosal mast cells are known communicate with nerves during asthma. However, it is still unclear how the mast cells in the lung interact with the nerves in childhood asthma. My research shows that mast cells express NT4 that is released upon degranulation. NT4 derived from mast cells increases cholinergic nerves in the neonatal lung. The increase in cholinergic nerves renders ASM persistently hyper contractile. This chapter describes in detail mast cell development and its neuro-modulatory role in childhood asthma.
2.2 Introduction

Mast cells are derived from multipotent hematopoietic progenitors in the bone marrow. Mature mast cells make large amounts of several different types of serine proteinases based on their location \(^{(51)}\). In the lung, there is a distinct difference in protease expression in connective tissue mast cells (CTMCs) and T-cell dependent mucosal mast cells (MMCs).

Mast cells are important immune effector cells that help link the innate and adaptive immune system. Studies have identified the mast cell as a pivotal component of the asthmatic response in both the acute and chronic disease models \(^{(52),(53),(54),(55)}\). Upon allergen exposure, mast cells release several mediators that have potent bronchoconstriction capacity \(^{(56)}\). In support, we show that mast cell deficient mice during early life allergen exposure display relatively lower AHR as compared to wild-type mice (Figure 3.4.6) \(^{(52),(53)}\). Studies have indicated increased recruitment of mast cell progenitors in mouse models of allergic asthma \(^{(57),(58)}\). In support, my study highlights a unique aspect of increased mast cell number in the neonatal lung as compared to the adult lung. It is likely that the increased mast cell numbers are associated with increased release of potent mediators of bronchoconstriction.

Mast cells via release of neuropeptides and neurotrophins act as a bridge between activation of the immune response, induction of neuronal stimulation and ASM contraction \(^{(50)}\). The spatial location of mast cells in lungs makes them key candidates for these neuro-immune
interactions. Studies have identified pathways whereby allergen exposure induces mast cell activation and serotonin release and results in initiation of bronchoconstriction by the ASM \(^{(59)}\). This chapter discusses mast cell biology, its inflammatory and neuro-regulatory roles during allergic asthma.

### 2.3 Mast cell development

Mast cells originate from the bone marrow and circulate in the blood as precursors. They undergo terminal maturation after reaching tissues that they reside in such as gut, lung, and other tissues \(^{(60)}\). Initially, it was speculated that mast cells originate from common myeloid progenitor cells. A second pathway shows the development of mast cells from lineage negative, multipotential progenitors. Studies in adult mouse bone marrow identified Lin− Kit\(^{+}\)Sca1\(^{+}\)−Ly6c\(^{−}\)−FceRI\(^{+}\)−CD27\(^{+}\)−\(\beta 7^{+}\)T1/ST2\(^{+}\) cell populations, as mast cell progenitors (MCps) \(^{(61)}\). This study suggests that mast cells arise from multipotential progenitor cells (MMPs). In mice, mast cell maturation depends upon both stem cell factor (SCF) and Interleukin-3 (IL-3) \(^{(62)}\) whereas the human mast cells completely mature upon KIT (SCF-induced tyrosine kinase receptor) activation. The KIT activation is brought about by SCF-induced KIT dimerization and autophosphorylation \(^{(63),(64)}\). Mature mast cells are identified by Fc\(\varepsilon\)RI and c-kit (CD117) receptors on its surface. Mast cells are tissue-specific cells and they differ in the mediators and the proteases they produce and secrete.
Several diseases show organ-specific increases in mast cell numbers \(^{(65),(66),(67)}\). These increases in mast cell numbers could be due to increased recruitment, proliferation of total mast cell progenitors \(^{(68)}\) or proliferation of the mature tissue-resident mast cells. Mature mast cells are terminally differentiated and hence have a lower potential for proliferation. Although studies indicate that MCps give rise to mature mucosal mast cells, further work needs to be done to establish the relative contribution of MCp to form CTMCs or MMCs.

The cytokines within the microenvironment of the tissue can influence the properties of mast cells and lead to phenotypic and functional differences. The functional role of mast cells in every tissue is dependent on the nature of their secretory granules. Specifically, mast cells in different tissues differ in the relative abundance of tryptase and chymase production.

### 2.4 Pulmonary mast cells

In mice, ova sensitization and challenge increases recruitment of MCp to the lung \(^{(57),(69)}\). The recruitment of MCp in the lung is highly dependent upon interactions with VCAM-1 expressed on the endothelium and \(\alpha_4\beta_1\) or \(\alpha_4\beta_7\) integrins and the CXCR2 receptor expressed on the surface of MCps \(^{(57)}\). Studies also show an important role of CD4\(^+\) T cells in the recruitment of MCp into the lung upon OVA administration \(^{(70)}\). Our studies in a neonatal mouse model show an increase in mast cell number as measured by toluidine blue staining on the lung tissue. The toluidine blue staining does not distinguish
between MCp and mature mast cells. Although allergen exposure increases the number of
mast cells in the lungs of both young and adult mice, the relative abundance of mast cells
to the age of the lung remains higher at P21 (~4.2 cells per 100X image) than adults (0.22
cells per 100X image) by approximately 20-30 fold. This age-related decrease in the
relative abundance of mast cells in our model is associated with changes in innervation in
lungs during early-life and not in adults.

Pulmonary mast cells have diverse function and characteristics owing to distinct
proteoglycan and proteinase contents. Pulmonary mast cells contain higher amounts of
chymase as compared to the mast cells in the gut. In the lung, there is a distinct difference
in serine protease expression in CTMCs and MMCs present in the airways and
intraepithelial locations (71). MMCs in the lung express tryptase and histamine and hence
are referred as MCₜ mast cells. CTMCs (referred as MCₜC) express chymase as well as
tryptase, carboxypeptidase A3 (Cpa3) and large amounts of histamine (72). There are
different types of tryptases mMCP-1, 2, 4, 6, 7,9 and 11. Human mast cell proteases beta-
1, beta-2 and beta-3 are similar to mMCP-7 like tryptase in mice. In mice, CTMCs and
MMCs both express mMCP-6 and mMCP-7 (73),(74),(75),(76). The CTMCs and MMCs both
contribute to airway pathology by different mechanisms (77). The lung CTMCs activate
the Th1 immune response by signaling through STAT 4 while the MMCs do not express
STAT 4 (78). Also, CTMCs play a major role in airway constriction by releasing serotonin
and high amounts of histamine. In contrast, the pulmonary MMCs maintain chronic
inflammation in the lungs of atopic patients by recruiting Th2 immune cells (79). Although
functions of CTMCs and MMCs in adult lungs are known, their specific roles in childhood asthma remain to be studied.

2.5 Mouse models for studying mast cell functions

Studies showing the functional role of mast cells in allergic asthma are based on mast cell deficient animal models. The most commonly used animal models are $Kit^{W}/Kit^{W-v}$ mouse and $Kit^{W-sh}/Kit^{W-sh}$ (80),(81),(82). The W-sash ($W^{sh}$) mutation is an inversion mutation in the promoter region of the Kit gene on mouse chromosome 5 (83). We used $Kit^{W-sh}/Kit^{W-sh}$ mice for our studies of a neonatal mouse model of asthma. We used this mouse model as it was readily available and had no fertility problems. The W-sash ($W^{sh}$) mutation rendered the coat color of the mice white with black spots in the ear and nose and thence eliminated the need for genotyping. Furthermore, this model did not show the presence of mast cells at P21 (Figure 3.4.6). Apart from mast cells, this mouse line has a deficiency in melanocytes and interstitial cells. In contrast, the $Kit^{W}/Kit^{W-v}$ transgenic mouse line has a point mutation in the exon-intron junction of Kit resulting in a truncated c-kit leading to fertility problems (84).

More recently non-kit dependent mast cell deficient models have been reported (85),(86). A novel mouse model for investigation of mast cell functions based on Cre/loxP recombination system has been generated. Cre was expressed under the mast cell protease 5 ($Mcpt-5$) promoter. Inducible deletion of CTMCs can obtained by crossing the
Mcpt-5 Cre line to the *ROSA-iDTR* strain, which expresses the high-affinity diphtheria toxin receptor (DTR) followed by administering diphtheria toxin. Another study has reported the generation of *Cpa3-Cre; Mcl*^{−/−} mice. Mcl is an anti-apoptotic factor myeloid cell leukemia sequence 1 (Mcl-1) which is required for the survival of mast cells. This mouse line has Cre recombinase expressed under the control of Cpa3 promoter. Crossing the *Cpa3-Cre* with *Mcl*^{−/−} causes a severe deficiency in mast cells and basophils (86).

Our future work aims to employ the *Mcpt-5* Cre mouse line crossed with the *ROSA-DTA* strain to further study the role of the mast cell in mediating neuroplasticity by releasing NT4 upon early-life allergen exposure. The recombination leads to expression of diphtheria toxin in Mcpt-5 producing mast cells leading to selective inhibition of CTMCs. We speculate that there might be problems associated with using the *Mcpt5-Cre* mouse line, as Mcpt-5, is expressed only in a subset of mast cells. Therefore, eliminating Mcpt5⁺ mast cells may not be sufficient to disrupt NT4 expression during allergic inflammation.

### 2.6 Role of mast cells in asthma

Mast cells are known to play a major role in allergic reactions that involve both innate and adaptive immune cells (87). The allergic cascade of events can be divided into two phases, early phase response and the late phase response. Early phase response involves the release of histamine, prostaglandin and proteases from mast cells upon IgE
degranulation which leads to ASM contraction in 30 minutes from allergen exposure (Figure 2.2) \(^{(65)}\) and mucous secretion before infiltration of immune cells \(^{(54)}\). The late phase response includes recruitment and activation of other immune cells such as T cells, B cells and eosinophils that last for up to 24 hours before subsiding. The activated Th2 cells, in turn, produce cytokines such as IL-4, IL-5 and IL-13. The release of IL-5 is associated with increased recruitment of eosinophils in asthmatic patients. Studies indicate a distinct role of IL-13 in mucous metaplasia \(^{(88)}\). Histamine and PGD\(_2\) can modulate DCs to induce the development of Th2 responses \(^{(89),(90)}\). Histamine binds to the histamine receptors H1 to H4 to exert its functions. H1 and H3 receptors induce dendritic cell activation \(^{(91)}\) whereas H2 leads to IL-10 secretion that in turn increases mast cell infiltration. Histamine can also lead to increased bronchoconstriction via H1 receptors on the airway smooth muscle.

Increased secretion of prostaglandins is shown to increase airway inflammation in mouse models \(^{(92)}\). Leukotrienes are lipid mediators released by mast cells and are known to affect airway inflammation in allergic asthma. These lipid mediators play an important role in the recruitment of T cells and DCs in the lung \(^{(93),(94)}\). Thus, mast cells play a major role in maintaining a chronic inflammatory environment during allergic asthma.

Apart from regulating inflammation in allergic asthma, mast cells have a neuro-modulatory role in asthma. Neuropeptides released from sensory neurons can degranulate mast cells to secrete various mediators that in turn influence neuronal activity. Thus, mast
cell facilitates bi-directional crosstalk between immune cells and nerves. However, the exact mechanism by which mast cells regulate nerve activity in childhood asthma is unknown. My study shows that mast cells release neurotrophin 4 on early-life allergen exposure leading to hyper cholinergic innervation of the ASM (Figure 2.2). The hyper-innervated ASM continues to have persistent AHR phenotype into adulthood. A study by Cyphert et al. shows that mast cells secrete serotonin, which activates cholinergic nerves to induce AHR. Their findings support our conclusion that mast cells play a critical non-inflammatory role in allergen-induced airway hyper reactivity by the crosstalk with nerves. Our study indicates that mast cells communicate with nerves to induce sustained AHR.

2.7 Mast cell-nerve interactions

Apart from being the key effector cell in allergic asthma, mast cells are known to interact with nerves in different tissues. The spatial location of mast cells in lungs makes them key candidates for the nerve-immune interactions (Figure 2.1). The mucosal mast cells are usually in close contact with the nerves and can directly modulate nerve activity. Mast cells synthesize and release NGF. The NGF released by mast cells bind to the Trk A receptors on the nerves and activate nerve growth. A functional NGF receptor Trk A is expressed on the surface of mast cells (45). Trk A activation by NGF leads to increased survival and proliferation of mast cells (95),(96). Therefore, NGF released by mast cells can further activate mast cells through a positive feedback loop. I show that in early-life upon allergen exposure, mast cells in neonatal mouse lung release NT4 causing an increase in
cholinergic neuroplasticity in the lung. My research shows that this role of mast cells is evolutionarily conserved in mice and non-human primates.

The proximity between mast cells and substance P positive nerve fibers have been studied in human and rat GI, peripheral rat lung and trachea \(^{(97)}\). Studies in adult mouse lungs show that mast cells release several neuropeptides such as Substance P \(^{(19)}\). Substance P acting via the NK-1 receptor on the mast cell surface can lead to degranulation of mast cells. In atopic dermatitis and psoriasis, there is an increase in sensory nerves releasing substance P and CGRP as well as an associated increase in degranulated mast cell number \(^{(98),(99),(100)}\). In inflammatory bowel disease, patients show an increase in mast cell number associated with increased sensory afferent nerves with high levels of Substance P \(^{(101)}\). Similarly, studies show that increases in mast cell numbers are associated with increased sensory afferent innervation in irritable bowel syndrome and interstitial cystitis \(^{(102)}\). Most of the studies show an association between mast cell numbers and nerve density.

Our previous work shows increases in NT4 levels upon early-life insults. However, how the NT4 levels were up regulated and its cellular source remained to be understood? Due to the spatial proximity of mast cells and ASM and its role in regulating nerve density in other organs, we decided to look at mast cell number and expression of NT4 upon allergen exposure in early-life. In our neonatal mouse model, we found that mast cells synthesize and express NT4 (Figure 3.2). Furthermore, we show that the NT4 expression
by mast cells is conserved in mice and primate lungs. The exact role of pulmonary mast cell-derived NT4 upon early-life allergen exposure is explained in detail in the next chapter.

2.8 Conclusion

It is evident that an extensive crosstalk exists between mast cells and nerves, which may differ in different species and tissues. During allergen exposure, the interaction between mast cells and nerves facilitates the involvement of peripheral and central nervous system in the regulation of inflammation. The role of mast cells in regulating neuro-inflammation during infection or disease is studied in detail in atopic dermatitis and inflammatory bowel disease. The potential role of the mast cell for induction of hyper innervation following allergen exposure in young children has not been addressed. Using the neonatal mouse and non-primate models of asthma, my research shows an essential role of mast cells in regulating cholinergic neuroplasticity. My study explains the mechanism by which activated mast cells release NT4 leading to hyper innervation of the ASM that causes persistent AHR.
Figure 2.1 Pulmonary mast cells in airways of different species. Toluidine blue staining of primate and mouse paraffin lung sections. Arrows point at toluidine blue positive mast cells. Scale bar: 100μm and 10μm respectively.
Figure 2.2 Mast cell-nerve interactions in the neonatal lung. Early-life allergen exposure triggers a cascade of events. 1) Repeated allergen exposure leads to crosslinking of IgE receptor inducing release of several pro-inflammatory mediators such as cytokines, histamine, leukotrienes, etc. causing chronic inflammation in the lung. 2) Mast cells release NT4 thereby increasing the NT4 levels in the lung. 3) NT4 released from mast cells bind to the Trk B receptor on the nerves causing hyper innervation of the ASM. 4) The NT4 released from the mast cells lead to increase in cholinergic nerves along the ASM. 5) The increased cholinergic nerves release acetylcholine that binds to the M3 receptor on the ASM, which in part, causes hyper constriction of the ASM that persists into adulthood.
CHAPTER THREE

Pulmonary mast cell-derived neurotrophin 4 mediates allergen-induced airway hyper innervation in early-life

3.1 Summary

Asthma often progresses from early episodes of insults. How early life events connect to long-term airway dysfunction remains poorly understood. We demonstrated previously that increased neurotrophin 4 (NT4) levels following early-life allergen exposure cause persistent changes in airway smooth muscle (ASM) innervation and airway hyper-reactivity (AHR) in mice. Herein, we identify pulmonary mast cells as a key source of aberrant NT4 expression following early insults. NT4 was selectively expressed by ASM and mast cells in mice, nonhuman primates and humans. We show in mice that mast cell-derived NT4 is dispensable for ASM innervation during development. However, upon insults, mast cells expand in number and degranulate to release NT4 and thus become the major source of NT4 under pathological condition. Adoptive transfer of wild-type mast cells, but not $NT4^{-/-}$ mast cells restored ASM hyper innervation and AHR in $Kit^{W-sh/W-sh}$ mice following early life insults. Notably, an infant primate model of asthma also exhibited ASM hyper innervation associated with the expansion and degranulation of mast cells. Together, these findings identify an essential role of mast cells in mediating ASM hyper innervation following early life insults by producing NT4. This role may be evolutionally conserved to link early insults to long-term airway dysfunction.
3.2 Introduction

Asthma is a chronic respiratory disease that often progresses from childhood to adulthood (103). Risk factors for asthma include early-life exposure to allergen, cigarette smoke, ozone and respiratory viral infection. As the lung continues to grow after birth, environmental insults during infancy and early childhood may cause prolonged changes in lung structure, function, and disease susceptibility (6),(15),(104),(104). However, the mechanism that connects early events to long-term airway dysfunction remains poorly understood. As a direct consequence, treatment strategies that prevent asthma in young children at high risk are lacking.

Previous studies in rodents and nonhuman primates showed that neural innervation in immature lungs is prone to change by insults. Respiratory syncytial virus (RSV) infection in neonatal guinea pig increases peptidergic, sensory nerves in the lower airway (36). Early-life exposure to ozone (O₃) or cigarette smoke in rats and mice similarly increases sensory and sympathetic innervation of the airway (37). Employing a neonatal mouse model of ovalbumin (OVA) and cockroach allergen exposure, we showed that ASM hyperinnervation is functionally linked to persistent AHR into adulthood (6). Furthermore, nonhuman primates exhibit changes in airway innervation following perinatal and neonatal exposure to O₃, house dust mite allergen (HDMA), or cigarette smoke (105),(106). In contrast, insults to mature adult lungs in animal models have no effect on airway innervation and elicit transient airway dysfunction (6),(3).
We showed in neonatal mice that allergen exposure elevates NT4 expression to induce ASM hyper innervation \(^{(6)}\). NT4 belongs to a nerve growth factor family that plays an essential role in the development of the nervous system \(^{(107)}\). During lung development, NT4 expressed by ASM serves as a target-derived neurotrophic factor for ASM innervation \(^{(6)}\). However, how NT4 expression is aberrantly upregulated following early-life allergen exposure is unknown. Consistent with a role of aberrant NT4 expression in long-term airway dysfunction in the neonatal mouse model, members of the NT family are over-expressed in lungs of infant nonhuman primates following exposure to cigarette smoke and in human infants who are infected with RSV \(^{(106)}\). In addition, serum levels of NT4 are positively correlated with disease severity in children with asthma \(^{(5)}\). These findings suggest that NT overexpression and associated airway hyper innervation may be evolutionarily conserved as early events that ultimately contribute to the pathogenesis of asthma.

Mast cells are known to interact with nerves and these interactions have been implicated in several diseases, such as multiple sclerosis, interstitial cystitis, irritable bowel syndrome and atopic dermatitis \(^{(108)}\). In the lung, pulmonary mast cells are often found in intra-epithelial and intramuscular spaces in close proximity to nerves in the airway \(^{(67),(109),(110)}\). The pulmonary mast cells are known to communicate with cholinergic nerves through serotonin secretion causing AHR in adult mice \(^{(111),(59)}\). Mast cells are also known to express NTs \(^{(44)}\). Whether mast cells contribute to changes in NT expression in neonatal mouse models and in childhood asthma is unknown.
In this study, we investigate how early-life allergen exposure in mice elevates the levels of NT4 to increase ASM innervation, which in turn causes AHR. This study is powered by parallel characterization of samples from mice, nonhuman primates and humans and is followed by in-depth mechanistic studies using mouse genetics and functional rescue assays. Herein, we identify NT4 released from pulmonary mast cells as the underlying mechanism of allergen-induced ASM hyperinnervation in neonatal mice and provide evidence that this neuro-modulatory role of mast cells may be conserved in primates.

3.3 Materials and Methods

3.3.1 Mice. Wild-type, *NT4*−/− (stock number 002497) and *Kit*W-sh/W-sh mice (stock number 012861) were purchased from The Jackson Laboratory. The double fluorescent, *SMA-GFP; NG2-dsRed* mice were described previously (112). All the mice lines were in the C57BL/6 background. The Institutional Animal Care and Use Committee approved all studies with mice.

3.3.2 Neonatal mouse model of allergic asthma. Neonatal pups were sensitized and challenged with ovalbumin (OVA) as described previously (113). Briefly, pups were sensitized at P5 and P10 by intraperitoneal injections of 10 µg OVA (A5503, Sigma) in Imject alum (#7761, Thermo Scientific). The sensitized pups were challenged daily with 3% aerosolized OVA solution between P18 and P20. Control pups were challenged with PBS. At day 21, mice were sacrificed for blood, BAL, and lung harvest. Serum levels of
OVA-specific IgE and lung IL-13 were measured with ELISA kits from Bio Products (M036005) and Life Technologies (KMC2221), respectively. BAL counts were performed as described.

3.3.3 Fluorescent staining and microscopy. Cells and tissue sections were fluorescently labeled using an established protocol: For endotracheal aspirates from patients at Boston Children’s Hospital, the aspirate was treated with collagenase I (10 μg/ml) for 15 min at 37°C to degrade the mucus before cells were spun onto a histology slide using Cytospin followed by antibody staining. Primary antibodies include mouse anti-NT4 (1:200, sc-365444, Santa Cruz Biotechnology), rat anti-tryptase βI/MCPT-7 (1:100, MAB1937, R&D systems), biotinylated mouse anti-neural class III β-tubulin antibody (TuJ1, 1:200, BAM1195, R&D Systems), and GFP-conjugated mouse anti-SMA antibody (1:500, F3777, Sigma). Isotype controls were rat IgG (1:100, ab37361, Abcam) and mouse IgG (1:200, sc-2025, Santa Cruz Biotechnology). The biotinylated TuJ1 antibody was detected by streptavidin-Cy3 (1:300, SA1010, Invitrogen). All secondary antibodies were purchased from Life Technologies and included donkey anti-mouse 546 (1:500, A10036), donkey anti-rat 488 (1:500, A21208), and donkey anti-rabbit 546 (1:500, A10040). Fluorescently stained cells and monkey sections were imaged with Axiovert 100M LSM 510 microscope (Zeiss). TuJ1 staining of mouse lung slices (100 μm in thickness) were imaged by confocal microscopy. The compressed z-stack images were quantified to determine the innervation density by dividing the TuJ1 immune-reactive area with the
perimeter of the airway measured. For quantification of axon density in rhesus monkey lungs, axon density was calculated by dividing TuJ1-immunoreactivity by SMA⁺ area.

3.3.4 Degranulation assay. Mast cells (2 x 10⁶) were cultured in a 24 well plate in 500 μl of DMEM. The cells were treated with mouse IgE (0.5 μg/ml, #553481, BD Biosciences) for 2 hours at 37°C. After washing, cells were incubated with anti-mouse IgE (1 μg/ml, #553413, BD Biosciences) for 2 hours at 37°C. The supernatant was collected before and after anti-IgE treatment. The supernatant was concentrated 10 fold using a spin column with 3kDa cutoff (#42404, Millipore).

3.3.5 Western blot analysis. The protein samples from lungs of P21 mice and lysates/ media from mast cell cultures were subjected to western blot analysis described previously.² Primary antibodies for VACHT (1:100, Abcam #AB68986) and GAPDH (1:10,000, Abcam #AB8245), NT4 (1:100, ANT004, Alomone Labs, Israel) were applied in blocking buffer. The secondary antibodies used were goat anti-rabbit HRP (1:1000, Santa Cruz Biotechnology #sc-2004) and goat anti-mouse HRP (1:5000, BD Biosciences #554002). The antigen-antibody complex was detected by SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Densitometry units for Individual protein bands were measured using Image J and normalized to its GAPDH levels.

3.3.6 Toluidine blue staining for mast cells. Left lung lobes were fixed in 4% paraformaldehyde/PBS at 4°C overnight. 5 μm paraffin sections were stained with 0.1%
Toluidine blue (pH 2.0) for 2-3 minutes after rehydration. The sections were washed by
dipping in water 3-5 times followed by dehydration in 100% ethanol. Data are presented
as an average of the mast cell number from 10 100X images (0.01 mm²) in each
condition.

3.3.7 Primary pulmonary mast cell culture. Primary pulmonary mast cells were derived
from the lungs of 4-week-old mice following a previously described protocol (114),(63).
Briefly, lungs were minced, dissociated by collagenase (50 U/ml in HBSS), and filtered
through a 40 μm filter. Cells were cultured in Dulbecco’s modified medium (DMEM)
containing 10% FBS, recombinant mouse IL-3 (10 ng/ml, 213-13; Peprotech), and 10
ng/mL recombinant SCF (455-MC-010; R &D Systems). By the end of 3 weeks, the non–
adherent population was enriched in mast cells, which was confirmed using flow
cytometric analysis of surface markers, CD117 (1:200, 553869; BD pharmingen™) and
FceR1 (1:500, 11-5898; eBioscience, San Diego, CA). An MC/9 mast cell line (ATCC
CRL-8306) was used as a positive control for flow cytometry.

3.3.8 Adoptive transfer of primary pulmonary mast cells and airway contraction assay.
20,000 mast cells were adoptively transferred into each K/ItW-sh/W-sh mouse at P15 via intra-
tracheal delivery. These mice were rested for 2 days and followed by OVA challenges. At
P21, the lungs were harvested. Precision-cut lung slices (250 μm in thickness) were
prepared and assayed similarly as previously described (113). The airway luminal area was
quantified at baseline and after methacholine treatment using Image J. Data were normalized to the pretreatment baseline value.

3.3.9 Cell sorting. Cell suspension and sorting from lungs from SMA-GFP; NG2-dsRed mice at P21 were performed as described previously (112). Antibodies against CD45 (1:100, 30-F11) and CD31 (1:100, MEC 13.3) were purchased from BD Pharmingen, San Diego, CA. Isotype antibodies were used as controls. Cells were sorted using a Moflo cell sorter (Beckman Coulter, Fullerton, CA). Cells from 5-6 mouse lungs were pooled prior to RNA extraction and gene expression analysis.

3.3.10 Flow cytometry. The lungs were dissociated for cell suspension as described previously (112). For intracellular staining, the cells were incubated with Golgi Stop (BD biosciences, #554724) for 4 hours at 37°C. Cells were then spun down at 1200 rpm for 5 min and fixed with Cytofix (BD biosciences, #554722) overnight at 4°C. The next day, the cells were washed with 1x Perm wash (BD biosciences, #554722) and then stained with the following antibodies: CD45-PERCP.CY5.5 (1:100 BioLegend #103132), CD117- PE (1:200, BD pharmingen™ #553869) and FcεR1-FITC (1:500 eBioscience, San Diego, CA #11-5898) and NT4-APC (1:50, Santa Cruz Biotech, #sc-365444 special order). Cells were assayed on an FACSCalibur flow cytometer. Data were analyzed using FlowJo software (Tree Star).
3.3.11 Statistics. All data are represented as mean±sem from a minimum of 3 separate experiments. Statistical analysis was performed with the 2-tailed Student’s t-test for comparisons between 2 conditions. For comparison between multiple variances in lung slice contraction assays, data were analyzed with 2-way, repeated measures ANOVA. A P value of ≤ 0.05 was considered to be significant.

3.4 Results

3.4.1 Early-life insults lead to an increase in ASM innervation in nonhuman primates

We showed in a neonatal mouse model that allergen exposure elevates NT4 levels to increase ASM innervation\(^{(113)}\). NTs are also overexpressed in lungs of RSV-infected infants and in severe childhood asthma\(^{(106)}\). However, whether airway innervation is altered in these young patients is unknown due to difficulties of obtaining tissue samples. To circumvent this issue, we assessed whether insults in infant rhesus monkeys increase ASM innervation using resources provided by California National Primate Research Center at University of California Davis (http://www.cnprc.ucdavis.edu/our-science/respiratory-diseases/). The infant primate model of O\(_3\) and house dust mite allergen (HDMA) exposure for 6 months after birth faithfully recapitulates the clinical hallmarks of asthma and disease progression (Figure 3.1 A). Proximal primate lung sections were double stained for nerves using a TuJ1 antibody and ASM using a smooth muscle actin (SMA) antibody (Figure 3.1 B). Axon density was calculated by normalizing TuJ1 immunoreactivity to the SMA\(^+\) area. Compared to filtered air-exposed
controls, O$_3$+HDMA exposure significantly increased ASM innervation by approximately 70% (Figure 3.1 C). These findings indicate that early-life insults increase ASM innervation in nonhuman primates.
Figure 3.4.1 Early-life allergen exposure increases airway innervation in nonhuman primate lungs. (A) Experimental scheme of O₃ and HDMA exposure in infant nonhuman primates. Controls were exposed to filtered air. (B) Assessment of ASM innervation by TuJ1 and alpha-SMA double staining of proximal lung sections from control and O₃+HDMA exposed infant rhesus monkeys at 6 months. Arrows indicate ASM and arrowheads indicate innervating nerves. Scale bar, 100 µm. (C) Quantification of axon density in the ASM of the lungs exposed to filtered air and O₃+HDMA. Axonal density is measured by normalizing the TuJ1 staining intensity to the SMA-positive area. A total of 25 sections, 5 from each infant rhesus monkey lung, were quantified. Data represent the mean and SEM.

***P<0.001.
3.4.2 Mast cells are a candidate source of NT4 in neonatal models of allergic asthma in mice and primates.

Given the central role of elevated NT4 levels in ASM hyper innervation and persistent AHR in the neonatal mouse model of allergen exposure, we set out to identify the cellular source of aberrant NT4 expression following early-life insults. We first assessed whether OVA exposure is increased NT4 expression in ASM. NT4 is expressed in ASM and serves as a target-derived neurotrophic factor for innervating nerves during postnatal development (113). Employing a previously characterized SMA-GFP; NG2-DsRed mice that permits separation of ASM from vascular smooth muscle, GFP+ ASM cells were isolated at postnatal day 21 (P21) after mice were subjected to OVA sensitization and challenge (Figure 3.2 A) (112). Comparison of NT4 mRNA levels in purified ASM cells yielded no significant difference between PBS and OVA exposure (Figure 3.4.2 B). Therefore, ASM is unlikely to be the source of elevated NT4 after OVA exposure in neonatal mice.

We next took an unbiased approach to narrow down candidate cell types that overexpressed NT4 after OVA exposure in neonatal mice. For this, P21 lungs were enzymatically dissociated to yield single cell suspension followed by cell sorting into 3 major groups, CD45+ hematopoietic cells (including mast cells), CD31+ endothelial cells, and CD45−; CD31− population (including ASM cells). We found that the only group of cells that had increased NT4 mRNA levels after OVA exposure was CD45+ hematopoietic cells (Figure 3.2 B). This finding was consistent with a lack of change in
NT4 gene expression in ASM, a constituent of the CD45+; CD31+ population (Figure 3.2 B).

Double staining of mouse lung sections at P21 using an antibody against tryptase, a specific marker of mast cells and TuJ1 antibody showed that mast cells were often in close proximity to the innervating nerves in the airways (Figure 3.2 C)(59). In addition, rat peritoneal mast cells were shown to express NTs (44). To test whether pulmonary mast cells and possibly other immune cell types express NT4, we stained dissociated lung cells for CD45, NT4 and mast cell-specific surface markers, c-kit (CD117) and FcεRI followed by flow cytometry. To ensure specific NT4 labeling, cells from NT4−/− mice were used for gating control (Figure 3.2 D). CD45+ immune cells accounted for approximately 25% total cell population of both wild-type and NT4−/− lungs at P21 (Figure 3.2 D). Among these hematopoietic cells, 3.09% cells were found to be NT4+ and most of them (90.1%) expressed c-kit (CD117) and FcεRI (Figure 3.2D), indicating NT4 was almost exclusively expressed by pulmonary mast cells within the immune cell population. To confirm this, we performed immunocytochemistry for NT4 using Bronchoalveolar lavage (BAL) collected from OVA-exposed mouse lungs at P21. NT4 was detected in a small percentage of cells with two distinct staining patterns (Figure 3.2 E). The punctate and diffusive cytoplasmic pattern of NT4 was confirmed to be the secretory granules of mast cells by double staining for tryptase, a mast cell marker (Figure 3.2 E). Very few other cells with a polarized NT4 staining pattern were likely macrophages that engulfed mast cells (Figure 3.2 E). Specificity of the NT4 monoclonal antibody and the tryptase
antibody for immunocytochemistry was validated by a lack of staining using IgG isotype controls and $NT4^+$ mast cells (Figure 3.2 F and Figure 3.4 C).

To test whether mast cells in primate lungs also express NT4, we characterized NT4 expression in the lungs of control, 6-month-old rhesus monkeys. We found that ASM and tryptase$^+$ mast cells are the only two cell types that express NT4 in lungs of nonhuman primates (Figure 3.2 F), similar to mice. We also characterized NT4 expression in human lungs by double staining cells in endotracheal aspirates of respiratory virus-infected children and on tissue sections from adult, healthy donor lungs. All NT4$^+$ cells in endotracheal aspirates were positive for tryptase (Figure 3.2 G). In addition, besides ASM that expressed NT4 (data not shown), almost all other NT4$^+$ cells were mast cells in human lungs (Figure 3.2 H). Together, pulmonary mast cells are the predominant immune cell source of NT4 in mice, nonhuman primates and humans.
Figure 3.4.2 Mast cells are a candidate source of increased NT4 levels in the lung after early-life allergen exposure. (A) Experimental protocol of OVA sensitization and challenge in neonatal mice. Controls received PBS challenges. (B) Comparison of NT4 gene expression in ASM and 3 major cell groups sorted from the lungs of PBS- and OVA-exposed mice at P21. ASM cells were isolated from SMA-GFP; NG2-dsRed mice and were pooled from 5-6 mouse lungs as one sample. n=3. (C) Double staining for mast cells (red) and nerves (green) in mouse lungs at P21 using a tryptase antibody and TuJ1 antibody. Scale bar, 50 µm. (D) Expression of NT4 in lung immune cells. CD45+ immune cells were gated for NT4 using NT4−/− cells as negative control. NT4+ immune cells were then gated for c-kit and FcεRI. (E) Double staining of the immune cells in BAL for NT4 and tryptase. BAL was collected from OVA-exposed mice at P21. The arrow indicates the double positive cells. * indicates a cell (likely macrophage) with polarized NT4 staining. The insert shows an enlarged image of a double positive mast cell. Scale bar, 25 µm. (F) NT4 and tryptase double staining of 6-month-old rhesus monkey lungs. Arrows indicate double positive mast cells. Arrowheads indicate NT4 expression in ASM. The IgG isotype controls showed no staining. The insert shows an enlarged image of a double positive mast cell. Scale bar, 50 µm. (G) Double staining of the cells in endotracheal aspirates from respiratory virus-infected children for NT4 and tryptase. The arrow indicates the double positive cell. Scale bar, 25 µm. (H) Double staining of adult human lung sections for NT4 and tryptase. The arrow indicates double positive mast cells. Scale bar, 50 µm. Nuclei were stained with DAPI in all images.
3.4.3 Mast cell dynamics after early-life allergen exposure

Mast cells are often found close to nerves (Figure 3.2 C). In addition to spatial proximity, we reasoned that in order to serve as a functional source of NT4, the number of pulmonary mast cells, NT4 expression, and changes in ASM innervation would be temporally coordinated during the course of insults. To address this issue, we characterized the correlation between the mast cell number and changes in ASM innervation following allergen sensitization and challenge in neonatal mice. At P15 when serum levels of OVA-specific IgE were increased after two rounds of sensitization (Supplemental Figure S1a), the number of mast cells within and close to ASM was doubled compared to PBS controls (Figure 3.3 A, B) (69). This was associated with an approximately 80% increase in ASM innervation and NT4 levels (Supplemental Figure S1b-c). At P21 after OVA challenges, the number of mast cells increased 4 fold compared to PBS controls (Figure 3.3 C, D), which was positively correlated with elevated levels of NT4 and lung innervation (113). In addition, toluidine blue staining showed spewed granules surrounding mast cells in OVA-exposed mouse lungs indicating mast cell degranulation (inserts, Figure 3.3 A, C), while granules were rarely found outside of mast cells in control lungs (Figure 3.3 A, C).

Similar to our findings in mice, O3+HDMA exposure in infant nonhuman primates almost doubled the number of tryptase+ mast cells in ASM when ASM was hyperinnervated (Figure 3.3 E, F). Human asthmatics also have increased infiltration of mast cells into ASM (67). The positive correlation between the number of mast cells and ASM
innervation supports mast cells as a candidate for aberrant NT4 expression and ASM hyper innervation in mice and nonhuman primates and possibly in humans.
Figure 3.4.3 Correlated changes in mast cell number and ASM innervation during the early-life insult. Toluidine blue staining and quantification of mast cells in control and OVA-exposed lungs at P15 (A, B) and P21 (C, D). Arrows point to stained mast cells. Scale bars, 10 µm. Inserts in (A, C) provide a zoomed-in view of spewed granules from a mast cell after OVA exposure. Data represent the average and SEM from 10 non-overlapping, 100X images (0.01 mm²) in each mouse lung and 5 mice for each condition. *P<0.05; ***P<0.001. (E) Tryptase staining of control and O3+HDMA exposed infant rhesus monkey lungs. Arrows indicate tryptase⁺ mast cells in ASM. Inserts provide a zoomed-in view of tryptase⁺ granules that were mostly inside of a mast cell of control lungs but got spewed from a mast cell in O3+HDMA exposed lungs. Scale bar, 50 µm. (F) Quantification of tryptase⁺ mast cells in ASM of control and O3+HDMA exposed infant rhesus monkey lungs. A total of 25 sections from 5 infant monkeys were quantified. Data represent the mean and SEM per 20X field (0.14 mm²). Scale bar, 50 µm. ***P<0.001.
3.4.4 NT4 release requires mast cell degranulation.

In allergic asthma, mast cells undergo IgE-mediated degranulation to release several inflammatory mediators (115). We speculated that NT4 release by degranulation might serve as a mechanism to regulate the bioavailability of NT4 to innervating nerves. To test this hypothesis, we assayed the secretion of NT4 from pulmonary mast cells by cross-linking IgE receptor FceR1. Primary pulmonary mast cells were obtained after cell suspension from dissociated lungs was treated with stem cell factor (SCF) and IL-3 over a prolonged period (Figure 3.4 A) (114,63). After 3 weeks, the culture was enriched in mature mast cells based on the expression of tryptase and cell surface markers, FceR1 and c-kit (CD117) that was indistinguishable from an established MC/9 mast cell line (Figure 3.4 B). NT4 staining showed that all wild-type primary mast cells expressed NT4 while the same mouse monoclonal antibody yielded no positive signal using primary NT4/− mast cells (Figure 3.4 C).

After confirming the purity of primary mast cell cultures, cultures were stimulated with mouse specific IgE followed by an anti-IgE antibody to induce crosslinking of FceR1. After 2 hours, wild type and NT4/− primary mast cells degranulated to the same extent assayed by Western blot for tryptase released into the media (Figure 3.4 D). This indicated that NT4 deficiency does not impact FceR1 mediated mast cell degranulation. Under the same conditions, the NT4 release was assessed using a polyclonal NT4 antibody. The specificity of this polyclonal NT4 antibody was determined by comparing cell lysates of wild-type and NT4/− primary mast cells (Figure 3.4 E). The antibody
detected specific protein bands representing NT4 in mature form (14 kDa), pro-form (22 kDa) and NT4 dimer (42 kDa) (Figure 3.4 E). Other protein bands at higher molecular weight, which were previously reported using rat peritoneal mast cells, were deemed non-specific, as they also appeared in NT4−/− cell lysates (Figure 3.4 E). Guided by these results, we evaluated NT4 in the conditioned media of primary pulmonary mast cell cultures. We did not detect any NT4 under IgE treatment alone (Figure 3.4 E). However, upon cross-linking of FcεRI to induce mast cell degranulation, NT4 in all three forms was released into the media (Figure 3.4 F). These findings demonstrate that NT4 release by mast cells is regulated by degranulation. In addition, NT4 itself is not required for degranulation. Consistently, there was no difference in mast cell number and allergen-induced degranulation between wild type and NT4−/− mice (Figure 3.4 S2).
Figure 3.4.4 NT4 release requires degranulation of mast cells. (A) Experimental protocol of primary pulmonary mast cell culture. (B) Flow cytometry analysis of c-kit and FcεRI expression by primary mast cells and MC9 mast cells. Inserts showed tryptase staining of cells in culture. (C) Staining of primary pulmonary mast cells for NT4. No NT4 staining was detected in NT4−/− primary mast cells. Nuclei were stained with DAPI. Scale bar, 10 μm. (D, E) Western blot analysis of tryptase and NT4 release in the medium from primary pulmonary mast cells treated with IgE alone (0.5 μg/ml) or with both IgE and anti-IgE (1 μg/ml). Data shown represent results from 3 independent experiments. (F) Validation of the specificity of NT4 antibody for Western blot analysis using cell lysates of wild-type and NT4−/− primary mast cells in culture.
3.4.5 Mast cells are required for allergen-induced ASM hyper innervation in early-life by releasing NT4

To study the role of pulmonary mast cells in NT4 expression and ASM innervation, we compared NT4 gene expression and ASM innervation between wild type and mast cell deficient, Kit<sup>W-sh/W-sh</sup> mice with and without early-life allergen exposure. Unlike other c-kit mutations, Kit<sup>W-sh/W-sh</sup> mice have normal levels of major classes of other differentiated hematopoietic and lymphoid cells (<sup>81</sup>,<sup>80</sup>,<sup>82</sup>). After OVA exposure, Kit<sup>W-sh/W-sh</sup> mice exhibited similar levels of inflammation as wild-type mice, assessed by BAL counts, serum levels of OVA-specific IgE and the Th2 cytokine IL-13 at P21 (Figure 3.5 A-C) (<sup>80</sup>,<sup>82</sup>). In addition, we found no change in baseline levels of NT4 protein expression or ASM innervation in Kit<sup>W-sh/W-sh</sup> mice at P21 (Figure 3.5 D-F), indicating that mast cells are not required for ASM innervation during development. This is consistent with a lack of NT4 release from mast cells at baseline and provides further evidence in support of ASM-derived NT4 as the target-derived neurotrophic factor for ASM innervation during normal development (<sup>113</sup>). Notably following OVA exposure, Kit<sup>W-sh/W-sh</sup> mice showed no increases in NT4 levels and ASM innervation (Figure 3.5 D-F). Subsequently, OVA allergen-exposed Kit<sup>W-sh/W-sh</sup> mice showed diminished AHR to increasing doses of methacholine (Figure 3.6 F), which was in sharp contrast to wild-type mice. Notably, these findings indicate that the c-kit-dependent cell population, which includes mast cells, is required for aberrant NT4 levels and ASM hyper innervation following early-life allergen exposure. Since mast cells are the only other cell type besides ASM that
expresses NT4, we speculated that mast cells might become a functional source of NT4 after the expansion of the cell pool and degranulation during insults.
Figure 3.4.5 Mast cells are required for increased ASM innervation after early-life OVA exposure. (A) Serum levels of OVA-specific IgE in PBS- and OVA-exposed, WT, and KitW+sh/W−sh mice at P21. n=9. (B) Differential BAL count of PBS- and OVA-exposed WT and KitW+sh/W−sh mice at P21. The numbers of eosinophils (Eos), lymphocytes (Lymph), neutrophils (Neut), and macrophages (Mac) are shown. n=9. (C) Serum levels of IL-13 in PBS- and OVA-exposed WT and KitW+sh/W−sh mice at P21 measured by ELISA. n=9. (D) Quantification of NT4 gene expression in the lungs of PBS- and OVA-exposed, WT and KitW+sh/W−sh mice at P21 by qPCR. n=9. (E) Quantification of the ASM innervation density in control and OVA-exposed, WT and KitW+sh/W−sh mice at P21. Data represent the average and SEM from 4 airways of each mouse and 10-12 mice for each condition. (F) Representative images of TuJ1 staining of the airway from control and OVA-exposed, WT and KitW+sh/W−sh mice at P21. Arrows indicate TuJ1+ axons. Scale bar, 50 μm. *P<0.05, ** P<0.01, ***P<0.001.
3.4.6. Adoptive transfer of pulmonary mast cells restores early-life allergen-induced ASM hyper innervation and AHR in \( \text{Kit}^{W-sh/W-sh} \) mice

In addition to mast cell deficiency, \( \text{Kit}^{W-sh/W-sh} \) mice have other non-mast cell related phenotypes (81). To definitively prove that mast cells were the functional source of aberrant NT4 levels for ASM hyper innervation after early-life allergen exposure, we tested whether adoptive transfer of wild-type mast cells, but not \( NT4^{-/-} \) mast cells would rescue the phenotypes in OVA-exposed \( \text{Kit}^{W-sh/W-sh} \) mice. After titrating, we determined that 20,000 mast cells via intratracheal instillation were sufficient to reconstitute the mast cell pool in \( \text{Kit}^{W-sh/W-sh} \) mice to the same levels as in wild-type mice at P21 (Figure 3.6 A, B). Wild-type and \( NT4^{-/-} \) mast cells were properly located in intra-epithelial and intramuscular spaces in \( \text{Kit}^{W-sh/W-sh} \) mice after engraftment and secreted out granules upon OVA exposure (Figure 3.6 B). Quantification of ASM innervation by TuJ1 staining showed that only wild-type pulmonary mast cells were able to fully restore ASM hyper innervation in \( \text{Kit}^{W-sh/W-sh} \) mice after OVA exposure while \( NT4^{-/-} \) mast cells had no such rescuing activities (Figure 3.6 C). Notably, wild-type mast cell reconstitution alone without allergen exposure had no effect on ASM innervation in \( \text{Kit}^{W-sh/W-sh} \) mice (Figure 3.6 C), further supporting our finding that NT4 release requires mast cell degranulation (Figure 4 E).

In addition to quantitative changes, we also evaluated qualitatively whether engraftment of wild-type mast cells restored specific types of innervation in the airway of OVA-
exposed Kit\textsuperscript{W-sh/W-sh} mice. Lungs were innervated mostly by sensory and parasympathetic nerves\textsuperscript{(116)}. Sensory nerves, labeled by calcitonin gene-related peptide (CGRP), were found unchanged in wild-type mice following OVA exposure in our previous study\textsuperscript{(113)}. We, therefore, measured the levels of vesicular acetylcholine transporter (VChT) in wild-type mice with and without OVA exposure at P21. VChT is a specific marker of parasympathetic nerves and mediates acetylcholine storage by synaptic vesicles. Compared to saline baseline, allergen exposure led to a 5-fold increase in VChT levels in wild-type mice at P21 assayed by Western blot (Figure 3.6 D). We then assessed whether engraftment of wild-type mast cells had a similar, inductive effect on parasympathetic lung innervation in Kit\textsuperscript{W-sh/W-sh} mice after OVA exposure. Western blot analysis showed that engraftment of wild-type mast cells, but not NT4\textsuperscript{−} mast cells, increased the levels of VChT in OVA-exposed, Kit\textsuperscript{W-sh/W-sh} mice by 3 fold above saline controls (Figure 3.6 E). These results indicate an essential role of mast cell-derived NT4 in airway hyper innervation by cholinergic nerves following allergen exposure.

We previously demonstrated that ASM hyper innervation following early-life allergen exposure is functionally connected to AHR\textsuperscript{(113)}. To assess AHR in Kit\textsuperscript{W-sh/W-sh} mice, we employed precision cut lung slices to measure ASM contraction in response to increasing doses of methacholine (Figure 3.6 F, G). Lung slices are largely devoid of humoral factors and free of complications associated with mucus blockade of the airway lumen and therefore, serve as an invaluable assay system for ASM contractility\textsuperscript{(117)}. OVA-exposed Kit\textsuperscript{W-sh/W-sh} mice, which had no increase in ASM innervation (Figure 3.5 E, F),
showed diminished AHR to increasing doses of methacholine as compared to OVA-exposed wild-type mice at P21 (Figure 3.6 F), consistent with previous reports (59),(52). In addition, \( \text{Kit}^{W-sh/W-sh} \) mice that were engrafted with wild-type pulmonary mast cells, but not \( NT4^- \) mast cells, recovered AHR to similar levels as wild-type mice following allergen exposure (Figure 3.6 G). Together, mast cells functionally contribute to early-life allergen-induced increases in ASM innervation and AHR by releasing NT4 in mice.
3.4.6 Reconstitution of the mast cell pool in the lungs of Kit<sup>W-sh/W-sh</sup> mice restores early-life allergen-induced increase in ASM innervation and AHR. (A) Experimental protocol of adoptive transfer of primary pulmonary mast cells (M.C.) during OVA exposure. Approximately 20,000 mast cells were installed intra-tracheal (I.T.) per mouse at P15. (B) Representative images of toluidine blue staining of mast cells in the lungs of Kit<sup>W-sh/W-sh</sup> mice with and without adoptive transfer of mast cells at P21. Arrows indicate pulmonary mast cells in the lungs. Scale bar, 10 µm. Inserts showed degranulation of engrafted mast cells. Quantification of mast cells in Kit<sup>W-sh/W-sh</sup> mice after adoptive transfer at P21 was shown in bar graph. Data represent the average and SEM from 10 non-overlapping, 100X images (0.01 mm<sup>2</sup>) in each mouse lung and 4 mice for each condition. (C) Representative images of TuJ1 staining of the airways from PBS- and OVA-exposed Kit<sup>W-sh/W-sh</sup> mice that received intratracheal instillation of WT or NT4<sup>−/−</sup> pulmonary mast cells. Arrows indicate TuJ1-labelled axons. n=6 mice from 3 independent experiments. Scale bars, 50 μm. The bar graph shows the quantification of the innervation density of ASM in PBS- and OVA-exposed Kit<sup>W-sh/W-sh</sup> mice with and without adoptive transfer of WT and NT4<sup>−/−</sup> mast cells. A total of 25 airways from 5 mice of each group were quantified. Data represent mean±SEM. (D) Western blot analysis of cholinergic innervation of the lungs at P21. Lung homogenates collected at P21 from PBS- and OVA-exposed wild-type mice were assayed for the levels of VaChT. Each lane represents 1 mouse. GAPDH was loading control. Data were normalized to PBS control mice. n=8. (E) Western blot analysis of cholinergic innervation in lungs of Kit<sup>W-sh/W-sh</sup> mice with and without reconstituted with primary mast cells at P21. Each lane represents 1 mouse. GAPDH was loading control. n=8. Data were normalized to PBS-exposed Kit<sup>W-sh/W-sh</sup> mice. (F) Measurement of airway contraction in response to increasing doses of methacholine.
using precision cut lung slices from OVA-exposed KitW-ski/ski mice with and without adoptive transfer of WT and NT4−/− mast cells. Lung slices from OVA-exposed wild-type mice were included as a positive control. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. Two-way ANOVA for multi-variance was used for statistical analysis. Statistically, significant differences between WT and NT4−/− mast cell transfer were marked. *P<0.05. **P<0.01. ***P<0.001.
Figure 3.4. S1 OVA-sensitized pups show increases in levels of serum IgE, airway innervation and NT4 levels at P15. (A) Serum levels of OVA-specific IgE in PBS- and OVA-exposed pups at P15 were measured by ELISA. Each marker represents a sample. Black horizontal line indicates mean of each group. (B) Western blot analysis of NF (L) and NT4 in lung homogenates collected at P15 from controls and OVA-exposed pups. GAPDH was used as loading control. Each lane represents 1 mouse. (C) Densitometry analysis of NF (L) signal in the Western blot assay in (B). Data were normalized to the GAPDH. (D) Densitometry analysis of NT4 signal in the Western blot assay in (B). Data were normalized to the GAPDH. ** P<0.01.
Figure 3.4 S2 NT4 deficiency has no effect on mast cell number at baseline and following OVA exposure. (A) Toluidine blue staining and quantification of mast cells in control and OVA-exposed lungs in NT4−/− mice at P21. Arrows point to stained mast cells. Scale bar, 10 µm. The insert provides an enlarged view of spewed granules from a mast cell after OVA exposure. (B) Quantification of mast cell number in NT4−/− mice at P21. Data represent the average and SEM from 10 non-overlapping, 100X images (0.015 mm²) in each mouse lung and 5 mice for each condition. NT4−/− mice have similar numbers of mast cells in lungs of wild-type mice at baseline and following allergen exposure at P21 (shown in Figure 3.3 C-D). ***P<0.001.
Figure 3.4 S3 Early-life exposure to HDMA increases the levels of cholinergic innervation and NT4 at P21. (A) Experimental scheme of HDMA exposure in neonatal mice. (B) Western blot analysis of NF(L) and VChT in lung homogenates collected at P21 from controls and HDMA-exposed mice. GAPDH was loading control. Each lane represents 1 mouse. (C, D). Densitometry analysis of VChT and NF(L) signal in the Western blot assay shown in (B). Data were normalized to GAPDH. (E) Quantitation of the NT4 levels in control and HDMA-exposed mice at P21 by Western blot assay. Data were normalized to GAPDH. *P<0.05
Figure 3.4 S4 Neonatal mice have more mast cells in proportion to the size of the lung than adult mice. (A) Representative images of toluidine blue staining of lung sections in control and OVA-exposed mice at P21 and in adulthood. Arrows point to stained mast cells. Scale bars, 100 µm. (B) Quantification of mast cell number in adult lungs. Data represent the average and SEM from 10 non-overlapping, 100X images (0.015 mm²) in each adult mouse lung and 3 mice for each condition. ***P<0.01. The relative abundance of mast cells in adult mouse lungs is approximately 20-30-fold lower than that in mouse lungs at P21 (shown in Figure 3.3 A-D).
3.5 Discussion

In this study, we identify a critical role of mast cells in NT4 overproduction, ASM hyperinnervation, and AHR following early life insult in mice. This role is only evident and essential under pathological conditions and is distinct from inflammatory functions traditionally associated with mast cells during allergic inflammation. In addition to OVA, we showed that repetitive intranasal exposure to cockroach allergen and HDMA similarly caused ASM hyperinnervation in neonatal mice (Figure 3.4 S3). These observations indicate that aberrant increases in ASM innervation are associated with early-life allergen exposure and are not administration route- or adjuvant- specific. In addition to allergens, O₃ also affects airway innervation in rats following neonatal exposure. In accordance, we showed that O₃ +HDMA caused an increase in ASM innervation in infant non-human primates (Figure 3.1). While the impact of HDMA and O₃ alone on ASM innervation in infant nonhuman primates warrants future work, we expect that the individual insult may have similar effects on ASM innervation based on previously reported epithelial hyperinnervation following individual or combined insults. Building upon our findings in both mice and nonhuman primates, we propose a model for ASM hyperinnervation following early life insults.

In our model, NT4 from ASM serves as an essential trophic factor for innervating nerves that express the NT4 receptor TrkB, thereby establishing ASM innervation during normal development (Figure 3.4.7). Following allergen exposure, NT4 expression by ASM is unchanged. However, mast cells increase in number and degranulate to release NT4,
thereby becoming a key source of aberrant NT4 expression that in turn causes ASM hyper innervation and AHR (Figure 3.4.7). Without mast cells, such as in Kit<sup>W-sh/W-sh</sup> mice, the only cellular source of NT4 in the lung is ASM. As a result, allergen exposure has no effect on ASM innervation and fails to elicit AHR (Figure 3.4.7). Notably, NT4 expression by ASM and pulmonary mast cells is conserved between mice and primates. In addition, the expansion of the mast cell pool and degranulation similarly occur in rodents, nonhuman primates and humans in response asthma. Therefore, mast cells may play a conserved role in ASM hyper innervation in the infant nonhuman primate model of asthma and thus, may contribute to the pathogenesis of asthma in human.
Figure 3.4.7 A model of pulmonary mast cells as a key source of elevated NT4 for early-life allergen-induced neuroplasticity. Allergen exposure increases the number of mast cells and triggers degranulation to release NT4, thereby increasing NT4-dependent ASM innervation. This, in turn, leads to AHR. Without mast cells in the lung, early-life allergen-induced neuroplasticity no longer happens. As a result, there is a lack of AHR in Kit<sup>W-sh/W-sh</sup> mice after early-life allergen exposure.
In allergic asthma, mast cells are known to degranulate due to the presence of high levels of IgE in the circulation. IgE-mediated degranulation leads to the release of several mediators such as tryptase and NT4 as shown in our study. It is unknown whether these mediators are released simultaneously because they are stored together within the same intracellular granules, or IgE triggers ubiquitous degranulation. Notably, NT4 is secreted in its mature form and pro-form upon IgE-mediated mast cell degranulation. This suggests that the proteolytic process to generate mature NT4 may occur both inside and outside of mast cells by serine proteases and matrix metalloproteinases. Notably, these proteolytic enzymes are abundant during allergic inflammation. Previous studies showed that beta-tryptase from human mast cells cleaved human pro-nerve growth factor (NGF) to mature NGF\(^{(118)}\).

The crosstalk between mast cells and nerves contributes to disease pathogenesis in multiple organs. In the lung, mast cells were shown to induce airway hyperreactivity by secreting serotonin to activate the cholinergic nerves in adult mice \(^{(59)}\). These previous studies employed Kit\(^{-/-}\)/W\(^{-/-}\) mice and provided evidence that mast cells have little effects on immune responses to an allergen \(^{(80),(81)}\). Similarly, our study found little evidence in support of a role of mast cells in allergic inflammation. However, rather than secreting serotonin, our studies in the neonatal mouse model indicate that mast cells communicate with innervating nerves by producing NT4, which leads to ASM hyper innervation by cholinergic nerves. We provided multiple lines of evidence in support of this unique role of mast cells. Firstly, mast cells are predominant immune cell type that expresses NT4.
Secondly, mast cell infiltration into ASM increases during repetitive allergen exposure. In addition, NT4 release requires mast cell degranulation. These two features enable mast cells to become a key source of aberrant NT4 levels following insults. Thirdly, the reconstitution experiment in Kit<sup>W-sh/W-sh</sup> mice showed that only wild-type mast cells, but not NT4<sup>−/−</sup> mast cells, were able to restore ASM hyper innervation and AHR following insults. These findings rule out the possibility that the phenotypes in allergen-exposed Kit<sup>W-sh/W-sh</sup> mice are caused by non-mast cell defects. Fourthly, NT4 deficiency has no effect on the number, differentiation, or degranulation of mast cells (Figure 3.4 S2, Figure 3.4 D). Lastly, the relative abundance of mast cells in immature mouse lungs is significantly higher than that in adult mouse lungs. Toluidine blue staining of lung sections showed that the density of mast cells is approximately 20-30 fold higher at P21 than in adult mice at both baseline and after allergen exposure (Figure 3.3 C, D, Figure 3.4 S4). Consistently, flow cytometry for mast cells using cell surface markers, c-kit and FcER1, showed that 0.6-0.9% of all lung cells are mast cells at P21 at the baseline in mice (Figure 3.2 D). In comparison, previous studies found only 0.021% of all cells in adult mouse lungs are mast cells. This age-related decrease in the relative abundance of mast cells may explain why mast cells in the neonatal lungs play a key role in allergen-induced NT4 over-expression and airway hyper innervation while they fail to do so in adult lungs following allergen exposure. This evidence collectively demonstrate a role of the mast cell in mediating NT4-induced ASM hyper innervation following early life insults. Together, both our study and previous studies highlight the impact of aberrant crosstalk between mast cells and
cholinergic nerves on airway reactivity under the pathological condition, although the mechanism underlying the crosstalk differs by age \(^{111,59}\).

Combining our findings from previous and current studies, mast cell degranulation and NT4 release serve as upstream events that ultimately trigger long-lasting changes in airway smooth muscle innervation and function following early life insults. These findings suggest that blockade of mast cell degranulation may be a preventative strategy for young children at high risk of asthma.
CHAPTER FOUR

Enhanced cholinergic signal in early-life leads to persistent airway hyper reactivity

4.1 Summary

Asthma is an inflammatory disease characterized by hyper reactive airway smooth muscle (ASM). Early-life respiratory insults often lead to persistent asthma in adulthood. Previously, our studies showed that early-life allergen exposure leads to NT4 mediated hyper innervation of the ASM, which is associated with persistent airway hyper reactivity (AHR) \(^6\). We now show that early-life allergen exposure leads to increases in NT4 mediated cholinergic innervation (Figure 3.6). Based on these findings, we hypothesize that enhanced cholinergic innervation in early-life leads to persistent AHR.

Cholinergic nerves release acetylcholine (Ach) that signals through M3 receptors present on the ASM leading to bronchoconstriction. We used a neonatal mouse model of repeated methacholine (Mch) exposure, an analog for Ach. Using this model, we show that methacholine challenge in early-life leads to persistent AHR in an inflammation-independent manner. In contrast, we found that methacholine exposure in adult life did not lead to persistent AHR. Notably, blockade of the M3 receptor using 4-DAMP in OVA-exposed pups prevented both acute and persistent AHR without affecting baseline airway function or inflammation. Furthermore, \textit{ex vivo} assays using lung slices from young and adult mice demonstrated enhanced cholinergic signaling in early-life but not in adults, leading to persistent AHR. Together, our findings show that enhanced cholinergic
signaling through M3 receptors plays a critical role in altering the ASM phenotype during postnatal growth. This study links early-life allergen exposure induced enhanced cholinergic innervation to persistent airway dysfunction. Our research findings suggest long-term M3R blockers as potential drugs for children with recurrent wheeze to alter asthma progression into adulthood.

4.2 Introduction

Asthma is a complex disease characterized by airway inflammation and reversible airway obstruction. It is known that pulmonary nerves alter airway tone. The ASM is innervated with sympathetic, parasympathetic and sensory neurons. We showed that early-life insults lead to an increase in innervation of the ASM causing persistent airway dysfunction (6). Previous data also indicated an increase in part, in cholinergic nerves upon-early-life allergen exposure (Figure 3.6 D). Also, the airways of asthmatic patients have increased responsiveness to cholinergic activity. However, the pathophysiological role of cholinergic nerves on the developing ASM remains unclear.

The cholinergic nerves signal through muscarinic receptors presents on the ASM. There are five muscarinic receptor subtypes (M1-M5), which belong to the family of seven transmembrane G-protein coupled receptors. M2 and M3 receptors have been associated with airway constriction in asthma models (119). The M3 receptors are found on the ASM while M2 receptors are found on parasympathetic nerves in the lung. The M2 receptor sequesters acetylcholine released by the nerve terminals, thus acting as a negative
feedback loop. The Gq-coupled M3 receptor stimulates the phospholipase C to convert phosphatidylinositol 4,5-biphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and sn-1, 2-diacylglycerol (DAG). IP3 triggers release of stored Ca\(^{+2}\) whereas DAG triggers protein kinase C (PKC). The increase in calcium levels activates the myosin light chain kinase. The myosin light chain kinase phosphorylates the myosin light chain leading to ASM contraction \(^{(120)}\). The ASM tone thus depends on agonist-induced increases in calcium levels in the cell. In addition to calcium-dependent pathways, calcium-independent pathways are also involved in ASM contraction referred to as calcium sensitization. The G\(_{12/13}\) M3 receptor can activate the Rho A/ Rho kinase pathway. The Rho Kinase activation leads to inhibition of myosin light chain phosphatase activity. Though Ca\(^{2+}\) dynamics in the ASM is studied extensively, the effect of enhanced parasympathetic tone on calcium dynamics in the neonatal ASM remains to be understood.

In mice, the parasympathetic neurons are known to play an important role in altering the ASM tone. The neuropeptides from the non-adrenergic non-cholinergic nerves are also associated with increased bronchoconstriction. Our study shows that an enhanced neural output in early-life leads to persistent AHR. However, how the early-life allergen regulates parasympathetic neuroplasticity in the neonatal ASM remains to be understood. Furthermore, it is essential to understand the regulation of the parasympathetic nerve pathways that control of the autonomic tone of neonatal ASM as compared to the adult ASM.
To understand the neural regulation of contraction and its effect on ASM physiology, we hypothesized that enhanced cholinergic signaling is sufficient to cause persistent AHR. In this study using a mouse model devoid of allergic inflammation, we investigated how methacholine exposure during early postnatal life affects airway function in adulthood. We demonstrate that an increase in acetylcholine/M$_3$R signaling functionally links early-life allergen exposure to persistent AHR by regulating ASM phenotype. We also show that the effect of enhanced cholinergic signaling is specific to the neonatal ASM as compared to the adult ASM. Our findings demonstrate a unique mechanism of early-life allergen-induced aberrant neuro-circuitry and its effect on the developing ASM. Furthermore, our data suggests there is a distinct difference in neonatal ASM phenotype as compared to the adult ASM in response to an enhanced cholinergic nerve output.

4.3 Methods

4.3.1 Western blot analysis. The lung samples from P21 mice were homogenized as described previously. Primary antibodies for VACHT (1:100, Abcam #AB68986) and GAPDH (1:10,000, Abcam #AB8245) were applied in blocking buffer (2.5% milk TBST). The secondary antibodies used were goat anti-rabbit HRP (1:1000, Santa Cruz Biotechnology #sc-2004) and goat anti-mouse HRP (1:5000, BD Biosciences #554002). The antigen-antibody complex was detected by SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Densitometry units for individual protein bands were measured using Image J and normalized to the GAPDH levels.
4.3.2 Airway slice contraction assay using lung slices. Mouse lungs were inflated by injecting 1ml of 2% warm (37° C) low-melting agarose-HBSS solution via a catheter followed by ½ cc of air. After complete solidification of agarose in the inflated lobes on ice, lung slices (250 μm thick) were cut perpendicularly to the airways with a vibratome (VF-300; Precision Instruments) at room temperature (RT) in HBSS. These slices were maintained overnight at 37° C and 5% CO2 in DMEM/F-12 supplemented with antibiotics. After overnight incubation, lung contraction studies were carried out. For this, the slices were removed to fresh HBSS and recovered for 20-30 minutes. The slices were secured in place within a standard 12 well culture plate using a ring of nylon mesh together with a metal washer. The slices were stimulated with increasing doses of methacholine (Sigma-Aldrich #A2251), Endothelin (Sigma Aldrich#E-7764). Mid-sized airways with a baseline luminal area between 14,000-20,000 μm² were assayed. Each airway was imaged every 30 seconds for a total duration of 2 minutes. From the acquired images, we quantified airway luminal area (Image J, NIH) and normalized to the pre-treatment baseline value. For ex vivo experiments, lung slices were treated with 100 μM of methacholine for 3 days in culture. The airway contraction measurements were performed on the treated slices on the fourth day after repeated washing with HBSS.

4.3.3 Airway resistance assay of airway. Measurement of airway resistance by FlexiVent was performed as described previously (113). Briefly, mice were anesthetized and the trachea was cannulated. The airway was ventilated via the cannula by the FlexiVent
ventilator. Baseline airway resistance was measured after airway delivery of nebulized vehicle, and similar measurements were performed at increasing concentrations of nebulized methacholine (5 mg/mL, 15 mg/mL, 25 mg/mL). After final measurements, the mouse was disconnected from the ventilator for organ and body fluid harvest.

4.4 Results

4.4.1 Early-life allergen exposure leads to increases in cholinergic innervation in the lung

Our previous studies indicated that early-life allergen exposure leads to NT4 mediated hyper innervation of the ASM leading to chronic airway dysfunction (6). We also showed that allergen exposure in early-life does not affect sensory innervation in the lung. To test whether early-life allergen exposure increased cholinergic innervation of the ASM, we analyzed lung lobes from allergen sensitized and challenged WT and NT4/− mice at P21 (Figure 4.1 A) for protein levels of vesicular acetylcholine transporter (VAChT). VAChT is responsible for transporting the neurotransmitter (acetylcholine) for parasympathetic nerves into secretory vesicles.

Western blot analysis showed a significant increase in the VAChT levels in the lungs of OVA-exposed WT mice as compared to the PBS controls. Furthermore, there was no change in the VAChT levels in the lungs of OVA exposed NT4/− mice at P21 (Figure 4.1 B). Similar increases in VAChT levels were seen in house dust mite allergen (HDM) immunized WT mice but not in NT4/− mice (Figure 4.1 D). To confirm that the increase
in NT4 mediated cholinergic innervation in the lung is specific to early-life allergen exposure and not adult age, we analyzed lung lobes for VACHT levels in PBS and OVA exposed adult mice (Figure 4.1 D). There were no significant changes in the VACHT levels between PBS and OVA exposed adult mice (Figure 4.1 E). These results indicate that the NT4 mediated hyper innervation of ASM is specific to early-life allergen exposure and is due to an increase in cholinergic nerves.

In addition, non-neuronal cells such as epithelial cells and immune cells express Ach and VACHT \(^{(12)}\). In our study, OVA exposure in both WT and NT4/− mice mount a similar immune response in the lung \(^{(6)}\). Furthermore, the array of the isolated epithelial cells does not show any increase in VACHT levels between PBS and OVA exposed WT mice. This indicates that only the neuronal cells contribute to the increase in VACHT levels in the OVA exposed WT mice. Thus, increases in VACHT levels correlate with increased innervation in OVA-exposed WT mice at P21.
Figure 4.4.1 Early-life allergen exposure leads to increases in cholinergic innervation in the lung at P21. (A) Schematic of the neonatal mouse model of asthma using OVA allergen. (B) Western blot analysis of cholinergic innervation of the lung at P21. Lung homogenates collected at P21 from PBS- and OVA-exposed wild type and NT4−/− mice were assayed for the levels of VaChT and GAPDH. Data were normalized to the GAPDH levels for each sample n=3. Each lane represents 1 mouse. (C) Schematic of the neonatal mouse model of asthma using HDM allergen. (D) Western blot analysis for VAChT in lungs of PBS and HDM exposed WT and NT4−/− mice at P21. Lung homogenates were assayed for the levels of VACHT and GAPDH. Each lane represents 1 mouse. Data were normalized GAPDH levels for each sample n=3. Each lane represents 1 mouse. (E) Schematic of the adult mouse model of asthma using OVA allergen (F) Western blot analysis for VACHT in lungs of PBS and OVA exposed WT adult mice. Lung homogenates were assayed for the levels of VACHT and GAPDH. Each lane represents 1 mouse. Data were normalized GAPDH levels for each sample n=3. P <0.01**, ***P<0.001.
4.4.2 M3 receptor signaling is required for early-life allergen-induced persistent AHR

We identified that early-life allergen exposure increases cholinergic innervation in the neonatal lung. We speculated that an enhanced cholinergic signal via M3 receptors might cause persistent AHR. To test this hypothesis, we blocked the cholinergic signal by intratracheal administration of M3 receptor specific blocker 4-DAMP (1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide) from P15 to P21 (Figure 4.2 A) (122). We performed lung slice contraction measurement to assess lung function at 8 weeks (117). The WT OVA mice that received the blocker showed diminished contraction at increasing doses of methacholine as compared to the WT OVA mice that did not receive the M3R blocker (Figure 4.2 B). Since endothelin-1 mediated contraction of ASM does not signal through the M3R, we investigated the response of these lung slices to increasing doses of endothelin-1. Similarly, we saw a significant reduction in airway contraction in WT OVA mice that received the M3R blocker drug as compared to the WT OVA mice without drug (Figure 4.2 C). These findings suggest that early-life allergen-induced enhanced cholinergic signaling through M3 receptors cause persistent AHR.
Figure 4.4.2 M3R Antagonist reduces early-life allergen-induced persistent AHR. (A) Experimental protocol of 4-DAMP (M3R antagonist) administration during OVA exposure. Approximately 100 μM M3R was administered intratracheally every day from P15 to P21. (B) (C) Measurement of airway contraction in response to increasing doses of methacholine and endothelin-1 respectively using precision cut lung slices from OVA-exposed WT mice with and without M3 receptor antagonist. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. Student t-test was used for statistical analysis. Statistically significant differences between OVA-treated mice that received M3R blocker and WT OVA mice were marked. *P<0.05. **P<0.01. ***P<0.001.
4.4.3 Aberrant NT4 mediated cholinergic signaling after early-life allergen exposure is sufficient to cause persistent AHR

To establish the causal relationship between NT4 mediated enhanced cholinergic signal in early-life and persistent AHR, we administered repeated methacholine aerosolized challenge from P15 to P21 in OVA-exposed NT4−/− mice (Figure 4.3 A). Lung slices from OVA-sensitized and challenged NT4−/− mice with or without methacholine exposure were analyzed for airway contraction. Airways from OVA-exposed NT4−/− mice that received methacholine challenges had a significant increase in their ASM contraction to increasing doses of methacholine as compared to the OVA-exposed NT4−/− mice that did not receive the methacholine challenge (Figure 4.3 B). The OVA-exposed WT mice were used as controls. Thus, repeated methacholine exposure between P15 to P21 in an OVA-treated NT4−/− mice was sufficient to rescue the AHR defects. In addition, we show that early-life allergen induces increases in cholinergic nerves in the lung (Figure 4.1 B, D). The blockade of the M3 receptor in allergen treated mice reduces persistent AHR. Thus, our findings suggest that early-life allergen-induced persistent AHR might be due to an increase in cholinergic nerve output.
Figure 4.4.3 Enhanced methacholine exposure in early-life leads to allergen-induced persistent AHR in NT4−/− mice. (A) Experimental protocol of methacholine administration during OVA exposure. Approximately 30mg/ml of Mch was administered every day from P15 to P21. (B) Measurement of airway contraction in response to increasing doses of methacholine using precision cut lung slices from OVA-exposed NT4−/− mice with and without Mch exposure. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. Student t-test was used for statistical analysis. Statistically significant differences between OVA-treated mice that did not receive Mch and WT OVA mice were marked. *P<0.05. **P<0.01. ***P<0.001.
4.4.4 Methacholine exposure in early-life, not in adults leads to persistent AHR independent of inflammation.

To dissociate the effect of early-life allergen-induced enhanced acetylcholine signaling from allergen-induced inflammation in persistent AHR, we used a mouse model of repeated methacholine exposure in neonatal mice from P15 to P21 without allergen sensitization or challenge (Figure 4.4 A)\(^{(123)}\). We then investigated the airway resistance of methacholine and PBS-exposed mice at 8 weeks. We found that repeated methacholine exposure in early-life led to increased AHR as compared to their PBS controls (Figure 4.4 B). Furthermore, we analyzed airway contraction in lung slices from the early-life methacholine and PBS exposed mice. The lung contraction studies from lung slices showed increases in airway contraction to increasing doses of methacholine in methacholine exposed mice as compared to their PBS controls (Figure 4.4 C). These results indicate that the hyper contractile ASM phenotype persists through adulthood. Our findings confirm that an enhanced cholinergic signal in early-life, without inflammation, is sufficient to cause persistent AHR.

To determine if persistent AHR was specific to cholinergic signaling, we administered substance P to neonatal WT mice from P15 to P21(Figure 4.4 D)\(^{(124)}\). Capsaicin pretreatment in adult allergic sheep and rabbits prevents airway hyper responsiveness\(^{(125),(126)}\). Substance P is a neuropeptide released by sensory afferent nerves and leads to ASM constriction. At 8 weeks, we investigated the airway resistance of substance P and
PBS exposed mice to increasing doses of methacholine. We found that repeated 
substance P exposure in early-life did not cause persistent AHR in adults (Figure 4.4 E). 
This data indicates the specificity of enhanced cholinergic signal in early-life and its role 
in causing persistent AHR.

To test if enhanced cholinergic signal in adult life leads to persistent AHR, 3 month-old 
mice were exposed to repeated methacholine challenges for 5 days (Figure 4.4 F). We 
then investigated airway contraction in adult methacholine-exposed mice to increasing 
doses of methacholine using lung slices, after 2.5 weeks recovery (adult ➔ 2.5-week 
recovery). There was no significant difference in the contraction of airways from 
methacholine and PBS- exposed adult mice after 3-week recovery to increasing doses of 
methacholine (Figure 4.4 G). These results indicated that enhanced cholinergic signaling 
to the adult lung does not lead to persistent AHR. This data indicates that the developing 
lung is more prone to long-term functional impairment by cholinergic signaling than the 
mature, adult lung.
Figure 4.4.4 Enhanced cholinergic signal in early-life, not adults, leads to persistent AHR in an inflammation-independent manner. (A) Experimental protocol of recurrent methacholine exposure in early-age. (B) Controls (n=5) and methacholine challenged pups (n=7) were allowed to grow for 5 weeks into adulthood. Airway resistance was measured using FlexiVent apparatus at 8 weeks of age. (C) Measurement of airway contraction in response to increasing doses of methacholine using precision cut lung slices from 8-week old mice with or without Mch exposure. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. Student t-test was used for statistical analysis. (D) Experimental protocol of recurrent Substance P exposure in early-age. (E) Controls (n=4) and Substance P challenged pups (n=4) were allowed to grow for 5 weeks into adulthood. Airway resistance was measured using FlexiVent apparatus at 8 weeks of age. (F) Experimental protocol of recurrent methacholine exposure in adult mice. (G) The adult mice were allowed to grow for 2.5 weeks after methacholine exposure. The mice were then sacrificed for measurement of airway contraction in response to increasing doses of methacholine using precision cut lung slices. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. Student t-test was used for statistical analysis. P<0.05*, P<0.01**, P<0.001***
4.4.5 *Ex vivo* Methacholine treatment in neonatal lung slices, not in adult lung slices leads to prolonged AHR.

In order to understand if the methacholine exposure in early-life led to changes in airway contraction, we treated lung slices from P21 and adult mice with methacholine for 2 days in culture. After one day of recovery in culture, on the third day, we analyzed airway contraction to increasing doses of methacholine. Only the neonatal slices treated with methacholine showed significantly increased AHR (Figure 4.5 A). Adult slices treated with methacholine showed no significant change in the airway contraction as compared to the untreated slices (Figure 4.5 B). These results confirmed that the neonatal, developing smooth muscle is vulnerable to change by an enhanced cholinergic signal. It further supports our finding that allergen exposure in early-life, not adulthood leads to persistent AHR through an enhanced cholinergic output (6). Furthermore, our data suggests that early-life allergen-induced enhanced cholinergic signaling via M3R lead to persistent AHR (Figure 4.2). Thus, we provide a novel neural mechanism, independent of inflammation capable of causing sustained decline in airway function.
Figure 4.4.5 *Ex vivo* Methacholine treatment in neonatal lung slices, not in adult lung slices leads to AHR. (A) The slices from mice at P21 were prepared and cultured with either 100 μM of methacholine for two days and cultured in DMEM/F-12 media overnight. On the third day, measurement of airway contraction in response to methacholine using precision cut lung slices were done. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. (B) Similarly, slices from an adult were prepared and cultured with either 100 μM of methacholine for two days and cultured in DMEM/F-12 media overnight. On the third day, measurement of airway contraction in response to methacholine using precision cut lung slices were done. The size of the airway lumen was normalized to the baseline before methacholine stimulation. Data represented mean±SEM from 30 airways of 3 mice for each condition. P<0.05*, P<0.01**, P<0.001***
4.5 Discussion

Previously, we demonstrated that early-life allergen exposure leads to NT4 mediated hyper innervation on the ASM causing persistent AHR\(^ {113}\). In this study, we show how early-life allergen exposure leads to a specific increase in NT4 mediated cholinergic innervation of ASM. Furthermore, by employing neonatal and adult mouse models of repeated methacholine exposures or pharmacologic blockade of M\(_3\)R, we found that increased cholinergic signaling in ASM after early-life allergen exposure is functionally linked to persistent alterations in ASM phenotype. In our model (Figure 4.6) early-life allergen exposure in WT mice leads to increases in cholinergic nerves around the ASM. This elevated cholinergic innervation alters the ASM phenotype causing persistent AHR (Figure 4.6). After early-life allergen exposure, blockade of M\(_3\)R cholinergic signaling prevents aberrant changes in ASM contractility (Figure 4.2) and thus prevents sustained AHR. In support, repeated methacholine exposure in OVA-exposed \(NT4^{−/−}\) mice leads to persistent AHR (Figure 4.3).
Figure 4.4.6 Model of how increased cholinergic innervation after early-life allergen exposure leads to persistent AHR. Early-life allergen exposure leads to NT4-activated TrkB signaling to increase ASM cholinergic innervation. Enhanced cholinergic signal (Acetylcholine) changes the ASM phenotype, ultimately resulting in persistent AHR.
Using \textit{ex vivo} lung slice assay, we show the neonatal ASM has intrinsically different properties than the adult ASM. Thus, our findings show that the developing ASM is more prone to alterations than the mature adult smooth muscle specifically in response to an enhanced cholinergic signal. The two major processes that determine the ASM contraction are the mechanisms involving calcium signaling and calcium sensitivity. The agonist-induced increases in calcium release from the sarcoplasmic reticulum lead to calcium oscillations in the ASM\textsuperscript{(120)}. The frequency of calcium oscillations correlates with ASM contraction. ASM contraction can occur through mechanisms independent of calcium signaling. The reduction in the activity of the myosin light chain phosphatase is associated with calcium sensitivity. The variability in the extent of contraction at a constant level of $[\text{Ca}^{2+}]_i$ is referred to as calcium sensitivity of the ASM. Thus, the hypercontractility in the neonatal ASM may be associated either with increased calcium signaling or increased calcium sensitivity to an enhanced cholinergic output. Further work needs to be done to understand the downstream signaling pathways that control $\text{Ca}^{2+}$ entry and release.

Furthermore, our study demonstrates that cholinergic signaling is not required for inflammation and suggests a physiological role of neural-dependent mechanisms in ASM contractility. This current study in the lung along with our previous study\textsuperscript{(6)} highlights the functional link between innervation and smooth muscle phenotypes. Our data suggests that deregulated release of neurotransmitters from innervating nerves due to early-life respiratory insults contribute to long-term changes in the ASM phenotype.
Altogether, these findings provide a novel mechanism of neuro-modulation during childhood asthma leading to a sustained decline in airway function into adulthood devoid of inflammation.

In summary, we identified an acetylcholine-M3R signaling mechanism that functionally links early-life allergen exposure, increased airway innervation, and persistent changes in ASM phenotype. This study further highlights a paradigm wherein insults to the developing lung can lead to irreversible changes in developing ASM phenotype thereby resulting in sustained airway dysfunction into adulthood. Thus, we propose that administration of long lasting anticholinergic drugs such as tiotropium in children with high risk of asthma to prevent asthma progression into adulthood.
CHAPTER FIVE

Unpublished Experiments

5.1 INTRODUCTION

This chapter will cover experiments in detail that have not been included in my publications for the following reasons:

- The experiments were designed to understand an important biological question but were beyond the scope of the paper although the results were informative.
- The experiments yielded negative results.
- The experiment designed yielded positive results but had non-specific targets.
- The experiments are a part of a new ongoing project.
5.2.1 Enhanced cholinergic signal in early-life is associated with airway remodeling.

Our study shows that early-life allergen exposure leads to increase in cholinergic innervation that is associated with persistent AHR. We hypothesize that increased airway constriction leads to matrix remodeling. It is shown that constriction of airways trigger release of Tgf-β by the epithelial cells which acts as a mitogenic signal for myofibroblasts \((127)\). The proliferation of myofibroblasts is associated with increases in ASM mass and collagen I deposition, collectively referred to as remodeling \((128)\). However, the exact role of cholinergic signaling in matrix remodeling following early-life allergen exposure remains to be understood.

Previously, we showed that early-life allergen exposure in WT mice leads to increased cholinergic innervation. In contrast, OVA exposure in \(NT4^{-/-}\) mice showed no increase in cholinergic nerves as compared to PBS controls. To determine if enhanced cholinergic innervation leads to remodeling changes in neonatal lung, we measured collagen I levels in PBS and OVA exposed lungs at P21. Western blot analysis showed that early-life OVA exposure increased collagen alpha I (COL-Iα) levels in WT mice as compared to PBS controls (Figure 5.2.1.A). In contrast, \(NT4^{-/-}\) mice showed no significant elevation in collagen I levels at P21 as compared to PBS controls following early-life allergen exposure (Figure 5.2.1.B). These data suggested that enhanced cholinergic signaling in early life is associated with increased collagen I deposition in neonatal lung. Future work
will focus on quantifying collagen I levels at eight weeks in the lungs of mice that were exposed to OVA in early-life.
Figure 5.2.1 Enhanced cholinergic signaling in early-life leads to increased collagen I deposition in the lungs at P21. (A) Western blot analysis of collagen I levels in the lungs of PBS and OVA exposed WT mice at P21. Each lane represents 1 mouse. GAPDH was loading control. n=2. Data were normalized to GAPDH levels for each sample. (B) Western blot analysis of collagen I level in the lungs of PBS and OVA exposed NT4-/- mice at P21. Each lane represents 1 mouse. GAPDH was loading control. n=2. Data were normalized to GAPDH levels for each sample.
5.2.2 Enhanced cholinergic signaling in early-life is associated with increased collagen I deposition at 8 weeks

Cholinergic signaling via M3R is associated with allergen–induced airway remodeling in mice (129). To test if blockade of M3R reduced collagen I deposition in neonatal lung, we administered intratracheal M3 receptor specific blocker 4-DAMP (1,1-Dimethyl-4-diphenylacetoxy-piperidinium iodide) from P15 to P21 (Figure 4.2 A) to OVA exposed WT mice (122). We analyzed lung samples for collagen I levels in OVA-exposed mice, with and without the drug at 8 weeks. We found that blocking M3R reduces collagen I deposition in OVA-exposed mice, at 8 weeks (Figure 5.2.2 A). These results confirm that enhanced cholinergic signaling in the neonatal lung is associated with increased deposition of collagen I. Thus, administration of long-acting anticholinergic drugs in children with severe asthma may prevent airway remodeling and subsequent decline in lung function.

Our data showed that early-life allergen exposure in NT4/− mice had no significant effect on cholinergic innervation as well as collagen I levels in the lung (Figure 5.2.1 B). To establish a causal relationship between NT4 mediated enhanced cholinergic signaling and airway remodeling, our future plan is to administer OVA exposed NT4/− mice with 30mg/ml of methacholine from P15 to P20. We plan to assess collagen I levels at 8 weeks in the lungs of OVA-exposed NT4/− mice with or without methacholine administration.
Figure 5.2.2 Enhanced cholinergic signaling in early-life is associated with increased collagen I deposition at 8 weeks. (A) Western blot analysis of collagen I level in the lungs of OVA exposed WT mice with and without the M3 receptor blocker at 8 weeks. Each lane represents 1 mouse. GAPDH was loading control. n=3. Data were normalized to GAPDH levels for each sample.
5.2.3 Methacholine exposure, in early life and not in adults leads to a persistent increase in collagen I levels.

Remodeling of airways is an important feature of asthma and includes extracellular matrix deposition of collagen I. Airway remodeling is known to occur as a result of excessive bronchoconstriction \(^{(123),(130)}\). Grainge and colleagues demonstrated that repeated methacholine challenges in patients with mild asthma were sufficient to induce airway remodeling without additional inflammation. They further showed that methacholine challenge increased collagen I deposition. We also demonstrated that repeated methacholine exposures in neonatal mice lead to increased AHR independent of inflammation (Figure 4.4). To confirm that an enhanced cholinergic signal is sufficient to increase collagen I deposition independent of inflammation, we analyzed lung samples at 8 weeks from mice exposed to methacholine nebulizations in early-age and as adults. Western blot analysis showed increased levels of collagen I in early-life methacholine treated mice as compared to PBS controls at 8 weeks (Figure 5.2.3 A). In contrast, adult mice treated with methacholine showed no increase in collagen I levels as compared to their PBS controls (Figure 5.2.3 B). These data indicated a distinct difference between the neonatal airway smooth muscle cells and the adult airways smooth muscle cells in response to enhanced cholinergic output.
Figure 5.2.3 Methacholine exposure in early-life, not adults, leads to persistent increases in collagen I deposition. (A) Western blot analysis of collagen I level at 8 weeks, in the lungs of early-life methacholine exposed mice and their PBS controls. Each lane represents 1 mouse. GAPDH was loading control. n=4. Data were normalized to GAPDH levels for each sample. (B) Western blot analysis of collagen I level at 8 weeks, in the lungs of adult mice that were exposed to methacholine. Each lane represents 1 mouse. GAPDH was loading control. n=4. Data were normalized to GAPDH levels for each sample.
To confirm that neonatal ASM and the adult ASM have an intrinsically different behavior to enhanced cholinergic signal, we performed *ex vivo* analysis of lung slices upon methacholine exposure from both ages. Lung slices provide a simple system to measure airway contraction and remodeling. We treated lung slices from P21 mice and adult mice with 100µM of methacholine for two days in culture. After overnight recovery, on the third day we analyzed the slices for collagen I levels using western blot. The results showed that methacholine treatment in lung slices from P21 mice showed increased levels of collagen I deposition as compared to their PBS controls (Figure 5.2.4 A). In contrast, lung slices from adult mice showed no significant increase in collagen I levels upon methacholine treatment (Figure 5.2.4 B). These results confirmed that the neonatal, developing ASM is vulnerable to change by an enhanced cholinergic signal as compared to the adult ASM.

Although the exact mechanism by which methacholine exposure leads to increased airway remodeling warrants further study, we plan to identify differentially expressed genes in ASM isolated from P21 and adults with and without methacholine exposure using *SMA GFP; NG2 dsRED* (112). Subsequent studies in our lab will focus on genes and pathways involved in increased airway constriction and remodeling in the neonatal ASM as compared to the adult ASM.
Figure 5.2.4 *Ex vivo* treatment in neonatal lung slices, not adults, with methacholine, leads to increases in collagen I levels. (A) Western blot analysis of collagen I level at 8 weeks, in the lungs of early-life methacholine exposed mice and their PBS controls. Each lane represents 1 mouse. GAPDH was loading control. n=2. Data were normalized to GAPDH levels for each sample. (B) Western blot analysis of collagen I level at 8 weeks, in the lungs of adult mice that were exposed to methacholine. Each lane represents 1 mouse. GAPDH was loading control. n=3. Data were normalized to GAPDH levels for each sample.
5.3 Cromolyn Sodium

We found that pulmonary mast cells are the major source of NT4 (Figure 3.2), which leads to hyper innervation of the airway smooth muscle. We also know that mast cells release NT4 upon degranulation (Figure 3.4). Cromolyn sodium (C.S) or nasalcrom is given to asthmatic patients and wheezing kids as an adjuvant therapy. C.S. is a mast cell stabilizing compound. We wanted to test the possibility if by blocking mast cell degranulation, can we prevent NT4 mediated hyper innervation in neonatal mice exposed to an allergen in early-life. To test this, neonatal mice that were subjected to OVA sensitization and challenge received the mast cell-stabilizing compound cromolyn sodium via intra-tracheal administration between P15 and P20 (Figure. 5.3 A). The efficacy of cromolyn sodium treatment to block mast cell degranulation was validated by reduced numbers of degranulated mast cells in lungs of OVA-exposed mice that received cromolyn sodium as compared to mice that received PBS at P21 (Figure 5.3 B). Cromolyn sodium treatment had no effect on the immune response induced by OVA exposure. Compared to mice that were treated with PBS, mice treated with cromolyn sodium exhibited similar allergic responses to OVA, shown by infiltration of eosinophils and neutrophils measured in BAL, increases in the levels of OVA-specific IgE and the Th2 cytokine IL-13 (Figure 5.3 C, D, E). However, TuJ1 staining of lung sections from PBS- and OVA–exposed WT mice with or without C.S. treatment showed that treatment with cromolyn sodium prevented OVA-induced changes in ASM innervation without affecting the baseline levels of innervation (Figure 5.3 G). These findings indicated that mast cell degranulation is crucial for early-life allergen-induced neuroplasticity.
Furthermore, although cromolyn sodium had no effect on the basal airway reactivity, mice treated with cromolyn sodium exhibited reduced OVA-induced airway hyperreactivity compared to PBS-treated mice (Figure 5.3 F). These findings were consistent with our previous results that increased ASM innervation after early-life allergen exposure is required for airway hyper reactivity\(^{(113)}\).

These outcomes suggest that blockade of mast cell degranulation in children at high risks of asthma may be a preventative strategy for young children at high risk of asthma but would have little beneficial effects in children with established asthma. This may provide some explanation for the conflicting outcomes of clinical studies using cromolyn in children\(^{(131),(132)}\). However, it is difficult to pinpoint the effect of cromolyn on mast cell release of NT4 in the treated mice due to a possible non-mast cell related activities of cromolyn \textit{in vivo} and conflicting findings of the effectiveness of cromolyn sodium as a mast cell stabilizer in mouse\(^{(133),(134)}\).
Figure 5.3 Cromolyn sodium blocks OVA-induced increases in ASM innervation at P21 without affecting inflammation. (A) Experimental protocol for cromolyn sodium administration in the neonatal mouse model of OVA. Controls received saline (PBS) I.T. B) Representative images of toluidine blue staining of mast cells in ASM of OVA-exposed mice that received saline or cromolyn sodium (C.S.). Arrows indicate mast cells. n=3. Scale bar, 10 μm. C) Serum levels of OVA-specific IgE in PBS- and OVA-exposed pups that received saline or Cromolyn sodium at P21, measured by ELISA. Each marker represents a sample. Black horizontal line indicates mean of the OVA-challenged group. D) Serum levels of IL-13 in PBS- and OVA-exposed WT mice with and without C.S. at P21 measured by ELISA. n=9. (E) Differential BAL counts of PBS- and OVA-exposed WT with and without cromolyn sodium mice at P21. The numbers of eosinophils (Eos), lymphocytes (Lymph), neutrophils (Neut), and macrophages (Mac) are shown. n=9. (F) Airway reactivity of control (n =6) and OVA-exposed (n =6) WT pups with and without Cromolyn sodium was assessed at P21 with the FlexiVent apparatus to increasing concentrations of methacholine. (G) Quantification of the ASM innervation density in control and OVA-exposed WT mice with and without C.S treatment at P21. Data represent the average and SEM from 4 airways of each mouse and 10-12 mice for each condition. P<0.05; ** P<0.01; ***P<0.001.
5.4 Mucin regulation by Mast cells

A central event after early-life allergen exposure is NT4 mediated hyper innervation of the airways. Juliana Barrios, a Ph.D. candidate in our laboratory, identified potential role of NT4 in innervation of the pulmonary neuroendocrine cell (PNECs). The PNECs are specialized airway epithelial cells that occur in clusters. They release several neuropeptides such as CGRP, bombesin, etc. Her studies show that aberrant PNEC innervation is associated with mucous metaplasia. Her thesis focuses on understanding how NT4 innervates the neuroendocrine bodies, which in turn release Gamma-Aminobutyric acid (GABA) that activates epithelial cells to cause mucus metaplasia.

My work identified mast cells as a key source of NT4. We have shown that pulmonary mast cells release NT4 upon degranulation, which leads to hyper innervation of the ASM. We also found that KitW-sh/W-sh mice are resistant to early-life allergen-induced innervation changes in the ASM. They also show reduced mucus metaplasia and AHR. We speculated that because mast cells are a key functional source of NT4, mast cell deficiency may lead to defects in the NEB innervation. We hypothesized that wild-type mast cell engraftment in KitW-sh/W-sh mice can recover NEB innervation, GABA hyper secretion, and rescue mucous metaplasia defects after early-life allergen exposure. To prove this hypothesis, we reconstituted OVA-challenged KitW-sh/W-sh mice with WT primary mast cells and NT4−/− primary mast cells at P15. We harvested the lungs at P21 and checked for reconstitution efficiency and the number of mast cells in reconstituted KitW-sh/W-sh mice was similar to that in OVA-exposed WT mice (Figure 3.4.6). Then we
measured *muc5ac* gene expression levels in the lungs of the reconstituted mice. Only the WT pulmonary mast cells significantly rescued the mucin defects in the OVA-treated *Kit*<sup>W-sh/W-sh</sup> mice (Figure 5.4 C, D). This was further confirmed using PAS stain on lung tissue sections from OVA-exposed *Kit*<sup>W-sh/W-sh</sup> mice that received WT and *NT4<sup>+/−</sup>* primary mast cells. These results indicate that the NT4 from mast cells might be responsible for innervating the NEBs. Although, further work needs to be done in order to characterize NEB innervation density in the PBS- and OVA- exposed *Kit*<sup>W-sh/W-sh</sup> mice and also between OVA-exposed *Kit*<sup>W-sh/W-sh</sup> reconstituted with WT and *NT4<sup>+/−</sup>* primary mast cells.
Figure 5.4 Mast cells are required for increases in NT4 levels, airway innervation and mucous metaplasia after OVA exposure at P21. (A) qRT-PCR analysis of the NT4 mRNA levels (B) and Western blot densitometry analysis of neurofilament (NF) in the lungs of wild-type and Kit\textsuperscript{W-sh/W-sh} mice with and without OVA exposure n=4, *p<0.05. (C) PAS staining of lung sections from wildtype and Kit\textsuperscript{W-sh/W-sh} mice. Scale bar, 50 μm. (D) qRT-PCR analysis of Muc5ac in the lungs of Kit\textsuperscript{W-sh/W-sh} mice reconstituted with WT and NT4\textsuperscript{-/-} P.P.M.C
CHAPTER SIX

Discussion

6.1 Overview

Asthma is associated with early-life insults and often persists into adulthood. Several studies indicate that allergen exposure in early-life is associated with impaired lung innervation \(^{(6),(37),(3)}\). Using a neonatal mouse model of asthma, our previous work showed that early-life environmental insults cause NT4 mediated innervation defects in the developing lung leading to persistent AHR. Given the central role of early-life allergen mediated innervation changes associated with hyper contractile ASM phenotype, I set out to determine how NT4 levels in the neonatal lung were increased upon allergen exposure. My work shows a novel role of mast cells in mediating innervation changes by releasing NT4 (Chapter 3).

Herein, I show that mast cells and ASM selectively express NT4 in mice, non-human primates as well as adult human lungs. My study highlights a unique aspect of mast cell number in the neonatal lung. I show that the relative abundance of mast cells in the neonatal lung is 20-30 fold higher than that in the adult lung. Upon repeated allergen exposure, there is mast cell expansion and degranulation in the lung, which lead to the release of NT4 causing hyper innervation of the ASM. Together, these findings suggest that mast cells are a key source of aberrant NT4 expression and cause hyper innervation of the ASM upon
early-life allergen exposure. This role of mast cells may be an evolutionarily conserved mechanism to bring about aberrant neuroplasticity upon allergen exposure.

In the second part, I showed that early-life allergen exposure at least in part increases cholinergic innervation in the lung. My data suggests that enhanced cholinergic activity during the developing lung stage can lead to persistent AHR (Chapter 4). Using repeated methacholine exposure in a neonatal mouse model, I showed that an enhanced cholinergic input in early-life is associated with a sustained increase in airway contraction to agonists in an inflammation-independent manner. Strikingly, repeated methacholine exposure in adult mice does not cause long term AHR. Together these studies propose a novel model, in which deregulation of neural circuits triggered by allergen exposure to the immature lung cause persistent AHR. A complete understanding of how deregulated neural circuits affect the developing lung ASM and the matrix is required to provide specific drug targets to prevent asthma progression in children. In this chapter, I am going to discuss our future plans for understanding how the enhanced neural regulation in the neonatal lung causes persistent changes in the ASM rendering muscle hyper contractile.

6.2 Effect of enhanced cholinergic signal on neonatal ASM

Previous studies in our laboratory show that increased innervation in early-life is associated with long-term airway dysfunction \(^6\). My work showed that early-life allergen exposure specifically increases parasympathetic cholinergic nerves in the lung. Several studies show that an increased parasympathetic tone is associated with COPD and asthma
Although increased cholinergic nerves might not translate into an enhanced signal, we show that recurrent methacholine exposure, an analog of acetylcholine in the postnatal developing lung leads to persistent AHR in an inflammation-independent manner (Figure 4.3). In contrast, recurrent methacholine exposure in adults does not cause persistent AHR (Figure 4.4). Furthermore, using a specific M3 blocker, 4-DAMP in the neonates, we blocked development of persistent AHR (Figure 4.2). Our studies bring out a novel role of enhanced cholinergic tone mediated increased ASM contraction in young mice. Clinical studies in humans with asthma have shown a positive effect of long-term tiotropium bromide in asthma\(^{(138)}\). Together, these findings suggest an essential role of cholinergic nerves in asthma. Thus, it becomes essential to understand the genes and pathways involved in altering developing ASM upon enhanced cholinergic signal. Furthermore, we want to understand what is the difference between the neonatal ASM and adult ASM?

### 6.3 Effect of enhanced cholinergic signal on matrix remodeling in neonatal lung

In addition to the intrinsic ASM changes, enhanced cholinergic signaling through M3 receptors on the fibroblasts can lead to changes in collagen deposition and thus affect matrix remodeling. Studies using tiotropium bromide showed reductions in airway remodeling in mouse models of asthma\(^{(139),(140),(141)}\). Our next aim is to understand how the increased ASM tone in neonatal life leads to remodeling changes. We speculate that increased airway constriction can lead to remodeling changes in neonatal lung structure. In support, Grainge and colleagues demonstrated that enhanced airway constriction...
without any additional inflammation can cause airway remodeling in atopic patients. In support of this reference, we found that enhanced cholinergic signaling during the neonatal age increases collagen IA levels in the lung (Figure 5.5.1). In contrast, there is no change in collagen IA levels in adult mice exposed to methacholine (Figure 5.5.3). The increase in collagen IA is associated with increased ASM contraction. These findings suggest that contraction mediated remodeling changes might occur due to an enhanced cholinergic output in early-life. To do this, we plan to first target collagen I integrin binding by using a competitive peptide in the ASM. Collagen-1 binds to integrin in order to mediate bronchoconstriction. We will use the peptide on methacholine treated lung slices and check for airway contraction after 3 days. The findings from these experiments will allow us to understand the role of collagen I in persistent AHR.

### 6.4 ASM array on contraction mediated remodeling

Our studies show a distinct difference in the response to cholinergic innervation by the developing ASM and the adult ASM. Furthermore, our previous study showed that early-life allergen exposure altered ASM phenotype (6). In order to understand the genes and pathways involved in the altering of ASM phenotype due to enhanced cholinergic output, we plan to perform an ASM array using the SMA-GFP; NG2-dsRed. The array will be performed on ASM cells isolated from neonatal mice at P21 and adult mice treated with methacholine (30 mg/ml) for 3 days ex vivo. These results will be further validated in the in vivo model of recurrent methacholine exposure in neonatal mice as well as adult mice. We will be specifically targeting pathways involved in increased collagen I a deposition.
These findings will be important in understanding contraction mediated remodeling changes that are clearly age-dependent. We then plan to use siRNA for specific genes and silence them using lung slices and measure contraction differences upon exposure to methacholine. This will help delineate the signaling mechanism by which increased cholinergic output leads to increased contraction and remodeling in neonatal age.

6.5 Summary

Asthma is on the increase worldwide. According to the CDC, around 9.3% of children in the USA suffer from asthma. Childhood asthma is a leading cause of hospitalization and school absence. Since asthma has an early-onset it becomes increasingly important to understand the mechanisms involved in the pathophysiology of childhood asthma which lead to its persistence into adulthood. The treatment options for children presenting with recurrent wheeze are limited and only provide symptom relief but do not prevent asthma progression. Children suffering from severe asthma are usually given corticosteroids as well as nebulizers using SABA (short acting beta agonists) almost daily (142). These drugs make the children immunosuppressed and many develop resistance to the beta agonists. Hence, there is a need for therapeutic targets specific to altering asthma progression in children.

Our studies provide an important time window for therapeutic intervention. We show that mast cells, upon allergen exposure release neurotrophin 4 causing hyper innervation of the ASM. Combining our findings from previous and current studies, mast cell
degranulation and NT4 release serve as upstream events that ultimately trigger long-lasting changes in ASM innervation and function following early-life insults. These findings suggest that blockade of mast cell degranulation may be a preventative strategy for young children (1-4 years of age) with recurrent wheeze but would have little beneficial effects in children with established asthma. This may provide some explanation for the conflicting outcomes of clinical studies using cromolyn in children. Furthermore, our studies show a novel age-dependent role of enhanced cholinergic output, in ASM alteration. Our data indicate that usage of long lasting anticholinergic drugs for children with a persistent wheeze to prevent asthma outcome. Together, my work shows a unique mechanism of crosstalk between mast cells, deregulated cholinergic nerves and ASM in early-life. Although asthma research has been carried out for numerous decades, several new pathways are now being discovered. My study demonstrates a unique role of mast cells in establishing enhanced cholinergic nerves in childhood asthma. Our findings provide potential drug targets that will improve the quality of life and asthma outcome in children.


CURRICULUM VITAE

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Year of Birth: 1987

EDUCATION

2011-Present  Ph.D. Candidate Molecular and Translational Medicine
Boston University School of Medicine
Advisor: Dr. Xingbin Ai
Thesis Title: “Role of pulmonary mast cells in Neurotrophin 4 mediated cholinergic neuroplasticity in neonatal asthma.”

2008-2010  Master of Science in Biotechnology
University of Pennsylvania, Philadelphia, PA

2002-2006  Bachelor of Science in Biotechnology
University of Mumbai, India

RESEARCH EXPERIENCE

2011-Present  Graduate Research, Boston University School of Medicine, MA/ Brigham and Women’s Hospital, MA
Advisor – Xingbin Ai (Advisor relocated to Brigham and Women’s Hospital since July 2014)
- Identified the role of mast cells as a key source of Neurotrophin 4 in a neonatal allergic asthma model. (1st author paper, published in Mucosal Immunology)
- Characterized the type of innervation in the airway smooth muscle after early-life allergen exposure and its effect on persistent asthma and remodeling. (manuscript in preparation)
- Incorporated precision cut lung slice contraction (PCLS) technique to build a system to test an array of neurotransmitters and identify the unique role of acetylcholine. (Co-author publication)
- Worked towards Identifying novel methods for studying smooth muscle cell contraction using individual isolated smooth muscle cells. (Co-author publication)
- Interacted with clinicians from the Pulmonary and Critical Care Medicine division at BWH and Harvard Medical School to understand the disease progression of asthma and provide better translational research tools.
- Collaborated on several projects with faculty from Boston University school of Medicine, Brigham and Women's (BWH) and Beth Israel Deaconess Medical Center Harvard Medical School.
- Mouse colony breeding and management.
- Involved in various volunteering and student activities as a member of graduate medical science student organization (GMSSO).
- Training of Ph.D. students, fellows, and interns in various techniques.

2009-2011  Research Assistant, Department of Pharmacology  IFEM, University of Pennsylvania
Advisor – Vladimir Muzykantov
The project focused on understanding the anti-thrombotic and anti-inflammatory properties on thrombomodulin in acute lung injury.

- Mammalian cell culture using S2 cells & RAW cells.
- Recombinant protein production followed by purification of protein using affinity chromatography
- *In vivo* and *in vitro* assays using the recombinant protein mainly ELISA

2008 - 2009
Lab Assistant, Hematology/Oncology Lab, University of Pennsylvania
Worked on various molecular biology techniques including PCR, PAGE, AGE, GST purification, cloning.

Aug 2009
Summer Intern, Gene Therapy and Gene Regulation, Wistar Institute, Philadelphia
Worked on determining the CTCF binding motifs in HSV genome. This involved using mammalian cell culture using Hela Vero cells, CHIP, EMSA and Western Blotting etc.

Aug 2008
Intern, Advanced Centre for Treatment, Research and Education in Cancer, India
- Understanding the role of chromatin involving the H2A.Z variant of histones during Hepatocellular carcinoma.
- Training was obtained in molecular biology techniques involving AUT-PAGE and Western blot.

March 2008
Intern, Reliable Analytical Laboratories, India
- Training in various instruments used commonly in an analytical laboratory.
- Gained hands-on experience gained in calibrating instruments such as Nephelometer, Ph. meter, Spectrophotometer, High Performance Liquid Chromatography and Gas Chromatography.

Dec 2005
Winter Intern, Mitcon Biotechnology Centre, Pune, India
- Plant culturing in-vitro was done including primary and secondary hardening of the plant.
- Algal, fungal and bacterial biofertilizers were prepared by inoculating and sub-culturing pure strains of the same.
- The formulation was done using lignite and talc powder and the biofertilizer and biopesticide thus prepared was quality tested for various parameters like ph., moisture content, and salmonella-shigella contamination.

June 2005
Summer Intern, BOKU University, Vienna Austria
- Genetic profiling of maize germplasm: Performed molecular biology techniques like polymerase chain reaction, DNA sequencing (by a Dideoxy method using ABI3100 capillary electrophoresis system), DNA extraction, data analysis, Agarose gel electrophoresis.
- Root development of Arabidopsis thaliana: Performed Histochemical techniques like GUS activity staining, real-time PCR, segregation analysis of transgenic plants (transgenic lines of ARABIDOPSIS thaliana T2 and T3 generation selection), confocal microscopy.

**TEACHING EXPERIENCE**
Fall 2010  
**University of Pennsylvania, PA**
Lab assistant for an undergraduate molecular biology lab course *BIOL 121/3* (Molecular biology Laboratory course)

Nov 2010  
**University of Pennsylvania, PA**
Tutored Undergraduate level organic chemistry to students.

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**PUBLICATIONS**


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**POSTERS AND PRESENTATIONS**


2. Presented a talk on “Mast cells are a source of NT4 for allergen-induced neuroplasticity in neonatal allergic asthma” at *Gordon Conference Neurotrophic factors- May 2013*. (Selected for oral presentation)
3. **Patel KR**, Aven Linh, Paez-Cortez J, et al. Mast cells are a source of NT4 in allergen-induced neuroplasticity in neonatal allergic asthma. *Russek day at Boston University School of Medicine, 2012-2013 (Poster presentation)*


**TECHNIQUES:**

- **Immunological techniques:**
  Flow cytometry, Intracellular cytokine (molecule) staining, ELISA.

- **Molecular biology techniques:**
  Cloning, liposome mediated transfection, RT-qPCR

- **Biochemical techniques:**
  Western blot, EMSA, protein purification using affinity chromatography

- **Animal handling:**
  Maintenance of different transgenic mice lines, breeding, dosing and immunization and familiar with different mouse models of allergic inflammation

- **Cell culture techniques:**
  Culturing primary mast cells, mammalian cell culture and proficient in handling different cell lines.

- **Microscopy:**
  Light and Confocal microscopy

- **Histology techniques:**
  Immunocytochemistry, HE staining, immunofluorescence, cryosection, paraffin embedding.

- **Lung slice culture:**
  Have proficiency in precision cut lung slicing after agarose infusion of the lung to determine the extent of airway contraction in response to different agonists.

**COMMUNITY SERVICE/VOLUNTEERING**

- Participated in Graduate Student Organization (GMSSO) activities
- Active participation in blood drive /Operation gratitude)
- Volunteer at soup Kitchen at Rosie’s and Haley house