The role of the TLR4-TRIF axis in allergic airway disease

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December 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Dedication

To God, for blessing me with the faculties, opportunities, drive and insight to carry out this work. May there be some value in it.

To my parents, Riitta Peltonen-Shalaby & Hamdy Shalaby, for being the most ardent, constant and loving supporters of me and my education.

To my high school biology teacher, Mr. John Switzer, for his inspiration and support of my natural inclination towards biology.

To my thesis supervisor, Dr. James G. Martin, for having faith in me and for his tireless mentorship, extraordinary selflessness and generous patience and positivity.

And to my loving wife, Suad Bushnaq, for her kindness and forbearance.

Acknowledgements

This thesis would not have been possible were it not for the contribution and support of countless individuals. I am particularly grateful to my supervisor, Dr. James Martin, for providing me the opportunity to work in his lab and to learn from him. Also, for maintaining faith in my capabilities, which is perhaps the greatest favour that I have received throughout this experience because it has helped me to have faith in myself and in the path that I am on. Dr. Martin's remarkable altruism and value of mentorship is evident, among other examples, in the numerous projects which he enabled me to be a part of or to pursue, whether directly related to my thesis or not, as well as the travels to scientific meetings. For example, the work presented in Chapter 2 was, in part, inspired by my trip in 2010 to the annual meeting of the American Academy of Allergy, Asthma and Immunology where I was exposed to the topic of allergen-associated danger signals. I truly believe that you gave me every possible opportunity to advance my intellect and career and finally, granted me the freedom and the time to realize my work as close as possible to what I had aspired to achieve. This is something that I am truly satisfied with and grateful for.

I would also like to recognize and give thanks to the experiences and learning opportunities with my colleagues. The Japanese fellows, Drs. Meiyo Tamaoka, Kimitake Tsuchiya, Nobuaki Hirota and particularly Taisuke Jo, always demonstrated a conscientiousness, composure and fertility in intellect and work ethic that one can only aspire to emulate. Working closely with Dr. Taisuke Jo, I also gained immensely in learning how to approach scientific problems and experimental design in a systematic manner. Drs. Paul Andre-Risse and Mauro Novali also taught me the importance of doing your best but also working with peace of mind. Dr. Muhannad Hassan as well as many other fellows and colleagues, including Emily Nakada, Alexandra Allard-Coutu, Mike O'Sullivan, Drs. David Prefontaine and Sana Siddiqui, Saba Al-Heialy and Toby McGovern were also a pleasure to work with.

I thank Dr. Clément Rioux and GlaxoSmithKline Biologicals North America (formerly I.D. Biomedical) for maintaining their support over the years by providing me with the mucosal adjuvant, Protollin, which was crucial to the majority of the research presented in this dissertation and permitting me to publish these results. Also, Dr. Salman Qureshi from the McGill University Health Centre Research Institute (MUHC RI) was instrumental in his collaboration and critical review of our work. Dr. Karim Maghni generously collaborated in a few projects. I thank Scireq Inc. and its staff, particularly Drs. Thomas Schuessler and Annette Robichaud, with whom I had the pleasure of working. Thank you for the technical and theoretical knowledge I was able to gain. I also benefited immensely from the technical training I received from Mr. Hossein Koohsari (GlaxoSmithKline Biologicals) and Dr. Marianne Raymond (Université de Montréal).

I am grateful to Dr. Martin's research associate, Barbara Tolloczko, for her reassuring demeanor, as well as technicians, Jamilah Saeed and Rosa Pantano, and administrative assistants, Maria Makroyanni, Angie Bentivegna and Stavroula Kalantzis for their regular help and camaraderie. Also, Mr. Normand Lavoie and Ms. Christina St-Louis were very helpful throughout the years.

I extend a gracious thank you to the research directors at the Meakins-Christie labs, in particular Drs. Qutayba Hamid and Becky Fixman, whom I know supported me immensely, beyond the regular valuable advice that I would receive from them. The Meakins-Christie labs offered an environment in which I felt at home and was glad to be a part of, thanks to the efforts of Dr. Martin, the research directors and other staff, fellows and students.

I thank the members of my supervisory committee; besides Drs. Martin and Rioux, Drs. Anne-Marie Lauzon, Christine McCusker and John Hanrahan provided constructive criticism and feedback relating to this work. I also thank Dr. Christian Giordano and Dr. Lauzon for their kind assistance with the translation of the abstract for this thesis. McGill University's international student differential fee waiver program provided great support, as did the Department of Physiology, in the form of resources, travel grants and awards; I thank the chairs

and program directors, Drs. John Orlowski, Cathleen Cullen and John White, as well as the Graduate Coordinator Christine Pamplin. Finally, this work would not have been possible without the funding agencies that provided me with financial backing throughout most of my PhD candidacy; the Canadian Lung Association (Canadian Thoracic Society), the Québec Health Respiratory Training Program of the Association des Pneumologues Pédiatriques du Québec, the McGill University Health Centre Research Institute and the Montréal Chest Institute. The Canadian Institutes of Health Research also provided my supervisor's grants which facilitated this work.

Contributions of Authors

This document has been prepared as a manuscript-based thesis, consisting of text from manuscripts that have either been published in peer-reviewed journals, submitted and are currently under review, or are to be submitted for publication.

CHAPTER 2. Inhaled birch pollen extract induces airway hyperresponsiveness via oxidative stress but independently of pollen-intrinsic NADPH oxidase activity, or the Toll-like receptor 4-TRIF pathway

Alexandra Allard-Coutu contributed to this project as an undergraduate summer student in Dr. Martin's lab under my supervision and participated in several aspects of experimentation, including measurement of lung function in mice and quantification of pulmonary inflammation, as well as data analysis.

Michael O'Sullivan (M.Sc. candidate) contributed to experimental work involving the measurement of NADPH oxidase activity in birch pollen extracts.

Emily Nakada (PhD candidate) contributed to the measurement of inflammatory mediators in tissue fluids.

Dr. Salman T. Qureshi (Investigator, McGill University) provided transgenic mice for this project and a constructive critical review of our manuscript.

Dr. Brian J. Day (Investigator, National Jewish Health, U.S.A.) provided reagents, equipment and technical support in his lab for the measurement of a lipid peroxidation product in lung tissues and critically reviewed our manuscript.

Dr. James G. Martin (Principal Investigator) contributed to the design of the study, data review and interpretation and writing of the manuscript.

CHAPTER 3. ICOS-expressing CD4 T cells induced via TLR4 in the nasal mucosa are capable of inhibiting experimental allergic asthma

Dr. Taisuke Jo (post-doctoral fellow, PDF) contributed to the optimization of protocols for quantification of cell surface markers by flow cytometry, as well as providing some assistance with tissue harvesting and processing.

Emily Nakada (PhD candidate) contributed to aspects of tissue processing and staining of cells for flow cytometric analysis.

Alexandra Allard-Coutu (undergraduate summer student) contributed to the quantification of airway inflammation and measurement of serum allergen-specific immunoglobulin E in some experiments.

Dr. Kimitake Tsuchiya (PDF) contributed to aspects of tissue processing, cell sorting and RT-PCR assays.

Dr. Nobuaki Hirota (PDF) contributed to some gene expression assays.

Dr. Salman T. Qureshi (Investigator, McGill University) provided transgenic mice for this project and a constructive critical review of our manuscript.

Dr. Karim Maghni (Investigator, Université de Montréal) provided reagents, equipment and technical support in his lab for measurement of inflammatory mediators in tissue fluids.

Dr. Clément R. Rioux (Senior Scientist level III, GlaxoSmithKline Biologicals North America) provided the principal compound employed in the study, as well as other reagents, equipment (for the measurement of allergen-specific IgE) and technical resources which contributed to the study design. He also contributed intellectually to the study in the early stages and critically reviewed our manuscript.

Dr. James G. Martin (Principal Investigator) contributed to the study design, data review and interpretation and writing of the manuscript.

CHAPTER 4. The TLR4-TRIF pathway protects against the development of experimental allergic asthma

Saba Al-Heialy (PhD candidate) contributed to aspects of tissue processing and cell sorting.

Dr. Kimitake Tsuchiya (PDF) contributed to aspects of tissue processing and cell sorting.

Dr. Salman T. Qureshi (Investigator, McGill University) provided transgenic mice for this project.

Dr. James G. Martin (Principal Investigator) contributed to the study design, data review and interpretation and writing of the manuscript.

APPENDIX. Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness

Leslie G. Gold contributed to methodological and technical aspects of the study.

Dr. Thomas F. Schuessler contributed to the conception and design of the study, as well as data interpretation and writing of the manuscript.

Dr. James G. Martin contributed to the design of the study, data review and interpretation, as well as writing of the manuscript.

Dr. Annette Robichaud contributed to the design, experimentation, data analysis and interpretation, as well as writing of the manuscript.

Table of Contents

Abstract

Epidemiological studies, genetic analyses, as well as clinical and experimental data indicate a potential for the innate immune pattern-recognition receptor, the toll-like receptor 4, and its bacterial ligand, lipopolysaccharide, to initiate, exacerbate, or conversely prevent or treat allergic airway disease, depending on conditions and mechanisms that are still being elucidated. Animal studies suggest that many of these pro- or anti-inflammatory events are driven by activation of a signaling pathway downstream of TLR4 which relies upon the intracellular adaptor protein 'myeloid differentiating factor 88' (MyD88), whereas the other major TLR4-activated signaling pathway controlled by the adaptor 'Toll, IL-1 receptor and Resistance protein (TIR) domain-containing adaptor inducing interferon-β' (TRIF), has not been explored in the context of allergic asthma. Here, we investigated the role of TLR4 and TRIF activation in different murine models of experimental allergic asthma. In Chapter 2, we studied its role in the context of exposure via the airways to an extract of a natural aeroallergen, the birch tree pollen, hypothesizing that this pathway might function to prime allergic sensitization and/or to facilitate the amplification of the allergic response. In Chapters 3 and 4, we examined the role of TLR4 and TRIF activation in the context of exposure to a mucosal adjuvant consisting of TLR4 and TLR2 bacterial ligands, intended for potential use in vaccination or allergen-specific immunotherapy, in order to determine whether this pathway was relevant to the inhibition of allergic airway disease development. In this latter context, we also examined cellular mechanisms that may be relevant to the prevention of allergic airway disease, namely the immunomodulatory effect of this adjuvant on the T cell response. We find that in both contexts, the TLR4-TRIF-dependent signaling pathway inhibits aspects of allergic airway disease and thus appears to have protective and potentially immunotherapeutic effects against the development of allergic asthma. We also determined that TLR4 activation and, to a significant degree, TRIF-dependent signaling, can influence the $CD4^+$ T cell response by enhancing the expression of a T cell-expressed co-stimulatory molecule, the

inducible co-stimulatory molecule (ICOS), and expanding $CD4^{+}ICOS^{+}$ cells which may contribute to mediating the TLR4-TRIF-dependent inhibition of allergic airway disease development. Thus, we identify TLR4-TRIF-dependent signaling as a potentially important pathway in allergic asthma and advance current knowledge regarding the adjuvant effect of TLR4 ligands upon T cells.

Résumé

Les études épidémiologiques, les analyses génétiques ainsi que les données cliniques et expérimentales indiquent un rôle potentiel pour le récepteur toll-like-4 (TLR4) et son ligand de source bactérienne, le lipopolysaccharide, dans l'initiation, l'exacerbation ou au contraire dans l'inhibition ou le traitement de maladies allergiques respiratoires dépendamment de conditions et de mécanismes qui demeurent encore à être élucidés. Des études réalisées chez l'animal suggèrent que plusieurs de ces événements pro- ou anti-inflammatoires sont induits par l'activation d'une voie de signalisation en aval de TLR4 qui dépend de la protéine adaptatrice 'myeloid differentiating factor 88' (MyD88), alors qu'une autre voie majeure activée par TLR4 et sous le contrôle de l'adaptateur 'Toll, IL-1 receptor and Resistance protein (TIR) domain-containing adaptor inducing interferon-β' (TRIF), n'a pas été encore étudiée dans le contexte de l'asthme. Dans cette étude, nous avons analysé le rôle de l'activation de TLR4 et TRIF dans différents modèles murins d'asthme. Au chapitre 2, nous avons étudié sont rôle suite à une exposition des voies aériennes à un extrait d'un aéroallergène naturel, le pollen de bouleau, dans l'hypothèse que cette voie de signalisation puisse être sollicitée afin d'initier la sensibilisation allergique et/ou faciliter l'amplification de la réponse allergique. Aux chapitres 3 et 4, nous avons examiné le rôle de l'activation de TLR4 et TRIF suite à une exposition à un adjuvant muqueux composé de ligands bactériens de TLR4 et TLR2, potentiellement destinés à un usage en vaccination ou dans le cadre d'une immunothérapie allergène-spécifique, afin de déterminer si cette voie de signalisation était pertinente pour contrer le développement de maladies allergiques respiratoires. Dans ce contexte, nous avons également examiné les mécanismes cellulaires qui pourraient s'avérer pertinents dans la prévention des maladies respiratoires d'origine allergique, à savoir l'effet immunomodulateur de cet adjuvant sur la réponse des lymphocytes T. Nous avons trouvé que, dans les deux cas, la voie de signalisation TLR4-TRIF bloque plusieurs aspects des maladies respiratoires d'origine allergique et ainsi semble avoir un effet protecteur et potentiellement immunothérapeutique contre le

développement de l'asthme. Nous avons également déterminé que l'activation de TLR4 et, dans une large mesure, la voie de signalisation dépendante de TRIF, peut influencer la réponse des cellules T CD4+ en augmentant d'une part l'expression d'une molécule co-stimulatrice exprimée par les cellules T, the inducible co-stimulatory molecule (ICOS), et d'autre part en augmentant la population de cellules $CD4^{+}ICOS^{+}$ qui contribuent à médier l'inhibition dépendante de TLR4-TRIF sur le développement des maladies allergiques respiratoires. Ainsi, nous avons identifié la voie de signalisation dépendante de TLR4-TRIF comme étant une voie potentiellement importante dans l'asthme et fait avancer les connaissances actuelles concernant l'effet adjuvant des ligands TLR4 sur les cellules T.

CHAPTER 1

Introduction & Literature Review

1 Overview of allergic asthma

1.1 Introduction

Asthma is a complex chronic airway disease with a range of clinical presentations that is characterized by recurrent episodes of coughing, wheezing and breathlessness associated with variable and reversible airflow obstruction, airway (bronchial) hyperresponsiveness (AHR) and an underlying peri-bronchial inflammation. Airway inflammation is evoked by repeated noxious exposures and can additionally manifest in permanent molecular or structural alterations of the airways, referred to as airway remodeling, that are possibly refractory to currently available treatments. This latter feature may be responsible for the fixed airflow obstruction and irreversible airflow limitation in some individuals, even after the resolution of inflammation, and has only in recent years been incorporated in the paradigm of asthma. Asthma is determined by the interaction of these processes that can lead to moderate or severe persistent disease. Although the importance of inflammation in asthma is well documented, the precise mechanisms responsible for specific pathophysiological features and clinical manifestations in asthma remain to be fully defined. Furthermore, asthma is known to have a significant genetic component, yet the relative contributions of either host (genes) or environment, and the precise interactions relevant to the initiation or persistence of disease remain to be elucidated. The variable contributions of these pathophysiological inflammatory mechanisms, genetic and environmental factors, as well as differences in response to therapies yield a range of clinical expressions of asthma and account for the heterogeneity of the disease (1;2).

1.2 Pathogenesis of Asthma

1.2.1 Epidemiology and Environmental Factors

Epidemiological studies such as the International Study of Asthma and Allergies in Childhood (ISAAC) and the European Community Respiratory Health Survey (ECRHS) have reported higher rates of asthma in the occident, as well as a global trend in recent decades of increasing asthma prevalence in more developed or affluent regions (3;4). Atopy, defined as the presence of elevated total and allergen-specific serum immunoglobulin E (IgE) leading to positive skin prick tests to common allergens and predisposition to allergic hypersensitivity reactions, is the single highest risk factor for asthma (5). Atopy has a strong hereditary component but also requires allergic sensitization and is classically associated with a T helper 2 (Th2) inflammatory immune response. However, whereas more than 40% of the Western population is atopic, only 7% go on to develop asthma (6). Moreover, the ISAAC and ECRHS studies reported that the proportion of asthma cases attributable to atopy was highly variable (with an overall estimate of about one third for all study centers) but increased with higher economic development (4;6). Standardized comparisons reveal a variable and tenuous correlation between atopy or allergen exposure levels and asthma prevalence (4), indicating that allergen exposure and atopy alone cannot explain the geographic patterns and time trends observed for asthma prevalence (7;8). Thus, one is inclined to conclude that a significant proportion of asthma is "non-atopic", wherein IgE-dependent allergic mechanisms are not thought to be involved due to the absence of systemic IgE and an obvious triggering antigen. Indeed, it has been demonstrated that inflammation and remodeling can occur independently of atopy (9). Furthermore, recent therapies aimed at inhibiting allergic sensitization (e.g. avoidance or allergen-specific immunotherapy) and Th2 inflammation (such as cyclosporine, or anti-IL-4 or IL-5 monoclonal antibody therapy) have variable efficacy and generally, in a limited proportion of patients (reviewed in (10)). Collectively, this has spurned debate over the role of atopy and Th2 inflammation as a cause for asthma and focused attention towards

identifying mechanisms that lead to the specific manifestation of atopy in the airways. The delineation of distinct asthma phenotypes, which is already underway (11;12), should help to clarify the precise role of atopy and Th2 inflammation in different forms of asthma and direct appropriate therapeutic interventions. Current epidemiological data imply that asthma is at some level an organ-specific disease in that, aside from atopy and allergen exposure, in most cases it must additionally involve an underlying defect(s) in immune regulation within the respiratory mucosa in response to environmental allergens. Such defects may be intrinsic to the respiratory mucosa (genetically determined) and furthermore influenced by non-allergenic environmental risk factors or deficiencies in protective factors, as will be discussed.

The 'hygiene hypothesis'

The preeminent theory postulated to explain the geographic and temporal trends of atopy and atopic asthma prevalence is the hygiene hypothesis, according to which growing up in a more sanitary environment with less childhood microbial exposure enhances the atopic Th2 immune response and the risk of developing allergic airway disease (13). Proposed by Strachan et al. following the finding of an inverse relationship between sibling number and hay fever or positive skin prick tests (14), numerous reports in the 1990s have since confirmed the inverse association of allergic disease and family size (reviewed in (13)). An additional protective effect of sharing a bedroom with another child, independent of family size, as well as lower socio-economic status was also reported ((15) and reviewed in (13)). This was proposed to be a reflection of increased exposure in childhood to microbial infections. The protective effect of sibship size on atopy appears to depend, however, on the absence of parental allergy, indicating that genetic predisposition outweighs environmental influences on atopy (15). Identification in the late 1980s of the Th2 helper cell subset that characterizes atopy, as well as the Th1 cells that are important in the response to infection (16), and more recently Th17 cells (17;18), of which the latter two subsets are potentially inhibitory of the Th2 immune response, rendered the hygiene hypothesis immunologically

plausible. The discovery of T regulatory cells that are important for the maintenance of immune homeostasis and tolerance (19;20) has also added a new dimension to the evolving hygiene hypothesis and one that is of particular interest given the rise in recent decades in Western countries of both Th2-mediated atopic diseases and Th1/Th17-mediated autoimmune diseases (illustrated in **Figure 1**).

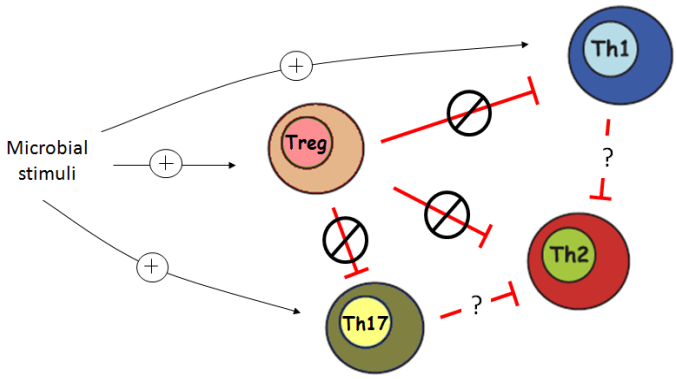


Figure 1. *According to the 'hygiene hypothesis' as well as supporting experimental data, microbial stimuli promote regulatory or pro-inflammatory immune responses that may suppress the development of allergic Th2 responses.*

In 2000, Strachan stated in a review titled, 'Family size, infection and atopy: the first 10 years of the "hygiene hypothesis"' (13), that the hypothesis suffered from a lack of more direct epidemiological evidence supporting an inverse association between infection and atopy, which remains to be the case to this day; studies assessing child day care attendance, as well as cross-sectional and longitudinal studies of common specific or non-specific infectious illness in infancy or childhood in relation to atopy have been largely inconsistent or inconclusive (21;22). Furthermore, support for the hypothesis has been built largely on observational studies rather than formally tested through controlled trials. However, Strachan argued that epidemiological data at the time supported the existence of a strong protective factor that is likely to be an infectious agent and that there may be a critical window in either immunological development or coinciding with initial exposure to a given allergen during which exposure to this agent would be significant. In agreement with the frequent dissociation of asthma from atopy, Strachan also pointed out that the epidemiology of asthma cannot be explained solely on the basis of patterns of allergic sensitization and that the household size and socio-economic status effects which are consistently observed

for hay fever and direct measures of atopy do not apply as clearly to asthma or wheezing illnesses. Thus, the hygiene hypothesis is unlikely to be the sole explanation for the ongoing asthma epidemic in industrialized nations. However, further supporting epidemiological evidence has accumulated over the past decade; in 2000, it was reported that farmers' children, and particularly those with more frequent contact with livestock, had a lower prevalence of hay fever, asthma and wheeze, compared to peers who were not living in an agricultural environment, suggesting that an anthroposophic lifestyle or possibly increased exposure to microorganisms or their components through farm animals is protective against allergic disease (23;24). It was simultaneously reported that the level of endotoxin, the lipopolysaccharide (LPS) found in the cell wall of Gram-negative bacteria, was higher in the homes and mattresses of children living on farms, particularly in families that had regular contact with livestock (25;26) and was associated with enhanced Th1 immunity (27), suggesting that environmental exposure to endotoxin is an important immunomodulatory protective determinant against the development of atopic disease in childhood. An inverse relationship between endotoxin levels in children's mattresses and the occurrence of hay fever, atopy and atopic asthma was subsequently demonstrated (28). The protective effect of endotoxin against the development of asthma in children has since been confirmed in numerous studies in both the rural and urban environments (29;30), although a positive correlation of endotoxin levels in inner-city homes with wheeze has also been reported (31). Subsequent studies also established a protective effect against atopy of prenatal exposure to a farming environment (via the pregnant mother) (reviewed in (32;33) and (34)), associated in some cases with augmented expression of molecules related to innate immunity (35;36), specifically the toll-like receptors (TLRs) 2, 4 and the co-receptor CD14, or enhanced cord blood T regulatory cell numbers and function (37). More recently, components of molds and gram-positive bacteria, β(1-3)-glucan, extracellular polysaccharides and muramic acid, have been associated with lower levels of allergy and asthma in both rural and urban populations (reviewed in (38)),

suggesting that several independent stimuli from different microbes, and not just one single factor, can be protective.

In recent years, focus has shifted towards characterization of the microbial composition of the lungs and gut, aided by technological advancements in pyrosequencing of the 16S gene of bacterial strains, and supported by expanding evidence of a gut-lung axis of immunoregulation (reviewed in (39;40)). Increases in specific bacterial species such as those in the pathogenic phylum *Proteobacteria* (*H. influenza*, *M. catarrhalis and S. pneumonia*) were found within the airways of both child and adult asthmatics compared to healthy subjects, as well as in neonates who went on to develop asthma in childhood (41-43). It is not clear, however, whether the disturbed microbiota of the asthmatic airways may be due to differences in antibiotic use, treatments (such as inhaled corticosteroids) or a defect in immunoregulation in the respiratory mucosa. Reduced gut bacterial colonization has also been associated with increased allergy (44), whereas specific non-commensal bacteria, such as the Gram-negative *H. pylori* and Gram-positive *Firmicutes* have been associated with protection (45-50). The prevailing question in the field is whether diversity, or rather exposure to or selection of specific kinds of bacteria is important for development of a healthy microbial flora (38). Animal experiments suggest that differences in just a single bacterial strain in the gut can strongly alter immune responses (51). A recent European study employing genome-wide interaction analysis concluded that common genetic polymorphisms are unlikely to explain the protective effects of a farming environment on childhood atopy and asthma, supporting the role of environmental exposures in mediating this protection (52). Another report showed that children living on farms are indeed exposed to a wider variety of microorganisms and that the protective effect of the farming environment can be explained to a large degree by the diversity of microbial exposure which was inversely related to asthma (53). In partial agreement, a recent study reported that reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease by school age, though there was no association with asthma (44). Research in the domain of the ‘Microflora Hypothesis’ (40) is

in its infancy and is emerging in parallel with studies of the immunomodulatory effects of antibiotics, prebiotics and probiotics on the intestinal and respiratory mucosa (reviewed in (39)), the hypothesis being that antibiotic use among pregnant mothers or children may alter homeostasis in the gut during an essential period in immunological development by depleting important immunostimulatory organisms (such as the commensal *Bacteroides fragilis*) (40;54-57). Conversely, prebiotics (non-digestible food components that stimulate healthy commensal bacterial strains) or probiotics (non-pathogenic bacterial strains shown to have favourable effects against digestive or respiratory infection, such as *Lactococcus* and *Bifidobacterium*) may stimulate mucosal immunity in a manner that protects against aberrant immune development (58-61).

The complex role of genetic variation in modulating immune responses to environmental stimuli is another aspect that will be discussed in subsequent sections and one that needs to be integrated into the hygiene hypothesis (39). A single unifying mechanism of disease is unlikely given the cumulative evidence in favor of a complex and variable interplay between genetic and environmental "programming" of the host immune response depending on the level, variety and timing of exposure to environmental stimuli, such as allergens, other risk factors and protective microbial signals (62). Nevertheless, progress is being made toward the identification of these interactions and development of appropriate microbe-derived therapeutic interventions; the concept of microbial signals modulating the adaptive immune response via stimulation of innate immunity, particularly in ways that prevent or promote allergic asthma, remains an expanding area of research and one upon which my own work is founded.

Additional Environmental Triggers or Protective Factors

Viral and Helminth Infection

Viral respiratory tract infections, particularly human rhinovirus (HRV) infections, are the major environmental triggers of asthma exacerbations (63-65). HRV is also found more frequently in the lower airways of asthmatics and is associated with increased disease severity (66). The Childhood Origins of Asthma (COAST) study revealed that wheezing episodes caused by respiratory syncytial virus (RSV) and particularly HRV infections in infancy are a major risk factor for the development of asthma by the age of 6 years (67). However, allergic sensitization has since been consistently determined to precede HRV-induced wheezing whereas the converse is not true, suggesting that atopy predisposes to rhinovirus infection and that the two 'insults' interact during infancy to incite the pathophysiological processes leading to asthma (68-70). A recent study also supports that severe RSV infection may be an indicator of a common genetic predisposition to asthma rather than a cause of the disease (71). Conversely, certain viral respiratory infections in early life, such as measles and even RSV, and non-respiratory viral infections, such as hepatitis A, have been associated with protection against asthma, as well as with the protective effects of sibship and day care attendance (reviewed in (13)).

Despite promoting robust Th2 immunity, specific intestinal parasitic helminth infections (particularly of the hookworm), may be implicated in the low prevalence of atopic wheeze in regions that are endemic to such infections (reviewed in (72) and (73-75)). It is unclear whether this may be related to the recently discovered capacity of helminths to induce regulatory B cell and Treg responses (76;77). The inverse relationship between helminth infection and skin test reactivity has been confirmed in multiple reports, though more recent randomized, placebo-controlled studies have not shown significant effects of helminth infection on allergic asthma (78).

Vaccination

The role of commercial vaccines became a public health concern in the 1990s upon reports of measles or whole cell *B. pertussis* immunizations potentially increasing the risk for atopy and asthma (reviewed in (13)). Subsequent studies have largely found no association or sometimes even an inverse association. On the other hand, Bacillus-Calmette-Guerin vaccination during infancy was suggested to be protective against atopy and asthma (79-81), though numerous studies have failed to detect an association, including the ISAAC Phase II study (22) and recent meta-analyses (82;83). The potential of vaccine adjuvants to stimulate atopic immune responses particularly after repeated exposure, such as aluminum hydroxide, a widely used commercial vaccine adjuvant that is also the standard choice for establishing systemic allergic sensitization and Th2 responses in animal models, has not garnered as much attention.

Pollutants, Irritants & Chemicals

Asthma can be elicited and/or exacerbated by exposure in the environment or the work place to pollutants (e.g. diesel exhaust and ozone), irritants (e.g. tobacco smoke, cleaning agents, aerosols, dusts and fumes), high molecular weight (food, plant and animal) proteins, or low molecular weight chemicals which can haptenate endogenous proteins causing them to become immunogenic (e.g. isocyanates and anhydrides) (reviewed in (84;85)). Work-related asthma, which is estimated to constitute about 15% of adult asthma cases, encompasses what is referred to as ‘occupational asthma’, defined rigidly as that which is caused by an exposure specific to the work place, ‘work-exacerbated asthma’, meaning aggravation of pre-existing or coincidental asthma by exposures at work, as well as variant syndromes that share similarities with asthma, such as eosinophilic bronchitis (reviewed in (86)). Occupational asthma can be further differentiated into that which is 'immunologic', in other words occurring after an asymptomatic latent period of sensitization and which is caused by IgE-dependent, or still unknown, mechanisms and that which is 'non-immunologic', occurring acutely (i.e. without a latent period) following either a single or multiple exposures to

high doses of irritants. This non-immunologic 'irritant-induced asthma' includes what is known as 'reactive airways dysfunction syndrome' (RADS) and 'not-so-sudden RADS' which has a delayed onset. Importantly, hundreds of sensitizing agents have been identified in occupational asthma (84). Furthermore, atopy has been consistently documented as a major risk factor for IgE-mediated occupational asthma caused by high molecular weight compounds (85). However, the observation that atopy is a prevalent trait whereas only a limited proportion of individuals develop occupational asthma suggests that additional genetic factors are likely to contribute to the individual differences in susceptibility.

Diet & Maternal Influences

The ISAAC Phase II study found an association of fish, vegetable and fruit consumption with a lower prevalence of wheeze and asthma, supporting the idea that reduced antioxidant and omega-3 fatty acid intake is linked to increased asthma prevalence (87;88). Vitamin D deficiency has also been associated with increased asthma prevalence and vitamin D supplementation may help to avert asthma exacerbations (89). Although both paternal and maternal atopy and history of asthma are predisposing factors for childhood development of these pathologies, evidence suggests that the maternal influence on atopy begins earlier than the paternal influence, even prior to birth (reviewed in (90)). A number of gene polymorphisms, epigenetic effects and gene-environment interactions, mostly related to maternal genetics or environmental exposures have been associated with an elevated risk for atopy and asthma in children, supporting the role of environmental cues transmitted through the mother during pregnancy or infancy in modifying asthma risk (91;92). Maternal immune factors, environmental exposures (tobacco smoke, household allergens, air pollution, or farms), diet (folate or vitamin D dietary supplementation during pregnancy), breast-feeding, or influences of the microbial flora of the gut, vagina or placenta (through the use of broad-spectrum antibiotics during pregnancy or delivery by caesarian section) may be responsible for either the maternal imprinting or prevention of allergic disease (34;93-102). Maternal atopy has been associated

with altered neonatal inflammatory mediator responses (103), impaired regulatory T cell numbers and function (37;104;105) and has been shown to modify the expression, function and effect of polymorphisms in innate immune receptors of the progeny (104;106).

Sex Differences & Comorbidities

Both atopy and asthma are more prevalent in boys in early life, but become more predominant in females during their reproductive years (5;107;108). Obesity is also believed to contribute primarily to non-allergic and early-onset asthma (109). These observations may be explained in large part by altered mechanics of breathing in obese individuals and between the two genders (due to developmental differences in lung structure and function), but are also a potential indication of hormonal influences, or unique inflammatory mediators in the case of obesity, upon allergic airway disease (110).

1.2.2 Host Factors and Genetics

Genome-wide association studies (GWAS) examine the relationship between allele frequencies and disease status across a large number of markers spaced throughout the genome in order to identify genetic variants, specifically single nucleotide polymorphisms (SNPs), which are causally related to complex traits (reviewed in (111)). Together with positional cloning studies, a large-scale GWAS for the 'GABRIEL' consortium (112), identified a number of genes which broadly function to alert the immune system of damage to the respiratory epithelium (e.g. the IL18R1, IL1RL1, IL33 locus and major histocompability complex genes), or regulate homeostatic and repair processes (e.g. SMAD3, ADAM33). The functions of other relevant genes are still being dissected. FcεRI, the high affinity IgE receptor, was significantly associated with higher IgE levels. A screen of select candidate SNPs in the ISAAC phase II study also revealed that the IL4R, TLR4, TLR9 and FcεRI genes were significantly associated with wheezing in children, whereas only the first two genes were additionally associated with allergen-specific IgE (113). However, apart from these genes, as

well as those of the Th2 cytokines, IL-4 and IL-13, identified in the GABRIEL meta-analysis, both of these studies concluded that genetic loci associated with elevated IgE levels were not associated with susceptibility to asthma or wheezing illness and hence suggested that atopy is secondary to asthma rather than a primary driver of the disease (though the authors stated that this does not exclude a significant role for atopic mechanisms in modulating the severity of the inflammation in asthmatic airways). A screen covering the type I IFN gene cluster was conducted in the genetically homogeneous and anthroposophic Hutterite population and demonstrated that variation in multiple type I IFN genes contributed to asthma and atopy susceptibility (114). SNPs in genes relating to antioxidant functions, as well as genotype combinations of IL-4R and CD14 SNPs have been associated with a type of occupational asthma (115). Overall, these studies confirm the genetic heterogeneity of asthma and support a potentially important role for gene-gene and gene-environment interactions, as well as epigenetic modifications (i.e. to heritable characteristics of the genome other than DNA sequences) in the pathogenesis of the disease. Although SNPs in the TLR2, TLR4, CD14 and NOD1 genes have been published to interact with the protective effect of farm-related exposures (116-119), a recent genome-wide analysis of childhood asthma failed to demonstrate strong interactions with farm exposures (only weak or partial effects) of these previously implicated candidate SNPs or the common genetic polymorphisms identified in the GABRIEL meta-analysis of urban populations (52). It was therefore concluded that the strong protective effect of a farming environment is a result of neither the genetic make-up of the farming population nor common genetic polymorphisms interacting with the studied exposures, implying that environmental exposures alone (or the lack of them) heavily determine the new onset of childhood asthma. To date, GWASs have failed to identify alleles that fully explain disease heredity, but have focused only on individual alleles and common SNPs. Combinatorial effects, as well as other factors such as repetitive elements and SNPs occurring in regulatory sequences will need to be examined in the future (39).

1.3 Pathophysiological mechanisms of response to allergen

1.3.1 Allergic sensitization

Allergic asthma is defined by aberrant immune responses to common environmental aeroallergen-borne proteins, caused by initial "sensitization" of the immune system, followed by repeated exposure to the allergen via inhalation. The inflammatory response in the respiratory mucosa may also be exacerbated by noxious environmental exposures, including respiratory infection or inhalation of viral, bacterial, or fungal components, as well as irritants such as chlorine, ozone or ambient particulate matter (120). Sensitization to an allergen may thus occur in genetically-predisposed individuals and/or due to concomitant exposure to other noxious stimuli, particularly during a critical window of immune development in early childhood, or due to the work or living environment. Thus, allergen exposure, allergic sensitization and respiratory tract infection function interactively in the development of asthma (121).

Common respiratory allergens known to produce positive skin-prick tests include insect allergens (e.g. house dust mite and cockroach), grass, weed or tree pollen (e.g. ragweed and birch tree), venoms and animal dander (e.g. cats and dogs). Fungal aeroallergens also exist, sensitization to which, is not as easily detected (122). Upon initial inhalation, allergen-associated or concomitant exogenous inflammatory signals trigger an innate immune response in the airway epithelium and intra-epithelial dendritic cells that sample the airway lumen and take up the allergen, instructing the dendritic cells to upregulate antigen-presentation machinery, costimulatory molecules and cytokines that promote adaptive Th2-type immunity. The epithelial-derived alarmins, thymic stromal lymphopoietin (TSLP), IL-1, IL-25, IL-33 and granulocyte macrophage colony-stimulating factor (GM-CSF) facilitate this process (illustrated in Figure 2) (123). DCs migrate to regional lymph nodes or mucosal lymphoid tissues and upregulate the major histocompability complex (MHC) class II molecules in the context of which, they present antigenic peptides (or 'epitopes') from processed allergen to $CD4^+$ T helper cells, via T cell receptor ligation. DC-expressed co-stimulatory molecules,

such as B7.1 (CD80), B7.2 (CD86) and CD40, provide additional activating signals to the adaptive immune cells to which they present antigen. These interactions support the differentiation of Th2 cells producing the signature cytokines IL-4, IL-5 and IL-13. B cells also recognize DC-presented epitopes via the B cell receptor and are then required to present the epitope to cognate T follicular helper or Th2 cells to become activated (124). These interactions with DCs and T cells promote survival and immunoglobulin class-switch recombination in B cells, leading to antigen-specific IgE production. Activated B cells then transform into memory B cells or IgE-producing plasma cells, the latter having the potential to reside in the bone marrow or inflamed tissues providing a long-term source of allergen-specific IgE (125). Allergic sensitization also serves to upregulate the high and low affinity IgE-receptors, FcεRI and FcεRII, respectively, in various inflammatory and structural cells, particularly B cells, dendritic cells, mast cells and basophils, the expression of which has been found to correlate with the elevated IgE levels in atopic individuals (126). Antigen sensitization was previously thought to occur predominantly in lymphoid germinal centers. However, recent evidence suggests that IgE is produced locally by B cells in the bronchial mucosa of both atopic and even non-atopic asthmatics who lack positive skin-prick tests and therefore, systemic IgE (127). Thus, the effects of allergic sensitization in relation to IgE production and priming of $CD4^+$ T cell responses is well-established and more recently, attention has turned to the role of epithelial-dendritic cell cross-talk (123). Besides this, however, little is known regarding the effects of sensitization on other inflammatory processes and populations.

In animal models of allergic airway disease, sensitization has been conventionally induced by systemic (intraperitoneal) injection of the experimental allergen, ovalbumin (OVA), admixed with an exogenous Th2-promoting adjuvant, most commonly aluminum hydroxide (128). Also, models of allergic sensitization to common, environmentally-prevalent allergens without adjuvant have permitted the study of allergen-intrinsic properties that may be relevant to natural sensitization mechanisms (129;130). Epidemiological evidence indicating that

allergic rhinitis is a strong risk factor for allergic asthma suggested that the upper and lower airways are immunologically linked. This concept of "united airways" has since been supported by experimental murine models of asthma demonstrating that repeated nasal/upper airway exposure to allergen can lead to corresponding lower airway inflammation and impaired lung function (131-133). Intratracheal instillation of allergen, by-passing the upper airways, has also been employed as a mode of sensitization (134). Furthermore, epidemiological evidence identifying the Th17-associated skin conditions, atopic dermatitis and eczema, as predisposing factors for the development of asthma (135), as well as the discovery that a loss-of-function mutation in the gene encoding the skin keratinocyte-expressed molecule, filaggrin, is associated with atopic diseases and asthma (136;137), have fueled recent efforts to develop animal models of epicutaneous or intradermal allergen sensitization that may reflect more Th17-biased allergic airway disease (138;139). Re-exposure to allergen within a few weeks of sensitization via intranasal or intratracheal administration with light anesthesia, or aerosol inhalation, permits its delivery into the lungs and consequent provocation of acute airway inflammation and hyperresponsiveness. Animal models that utilize natural allergens or modes of sensitization may thus be particularly useful for examining mechanisms that may be relevant to the pathogenesis of allergic airway disease. On the other hand, models that rely on adjuvants and systemic sensitization may be limited in this respect, but are perhaps better suited for modeling the atopic development of asthma and remain useful in evaluating the capacity of prophylactic or therapeutic interventions to inhibit maladaptive type 2 immune responses and features of allergic airway disease. However, the physiological relevance to human disease of the dose, timing and frequency of allergen or therapeutic administrations may be limited and difficult to ascertain.

1.3.2 Allergic inflammation

Allergic sensitization primes the immune system to mount a robust inflammatory response upon secondary exposure to allergen, analogous to how a primary

infection or vaccination elicits powerful immunological memory. Inhaled allergen again activates the airway epithelium to produce Th2-biasing inflammatory mediators and is mopped up by IgE bound to FcεRI-bearing dendritic cells, mast cells and basophils in the airway mucosa, triggering the early inflammatory response, namely the events of the first few hours following allergen challenge (126). This is characterized by mast cell degranulation and release of inflammatory and lipid mediators, such as histamine, serotonin, cysteinyl leukotrienes and prostaglandins, that particularly target airway smooth muscle cells causing bronchoconstriction and epithelial cells, inducing growth factors and genes related to goblet cell differentiation and mucus production. Maturing dendritic cells begin to home to the lung-draining lymph nodes where they attain maximal antigen-presenting capacity to promote further IgE production by B cells and Th2 cell expansion, while activated dendritic cells may also present antigen to T cells that they encounter along the way (140). Basophils are additionally recruited to the lymph nodes and augment the Th2 response (141). Eosinophil infiltration of the lungs, another characteristic feature of allergic asthma which promotes Th2 responses and remodeling, has been associated with the early response and particularly the late phase of the allergic response occurring after the first few hours following allergen exposure (142). Inflammatory signals are also sent to the bone marrow from which large numbers of FcεRI-bearing dendritic cells, monocytes, as well as eosinophils and basophils are released into the circulation that home to the lungs to sustain the adaptive B cell and T cell responses (143), the latter mediating the AHR that is observed in animal models starting 24-48 hrs after allergen challenge. Significant progress has been made in recent years in our understanding of the contribution of other innate immune cells to the allergic inflammatory response; invariant natural killer T cells producing the key Th2 effector cytokine, IL-13, have been linked to asthma (144) and innate helper type 2 (Ih2) cells, also called nuocytes, have been recently described, that are likewise bone marrow-derived but depend on epithelial cell-derived signals, rather than activation by IgE, to produce IL-13 (145). Alveolar macrophages may also intensify allergic inflammation by expressing Th2-cytokine promoting

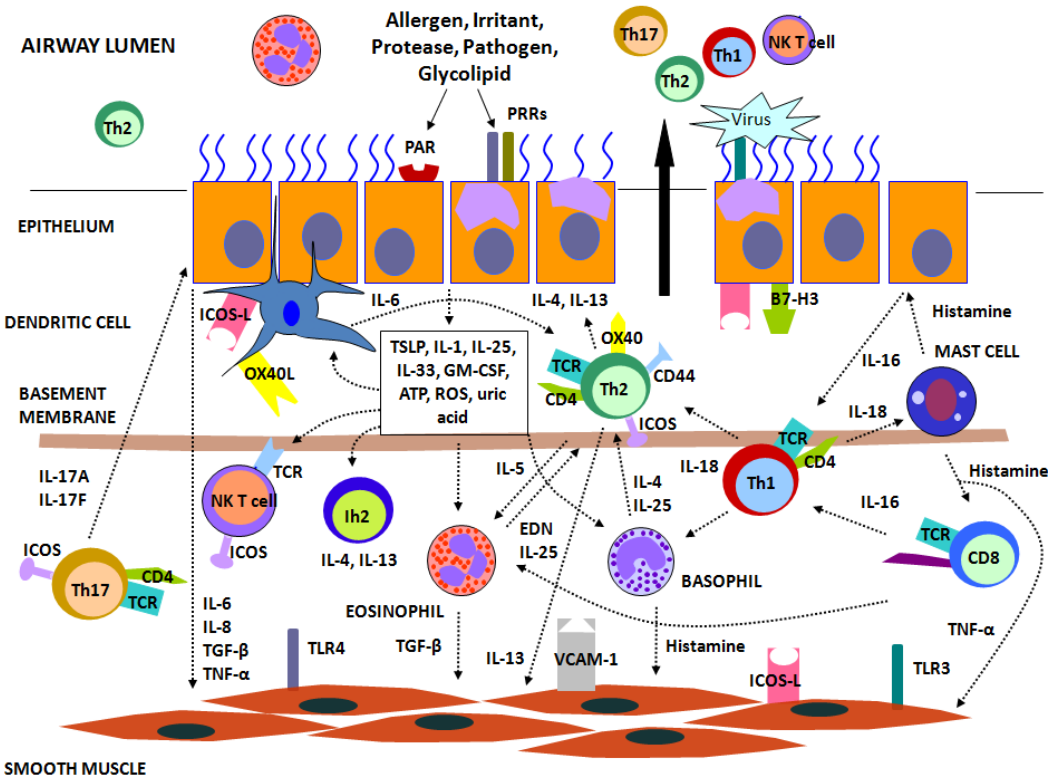
factors and have been associated, along with neutrophils, with more severe, steroid-resistant asthma (146;147).

Bidirectional cross-talk between airway nerves and the inflammatory milieu also contributes to the inflammatory response in the airways; endogenous inflammatory mediators or exogenous airborne allergens and irritants can activate sensory nerve fiber endings in the airways, particularly C-fiber afferents, causing a neurogenic inflammatory response that is orchestrated by a variety of pro-inflammatory neuropeptides acting upon receptors on immune cells, blood vessel walls, bronchial smooth muscle, airway epithelium and mucus glands (148). Sensory nerves can be additionally activated by factors such as temperature, pH and osmolarity and can also elicit bronchoconstriction via vagus nerve stimulation. Experimental work, including some of our own unpublished collaboration, suggest that antagonism of neurokinin receptors may have therapeutic potential in allergic asthma (149). While there are considerable data regarding the role of airway innervation and neurogenic inflammation in experimental animal models of airway inflammation, little is known about its precise involvement in human airway disease due to the heterogeneity of mammalian sensory innervation of the respiratory tract.

In summary, bronchial inflammation in asthma is pleiomorphic involving both the innate and adaptive arms of the immune system, as well as neurogenic inflammation and a systemic component involving signals to the bone marrow. Much of the inflammation may perhaps be attributed to T cell activation by dendritic cells that are instructed by the epithelium (**Figure 2**) (150). However, the recent surge in novel innate immune cell subsets suggests that the influence of these cells, particularly upon the chronicity or exacerbation of asthma, may have been underestimated. Structural cells, such as the epithelium, smooth muscle cells, fibroblasts and vascular endothelial cells, may additionally secrete pro-inflammatory molecules that recruit the inflammatory cells across a gradient from the blood to the airway mucosa and lumen (151). The implicated cell types and their mediators, which will be discussed in greater detail in the ensuing sections, are variably sustained in the airway mucosa by recurrent exposure to allergens

and other environmental triggers. Airway inflammation in asthma may perhaps also be self-perpetuated by innate immune responses related to tissue damage-associated endogenous molecules (152), and may occur in parallel with, or eventually leads to structural remodeling of the airway wall, namely goblet cell hyperplasia, mucous gland hypertrophy, epithelial-mesenchymal transition, thickening of the basement membrane, extracellular matrix deposition, as well as smooth muscle hyperplasia, hypertrophy and possible migration of contractile elements towards the airway epithelium (153-158).

Figure 2. *Overview of inflammatory processes and T cell subsets in the asthmatic airways. Cells: Th, T helper; Ih, innate helper; NK, natural killer. Receptors: PRRs, pattern recognition receptors (e.g. TLRs, Toll-like receptors); PAR, protease-activated receptor; TCR, T cell receptor. Co-stimulatory molecules: ICOS, inducible costimulatory molecule; OX40-ligand; B7-H3. Alarmins and pro-inflammatory mediators: TSLP, thymic stromal lymphopoietin; GM-CSF, granulocyte macrophage colony stimulating factor; ATP, adenosine triphosphate; ROS, reactive oxygen species; Il, interleukin; EDN, eosinophil-derived neurotoxin; TGF, transforming growth factor; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule.*



1.4 Inflammatory cells and mediators

1.4.1 Dendritic cells

Dendritic cells (DCs) have the pivotal function of sensing stimuli in the microenvironment and orchestrating appropriate adaptive immune responses given their antigen-presenting capacity to B cells and T cells. They also ensure balanced immunity by maintaining central tolerance to autoantigens and peripheral tolerance to environmental antigens. Thus, depending on the microenvironmental stimuli *in situ*, DCs may be induced to express different costimulatory molecules and release various cytokines, such as IL-4, IL-12, IL-23, IL-6, IL-10 and TGF-β, to polarize the differentiation of T cells. DCs are the primary antigen-presenting cell in the response to allergen, shown to be responsible for both initiation and maintenance of allergic inflammatory responses (reviewed in (152)). Upon taking up allergen and receiving additional Th2-promoting signals, specific DC subsets will secrete IL-4, IL-10 and particularly IL-6, in order to direct Th2 differentiation (159). Bronchial biopsy specimens of asthmatic subjects contain higher numbers of DCs expressing the high affinity IgE receptor FcεRI (160) (and the activation marker MHC II (161)), the precursors of which are abundantly released into the blood during asthma exacerbation and migrate from the bone marrow to the airway (70). Both human and animal studies have highlighted the importance of IgE and FcεRI expression on DCs in facilitating antigen presentation and DC-dependent T cell effector cytokine responses (162). There are at least five distinct DC subsets that may differentially modulate allergic airway inflammation; three subsets of $CD11c^{hi}$ myeloid DCs (mDCs), one population of $CD11c^{int}$ plasmacytoid DCs (pDCs), and the skin-derived Langerhans DCs (163). Residing under the basal membrane, mDCs are crucial to the initiation of allergic sensitization and promotion of Th2 responses in mice in response to inhaled allergen, whereas the type I interferon (IFN)-secreting pDCs have tolerogenic activity and can counter-regulate mDC-driven T cell responses (152;164). Lung DCs secreting IL-10 have also been demonstrated to induce tolerance in mice (165), while in humans, diminished IL-10 production by

DCs and reduced numbers of circulating pDCs have been associated with atopy and increased asthma susceptibility, respectively (166;167).

DC function is also influenced by the expression of pattern-recognition receptors that detect specific pathogen-associated and endogenous molecules, Notch receptors and their ligands, a family of transmembrane proteins that regulate cell fate decisions, as well as surfactant protein and complement receptors. The significant influence of allergen-associated and endogenous danger signals, as well as epithelium-derived mediators in modulating DC function has risen to the forefront of DC research. Our understanding of the factors that are important to the initiation of Th2 responses has advanced markedly in recent years. DCs are not a major source of the key Th2-polarizing cytokine IL-4 (141). More recently, IL-4 production by accessory innate immune cells, such as natural killer T cells, mast cells, basophils and eosinophils, and release of the innate pro-Th2 cytokines TSLP, IL-1, IL-25, IL-33 and GM-CSF by bronchial epithelial cells has been shown to play a major role in instructing DCs to promote Th2 responses to allergen (152;168). The epithelium-derived TSLP, in particular, has emerged as a key mediator of DC function, and has thus been anointed as a "master switch" of Th2 allergic inflammation at the epithelial/DC interface. DCs are considered a major target for asthma immunotherapy given their primary role in directing adaptive immune responses and propensity for modulation by innate immune signals (169).

1.4.2 T cells

Asthma is associated with activated T cells in the airway wall (Figure from T cell review). Allergy-driven airway disease is typically dominated by the Th2 cell subset (170-174) but other T cell subsets such as Th1, Th17 and invariant NK T (iNKT) cells may also contribute to disease pathogenesis and chronicity. Resolution of inflammation may be controlled in part by Tregs. Therapies directed at T cells and their cytokines in asthma have, however, been underwhelming. This suggests that the induction of asthma may be T cell mediated whereas disease may be sustained and exacerbated by other

mechanisms. Asthmatics have elevated lymphocyte numbers, consisting of both $CD4^+$ ("helper") and $CD8^+$ ("cytotoxic") T cells, in their bronchoalveolar lavage (BAL) fluid (175). To date, the link between T cell cytokines and airway narrowing in asthma has not been elucidated but T cell cytokines may also upregulate enzymes of the 5-lipoxygenase pathway favouring leukotriene synthesis (176). T cells and their cytokines may affect structural cells of the airways, potentially contributing to AHR (177) and remodeling (178-181).

$CD4^+$ T cells

Mosmann and colleagues identified distinct cytokine profiles in the $CD4^+$ T cells of mice and termed these Th1 (interferon-rich) and Th2 (IL-4 and IL-5-rich) cells (182). The CD4 surface antigen is expressed by a large number of T cell subsets, including αβ-T cell receptor (TCR)-expressing Th1, Th2, Th17, Treg and the recently characterized T follicular helper (Tfh), Th9 and Th22 subsets, as well as some γδ-TCR-expressing cells. Th1 cells are important for immunity against viruses and intracellular bacteria and Th17 cells mediate protection against extracellular bacteria and fungi. Th2 cells are crucial for the expulsion of parasites, such as helminths (reviewed in (183)). Activation of distinct gene and microRNA repression programs controls the expression of key T cell lineage-specifying transcription factors and molecules (184), though many of the effector T cell subsets have a capacity for phenotypic plasticity.

T helper 2 cells

Th2 cells are strongly associated with IgE synthesis and eosinophil-rich inflammation. Th2 differentiation depends upon the activation of the transcription factors GATA-3 and Signal Transducer and Activators of Transcription 6 (STAT6) (as well as STAT5). GATA-3 is recognized as the 'lineage-defining' transcription factor for Th2 differentiation, although it has also been found to be highly expressed in innate helper type 2 cells and to a lesser degree in NK T cells, Tregs, basophils and epithelial cells (185). Conditional knockout of GATA-3 in mice demonstrated its importance in both IL-4-dependent and independent Th2

differentiation, regulation of the signature Th2 cytokines, IL-4, IL-5 and IL-13, Th2 proliferation and inhibition of Th1 programming (186). Th2 cytokines have been implicated in many aspects of the allergic response. IL-4 is essential for the initiation of sensitization (187) and can also induce IL-13-independent airway inflammation and AHR (188). A recent study has shown that human IL-4+IL-13+ Th2 cells differentially express IL-5 and that IL-5 expression signifies a more highly differentiated minority of Th2 cells that has augmented GATA-3, IL-4 and IL-13 expression (189). The pleiotropic Th2 cytokine IL-13 is elevated in the BALF and in lung tissues of asthmatics and has been implicated in AHR and goblet cell hyperplasia (reviewed in (190)). The principal transcription factor responsible for mediating signaling downstream of the IL-4 and IL-13 receptors is STAT6, which is expressed by various immune and structural cells, promoting the transcription of numerous genes that are significant in asthma (191). Humanized monoclonal antibodies, soluble receptors, or receptor antagonists against the cytokines IL-4, IL-5 and IL-13 have thus far failed to demonstrate broad efficacy in clinical trials, but generally have had some beneficial effects in severe asthmatics (reviewed in (10;192)).

T helper 1 cells

Th1 cells were believed to potentially be inhibitory of asthmatic airway inflammation. The Th1 lineage-defining transcription factor, T-bet, and GATA-3 are known to reciprocally regulate each other. Numerous studies have demonstrated that the Th1-associated cytokines IL-12 and interferon (IFN)-γ may inhibit antigen-induced AHR in mice (193-195), rats (196), and guinea pigs (197). This is likely due to the inhibition of Th2 cytokine responses. Mice deficient in T-bet develop spontaneous AHR and augmented airway eosinophilia, dependent on IL-13 (198;199). However, Th1 cells are found in elevated numbers in the airways of asthmatics and more recent evidence suggests that these cells and their cytokines, such as IL-18, may contribute to the development and severity of allergic airway disease (200-203).

T helper 17 cells

Th17 cells are defined by the expression of the transcription factor Retinoic acid-related Orphan Receptor (ROR)γt and STAT-3 is also important in the differentiation of these cells (204). The IL-17 family of cytokines consists of IL-17A, E (also known as IL-25) and F. Th17 cells secrete primarily IL-17A and F, promoting neutrophil responses with the purpose of clearing bacterial and fungal infections (205). Interestingly, the AHR and enhanced airway inflammation observed in T-bet knockout mice, is due not only to impaired regulation of the Th2 response, but also IL-17A-mediated neutrophilia (206-208). IL-17A and F are key cytokines in neutrophil migration (209;210). They stimulate human vascular endothelial cells, lung fibroblasts (211), smooth muscle (212) and epithelial cells (212;213) to secrete chemokines, such as CXCL1 (or KC in mice), CXCL8 (or IL-8) and IL-6, important in neutrophil and T cell migration, as well as IL-11, a cytokine that increases goblet cell mucus production and lung fibrosis (214). IL-17- and IL-25-dependent AHR has also been reported and the latter cytokine augments Th2 responses (215-220). Moreover, Th17 cells co-expressing RORγt, GATA-3, IL-17 and IL-13 can be induced in the lungs of mice (221). Thus, IL-17 cytokines can contribute to the enhancement of Th2 cell responses (208), but can also mediate IL-4- and IL-13-independent AHR (222). However, IL-17 was found to be critical in the development of murine allergic asthma to ovalbumin, but was inhibitory of established disease (223). In humans, IL-17 expression in sputum has been shown to correlate with AHR (224) and is associated with severe asthma (210;225). Furthermore, Th17-mediated experimental allergic asthma in mice is steroid-resistant (226), as is IL-17 expression in "asthmatic" rats (227). Thus, Th17 cells may potentially be implicated in steroid-resistant, severe asthma.

Regulatory T cells

$CD4^+$ Tregs exist in a number of subtypes, including the naturally-occurring $CD4^+CD25^+Foxp3^+$ Tregs, adaptive $Foxp3^+$ Tregs that derive from naïve T cells in the periphery, inducible $Foxp3^-$ Tr1 cells, and mucosal Th3 cells (reviewed in

(228)). Naturally-occurring Tregs are a thymus-derived population that constitutively expresses the IL-2 receptor alpha chain, CD25, as well as Foxp3, a transcription factor that is induced early in thymic T cell development which confers regulatory/suppressive activity to the $CD4^+CD25^+$ T cell population. Tregs have the capacity to inhibit $CD4^+$ T cell differentiation and expansion, as well as $CD8^+$ T cell cytotoxic function, in addition to other cell types (229), although the suppressive mechanisms may vary *in vitro* and *in vivo* and according to the different Treg subtypes. *In vivo*, Treg cells mediate suppression by inhibiting the capacity of antigen-presenting cells to prime T cell responses (230). Recent data have established a high degree of plasticity and reciprocal regulation in the expression of transcription factors in Tregs, which can undergo conversion to a Th17 or Tfh phenotype (231-235). The loss of Foxp3 was also shown to result in a default developmental polarization of Tregs towards the Th2 phenotype (236), whereas TGF-β-induced gain of Foxp3 expression inhibits Th17 differentiation (237). IL-10 is also an important effector of Treg-mediated homeostasis at mucosal surfaces (238) and suppression of Th2 (239) or Th17 responses (240;241). IL-10-producing Tregs have been shown to develop from both $Foxp3^+$ and $Foxp3^-$ T cells (242). Although $Foxp3^+$ Tregs are recruited to the lungs of asthmatic subjects (243) and mice (244) upon allergen provocation, they were not reported to inhibit the development of airway inflammation acutely. However, adaptive (inducible) $Foxp3^+$ Tregs, in particular, establish tolerance to chronic aeroallergen exposure (244-247) and mediate mucosally-induced tolerance to antigen (244;248). A recent report demonstrated that whereas thymically-derived naturally-occurring Tregs are necessary and sufficient for systemic, multi-organ homeostasis, extrathymically-differentiated inducible (i)Tregs are specifically critical to the maintenance of mucosal homeostasis, preventing allergic-type airway disease in mice (249). There is increasing evidence implicating defective Treg maturation or function in the deficient immunoregulation observed in asthmatics (250-252). Conversely, the clinical benefits of allergen-specific immunotherapy have been associated with enhancement of Treg activity (253-258).

Natural Killer T cells

NKT cells recognize glycolipid, rather than peptide antigens, via a CD1d-restricted invariant T cell receptor. It has been postulated that environmental agents or microorganisms entering the lungs might directly activate NKT cells. For example, lipids from cypress tree pollen and glycolipids from bacteria such as *B. burgdorferi* and *S. paucimobilis* can activate NKT cells through direct or indirect mechanisms (259-261). Recently, iNKT cells were also shown to recognize and become activated by an abundant endogenous lipid in both mice and humans, in response to infection or TLR agonists, indicating that even in the absence of foreign lipid antigen, these cells may be activated by lipid self antigen (262). Reports of the proportions of these cells vary between < 2% and up to 60% of T cells in the BAL of asthmatic patients, correlating with disease severity and control (263;264). Collectively, animal studies show that allergic sensitization and AHR can be induced by these innate immune cells independently of adaptive Th2 immunity (265-269) and that specific subsets of NKT cells ($CD4^+IL\text{-}13^+$ (267;268), $CD4^-IL\text{-}17^+$ and $CD4^-IL\text{-}13^+$ (265)) can promote airway disease induced by α-galactosylceramide (266;270), allergen (267;268) ozone (269) or respiratory viral infection (265).

$CD8^+$ T cells

These cells are also increased in the airways after allergen challenge and the annual decline in FEV_1 in asthmatics may be predicted from the bronchial $CD8^+$ T cell infiltrate (271). Depletion of $CD8^+$ T cells leads to an augmentation of allergic responses in the rat (272), however the inhibitory effect appears to be mediated by $CD8^+$ γδ T cells (273) and adoptive transfer of αβ-TCR $CD8^+$ cells indicates that they may promote allergic type 2 inflammation (274). However, it appears that $CD4^+$ T cells play the predominant role in establishing AHR and airway eosinophilia while $CD8^+$ T cells act in concert with these cells (275).

γδ T cells

The γδ TCR-bearing cells are trophic for epithelia but are relatively uncommon cells in the airway mucosa. They produce large quantities of IFN-γ, potently down-regulate IgE responses to sensitization and are important mediators of allergen tolerance (276;277). IFN-γ production by $CD8^+$ γδ T cells is potently inhibitory of late allergic airway responses in the rat (273). γδ T cell subsets in the mouse are both pro- and anti-inflammatory; Vγ1-expressing γδ T cells enhance (278), whereas Vγ4-expressing cells negatively regulate IgE, AHR (279), the Vγ1 subset (280;281) as well as NK T cells (282). The γδ T cell has emerged in recent years as a major source of IL-17, particularly in a murine model of experimental asthma, in which it was found to mediate the resolution of AHR and inflammation (283).

1.4.3 B cells and Immunoglobulin E

Activated B cells can differentiate either into extrafollicular antibody-secreting plasma cells by default, or germinal center B cells and recirculating early memory B cells (124). The inflamed lung tissues may also provide a survival niche for long-lived IgE-producing plasma cells, potentially accounting for the "organ-specificity" of asthma and it is speculated that the IgE detected in peripheral blood, lower than any other immunoglobulin isotype, may be that which escapes from the site of disease (reviewed in (126)). IgE plays a primary role in the early immediate hypersensitivity response to allergen involving mast cells and basophils, mediating processes such as bronchoconstriction and mucus hypersecretion, as well as facilitating antigen-presentation to T cells and thereby, the initiation of the adaptive immune response. Allergic asthma is associated with elevated numbers of B lymphocytes expressing the low affinity IgE receptor, FcεRII (CD23), in the airways (284). FcεRII expression correlates with peripheral blood IgE levels, although it has been shown to both positively and negatively regulate IgE production. This C-type lectin exists in a membrane-bound or soluble form and its expression on epithelial cells is thought to amplify IgE-mediated responses by promoting the transcytosis or migration of IgE and antigen-IgE

complexes from the airway lumen across the respiratory epithelium, which can then bind to FcεRI on inflammatory cells (126). Thus, FcεRII can augment the activation of these cells and enhance processes such as dendritic cell maturation, migration and antigen-presentation, facilitating allergic sensitization to further antigens. In addition, FcεRII plays a crucial role in a process known as facilitated antigen presentation (FAP). Whereas initial recognition of an epitope via the membrane-associated BCR invariably leads to the presentation of the equivalent epitope by B cells to cognate T cells (285), in FAP, antigen recognized by secreted IgE (forming antigen-IgE complexes) can be bound and endocytosed by any antigen-activated, FcεRII-expressing B cell, regardless of the specificity of its BCR (126). Thus, FcεRII augments antigen-presentation and additionally permits the IgE-mediated presentation of diverse epitopes, derived from related or unrelated antigen, by B cells to cognate T cells (or by delivering the antigen to DCs (286)). This is referred to as epitope spreading, in which the presence of an antibody response to one epitope can result in the generation of antibodies to other epitopes on the same or unrelated antigen. Epitope spreading may contribute to the progressive development of allergies to multiple antigens in allergic individuals, as well as the 'atopic march', wherein individuals who present in early childhood with atopic dermatitis later go on to develop allergic rhinitis and asthma (135). B cells, such as the IL-10-producing 'regulatory B cells', can also possess anti-inflammatory functions (76). Furthermore, IgA and IgG4 are anti-inflammatory neutralizing immunoglobulins that have been associated with successful allergen-specific immunotherapy (287-291).

1.4.4 Mast cells & Basophils

Mast cells play a major role in the acute manifestations of asthma given that they are one of the first responders to inhaled allergen. Mast cells are activated by cross-linking of allergen-bound IgE on surface FcεRI receptors (or additionally by monomeric IgE and immunoglobulin light chains (126)), triggering an immediate hypersensitivity reaction involving their degranulation and thus, release of preformed mediators (histamine and serotonin) and synthesis of lipid mediators

(prostaglandin D2 and cysteinyl leukotrienes). In concert with mast cell-derived serine proteases (tryptase and chymase), peptidases, cytokines (TNF-α, IL-4 and IL-13), chemokines and growth factors (amphiregulin and fibroblast growth factor), these mediators promote airway smooth muscle cell mitogenesis and contractility, goblet cell metaplasia, mucous gland hypertrophy and Th2 immune responses causing airway hyperresponsiveness, mucus overproduction, as well as leukocyte recruitment, thereby facilitating the 'late' inflammatory response. Mast cells can also be activated by diverse stimuli independently of IgE and antigen, such as cytokines (IL-9), proteases, complement, adenosine, sphingosine-1-phosphate, TLR ligands, neuropeptides and hyperosmolarity (reviewed in (292;293)). The formerly orphan Th2-associated receptor ST2, now known to be the receptor for the cytokine IL-33, is also a lineage marker for mast cells. IL-33 promotes mast cell survival and differentiation from progenitor cells, synergizes with IgE receptor cross-linking to augment mast cell chemokine production and increases IgE-mediated degranulation and leukotriene synthesis by primary human mast cells (294). Thus, mast cells and IgE can have both interdependent, as well as independent actions in the pathophysiology of asthma (126). The localization of mast cells in the airway wall appears to be of particular importance to asthma pathophysiology. Mast cells are the predominant inflammatory cell within human airway smooth muscle bundles and there is evidence to suggest that the activation of mast cells in close proximity to responsive structural cells in the airway wall may play a key role in the development of airway dysfunction and remodeling and is associated with the chronicity, severity and morbidity of allergic asthma (295-298). Basophils are the least abundant granulocyte in the airways and are highly similar to tissue-resident mast cells in terms of their morphology and secreted products (299). They have long been recognized as an effector cell of Th2 and allergic inflammation. Although recent studies have drawn attention to a possible antigen-presenting capacity of basophils (300), inflammatory dendritic cells, not basophils, were reported to be necessary and sufficient for induction of Th2 responses to inhaled house dust mite allergen, whereas basophils were rather important in facilitating and augmenting Th2

inflammation via IL-4 production. Thus, the *in vivo* functions of basophils are receiving greater appreciation, though their antigen-presenting capacity remains controversial (summarized in (141;299)).

1.4.5 Eosinophils

Eosinophils are prominent in allergic asthma, constituting a large fraction of the lung inflammatory infiltrate in humans and in animal models of allergic asthma. They are present in the airway wall, but also in the sputum and bronchoalveolar lavage fluid of uncontrolled asthmatics (2). According to the Local Immunity And/or Remodeling/Repair (LIAR) hypothesis, eosinophil accumulation is proposed to ensure homeostasis in various tissues (142). They are a significant source of IL-4, TGF-β and cysteinyl leukotrienes and their granules contain proteins with anti- parasitic, bacterial and viral activity, such as major basic protein, eosinophil peroxidase (EPO), eosinophil cationic protein and eosinophil-derived neurotoxin, superoxide and hypobromous acid, but which promote bronchoconstriction and tissue remodeling in asthma. Human eosinophils express ST2 through which IL-33 can augment superoxide production, eosinophil degranulation and survival (294). IL-3, GM-CSF, IL-5 and the chemokine, eotaxin 1, are the principal mediators of eosinophil haematopoiesis. IL-5 is also responsible for terminal differentiation, survival and function of eosinophils (301). Communication between the lungs and bone marrow leading to mobilization of CD34+IL-5R+CCR3+ eosinophil progenitor cells, their presence in the locale of the airways and their ability to undergo differentiation *in situ* in response to allergen has been postulated as a potentially important pathophysiological mechanism in allergic asthma; these cells are elevated specifically in atopic subjects who develop asthma (302). The eotaxin proteins bind CCR3, promoting eosinophil haematopoiesis as well as airway and blood eosinophilia. Importantly, eotaxin or CCR3 antagonism in mice has not always yielded total depletion of eosinophils or successful protection against, or inhibition of, allergen-induced AHR (301). Furthermore, IL-5 deficiency or selective ablation of eosinophils abolishes allergen-induced AHR in C57Bl/6J

mice but not in the Balb/c strain, despite reductions in eosinophils and other inflammatory outcomes in both strains. The collective evidence suggests that the threshold of eosinophilic airway inflammation, the background strain of the mice and the experimental model are important determinants of AHR. The eosinophil-deficient mouse strains have demonstrated the important role of eosinophils as effectors of Th2 cell recruitment, airway inflammation and remodeling (reviewed in (303)).

1.4.6 Neutrophils

Neutrophils are among the first cells to be recruited to the airways by exogenous and endogenous danger signals associated with allergen-, microbial- or irritant-induced innate immune responses or tissue damage. For example, airway neutrophilia is exacerbated by viral and bacterial respiratory tract infection, LPS, bacterial flagellin, environmental or cigarette smoke exposure and chlorine. Neutrophils are a major source of superoxide anion and hydrogen peroxide, from which neutrophil myeloperoxidase activity generates the tissue-damaging oxidant, hypochlorous acid (reviewed in (304)). They also produce a diverse array of mediators, such as proteases (e.g. neutrophil elastase), cytokines, chemokines and growth factors (IL-1β, IL-6 IL-8, IL-17, TNF-α, IFN-γ, growth-related oncogene α, macrophage inflammatory protein 1α and β), matrix metalloproteinases 8 and 9, lipid mediators (platelet-activating factor, leukotriene B_4 (LTB_4)), lactoferrin and granulocyte proteins (eosinophil cationic protein (ECP), S100A8 and A9) that augment neutrophil and eosinophil recruitment, activation and degranulation and cause tissue damage and remodeling. Neutrophils also feature prominently in heaves, a naturally-occurring antigen-driven respiratory disease in horses that additionally parallels human asthma in aspects of airway dysfunction and remodeling, in which neutrophils have been shown to be responsive to IL-4 (305-307). Many neutrophil processes and mediators can be augmented FcεRI and IgE-dependently, are elevated in the airways of atopic and asthmatic individuals and are sensitive to anti-histamines, corticosteroids and immunotherapy (304). However, other mediators, such as S100A8 and LTB_4 appear to be corticosteroid-

refractory (308;309). Furthermore, unlike eosinophils, neutrophils are resistant to corticosteroids and are actually inhibited from undergoing apoptosis (304). Significantly, neutrophilic airway inflammation has been associated with the chronicity and severity of asthma, steroid-resistance and frequency of asthma exacerbations (12;310-312).

1.4.7 Macrophages

Pro-inflammatory and tissue damaging 'M1' macrophages can become activated by tissue injury, such as airway epithelial injury caused by noxious stimuli or infection, but can be controlled by what are thought to be anti-inflammatory and tissue repairing 'M2' macrophages (or 'alternatively activated macrophages') (reviewed in (313;314)). *In situ*, IL-4 stimulates proliferation of both subsets and can also promote the conversion of M1 macrophages to the M2 phenotype. M2 macrophages secrete IL-13 and IL-33 and can promote Th2, mast cell, basophil and eosinophil trafficking and/or CD4 T cell-independent AHR in response to respiratory viral infection, glycolipid-activated NK T cells or epithelium-derived IL-33 (reviewed in (147)). They additionally produce growth factors, such as the vascular endothelial and platelet-derived growth factors (VEGF and PDGF) and TGF-β, as well as matrix metalloproteinases and arginase 1 that promote tissue repair or remodeling. Though the role of macrophages in human asthma is obscure, they have generally been associated with more severe asthma and in experimental models, with steroid resistance (147). In contrast, other studies have suggested a regulatory role for M2 macrophages; defective phagocytosis or reduced IL-10 production has been associated with severe asthma (313).

1.4.8 Innate helper type 2 cells

A novel innate cell subset was identified as the primary early source of IL-13 (and IL-5) that is necessary for expulsion of the helminth parasite, *Nippostrongylus brasiliensis* (315). Named 'nuocytes', these cells were found to be induced by IL-25 or IL-33, independently of adaptive immunity. However, they required the presence of T cells to be sustained and only eradicated the parasite upon

amplification of Th2 cell responses, indicating co-operation of nuocytes with T cells. Subsequent studies described phenotypically and functionally similar 'innate helper type 2 (Ih2) cells' that did not require T cells to induce B cell responses and goblet cell hyperplasia in adipose-associated lymphoid tissues (316). Although these cells do not express lineage-specific surface markers, they have recently been shown to originate from bone marrow lymphoid progenitors (317). A recent study elegantly established divergent cellular expression of IL-4 and IL-13; whereas Th2 cells were capable of producing both IL-4 and IL-13, Ih2 cells produced only IL-13 and T follicular helper cells and basophils produced only IL-4 (185). IL-13 production depended on the elevated degree of GATA-3 expression among Th2 and Ih2 cells. Recently, fungal aeroallergen-induced epithelial IL-33 (318;319), virus- (320), parasite- (321), or protease-induced IL-33 production (322), or intranasal administration of recombinant IL-33 (318;323) have been reported to promote AHR via Ih2 cells, independently of IL-4, IgE, and adaptive immunity (324-327). Thus, there is increasing evidence implicating Ih2 (now also referred to as innate lymphoid type 2; ILC2) cells in experimental allergic airway disease and their induction particularly by inflammatory signals affecting the airway epithelium. Ih2 cells have also been identified in humans and CD34+ST2 (IL-33R)+IL-5+IL-13+, possibly Ih2 cells, have been noted in the sputum of asthmatic subjects (328-330).

1.4.9 Structural cells; airway epithelial & smooth muscle cells

The airway-lining epithelium is the first line of defense against inhaled environmental insults. Thus, its role in innate immunity has gained considerable attention in recent years; the epithelium can become activated via numerous innate immune receptors, including classical pattern-recognition receptors (discussed later) as well as protease-activated or ATP-activated (purinergic) receptors, consequently generating various Th2-promoting alarmins and growth factors associated with airway remodeling (reviewed in (168;331)). Numerous studies have shown that non-haematopoietic or specifically epithelial expression of innate immune receptors or factors associated with type 2 immunity is

fundamental to the development of allergic airway disease in animal models (332-335). Furthermore, GWAS screens have revealed that the majority of genes associated with asthma are predominantly expressed within the respiratory epithelium and serve diverse defense and barrier functions (111). Indeed, the asthmatic airway epithelium has been determined to be structurally and immunologically compromised, as well as impaired in differentiation, proliferation and repair, also resulting in pathological processes such as epithelial-mesenchymal transition which may contribute to the inflammation and altered mechanical properties of the airways (336). Thus, the airway epithelium has emerged as a central player in asthma research. The airway smooth muscle (ASM) is also of interest given that smooth muscle hyperplasia and hypertrophy have been associated with the severity of human asthma and repeated allergen challenge in experimental animal models. Differences at the molecular level may render asthmatic ASM intrinsically hypercontractile. ASM has also been shown to exhibit inflammatory and particularly migratory potential which may contribute to the thickening of the airway wall and narrowing of the airways (337;338).

1.5 Pathophysiology of Asthma

1.5.1 Airway Hyperresponsiveness (AHR) & Airflow Obstruction

Airflow obstruction and AHR are the primary diagnostic criteria for asthma, commonly measured by spirometry (1). Subjects are required to perform a maximal expiration following inhalations of increasing concentrations of a bronchoconstrictor, methacholine (MCh), during which the volume of air that is expired within 1 second is measured, referred to as the forced expired volume in 1 second (FEV1) (339). Asthmatics experience a fall in FEV1 of greater or equal to 20% at concentrations of MCh below 16 mg/ml, the formal definition of AHR. Airway narrowing causes impairment or obstruction to expiratory flow resulting in hyperinflation in asthmatics. In small animals, AHR is commonly assessed by an invasive procedure that involves tracheotomy (340). Using the forced oscillation technique, airway responses are measured during tidal breathing

following administrations of increasing doses of intravenously injected, or now more commonly, aerosolized bronchoconstrictor, such as MCh (serotonin or histamine). This entails measuring pressure changes near the airway opening (or trachea) of paralyzed, mechanically-ventilated animals, which reflects pressure changes across the lungs that are related to changes in lung volume and airflow (340). These parameters are fit into the simple linear equation of motion (Pressure = Resistance * Flow + Elastance * Volume) and analyzed by multiple linear regression to derive total respiratory system resistance and elastance values of lung mechanics (341). This equation is related to the simplest model of lung function, according to which the lungs behave like a uniform single compartment. Resistance reflects the obstruction against the flow of air within the airways (also the lung tissues) and elastance, which is the inverse of compliance, is an indication of the elastic properties or "stiffness" of the lung tissues. Thus, airway narrowing such as that caused by smooth muscle contraction or edema and leakage of proteins across the airway epithelium into the lumen in response to an agent such as MCh increases the resistance against the flow of air within the airways of the lungs and causes an increase in Rrs. Also, the frictional heat generated by the distortion of lung tissue constituents contributes to Rrs. Peripheral small airway closure caused either by actual collapse of these airways, or more likely, the formation of "liquid bridges" - mucus and fluid plugs that block the airways - would result in a reduction in the lung volume contributing to tidal volume excursions and an apparent increase in stiffness or Ers of the lungs (342). This "stiffening" of the lungs is due to the increased pressure required to reopen such airways. Therefore, the main events taking place during bronchoconstriction are narrowing of the conducting airways due to contraction of smooth muscle in the airway wall, as well as inhomogeneous airway closure across the lungs. Asthma is a disease of the lower airways, i.e. from the trachea to the terminal respiratory airways; AHR in asthma, understood as meaning bronchial hyperresponsiveness (excluding the upper airways), may thus be identified as an exaggerated bronchoconstriction causing a more significant increase in respiratory pressure swings, Rrs and/or Ers than in healthy individuals

or animals. This is characterized by a leftward/upward shift in the dose response curve to MCh, which reflects two aspects of AHR, namely *airway hypersensitivity*, or a reduction in the dose of agonist required to elicit a response significantly above baseline, and *airway hyperreactivity*, an augmented maximal response to the highest dose of agonist. AHR was also frequently assessed non-invasively in conscious, unrestrained mice using whole body plethysmography, a technique which measures changes in airflow and pressure that can be related to the animal's breathing within a closed container. However, these measurements cannot provide intuitively interpretable parameters of lung mechanics such as Rrs and Ers and most significantly, were shown to be potentially strongly influenced by changes in airflow within the upper airways of the animal (as well as temperature and gas compression within the container). Therefore, this technique is no longer considered a valid method of measuring bronchoconstriction or lower airway mechanics in mice, although the non-invasive 'head-out' body plethysmograph is accepted (343-345). In the interest of establishing an accurate, reproducible measure of forced expired volume in mice, to provide a parameter of pulmonary function that is more comparable to that which is most commonly used in humans, we have contributed to the development of a technique that uses forced oscillation, or more specifically changes in a parameter such as Rrs or Ers, to guide the timing of a negative pressure-driven forced expiratory maneuver in mice such that it occurs concurrently with the maximal bronchoconstriction to a given dose of MCh (Appendix) (346). This technique also utilizes forced oscillation in combination with whole body plethysmography and provides read-outs of both lung mechanics associated with resistance and elastance, and pulmonary function associated with spirometry.

2 Role of the Toll-like Receptors in Allergic Asthma; Allergenicity vs Adjuvanticity

2.1 Pattern-Recognition Receptors

About two decades ago, Charles Janeway Jr. developed a synthesis that placed the innate and adaptive arms of the immune system, which had been studied largely independently to that point, in a clear biological framework (reviewed in (347)). He proposed the existence of pathogen-sensing receptors through which the innate immune system instructs the initiation of adaptive immune responses to antigens of foreign microbial origin, thus placing the activation of adaptive immunity under the control of innate immune pathogen-sensing mechanisms and also providing an explanation for the well-established immunostimulatory and adjuvant effects of certain microbial molecules, such as lipopolysaccharide (LPS). These innate "pattern recognition receptors" (PRRs) are germline-encoded and recognize conserved microbial structural or biosynthetic products, known as pathogen-associated molecular patterns (PAMPs), in contrast to the receptors associated with adaptive immunity (T cell receptors and B cell immunoglobulins), which are generated *de novo* by random gene rearrangement, recognize unique antigen-specific epitopes and are clonally distributed (347). Importantly, he hypothesized that activated PRRs would be capable of stimulating pro-inflammatory pathways, such as Nuclear Factor κB (NFκB) signaling, and costimulatory molecule expression in antigen-presenting cells such as dendritic cells and macrophages, thus permitting the immune system to distinguish infectious non-self from non-infectious self antigens under normal physiological conditions. Genetic sequencing for a cytoplasmic domain resembling that of the IL-1 receptor, also known to occur in the *Drosophila* Toll protein and a resistance protein from tobacco, thus named a TIR domain (for Toll, IL-1R and Resistance protein) lead to the discovery of the Toll-like Receptors (TLRs), which recognize a variety of bacterial, viral, fungal and protozoal ligands, including DNA, RNA, carbohydrates, lipopeptides and glycolipids (347;348). The resulting activation of TLRs can promote the production of pro-inflammatory cytokines, chemokines,

type I interferons (IFNs), costimulatory and adhesion molecules, major histocompatibility complex (MHC) expression and antibody production to ensure the stimulation of rapid and robust immune responses against pathogenic environmental signals (348). However, PRRs play an even more expansive role in regulating immunity in that their activation can additionally be triggered by non-pathogenic organisms, including commensals, as well as endogenous ligands or damage-associated molecular patterns (DAMPs) that are exposed in the context of cellular stress, tissue injury, or autoimmunity such as host DNA, the intracellular high mobility group box protein 1, heat shock proteins and the extracellular matrix components, hyaluronan, biglycan and versican (347;348). Thus, TLR signaling occurs constitutively within normal physiological conditions and is further induced by foreign or endogenous ligands within pathophysiological contexts. It is thought that microbial and endogenous TLR ligands may influence distinct signaling pathways via their differential compartmentalization and engagement of co-receptors (348;349).

Other families of PRRs have also been identified; the cell-surface C-type lectin receptors (CLRs; or Dectins) participate particularly in anti-fungal immunity. The cytosolic family of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) detect bacterial and viral components and partake in multiprotein complexes, inflammasomes, which activate IL-1 production in response to pathogens as well as non-infectious stimuli that compromise cellular membrane integrity (347;348). Such mechanisms that permit the immune system to respond indirectly to “disturbances” in specific biochemical or cellular processes (the ‘guard theory’ (350;351)) thus also act in concert with PRRs (349). The detection of proteolytic activity by the protease-activating receptors (PARs) may be part of this system and may also be implicated in the response to aeroallergen-associated proteases. More recently, intracellular RNA-sensing retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and DNA-sensing PRRs which participate in anti-viral and bacterial immunity have also been discovered. The pentraxins (C-reactive protein, serum amyloid protein and pentraxin 3), as well as the lectin families of ficolins and collectins (including

mannan-binding lectin and surfactant proteins A and D) are secreted, extracellular PRRs that activate the complement system and opsonize pathogens for phagocytosis (347;348), accounting for most of the remaining pathogen-sensing capabilities of the mammalian host. Thus, PRRs can occur on the cell surface, in intracellular compartments, or can be secreted into the bloodstream and tissue fluids (352) and their localization is an important determinant of the ligands that they detect and the ensuing signaling pathways that are activated (353). Most, if not all, PRRs that activate the transcription factors NFκB, interferon regulatory factors (IRFs) or nuclear factor of activated T cells (NFAT) are sufficient to induce T and B cell responses, whereas the secreted PRRs and some phagocytic PRRs (scavenger receptors) are not (348). The role of PRRs in the immune response to allergens is beginning to be defined. Although the term 'danger signal' was originally coined in reference to ligands of a strictly endogenous origin (according to Matzinger's 'Danger Theory') (354;355), it will be subsequently used to refer to any exogenous (pathogen, allergen, or irritant-associated), or endogenous (tissue damage-, or autophagy-associated) inflammatory stimuli.

2.2 TLR signaling; the importance of structure and localization

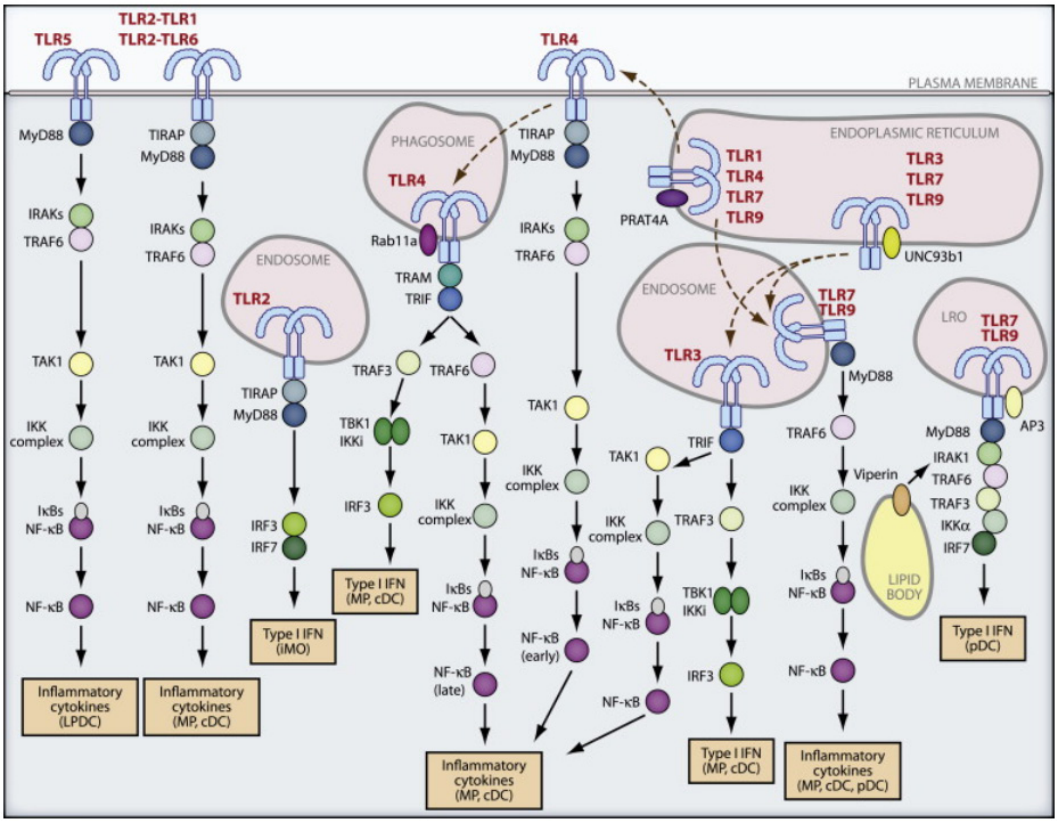
TLRs are the most extensively studied family of PRRs and to date, 10 and 12 functional TLRs have been identified in human and mouse, respectively (349). TLRs 1, 2, 4, 5, 6, and probably TLRs 11 and 12 of mice and TLR10 of humans are expressed largely on the cell surface; TLR4 recognizes LPS of Gram-negative bacteria, TLR2 (associated with either TLR1, 6 or 10) mainly recognizes lipoteichoic acids and peptidoglycans of Gram-positive bacteria, as well as bacterial lipoproteins, while TLR5 detects the bacterial protein, flagellin (348). The intracellular TLRs (TLRs 3, 7, 8, 9 and mouse TLR13) are transported by chaperones such as UNC93B1 from the endoplasmic reticulum, via the Golgi apparatus, to endolysosomal compartments where they encounter their ligands and are able to become activated by the acidic microenvironment (356). These TLRs mainly detect microbial nucleic acids, such as double-stranded RNA

(dsRNA) (TLR3), single-stranded RNA (TLR7 (and TLR8 in humans (357))) and DNA (TLR9) (348). TLRs are type I transmembrane glycoproteins, thus consisting of a ligand recognition 'ectodomain', a transmembrane domain and an intracellular signaling (TIR) domain which, upon activation, interacts with the TIR domains of adaptor proteins (358). TLR4 is unique in that it appears to utilize all four key adaptor proteins which operate in functional pairs: MyD88 with TIRAP (also known as Mal) and TRIF with TRAM. MyD88 (Myeloid differentiating factor 88) and TRIF (TIR domain-containing adaptor inducing interferon-β) trigger distinct signaling pathways and constitute the two major adaptor proteins through which all TLR signaling occurs (359) (summarized in **Figure 3**). Mice that are genetically deficient in both MyD88 and TRIF cannot signal via any TLRs or the structurally-related IL-1, IL-18 and IL-33 receptors (360). All TLRs signal via MyD88, with the exception of TLR3, which relies exclusively upon TRIF. TLR4 was thought to be the only TLR that could induce both MyD88 and TRIF-dependent signaling, though recently TLR2 and TLR5 have also been shown to be capable of recruiting TRIF (361;362). Ligation of TLRs initiates homodimerization of two ectodomains (or heterodimerization, as in the case of TLR2 with TLRs 1, 6 or 10), which is believed in turn to bring together the cytoplasmic TIR domains, thereby permitting the aggregation and interaction of adaptor molecules, such as the assembly of MyD88-IRAK4-IRAK2 (363). The IL-1R-associated kinases (IRAKs) are then phosphorylated, initiating a cascade of protein modifications (ubiquitylation, phosphorylation and proteolysis) including the eventual phosphorylation of IκB kinase, an inhibitory subunit, the subsequent degradation of which results in the release and translocation of the transcription factor NFκB to the nucleus. Mitogen-activated protein kinases (MAPKs) are also triggered leading to the activation of c-Jun N-terminal kinases (JNK), p38 MAPK and extracellular signal- regulated kinases (ERK), culminating in the activation of the transcription factor, AP-1. Together, with the additional activation of the interferon regulatory factors (IRFs) 1 and 5, AP-1 and NFκB ultimately promote the transcription of various pro-inflammatory cytokines and chemokines (359). Interestingly, certain mutations in MyD88 produce TLR

receptor-selective effects (364) and not all TLRs employ the same accessory proteins, indicating a high degree of complexity in MyD88 signaling. TRIF can also induce pro-inflammatory mediators through its association with the TNF receptor associated factor (TRAF)6 which leads to the activation of "late" NFκB and MAPK responses. Importantly, TRIF also associates with TRAF3 leading to the phosphorylation of IRF-3 (and IRF-7) and resultant production of type I interferons, particularly IFN-β (359). TLR4 can signal through TRIF in the same manner, but additionally requires receptor internalization and the participation of TRAM (365;366). Upon binding its ligand on the cell surface, TLR4 recruits and signals via MyD88 but is then internalized into early endosomes halting MyD88-dependent signaling. Instead, TRIF-dependent signaling is triggered, which is later arrested by additional accessory proteins in the late endosomes. Thus, TLR4 signaling via MyD88 and TRIF occurs sequentially. TLR7 and TLR9 also promote pro-inflammatory cytokines such as TNF, IL-6 and IL-12, but have received particular attention due to the discovery of an additional MyD88-dependent signaling pathway that is uniquely present in plasmacytoid dendritic cells and not in conventional DCs or macrophages that leads to the phosphorylation of IRF7 and abundant production of the type I IFNs, particularly IFN-α (367;368). Bacterial TLR2/1 or TLR2/6 ligands have recently been shown to induce receptor internalization and endolysosomal acidification resulting in type I IFN production via MyD88-dependent activation of IRF1/IRF-7 or IRF3/IRF7 (369;370). TLR7 activation by phagosomal bacteria was also recently shown to activate the former signaling pathway (371). Thus, while TRIF signals strictly from an intracellular location, MyD88 can signal from both the plasma membrane as well as within the endolysosomal compartment and there is increasing evidence to suggest that the localization of the receptor dictates the signal that is produced (353). In summary, all type I IFN production, resulting from TLR3 or TLR4 signaling via TRIF-IRF3(/IRF7) or TLR2, TLR7 or TLR9 signaling via MyD88-IRF7(/IRF3/IRF1), occurs intracellularly, within phagosomal or endolysosomal compartments depending on the individual TLR (due to the specific compartmentalization of adaptor molecules), and thus,

requires internalization of the pathogen (372-374). Mast cells, for example, are able to produce TLR-mediated type I IFNs in response to viruses, but can only generate pro-inflammatory cytokine responses to bacteria due to their inability to undergo phagocytosis (375).

Figure 3. *TLR trafficking and signaling. Individual TLRs initiate overlapping and distinct signaling pathways from various cell surface and intracellular compartments and in various cell types, such as macrophages (MP), conventional DCs (cDC), plasmacytoid DCs (pDC), lamina propria DCs (LPDC) and inflammatory monocytes (iMO)* (*schematic adapted from Kawai, T and Akira, S Immunity, 2011* (349)).



For certain TLRs their differential compartmentalization and localization, rather than structure, of the ligands is a crucial determinant of activation, such as in the case of TLR9, which can recognize self DNA in pathological circumstances in which it is not properly sequestered and degraded (356). However, the elucidation of numerous TLR crystal structures in recent years has indicated that TLR

signaling is also crucially dependent on ligand structure (reviewed in (358;376)). LPS is a glycolipid consisting of a hydrophobic lipid A moiety, a core oligosaccharide and a hydrophilic *O*-polysaccharide tail. The lipid A portion is composed of a diphosphorylated glucosamine backbone and four to seven acyl chains and has been demonstrated to be the main inducer of biological responses to LPS (376). Synthetic variants of *E. coli* lipid A have demonstrated that the presence of 6 acyl chains is optimal for endotoxicity, whereas five or seven acyl chains render the lipid A 100 times less inflammatory. Derivatives possessing only four acyl chains, rendering them potent TLR4 antagonists, such as Eritoran, a molecule derived from the lipid A component of the non-pathogenic LPS of *Rhodobacter sphaeroides*, or Lipid IVa, an intermediate of LPS biosynthesis, can thus be utilized for clinical purposes. The phosphorylation of the glucosamine backbone of lipid A is also an important determinant of its potency given that diphosphorylated lipid A (DPLA) is more than 1000-fold more active than monophosphorylated lipid A (MPLA). At lower doses, synthetic *E. coli*-derived and natural *Salmonella minnesota* MPLA have both been shown to retain equivalent TRIF-dependent signaling and adjuvant properties compared to DPLA but impaired MyD88-dependent signaling and toxicity (377;378).

The polysaccharide '*O*-chain' consists of repeating, highly variable oligosaccharide units, conferring almost unlimited diversity to LPS (reviewed in (376)). The length of the *O*-polysaccharide tail determines the colony morphology of microbial variants; those with long *O*-polysaccharide chains form 'smooth' colonies, while the lack of such chains results in 'rough' colonies, hence the designations smooth and rough LPS (379). Some biological activities of these LPS 'chemotypes' and apparently how they are sensed by the TLR4 receptor complex have been revealed to differ. The TLR4 ectodomain recognizes LPS only through its binding to an interacting co-receptor, MD-2 (380;381). TLR4 also utilizes two additional accessory proteins, LPS-binding protein (LBP) and CD14 (382;383). LBP belongs to the lipid transfer family of proteins and is required for extracting LPS from the outer-membrane of bacteria or their liberated vesicles and its transfer to CD14. CD14 is found in soluble or membrane-bound form but has

no intracellular signaling domain and so transfers LPS to the TLR4-MD-2 complex. However, in addition to concentrating the LPS signal at the TLR4 receptor to facilitate MyD88-dependent responses to low level LPS (381;383-386), CD14 was found to be absolutely necessary for both rough and smooth LPS-induced activation of the TRIF pathway (379). In the absence of CD14, TLR4 was either fully inactive (in response to smooth LPS) or MyD88-restricted (to rough LPS or lipid A), thus indicating that CD14 enables specific biological activities of both LPS chemotypes and their discrimination by TLR4 (379). In addition, CD14 was recently revealed to mediate LPS-induced TLR4 endocytosis which was necessary for TRIF-dependent type I IFN production but had no effect on MyD88-dependent signaling (387). Finally, CD14 has also been shown to operate an additional TLR4 signaling-independent mechanism inducing NFAT activation in DCs in response to LPS stimulation, which was proposed to be important in limiting T cell responses by maintaining DC self-tolerance (388). Collectively, CD14 has at least two roles, firstly in transporting LPS to the TLR4 receptor, thereby facilitating MyD88 signaling to low levels of LPS, a function which is not necessary at higher levels of LPS. Secondly, CD14 is rate-limiting in the LPS-induced endocytosis of TLR4 which is a prerequisite for TRIF-TRAM signal transduction. This may have significant biological implications given that certain cell types, such as mast cells and B cells lack CD14 expression (387;389) and that single nucleotide polymorphisms in CD14 have been associated with disease, namely allergic asthma (390). That divergent T cell polarization can be induced by LPS from different bacterial strains (391), or by a bacterial antigen that triggers differential TLR4 signaling via MyD88 and TRIF (392), has also been documented. In summary, TLR and particularly TLR4 signaling is fairly well characterized, diverse and considerably sophisticated, apart from even considering the negative regulation of these pathways; their role in diseases such as asthma may be even more convoluted.

2.3 TLRs in immune disease; an issue of dose, timing and route of exposure

TLRs and their coreceptors are variably expressed by numerous structural and innate immune cells, but also by T and B lymphocytes. Direct TLR7 and 9 signaling on B cells has been shown to be critically involved in antibody-dependent autoimmunity, such as systemic lupus erythematosus and multiple sclerosis (393). Genetic variations in TLR genes have been associated with susceptibility to sepsis and infectious diseases. Also, five primary human immunodeficiencies causing impaired TLR signaling have been identified; the autosomal-recessive MyD88, IRAK4 and UNC93B1 and the autosomal-dominant TLR3 and TRAF3 deficiencies (reviewed in (349;394)). The relationship between TLRs and allergic asthma is evidently complex. Despite epidemiological and experimental evidence supporting the hygiene hypothesis and a protective role for TLRs, there are also paradoxical data suggesting that TLRs may perhaps be involved in the pathogenesis or exacerbation of allergic disease. Association studies have linked numerous single nucleotide polymorphisms (SNPs) in TLRs and their associated molecules, including TLR4 and CD14, with atopy and asthma phenotypes (reviewed in (395;396)). Overall, it is difficult to formulate a collective synthesis of the studies of these SNPs other than that there is an association between TLRs and atopy, in particular. It is clear that single genetic polymorphisms confer only a small risk factor each and a number of gene-gene, as well as gene-environment interactions have been discovered in recent years. For example, ambient house dust mite allergen or endotoxin levels, as well as farm living have been shown to modify the effect of CD14 SNPs in atopy (116;118;397-400), as will be discussed more extensively. Collectively, the epidemiological, genetic, clinical and experimental data point to a complex role of TLRs in potentially (1) initiating or (2) exacerbating allergic airway disease. However, there is also evidence of the potential of TLRs to (3) prevent the development of type 2 airway disease, as well as (4) inhibit established disease. The complexity of TLR responses is likely due to several factors, including the

capacity of allergens or concomitant noxious stimuli to activate multiple TLRs, the relative abundance of TLR expression on various cells, the structure of the specific TLR ligand and the functional consequences of gene polymorphisms. Also, the dose, route and timing of exposure relative to the biological development of the immune system, or disease onset or exacerbation, are important determinants of whether TLRs counter-regulate or promote allergic airway disease (reviewed in (401)).

2.4 Why is an allergen an allergen?

Increasing attention is being directed towards understanding the properties that confer allergenicity to an antigen, or in other words, to elucidating the mechanisms that cause ubiquitous, otherwise innocuous environmental proteins to evoke potent Th2 immune responses. Most individuals are allergic to only a minute fraction of the environmental antigens that they are exposed to on a daily basis, whether via skin contact, ingestion or inhalation, and tend to respond to the same allergens in the same manner. Therefore, allergens have been proposed to share common structural motifs or conformational sequence patterns endowing them with a capacity to activate the innate immune system (reviewed in (120)). Although work over the past few decades focused on elucidating allergenic epitopes recognized by T and B cells, there is no compelling evidence to support the existence of common structural characteristics among them, or that the presence of such epitopes is sufficient to endow a protein with allergenic "sensitizing" potential (120;402). Thus, there are growing research efforts in the area of allergic asthma to employ naturally prevalent aeroallergens and to identify whether they may be inherently associated with common or allergen-specific danger signals. Moreover, delineation of allergen-triggered PRRs and their signaling pathways may yield therapeutic benefits.

Danger signals have been shown to be important for the initiation of allergic immune responses, given that the presentation of exogenous antigens by dendritic cells to T cells in the absence of such signals leads to tolerance. For example,

when depleted of LPS, the experimental allergen OVA does not elicit allergic sensitization via the airways without adjuvant (403). Moreover, either Th2 or Th1 responses to OVA can be induced *in vivo* depending on the adjuvant that is selected, such as alum or complete Freund's adjuvant, respectively (128;404). There is evidence to suggest that once a Th2 response has been evoked, adaptive immunity can elicit responses to other antigens in the absence of classical innate immune-triggering signals by a mechanism referred to as 'Th2 collateral priming' (405). Nonetheless, TLRs may play a variable role in either the initiation of immune responses to allergen during sensitization, or the amplification of inflammatory responses upon secondary exposure to allergen in pre-sensitized subjects. Animal studies that have investigated the effects of LPS are quite variable in this respect; LPS has been given prior to, at the same time, or after sensitization or challenge with an allergen (401). For the sake of clarity, these two "events" of allergic airway disease will be discussed separately in the ensuing sections, and additionally, with particular emphasis on the role of TLR4-dependent MyD88 or TRIF signaling. This is relevant to the second chapter of the thesis which addresses the question of danger signals associated with a natural aeroallergen, the birch tree pollen.

2.5 'The sharp edge of the sword';

2.5.1 TLRs in allergic sensitization

Bottomly and colleagues were the first to show that the role of TLR4 stimulation in priming allergic inflammation via the airways is dependent upon the dose of agonist, LPS (403). In a model of mucosal OVA sensitization without exogenous adjuvant, intrapulmonary co-administration of OVA with a low dose of LPS (100 ng) primes TLR4-dependent, Th2-polarized airway pathology, whereas a high concentration of LPS (100 μg) promotes a Th1 immune response (403), airway neutrophilia and IFN-γ-dependent AHR (measured non-invasively) (406). It has subsequently been shown that low levels of LPS (100 ng) additionally prime Th17 immunity and mixed IL-17/IL-13- and neutrophil-dependent AHR (134). These

findings are also in line with the LPS dose effects observed in epidemiological and human challenge studies ((407) and reviewed in (401)). More recently, it was demonstrated using bone marrow chimeric mice that TLR4 expression in the hematopoietic compartment was necessary and sufficient to drive Th1 responses to high dose LPS-laden OVA, but was also required for Th2 responses to low dose LPS-laden OVA (334). TLR4 expression in the non-hematopoietic ("stromal") compartment was important only for the development of Th2 allergic responses. Interestingly, low level LPS did not drive either Th2 or Th1 responses to OVA when TLR4 was expressed only in the non-hematopoietic compartment. By contrast, Lambrecht and colleagues reported that TLR4 signaling in the stromal compartment alone was sufficient to promote AHR and type 2 airway inflammation to house dust mite allergen (HDM) extracts containing only low levels of contaminating endotoxin (332), suggesting the presence of endogenous TLR4 agonists in the extracts. Indeed, Der p2, a major antigen in HDM extracts, was revealed to have both structural and functional homology with the LPS-binding co-receptor in the TLR4 signaling complex, MD-2, and importantly, was capable of reconstituting LPS-driven TLR4 signaling in the absence of MD-2 both *in vitro* and *in vivo* (408). Overall, these studies suggest that the airway epithelium requires a specific threshold of TLR4 signaling in order to elicit a Th2 immune response to an antigen and that components of complex allergens may be capable of augmenting TLR4 signaling to the required level. Interestingly, TLR4 expression on human airway epithelial cells has been detected, although not at a high level, while under homeostatic conditions, little to no MD-2 has been found (409). In her review on this topic, Wills-Karp wrote "Collectively, these studies suggest that exposure to naturally-occurring components of complex allergens under low ambient levels of bacterial product exposure such as those associated with increasing rates of aeroallergy in the urban, Westernized world, may shift the TLR4-response curve from the tolerizing into the Th2-inducing range through their ability to directly activate the TLR4 signaling complex on stromal cells in the airway (presumably epithelial cells)" (120). A recent report indicated that mucosal sensitization to cockroach allergen extract was mediated independently

of TLR4 but rather required protease-activated receptor (PAR) signaling (410), implying that TLR4 stimulation may be an allergen-specific rather than a universal mechanism of evoking airway Th2 responses.

Importantly, the aforementioned investigators, along with others, also demonstrated that the induction of type 2 allergic airway disease via the respiratory mucosa by OVA (411) and HDM extract in the absence of an exogenous adjuvant depended on TLR4-MyD88 signaling (141;412;413). Interestingly, whereas the recruitment of IL-4-competent basophils and inflammatory DCs in response to intradermal cysteine protease, papain, injection has been reported to be TLR4-TRIF-dependent (414), their recruitment in response to inhaled HDM extract, which also includes cysteine protease activity, was TLR4-MyD88-dependent (141). Whether this discrepancy is due to the difference in the targeted tissues or due to additional constituents of HDM is unclear. LPS-induced IL-4 production in macrophages has been shown to depend on both MyD88 and TRAM (415), while MyD88 deficiency in dendritic cells caused LPS to promote T cell IL-4 rather than IFN-γ production (416), indicating that TLR4-TRIF signaling can support Th2 immunity. Whether these effects are LPS dose-dependent is not known. Concomitant inhalation of LPS with OVA breaks airway tolerance by increasing costimulatory molecule, OX40-ligand (OX40-L), expression on DCs relieving Treg-mediated immune repression (417). TLR4-MyD88 signaling has also been shown to impact upon allergic airway disease in mice by augmenting DC expression of the Th2-inducing Notch ligand Jagged-1 relative to Delta-4 and by inducing epithelial and inflammatory cell production of TSLP, IL-25, IL-33, GM-CSF, TNF-α, IL-4, IL-6, IL-12, KC and vascular endothelial growth factor (332;334;418-423). Furthermore, IL-33 requires MyD88 to signal (327;424;425) and may preferentially potentiate MyD88- rather than TRIF-dependent signaling in murine macrophages (426).

Natural allergens other than HDM have also been demonstrated to provoke allergic airway disease via MyD88; the early immune response to German cockroach frass leading to a mixed Th2/Th17 profile of airway inflammation and AHR involves MyD88 signaling in mDCs (427). Short ragweed pollen and its

extract require TLR4 and MyD88 for the induction of epithelial TSLP expression and Th2 responses (428). Complex carbohydrates, such as the fucosylated glucans, a diverse class of polysaccharides that are widely expressed in the cell walls of fungi, multicellular parasites, some bacteria and allergens (such as peanut, HDM and Bermuda grass pollen), but that are not found in mammalian cells, also have the capacity to promote Th2 immune responses via CLRs, synergistically with TLR4 (120). β1,3-glucans were shown to promote iNK T cell responses to several fungal species through Dectin-1 and MyD88 (429). Thus, complex allergens, such as house dust mite, and even individual antigens within allergen extracts, such as Der p1 and Der p2, or microbial components, such as the β1,3-glucans, can trigger multiple immune recognition mechanisms simultaneously.

Furthermore, MyD88-dependent signaling has not only been demonstrated to mediate allergic sensitization caused by allergen-intrinsic danger signals, but also that which is triggered by microbial components, such as in the context of aeroallergen exposure occurring concurrently with respiratory infection (e.g. *Chlamydia pneumoniae* (430)). Activation of TLR2 or TLR5 can also promote MyD88-dependent $CD4^+$ Th2 responses (431;432) and experimental asthma (433;434). RNAs of the human RSV and rhinovirus, risk factors for the development of asthma, stimulate TLR3 or TLR7 activation (435-439); synthetic double-stranded RNA which mimics viral dsRNA has been shown to prime sensitization to inhaled OVA via TLR3 (with similar dose-dependent Th2 or Th1-promoting effects as LPS) (440) and amplify OVA/alum sensitization (441). A recent study demonstrated that concomitant inhalation of only synthetic dsRNA, not a TLR7 ligand, with OVA promoted allergic sensitization (442), whereas another study suggested that TLR7 activation within a specific window of susceptibility (neonatal exposure) may promote allergic airway disease (443).

Finally, TLR4 may be additionally involved in the response to allergen via indirect mechanisms that do not require direct interaction of the antigen itself, or concurrent allergen- or microbe-associated danger signals, with the receptor complex, as described thus far. Oxidative stress has been frequently associated

with TLR4 activation. For example, augmentation of draining lymph node Th2 responses by intradermal papain injection occurs via the generation of reactive oxygen species. This was suggested to yield oxidized phospholipids that could trigger TLR4-TRIF signaling to induce TSLP production in the epidermis (414). The ability of oxidized phospholipids to influence TLR4 activation in a cell type-specific manner, both positively and negatively, has been known for some time; they are thought to promote TRIF rather than MyD88 signaling but have also been reported to inhibit TLR2, 3, 4 and 9 signaling ((444;445) and reviewed in (446)). Heme oxygenase I, a major antioxidant gene, has also been demonstrated to be necessary for the activation of IRF3 and the expression of its primary target genes following TLR3 or TLR4 stimulation, as well as viral infection (447;448), suggesting an interplay between oxidative stress and TRIF activation. Moreover, mouse macrophages lacking a master transcription factor that regulates antioxidant genes, Nrf2, therefore having impaired antioxidant defense, exhibited augmented LPS-induced NADPH oxidase activity and ROS production, which in turn enhanced TLR4 signaling via both MyD88 and TRIF (449). Ozone has been shown to promote type 2 allergic airway disease on its own, characterized by IL-13/IL-17-dependent AHR, and additionally has the capacity to prime immune responses to low levels of inhaled LPS (269;450-453). Ozone-induced AHR and priming of LPS responses are mediated by the same mechanism, fragmentation of hyaluronan, an abundant extracellular matrix protein, into low molecular weight fragments that elicit TLR4-MyD88-signaling. The above are examples of how the guard and danger theories discussed earlier may potentially act in tandem with pattern recognition in initiating the response to allergens.

In summary, there is considerable evidence in the literature linking TLR4-MyD88 signaling or MyD88 signaling via other TLRs in the induction of ‘Th2’ immune responses, allergic sensitization and type 2 airway disease. TRIF-dependent mechanisms have also been described, through TLR3 (not TLR4), in relation to allergic airway disease. The dependence of allergic sensitization on TLR signaling is, however, contingent upon the specific allergen that is studied and the experimental model, but also the mode of sensitization employed in animal

models of experimental asthma. Whereas intranasal sensitization to LPS-laden OVA is mediated by TLR4 and MyD88 signaling, this requirement can be by-passed if an exogenous adjuvant, such as alum, is used intraperitoneally to elicit allergic sensitization (128). Alum can induce a Th2 response to OVA even in TLR4- and MyD88-deficient mice (411;454) and has been confirmed to possess alternative immunostimulatory properties that may undermine the significance of TLR signaling from allergen-associated danger signals (455), a factor which must be taken into consideration in the experimental design. In the second chapter, we investigate the role of oxidative stress, TLR4 and TRIF in mediating sensitization or the secondary inflammatory response to an extract of the natural aeroallergen birch tree pollen administered exclusively via the airways without adjuvant.

2.5.2 TLRs in the secondary response to allergen

Controlled human challenge studies have demonstrated that LPS exposure can exacerbate existing airway disease in sensitized individuals (reviewed in (390;401)). Inhalation of low dose LPS enhanced the response to HDM allergen, as evidenced by reduced lung function. In another study, low dose LPS (2 ng/kg) also augmented mDC influx, proinflammatory cytokines and airway inflammation in asthmatic subjects when combined with segmental HDM allergen challenge. A number of studies have assessed the effect of intranasal LPS administration in mice coinciding with OVA challenge, following OVA/alum systemic sensitization. The majority of these studies indicate LPS-mediated augmentation of airway eosinophilia, but the effects on Th2 cytokines, serum IgE and AHR have been more variable (reviewed in (401)). Mast cells appear to be important in the LPS-mediated exacerbation of Th2/eosinophilic airway inflammation and serum OVA-specific IgE (456;457). Rather than administering LPS exogenously, our lab has also investigated its role in modifying the secondary response to inhaled allergen, using a similar protocol of OVA/alum sensitization followed by intranasal challenge of mice with either LPS-rich or LPS-depleted OVA. Our unpublished data also indicate that the contaminating LPS in OVA augments Th2 and eosinophilic airway inflammation, but does not affect AHR, and additionally

accounts entirely for the observed increase in Th1 cells, neutrophils and Tregs in the airways. In a rat model, our lab also found that the contaminating LPS augmented macrophage and neutrophilic inflammation in the airways, as well as AHR, but did not affect Th1 and Th2 cytokines, or airway remodeling in the form of goblet cell or smooth muscle hyperplasia (458). Collectively, these studies that have used relatively similar sensitization and challenge protocols (but in which the LPS concentration coinciding with allergen challenge has been more variable) have demonstrated variable effects of LPS in amplifying different aspects of the secondary response to antigen.

Inhaled LPS is known to promote Th1 cell trafficking to the airways and augments antigen-induced trafficking of adoptively-transferred OVA-specific Th1 cells, which in turn, promotes IFN-γ-dependent airway neutrophilia (459). Severe asthmatics have an increased percentage of IFN-γ-expressing cells in their airways and induced sputum (406), while LPS levels have been reported to be high in the BAL fluid of steroid-resistant asthmatics and seem to correlate with disease severity (460-462). Recently, LPS inhalation with OVA was shown to produce glucocorticoid-resistant AHR in mice, mediated by pulmonary macrophages depending on co-operative TLR4-MyD88-induced signaling between IFN-γ and IL-27 (463;464). MyD88 is also involved in the expression and or signaling of other mediators associated with severe asthma and corticosteroid resistance, such as IL-33 and S100A8 (308;465-467). Overall, the preponderance of the evidence suggests that exposure to LPS via the airways, when exactly coinciding with either sensitization or secondary exposure to an antigen, is pathophysiological and that the dose of LPS dictates whether the ensuing pro-inflammatory response is Th2/eosinophil-dominated, or Th1/Th17/neutrophil/macrophage-abundant. The only indications of TRIF-mediated mechanisms are reports of intranasal dsRNA exacerbating allergic airway disease in sensitized mice (TLR3-TRIF-dependently) (468), as well as enhancing airway inflammation and remodeling in a rat model of bronchial asthma (469).

2.6 'The blunt edge of the sword';

2.6.1 TLRs in the protection against allergic disease development

As discussed in earlier sections, there is considerable epidemiological evidence linking endotoxin exposure with protection against the development of allergic disease. Several studies have associated higher LPS exposure in farming environments with protection against atopy and have identified a weak effect of high levels of LPS in urban homes in reducing the risk of atopy (though several studies have also failed to confirm this association) (reviewed in (32;390)). These observations have been more consistently reproduced when gene-environment interactions are taken into account. The most extensively studied example is a common SNP occurring in the promoter region of CD14 rs2569190, a C-to-T transition at -260 bp from the translation start site and at -159 bp from the transcription start site (thus referred to as both CD14/-260 or CD14/-159) (390). About 50% of the general population is heterozygous for the C and T alleles, while the remainder is fairly evenly divided between CC and TT homozygotes. The direction of the association of this SNP with atopy was inconsistent, similar to the association of endotoxin exposure with atopy, therefore prompting an analysis of the CD14 genotype and atopy in the context of domestic endotoxin exposure (470). High level LPS exposure was thus convincingly associated with protection against the development of atopy in individuals that are homozygous for the CC genotype of the CD14 gene (118;390;470-473). In low ambient LPS conditions, however, the same genotype actually appears to confer the highest risk of allergic sensitization, indicating a capacity of CD14 to influence allergen-induced immune responses in opposite directions depending on the microbial load. The functional consequence of the CD14 genotype upon TLR4 signaling and sCD14 levels is not entirely clear (390). Recently, compared to children of 2 years of age, 10 year-olds were reported to have significantly increased methylation of the CD14 gene, which inversely correlated with serum sCD14 levels (474). Interestingly, DNA methylation in the CD14 promoter region was significantly lower in the placentas of mothers living on a farm compared to

mothers not living on a farm and correlated with differential CD14 expression between these groups (475). It is unknown whether there is a possibility that polymorphisms or epigenetic regulation of CD14 may affect signaling by different LPS chemotypes and via MyD88 and TRIF, such as the mutation in mice. Two common variants in the TLR4 gene, suggested to confer hyporesponsiveness to inhaled LPS have also been associated with more severe atopy and asthma (390;476), though more investigations taking into account the effect of LPS exposure appear to be necessary. Overall, these findings impart an additional level of complexity to the model in which the degree of LPS exposure modifies the propensity of the immune system to mount an allergic response.

In animals, a study that evaluated the effects of intraperitoneal sensitization (in the absence of adjuvant) followed by aerosol challenge with either commercial OVA on its own, or OVA that is purified of LPS or supplemented with LPS, surprisingly indicated that the purified OVA elicited greater allergic sensitization and airway responses, whereas the contaminating or supplemented LPS inhibited these responses (477). These findings seem incongruous with the earlier mentioned studies pertaining to intranasal sensitization by LPS and the postulated pattern of simultaneous intrapulmonary exposure to LPS and antigen being pathological. It is possible that the inhibitory effect of LPS on the immune response to OVA may be related to the systemic co-exposure to LPS during sensitization rather than the pulmonary exposure during challenge (477). However, neonatal mice receiving repeated applications restricted to the nasal compartment (and not the lungs) of either LPS alone, or in combination with OVA (LPS/OVA) were protected from developing subsequent allergic airway disease (478). In another study, whole body exposure by aerosol throughout infancy to combined LPS/OVA, but not LPS on its own, prior to systemic OVA/alum sensitization, inhibited the development of allergic airway responses (479). The combination of LPS/OVA was associated in both of these studies with a mixed IFN-γ/IL-10 T cell response (478;480) demonstrating that in addition to having the capacity to promote Th2, Th17, or Th1 responses, LPS can also prime T cell IL-10 responses to antigen via the airways, possibly Treg responses.

However, in both of the latter studies similar application of OVA, even without LPS, was also sufficient to prevent subsequent allergic airway disease, limiting one to conclude that during the early developmental period, LPS exposure via the airways coinciding with antigen, at the very least, does not evoke or enhance allergic inflammatory responses. Alternatively, these results may be a reflection of tolerance induction via the nasal or oral routes (due to swallowing of antigen), or that which occurs with repeated allergen exposure (481-483). There are additional studies to support that exposure to LPS in the absence of concurrent antigen is inhibitory of the development of aspects of allergic airway disease. Maternal exposure to LPS before and during pregnancy inhibited subsequent OVA-induced airway inflammation but not AHR (484), as did prenatal exposure of rats to LPS (485). Combined pre- and post-natal LPS exposure inhibited later allergic airway disease and was associated with increased T-bet expression in the mouse lungs, as well as *in vitro* Th1 responses (486). Moreover, both systemic LPS given prior to intraperitoneal OVA/alum sensitization and intrapulmonary LPS given between sensitization and challenge inhibited the development of airway Th2 inflammation, eosinophilia and serum IgE in an IL-12-dependent manner, suggesting Th1-mediated inhibition (487). However, AHR (measured non-invasively) was unaffected by either LPS exposure. Conversely, the adsorption of LPS or a synthetic TLR4 agonist to alum during intraperitoneal OVA sensitization was shown to prevent allergen challenge-induced AHR and Th2 airway inflammation, TLR4/MyD88- and IL-12/IFN-γ-dependently (488). Overall, the pattern that emerges is that airway exposure to LPS is most likely to be inhibitory of the allergic airway response when it does not exactly overlap with the allergen exposure and furthermore, that compared to intrapulmonary LPS, systemic administration has appeared to more consistently result in Th2-inhibitory effects, even when coinciding with allergen sensitization or challenge. Whether this may be due to the pro-inflammatory effects occurring remotely from the lungs, by-passing of the Th2-promoting effects of the airway epithelium, differential availability of TLR co-receptors in different tissues, or an indication that non-respiratory and respiratory bacterial infections coinciding with allergen

exposure may have divergent effects upon the development of atopy and asthma is an intriguing question.

As mentioned, the induction of IL-12, IFN-γ and/or IL-10 has been associated in a number of studies with the inhibition of allergic airway disease by LPS. Therefore, it is commonly believed that the inhibition of allergic airway disease by LPS likely involves Th1 and/or regulatory T cell responses, such as the $Foxp3^+$ Treg or $Foxp3^-$ IL-10-producing Tr1 cells (489). However, only IL-12 and IFN-γ neutralization *in vivo* have been confirmed to abolish the inhibitory effects of LPS in allergy/asthma models (487;488). To date, there is no evidence of Treg depletion or IL-10 neutralization *in vivo*, to directly prove a role for these in the LPS-mediated inhibition of allergic airway disease. Moreover, there is evidence to suggest that T cells are not the only players to mediate the Th2-inhibitory effects of LPS. DCs stimulated by LPS or TLR2 ligand can provide a MyD88-dependent negative signal directly to T cells that suppresses their polarization towards the Th2 phenotype and that is independent of Th1 cells or their cytokines, IL-12, IL-18, IL-23, IL-27 or IFN-γ (490;491). LPS-stimulated DCs adoptively transferred prior to allergen challenge also prevented Th2 airway inflammation, suggesting that DCs are capable of mediating LPS-induced inhibition of Th2 development, in which IL-12 is not a requirement (492). IL-10-producing DCs, on the other hand, failed to inhibit allergic airway disease, suggesting that the DC-derived Th2-inhibitory signal is also unlikely to be IL-10 (493). Given simultaneously only with the second of two allergen challenges, systemic LPS (20 μg) inhibited the development of IgE-mediated and mast cell-dependent passive cutaneous anaphylaxis and all aspects of experimental asthma, including AHR. An equivalent dose of intranasal LPS reduced Th2 cytokines, eosinophilia and mucus production but caused dramatic airway neutrophilia and exacerbated and prolonged AHR (measured non-invasively). These effects also did not require IL-12 and IFN-γ but were dependent on functional TLR4 and nitric oxide synthase 2 (494). A recent study showed that repeated inhalational exposure to LPS (10 μg) inhibited subsequent development of airway inflammation (AHR was not measured) caused either by HDM allergen or adoptively transferred Th2-skewed

OVA-specific T cells and promoted the development of lung-resident $CD11b^{+}Gr1^{int}F4/80^{+}$ cells resembling myeloid-derived suppressor cells, in a TLR4 and MyD88-dependent manner (495). These cells produced significant quantities of nitric oxide, but not IFN-γ, suppressed the activation of Th2 cells *in vitro* by producing IL-10 and also inhibited the development of allergic airway inflammation when adoptively transferred. Interestingly, in a very similar study, inhalation of OVA containing low levels of contaminating LPS was shown to induce F4/80-expressing interstitial lung macrophages that could counter-regulate the Th2-stimulatory effects of LPS on DCs by producing IL-10. Depletion of $F4/80^{+}$ cells triggered an allergic airway response to the low LPS-laden OVA (496).

Sterile house dust extracts also exhibit dose-dependent tolerogenic effects upon the development of type 2 airway inflammation that partially depend upon TLR4, and can promote airway neutrophilic inflammation (497). Also in humans, exposure to organic dusts in the agricultural environment has been associated with reduced IgE levels and protection against allergic diseases, although persistent exposure causes low-grade chronic airway inflammation which often leads to non-atopic wheeze (reviewed in (498)). These dusts contain high levels of both Gram -negative and -positive bacterial cell wall components (namely endotoxin and peptidoglycan) and are capable of inducing non-allergic pulmonary inflammation and dysfunction in mice partially via TLR4 and TLR2 (499). Moreover, experiments examining the effects of foreign and commensal microbes, rather than just their individual components, also indicate the importance of MyD88-dependent TLR signaling in preventing type 2 inflammation. Oral treatment with a commercial bacterial extract prevented experimental asthma in mice by MyD88- and IL-10-dependent mechanisms that enhanced T cell conversion to the Foxp3-expressing Treg phenotype (500). Another study demonstrated that oral administration of a probiotic preparation consisting of *Bifidobacterium*, *Lactobacillium* and *Streptococcus* strains, prevented OVA-induced experimental asthma in the immune-compromised non-obese diabetic (NOD) mouse strain and that the probiotic effects were MyD88-

dependent (501). Furthermore, genetic ablation of MyD88 in the NOD mice caused spontaneous exacerbation of the experimental allergic asthma, altogether supporting the existence of MyD88-dependent tonic, as well as inducible, immunoregulatory signals. Interestingly, disruption of commensal-derived signals by oral antibiotic treatment resulted in enhanced basophil haematopoiesis and IgE overproduction; B cell-intrinsic MyD88 expression was required to limit these pathologies (502). Finally, *Acinetobacter iwoffii* F78, a non-pathogenic gram-negative bacterium found to be prevalent in the farming environment by sequencing and phylogenetic analysis of mattress dust and cow, swine, horse, or chicken-shed dust samples (503), was determined to provide protection against allergic airway inflammation in mice (504) and to induce a Th1-polarizing program in human DCs which depended on its LPS component (505). Notably, respiratory exposure of pregnant mice to this bacterium was shown to protect against the development of experimental asthma in the progeny, which was dependent upon maternal TLR signaling, given that protection against asthma was not transferred from mothers that were simultaneously deficient in TLR2/4/7 and 9 (506). IFN-γ expression in the offspring and epigenetic enhancement of the IFN-γ promoter of their $CD4^+$ T cells was subsequently revealed to mediate the protective effects (507). These findings provide further experimental support to the hygiene hypothesis and indicate that microbes operating through TLRs can modulate epigenetic mechanisms to influence the development of allergic airway disease. They also indicate that TLRs relying on MyD88-dependent mechanisms, other than just TLR4, can also prevent the development of Th2 responses and/or experimental allergic airway disease.

TLR2 ligands, such as synthetic (508;509) and bacterial (510) lipopeptides, inhibit IgE and features of experimental asthma in mice by promoting Th1 and MyD88-dependent IFN-γ/IL-10 T cell responses. A gene variant of TLR2 was associated with reduced diagnosis of asthma and atopic sensitization among children of farmers (117). A recombinant flagellin (TLR5)-OVA fusion protein suppressed both Th1 and Th2 responses *in vitro* MyD88-dependently (511). TLR9 activation by synthetic immunostimulatory CpG motif-containing

oligodeoxynucleotides (ODN) can also prevent the development of allergic airway disease and airway remodeling (512;513), including in primates (514) and in some cases providing long-term protection (515), when the ligand is administered either systemically, orally or into the lungs around the time of allergen sensitization (516), or to presensitized mice prior to or during allergen challenge (517;518). CpG ODNs can also prevent Th2 'phenotype spread' or spread of sensitization to a second allergen (519) as well as the maternal transmission of 'asthma' susceptibility in a murine model (520). Although Th1 cytokine responses were initially implicated in TLR9-mediated inhibition of experimental asthma (515;521), accumulating evidence suggests that alternative mechanisms may also be involved (522), such as IL-10 production by macrophages (523) and the induction of the T cell-inhibitory indoleamine-2,3-dioxygenase (IDO) enzyme (524). More recent data also suggest a role for type I IFNs in the inhibition of allergic airway disease by TLR9 activation (525).

TLR7 engagement by systemically administered synthetic small molecules belonging to the imidazoquinoline family has also been demonstrated to prevent the development of experimental asthma, including airway remodeling (526), with anti-inflammatory effects upon both Th2 and Th1 cytokine responses (527;528). Intraperitoneal or subcutaneous administration of TLR3 or TLR7 ligand around the time of sensitization prevented later OVA-induced experimental asthma while inducing IL-10 and Th1 cytokines (529). However, local rather than systemic administration of TLR7 ligands may be a preferred treatment option for the clinic given reports of systemic administration causing toxic effects in humans (530). Prophylactic intranasal administration following allergen sensitization of mice was also shown to induce long-term protection against the development of experimental asthma (531). The early effects were IFN-γ-independent but rather required type I IFN production from the hematopoietic cell compartment and could be reproduced by administration of recombinant IFN-α, whereas the long-term protective effects were dependent upon $CD8^+$ T cell IFN-γ production, suggesting that a classical anti-viral response induced by TLR7 activation in the airways, characterized by initial type I IFN production and later $CD8^+$ T cell/IFN-

γ responses, could be protective against the development of allergic asthma. Other studies have shown TLR7-mediated suppression of allergic inflammation or AHR driven by invariant NK T cells producing IFN-γ (532;533). A recent study systematically evaluated the dose effects of intratracheally administered TLR2, 3, 4, 7 and 9 ligands upon OVA-induced airway inflammation and determined that when the ligand exposure coincided with OVA, all ligands with the exception of TLR2 and TLR4, inhibited airway eosinophilia, while also simultaneously inducing significant macrophage and neutrophilic inflammatory responses in the airways (with the exception of TLR7 ligand) (534). The inhibitory effects of the ligands in this setting were independent of IL-10, IFN-γ or Tregs, while type I IFNs were undetectable. When the ligand exposure preceded OVA inhalation by a few days, only the TLR4 and TLR9 ligands successfully inhibited airway inflammation as well as AHR, while inducing milder inflammation. This study highlights the importance of the timing of the exposure to TLR agonists, as well as the substantial inflammatory potential of these ligands when administered via the intrapulmonary route and indicated that TLR7 agonists may pose the least danger in this respect.

2.6.2 TLRs inhibiting established disease

Studies investigating the therapeutic effects of TLR activation upon allergic airway disease have shown that administration of TLR3, 4, 7 and 9 ligands following allergen challenge can inhibit experimental asthma (534). Whereas wild-type and tlr4-deficient mice challenged acutely with OVA developed a similar degree of airway disease, extended allergen challenge caused the tlr4-deficient mice to develop more severe airway disease and IgE (535). TLR9 activation, in particular, by systemically, orally or intranasally administered ODNs can reverse established allergic airway disease in mice (516;536;537) and even airway remodeling (538;539). These effects were associated mostly with IFN-γ (536;540) and IL-10 responses (538;541;542), though not conclusively. TLR7-mediated suppression of established asthma symptoms was shown in one study to be related to Tregs (543). Systemic TLR3 or TLR7 ligand also reversed

established asthma symptoms in another study via IL-12 and IL-10-dependent mechanisms (529). Intranasal TLR7 ligand delivery to mice that were already suffering from airway inflammation also prevented the deterioration of airway disease caused by further allergen challenge (531).

2.7 TLRs in Immunotherapy

As discussed, TLRs have the capacity to promote pathogenic or tolerogenic immune responses to an allergen. Thus, continued efforts are necessary to define the precise mechanisms that underlie this allergenicity or immunogenicity of microbial exposures and the relevant TLR ligands such that they may be harnessed for therapeutic purposes. Allergen-specific immunotherapy (SIT) is the oldest form of treatment for asthma, having existed for nearly a century (Freeman J Lancet, 1914, cited by (544)) and is the only existing treatment that can modify the underlying atopic condition and natural history of the disease. SIT has been reported to dampen both early- and late-phase allergic responses in the skin, nose and lungs (545;546) and has demonstrated successful immunomodulation of ragweed, grass pollen, birch pollen, cat and dust mite allergen sensitization (547-550). Randomized controlled trials and recent meta-analyses have shown that SIT is effective in reducing symptoms of allergic rhinitis and asthma (551-560) and can also prevent new sensitization to further allergens (561) as well as the progression of upper airway disease to asthma (562-566). The protective effects conferred by SIT can also be long-lasting, having been sustained up to 7 years after completion of the therapeutic regimen (562;566). Traditionally, SIT aimed to achieve desensitization by subcutaneous injection of incrementally increasing doses of allergen extract over an extended period of time (567). This was associated with reductions in allergen-specific IgE levels in the blood (548;568;569) but was subsequently shown to inhibit $CD4^+$ Th2 responses (570;571). SIT was suspected to elicit immune deviation of T cell responses towards the Th1 phenotype (570;572). However, later studies indicated that the normal immune response of healthy individuals to allergen and also successful SIT in allergic subjects, were associated with suppression of both antigen-specific

Th1 and Th2 responses, and were rather characterized by T cell production of IL-10 and TGF-β cytokines, as well as B cell production of IgA and IgG4 antibodies (287-289). Furthermore, bee keepers who became naturally desensitized to bee venom as a result of recurrent insect stings, exhibited enhanced IL-10 cytokine production from T cells and elevated numbers of T cells of the regulatory phenotype (255). Therefore, successful SIT may promote immune suppression, more so than immune deviation, that is frequently associated with the enhancement of both $Foxp3^+$ Treg (257;258) and inducible Tr1 responses (253;254;256) involving IL-10 and/or TGF-β, which contribute to B cell production of the mentioned neutralizing antibodies (290;291). One of the potential dangers of SIT is adverse anaphylactic reactions to the injected allergen and therefore alternative strategies are in development to enhance the immunogenicity, or promotion of favourable immune responses towards the allergen, and to curtail allergenicity. For example, recombinantly engineered hypoallergenic derivatives (573-576) may be preferable over the more crude allergenic extracts. Peptide immunotherapy, or application of short T cell epitopes rather than the intact antigen, is also aimed to generate tolerogenic immune responses while avoiding the cross-linking of the antigen on IgE molecules which triggers adverse reactions (577). Additionally, innate immune stimuli such as TLR ligands may be administered either admixed, or upon conjugation, with the allergen to enhance the immunogenicity of the treatment while permitting lower doses of allergen injection. TLR7 and 9 ligands, in particular, can generate protective immune responses against developing or even established type 2 inflammation while also appearing to carry the lowest potential of adverse pro-inflammatory immune responses (534). CpG ODN (578), TLR2 ligands (579;580) and heat-killed *Mycobacterium vaccae* (581) have demonstrated efficacy in murine models of SIT. Thus far, only the TLR9 immunostimulatory CpG ODN sequences (582;583) and the TLR4 ligand monophosphoryl lipid A (584-587) have progressed to clinical trials.

Another important consideration in immunotherapeutic regimens is the route of administration. Subcutaneous allergen-specific immunotherapy (SCIT) is the

traditional modality, although sublingual immunotherapy (SLIT) is emerging as a safer clinical alternative (588-590). In animal models of disease, intranasal and oral IT are most common (591). Mucosal IT may be advantageous given that the mucosal immune network of the respiratory, gastrointestinal and reproductive tracts serves as a connected gateway to foreign substances; antigen-specific IgA responses can be detected as remotely as the vaginal and rectal mucosa of mice following intranasal vaccine delivery (592;593). The nasal mucosa is a potent inductive site for both humoral and cell-mediated immunity. It is also important in the induction of antigen-specific tolerance to nasally deposited antigen (482;594) and can mediate the downregulation of antigen-specific IgE and delayed-type hypersensitivity (594-596). Thus, the nasal mucosa supports both potentiating and tolerogenic regulatory signals for the immune system (597). In rodents, the primary immunologically relevant organized structures in the nasal mucosa are the nasopharynx-associated lymphoid tissues (NALT), found dorsal to the cartilaginous soft palate. The functionally comparable structure in humans is considered to be the Waldeyer's ring, which is comprised of the unpaired nasopharyngeal tonsils (adenoids) and paired palatine and lingual tonsils (reviewed in (598)). Human NALT is more strategically situated such that it is exposed to both airborne and alimentary antigens. NALT consists of follicle-associated epithelium with antigen-sampling M cells, dendritic cells, macrophages and T and B cell-rich areas. Recently, the antigen-sampling M cells that are also common to the gut-associated lymphoid tissues, have been shown to be capable of inducing immune responses on their own, even in Id2 knockout mice in which NALT formation is absent (599). Furthermore, the draining cervical lymph nodes have been shown to be critical in propagating immune responses from the NALT to other effector sites in the respiratory mucosa and can also directly receive incoming soluble antigens that cross the nasal epithelium not associated with the NALT ((594) and reviewed in (600)). The NALT is preferentially primed to mount secretory IgA-producing B cell responses (601) and accumulating evidence also supports a major role for NALT in humoral immunity of the respiratory tract (598). Depending on the specific mucosal adjuvant or infectious agent, nasal

immunization has also been shown to induce antigen-specific Th1, Th2 or Th17 responses (592;602-606). Furthermore, Treg responses can be induced by antigen stimulation of the nasal mucosa (482;607). Thus, the nasal mucosa is capable of serving as a potent inductive site for the generation of diverse T cell and antibody responses by mucosal adjuvants. With this in mind, in the third and fourth chapters, we investigate the potential of a mucosal vaccine adjuvant, Protollin^{TM}, consisting of TLR2 and TLR4 ligands, to prevent the development of acute experimental asthma in pre-sensitized mice.

2.8 Mechanisms of TLR regulation of adaptive immunity

LPS is an example of a TLR ligand that can support the development and/or function of each major $CD4^+$ T cell subset (Th1, Th2, Th17, Tfh and Treg) depending on the route of its administration, dose and differences in the cytokine microenvironments of various tissues, such as the lungs, spleen, lymph nodes and the gut ((489;608;609) and reviewed in (610)). Inhalation of increasing doses of LPS primes Th2 (403), Treg (unpublished data from our lab), Th17 (134), or Th1 (406) responses to allergen, though the precise thresholds for such responses are difficult to pinpoint. As correctly proposed by Janeway, it is now well recognized that a primary mechanism through which TLRs accomplish their modulation of adaptive immunity is by facilitating antigen presentation through MHC II (372;611;612) and inducing the synthesis of costimulatory or coinhibitory molecules on APCs (also on various structural cells; epithelial, smooth muscle, fibroblast and endothelial cells), such as CD40 (613), OX40-L (417) and those belonging to the B7 superfamily (B7.1, B7.2, B7-H1 (or PD-L1), B7-H2 (or ICOS-L), B7-H3) (614-617). These molecules are selectively regulated by MyD88 and TRIF-dependent pathways. TLRs also influence the expression on APCs of molecules belonging to the Notch family (Notch 1, Jagged-1, Delta-1 and Delta-4), shown to rely on MyD88-dependent signaling (418;491;618). In tandem with TLR-induced cytokines, these molecules dictate whether the APCs instruct immunomodulatory T cell responses, in other words biasing towards the Th1, Th2, or Th17 phenotype, or immunosuppressive responses, involving anergy

or Tregs. However, TLR ligands can also stimulate other innate immune cells, such as macrophages, mast cells, eosinophils, neutrophils and basophils to influence adaptive immunity and can additionally act directly upon B cells (619) or T cells (620;621) to affect their function. Various murine B cell subsets, including truly naïve B cells, have been shown to express multiple TLRs, the direct stimulation of which *in vitro* results in their proliferation and antibody secretion even in the absence of antigenic stimulation (or B cell receptor cross-linking), DC activation, or T cell help (622). Mice that are deficient specifically in B cell TLR signaling fail to mount antibody responses to protein antigens given with adjuvant, although this depends on the nature of the antigen, adjuvant and the specific antibody isotype (623;624). Direct stimulation of TLRs on T cells has been explored primarily in Tregs, perhaps due to the elevated level of expression of TLRs in this population (reviewed in (625)). Although human $CD4^+$ and $CD8^+$ T cell subsets express transcripts for all TLRs, as well as MyD88, MD-2 and CD14 (TRIF was not analyzed), the level of expression is 10-1000 times lower than that in B cells or monocytes (626). However, expression of TLRs 4, 5, 7 and 9 has been demonstrated in both human and murine $CD4^+$ T cells (though this may be contingent upon activation of the cells) and are preferentially expressed by Tregs (229;627-629). TLRs 1, 2 and 6 are more broadly expressed by T cells but also directly affect Tregs (630;631). MyD88-dependent mechanisms appear to be important for both B cell and T cell-intrinsic TLR signaling, though a role for TRIF is also beginning to be described (502;632-637). By examining Protollin's effects upon allergic airway disease, we additionally attempt to provide, in the third and fourth chapters, novel insight into the mechanisms of TLR-mediated control of adaptive immune responses.

3 Aims & Hypotheses

(1) To investigate the role of oxidative stress and the TLR4-TRIF pathway in the pathogenesis (allergic sensitization) and pathophysiology (secondary response) of birch pollen-induced airway disease.

Hypothesis: Oxidative stress is an important process in allergic sensitization as well as amplification of the inflammatory response upon secondary exposure to allergen due to its activation of the TLR4-TRIF pathway.

(2) To investigate the immuno-regulatory or -modulatory potential of a TLR2/4 ligand, Protollin, in acute experimental asthma and to characterize the TLR-mediated $CD4^+$ T cell response.

Hypothesis: Protollin prevents the development of type 2 airway disease in mice TLR-dependently and by promoting Treg or Th1 responses.

(3) To determine the role of the MyD88 and TRIF signaling pathways in Protollin's immunomodulation of the $CD4^+$ T cell response and experimental allergic asthma.

Hypothesis: Protollin's inhibition of type 2 airway disease and modulation of $CD4^+$ T cell responses is MyD88-dependent.

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CHAPTER 2

Inhaled birch pollen extract induces airway hyperresponsiveness via oxidative stress but independently of pollen-intrinsic NADPH oxidase activity, or the Toll-like receptor 4-TRIF pathway

2.1 Prologue

In this study, we aimed to examine the danger signals associated with the birch pollen allergen, specifically which would evoke allergic sensitization and/or promote the secondary inflammatory response to an extract of this natural aeroallergen. A number of studies have shown that TLR4-MyD88 signaling is important in the development of experimental allergic airway disease to house dust mite allergen extract or ovalbumin that is contaminated with LPS. Furthermore, pollen-intrinsic NADPH oxidase activity has been proposed as another mechanism which can promote a type 2 immune response to an allergen, while oxidative stress and cysteine proteases may also be capable of stimulating a Th2 response through TLR4-TRIF activation. Therefore, using a murine model of allergic sensitization and challenge exclusively via the airway mucosa without adjuvant, and an allergen extract which should not be contaminated with significant levels of LPS, but which is known to possess intrinsic NADPH oxidase and cysteine protease activity, we hypothesized that oxidative stress would be an important factor in the allergic disease process and possibly due to its activation of the TLR4-TRIF pathway. We were particularly interested in whether these mechanisms prime the immune response to elicit sensitization given that this area has been less extensively investigated.

2.2 Abstract

Oxidative stress in allergic asthma may result from oxidase activity or pro-inflammatory molecules in pollens. Signaling via TLR4 and its adaptor TRIF has been implicated in reactive oxygen species (ROS)-mediated acute lung injury and in T helper 2 immune responses. We investigated the contributions of oxidative stress and TLR4/TRIF signaling to experimental asthma induced by birch pollen exposure exclusively via the airways. Mice were exposed to native or heat-inactivated white birch pollen extract intratracheally and injected with N-acetyl-L-cysteine (NAC) prior to sensitization, challenge, or all allergen exposures, to assess the role of oxidative stress and pollen-intrinsic NADPH oxidase activity in allergic sensitization, inflammation and airway hyperresponsiveness (AHR). Additionally, TLR4 signaling was antagonized concomitantly with allergen exposure, or the development of allergic airway disease was evaluated in TLR4 or TRIF knockout mice. NAC inhibited eosinophilic airway inflammation and AHR induced by inhaled birch pollen extract, even when administered only with the allergen challenge, but not when given exclusively during sensitization. Heat-inactivation of the pollen had no effect on the development of allergic airway disease and oxidative stress-mediated AHR was TLR4- and TRIF- independent. However, TLR4 deficiency reduced the birch pollen-induced airway inflammation and Th2 response, whereas TRIF deficiency augmented airway inflammation. In conclusion, oxidative stress mediates selective aspects of allergic sensitization to pollen via the airway mucosa, and promotes AHR to inhaled birch pollen independently of the pollen-intrinsic NADPH oxidase activity and TLR4-TRIF signaling. Pollen-induced airway inflammation is enhanced by TLR4 activation and restrained by TRIF-dependent pathways.

2.3 Introduction

Allergic asthma is a chronic airway disease triggered by an aberrant inflammatory response to inhaled allergens, resulting in airway inflammation, hyperresponsiveness to inhaled bronchoconstrictors and eventual progression to airway remodeling. Allergic asthma is characterized by a T helper (Th) 2-predominant T cell response. There is emerging interest in identifying common intrinsic properties of allergens, or allergen-associated danger signals that impart allergenicity to certain proteins. Oxidative stress has been implicated in airway diseases, such as asthma (1) and may be a critical mechanism evoking allergic sensitization upon initial allergen exposure and/or amplifying inflammatory responses upon secondary allergen exposure. Markers of oxidative stress have been detected in the lungs of patients with asthma caused by the release of reactive oxygen and nitrogen species (ROS & RNS) from airway epithelial cells (2), upon direct exposure to allergens or environmental pollutants, such as cigarette smoke, diesel exhaust (3) and ozone, as well as from inflammatory cells responding to these environmental stresses. Lower levels of the antioxidant, glutathione (4), as well as reduced activity of major antioxidant enzymes, such as superoxide dismutase (5), are also reported in asthmatics, and associated with airflow obstruction, airway hyperresponsiveness (AHR) and airway remodeling.

Oxidative stress may result not only from activation of inflammatory cells and structural cells but also pollens, such as ragweed and birch, that have intrinsic NADPH oxidase activity and have been shown to rapidly trigger the production of ROS within the airway epithelium upon topical exposure (6). Using a systemic sensitization of mice with ragweed and aluminum hydroxide adjuvant, followed by subsequent intranasal exposure to ragweed, Boldogh et al. found that a pollen-intrinsic capacity to generate oxidative stress amplified allergic airway inflammation (6). This effect could be prevented by concomitant intranasal administration of antioxidants (7). To date, it has not been investigated whether pollen-intrinsic NADPH oxidase activity is important in eliciting allergic sensitization.

The Toll-like receptor (TLR) 4 has also been implicated in mediating inflammatory responses in experimental allergic asthma. Der p 2, an antigen found in house dust mite allergen extracts (HDM), was demonstrated to mimic a component of the TLR4 signaling complex, MD2, both structurally and functionally (8). Importantly, TLR4 expression and signaling specifically on airway structural cells was shown to be necessary for the establishment of inflammation and AHR in response to inhaled HDM (9). TLR4 functionality on stromal cells was also found to be critical for the development of Th2-mediated airway disease induced by inhaled lipopolysaccharide (LPS)-laden ovalbumin (OVA) (10). Thus, there is evidence to indicate that TLR4 plays a significant role in compromising mucosal tolerance to elicit allergic sensitization, and/or in driving inflammatory responses to antigens via the airways.

The pathways of oxidative stress and TLR4 activation may also be interlinked. For example, studies in mice have revealed a role for TLR4 in mediating ozone-induced airway disease (11;12). Recently, intradermal injection of the cysteine protease papain was shown to induce the production of ROS in the skin epithelium and dermal dendritic cells of mice, eliciting a potent antioxidant (N-acetyl cysteine)-sensitive Th2 and basophil response via the TLR4 pathway and its adaptor, Toll-Interleukin 1 Receptor domain-containing adaptor inducing Interferon-β (TRIF) (13). The TLR4-TRIF pathway was suggested to be activated by oxidized phospholipids produced in the lungs of humans and animals infected with SARS, anthrax or H5N1 and implicated in triggering TLR4-TRIF-mediated acute lung injury induced by acid or inactivated H5N1 avian influenza virus (14). Interestingly, pollens such as white birch and ragweed, contain readily releasable cysteine (as well as serine) proteases, the role of which is undefined in the allergic airway response (15). In addition, pollens also release bioactive lipids such as the prostaglandin-like phytoprostanes (16). These are formed via a series of auto-oxidation steps, initiated by free radical attack of α-linolenic acid. Notably, concentrations of linolenic and linoleic acid in pollen are high, lending support to the possibility of the respiratory tract encountering biologically significant concentrations of oxidized derivatives of these fatty acids (17). Whether these

factors, as well as the protease activity of pollens, are capable of triggering TLR4 signaling via TRIF, and the relevance of this pathway to the development of allergic airway disease has not been examined. Thus, we hypothesized that oxidative stress, potentially activating the TLR4-TRIF pathway, is important in mediating allergic sensitization via the airway mucosa, and promoting experimental asthma to inhaled birch pollen allergen. We aimed to determine whether oxidative stress has a role in mediating airway disease when animals are exposed to pollen exclusively via the airways, in the absence of systemic sensitization with an exogenous adjuvant, such as alum. Secondly, we wished to delineate whether pollen-intrinsic NADPH oxidase activity and oxidative stress were critically involved in the initial stages of sensitization, or played a greater role in driving aspects of allergic inflammation during secondary allergen exposure of already sensitized animals. Thirdly, we examined the role of TLR4-TRIF signaling in the processes of sensitization and responses to challenge. We determined that oxidative stress does not mediate mucosal sensitization to pollen leading to AHR and eosinophilic airway inflammation, but rather is critical during secondary allergen exposure for the development of these aspects of airway disease. AHR develops independently of pollen-intrinsic NADPH oxidase activity, TLR4 and TRIF, whereas pollen-induced airway inflammation is enhanced by TLR4 activation and dampened by TRIF-dependent pathways.

2.4 Methods

Animal Treatments

Six to eight week-old, female BALB/c mice were purchased from Charles River, Canada. TLR4 knockout mice on a BALB/c background were bred by Dr. S.T. Qureshi in the Animal Care Facilities of the McGill University Health Centre. TRIF knockout mice on a C57Bl/6J background, as well as wild-type controls, were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in a conventional or specific pathogen-free animal facility under a 12 hour light/dark cycle with free access to food and water. Animals received *Betula populifolia*, white birch pollen extract (BPEx; Greer Laboratories, Lenoir, NC, U.S.A.) exclusively via intratracheal (i.t.) administration under isoflurane anesthesia; anesthetized mice were administered 100 µg of BPEx into the posterior pharynx in a volume of 60 µl sterile PBS (18). In this manner, animals were sensitized on days 0 and 7 and challenged on days 14 and 15. Alternatively, control mice received only PBS on each of these days. Experimental procedures were approved by the McGill University Animal Care Committee. The endotoxin concentration of BPEx, assessed by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA), was 150 EU/mg protein (less than $1/10^{th}$ of the level in ovalbumin).

Antioxidant administration concomitant with allergen exposure, sensitization, or challenge

To study the role of oxidative stress in eliciting and/or promoting airway disease to inhaled BPEx, the antioxidant N-acetyl-L-cysteine (NAC; Sigma-Aldrich, U.S.A.) was administered intraperitoneally 1 hour prior to each i.t. BPEx exposure, prior to the sensitizations (days 0 and 7) only, or prior to the challenges (days 14 and 15) only, at a dose of 150 mg/kg in 0.2 ml PBS (comparable to previously reported doses that have effectively inhibited oxidative stress in mice (7;19;20)). Control animals were injected only with sterile PBS. We also tested

150 mg/kg NAC administered topically and concomitantly with the allergen, in the same intratracheal 60 µl bolus with BPEx.

Assessment of BPEx NADPH oxidase activity

To confirm the presence of NADPH oxidase activity in BPEx, aliquots prepared at 100 µg/ 60 µl sterile PBS, stored at -20^{o}C, were thawed and 100 µg of BPEx was incubated at 37^{o}C for 30 minutes with 2 mM nitroblue tetrazolium (NBT), in the presence or absence of 1 mM NADPH, in a total volume of 0.4 ml PBS. Alternatively, the NADPH oxidase inhibitor diphenylene iodonium, DPI (100 µM), was added during the incubation of BPEx with NBT and NADPH, or BPEx was heat-inactivated at 95^{o}C for 15 minutes prior to this incubation. Solutions were then centrifuged at 8000 g for 4 minutes, washed once with 1 ml distilled water, after which the pellet was allowed to dissolve within 5 minutes in 0.4 ml methanol. Following centrifugation, the supernatants were plated and read using the Infinite M1000 PRO microplate reader (Tecan, Männedorf, Switzerland) at an absorbance of 530 nm.

Sensitization or challenge with heat-inactivated birch pollen extract

To investigate the role of birch pollen-intrinsic enzymatic, particularly NADPH oxidase activity, in mediating allergic sensitization via the airway mucosa, as well as in the secondary exposure to allergen, animals received i.t. administrations of heat-inactivated BPEx (BPEx^{H}; 95^{o}C, 15 mins) either on days 0 and 7, or on days 14 and 15. The same groups of animals received intact BPEx on days 14 and 15, or on days 0 and 7, respectively, and thus, enzymatic activity of the pollen extract was abolished either during the sensitization or challenge, respectively. Control animals received PBS on days 0 and 7 and intact BPEx on days 14 and 15, or were given intact BPEx throughout all of the exposures.

Airway TLR4 antagonism concomitant with allergen exposure, sensitization, or challenge

In order to study the effect of blocking TLR4 signaling during each airway exposure to pollen extract, in some experiments, PBS or BPEx (100 μg) was delivered i.t. to wild-type mice in combination with 1 μg TLR4-antagonizing LPS, *Rhodobacter sphaeroides* LPS (LPS-RS; Invivogen, San Diego, CA, U.S.A.) on days 0, 7, 14 and 15. A dose of 1 μg TLR4-antagonizing LPS has been previously shown to prevent the induction of HDM-induced airway disease (9). To investigate the role of TLR4 signaling exclusively during the sensitization or challenge phase, some mice received BPEx with LPS-RS only on days 0 and 7 (group identified as αTLR4 BPEx/ BPEx), or 14 and 15 (BPEx/ αTLR4 BPEx).

Assessment of allergen-induced airway hyperresponsiveness

On day 17, 48 hrs after the final allergen challenge, mice were anesthetized with an injection of xylazine hydrochloride (10 mg/kg, i.p.) followed by sodium pentobarbital (32 mg/kg, i.p.). Mice were tracheostomized using a 19G metal cannula and ventilated with a small animal ventilator (FlexiVent, SCIREQ Inc., Montreal, Canada) at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end-expiratory pressure of 3 cmH_2O. The mice were paralyzed with pancuronium bromide (1 mg/kg, i.p.) and subjected to lung inflations to a transrespiratory pressure of 30 cm H_2O to standardize volume history prior to the measurement of baseline respiratory mechanics. A 1.2 second, 2.5 Hz single-frequency forced oscillation maneuver was performed at 10 sec intervals and respiratory system resistance (Rrs) and elastance (Ers) were calculated with commercial software (SCIREQ Inc.). Doubling concentrations of methacholine (MCh; acetyl-β-methylcholine; Sigma-Aldrich, USA) from 15.6 – 250 mg/kg were delivered to the mouse as an aerosol using a 4s nebulization period synchronized with inspiration, at a nebulization duty cycle of 50%. Allergen-induced AHR was assessed by MCh-induced bronchoprovocation by recording the peak Rrs and Ers for each dose of MCh administered.

Airway Inflammation

On day 17, following lung function measurements, bronchoalveolar lavage (BAL) was performed using saline containing 10% fetal bovine serum. The recovered cell pellet was used to measure the total number of cells in the BAL and cytospins were prepared and stained with Diff-Quik stain (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany) for differential cell counts. Cytokines in BAL supernatants were assayed using an ultrasensitive Th1/Th2 multiplex and SECTOR Imager 2400 (Meso Scale Discovery, Gaithersburg, MD, U.S.A.), according to the manufacturer's instructions. The lower limit of detection (LLOD) for each represented cytokine was as follows: IL-4, 0.87 pg/ml; IL-5, 0.70 pg/ml; IL-12p70, 5.3 pg/ml; KC, 2.9 pg/ml; IL-10, 11 pg/ml. Eotaxin was measured in BAL supernatants using a human eotaxin 1 ELISA that is 100% cross-reactive with mouse eotaxin 1 (PeproTech Canada, Dollard-des-Ormeaux, QC, Canada; LLOD of 23 pg/ml).

Measurement of BPEx-specific Serum IgE

On day 17, blood was collected from mice by exsanguination in serum separator tubes and left at room temperature to clot. Samples were centrifuged at 4000 g for 5 minutes and the serum was collected and stored at -20^{o}C. BPEx-specific serum IgE was measured by ELISA using a modified commercial assay (Biolegend, San Diego, CA, U.S.A.) such that the plates were coated overnight with 20 µg/ml BPEx rather than anti-IgE capture antibody and samples were diluted ten times in assay diluent prior to their incubation. We tested two concentrations (20 or 40 µg/ml) of BPEx to coat the plates as well as IgG precipitating agarose beads. Both concentrations of BPEx gave similar results and IgG precipitation did not alter the sensitivity of the assay. An internal positive control sample was included.

Assessment of Allergen-Induced Oxidative Stress

To confirm that exposure of animals to birch pollen exclusively via the airways resulted in measurable oxidative stress, an end-product of lipid peroxidation, 4-hydroxynonenal (4-HNE) was quantified by gas chromatography-mass spectrometry as previously described (21) in snap-frozen lungs harvested either 30 minutes after a single intratracheal BPEx exposure to assess immediate allergen-induced oxidative stress occurring prior to the recruitment of inflammatory cells to the lungs, or 24 hrs after final allergen challenge (day 16).

Statistical Analysis

Airway responses to MCh bronchoprovocation were analyzed in GraphPad Prism Version 5 by two-way ANOVA followed by Bonferroni post-tests comparing all experimental groups to each other. One-way ANOVA and post-hoc Newman-Keuls' tests were used for all other analyses, such as BAL inflammatory cell and cytokine data, involving three or more groups, or unpaired Student's t-test was used in the case where only two experimental groups were compared. Data were log-transformed prior to statistical analysis when not normally distributed.

2.5 Results

Oxidative stress mediates inhaled allergen-induced AHR during secondary allergen exposure

To examine whether oxidative stress associated with sensitization and challenge with BPEx was necessary for the development of AHR, NAC was administered intratracheally in the same bolus with BPEx either during sensitization (days 0 and 7) or challenge (days 14 and 15). Otherwise, these animals received BPEx alone during challenge or sensitization, respectively. All animals unexpectedly exhibited respiratory distress and died within 72 hrs of intratracheal NAC exposure. pH-adjusted (pH 7), sterile-filtered NAC was better tolerated but augmented BPEx-induced airway inflammation, particularly when administered with BPEx challenge, thus demonstrating irritant effects upon the airways at this dose (Supplementary Figure 1). Therefore, we proceeded to administer NAC by the more common intraperitoneal route, prior to each intratracheal BPEx exposure on days 0, 7, 14 and 15. Mice receiving BPEx following PBS (sham) i.p. injection, exhibited significant AHR to methacholine compared to mice that received just PBS inhalations (Figure 1A, B) (Note: PBS + NAC treated animals (i.e. animals injected with NAC 1 hr prior to PBS inhalations) had equivalent methacholine and airway inflammatory responses as animals treated with PBS only (see Figures 4 and 5)). Notably, mice injected with NAC 1 hr prior to each BPEx exposure (BPEx + NAC) had significantly lower airway responses (lower Rrs and Ers values) compared to BPEx mice. Furthermore, BPEx + NAC mice had no significant difference in Rrs compared to PBS + NAC mice (Figure 1A), and only had an elevated Ers at the highest dose of MCh (Figure 1B), indicating a slightly augmented peripheral airway response. Thus, the administration of NAC prior to each allergen exposure essentially prevented the development of AHR.

To assess whether oxidative stress specifically during secondary allergen exposure was necessary for the induction of AHR by BPEx, we administered NAC prior to challenges only, on days 14 and 15. This BPEx/ BPEx + NAC group also had significantly reduced Rrs and Ers responses compared to BPEx

mice (Figure 1A, B). The BPEx/ BPEx + NAC group also showed no difference in Rrs and only an elevated Ers at the highest MCh dose, compared to PBS + NAC mice, indicating that the administration of NAC during the challenge, or secondary exposure to allergen in pre-sensitized mice, was sufficient to achieve almost complete inhibition of AHR. In contrast, NAC administration exclusively prior to BPEx sensitization on days 0 and 7 (BPEx + NAC/ BPEx) did not significantly reduce airway responsiveness compared to BPEx mice. Also, both Rrs and Ers were significantly elevated in these mice compared to PBS + NAC mice. Thus, NAC administration during allergic sensitization alone did not prevent the development of AHR to inhaled BPEx.

BPEx-specific serum IgE was significantly augmented in BPEx-exposed mice compared to PBS-exposed mice or mice that received NAC prior to each BPEx exposure (Figure 1C). Furthermore, NAC injection during sensitization or challenge alone was sufficient to prevent the BPEx-induced augmentation in IgE, indicating that oxidative stress in both sensitization and challenge was necessary for the induction of IgE. All groups that received NAC had comparable BPEx-specific serum IgE levels to PBS-exposed mice.

Oxidative stress influences airway inflammation primarily during secondary allergen exposure and not sensitization

Total BAL inflammatory cell numbers were significantly lower in PBS + NAC and BPEx + NAC mice compared to BPEx mice, indicating that the injection of NAC prior to each BPEx exposure reduced airway inflammation (Figure 2A). NAC administration throughout the course of BPEx exposures, or exclusively during the challenge period (days 14 and 15), significantly reduced BAL eosinophilia, whereas NAC injection during the sensitization phase alone failed to do so (Figure 2B). Compared to sham (PBS)-treated mice, however, all groups had airway eosinophilia indicating that antioxidant administration only partially inhibited eosinophilic inflammation.

Neutrophil numbers were significantly elevated in all groups compared to PBS + NAC and, unlike eosinophilia, BAL neutrophilia was unaffected by antioxidant

administration (Figure 2C). Lymphocytic inflammation, however, was influenced by oxidative stress in both sensitization and challenge phases, as NAC injection during either of these periods reduced the influx of lymphocytes to the airway lumen (Figure 2D). Thus, oxidative stress during allergic sensitization influences only later lymphocytic airway inflammation, without impact upon the development of eosinophilic and neutrophilic inflammation. The relative percentages of macrophages, neutrophils and lymphocytes in the BALs of all NAC injected mice were similar to those of BPEx mice (data not shown); oxidative stress during the secondary response to allergen particularly influenced eosinophilic inflammation, as the proportion of BAL eosinophils was greater than 20% in BPEx mice, compared to about 10% in BPEx/ BPEx + NAC mice (Supplementary Figure 2A). Also, BPEx/ BPEx + NAC mice had a significantly lower percentage of BAL eosinophils compared to BPEx + NAC/ BPEx mice.

Intratracheal BPEx instillation resulted in the induction of a number of pro-inflammatory cytokines in the mouse BAL fluid, including IL-4, IL-5, IL-12 and KC (Figures 2E-H). Systemic NAC injection coinciding with all BPEx exposures completely abrogated the induction of IL-4. However, BAL IL-5, IL-12 and KC, although significantly inhibited by NAC administration, remained slightly elevated compared to PBS + NAC mice. BPEx-induced TNF-α production in the airways was insensitive to NAC (data not shown).

BPEx-intrinsic NADPH oxidase activity does not contribute to allergic sensitization via the airway mucosa or airway disease

BPEx-intrinsic NADPH oxidase activity and its capacity to generate ROS was confirmed by NBT assay. BPEx generated significant ROS production only in the presence of NADPH substrate (Figure 3A). ROS production by BPEx was diminished in the presence of the NADPH oxidase inhibitor, DPI, and completely abrogated by prior heat-inactivation of BPEx ($BPEx^{H}$). ROS levels in the presence of DPI or $BPEx^{H}$ were not significantly different from levels in PBS. Thus, in the absence of cellular material, the BPEx-intrinsic capacity to generate ROS was entirely NADPH oxidase-dependent. To investigate the importance of

this property of BPEx in sensitization via the airway mucosa, or in promoting the inflammatory response to secondary allergen exposure, animals received $BPEx^{H}$ either during sensitization or challenge and otherwise received intact BPEx. We confirmed that exposure to BPEx on days 0 and 7 is necessary for sensitization leading to the development of airway disease upon secondary allergen exposure on days 14 and 15, as control animals that received PBS on days 0 and 7 and intact BPEx on days 14 and 15 (PBS /BPEx) exhibited significantly lower airway responses to MCh compared to animals that received BPEx throughout both sensitization and challenge (BPEx /BPEx) (Figure 3B). Sham-sensitized animals also had lower BAL eosinophilia (Figure 3D) and lymphocytosis (Figure 3F), but dramatically elevated neutrophilia (Figure 3E), compared to BPEx/BPEx animals. Neither sensitization of animals with $BPEx^{H}$, nor challenge with $BPEx^{H}$ altered airway responsiveness or inflammation compared to BPEx/BPEx animals. Thus, the enzymatic activity of BPEx, or NADPH oxidase activity, made no contribution to allergic sensitization via the airway mucosa or alternatively, to the promotion of airway disease upon secondary allergen exposure in animals sensitized with intact BPEx.

Furthermore, we sought evidence of early oxidative stress in the lungs occurring within 30 mins of a single intratracheal BPEx exposure, prior to the recruitment of inflammatory cells and potentially elicited by the pollen-intrinsic oxidase activity, through quantification of 4-HNE. However, at this early time point no measurable increase in lung 4-HNE was found in BPEx- compared to PBS-exposed mice (Figure 3C). In contrast, lung 4-HNE levels were significantly augmented on day 16, following BPEx sensitization and challenge, as compared to in PBS-exposed, or NAC-injected mice. Thus, repeated BPEx inhalation was confirmed to induce significant oxidative stress in the lungs, which was completely prevented by systemic antioxidant administration prior to each allergen exposure.

Oxidative stress-mediated AHR to inhaled pollen allergen is independent of TLR4

To investigate the role of TLR4 signaling in the development of allergic airway disease exclusively via airway exposure to BPEx, responses to MCh were assessed in mice in which the TLR4 receptor was antagonized, using *Rhodobacter sphaeroides* LPS, concomitantly with each exposure to BPEx (identified as αTLR4) (Figures 4-6). Administration of αTLR4-LPS with PBS (αTLR4 PBS) had no effect on airway responsiveness compared to animals that just received PBS (Figure 4A, B). Both of these groups, however, had significantly lower Rrs and Ers responses to MCh compared to animals receiving BPEx alone (BPEx), or αTLR4-LPS with BPEx (αTLR4 BPEx). Unlike NAC administration, TLR4 antagonism did not prevent BPEx-induced AHR and in fact, resulted in significantly higher Rrs and Ers ($p < 0.001$ and < 0.05, respectively) at the highest dose of MCh, compared to BPEx animals (Figure 4A, B). TLR4 antagonism concomitant with BPEx sensitization or challenge alone produced no differences in AHR (Figure 6A, B). Furthermore, intratracheal BPEx still produced significant AHR in TLR4 KO mice compared to PBS administration (Figure 4C, D).

TLR4 amplifies airway inflammation caused by inhaled pollen allergen

Pollen-induced airway inflammation was significantly blunted in the absence of functional TLR4, as total BAL fluid inflammatory cell numbers were lower as a result of TLR4 antagonism, as well as in TLR4 KO mice, compared to wt BPEx mice (Figure 5A). Eosinophil numbers in the BAL fluid were significantly lower as a result of TLR4 antagonism, as well as in TLR4 KO mice exposed to BPEx, but still remained elevated compared to the respective negative controls (PBS-exposed mice) (Figure 5B). The percentage of eosinophils in the BAL fluid was also significantly, albeit partially, reduced from nearly 30% in wt mice to 20% in the absence of functional TLR4 (Supplementary Figure 2B). Interestingly, BPEx-induced neutrophil and lymphocyte recruitment to the airway lumen was absent in TLR4 KO mice, but remained significant in αTLR4 BPEx mice in which TLR4 was antagonized concomitantly with BPEx exposures (Figures 5C and D); BPEx

challenged TLR4 KO mice had significantly lower neutrophil and lymphocyte numbers compared to αTLR4 BPEx mice. Thus, functional TLR4 is critical for neutrophil and lymphocyte recruitment to the airway lumen and plays a considerable role in promoting airway eosinophilia following birch pollen inhalation. Finally, TLR4 antagonism concomitant with BPEx challenge resulted in significantly lower eosinophilia compared to TLR4 antagonism exclusively with BPEx sensitization, although no difference was observed in any of the other inflammatory cell types (Figures 6C, D and data not shown). This was accompanied by reduced BAL eotaxin levels as compared to that resulting from TLR4 antagonism exclusively with sensitization (Figure 6E). Thus, TLR4 signaling during secondary allergen exposure, but not sensitization, contributed to eosinophilic airway inflammation.

As in the earlier experiments (Figures 2E-H), intratracheal BPEx instillation significantly augmented IL-4, IL-5, IL-12 and KC levels in the mouse BAL fluid (Figures 5E-H). Surprisingly, the Th1-inducing cytokine IL-12 was still induced by BPEx in the absence of functional TLR4 and was even elevated in TLR4 KO mice, compared to αTLR4 BPEx mice (Figure 5G). Another pro-inflammatory molecule that might have been expected to be inhibited in the absence of TLR4 signaling, KC was also still induced in TLR4 KO mice exposed intratracheally to BPEx (Figure 5H). Induction of IL-1β and TNF-α was not affected by the absence of TLR4, and IFN-γ and IL-2 also showed a similar tendency to be unaffected by lack of TLR4 signaling (data not shown). BAL fluid IL-5 levels were lower in αTLR4 BPEx mice compared to BPEx mice, whereas IL-5 was induced to a similar degree in wt and TLR4 KO mice exposed to BPEx (Figure 5F). Thus, the BAL inflammatory cell and cytokine data show that the induction of the indicated pro-inflammatory cytokines by inhaled birch pollen was partially independent of TLR4 signaling. Only allergen-induced IL-4 and IL-10 were significantly reduced in TLR4 KO mice, or as a result of TLR4 antagonism (Figures 5E and I).

TRIF tempers airway inflammation caused by inhaled pollen allergen

Elevated mRNA expression of the TRIF-inducible type I interferon, IFN-β, was detected in lung tissues of Balb/c mice harvested 30 mins after a single BPEx exposure (Supplementary Figure 2C). TRIF KO mice exposed to BPEx developed AHR comparably to their wt C57Bl/6J counterparts (Figure 7A). However, in contrast to TLR4 deficiency which resulted in reduced airway inflammation, TRIF deficiency caused total BAL inflammatory cells (data not shown), specifically macrophages, eosinophils and lymphocytes (Figures 7B-E), to be significantly augmented in response to BPEx, compared to wt C57 BPEx-exposed mice. This was accompanied by increases in the pro-inflammatory cytokine IL-12 and chemokines KC and eotaxin in the BAL fluid of TRIF knockout mice (Figures 7F-I and Supplementary Figure 2D). Thus, TRIF deficiency resulted in exacerbated airway inflammation.

Figure 1. Antioxidant administration during challenge, but not sensitization, prevents inhaled allergen-induced AHR. Airway responses (respiratory system resistance, Rrs (A) and respiratory system elastance, Ers (B)) were measured in response to increasing doses of aerosolized MCh. Animals were injected i.p. with PBS 1 hr prior to intratracheal BPEx (BPEx), or alternatively, received 150 mg/kg NAC i.p. 1 hour prior to intratracheal PBS or BPEx (PBS + NAC or BPEx + NAC, respectively). Some mice were injected with NAC prior to BPEx sensitization only (days 0 and 7; BPEx + NAC/ BPEx), or challenge only (days 14 and 15; BPEx / BPEx + NAC). The effect of NAC injection prior to BPEx exposures on the induction of specific serum IgE levels (C) was determined by ELISA (n=8-11 animals per group pooled from at least 3 independent experiments). (For clarity, not all statistically significant differences between groups are indicated).

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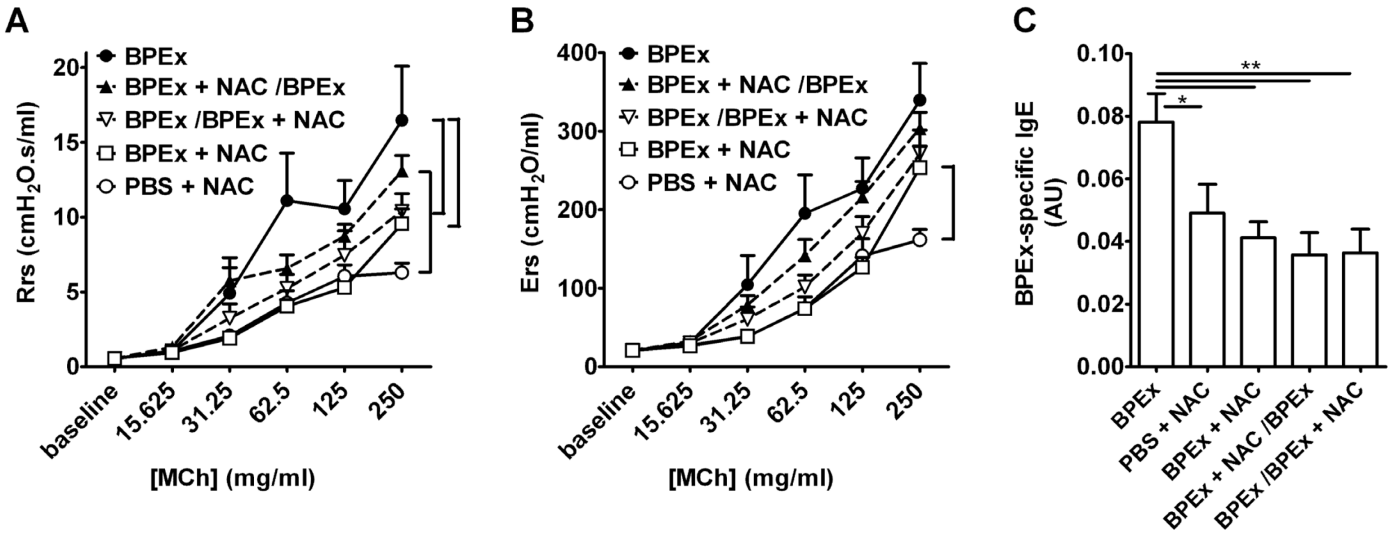
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BPEx + NAC
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baseline
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31.25
62.5
125
250
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BPEx /BPEx + NAC
BPEx + NAC
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300
200
100
0
baseline
15.625
31.25
62.5
125
250
[MCh] (mg/ml)
C
BPEx-specific IgE (AU)
0.10
0.08
0.06
0.04
0.02
0.00
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*
BPEx
PBS + NAC
BPEx + NAC
BPEx + NAC /BPEx
BPEx /BPEx + NAC

Figure 2. Differential effects of antioxidant administration during sensitization or challenge on allergen-induced airway inflammation. Total BAL inflammatory cells (A), eosinophils (B), neutrophils (C) and lymphocytes (D) were determined following intratracheal PBS or BPEx exposure that had been preceded by i.p. NAC injection 1 hr prior to all exposures, sensitization only, or challenge only. Pro-inflammatory cytokines, IL-4 (E), IL-5 (F), IL-12 (G) and KC (H) were measured by an ultrasensitive multiplex assay in the BALs of mice that received either PBS or NAC prior to intratracheal BPEx and control mice that received PBS and NAC, (n=7-8 animals per group pooled from at least 3 independent experiments). (* indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$; # indicates significant difference from all other groups, $p < 0.001$).

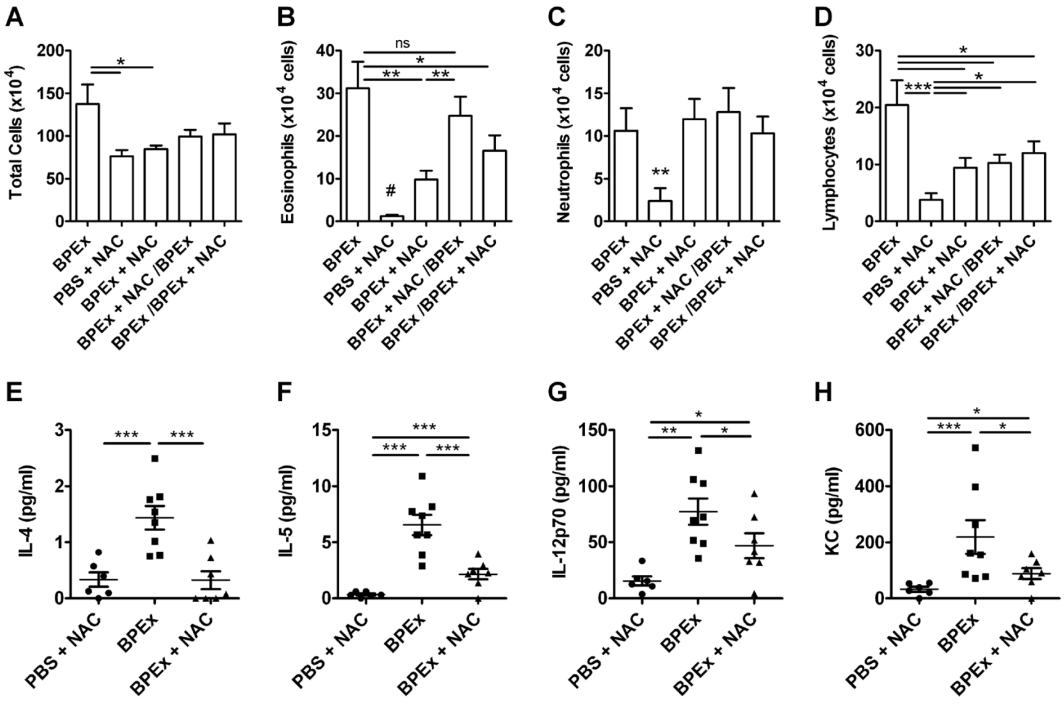

Figure 3. Pollen-intrinsic NADPH oxidase activity does not mediate mucosal sensitization to BPEx or the inflammatory response to secondary allergen exposure. Pollen-intrinsic NADPH oxidase activity was measured by NBT assay and verified by incubation of BPEx with the NADPH oxidase inhibitor DPI, or by prior heat-inactivation ($BPEx^{H}$) (A) (Data pooled from 4 independent experiments). Animals were sensitized or challenged with $BPEx^{H}$ and otherwise received intact BPEx in order to study the contribution of pollen-intrinsic NADPH activity in sensitization or challenge to the development of AHR (B) or BAL eosinophilia (D), neutrophilia (E), or lymphocytosis (F) (n=6-7 animals per group pooled from 3 independent experiments). 4-HNE, a marker of oxidative stress, was measured in lung tissues of mice within 30 mins of a single intratracheal PBS or BPEx exposure, after repeated PBS or BPEx exposure (sensitization and challenge), or after repeated BPEx exposure preceded by i.p. NAC injections (C) (n=5-8 animals per group from two independent experiments).

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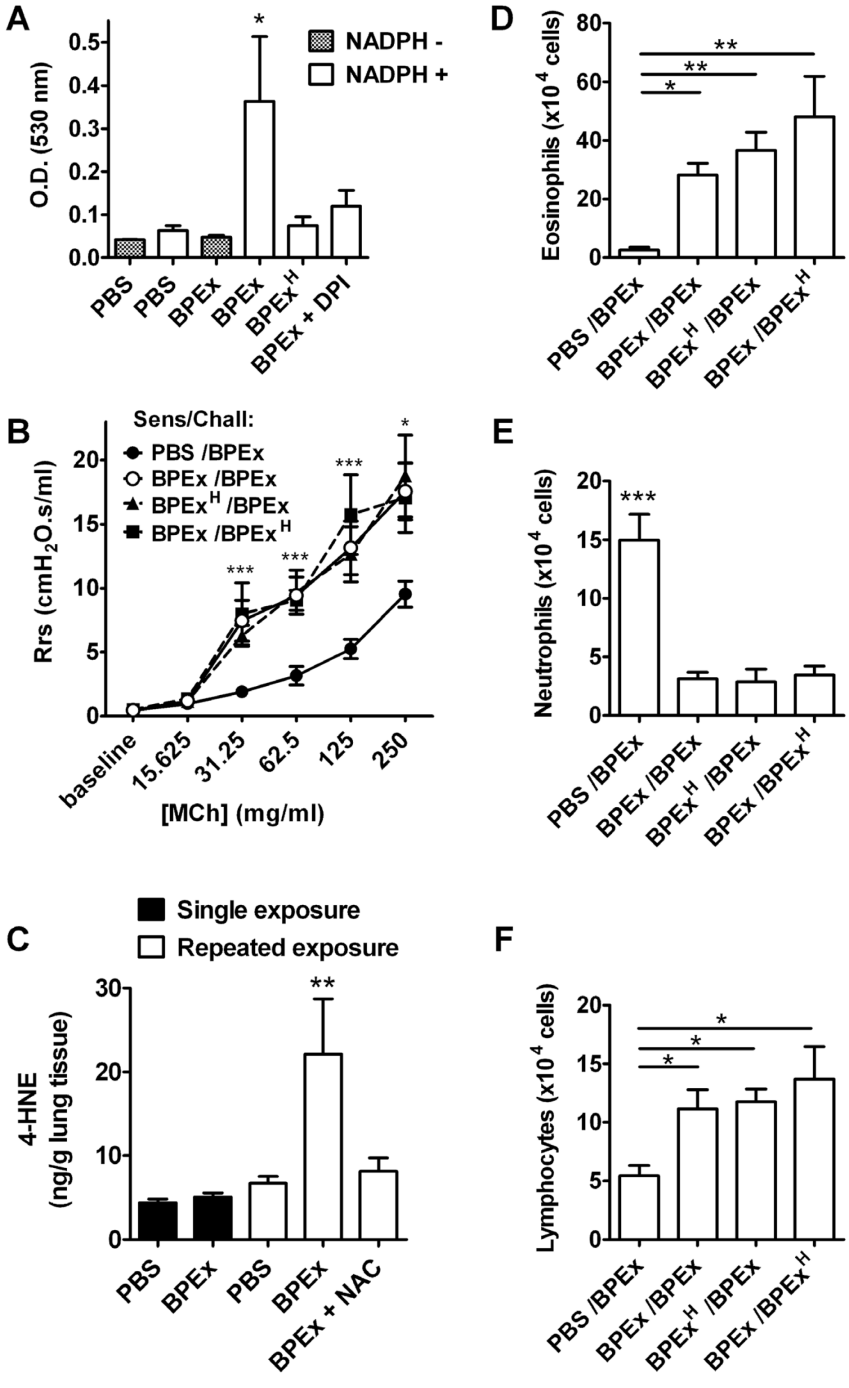
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NADPH +
PBS
PBS
BPEx
BPEx
BPEx H
BPEx + DPI
B
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BPEx /BPEx
BPEx H /BPEx
BPEx /BPEx H
Rrs (cmH2O.s/ml)
baseline
15.625
31.25
62.5
125
250
[MCh] (mg/ml)
C
Single exposure
Repeated exposure
4-HNE
(ng/g lung tissue)
PBS
BPEx
PBS
BPEx
BPEx + NAC
D
Eosinophils (x10 4 cells)
PBS /BPEx
BPEx /BPEx
BPEx H /BPEx
BPEx /BPEx H
E
Neutrophils (x10 4 cells)
PBS /BPEx
BPEx /BPEx
BPEx H /BPEx
BPEx /BPEx H
F
Lymphocytes (x10 4 cells)
PBS /BPEx
BPEx /BPEx
BPEx H /BPEx
BPEx /BPEx H

Figure 4. TLR4 is not necessary for the development of AHR to inhaled BPEx. The effect of TLR4 antagonism coinciding with intratracheal BPEx exposures on airway responses of animals to increasing doubling doses of aerosolized MCh was measured (A, B). Animals received PBS or BPEx intratracheally, with or without 1 μg TLR4-antagonizing *Rhodobacter sphaeroides* LPS (αTLR4 PBS or αTLR4 BPEx) (n=8-12 animals per group pooled from at least 3 independent experiments). Airway responses to MCh were also assessed in TLR4 knockout mice sensitized and challenged intratracheally with BPEx, as compared to PBS (C, D) (n=7 animals per group pooled from two independent experiments).

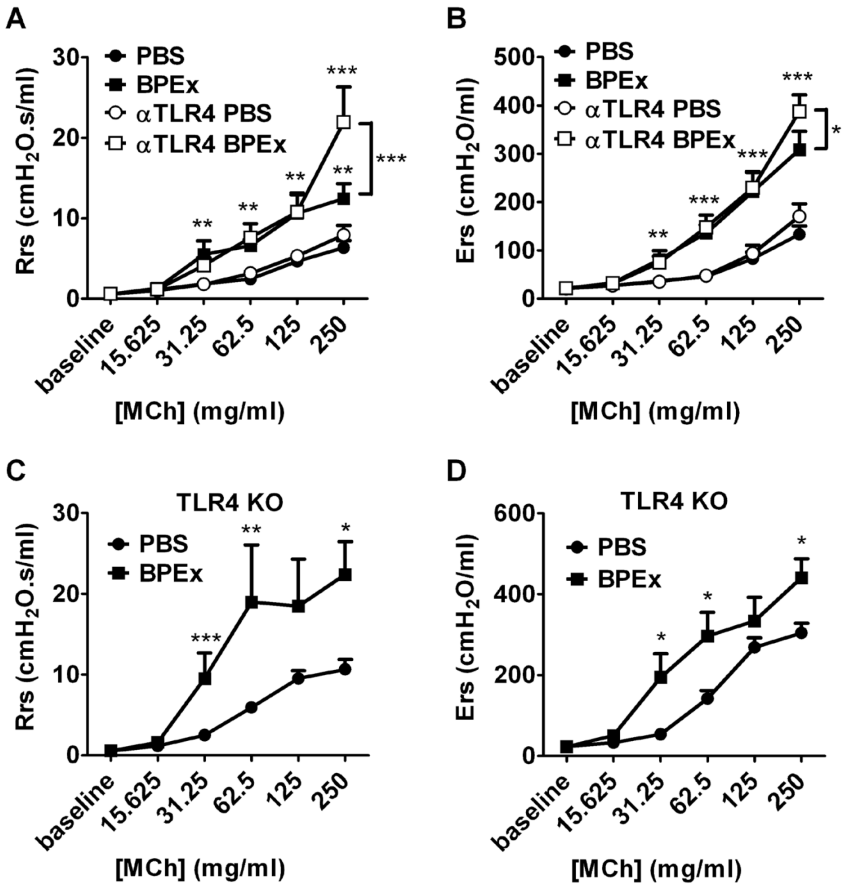

Figure 5. TLR4 contributes to the inflammatory response to inhaled BPEx. Total BAL inflammatory cells (A), eosinophils (B), neutrophils (C) and lymphocytes (D) were determined following intratracheal BPEx exposure of TLR4-antagonized or TLR4-deficient (TLR4 KO) mice (n=7-12 animals per group pooled from at least 2 independent experiments). Pro-inflammatory cytokines, IL-4 (E), IL-5 (F), IL-12 (G), KC (H) and IL-10 (I), were measured in the BAL fluid of PBS- or BPEx-exposed TLR4-antagonized or TLR4-deficient (TLR4 KO) mice (n=5-7 animals per group pooled from at least 2 independent experiments).

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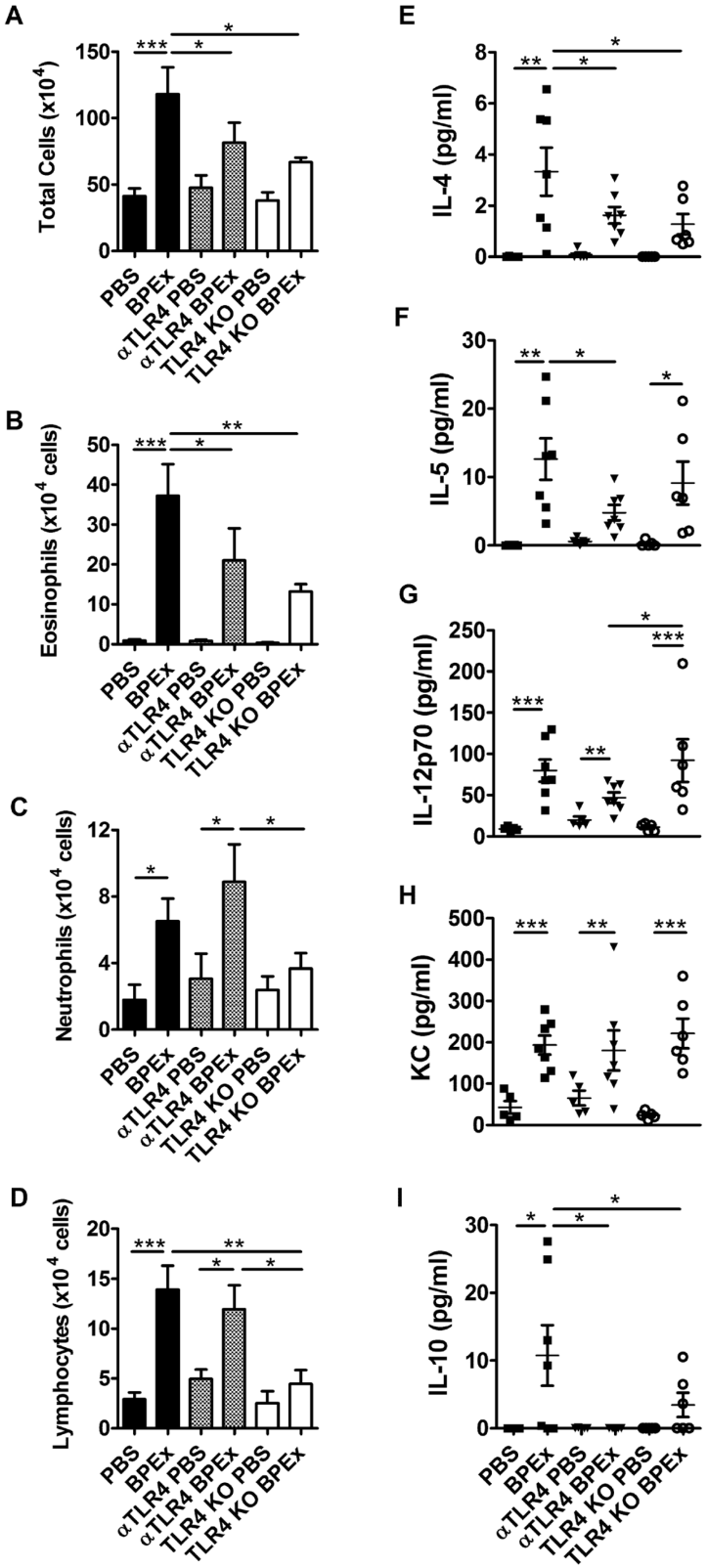
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100
50
0
***
*
*
B
Eosinophils (x10^4 cells)
50
40
30
20
10
0
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*
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C
Neutrophils (x10^4 cells)
12
8
4
0
*
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*
D
Lymphocytes (x10^4 cells)
20
15
10
5
0
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*
*
E
IL-4 (pg/ml)
8
6
4
2
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F
IL-5 (pg/ml)
30
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G
IL-12p70 (pg/ml)
250
200
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KC (pg/ml)
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400
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IL-10 (pg/ml)
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PBS
BPEx
αTLR4 PBS
αTLR4 BPEx
TLR4 KO PBS
TLR4 KO BPEx

Figure 6. Functional TLR4 is particularly important during the secondary inflammatory response to inhaled BPEx, rather than sensitization, for the development of eosinophilic airway inflammation. Airway responses to aerosolized MCh (A, B), total BAL inflammatory cells (C), eosinophils (D) and BAL eotaxin levels (E) were assessed in mice that received TLR4-antagonizing *Rhodobacter sphaeroides* LPS concomitantly with either sensitization only (αTLR4 BPEx / BPEx) or challenge only (BPEx / αTLR4 BPEx) (n=8 animals per group pooled from at least 3 independent experiments).

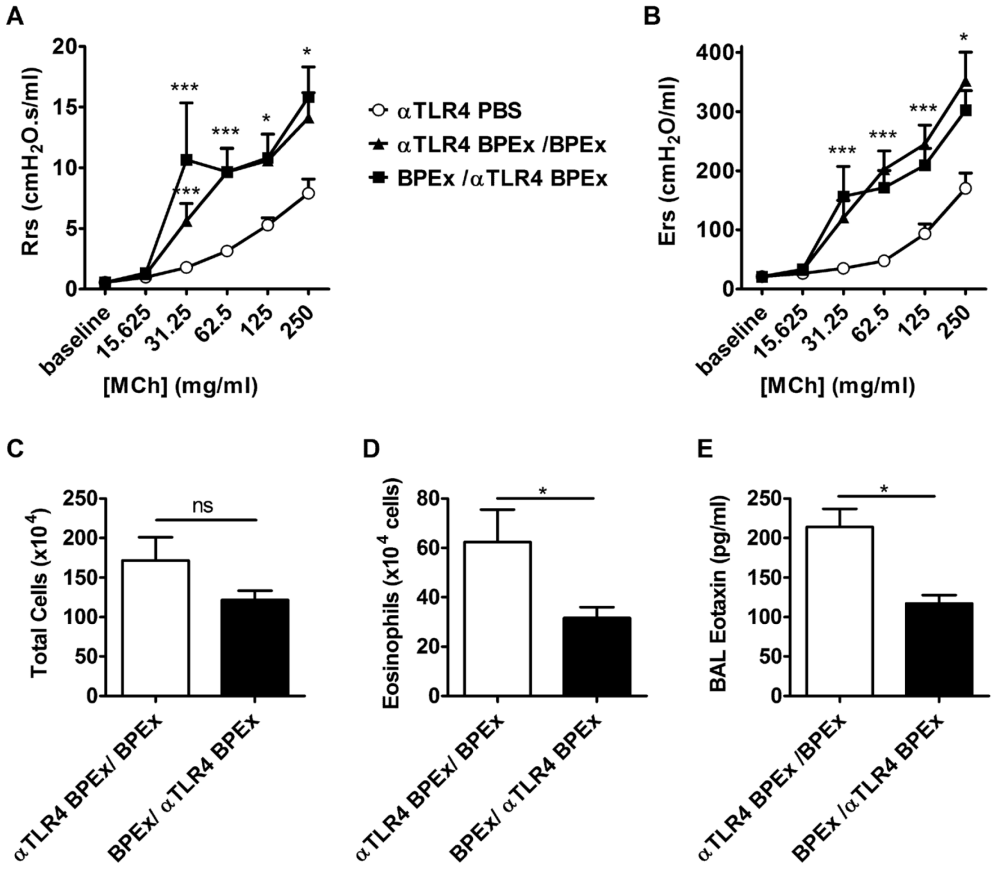
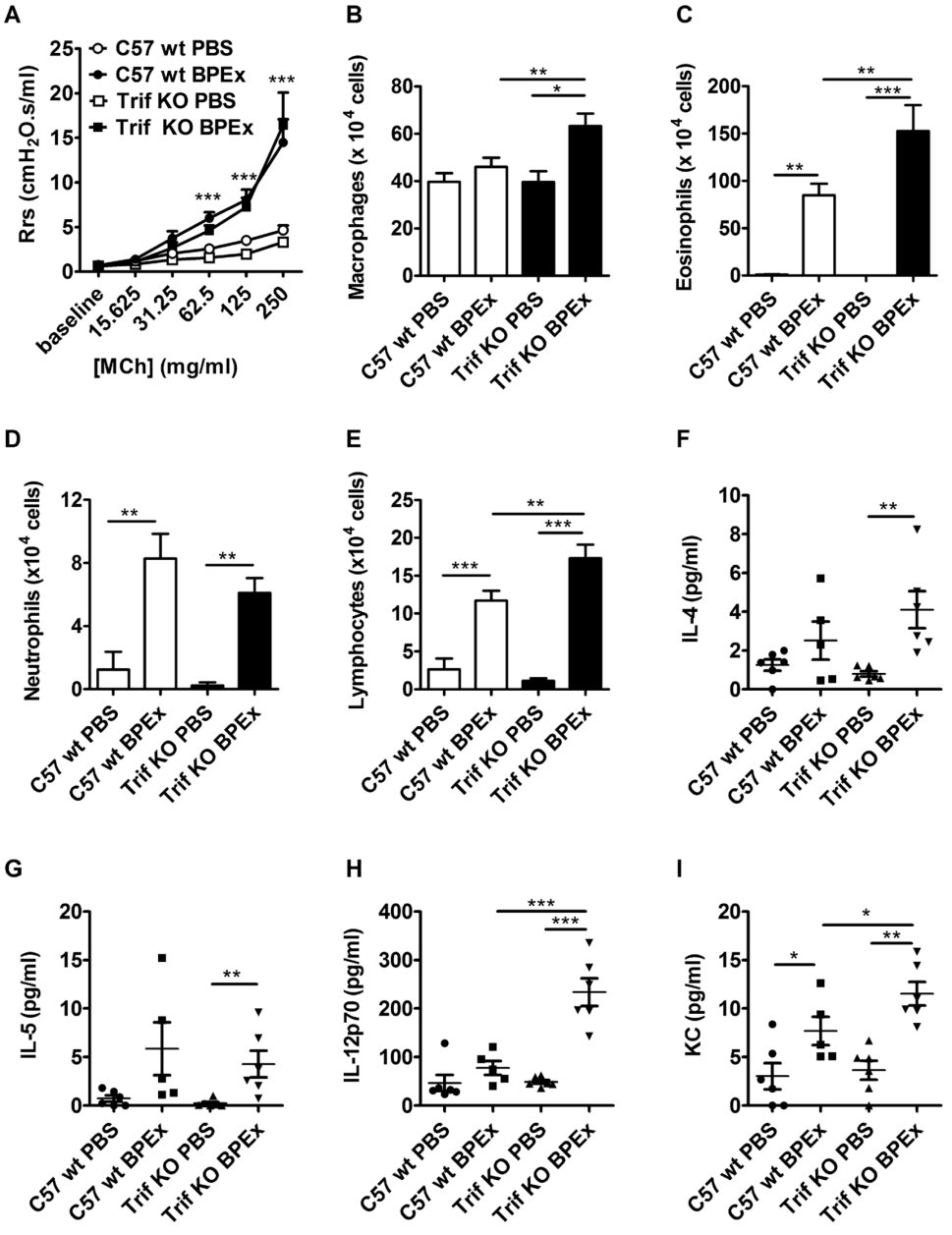
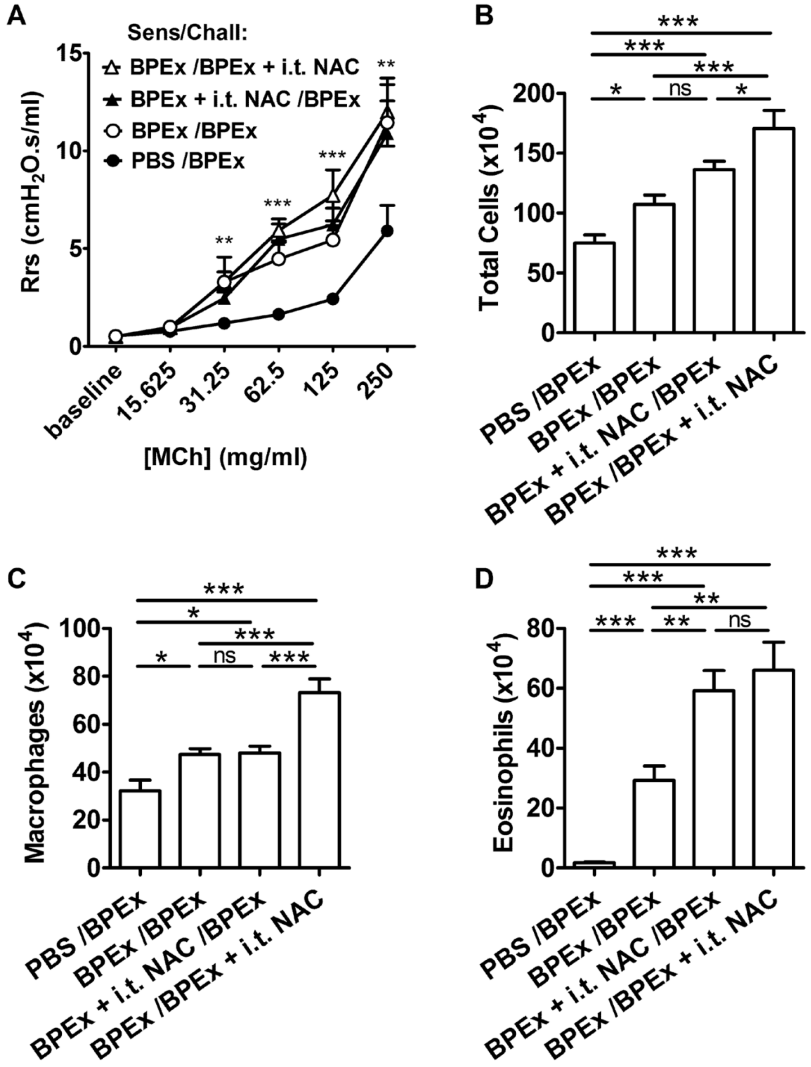


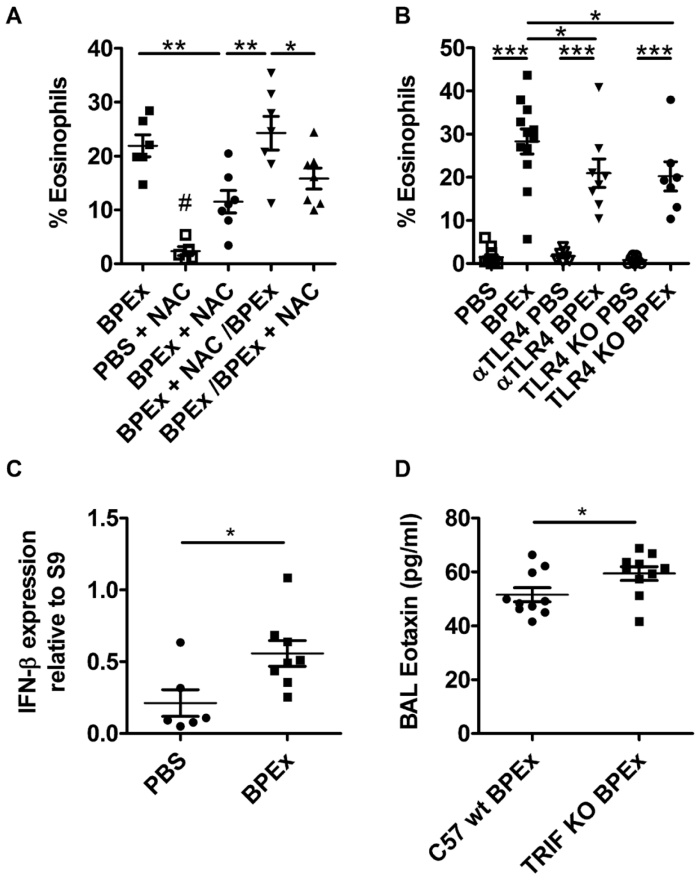
Figure 7. TRIF signaling does not influence AHR, but tempers allergen-induced airway inflammation. Airway responses to aerosolized MCh (A), macrophages (B), eosinophils (C), neutrophils (D) and lymphocytes (E) were measured after intratracheal PBS or BPEx exposure of C57 wt or Trif KO mice (n=6-16 animals per group pooled from at least 3 independent experiments). Pro-inflammatory cytokines, IL-4 (F), IL-5 (G), IL-12 (H) and KC (I) were quantified in the BAL fluid of PBS- or BPEx-exposed C57 wt or Trif KO mice (n=5-6 animals per group pooled from at least 3 independent experiments).



Supplementary Figure 1. Intratracheal administration of 150 mg/kg NAC irritates the airways causing enhanced airway inflammation. Airway responses (Rrs) to increasing doses of aerosolized MCh (A), total BAL cells (B), BAL macrophages (C) and eosinophils (D) of Balb/c mice that received 150 mg/kg of pH-adjusted, sterile-filtered NAC intratracheally, concomitantly with either BPEx sensitization (BPEx + i.t. NAC /BPEx) or challenge (BPEx /BPEx + i.t. NAC) (n=8-9 animals per group pooled from four independent experiments; * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).



Supplementary Figure 2. BPEx-induced airway eosinophilic inflammation is partially dependent upon oxidative stress and TLR4 and is also regulated by TRIF-dependent mechanisms. Frequency of eosinophils among total BAL cells on day 17, 48 hrs after final intratracheal PBS or BPEx exposure preceded by i.p. NAC injection (150 mg/kg) prior to all BPEx exposures, sensitization only, or challenge only (A). Frequency of BAL eosinophils following intratracheal PBS or BPEx exposures of TLR4-antagonized or TLR4 KO mice (B) (n=5-12 animals per group pooled from at least two independent experiments). mRNA expression of the TRIF-inducible type I interferon, IFN-β (normalized to the house-keeping gene, S9) in lung tissues of wild-type Balb/c mice harvested 30 mins after a single PBS or BPEx exposure (C) (n=6-8 animals per group from two independent experiments). Eotaxin 1 protein levels in BAL fluid of wild-type or Trif KO C57Bl/6J mice on day 17, 48 hrs after final BPEx challenge (D) (n=9-10 animals per group pooled from at least 3 independent experiments).



2.6 Discussion

Allergenic proteins possess inherent properties or are associated with danger signals that target receptors mediating allergic inflammatory responses (22). Importantly, these danger signals and receptors may be allergen-specific, or may confer allergenicity to a broad range of environmental proteins. Oxidative stress has been implicated in the pathogenesis of allergic airway disease and there is evidence of its association with innate immunity, specifically the TLR4 pathway, in mediating airway disease. We wished to determine the role of these mechanisms in evoking mucosal sensitization to pollen and/or promoting allergic airway disease upon secondary allergen exposure. We have found that oxidative stress is crucial in the development of airway hyperresponsiveness to birch pollen via the airway mucosa, whereas TLR4 signaling is not. The pollen-intrinsic NADPH oxidase activity was not relevant to either mucosal sensitization or amplification of the secondary response, as administration of heat-inactivated BPEx during either of these periods did not reduce subsequent airway responses and inflammation. We also confirmed in this model that oxidative stress contributes significantly to the recruitment of eosinophils and lymphocytes to the airway mucosa, whereas allergen-induced airway neutrophilia occurs independently of oxidative stress. Although TLR4 signaling does not contribute to AHR induced by inhaled pollen extract, it significantly augments airway eosinophilia, neutrophilia and lymphocytosis. In contrast, signaling via its adaptor TRIF tempers the inflammatory response to inhaled pollen extract. Oxidative stress appears to influence a broader spectrum of BAL fluid cytokines that are induced by inhaled pollen, including Th1, Th2, and other inflammatory mediators, compared to TLR4 signaling. Finally, we found that oxidative stress contributes to specific features of allergic sensitization via the airway mucosa (serum IgE, BAL lymphocytosis), but does not mediate all aspects of sensitization, such as those leading to AHR and eosinophilic airway inflammation, given that administration of antioxidant exclusively during sensitization failed to have an impact on these parameters of allergic airway disease.

There is evidence in the mouse (20), rat (23) and guinea pig (24) that following intraperitoneal sensitization with the experimental allergen OVA, and adjuvant alum, the administration of antioxidant, such as NAC (or its derivatives), around the time of OVA challenge can prevent the development of airway inflammation. A few studies have also shown that AHR can be prevented (25;26). Boldogh et al. demonstrated that intrinsic NADPH oxidase activity in ragweed pollen extract generates ROS in the airway lining fluid and epithelium within minutes of allergen challenge of mice, prior to the recruitment of inflammatory cells and independent of IgE, mast cells and adaptive immunity (6). This pollen-intrinsic NADPH oxidase activity was responsible for the augmentation of antigen-induced eosinophil recruitment to the airways and systemic IgE, which could be prevented by the administration of NAC during allergen challenge (7) (AHR was not evaluated in this case). Thus, studies to date have shown in animals that have been presensitized systemically with allergen and adjuvant, that NAC administration during allergen challenge is effective in inhibiting allergic airway disease, suggesting that oxidative stress is important in amplifying airway disease independently of sensitization. However, whether oxidative stress is a mechanism that is key to allergic sensitization in the first place, and via the natural route of the airway mucosa, has not been addressed. Using white birch, a pollen known to possess intrinsic NADPH oxidase (27), and cysteine protease activity (15) which may also contribute to ROS production, we investigated whether in the context of sensitization via the airway mucosa, oxidative stress would play an equally important role in augmenting airway inflammation and promote AHR. We determined that oxidative stress is critical to the development of AHR and airway inflammation to inhaled pollen extract, as antioxidant administration 1 hr prior to each BPEx exposure prevented AHR, blunted BAL eosinophilia and lymphocytosis, and inhibited inflammatory mediators such as KC, IL-12, IL-4 and IL-5.

Given that we did not sensitize animals by intraperitoneal injection with adjuvant, we expected pollen-induced oxidative stress to be critical for allergic sensitization. ROS production in the dermal epithelium elicited by the cysteine

protease papain triggers the expression and production of thymic stromal lymphopoietin (TSLP), a cytokine that promotes mast cell and basophil responses and, importantly, stimulates dendritic cells to prime Th2 responses (13). Recently, IgE cross-linking on mast cells has been shown *in vitro* to elicit ROS-mediated and NAC-sensitive induction of the leukotriene B_4 receptor, BLT2, which promotes Th2 responses (28). Furthermore, pollen-associated oxidative stress stimulates dendritic cell maturation and proinflammatory cytokine production (29;30) and has been reported to promote NAC-sensitive Th2 responses or effector T cell responses of mixed cytokine profile (13;30;31). Other components of pollen grains besides the intrinsic NADPH oxidase activity, such as the PGE_2-resembling pollen-derived E_1-prostanes have also been shown to contribute to this (16;32). Therefore, we expected sensitization to birch pollen via the airway mucosa to be mediated by oxidative stress. We confirmed that the intratracheal BPEx sensitizations on days 0 and 7 were indeed necessary for the development of airway disease, as PBS-sensitized, BPEx-challenged mice failed to develop AHR, BAL eosinophilia and lymphocytosis in comparison to BPEx-sensitized and challenged mice. However, we found that NAC administration coinciding only with sensitization was not sufficient to prevent AHR and BAL eosinophilia, while blunting only lymphocyte recruitment to the airways and systemic BPEx-specific serum IgE. Thus, airway disease developed in spite of antioxidant administration during allergic sensitization. Oxidative stress is known to activate the epidermal growth factor receptor (EGFR) leading to the elaboration of pro-inflammatory cytokines, such as IL-8, by epithelial cells (33). Similar to our current findings, inhibition of EGFR phosphorylation exclusively during allergic sensitization of rats limits the recruitment of lymphocytes to the airways, without affecting the development of AHR, or other aspects of inflammation (34). To our knowledge, we are the first to report that although oxidative stress mediates AHR and augments airway inflammation to inhaled pollen extract, it plays a limited role in allergic sensitization via the airway mucosa.

Thus, our data suggest that there exist undefined aspects of sensitization, possibly independent of adaptive immunity, that are not mediated by oxidative stress, or

that are insensitive to antioxidant. The recently described ATP- and purinergic signaling-dependent induction of IL-33 by airway epithelial cells and promotion of innate helper type 2 cell responses in response to aeroallergen may be one such mechanism (35). This is supported by our observation that heat-inactivation of BPEx had no effect either in sensitization or challenge on the subsequent development of AHR or airway inflammation, indicating a negligible role for birch pollen-intrinsic enzymatic activity, and specifically NADPH oxidase activity, in mucosal sensitization or amplification of the secondary inflammatory response. Furthermore, we found no measurable increase in lung 4-HNE levels immediately following a single BPEx exposure, but only after repeated BPEx sensitization and challenge, indicating that oxidative stress resulting directly from the pollen-intrinsic NADPH oxidase (or other enzymatic) activity is minimal compared to that which is generated by the recruitment of pro-inflammatory cells with repeated allergen exposure. This does not preclude that allergens with greater oxidative stress-capacity, such as ragweed, reduced antioxidant status of the animal, or concomitant exposure to irritants that amplify ROS in the airways would not enhance the significance of oxidative stress in allergic sensitization. Interestingly, despite the regulation of KC by NAC administration, neutrophil recruitment to the airways and allergen-induced TNF-α production were unaffected, suggesting that other neutrophil chemoattractants are induced independently of oxidative stress. Thus, the insensitivity of neutrophil recruitment may be specific to NAC, or suggests that antioxidants may be ineffective therapeutically in neutrophil-abundant airway diseases, such as COPD or severe asthma. Furthermore, intratracheal administration of the same dose of NAC together with BPEx, unexpectedly, was toxic or pro-inflammatory to the animals. Delivery of NAC into the airways is known to have the potential to induce bronchospasm and irritant effects (36;37).

There is considerable evidence indicating that oxidative stress can promote airway disease by activating innate immune signalling pathways (38;39). Hollingsworth et al. demonstrated that TLR4 KO mice fail to develop AHR, but still exhibit neutrophilic inflammation after ozone exposure (11), whereas another study has

implicated TLR2, TLR4 and MyD88 in the development of AHR caused by ozone, and MyD88, in particular, regulating ozone-induced airway neutrophilia (40). These TLRs have been proposed to be activated by lipid ozonation products or surfactant-derived oxidized lipids. Recently, ozone-induced fragmentation of hyaluronan, a component of pulmonary extracellular matrix has been confirmed to prime TLR4 responses (41;42). Similar to allergic airway disease, ozone-induced airway inflammation is characterized by Th2-type inflammation (IgE, IL-4 and IL-13) (43), as well as IL-17 and natural killer T cell responses (44). NADPH oxidase-induced ROS reportedly also amplifies TLR4 signaling in sepsis (45). Thus, we hypothesized that oxidative stress-mediated AHR and airway inflammation elicited by inhaled birch pollen may require signaling through the TLR4 pathway. Mice were given TLR4-antagonizing LPS-RS concomitantly with BPEx exposure, at a dose described by Hammad et al. to have successfully inhibited house dust mite allergen-induced experimental asthma (9). Unlike HDM-induced AHR, pollen-induced AHR still developed in spite of TLR4 antagonism, as well as in TLR4 KO mice. We conclude that TLR4 is not necessary for the development of AHR caused by inhaled pollen-induced oxidative stress. Our data demonstrate that pollen-induced oxidative stress mediates airway disease via mechanisms that differ from those that are activated by ozone, or intradermal papain injection, indicating that oxidative stress-induced airway disease is specific to the distinct nature or magnitude of the stress.

Despite the lack of effect on AHR, TLR4 does contribute to airway inflammation induced by inhaled pollen, augmenting eosinophilic inflammation and IL-4 and IL-10 cytokine production, in agreement with other studies that have described a role for TLR4 in promoting allergic Th2-type inflammation (9;10;13). The absence of functional TLR4 did not affect the induction of a number of Th1-inducing and pro-inflammatory cytokines by pollen despite blunting eosinophilic, neutrophilic and lymphocytic airway inflammation, suggesting a selective dissociation of airway cytokine expression levels from the recruitment of these cells. Our data from TLR4 KO mice indicate that neutrophil and lymphocyte recruitment to the airways in response to inhaled pollen is entirely dependent on

TLR4 signaling, whereas TLR4 antagonism did not significantly influence these inflammatory responses. The apparent disparity between pharmacological TLR4 antagonism and genetic ablation of TLR4 function may be due to a sub-maximal antagonism of TLR4 by the dose of LPS-RS used. It is also noteworthy however, that Tan et al. reported that whereas TLR4 expression on haematopoietic cells was necessary and sufficient for Th1-type, and contributed to Th2-type inflammatory responses to inhaled LPS-laden OVA, TLR4 expression on non-haematopoietic cells was important for the development of Th2 allergic responses, not Th1, to the same antigen (10). Thus, in contrast to the TLR4 knockout mice that completely lack functional TLR4 in both compartments, it is possible that LPS-RS delivered intratracheally exclusively or primarily antagonizes TLR4 on the airway epithelial and stromal cells, depending on whether it can cross this barrier. In this manner, the administration of LPS-RS perhaps may largely affect the Th2-type and eosinophilic inflammatory response, without a significant effect on neutrophilic and Th1 inflammation.

Little is known regarding the role of TLR4-TRIF signaling in asthma pathogenesis. TRIF signaling via TLR3 has been demonstrated to mediate double-stranded RNA exacerbation of ovalbumin-induced airway inflammation (46). However, whether TRIF signaling is involved in the development of airway disease solely to allergen has not been investigated to date. As in TLR4 knockout mice, AHR was not affected in TRIF knockout mice. Unexpectedly, TRIF deficiency exacerbated BPEx-induced airway macrophage, eosinophilic, and lymphocytic inflammation, as well as the secretion of pro-inflammatory mediators IL-12, KC and eotaxin compared to wild type mice. Thus, while TLR4 activation is pro-inflammatory, TRIF signaling appears to dampen pollen-induced airway inflammation. These findings suggest that MyD88-dependent signaling may promote Th2 and eosinophilic inflammation to inhaled pollen extract, whereas TRIF signaling may be anti-inflammatory, or may competitively or negatively regulate TLR4/MyD88 pathways; however, the precise mechanisms that account for these observations remain to be resolved. Consistent with the present observations is recent *in vivo* evidence that TRIF may be anti-inflammatory in

non-infectious lung disease (47) and that TRIF can negatively regulate TLR4-MyD88-induced activation of DCs, NK, T, and B cells, as well as pro-inflammatory cytokines and chemokines, including IL-12 (48). Whether TRIF-biased adjuvants, such as monophosphoryl lipid A, may be advantageous in the treatment of pollen-induced airway inflammation remains to be investigated.

Commercially available pollen extracts are delipidated to facilitate the extraction process and to permit re-suspension in aqueous media (49). The significance of this modification relative to natural exposure to pollens is unclear, as the fraction of pollen that is spontaneously releasable in the airways is likely more substantial in relation to physiological exposure than the non-aqueous fraction (16). However, CD1d-restricted human γδ T cells have been demonstrated *in vitro* to recognize, and become activated by, lipid constituents of pollen (50). Also, concentrations of linolenic and linoleic fatty acids which upon oxidation, may potentially trigger the TLR4 pathway, are 15 times higher in the organic than aqueous fractions of pollen (17). Thus, it cannot be excluded that oxidative stress and the TLR4 pathway may have a greater influence towards the development of airway disease in natural pollen exposure, compared to the inhalation of processed and extracted pollen. On the other hand, the sonication of pollen during the extraction process releases protein antigens, proteases and other pollen constituents that may not be readily released in natural exposure to pollen grains.

In conclusion, our data show that oxidative stress only partially impacts upon allergic sensitization to birch pollen extract via the airway mucosa, but not critically in relation to the promotion of AHR and eosinophilic airway inflammation. Oxidative stress is necessary, however, in the secondary response to allergen that promotes AHR and this is independent of the TLR4-TRIF pathway. Importantly, intrinsic NADPH oxidase activity, or other enzymatic activity, is not a universal mechanism conferring allergenicity to pollens. Finally, we also show that the TLR4 pathway significantly contributes to airway inflammation caused by inhaled pollen extract, whereas TRIF signaling limits the extent of the inflammatory response, but neither molecule is required for mucosal sensitization to pollen. Select aspects of the secondary inflammatory response to

inhaled pollen induced by oxidative stress, such as airway eosinophilia, lymphocytosis and IL-4, may indeed be mediated via TLR4.

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CHAPTER 3

ICOS-expressing CD4 T cells induced via TLR4 in the nasal mucosa are capable of inhibiting experimental allergic asthma

3.1 Prologue

This study was designed to investigate the benign edge of the TLR4 sword in relation to allergic asthma; specifically, to evaluate the efficacy of a compound named Protollin, composed of TLR2 and 4 ligands, as a mucosal adjuvant for immunotherapy against the development of experimental allergic airway disease and to delineate the relevant TLRs and modulatory effect on the T cell response. For this purpose, we used an acute model of experimental asthma induced by systemic alum sensitization with very low dose birch pollen extract, in order to minimize TLR4 stimulation by the allergen itself and to negate its impact upon the development of the asthma model, such that the role of TLR activation by Protollin could be clearly dissected.

3.2 Abstract

Modulation of adaptive immune responses via the innate immune pattern recognition receptors, such as the Toll-like Receptors (TLRs), is an emerging strategy for vaccine development. We investigated whether nasal rather than intrapulmonary application of Protollin, a mucosal adjuvant composed of TLR2 and 4 ligands, is sufficient to elicit protection against murine allergic lower airway disease. Wild-type, Tlr2 -/- or Tlr4 -/- Balb/c mice were sensitized to a birch pollen allergen extract (BPEx), then received either intranasal or intrapulmonary administrations of Protollin or Protollin admixed with BPEx (Pro/BPEx), followed by consecutive daily BPEx challenges. Nasal application of Protollin or Pro/BPEx was sufficient to inhibit allergic lower airway disease with minimal collateral lung inflammation. Inhibition was dependent upon TLR4 and was associated with the induction of ICOS in cells of the nasal mucosa and on both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells of the draining lymph nodes, as well as their recruitment to the lungs. Adoptive transfer of cervical lymph node $CD4^+ICOS^+$ but not $CD4^+ICOS^-$ cells inhibited BPEx-induced airway hyperresponsiveness and BAL eosinophilia. Thus, our data indicate that expansion of resident ICOS-expressing $CD4^+$ T cells of the cervical lymph nodes by nasal mucosal TLR4 stimulation may inhibit the development of allergic lower airway disease in mice.

3.3 Introduction

Allergic asthma is a chronic disease characterized by airway hyperresponsiveness (AHR), airway inflammation and intermittent obstruction, as well as airway remodeling. Immunologically, allergic asthma is T helper (Th) 2 cell-dominated, marked by expression of cytokines such as IL-4, IL-5, and IL-13, as well as peri-bronchial inflammatory cell infiltrates including eosinophils and neutrophils (1). Inhibition of allergic asthma by immunomodulation or regulation of the adaptive immune system via manipulation of innate immune processes is an attractive therapeutic strategy. Ligands of the Toll-like receptor (TLR) family, such as the synthetic lipoprotein PAM3CSK4 (2;3), lipopolysaccharide (LPS) (4-6), resiquimod (7;8), and CpG oligodeoxynucleotides (9;10), that activate TLRs 2, 4, 7/8 and 9 respectively, are capable of inhibiting experimental allergic asthma in animal models. In this context, we examined the immunomodulatory potential of Protollin^{TM}, a mucosal vaccine adjuvant composed of TLR2 and 4 ligands, in a murine model of experimental allergic asthma (11). Protollin was determined to be safe and well-tolerated up to a dose of 1.5 mg LPS when administered via the intranasal route in Phase I (12) and II (13) human clinical trials and has been tested as an intranasal vaccine adjuvant in animal models of influenza (14), respiratory syncytial virus (15), SARS (16), plague (*Yersinia pestis*) (17) and measles (18) infection, as well as in models of Alzheimer's disease (19). Protollin consists of LPS molecules (TLR4 ligand) from *Shigella flexneri,* a gram-negative bacterium, non-covalently incorporated in nanomolecular vesicles formed by proteosomes, consisting of purified hydrophobic outer-membrane proteins from *Neisseria meningitidis* (shown to signal via a TLR2/1 heterodimer complex) (20;21).

Harnessing the therapeutic potential of adjuvants while minimizing toxicity is a central aspect of vaccine design. Administration of TLR2 or TLR4 ligands into the lower airways carries the potential danger of exacerbating lower airway inflammation by promoting monocytic/ neutrophilic inflammation (22). Alternatively, the nasal mucosa is populated by lymphoid tissues (23), as well as

the recently described M cells (24), that can serve as effective inductive sites for immune responses, while minimizing exposure of the lower airways to TLR ligands. Thus, we assessed the immunomodulatory potential of nasal application of Protollin as an adjuvant in intranasal immunotherapy of lower airway disease, in a murine model of birch pollen allergic asthma, and focused on the changes in $CD4^+$ T cell responses induced by Protollin *in vivo* via the nasal mucosa.

TLR adjuvants can provide signals that suppress or divert the T cell response to an allergen away from the Th2 inflammatory response. The specific anatomical microenvironment targeted by the adjuvant, the activation state of dendritic cells (DCs) and the cytokine milieu provide the signals that direct lineage-specific differentiation of naive T cells into Th1, Th2, Th17, T follicular helper (Tfh), or T regulatory (Treg) subsets (25). LPS is a TLR ligand that can support the development of diverse T helper responses and in fact, has been shown to promote the differentiation of each of these T cell lineages depending on the particular tissue compartment (26-29). The dose of LPS is also an important determinant of the ensuing T cell response (28;30), and the timing of LPS exposure in relation to disease onset or development, as well as the age of animals at the time of exposure influences whether LPS amplifies or attenuates allergic disease (31). We were interested in characterizing the T cell response associated with the inhibition of experimental allergic asthma by Protollin. The induction of IL-12 (32), IFN-γ (4) and/or IL-10 (33;34) has been associated with the inhibition of allergic airway disease by LPS; TLR2 ligands have also been shown to inhibit experimental asthma in mice by promoting a Th1 response (3). Thus, we hypothesized that nasal application of Protollin would inhibit experimental asthma by promoting Th1 or Treg responses. The inducible costimulatory molecule (ICOS) is expressed by all of the aforementioned T cell subsets and is important in the differentiation (35), effector (36;37), proliferative (38), migratory (39) and memory (40) functions of T cells. A number of recent reports have shown that ICOS expression on Tregs enhances the function, proliferation and survival of these cells and has critical immunoregulatory implications (41). Our data demonstrate that nasal application of Protollin is sufficient to inhibit the

development of allergic lower airway disease, mediated by TLR4-dependent stimulation of the nasal mucosa and potential amplification of ICOS-expressing $CD4^+$ T cell populations.

3.4 Methods

Animal treatments

Six to eight week-old, female wild-type (wt) Balb/c mice were purchased from Charles River, Canada. Tlr2 knockout mice on a Balb/c background were generated in the Animal Care Facilities of the McGill University Health Centre and Tlr4 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Animals were housed in a conventional or specific pathogen-free animal facility under a 12 hour light/dark cycle with free access to food and water. All animals were sensitized on day 0 with a single 0.15 ml intraperitoneal (i.p.) injection of 20 protein nitrogen units (PNU) birch pollen allergen extract (BPEx; Greer Laboratories, Lenoir, NC, U.S.A.) and 3 mg alum hydrogel (Alum hydrogel 2%, Brenntag Biosector, Frederiksund, Denmark). This BPEx extract is used for clinical purposes in intradermal desensitization and thus is low in endotoxin (only 114 EU /mg protein, equivalent to a dose of about 1.5 EU /kg mouse body weight). Experimental procedures were approved by the McGill University Animal Care Committee.

Experimental asthma and nasal immunotherapy protocol

On each of days 7, 10, and 13 following sensitization (Figure 1), awake mice received nasal applications, i.e. without anesthesia, of 15 ± 5 µl of either phosphate-buffered saline (PBS), BPEx (25 PNU), Protollin alone ('Pro alone', 15 µg), or Protollin admixed with BPEx ('Pro/BPEx', 15 µg/ 25 PNU). Previously, nasal application of similar volumes of Evans blue dye indicated that >90% deposits in the nose/upper airways, with the remaining portion depositing largely in the gastrointestinal tract with very little in the lungs (42). Protollin consisted of a 1:1.1 ratio of Neisserial proteins to Shigella LPS at a concentration of 1 µg/µl LPS, resulting in an intranasal dose of≈ 15 ± 5 µg of LPS and Neisserial proteosomes on each of the indicated days. To test the effects of Protollin deposited in the lower airways, some mice were administered an equivalent dose of 15 µg, or a lower dose of 2 µg, Protollin alone or Pro/BPEx, in

a volume of 20 µl sterile PBS, intranasally under light isoflurane (4%) anesthesia. On days 15, 16 and 17, mice were also allergen challenged intranasally under light isoflurane anesthesia with a dose of 25 PNU BPEx in a volume of 36 µl sterile PBS. Control mice were sham-challenged with sterile PBS only.

Assessment of allergen-induced airway hyperresponsiveness

On day 19, 48 hrs after the final allergen challenge, mice were anesthetized with an injection of xylazine hydrochloride (10 mg/kg, i.p.) followed by i.p. administration of sodium pentobarbital (32 mg/kg). Once anesthesia was achieved, as assessed by loss of response to external stimuli, in some experiments a catheter was inserted in the jugular vein of the mouse (for intravenous (i.v.) methacholine delivery only). In all experiments, the mice were tracheostomized using a 19G metal cannula and connected via the endotracheal cannula to a commercial small animal ventilator (FlexiVent, SCIREQ Inc., Montreal, Canada). The animal was ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure of 3 cmH_2O. The mouse was then paralyzed with a 1 mg/kg pancuronium bromide i.v. or i.p. injection prior to the measurement of baseline respiratory mechanics. A 1.2 second, 2.5 Hz single-frequency forced oscillation maneuver was performed at 10 sec intervals and respiratory system resistance (Rrs) and elastance (Ers) were calculated with commercial software. In some experiments, doubling doses of methacholine (MCh; acetyl-β-methylcholine; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) from 20 – 640 µg/kg were delivered i.v. In other experiments, doubling concentrations of MCh from 15.6 – 125 mg/kg were delivered to the mouse as an aerosol using a 4 second nebulization period synchronized with inspiration at a nebulization duty cycle of 50%. Allergen-induced AHR was assessed by MCh-induced bronchoprovocation by recording the peak Rrs and Ers for each dose of MCh administered.

Assessment of airway inflammation

On day 19, bronchoalveolar lavage (BAL) was performed using saline and a protease-inhibitor cocktail. The recovered cell pellet was used to measure the total number of cells in the BAL and cytospins were prepared and stained with Diff-Quick stain (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany) for differential cell counts. A panel of cytokine proteins was assayed in the BAL supernatant using a Bioplex system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

Measurement of BPEx-specific serum IgE

On day 19, blood was collected by exsanguination into serum separator tubes and left at room temperature to clot. Samples were centrifuged at 4000 g for 5 minutes and the serum was collected and stored at -20^{o}C. Plates were coated overnight with 20 μg/ml BPEx for measurement of BPEx-specific serum IgE by ELISA (Biolegend, San Diego, CA, U.S.A.), according to the manufacturer's instructions.

mRNA analysis of nasal-associated lymphoid tissues

On day 14 (Figure 1), 24 hrs after the final nasal application of PBS, BPEx, Pro, or Pro/BPEx, nasal-associated lymphoid tissues (NALT) were harvested, immersed in *RNA later* and then stored at -80^{o}C. After thawing, tissues were homogenized and RNA was extracted using the RNeasy Minikit (Qiagen, Toronto, ON, Canada). Reverse transcription was performed using Superscript II enzyme (Invitrogen, Life Technologies Inc., Burlington, ON, Canada). Real-time polymerase chain reaction was used to measure the expression (relative to the housekeeping gene, S9) of cytokines relevant to T helper and T regulatory cells, interleukin (IL)-4, interferon (IFN)-γ, IL-17A, IL-10, IL-27 and transforming growth factor (TGF)-β, transcription factors GATA-3, T-bet, RORγt, Foxp3 and Bcl6, as well as co-stimulatory molecules, ICOS, CTLA-4 and PD-1 mRNA.

Flow cytometric analysis of cervical lymph node and lung T helper and regulatory cells

In order to further characterize the $CD4^+$ T helper cell and regulatory T cell response induced by Protollin, cervical lymph nodes and lungs were harvested from BPEx-sensitized mice on day 16 or 17 (Figure 1), 24 hrs after one or two BPEx allergen challenges respectively, following 3 administrations (days 7, 10 and 13) of either PBS or Protollin alone. Superficial cervical lymph nodes were isolated and placed in RPMI-1640 medium, containing 8% heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml gentamicin and 10 mM HEPES. The mouse was then tracheostomized, cannulated and the lungs were inflated with 1 ml sterile PBS (Invitrogen), supplemented with 0.2 Wunsch units/ml Collagenase from *Clostridium histolyticum* (Type XI-S), 1000 Dornase units/ml DNAse I (Type II-S) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and 0.5 mM Ca^{2+}. Lungs were then excised, minced with forceps and a scalpel blade and incubated on an orbital shaker at 37^{o}C for 1 hour. The digestion was inhibited by the addition of cold complete RPMI medium with 2 mM EDTA (Invitrogen) and 50 μM β-mercaptoethanol. The lung digest was then repeatedly passed through a 16G needle and then through a 70 μm BD Falcon cell strainer and centrifuged. Red blood cells were lysed with ammonium chloride solution. Cells were counted using a Beckman Coulter A^c.T Counter, plated in a 96-well culture plate and incubated for 15 min on ice with mouse BD Fc Block (BD Biosciences, Mississauga, ON, Canada). The cells were then stained for 20 min with FITC-conjugated rat anti-mouse CD4 mAb followed by PE anti-CD25 (BD Biosciences, Mississauga, ON, Canada) or PE anti-ICOS (Biolegend, San Diego, CA, U.S.A.), or the appropriate isotype control Ab. Cells were then fixed with BD Cytofix/Cytoperm solution, incubated with 1% BSA in BD Perm/Wash solution and finally stained with APC anti-Foxp3 mAb or isotype control Ab (eBioscience Inc., San Diego, CA, U.S.A.). Cell acquisition was performed using the BD FacsCalibur and analyzed with Cell Quest Pro software (BD Biosciences, Mississauga, ON, Canada). For flow cytometric analysis of CD4+ T cell cytokines, cells isolated from cervical lymph nodes and lungs were stimulated for

5 hrs with phorbol myristate acetate (10 ng/ml), ionomycin (250 ng/ml) and BD GolgiStop in RPMI-1640 medium containing 8% heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml gentamicin and 10 mM HEPES at 37 ^{o}C. The cells were then stained with anti-mouse CD4 FITC, ICOS PE and either IFN-γ PE-Cy7, IL-4 APC, or IL-10 APC (BD Biosciences, Mississauga, ON, Canada).

In vivo neutralization of IL-10

Coinciding with their first administration on day 7, mice receiving Protollin alone were injected i.p. with 50 μg of either isotype control, or anti-IL-10 mAb (BD Biosciences, Mississauga, ON, Canada). Additionally, the mice received intranasal administrations, under isoflurane anesthesia, of 25 μg of either antibody on days 7 and 10. Following Protollin and antibody administrations, mice were challenged with BPEx on days 15-17 and underwent lung function testing as well as blood and BAL collection on day 19.

Adoptive transfer of FACS-sorted $CD4^{+}ICOS^{+}$ or $CD4^{+}ICOS^{-}$ cells

On day 14, cervical lymph nodes were harvested and pooled from BPEx-sensitized mice that received nasal applications on days 7, 10 and 13 of either PBS or Protollin alone, and isolated cells were stained with anti-CD4 FITC and anti-ICOS PE mAb. $CD4^{+}ICOS^{+}$ or $CD4^{+}ICOS^{-}$ cells were then sorted by flow cytometry using a Beckman Coulter MoFlo cell sorter. Due to the rarity of $CD4^{+}ICOS^{+}$ cells, a maximum of 0.3-0.4 million cells of either of the sorted populations were adoptively transferred i.p. in 0.3 ml sterile PBS (on day 14) to mice that had been sensitized with BPEx in parallel with the sacrificed animals but that had not received any intranasal treatments. Control animals received an injection of only sterile PBS as a sham adoptive transfer ('Mock AT'). Mice were then challenged on days 15-17 and underwent lung function testing in response to MCh, as well as BAL collection on day 19.

Statistical analysis

Airway responses to MCh bronchoprovocation were analyzed in GraphPad Prism Version 5 (GraphPad software, San Diego, CA, U.S.A.) by two-way ANOVA followed by Bonferroni post-tests comparing all experimental groups to each other. One-way ANOVA and post-hoc Newman-Keuls' tests were used for all other analyses involving three or more groups, or unpaired Student's t-test was used in the case where only two experimental groups were compared. Data were log-transformed prior to statistical analysis when not normally distributed.

3.5 Results

Nasal application of Protollin prevents allergen-induced lower airway inflammation

The effects of intranasal instillation of Protollin, with or without anesthesia, on allergen-induced airway inflammation were compared. Protollin (15 μg) administered to awake animals without anesthesia, prior to the BPEx challenge (Figure 1), significantly reduced BAL eosinophil and neutrophil numbers on day 19 compared to mice that received PBS prior to the challenge (Figure 2A (ii-iii)). BAL lymphocyte numbers, however, were not significantly reduced (Figure 2A (iv)). In contrast, Protollin (15 μg) administered with anesthesia prior to the BPEx allergen challenge, caused significant airway inflammation as indicated by substantially higher total BAL cell counts compared to mice given PBS or an equivalent dose of Protollin without anesthesia, followed by BPEx challenge (Figure 2A (i)). Differential cell counts of BAL fluid from mice that received Protollin with anesthesia demonstrated marked neutrophilia (Figure 2A (iii)) and elevated macrophage (Supplementary Figure 1A) and lymphocyte (Figure 2A (iv)) numbers, whereas eosinophils (Figure 2A (ii)) were virtually absent. Administration of a lower dose of Protollin (2 μg) to anesthetized animals prior to the BPEx challenge also resulted in airway inflammation that was characterized by increased macrophage numbers (Supplementary Figure 1A), as well as lymphocytes (Figure 2A (iv)); in this case, neutrophil numbers were not increased beyond those of PBS-treated, BPEx challenged mice and eosinophilia was still completely inhibited (Figure 2A (ii-iii)). Overall, delivery of Protollin into the mouse lungs, even at the lower dose, was sufficient to cause considerable airway inflammation.

When Protollin was delivered admixed with BPEx (Pro/BPEx /BPEx), intranasally without anesthesia, mice had significantly lower BAL eosinophil and neutrophil numbers compared to mice given nasal applications of BPEx alone prior to the BPEx allergen challenge (BPEx /BPEx; Figure 2B (ii-iii)). Pro/BPEx

delivered with anesthesia caused severe airway inflammation similar to that observed when Protollin was administered alone with anesthesia (Figure 2B (i)), except that eosinophils were also increased in the BAL fluid (Figure 2B (ii)). Thus, we determined that nasal application of Protollin was sufficient to inhibit allergen-induced lower airway eosinophilia and neutrophilia, while evoking less inflammation compared to the intrapulmonary administration.

Nasal application of Protollin is sufficient to inhibit AHR

To determine whether nasal application of Protollin as an adjuvant with BPEx inhibited the development of AHR to subsequent allergen challenge, airway responses to increasing doses of MCh were assessed. In animals that received only nasal applications of PBS, those that were BPEx allergen challenged exhibited significantly greater increases in Ers (Figure 3A), as well as Rrs (Supplementary Figure 1B), in response to increasing doses of i.v. MCh compared to control animals sham-challenged with PBS. Nasal application of BPEx alone prior to the BPEx challenge significantly reduced airway responsiveness at the highest dose of MCh compared to animals that received PBS prior to the challenge, indicating tolerogenic effects of the allergen extract itself mediated via the nasal mucosa. Mice given Protollin mixed with BPEx (Pro/BPEx) had significantly reduced MCh-induced changes in Ers and Rrs compared to mice that received nasal applications of PBS or BPEx alone. Thus, the combined nasal administration of Protollin and BPEx to conscious animals prior to BPEx challenge inhibited the development of allergen-induced AHR. In a separate experiment, mice received nasal instillations of PBS or Protollin alone (Pro alone) prior to the BPEx challenge and airway responses to aerosolized MCh were determined. Even on its own, nasal Protollin administration prevented the development of AHR as reflected in changes in Ers (Figure 3B) and Rrs (Supplementary Figure 1C).

Nasal application of Protollin inhibits allergen-induced airway Th2 cytokines and serum birch pollen specific-IgE

Cytokine measurements in BAL fluid collected 48 hrs after the final challenge (day 19) indicated that nasal administration of the allergen extract, BPEx, on its own, without anesthesia, partially but significantly, reduced the allergen-induced Th2 cytokines IL-4 and IL-5 (Figure 3D). Mice that received Protollin mixed with BPEx or Protollin alone prior to BPEx challenge had even lower IL-4 and IL-5 levels in the BAL fluid, comparable to levels in sham (PBS) challenged mice. The neutrophil chemoattractant, KC, was also significantly lower in all experimental groups compared to its level in PBS-treated, BPEx-challenged mice. Despite trends indicating that nasal application of Protollin resulted in lower allergen-induced IL-17 (Figure 3D) and IFN-γ levels (data not shown), there was no statistically significant change in these cytokines. IL-2, IL-10 and TNF-α were below the level of detection (not shown). Thus, congruent with the paucity of inflammatory cells in the BAL fluid of Pro /BPEx or Pro alone-treated mice, these mice also had reduced airway Th2 cytokines and KC, without indication of induction of alternative pro-, or anti-inflammatory cytokines. Furthermore, nasal application of BPEx alone, Protollin mixed with BPEx, or Protollin alone significantly reduced serum BPEx-specific IgE production associated with allergen challenge (Figure 3C). The combination of Protollin with BPEx did not further reduce BPEx-specific IgE compared to BPEx or Protollin administration alone.

Nasal application of Protollin inhibits allergic airway disease via TLR4 and not TLR2

Protollin was administered intranasally to conscious allergen-sensitized Tlr2 or Tlr4 knockout mice, prior to BPEx allergen challenge, as indicated for wt mice (Figure 1). Protollin significantly inhibited AHR (Figure 4A), BAL eosinophilia (Figure 4C) and serum IgE (Figure 4D) in Tlr2 -/- mice, to a comparable degree as in wt mice. However, Protollin failed to inhibit AHR and BAL eosinophilia in Tlr4 -/- mice (Figure 4B, C). Serum IgE was not significantly induced in BPEx-

challenged Tlr4 -/- mice, indicating that TLR4 additionally contributes to aspects of experimental allergic asthma in this model.

Nasal application of Protollin affects T cell transcription factor and co-stimulatory molecule expression in nasal-associated lymphoid tissues

Given that nasal application of Protollin inhibited the allergen challenge-associated Th2 response, we wished to assess the early effects of Protollin administration on the local T cell response in the nasal mucosa, by quantifying T cell transcription factor and co-stimulatory molecule expression. To this end, we harvested NALT from mice on day 14 (Figure 1), 24 hrs after the final nasal application of either PBS, Protollin alone, BPEx alone, or Protollin mixed with BPEx, in the absence of allergen challenge. Nasal application of BPEx alone, Protollin alone, or Protollin mixed with BPEx significantly inhibited the expression of the Th2 master transcription factor GATA-3 (Figure 5A). The expression of IL-4, Th1-associated IFN-γ and T-bet, as well as TNF-α and T-regulatory cell-associated IL-27, TGF-β (data not shown) and Foxp3 (Figure 5A) was unaffected by the application of Protollin to the nares. We also found no significant induction of the co-inhibitory molecules CTLA-4 or PD-1 that have been associated with negative regulation of T cell responses and allergic airway disease (data not shown). Protollin also did not induce the inhibitor of T cell proliferation, indoleamine-2,3-dioxygenase, or the transcription factor, Egr-2, associated with Tr1 regulatory cells and T cell anergy (data not shown). However, Protollin administration alone, or in combination with BPEx, significantly induced the expression of ICOS, as well as the anti-inflammatory cytokine IL-10 (Figure 5A). The induction of these molecules and inhibition of GATA-3 expression indicated early immunomodulatory effects of Protollin on the local T cell response in the nasal mucosa, preceding airway exposure to allergen.

Nasal application of Protollin induces ICOS on $CD4^+$ T cells in the cervical lymph nodes and recruitment to the lungs upon allergen challenge

To assess the effects of intranasal Protollin on the induction of ICOS and Treg cells in the superficial cervical lymph nodes (LNs) that drain the nasal mucosa, as well as in the lungs, these tissues were harvested on day 16, 24 hrs after a single BPEx challenge following intranasal applications of either PBS or Protollin on days 7, 10 and 13 in order to examine by flow cytometry the frequency of $CD4^+$ T cells expressing either ICOS or CD25, as well as the transcription factor Foxp3. Protollin caused an increase in the total number of cells in the LNs, but not in the lungs (Figure 5B), and significantly increased ICOS expression among $CD4^+$ cells in the LNs compared to animals that received only PBS prior to sham or allergen challenges (Figure 6A, C (i)). Although both $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ cells were increased in the LNs (Figure 6B, C (i-ii)), Protollin primarily induced $ICOS^+Foxp3^-$ cells. However, the ratio of LN $CD4^+ICOS^+Foxp3^+$ cells to $CD4^+ICOS^-Foxp3^+$ cells was significantly increased as compared to that in PBS-treated sham or BPEx-challenged mice, further affirming the induction of ICOS on Tregs of Protollin-exposed mice (Figure 7A). In comparison, absolute numbers of LN $CD4^+CD25^+Foxp3^+$ and $Foxp3^-$ cells were similarly augmented in BPEx challenged mice pre-treated with either PBS or Protollin, as compared to sham-challenged mice (Figure 7B), and the relative ratios of these populations were not significantly affected by Protollin exposure (Figure 7A). Overall, Protollin did not increase Foxp3-expressing Treg cells at this time point beyond those found in PBS-exposed, allergen challenged mice, whether in the LNs (Figure 7B) or the lungs (data not shown). Importantly, in contrast to wt Balb/c mice, nasal application of Protollin to Tlr4 knockout mice failed to increase either the percentage (Figure 6C (iii)), or absolute number (Figure 6C (iv)) of draining cervical lymph node $CD4^+ICOS^+$ cells, indicating a TLR4-dependent induction of ICOS that correlated with the inhibition of allergic airway disease.

In contrast to wt LNs, there was no concomitant increase in ICOS expression on wt lung $CD4^+$ cells of Protollin-treated mice after a single allergen challenge (data not shown). However a significant increase was observed 24 hrs after two BPEx challenges (Figure 8), supporting a recruitment of ICOS-expressing $CD4^+$ cells to the lungs. ICOS expression among lung $CD4^+Foxp3^+$ cells appeared to have been equivalently induced in BPEx-challenged mice whether or not they had received prior Protollin (Figure 8A). However, Protollin significantly increased the absolute number of $CD4^+ICOS^+Foxp3^+$ cells (Figure 8C). Protollin also significantly increased ICOS expression among $CD4^+Foxp3^-$ lung cells (Figure 8B and D) and both $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ populations were increased by Protollin relative to $CD4^+ICOS^-Foxp3^-$ cells (Figure 8E and F, respectively). Thus, nasal applications of Protollin prior to allergen challenge induced ICOS-expressing $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells, initially in the cervical lymph nodes draining the nasal mucosa, and later in the lungs.

Nasal application of Protollin inhibits allergic airway disease independently of IL-10

Our finding that intranasal Protollin augmented both ICOS and IL-10 mRNA levels in the NALT, together with reports of high ICOS expression being associated with IL-10-producing T cells, led us to investigate whether the $CD4^+ICOS^+$ cells induced by Protollin preferentially expressed IL-10. Flow cytometric analysis of cervical LN intracellular cytokines was performed 24 hrs after a single BPEx challenge (day 16). No changes were found in IL-4 or IFN-γ expression in $CD4^+$ cells, or the $CD4^+ICOS^+$ subset (data not shown); however, Protollin exposure increased the number of IL-10-expressing $CD4^+ICOS^+$ cells and the ratio of IL-10- to IFN-γ-expressing $CD4^+ICOS^+$ cells in the cervical LNs (Figure 9A). No concomitant changes in IL-4, IFN-γ, or IL-10 expression were found in the lungs, in either the $CD4^+$ or $CD4^-$ populations (data not shown), further indicating the absence of a direct effect of Protollin on the lungs. 24 hrs after two allergen challenges, Protollin increased lung $CD4^+ICOS^+IL\text{-}10^+$ cells relative to PBS-treated sham-challenged, but not allergen-challenged mice (Figure

9B). Furthermore, although administration of anti-IL-10 mAb during the period of Protollin administrations increased BAL lymphocyte numbers (Figure 9C (iii)) compared to injection of isotype control antibody, airway responses to MCh (Figure 9C (i)) and BAL eosinophilia (Figure 9C (ii)) were unaltered following BPEx challenges. Thus, *in vivo* neutralization of the IL-10 cytokine did not abolish Protollin's inhibition of experimental asthma.

ICOS induced via the nasal mucosa plays a role in inhibiting allergic airway disease

To assess whether the TLR4-dependent induction of ICOS by Protollin was involved in the inhibition of allergic airway disease, on day 14 following BPEx-sensitization, mice received a sham adoptive transfer, an adoptive transfer of FACS-sorted $CD4^+ICOS^+$ or $CD4^+ICOS^-$ cells isolated from the pooled superficial cervical lymph nodes of BPEx-sensitized, intranasal PBS-treated mice, or $CD4^+ICOS^+$ cells from Protollin-treated mice. Following BPEx challenges, mice that had received prior adoptive transfer of LN $CD4^+ICOS^+$ cells from either Protollin- or PBS-treated mice had significantly lower airway responses to MCh compared to those that received $CD4^+ICOS^-$ cells from PBS-treated mice or that underwent sham adoptive transfer (Figure 10A, B). Thus, specifically ICOS-expressing $CD4^+$ cells from the lymph nodes draining the nasal mucosa had the capacity to inhibit allergen-induced AHR. Furthermore, adoptive transfer of LN $CD4^+ICOS^+$ cells from mice that received nasal administrations of Protollin significantly reduced total BAL inflammatory cell numbers (Figure 10C) and specifically eosinophils (Figure 10D).

Figure 1. Acute experimental asthma protocol. All animals were sensitized with BPEx/alum (i.p.) on day 0. Animals received nasal applications, while conscious (i.e. without anesthesia), of either PBS, Pro alone, BPEx, or Pro/BPEx, indicated by thin arrows, followed by intranasal challenge with light anesthesia of PBS or BPEx, indicated by thick arrows. Dashed lines and 'X' indicate days on which samples were harvested and/or airway responses to MCh were assessed; Day 14: NALT harvested for RNA extraction; Day 16: cervical lymph nodes and lungs harvested for flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ (Treg) cells, or $CD4^+ICOS^+Foxp3^+$ cells, as well as intracellular cytokine staining for IL-4, IL-10 and IFN-γ; Day 17: lungs harvested for flow cytometric analysis of Tregs or $CD4^+ICOS^+Foxp3^+$ cells; Day 19: assessment of airway responses to MCh using the flexiVent, followed by blood and BAL collection.

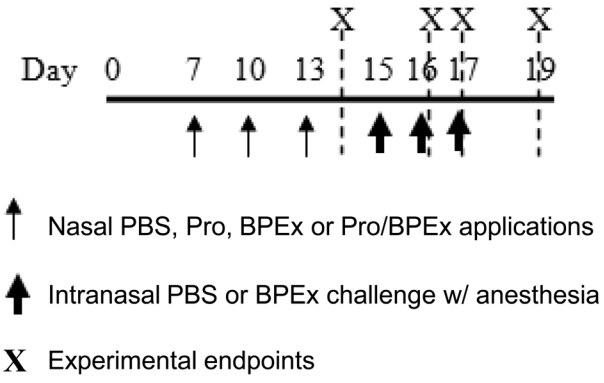

Figure 2. Intranasal application of Pro/BPEx or Protollin alone to conscious animals is sufficient to inhibit allergen-induced airway inflammation while evoking less collateral inflammation in comparison to intrapulmonary administration. Total inflammatory cells (A and B (i)), eosinophils (A and B (ii)), neutrophils (A and B (iii)) and lymphocytes (A and B (iv)) in BALs collected on day 19 after nasal application without anesthesia of PBS, Protollin alone, BPEx, or Protollin combined with BPEx (Pro/BPEx), or intranasal application with anesthesia of Protollin , 15 or 2 μg, or Pro/BPEx, followed by sham (PBS) or allergen (BPEx) challenge; n=5-19 animals/group from more than 3 independent experiments (# and ## indicates significant difference from other groups ($p < 0.01$ and $p < 0.001$, respectively); * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).

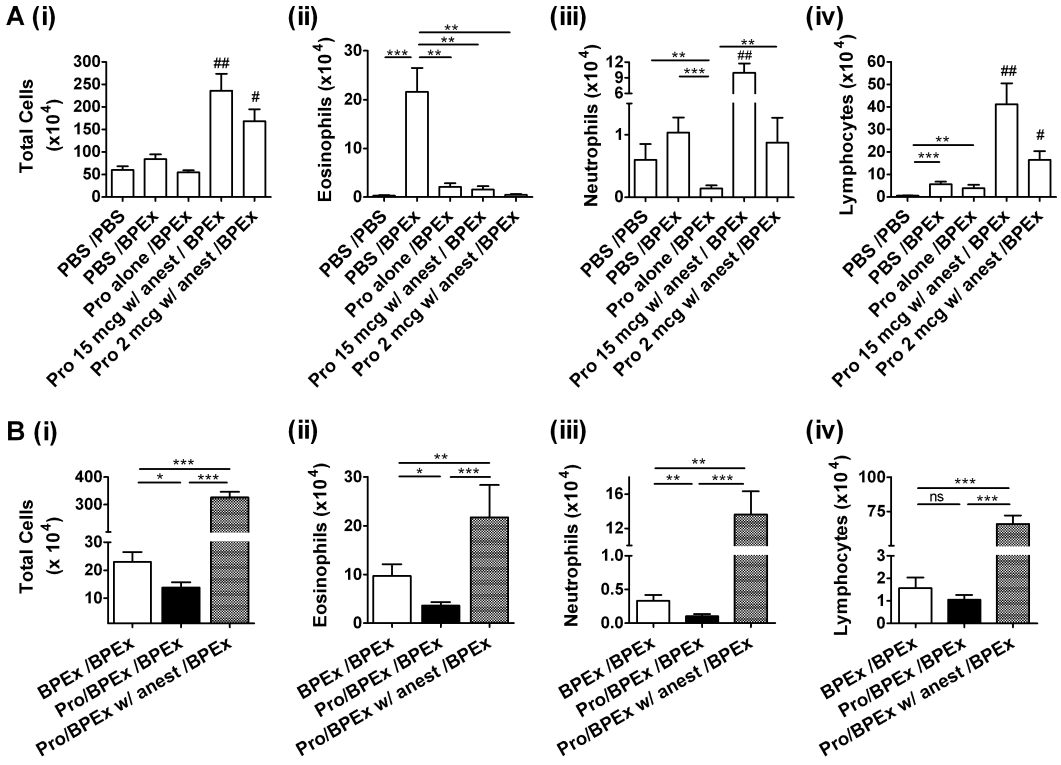

Figure 3. Intranasal application of Pro/BPEx or Protollin alone to conscious animals prior to allergen challenge is sufficient to prevent allergic airway disease. Airway responses (respiratory system elastance) to i.v. (A; n=8-19 animals/group from more than 3 independent experiments) or aerosolized (B; n=6-8 animals/group from > 3 independent experiments) MCh 48 hrs after intranasal sham (PBS) or allergen (BPEx) challenges following nasal application to conscious mice of either PBS, BPEx, Pro alone, or Pro/BPEx (** and *** in Figure 3B indicate significant difference of PBS /BPEx from Pro alone /BPEx). BPEx-specific serum IgE quantified by ELISA; all groups were BPEx sensitized, except 'Naïve' (C); n=4-9 animals/group from > 3 independent experiments, and cytokines in the BAL fluid quantified by multiplex assay (D) 48 hrs after PBS or BPEx challenges; n=6 animals/group from 4 independent experiments ($^{\infty}$ indicates significant difference from all other groups).

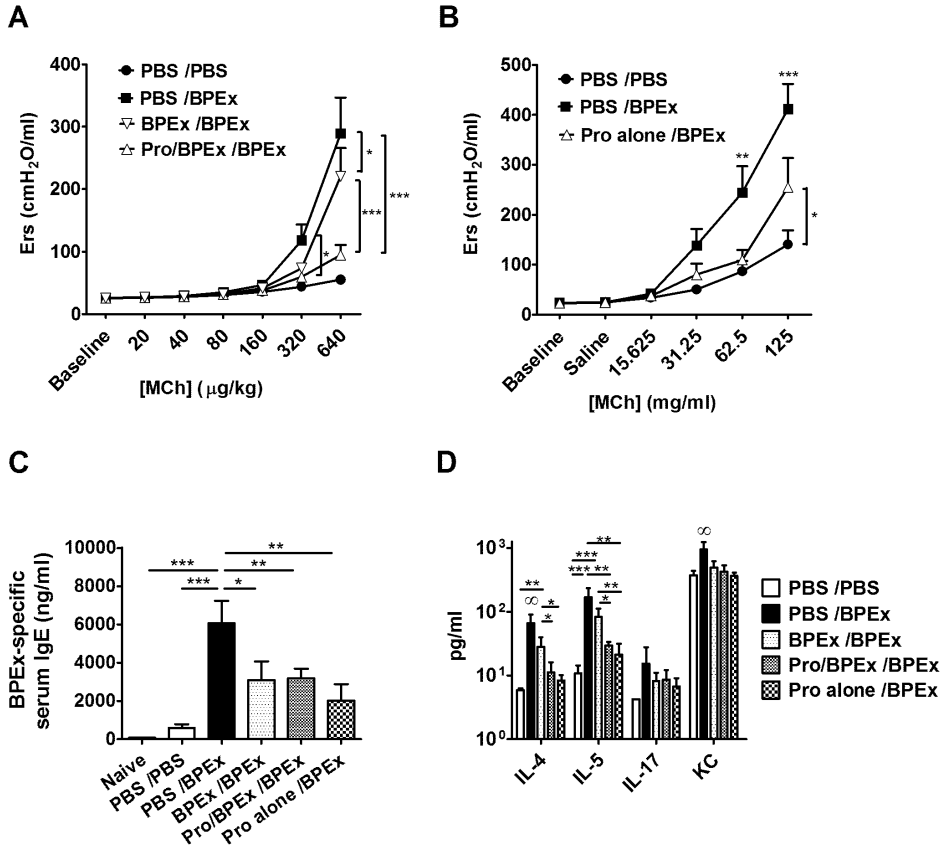

Figure 4. The inhibition of allergic airway disease by intranasal Protollin application is TLR4- and not TLR2-dependent. Airway responses (respiratory system elastance) to aerosolized MCh in Tlr2 -/- (A; n=10-13 animals/group from > 3 independent experiments) or Tlr4 -/- (B; n=6-8 animals/group from 4 independent experiments) mice 48 hrs after intranasal sham (PBS) or allergen (BPEx) challenges following nasal application to conscious mice of either PBS or Protollin alone. BAL eosinophil numbers (C; n=8-12 animals/group) and BPEx-specific serum IgE (D; n=7-11 animals/group) in Tlr2 or Tlr4 -/- mice after nasal applications without anesthesia followed by sham or allergen challenge.

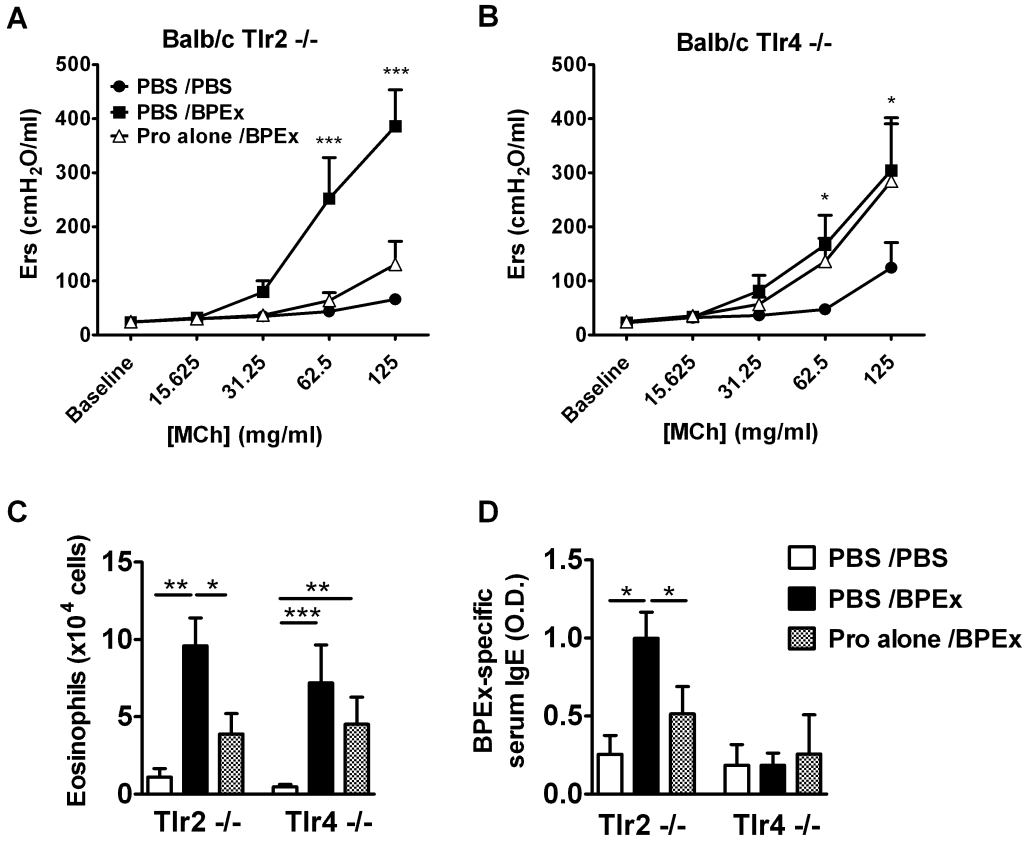

Figure 5. Intranasal Protollin application has an immunomodulatory effect preceding allergen exposure on T cells in the nasal mucosa and causes draining cervical lymph node cell counts to increase. Expression of T cell transcription factors, co-stimulatory molecules and cytokines quantified by real-time RT-PCR in nasal-associated lymphoid tissues harvested from BPEx-sensitized mice (day 14) 24 hrs after final nasal application without anesthesia of PBS, BPEx, Protollin combined with BPEx (Pro/BPEx), or Protollin alone (A; n=4-5 samples/group from > 3 independent experiments, each sample consisting of NALT pooled from 3 animals). Total cell numbers from cervical lymph nodes (LNs; n=16-18 samples from > 3 independent experiments) or lungs (n=15 samples/group from > 3 independent experiments) harvested (day 16) 24 hrs after a single PBS or BPEx challenge following nasal applications without anesthesia of either PBS or Protollin (B).

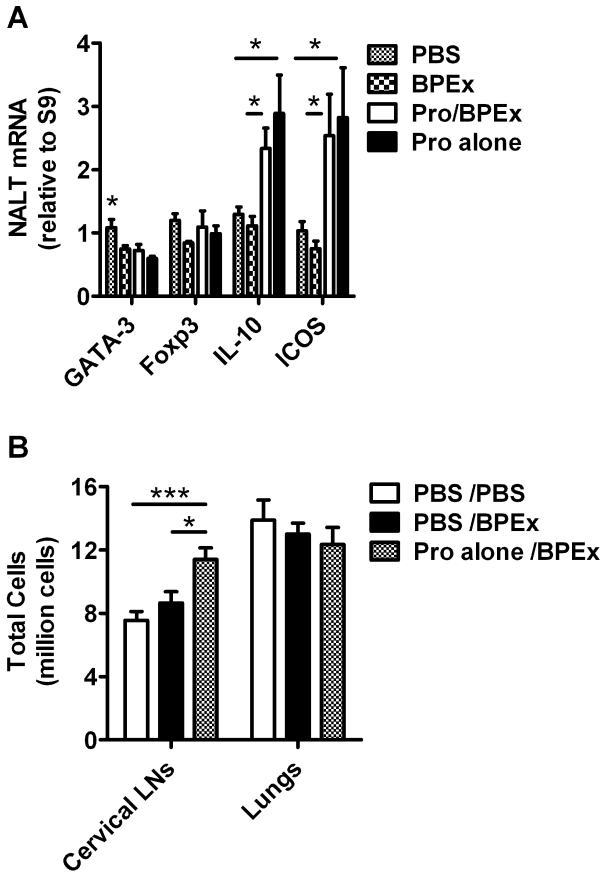

Figure 6. Intranasal Protollin application elicits TLR4-dependent induction of ICOS expression and amplification of ICOS-expressing $CD4^+$ T cell populations in the draining cervical LNs. Representative dot plots of CD4-gated LN cells from wt Balb/c mice stained for CD4 and ICOS (A), as well as Foxp3 (B) harvested on day 16, 24 hrs after a single PBS or BPEx challenge following nasal applications of either PBS or Protollin alone. Percentage of LN CD4-gated cells that are $ICOS^+$, $ICOS^+Foxp3^+$, $ICOS^+Foxp3^-$ and absolute cell numbers from wt Balb/c mice (C (i) and (ii), respectively) or Tlr4 -/- mice (C (iii) and (iv)); n=6-8 samples/group from at least 3 independent experiments.

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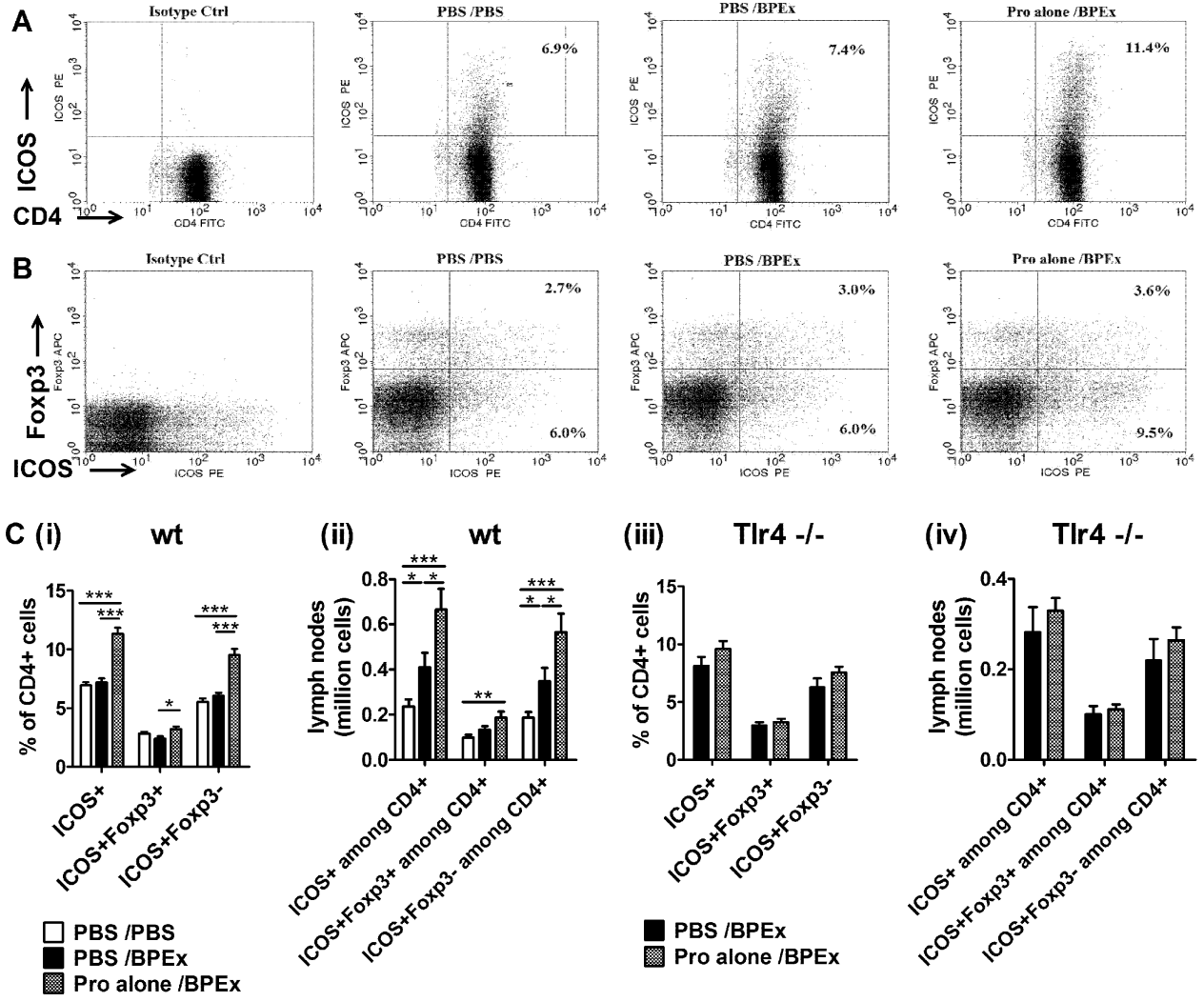
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Isotype Ctrl
PBS /PBS
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ICOS
CD4
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6.9%
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B
Foxp3
ICOS
Foxp3 APC
ICOS PE
2.7%
3.0%
3.6%
6.0%
6.0%
9.5%
C (i) wt
(ii) wt
(iii) Tlr4 -/-
(iv) Tlr4 -/-
% of CD4+ cells
lymph nodes (million cells)
ICOS+
ICOS+Foxp3+
ICOS+Foxp3-
ICOS+ among CD4+
ICOS+Foxp3+ among CD4+
ICOS+Foxp3- among CD4+
PBS /PBS
PBS /BPEx
Pro alone /BPEx

Figure 7. Intranasal Protollin application significantly affects the relative proportion of cervical LN $CD4^+ICOS^+Foxp3^+$ and $ICOS^+Foxp3^-$ cells but does not affect $CD4^+CD25^+Foxp3^+$ cells. Ratio of LN $CD4^+ICOS^+Foxp3^+$ to $CD4^+ICOS^-Foxp3^+$ cells, as well as ratio of $CD4^+CD25^+Foxp3^+$ to $CD4^+CD25^-Foxp3^+$ cells (A). Absolute numbers of lymph node $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$ or $Foxp3^-$, or total $CD4^+Foxp3^+$ cells (B); n=6 samples/group from > 3 independent experiments.

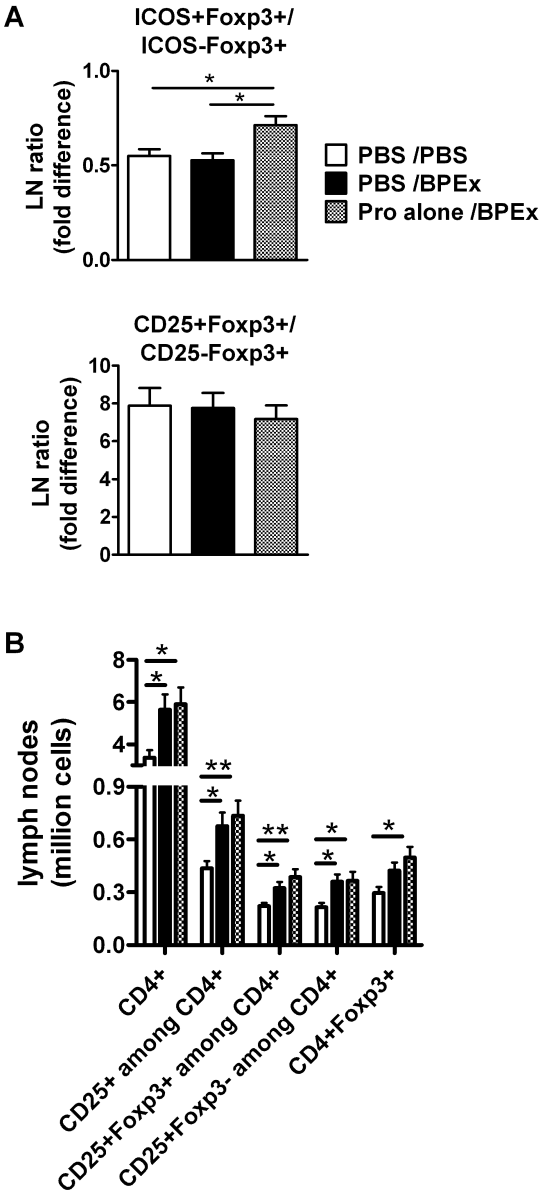


Figure 8. Intranasal Protollin application may promote the recruitment of ICOS-expressing $CD4^+$ T cell populations to the lungs. Percentage of lung $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells expressing ICOS (A and B, respectively), harvested from wt Balb/c mice on day 17, 24 hrs after two PBS or BPEx challenges following nasal applications of either PBS or Protollin alone, as well as absolute cell numbers (C and D, respectively). Proportion of $CD4^+ICOS^+Foxp3^+$ or $ICOS^+Foxp3^-$ cells relative to $CD4^+ICOS^-Foxp3^-$ cells (E and F); n=7-8 samples/group from 3 independent experiments.

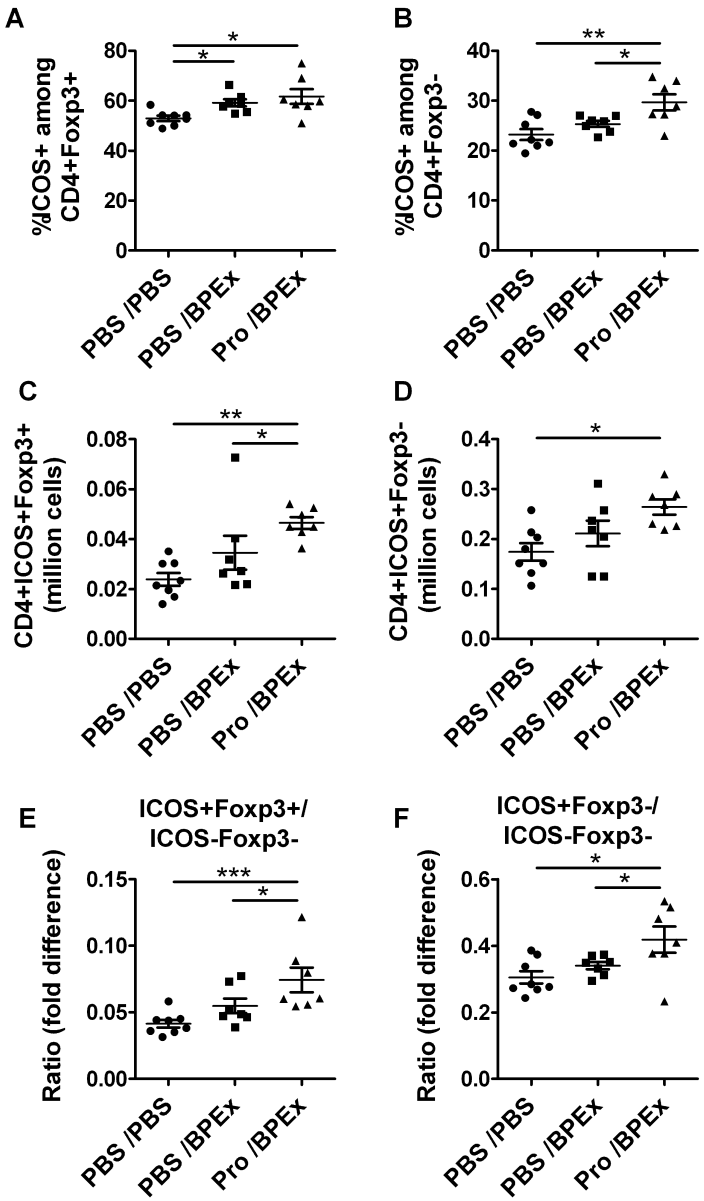


Figure 9. Intranasal Protollin application induces IL-10-expressing $CD4^+ICOS^+$ T cells but IL-10 is dispensable for the inhibition of allergic airway disease. Intracellular cytokine staining and flow cytometry was used to assess IL-10 expression among αCD4, αICOS Ab-stained cervical lymph node cells harvested on day 16, 24 hrs after a single sham (PBS), or allergen (BPEx) challenge of mice following nasal applications of PBS or Protollin. The ratio of $IL\text{-}10^+$ to $IFN\text{-}\gamma^+$ cells among LN $CD4^+ICOS^+$ cells is also shown (A); n=6 animals/group from > 3 independent experiments. Absolute number of $CD4^+ICOS^+IL\text{-}10^+$ cells harvested from the lungs on day 17, 24 hrs after two PBS or BPEx challenges following nasal applications of PBS or Protollin (B); n=7-8 animals/group from 3 independent experiments. Airway responses to MCh (Ers) (C (i)), BAL eosinophil (C (ii)) and lymphocyte (C (iii)) numbers evaluated after BPEx challenge of mice that received *in vivo* administrations of either neutralizing αIL-10 monoclonal Ab or isotype control Ab coinciding with intranasal Protollin administrations; n=4 animals/group from 2 independent experiments.

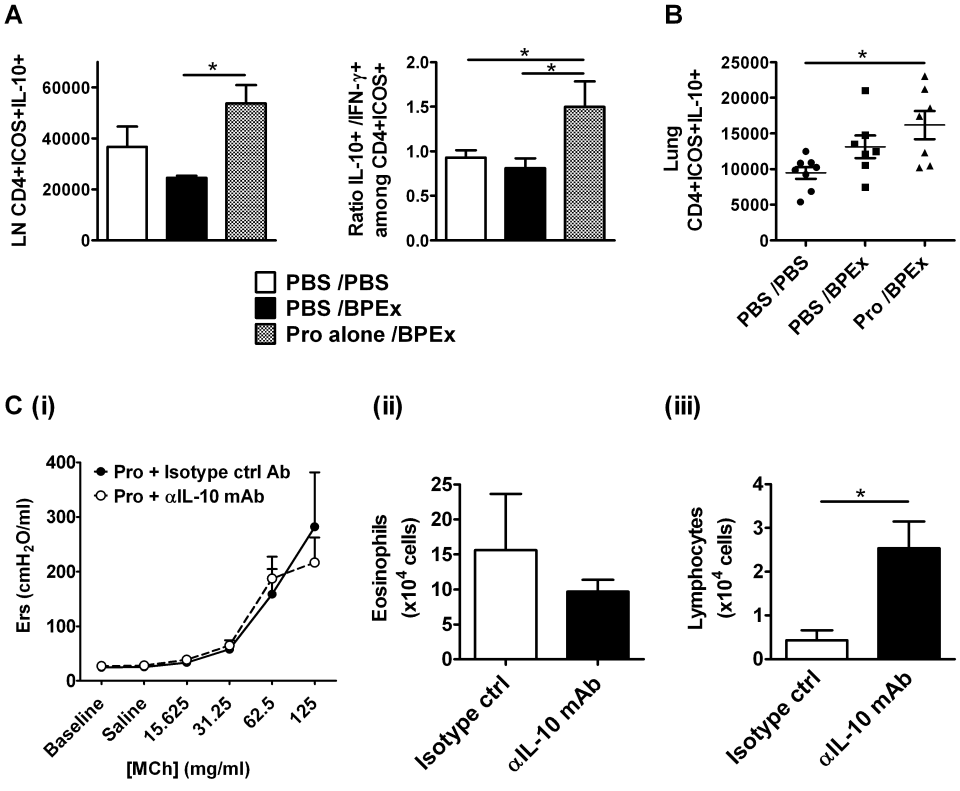
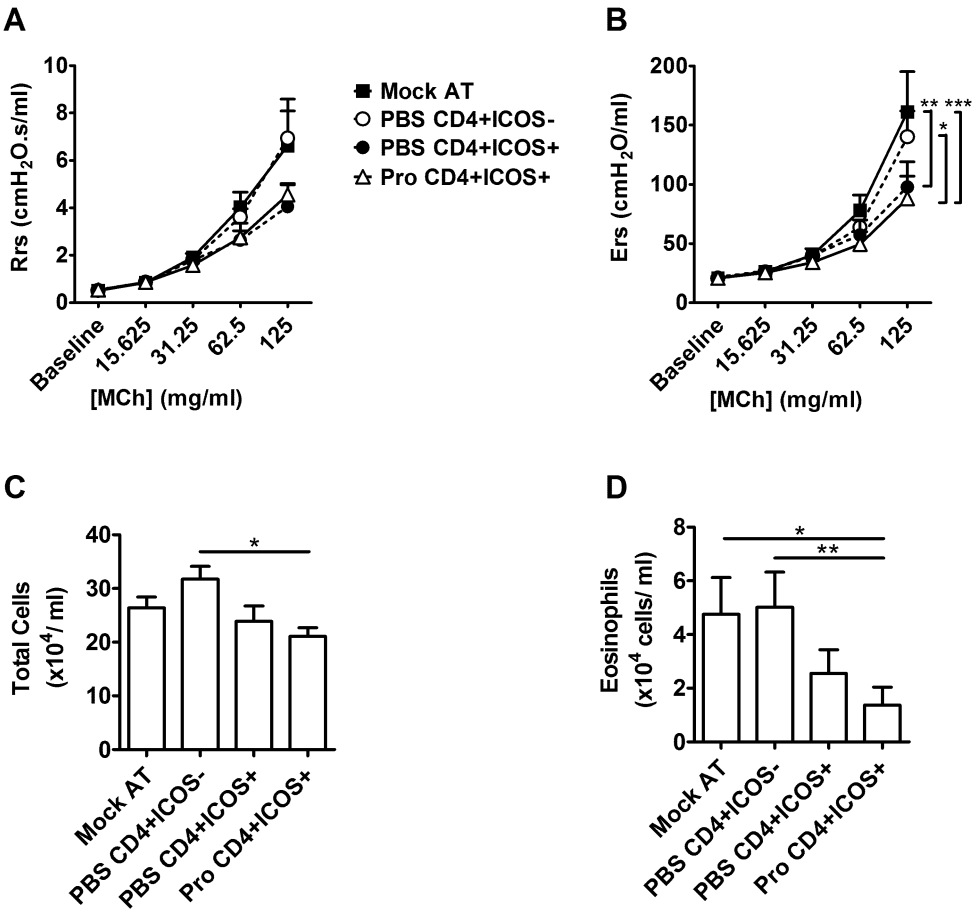
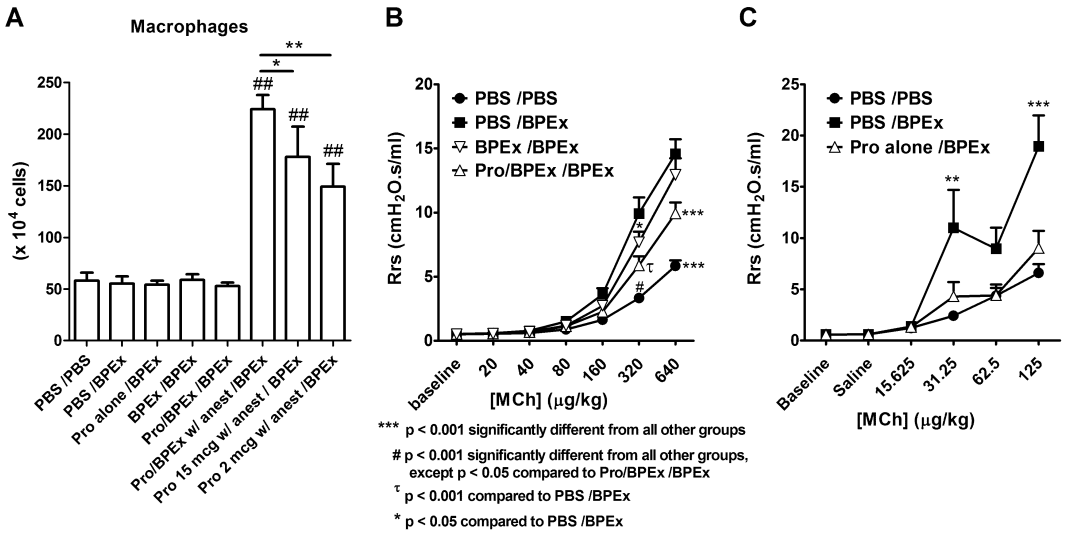


Figure 10. ICOS-expressing $CD4^+$ cells of the cervical LNs have the capacity to inhibit allergic airway disease. Airway responses (Rrs and Ers) to aerosolized MCh of allergen-challenged wt Balb/c mice receiving prior injection of sterile PBS (Mock AT), or adoptive transfer of $CD4^+ICOS^+$ or $CD4^+ICOS^-$ cells pooled from the cervical LNs of either intranasal PBS- or Protollin-treated mice (A and B, respectively). Total inflammatory cells and eosinophils (C and D, respectively) in BAL fluid collected immediately following assessment of airway responses; n=5-7 animals/group from 3 independent experiments.



Supplementary Figure 1. Intranasal application of Protollin does not induce significant inflammation in the lungs, as compared to intrapulmonary administration of an equivalent or lower dose, and is sufficient to inhibit AHR (respiratory system resistance) to increasing doses of MCh. Macrophage numbers (A) in BALs collected on day 19 after nasal application without anesthesia of PBS, Protollin alone, BPEx, or Protollin combined with BPEx (Pro/BPEx), or intranasal application with anesthesia of Protollin , 15 or 2 μg, or Pro/BPEx, followed by sham (PBS) or allergen (BPEx) challenge; n=5-19 animals/group from > 3 independent experiments. All groups receiving intranasal applications of Protollin alone or Pro/BPEx with anesthesia were significantly different from groups receiving nasal applications without anesthesia ($^{\#\#}$ $p < 0.001$). Airway responses (respiratory system resistance) to i.v. (B; n=8-19 animals/group from > 3 independent experiments) or aerosolized (C; n=6-8 animals/group from > 3 independent experiments) MCh 48 hrs after intranasal sham (PBS) or allergen (BPEx) challenges following nasal application to conscious mice of either PBS, BPEx, Pro alone, or Pro/BPEx (* indicates significant difference of BPEx /BPEx from PBS /BPEx; $^{\tau}$ indicates difference of Pro/BPEx /BPEx from PBS /BPEx; $^{\#}$ indicates difference of PBS /PBS from all other groups in Supp. Fig. 1B; ** and *** in Supp. Fig. 1C indicate significant difference of PBS /BPEx from Pro alone /BPEx).



3.6 Discussion

Modulation of adaptive immune responses via the innate immune pattern recognition receptors, such as the TLRs, is an emerging strategy for vaccine development. Defining the conditions and mechanisms by which these receptors can regulate inflammation in a safe manner is complicated by the diversity of immunological effects propagated by their cognate ligands. Here, we show that nasal application of Protollin, a mucosal adjuvant that has been determined safe in human clinical trials and composed of TLR2 and 4 ligands, is sufficient to inhibit the development of allergic lower airway disease. Compared to intrapulmonary delivery, nasal application of Protollin is associated with less collateral lung inflammation and may confer reduced toxicity and an increased safety profile. Interestingly, the inhibition of experimental asthma by nasal application of Protollin is dependent upon TLR4 and not TLR2 and is associated with elevated numbers of ICOS-expressing $CD4^+$ T cells in the draining cervical lymphoid tissues and lungs, with no increase in $CD4^+CD25^+Foxp3^+$ lymphocytes. Adoptive transfer of $CD4^+ICOS^+$ cells from the cervical LNs of either PBS- or Protollin-treated mice prior to allergen challenge inhibits subsequent allergic airway disease, suggesting that these cells have the capacity to inhibit the development of allergic airway disease and can be amplified by TLR4 stimulation of the nasal mucosa.

Epidemiological evidence supports a role for microbial exposure in ensuring the maturation of the immune system in a balanced manner that prevents predisposition to Th2 associated responses (43-45). LPS and its receptor TLR4 have been associated with specific gene-environment interactions that may either protect or exacerbate allergic disease (46;47), demonstrating the complexity of the relationship between microbial exposure and immune responses. The biological activity of LPS and its potential toxicity for the airway epithelium is due to its diphosphorylated lipid A portion (48); thus, derivatives with lower inflammatory capacity such as the monophosphoryl lipid A (49) are required for use in vaccines. Although the hydrophobic lipid A portions of LPS are less toxic because they are

shielded within vesicles formed by the neisserial proteosomes in Protollin (11), we found that intrapulmonary administration of 15 μg of Protollin prior to allergen challenge caused marked monocytic, neutrophilic and lymphocytic lung inflammation, while inhibiting eosinophilia. In addition, combined Protollin/BPEx administration with anesthesia prior to challenge amplified all types of inflammatory cells in the BAL, including eosinophils. Thus, intranasal, rather than intrapulmonary, administration appears to be a safer modality for mucosal vaccines, such as Protollin, given that nasal Protollin application to conscious animals was sufficient to inhibit experimental lower airway allergic disease while evoking less collateral pulmonary inflammation compared to an equivalent dose administered with anesthesia.

In rodents, the nasal mucosa contains nasopharynx-associated lymphoid tissues (NALT) that are found dorsal to the cartilaginous soft palate; analogous structures exist in the human upper airways (50). The NALT also mediates antigen-specific tolerance to nasally deposited antigen (51), and thus, supports both positive and negative regulatory signals for the immune system that are propagated by the draining cervical lymph nodes (52). We found that nasal application of the allergen extract BPEx, on its own, prior to the allergen challenge resulted in partially reduced pulmonary responsiveness to methacholine, BAL Th2 cytokines and BPEx-specific serum IgE, indicating that intranasal BPEx administration mediates tolerogenic lower airway and systemic effects. Nasal application of Protollin on its own also inhibited allergic airway disease in a TLR4-dependent manner, suggesting that LPS is the active component of the adjuvant in this model. Protollin's protective effects in another murine model, as a component of an intranasal respiratory syncytial virus vaccine, were also mediated by TLR4-MyD88 signaling and not TLR2 (53). Interestingly, the *N. meningitidis* outer membrane vesicle vaccine which is related in composition to Protollin has also been shown to depend on TLR4 and not TLR2 activation (54). The combined nasal application of BPEx with Protollin produced a greater inhibition of AHR compared to BPEx on its own and reduced BAL granulocyte numbers. Intra-pulmonary LPS administration to mice has been shown to prevent experimental

allergic asthma, associated with an absence of allergen-specific Th2 or Th1 cytokines, suggested to be anergy (55). Other studies have implicated Th1 responses, due to elevated expression of the transcription factor T-bet (56) or a requirement for IL-12 (32), and/or IL-10-producing $CD4^+$ cell responses (34;57), in the inhibition of Th2-mediated allergic airway disease, depending on the timing of intra-pulmonary LPS exposure in relation to disease onset or development. We aimed to characterize the CD4 T cell response associated with the TLR4-dependent inhibition of experimental allergic airway disease via the nasal mucosa by nasal Protollin administration, but found no induction of IFN-γ mRNA in the NALT, or protein in $CD4^+$ cells of the superficial cervical LNs or lungs, as well as in BAL fluids. Expression of T-bet in the NALT was also unaffected by Protollin (data not shown). We explored an alternative immunoregulatory mechanism, namely the induction of Tregs by Protollin in the NALT, cervical lymph nodes and lungs. Neither Foxp3 expression nor $CD4^+CD25^+Foxp3^+$ Treg numbers were significantly altered; however, we found the exclusively T cell-expressed inducible co-stimulatory molecule (ICOS) to be significantly induced in the NALT following nasal application of Protollin, revealing an immunomodulatory effect on T cells in the nasal mucosa. We confirmed by flow cytometry that ICOS expression was substantially increased in $CD4^+$ cells of the superficial cervical LNs, but not of the lungs following Protollin administrations and a single allergen challenge. At a later time point, after two allergen challenges, we also found elevated ICOS expression among lung $CD4^+$ cells, as well as an induction in both $CD4^+ICOS^+Foxp3^+$ and $ICOS^+Foxp3^-$ populations, indicating possible migration of these cells from the draining lymph nodes. This latter observation is supported by a recent report that ICOS expression enhances CD4 T cell migration to the lungs (39).

ICOS expression has been identified as a distinguishing marker conferring enhanced function and survival among human (58) and murine (41) Tregs. Interestingly, Protollin increased the proportion of LN $CD4^+ICOS^+Foxp3^+$ cells to $ICOS^-Foxp3^+$ cells, without a simultaneous impact on the ratio of $CD4^+CD25^+Foxp3^+$ to $CD25^-Foxp3^+$ cells, indicating a selective induction of

ICOS among Tregs. We also observed a specific induction of $CD4^+ICOS^+Foxp3^+$ and not $CD4^+CD25^+Foxp3^+$ cells in the lungs. Thus, our data establish the induction of ICOS rather than $CD4^+CD25^+Foxp3^+$ Tregs as a correlate of the inhibition of AHR. The increased relative ICOS, but not CD25 expression among $CD4^+Foxp3^+$ cells from Protollin-treated mice suggests that these $CD4^+$ cells may consist of distinct subpopulations that are differentially modulated by Protollin, or that the induced ICOS-expressing cells constitute only a minute subset of the $CD4^+CD25^+Foxp3^+$ population. An alternative explanation may be that Protollin induces ICOS equivalently among $CD25^+Foxp3^+$ and $CD25^-Foxp3^+$ Tregs.

Protollin-induced ICOS-expressing $CD4^+$ cells in the cervical LNs were, however, predominantly $Foxp3^-$. High ICOS expression has been reported in IL-10-producing cells such as the peripheral regulatory Tr1 cells (59). Interestingly, IL-10 expression in the NALT was increased by Protollin administration compared to PBS, and by combined Protollin/BPEx administration compared to BPEx alone. Additionally, nasal application of Protollin prior to allergen challenge increased the numbers of $CD4^+ICOS^+IL\text{-}10^+$ cells in the cervical LNs, as well as their proportion relative to IFN-γ^+ cells, without concomitant increases in lung $CD4^+ICOS^+IL\text{-}10^+$ cells. Intrapulmonary administration of LPS at a dose that is comparable to our intranasal application has been reported to inhibit experimental ovalbumin-induced asthma by inducing lung resident myeloid-derived suppressor cells to produce IL-10 (33). We also found no increase in IL-10 expression by $CD4^-$ cells in the lungs at any time point (data not shown), further supporting the absence of a direct effect of Protollin on the lungs. After two allergen challenges, Protollin-treated mice had higher numbers of $CD4^+ICOS^+IL\text{-}10^+$ cells in the lungs; however, this was not significantly different compared to PBS-treated, allergen challenged mice, and no increase in IL-10 protein levels could be detected in the BAL fluid at a later time point. Finally, the administration of anti-IL-10 mAb had no effect on the inhibition of AHR or BAL eosinophilia by Protollin, despite significant effects on other inflammatory cell populations in the BALs. Thus, despite preferential expression of IL-10 by Protollin-induced $CD4^+ICOS^+$ cells of the lymph nodes draining the nasal

mucosa, this cytokine did not appear to mediate the inhibitory effects of Protollin in this model.

ICOS deficiency in humans precipitates either immunodeficiency or autoimmunity, indicating its importance in immunoregulation (37). Through adoptive transfer studies, we confirmed that ICOS-expressing $CD4^+$ cells from the cervical LNs of Protollin-treated mice were capable of inhibiting the development of allergic airway disease, indicating that the TLR4-dependent induction of ICOS via the nasal mucosa can inhibit lower airway allergic disease. However, we did not confirm whether the $CD4^+ICOS^+Foxp3^+$ or $CD4^+ICOS^+Foxp3^-$ populations mediated this inhibition and whether their recruitment to the lungs was necessary for protection. There is evidence to suggest that either population could be inhibitory, given that ICOS-ICOS-L interaction alone has been shown to induce immunoregulatory negative feedback (60). Consistent with our findings, $ICOS^+$ cells inducing T cell anergy without a requirement for IL-10 have been identified in both humans and mice (61;62). Interestingly, the ICOS-ICOS-L interaction was reported to be necessary in only one of these reports, indicating that ICOS ligation is not always required for the inhibitory function of $ICOS^+$ cells. T cells relying on IL-10 and ICOS-ICOS-L interactions have also been shown to inhibit AHR and allergic airway inflammation (63); however, Foxp3 expression was not reported in any of these studies. Significantly, a recent report by Whitehead et al. demonstrated that induction of $ICOS^+Foxp3^+$ T cells mediated the natural suppression of IL-17-dependent allergic airway disease that occurs with repeated allergen challenge. This was IL-10-independent, despite its expression by these cells, and was rather mediated by the cytokine IL-35 (64). The authors proposed that targeting this pathway might be of therapeutic value for the treatment of allergic asthma in humans. Interestingly, both aforementioned studies that demonstrated ICOS-dependent spontaneous resolution of murine allergic airway disease following allergen challenge employed the experimental allergen ovalbumin, known to be tainted with significant levels of LPS. Whether the contaminating LPS has any bearing on the induction of ICOS in these studies was not described. Here, we show that the mucosal adjuvant Protollin induces ICOS

among $CD4^+Foxp3^+$ and $Foxp3^-$ cells via the nasal mucosa in a TLR4-dependent manner. TLR4 stimulation is known to enhance ICOS-ligand expression in innate immune cells (65;66); our finding that TLR4 induces ICOS provides additional insight into the regulation of CD4 T cell responses by innate immune stimuli. Finally, that the adoptive transfer of equivalent numbers of $CD4^+ICOS^+$ cells pooled from intranasal PBS or Protollin-treated mice appeared to yield the same inhibitory effects, suggests that the cervical LNs inherently contain resident $CD4^+ICOS^+$ cells that possess an intrinsic regulatory capacity and that stimulation of the nasal mucosa via TLR4 serves to amplify this population to a threshold that permits the inhibition of allergic airway disease in this model. Our work is supported by a report that specifically proliferating $CD4^+CD25^+$ and $CD25^-$ T cell subsets isolated from mouse cervical LNs possessed the capacity to transfer tolerance to delayed type hypersensitivity (51).

In conclusion, we find that nasal application of Protollin is sufficient to prevent the development of lower airway allergic disease in response to subsequent allergen exposure, with minimal inflammation of the lungs, and that this inhibition is dependent upon TLR4 but not TLR2. It is associated with an induction of ICOS expression in the nasal mucosa and on $CD4^+$ T cells of the draining lymph nodes and lungs. Finally, we show that resident $CD4^+ICOS^+$ cells of the cervical lymph nodes have the capacity to inhibit allergic airway disease in mice and can be amplified via TLR4 stimulation of the nasal mucosa.

3.7 Acknowledgments

We would like to thank Dr. Elizabeth Fixman for editing our manuscript and Dr. Meiyo Tamaoka, Mr. Hossein Koohsari, Ms. Rosa Pantano and Ms. Jamilah Saeed for their technical assistance, as well as Ms. Julie Lord and Mr. Eric Massicotte from the Institut de Recherches Cliniques de Montreal for cell sorting services.

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CHAPTER 4

The TLR4-TRIF pathway protects against the development of experimental allergic asthma

4.1 Prologue

To extend our analysis of the TLR4 signaling mechanisms pertinent to the inhibition of experimental allergic airway disease and expansion of ICOS-expressing $CD4^+$ T cell responses by Protollin via its action upon the nasal mucosa, here we evaluate the role of the adaptors MyD88 and TRIF, which are positioned at the apex of the two major signaling cascades downstream of TLR4 activation. Given the extensively documented importance of MyD88 to various TLR4-mediated processes, we expected MyD88-dependent signaling to play a significant role in the inhibition of allergic airway disease development by Protollin.

4.2 Abstract

The Toll-like Receptor (TLR) adaptor proteins Myeloid differentiating factor 88 (MyD88) and Toll, IL-1 Receptor and Resistance protein (TIR) domain-containing adaptor inducing interferon-β (TRIF) comprise the two principal limbs of the pleiotropic TLR signaling network. The diverse engagement of these pathways, influenced by receptor/adaptor polymorphisms, ligand dose and structure, may explain the complex role of TLR4 in allergic asthma. We studied the role of these adaptors in the TLR4-dependent inhibition of allergic airway disease development and induction of $CD4^+ICOS^+$ T cells by nasal application of Protollin, a mucosal adjuvant composed of TLR2 and 4 agonists. Wild-type (wt), *Trif* -/- or *Myd88* -/- mice were sensitized to birch pollen extract (BPEx), then received intranasal Protollin followed by consecutive BPEx challenges. Protollin's protection against allergic airway disease was TRIF-dependent and MyD88-independent. TRIF-deficiency diminished the $CD4^+ICOS^+$ T cell subsets in the lymph nodes draining the nasal mucosa, as well as their recruitment to the lungs. Overall, TRIF-deficiency reduced the proportion of cervical lymph node and lung $CD4^+ICOS^+Foxp3^-$ cells in particular. *In vitro*, both Protollin and the TRIF-biased adjuvant monophosphoryl lipid A also stimulated ICOS expression preferentially among $CD4^+Foxp3^-$ rather than $CD4^+Foxp3^+$ splenocytes. Adoptive transfer of cervical lymph node cells supported a role for Protollin-induced $CD4^+ICOS^+$ cells in the TRIF-dependent inhibition of AHR. Thus, our data demonstrate that stimulation of the TLR4-TRIF pathway can protect against the development of allergic airway disease and that a TRIF-dependent adjuvant effect on $CD4^+ICOS^+$ T cell responses is a contributing mechanism.

4.3 Introduction

Epidemiological, genetic, clinical and experimental data collectively indicate a potential for the toll-like receptor (TLR) 4 to initiate, exacerbate, or conversely prevent allergic airway disease (1;2). These apparently contradictory findings are likely due to several factors, such as the capacity of an allergen and influential immunomodulatory microbial exposures to activate multiple TLRs or interacting pattern recognition receptors simultaneously, the effect of gene polymorphisms upon the host response to the microbial stimuli, the structure of the specific TLR ligand(s), as well as the dose and the timing of exposure relative to the biological development of the immune system, disease onset or exacerbation. Elucidating the role of specific TLR signaling pathways in regulating the predominantly type 2 inflammatory response of the airways to aeroallergens that is characteristic of allergic asthma may also be an important step towards defining the specific conditions and mechanisms by which TLRs influence allergic disease. We have recently demonstrated that selective intranasal application of Protollin^{TM}, a mucosal adjuvant composed of purified bacterial TLR2 and 4 ligands, vesicles of hydrophobic outer-membrane proteins from *Neisseria meningitidis* and lipopolysaccharide (LPS) from *Shigella flexneri*, to sensitized animals prior to the allergen challenge prevented the development of allergen-induced airway hyperresponsiveness (AHR) and inflammation (3). The inhibition of allergic airway disease in mice was TLR4- and not TLR2-dependent and was associated with an expansion of $CD4^+$ T cells in the cervical lymph nodes draining the nasal mucosa expressing the inducible costimulatory molecule (ICOS), as well as an increase in lung $CD4^+ICOS^+$ cells. The induction of ICOS occurred in both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells and adoptive transfer of lymph node $CD4^+ICOS^+$ cells to sensitized mice prior to their challenge also prevented the development of allergic airway disease, suggesting that the expansion of these cells mediates the TLR4-dependent inhibition.

The adaptor proteins Myeloid differentiating factor 88 (MyD88) and Toll, IL-1 Receptor and Resistance protein (TIR) domain-containing adaptor inducing

interferon-β (TRIF) mediate distinct, but interacting, signaling cascades downstream of TLR ligation, ultimately leading to the production of pro-inflammatory mediators and type I interferons (IFNs) (4;5). Mice that are deficient in both proteins cannot signal via any of the 13 TLRs discovered to date (6). All TLRs are thought to signal via MyD88, with the exception of TLR3 which relies solely upon TRIF (7). Until recently, TLR4 was thought to be the only TLR that signals through both adaptors but TLRs 2 and 5 can also do so in certain conditions (8;9). To date, the TLR4-MyD88 pathway has been frequently implicated in allergic airway disease particularly in allergic sensitization via the respiratory mucosa or induction of type 2 immunity by inhaled antigens such as ovalbumin (OVA) (10), house dust mite allergen extract (11-13), German cockroach frass (14), short ragweed pollen (15) and the oxidizing pollutant ozone (16). TLR4-MyD88 signaling augments Th2-promoting molecules on dendritic cells, such as OX40-ligand and Jagged-1 (17;18) and epithelial and inflammatory cell production of thymic stromal lymphopoietin, IL-4, -6, -12, -25 and -33, GM-CSF, TNF-α, KC and vascular endothelial growth factor (19-22). MyD88 is also involved in the signal transduction of mediators associated with severe asthma and/or corticosteroid resistance such as IL-33 and S100A8 (23;24), while LPS inhalation with OVA can promote TLR4-MyD88-dependent glucocorticoid-resistant AHR in mice (25). Conversely, TLR4-MyD88 signaling has also been documented to inhibit the development of AHR and/or type 2 airway inflammation by LPS administered systemically (26) or by repeated inhalational exposure (27). Oral or respiratory exposure to a non-pathogenic cowshed bacterium (28;29), commercial bacterial extracts (30), or probiotic strains (31) also offers MyD88-dependent protection against the development of allergic airway disease.

Elucidating the relevance of the TLR4-TRIF pathway to allergic asthma may enhance our understanding of the complex relationship between TLR4 and this disease. Activation of TLR3 by the synthetic double-stranded RNA, polyinosinic-polycytidylic acid (poly(I:C)), was confirmed to elicit (32) as well as exacerbate (33) type 2 airway disease in animals TRIF-dependently. However, poly(I:C) was

also reported to inhibit experimental allergic asthma in mice (34), but it was not confirmed whether this was TRIF-dependent and poly(I:C) can also activate the TRIF-independent RNA-sensing protein kinase R (PKR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (35-37). Moreover, TLR4-TRIF signaling dampens non-infectious, hyaluronan-induced, lung inflammation (38). However, there are no reports to date directly confirming an anti-inflammatory role of the TRIF pathway in allergic asthma. In the current study, we sought to elucidate the roles of MyD88 and TRIF in mediating the TLR4-dependent inhibition of allergic airway disease development by intranasal Protollin. Our data indicate that activation of TLR4 signaling via the TRIF pathway is capable of preventing the development of allergic airway disease in mice and that the recruitment of $CD4^{+}ICOS^{+}$ cells to the lungs may be one contributing TRIF-dependent mechanism.

4.4 Methods

Animal treatments

Six to nine week-old, female *Myd88* -/- mice on a Balb/C background supplied by Dr. Qureshi and breeding pairs of C57Bl/6J Ticam^{1}/Lps^{2} (*Trif* -/-) mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were bred in the Animal Care Facilities of the McGill University Health Centre. Wild type (wt) C57Bl/6J mice were also purchased from Jackson Laboratories. All animals were housed in a specific pathogen-free animal facility under a 12 hour light/dark cycle with free access to food and water. All animals were sensitized on day 0 with a single 0.15 ml intraperitoneal (i.p.) injection of 20 protein nitrogen units (PNU) birch pollen allergen extract (BPEx; Greer Laboratories, Lenoir, NC, U.S.A.) and 3 mg alum hydrogel (Alum hydrogel 2%, Brenntag Biosector, Frederiksund, Denmark). This BPEx extract is used for clinical purposes in intradermal desensitization and thus is low in endotoxin (< 5 EU /ml; equivalent to < 0.05 EU /kg body weight). Experimental procedures were approved by the McGill University Animal Care Committee.

Experimental asthma protocol and nasal immunomodulation

Allergic airway disease was induced in mice as described previously (3). Following sensitization, on each of days 7, 10 and 13, awake mice received nasal applications, without prior anesthesia, of 15 µl of either phosphate-buffered saline (PBS) or Protollin (15 µg). Previously, it has been confirmed that >90% of fluid administered in this manner deposits in the nose/upper airways and the remaining portion is found largely in the gastrointestinal tract with very little in the lungs (39). Protollin consisted of a 1:1.1 ratio of Neisserial proteins to Shigella LPS at a concentration of 1 µg/µl LPS, resulting in an intranasal dose of approximately 15 µg of LPS and Neisserial proteosomes on each of the indicated days. On days 15, 16 and 17, mice were allergen challenged intranasally under light isoflurane anesthesia (4%) with a dose of 25 PNU BPEx in a volume of 36 µl sterile PBS. Control mice were sham-challenged with sterile PBS only.

Assessment of allergen-induced airway hyperresponsiveness

Airway responses to aerosolized doubling concentrations of the bronchoconstrictor, methacholine (MCh; acetyl-β-methylcholine; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) were assessed on day 19, 48 hrs after the final allergen challenge. Mice were anesthetized with an injection of xylazine hydrochloride (10 mg/kg, i.p.) followed by i.p. administration of sodium pentobarbital (32 mg/kg). Once anesthesia was achieved, mice were tracheostomized using a 19G metal cannula and connected via the endotracheal cannula to a commercial small animal ventilator (FlexiVent, SCIREQ Inc., Montreal, Canada). The animal was ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure of 3 cmH_2O. The mouse was then paralyzed with a 1 mg/kg pancuronium bromide i.p. injection prior to the measurement of baseline respiratory mechanics. A 1.2 second, 2.5 Hz single-frequency forced oscillation maneuver was performed at 10 sec intervals and respiratory system resistance (Rrs) and elastance (Ers) were calculated with commercial software. Doubling concentrations of methacholine (MCh; acetyl-β-methylcholine; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) from 15.6 – 250 mg/kg were delivered to the mouse as an aerosol using a 4 second nebulization period synchronized with inspiration at a nebulization duty cycle of 50%. Allergen-induced AHR was assessed by recording the peak Rrs and Ers following each dose of MCh administered.

Assessment of airway inflammation

On day 19, bronchoalveolar lavage (BAL) was performed using saline containing 10% fetal bovine serum. The recovered cell pellet was used to measure the total number of cells in the BAL and cytospins were prepared and stained with Diff-Quik stain (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany) for differential cell counts.

Measurement of BPEx-specific serum IgE

On day 19, blood was collected by exsanguination into serum separator tubes and left at room temperature to clot. Samples were centrifuged at 4000 g for 5 minutes and the serum was collected and stored at -20^{o}C. BPEx-specific serum IgE was measured by ELISA, according to the manufacturer's instructions (Biolegend, San Diego, CA, U.S.A.).

Flow cytometric analysis of cervical lymph node and lung CD4^{+} T cell ICOS expression

In order to characterize Protollin's adjuvant effect on the CD4^{+} T helper cell and regulatory T cell response, cervical lymph nodes and lungs were harvested from BPEx-sensitized mice on day 16 and 17 respectively, 24 hrs after one or two BPEx allergen challenges, following 3 administrations (days 7, 10 and 13) of either PBS or Protollin alone. Superficial cervical lymph nodes were isolated and placed in RPMI-1640 medium, containing 8% heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml gentamycin and 10 mM HEPES. A single cell suspension was obtained by mincing and crushing the tissue on a 70 μm BD Falcon cell strainer. Lungs were injected and minced *ex vivo* in a solution of RPMI-1640 (Invitrogen, Life Technologies Inc., Burlington, ON, Canada), supplemented with 0.2 Wunsch units/ml Collagenase from *Clostridium histolyticum* (Type XI-S), 1000 Dornase units/ml DNAse I (Type II-S) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and 0.5 mM Ca^{2+}. The tissue was then incubated on an orbital shaker at 37^{o}C for 1 hour after which digestion was inhibited by the addition of cold complete RPMI medium with 2 mM EDTA and 50 μM β-mercaptoethanol. The lung digest was then repeatedly passed through a 16G needle and then through a 70 μm BD Falcon cell strainer and centrifuged and red blood cells were lysed with ammonium chloride solution. Isolated lymph node or lung cells were stained as described previously. Briefly, cells were stained with anti-CD4 FITC, anti-ICOS PE, and anti-Foxp3 APC monoclonal antibodies (mAb) or the appropriate isotype controls. Cell acquisition was performed using either the BD FacsCalibur or LSRII and analyzed with Cell Quest Pro or FlowJo

software, respectively (BD Biosciences, Mississauga, ON, Canada). The percentage of ICOS-expressing $CD4^+Foxp3^+$ or $CD4^+Foxp3^-$ cells was analyzed according to the gating strategy shown in Supp. Fig. 1.

In vitro splenocyte stimulation with Protollin to assess ICOS induction in $CD4^+$ *T cells*

To verify the MyD88 or TRIF-dependency of ICOS induction on $CD4^+$ cells by Protollin *in vitro*, splenocytes were harvested from wt C57Bl/6J mice and were cultured at 5 million cells/ ml with either complete RPMI medium, or increasing concentrations (0.01 – 10 μg/ml) of Protollin or synthetic VacciGrade monophosphoryl lipid A of the *E. coli* chemotype (MPLA; Invivogen, San Diego, CA, U.S.A.), a low-toxicity derivative of LPS and TLR4 ligand approved as a commercial vaccine adjuvant with demonstrated TRIF-biased activity (40). After 1, 3, or 5 days of culture, total splenocytes were stained as described earlier to assess ICOS expression on $CD4^+Foxp3^+$ regulatory T cells or $CD4^+Foxp3^-$ helper T cells by flow cytometry. The chosen time point and dose of Protollin and MPLA were determined based on a time course (up to 5 days) and dose response evaluation (0.1-10 μg/ml Protollin or MPLA) of wt splenocytes and sorted $CD4^+$ cells.

Adoptive transfer of FACS-sorted wt $CD4^+ICOS^+$ *cells to Trif knockout mice*

On day 14, cervical lymph nodes were harvested and pooled from wt C57Bl/6J BPEx-sensitized mice that received nasal applications of Protollin on days 7, 10 and 13, and isolated cells were stained with anti-CD4 FITC and anti-ICOS PE mAb. $CD4^+ICOS^+$ cells were sorted by flow cytometry using a Beckman Coulter MoFlo cell sorter and either 0.1 or 0.3 million cells were adoptively transferred (i.p.) in 0.3 ml sterile PBS to *Trif* -/- mice that had been sensitized with BPEx in parallel with the wt animals but that were otherwise untreated (an adoptive transfer of 0.3 million $CD4^+ICOS^+$ cells was previously shown to be effective in inhibiting the development of AHR and airway inflammation in wt Balb/C mice).

Mice were then challenged on days 15-17 and underwent lung function testing in response to MCh, as well as BAL collection on day 19 (Fig. 7A).

Adoptive transfer of MACS-sorted total $CD4^+$ cells to wt mice

On day 14, cervical lymph nodes were harvested and pooled from wt or *Trif* -/- BPEx-sensitized mice that received nasal applications of either PBS or Protollin on days 7, 10 and 13. $CD4^+$ cells were isolated by positive selection using mouse CD4 (L3T4) MicroBeads and magnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA, U.S.A.). 3 million sorted cells from wt PBS-treated mice or wt or *Trif* -/- that were treated with Protollin were then adoptively transferred (i.p.) in 0.3 ml sterile PBS to wt mice that had been sensitized in parallel but that were otherwise untreated. Mice were then challenged on days 15-17 and underwent lung function testing in response to MCh, as well as BAL collection on day 19 (Fig. 8A).

Statistical analysis

Airway responses to MCh bronchoprovocation were analyzed in GraphPad Prism Version 5 (GraphPad software, San Diego, CA, U.S.A.) by two-way ANOVA followed by Bonferroni post-tests comparing all experimental groups to each other. One-way ANOVA and post-hoc Newman-Keuls' tests were used for all other analyses involving three or more groups, or unpaired Student's t-test was used in the case where only two experimental groups were compared. Data were log-transformed prior to statistical analysis when not normally distributed.

4.5 Results

TRIF signaling can prevent the development of experimental allergic airway disease

We have previously reported that the inhibition of allergen-induced AHR and BAL eosinophilia in Balb/C mice by nasal Protollin administration prior to allergen challenge was TLR4- and not TLR2-dependent (3) and proceeded to study the effects of Protollin in *Myd88* -/- mice. Mice that received Protollin exhibited significantly lower respiratory system resistance (Rrs) and elastance (Ers) values in response to increasing doses of MCh (Fig. 1A) and serum BPEx-specific IgE (Fig. 1C) compared to mice that were BPEx-challenged but that had not received Protollin. Total inflammatory cells and eosinophils were not significantly lower in the BALs of Protollin-treated mice (Fig. 1B), suggesting a potential contribution of the MyD88 pathway to the inhibition of airway inflammation. However, these data collectively indicated that Protollin retained its capacity to inhibit the airway dysfunction associated with allergic airway disease even in the absence of MyD88. *Trif* -/- mice were only available on the C57Bl/6J background; Intranasal Protollin also potently inhibited AHR in wt mice of this strain (Fig. 2A) and significantly reduced total BAL cell counts and eosinophils (Fig. 2B). On the other hand, Protollin failed to inhibit AHR (Fig. 2C) and airway inflammation (Fig. 2D) in *Trif* -/- mice. Serum BPEx-specific IgE levels in either strain of C57Bl/6J mice did not exceed the lower limit of detection and allergen-associated increases could not be detected to allow confirmation of whether Protollin's inhibition of IgE synthesis was also TRIF-dependent.

In vivo, induction of ICOS in $CD4^+$ cells and expansion of $CD4^+ICOS^+$ T cells is mediated by both MyD88 and TRIF

Nasal application of Protollin to conscious animals was previously shown to increase ICOS mRNA expression in the nasal-associated lymphoid tissues, as well as protein expression in both the $CD4^+Foxp3^+$ regulatory cell and $CD4^+Foxp3^-$ T cell populations in the cervical lymph nodes draining the nasal mucosa. The

induction of ICOS and expansion of these cells was TLR4-dependent. Here, we assessed the effects of Protollin upon the cervical lymph nodes at the same time point, following intranasal PBS or Protollin administrations and a single intrapulmonary allergen challenge, after which total lymph node cell numbers were similarly augmented by Protollin in wt and *Myd88* -/- Balb/C mice, but were unaltered in *Tlr4* -/- mice, suggesting that the TLR4-dependent lymphoproliferation was intact even in the absence of MyD88 (Supp. Fig. 2A). Total lymph node cell numbers were also significantly augmented in wt C57Bl/6J mice and seemingly so in the *Trif* -/- strain, though not significantly(Supp. Fig. 2A). Interestingly, in *Myd88* -/- mice on the Balb/C background, ICOS was significantly induced by Protollin within the $CD4^+Foxp3^+$ population (Fig. 3B). A significant induction of ICOS in the cervical lymph node Tregs of the C57Bl/6J strains was not detected. However, the absolute number of $CD4^+ICOS^+Foxp3^+$ cells and the relative proportion of $CD4^+ICOS^+Foxp3^+$ to $CD4^+ICOS^-Foxp3^-$ cells were increased by Protollin in both *Myd88* -/- and wt C57Bl/6J mice but were not significantly increased in *Trif* -/- mice (Fig. 3C and Fig. 6C), indicating that the expansion of these cells is TRIF-dependent. We previously reported that Protollin primarily augmented the numbers of $CD4^+ICOS^+Foxp3^-$ cells (3). Here, Protollin significantly increased the percentage of $ICOS^+$ cells among the $CD4^+Foxp3^-$ population even in the absence of MyD88 signaling, but also in the absence of functional TRIF, though the degree of induction of ICOS in the *Trif* -/- mice was significantly lower than in the wt C57Bl/6J mice (Fig. 3A and D) (the degree of induction in the *Myd88* -/- mice also appeared to be incomplete). Finally, Protollin increased the absolute number of $CD4^+ICOS^+Foxp3^-$ cells in the lymph nodes of all three strains (Fig. 3E), indicating either redundancy between the MyD88 and TRIF pathways in the induction of ICOS, or a contribution from both pathways. Overall, however, TRIF deficiency resulted in a reduced proportion of $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells expressing ICOS in the cervical lymph nodes of Protollin-treated mice (Fig. 3D and Fig. 6A,C).

*In vitro, Protollin induces ICOS primarily in CD4$^+$Foxp3$^-$ cells and expands CD4$^+$ICOS$^+$*Foxp3$^-$ *cells in splenocyte cultures*

To further examine the role of MyD88 and TRIF in the induction of ICOS, splenocytes from naïve wt C57Bl/6J mice were harvested and stimulated with increasing concentrations of Protollin or the TLR4-TRIF-biased adjuvant, synthetic monophosphoryl lipid A (MPLA). Only the highest concentration of Protollin significantly increased the percentage of ICOS-expressing cells within the CD4$^+$Foxp3$^+$ population, which occurred rapidly (within one day of culture) but was not sustained (Fig. 4B). Interestingly, the relative proportion of ICOS expression among Tregs spontaneously increased even in the absence of either adjuvant during the course of the culture (Fig. 4B). Also, only the highest concentration of Protollin augmented the absolute number of CD4$^+$ICOS$^+$Foxp3$^+$ cells by day 5 of culture, relative to the numbers found among splenocytes that were cultured with medium alone (Fig. 4D). On the other hand, multiple doses of Protollin, but not MPLA, were capable of inducing ICOS among CD4$^+$Foxp3$^-$ cells and expansion of CD4$^+$ICOS$^+$Foxp3$^-$ cells, as early as within a single day of stimulation (Fig. 4A, C and E). The proportion of CD4$^+$Foxp3$^-$ cells expressing ICOS normalized within 3 days of culture but was most dramatically elevated at day 5 by the lowest concentration of Protollin (0.01 μg/ml) and highest two concentrations of MPLA (1 and 10 μg/ml). This was partially due to the emergence of a new discrete population of CD4$^+$ICOS$^+$ cells (see the representative dot plots in Fig. 4A), different from what was observed *in vivo*. Thus, whereas the absolute numbers of CD4$^+$ICOS$^+$Foxp3$^-$ cells cultured in medium alone declined rapidly over the course of the 5 days, all doses of Protollin induced an early and sustained expansion of these cells while MPLA promoted a significant increase only at higher concentrations and at a later time point (Fig. 4E). Overall, the data indicate that Protollin and MPLA selectively and preferentially stimulate the induction of CD4$^+$ICOS$^+$Foxp3$^-$ rather than CD4$^+$ICOS$^+$Foxp3$^+$ cells among total splenocytes *in vitro*. Furthermore, the lower doses of Protollin and higher doses of MPLA which were particularly adept at inducing ICOS-expressing CD4$^+$Foxp3$^-$ cells on day 5, also potently stimulated

ICOS expression among $CD4^-$ cells (Supp. Fig. 4), an observation that was not apparent *in vivo*.

TRIF signaling is necessary for the recruitment of $CD4^+ICOS^+$ *cells to the lungs*

We have previously shown that $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ cell numbers are elevated in the lungs of Protollin-treated wt Balb/C mice after successive allergen challenges, implying a recruitment of these cells to the lungs. In wt C57Bl/6J mice, Protollin exposure increased only the percentage of $CD4^+Foxp3^-$ cells expressing ICOS (Fig. 5B) and not $CD4^+Foxp3^+$ cells (Fig. 5A), as we observed in the lymph nodes. However, in contrast to the earlier mentioned Balb/C data and C57Bl/6J lymph node data (Fig. 3C), only $CD4^+ICOS^+Foxp3^-$ cells (Fig. 5D) and not $CD4^+ICOS^+Foxp3^+$ cells (Fig. 5C) were increased in absolute numbers by Protollin. Significantly, the proportion of $CD4^+Foxp3^-$ cells expressing ICOS (Fig. 5B and Fig. 6B), as well as the absolute number of $CD4^+ICOS^+Foxp3^-$ cells in the lungs (Fig. 5D), was not increased by Protollin in *Trif* -/- mice, despite the expansion of these cells in the lymph nodes, supporting a role for TRIF signaling in mediating the recruitment of the $CD4^+ICOS^+$ cells to the lungs.

$CD4^+ICOS^+$ cells contribute to the TRIF-dependent inhibition of allergen-induced airway hyperresponsiveness

$CD4^+ICOS^+$ cells isolated from the cervical lymph nodes of Protollin-treated wt Balb/C mice have the capacity to inhibit the development of allergen-induced airway disease upon adoptive transfer (3). To confirm the immunoregulatory potential of these cells in the C57Bl/6J strain, FACS-sorted $CD4^+ICOS^+$ cells isolated from the cervical lymph nodes of Protollin-treated wt C57Bl/6J mice were adoptively transferred to *Trif* -/- recipients (Fig. 7A). An adoptive transfer of 0.3 million $CD4^+ICOS^+$ cells potently inhibited AHR (Fig. 7B) but, surprisingly, was ineffective in reducing airway inflammatory cell numbers (Fig. 7C). Therefore, these data indicate that wt $CD4^+ICOS^+$ cells adoptively transferred via the systemic route can by-pass the requirement for TRIF to inhibit AHR.

Given that the proportion of $CD4^+$ cells expressing ICOS was lower in the cervical lymph nodes of sensitized wt PBS-treated mice and *Trif* -/- Protollin-treated mice compared to wt Protollin-treated mice (Fig. 3D and Fig. 6A,C), we proceeded to adoptively transfer an equivalent number of lymph node $CD4^+$ cells from each of these groups of mice to wt C57Bl/6J recipients prior to their allergen challenge to determine the capacity of these cells to inhibit allergen-induced airway disease (Fig. 8A). Notably, only the $CD4^+$ cells from Protollin-treated wt C57Bl/6J and not from *Trif* -/- donors inhibited AHR; mice that received $CD4^+$ cells from wt Protollin-treated mice had significantly reduced Rrs and Ers responses to methacholine compared to those that received $CD4^+$ cells from either wt PBS- or *Trif* -/- Protollin-treated mice (Fig. 8B), consistent with the notion that $CD4^+ICOS^+$ cells contribute to the TRIF-dependent inhibition of AHR. Surprisingly, the $CD4^+$ cells from both wt and *Trif* -/- Protollin-treated donors reduced airway inflammation, namely BAL total inflammatory cells and eosinophils (Fig. 8C), as compared to the effects of $CD4^+$ cells adoptively transferred from wt PBS-treated mice. Collectively, this suggests that Protollin is also evoking MyD88-dependent modulation of $CD4^+ICOS^-$ cells in the cervical lymph nodes which are capable of inhibiting allergic airway inflammation in wt C57Bl/6J mice when adoptively transferred via the systemic route.

Figure 1. The inhibition of allergic airway disease by intranasal Protollin is MyD88-independent. Airway responses (respiratory system resistance and elastance) to aerosolized MCh (A), BAL fluid total inflammatory cells and eosinophils (B) and serum BPEx-specific IgE (C) were measured in *Myd88* -/- mice following nasal application to conscious mice of either PBS or Protollin and intranasal sham (PBS) or allergen (BPEx) challenge with light anesthesia (n=6-9 animals/group pooled from > 3 independent experiments).

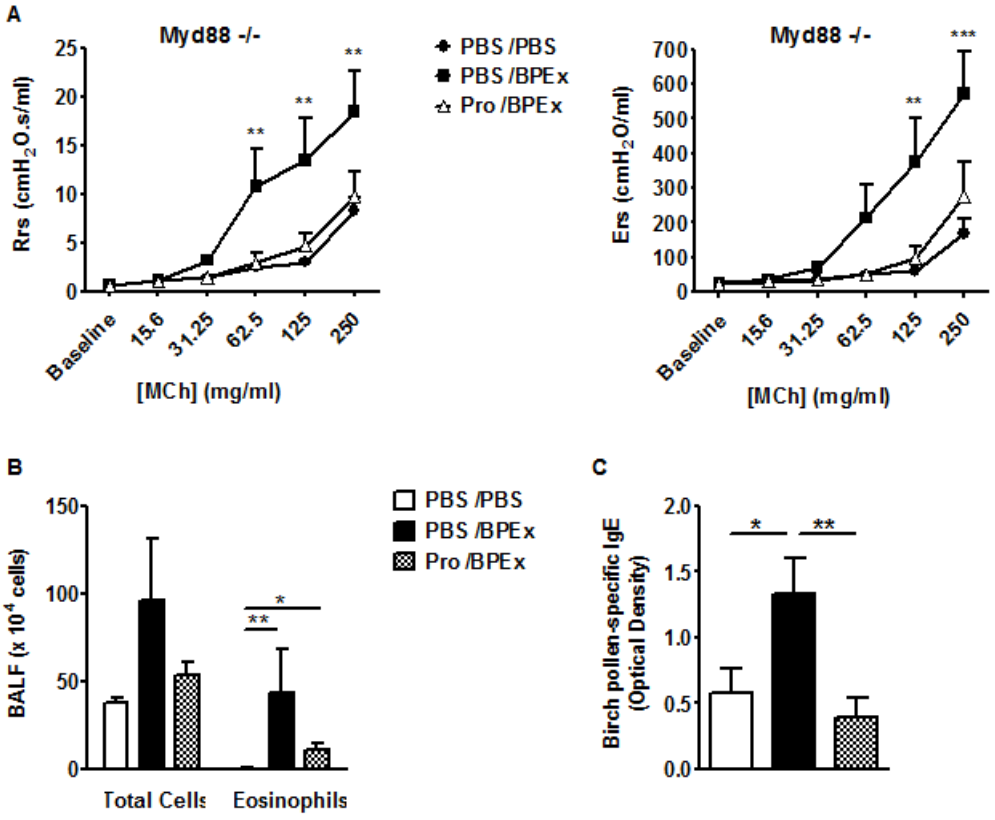

Figure 2. The inhibition of allergic airway disease by intranasal Protollin is TRIF-dependent. Airway responses (respiratory system resistance and elastance) to aerosolized MCh and BAL fluid total inflammatory cells and eosinophils were quantified in C57Bl/6J wt mice (A and B; n=10-14 animals/group from > 3 independent experiments) or *Trif* -/- mice (C and D; n=7-8 animals/group from > 3 independent experiments) following nasal PBS or Protollin applications and intranasal PBS or BPEx challenges.

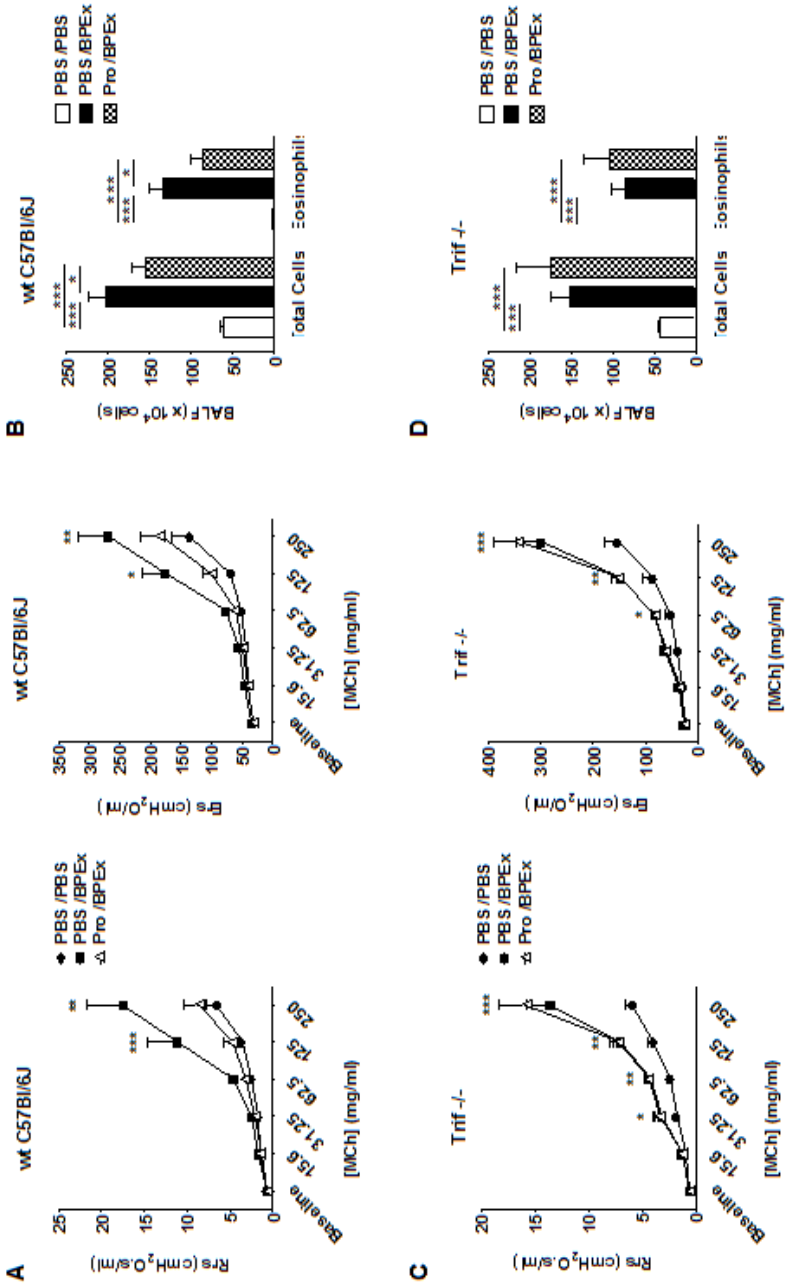

Figure 3. The induction and expansion of ICOS-expressing $CD4^+$ T cells in the cervical lymph nodes is significantly dependent on TRIF. Lymph node cells harvested from C57Bl/6J wt or *Trif* -/- mice or Balb/C *Myd88* -/- mice on day 16, 24 hrs after a single PBS or BPEx challenge following nasal applications of either PBS or Protollin were stained for CD4, ICOS and Foxp3. Representative dot plots show ICOS expression among gated $CD4^+Foxp3^-$ cells (A). Percentage of ICOS-expressing $CD4^+Foxp3^+$ (B) or $CD4^+Foxp3^-$ cells (D). Absolute number of lymph node $CD4^+ICOS^+Foxp3^+$ (C) and $CD4^+ICOS^+Foxp3^-$ cells (E). (Balb/C *Myd88* -/-, n=9-10 animals/group from > 3 independent experiments; C57Bl/6J wt or *Trif* -/-, n=7 animals/group from at least 2 independent experiments per strain).

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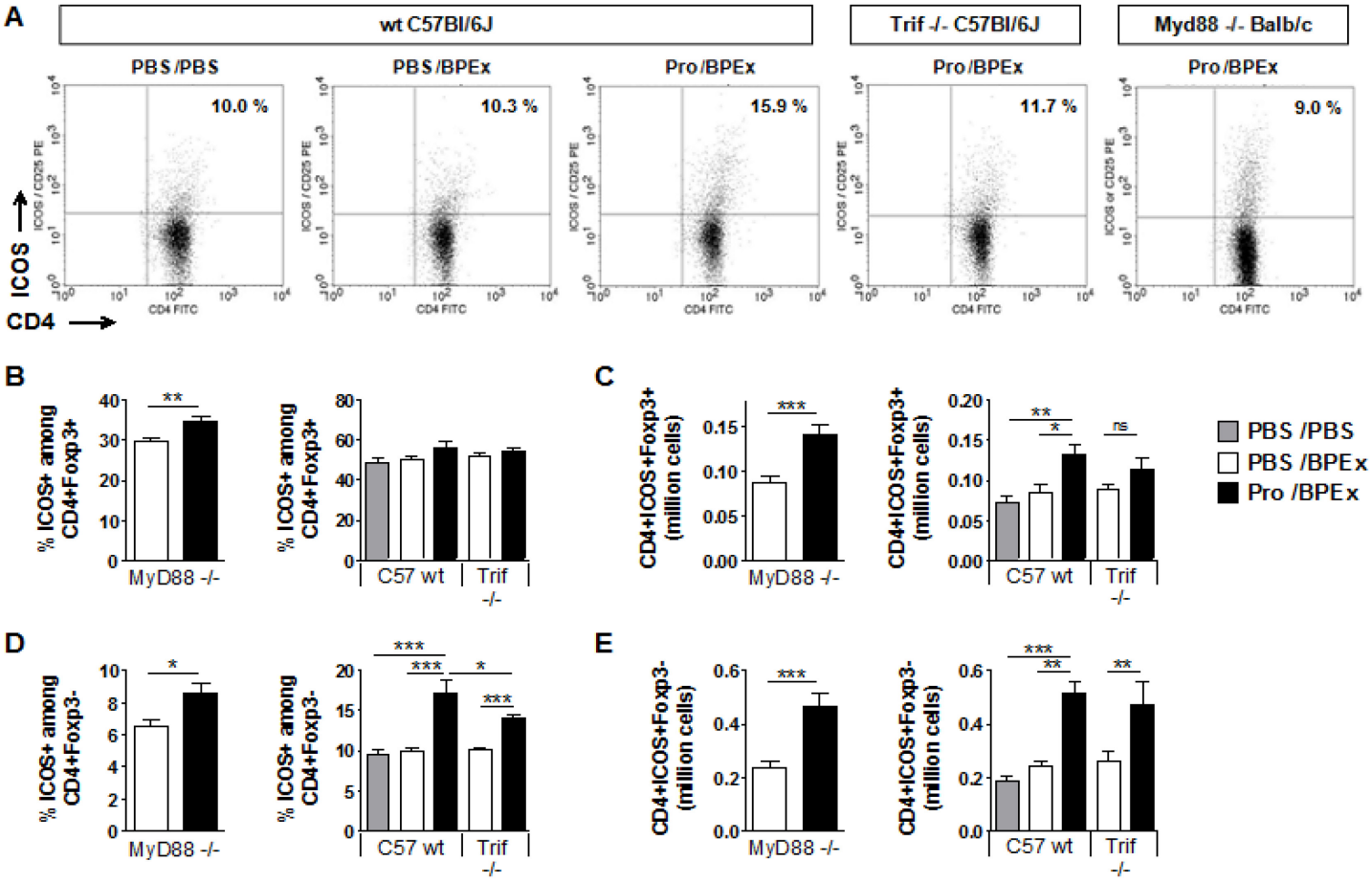
A
wt C57Bl/6J
Trif -/- C57Bl/6J
Myd88 -/- Balb/c
PBS /PBS
PBS /BPEx
Pro /BPEx
Pro /BPEx
Pro /BPEx
10.0 %
10.3 %
15.9 %
11.7 %
9.0 %
ICOS
CD4
B
% ICOS+ among CD4+Foxp3+
MyD88 -/-
C57 wt
Trif -/-
C
CD4+ICOS+Foxp3+ (million cells)
MyD88 -/-
C57 wt
Trif -/-
PBS /PBS
PBS /BPEx
Pro /BPEx
D
% ICOS+ among CD4+Foxp3-
MyD88 -/-
C57 wt
Trif -/-
E
CD4+ICOS+Foxp3- (million cells)
MyD88 -/-
C57 wt
Trif -/-

Figure 4. *In vitro* stimulation of total splenocytes with Protollin or MPLA induces ICOS predominantly on $CD4^+Foxp3^-$ cells. Splenocytes harvested from naïve wt C57Bl/6J mice were cultured up to 5 days with complete RPMI medium and a range of concentrations of Protollin or MPLA (0.01 – 10 μg/ml). Cells were stained after 1, 3 and 5 days of culture to assess CD4, ICOS and Foxp3 expression. Representative dot plots show ICOS expression among gated $CD4^+Foxp3^-$ cells (A) and the emergence of a distinct population of ICOS-expressing cells particularly after 5 days of culture with Protollin or MPLA (circled**). Percentage of ICOS-expressing $CD4^+Foxp3^+$ (B) or $CD4^+Foxp3^-$ cells (C). Absolute number of lymph node $CD4^+ICOS^+Foxp3^+$ (D) and $CD4^+ICOS^+Foxp3^-$ cells (E). (n=3 animals per culture condition per day from 1 experiment).

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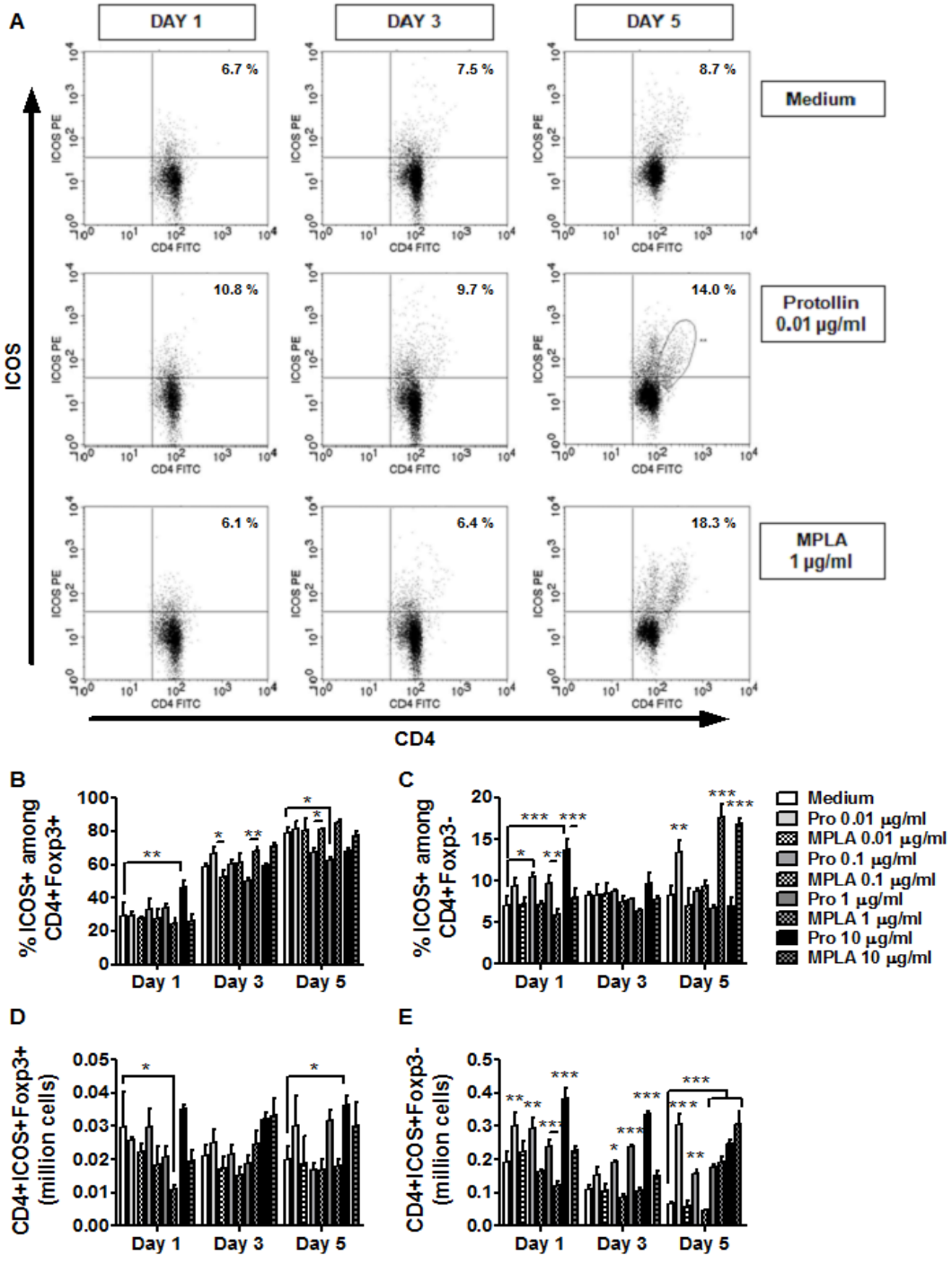

A
DAY 1
DAY 3
DAY 5
6.7 %
7.5 %
8.7 %
Medium
10.8 %
9.7 %
14.0 %
Protollin
0.01 µg/ml
6.1 %
6.4 %
18.3 %
MPLA
1 µg/ml
ICOS
CD4
ICOS PE
CD4 FITC
B
% ICOS+ among
CD4+Foxp3+
Day 1
Day 3
Day 5
C
% ICOS+ among
CD4+Foxp3-
Medium
Pro 0.01 µg/ml
MPLA 0.01 µg/ml
Pro 0.1 µg/ml
MPLA 0.1 µg/ml
Pro 1 µg/ml
MPLA 1 µg/ml
Pro 10 µg/ml
MPLA 10 µg/ml
D
CD4+ICOS+Foxp3+
(million cells)
E
CD4+ICOS+Foxp3-
(million cells)

Figure 5. Protollin increases the percentage and absolute number of ICOS-expressing $CD4^+Foxp3^-$ T cells but not $CD4^+Foxp3^+$ Tregs in the lungs, which occurs TRIF-dependently. Percentage of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells expressing ICOS (A and B, respectively) and absolute numbers of $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ cells (C and D, respectively) in lungs harvested from C57Bl/6J wt or *Trif* -/- mice on day 17, 24 hrs after two PBS or BPEx challenges following nasal applications of either PBS or Protollin (n=6-8 animals/group from at least 2 independent experiments per strain).

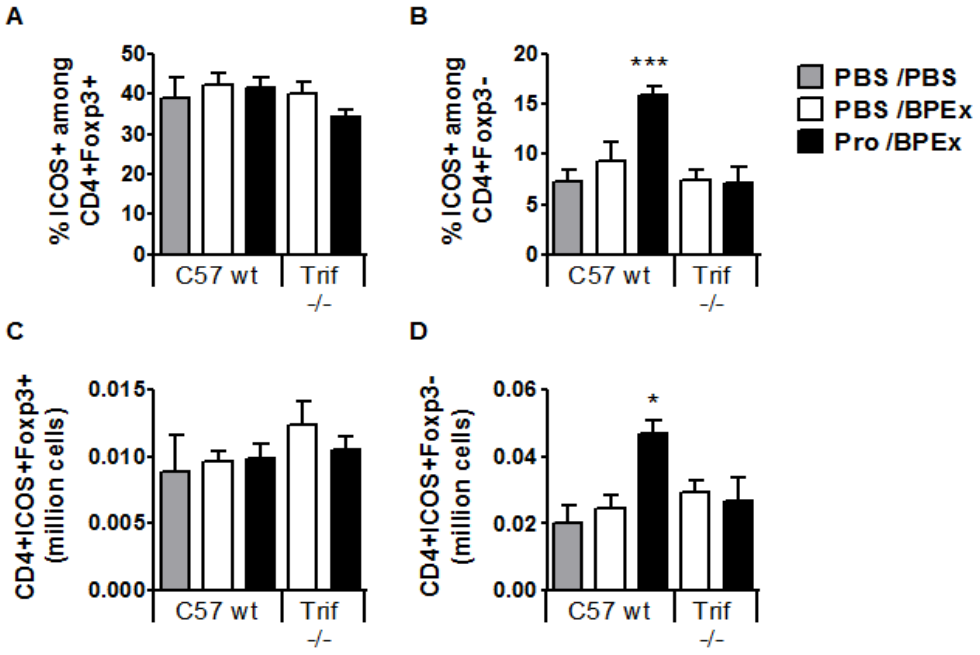


Figure 6. Protollin increases the relative proportion of $CD4^+ICOS^+$ cells in the lymph nodes and lungs of C57Bl/6J mice in TRIF-dependent fashion. Ratio of $CD4^+ICOS^+Foxp3^-$ (A and B) or $ICOS^+Foxp3^+$ (C and D) cells relative to $CD4^+ICOS^-Foxp3^-$ cells in the cervical lymph nodes and lungs of C57Bl/6J wt or *Trif* -/- mice following nasal PBS or Protollin applications and BPEx challenges (n=6-8 animals/group from at least 2 independent experiments per strain).

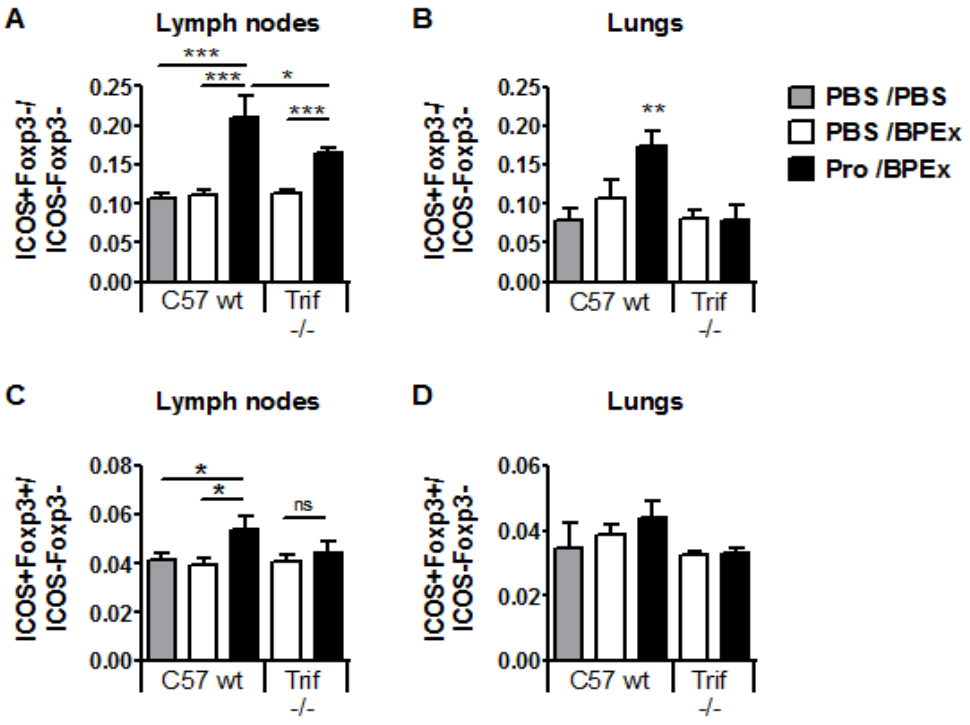


Figure 7. $CD4^+ICOS^+$ cells from the cervical lymph nodes of Protollin-treated wt mice have the capacity to inhibit AHR when adoptively transferred to *Trif* -/- mice. $CD4^+ICOS^+$ cells sorted by FACS from lymph nodes of wt Protollin-treated mice on day 14 were adoptively transferred (0.1 or 0.3 million cells, i.p.) to *Trif* -/- mice that had been sensitized in parallel but that did not receive Protollin, after which the recipient mice were challenged with BPEx allergen, as described in (A). Airway responses to aerosolized MCh (B) and BAL fluid total inflammatory cells and eosinophils (C) were quantified on day 19 as in prior experiments (n=5 animals/group from 3 independent experiments).

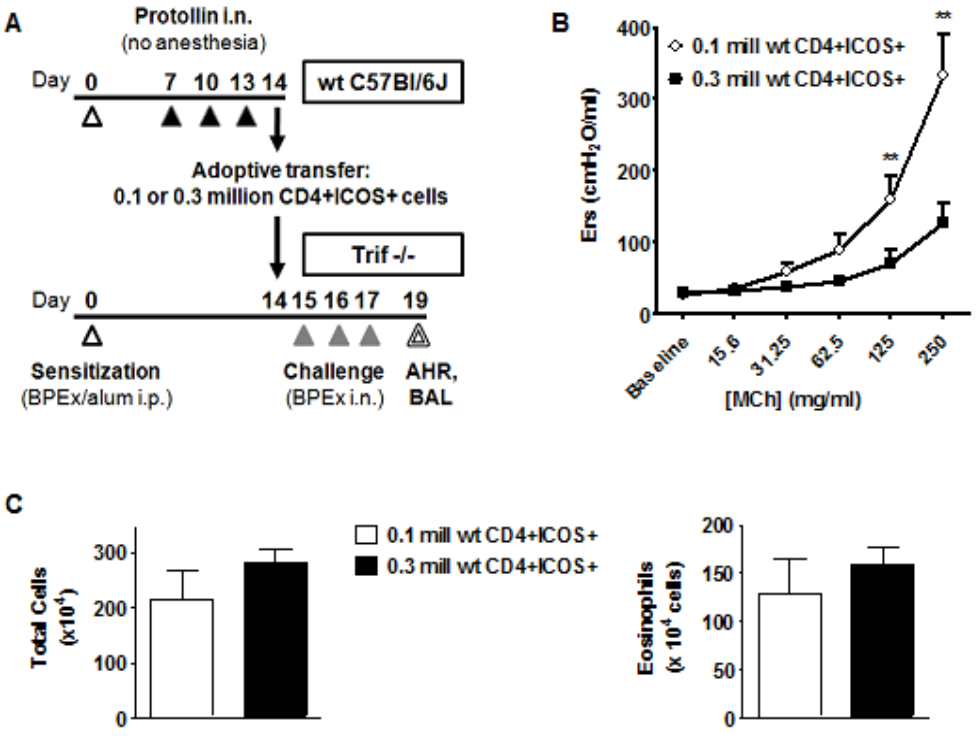
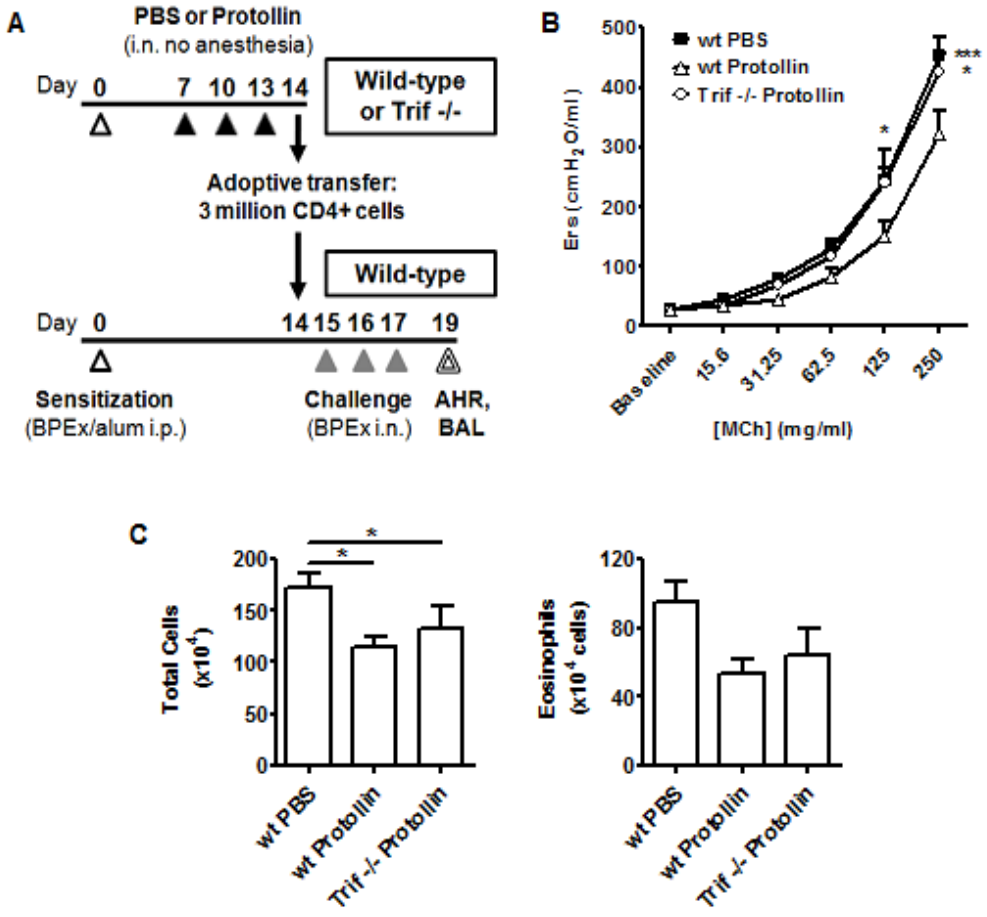
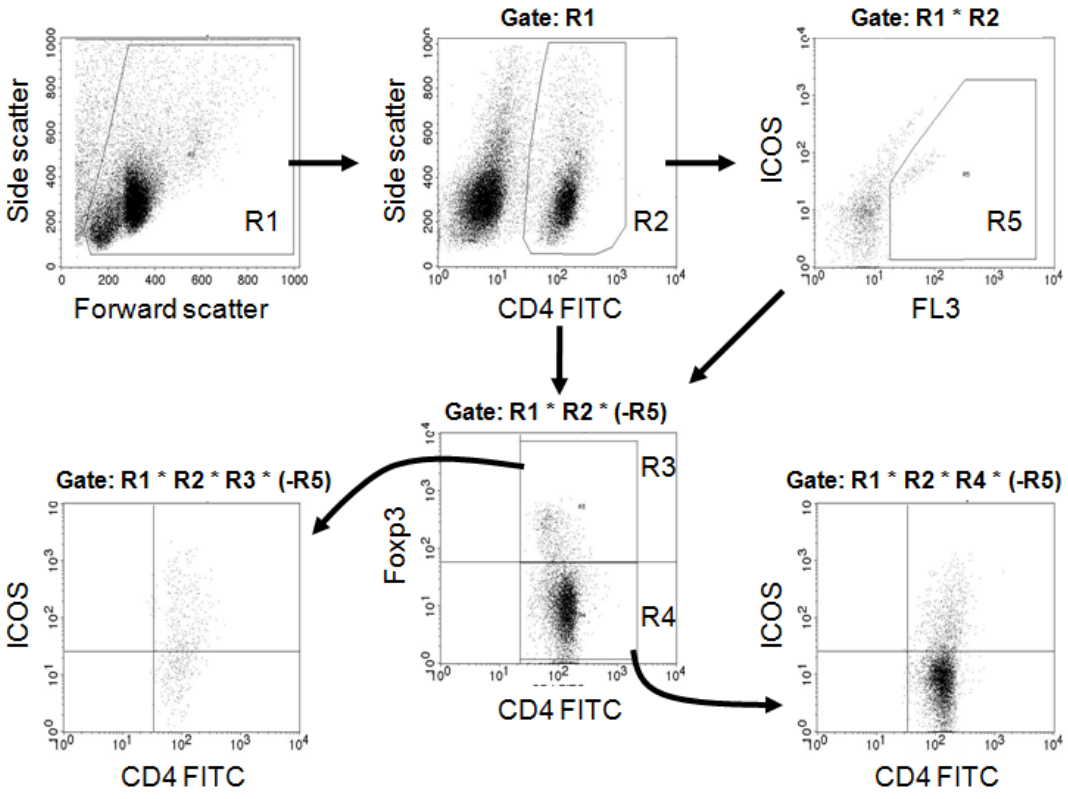


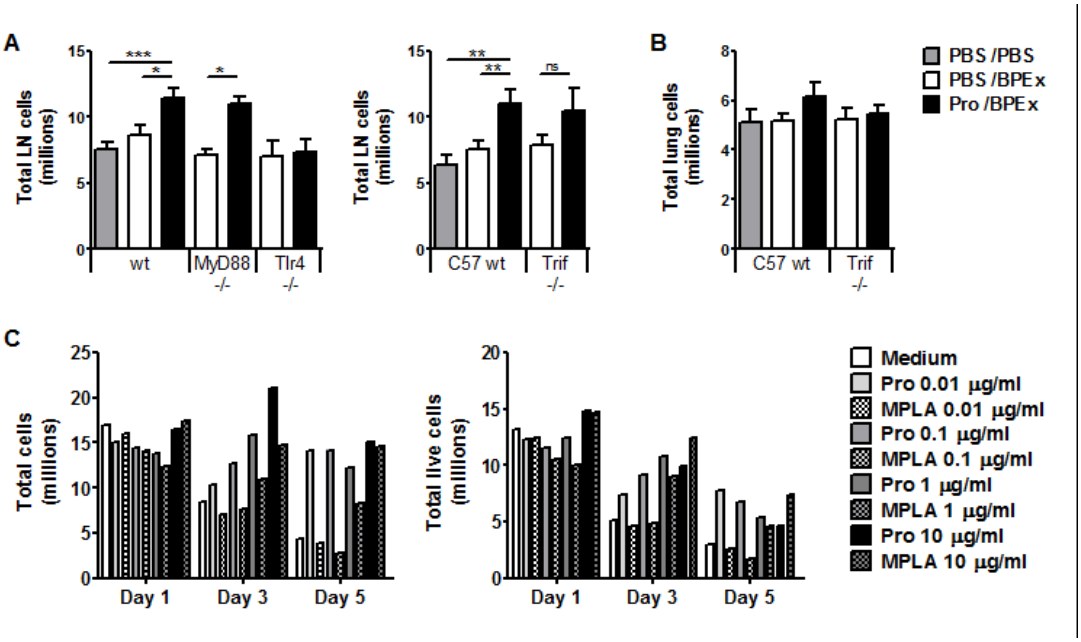
Figure 8. CD4$^+$ cells from lymph nodes of Protollin-treated wt mice are capable of significantly inhibiting AHR upon adoptive transfer whereas CD4$^+$ cells from Protollin-treated *Trif* -/- mice are not. Total CD4$^+$ cells were sorted by MACS from lymph nodes of wt Protollin-treated, PBS-treated, or *Trif* -/- Protollin-treated mice on day 14 and were adoptively transferred (3 million cells, i.p.) to wt C57Bl/6J mice that had been sensitized in parallel but that were otherwise untreated, after which the recipient mice were challenged with BPEx allergen, as described in (A). Airway responses to aerosolized MCh (B; n=6-7 animals/group from 3 independent experiments) and BAL fluid total inflammatory cells and eosinophils (C; n=12-14 animals/group from > 3 independent experiments) were quantified on day 19 as in prior experiments.



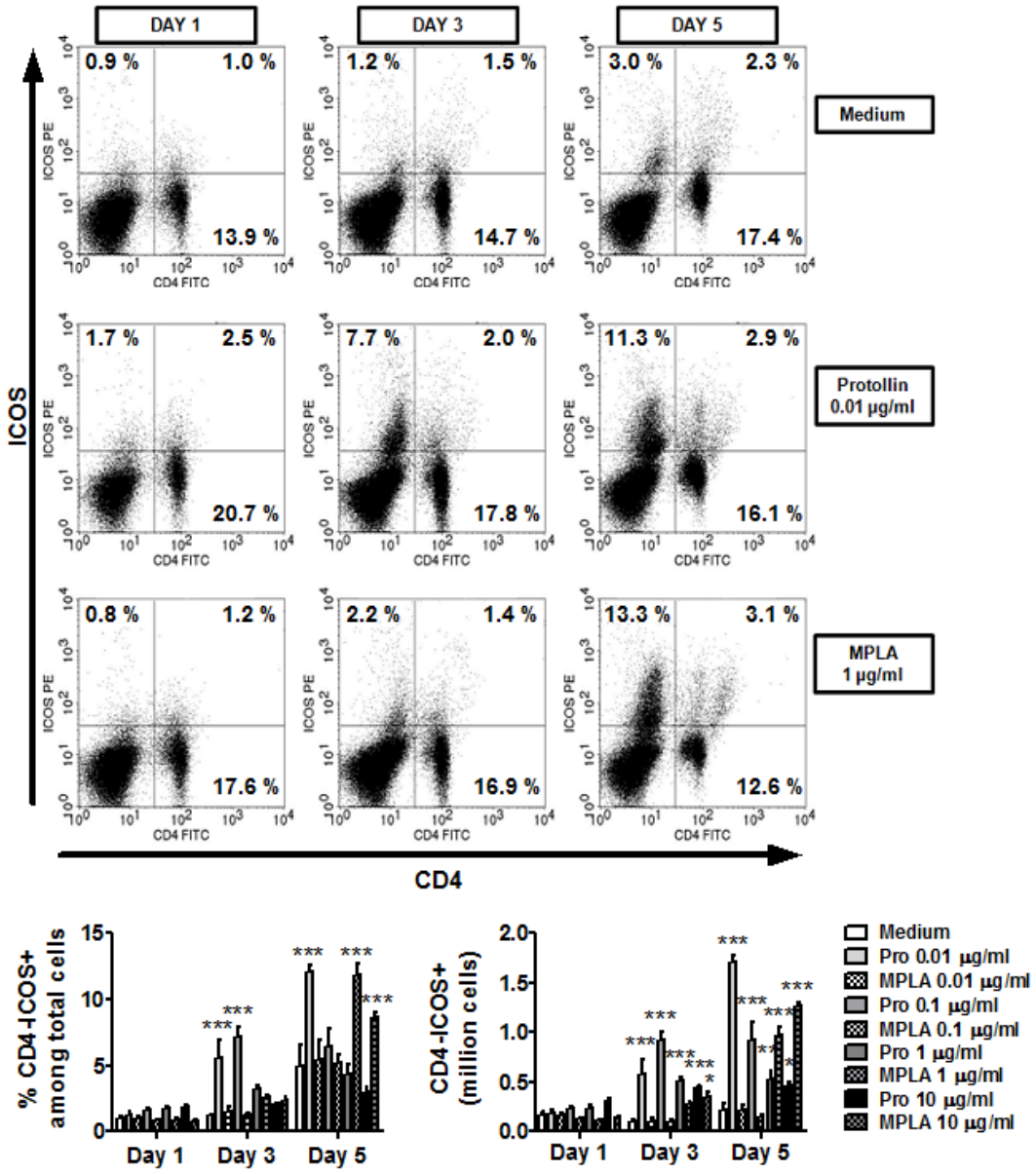
Supplementary Figure 1. Gating strategy for assessment of ICOS expression among $CD4^+Foxp3^+$ or $CD4^+Foxp3^-$ cells by flow cytometry. FACS analyses for all *in vivo* (lymph node and lung) and *in vitro* (splenocyte) experiments were performed as shown; a large gate (R1) was used to select the vast majority of cells (excluding small debris) which were plotted as CD4 vs side scatter. $CD4^+$ cells were then gated (R1 * R2) and plotted vs Foxp3 to discriminate between $CD4^+Foxp3^+$ (R3) and $CD4^+Foxp3^-$ cells (R4) which were then plotted vs ICOS to allow its quantification among $CD4^+Foxp3^+$ (R1 * R2 * R3) and $CD4^+Foxp3^-$ (R1 * R2 * R4) cells. For lung and *in vitro* FACS experiments which were subject to more substantial autofluorescent signals, gated $CD4^+$ cells were plotted as ICOS vs unused fluorescent channel (i.e. a channel for which there was no fluorochrome; FL3), which permitted the true fluorescent ICOS signal to be differentiated from the autofluorescent signal. The latter signal was gated (R5) and eliminated from further analyses (e.g. R1 * R2 * R3 * (-R5)).



Supplementary Figure 2. Protollin induces significant lymphoproliferation (based on total cell numbers) *in vivo* within the cervical lymph nodes draining the nasal mucosa (A), as well as *in vitro* in cultured splenocytes (C). *In vivo*, intranasal Protollin application does not have a significant and direct impact on lung digest total cell numbers (after 2 challenges) (B; n=7 animals/group from at least 2 independent experiments per strain). However, Protollin-induced lymphoproliferation of the cervical lymphoid tissues appears intact in *Myd88* -/- mice but is entirely absent in *Tlr4* -/- mice and highly variable in *Trif* -/- animals (A; n=7-18 animals/group from at least 2 independent experiments per strain), implying at least some degree of redundancy between the MyD88 and TRIF pathways in promoting the lymphoproliferation. *In vitro*, Protollin appeared to dose-dependently stimulate splenocyte proliferation, as did higher levels of MPLA (C; total cell counts were determined each day by pooling cells equally from the wells of the 3 animals in each condition and thus doing one average cell count for the 3 mice per condition; n=1 per culture condition per day from 1 experiment). Cell viability progressively declined in all culture conditions relative to day 1.



Supplementary Figure 3. ***In vitro*****, Protollin and MPLA also promote substantial and progressive induction of ICOS among CD4$^-$ cells and expansion of CD4$^-$ ICOS$^+$ cells.** Particularly the lower concentrations of Protollin and higher concentrations of MPLA induced a dramatic increase in the percentage of ICOS-expressing CD4$^-$ cells, although all concentrations of Protollin significantly enhanced the absolute numbers of CD4$^-$ICOS$^+$ cells (n=3 animals per culture condition per day from 1 experiment).



4.6 Discussion

The regulation of atopy and asthma by lipopolysaccharide and its receptor TLR4 may be the most extensively studied example of an environmental innate immune stimulus modulating type 2 immunity in the respiratory mucosa, yet our understanding of the preconditions and mechanisms, including the role of specific signaling pathways, through which TLR4 influences allergen-induced airway disease and adaptive immune responses is incomplete. In the current study, we sought to identify the role of the primary TLR adaptor proteins, MyD88 and TRIF, in mediating the previously described TLR4-dependent inhibition of allergic airway disease development and induction of $CD4^+ICOS^+$ T cells (3). We report that TRIF signaling via TLR4 can prevent the development of experimental allergic airway disease in mice in the context of nasal application of a TLR4-stimulating mucosal adjuvant, Protollin. Our data also support that $CD4^+ICOS^+$ cells contribute to the TLR4-TRIF-dependent inhibition of AHR in this model.

Using a model of birch pollen allergen extract (BPEx)-induced experimental asthma, we have shown that the application of Protollin in a manner that was restricted to the nares prevented AHR and production of BPEx-specific serum IgE independently of TLR2 (3) and MyD88 (current study). Also, airway inflammation consisting primarily of eosinophilia was significantly reduced by Protollin in *Tlr2* -/- mice and showed a statistically insignificant trend to be reduced in *Myd88* -/- mice. However, AHR and airway inflammation were totally unaffected by Protollin administration in *Tlr4* -/- (3) or *Trif* -/- mice (current study) indicating that the TLR4-TRIF pathway is capable of inhibiting allergen-induced type 2 airway disease. This finding suggests that the TLR4-TRIF pathway is a promising avenue for adjuvant-based immunotherapy, although an additional caveat is that the stimulus to the pathway needs to be limited to the nasal cavity as it is potently pro-inflammatory when administered at an equivalent dose to the lower airways (3). Whether it is a relevant mechanism for the effects of TRIF-biased adjuvants such as MPLA in allergy therapy remains to be investigated (41). It is well documented epidemiologically, clinically and

experimentally that the dose of LPS has a substantial influence on the development of allergic airway disease (1;2;42). Thus in the broader context, given that we have administered a moderate-high dose of agonist to the nares, our finding suggests that the TLR4-TRIF pathway may perhaps be important in the protective effects of higher ambient levels of endotoxin upon allergic disease, such as those associated with living in a farming environment. Conversely, diminution of TLR4-TRIF signaling might rather abrogate the effect of protective environmental stimuli.There is substantial evidence linking atopy and allergic asthma with gene-environment interactions between polymorphisms of the TLR4 accessory molecule CD14 gene and varying levels of LPS exposure (1;43). It has only relatively recently been identified that CD14 is of paramount importance to LPS-induced TLR4 signaling via the TRIF pathway by facilitating TLR4 endocytosis and thus permitting TRIF signaling. This process is impaired in specific cells in the absence of CD14 unless the ligand can be internalized by alternative mechanisms (44;45). Whether our data permit the inference that a CD14 genotype which offers protection against the development of allergic asthma might do so by conferring enhanced TRIF-dependent signaling is an interesting possibility. Furthermore, atopic asthmatics have been reported in recent years to display impaired type I IFN production from airway epithelial, bronchoalveolar lavage, peripheral blood mononuclear and plasmacytoid dendritic cells (46-50) and greater susceptibility to respiratory viral infection with allergic asthma (50;51). Though investigations have focused primarily on TLR3 and TLR7-mediated type I IFN responses, there is increasing evidence to suggest that TLR4-TRIF-dependent signaling is also important in respiratory immunity against viruses, such as respiratory syncytial virus (52) and H5N1 influenza virus (53), as well as bacteria (54;55). Although MyD88 can also induce type I IFNs through other TLRs (56), TLR4 signaling via MyD88 results only in the translocation of the transcription factors NFκB and AP-1 to the nucleus and thus, exclusively the production of pro-inflammatory mediators, such as TNF-α, KC, IL-1β, IL-6, -8, -12 and IFN-γ (57). TRIF can also induce pro-inflammatory mediators through its association with the TNF receptor-associated factor (TRAF) 6 which leads to the

activation of "late" NFκB and MAPK responses. TRIF additionally associates with TRAF3 leading to the phosphorylation of the interferon regulatory factor (IRF)-3 (and IRF-7) and resultant production of type I interferons, particularly IFN-β (57). That the inhibition of allergic airway disease by Protollin was TRIF-dependent and appeared to be largely, if not completely, MyD88-independent would suggest that the TLR4-TRIF pathway leading to type I IFNs rather than pro-inflammatory cytokines is playing a major role in Protollin's inhibitory capacity in this model, though we have not tested this directly. Type I IFN-dependent inhibition of experimental allergic airway disease by TLR7 or TLR9 ligands has previously been reported (58;59) and there is evidence to indicate that type I IFNs can suppress and even reverse Th2 immunity (60;61).

The role of MyD88 and TRIF in stimulating the adjuvant effect of TLR4 ligands is also an area of active investigation. Interestingly, the immunostimulatory effects of the *N. meningitidis* outer membrane vesicle vaccine that is relatively similar in structure to Protollin and the whole cell *Pertussis* vaccine, both of which contain LPS and lipoproteins that can activate TLR4 and TLR2 respectively, were shown to be TLR2-independent but rather TLR4- and TRIF-dependent, supporting the TLR4-TRIF pathway as an attractive target for vaccine adjuvants (62). Priming of T cell responses by TLR4 agonists can occur MyD88-independently (63-65), whilst *in vivo* clonal expansion of $CD4^+$ and $CD8^+$ T cells by MPLA (63), as well as upregulation of costimulatory molecules, such as CD40, CD80 and CD86 on antigen presenting cells by LPS has been shown to be TRIF-dependent and MyD88-independent (66). We extended our prior observation of TLR4-mediated induction of the T cell-expressed co-stimulatory molecule belonging to the CD28 family, ICOS, and expansion of $CD4^+ICOS^+$ cells. Our data demonstrate that *Trif* -deficient mice exhibit impaired expansion of $CD4^+ICOS^+Foxp3^+$ cells *in vivo* in the cervical lymph nodes draining the nasal mucosa and a reduced proportion of ICOS-expressing $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells. Although *Myd88*-deficient mice demonstrated significant induction and expansion of $ICOS^+$ cells within both of these populations, the degree of induction appeared less robust than in wt mice, further supporting that

MyD88 also contributes to the induction of ICOS. Moreover, *in vitro*, Protollin stimulated rapid and preferential induction of ICOS among $CD4^+Foxp3^-$ cells within 24 hours, similar to what has been reported with anti-CD3/CD28 stimulation by others (67;68), and sustained higher numbers of $CD4^+ICOS^+Foxp3^-$ cells over several days even in the absence of exogenous antigen in culture. Similar to our observation of predominantly $CD4^+ICOS^+Foxp3^-$ rather than $Foxp3^+$ expansion both *in vivo* and *in vitro*, the priming of *in vivo* antigen-specific T cell responses to OVA by systemic LPS administration was recently associated with selective induction of ICOS, which played a particularly important role in $CD4^+$ T cell expansion rather than functional activation, and preferential amplification of effector rather than regulatory T cells (69). Interestingly, the authors showed that the absence of ICOS costimulation particularly inhibited the number (and subsequent survival) of T cells about 5 days after LPS injection, the same time point at which we observed the highest levels of ICOS by Protollin or MPLA *in vitro*. Higher concentrations of Protollin induced greater ICOS expression at earlier time points and lower viability at day 5 (supplemental data), indicating differential dose-dependent dynamics and possible exhaustion of the cells in the absence of antigen in the *in vitro* conditions. We also observed a spontaneous increase in the proportion of $CD4^+Foxp3^+$ Tregs expressing ICOS and sustained numbers of $CD4^+ICOS^+Foxp3^+$ cells in the unstimulated (medium only) culture condition over the course of the 5 days, whereas no such increase was evident in the $CD4^+Foxp3^-$ population, further supporting that ICOS is differentially inducible among Tregs and effector T cells, in line with our *in vivo* observations. $CD4^+Foxp3^+$ cells lacking ICOS declined dramatically in number (data not shown), consistent with a recent report that ICOS expression distinguishes surviving and death-prone Treg subsets (70).

Unlike our *in vivo* lymph node observations, we also found a substantial induction of ICOS in $CD4^-$ cells (possibly $CD8^+$ T cells) by the corresponding doses of Protollin and MPLA that induced the highest levels of ICOS in $CD4^+$ cells; we also observed an emergence of a distinct population of ICOS-expressing

$CD4^+Foxp3^-$ cells, indicating possible differences in the *in vivo* and *in vitro* responses. Notably, $CD8^+$ T cell expansion in response to antigen plus natural MPLA or LPS was reported to be TRIF-dependent by Mata-Haro et al. (63) and McAleer et al. (71). However, in our experiments MPLA only promoted late expansion of $ICOS^+$ cells and only at higher doses that may also be fully sufficient in MyD88-dependent signaling (40). Furthermore, ICOS gene expression induced by T cell receptor/CD28 or cytokine stimulation was recently shown to rely on the transcription factor AP-1 (72), which can also be activated by both MyD88 and TRIF pathways and hence may be a plausible mechanism for the induction of ICOS by Protollin and MPLA. These observations imply that the induction of ICOS and expansion of $CD4^+ICOS^+$ cells may be tied to the pro-inflammatory mediators downstream of the MyD88 and TRIF pathways rather than exclusively to the TRIF-dependent type I IFN production, suggesting that the induction of ICOS may be functionally irrelevant for, but rather a correlating marker of, what appears to be strictly TRIF-dependent MyD88-independent, and hence likely to be type I IFN-associated, inhibition of allergic airway disease. On the other hand, it is well documented that the structure of the LPS molecule which can be highly variable even among similar species (or even strains) of bacteria such as *E. coli* and *S. flexneri* both of which are gram-negative enteropathogens but which contain predominantly hexaacylated and tetraacylated lipid A respectively (44;73-75), as well as phosphorylation of the lipid A moiety (40), plays a significant role in dictating TLR4 signaling. Consequently, selective TRIF-dependent activation of genes that are associated with both MyD88 and TRIF pathways and active suppression of MyD88 signaling has been demonstrated (40;76). It is also possible that Protollin's vesicular structure may endow the compound with greater propensity for internalization than MPLA and consequently enhanced intracellular TLR4 signaling that relies on the TRIF pathway (45). Therefore, it remains unclear whether the TRIF-dependent induction of type I IFNs is essential in the induction of ICOS by Protollin. Furthermore, it is not clear whether ICOS-ICOS-L interaction is imperative for the observed tolerogenic effects of Protollin, or only for the initial expansion of the relevant cells in the nasal mucosa and

draining lymphoid tissues, after which effector functions that are independent of ICOS-L may take over. To this effect, Lischke et al. demonstrated that the absence of ICOS-L did not affect the induction of ICOS itself by LPS or general T cell effector functions, but rather specifically T cell expansion and IL-17 production (69). Whereas they described the induction of ICOS as a distinguishing marker of a pro-inflammatory LPS-driven immune response (69), our data indicate that similar processes elicited by LPS via the nasal mucosa may be anti-inflammatory in the context of allergen-induced type 2 airway disease.

ICOS expression in $CD4^+Foxp3^+$ cells has also recently been demonstrated to be important in limiting airway inflammation in a model of intranasal allergen-induced tolerance (77). We have previously shown that $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ cells are detected in higher numbers in the lungs of wt Balb/C mice at a later time point than in the cervical lymph nodes, suggesting that these cells migrate towards the lungs. In C57Bl/6J mice, we were only able to detect $CD4^+ICOS^+Foxp3^-$ cells in higher numbers in the lungs of wt Protollin-treated mice and additionally found this to be TRIF-dependent. That *Trif* -/- mice displayed similar absolute numbers of $CD4^+ICOS^+Foxp3^-$ cells in the cervical lymph nodes as compared to wt mice but lower numbers in the lungs, supports that these cells likely traffic towards the lungs and that this process is impaired in the absence of functional TRIF. This is consistent with a report by McAleer et al. demonstrating that TRIF potentiated effector T cell migration to non-lymphoid tissues, including the lungs, following intraperitoneal LPS injection, whilst T cell accumulation in lymphoid tissues was normal (71). This was associated with impaired chemokine receptor expression in $CD4^+$ T cells, whose migration to non-lymphoid tissues could not be rescued by supplementary CD40 costimulation. Meanwhile, ICOS-L -/- mice displayed reduced accumulation of $CD4^+$ T cells to the lungs following systemic LPS boosting of the T cell response to OVA and subsequent intranasal OVA/LPS challenge (69). However, it is unclear whether this is related to a migratory deficiency or more likely to the reduced expansion of $CD4^+$ T cells observed systemically in LPS-primed ICOS-L -/- mice. That we observed reduced accumulation of $CD4^+ICOS^+Foxp3^-$ cells in the lungs of *Trif* -

deficient mice despite normal numbers in the lymph nodes further suggests that ICOS is not a sufficient costimulatory signal for Protollin-induced migration of $CD4^+$ T cells to the lungs and that additional TRIF-dependent stimuli mediate this process.

We additionally determined that adoptive transfer of lymph node $CD4^+ICOS^+$ cells from wt Protollin-treated mice was capable of rescuing the inhibition of AHR in *Trif* -/- mice. Finally, MACS-sorted $CD4^+$ cells from the lymph nodes of wt Protollin-treated mice but not from wt PBS- or *Trif* -/- Protollin-treated mice, which contained a lower proportion of ICOS-expressing cells compared to the preceding group, could inhibit AHR to a significant, albeit partial, degree. Overall, these results indicate a capacity for, and strong likelihood that $CD4^+ICOS^+$ cells contribute at least partially to the TLR4-TRIF-dependent inhibition of AHR. However, wt $CD4^+ICOS^+$ cells failed to suppress BPEx-induced airway inflammation in *Trif* -/- mice while MACS-sorted $CD4^+$ cells from both wt and *Trif* -/- mice were capable of inhibiting airway inflammation when transferred to wt mice, collectively indicating that these particular inflammatory outcomes are regulated independently of AHR and implying that the ICOS-negative constituency of $CD4^+$ cells possesses the relevant inhibitory capacity when adoptively transferred intraperitoneally. We have not definitively concluded whether migration to the lungs is occurring/necessary for the inhibition of AHR or airway inflammation following the adoptive transfers. Therefore, it is unconfirmed whether Protollin-induced $CD4^+ICOS^+Foxp3^-$ cell migration to the lungs may be more pertinent than the increase in lymph node $CD4^+ICOS^+Foxp3^+$ cells, both of which appear to be impaired in *Trif* -deficient mice. Also, that MACS-sorted $CD4^+$ cells from *Trif* -deficient mice were capable of reducing airway inflammation when adoptively transferred to wt mice prior to their allergen challenge supports a role for MyD88-dependent signaling in additionally modulating $CD4^+$ cell function by Protollin and that systemic adoptive transfer of the cells may be circumventing their potential migratory impairment in *Trif* -/- mice. Overall, our data support that discrete TLR4-TRIF-dependent signals influence the induction and migration of $CD4^+ICOS^+$ cells that possess the

capacity to contribute to the inhibition of AHR and that additional mechanisms are also likely to be involved in the TLR4-TRIF-mediated protection against the development of allergic airway disease.

4.7 Acknowledgments

We kindly thank Dr. Clément Rioux and GlaxoSmithKline Biologicals North America (Laval, Canada) for generously providing us with Protollin. We also thank Ms. Jamilah Saeed for outstanding technical assistance, as well as Ms. Julie Lord and Mr. Eric Massicotte from the Institut de Recherches Cliniques de Montreal for cell sorting services.

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CHAPTER 5

Discussion

5.1 Discussion

The innate arm of the immune system plays a complex role in relation to the predominantly type 2 inflammatory response of the airways to aeroallergens that is characteristic of allergic asthma. Epidemiological, genetic, clinical and experimental data collectively indicate a potential for TLR4 to initiate, exacerbate, or conversely prevent or treat allergic airway disease (1;2). Elucidating the role of specific TLR signaling pathways may enhance our understanding of the conditions and mechanisms by which TLRs influence allergic airway disease and direct the development of successful TLR-targeting immunotherapeutic strategies. Five major conclusions can be drawn from the enclosed manuscripts regarding the involvement of TLR4 and TLR4-TRIF signaling in experimental allergic airway disease and the modulation of adaptive immunity:

(1) *TLR4 is not a requirement for sensitization and development of airway disease to inhaled birch pollen extract but is indeed implicated in the amplification of aspects of the Th2 immune response during the secondary exposure to allergen* (shown in Chapter 2).

(2) *The TRIF signaling pathway of TLR4 appears to have protective and potentially immunotherapeutic effects against the development of allergic airway disease in that (a) it tempers airway inflammation induced by inhalation of a natural aeroallergen* (Chapter 2) *and (b) mediates adjuvant-induced TLR4-dependent protection against the development of experimental allergic asthma in sensitized animals* (Chapter 4).

(3) *TLR4 signaling induces ICOS expression and amplifies ICOS-expressing $CD4^+$ T cells* (Chapter 3)*, an additional modality by which innate immunity can potentially influence adaptive T cell responses in that TLR4 not only modulates APC but also T cell expression of co-stimulatory molecules.*

(4) *Lymph node-resident ICOS-expressing $CD4^+$ T cells, inducible via TLR4 stimulation of the nasal mucosa, contribute to the TRIF-dependent suppression of allergic airway disease* (Chapters 3 and 4).

(5) *The capacity to induce ICOS is not specific to Protollin but is also relevant to the TLR4 ligand, synthetic monophosphoryl lipid A* (Chapter 4).

These conclusions will be individually discussed and elaborated upon in the ensuing sections.

(1) TLR4 is not a requirement for sensitization and development of airway disease to inhaled birch pollen extract but is indeed implicated in the amplification of aspects of the Th2 immune response during the secondary exposure to allergen (shown in Chapter 2).

Pollens, such as ragweed and birch, have been shown to contain components possessing NADPH oxidase, as well as cysteine and serine protease activity (3;4). When injected intradermally, the cysteine protease papain, was demonstrated to induce Th2 immune responses in the skin and draining lymph nodes through the generation of reactive oxygen species resulting in the activation of the TLR4-TRIF pathway (5). As discussed in the introduction, there are numerous other examples of oxidative stress provoking innate and adaptive immune responses in a manner that depends on activation of TLR4 and either the MyD88 or TRIF pathway (6-10). Therefore, we hypothesized that an extract of birch tree pollen primes allergic sensitization via the airway mucosa by promoting oxidative stress and activating the TLR4-TRIF pathway. Using a model of repeated inhalational allergen exposure without additional exogenous immune stimulation by an adjuvant, we were able to distinguish and thus, independently study the phases of allergic sensitization and secondary allergen exposure. Numerous studies have demonstrated a role for oxidative stress in amplifying the inflammatory response that occurs with secondary allergen exposure (3;11-16). However, this model

enabled us, most importantly, to study its role in the more natural context of allergic sensitization via the airway mucosa. Our results indicate that oxidative stress does not mediate all aspects of sensitization, such as undefined processes that eventually lead to AHR and airway eosinophilia upon secondary allergen exposure, but is a critical component of the secondary inflammatory response leading to AHR and eosinophilia. In other words, oxidative stress does not initiate all aspects of allergic airway disease in our model but seems to be subsequently important in driving most fronts of the disease. We are still, however, in the process of substantiating these findings, specifically, clarifying the relevance to our results of the acidity of the antioxidant NAC solution (a point raised by a reviewer of our manuscript) and may need to provide supporting evidence using another antioxidant. Nevertheless, our data also indicate that neither TLR4, nor TRIF, are necessary for allergic sensitization to birch pollen extract via the airway mucosa leading to the development of AHR, supporting that TLR4 activation, like oxidative stress, is also not a universal mechanism by which common aeroallergens evoke sensitization. However, we did not measure IgE in these strains of mice and therefore, cannot comment on the role of TLR4/TRIF in this aspect of allergic sensitization. Nonetheless, our results are not in complete agreement with those of Lambrecht's and Bottomly's groups showing that TLR4 function is necessary for all aspects of allergic airway disease provoked by inhaled house dust mite allergen extract or LPS-laden OVA (17;18). Our results are more in line with a study showing that TLR4 was not required for mucosal sensitization to a cockroach allergen extract which was rather mediated by protease-activated receptors (19). We did find however that TLR4 activation significantly augmented the inflammatory response, particularly aspects of the Th2 response, such as cytokine synthesis and airway eosinophilia. Thus, the discrepancy between our results and those of Lambrecht's and Bottomly's is confusing given that in all cases TLR4 contributed to Th2 inflammation. It is unclear whether AHR may be more dependent on Th2 inflammation in their models of house dust mite allergen extract- or OVA-induced airway disease, or whether there is a difference in the magnitude of TLR4 stimulation.

TRIF surprisingly appeared to control the magnitude of BPEx-induced airway inflammation given that Trif-deficient mice displayed augmented inflammation. Thus, despite the demonstrated capacity to elicit oxidative stress via its NADPH oxidase activity and its reported cysteine and serine protease activity, we did not find that a mechanism of oxidative stress promoting TLR4-TRIF activation, such as that described for papain's action in the skin, was applicable to the development of allergic disease through the airway mucosa. Based on the observed reduction in BPEx-induced BAL IL-4 and eosinophilia either by antioxidant treatment or genetic/pharmacological inhibition of TLR4 activity, our data indicate the plausibility that an oxidative stress-TLR4 axis is relevant strictly in relation to Th2 inflammation, as shown with papain, but not for overall allergic airway disease and not in a manner that depends on TRIF signaling. Also, oxidative stress associated with ozone inhalation promotes AHR and airway inflammation TLR4-MyD88-dependently (9;10). Therefore, it remains to be clarified whether the apparent disparity in TLR4 signaling mediated by papain, ozone, or birch pollen- associated oxidative stress may potentially be due to divergent production of oxidized phospholipids or utilization of TLR4 co-receptors resulting in differential engagement of the MyD88 or TRIF signaling pathways (7). For example, unlike LPS which requires TLR4 and CD14, low molecular weight hyaluronan fragments induced by ozone interact with TLR4 and CD44 (20;21).

(2) *The TRIF signaling pathway of TLR4 appears to have protective and potentially immunotherapeutic effects against the development of allergic airway disease in that (a) it tempers airway inflammation induced by inhalation of a natural aeroallergen* (Chapter 2) *and (b) mediates adjuvant-induced TLR4-dependent protection against the development of experimental allergic asthma in sensitized animals* (Chapter 4).

That TLR4 contributes to allergic inflammation, whereas TRIF appears to temper it, implies that TLR4-MyD88 signaling contributes to the amplification of the inflammatory response to BPEx, though we have not tested this directly. This is in

agreement with the various reports indicating a role for MyD88-dependent signaling in eliciting and augmenting allergic airway inflammation, such as that caused by inhalation of house dust mite allergen extract (22-24), LPS-laden OVA (25), cockroach frass (26), short ragweed extract (27), or the recently published effect of bacterial flagellin in priming TLR5-MyD88-dependent type 2 airway disease to OVA (28). Possibly the most important implication of our data is that they support a dual role for TLR4 in relation to birch tree pollen allergy of the airways; a pro-inflammatory effect of TLR4-MyD88-dependent signaling and an anti-inflammatory effect of TLR4-TRIF-dependent signaling. Whether this also applies to other allergens which promote TLR4-MyD88-dependent inflammatory responses is of interest and should be explored. The mechanisms by which TRIF activation may potentially counter-balance MyD88 activation, as the data discussed thus far suggest, will be elaborated upon momentarily.

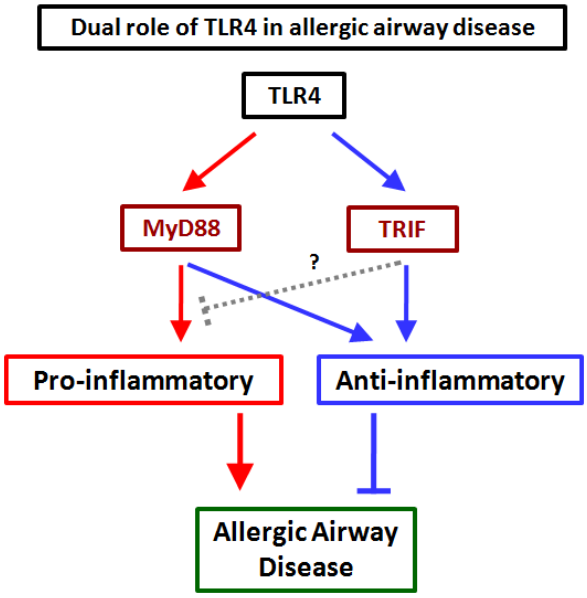
First, a word on Protollin, a mucosal adjuvant determined to be safe in human clinical trials and composed of purified bacterial TLR2 and 4 ligands, namely vesicles of hydrophobic outer-membrane proteins from *Neisseria meningitidis* and incorporated LPS molecules from *Shigella flexneri* (29-31). We reported that intranasal application of Protollin^{TM} in a manner that was restricted to the nares of the sensitized animals, prior to their allergen challenge, was sufficient to prevent the development of allergen-induced AHR, inflammation and production of BPEx-specific IgE (Chapter 3) (32). The advantage of the selective exposure of Protollin to the nasal mucosa was that it protected against the development of allergic airway disease without causing collateral lung inflammation such as that associated with intrapulmonary Protollin administration. Rather than expanding our investigation of the potential of Protollin to elicit long-term protective or therapeutic immunity against allergic airway disease, which is of no lesser merit, we set out on a more mechanistic analysis of Protollin's effects in this acute asthma model to identify the role of TLR2- and TLR4-mediated signaling. For this model, we utilized intraperitoneal sensitization with alum adjuvant which has been shown with other allergens to bypass a requirement for TLR4 in the sensitization process (25;33;34) and more importantly, which permitted the

development of allergic airway disease with repeated intranasal challenges of a very low dose of allergen (25 PNU, i.e. in the nanogram range), unlike in the previous study (Chapter 2) in which the animals received a high dose of allergen intratracheally (100 μg). Given that we wished to study the role of the TLRs in response to Protollin, this enabled us to largely circumvent the issue raised in Chapter 2 of a role for TLR4 in the actual response to the allergen itself and hence, potential differences in the development of the asthma model in the various mouse strains. In Chapters 3 and 4, we showed that Protollin prevented the development of AHR and production of BPEx-specific IgE independently of TLR2 and MyD88, suggesting that this inhibition required TLR4 and TRIF which indeed we confirmed for AHR. Protollin also prevented eosinophilic airway inflammation independently of TLR2, whereas TLR4 and TRIF were necessary for this inhibition. The reduction of airway eosinophilia was also not statistically significant in the absence of MyD88, suggesting a potential additional contribution of this pathway. Overall, these data indicate that the protective effect of Protollin against the development of allergic airway disease is predominantly, if not entirely, dependent on TLR4-TRIF signaling, providing further evidence, in addition to that which was described in Chapter 2, of an anti-inflammatory capacity of this pathway in allergen-induced type 2 airway disease. Therefore, we show in Chapters 3 and 4 that stimulation of the TLR4-TRIF pathway via the nasal mucosa can offer protection against allergic lower airway disease and may be a promising avenue for adjuvant-based immunotherapy.

Although the data collectively support a protective role for the TLR4-TRIF pathway in relation to allergic airway disease, there remains a clear distinction between Chapter 2 which implies that TRIF signaling antagonizes the pro-inflammatory, allergen-induced TLR4-MyD88-dependent signals (which occurs only to a limited extent given that the net effect of TLR4 function in response to BPEx is amplification of allergic inflammation) and the Protollin-related studies (Chapters 3 and 4) which show that stimulation of the TLR4-TRIF pathway can inhibit type 2 airway disease beyond merely counter-balancing TLR4-MyD88-dependent pro-allergic signals. Therefore, whether the TLR4-TRIF-dependent

regulatory mechanisms relevant to these studies are one and the same requires further exploration. More specifically, the question is whether the TLR4-TRIF-dependent signals directly interfere with the TLR4-MyD88 pathway in Chapter 2, or whether the apparent anti-inflammatory effect is due to the same MyD88-independent Th2-inhibitory activity of TRIF signaling observed in Chapters 3 and 4 (**Figure 1**).

Figure 1.

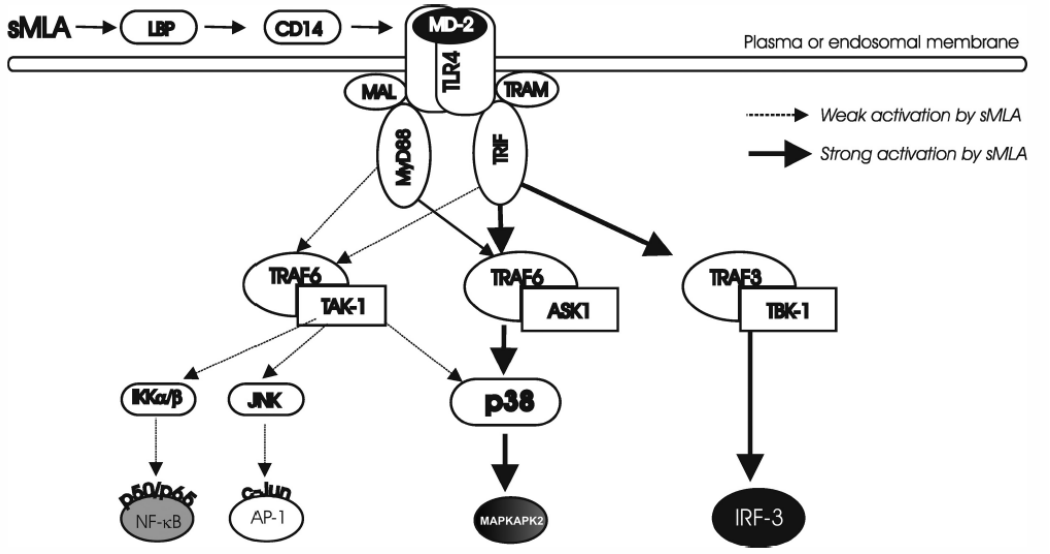


Current literature supports that the TLR4-MyD88 pathway can provide both pro- and anti-inflammatory stimuli in relation to the development of allergic airway disease (as described in Chapter 1). The data presented in this thesis demonstrate that in the context of exposure to a birch pollen allergen extract via the airways, TRIF activity is anti-inflammatory (Chapter 2), perhaps by restraining/ counter-balancing 'pro-allergic' TLR4-MyD88-dependent signals (dotted grey line), whereas adjuvant-induced TLR4 activation in the nasal mucosa also promotes anti-inflammatory TLR4-TRIF-dependent processes that are independent of the MyD88 pathway (Chapters 3 and 4).

Most studies (including ours) are still at the stage of describing a potential antagonistic capacity of TLR4-TRIF signaling upon the MyD88 pathway or type 2 immunity, whereas the relevant mechanisms remain to be defined. Seregin et al. reported that TRIF negatively regulated the MyD88-dependent activation of pro-inflammatory mediators from dendritic cells stimulated *in vitro* with TLR4 (LPS), TLR7/8 (R848) or TLR9 (ODN2006) ligands, as well as an antigen from the protozoa *Eimeria tenella* (a suspected TLR11 ligand) (35), suggesting a more expansive role of TRIF in the suppression of pathogen-induced innate immune responses, to the extent of influencing TLRs that are not known to utilize the

TRIF pathway. TRIF has been shown to promote proteolytic degradation of TLR5 (and possibly many additional TLRs) (36). The induction of endotoxin tolerance, the phenomenon of repeated exposure to endotoxin or other TLR ligands causing progressive hyporesponsiveness to subsequent endotoxin challenge, is perhaps another possible mechanism by which TRIF can negatively regulate the TLR4-MyD88-dependent adjuvant effects of LPS upon allergens (37;38). However, these mechanisms suggest that TRIF might antagonize or at least set boundaries to pro-allergic microbial stimuli rather than actually evoking immunostimulatory processes that are inhibitory of type 2 immune responses, which may be counter-intuitive to the paradigm of the Hygiene Hypothesis discussed in the introduction, of diverse microbial exposure in early life being negatively associated with atopic asthma. In studying the differential signaling outcomes of synthetic MPLA and DPLA, Cekic et al. demonstrated "TRIF-biased" activation of macrophages by sMPLA, caused by generally weak or impaired MyD88 signaling (39). The authors showed that the expression of certain TRIF- and MyD88-associated genes by sMPLA was due to selective p38 MAPK activation, demonstrating that the lack of a single phosphate group on lipid A results in strong p38 MAPK but weak JNK activation (as depicted in **Figure 2**), to which both adaptors, MyD88 and TRIF, contributed. In contrast, unidirectional TRIF-dependent synergy of MyD88-dependent activation of DCs and OVA-specific $CD8^+$ T cell responses has also been described, which is mediated by activation of the JNK pathway (40). These results serve as a reminder that the notion of strictly independent MyD88 or TRIF signaling may be an over-simplification and that the signaling events downstream of these adaptors are rather heterogeneous in nature and ligand-specific.

Figure 2. *Summary of TRIF-biased signaling and selective p38 activation by sMPLA (Cekic et al. J Biol. Chem. Vol: 284 (46) p.31982–31991, 2009) (39).*



Although the basic biology, let alone reciprocal regulation, of MyD88- and TRIF-dependent signaling processes are still being unraveled, there has been recent progress in identifying the molecular mechanisms that control the compartmentalization of TLR4 and switching between TIRAP-MyD88 and TRAM-TRIF signaling. Engagement of the MyD88 and TRIF pathways has been established to occur sequentially and TLR4 internalization is an important step in the arrest of MyD88 signaling and initiation of TRIF activation (41;42). CD14 mediates LPS-induced TLR4 endocytosis through a clathrin-mediated mechanism that does not require actual signaling by TLR4 itself, but rather phospholipase Cγ2 and spleen tyrosine kinase signaling, which is necessary for TRIF-dependent type I IFN production (42). Furthermore, it was recently shown that a member of the phosphatidylinositol-3-OH kinase (PI(3)K) class I, p110δ, plays a significant role in removing a TIRAP-anchoring plasma membrane lipid in DCs, thereby promoting the dissociation and degradation of TIRAP, endocytosis of TLR4 and switching to endosomal TRAM-TRIF signaling (43). Genetic or pharmacological inactivation of p110δ prolonged TIRAP-MyD88 signaling and production of pro-inflammatory cytokines while diminishing TRAM-TRIF signaling and production

of IFN-β and IL-10, resulting in greater endotoxin-induced death in mice. This PI(3)K class I isoform was therefore proposed to be a critical balance between pro- and anti-inflammatory TLR4 signaling. Some of the hyperproduction of proinflammatory cytokines, such as IL-12, which we also observed in allergen-exposed Trif-deficient mice in Chapter 2, was suggested to be the indirect result of diminished IL-10 production. However, Seregin et al. observed that IL-10 levels were indistinguishable in Trif-deficient and wt mice treated with TLR agonists, despite augmented IL-12 production in the absence of TRIF (35). We also observed no difference in BAL fluid IL-10 levels in either strain following repeated BPEx inhalation (Chapter 2). Also, our attempts to neutralize IL-10 *in vivo* in Protollin-treated mice (Chapter 3) did not abrogate Protollin's inhibitory effect upon AHR and eosinophilic airway inflammation and we were unable to detect a Protollin-induced increase in IL-10 in the BAL fluid or in isolated lung cells. We were also unable to detect an increase in pro-inflammatory cytokines, such as IFN-γ or IL-17A, either in the BAL fluid or in isolated lung cells as a result of nasal Protollin applications. Alternatively, that the inhibition of allergic airway disease by Protollin was TRIF-dependent and appeared to be largely, if not completely, MyD88-independent may suggest that the TLR4-TRIF pathway leading to type I IFNs rather than pro-inflammatory cytokines is playing a major role in Protollin's inhibitory capacity in this model, which is a major unresolved question. Though other reports exist of type I IFN-dependent inhibition of allergic airway disease by ligands of TLR7 and TLR9 (44;45), the precise mechanisms by which these cytokines inhibit type 2 immune responses are unclear.

The type I IFNs have garnered significant attention in recent years due to their importance in defense against respiratory viral infection (46;47) and particularly due to a number of seminal studies revealing that allergic asthma is associated with type I IFN deficiency and greater susceptibility to respiratory viral infection (48-51). Interestingly, respiratory viruses (52;53), as well as Th2 immunity such as that associated with pregnancy, allergy or atopic asthma (54-57), have been reported to negatively regulate TLR3, TLR7 and/or TLR9 activation in pDCs and airway epithelial cells and the resultant production of type I IFNs. It is not clear at

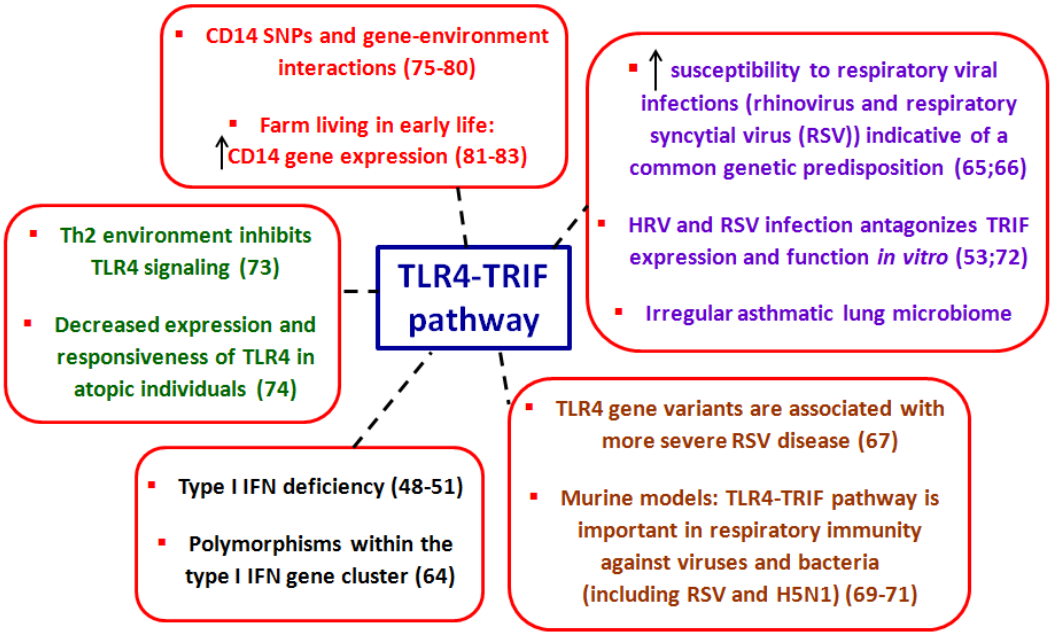
the moment whether the deficiency in type I IFNs is an instigator or consequence of the allergic airway Th2 environment in asthmatics. Conversely, recent evidence supports that type I IFNs are capable of suppressing Th2 immune responses. Neutralization of these cytokines in healthy human rhinovirus-stimulated PBMCs, or depletion of pDCs producing IFN-α, selectively enhances T cell-derived IL-13 but not IFN-γ, IL-10 or IL-17, indicating that type I IFNs can constrain the Th2 immune response to rhinovirus (58). Another study showed that type I IFNs can even reverse human Th2 commitment by inhibiting GATA-3 expression (59). Reciprocal regulation of IgE-dependent mechanisms and TLR7 and 9 expression/ activation in human pDCs has also been demonstrated (55;57;60;61). Furthermore, IFN-α modulates FcεRI expression on DCs, upregulating the γ chain whilst at sufficiently high levels, inhibiting Th2 cytokine-induced upregulation of the α chain and thus, has been proposed to play a dual role in the initiation and termination of inflammation induced by respiratory viral infection of atopic children (62). Interestingly, IFN-β gene deficiency in mice exacerbates OVA-induced IgE, Th2 and eosinophilic airway inflammation (63). Moreover, polymorphisms in multiple genes within the type I IFN gene cluster have been associated with allergic sensitization to molds, HDM, cockroach and pollen allergens, as well as asthma and bronchial hyperresponsiveness in the genetically homogeneous anthroposophic Hutterite population (64). Therefore, it remains to be answered whether the impairment in type I IFNs is only relevant to viral exacerbation of asthma or more broadly significant for the development of allergic asthma. In this regard, studies have suggested that susceptibility to respiratory syncytial virus (RSV) infection and asthma may be due to a common genetic predisposition (65;66).

Notably, TLR4 gene variants have been epidemiologically associated with more severe RSV disease in children (67) and the TLR4 polymorphism Asp(299)Gly, but not Thr(399)Ile, was recently shown to diminish both MyD88- and TRIF-dependent signaling without altering TLR4 expression (68). There is increasing evidence from murine models to suggest that TLR4-TRIF-dependent signaling is important in respiratory immunity against viruses, such as RSV and H5N1

influenza (69), as well as bacteria (70;71). Synthetic dsRNA, human rhinovirus or RSV infection have been shown to antagonize TRIF expression and function in various cells *in vitro*, including airway epithelial cells, resulting in the suppression of subsequent TLR3/4-mediated type I IFN production (53;72). Atopy and type 2 immunity have also been associated with decreased expression and responsiveness of TLR4 (73;74). Interestingly, multiple CD14 SNPs have been shown to influence the effect of the farming environment and farm-related exposures, such as raw milk consumption, on the development of atopy, atopic asthma as well as non-atopic wheeze, in some cases depending on the level of endotoxin exposure (75-80). Furthermore, farm living in early life is associated with enhanced CD14 gene expression in children's peripheral blood cells (81;82) as well as altered epigenetic regulation of placental CD14, also resulting in higher gene expression (83).

Taken together, that allergic asthma is associated with deficiency in type I IFN production, reduced TLR4 expression and signaling in certain cells, altered CD14 gene expression and gene-environment interactions, enhanced susceptibility to specific respiratory infections and an irregular lung microbiome (**Figure 3**) suggests the possibility that in the broader scheme, the TLR4-TRIF pathway may be highly pertinent to allergic airway disease. The findings presented in this dissertation support such a conclusion.

Figure 3. *Summary of epidemiological and experimental findings which support the potential relevance of the TLR4-TRIF pathway to atopic asthma (references in parentheses).*



A scenario in which reduced TLR4-TRIF activity (perhaps caused by pre-existing atopy and/or loss-of-function gene polymorphisms, such as in TLR4, CD14, or other signaling proteins) augments susceptibility to atopic asthma, as well as respiratory viral and bacterial infections that are common to the disease is plausible and may contribute to both the pathogenesis and pathophysiology of allergic airway disease. Our results which indicate that TLR4-TRIF signaling is protective against the development of allergic airway disease in mice are a step towards clarifying the precise relevance of this pathway to the stated phenomena. Moreover, given that we have administered a moderate-high dose of agonist to the nares, one might be inclined to speculate that our results suggest that the TLR4-TRIF pathway may be important in the protective effects of higher ambient levels of endotoxin upon allergic disease, such as those associated with living in a farming environment, and perhaps that a CD14 genotype which offers protection against the development of allergic asthma might do so by conferring enhanced TRIF-dependent signaling. However, in our first study (Chapter 2) we assess the

role of TRIF signaling in the context of an allergen exposure associated with minimal LPS and hence low level TLR4 stimulation. In agreement with other studies (17;18;23;24;27;84), we show that TLR4 activation in this context is 'pro-allergic', at least with respect to Th2 inflammation, whereas in our Protollin studies, we examine a context of what is strong TLR4 stimulation and which we have shown to be 'anti-allergic' or inhibitory of airway Th2 inflammation and AHR. Yet, in both conditions we find signaling via the TRIF pathway to be inhibitory of type 2 airway inflammation, supporting the view that while TLR4 (and TLR4-MyD88) signaling can be either pro- or anti-allergic, TLR4-TRIF signaling is anti-allergic regardless of the level of TLR4 stimulation (or LPS dose). It is not clear, however, how this uniformity in our results may be reconciled with other reports showing that LPS inhalation inhibits allergen-induced type 2 airway disease MyD88-dependently (85-87), while dsRNA can prime and potentiate allergic airway disease TLR3-TRIF-dependently (88;89). Furthermore, Cyr et al. showed that intrapulmonary administration of Protollin generated a TLR4-MyD88-dependent protective immune response in a model of RSV infection (90). Additionally, Piggott et al. who published the study describing that the priming of Th2 responses to OVA via the airway mucosa by low dose LPS (84), was dependent on functional TLR4-MyD88 signaling, observed that whereas a TLR9 ligand was capable of inducing IFN-β expression in the lungs, LPS was not, and therefore concluded that LPS-induced induction of the TRIF pathway was defective in the pulmonary environment (25). It should also be noted, however, that Piggott et al. did not evaluate the effects of LPS inhalation in TRIF-deficient mice and only measured the induction of IFN-β in mice following low dose LPS inhalation which may have been insufficient to induce significant stimulation of the TRIF-type I IFN pathway. These observations emphasize the potential importance of the dose and localization of the stimulus to TLR4, particularly the difference between the nasal vs lower airway mucosa. We also reported that an equivalent dose of Protollin administered into the lungs, despite dramatically inhibiting eosinophilic airway inflammation, was severely pro-inflammatory and that this was also dependent

upon whether Protollin was given on its own, or together with allergen, given that combined administration into the lungs of Protollin admixed with allergen, prior to the allergen challenge, additionally appeared to augment type 2 airway inflammation based on its effect on eosinophil numbers (Chapter 3) (32). Therefore, it may not be appropriate to extrapolate the conclusion that the observed TLR4-TRIF-dependent protection against experimental allergic airway disease via the nasal mucosa is necessarily applicable to the postulated protective effect of higher ambient levels of endotoxin.

Nevertheless, for the sake of synthesizing a clear hypothesis and provided the assumption that the observed TLR4-TRIF-dependent protection is not confined to the nasal mucosa, one might propose that there exists a threshold such that at low levels of TLR4 stimulation of the lower respiratory tract, the net effect of TLR4 stimulation is in favor of pro-Th2 MyD88-dependent signaling due to the insufficiency of TRIF-dependent signals, whereas at moderate levels that are inhibitory of Th2 inflammation without causing substantial collateral lung inflammation (less than 1 μg), TRIF signaling would increase to a threshold at which the net effect of TLR4 stimulation favored TRIF. At higher levels (beyond 1 μg), however, the magnitude of the pro-inflammatory (but no longer pro-Th2) MyD88-dependent signals might overwhelm the TRIF-dependent anti-inflammatory signals, or the two pathways may synergize to promote a maximal immune response (91). The equivalent of the high end of this spectrum in terms of human exposure may be, for example, the chronic low-grade, yet Th2-inhibitory, inflammation associated with agricultural organic dust exposure (92) and beyond this, the Th1/Th17 inflammatory phenotype characterizing steroid-resistant severe asthma (93-97). Thus, the development and expression of allergic asthma would be confined to the fringes of this spectrum, namely low and high level endotoxin inhalation, and depending on whether it is sufficiently proximal temporally to the allergen exposure. According to this depiction, the protective effects of the endotoxin exposure levels in between would be mediated by TRIF-dependent signaling at the lower end and more so by MyD88-dependent signaling at the higher end, though this may also be dependent upon ligand structure. Another

assumption that I will make is that most individuals are unlikely to be acutely exposed to the high levels of endotoxin employed in animal models, or chronically to levels such as those relevant to agricultural organic dusts. Therefore, the vast majority are likely to be in the low-moderate category, even within studies that differentiate between 'low' and 'high' endotoxin exposure such as those investigating CD14 gene-environment interactions. Thus, a CD14 genotype which would enhance CD14 expression and soluble CD14 levels (and thus presumably overall CD14 function) would primarily be expected to augment MyD88-dependent responses in the context of low level LPS exposure, but might conversely enhance TRIF-dependent responses to moderate LPS levels, as extrapolated from *in vitro* studies (21;42;98-100). In effect, such a CD14 genotype might augment allergic immune responses to low level LPS, but could inhibit type 2 immunity to moderate level LPS, in line with the dual relationship of the CD14/-260 polymorphism with atopy depending on the level of LPS exposure, or the capacity of CD14 to influence allergen-induced immune responses in opposite directions depending on the microbial load (101;102). The effects of gene-environment interactions between this polymorphism and the level of endotoxin exposure at 6 months of age (estimated from dust samples in the home) were evaluated in relation to total serum IgE and $CD4^+$ lymphocyte numbers in the blood at 12 months of age (103). Interestingly, increasing endotoxin exposure at 6 months, was associated with significantly lower IgE levels at 1 year in children expressing the CC genotype, as has been corroborated by various groups, but was also associated with higher numbers of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells in the blood, though the relationship was statistically significant only for $CD4^+Foxp3^-$ lymphocyte numbers. In general, the pattern of the gene-environment interactions between children's CD14/-260 genotype and dust endotoxin exposure on total IgE levels 6 months later, was opposite to that observed for $CD4^+$ lymphocyte numbers, suggesting reciprocal relationships. Our research addressing the importance of the immunostimulatory capacity of TLR4 upon $CD4^+$ lymphocyte responses is further discussed below.

(3) TLR4 signaling induces ICOS expression and amplifies ICOS-expressing $CD4^+$ T cells (Chapter 3), an additional modality by which innate immunity can potentially influence adaptive T cell responses in that TLR4 not only modulates APC but also T cell expression of costimulatory molecules.

The promotion of effective T cell immunity is a key property of vaccines and the quantity, quality and localization of the T cell response are important elements of vaccine design. TLR-mediated activation of dendritic cells (DCs) is thought to be a primary mechanism by which TLR adjuvants influence the clonal expansion and functional differentiation of specific T cell subsets, which in turn, act as the effectors of the adaptive immune response. The specific properties of the ligands in the adjuvant, the targeted anatomical microenvironment, activation state of DCs and the cytokine milieu provide the signals that direct lineage-specific differentiation of T cells into Th1, Th2, Th17, T follicular helper (Tfh), or Treg subsets (reviewed in (104)). In Chapter 3, we show that the capacity of nasally administered Protollin to prevent the development of lower airway allergic disease in Balb/c mice was associated with the induction of ICOS in cells of the nasal mucosa and on both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells of the draining lymph nodes (LNs), as well as their recruitment to the lungs. Furthermore, the induction of ICOS in the cervical lymph node populations was determined to be TLR4-dependent. That these cells are T cells is highly likely given the high percentage of CD3 expression (> 95 %) within the CD4-sorted population of the lymph nodes (data not shown) and given that ICOS is considered a mainly T lymphocyte-expressed co-stimulatory molecule (recently its expression was also reported in innate helper type 2 cells (or nuocytes) which are also of lymphoid origin but do not express CD4 or other lineage-specific markers (105)). Therefore, this finding draws attention to perhaps an overlooked aspect of TLR function, in that TLRs not only modulate adaptive immune responses by inducing co-stimulatory molecules on DCs, but can also influence this aspect of $CD4^+$ T cell biology.

ICOS belongs to the expanding CD28 immunoglobulin family of co-stimulatory (and co-inhibitory) molecules, of which CD28 is the most prominent member (reviewed in (106)). ICOS is the second most extensively studied molecule due to its broad expression (ICOS is expressed by all of the aforementioned T cell subsets, as well as unpolarized $CD4^+$ T cells), its implication in numerous immune disorders and the occurrence of ICOS-deficiency in humans which results in common variable immunodeficiency (107). Furthermore, ICOS plays a substantial role in T cell co-stimulation as it has the capacity to compensate for many of the functions of CD28 but also possesses complementary activities (108-112). ICOS has a single cognate ligand, ICOS-L (CD275; B7h; B7-H2; B7RP-1) (113-115), which is constitutively expressed and inducible on B cells, DCs, macrophages as well as non-lymphoid tissues, such as endothelial and airway epithelial and smooth muscle cells (114;116-118). The name 'inducible costimulatory molecule' derives from the fact that ICOS is chiefly expressed on activated T cells. It is well established that ICOS is pivotal in Th2 responses and allergic airway inflammation since it can contribute to Th2 cell differentiation, expansion, migration and effector function (IL-4, IL-5 and IL-13 production) (119-124). A SNP in the ICOS promoter region is associated with increased atopy in the Hutterite population (125). ICOS is a potent inducer of IL-10 and appears to be important in T cell IFN-γ and IL-2 production as well (126-131). Varying surface expression levels of ICOS have been correlated with distinct $CD4^+$ T cell cytokine profiles, with IFN-γ-producing Th1 cells having low expression, IL-4 and IL-5-producing Th2 cells displaying moderate expression and IL-10-producing T cells exhibiting high levels of ICOS (132). Its crucial role in humoral immune responses, including T-dependent B cell help, antibody class switching and germinal center formation has long been known (120;128;130;133-136) but ICOS was only recently shown to promote Tfh cell differentiation and expansion, as well as production of the IL-21 cytokine which is essential to these processes (137-142). ICOS is also expressed by Th17 cells and is essential in their proliferation, maintenance and function (138). More recently, ICOS has been shown to play a critical role in the function and survival of $CD4^+$ invariant NK T

cells and in their exacerbation of experimental asthma in mice (143-145), whereas ICOS expression in IL-10-secreting Tr1-like cells, Tregs or anergic T cells induced by tolerogenic DCs mediates mucosal tolerance to inhaled antigen and suppression of experimental asthma (146-150), contact hypersensitivity (151), atherosclerosis (152;153), autoimmune diabetes (154;155) and encephalomyelitis (148;156). Although ICOS does not appear to play a role in the peripheral conversion of Tregs from $CD4^+Foxp3^-$ cells (157), its expression endows Tregs with enhanced proliferative, survival and suppressive capacity in the form of both IL-10 and TGF-β activity rather than just TGF-β alone (158;159). In summary, ICOS expression in T cells has been implicated in both the promotion and inhibition of inflammatory and autoimmune disorders in experimental murine models (160-164), consistent with the noted susceptibility of ICOS-deficient patients to a broad spectrum of pathologies, including inflammatory or autoimmune conditions as well as malignant disease and recurrent infection (reviewed in (165)). Thus, ICOS is important in facilitating both immunostimulatory and tolerogenic responses. Though the underlying mechanisms are still being delineated, the level, persistence and cell-specificity of its expression may be important determinants.

Perhaps the most clearly established function of ICOS is its role in the expansion and survival, rather than early activation and differentiation, of various T cell subsets and therefore, in controlling the pool size of effector, memory and regulatory T cells (166-168). This is probably due to the exceptional potency of ICOS signaling, compared to CD28, in activating Akt, a known T cell survival factor (reviewed in (106)). We determined that Protollin was capable of inducing ICOS in Foxp3-expressing Tregs *in vivo* and amplifying ICOS-expressing Tregs in the lymph nodes draining the nasal mucosa in both Balb/c and C57Bl6/J strains of mice (Chapters 3 and 4). Protollin primarily induced ICOS within $CD4^+Foxp3^-$ cells and caused their expansion in the LNs. However, beyond demonstrating that the Protollin-induced LN $CD4^+ICOS^+$ cells preferentially expressed IL-10 compared to IFN-γ (Chapter 3), we did not further characterize the specific phenotype of the $CD4^+Foxp3^-$ cells. Lung $CD4^+ICOS^+$ cells from Protollin-treated

mice did not express significantly greater intracellular levels of IL-4, IL-10, IFN-γ, or IL-17 compared to cells from PBS-treated, allergen-challenged mice. Also, despite reducing GATA-3 gene expression in the nasal-associated lymphoid tissues, Protollin did not enhance T-bet, RORγt, Foxp3, or Egr-2 expression, transcription factors associated with Th1, Th17, Treg, or Tr1 cells, respectively. Protollin also induced no change in the master Tfh cell transcription factor, Bcl-6, or the characteristic chemokine receptor, CXCR5. Therefore, whether these cells are helper T cells, unpolarized or anergic T cells, regulatory T cells of the Tr1 phenotype, or even innate NK T cells or γδ T cells remains to be resolved.

Another unresolved question is how Protollin and sMPLA might be inducing ICOS *in vivo* and *in vitro*. Maximal ICOS expression requires CD28 co-stimulation in addition to TCR ligation (106;169), which is why ICOS is thought to enhance and maintain T cell responses activated by CD28. Recent work has revealed a significant complexity to the expression of ICOS in that it is regulated by different T cell lineage-specific mechanisms (reviewed in (106)). That ICOS expression depends on the polarization of the individual T cell subsets may explain the varying level of surface expression in different lineages and is further indication that ICOS primarily facilitates the development of the predetermined T cell response. ICOS has thus been shown to costimulate distinct T cell effector functions (cytokines) depending on the nature of the antigen and localization of the immune response (127). Furthermore, a recently published study by Watanabe et al. showed for the first time that numerous cytokines could on their own induce ICOS expression, independently of TCR/CD28 ligation, and that both mechanisms relied upon the activation of the transcription factor AP-1 and its binding to the ICOS promoter (170). Moreover, the authors showed that the individual cytokines had different capacities to induce surface expression of ICOS despite yielding a similar intensity of AP-1 binding to its response element and that the upregulation of ICOS also depended on whether the cells were naïve, activated or memory T cells. In relation to our study, these findings suggest that the capacity of TLR4 signaling to induce ICOS on $CD4^+$ or $CD8^+$ T cells and hence to modulate their activation and expansion may depend on the level of

TLR4 stimulation (in other words dose of ligand) and the associated signaling and cytokine responses. This may explain some of the discrepancies between our *in vivo* and *in vitro* findings, namely the differential induction of ICOS among Tregs, the appearance of a novel subset of ICOS-expressing $CD4^+Foxp3^-$ cells and the induction of ICOS among $CD4^-$ cells.

Another consideration is that the TLR4 stimulation and induction of ICOS *in vivo* and *in vitro* is either preceding or lacking antigen exposure, respectively, and is therefore driven independently of TCR and CD28 co-stimulation, but rather potentially through cytokine elaboration by Protollin or sMPLA-responsive cells or perhaps direct stimulation of T cells by Protollin or sMPLA. Therefore, in the presence of antigen TLR4 stimulation may potentially elicit far greater upregulation of ICOS. Interestingly, our preliminary data suggest that Protollin, but not sMPLA, was capable of directly stimulating ICOS expression in MACS-sorted $CD4^+$ cells (data not shown) which, if substantiated, would be a novel observation, the implication being that innate immune signals are capable of modulating adaptive T cell responses also through direct enhancement of a co-stimulatory molecule on $CD4^+$ T cells, a phenomenon that is less well characterized compared to direct TLR stimulation of B cells, for example. Another implication of this mechanism of direct ICOS induction on T cells would be its dependence on TLR4 expression by the T cells, which is a controversial issue; TLR4 expression has been more consistently described in Tregs and in activated or memory $CD4^+$ T cells than in naïve cells (171-177). This would imply that Tregs and pre-activated/memory $CD4^+$ splenocytes would presumably preferentially undergo ICOS upregulation by Protollin or sMPLA. Our preliminary data indicate that, unlike in the total splenocyte cultures, direct stimulation of sorted $CD4^+$ cells by Protollin induced ICOS in both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ populations, though not preferentially in Tregs, and furthermore imply that Protollin induces inhibitory signals from non-$CD4^+$ cells that override the induction of ICOS among $CD4^+Foxp3^+$ cells which would otherwise occur from direct stimulation of these cells.

To summarize, we know very little at this point regarding the phenotype of the $CD4^+ICOS^+$ cells induced by Protollin and the factors that are involved in the upregulation of ICOS, other than that we have confirmed that it is TLR4-dependent *in vivo* and relies significantly on the TRIF pathway, as well as partially on the MyD88 pathway. In addition to finding an upregulation of ICOS and IL-10 mRNA in the NALT following Protollin applications (Chapter 3), we also observed a marked (20-fold) increase in IL-17A gene expression, raising the question of whether this cytokine may be involved in precipitating the induction of ICOS (IL-17A was not included in the panel of cytokines tested by Watanabe et al. (170)). The increase in IL-17A expression was not accompanied by changes in RORγt expression in the NALT or IL-17 in the lungs, suggesting that the augmented expression of IL-17 may be limited to the nasal mucosa and is likely related to innate immune sources. Interestingly, innate $CD3^-CD4^+CD45^+$ lymphoid tissue inducer cells which may be a significant source of IL-17 and IL-22 cytokines have been shown to populate the NALT and regulate its organogenesis (178-180), while IL-17 production by $CD4^+$ cells has also been shown to be responsible for the formation of ectopic or tertiary lymphoid structures (181), suggesting that this cytokine could therefore potentially be relevant to the induction of ICOS. We also have not clarified whether TRIF-dependent type I IFNs play a role in the induction of ICOS. Contrasting effects of type I IFNs on ICOS-L expression and costimulatory function in B cells, peritoneal macrophages and pDCs have been reported (182;183). LPS stimulation of mast cells, which lack TRIF signaling as well as type I IFN production (184), can also upregulate ICOS-L expression (185). Therefore, the regulation of ICOS or ICOS-L expression is multifactorial and cell-specific and the role of type I IFNs requires further clarification. Clay et al. found that APCs of ICOS-deficient mice, whether naïve or inflamed, had augmented surface expression of ICOS-L compared to those of wt C57Bl/6J mice (119). Furthermore, they elegantly demonstrated that the adoptive transfer of ICOS -/- T cells compared to wt T cells to T cell-deficient (Rag -/-) mice augmented ICOS-L expression in the recipients' DCs. Downregulation of ICOS-L by interaction with ICOS has also been reported

elsewhere as a potential immunoregulatory negative feedback mechanism (186). In contrast, Lischke et al. recently reported that ICOS-L-deficient mice did not exhibit altered expression or upregulation of ICOS itself (167), therefore suggesting unidirectional regulation of ICOS-L surface expression depending on ICOS expression.

(4) *Lymph node-resident ICOS-expressing $CD4^+$ T cells, inducible via TLR4 stimulation of the nasal mucosa, contribute to the TRIF-dependent suppression of allergic airway disease* (Chapters 3 and 4).

In Chapter 3, we show that the TLR4-dependent induction of ICOS by nasally administered Protollin in $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells of the cervical LNs was associated with the TLR4- and not TLR2-dependent inhibition of lower airway allergic disease development in Balb/c mice (**Figure 4**, p.310). Adoptive transfer of sufficient numbers of cervical LN $CD4^+ICOS^+$, even from PBS-treated mice, but not equivalent numbers of $CD4^+ICOS^-$ cells inhibited BPEx-induced AHR and BAL eosinophilia. This suggested that $CD4^+ICOS^+$ cells of the cervical LNs possess an intrinsic regulatory capacity and that stimulation of the nasal mucosa via TLR4 serves to amplify this population to a threshold that permits the inhibition of allergic airway disease in this model. We also observed that Protollin caused an increase in $CD4^+ICOS^+Foxp3^+$ and $CD4^+ICOS^+Foxp3^-$ cells in the lungs but we did not confirm whether the migration of the cells or which of these populations was necessary for Protollin's regulatory function.

We extended these findings in Chapter 4, showing that MyD88 signaling also was not necessary for Protollin's inhibition of AHR but perhaps may have participated in the inhibition of BAL eosinophilia, whereas TRIF-dependent signaling was clearly essential to both processes. Unexpectedly, however, we found that the numbers of $CD4^+ICOS^+Foxp3^-$ cells in the cervical LNs of Trif-deficient mice were equivalent to those of wild-type mice, despite a lower proportion (percentage) of ICOS-expressing cells among the $CD4^+Foxp3^-$ subset, potentially suggesting (a) that the Protollin-induced expansion of $CD4^+ICOS^+Foxp3^-$ cells is intact in TRIF-deficient mice and that the absence of TRIF is rather enhancing a

MyD88-dependent $CD4^+ICOS^-Foxp3^-$ population, or (b) perhaps that despite reduced induction of ICOS among $CD4^+Foxp3^-$ cells, expansion of these cells is actually elevated in Trif-deficient compared to wt mice such that the absolute numbers of the cells are no different in the two strains. The first interpretation would indicate normal induction and expansion of ICOS in these particular cells in the LNs of Trif-deficient mice and augmentation of other cells via supernormal MyD88 signaling, whereas the second interpretation implies diminished induction of ICOS due to TRIF deficiency and supernormal expansion of this smaller initial $ICOS^+$ population. Interestingly, Zhou et al reported that LPS-induced ICOS-L surface expression on murine peritoneal macrophages and splenic B cells is higher in Trif-deficient than in wt mice (183). Furthermore, ICOS-L surface expression is inversely regulated by ICOS expression, as mentioned earlier (119;186). These findings potentially provide an explanation for our second interpretation of a compensatory mechanism, i.e. enhanced ICOS-L expression, overcoming the reduced induction of ICOS, though this is predicated on the assumption that both ICOS and ICOS-L expression are rate-limiting in the expansion of ICOS-expressing T cells. We have not confirmed that ICOS-L expression is similarly augmented in the LNs of Trif-deficient animals and it has not been proven to necessarily equate to enhanced ICOS-ICOS-L interaction and supernormal T cell expansion.

To further complicate matters, we additionally found that the adoptive transfer of LN $CD4^+ICOS^+$ cells from wt C57Bl/6J Protollin-treated mice to Trif-deficient animals prior to their allergen challenge only prevented the development of AHR but not eosinophilic airway inflammation, unlike in our Balb/c experiments in Chapter 3 in which transfer of wt $CD4^+ICOS^+$ cells to wt recipients inhibited both AHR and inflammation. This suggests that the phenotype or function of the $CD4^+ICOS^+$ cells may be strain-dependent, which is plausible given other reports of strain-dependent ICOS expression (187) and the suggestion by Watanabe et al. discussed in the prior section, that the cytokine-dependent induction of ICOS may be influenced by differential cytokine receptor expression on various cells, which could also be strain-dependent (170). The discrepancies in our experiments could

alternatively suggest an as yet unidentified role for TRIF expression in the recipient mice in modulating the effects of $CD4^+ICOS^+$ cells in relation to airway inflammation. Furthermore, when we adoptively transferred equivalent numbers of total LN $CD4^+$ cells to wt mice we found that cells from wt inhibited both AHR and BAL eosinophilia, whereas cells from Trif-deficient mice were, unexpectedly, not completely dysfunctional and actually inhibited BAL eosinophilia. Taken together, that $CD4^+ICOS^+$ cells from wt Protollin-treated mice only inhibited AHR and not airway eosinophilia upon transfer, whereas total $CD4^+$ cell transfer from Protollin-treated Trif-deficient mice which include a smaller proportion of ICOS-expressing cells inhibited only inflammation but not AHR, implies a role for MyD88-dependent induction of $CD4^+ICOS^-$ cells that contribute strictly to the inhibition of airway inflammation. Therefore, our data collectively support that in the C57Bl/6J strain $CD4^+ICOS^+$ cells in the LNs draining the nasal mucosa are functionally equipped to and likely to contribute only to Protollin's TLR4-TRIF-dependent inhibition of AHR, while $CD4^+ICOS^-Foxp3^-$ cells induced via the TLR4-MyD88-dependent pathway have the capacity to and may also contribute to the inhibition of airway eosinophilic inflammation. Returning to the discussion in the previous paragraph, this would support the first interpretation of our data pertaining to the reduced percentage but equivalent absolute number of ICOS-expressing $CD4^+Foxp3^-$ cells in the cervical LNs of Trif-deficient compared to wt mice; in other words, that the induction and expansion of $CD4^+ICOS^+$ cells is normal in the absence of TRIF, but that TRIF deficiency does not interfere with, or perhaps even enhances MyD88-dependent expansion of $CD4^+ICOS^-Foxp3^-$ cells. We cannot confirm, however, that the MyD88-dependent expansion of these $CD4^+ICOS^-Foxp3^-$ cells is supernormal in Trif-deficient mice. In conclusion, it is not clear whether TRIF deficiency causes diminished expression of ICOS but increased co-stimulation via ICOS-L which could enhance the expansion of a smaller proportion of ICOS-expressing cells, or enhances MyD88-dependent expansion of $CD4^+ICOS^-Foxp3^-$ cells. However, the sum of our data, as well as evidence from the literature, supports both hypotheses; that Protollin promotes MyD88-dependent expansion of $CD4^+ICOS^-Foxp3^-$ cells in the cervical LNs

which at the very least is not compromised in Trif-deficient mice and furthermore, that TRIF deficiency may result in diminished ICOS expression which could potentially be compensated by enhanced ICOS-L expression.

In any case, what is clear from our analysis of the percentage and absolute numbers of $CD4^+ICOS^+Foxp3^-$ cells in the cervical LNs is that the expansion of this cell subset is either intact or above normal in the absence of TRIF and thus, that the expansion of these LN cells *per se* is not sufficient to mediate the inhibition of allergic airway disease. Hence, our conclusion in Chapter 3, that the expansion of these cells via TLR4 stimulation may mediate the inhibition of allergic airway disease is either inaccurate or incomplete. Interestingly, we also found that Trif-deficient mice exhibited a lower absolute number of $CD4^+ICOS^+Foxp3^+$ cells in the cervical LNs following Protollin treatment compared to wt mice. (Why this is the case is unclear given our previous discussion of potentially enhanced ICOS-L costimulation and expansion of $CD4^+ICOS^+Foxp3^-$ cells). As we have established that $CD4^+ICOS^+$ cells are highly likely to contribute to the inhibition of AHR in this model (though we cannot definitively confirm this using ICOS- or ICOS-L-deficient mice due to the importance of ICOS costimulation to the development of the experimental asthma model itself), like in Chapter 3, we are unable to confirm whether the $CD4^+ICOS^+Foxp3^-$ or $CD4^+ICOS^+Foxp3^+$ cells are important to the inhibition of AHR. Another factor to consider in addition to the expansion of either of these populations, is the potential importance of migration of these cells to the lungs. In Chapter 3, we showed that the inhibition of allergic airway disease development by Protollin in Balb/c mice was associated with an increase in both of these populations in the lungs. In Chapter 4, we observed a significant Protollin-induced increase in only $CD4^+ICOS^+Foxp3^-$ cells in the lungs of wt C57Bl/6J mice which was absent in Trif-deficient mice (we did not evaluate ICOS expression in lung cells of MyD88-deficient mice). That Trif -/- mice displayed similar absolute numbers of $CD4^+ICOS^+Foxp3^-$ cells in the cervical LNs as compared to wt mice but lower numbers in the lungs, supports that these cells likely traffic towards the lungs and that this process is impaired in the absence of

functional TRIF. This is consistent with a report demonstrating TRIF's importance in effector T cell migration specifically to non-lymphoid tissues such as the lungs (188). The authors were unable to identify the LPS-induced TRIF-dependent co-stimulatory mechanisms promoting such migration of $CD4^+$ T cells and showed that it was independent of CD40 stimulation. Meanwhile, ICOS-L -/- mice were recently shown to display reduced accumulation of $CD4^+$ T cells to the lungs following systemic LPS boosting of the T cell response to OVA and subsequent intranasal OVA/LPS challenge, suggesting that ICOS co-stimulation is perhaps the driving mechanism for LPS-induced TRIF-dependent migration of $CD4^+$ T cells to the lungs (167). However, it was not confirmed whether the reduced recruitment was the result of a migratory defect or the reduced expansion of $CD4^+$ T cells observed systemically. Moore et al. attempted to address the importance of ICOS co-stimulation to the migration, beyond the expansion, of Th2 cells in a model of allergic airway disease not involving exogenous LPS (122). They concluded that ICOS co-stimulation enhances antigen-induced migration of activated $CD4^+$ T cells to the lungs and spleen by downregulating homing receptors which direct migration to the draining lymph nodes. In contrast, Tesciuba et al. reported that ICOS co-stimulation increases naïve lymphocyte migration towards the draining lymph nodes by augmenting specific chemoattractants (189). That we observed reduced accumulation of $CD4^+ICOS^+Foxp3^-$ cells in the lungs of Trif-deficient mice despite normal numbers in the lymph nodes suggests that ICOS alone is not a sufficient co-stimulatory signal for Protollin-induced migration of $CD4^+$ T cells to the lungs and that additional TRIF-dependent stimuli mediate this process. We have not answered whether migration to the lungs is occurring/ necessary for the inhibition of AHR or airway inflammation following the adoptive transfers or why adoptive transfer via the intraperitoneal route would circumvent the migratory defect from the cervical LNs. Therefore, it is unconfirmed whether $CD4^+ICOS^+Foxp3^-$ cell migration to the lungs may be more pertinent than the increase in lymph node $CD4^+ICOS^+Foxp3^+$ cells, both of which appear to be impaired in Trif-deficient mice. Overall, our data indicate that discrete TLR4-TRIF-dependent signals

influence the expansion and migration of $CD4^+ICOS^+$ cells that possess the capacity to contribute to the inhibition of AHR and that additional mechanisms are also likely to be involved in the TLR4-TRIF-mediated protection against the development of allergic airway disease (**Figure 4**, p.310).

(5) *The capacity to induce ICOS is not specific to Protollin but is also relevant to the TLR4 ligand, synthetic monophosphoryl lipid A.*

In an effort to gain greater clarity in relation to the MyD88- or TRIF-dependency of the induction of ICOS expression and expansion of $CD4^+ICOS^+$ cells, as well as its specificity to Protollin or potentially broader relevance to other TLR4 ligands, we began to examine these processes *in vitro*. Furthermore, we hypothesized that the TRIF-biased adjuvant, synthetic MPLA (sMPLA), might provide further confirmatory evidence of the TRIF-dependency of ICOS responses. Although Protollin consists of both TLR4 and TLR2 ligands, the effects of Protollin in preventing allergic airway disease were TLR2-independent, so we did not analyze further the role of TLR2 stimulation in relation to ICOS expression. It is unlikely to contribute significantly to the induction of ICOS *in vivo* since we demonstrated the dependence of ICOS on TLR4 stimulation and given our data supporting a primary role for the TRIF pathway, which the Neisserial proteosomes should not activate. However, there is a possibility that the mechanisms of ICOS induction *in vitro* may be different. Furthermore, TLR ligand structure is known to be an important determinant of activation of various cell types and signaling events, raising the question of whether Protollin's structure confers upon it an enhanced capacity to induce ICOS. Importantly, CD14 allows TLR4 to discriminate between structural variants of LPS and the consequent activation of the MyD88 and TRIF pathways, as discussed in the introduction (42;190). It is marginally important for MyD88 signaling (only at low LPS concentrations) but is rate-limiting in LPS-induced endocytosis of TLR4 which is a prerequisite for TRIF-TRAM signal transduction (21;42;98-100;190). Zanoni et al. showed that maturing DCs enhance their expression of CD14 and that its cell-specific expression determines which cell types can internalize TLR4

as well as extracellular macromolecules in response to LPS (for example, B cells cannot) (42). Importantly, they also demonstrated that CD14 simply functions to internalize TLR4 into endosomes where TRIF signaling can occur, such that if TLR4 can be non-specifically internalized, via phagocytosis for example, then the requirement for CD14 can be bypassed. The ability of phagocytic cargo, such as LPS-coated beads and *E. coli,* as opposed to soluble LPS, to bypass the need for CD14 in promoting IFN expression was not ubiquitous to all cell types, however. Nevertheless, the authors speculated that "the TLR4 network is designed such that any means of generating a TLR4-containing endosomal vesicle is sufficient to induce TRIF signaling".

These findings raise the interesting possibility that despite the absence of a requirement for TLR2 signaling for Protollin's effects in our model, the nanomolecular vesicular structure of Protollin which depends on its proteosomes may still confer some degree of advantage that permits the inhibition of allergic airway disease and induction of ICOS on $CD4^+$ T cells, perhaps circumventing the requirement for CD14 and facilitating greater internalization of the LPS molecules into endosomal compartments, thereby permitting enhanced TRIF-dependent signaling. Moreover, whether LPS on its own has the capacity to induce ICOS and whether the degree of ICOS expression may depend on different chemotypes or concentrations of LPS remains to be determined. However, our data indicate that sMPLA from *E. coli* dose-dependently increases ICOS expression among $CD4^+$ and $CD4^-$ (possibly $CD8^+$) cells in total splenocyte cultures, similarly to Protollin at later time points, but does not induce equivalent upregulation at earlier time points. Nonetheless, this observation establishes that ligands that are strictly TLR4-specific have the capacity to induce ICOS. Interestingly, that only Protollin but not sMPLA induced ICOS when directly stimulating sorted $CD4^+$ cells suggests that Protollin's capacity to stimulate TLR2 or more substantial MyD88-dependent signaling compared to the TLR4-TRIF-biased synthetic agonist is relevant to the observed induction of ICOS in this context, particularly given that the literature tends to suggest that TLR4 signaling is either completely absent in murine $CD4^+$ T cells or serves only to enhance Treg

functions (171;173;175-177). Moreover, it is unclear to what extent human and, particularly, murine $CD4^+$ T cells express CD14 (191;192); inadequate CD14 expression might render sMPLA MyD88-restricted in its signaling capacity in T cells, whereas Protollin's vesicular structure could perhaps facilitate its internalization into endosomal compartments from which it could activate TLR4-TRIF-dependent induction of ICOS.

The induction of ICOS by Protollin *in vivo* in the cervical lymph nodes was, however, at least partially independent of TRIF. Moreover, the observation that sMPLA, at concentrations which have been shown to induce mainly TRIF-dependent signaling (39;193), did not augment ICOS expression in total splenocyte cultures, but only at higher concentrations that were reportedly MyD88-sufficient (at least in the measured signaling outcomes), would also suggest a significant dependence of ICOS induction on MyD88 signaling. In summary, our findings indicate that the induction of ICOS, whether *in vivo* in the cervical lymph nodes of Protollin-treated animals or *in vitro* in sMPLA-stimulated splenocyte cultures appears to occur, at least partially independently of TRIF. The implication is that the induction of ICOS on $CD4^+$ T cells and expansion of $CD4^+ICOS^+$ T cells is not a major contributing factor for, but rather a correlating marker of, what appears to be predominantly TRIF-dependent, MyD88-independent inhibition of allergic airway disease. Our discussion in the previous section supports that the $CD4^+ICOS^+$ cells may contribute only partially and exclusively to the inhibition of AHR by Protollin. However, the precise role of MyD88 and TRIF in the context of our *in vitro* experiment requires clarification and may not necessarily be translated to the *in vivo* setting. Moreover, based on the literature discussed previously, the induction of ICOS *in vivo* as well as in our total splenocyte or isolated $CD4^+$ T cell cultures may rely on mechanisms that are specific to each context and is likely to be dependent on the sum of the differential capacity of Protollin or sMPLA to stimulate various cells capable of providing important signals to the T cells, to generate mediators which would influence ICOS expression, as well as to intrinsically stimulate the T cells.

Therefore, it would be premature to conclude at this point that TRIF- and type I IFN-dependent signaling by Protollin is not relevant to the induction of ICOS.

However, the possibility of predominant or partial MyD88-dependency of ICOS induction may also have broader implications with respect to the adjuvant properties of TLR4 ligands and particularly MPLA, which is of significant interest given its approval as a commercial vaccine adjuvant. Natural MPLA demonstrated TRIF-biased signaling in that it was shown to promote intact TRIF-dependent mediator responses in macrophages but diminished MyD88-dependent responses (194). Additionally, when employed as an adjuvant *in vivo*, natural MPLA enhanced OVA-specific $CD4^+$ T cell expansion TRIF-dependently and MyD88-independently. Although the TRIF-biased production of inflammatory mediators by macrophages was also confirmed for sMPLA, $CD4^+$ T cell responses were not analyzed (39). Assuming that the expansion of $CD4^+$ T cells by sMPLA is similarly strictly TRIF-dependent as with natural MPLA, the possibility of MyD88-dependent ICOS induction and $CD4^+$ T cell expansion by sMPLA based on our *in vitro* stimulation of naïve splenocytes in the absence of antigen, suggests that the induction of ICOS is neither a requirement for, nor an epiphenomenon of the promotion of largely intact TRIF-dependent $CD4^+$ T cell responses by MPLA. If correct, what may be the function of ICOS induction by TLR4 stimulation if not to augment effector T cell responses? Could it be that the induction of ICOS differentiates between an over-exuberant, inflammatory LPS-adjuvanted immune response versus an appropriate, controlled MPLA-adjuvanted response? This seems unlikely given that sMPLA did induce substantial ICOS expression and expansion of $CD4^+ICOS^+$ as well as $CD4^-ICOS^+$ T cells in total splenocyte cultures at an equivalent dose at which natural MPLA stimulated TRIF-dependent $CD4^+$ and $CD8^+$ T cell responses (194). Is the induction of ICOS then a contributing factor to the inflammatory response or a regulatory mechanism? Although innate immune responses precede adaptive responses to infection by several days, suggesting that adaptive immunity should not affect the early innate response, evidence exists of a requirement for adaptive immune suppression, mediated by both Tregs and conventional $CD4^+$ T cells, of over-

zealous early innate responses; Kim et al. demonstrated that these cells were both necessary and sufficient to prevent the cytokine storm and subsequent death triggered by viral infection or TLR stimulation (195). This is consistent with the earlier-discussed notion that TRIF-dependent signaling is important in restraining pro-inflammatory MyD88-dependent signaling events. That the $CD4^+ICOS^+$ cells have the capacity to inhibit AHR suggests that the induction of ICOS may serve a regulatory function, though the apparent additional inhibitory capacity of Protollin-induced $CD4^+ICOS^-$ cells, indicated in Chapter 4 in relation to inhibition of allergic inflammation, confounds the conclusion that specifically ICOS induction via TLR4 serves a regulatory purpose.

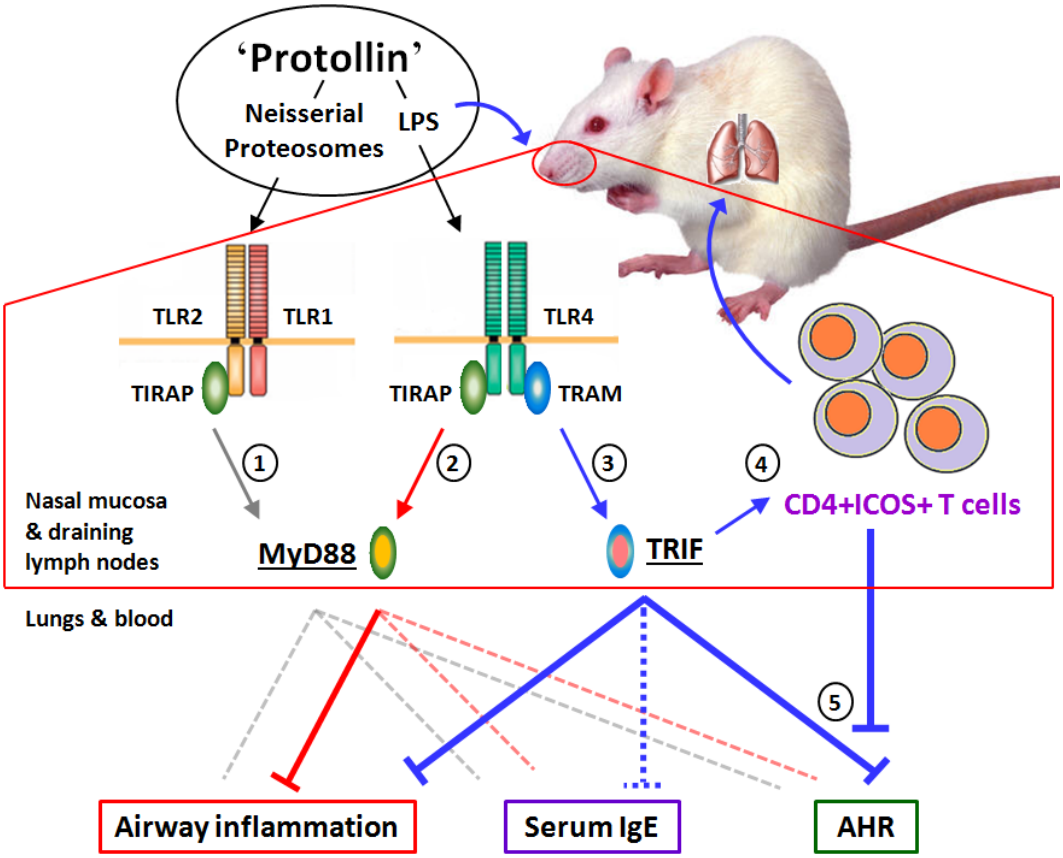
Furthermore, using gene array analysis, Lischke et al. described that ICOS induction was a distinguishing marker of a pro-inflammatory LPS-induced response, as opposed to a tolerogenic antigen-induced response (167). Given that our *in vivo* and *in vitro* data show a preferential expansion of $CD4^+ICOS^+Foxp3^-$ cells compared to $CD4^+ICOS^+Foxp3^+$ cells by Protollin, similar to the LPS-induced ICOS-ICOS-L interaction-dependent increase in T effector to Treg ratio observed by Lischke et al., it appears that we may be studying a similar TLR4-dependent, essentially pro-inflammatory, phenomenon, but which may possess anti-inflammatory potential in the context of type 2 allergic disease. Furthermore, we found no significant induction by Protollin in NALT tissues of the co-inhibitory molecules CTLA-4 or PD-1, or the tryptophan metabolite, indoleamine-2,3-dioxygenase that have been associated with negative regulation of immune responses and allergic airway disease, or the transcription factor, Egr-2, which has been associated with Tr1 cells and shown to mediate T cell anergy. The pro- or anti-inflammatory potential of the induction of ICOS via TLR4 may thus depend on the underlying immune status of the host and possibly the differential induction of ICOS on Tregs or T effectors. Our data indicate, however, that the expansion of $ICOS^+Foxp3^+$ Tregs was indeed TRIF-dependent. Therefore, the possibility that the induction of ICOS among T effector and Treg cells is differentially regulated by MyD88 and TRIF-dependent signaling and may be dose or ligand-dependent is certainly intriguing and of relevance to the

development of vaccine adjuvants. Alternatively, it is also possible that the induction of ICOS, regardless of whether on Tregs or T effectors, may be an important anti-inflammatory mechanism for the control of innate immune responses given that it has been shown that ICOS-ICOS-L interaction provides negative regulatory feedback to APCs by downregulating their expression of ICOS-L, as mentioned previously (186). Whether this is a relevant mechanism to the inhibition of experimental allergic asthma in our model is unclear given that we have not tested ICOS-L interaction. In addition, it would be interesting to test the effect of antagonism of the ICOS-ICOS-L interaction upon the expansion of both innate immune cells and T cells in our *in vitro* culture system to determine whether the induction of ICOS is exclusively associated with immunostimulatory or also anti-inflammatory effects of TLR4 on these populations.

Therefore, the major questions that arise from the findings presented in this dissertation are:

1. What is the role of type I IFN production by Protollin in the inhibition of allergic airway disease development and induction of ICOS?
2. What are the precise roles of MyD88 and TRIF in the induction of ICOS by Protollin and MPLA and are they dependent on whether the T cell is $CD4^+$, $CD8^+$, effector, Treg, naïve or antigen-activated?
3. What are the mechanisms and conditions for the induction of ICOS by TLR4 ligands, e.g. structure-, dose-, cytokine- and cell- specificity?
4. What is the significance of ICOS induction in relation to the T cell immunostimulatory properties of TLR4 ligands, as well as the moderation of innate immune responses?
5. What is the significance of ICOS induction by TLR4 ligands in relation to phenotypic differentiation of T cells and effector capacity, such as migratory function, etc.?

Figure 4. *Schematic summary of the data presented in Chapters 3 and 4 regarding the mechanisms by which the nasal application of a mucosal adjuvant, Protollin, consisting of TLR2 and 4 ligands protects against the development of features of allergen-induced airway disease. TLR2 signaling (1) was eliminated from being important in the inhibition of airway inflammation, hyperresponsiveness (AHR), or systemic IgE production (dashed grey lines), whereas MyD88-dependent signaling was shown to be dispensable for the inhibition of IgE and AHR only (dashed grey and red lines), suggesting a contribution of the TLR4-MyD88 pathway (2) exclusively to the suppression of allergen-induced airway inflammation by Protollin (thick red line). TLR4-TRIF signaling (3) was necessary for the inhibition of airway inflammation and AHR (thick blue lines), but its requirement for attenuation of IgE production could not be confirmed or excluded (dotted blue line). The TLR4-TRIF-dependent inhibition of AHR (5) is mediated at least in part by the induction in cervical lymph nodes (and possibly migration to the lungs) of CD4+ICOS+ T cells (4).*



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CHAPTER 6

Summary & Conclusions

The concept that microbial exposures, particularly early in life, are important in stimulating the immune system in a manner that protects against the development of exuberant type 2 inflammatory immune responses that typify allergic disease, including allergic asthma, has gained support over the past few decades. Consequently, there have been substantial efforts to identify the specific environmental conditions, microbial organisms or their components and the pertinent immune mechanisms, as well as to potentially harness these mechanisms for immunotherapy of allergic disease. The discovery of innate immune receptor families that recognize components of both invading and endogenous microbes, consequently stimulating protective or homeostatic immune responses has been a major break-through in our understanding of the regulation of the immune system and the immunological interface between the host and environment in the context of allergic disease. However, the central role of these receptors in alerting the immune system to antigens of foreign origin has in recent years shifted emphasis to the capacity of these receptors and their ligands to compromise immunological tolerance, such as at the respiratory mucosal surface, and thereby elicit excessive inflammatory responses to allergens. Thus, the 'allerginicity' of specific proteins, such as aeroallergens, is thought to depend on common molecular features which endow these antigens with the capacity to engage such mechanisms. The TLRs are one such family of innate immune receptors and there are significant indications from epidemiological studies, genetic analyses, as well as clinical and experimental data that indeed indicate a potential for the TLR4 isoform and its bacterial ligand, lipopolysaccharide, in particular, to initiate, exacerbate, or conversely prevent or treat allergic airway disease, depending on conditions and mechanisms that are still being elucidated. Animal studies suggest that many of these pro- or anti-inflammatory events are driven by activation of a signaling pathway downstream of TLR4 which relies upon the intracellular adaptor protein MyD88, whereas the other major TLR4-activated signaling pathway controlled by the adaptor TRIF, has not been explored in the context of allergic asthma. Here, we investigated the role of TLR4 and TRIF activation in different murine models of experimental allergic asthma. We have presented data supporting that (a) TLR4

is not universally important in the development of all aspects of allergic airway disease but rather may be an 'allergen-specific' pattern recognition receptor and (b), our primary finding, that the TRIF signaling pathway of TLR4 may have protective and potentially immunotherapeutic effects against the development of allergic airway disease in the context of exposure via the airways to a natural aeroallergen and adjuvant-induced TLR4-dependent protection, respectively. We also demonstrate (c) that TLR4 stimulation induces ICOS expression and amplifies ICOS-expressing $CD4^+$ T cells, (d) that this may be broadly applicable to TLR4 agonists and not exclusively Protollin and (e) that these cells partially contribute to aspects of the TLR4-TRIF-dependent inhibition of experimental allergic airway disease. We also present the argument that these findings may be of broader significance to the pathogenesis or pathophysiology of allergic asthma, as well as the modulation of adaptive immune responses by TLR4 ligands.

APPENDIX

Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness

RESPIRATORY RESEARCH

RESEARCH Open Access

Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness

Karim H Shalaby[1], Leslie G Gold[2], Thomas F Schuessler[2], James G Martin[1] and Annette Robichaud*[2]

Abstract

Background: Pulmonary function has been reported in mice using negative pressure-driven forced expiratory manoeuvres (NPFE) and the forced oscillation technique (FOT). However, both techniques have always been studied using separate cohorts of animals or systems. The objective of this study was to obtain NPFE and FOT measurements at baseline and following bronchoconstriction from a single cohort of mice using a combined system in order to assess both techniques through a refined approach.

Methods: Groups of allergen- or sham-challenged ovalbumin-sensitized mice that were either vehicle (saline) or drug (dexamethasone 1 mg/kg ip)-treated were studied. Surgically prepared animals were connected to an extended *flexiVent* system (SCIREQ Inc., Montreal, Canada) permitting NPFE and FOT measurements. Lung function was assessed concomitantly by both techniques at baseline and following doubling concentrations of aerosolized methacholine (MCh; 31.25 - 250 mg/ml). The effect of the NPFE manoeuvre on respiratory mechanics was also studied.

Results: The expected exaggerated MCh airway response of allergic mice and its inhibition by dexamethasone were detected by both techniques. We observed significant changes in FOT parameters at either the highest (Ers, H) or the two highest (Rrs, R_N, G) MCh concentrations. The flow-volume (F-V) curves obtained following NPFE manoeuvres demonstrated similar MCh concentration-dependent changes. A dexamethasone-sensitive decrease in the area under the flow-volume curve at the highest MCh concentration was observed in the allergic mice. Two of the four NPFE parameters calculated from the F-V curves, $FEV_{0.1}$ and FEF50, also captured the expected changes but only at the highest MCh concentration. Normalization to baseline improved the sensitivity of NPFE parameters at detecting the exaggerated MCh airway response of allergic mice but had minimal impact on FOT responses. Finally, the combination with FOT allowed us to demonstrate that NPFE induced persistent airway closure that was reversible by deep lung inflation.

Conclusions: We conclude that FOT and NPFE can be concurrently assessed in the same cohort of animals to determine airway mechanics and expiratory flow limitation during methacholine responses, and that the combination of the two techniques offers a refined control and an improved reproducibility of the NPFE.

Background

An excessive airway response to agonists such as methacholine (MCh) or histamine is widely employed as a diagnostic criterion for asthma [1]. Response is generally measured in human subjects through the spirometric assessment of maximal forced expiratory manoeuvres following the administration of progressively increasing concentrations of the constrictive agonist [1]. Forced expiratory manoeuvres have been favoured because of their relative technical simplicity and the widespread availability of inexpensive equipment. However, forced expirations are dependent on patient cooperation, which is not possible to obtain in very young patients [2], and techniques such as forced oscillatory mechanics [3] and the squeeze technique for forced expirations have been applied in these circumstances [4-6].

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In experimental animals, airway responsiveness is commonly assessed using measurements of lung mechanics acquired during tidal breathing or using forced oscillation with volumes less than tidal volume. Forced expiratory manoeuvres have also been used to successfully assess airway hyperresponsiveness in the mouse [7-11] and rat [10,12]. In these experiments, rapid forced expiration was induced by subjecting the tracheostomized animals to a large negative pressure. Direct comparisons of the two approaches of measuring airway responsiveness have been reported in mice using either separate groups of animals or separate equipment. The objective of this study was to obtain lung function measurements at baseline and following bronchoconstriction from both techniques using a single cohort of mice and a single system. More specifically, negative pressure-driven forced expiratory (NPFE) and forced oscillation technique (FOT) manoeuvres were concurrently performed using a single combined setup in groups of allergen- or sham-challenged ovalbumin-sensitized mice. We studied the performance of these tests at baseline and following increasing aerosolized MCh challenges as well as in the context of a therapeutic intervention with dexamethasone, a drug known to inhibit allergen-induced airway hyperresponsiveness. The impact of NPFE on respiratory mechanics was investigated as well.

Methods

Animals

Six to eight week-old, female Balb/c mice, ranging in weight between 17 and 22 grams at the time of study, were purchased from Charles River, Canada. The mice were housed in a conventional animal facility under a 12 hour light/dark cycle with free access to food and water. Experimental procedures were approved by McGill University Institutional Animal Care Committee.

Experimental procedures and protocol

Animals were divided in four experimental groups: (i) vehicle-treated, saline-challenged (Veh/Sal), (ii) dexamethasone-treated, saline-challenged (Dex/Sal), (iii) vehicle-treated, OVA-challenged (Veh/OVA), and (iv) dexamethasone-treated, OVA-challenged mice (Dex/OVA). All mice received two intraperitoneal (ip) sensitizations, one week apart (Day 0 and 7), of 10 μg ovalbumin (OVA grade V; Sigma-Aldrich, USA) and 1 mg aluminum hydroxide (Sigma-Aldrich, USA) in 0.2 ml sterile saline. The mice were challenged one week later on three consecutive days (Day 14, 15, 16) by intranasal instillation of either sterile saline, or 10 μg OVA/day (in 36 μl) under light isoflurane anesthesia. One day prior to OVA- or saline-challenge (Day 13), animals began receiving daily ip injections of either sterile saline (vehicle) or 1 mg/kg dexamethasone, until one day after the final challenge (Day 17). All measurements were obtained 48 hours following the final challenge (Day 18). On the day of the experiment, mice were weighed and anesthetized with an injection of xylazine hydrochloride (10 mg/kg, ip) followed 5 minutes later by the administration of sodium pentobarbital (32 mg/kg, ip). Once the desired level of anesthesia was reached, as assessed by loss of withdrawal reflex and absence of response to external stimuli, the mouse was tracheostomized using an 18G metal cannula. The animal was then placed in a flow-type body plethysmograph and connected via the endotracheal cannula to a *flexiVent* system (SCIREQ Inc., Montreal, Canada). After initiating mechanical ventilation, the mouse was paralyzed with a 1 mg/kg pancuronium bromide ip injection and subjected to a deep lung inflation (DI; slow inflation to a pressure of 30 cmH_2O held for 3 seconds) before the plethysmograph was sealed for the rest of the experiment. The animal was ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure (PEEP) of 3 cmH_2O.

Experimental Setup

To permit NPFE and FOT measurements in the same setup, we extended a standard *flexiVent* system as follows (Figure 1). The inspiratory arm of the Y-tubing contained a computer-controlled nebulizer (Aeroneb Lab, standard mist model, Aerogen Ltd, Ireland) as well as a computer-operated pinch valve that isolated the nebulizer from high negative pressures during NPFE manoeuvres. A T-piece in the expiratory limb of the ventilator connected the mouse airways to a negative pressure reservoir via a second computer-operated fast response (typical opening time < 4 ms) solenoid shutter valve. The reservoir pressure and the air flow into the plethysmograph were recorded during NPFE manoeuvres via precision differential pressure transducers attached, respectively, to the pressure reservoir (SCIREQ UT-PDP-100; 10 kPa nominal range) and the pneumotachograph mounted on the plethysmograph chamber (SCIREQ UT-PDP-02; 0.2 kPa nominal range). This was done in addition to the signals typically recorded by the *flexiVent*, i.e. volume displaced by piston, pressure in the cylinder and pressure at airway opening. All data were digitized at a rate of 256 Hz with 12 bit accuracy. The mechanical cut-off frequency of the plethysmograph chamber was over 300 Hz. The spectra of the forced expired flow signals we collected did not contain any significant power at frequencies above 50 Hz.

Forced Oscillation Measurements

Respiratory mechanics were assessed using a 1.2 second, 2.5 Hz single-frequency forced oscillation manoeuvre (SFOT; using the SnapShot-150 perturbation) and a 3 second, broadband low frequency forced oscillation manoeuvre containing 13 mutually prime frequencies

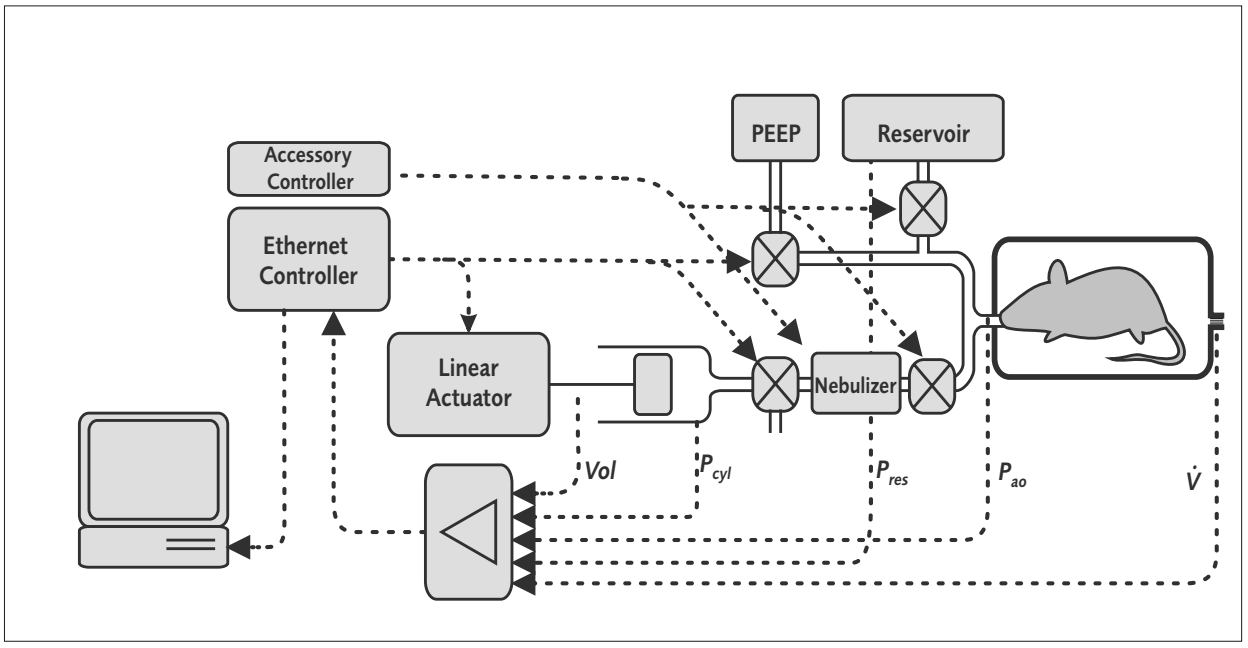

Figure 1 Block diagram of *flexiVent* system with extensions for negative pressure-driven forced expiration manoeuvres. During a negative pressure-driven forced expiration manoeuvre, the reservoir pressure (P_{res}) as well as the air flow into the plethysmograph ($\dot{V}$) were recorded via precision differential pressure transducers attached respectively to the negative pressure reservoir and the pneumotachograph mounted on the plethysmograph chamber. These signals were collected in addition to the volume displaced by the piston (Vol), the pressure in the cylinder (P_{cyl}) and the pressure at airway opening (P_{ao}) typically recorded by the *flexiVent*. PEEP stands for positive end expiratory pressure.

between 1 and 20.5 Hz (LFOT; using the Quick Prime-3 perturbation). The settings of both perturbations were configured to ensure that onset transients were omitted and the oscillations had reached steady state in the analyzed portions of the manoeuvres. Respiratory system resistance (Rrs) and elastance (Ers) were calculated in the *flexiVent* software by fitting the equation of motion of the linear single compartment model of lung mechanics to the SFOT data using multiple linear regressions. Respiratory system input impedance was calculated from the LFOT data and Newtonian resistance (R_N), tissue damping (G) and tissue elastance (H) were determined by iteratively fitting the constant-phase model [13] to input impedance. Both FOT manoeuvres were executed every 15s in alternation after each MCh aerosol challenge to capture the time course and the detailed response of the MCh-induced bronchoconstriction.

Forced Expiratory Measurements

In preparation for each NPFE manoeuvre, the negative pressure reservoir was adjusted to a given negative target pressure by retracting a sufficiently large syringe. Once the manoeuvre was initiated, the *flexiVent* was programmed to gradually inflate the mouse lungs to a pressure of 30 cmH_2O over 1 second and hold this pressure for 2 seconds before opening the shutter valve to connect the animal's airway opening to the negative pressure reservoir for 2 seconds. The negative pressure gradient generated a rapid deflation of the mouse lungs and the ensuing flow of air into the body box associated with the animal chest wall movement was measured. From that signal, we calculated the forced expired volume over 0.1 second ($FEV_{0.1}$), forced vital capacity (FVC), peak expiratory flow (PEF) and forced expiratory flow at 50% of FVC (FEF50). In order to study the relationship between the expiratory flow and the driving pressure, we repeated this procedure with increasingly negative pressures in 10 cmH_2O increments from -15 to -65 cmH_2O.

Impact of NPFE on respiratory mechanics

During pilot experiments and to assess the effect of NPFE manoeuvres on lung function, we measured respiratory mechanics using the FOT immediately and 1, 3, 5 and 10 minutes after a NPFE manoeuvre performed with a reservoir pressure of -35 cmH_2O and a PEEP of 2 cmH_2O. Then, we administered a DI and obtained another set of FOT data. Similar data were obtained in our main experiments in OVA-sensitized, vehicle pre-treated, sham-or OVA-challenged animals over a time frame of one minute after NPFE performed with a reservoir pressure of -55 cmH_2O and a PEEP of 3 cmH_2O.

Assessment of allergen-induced airway hyperresponsiveness by FOT and NPFE

Following DI and baseline measurements, saline solution was delivered to the mouse as an aerosol using a 4s nebu-

lization period synchronized with inspiration at a nebulization rate of 50%. FOT measurements were then used to monitor the time-course of the ensuing response, as described above. Immediately upon observing a peak in Rrs reported by the software, a single NPFE manoeuvre was applied, as previously described, using a negative pressure of -55 cmH_2O. Measurements of FOT parameters resumed immediately following the NPFE manoeuvre for a period of one minute. To ensure a return to baseline, the mouse underwent repeated DIs followed by default ventilation and respiratory mechanics measurements prior to the administration of an initial MCh-induced bronchoprovocation (31.25 mg/ml acetyl-β-methylcholine; Sigma-Aldrich, USA). In this manner, doubling concentrations of MCh were administered up to 250 mg/ml and a NPFE manoeuvre was performed at the peak response to a given concentration (Figure 2).

Statistical analysis

The results are expressed as mean ± SD with *n* being the number of animals per group. For statistical analyses, responses were converted to their logarithms (log_{10}) and differences between groups were analysed using analyses of variance for repeated measurements (ANOVA) followed by Bonferroni or Tukey's multiple comparisons with $p < 0.05$ considered statistically significant (GraphPad Prism version 5; GraphPad Software, San Diego, USA) [14].

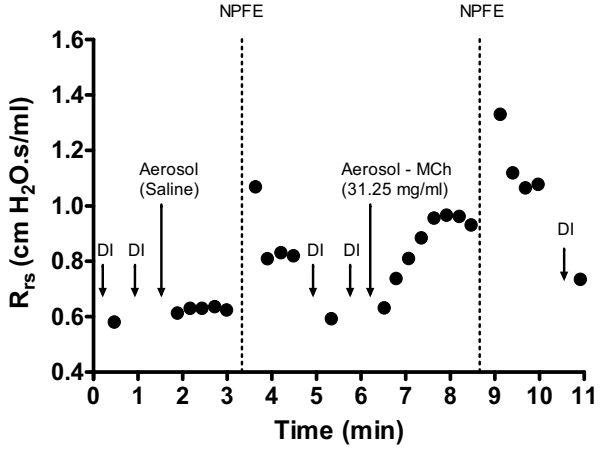

Figure 2 Measurement protocol. Experimental trace in a sham control mouse illustrating the timing of a negative pressure-driven forced expiration (NPFE) manoeuvre following saline and methacholine (31.25 mg/ml) aerosol challenge, using closely-spaced (15s) single-frequency forced oscillation parameter Rrs to follow the time-course of the response. Rrs = respiratory system resistance; DI = deep lung inflation (30 cmH_2O); MCh = methacholine.

Results

Impact of NPFE on respiratory mechanics

Following the application of a negative pressure to perform NPFE manoeuvres in naïve mice in the absence of MCh challenge (-35 cmH_2O and a PEEP of 2 cmH_2O), we observed a sustained increase in Rrs, Ers, G and H, but not in R_N (Figure 3). This effect did not spontaneously reverse during a period of 10 minutes of tidal ventilation, but respiratory mechanics returned to baseline after DI. Given this impact of NPFE manoeuvres on lung mechanics in our pilot experiments, DI was performed following all subsequent NPFE manoeuvres. We also investigated whether the effect of the NPFE manoeuvre on respiratory mechanics was amplified in vehicle-treated allergen-sensitized and challenged animals studied for one minute post-NPFE manoeuvre following saline aerosol administration. The adverse effect of the NPFE was reproduced, but not significantly augmented, in OVA-challenged, compared to sham-challenged mice.

The pressure-dependence of expiratory flow

Mean flow-volume curves obtained over a range of negative pressures from -15 to -65 cmH_2O, for both sham-challenged and OVA-challenged allergic mice are shown in Figure 4. As expected, the mean peak expiratory flow was pressure-dependent at lower pressures. Negative pressures of -15 and, to a lesser extent, -25 cmH_2O produced sub-maximal peak expiratory flows and altered flow-volume loops when compared to higher pressures both in sham- and OVA-challenged mice. In sham-challenged, as well as, in OVA-challenged mice, negative pressures of -35, -45, -55 and -65 cmH_2O produced virtually identical flow-volume loops, indicating that maximal expiratory flow had been reached. In all subsequent NPFE manoeuvres, a negative pressure of -55 cmH_2O was used to ensure that a maximal effect was evoked.

Assessment of airway responsiveness to methacholine

Mean baseline lung function parameters for the different experimental groups did not differ significantly whether assessed by FOT or by NPFE parameters (Figures 5 and 6). However, as expected, the group of OVA-challenged allergic mice demonstrated a dexamethasone-sensitive hyperresponsiveness to MCh compared to its respective control group, as illustrated by significant increases in all FOT parameters after the 125 and/or 250 mg/ml aerosol bronchoprovocation and reversal following drug treatment (Figure 5).

The flow-volume curves obtained from NPFE manoeuvres also demonstrated MCh concentration-dependent

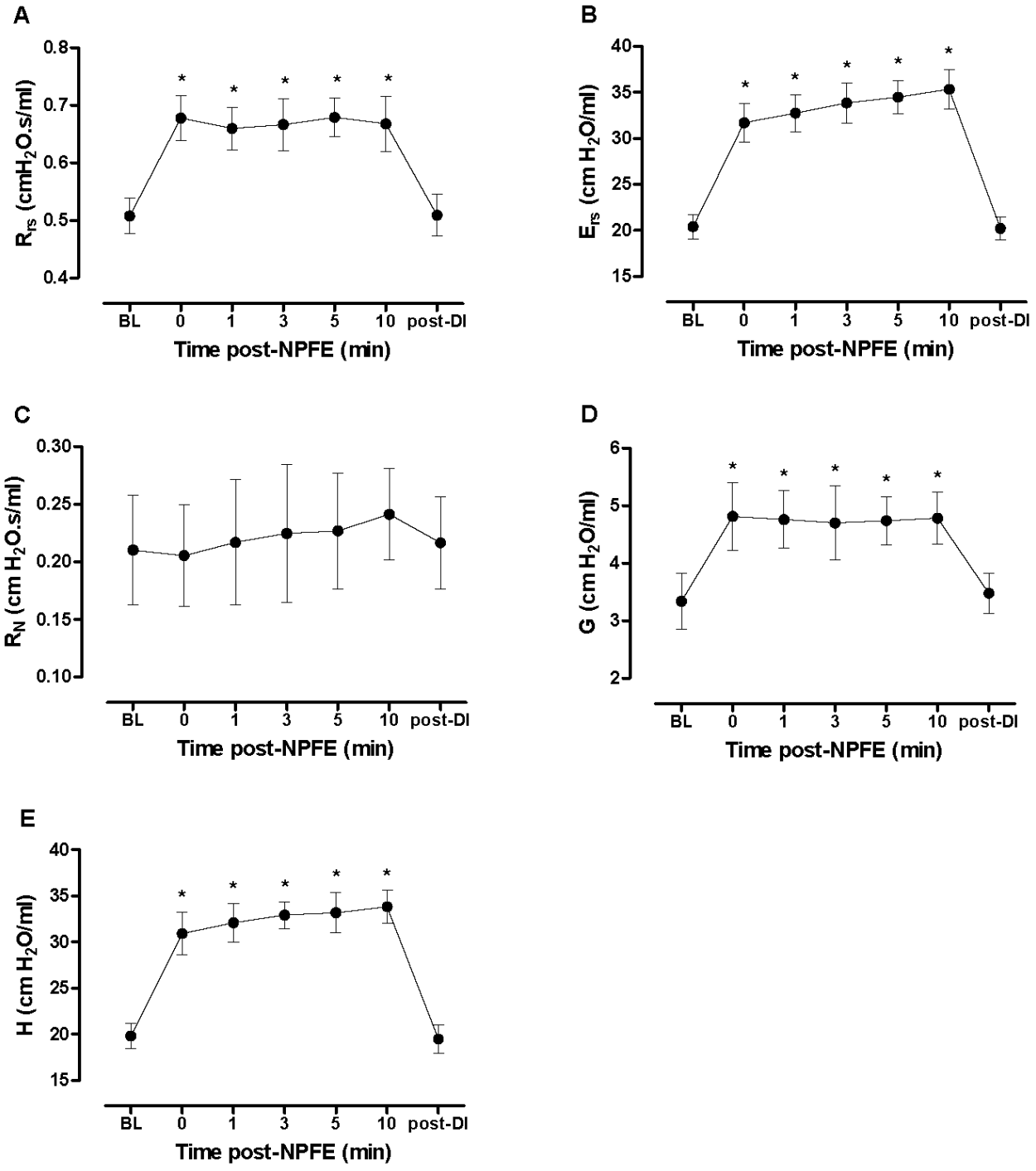

Figure 3 Impact of negative pressure-driven forced expiration manoeuvres on respiratory mechanics. Respiratory mechanics in naïve mice at baseline (BL), following the application of a negative pressure-driven forced expiration (NPFE) manoeuvre and following deep lung inflation (post-DI; 30 cmH_2O). Values are mean ± standard deviation from a group of 12 mice that were each studied once in the absence of methacholine challenge. (*$p < 0.05$; ANOVA).

changes with a decrease in the area under the flow-volume curve that was more pronounced at the highest MCh concentration in the OVA-challenged allergic mice and reversible by dexamethasone treatment (Figure 7D). From the four NPFE parameters calculated, an exaggerated response to methacholine was significantly detected in the OVA-challenged mice with $FEV_{0.1}$ and FEF50 at the highest concentration (Figure 6C, D). Normalization of

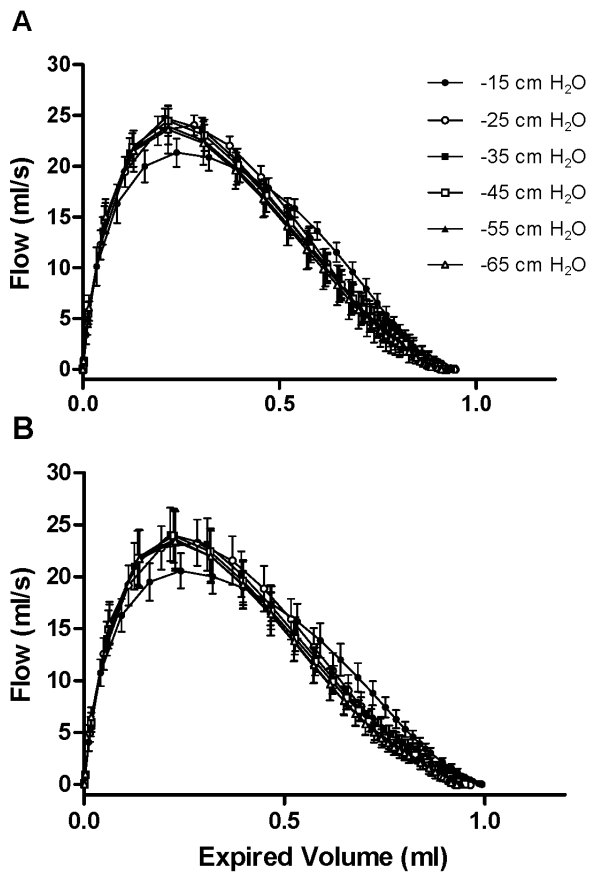

Figure 4 Pressure-dependence of expiratory flow. Mean flow-volume curves from ovalbumin-sensitized and sham-challenged mice (A) and ovalbumin-sensitized and challenged mice (B) at varying negative pressures. Values are mean ± standard deviation from groups of 7-9 mice (1 determination per animal at each negative pressure).

$FEV_{0.1}$ to FVC extracted from the same manoeuvre did not improve the sensitivity with which airway hyperresponsiveness was detected (Figure 6E). However, normalization to baseline permitted hyperresponsiveness of the OVA-challenged mice relative to the sham-challenged animals (Veh/OVA vs Veh/Sal) to be detected at a lower concentration of MCh (125 mg/ml) (Figure 8C). Also, when NPFE parameters were expressed as % of baseline, airway hyperresponsiveness of the OVA-challenged mice was captured by all four parameters calculated but mostly at the highest MCh concentration (Figure 8). Normalization to baseline had a minimal impact on FOT results (Figure 9). Finally, the effect of the drug treatment on preventing airway hyperresponsiveness (Dex/OVA vs Veh/OVA) was detected by both techniques (Figures 5, 6, 7, 8, and 9).

Discussion

In this study, we obtained measurements of NPFE and FOT from the same cohorts of animals using a setup that combined both techniques. NPFE manoeuvres in mice, unlike spirometry in humans, are invasive procedures. As with FOT measurements, NPFE manoeuvres require that the animals undergo anaesthesia, tracheotomy or intubation, and mechanical ventilation. The combination of the two techniques in a single set-up allowed us to study the performance of both tests through a refined approach. In the present study, we measured airway responsiveness to MCh in a mouse model of allergen-induced airway hyperresponsiveness using concurrent NPFE and FOT manoeuvres and examined whether one technique offered practical advantages or was informative in ways that the other was not.

As expected, we found forced expiration to be pressure-dependent at lower negative pressures but pressure-independent at higher negative pressures. In our animals, a negative pressure of -35 cmH_2O or greater was required to reliably produce a maximal forced expiration (Figure 4). Above this threshold, expiratory flow became independent of the driving pressure, indicating that maximal flow was produced and that expiratory flow limitation (EFL) played an important role in determining the forced expiratory flow.

In the present model of allergen-induced airway hyperresponsiveness, the four experimental groups studied were indistinguishable under baseline conditions by FOT or NPFE. Baseline values of calculated parameters from either measurement technique were comparable to those reported in the literature (Figures 5, 6) [7,9,11].

Under our experimental conditions, we were able to detect airway hyperresponsiveness to MCh in vehicle-treated allergen-challenged mice compared to the sham-challenged or drug-treated mice by both techniques. In addition to significant increases in FOT parameters following MCh provocation, we also observed significant changes when using the NPFE technique. Therefore, we found, as in previous studies [7-12], that NPFE can be used as an indicator of bronchoconstriction in mice.

However, compared to FOT, the sensitivity at which NPFE parameters significantly detected the MCh-induced changes was lower. Normalization to baseline improved this sensitivity while having minimal impact on FOT responses. This discrepancy could highlight the fact that the two measurement techniques are determined by different factors or alternatively, that the distribution of a specific determinant of NPFE (perhaps lung volumes) was unequal between groups and that the normalization of NPFE parameters in terms of the initial lung condition provided an adjustment [15]. Since good statistical practice in pharmacology generally recommends looking at data in its raw form before any normalization [16], our results highlight a potential shortcoming of the NPFE technique compared to FOT. Normalization to baseline could prove to be difficult in chronic or longitudinal studies where baseline recordings are collected an extended period of time before the measurements.

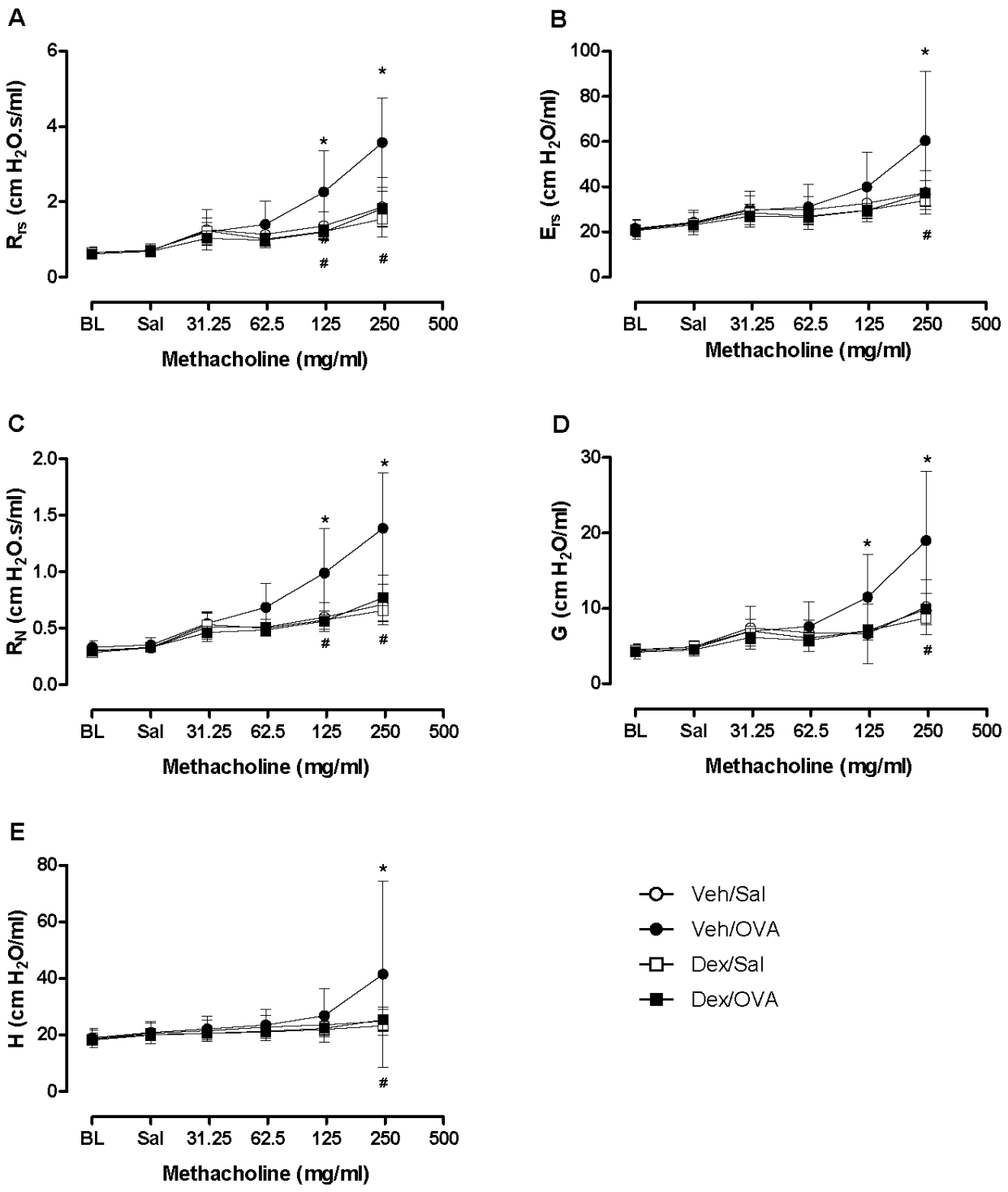

Figure 5 Assessment of allergen-induced airway hyperresponsiveness by the forced oscillation technique. Forced oscillation parameters at peak Rrs response to each concentration of aerosolized methacholine in ovalbumin- and saline-challenged OVA-sensitized mice that were either vehicle- or dexamethasone-treated. Values are mean ± standard deviation from groups of 5-7 mice. (*$p < 0.05$ Veh/OVA vs Veh/Sal, #$p < 0.05$ Veh/OVA vs Dex/OVA; ANOVA).

The interpretation and structural correlation of human spirometry is fairly complex since it has been shown to be influenced by a variety of factors, including upper airway resistance, EFL, elastic lung and chest wall recoil, patient characteristics, health status or effort [17]. However, not all these confounding factors apply to the NPFE manoeuvres we performed in mice since some were controlled by the machine or the experimental protocol. The animals

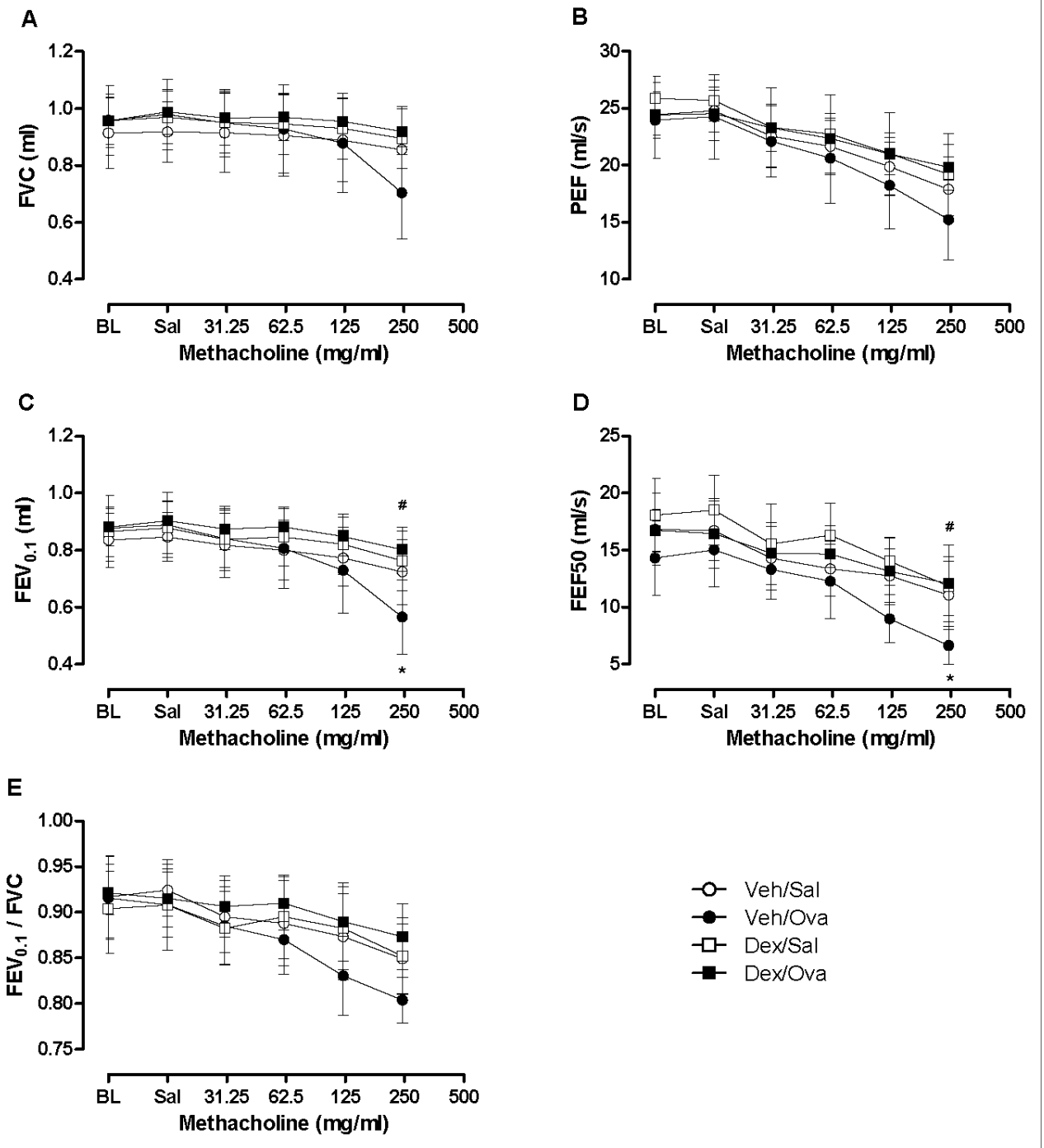

Figure 6 Assessment of allergen-induced airway hyperresponsiveness by negative pressure-driven forced expiratory parameters. Forced expiration parameters at baseline (BL) and following aerosolized saline (Sal) or increasing methacholine concentrations in vehicle (Veh)- or dexamethasone (Dex)-treated, sham (Sal)- and ovalbumin (OVA)-challenged ovalbumin-sensitized mice. Values were obtained at peak response to each concentration of aerosolized methacholine and are expressed as mean ± standard deviation from groups of 4-6 mice where each animal was studied once. (*$p < 0.05$ Veh/OVA vs Veh/Sal, #$p < 0.05$ Veh/OVA vs Dex/OVA; ANOVA).

were anaesthetized, tracheostomized and passive, so their upper airways were bypassed and effort or muscular pressure was eliminated. Furthermore, prior to a forced expiration manoeuvre, the mouse lungs were inflated to a controlled and highly reproducible inflation pressure of 30 cmH_2O, which contributed to standardize the driving pressure for the manoeuvre, to minimize the variations in elastic recoil and to achieve maximal expiration. This

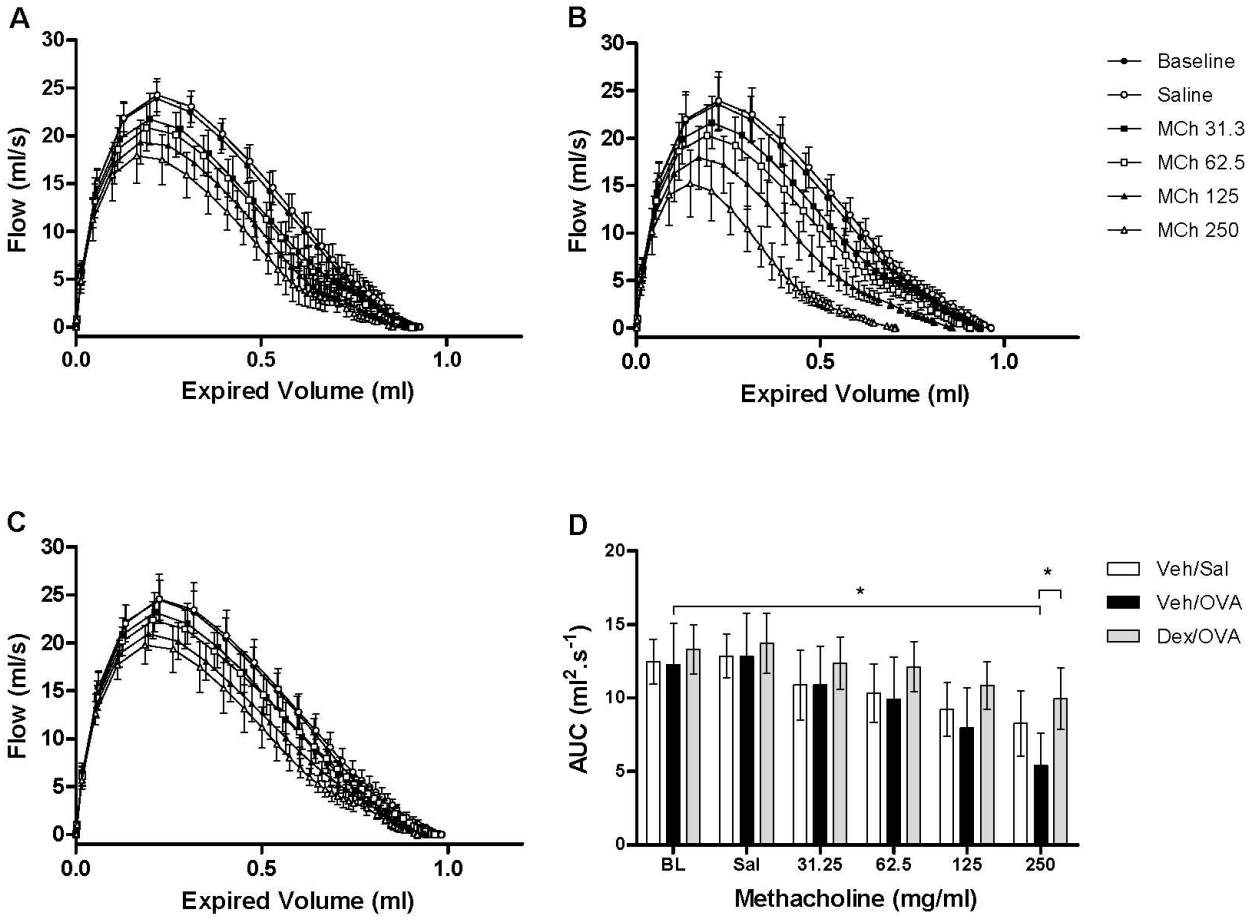

Figure 7 Flow-volume curves following increasing aerosolized methacholine concentrations. Mean flow-volume curves (mean ± standard deviation) from vehicle-treated saline- (A; Veh/Sal) and ovalbumin- (B; Veh/OVA) challenged ovalbumin-sensitized mice as well as from dexamethasone (1 mg/kg)-treated ovalbumin-sensitized and challenged mice (C; Dex/OVA) at baseline (BL) and following aerosolized saline (Sal) or increasing methacholine concentrations (MCh 31.25-250 mg/ml). Figure 7D represents the mean and standard deviation of the area under the flow-volume curves (AUC) under the varied experimental conditions. (*$p < 0.05$; ANOVA, $n = 5$-6 mice/group).

leaves EFL as one of the remaining factors governing the flow-volume loops obtained from NPFE in mice. While DI contributes in this manner to lower variability between animals, it may influence the magnitude of the MCh-induced bronchoconstriction by opening airways immediately prior to the forced expiration, which would be expected to reduce the airway resistance [18].

In previous assessments of airway responsiveness by NPFE, manoeuvres were often performed at pre-determined times following MCh administrations [8,9,11]. In the present study, the combination with FOT allowed us to measure respiratory mechanics in real-time leading up to, and following the NPFE manoeuvre, thus avoiding added variance related to the timing of the NPFE measurement. Consequently, reproducible flow-volume curves with relatively small within-group variability were obtained, compared to what has been previously reported [7,11], despite the small group sizes and single NPFE manoeuvres that were used.

Using closely spaced (15 seconds apart) repeated FOT measurements to capture the physiological response to the inhaled MCh challenge, Rrs was used to select the moment at which the NPFE manoeuvre was performed. However, in addition to the ability to follow the time-course of the bronchoconstrictor response, FOT also offers the possibility to distinguish between central and peripheral respiratory mechanics. The mathematical models used in the analysis of FOT data, specifically the constant-phase model [13], can provide valuable information pertaining to the heterogeneity of the respiratory response and whether it is dominated by resistance of the conducting airways, peripheral airway closure or tissue resistance [19]. Ultimately, any FOT parameter could serve as a guide to refine the experimental design.

The combination of both techniques in a single setup also allowed us to study the impact of NPFE on respiratory mechanics and to investigate the underlying mechanisms. Our data indicated that the NPFE manoeuvre

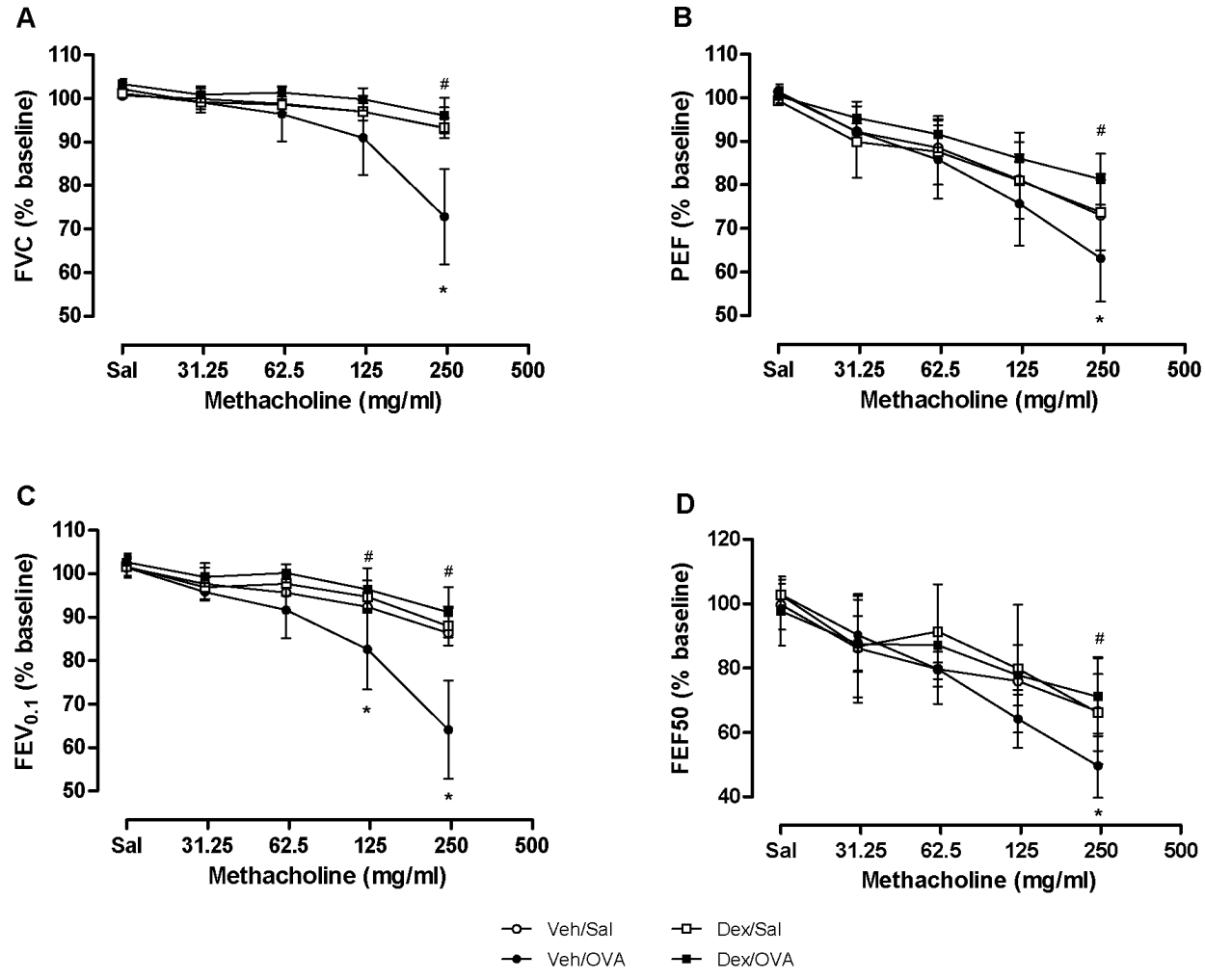

Figure 8 Normalized forced expiratory parameters. Forced expiration parameters normalized to baseline values at each concentration of aerosolized methacholine in ovalbumin-challenged (OVA) and sham-challenged (Sal) ovalbumin-sensitized mice that were either vehicle (Veh)- or dexamethasone (Dex)-treated. Values were normalized to individual baseline and expressed as mean ± standard deviation for each group (n = 4-6 mice/group, each mouse studied once). (*$p < 0.05$ Veh/OVA vs Veh/Sal; #$p < 0.05$ Veh/OVA vs Dex/OVA; ANOVA).

itself affected the respiratory mechanics (Figure 3). Namely, it caused a significant increase in all FOT parameters, except R_N. The proportional increases in G and H suggest that the NPFE manoeuvre causes a uniform derecruitment of peripheral lung units [20]. R_N represents the resistance of the conducting airways, which is dominated by the larger proximal airways. Therefore, this finding suggests that the loss of lung units is restricted to the periphery, possibly caused by small airway closure or alveolar collapse. It is interesting to note that while R_N was not altered following a NPFE manoeuvre, Rrs was. Since Rrs is still commonly interpreted as a surrogate of airway resistance, it is worth pointing out that our finding that Rrs is altered under these circumstances confirms that this parameter is also coupled to the resistive properties of the lung tissues and that therefore it does not solely reflect airway resistance.

The sustained airway closure caused by NPFE was reversible by deep inflation. Thus, for the assessment of MCh responsiveness, this limitation of the technique was addressed by performing DI following each NPFE manoeuvre to ensure automated and reproducible lung recruitment. However, the post-NPFE DI interfered with the ability to perform closely spaced repeated NPFE measurements, to measure cumulative bronchoconstrictor dose-responses to MCh using NPFE or to follow by FOT the course of the bronchoconstrictive response after a NPFE manoeuvre.

Expiratory flow limitation is a major nonlinear effect in the lungs that may play an important role in many disease models. In the current study, the NPFE data mostly mirrored the FOT data and provided no complementary information. However, Vanoirbeek *et al.* [11] recently reported EFL at baseline in an emphysematous mouse

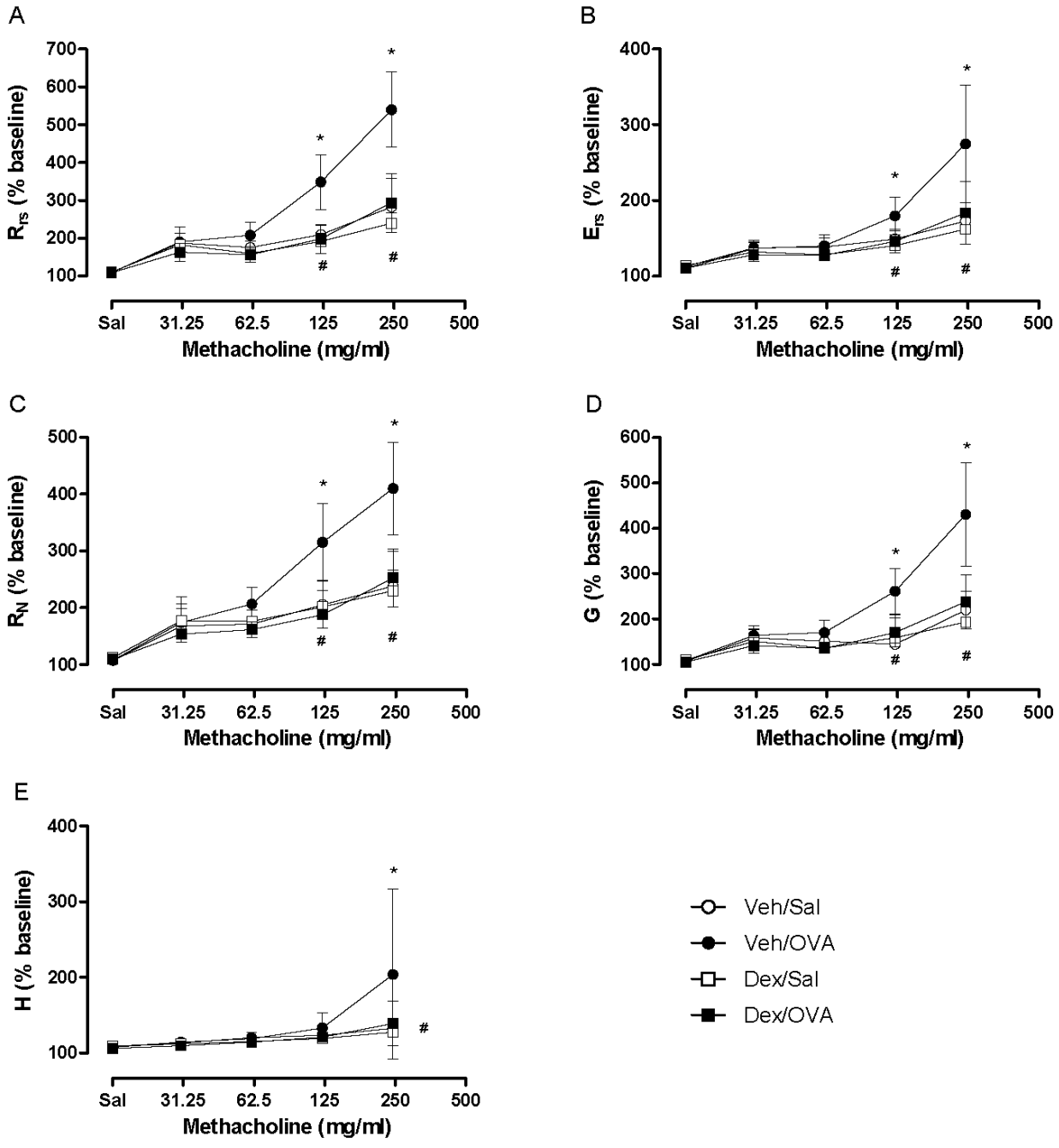

Figure 9 Assessment of drug effect via normalized parameters of the forced oscillation technique. Forced oscillation technique parameters normalized to baseline values at each concentration of aerosolized methacholine in vehicle treated- (Veh/OVA; closed circles) and dexamethasone treated- (Dex/OVA; closed squares) ovalbumin sensitized and challenged mice. Values were normalized to individual baseline and expressed as mean ± standard deviation. (*$p < 0.05$ Veh/OVA vs Veh/Sal; #$p < 0.05$ Veh/OVA vs Dex/OVA; ANOVA; n = 5-7 mice/group).

model, indicating that EFL assessment may prove valuable for other protocols and disease models. It is worth mentioning that the pattern of methacholine responsiveness observed in the present study differed from previous reports where a dominant peripheral lung response was noted following challenge in a similar model of allergen-induced airway hyperresponsiveness [14,19,21]. While previous studies also employed the forced oscillation technique to assess airway responsiveness, different nebulizers and nebulisation patterns were used in addition to variations in the ventilation circuitry around the nebulizer. These variations may account for the discrepancies

as the intra-pulmonary dose of methacholine and/or its site of deposition could have been influenced.

Finally, although the extracted NPFE parameters in mice resemble those obtained in humans, the abovementioned differences in how these measurements are obtained in both species are sufficiently important that caution should be applied when directly comparing their outcomes, limitations or shortcomings until the validity of such comparisons has been established.

Conclusions

In summary, we obtained concurrent FOT and NPFE measurements from the same cohort of mice using an extended *flexiVent* system that combined both techniques with the aim of assessing allergen-induced airway hyperresponsiveness as well as post-NPFE respiratory mechanics. The allergen-induced changes in lung function and their prevention by dexamethasone were detected by parameters of both techniques. Although in the context of the current protocol, NPFE provided no complementary information over and above FOT, NPFE as a method to assess EFL ultimately may complement FOT. Studying the mechanisms of NPFE-induced changes in respiratory mechanics broadened our understanding of the manoeuvre and allowed us to improve the way measurements were performed in order to get meaningful results. The combination of the two techniques represents an experimental design refinement applicable to a variety of respiratory disease models.

Abbreviations

ANOVA: analysis of variance; DI deep lung inflation; EFL: expiratory flow limitation; Ers: respiratory system elastance; FEF50: forced expiratory flow at 50% of forced vital capacity; $FEV_{0.1}$: forced expired volume over 0.1 second; FVC: forced vital capacity; FOT: forced oscillation technique; G: tissue damping; H: tissue elastance; LFOT: broadband low frequency forced oscillation technique; ip: intraperitoneal; MCh: methacholine; NPFE: negative pressure-driven forced expiration; OVA: ovalbumin; PEEP: positive end expiratory pressure; R_N: Newtonian resistance; Rrs: respiratory system resistance; s: second; SFOT: single-frequency forced oscillation technique.

Competing interests

LGG, TFS and AR are employed by SCIREQ Scientific Respiratory Equipment Inc. TFS also owns stock. KHS and JGM declare that they have no competing interests.

Authors' contributions

KHS participated in the design of the study, the data acquisition and interpretation, drafted the manuscript and revised it critically for scientific content. LGG participated in the conception of the extended *flexiVent* system functionalities, data acquisition, analysis and interpretation, and revised the manuscript critically for scientific content. TFS conceived the extended *flexiVent* system, designed part of the study, participated in the data acquisition, performed the NPFE signal analyses and participated in result interpretation, manuscript writing and critical revision. JGM participated in the study design, the interpretation of results, manuscript writing and critical revision. AR designed part of the study, participated in the data acquisition, analysis and interpretation, and took part in manuscript writing and critical revision. All authors read and approved the final manuscript.

Acknowledgements

The study was funded by the JT Costello Memorial Fund, CIHR and SCIREQ Inc. The authors wish to thank Mr. Patrick Lefebvre for calculating the mechanical cut-off frequency of the plethysmograph chamber.

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Received: 5 January 2010 Accepted: 21 June 2010
Published: 21 June 2010

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doi: 10.1186/1465-9921-11-82
Cite this article as: Shalaby *et al.*, Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness *Respiratory Research* 2010, **11**:82

Statement of Originality

This thesis contains original experimental approaches, findings and hypotheses described below. In Chapter 2, we examined the role of oxidative stress in the sensitization process to a natural aeroallergen, the birch tree pollen, occurring through the airway mucosa and determined that oxidative stress did not mediate all aspects of sensitization to this allergen, such as those leading to airway hyperresponsiveness, a cardinal feature of asthma. To our knowledge, we are the first to examine the role of oxidative stress in such a context and to differentiate between the sensitization and secondary inflammatory phases of the allergic response using such a model of repeated allergen inhalation without adjuvant. We also demonstrated that the pollen-intrinsic NADPH oxidase activity did not have a significant impact upon either allergic sensitization or amplification of the secondary inflammatory response, unlike reports that have described it to be important in relation to another type of pollen. Furthermore, we determined that although the toll-like receptor 4 (TLR4) contributed to airway inflammation it did not appear to influence the processes relevant to AHR in this model, indicating that the significance of TLR4 to the development of allergic airway disease is also likely dependent on the specific allergen in question. To our knowledge, we additionally examined for the first time the role of the TRIF-dependent signaling pathway downstream of this receptor in the context of airway disease caused by allergen inhalation and determined that the functional deletion of this pathway augmented allergen-induced airway inflammation.

In Chapter 3, we demonstrated that the application of an adjuvant consisting of TLR2/4 ligands in a manner that was restricted to the upper airways of pre-sensitized animals was capable of inhibiting subsequent allergen-induced lower airway disease, without eliciting collateral and potentially dangerous airway inflammation as is often observed with intrapulmonary administration of such ligands. This finding may have important therapeutic implications. Moreover, the suppression of airway disease development was shown to be TLR4-dependent and TLR2-independent and was associated with an induction in the expression of a

key T cell co-stimulatory molecule, the inducible co-stimulatory molecule (ICOS) in the nasal mucosa, as well as expansion of ICOS-expressing $CD4^+$ T cells in the draining lymph nodes and potential migration to the lungs. The induction of ICOS and expansion of these cells was also TLR4-dependent and adoptive transfer experiments suggested that ICOS-expressing cells in the naso-pharynx-draining cervical lymph nodes are inherently capable of suppressing allergic airway disease.

In Chapter 4, we examined the role of the primary TLR4 adaptor molecules, MyD88 and TRIF, in mediating the observed effects of this adjuvant and determined that its potential to inhibit the development of allergic airway disease was largely, if not completely, TRIF-dependent and MyD88-independent. Also, various aspects of the induction and migration of ICOS-expressing $CD4^+$ T cell subsets were found to be impaired in the absence of TRIF signaling. Novel adoptive transfer experiments supported that the induction and perhaps migration of ICOS in $CD4^+$ T cells *in vivo* contributed to the TLR4-TRIF-dependent inhibition of allergic airway disease by this adjuvant and were likely mediated by discrete TRIF-dependent signals. We also demonstrated *in vitro* that the capacity to induce ICOS in $CD4^+$ T cells is more broadly applicable to TLR4 ligands and thus, may be an important aspect of the potent adjuvant activity of such ligands upon T cell responses.

Our demonstration that the TLR4-TRIF pathway can protect against the development of allergic airway disease in the context of inhalational exposure to allergen, as well as adjuvant-mediated suppression of allergic airway responses, is the primary novel finding presented in this thesis and advances current knowledge pertaining to the role of TLR4 activation and signaling mechanisms in allergic airway disease. The concluding discussion in Chapter 5 also contains novel intellectual contribution in the synthesis of the current state of knowledge regarding the topic of TLR4-dependent mechanisms in allergic airway disease and hypothesis of the significance of the TRIF pathway within this framework.

Finally, the findings presented in the Appendix provide a technological advancement in existing methodologies employed to measure lung function in small animals, which may also enhance current capacities to translate animal models of human lung disease.