The genetic control of airway responsiveness and the effect of resiquimod treatment on allergic asthma

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# List of Abbreviations

- ADHD: Attention Deficit Hyperactivity Disorder
- BHR: Bronchial Hyperreactivity
- COPD: Chronic Obstructive Pulmonary Disease
- ERS: External Resonant System
- FEV1: Forced Expiratory Volume in 1 second
- FENO: Fractional Exhaled Nitric Oxide
- LTL: Long-Term Treatment
- OVA: Ovalbumin
- PEF: Peak Expiratory Flow
- OR: Odds Ratio
- SPT: Skin Prick Test
- T2D: Type 2 Diabetes
- VAD: Ventilation Assist Device

## Preface and Contribution of Authors

This section provides an introduction to the work presented in the document, including contributions from various authors.

## Abstract

A brief summary of the document's main findings and conclusions.

## Résumé

A French translation of the abstract.

## General Introduction

Overview of the topic and its significance.

## Chapter 1 - Literature Review

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2. Role of Toll-Like Receptors (TLR) Signalling in the Pathogenesis of Asthma
3. Future Directions

## Acknowledgements

Gratitude to all contributors and supporters.

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<td>A/A/J</td>
<td>A disintegrin and metalloprotease 33</td>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease 33</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>AR</td>
<td>Airway responsiveness</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>B</td>
<td>C57BL/6</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>BALF</td>
<td>BAL fluid</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<tr>
<td>CAMS</td>
<td>Cell adhesion molecules</td>
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<tr>
<td>CCAC</td>
<td>Canadian council on animal care</td>
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<tr>
<td>Ccl</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CIHR</td>
<td>Canadian institutes of health research</td>
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<tr>
<td>CpG</td>
<td>Unmethylated CG DNA</td>
</tr>
<tr>
<td>CR</td>
<td>Congo red</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<tr>
<td>DPP10</td>
<td>Dipeptidyl-peptidase 10</td>
</tr>
<tr>
<td>DSO</td>
<td>Donor strain of origin</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>Ers</td>
<td>Respiratory system elastance</td>
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<tr>
<td>FcεR</td>
<td>Fc receptor, IgE</td>
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<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPRA</td>
<td>G protein-coupled receptor for asthma susceptibility</td>
</tr>
<tr>
<td>GXD</td>
<td>Gene expression database</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>IBD</td>
<td>Identical by descent</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroids</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Klr</td>
<td>Killer cell lectin-like receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>----------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MAL</td>
<td>Myelin and lymphocyte protein, T-cell differentiation protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Mapkapk2</td>
<td>Mitogen activated protein kinase activated protein kinase 2</td>
</tr>
<tr>
<td>MGI</td>
<td>Mouse Genome Informatics</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MK2</td>
<td><em>Mapkapk2</em></td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Myd88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFXB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKCMC</td>
<td>NK cell mediated cytotoxicity</td>
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<tr>
<td>Nrramp1</td>
<td>Natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>NSERC</td>
<td>Natural Sciences and Engineering Council</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
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<tr>
<td>Pbm</td>
<td>Perimeter of the basement membrane</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>Penh</td>
<td>Enhanced pause</td>
</tr>
<tr>
<td>PHF11</td>
<td>PHD finger protein 11</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RCS</td>
<td>Recombinant congenic strain</td>
</tr>
<tr>
<td>RES</td>
<td>Resiquimod</td>
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<tr>
<td>Rs</td>
<td>Respiratory system resistance</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase – polymerase chain reaction</td>
</tr>
<tr>
<td>S28</td>
<td>Resiquimod, R-848</td>
</tr>
<tr>
<td>S28463</td>
<td>Resiquimod, R-848</td>
</tr>
<tr>
<td>SAV</td>
<td>Small animal ventilator</td>
</tr>
<tr>
<td>Slc11a1</td>
<td>Solute carrier family 11, member 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPAR</td>
<td>Sandler Program for Asthma Research</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;1</td>
<td>Type 1 T helper cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;2</td>
<td>Type 2 T helper cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;3</td>
<td>Type 3 T helper cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;17</td>
<td>Type 17 T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;R&lt;/sub&gt;1</td>
<td>Type 1 T regulatory cell</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
</tr>
<tr>
<td>Trif</td>
<td>TIR domain containing adaptor inducing interferon-beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;Reg&lt;/sub&gt;</td>
<td>Regulatory T cell</td>
</tr>
</tbody>
</table>
Preface and Contribution of Authors

As described in the *Thesis Preparation Guidelines*, the author will present a manuscript-based thesis. The work described in this thesis was performed under the supervision of Dr. Danuta Radzioch at the Research Institute of the McGill University Health Center.

The literature review presented in chapter 1 is adapted and updated from an article published in *Current Pharmaceutical Design* [1]. The thesis author wrote and performed the literature search for the bulk of the manuscript. Jacques Moisan contributed to the section on Toll-like receptor ligands and signalling, Jill Hénault contributed the illustration, and all authors contributed to the editing and drafting of the manuscript.

The work presented in chapter 2 was published in the *American Journal of Physiology – Lung Cellular and Molecular Physiology* [2]. The thesis author, as well as Jacques Moisan, participated in all experimental design, experiments, and data analysis. As well the thesis author wrote a significant portion of the manuscript. T. Thuraisingam participated in experiments involving MK2 knock-out mice. H. Koohsari was responsible for the measurements of airway responsiveness. D. Marion and M.L. Boghdady participated in the histological analysis of collected samples. All other authors participated in experimental design and the editing of the manuscript.

The work presented in chapter 3 was published in the *American Journal of Respiratory and Critical Care Medicine* [3]. The thesis author was involved with all aspects of experimental design, the processing of all samples, most of the analysis, and wrote the manuscript. Meiyo Tamaoka generated the rat model of chronic asthma and was responsible for the sensitization, challenge, euthanasia of the animals and sample collection. Mohanad Hassan performed the measurements of airway smooth muscle mass and PCNA\(^+\) cells. All other authors...
contributed to experimental design, some sample manipulations, and/or editing of the manuscript.

At the time of the writing of this thesis, the work presented in chapter 4 was accepted for publication, and in press, in *Physiological Genomics*. The thesis author performed all experiments except the RT-PCR gene expression validation (which was performed by C. Kanagaratham) and microarray hybridizations (performed by the McGill University and Genome Quebec Innovation Center), as well as all the analysis presented in the chapter. The thesis author also wrote the manuscript. All authors contributed to the experimental and analytical design as well as edited the manuscript.

At the time of the writing of this thesis, the data presented in chapter 5 has been prepared for submission to a peer-reviewed journal. The thesis author collected most of the phenotyping data, performed all the statistical analyses, and wrote the manuscript. R. Marino assisted with the phenotyping, and all other authors participated in the experimental design and interpretation of results.
Abstract

Asthma is a heterogeneous airway disease caused by a mixture of genetic and environmental factors which result in improper immune responses to innocuous antigens. Toll-like receptors (TLR) are pathogen associated pattern recognition receptors which form homo- or heterodimers which bind specific ligands leading to activation and modulation of immune responses. The present study examined the effect of resiquimod, a synthetic toll-like receptor 7 ligand, on the development of allergic asthma pathology in animal models. Resiquimod treatment of ovalbumin sensitized mice prevented the subsequent development of airway hyperresponsiveness and inflammation, increased plasma IgE levels, and both Th1 and Th2 cytokine production. This effect was independent of the Mapkapk2 gene but was ineffective in Myd88 knockout mice.

A defining feature of asthmatic airways is airway wall remodelling which is characterized by an increase in airway smooth muscle mass, goblet cell hyperplasia, and the deposition of extra-cellular matrix components. The effects of resiquimod treatment on the development of airway remodelling were examined in Brown Norway rats. Resiquimod treatment prevented the increase in airway smooth muscle mass and goblet cell hyperplasia observed in control animals. These effects were associated with a reduction in the number of proliferating airway cells and were preceded by an abrogation of the allergic inflammatory reaction.

Employing gene expression microarray analysis, the transcriptome of resiquimod treated, and untreated asthmatic A/J and C57BL/6 mice, was characterized. Asthma induction resulted in the up-regulation of genes involved with the control of cell cycle progression, the complement and coagulation cascades, and chemokine signalling, findings which are consistent with previous reports. Treatment with resiquimod resulted in the normalization of asthma induced genes related to airway remodelling and chemokine signalling.
Additionally, treatment resulted in the induction of cell adhesion genes, and genes involved in natural killer (NK) cell-mediated cytotoxicity. Furthermore, NK cell recruitment to the lungs and livers of resiquimod treated mice was demonstrated, though treatment efficacy was not dependent on these cells.

The difference in asthma susceptibility between A/J and C57BL/6 mice was further explored at the genetic level. Specifically, airway responsiveness, a predisposing factor for the development of asthma in humans, was assessed using a panel of 33 recombinant congenic strains of mice derived from A/J and C57BL/6 parental strains. A genotype-phenotype association analysis was then performed and identified 16 chromosomal regions as significantly associated with airway responsiveness. Of these 16 regions, 8 are novel while the remainder have previously been linked with airway responsiveness. Several likely candidates have been identified from these 16 regions, but further study will be required in order to determine if these genes have any causal relationship with airway responsiveness.

Overall, the data presented in this thesis demonstrate and characterize the protective effect of resiquimod treatment against both the acute and chronic pathological changes associated with the development of asthma. Furthermore, genetic factors which are associated with a predisposition to the development of asthma and with asthma pathology have been described at the genetic and transcriptional levels, respectively. Taken together, these findings further our understanding of the molecular basis of asthma pathology and will aid in the development of new therapeutic strategies.
Résumé

L’asthme est une maladie des voies respiratoires causée par une combinaison de facteurs génétiques et environnementaux qui entraîne une réponse immunitaire inappropriate contre des antigènes bénins. Les TLRs (récepteurs ressemblant à Toll) sont des récepteurs qui reconnaissent des motifs dérivés de pathogènes qui forment des homo- ou hétérodimères se liant à des ligands spécifiques qui amènent à l’activation et la modulation de diverses réponses immunitaires. La présente étude a examiné l’effet du composé resiquimod, un ligand synthétique de TLR-7, dans le développement pathologique de l’asthme allergique dans des modèles animaux. Chez les souris sensibilisées avec l’ovalbumine, le traitement avec resiquimod a prévenu le développement subséquent de l’hyperréactivité et l’inflammation des voies aériennes, l’augmentation des niveaux d’IgE, ainsi que la production de cytokines de type T_h1 et T_h2. Cet effet est indépendant du gène Mapkapk2 mais requiert la présence du gène Myd88.

Une caractéristique des voies aériennes asthmatiques est le remodelage de la paroi des voies respiratoires; ceci inclut une augmentation de la masse des muscles lisses, l’hyperplasie des cellules caliciformes, et le dépôt des composantes de la matrice extracellulaire. Les effets du traitement avec resiquimod sur le remodelage des voies respiratoires ont été examinés chez les rats brun norvégien. Ce traitement a prévenu l’augmentation de la masse des muscles lisses des voies respiratoires ainsi que l’hyperplasie des cellules caliciformes chez les témoins. Ces effets étaient associés avec une diminution de la prolifération des cellules des voies respiratoires qui était précédée par l’élimination de la réaction inflammatoire allergique.

Le transcriptome de souris A/J et C57BL/6 traité et non-traité avec resiquimod fut analysé en utilisant des puces à ADN pour l’analyse de l’expression des gènes. Le déclenchement de l’asthme a provoqué l’induction de gènes impliqués dans le
contrôle de la progression du cycle cellulaire, la cascade du complément et de la coagulation, et la signalisation des chimioxines. Ces résultats sont conformes avec les études antérieures. Le traitement avec resiquimod a entraîné la normalisation des gènes induits par l’asthme liés au remodelage des voies respiratoires et la signalisation des voies chimioxines. Par ailleurs, le traitement a abouti à l’induction de gènes reliés à l’adhérence cellulaire et des gènes impliqués dans la cytotoxicité médiée par les cellules tueuses naturelles (NK). De plus, le recrutement des cellules NK dans les poumons et le foie a été démontré chez des souris traitées avec resiquimod. Cependant, l’efficacité du traitement n’était pas dépendante des cellules NK.

La différence dans la susceptibilité de l’asthme entre les souris A/J et C57BL/6 fut exploré davantage au niveau génétique. Plus spécifiquement, la réactivité des voies respiratoires, un facteur prédisposant pour le développement de l’asthme chez les humains, fut évalué à l’aide d’un panneau de 33 souches de souris congéniques recombinantes qui était dérivé de souris parentales A/J et C57BL/6. Une analyse de l’association entre le génotype et le phénotype fut ensuite effectuée et 16 régions chromosomiques qui sont associées de façon significative à la réactivité des voies respiratoires ont été définies. De ces 16 régions, 8 sont nouvelles alors que les autres ont déjà été lié à la réactivité des voies respiratoires. De ces 16 régions, plusieurs candidats potentiels furent identifiés. Cependant, des recherches additionnelles seront requises afin de déterminer si ces gènes ont une relation de cause à effet avec la réactivité des voies respiratoires.

En conclusion, les données présentées dans cette thèse démontrent et caractérisent l’effet protecteur apporté par le traitement avec resiquimod contre les changements pathologiques associés à l’asthme chronique et aigu. Par ailleurs, les facteurs génétiques qui sont associés à une prédisposition du développement de l’asthme et de la pathologie de l’asthme ont été décrits au niveau génétique et transcriptionnel, de façon respective. Prises ensemble, ces
découvertes avancent notre compréhension au point de vue moléculaire de la pathologie de l’asthme et aideront au développement de nouvelles stratégies thérapeutiques.
General Introduction

Asthma is a common disease, often appearing in childhood, whose prevalence is increasing, and which results in significant morbidity and health care costs [4-7]. The disease is characterized by intermittent episodes of reversible airway obstruction whose pathology is caused by airway inflammation and characterized by airway smooth muscle and goblet cell hyperplasia, excessive mucous production, and hyperresponsiveness to bronchoconstricting stimuli [8;9]. In allergic asthma, the airway inflammation is caused by repeated airway exposure to allergens which trigger IgE dependent signalling resulting in the recruitment of monocytes, lymphocytes, and eosinophils as well as the production of Th2 cytokines. These cells and cytokines then promote isotype switching to, and production of, IgE, which leads to further allergen sensitization and amplifies the allergic response.

Current asthma therapy involves the use of short- and long-acting β2 agonists, bronchodilators which relieve the airway obstruction symptoms, as well as inhaled corticosteroids (ICS) to reduce airway inflammation [10]. While ICS are effective in preventing further inflammation, they have several potential side effects and do not resolve the allergic reaction which is the root cause of disease.

Allergic reactions are mediated by the Th2 branch of the adaptive immune system. As described in greater detail in chapter 1, the mediators of Th2 type immunity can be repressed by Th1 and Th17 mediators. Toll-like receptors (TLR) are a family of pathogen associated molecular pattern receptors which play an important role in the initial activation of immune responses [11;12]. Signalling through these receptors can in fact polarize the adaptive immune response when triggered by different types of pathogens. Therefore, TLR which recognize viral molecular patterns, such as double stranded RNA (TLR3) or single stranded RNA of viral origin (TLR7/8), can induce a Th1 response which could suppress the Th2 mediators which result in the allergic inflammation associated with asthma.
The second chapter of this thesis describes the effects of resiquimod treatment on the development of asthma pathology in a standard mouse model of acute ovalbumin induced allergic asthma. Resiquimod is a TLR7 ligand (TLR7/8 ligand in humans) which had previously been demonstrated to induce T_{H}1 biased immune responses [13;14]. Our data demonstrates that resiquimod treatment of antigen sensitized animals prior to airway antigen exposure prevents the development of pulmonary inflammation and antigen induced airway hyperresponsiveness while the levels of both T_{H}1 and T_{H}2 cytokines, as well as total plasma IgE, were also repressed following antigen challenge in treated animals when compared to untreated positive controls.

The products of several genes modulate or are involved in the signalling pathways which lead to the biological effects of resiquimod treatment. One of these is \textit{Slc11a1} (Nramp1), a gene which encodes a macrophage specific gene involved with host resistance and affects the activation of protein kinase C zeta and p38 mitogen-activated protein kinase following resiquimod treatment [15]. The A/J and C57BL/6 mice employed in the study presented in chapter 2 differ at the \textit{Slc11a1} locus (the C57BL/6 allele renders the gene non-functional while the A/J allele is fully functional [16]) yet both strains efficiently respond to resiquimod treatment indicating either that macrophage response to resiquimod is not necessary for treatment efficacy, or that the subset of signalling molecules induced in the macrophages of both strains is sufficient. Furthermore, the efficacy of resiquimod treatment was also assessed in mice deficient for \textit{Myd88} and \textit{Mapkapk2} (MK2), both genes involved in resiquimod signalling. The data presented in chapter 2 demonstrates that the efficacy of resiquimod treatment requires a functional \textit{Myd88} gene but is independent of the MK2 gene product.

In the next chapter the effects of resiquimod treatment on the development of asthma pathology are further explored. Having demonstrated that resiquimod treatment can prevent the development of the asthma associated allergic inflammation reaction which occurs following acute airway antigen
exposure; we examined the effect of resiquimod treatment on the subsequent development of pathology associated with chronic asthma. Due to the difficulty in analyzing airway remodelling in most mouse models of chronic asthma [17], we employed a Brown Norway rat model of chronic asthma developed by our collaborators Dr. M. Tamaoka and Dr. J. Martin at the Meakins Christie Laboratories. The data presented in chapter 3 demonstrates that resiquimod treatment prevents the development of allergic inflammation in both acutely and chronically allergen challenged rats. Furthermore, resiquimod treatment prevents the development of chronic airway remodelling, as assessed by smooth muscle mass and goblet cell hyperplasia.

In chapter 4, data is presented which was obtained from expression microarrays derived from resiquimod treated and untreated A/J and C57BL/6 mice. This data allowed the identification of several molecular processes altered by both antigen exposure of sensitized animals, and by resiquimod treatment. Genes involved in cell cycle progression, complement and coagulations cascades, and chemokine signalling were induced by antigen challenge in both strains, as well as in other expression microarray studies employing C57BL/6 and BALB/c mice. More extensive transcriptional changes were observed in resiquimod treated mice. As well as normalizing the expression of asthma induced genes involved in airway remodelling and chemokine signalling, resiquimod treatment induced many genes involved with various immunological processes. In particular, many genes involved in NK cell function were up-regulated following resiquimod treatment and the data presented in chapter 4 demonstrates that this was accompanied by a significant pulmonary and hepatic NK cell recruitment.

Recombinant congenic mice, derived from the A/J and C57BL/6 strains (the same strains which were used in the previous chapters), were employed to dissect the genetic factors underlying a single aspect of asthma pathogenesis, namely, airway responsiveness. This data is presented in the final chapter and
demonstrates that there is evidence that at least 16 loci underlie the difference in airway responsiveness observed between the A/J and C57BL/6 strains. While there is prior evidence supporting a role in the control of airway responsiveness for 8 of the 16 chromosomal regions we identified, the association of airway responsiveness to the remaining 8 regions was never previously reported. Furthermore, several likely candidate genes were identified from these regions based on published expression and sequence variance data.

Overall the studies presented in this thesis demonstrate the protective effects of treatment with resiquimod, a Toll-like receptor 7 ligand, against the development of both acute and chronic asthma pathology. While we have established the role of certain molecules that are involved in mediating the effects of resiquimod, and characterized the molecular and cellular consequences of treatment; further studies aimed at dissecting the mechanism of action of resiquimod treatment have the potential to broaden our understanding of the development of asthma as well as identify novel targets for therapeutic intervention. Furthermore we have presented data which describes a reproducible set of genes whose expression is altered following the onset of asthma pathology as well as identified several chromosomal regions which contain genes that control susceptibility of airway responsiveness, an important phenotype of asthma, in inbred mice. These findings have the potential to lead to a better understanding of the natural history of asthma and the further identification of new therapeutic approaches.
Chapter 1 - Literature Review

Adapted from:


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Abstract

Asthma is a complex disease caused by a poorly characterized set of genetic and environmental factors whose pathology is a result of immune dysregulation. Toll-like receptors are pathogen associated molecular pattern receptors expressed by many airway and pulmonary tissues as well as cells of the innate and adaptive immune system. Ligation of toll-like receptors can lead to a change in the expression levels of multiple inflammatory and anti-inflammatory mediators which are involved, or are thought to be involved, in the pathogenesis of asthma. These ligands and their receptors are therefore prime candidates in the search for immunotherapeutic treatments of asthma. The use of murine models of allergic asthma as tools for the genetic dissection of this disease should allow the molecular mechanisms underlying asthma to be identified and possibly used as further immunotherapeutic targets.
**1. Epidemiology and Pathogenesis of Asthma**

Asthma is a common disease, which frequently appears in childhood, affects millions of people worldwide, and is a leading cause of hospitalization of children in westernized countries. While asthma remains more common in industrialized and urbanized nations, its incidence has increased around the world over the last 20 years [4-7]. Asthma is characterized by episodes of intermittent reversible airway obstruction, excessive airway mucous production, airway smooth muscle hyperplasia and chronic inflammation of the bronchi involving infiltration of the submucosa by neutrophils, monocytes and eosinophils [8]. There is convincing evidence that the development and progression of asthma depends on the expression of several genes interacting with multiple environmental factors. Airway hyperresponsiveness (AHR) and atopy represent two important genetically-regulated phenotypes characteristic of asthma.

**1.1. Airway Hyperresponsiveness (AHR)**

Airway responsiveness is the ability of the airways to respond to bronchoconstricting stimuli by reducing their diameter. AHR is the term used to describe overly forceful airway responses to direct and indirect bronchoconstricting stimuli. Direct stimuli induce a response by ligating airway smooth muscle cell receptors [18] and include histamine, cholinergic agonists such as acetylcholine and methacholine, prostaglandin D₂ [19] and F₂ [20], as well as leukotrienes C₄ and D₄ [21]. Indirect stimuli elicit a response by stimulating the release of bronchoconstrictors from airway cells [18] and include exercise [22], cold air [23], distilled water, adenosine monophosphate [24], pollutants and allergens [25].

AHR in response to direct stimuli has long been known to be a risk factor for the development of asthma [26]. In fact, it has been demonstrated that AHR in response to histamine, in infants as young as 4 weeks old, is significantly associated with the development of asthma by age 6 [27]. It is well documented that children with AHR are more likely to develop asthma than children with low
airway responsiveness [28]. Furthermore, there are several lines of evidence which indicate that AHR is a heritable trait in both man and mouse. For over a decade, AHR has been known to precede the development of asthma [29] and for many years it has been known that nonasthmatic parents of asthmatic children have higher levels of AHR than nonasthmatic adults with no familial history of asthma [30;31]. More recently, several studies demonstrated genetic linkage of specific loci and AHR in response to histamine and methacholine [32;33]. Taken together, these findings demonstrate that AHR is genetically determined and often precedes the development of asthma. In mice, clear evidence of the heritability of AHR comes from several groups who have compared the AHR of various inbred strains of mice [34;35].

However, it is important to keep in mind that, in humans and in murine models of asthma, AHR is also affected by environmental factors. For example, in virtually all mouse models of allergic asthma, an increase in airway reactivity is observed after the animal has been antigen-sensitized and challenged [36], and allergen challenge in humans similarly leads to an increase in AHR [37]. It has been hypothesized that the increase in AHR seen in asthmatics results from structural changes of the large and small airways, and that these structural changes are a result of acute-on-chronic inflammation caused by repeated exposure to allergens or agents which induce inflammation [18].

1.2. Atopy and Adaptive Immunity

Atopy is a predisposition of individuals to produce an immunoglobulin E (IgE) antibody-mediated response to common, innocuous antigens. Like AHR, there is strong evidence that atopy is in large part genetically determined in both human subjects and murine models of allergic disease [38-41]. Atopy, and its markers, are associated with a heightened risk of developing asthma in man [42;43].
1.2.1. Effector T-Cells

A defining characteristic of atopy is the shift in the T helper cell balance in favour of T\textsubscript{H}2 cells and mediators favouring IgE production. CD4\textsuperscript{+} T cells can be divided into several separate subpopulations, including T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, and regulatory T cells, based on their cytokine production profiles [44]. The different cytokine production profiles of the CD4+ T cell subpopulations allow each to exhibit distinct functional properties. Furthermore, the cytokines produced by the T\textsubscript{H}1, T\textsubscript{H}2, and T\textsubscript{H}17 subpopulations promote the growth and function of that T helper cell subpopulation while antagonizing the growth and function of the others [45]. Thus, T\textsubscript{H}1 cytokines promote T\textsubscript{H}1 cell growth and function while inhibiting T\textsubscript{H}2 and T\textsubscript{H}17 cell growth and function, and vice versa for each of the other helper T helper cell populations.

T\textsubscript{H}1 cells are known to produce interferon-\gamma (IFN-\gamma) and interleukin (IL)-2, and they are principally involved in fighting intracellular pathogens such as viruses and certain bacteria. Defence against intracellular pathogens is achieved by providing help to B cells which leads to isotype switching favouring opsonizing antibodies, and by activating macrophages to kill bacteria, such as Mycobacterium tuberculosis, efficiently. T\textsubscript{H}1 mediated immune responses commonly involve a strong inflammatory reaction that may lead to tissue damage and destruction (reviewed in [46]).

T\textsubscript{H}2 cells characteristically produce IL-4, IL-5, IL-6, IL-10 and IL-13 and they are principally involved in defence against helminths. T\textsubscript{H}2 cells also provide B cell help, but they induce isotype switching to a different set of isotypes, including IgE, which is partially mediated by IL-4. In addition, several T\textsubscript{H}2 cytokines, such as IL-10, have strong anti-inflammatory properties while IL-13 is necessary for the development of AHR, goblet cell hyperplasia, and possibly subepithelial fibrosis (reviewed in [47]). Finally, IL-5 is a potent growth and differentiation factor for eosinophils (reviewed in [46]). Because of their ability to promote IgE
production and eosinophilic inflammation \(T_h2\) cell mediated immunity underlies atopic disorders including allergic asthma.

More recently, \(T_h17\) cells which characteristically produce IL-6, IL-17, IL-17F, IL-22, and TNF-\(\alpha\) have been described. The cytokines produced by \(T_h17\) cells are strongly pro-inflammatory and promote neutrophil recruitment (reviewed in [48]). While \(T_h17\) mediated inflammation does not appear to contribute to the \(T_h2\) driven eosinophilic inflammation observed in typical asthmatics, there is evidence that inflammatory mediators produced or induced by \(T_h17\) cells contribute to disease severity and progression in a subset of severe asthmatics with steroid resistant neutrophilic airway inflammation asthma (reviewed in [49]).

**1.2.2. Regulatory T Cells**

In healthy individuals, inappropriate inflammatory reactions to innocuous antigens are held in check by several different CD4\(^+\) regulatory T cells which can suppress \(T_h2\) mediated reactions leading to allergy as well as \(T_h1\) and \(T_h17\) mediated reactions leading to autoimmunity [50]. \(T_h3\) cells were identified due to their ability to suppress experimental autoimmune encephalitis (EAE) in a TGF-\(\beta\) dependent manner [51]. Generated in the periphery in-vivo following the induction of oral tolerance, these cells promote IgA class switching in B cells and suppress inflammatory T cell responses in the gut mucosa. A second group of CD4\(^+\) regulatory T cells generated in the periphery are the type 1 regulatory T cells (\(T_r1\)). These cells exert their suppressive effects primarily through the production of IL-10 and have been shown to possess the ability to prevent antigen-specific immune responses and to suppress pathological immune responses in mice [52].

Unlike \(T_h3\) and \(T_r1\) regulatory T cells, natural CD4\(^+\) CD25\(^+\) Foxp3\(^+\) regulatory T cells (\(T_{Reg}\)) are generally generated in the thymus [53;54]. It is well established that these cells can inhibit effector T cell proliferation and prevent the
development of autoimmune diseases including diabetes and inflammatory bowel disease [55]. More recently, it has been demonstrated that T_{Reg} cells inversely correlate with IgE levels and eosinophilia in allergic (atopic dermatitis and bronchial asthma) patients, who, compared to healthy controls, also exhibit lower levels of CD4^+ Foxp3^+ cells [56]. Similarly, children who underwent spontaneous remission or “outgrew” a cow’s milk allergy had elevated T_{Reg} cell levels and lower ex-vivo proliferative responses to antigen stimulation. Furthermore, depletion of the CD25^+ cells resulted in a significant increase in the ex-vivo proliferative response [57].

While all these cells are believed to be capable of regulating autoimmune and allergic diseases, the potential role of CD4^+ regulatory T-cells in the pathogenesis or treatment of asthma [58-61] has not yet been fully elucidated.

### 1.3. Immunopathogenesis of Asthma

#### 1.3.1. Atopy and AHR

Several studies using human clinical samples [62-64] and experimental animal models [65-67], together, strongly implicate high levels of T_{H2} cells and their soluble products in the pathology of asthma. The first mechanism by which T_{H2} cells are thought to contribute to the pathology of allergic asthma, and in particular AHR, involves mast cell activation. In this model, T_{H2} cells produce IL-4 and provide B-cell help leading to the production of allergen-specific IgE antibodies, which subsequently bind to FcεR expressed on the surface of mast cells. In the presence of allergen, or anti-IgE antibodies, FcεR-mediated mast cell degranulation is able to induce AHR in an IL-5 and eosinophil independent manner [68]. It had been postulated that the mast cells, which secrete IL-4 following FcεR activation, were the initial source of IL-4 [69]. However, the results from reconstitution experiments using IL-4 deficient mice, in which only transferred T cells could produce IL-4, indicate that IL-4 producing T cells are sufficient to restore the ability to develop AHR [70].
The second mechanism through which Th2 cells are thought to contribute to AHR is by the recruitment of eosinophils to the airways, mediated principally by IL-5. The release of mediators, including major basic protein and IL13 [71], from the eosinophils then leads to AHR [72;73]. However, eosinophils have not universally been recognized to be required for the development of airway hyperresponsiveness or goblet cell hyperplasia [74]. The central importance of T cells in eosinophil recruitment to the airways, as well as to the development of AHR after methacholine challenge, has been demonstrated by several experiments which showed that both eosinophil recruitment and AHR were abrogated in mice whose T cells were depleted after monoclonal anti-CD4 antibody treatment [75].

While asthma pathology is strongly associated with Th2 immunity, it is clear that a large minority of asthma cases, and possibly the majority of corticosteroid resistant cases, are not associated only with eosinophilic inflammation, but rather with neutrophilic inflammation (with or without eosinophilia) [76]. Recent studies in mice have revealed that Th17 mediated immune responses characterized by the recruitment of neutrophils to the lungs are also capable of causing AHR [77]. While the role of Th17 mediated inflammatory reactions in asthma have yet to be clearly defined, it is likely that this branch of adaptive immunity also plays an important role in disease development and progression in a subset of asthmatics.

1.3.2. Hygiene Hypothesis

As mentioned above, Th1 cytokines have the ability to downregulate Th2 responses. Given that Th2 cells and their soluble mediators are known to be necessary for the development of allergic asthma pathology, it has been suggested that a relatively lower number of Th1 cells, or decreased Th1 cell activation, and a relatively enhanced number of Th2 cells, or augmented Th2 cell function, could at least partly be responsible for the development of AHR and atopy.
Asthma is not the only atopic disease in which T\(_H\)2 cells have been implicated. The fact that many allergic disorders are mediated by T\(_H\)2 cell products and that the incidence of these diseases has been increasing in westernized countries has led to the proposal that this increase is due to an imbalance between the numbers of T\(_H\)1 and T\(_H\)2 cells caused by environmental factors.

The hygiene hypothesis states that a lack of childhood exposure to infectious microorganisms will increase susceptibility to allergic diseases [78]. Since many bacteria and viruses elicit T\(_H\)1 responses, it was originally thought that insufficient stimulation of T\(_H\)1 immunity by these organisms led to insufficient T\(_H\)1-mediated downregulation of T\(_H\)2 immune responses which in turn led to allergic disease [79]. There are at least two observations which contradict this mechanistic explanation of the hygiene hypothesis. The first is that the incidence of T\(_H\)1 mediated autoimmune diseases such as multiple sclerosis (MS) [80;81], type I diabetes [82] and inflammatory bowel disease [83] have increased concurrently with an increase in T\(_H\)2-mediated allergic diseases in westernized countries. The second is that the introduction of antigen-specific T\(_H\)1 cells into an antigen sensitized and challenged mouse not only failed to reduce AHR but led to an increase in airway inflammation [84].

An alternative mechanistic explanation for the hygiene hypothesis has been suggested to address the contradictions of the original formulation. The mechanism proposed is one in which insufficient downregulation of T helper cell-mediated immune responses by (as yet unspecified) regulatory T cells leads to allergic or autoimmune disease by allowing the response to escalate out of control, and that colonization or infection by certain microorganisms can induce the development, or promote the function, of regulatory T cells [85-88]. In other words, the imbalance would occur between T\(_H\)1 or T\(_H\)2 cells and regulatory T cells; T\(_H\)1 cells and regulatory T cells in the case of autoimmunity, and T\(_H\)2 cells and regulatory T cells in the case of allergic diseases.
2. Role of Toll-Like Receptors (TLR) Signalling in the Pathogenesis of Asthma

2.1. TLR Ligands

During the past decade, the TLR family has been shown to play a crucial role in both innate and adaptive immune responses [89;90]. They have the ability to modulate the immune response through the induction of cellular activation, as well as cytokine secretion. These receptors are considered to be sensors of the extracellular environment which trigger an immune response to conserved motifs on the surface of invading pathogens, hence their classification as pattern recognition receptors (PRR). Toll-like receptors are therefore some of the first response modulators to interact with environmental immunological stimuli. The TLR family of receptors is broadly expressed in many tissues and cell types, although the exact combination expressed by any given cell can vary greatly. TLR expression in the lungs as well as in immune cells known to traffic to this organ will be discussed here because of its relevance to asthma [91].

To date, eleven TLR family members have been characterized in mammals (TLR1-TLR11). Mice express TLR1 to TLR9 as well as TLR11. Humans express TLR1 to TLR10; however, TLR11 contains a potential stop codon in the coding region that would lead to a truncated protein [92]. Most of the ligands defined for these receptors are pathogen-derived products. TLR4 is crucial for the response to lipopolysaccharides (LPS) from Gram-negative bacteria [93], and TLR2 transduces signals from lipoprotein and cell wall products of Gram-positive bacteria such as peptidoglycans and lipoarabinomannan (LAM) from mycobacteria [94;95]. TLR5, complexed to TLR4, recognizes flagellin derived from Gram-negative bacteria [96]. TLR9 has been found to be involved in the response to CpG oligodeoxynucleotides (ODN) [97]. Three categories of CpG ODN have been defined based on their backbone (phosphodiester or phosphorothioate), their sequences and the cell types mediating the response to these oligonucleotides.
Interestingly, TLR7 knockout (KO) mice were unresponsive to a class of pharmaceutical compounds belonging to the imidazoquinoline family and failed to induce cytokines in the presence of these compounds [99]. In humans, TLR8 might also play a role in fine-tuning the response to these compounds [100]. Recently, TLR7 and TLR8 have been shown to mediate the response to viral RNA sequences [101-103]. In mice, TLR11 knockout mice have been described as being susceptible to uropathogenic bacteria, which suggests a specific ligand is found within this class of bacteria [92].

MyD88 is a universal adapter protein that is essential for most of the responses transduced by Toll-like receptors [104]. The initial observation that dendritic cells could mature in response to LPS even in the absence of MyD88 suggested that other adapters were important for responsiveness to LPS. The topic of adapter proteins in TLR signalling is reviewed elsewhere [105]. Briefly, TLR4 signalling is dependent on two branches of adaptor proteins. One is the MAL/Myd88 pathway which leads to early NFκB activation. The TRAM/Trif pathway leads to both IRF3 activation and late NFκB activation. TLR2 uses MAL/MyD88 to mediate cellular activation. On the other hand, TLR7 and TLR9 immunomodulatory activity depends solely on the presence of MyD88. Correspondingly, MyD88 KO mice and the bone marrow-derived macrophages (BMM) from these mice were completely unresponsive to imidazoquinolines and CpG ODN ([99], our unpublished observations). TLR3 responses are dependent on the Trif adaptor protein. Following ligand engagement, a protein complex is assembled at the receptor and members of the IRAK kinase family, and TRAF6, are recruited and activated, which leads to NFκB activation [106]. MAPK have also been shown to be activated following TLR activation [106].

2.2. TLR and the Pathogenesis of Asthma

Most studies analyzing the expression of TLRs are based on mRNA expression analyses and only a few have corroborated mRNA expression with protein analysis. Human lungs have been shown to express most TLRs with varying
levels of expression (high levels of TLR-3, 5, 7, and 8 and low levels of TLR9 and 10) [107]. TLR1-5, as well as TLR7 and TLR8, have been shown to be expressed at relatively high levels. Primary human airway epithelial cells express TLR2-6 as well as TLR9 [108;109]. Tracheal smooth muscles have been shown to express TLR2 and TLR4, with the former mediating responses to lipoteichoic acid [110]. Alveolar macrophages have been described as being able to respond to TLR2, TLR4 and TLR9 ligands, although responses to the ligands of other receptors were not tested for [111]. An mRNA expression study indicated that the expression of various combinations of TLR1-7 and TLR9 was possible in murine macrophages [112]. Mouse neutrophils have been shown to respond to LPS through TLR4 [113] and expression studies using human neutrophils indicate that they express mRNA transcripts for TLR1, TLR2, and TLR4-10 [114].

Of particular interest in relation to asthma, human mast cells have been shown to respond to TLR2 and TLR4 ligands [115] and murine mast cells were shown to express mRNA transcripts for TLR1, 2, 4 and 6 [112]. It has also been demonstrated that human primary eosinophils respond to resiquimod (S28463, R-848), a member of the imidazoquinoline family and a TLR7 ligand, by altering adhesion molecule expression, inducing superoxide production, and prolonging survival [116]. Dendritic cells, which are involved in allergen presentation, express a wide range of TLRs (1-7 and 9) [112] and can respond to several of their ligands, including CpG ODN (TLR9 ligand) [117] and resiquimod and imiquimod (TLR7 ligands) [118].

B-cells are very important in the pathogenesis of asthma as a result of their ability to produce IgE antibodies. It has been demonstrated that both human and mouse B-cells respond to CpG ODN through TLR9 [117], and murine B-cells have been shown to respond to LPS [119]. Finally, there is evidence that several T-cell subsets express TLRs and are capable of responding to TLR ligands [112]. Human T-cells that are known to respond to TLR ligands include CD8+ T-cells,
which can respond to imiquimod through TLR7 [120], and CD4^+CD25^+ regulatory
T-cells, which can respond to LPS through TLR4 [121].

Taken together, these observations suggest that the lung constitutes an
environment where TLR ligands can modulate the responses of various cell types
and potentially alter the asthmatic phenotype.

2.3. TLR Signaling and Immunotherapy of Asthma

Dendritic cells express several TLRs (see above), are at the forefront of the
innate immune system, and play a pivotal role in activating and directing both
the innate and adaptive immune responses. In the periphery, dendritic cells are
highly endocytic and efficiently take up antigen. Upon activation, most
commonly by a pathogen component signalling through a TLR, these cells
migrate to a local lymph node and present antigen (in the context of MHC
molecules) and co-stimulatory molecules to naïve T-cells [122]. A dendritic cell’s
ability to direct the activation of T\textsubscript{H} cells to a particular subtype depends on the
types of co-stimulatory molecules expressed and the cytokines secreted. These
in turn depend on the type of dendritic cell and the microbial environment in
which the dendritic cell was activated. Specifically, myeloid dendritic cells
secrete the T\textsubscript{H}1 cytokine IL-12 in response to TLR7 or TLR9 ligation while
plasmacytoid dendritic cells secrete IFN\alpha [122]. With this in mind, several TLR
ligands have been used in immunomodulatory treatment of murine models of
asthma over the past few years in an effort to direct the immune response away
from the allergic T\textsubscript{H}2 arm of the adaptive immune system. It important to note
that, as discussed in Section 2.2, many immune and non-immune cells also
express TLRs. While dendritic cells are well characterized with respect to their
expression and response to TLR ligands, they are by no means the only cell type
that could be affected by TLR ligand treatment. We attempt here to briefly
summarise the key findings of these experiments.
Several groups have used murine models of asthma, principally in the BALB/c and C57BL/6 strains, to demonstrate the protective effect of TLR ligands on the development of the asthma phenotype. There are almost as many murine models of asthma used as there are groups using them, but most involve sensitization of the animal with one to three weekly injections of ovalbumin (OVA), though other antigens are sometimes used, in aluminium hydroxide. The sensitization(s) are followed by one or more challenges of the airways, either by aerosol or intranasal delivery, with a solution of OVA in normal saline. These protocols of sensitization and challenge have led to the development of an asthma-like phenotype which includes allergen-induced AHR, inflammation of the lungs and airways, the production of Th2 cytokines and an increase in serum IgE levels.

2.3.1. Toll-Like Receptor 2

Like TLR4, the role of TLR2 signalling in the development and pathogenesis of asthma remains controversial. Using a mouse model, some studies have shown that the activation of TLR2 aggravates asthma by inducing the Th2 cytokines IL-13 and IL-5, inhibiting the Th1 cytokines IL-12 and IL-18 and increasing AHR [123]. On the contrary, other studies have shown that, in vitro, TLR2 ligands induce the production of the Th1 cytokine IFN-γ but not the Th2 cytokines IL-4 and IL-5. In addition, the use of TLR2 ligands inhibited IgE production in an allergic model in vivo [124].

2.3.2. Toll-Like Receptor 3

Evidence of the effect of TLR3 signalling in asthma is beginning to accumulate. In vitro studies have shown that TLR3 ligands can enhance murine tracheal smooth muscle responses to bradykinin, suggesting that TLR3 signalling could increase AHR [125]. The induction of RANTES expression by airway epithelial cell lines, in response to TLR3 ligands, has also been demonstrated in vitro [126]. Furthermore, in-vivo studies in mice have demonstrated that the administration of TLR3 ligands to the airways at high and low doses results in Th2 and Th1
inflammation, respectively [127]. Unlike TLR7 and TLR9, TLR3 signals, based on these observations, appear to aggravate the asthma phenotype and in fact, several respiratory viruses, such as Rhinoviruses which have dsRNA genomes that can interact with TLR3, are known to exacerbate asthma in human adults [128]. This would suggest that blockade of TLR3 activation is a viable therapeutic approach to the treatment of virus-induced exacerbations of asthma.

2.3.3. Toll-Like Receptor 4

Epidemiological evidence, though often contradictory [129-131], suggests a role for LPS in the pathology of asthma. In vitro, airway macrophages are activated, in a TLR4 dependent manner, by the low levels of bacterial products that contaminate air pollution particles [132]. Taken together, this suggests that environmental endotoxin is capable of exerting physiologically relevant effects on the airways; however, the circumstances under which, as well as the level of, exposure required to protect against asthma have yet to be established (reviewed in [133]). Like the epidemiological evidence, the experimental evidence for the role of LPS in asthma is contradictory. Studies have found that LPS administered with antigen during the sensitization or challenge phase, in a murine model of asthma, prevented the development of AHR and inflammation of the lungs [134;135]. On the other hand, TLR4-defective mice, which cannot respond to LPS, also develop very little AHR and airway inflammation [136]. These dichotomous results cannot simply be explained by the existence of two independent signalling pathways. While MyD88-dependent TLR4 signalling has recently been demonstrated to be required for the development of a T\textsubscript{H}2 mediated allergic response in a mouse model of asthma, in which sensitization is carried out by intra-nasal delivery of OVA containing LPS [137], other models clearly demonstrate that a lack of MyD88 leads to an increase in T\textsubscript{H}2 cytokine production [138]. These disparate results might be reconciled by the observation that, in some murine asthma models, low levels of LPS in the antigen preparation are required to induce the T\textsubscript{H}2 response characteristic of asthma while high
levels of LPS inhibit it [139]. While TLR4 signal modulation may turn out to have therapeutic potential, it is unclear, at the present time, how TLR4 signalling could be used to alleviate asthma.

2.3.4. Toll-Like Receptor 7

In vitro stimulation of \( \text{T}_{h2} \) cells with the TLR7 ligand resiquimod can inhibit the production of IL-4 and induce the production of IFN-\( \gamma \), resulting in cells with a \( \text{T}_{h1} \) profile of cytokine expression [140]. Other in vitro studies of human peripheral blood mononuclear cells [141], as well as studies employing mouse splenocytes [142], indicate that IgE production can be inhibited by resiquimod treatment in an, at least partially, IFN-\( \gamma \) or IL-12 dependent manner.

Furthermore, resiquimod has been shown by our group [143] and others [144] to have a beneficial effect in murine models of asthma using C57BL/6 and BALB/c mice, respectively. Intraperitoneal or intranasal administration of the compound before allergen challenge inhibited the development of AHR and allergen induced lung inflammation. Furthermore, the administration of the compound was shown to inhibit the production of \( \text{T}_{h2} \) cytokines and to decrease IgE levels in serum and separate studies have shown that these effects depend on the additive actions of IL-10 and IL-12 [145]. Several animal and human studies have been conducted which used resiquimod as an immunomodulator. This fact, combined with the recent findings of ours and others, imply an important therapeutic potential of imidazoquinolines for the treatment of allergic asthma.

2.3.5. Toll-Like Receptor 9

Treatment of sensitized animals with CpG ODN, a TLR9 ligand, either systemically by injection or by tracheal instillation before, or concurrently with, allergen challenge, leads to a decrease in airway inflammation that can be observed by histological analysis or by quantifying the number of inflammatory cells in broncho-alveolar lavage fluid (BALF) [146;147]. Production of the \( \text{T}_{h2} \)
cytokines IL-4 and IL-5 was abrogated, following allergen challenge, while levels of the Th1 cytokines IFN-γ and IL-12 were increased and total serum IgE levels decreased [146;147]. Finally, the increase in AHR and airway remodelling induced by allergen challenge was also greatly reduced in treated animals [147;148]. Follow-up studies indicate that a single CpG ODN administration can protect sensitized animals from allergen challenge for 6 – 8 weeks following treatment [149;150]. Evidence from some studies suggest that dendritic cells may be involved in promoting a Th1 response and inhibiting the Th2 response after TLR9 ligand treatment [151]. Others have shown increased levels of transforming growth factor β (TGF-β) in the BALF of treated animals [152], which indicates that regulatory T-cells may be involved. Recently, Hayashi et al. have demonstrated that, in a BALB/c model of acute allergic asthma, TLR9 ligation induces indoleamine 2,3-dioxygenase (IDO) activity in the lung and in CD11c+ splenocytes. This enzyme is involved in tryptophan catabolism and has been shown to induce regulatory T-cells [153]. In this model, the TLR9 induced increase in IDO activity mediated the suppression of allergic inflammation and AHR [154].

Agrawal et al. have generated immunomodulatory oligonucleotides based on the active structures of classical CpG ODN, which appear to have more potent but similar biological activity, suggesting that compounds exhibiting an improved therapeutic potential could be generated [155]. A potential caveat to the successful treatment of asthma by TLR9 ligands in humans is that human lungs appear to express relatively little TLR9 compared to other Toll-like receptors, such as TLR2 and TLR7 [107].

As a whole, there is strong evidence which suggests that the modulation of TLR signalling can result in a reduction of asthma development and pathology Fig.(1). Natural and artificial ligands for these receptors have successfully been used to treat murine models of asthma and are prime candidates for continued development into therapeutics for human patients.
3. Future Directions

3.1. Identification of Human Asthma Susceptibility Genes

While TLR signalling has been empirically demonstrated to modulate asthma pathology, the mechanisms through which these receptors exert their influence are poorly understood. In fact, the molecular mechanisms involved in the development and maintenance of asthma pathology are generally poorly understood. As previously mentioned, asthma pathogenesis is known to develop as a result of the interactions of several genes with many environmental factors. Recent years have seen great progress made towards the identification of the genes involved in the pathogenesis of asthma. This topic is covered in detail elsewhere [40;156]; for the purpose of this review, we will briefly summarize the state of the field and point out findings relevant to the present discussion.

Several new human asthma susceptibility genes have been identified in the past few years. The first to be identified was ADAM33 [157]; the protein encoded by this gene is part of a family of membrane-bound metalloproteinases and is expressed in human airway fibroblasts and epithelium. Its role in asthma pathogenesis remains unknown at the present time. Following ADAM33, PHF11 was identified and linked to atopy and serum IgE levels [92]. PHF11 contains two zinc-finger motifs and is therefore believed to be a transcription factor. DPP10 was identified shortly thereafter. Polymorphisms of this protein have been linked to skin prick test reactivity and atopy while certain haplotypes have been shown to be correlated with the severity of asthma [158]. Based on sequence homology, the authors have suggested that the protein encoded by this gene is a cytokine N-dipeptidase. Finally, haplotypes of GPRA, a G protein-coupled receptor of unknown function, was recently identified and associated with high serum IgE levels [159].

All of the above genes were identified after asthma-related loci, previously identified in whole-genome scans, were mapped and associations between
single-nucleotide polymorphisms and asthma were found in family-based transmission disequilibrium tests. These studies often require hundreds of volunteer families to complete and usually provide very little information about the mechanism of action of the identified genes. Genetic studies can also be performed in mice and, over the last decade, just as in humans, several asthma susceptibility loci have been identified that are associated with high serum IgE, AHR and atopy [160-164]. While there is no guarantee that asthma susceptibility genes identified in mice will have a human analogue or functional equivalent, there are several reasons for using mouse models in the genetic dissection of asthma susceptibility. Firstly, the environmental variables can be closely regulated, minimizing the variability of gene-environment interactions. Secondly, the genetic makeup of animals can be controlled through selective breeding and genetic manipulation techniques. Finally, once specific genes have been identified, their function can be elucidated with relative ease through the use of techniques (e.g. transgenic mice) which, for obvious reasons, are not available in human genetics.

3.2. Recombinant Congenic Strains of Mice as a Tool for the Genetic Dissection of the Mechanisms of Asthma

Of the available techniques for performing murine genetic analyses, recombinant congenic strains of mice (RCS) in combination with microarray expression analysis, as employed by Karp et al. [165], provide a powerful tool for the genetic dissection of complex diseases. RCS for the study of asthma are generated from two inbred strains of mice, one exhibiting one or more asthma-like phenotypes and the other exhibiting non-asthma-like phenotypes. RCS of this type have been generated from A/J (A) mice, which have high basal and antigen-induced AHR, and C57BL/6 (B) mice, which have low AHR [34;166]. These RCS were generated as described in [167]. Briefly, the RCS used were generated by creating an A/J × C57BL/6 F1 cross, followed by two backcrosses to the parental strains. The resulting progeny generated by backcrossing to A, [(F1 × A)
× A] is referred to as AcB, whereas the progeny resulting from a backcross to B, [(F1 × B) × B] is referred to as BcA. Both AcB and BcA mice were subsequently inbred for 30 – 40 generations. This strategy of breeding resulted in 15 distinct AcB strains, containing on average 13.25% of the B genome on an A background (on average 84.4% of the A genome) as well as 22 distinct BcA strains, containing on average 13.24% of the A genome on a B background (on average 85.37% of the B genome) (40). Genotyping of these strains using 625 microsatellite markers which are polymorphic for the A and B strains, with an average spacing of 2.6 cM, showed that 79% of the B genome is represented in AcB strains and that 84% of the A genome is represented in BcA strains (167). AcB strains with a B phenotype and BcA strains with an A phenotype are called informative strains because their phenotype is controlled by their minor genetic donor, immediately localizing the genes responsible for the trait to a fraction of the genome. By performing quantitative trait loci (QTL) analysis on the backcrosses of informative strains with their major genetic donor, narrow susceptibility loci can be defined. Using microarray expression analysis, genes with significantly different expression levels can be identified in mice phenotypically similar to A versus mice phenotypically similar to B. Candidate genes with a high probability of being relevant to the phenotype in question can then be identified as those genes whose expression level is significantly different between phenotypes and which are found at loci identified through QTL analysis.

3.3. Future Therapeutic Prospects

Elaboration of the mechanism of action of genes identified in this way can then be undertaken in an in vivo murine model of asthma. This allows investigators to use modern genetic tools, such as transgenic animals, reconstitution studies, treatments aimed at depleting certain cells or mediators, and other invasive experimental procedures that are required to demonstrate mechanisms but cannot be performed in humans for ethical reasons. The
identification of the mechanisms underlying the development and maintenance of asthma can then provide novel therapeutic and immunotherapeutic targets.

Successful therapy of asthma will only be achievable when the mechanisms underlying its onset and pathology have been elucidated. Progress is being made in our understanding of both the genetic and environmental factors involved in the disease. The use of advanced genomic approaches, such as those described in this article, will allow the dissection of the genetic factors involved in a disease as complex as asthma. Also, TLRs, which direct the interactions of the innate immune system with the adaptive immune system, which in turn is responsible for much of the pathology of asthma, may be an important link between environmental factors and asthma pathology. Furthermore, the relevance of studies exploring the mechanism of TLR ligand-mediated effects on allergic asthma is that they may explain how environmental factors impact the adaptive immune system protecting the host against asthma (hygiene hypothesis). It is also quite possible that modulation of the interaction between TLRs and their ligands will allow significant therapeutic effects to be potentiated.
Acknowledgements

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Figure 1: TLR, the Immune System, and Asthma

Toll-like receptor ligands bind to their cognate receptors on respiratory system cells, and cells of the innate and adaptive immune system. Cell receptor ligation of these cells causes expression of numerous immune mediators and surface molecules which regulate the innate and adaptive immune response leading to modulation of the asthma phenotype.
Chapter 2 - TLR7 Ligand Prevents Allergen-Induced Airway Hyperresponsiveness and Eosinophilia in Allergic Asthma by a Myd88-dependent and Mapkapk2-independent Pathway

Adapted from:

Preface

Toll-like receptor 7 ligands have been shown to promote anti-viral immunity in a number of hosts through the induction of a T\textsubscript{H}1 biased immune response. Given that allergic asthma aetiology is associated with a T\textsubscript{H}2 biased immune response and given that T\textsubscript{H}1 response suppresses T\textsubscript{H}2 responses, the goal of the studies presented in this chapter was to determine if treatment with the toll-like receptor 7/8 ligand resiquimod could prevent the development of allergic asthma. The results of the studies described in chapter 2 demonstrate that resiquimod treatment can prevent the development of allergic asthma in mice, but that the protective effect is not accompanied by an induction of T\textsubscript{H}1 biased immune response.
Abstract

Asthma is one of the leading causes of childhood hospitalisation and its incidence is on the rise throughout the world. Currently, the standard treatment for asthma is the use of corticosteroids to try to suppress the inflammatory reaction taking place in the bronchial tree. Using a murine model of atopic allergic asthma employing a methacholine hyperresponsive (A/J) as well as a hyporesponsive (C57BL/6) strain of mice sensitized and challenged with ovalbumin, we show that treatment with a synthetic Toll-like receptor 7 ligand (resiquimod, also known as S28463 and R-848, a member of the imidazoquinoline family) prevents development of the asthmatic phenotype. Treatment with resiquimod resulted in a reduction of airway resistance and elastance following ovalbumin sensitization and challenge. This was accompanied by a dramatic reduction in infiltration of leukocytes, especially eosinophils, into the lungs of both C57BL/6 and A/J mice following OVA challenge. Treatment with resiquimod also abolished both the elevation in serum IgE level as well as the induction of IL-4, IL-5 and IL-13 by OVA challenge. The protective effects of resiquimod were also observed in MK2 knockout, but not Myd88 knockout mice. We did not observe a switch in cytokine profile from Th2 to Th1, as both IL-12p70 and IFN-γ levels were reduced following resiquimod treatment. These results clearly demonstrate the anti-inflammatory effect of imidazoquinolines in an allergic asthma model as well as the clinical potential of TLR7 ligands in the treatment of allergic diseases.
**Introduction**

Allergic asthma is a complex and heterogeneous respiratory and immune disorder. It is a leading cause of childhood hospitalization, whose prevalence is increasing in westernized countries [4]. Asthma is generally defined as a chronic inflammation of the bronchial airways, characterized by intermittent episodes of reversible airway obstruction, excessive mucus production and infiltration of the airway submucosa by activated lymphocytes, monocytes, neutrophils and especially eosinophils [8].

One of the most important pathological features of asthma is airway (or bronchial) hyperresponsiveness (AHR). AHR is an increased sensitivity, exhibited by asthmatic subjects, to a variety of stimuli including allergens, pollutants, cold air, exercise, distilled water and methacholine which cause bronchoconstriction [168]. The AHR seen in human asthmatic patients is associated with airway remodelling characterized by the deposition of excess collagen in the subepithelial layer of the airway mucosa due to chronic inflammation of the bronchial airway wall and airway smooth muscle hyperplasia [169;170].

Attempts at controlling the inflammatory reaction have mainly consisted of approaches targeting the effector inflammatory symptoms present in asthmatic patients. Present medications which require chronic dosing are not fully effective and were reported to have some side effects. The majority of treatments do not target the early upstream events in the pathways of allergic inflammation and bronchospasm which has prompted the search for new therapeutic targets [171]. One of these new strategies involves modulation of the immune response through Toll-like receptor (TLR) activation. Most of the ligands defined for these receptors are pathogen derived products. TLR4 is important for the response to LPS from gram-negative bacteria [172]. TLR2 transduces signals for lipoprotein and cell wall products from gram positive bacteria as well as peptidoglycan and lipoarabinomannan (LAM) from mycobacteria [173;174]. TLR9 has been found to
be responsible for the response to unmethylated CpG oligodeoxynucleotides in both mice and humans [175;176]. Resiquimod, a member of the imidazoquinoline family also called R-848 and S28463, has been shown to be a TLR7/8 ligand in humans [177]. The imidazoquinolines are potent inducers of a whole spectrum of cytokines such as IL-1, IL-6, IL-8 and TNFα in a number of animal species such as the mice, guinea pigs and monkeys [178]. TLR7 and 8 have also been implicated in recognising single stranded RNA of viral origin [101;179]. Although mice express both TLR7 and TLR8, only TLR7 appears to be functional [177]. Furthermore, although TLR4 signal transduction can occur through an MYD88-dependent and independent pathway, TLR7-mediated immunomodulatory effects are solely dependent on the MYD88 pathway [180].

TLR stimulation can affect various aspects of the asthmatic phenotype. Unmethylated CpG oligodeoxynucleotides have been shown to reverse allergen-induced inflammation and prevent airway remodelling and AHR in response to allergen in a mouse model of asthma [146;147]. Inoculation of mice with M. bovis BCG and M. vaccae, whose cell wall components are TLR2 ligands, have also been shown to reduce AHR in response to allergens [181;182].

Using mice sensitized and challenged with ovalbumin, we demonstrate that imidazoquinoline treatment prevents increased lung resistance and elastance in mice that exhibit low (C57BL/6) and high (A/J) naive lung AHR. TLR7 ligand administration also led to a decrease in serum IgE levels. TLR7 activation prevented lung cellular infiltration; completely abrogating lung eosinophil recruitment. We also demonstrate that the protective effect of Toll-like receptor 7 ligands is dependent on MYD88, but independent of MAPKAP-2. Furthermore, complete inhibition of Th2 as well as Th1 cytokines following imidazoquinoline treatment in OVA-challenged animals suggests that TLR7 triggering in the context of allergic asthma plays an anti-inflammatory role.
Material and Methods

Mice

Eight to ten-week-old male A/J and C57BL/6 mice were purchased from Harlan (Indianapolis, IN). C57BL/6 Mapkapk2 knockout mice previously generated by Kotlyarov et al. [183] were bred at the Research Institute of the McGill University Health Center. Myd88−/− mice were generated in C57BL/6 x 129/SvJ [184] and subsequently backcrossed six times to the C57BL/6 background at the Weill Medical College of Cornell University. All animals were specific pathogen free and were maintained according to the standards of the Animal Care Committee of the McGill University Health Center.

Challenge Protocol and Respiratory System Physiology

Mice were sensitized once a week for three consecutive weeks by intraperitoneal injections of 100 µg ovalbumin adsorbed to 1.5 mg of aluminium hydroxide (Imject Alum, Pierce, Rockford, IL) in a total volume of 0.2 mL of sterile PBS. Seven days following the final sensitization mice were challenged on three consecutive days by aerosol exposure to either a 1% ovalbumin solution (OVA-OVA and OVA-S28 group) or PBS alone (OVA-PBS group) for 30 minutes. One group of mice was injected intraperitoneally with 100 µg of resiquimod (generously provided by Dr. R. Miller, 3M Pharmaceuticals, St. Paul, MN) on 3 consecutive days starting one day prior to the first ovalbumin challenge (OVA-S28 group). Measurements of respiratory system resistance (Rrs) and elastance (Ers) were measured using a Flexivent small animal ventilator (SAV) (Scireq, Montreal, PQ, Canada). Briefly, 48 hours after final challenge, animals were anaesthetized, tracheotomized and connected to the ventilator. Mechanical ventilation was carried out and peak resistance and elastance were measured after intra-jugular administration of increasing doses of methacholine (0-320 µg/kg).
**Histology**

Immediately following sacrifice of the mouse, the lungs were removed, inflated with 10% buffered formalin, dehydrated, mounted in paraffin and sectioned. Deparaffinized and hydrated sections were stained with hematoxylin and eosin (H&E) and Congo red (CR) stains using standard procedure. For quantitation, 300 infiltrating cells in the lungs were counted by two blinded observers and the number of Congo red positive cells was quantitated.

**Bronchial Alveolar Lavage Fluid Analysis**

After the final antigen challenge, mice were sacrificed and the BAL analysis was performed. Following insertion of a catheter into the trachea, the lungs of the animal were lavaged using 1.2 mL of Hank’s Balanced Salt Solution (Invitrogen, New York) and the samples were kept on ice for further processing. The lavage fluid was centrifuged at 250×g and the cell pellet was resuspended in RPMI media (Invitrogen, New York). Cells were then loaded onto a cytopsin machine and spun onto slides at 350 rpm. The slides were subsequently stained with a Diff-Quik stain set (Dade Behring, Newark) and a differential cell count was performed to determine the cellular makeup of the inflammatory cells.

**Cytokine and IgE Measurements**

Immediately following sacrifice of the mouse, the lungs of the animals were removed and placed in PBS containing protease inhibitors (Complete inhibitor, Roche Diagnostics, Laval, PQ, Canada). Shortly thereafter, the lungs were homogenized using a polytron and the homogenates were stored at −80°C. Cytokine production was measured 3 and 6 hours after the last OVA challenge. IL-4, IL-5, IL-6, CCL5 (RANTES) and IL-10 levels were quantitated using Lincoplex murine cytokine analysis (Linco Research Inc., St. Charles, MO). IL-12p70, IL-13 and IFNγ production was measured using Beadlyte cytokine kits (Upstate, Charlottesville, VA). Serum IgE levels were measured 48 hours following the last challenge using the BD OptEIA ELISA kit (BD Biosciences, Missisauga, ON, Canada) according to the manufacturer’s protocol.
RNA Analysis

Total lung RNA was purified using Trizol reagent (Invitrogen). Following DNase digestion of 4 µg of total RNA (DNA-free, Ambion, Austin, TX), reverse transcription was performed using random primers (Stratascript First Strand cDNA synthesis kit, Stratagene, LaJolla, CA). *Ccl5, Ccl11, Ccl17* and *Ccl24* mRNA levels were determined by real-time PCR analysis performed using Brilliant SYBR Green QPCR kit according to manufacturer’s protocol (Stratagene) on Stratagene MX-4000 apparatus using the following cycling conditions: denaturation at 95°C for 30 seconds, annealing at 56°C for 60 seconds and extension at 72°C for 30 seconds. Primer sequences employed in this study are presented in Table 1. Levels of chemokine mRNA were adjusted for differences in GAPDH expression and normalised to the levels in the A/J OVA-PBS group.

Statistical Analysis

A Mann and Whitney non parametric test was performed using the SigmaStat software (SPSS, Chicago, Illinois) to calculate statistical significance for all analyses except gene expression where an unpaired t test using Welch correction was applied (Prism 4, GraphPad Softwares, San Diego, CA). Differences among treatments are considered significant if p≤0.05.
Results

Imidazoquinoline Treatment Prevents Ovalbumin-Induced Increases in Airway Hyperresponsiveness

Previous studies have indicated the potential of TLR signalling in modulating allergen-induced airway hyperresponsiveness. TLR4 and 9 ligands have been shown to down regulate allergic immune responses in murine models of asthma [134;147;185;186]. In this study, the potential of TLR7 ligands to regulate allergic diseases was investigated in A/J and C57BL/6 mice. All animals were sensitized once a week for 3 consecutive weeks. Mice were then separated into three groups receiving either a challenge with PBS, with 1% OVA or treatment with resiquimod followed by challenge with OVA. Treatment with OVA induced an increase in lung responsiveness that could be prevented by TLR7 ligand treatment (Figure 1 panel A). There was a statistically significant increase in lung resistance values in mice receiving OVA challenge versus PBS at 320 µg/kg of methacholine (30.4±4.2 and 12.5±3.1 cmH₂O/ml/s, respectively, p<0.05). Interestingly, the animals receiving resiquimod treatment prior to OVA challenge had significantly lower lung resistance than the OVA challenged group at the same methacholine dose (30.4±4.2 for OVA-OVA and 17.3±3.6 cmH₂O/ml/s for the OVA-S28 group, p<0.05). This reduction in lung resistance by resiquimod could also be observed at the 160 µg/kg methacholine dose (12.1±3.2 for the OVA-OVA group and 4.6±0.7 cmH₂O/ml/s for the OVA-S28 group, p<0.05). Similar effects were observed in C57BL/6 mice treated with TLR7 ligand and challenged with OVA (data not shown). Concurrently with modulation of airway resistance, OVA challenge also increased lung elastance which could also be prevented by resiquimod treatment (Figure 1 panel B). These data suggest that TLR7 signalling is able to prevent airway hyperresponsiveness following allergen challenge in a murine model of asthma.
**TLR7 Signalling Abrogates Increases in Serum IgE Levels Following OVA Challenge**

One of the hallmarks of asthma and other atopic diseases is the presence of increased levels of IgE [187]. A/J mice sensitized and challenged with OVA displayed increased levels of serum IgE compared to naïve animals (Figure 2). Challenge with ovalbumin resulted in an increase in the levels of IgE compared to the group receiving PBS challenge (9.0 ± 1.8 μg/mL versus 3.2 ± 0.7 μg/mL, respectively). Imidazoquinoline treatment resulted in a dramatic reduction in IgE levels compared to the OVA challenged group (1.3 ± 0.3 μg/mL and 9.0 ± 1.8 μg/mL, respectively), bringing the levels into the range of the PBS-challenged group. Interestingly, both the A/J and C57BL/6 strains display a similar pattern of response, except that OVA challenge induced 4 to 5 times more IgE in A/J as compared to C57BL/6 mice. This data demonstrates that TLR7 signalling can reduce the increase in IgE levels induced by OVA challenge even in a genetically predisposed strain of mice such as A/J.

**Treatment with TLR7 Ligand Prevents Induction of both T\textsubscript{H}2 and T\textsubscript{H}1 Cytokines Following OVA Sensitization and Challenge**

Cytokine profiling in asthmatic patients has pointed to a role for T\textsubscript{H}2 cytokines such as IL-4, 5 and 13 in the pathology of this disease [188]. Studies with TLR ligands have shown a tendency to skew the asthmatic response from a T\textsubscript{H}2 to a T\textsubscript{H}1 immune response resulting in prevention of the asthmatic phenotype in animal models. We assessed the ability of TLR7 ligands to modulate cytokine production in animals sensitized and challenged with OVA. IL-4, 5, 6 and 13 were all strongly induced 6 hours following OVA challenge in both A/J and C57BL/6 mice (Figure 3 panel A-D). PBS challenge did not lead to any increase in these cytokines. Treatment with resiquimod completely prevented induction of these cytokines following OVA challenge. TLR7 activation did not lead to induction of T\textsubscript{H}1 cytokines as both IFN\textsubscript{γ} and IL-12p70 levels were reduced by resiquimod treatment (Figure 3 panel E-F). Levels of IL-10 were only slightly elevated.
following OVA challenge and returned to PBS levels in animals treated with imidazoquinolines (Figure 3 panel G). Imidazoquinoline treatment also led to a significant induction of Ccl5 (RANTES) protein levels in the lungs of challenged animals (Figure 3 panel H) suggesting that the mode of action of these compounds is not through induction of a general immunosuppressed state. Furthermore, mRNA expression analysis in lungs from A/J and C57BL/6 mice revealed an upregulation by TLR7 ligand treatment of Ccl5 (RANTES) mRNA (Figure 4 panel A) as well as chemokines normally associated with a T\(_{H2}\) response (Ccl4, data not shown). Moreover, we found that imidazoquinoline treatment led to inhibition in the mRNA levels of chemokines normally involved in eosinophil recruitment and T\(_{H2}\) cell infiltration (Ccl11, Ccl17 and Ccl24) following OVA challenge (Figure 4 panel B, C and D, respectively). Taken together, our data demonstrate that TLR7 ligand treatment results in an almost complete prevention of the inflammatory reaction following OVA sensitization and challenge; characterized by an inhibition of induction of several T\(_{H2}\) and T\(_{H1}\) cytokines in the lungs.

**TLR7 Ligand Treatment Prevents Airway Inflammation**

Proinflammatory cytokines such as IL-5 are known to be important in the recruitment of inflammatory cells to the lung [189]. The inhibition of cytokine induction by OVA challenge in imidazoquinoline treated animals suggested that this might lead to a change in the cellular influx to the lungs of antigen challenged animals. OVA challenge induced a significant increase in the amount of cells in the BAL. Total BAL cell numbers went from 28,295±18,942 in the PBS group to 115,682±35,825 for OVA challenged A/J mice and from 13,766±1,995 to 283,807±138,121 in C57BL/6 (Figure 5 panel A). Treatment with imidazoquinolines completely prevented cellular infiltration in the BAL of both strains of mice. Analysis of cellular composition revealed that OVA challenge induced a significant increase in eosinophil composition of the BAL representing 31±7% in A/J (Figure 5 panel B) and 30±8% in C57BL/6 mice (Figure 5 panel C).
Treatment with a TLR7 ligand completely prevented eosinophil recruitment in both strains of mice. OVA challenge also increased the relative amounts of lymphocytes in the BAL of both strains of mice which was not prevented by imidazoquinoline treatment. We did not observe any significant modulation of neutrophil levels by any of the treatments (data not shown). These results demonstrate the ability of imidazoquinolines to prevent influx of inflammatory cells, mainly eosinophils, to the lumen of airways in both A/J and C57BL/6 mice.

**Imidazoquinoline Treatment Prevents Lung Inflammatory Cell Recruitment in OVA-Challenged Mice**

Paraffin embedded lungs from animals sensitized and challenged with OVA displayed increased cell infiltration in both strains of mice as shown by H&E staining (Figure 6). The recruited cells tended to accumulate around the blood vessels close to airways (Figure 6 panel B and E). Imidazoquinoline treatment almost completely abrogated the recruitment of cells to the lungs around both blood vessels and the surrounding airways (Figure 6 panel C and F). Challenge with PBS alone did not induce any significant cellular recruitment to the lungs (Figure 6 panel A and D). Flow cytometry analysis of digested lungs revealed an increased influx of CD45-positive cells following OVA challenge that could be prevented by imidazoquinoline treatment (data not shown), further confirming these observations. In addition, *Myd88*−/− mice displayed marked cellular infiltration to the lungs following OVA challenge which was not prevented with imidazoquinoline treatment. These findings further confirm the involvement of Toll-like receptors in the mechanism of action of imidazoquinolines in the treatment of asthma (Figure 6 panels H and I, respectively). Interestingly, although very different in their lung AHR, A/J and C57BL/6 mice exhibit very similar cellular infiltration following OVA challenge.
Eosinophil Infiltration is Abrogated Following TLR7 Ligand Treatment

Eosinophilic infiltration has been implicated in many of the pathological features of asthma. They release mediators such as leukotriene C4, major basic protein as well as many cytokines which contribute to the asthmatic phenotype [189]. Histological analysis of lungs following OVA challenge revealed a significant increase in eosinophilic infiltration (Figure 7). Eosinophils represented 42.2±5.7% of infiltrating cells in the lungs of A/J mice and 39.8±4.7% in C57BL/6. Treatment with resiquimod completely prevented eosinophil infiltration into OVA-challenged lungs in both strains of mice. Myd88−/− mice sensitized and challenged with OVA displayed a similar increase in lung eosinophilia which could not be prevented by imidazoquinoline treatment (Figure 7). Interestingly, Mapkapk2 (MK2) knockout mice responded normally to resiquimod treatment as shown by a reduction in airway eosinophilia (Figure 7) as well as AHR (data not shown). PBS challenge did not induce any detectable levels of eosinophils in any of the strains tested. These data demonstrate that Toll-like receptor 7 ligand treatment prevents eosinophil recruitment to the lungs leading to attenuated AHR levels in both A/J and C57BL/6 mice which is dependent on Myd88, but independent of MK2.
Discussion

The results presented in this study demonstrate that TLR7 ligand treatment in the context of atopic allergic asthma leads to a reversal of the acute inflammatory state. The inhibition of both $\text{T}_\text{H}2$ (IL-4, 5, 6 and 13) and $\text{T}_\text{H}1$ (IL12p70 and IFN$\gamma$) cytokines prevents infiltration of inflammatory cells, especially eosinophils, to the lungs of animals sensitized and challenged with OVA. The inhibition of early inflammatory events associated with OVA challenge led to a reduction in serum IgE levels as well as a reduced lung response to methacholine challenge in both A/J and C57BL/6 mice. Furthermore, the protective effect of imidazoquinolines was found to be dependent on the presence of MYD88, but not on the MK2 pathway.

In both A/J and C57BL/6 strains, we did not observe any induction of $\text{T}_\text{H}1$ cytokines following imidazoquinoline treatment. A previous study assessing the effect of resiquimod treatment of allergic asthma in BALB/c mice has shown a decrease in Penh induction following OVA challenge associated with a shift in cytokine profile from $\text{T}_\text{H}2$ to $\text{T}_\text{H}1$ [144]. The possible discrepancy in cytokine profile can be attributed to the fact that cytokine production was measured from $\text{in vitro}$ cultured lung cells in the previous study rather than direct lung measurement as presented here. It is of great clinical importance whether TLR7 ligand treatment induces a shift in cytokine production or a complete block of the inflammatory reaction. We have used two different mouse strains, directly measured lung parameters, as well as measured cytokines $\text{in vivo}$ which we believe gives a better approximation of the physiological effect of TLR7 ligand in the lungs.

Both TLR4 and TLR9 activation have been associated with positive outcomes in allergic asthma models [134;185]. Initial observations had suggested that the mechanism of action involved a shift in cytokine production, although further analysis revealed that $\text{T}_\text{H}1$ cytokines could not account for the observed
beneficial effect associated with both LPS and CpG treatment of allergic asthma. This suggests that TH2 cytokine inhibition and induction of regulatory mechanism account for the observed amelioration [134;185]. IL-10 has been shown to be crucial in mediating CpG efficacy in preventing allergic asthma [190]. We saw very little modulation in the levels of this cytokine suggesting an alternative mechanism for TLR7 ligand action in allergic asthma treatment. The fact that imidazoquinolines have been employed in humans for the treatment of human papilloma virus (HPV)-induced warts and the observation that human lungs have tenfold higher mRNA levels for TLR7 than TLR9 suggests a greater clinical applicability of imidazoquinolines for asthma treatment [107].

Although A/J and C57BL/6 mice displayed comparable inflammatory responses following OVA sensitization and challenge, serum IgE levels were found to be differentially induced in these animals. OVA sensitization induced five times more serum IgE in A/J mice compared to C57BL/6. OVA challenge maintained the difference in serum IgE between the two strains. Levels of serum IgE are also associated with differences in airway resistance and elastance between A/J and C57BL/6 following OVA challenge. Imidazoquinoline treatment prevented increases in IgE levels as well as improved the lung physiology in both strains of mice. Using resiquimod as an adjuvant in conjunction with Alum, it had been reported that TLR7 ligands tended to favor IgG2a production and led to reduced IgE production in a murine model [191]. Human PBMC stimulated in vitro with CD40 and IL-4 also produced significantly less IgE in the presence of TLR7 ligands [141]. We demonstrate that administration of TLR7 ligands, even after sensitization, leads to a dramatic reduction in serum IgE levels induced following OVA challenge.

OVA challenge induced a dramatic influx of cells both in the BAL and in the interstitial lung environment. The main recruited cell types were eosinophils and macrophages. OVA challenge also led to a significant increase in lung responsiveness to methacholine in A/J mice following OVA challenge. Both
effects were greatly diminished by imidazoquinoline treatment. This was also associated with a reduction in CD45 positive lung cells in both A/J and C57BL/6 animals (data not shown). The complete lack of eosinophils as well as IL-5 and IL-13 can account for the observed reduction in lung AHR levels [189;192]. Because of their role in airway remodeling and lung inflammation [193], inhibition of eosinophilia through lack of cytokine (IL-5) and chemokine (Ccl11, Ccl17 and Ccl24) induction following TLR7 ligand treatment provides a plausible mechanism for the protective effect of imidazoquinolines in the treatment of allergic asthma [194].

The mechanism of action of imidazoquinoline treatment in allergic asthma was also addressed using mice lacking important signal transduction components which have been previously shown to play a major role in immunomodulatory effects induced by various TLR. Myd88 is a crucial adapter molecule linking the TLR with other signal transduction components. TLR7 has been previously shown to be dependent on the presence of Myd88 for signal transduction to be initiated following imidazoquinoline stimulation [180]. We demonstrate that in the context of atopic allergic asthma, the protective effect of imidazoquinoline on lung cellular infiltration and eosinophilia is dependent on the presence of a functional Myd88. The P38 MAPK substrate, Mapkapk2 (MK2), has previously been shown to be important in cytokine induction by TLR [183]. Using MK2 KO mice, we demonstrate that this kinase was not required for the protective effect of imidazoquinoline in allergic asthma as measured by both lung eosinophilia and AHR. A recent study has demonstrated a role for P38 MAPK in the induction of allergic asthma [195]. MK2 KO mice displayed normal increases in lung resistance following OVA challenge (data not shown). Furthermore, MK2 KO mice exhibited increased lung eosinophilia following OVA challenge. This suggests that P38 MAPK, but not MK2 is important for allergic asthma induction in mice. This is the first study describing the role of various signal transduction
molecule in the protective effect of Toll-like receptor 7 ligands in the treatment of allergic asthma.

Previous studies in our laboratory have pointed to a role of *Nramp1* (Natural Resistance-Associated Macrophage Protein 1 or *Slc11a1*) in modulating responsiveness to TLR7 ligands in mycobacterial infections [196]. Mice carrying the wild type (or resistant) allele of *Nramp1* displayed increased responsiveness to imidazoquinoline treatment than mice carrying the susceptible allele of this gene. NRAMP1 expression in mice is restricted to macrophages. In the present study, both A/J and C57BL/6 mice responded equally to resiquimod treatment even though they carry different *Nramp1* alleles. This might suggest that the response of macrophages to TLR7 ligands in the lungs might not be critical for its protective effect against allergic asthma.

Taken together, we demonstrate that the ability of TLR7 to prevent acute atopic allergic asthma through inhibition of cytokine production as well as cellular infiltration leading to a reduction in airway hyperresponsiveness is dependent on *Myd88*, but does not require functional MK2. The data presented in this study warrant further clinical investigation of the potential for TLR7 ligands in the treatment of allergic asthma.
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**Figure 1:** Treatment with Resiquimod Abrogates Allergen Induced AHR.

*A/J mice were sensitized with OVA and challenged by aerosol exposure to PBS (OVA-PBS) or OVA (OVA-OVA), as described in the *Materials and Methods* section. One group of mice received 100 μg of resiquimod (S28) intraperitoneally one day prior to each OVA challenge (OVA-S28). Respiratory system resistance (Rrs) (Panel A) and elastance (Ers) (Panel B) in response to increasing doses of methacholine (MCh) was measured 48 hours after the last PBS or OVA aerosol challenge. Results are presented as mean ± SEM from three independent experiments (n=10) (* represents p<0.05 between OVA-PBS and OVA-OVA group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).*
Figure 2: IgE Levels in OVA Challenged A/J and C57BL/6 mice.

Mice were sensitized with OVA and challenged with PBS (OVA-PBS) or OVA (OVA-OVA) and serum was collected as described in the Materials and Methods section. One group of mice received 100µg of resiquimod (S28) intraperitoneally one day prior to each OVA challenge (OVA-S28). Total serum IgE levels were determined by ELISA 48 hours after PBS or OVA challenge. OVA challenge in A/J mice led to a significant increase in IgE levels (n=13) compared to PBS challenge (n=11). Resiquimod treatment led to a dramatic reduction in serum IgE levels (n=12). Similar pattern was observed in C57BL/6 mice for OVA-PBS (n=24), OVA-OVA (n=26) and OVA-S28 (n=25) group. Results are presented as mean ± SEM from 5 independent experiments (* represents p<0.05 between OVA-PBS and OVA-OVA group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).
Figure 3: Cytokine Levels Following OVA Challenge.

Following sensitization and challenge with OVA or PBS, whole lung tissue homogenates from A/J (solid bars) and C57BL/6 (open bars) were collected as described in the *Materials and Methods* section. Lung IL-4 (Panel A), IL-5 (Panel B), IL-6 (Panel C), IL-10 (Panel G) and RANTES (Panel H) levels were determined by Lincoplex murine cytokine array 6 hours after PBS or OVA challenge. IL-13 (Panel D), IL-12p70 (Panel E) and IFNγ (Panel F) levels were quantitated using the Beadlyte cytokine kits. Results are represented as mean ± SEM from four animals (* represents p<0.05 between OVA-PBS (PBS) and OVA-OVA (OVA) group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).
Figure 4: Chemokine mRNA Expression Following OVA Challenge.

Total lung RNA from A/J (solid bars) and C57BL/6 (open bars) mice was purified using Trizol reagent. Real-time PCR analysis was performed using the Statagene MX-4000 apparatus. Levels of CCL5 (Panel A), CCL11 (Panel B), CCL17 (Panel C) and CCL24 (Panel D) mRNA were adjusted for differences in GAPDH expression and normalised to the levels of the A/J OVA-PBS group. Results are represented as mean ± SEM from three animals for each group (* represents p<0.05 between OVA-PBS and OVA-OVA group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).
**Figure 5: BAL Analysis.**

Analysis of inflammatory cells found in bronchoalveolar lavages following OVA challenge. Total number of cells was increased in both A/J and C57BL/6 following OVA challenge (Panel A). Treatment with imidazoquinoline significantly inhibited influx of inflammatory cells to the lungs. Analysis of cell types in BAL from A/J (Panel B) and C57BL/6 (Panel C) revealed influx of eosinophil following OVA challenge which was prevented by TLR7 ligand treatment. Results for total BAL cell count are represented as mean ± SEM from three independent experiments (n=8 for all groups) (* represents p<0.05 between OVA-PBS and OVA-OVA group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).
Figure 6: Histological Analysis of Lungs from OVA Challenged mice.

Pictures illustrate representative hematoxylin- and eosin- (H&E)-stained lung sections prepared from mice sensitized and challenged as described in the Materials and Methods section. The three top panels are from A/J mice, the middle panels represent lungs from C57BL/6 and the lower panels represent lungs from MYD88 knockout mice. The panels are representative lung sections of H&E stains (200X magnification) from OVA-PBS (Panel A, D and G), OVA-OVA (Panel B, E and H) and OVA-S28 (Panel C, F and I) mice 48 hours after challenge. OVA challenged A/J and C57BL/6 mice displayed a markedly increased cellular infiltration around blood vessels (indicated by black arrows) and airways when compared to both OVA-PBS and resiquimod treated lungs. Lungs from MYD88−/− mice displayed a marked cellular infiltration following OVA challenge that could not be reversed by resiquimod treatment.
Figure 7: Lung Eosinophilia Following OVA Challenge is Inhibited by Resiquimod.

Imidazoquinoline treatment resulted in a dramatic reduction in eosinophil level following OVA challenge (Figure 7). Quantitation of Congo red positive cells revealed a similar amount of eosinophil infiltration in both A/J (n=8 for PBS, n=9 for OVA and n=7 for OVA-S28) and C57BL/6 (n=9 for PBS, n=8 for OVA and n=9 for OVA-S28) mice following OVA challenge that was prevented by resiquimod treatment. MYD88−/− mice also displayed similar eosinophil infiltration following OVA challenge that could not be prevented by imidazoquinoline challenge (n=3 for all groups). MAPKAP-2 KO mice responded normally to resiquimod treatment resulting in reduction of airway eosinophilia following OVA challenge (n=3 for PBS, n=3 for OVA and n=8 for OVA-S28). Data for A/J and C57BL/6 are represented as mean ± SEM from three experiments (n=8 for all groups) (* represents p<0.05 between OVA-PBS and OVA-OVA group. ** represents p<0.05 between OVA-OVA and OVA-S28 group).
Chapter 3 - Chronic Asthma-induced Airway Remodeling is Prevented by Toll-like Receptor-7/8 Ligand Resiquimod

Adapted from:

Preface

The results of the studies presented in the preceding chapter demonstrated that resiquimod treatment could prevent the development of acute inflammation, airway hyperresponsiveness, and IgE production in a mouse model of allergic asthma. However, the effect of resiquimod treatment on the development of chronic aspects of asthma pathology had not yet been investigated. The studies employing a Brown Norway rat model of chronic allergic asthma presented in chapter 3 demonstrate that resiquimod treatment inhibits the increase in airway smooth muscle mass and the development of goblet cell hyperplasia which are associated with chronic allergic asthma.
Abstract

Rationale: Allergic asthma is a heterogeneous disease whose pathology is a result of improper immune responses to innocuous antigens. We and others have previously shown that one of the Toll-like Receptor (TLR) 7/8 ligands, the synthetic compound resiquimod (S28463, R-848) is able to inhibit acute allergic asthma in mice. Objectives: Given that the efficiency of this pharmacological compound against the smooth muscle mass increase and goblet cell hyperplasia that are characteristic of chronic allergic asthma has not been previously assessed, we investigated the ability of this compound to prevent these aspects of chronic airway remodelling. Methods: The impact of resiquimod treatment was assessed in a Brown Norway rat model of chronic asthma by histological, morphometric and molecular techniques. Main Results: We demonstrate that treatment with resiquimod is able to prevent the development of goblet cell hyperplasia and increases in airway smooth muscle mass, and that this effect is at least partially mediated by inhibiting proliferation of goblet and smooth muscle cells, respectively. Furthermore, we show that the abrogation of airway remodeling is preceded by the inhibition of the inflammatory reaction normally occurring in response to allergen challenge in sensitized animals. This inhibition was associated with a reduction of both T\textsubscript{H}1 and T\textsubscript{H}2 cytokine protein expression in the lungs, demonstrating the potent anti-inflammatory effect of this pharmaceutical compound in the context of allergic reactions. Conclusion: Taken together, our results indicate great potential for the use of resiquimod as an anti-inflammatory therapeutic agent for the management of chronic asthma.
**Introduction**

Allergic asthma is a heterogeneous disease affecting the airways and is characterized by a chronic inflammatory response involving several cellular and humoral mediators. Prototypical asthmatic airway inflammation is believed to be triggered by IgE-dependent mast cell degranulation that results in the recruitment of monocytes, lymphocytes, and particularly, large numbers of eosinophils as well as the induction of several T\(_H\)2 cytokines. This chronic inflammatory response is believed to be the underlying cause of airway remodeling; characterized by sub-epithelial fibrosis, goblet cell hyperplasia, and increased smooth-muscle mass [9], and has been associated with increased asthma severity [197].

Current asthma management strategies principally involve the use of bronchodilators in the form of short- and long-acting β2 agonists, as rescue medication during exacerbations and to prolong the time to exacerbation, respectively, and inhaled corticosteroids to minimize the underlying inflammation. Despite having been shown to be very effective in preventing inflammation [10] by inhibiting cytokine transcription [198] and eosinophil survival [199;200], inhaled corticosteroids have been relatively under-prescribed because of concerns, by primary-care physicians and patients alike, over potential side effects which may include oral candidiasis, dysphonia, growth retardation, skin thinning/bruising, and loss of bone mineral density [201]. There is therefore a need for alternative approaches to the treatment of the inflammation underlying asthma pathology.

Considerable effort has recently been devoted to exploring the potential of immunomodulatory treatment of asthma by means of Toll-like receptor (TLR) activation [1]. TLRs are a family of molecules which mediate pathogen associated molecular pattern recognition and which play a crucial role in both innate and adaptive immunity [11;12]. These widely expressed proteins are
some of the first molecules to be involved in generating immune responses to environmental stimuli, a process that is mediated by cellular activation and stimulation of cytokine production [91]. Furthermore, several TLRs or TLR ligands have been associated with asthma. Elevated levels of lipopolysaccharides, which are TLR4 ligands, have been negatively correlated with the incidence of asthma in several studies [129;133], while CpG oligodeoxynucleotides, which are TLR9 ligands, have been used, with varying degrees of success, in the treatment of asthma in animal models [146;147;149;150].

The natural ligands of TLR7 are believed to be a subset of single–stranded RNA molecules of viral origin [101;102;179], however, this receptor, as well as TLR8, are also known to be ligated by members of the imidazoquinoline family of pharmaceutical compounds [180;202]. Resiquimod (also known as S28463 and R-848) is a member of the imidazoquinoline family and has been shown to induce the expression of a wide range of cytokines in many species from rodents to primates [178]. Our group [2;143], and others [144;203] have previously demonstrated that resiquimod treatment can prevent the development of lung pathology, including airway hyperresponsiveness and eosinophilia, in mouse models of acute asthma. Based on studies using BALB/c mice, Quarcoo and colleagues suggested that treatment with resiquimod causes a shift from an allergic T\textsubscript{H}2 to a non-allergic T\textsubscript{H}1 biased immune response [144]. In two other strains of mice (C57BL/6 with very low airway hyperresponsiveness and A/J with a very high airway hyperresponsiveness) treatment with S28643 resulted in a dramatic inhibition of the acute inflammatory reaction that results from allergen exposure in properly sensitized animals and this inhibition was accompanied by a concurrent inhibition of both T\textsubscript{H}1 and T\textsubscript{H}2 cytokines [2].

Given that resiquimod treatment could prevent the acute inflammatory reaction associated with allergen challenge in several genetically diverse strains of mice, we postulated that this compound could also inhibit the acute
inflammatory response in other species. Furthermore, we hypothesized that if
treatment with resiquimod was capable of preventing the acute inflammatory
reaction caused by allergen exposure, it might also prevent the development of
the chronic remodeling associated with repeated allergen exposure. The
potential of Toll-like receptor 7 ligands in preventing changes associated with
chronic asthma remains to be addressed.

The Brown Norway rat has been shown to develop airway remodeling
consistent with asthma after only two weeks of allergen challenge [204;205]
making this species a convenient experimental model for the study of the
chronic aspects of the disease. Utilizing an acute ovalbumin (OVA) sensitized and
challenged Brown Norway rat model of asthma; we demonstrate that
resiquimod treatment is able to prevent the development of allergen induced
inflammation and that this effect is associated with a reduction of both T$_H$1 and
T$_H$2 cytokine levels and in a dramatic reduction of inflammatory cell infiltration
into the lungs. Furthermore, using a chronic model of OVA sensitized and
challenged rats; we demonstrate that continued treatment with resiquimod is
capable of preventing the increase in smooth muscle mass and goblet cell
hyperplasia associated with airway remodeling in asthma. We also demonstrate
that this effect is mediated, at least partially, by inhibiting the proliferation of
epithelial and subepithelial airway cells.
Materials and Methods

Animals

All animals used in this study were eight to ten week old male Brown Norway rats (Harlan, Bicester, UK) and all procedures performed on the animals were in compliance with the Canadian Council of Animal Care (CCAC) guidelines and were approved by the McGill University Animal Care Committee.

Sensitization and Challenge for the Assessment of the Acute Inflammatory Response

Ovalbumin (OVA) sensitization was performed by concurrent subcutaneous injection of 1mg OVA (Grade V, Sigma, Oakville, Ontario, Canada) adsorbed to 100mg of aluminium hydroxide (EM Industries Inc., Hawthorne, NY) in 1mL of saline, and intra-peritoneal injection of $5 \times 10^8$ heat killed *Bordetella pertussis* bacilli (provided by Dr. Thomas Issekutz, Dalhousie University, Halifax, Nova Scotia, Canada) in 0.5mL of saline as adjuvant. Fourteen days following sensitization, the animals were challenged by aerosol exposure to either a 1% OVA solution, OVA-OVA and OVA/S28 groups, or to PBS vehicle only (OVA-PBS and OVA-S28/PBS groups), for 30 minutes. Twenty-four hours before the antigen challenge, two groups of rats, OVA-S28/OVA and OVA-S28/PBS, were treated by an intra-peritoneal injection of 1mg of resiquimod (resiquimod is generously provided by Dr. Richard Miller; 3M Pharmaceutical, Saint-Paul, MN). All animals were sacrificed 24 hours after antigen challenge, $n = 6$ for all groups, pooled from three separate experiments.

Sensitization and Challenge for the Assessment of Chronic Airway Remodeling

OVA sensitization was induced as described above. Thereafter, three antigen challenges were performed, at 5 day intervals and beginning on the $14^{th}$ day following sensitization, by intra-tracheal instillation of a 5% solution of OVA ($n = 6$) in saline or with saline vehicle only ($n = 5$). One group of rats ($n = 6$) received
Bronchoalveolar Lavage (BAL) Fluid and Cellular Analysis

Upon sacrifice, 24 hours following the final allergen challenge, 25mL of saline was instilled into the lungs of the animals in 5 equal portions of 5mL. The total cell counts were determined by use of a hemacytometer. Three hundred cells were identified on each BAL cytospin slide using the Diff-Quik Stain Set (Dade Behring Inc., Newark, DE).

Plasma IgE

Upon sacrifice of the animals, 24 hours following the final challenge, blood was collected by cardiac puncture and clotting was prevented by the addition of EDTA. Plasma was recovered after centrifugation of the blood samples and the total IgE levels were measured by ELISA. The ELISA was performed on Immulon 2 HB 96-well plates (Fisher, Ottawa, ON) coated with mouse anti-rat IgE monoclonal antibodies (BD Biosciences, Mississauga, ON). Rat IgE protein was detected with biotin-conjugated mouse anti-rat IgE monoclonal antibodies (BD Biosciences, Mississauga, ON) and streptavidin-HRP (R&D Systems, Minneapolis, MN). Rat IgE monoclonal isotype standard (BD Biosciences, Mississauga, ON) was used as a standard.

Cytokine Analysis

Upon sacrifice of the animals, 24 hours following the final challenge for the acute model and 48 hours following the final challenge for the chronic model, the right lung was dissected, frozen, and stored at -80°C. A single lobe from each sample was then homogenized in 4mL of PBS containing a protease inhibitor cocktail (Complete Inhibitor, Roche Diagnostics, Laval, PQ, Canada) and the total protein concentrations were adjusted using the Bio-Rad protein assay dye (BioRad Laboratories, Hercules, CA). Samples were concentrated 20-fold with
Microcon YM-3 centrifugal filters (Millipore, Billerica, MA) before analysis using a custom Lincoplex rat cytokine kit (Linco Research, St. Charles, MO) on a Luminex 100 LS apparatus with software version 2.3 (Luminex Corporation, Austin, TX). The cytokine protein levels reported refer to the cytokine levels of the samples in the lung homogenates as they were originally prepared, and not the 20-fold concentrated homogenates as the samples were assayed.

**Preparation of Histological Samples**

For preparation of the histological sections, the left lung was dissected and fixed with 10% formalin overnight with a pressure of 25cmH$_2$O. The paraffin embedded tissues were cut into 5 µm sections for histological analysis, deparaffinized, hydrated, and then stained with a standard hematoxylin and eosin (H&E) procedure.

**Measurement of Chronic Inflammation**

Chronic inflammation was assessed by histological analysis of haematoxylin and eosin stained lung sections prepared as described above. Quantification of the inflammation present was accomplished by counting the number of cells in the peribrochial space surrounding each airway and standardizing for airway size by dividing the count by the square of the perimeter of the basement membrane ($P_{bm}^2$). Eosinophils were identified on the basis of nuclear morphology and the presence of eosin staining in the cytoplasm.

**Measurement of Airway Smooth Muscle (ASM) Mass**

The mass of ASM was estimated from the measurement of the area of smooth muscle specific $\alpha$-actin ($\alpha$-SMA) immunoreactivity as previously described [206]. Briefly, sections, as described above, were stained with a mouse monoclonal antibody to $\alpha$-SMA (clone 1A4) (Sigma-Aldrich) and a biotinylated horse anti-mouse IgG (Vector Laboratories). The signal was detected with Vector Red (Vector Laboratories). The area of ASM was traced using a camera lucida side arm attachment to the microscope and digitized [206]. The mass of ASM
was standardized for airway size by dividing the area of ASM by the square of the perimeter of the basement membrane ($P_{bm}^2$).

**Goblet Cell Hyperplasia**

Goblet cell hyperplasia was assessed by histological analysis of periodic acid-Schiff (PAS) stained sections of lungs prepared as described above. Goblet cell hyperplasia quantification was accomplished by counting the number of PAS$^+$ cells, and standardizing for airway size by dividing the count by the perimeter of the basement membrane ($P_{bm}$), for at least five different airway cross-sections per animal.

**Proliferating Airway Epithelial Cell and Sub epithelial Cell Quantification**

Proliferation of airway epithelial and sub epithelial cells was investigated using immunostaining for proliferating cell nuclear antigen (PCNA) using a monoclonal antibody to PCNA (Calbiochem). Staining was preceded by high-temperature epitope unmasking in antigen retrieval solution (Vector Laboratories) and permeabilization in 0.2% Triton X-100 (Sigma-Aldrich). Sections were then blocked with 20% horse serum (Vector Laboratories) in universal blocking solution (Dako Cytomation), and a monoclonal antibody to PCNA was detected with biotinylated horse anti-mouse IgG, avidin/avidin-alkaline phosphatase complex, and BCIP/NBT chromogen substrate (Vector Laboratories), followed by methyl green (Sigma-Aldrich) counterstain. The number of PCNA positive airway epithelial and sub epithelial cells was counted and corrected for airway size by dividing by $P_{bm}$ and $P_{bm}^2$ respectively.

**Statistical Analysis**

All data were analyzed with GraphPad Prism 4 (version 4.03, GraphPad Software Inc.) using a parametric one-way analysis of variance (ANOVA) method followed by a Bonferroni post-test procedure. Data were normalized prior to
analysis by logarithmic or square root transformation where appropriate. A p-value $\leq 0.05$ was considered significant.
Results

Treatment with resiquimod prevents the influx of inflammatory cells into the BALF

Having previously established that resiquimod prevents acute airway inflammation in a mouse model of allergic asthma [2], in this study we investigated if the compound had an effect on the development of airway remodeling in a rat model of chronic asthma. We first established the effectiveness of resiquimod treatment in preventing the acute inflammatory reaction observed after a single allergen challenge. A prototypical finding in allergic asthma and its models is an influx of inflammatory cells, composed primarily of eosinophils but often also including neutrophils and macrophages, into the bronchoalveolar space. As expected (Fig. 1A), there was a dramatic increase in the number of cells recovered in the BALF of sensitized and OVA challenged (OVA) rats when compared with rats that were challenged with vehicle only (PBS). Treatment with resiquimod (S28/OVA) significantly reduced the number of inflammatory cells recovered in the BALF of OVA challenged rats while not causing a significant difference in sham challenged animals (S28/PBS). While both the number of macrophages and lymphocytes is increased in the BALF of OVA challenged animals (Fig. 1B), eosinophils, and to a lesser extent neutrophils, account for the majority of the increase. The dramatic increase in eosinophil numbers was significantly inhibited by treatment with resiquimod while neutrophil and lymphocyte numbers trended downwards, but the observed decrease did not reach statistical significance.

Treatment with resiquimod prevents airway inflammation

It has been previously observed that, in human subjects treated with anti-IL-5 antibodies [207], it is possible to affect eosinophil levels in the BALF compartment to a greater extent than in the tissue compartment. We therefore evaluated inflammatory cell infiltration of the area surrounding the airways and blood vessels in the lung by histological analysis. Sensitization with OVA
followed by sham PBS challenge (Fig. 2A), or followed by resiquimod treatment and sham challenge (Fig. 2B), did not cause any noticeable inflammation. The lungs of animals that were sensitized and challenged with OVA contained large numbers of inflammatory cells (Fig. 2C) concentrated near the airways and in the perivascular areas between the airways and blood vessels. Treatment with resiquimod prior to allergen challenge with OVA results in a marked decrease of inflammatory cell infiltration (Fig. 2D).

**Treatment with resiquimod causes a decrease in both T\textsubscript{H}1 and T\textsubscript{H}2 cytokine levels**

Allergic asthma is an atopic disease, a class of diseases characterized by elevated blood IgE levels. OVA sensitized and challenged animals treated with resiquimod (Fig. 3) had significantly lower plasma IgE levels than those animals which were not treated with the compound.

The predisposition towards an IgE mediated immune response in individuals suffering from allergic asthma is closely related to an elevated number of T\textsubscript{H}2 cells and their associated cytokines [188;208]. Furthermore, TLR7 ligation has been associated with the induction of T\textsubscript{H}1 cytokines in several models [144;209]. We therefore used a cytokine protein multiplex assay to assess the production of both T\textsubscript{H}1 and T\textsubscript{H}2 cytokines elicited by OVA challenge of sensitized rats when treated with resiquimod.

Both the T\textsubscript{H}2 cytokines IL-4 (Fig. 4A), IL-5 (Fig. 4B) and IL-13 (Fig. 4D) and the T\textsubscript{H}1 cytokine IFN-\gamma (Fig. 4E) were strongly induced (16, 85, 10, and 32 fold relative to PBS control, respectively) 48 hours after OVA challenge (OVA) when compared to mock challenged animals (PBS, Fig. 4A, B, D, and E, respectively). The inflammatory cytokines IL-1\alpha (Fig. 4F), IL-1\beta (Fig. 4G), and IL-6 (Fig. 4C) were also found to be induced to a much greater extent in the OVA challenged animals than in the PBS group (Fig. 4C, F, and G). Furthermore, the chemokines CXCL-1
(Fig. 4H) and MIP-1α (Fig. 4F) were also elevated in OVA challenged animals, but to a lesser extent than the other cytokines.

Treatment with resiquimod resulted in a dramatic decrease of cytokine production associated with OVA challenge. Production of the T\textsubscript{H}2 cytokines IL-4, 5, and 13 following OVA challenge was significantly lower (Fig. 4A, B, and D, respectively) in the resiquimod treated group (S28/OVA) than in the untreated controls. Similarly, production of the inflammatory cytokine IL-6 (Fig. 4C) following antigen challenge was significantly lower in resiquimod treated rats, while IL-1α and IL-1β (Fig. 4F, and G, respectively), as well as CXCL-1 (Fig. 4H) levels trended downwards but did not reach statistical significance. Importantly, the production of the T\textsubscript{H}1 cytokine IFN-γ (Fig. 4E) induced by antigen challenge was also significantly reduced in animals treated with resiquimod.

The cytokine profile (Fig. 4) of animals that received a mock challenge (PBS) was very similar to the profile of the animals who received both the resiquimod treatment and the mock challenge (S28/PBS) as evidenced by the fact that none of the cytokine levels tested was significantly different. IL-9 and eotaxin were not found to be significantly modulated by any treatment (data not shown).

**Treatment with resiquimod prevents the airway inflammation associated with repeated allergen challenge**

After characterizing acute inflammatory responses to allergic stimulation with OVA, we then assessed the ability of resiquimod to prevent the development of airway remodeling and inflammation induced by repeated allergen challenge. The presence of inflammatory cells surrounding the airways of animals after repeated allergen challenge was evaluated by histological analysis (Fig. 5). Sensitization with OVA followed by repeated allergen (OVA) challenges resulted in significant inflammatory cell infiltration in the area surrounding airways (Fig. 5B). Comparatively few inflammatory cells could be found surrounding the airways of animals that had been sensitized with OVA but sham challenged with
PBS (Fig. 5A). Similarly, the airways of OVA sensitized and challenged animals that had received resiquimod treatment displayed a markedly lower numbers of inflammatory cells (Fig. 5C).

The level of inflammation was quantified by counting the number of cells in the peribronchial area surrounding the airways of haematoxylin and eosin stained lung sections and normalizing this count for airway size by dividing by the square of the perimeter of the basement membrane ($P_{bm}^2$). Sensitization and challenge with OVA leads to a significant increase in the number of inflammatory cells with an important eosinophil component accounting for 37% of all inflammatory cells surrounding the airways (Fig. 5D). Treatment with resiquimod leads to a complete abrogation of the increase in inflammatory cells (Fig. 5D). Additionally, treatment with resiquimod leads to a dramatic decrease in eosinophil numbers when compared to OVA sensitized and challenged animals that did not receive treatment.

**Treatment with resiquimod prevents the increase in airway smooth muscle (ASM) specific $\alpha$-actin positive cell mass**

One of the key structural changes observed in asthmatic lungs is an increase in ASM mass. Sensitization and repeated challenge of an animal leads to more ASM specific $\alpha$-actin staining (Fig. 6B) in lung sections than can be observed in animals that are mock challenged (Fig. 6A). The lungs of animals that were treated with resiquimod (Fig. 6C) were not visually distinguishable from the mock challenged lung sections and showed less $\alpha$-actin immunoreactivity than the OVA sensitized and challenged lung sections (Figure 6).

ASM $\alpha$-actin positive cell mass was quantified as the area of ASM specific $\alpha$-actin staining cells divided by the square of the perimeter of the basement membrane ($P_{bm}^2$). $P_{bm}^2$ was used to correct measurements for airway size. Quantitation of ASM $\alpha$-actin positive cell mass confirmed our observations by visual inspection. OVA sensitized and challenged rats have almost three-fold
greater ASM α-actin positive cell mass than rats that were PBS challenged while resiquimod treatment leads to an essentially complete abrogation of the ASM α-actin positive cell mass increase (Fig. 6D).

**Goblet cell hyperplasia is prevented by resiquimod treatment**

A second alteration of airway structure characteristic of the chronic remodeling changes in asthma is goblet-cell hyperplasia. Goblet cells can be easily identified using the periodic acid-Schiff (PAS) method which stains mucus glycoproteins. Only a relatively few and isolated PAS⁺ cells can be observed in the airway epithelium of OVA sensitized and mock challenged lungs (Fig. 7A). OVA challenge leads to an easily observable increase in airway PAS⁺ cells (Fig. 7B) which was inhibited in animals that were treated with resiquimod (Fig. 7C).

Goblet cell hyperplasia was quantified by counting the number of PAS⁺ airway epithelial cells and dividing this number by $P_{bm}$. Confirming the observations made by visual inspection of the slides, OVA sensitized and challenged animals had an elevated number of PAS⁺ airway epithelial cells compared to mock challenged animals (Fig. 7D). Again, resiquimod treatment prevented the increase in PAS⁺ airway epithelial cell numbers induced by repeated allergen challenge (Fig. 7D).

**Resiquimod treatment prevents the proliferation of airway cells in response to repeated allergen challenge**

One of the mechanisms believed to contribute to ASM mass increases in asthma is ASM cell proliferation [210;211]. Similarly, proliferation may account for the increase in the number of goblet cells found in the airways. We therefore evaluated the expression of proliferating cell nuclear antigen (PCNA) in epithelial cells and in the subepithelial cells corresponding to the smooth muscle zone to determine if proliferation might be involved in the goblet cell hyperplasia or ASM mass increase respectively.
The airways of PBS challenged rats only contain relatively low number of PCNA-positive epithelial (Fig. 8A, red arrows) and subepithelial (Fig. 8A) cells scattered along the airway wall. OVA challenge led to a clear increase in the number of PCNA-positive airway epithelial cells (Fig. 8B, red arrows) visible in the histological samples. Furthermore, OVA challenge led to a dramatic increase in the number of subepithelial PCNA⁺ cells which could be observed in the ASM zone (Fig. 8B, blue arrow). Treatment with resiquimod prior to allergen challenge prevents the increase in both epithelial and subepithelial PCNA⁺ cells, as can be observed by inspection of the lung sections, which reveal relatively few isolated epithelial PCNA⁺ cells and even fewer PCNA⁺ subepithelial cells (Fig. 8C).

Quantification of epithelial and subepithelial PCNA⁺ cells was accomplished by counting the number of epithelial or ASM zone subepithelial cells that were positively stained and dividing this number by \( P_{\text{bm}} \) and \( P_{\text{bm}}^2 \) respectively. Quantitative analysis of the histology indicates that repeated allergen challenge leads to an almost four-fold greater number of PCNA⁺ airway epithelial cells than that found in the mock challenged control (Fig. 8D). Furthermore, there was a significant eight-fold increase in the number of PCNA⁺ subepithelial cells located in the ASM zone in OVA challenged animals when compared to PBS challenged animals (Fig. 8D). Treatment with resiquimod prior to allergen challenge completely abrogates the increase in both epithelial and subepithelial PCNA⁺ airway cells (Fig. 8D).

**Cytokine levels in the lungs of chronically challenged animals**

As illustrated in Fig. 4 and Table 1, we found a significant induction of both T\( _{\text{H}}1 \) and T\( _{\text{H}}2 \) cytokines in the lungs of OVA sensitized and challenged rats 24 hours after the first allergen challenge. 48 hours after the final allergen challenge, however, we did not detect elevated levels of any cytokine (Table 1). The levels of the cytokines in the rats that were chronically challenged with allergen (OVA) did not remain significantly elevated, when compared to the mock challenged animals (PBS), when assessed at the 48 hour time point. Both
of these groups expressed the measured cytokines at levels comparable to those found in the lungs of mock challenged animals 24 hours after the initial exposure.
Discussion

This study was initiated in order to characterize the effect of treatment, with the TLR7 ligand resiquimod, on the early inflammatory responses to allergen challenge in a rat model of asthma. We found that, as in the mouse model, resiquimod was able to effectively inhibit the inflammatory reaction in response to allergen. These encouraging findings prompted us to test if resiquimod could prevent the development of chronic airway remodeling. The results presented clearly demonstrate that continued treatment with this TLR7 ligand could prevent the development of chronic airway remodeling.

Sensitization and challenge of the Brown Norway rats resulted in a significant recruitment of cells, composed mainly of eosinophils and to a lesser extent neutrophils, into the airway tissue and alveolar space. Animals that were treated with resiquimod one day prior to allergen challenge in this acute model of allergic asthma showed greatly reduced eosinophilic inflammation, consistent with previous observations in murine models. Furthermore, we found that, during the initial phase of the allergic response to allergen exposure, total IgE levels were lowered by resiquimod treatment, which is also consistent with the published results of experiments performed in murine models [2;144]. We also observed an increase in the T\textsubscript{H}2 cytokines IL-4, IL-5, and IL-13, the T\textsubscript{H}1 cytokine IFN-\gamma, as well as an increase in IL-6 following allergen challenge which was prevented by resiquimod treatment.

Previous studies employing TLR7 ligands have demonstrated that signaling through this receptor can stimulate the production of the T\textsubscript{H}1 cytokines IL-12, IFN-\alpha, and IFN-\gamma in a CD4\textsuperscript{+} lymphocyte and macrophage co-culture [209]. The cell type responsible for the bulk of the IL-12 production was determined to be the macrophage and additionally, resiquimod treatment of these cultures resulted in an inhibition of IL-5 production which was only partially mediated by IFN-\alpha [209]. Furthermore, studies conducted using human cells have shown that
resiquimod can directly induce proliferation of, and up-regulate IFN-γ production in, effector memory CD4+ T-cells [212]. Neither of these mechanisms, however, would fully account for the available data.

The natural resistance-associated protein 1 (Nramp1, now Slc11a1) gene is macrophage-restricted, involved in modulating macrophage responsiveness to TLR7 agonists [15], and has been shown to be necessary for the TLR7 ligand, resiquimod, induced activation of macrophages in a murine model of Mycobacterium bovis BCG infection [196]. In the BCG model, resiquimod treatment allows wild-type Nramp1+/− mice to clear the infection of the intracellular parasite, on the other hand, Nramp1−/− or Nramp1S (susceptible) mice did not respond to resiquimod treatment. Our previous results demonstrated that resiquimod treatment in an acute model of allergic asthma was effective in both Nramp1+/− and Nramp1S mice [2]. These findings suggest that prevention of the acute allergic inflammation of the airways seen in asthma is not dependent on macrophage responsiveness to TLR7 ligands.

While treatment with resiquimod in a model of allergic asthma in the BALB/c mouse appears to result in an increase in Th1 cytokines, particularly IFN-γ [144], we have previously shown that, in both A/J and C57BL/6 mice, a similar treatment which successfully prevents the development of airway inflammation and hyperresponsiveness does not cause an increase in IFN-γ or IL-12p70 [2]. The present study indicates that treatment in the Brown Norway rat produces a similar result. In fact, in both the present study and in our previous work [2] we find that IFN-γ is elevated following allergen challenge and that TLR7 ligand treatment prevents this induction. These findings suggest that it is unlikely that resiquimod exerts its effect simply by redirecting the Th2 immune response typical of allergic asthma towards a Th1 type response.

Th2 responses and activated CD4+ cells generated by the acute inflammatory reaction have both been implicated in the development of chronic airway
remodeling [205;213]. It is therefore not surprising, but nonetheless important, that TLR7 ligand treatment can suppress the development of airway remodeling given the effectiveness with which it prevents the development of the initial inflammatory events.

Goblet cell hyperplasia may contribute to airway obstruction through excessive mucus production [214]. We have demonstrated in this study that continued treatment with the TLR7 ligand resiquimod prevents the development of goblet cell hyperplasia. Another important aspect of airway remodeling, which has been linked to asthma severity and is assumed to contribute to the development of airway hyperresponsiveness [197], is the increase in smooth muscle mass in the asthmatic airways. Given that eosinophils have been shown to play a crucial role in the disease process leading to the marked increase in smooth muscle area [215], preventing the development of the eosinophilic inflammation after allergen challenge would be expected to prevent the increase in smooth muscle mass observed in allergen sensitized and challenged animals. TLR7 ligand treatment behaved as expected in this respect as we find no evidence of an increase in smooth muscle mass in the resiquimod treated animals.

The increase in ASM α-actin positive cell mass can occur through hyperplasia or hypertrophy. We have observed an increase in PCNA+ cells in the subepithelial smooth-muscle zone of OVA sensitized and challenged rats. We therefore believe that an increase in proliferation of ASM α-actin positive cells is at least partially responsible for the ASM mass increase in our model. Similarly, we observe an increase in epithelial PCNA+ cells which leads us to believe that the goblet cell hyperplasia we observe in the chronically challenged animals is also at least partially caused by an increase in the proliferation of goblet cells or their precursors.
In summary, our data demonstrates that treatment with resiquimod, a TLR7/8 ligand, can prevent the development of the inflammatory reaction that results from allergen exposure in sensitized rats and that this was accomplished through inhibition of both Th1 and Th2 cytokines. Our data also demonstrated that continued treatment with resiquimod can prevent the development of chronic airway remodelling including goblet cell hyperplasia and airway smooth muscle hyperplasia. The ability of TLR7 signalling to modulate both acute and chronic asthma pathology makes this receptor a prime target for a new class of therapies aimed at minimizing the pathology associated with chronic inflammation in asthma. Furthermore, elucidation of the mechanism by which TLR7 modulates the immune response in the context of asthma should provide us with invaluable insight into the processes that lead to the development of the disease.
Acknowledgements

We kindly acknowledge the contributions of Dr. Yasuyuki Yoshizawa from the Department of Geriatric and Pulmonary Medicine, Tokyo Medical and Dental University, Tokyo. This work was supported by a Senior Fellow Award from the American Asthma Foundation [formerly the Sandler Program for Asthma Research (SPAR)] awarded to Dr. D. Radzioch and a grant from the Canadian Institutes of Health Research (MT 10381) to Dr. J. Martin. Resiquimod (S28463, R-848) was kindly provided by Dr. Richard Miller (3M Pharmaceutical). Pierre Camateros had been a recipient of a Natural Sciences and Engineering Council (NSERC) Postgraduate Scholarship and a Canadian Graduate Scholarship Doctoral Award from the Canadian Institutes of Health Research (CIHR). Dr. Jacques Moisan was a recipient of a Doctoral Research Award from the CIHR.
Table 1: Lung Cytokine Levels 24 and 48 Hours Post Challenge

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>24h After Challenge</th>
<th>48h After Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>OVA</td>
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<tr>
<td>IL-4</td>
<td>0.22 ± 0.03</td>
<td>3.5 ± 0.7</td>
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<tr>
<td></td>
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<tr>
<td>IL-5</td>
<td>0.17 ± 0.02</td>
<td>14.6 ± 4.2</td>
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<tr>
<td>IL-6</td>
<td>1.3 ± 0.2</td>
<td>19.3 ± 4.6</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>1.8 ± 0.3</td>
<td>19.3 ± 4.3</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>IFN-γ</td>
<td>0.19 ± 0.07</td>
<td>6.1 ± 1.2</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>IL-1α</td>
<td>41.8 ± 6.4</td>
<td>323 ± 70</td>
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<td></td>
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<td></td>
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<tr>
<td>IL-1β</td>
<td>4.7 ± 0.6</td>
<td>28.6 ± 5.0</td>
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<td></td>
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<td></td>
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<tr>
<td>CXCL-1</td>
<td>142 ± 22</td>
<td>301 ± 91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>4.3 ± 0.2</td>
<td>7.8 ± 0.6×</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Cytokine levels in the lung homogenate of sensitized and challenged rats. Rats were OVA sensitized and challenged three times at 5 day intervals, with either OVA (OVA and S28/OVA) or PBS (PBS) with (S28/OVA) or without (PBS and OVA) prior treatment with resiquimod. The animals were sacrificed 24 hours after the first challenge or 48 hours after the final challenge. Data presented as mean ± SEM in pg/mL (n = 6 for all groups). † signifies p < 0.05 and † † signifies p < 0.001 for OVA vs. PBS groups. ‡ signifies p < 0.05 and ‡ ‡ signifies p < 0.001 for OVA vs. S28/OVA groups.
Figure 1: Inflammatory Cells in the BALF

Analysis of inflammatory cells found in bronchoalveolar lavage fluid (BALF) following imidazoquinoline treatment (S28/OVA and S28/PBS) and OVA (OVA and S28/OVA) or PBS (PBS and S28/PBS) challenge. Total number of inflammatory cells recovered in the BALF of the animals (A). Differential count of the inflammatory cells recovered (B). Results represented as mean ± SEM (* designates significance between PBS and OVA, ** designates significance between OVA and S28/OVA, n = 5 for S28/PBS group, n = 6 for all other groups).
**Figure 2: Inflammation in Lung Tissues**

Representative Haematoxylin and Eosin stained lung sections collected 24 hours after challenge from animals acutely sensitized with OVA and challenged with PBS (A and B) or OVA (C and D) with prior treatment with (B and D) or without (A and C) resiquimod treatment.
Figure 3: Plasma IgE Levels.

Total blood plasma IgE levels in rats following imidazoquinoline treatment (S28/OVA and -S28/PBS) and OVA (OVA and S28/OVA) or PBS (PBS and OVA-S28/PBS) challenge. Data represented as medians ± interquartile range (n = 6 for all groups).
Figure 4: Cytokine Levels Following Acute Antigen Challenge

Cytokine levels in lung homogenates of OVA sensitized animals following challenge with PBS (PBS and S28/PBS) or OVA (OVA and S28/OVA) with (S28/PBS and S28/OVA) or without (PBS and OVA) prior imidazoquinoline treatment. Data presented as mean ± SEM (* designates p < 0.05, *** designates p < 0.001, n = 6 for all groups).
Figure 5: Inflammation in the Lungs of Chronically Challenged Rats

Representative Haematoxylin and Eosin stained lung sections collected 48 hours after challenge from animals sensitized with OVA and chronically challenged with PBS (A) or chronically challenged with OVA (B and C) with prior treatment with (C) or without (A and B) resiquimod. Peribronchial inflammation was quantified (D) by counting the number of inflammatory cells, or eosinophils, surrounding airways and normalizing for airway size by dividing by the square of the perimeter of the basement membrane ($P_{bm}^2$). Data presented as mean ± SEM (* designates $p < 0.01$ between PBS and OVA, ** designates $p < 0.01$ between OVA and S28, *** designates $p < 0.001$ between OVA and S28, n = 6 for all groups).
**Figure 6: Smooth Muscle Mass of Chronically Challenged Rats**

Representative airway smooth muscle specific α-actin stain (red stain) from OVA sensitized animals chronically challenged with PBS (A), OVA (B), or OVA following resiquimod treatment (C). Smooth muscle specific α-actin staining area was standardized for airway size by dividing by the square of the perimeter of the basement membrane of the airway ($P_{bm}^2$). Data presented as mean ± SEM ($n = 5$ for OVA group, $n = 4$ for all other groups).
Figure 7: Goblet Cell Hyperplasia in Chronically Challenged Rats

Representative PAS stain from OVA sensitized animals chronically challenged with PBS (A), OVA (B), or OVA following resiquimod treatment (C). Goblet cells stain magenta in colour. Goblet cell hyperplasia was determined by counting the number of PAS⁺ cells per airway and standardized for airway size by dividing by the perimeter of the basement membrane (P_{bm}). Data presented as mean ± SEM (n = 6 for all groups).
**Figure 8: Proliferation of Airway Cell in Response to Chronic Challenge**

Representative proliferating cell nuclear antigen (PCNA)-specific stain from OVA sensitized animals chronically challenged with PBS (A), OVA (B), or OVA following resiquimod treatment (C). The nucleus of PCNA-positive cells stain dark blue. PCNA-positive epithelial cells are indicated by red arrows and PCNA-positive subepithelial cells by a blue arrow. Quantification of PCNA$^+$ cells (D) of the airway epithelium and sub-epithelium (smooth muscle zone). The number of PCNA$^+$ cells were counted in each airway and standardized for airway size by dividing by the square of the perimeter of the basement membrane ($P_{bm}^2$, subepithelial cells) or the perimeter of the basement membrane ($P_{bm}$ epithelial cells). Data presented as mean ± SEM (* designates $p < 0.01$ between PBS and OVA, ** designates $p < 0.01$ between OVA and S28/OVA, $n = 6$ for PBS, $n = 4$ for OVA, $n = 5$ for S28).
Chapter 4 - Modulation of the Allergic Asthma Transcriptome Following Resiquimod Treatment

Adapted from:

Preface

The data presented in the two preceding chapters demonstrated that resiquimod treatment could prevent the pathology associated with both acute and chronic allergic asthma. The molecular processes which were altered following resiquimod, however, remained poorly characterized. The data presented in this chapter sheds light on the transcriptional consequences of resiquimod treatment and provides important clues regarding the mechanism of resiquimod action.
Abstract:

Resiquimod is a compound belonging to the imidazoquinoline family of compounds known to signal through Toll-like receptor 7. Resiquimod treatment has been demonstrated to inhibit the development of allergen induced asthma in experimental models. The aim of the present study was to elucidate the molecular processes which were altered following resiquimod treatment and allergen challenge in a mouse model of allergic asthma. Employing microarray analysis, we have characterized the “asthmatic” transcriptome of the lungs of A/J and C57BL/6 mice and determined that it includes genes involved in the control of cell cycle progression, the complement and coagulation cascades, and chemokine signalling. Our results demonstrated that resiquimod treatment resulted in the normalization of the expression of genes involved with airway remodelling, and generally, chemokine signalling. Resiquimod treatment also altered the expression of cell adhesion molecules, and molecules involved in natural killer (NK) cell-mediated cytotoxicity. Furthermore, we have demonstrated that systemic resiquimod administration resulted in the recruitment of NK cells to the lungs and livers of the mice, although no causal relationship between NK cell recruitment and treatment efficacy was found. Overall, our findings identified several genes, important in the development of asthma pathology, that were normalized following resiquimod treatment, thus improving our understanding of the molecular consequences of resiquimod treatment in the lung milieu. The recruitment of NK cells to the lungs may also have application in the treatment of virally induced asthma exacerbations.
Introduction

Asthma is a disease of the respiratory system, characterized by intermittent reversible airway obstruction, which is estimated to affect 300 million worldwide and results in a quarter million deaths annually [6]. While the airflow obstruction in asthma results from contraction of airway smooth muscles, the underlying pathology in asthmatic airways is inflammatory in nature. In allergic asthma the inflammatory reaction is typically driven by the IgE mediated recognition of environmental allergens resulting in the production of Th2 cytokines. These cytokines, in turn, participate in the recruitment of inflammatory cells such as CD4$^+$ T-cells, mast-cells, and especially eosinophils, to the airways [216].

Toll-like receptors (TLR) belong to a family of conserved pathogen associated molecular pattern recognizing receptors involved in the activation of innate and adaptive immune responses [91]. Each member of the TLR family (or combinations of two distinct TLR molecules) recognizes molecules derived from different pathogens; for example TLR4 recognizes lipopolysaccharides from gram-negative bacteria and TLR3 recognizes double stranded RNA molecules produced by some viruses during replication. The interaction between TLR ligands and the TLRs on innate immune cells, such as dendritic cells, leads to cellular activation and the production of cytokines resulting in a Th1 or Th2 polarized adaptive response [12;217;218]. TLR expression and signalling also occurs in non-immune cells, such as airway epithelial and smooth-muscle cells, where it again results in cytokine production [108-110]. Furthermore, several studies have demonstrated that TLR ligands are capable of modulating the expression of several asthmatic phenotypes [1].

Resiquimod (R-848, S-28463) belongs to the imidazoquinoline family of compounds and is known to signal, in an Myd88 dependent manner, through TLR7 in mice [180], and TLR7 and TLR8 in humans [177]. The natural ligands for
these TLRs are single stranded RNA molecules of viral origin \[101;102;179\] and, as might be expected based on this fact, TLR7/8 and their ligands in vitro \[209\] and in animal models \[13;14\] contribute to viral resistance through the production of type I interferons and T\(\text{H}1\) cytokines including IL-12 \[219\].

A fundamental mechanism which underlies allergic asthma pathology is a T\(\text{H}2\)-skewed immune response. The use of TLR7/8 ligands in the treatment of asthma, and in particular the use of resiquimod, which is both more potent and water soluble than many other TLR7 or TLR8 ligands, have been investigated by several groups \[2;3;143-145;203\] because these ligands were shown to promote the development of T\(\text{H}1\)-skewed immune responses both \textit{in vitro} \[209;220-222\] and \textit{in vivo} \[219;220\]. Studies in models of allergic asthma in mice \[2;144;145\] demonstrated that resiquimod treatment during or after antigen sensitization could prevent the development of airway inflammation and hyperresponsiveness, as well as IgE and mucus production. Furthermore, resiquimod treatment also prevented the development of airway remodelling, in a chronic asthma model in the rat \[3\], as assessed by airway smooth-muscle mass and goblet cell hyperplasia.

Despite the demonstration that resiquimod treatment is effective in preventing the development of allergic asthma in rodent models, the molecular and cellular consequences of this treatment remain poorly characterized. \textit{Ex vivo} resiquimod treatment of human T\(\text{H}2\) cells was able to stimulate IFN-\(\gamma\) production \[140\] and when using BALB/c models of allergic asthma, both pulmonary IFN-\(\gamma\) \[144\] and IL-12 \[145\] were found to be induced following systemic resiquimod treatment. Again in BALB/c mice, resiquimod treatment was found to be partially effective in IL-12p35\(^{-/-}\) mice unless IL-10 was simultaneously ablated \[145\], in which case treatment had no effect. Furthermore, \textit{in vitro} studies of human peripheral blood mononuclear cells \[141\] and mouse splenocytes \[142\] indicate that IgE production can be inhibited by resiquimod treatment in an (at least partially) IFN-\(\gamma\) or IL-12 dependent manner. However, neither IFN-\(\gamma\) nor IL-
12 was found to be increased in the lungs of similarly treated A/J or C57BL/6 mice. In fact, in the lungs of both A/J and C57BL/6 mice [2] and Brown Norway rats [3], IFN-γ levels were lower in treated animals and the treatment appeared to have systemic anti-inflammatory effects. While methodological differences between these studies may in large part explain the differences in TH1 cytokine modulation, all the studies demonstrated significant drops in TH2 cytokine levels in the lungs of treated animals.

Given the dramatic and positive effects of resiquimod treatment on the development of phenotypes associated with allergic asthma, and the relative paucity of data regarding the mechanism by which these effects are mediated, the aim of the present study was to elucidate the cellular and molecular processes which were altered following resiquimod treatment and antigen challenge in a mouse model of allergic asthma. To evaluate the changes in pulmonary gene expression resulting from resiquimod treatment in the context of a previously described model of allergic asthma in both A/J and C57BL/6 mice [2], we employed microarray analysis to identify the molecular pathways altered by resiquimod treatment.
Materials and Methods

Antigen Sensitization and Challenge, and Drug Treatment Protocol

A/J and C57BL/6 mice underwent an antigen sensitization, challenge and drug treatment protocol as previously described [2]. Briefly, animals were sensitized to ovalbumin (Grade VI, Sigma) by three weekly intra-peritoneal injections of ovalbumin in Imject Alum (Pierce). One week after the final sensitization injection, mice received either a 1% ovalbumin aerosol challenge (OVA, and RES) or a mock challenge (PBS) with a PBS aerosol, every 24 hours for three consecutive days. One group of mice (RES) received 100μg of resiquimod (in 0.2 mL of PBS by intra-peritoneal injection) one day prior to each antigen challenge. All procedures were reviewed and approved by the Montreal General Hospital and McGill University Animal Care Committees in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Microarray analysis

A/J and C57BL/6 mice (3 mice from each experimental group) were euthanized 3 hours after the final antigen aerosol challenge, at which point the whole lungs were dissected and immediately frozen in liquid nitrogen. Total lung RNA was extracted and purified using TRIzol (Invitrogen) according to the manufacturer’s instructions and subsequently used with Affymetrix Mouse430 v2.0 microarrays. The preparation of probes, the hybridization and the scanning of the microarrays were performed at the McGill University and Génome Québec Innovation Centre according to the manufacturer’s instructions.

Background correction and normalization were performed in the R environment (version 2.4.1, www.r-project.org) with the Bioconductor packages (version 1.12.2, www.bioconductor.org) using the robust multiarray average (RMA) method [223] of the affy package. Differential expression analysis was performed using the LIMMA package [224] (version 2.9.13). The data was encoded in a design matrix with all six strain/treatment combinations (A/J (PBS,
OVA, and RES) and C57BL/6 (PBS, OVA, and RES)) and then fitted to a linear model. The data was then first analyzed by making pairwise comparisons between the two strains for each treatment, between the PBS and OVA treatment groups for each strain separately, and between the OVA and RES treatment groups for each strain separately. The effects of ovalbumin challenge (PBS vs. OVA) and resiquimod treatment (OVA vs. RES) were then analyzed jointly in both strains by using the contrast representing the mean of the expression differences, between the treatment groups, of each strain. In all cases a multiple test correction was then performed using the Benjamini-Yekuteli method [225] of the LIMMA package [224] and finally, significant (at a false discovery rate = 0.05) genes were then filtered for a minimum expression fold difference of 1.5 (in the joint analysis, a minimum 1.5 fold difference in each of the strains). All microarray data has been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE13032.

Pathway analysis of genes identified as significantly altered between experimental groups was performed with EASE [226]. Over-representation of significant genes, amongst the population of genes which were present on the microarray, in Kyoto Encyclopedia of Genes and Genomes pathways (KEGG, www.genome.jp/kegg/) [227] was assessed using the EASE score and corrected for multiple testing by bootstrapping ($1 \times 10^4$ iterations) all probabilities. A pathway was considered significantly overrepresented if, after multiple testing correction, $p \leq 0.05$.

Fold changes used throughout this article represent the mean ratio of gene expression in the high expressing group to the low expressing group (the mean of the gene expression ratio of each strain). In comparisons of the sham challenged (PBS) group to the untreated control group (OVA), positive fold change values indicate that the OVA group is the high expressing group and negative values signify that the PBS group is the high expressing group. Similarly,
for comparisons involving the untreated control group (OVA) and the resiquimod treated group (RES) positive values represent higher expression in the RES group and negative values represent higher expression in the OVA group. In all cases, the median fold change value of all significantly altered probesets of any given gene is reported.

One outlier in the data was the 130 fold difference in expression of H2-D1 between A/J and C57BL/6 mice. The unusually large difference in H2-D1 appears to be caused by a pair of probesets (1427651_x_at and 1452544_x_at) whose target sequence in the H2-D1 gene contains a pair of polymorphic SNPs (between A/J and C57BL/6). These probesets were removed and not used in the subsequent analysis of strain differences. Of the next 45 genes with the largest absolute difference in expression between the strains, only one gene contained a SNP in the target sequence covered by a probeset. This SNP affected only one of the 11 probes within one probeset for this gene. Overall, this indicates that polymorphisms account for only a very small portion of the differences in expression between the strains and can generally be safely ignored.

**Real Time RT-QPCR validation of microarray data**

In an independent experiment, A/J mice were sensitized, challenged, and treated in an identical manner to the animals on which the microarray analysis was performed. The lungs of the experimental animals were snap-frozen in liquid nitrogen 3 hours after the final antigen aerosol challenge and homogenized in TRIzol reagent (Invitrogen) for RNA isolation. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturers recommendations. Quantitative PCR was performed using the Brilliant QPCR SYBR Green QPCR Master Mix kit (Stratagene) on the Mx4000 apparatus (Stratagene) with the following cycling conditions: denaturation 30s at 95°C, annealing 60s at 56°C, and extension for 30s at 72°C. Supplemental table 1 lists the primers used in the analysis.
Flow cytometry analysis

A/J, C57BL/6 and B6.129S2-Cd8a\textsuperscript{tm1Mak}/J (CD8 knock-out mice backcrossed 13 generations to C57BL/6) mice were euthanized 3 hours following the final antigen aerosol challenge, as described above, in order to confirm NK cell recruitment following resiquimod treatment in the asthma model, and for the NK cell depletion experiments described below. C57BL/6 mice were euthanized without antigen sensitization or challenge prior to receiving resiquimod, 3 hours after receiving the first, second, or third daily resiquimod dose, or 24, 48, or 96 hours following the third resiquimod dose in order to determine the effects of resiquimod treatment on NK cell recruitment. In all cases the lungs and livers were removed, placed in media, and finely cut using a scalpel blade. The finely cut pieces were placed in a digestion buffer containing 2mg/mL of collagenase 4 and 10μg/mL of DNase (Worthington Biochemical Corporation) then incubated for one hour at 37°C with continuous mixing. The resulting cell suspension was decanted and lymphocytes were extracted with Lympholyte M (Cedarlane Laboratories) according to the manufacturer’s instructions.

The cells were then washed and resuspended in PBS. Cells were pre-incubated with rat anti-mouse CD16/CD32 (Fcy III/II Receptor) (clone 2.4G2, BD Biosciences), then double stained with phycoerythrin conjugated rat anti-mouse CD49b (clone DX5, BD Biosciences) and fluorescein isothiocyanate conjugated rat anti-mouse CD11b (clone M1/70, BD Biosciences). The cells were subsequently analyzed using a FACScan (BD Biosciences) machine. Cell counts were gated for lymphocytes based on front and side scatter and NK cells were identified as gated CD49b and CD11b double positive cells.

NK cell depletion

NK cell depletion was accomplished by intra-peritoneal injection of 100μg of anti- NK-1.1 antibody (clone PK136, BioExpress Cell Culture Services) in a total volume of 200μL of PBS. The injection was carried out 24 hours prior to the first resiquimod treatment (as described above). NK cell depletion efficiency was
assessed by FACS analysis, as described below, and was found to result in a
greater than 90% depletion of NK cells in the lungs of the mice (Supplemental
figure 1).

**Bronchoalveolar lavage (BAL) fluid analysis**

Upon euthanasia, 48 hours following the final antigen-challenge, a blunt 18G
needle was inserted into the trachea of the animal. The lungs were then lavaged
with 1.2 mL of PBS (Invitrogen) which was subsequently kept on ice. The lavage
fluid was then centrifuged at 250 × g, the cell pellet was resuspended in 100 μL
of RPMI (Roswell Park Memorial Institute) medium (Invitrogen), and loaded into
a cytospin centrifuge which was used to spin (at 350 rpm) the cells onto glass
slides (Shandon Cytoslide, Thermo Scientific). The slides were fixed and stained
with the DiffQuik stain set (Dade Behring) and a differential count was
performed by a blinded observer.

**Other statistical analysis**

All flow cytometry, bronchoalveolar lavage fluid (BALF) cell counts, and RT-
QPCR data were analyzed with a one-way ANOVA followed by a Bonferroni post-
test. For BALF cell analysis, outliers which were more than two standard
deviations above or below the mean were discarded. All data expressed as mean
± SEM.
Results

Identification of genes differentially expressed between A/J and C57BL/6 mice

In order to evaluate the transcriptional differences induced by resiquimod treatment in antigen sensitized and challenged mice we performed a microarray analysis on the whole lung RNA of both A/J and C57BL/6 mice following antigen sensitization and mock challenge (PBS), antigen sensitization and challenge (OVA), or antigen sensitization and challenge with resiquimod treatment (RES). We have previously shown that, in these strains, this sensitization and challenge protocol results in the induction of asthmatic phenotypes and that the resiquimod treatment ablates these phenotypes [2]. For each treatment protocol, the lungs of three mice of each strain were collected three hours following the last antigen (or sham) challenge and analyzed with Affymetrix Mouse430 v2.0 microarrays.

The first set of comparisons performed compared gene expression in the lungs of A/J mice to the expression in the lungs of C57BL/6 mice in each treatment group (PBS, OVA, and RES). To assess the significance of differences in gene expression, genes were first selected on the basis of a p-value meeting the threshold for a 0.05 false discovery rate (as described in the Materials and Methods section). In our data, the threshold p-value was 1.91×10^{-4} when comparing A/J mice to C57BL/6 mice in the PBS group, 1.81×10^{-4} in the OVA group, and 2.97×10^{-4} in the RES group. Genes whose expression was significantly different were defined as those that met this p-value cut-off and had an absolute fold change, between the groups being compared, of 1.5 or more.

Using these criteria, we found that the expression of 1043, 1053, and 1483 genes was significantly different between the strains in the PBS, OVA, and RES groups respectively (Figure 1, Supplemental tables 2, 3, 4). Of the 1043 genes found to be differentially expressed in the PBS group, only 13% (136 genes) had
significant differences in expression only in the PBS group, a large majority (63% or 654 genes) were differentially expressed in all treatment groups, and the remainder (24%) were differently expressed in two groups including PBS. Similarly, the majority of genes found to be differentially expressed in the OVA group (62% of the 1053 genes) were differentially expressed in all three treatment groups. Furthermore, the majority of genes found to be differentially expressed between strains in more than one treatment group were differentially expressed by a similar ratio in all groups. It therefore appears that most of the initial differences in gene expression between the strains were maintained regardless of allergen challenge and resiquimod treatment.

The 1043 genes which were differentially expressed in the PBS group are involved in a multitude of biological processes and KEGG pathway overrepresentation analysis using EASE did not identify any significantly overrepresented pathways. On the other hand, a similar analysis of the 1053 genes which were found to be differentially expressed in the OVA group identified two overrepresented pathways: antigen processing and presentation, and cytokine-cytokine receptor interaction (Table 1, Supplemental table 5). Six of the 8 genes in the antigen processing and presentation pathway are histocompatibility 2 genes of which 1 was more highly expressed in A/J mice and 5 in C57BL/6 mice. The other two genes are Lgmn (a.k.a. AEP), a lysosomal protein involved in cathepsin L processing [228] with a role in extracellular matrix (ECM) remodelling in the kidneys [229] whose expression is higher in A/J mice, and Klrd1 (CD94) whose expression is higher in C57BL/6 mice and, as a dimer with Klrc1 (NKG2A), acts as an inhibitory receptor for NK cells. Genes in the cytokine-cytokine receptor pathway were evenly divided with respect to which strain had the highest level of expression. Most of these genes were chemokines with A/J mice having higher transcript levels for Ccl6, Ccl11 (eotaxin), Ccl24 (eotaxin-2), and Cxcl12 (SDF) and C57BL/6 mice for Ccl19 (MIP-3Î²), Ccl21a (SLC), Ccl27 (CTACK), Cxcl7, and Cxcl14 (BRAK, MIP-2Î±).
Most (60%) of the strain differences observed in the resiquimod treatment group were genes whose expression was also different in either the OVA or PBS groups. These genes also had the same differences in expression across the different treatments, indicating that most of these differences were unlikely to have been caused by resiquimod. However, a significantly larger fraction of the genes identified as differentially expressed in the RES group were found to be differentially expressed only in resiquimod treated animals. In fact, a full 40% of the differently expressed genes in resiquimod treated animals were only differentially expressed in these animals (versus 13% and 15% for the PBS and OVA groups respectively).

KEGG pathway analysis with EASE identified four significantly overrepresented pathways: antigen processing and presentation, cell adhesion molecules, type I diabetes mellitus, and natural killer (NK) cell mediated cytotoxicity (Table 1, Supplemental table 6). The antigen processing and presentation pathway contains many of the same histocompatibility genes which were already differentially expressed after OVA treatment. Furthermore, the relative difference in expression of these genes between the strains is maintained at similar levels. Four additional histocompatibility genes (H2-Dma, H2-Q1, H2-Oa, and H2-Eb1) as well as the CD8 receptor (Cd8a and CD8b1) and the CD94/NKG2A receptor (klrc1 and krld1) genes had higher expression in C57BL/6 mice following resiquimod treatment. Of the 20 cell adhesion pathway genes, the same ten histocompatibility genes and the CD8 receptor genes which were found in the antigen processing and presentation pathway were present. In addition, several genes involved in modulating cell adhesion (ltgb7, lthal, Cd34, and Spn) were found to be expressed in the resiquimod treated C57BL/6 mice at higher levels than in the A/J mice. However, this pathway also contained several claudin genes (Cldn7, 8, 10, 23), a family of genes involved in the formation of tight junctions, which had higher transcript levels in the resiquimod treated A/J mice. The type I diabetes mellitus pathway contains the same 10 histocompatibility
genes as the two other pathways along with granzyme B, a gene involved in cytotoxic T-cell and NK cell mediated cytotoxicity. Finally, all the genes in the NK cell mediated cytotoxicity pathway were expressed at higher levels in resiquimod treated C57BL/6 mice than in A/J mice. The 17 genes belonging to this pathway and, found to be differentially expressed between resiquimod treated mice of the two strains, include the CD94/NKG2A (klrc1 and Klrd1) genes and several other NK cell receptors (Klrα3, Klrβ1c, Klrκ1, Klrα7, Klrα8, and Cd244), the integrin Itgal, the antibody receptors Fcγr3a and Fcεr1g, genes involved in signal transduction (Tyrobp, Sh2d1a, Pik3r4, and Nfatc1), the histocompatibility gene H2-T23, and granzyme B.

**Identification of genes differentially expressed following antigen challenge**

Changes in gene expression resulting from antigen challenge (PBS group vs. OVA group) were then investigated. Differentially expressed genes were first identified in each strain separately, as described in the Materials and Methods section. Ovalbumin challenge led to changes in expression of 268 genes in the A/J strain (Supplemental table 7) and 148 genes in the C57BL/6 strain (Supplemental table 8). Of these, 116 genes, or 39% of the combined list, were found to be differentially expressed independently in both strains. While the 32 genes whose expression was found to be altered only in C57BL/6 mice do not exhibit any clear pattern or groupings, two groups of genes are easily observable in the 152 genes which were only identified in the A/J strain (Table 2). The first group includes a number of genes which may be involved in the regulation of the extracellular matrix and includes several procollagenase genes (Col1α1, Col1α2, Col3α1, Col5α2, and Col6α2), cathepsins (Ctsk, Cts, Ctsk), tenascin (Tnc), and thrombospondin 1 (Thbs1), all of which were induced following OVA challenge. The second group consists of cytokines and cytokine receptors and includes interleukin-6 (Il6), the C-C chemokines Ccl6 and Ccl22, the C-X-C chemokine Cxcl12, BAFF (Tnfsf13b), as well as several receptors (Ccr1, Cx3cr1, Csf2rb1,
Csf2rb2, and Tnfrsf9). With the exception of the fractalkine receptor (Cx3cr1), the cytokines and receptors were all found to be expressed at higher levels in OVA challenged A/J mice than PBS challenged A/J mice.

Given that the transcriptional changes induced by antigen challenge significantly overlapped in the two tested strains, and given that the experimentally induced allergic asthma that results from antigen challenge is similar in both strains, the data generated from both strains was analyzed jointly (as described in the Materials and Methods section). This analysis identified 197 genes as differentially expressed following antigen challenge, of which 156 (79%) were previously identified employing only the A/J strain, 122 (62%) were identified employing only the C57BL/6 strain, 116 (59%) were identified in each strain independently, and 35 (18%) were not identified when employing only the A/J or only the C57BL/6 strain.

Using the pooled analysis incorporating data from both strains, the genes whose expression was significantly altered by antigen challenge were ranked based on the absolute value of the fold change in expression. The top 30 genes, in terms of absolute fold change, between the antigen-challenged and the sham-challenged lungs are listed in table 3. The direction of differential gene expression in the lungs of antigen-challenged mice when compared to the sham-challenged mice is immediately apparent from table 3. Of the 30 genes with the largest fold change in expression, all were up-regulated in the antigen-challenged mice. Furthermore, the extent of up-regulation was important with 18 genes having a greater than 5-fold increase in expression and 37 genes with a greater than 3-fold increase in expression. By comparison, of the 7 genes which were down-regulated after antigen-challenge, only two had a greater than two-fold change in expression (whereas 89 genes were up-regulated by more than 2-fold).

An examination of the genes listed in table 3 reveals the presence of eight chemokines (Ccl7, Ccl8, Ccl11, Ccl12, Ccl17, Ccl24, Cxcl9, and Cxcl10). Most of
the other genes in the table have been associated with asthma. For example, *Ear11* is an eosinophil-associated ribonuclease which has been observed in the lungs of asthmatic mice [230] and *Clca3* (Gob-5) has been shown to cause mucus overproduction and airway hyperresponsiveness in mice [231].

In order to identify features in the data which were unlikely to be present by chance alone, EASE was used to identify pathways found in the Kyoto Encyclopedia of Genes and Genomes (KEGG) in which differentially regulated genes were overrepresented. When the list of 197 genes, whose expression was significantly altered following antigen-challenge, was analyzed with EASE, significantly altered genes were found to be overrepresented in three pathways (Table 4). In all three cases (Cell cycle, Cytokine-cytokine receptor interaction, and Complement and coagulation cascades), all the differentially expressed genes in the pathway were up-regulated (supplementary table 9). Genes involved in cell cycle control and proliferation represent one of the largest groups to be up-regulated in the asthmatic transcriptome and include the cyclins *Ccna2*, *Ccnb1-rs1*, *Ccnb2*, and *Ccne2*, and the cell division cycle genes *Cdc2a*, *Cdc20*, *Cdca8*, *Mcm2*, *Mcm5*, and *Mcm6*. All the differentially expressed genes in the cytokine-cytokine receptor interaction pathway were chemokines and, in one case, a chemokine receptor.

**Identification of genes differentially expressed following resiquimod treatment**

The transcriptional consequences of resiquimod treatment in the context of the allergic asthma model were then explored. First, the strains were analyzed separately as described in the Materials and Methods section. Using those criteria, the expression levels of 565 genes in the A/J mice (Supplemental table 10), and 697 genes in the C57BL/6 mice (Supplemental table 11), were found to be altered by resiquimod treatment. Of the combined set of genes, the expression of only 260, or 26%, were found to be altered in both strains independently. The genes which were only identified as differentially expressed
in A/J mice were generally down-regulated following resiquimod treatment (200 genes, or 66%) and represented a wide assortment of genes in which we identified no informative motifs (Supplemental table 8). The 437 genes which were identified only in the C57BL/6 mice (Supplemental table 9), however, were mostly up-regulated (372 genes, or 85%) and contained several significantly overrepresented KEGG categories, namely, B cell receptor signalling pathway, NK cell mediated cytotoxicity, cell cycle, leukocyte transendothelial migration, and cell adhesion molecules (Table 4, Supplemental Table 12).

Despite the fact that the two strains present important differences in their response to resiquimod treatment, this treatment is effective in preventing the development of experimental allergic asthma in both strains. This suggests that a joint analysis of both strains has the potential to reveal universal effects of resiquimod administration as well as its effect on asthma development. To this end, the data from both strains was pooled and an analysis was performed (as described in the Materials and Methods section) identifying 386 genes as differentially expressed following antigen challenge, of which 283 (73%) were identified as differentially expressed when the A/J strain was analyzed independently, 310 (80%) were identified as differentially expressed when the C57BL/6 strain was analyzed independently, 250 (65%) were identified as differentially expressed in each strain independently, and 43 (11%) were not identified when the strain were analyzed separately.

Using the pooled analysis, which incorporates data from both strains, the genes whose expression was significantly altered by resiquimod treatment were ranked based on the absolute value of the average fold change in expression of the two strains. In contrast to the genes listed in table 3, the 30 genes with the largest absolute fold-changes in expression in the lungs of resiquimod treated mice, compared to mice which had only been antigen-challenged, are evenly divided with respect to up- and down-regulation (Table 5). However, the magnitude of the changes in gene expression is similar with 28 genes whose
expression was altered by more than 5-fold (14 up-regulated and 14 down-regulated) and 69 genes with a greater than 3-fold change in expression (41 up-regulated and 28 down-regulated).

When the down-regulated genes are examined, it can be observed that 10 of the 15 most down-regulated genes (Ear11, Mgl2, Slc26a4, Chi3l4, Cd209e, Ccl24, Ccl17, Clica3, Fcgbp, and Ccl11) following resiquimod treatment were found to have been up-regulated by antigen-challenge alone (Table 5). One of the most interesting features of the up-regulated genes is that 7 of the 15 genes (Gzma, Gzmb, Prf1, Nkg7, Klra3, Klrd1, and Ncr1) are associated or involved with cell-mediated cytotoxicity, or killer cell receptors. The remaining most highly up- and down-regulated genes are involved in an assortment of known and unknown processes and no clear features can readily be identified from a simple cursory inspection of the data.

EASE was again used to identify KEGG pathways in which differentially regulated genes were overrepresented. Significantly altered genes were found to be overrepresented in four pathways when the list of 386 genes, whose expression was significantly altered following resiquimod treatment, was analyzed (Table 4, Supplemental table 13). All four of the pathways (NK cell mediated cytotoxicity (NKCMC), Cytokine-cytokine receptor interaction, Type I diabetes mellitus, and Cell adhesion molecules (CAMS) are related to immune function and all four contain predominantly, or uniquely, up-regulated genes. The cytokine-cytokine receptor interaction pathway, with seven down-regulated genes, was the pathway with the most down-regulated genes. All of these were chemokine genes (Ccl8, Ccl11, Ccl12, Ccl17, Ccl22, and Ccl24, and Cxcl12) which, with the exception of Ccl22 (MDC), were found to be induced by antigen-challenge in the absence of resiquimod treatment (although even Ccl22 was significantly elevated in the A/J mice and non-significantly elevated in C57BL/6 mice).
By several orders of magnitude, the pathway in which differentially expressed genes are most significantly over-represented (as assessed by the magnitude of the EASE score) is the NKCMC pathway. All differentially expressed genes in the NKCMC pathway were up-regulated (Figure 2). In fact, the fold changes in Gzmb and Prf1 expression were the second and third largest fold changes, respectively, induced by resiquimod treatment; while the related gene Gzma, which is not listed in the KEGG NKCMC pathway, had the largest fold change. Overall, the magnitude of the changes in expression of the genes in the NKCMC pathway, in which 8 and 6 of the 21 genes displayed a greater than 3- and 5-fold change in expression, respectively 38% and 29%, was far greater than the magnitude of the changes in expression for the entire population of genes which were differentially expressed following resiquimod treatment. In the latter case, 69 (18%) and 28 (7%) of the 386 genes had a greater than 3- and 5-fold change in expression, respectively.

**Real-time RT-QPCR validation of differentially expressed genes**

To validate the microarray expression measurements we performed real-time quantitative reverse transcriptase PCR on whole lung RNA samples obtained from an independent set of six A/J mice per experimental group. A total of fourteen genes were selected which represented approximately 2 to 20 fold differences in expression between both the sham-challenged and antigen-challenged groups, and the antigen-challenged and resiquimod-treated groups. As can be observed in figure 3A, the expression changes of the 9 genes whose expression was increased following antigen-challenge and decreased following resiquimod treatment were confirmed. Furthermore, changes in the 5 genes whose expression was induced following resiquimod treatment (Figure 3B) were also confirmed.

**Recruitment of natural killer cells following resiquimod treatment**

In order to determine whether the increased expression of genes contained within the NKCMC pathway were the result of the recruitment of NK cells into
the lungs of resiquimod treated mice; we assessed the relative abundance of NK cells in the lungs of sham-challenged A/J mice as well as antigen-challenged mice with or without resiquimod treatment. The mice were euthanized, at the same time-point which was used to generate the microarray data, three hours following the final antigen challenge. Cells were then isolated from the lungs, as described in the Materials and Methods section, and the relative number of NK cells was assessed. In antigen sensitized animals, antigen aerosol challenge did not significantly alter the relative number of NK cells (Figure 4, OVA vs. PBS). However, NK cell numbers increased almost 5-fold following resiquimod treatment (RES vs. OVA).

In order to confirm that the NK cell recruitment observed in the experimental asthma model was a result of resiquimod treatment alone, we evaluated the influx of NK cells following resiquimod treatment in the lungs of C57BL/6 mice (Figure 5). We observed that the proportion of NK cells trends upwards as soon as 3 hours following the first injection and is statistically significantly elevated 3 hours after both the second and third doses (Figure 5A). These elevated lung NK cell levels remain relatively stable for 48 hours after the last dose of resiquimod before starting to return to basal levels after 96 hours (Figure 5B). To determine whether the influx of NK cells was specific to the lungs, NK cells were also assessed in the livers and spleens of the same animals. The NK cell levels in the liver, which increase following each dose of resiquimod, mirror those observed in the lungs of the same animals (Figure 5A). As in the lungs, NK cell levels peak between 3 and 24 hours following the final resiquimod dose. However, NK cell levels in the livers fully returned to basal levels 96 hours after the last dose and demonstrated a greater decline than that which was observed in the lungs after 48 hours (Figure 5B).

**The role of cytotoxic cells in resiquimod treatment of allergic asthma**

The role of the recruited NK cells on the ability of resiquimod treatment to prevent the development of allergic asthma, as measured by the eosinophil
content of bronchoalveolar lavage fluid recovered 48 hours following the final antigen challenge, was then assessed. As previously described [2], antigen sensitization and challenge results in eosinophilic infiltration of the lungs. This infiltration can be observed in BAL fluid and is prevented by resiquimod treatment (Figure 6A). Anti-NK1.1 antibodies were used to deplete NK cells in order to determine if this effect could be observed in the absence of NK cells. Resiquimod treatment of antigen-challenged mice, despite NK cell depletion, prevented eosinophil recruitment (Figure 6B). Changes in airway responsiveness were also assessed; however, depletion of NK1.1+ cells prevented the development of hyperresponsiveness making it difficult to draw any further conclusions on the role of these cells in resiquimod treatment of allergic asthma in these mice (data not shown).

In order to determine if the cytotoxic activity of cytotoxic T-lymphocytes, rather than the cytotoxic acivity of NK cells, is necessary in order for resiquimod treatment of allergic asthma to be effective, resiquimod treatment was assessed in Cd8 knock-out mice. The absence of CD8 T-cells did not reduce the effectiveness of resiquimod treatment as assessed by the eosinophilic content of the BAL fluid (Figure 6C).
Two strains of mice were employed in our study. The A/J strain is considered asthma-susceptible (high airway responsive to methacholine and a predisposition towards the production of IgE antibodies) while the C57BL/6 strain is considered asthma-resistant (low airway responsiveness to methacholine and low IgE production). However, experimental allergic asthma can be induced in both strains and despite carrying different versions of the Slc11a1 gene (formerly Nramp1), which has been shown to modulate responsiveness to resiquimod treatment in a mycobacterium infection model [196], both strains respond to resiquimod treatment in the context of asthma [2].

After challenging the lungs with the antigen, differences in gene expression emerge from which clear patterns can be observed. In particular, changes in antigen processing and presentation and in cytokine-cytokine receptor interactions are prominent. Interestingly, we see differences in chemokine expression where the A/J mice express asthma associated Ccr3 binding chemokines highly, consistent with the more atopic phenotypes of the A/J mice. Furthermore, up-regulation of genes involved in airway remodelling, such as procollagenases, cathepsins, and tenascin, were only observed in A/J mice leading us to speculate that these genes may contribute to the more severe airway hyperresponsiveness phenotype observed in this strain [161].

By identifying genes whose expression is consistently altered by the induction of experimental asthma in mice (either by OVA or Aspergillus fumigatus), a core and reproducible “asthmatic” transcriptome can be characterized. We have compared here the genes identified in our study, using an OVA induced model in both A/J and C57BL/6 mice, to the genes identified by Zimmermann et al., using both an OVA and an A. fumigatus induced model in BALB/c mice, as well as by
Kuperman et al. using an OVA induced model in BALB/c mice (summarized in Table 6).

Despite the use of different microarrays, statistical analyses, sensitization and challenge protocols, allergens, and strains of mice, there was significant overlap between the genes identified in all studies. Nearly half (42%, or 82 out of 197 genes) of the genes identified as significantly altered in the present study (OVA induced model of asthma in both A/J and C57BL/6 mice) were also identified as significantly altered in both the *A. fumigatus* induced model of asthma, and in at least one of the OVA induced models, in BALB/c mice (supplemental table 14) [232;233]. Furthermore, in every study, the overwhelming majority of genes with significantly altered expression, including those identified in only one or two studies, were up-regulated.

Three groups of genes are prominent in this asthmatic transcriptome: genes involved with cell cycle control and progression, genes involved in the complement and coagulation cascades, and chemokines and their receptors. The activation of cell cycle control genes has been identified as a common lung expression response, to parasites and allergens, in several studies [234] and although it is impossible, from whole lung microarray data, to determine which cell types are responsible for this increased expression, likely candidates would include airway epithelial cells, which are responsible for the goblet cell hyperplasia and mucus overproduction [235;236], which are defining features of the airway remodelling characteristic of asthma in both human patients [237] and mouse models [238]. Supporting this hypothesis, several of the most highly up-regulated genes identified in our assay were involved with mucus production. In our study, the level of *Clca3* RNA was found to be 20 times higher in OVA-challenged mice than in control animals. *Clca3* has consistently been identified as one of the most highly up-regulated genes in mouse models of asthma whether in response to OVA [232;233;239], *A. fumigatus* [233;239], or IL-13 [232;239]. Furthermore, increased expression of *CLCA1*, the human ortholog of
*Clca3*, has been observed in the airways of asthmatic patients where it is associated with mucus production [240;241]. *Clca3* expression is stimulated by the T\(_h2\) cytokines *IL-4*, *IL-9*, and *IL-13* [242], and it is expressed by goblet cells, where its protein product has been observed in mucin granules [243]. Expression of *Muc5ac* and *Muc5b*, the main gel forming components of mucus [244;245], was also highly up-regulated following OVA challenge and has been observed almost universally in previous studies [232;233;239]. Other than goblet cell hyperplasia, the remodelling associated with asthma is also characterized by an increase in airway smooth-muscle (ASM) mass and sub-epithelial fibrosis. ASM mass increase is caused by hyperplasia, and possibly, hypertrophy of ASM cells [246], while the sub-epithelial fibrosis is characterized by collagen deposition and alterations of the ECM. A weakness inherent in most mouse models of OVA-induced allergic asthma is that increases in airway smooth muscle mass are not normally observed within the timeframes typically employed [238;247]. While this was also the case with the model employed in the present study, a large number of genes associated with sub-epithelial fibrosis were significantly upregulated in the A/J strain in our study.

The expression of coagulation factors 7 (*F7*) and 10 (*F10*), as well as tissue factor pathway inhibitor 2 (*Tfpi2*) which inhibits the activation of *F10*, were all consistently up-regulated and may contribute to airway remodelling, and eventually airway hyperresponsiveness, through the production of fibrin [248], or by other mechanisms [249]. As far as complement genes are concerned, the expression of *C1qb*, *C1qc*, and *C3* as well as the complement 3a receptor 1 (*C3ar1*) were similarly found to be universally up-regulated regardless of the asthma model used. Several mechanisms are known to induce *C3* expression and lead to airway hyperresponsiveness including the T\(_h2\) cytokines *IL-4* [250] and *IL-13* [251], and several airborne pollutants including diesel exhaust particles [252;253], ozone [254], and cigarette smoke [255]. An important difference between the A/J and C57BL/6 mice is that the former harbours a mutation in the
haemolytic complement (Hc or C5) gene which renders these mice C5 deficient. While there is evidence implicating this gene in the differential asthma susceptibility of the A/J and C57BL/6 strains, and that the balance of the C5a and C3a anaphylatoxins modulates the response to allergens during the sensitization phase [256], the C5a protein is believed to have pro-inflammatory effects during the challenge phase. The use of intraperitoneally administered antigen combined with adjuvant appears to overcome the protective effect of C5a as the C57BL/6 mice are efficiently sensitized against the antigen in our model as previously described [2]. Furthermore, we observed no modulation of Hc (C5) transcripts across any of the C57BL/6 experimental groups suggesting that in the challenge phase of this model of allergic asthma, C5 status does not significantly affect outcome.

The most common receptors for the 6 chemokines which were consistently found to be up-regulated across the different genome-wide expression studies were Ccr1, Ccr2, and Ccr3. While the Ccr1 receptor agonists include Ccl7 (MCP-3), Ccl8 (MCP-2), and Ccl9 (MIP-1γ)[257;258], there are at least 8 other chemokines which can act as Ccr1 agonists. Given this high level of polygamy, it is not surprising that many of the chemokines found to be induced in experimental asthma, by genome wide expression analyses, can act as agonists for this receptor. There is, however, a large body of evidence which implicates Ccr1 signalling in the pathogenesis of asthma including the observed dependence on Ccr1 of respiratory syncytial virus induced asthma exacerbations in a BALB/c model [259], the protective effect of a Ccr1 antagonist in a mouse model of chronic asthma [260], and elevated Ccr1 expression by airway smooth muscle cells [261] and mast cells [262] in human asthma patients.

Most of the chemokines known to be Ccr2 agonists were found to be induced, following the induction of experimental asthma, in every genome-wide expression study strongly suggesting that this receptor is involved in allergic asthma in mouse models. Ccr2 is the only known receptor for Ccl2 (MCP-1), and
the presence of this chemokine is known to be able to induce the polarization of naive T-cells towards the T_{H}2 arm of adaptive immunity [263;264]. Furthermore, a recent meta-analysis of microarray data from mouse asthma models has also identified Ccl2 as a central regulator of a gene expression module which includes many genes, such as Chia, Chi3l3, and Adam8, which are consistently activated in asthmatic mice [265]. The literature surrounding the role of Ccr2 in asthma is, however, quite contradictory, since studies in Ccr2 knock-out mice have shown that deletion of Ccr2 had either no effect [266] on asthmatic phenotypes, or that it resulted in increased airway responsiveness and eosinophilia and increased IgE production [267]. A study in primates however, demonstrated that administration of a Ccr2 antagonist could prevent the development of asthma [268].

Finally, Ccr3 is another chemokine receptor with a large number of known agonists. Of these, Ccl8 and Ccl11 (eotaxin) were consistently observed to be induced following the induction of experimental asthma, and were found to be amongst the most highly up-regulated genes in the present study (along with Ccl24, also known as eotaxin-2, which was not found to be significantly induced in the studies by Zimmerman et al. or Kuperman et al.). Ccr3 is expressed by several types of cells implicated in asthma pathology including eosinophils [269], mast cells [270], and selectively by T_{H}2 T cells [271] and there is evidence that Ccr3 plays a role in the development of asthma in both mice [272;273] and humans [274].

The role of these consistently altered genes in the pathophysiology of experimentally induced allergic asthma in mice, or of allergic asthma in human patients, remains uncertain. However, there is strong evidence that at least several may contribute to disease development and progression.

Resiquimod treatment had a significant impact on transcription in the lungs of treated animals resulting in the altered expression of 386 genes in both A/J and
C57BL/6 mice. Interestingly, resiquimod treatment resulted in similar numbers of up- and down-regulated genes (215 and 171, respectively) whereas antigen challenge alone resulted almost exclusively in the up-regulation of gene expression. The vast majority of genes which were identified as both altered following antigen challenge, and following resiquimod treatment, were up-regulated following antigen challenge and down-regulated following resiquimod treatment.

Genes whose expression was significantly altered following resiquimod treatment can be divided into two groups. The first is composed of those genes whose expression is normalized by resiquimod treatment, that is, those genes whose expression was induced following antigen challenge and whose expression was comparatively repressed after resiquimod treatment. The second category includes genes whose expression is specifically altered by treatment and which were not initially up-regulated following asthma induction. Of the 197 genes whose expression was altered by antigen challenge, the expression of 55 (or 28%) was normalized by resiquimod treatment. However, most of the genes (83%) whose expression was affected by resiquimod treatment were not affected by antigen challenge alone.

Of the 12 chemokines whose expression was up-regulated following antigen challenge, the expression of 6 (Ccl8, Ccl11, Ccl12 (MCP-5), Ccl17 (TARC), Ccl24, and Cxcl12 (SDF)) was normalized following resiquimod treatment. Of the six remaining chemokines, the expression of five was not significantly altered in the treated versus untreated animals and the expression of one, Cxcl9, was induced by antigen challenge and further induced by resiquimod treatment. Most of the down-regulated chemokines were Ccr2 or Ccr3 agonists, except for the T\(_{\text{h}2}\) chemokines Ccl17 and Ccl22 (MDC), which bind Ccr4.

The expression of several genes that were highly up-regulated following antigen challenge, such as Ear11 or Slc26a4, as well as genes whose expression
was consistently altered across different microarray studies [232;233], such as *Chia*, *Agr2*, or *Scin*, were normalized by resiquimod treatment. However, the 55 genes whose expression was normalized by resiquimod treatment represent only 14% of the 387 genes whose expression was altered by resiquimod treatment. Furthermore, these 55 genes represent 85% of the 65 genes whose expression was altered both by antigen challenge and resiquimod treatment.

Interestingly, none of the complement cascade genes whose expression was induced by antigen challenge were normalized by resiquimod treatment, and the expression of a few of these genes, including those for complement factor B (*Cfb*) and the C1q β polypeptide (*C1qb*), as well as the gene for the C3a receptor (*C3ar1*), were further induced by resiquimod treatment. Despite the consistency with which coagulation and complement cascade genes were identified in genome wide expression studies, and the literature addressing the role of these factors in asthma [249;251], these findings suggest that expression of complement cascade genes, while strongly associated with, and possibly functionally implicated in the development of, experimental asthma pathology in mouse models, are not sufficient for the development of this pathology.

While resiquimod treatment led to the suppression of several chemokines which were up-regulated by antigen challenge, it also induced the expression of others. The expression of the C-C motif chemokines *Ccl3* (MIP-1α), and *Ccl5* (RANTES), and the C-X-C motif chemokines *Cxcl9* (MIG), and *Cxcl13* (BLC, BCA-1) was induced in the lungs of resiquimod treated animals. *Ccl3* has been shown to induce Th1 differentiation of naive T cells in the presence of antigen [263] while *Cxcr3*, the *Cxcl9* receptor, is preferentially expressed on Th1 cells [275]. These findings are consistent with the known immunomodulatory activities of resiquimod [220] and studies in which resiquimod was used in a mouse model of allergic asthma [144;145], although the latter finding was not observed in all studies [2]. Furthermore, *in vitro* microarray analysis of purified plasmacytoid dendritic cells also found that several chemokines and chemokine receptors
were induced by resiquimod treatment. In particular, \textit{Ccl3}, \textit{Ccl5}, \textit{Cxc19}, and the \textit{Ccr5} and \textit{Cxcr3} receptors, were found to be induced in isolated dendritic cells [276], suggesting that this cell type is at least partially responsible for the chemokine production observed in our model.

In contrast to the large number of chemokines which were affected by resiquimod treatment, the expression of relatively few other cytokines, including only a single interleukin, were significantly altered. The single interleukin, \textit{IL-16}, was significantly induced by resiquimod treatment. This cytokine is known to be chemotactic for \textit{Cd4+ Ccr5+} double positive cells. Because \textit{Ccr5} is preferentially expressed on \textit{T}_{H1} cells, \textit{IL-16} preferentially induces migratory responses in \textit{T}_{H1} cells when compared to \textit{T}_{H2} cells [277]. The expression of several cytokine receptor genes, including the interleukin receptor genes \textit{IL2rb}, \textit{IL3ra}, \textit{IL10ra}, \textit{IL17ra}, and \textit{IL22ra}, the interleukin 18 receptor associated protein \textit{IL18rap}, and the tumour necrosis subfamily receptor genes \textit{Tnfrsf13b}, and \textit{Tnfrsf13c}, however, were induced by resiquimod treatment.

Many genes associated with B cells were altered by resiquimod treatment. The cytokine \textit{Tnfsf1b} (BAFF) signals through \textit{Tnfrsf13b} (TACI) [278] and \textit{Tnfrsf13c} (BAFFR) [279;280], both of which are expressed on B-cells, the latter being necessary for B cell survival. The expression of both receptors was induced by resiquimod treatment. A dichotomy exists, however, for B cell receptor (BCR) signaling. \textit{Cd72} is an inhibitor of BCR signaling [281;282] and induced by resiquimod treatment. On the other hand, \textit{Cd79a}, and \textit{Cd79b}, genes which encode components of the BCR complex, as well as several genes involved in BCR signaling including \textit{Blnk}, \textit{Rac2}, and \textit{Vav1}, were also induced by resiquimod treatment. It is possible that the induction of inhibitory genes and the suppression of receptors necessary for B cell survival was part of a negative feedback mechanism as resiquimod has been shown to be a potent mediator of B cell activation [283].
Amongst other genes related to immunity, resiquimod treatment also induced the expression of a number of cell adhesion molecules including integrins, cadherins, and selectins, several histocompatibility 2 genes, as well as a large number of genes associated with NK cells, or involved in NK cell mediated cytotoxicity. The number of transcripts of both NK cell receptors including Ncr1 (Nkp46) and a number of killer cell lectin-like receptors (Klra2, Klra3, Klra8, KlrC1, KlrD1, and KlrK1), as well as several genes involved in NK cell mediated cytotoxicity such as perforin (Prf1), Fas ligand (Fasl), and granzymes (Gzma, Gzmb) were much higher in the lungs of resiquimod treated animals.

Since it is known that resiquimod can lead both to the activation of NK cells and enhancement of NK cell mediated cytotoxicity, at least in vitro [284;285], we determined whether the increased expression of NK cell associated genes was due to the recruitment of NK cells into the lungs of the treated mice. By flow cytometry we found that the lungs of resiquimod treated animals did indeed contain elevated levels of NK cells, and that these levels were sustained for at least 48 hours. Furthermore, NK cell recruitment, when resiquimod is administered systemically by intraperitoneal injection, was not limited to the lungs as it was also observed in the liver. Employing anti-NK1.1 antibodies we demonstrated that that NK cells were not required in order for resiquimod to prevent the development of experimental allergic asthma in mice. Similarly, using Cd8 knockout mice, we demonstrated that resiquimod treatment does not depend on Cd8-positive T cells.

The majority of the morbidity and mortality associated with asthma are caused by exacerbations, and as many as 85% of these exacerbations are associated with viral infections[128;286]. As present therapies are only moderately effective at preventing asthma exacerbations, new approaches are needed [287]. Through IFN-γ production and the lysis of infected cells, NK cells are important in the control of viral infections [288]. Due to its ability to inhibit the development of asthma pathology, recruit NK cells to the lungs for several
days, and the known anti-viral activities of the imidazoquinoline family of compounds; resiquimod represents a promising new approach for the prevention and treatment of asthma exacerbations which warrants further investigation.

In summary we have shown that there is a common set of genes which are induced following antigen challenge in the mouse models of allergic asthma currently in use. In particular, groups of genes which are reproducibly and significantly up-regulated include genes involved in the control of cell cycle progression, the complement and coagulation cascades, and chemokine signalling. As well, there were several genes of uncertain function which were consistently found to be highly induced. We have shown that resiquimod treatment, which completely inhibits the development of experimental allergic asthma, had wide ranging effects on the transcriptional environment of the lung. While resiquimod treatment normalized the expression of genes involved in airway remodelling and, for the most part, chemokine signalling, it also induced the expression of a number of genes involved in a variety of immunological processes. Furthermore, systemic administration of resiquimod resulted in the recruitment of a large number of NK cells to the lungs and liver, but not to the spleen. While the NK cell recruitment was dispensable in the context of asthma therapy per se, the relatively strong and sustained increase in pulmonary NK cell levels may be beneficial in the treatment of a multitude of viral respiratory infections, particularly in the context of preventing or treating asthma exacerbations.
Acknowledgements

We thank Graceway Pharmaceuticals for providing the resiquimod used in this study. This research was supported by a Senior Fellow Award from the American Asthma Foundation [formerly the Sandler Program for Asthma Research (SPAR)] and a grant from the Canadian Institutes of Health Research (CIHR) awarded to Dr. D. Radzioch. P. Camateros is supported by a Doctoral Research Award from the CIHR and C. Kanagaratham was supported by a Natural Science and Engineering Research Council scholarship.
### Table 1: Pathways Different Between A/J and C57BL/6 Mice.

Table 1: Pathways whose genes are overrepresented amongst the genes whose expression was significantly different between A/J and C57BL/6 mice in the OVA and RES treatment groups.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Hits</th>
<th>Assayed</th>
<th>A/J High</th>
<th>C57BL/6 High</th>
<th>EASE Score</th>
<th>Bootstrap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine-Cytokine Receptor Interaction</td>
<td>16</td>
<td>221</td>
<td>8</td>
<td>8</td>
<td>1.6 x10^-3</td>
<td>0.03</td>
</tr>
<tr>
<td>Antigen Processing and Presentation</td>
<td>8</td>
<td>66</td>
<td>2</td>
<td>6</td>
<td>3.7 x10^-4</td>
<td>0.05</td>
</tr>
<tr>
<td>OVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen Processing and Presentation</td>
<td>14</td>
<td>66</td>
<td>1</td>
<td>13</td>
<td>3.2 x10^-5</td>
<td>8.0 x10^-4</td>
</tr>
<tr>
<td>Cell Adhesion Molecules (CAMS)</td>
<td>20</td>
<td>130</td>
<td>5</td>
<td>15</td>
<td>4.4 x10^-5</td>
<td>9.0 x10^-4</td>
</tr>
<tr>
<td>Type I Diabetes Mellitus</td>
<td>11</td>
<td>47</td>
<td>1</td>
<td>10</td>
<td>1.4 x10^-4</td>
<td>2.6 x10^-3</td>
</tr>
<tr>
<td>Natural Killer Cell Mediated Cytotoxicity</td>
<td>17</td>
<td>110</td>
<td>0</td>
<td>17</td>
<td>1.9 x10^-4</td>
<td>3.6 x10^-3</td>
</tr>
<tr>
<td>RES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The pathways listed are those contained in the KEGG[227] mouse pathway database that were significantly overrepresented after antigen challenge of sensitized animals (OVA), or resiquimod treatment (RES), as assessed by EASE[226] (detailed in the Materials and Methods section). The Hits column represents the number of genes with significant differences in expression, where significance was assessed as described in the Materials and Methods section. The Assayed column represents the number of genes belonging to the pathway which were assayed by the Affymetrix Mouse 430v2 chips. The "A/J High" and "C57BL/6 High" columns represent the number of genes whose expression was higher in the A/J and C57BL/6 mice respectively. The EASE score of each pathway is indicated. The threshold of significance was set at $p \leq 0.05$ after correction by the bootstrap method incorporated in EASE. Bootstrap $p$ values are reported in the Bootstrap column.
#### Table 2: Genes Altered After Antigen Challenge in A/J Mice Only

Table 2: Selected genes with significant changes in expression, following OVA challenge, only in A/J mice.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>p-value</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctsk</td>
<td>Cathepsin K</td>
<td>$1.9 \times 10^{-7}$</td>
<td>3.0</td>
</tr>
<tr>
<td>Thbs1</td>
<td>Thrombospondin 1</td>
<td>$1.2 \times 10^{-6}$</td>
<td>2.8</td>
</tr>
<tr>
<td>Ccl22</td>
<td>Chemokine (C-C motif) ligand 22</td>
<td>$2.7 \times 10^{-10}$</td>
<td>2.6</td>
</tr>
<tr>
<td>Il6</td>
<td>Interleukin 6</td>
<td>$2.7 \times 10^{-5}$</td>
<td>2.3</td>
</tr>
<tr>
<td>Csf2rb2</td>
<td>Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)</td>
<td>$9.7 \times 10^{-6}$</td>
<td>2.2</td>
</tr>
<tr>
<td>Col6a2</td>
<td>Procollagen, type VI, alpha 2</td>
<td>$4.8 \times 10^{-8}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Tnc</td>
<td>Tenascin C</td>
<td>$2.7 \times 10^{-5}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Col1a1</td>
<td>Procollagen, type I, alpha 1</td>
<td>$1.1 \times 10^{-5}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Ccr1</td>
<td>Chemokine (C-C motif) receptor 1</td>
<td>$9.7 \times 10^{-6}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Tnfrsf9</td>
<td>Tumor necrosis factor receptor superfamily, member 9</td>
<td>$1.1 \times 10^{-6}$</td>
<td>2.0</td>
</tr>
<tr>
<td>Tnfsf13b</td>
<td>Tumor necrosis factor (ligand) superfamily, member 13b</td>
<td>$3.0 \times 10^{-6}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Ccl6</td>
<td>Chemokine (C-C motif) ligand 6</td>
<td>$6.3 \times 10^{-6}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Col3a1</td>
<td>Procollagen, type III, alpha 1</td>
<td>$1.5 \times 10^{-5}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Cxcl12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
<td>$2.2 \times 10^{-6}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Csf2rb1</td>
<td>Colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macrophage)</td>
<td>$9.5 \times 10^{-8}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Col1a2</td>
<td>Procollagen, type I, alpha 2</td>
<td>$5.6 \times 10^{-6}$</td>
<td>1.8</td>
</tr>
<tr>
<td>Col5a2</td>
<td>Procollagen, type V, alpha 2</td>
<td>$5.7 \times 10^{-7}$</td>
<td>1.8</td>
</tr>
<tr>
<td>Ctsz</td>
<td>Cathepsin Z</td>
<td>$1.9 \times 10^{-7}$</td>
<td>1.7</td>
</tr>
<tr>
<td>Col6a1</td>
<td>Procollagen, type VI, alpha 1</td>
<td>$1.8 \times 10^{-6}$</td>
<td>1.6</td>
</tr>
<tr>
<td>Ctsz</td>
<td>Cathepsin Z</td>
<td>$9.4 \times 10^{-7}$</td>
<td>1.5</td>
</tr>
<tr>
<td>Cx3cr1</td>
<td>Chemokine (C-X3-C) receptor 1</td>
<td>$4.0 \times 10^{-5}$</td>
<td>-1.7</td>
</tr>
</tbody>
</table>

Differential gene expression following antigen challenge in sensitized mice. Positive and negative fold changes represent up- and down-regulation of genes, respectively, in antigen-challenged mice versus sham challenged mice. Unadjusted p values are shown and only genes with significantly altered expression are included (statistical assessment is described in the Materials and Methods section).
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Affymetrix probeset ID</th>
<th>GenBank Accession</th>
<th>p Value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear11</td>
<td>Eosinophil-associated, ribonuclease A family, member 11</td>
<td>1425295_at</td>
<td>BC020070</td>
<td>1.0 x 10^-11</td>
<td>22</td>
</tr>
<tr>
<td>Clca3</td>
<td>Chloride channel calcium activated 3</td>
<td>1416306_at, 1459889_at</td>
<td>NM_017474</td>
<td>2.8 x 10^-12</td>
<td>20</td>
</tr>
<tr>
<td>S1c26a4</td>
<td>Solute carrier family 26, member 4</td>
<td>1419725_at</td>
<td>NM_011867</td>
<td>2.4 x 10^-15</td>
<td>18</td>
</tr>
<tr>
<td>Chi3l4</td>
<td>Chitinase 3-like 4</td>
<td>1425450_at, 1425451_s_at</td>
<td>AK065557</td>
<td>2.4 x 10^-13</td>
<td>17</td>
</tr>
<tr>
<td>Retnla</td>
<td>Resistin like alpha</td>
<td>1449015_at</td>
<td>NM_020509</td>
<td>2.7 x 10^-16</td>
<td>8.0</td>
</tr>
<tr>
<td>Fcgbp</td>
<td>Fc fragment of IgG binding protein</td>
<td>1426872_at</td>
<td>BC026653</td>
<td>1.9 x 10^-14</td>
<td>7.7</td>
</tr>
<tr>
<td>Ccl11</td>
<td>Mucin 5, subtypes A and C, tracheobronchial/gastric</td>
<td>1430899_at</td>
<td>AK008656</td>
<td>1.1 x 10^-11</td>
<td>7.2</td>
</tr>
<tr>
<td>Cd209e</td>
<td>CD209e antigen</td>
<td>1420582_at</td>
<td>AF373412</td>
<td>1.5 x 10^-12</td>
<td>7.1</td>
</tr>
<tr>
<td>Scin</td>
<td>Scinderin</td>
<td>1450276_a_at</td>
<td>NM_009132</td>
<td>1.6 x 10^-11</td>
<td>6.9</td>
</tr>
<tr>
<td>Tff2</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>1418652_at</td>
<td>NM_008599</td>
<td>1.3 x 10^-13</td>
<td>6.6</td>
</tr>
<tr>
<td>Cxld9</td>
<td>Cholesterol 25-hydroxylase</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>6.8</td>
</tr>
<tr>
<td>Timp1</td>
<td>Timp1</td>
<td>1419282_at</td>
<td>BC011134</td>
<td>2.0 x 10^-11</td>
<td>5.5</td>
</tr>
<tr>
<td>AA467197</td>
<td>Expresses sequence AA467197</td>
<td>1424923_at</td>
<td>BC002065</td>
<td>2.9 x 10^-9</td>
<td>5.1</td>
</tr>
<tr>
<td>Serpina3g</td>
<td>Serine (or cysteine) peptidase inhibitor, clade A, member 3G</td>
<td>1419413_at</td>
<td>BC011134</td>
<td>2.0 x 10^-11</td>
<td>5.5</td>
</tr>
<tr>
<td>Ccl17</td>
<td>Matrix metalloproteinase 12</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Mmp12</td>
<td>Metalloproteinase 12</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Itlna</td>
<td>Intelectin a</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Ccl24</td>
<td>Chemokine (C-C motif) ligand 24</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Agr2</td>
<td>Anterior gradient 2 (Xenopus laevis)</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Ccl12</td>
<td>Chemokine (C-C motif) ligand 12</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Birc5</td>
<td>Baculoviral IAP repeat-containing 5</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
<tr>
<td>Top2a</td>
<td>Topoisomerase (DNA) II alpha</td>
<td>1419413_at</td>
<td>AF128193</td>
<td>1.1 x 10^-10</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Differential gene expression following antigen challenge in sensitized mice. The table contains the 30 transcripts showing the largest absolute fold change between antigen sensitized animals following antigen or and sham challenge. Positive and negative fold changes represent up- and down-regulation of genes, respectively, in antigen-challenged mice versus sham challenged mice. No genes were found to be down-regulated by 3.4 fold or more. Unadjusted p values are shown and only genes with significantly altered expression are included (statistical assessment is described in the Materials and Methods section).
Table 4: Pathways Altered by Antigen Challenge or Resiquimod Treatment

Table 4: Pathways overrepresented by genes whose expression was altered by antigen challenge or resiquimod treatment.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Hits</th>
<th>Assayed</th>
<th>Up</th>
<th>Down</th>
<th>EASE</th>
<th>Bootstrap</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OVA (Joint Analysis)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>12</td>
<td>103</td>
<td>12</td>
<td>0</td>
<td>1.5 x 10^-7</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>Cytokine-Cytokine Receptor Interaction</td>
<td>13</td>
<td>221</td>
<td>13</td>
<td>0</td>
<td>5.5 x 10^-5</td>
<td>7 x 10^-4</td>
</tr>
<tr>
<td>Complement and Coagulation Cascades</td>
<td>7</td>
<td>67</td>
<td>7</td>
<td>0</td>
<td>3.7 x 10^-4</td>
<td>5.2 x 10^-3</td>
</tr>
<tr>
<td><strong>RES (Joint Analysis)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural Killer Cell Mediated Cytotoxicity</td>
<td>21</td>
<td>110</td>
<td>21</td>
<td>0</td>
<td>4.9 x 10^-10</td>
<td>1.0 x 10^-4</td>
</tr>
<tr>
<td>Cytokine-Cytokine Receptor Interaction</td>
<td>21</td>
<td>221</td>
<td>14</td>
<td>7</td>
<td>1.1 x 10^-6</td>
<td>2.0 x 10^-4</td>
</tr>
<tr>
<td>Type I Diabetes Mellitus Cell Adhesion Molecules (CAMS)</td>
<td>10</td>
<td>47</td>
<td>10</td>
<td>0</td>
<td>8.0 x 10^-5</td>
<td>1.1 x 10^-3</td>
</tr>
<tr>
<td>Cell Adhesion Molecules (CAMS)</td>
<td>15</td>
<td>130</td>
<td>12</td>
<td>3</td>
<td>1.6 x 10^-4</td>
<td>2.2 x 10^-3</td>
</tr>
<tr>
<td><strong>RES (in C57BL/6 only)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B Cell Receptor Signalling Pathway</td>
<td>17</td>
<td>68</td>
<td>17</td>
<td>0</td>
<td>3.3 x 10^-9</td>
<td>1.0 x 10^-3</td>
</tr>
<tr>
<td>Natural Killer Cell Mediated Cytotoxicity</td>
<td>21</td>
<td>110</td>
<td>21</td>
<td>0</td>
<td>4.0 x 10^-9</td>
<td>1.0 x 10^-3</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>15</td>
<td>103</td>
<td>13</td>
<td>2</td>
<td>3.5 x 10^-5</td>
<td>1.0 x 10^-3</td>
</tr>
<tr>
<td>Leukocyte Transendothelial Migration</td>
<td>13</td>
<td>110</td>
<td>12</td>
<td>1</td>
<td>1.0 x 10^-3</td>
<td>2.4 x 10^-3</td>
</tr>
<tr>
<td>Cell Adhesion Molecules (CAMS)</td>
<td>14</td>
<td>130</td>
<td>14</td>
<td>0</td>
<td>1.4 x 10^-3</td>
<td>3.8 x 10^-3</td>
</tr>
</tbody>
</table>

The pathways listed are those contained in the KEGG[227] mouse pathway database which were overrepresented by genes whose transcript levels were significantly affected by antigen challenge (OVA) or resiquimod treatment (RES) in the joint analysis of both the A/J and C57BL/6 strains, or which were significantly affected by resiquimod treatment in the C57BL/6 strain, but not the A/J strain, when the strains were analyzed separately. The Hits column represents the number of genes significantly affected by antigen challenge (OVA) or resiquimod treatment (RES) treatment, relative to non-challenged controls (OVA) or untreated animals (RES), respectively. The significance of differences was assessed as described in the materials and methods. The Assayed column represents the number of genes belonging to the pathway which were assayed by the Affymetrix Mouse 430v2 chips. The Up and Down columns represent the number of genes which were induced and repressed, respectively. The EASE[226] score of each pathway is indicated. The threshold of significance was set at \( p \leq 0.05 \) after correction by the bootstrap method incorporated in EASE. Bootstrap \( p \) values are reported in the Bootstrap column.
Table 5: Top 30 Genes Altered by Resiquimod Treatment

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Affymetrix probeset ID</th>
<th>Genbank Accession</th>
<th>P Value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gzma</td>
<td>Granzyme A</td>
<td>1417898_a_at</td>
<td>NM_01037</td>
<td>8.5 x 10^-19</td>
<td>21</td>
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Differential gene expression in resiquimod treated animals. The table contains the 30 transcripts showing the largest absolute fold change between resiquimod treated and untreated animals following antigen challenge. All animals were sensitized to the antigen. Positive and negative fold changes represent up- and down-regulation of genes, respectively, in treated mice versus untreated mice. Unadjusted p values are shown and only genes with significantly altered expression are included (statistical assessment is described in the Materials and Methods section).
**Table 6: Significantly Altered Genes Following Antigen Exposure in Different Studies**

Table 6: The number of significantly altered genes following allergen exposure in different studies

<table>
<thead>
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<th>Study/Studies</th>
<th>Number of Genes</th>
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<tr>
<td>Present Study</td>
<td>197</td>
</tr>
<tr>
<td>BALB/c OVA-Induced Model 1</td>
<td>436</td>
</tr>
<tr>
<td>BALB/c OVA-Induced Model 2</td>
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<tr>
<td>BALB/c A. fum. -Induced Model</td>
<td>387</td>
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<tr>
<td>Present Study, A. fum. Model, and either BALB/c OVA-Induced Model</td>
<td>82</td>
</tr>
<tr>
<td>All Studies</td>
<td>40</td>
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</tbody>
</table>

Comparison of the number of genes whose expression was found to be significantly altered, by allergen challenge of sensitized mice, in the present study, in the BALB/c ovalbumin- and *A. fumigatus*-induced models used by Zimmermann et al.[233] (BALB/c OVA-Induced Model 1 and BALB/c A. fum.-Induced Model respectively), and in the BALB/c ovalbumin-induced model used by Kuperman et al.[232] (BALB/c OVA-Induced Model 2). Also shown is the number of genes which were identified by the present study (A/J and C57BL/6J ovalbumin-induced model), the BALB/c A. fumigatus-induced model, and either one of the BALB/c ovalbumin-induced models and the number of genes which were identified by all studies.
Figure 1: Summary of the Total Numbers of Differentially Expressed Genes

Venn diagram summarizing the number of genes on the microarrays with statistically significant differences in expression (as described in the Materials and Methods section) between A/J and C57BL/6 mice in each treatment group. All mice were sensitized with ovalbumin. The PBS group received 3 daily mock aerosol challenges of phosphate buffered saline. The OVA and RES groups received 3 daily ovalbumin aerosol challenges. The RES group was also treated with 100μg of resiquimod 24 hours prior to each challenge.
Figure 2: Genes NK cell mediated cytotoxicity pathway altered by resiquimod treatment

Represented is a portion of the Natural Killer Cell Mediated Cytotoxicity pathway (04650mmu) adapted from the KEGG[227] database. The 21 genes contained within this portion of the pathway with significant changes in pulmonary expression, when comparing resiquimod treated mice to untreated mice, are highlighted (yellow ≤ 3 fold, orange ≤ 6 fold, pink ≤ 9 fold, and red > 9 fold increase in expression). The pathway contains a total of 128 genes, 110 of which were assayed, and 21 of which were significantly altered. All mice were antigen sensitized and challenged, and treated mice received 100μg of resiquimod 24 hours prior to each antigen challenge. Fold changes in gene expression are expressed as the ratio of expression in the resiquimod treated mice to the untreated asthmatic mice. The significance of changes in expression was determined as described in the materials and methods.
Figure 3: Real-time RT-QPCR Validation

Microarray results were validated by real-time RT-QPCR. 14 genes with statistically significant change in expression following antigen challenge and/or resiquimod treatment were chosen to represent approximately -20 to -2 and 2 to 20 fold changes in expression between antigen challenge and control animals (OVA vs. PBS) and between resiquimod treated and untreated animals (RES vs. OVA). The real-time RT-QPCR data of the 9 genes whose expression was both significantly higher in OVA than PBS A/J mice and significantly lower in RES than OVA A/J mice (based on the microarray data) is illustrated in panel A. The real-time RT-QPCR data of the 5 genes whose expression was significantly higher in RES than OVA mice is illustrated in panel B. All significant differences were confirmed by the real-time RT-QPCR data. * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001, N = 5 or 6 for each group.
Figure 4: Resiquimod Treatment Results in Pulmonary NK-Cell Recruitment

FACS analysis of the cellular content of the lungs of animals 3 hours following the final antigen challenge showing an increase in NK cells in resiquimod treated versus untreated mice. All mice were antigen sensitized; OVA and RES mice received an antigen challenge whereas PBS mice were mock challenged with PBS. Furthermore, RES mice were treated with 100μg of resiquimod 24 hours prior to each antigen challenge. Cell counts were gated for lymphocytes based on front and side-scatter. NK cells were identified as gated DX5\(^+\) and CD11b\(^+\) double positive cells. Data is representative of 2 separate experiments. FACS analysis was performed on cells isolated from 6 animals which were pooled into 3 samples containing cells isolated from 2 mice.
Figure 5: Pulmonary and Hepatic NK Cell Recruitment Following Resiquimod Treatment

FACS analysis of the cellular content of the lungs and livers of C57BL/6 mice. (A) NK cell numbers in the organs of untreated (Ctrl) mice and from the organs of resiquimod treated mice collected 3 hours following the first, second, or third daily dose (100μg i.p.) of resiquimod. (B) A similar analysis of NK cell numbers in untreated mice (Ctrl) and 24, 48, and 96 hours following the third daily resiquimod treatment. A similar analysis was performed with the spleens where no significant changes were observed (data not shown). Cell counts were gated for lymphocytes based on front and side-scatter. NK cells were identified as gated DX5+ and CD11b+ double positive cells. Data is pooled from 2 independent experiments with a total N of 5-6, * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001.
Figure 6: Effect of NK Cell Depletion and CD8 ablation on the efficacy of resiquimod treatment

Analysis of the prevalence of eosinophils in the bronchoalveolar lavage fluid (BALF) of C57BL/6J (A), NK cell depleted (B), and CD8 knock-out (C) mice 48 hours after the final antigen aerosol challenge. Both OVA and RES mice were antigen sensitized and challenged daily for three consecutive days, while only RES mice were treated with 100μg of resiquimod 24 hours prior to each antigen challenge. Eosinophils were counted from Diff-quik stained cytospin slides prepared from the BALF of the mice. Values are expressed as the percentage of the BALF inflammatory cells identified as eosinophils, N = 3-7.
A C57BL/6J

B NK Cell Depleted

C CD8 Knock-out

BAL Eosinophils (% of Inflammatory Cells)

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p = 0.0006

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p < 0.0001
Chapter 5 - Identification of Novel Chromosomal Regions Associated with Airway Hyperresponsiveness (AHR) in Recombinant Congenic Strains of Mice

Adapted from:

Preface

In the fourth chapter data was presented describing the transcriptional changes induced by the onset of asthma in A/J and C57BL/6 mice. While both A/J and C57BL/6 mice develop similar pathology following antigen sensitization and challenge, which results in similar transcriptional changes, there are several important inter-strain differences. The airways of A/J mice, like the airways of atopic asthmatics and individuals at risk of developing asthma, are hyperresponsive to bronchoconstricting stimuli, including methacholine, while the airways of C57BL/6 mice are hyporesponsive. Understanding the genetic factors controlling airway responsiveness to methacholine is critical as these differences underlie airflow obstruction, the cardinal symptom of asthmatic disease.

The study presented in the following chapter describes the identification of chromosomal regions, using recombinant congenic strains of mice generated from A/J and C57BL/6 mice, which are associated with the differences in airway responsiveness.
Abstract

Airway responsiveness (AR) is the ability of the airways to respond to bronchoconstricting stimuli by reducing their diameter. Airway hyperresponsiveness has been associated, in both humans and murine models, with asthma susceptibility and it has been shown to be a complex and heritable trait. In particular the A/J mouse strain is known to have hyper-responsive airways, while the C57BL/6 strain is known to be relatively refractory to bronchoconstricting stimuli. We analyzed recombinant congenic strains (RCS) of mice generated from these hyper- and hypo-responsive parental strains, to identify genetic loci underlying the trait of AR in response to methacholine as assessed by whole-body plethysmography. Our screen identified 16 chromosomal regions significantly associated with AHR (adjusted p<=0.05); 8 are supported by independent and previously published reports while 8 are entirely novel. Regions, which overlap with previous reports, include two regions on chromosome 2, three on chromosome 6, one on chromosome 15, and two on chromosome 17. The 8 novel regions are located on chromosome 1 (92 – 100cM), chromosome 5 (> 73cM), chromosome 7 (> 63cM), chromosome 8 (52 – 67cM), chromosome 10 (3 – 7cM and > 68cM), and chromosome 12 (25 – 38cM and >52cM). Our data identifies several likely candidate genes from the 16 regions including Ddr2, Hc, Fbn1, Flt3, Utrn, Enpp2, and Tsc.
Introduction

Asthma is a common disease of the airways characterized by episodes of reversible airway obstruction, as well as airway hyperresponsiveness and inflammation [8]. While there is convincing evidence that genetic factors are in part responsible for the development of asthma, most genetic studies have focused on intermediate phenotypes such as total and allergen specific IgE levels, skin-prick test responses to a number of common allergens, eosinophil counts, and airway responsiveness to methacholine [156]. There are several advantages to using intermediate phenotypes, rather than a diagnosis of asthma, as traits in linkage and mapping studies. The intermediate phenotypes are objective and quantitative, each is likely to only be affected by a subset of the genetic factors involved in the etiology of asthma [39], and they can be readily modeled in animals [289].

A commonly studied intermediate phenotype of asthma is airway hyperresponsiveness. Airway responsiveness is the ability of airways to respond to direct or indirect bronchoconstricting stimuli through airway smooth muscle contraction which results in a reduced airway diameter. The airways of asthmatic patients, however, are hyperresponsive in that they respond more forcefully to bronchoconstricting stimuli. Airway responsiveness has been used in many studies aimed at identifying asthma susceptibility genes because it is both a heritable trait, and precedes the development of asthma [26-29]. To date, several chromosomal regions have been linked to airway responsiveness in human studies including regions on chromosome 2 [290], 4 [291], 5 [33], 7 [291], and 12 [292]. However, despite some successes, including DPP10 [158], identification of the genes responsible for these associations in human populations has proven to be difficult. While large-scale multicenter whole-genome association studies which are now underway promise to lead to the identification of significantly more candidate genes from these regions in human populations [293], animal models, and in particular mice, are necessary to study
the molecular networks responsible for specific phenotypes. Studying the genetic regulation of asthmatic responses in animal models offers an alternative approach for the identification of genes in smaller scale studies where genetic and environmental heterogeneity can be more tightly controlled.

Airway responsiveness is not only a heritable trait in humans but also in mice, as evidenced by the fact that it varies considerably among inbred strains [294]. The A/J strain has long been known to be one of the most hyperresponsive amongst the common laboratory strains, and for this reason, crosses involving A/J mice and either the C57BL/6 or C3H hyporesponsive strains have been the most commonly used in whole-genome association studies of airway responsiveness. Significant or suggestive QTL for airway responsiveness have been identified on chromosomes 2 [161], 6 [162;295], 7 [295], and 17 [295] by employing F2 intercrosses generated from A/J and C3H mice while QTL or significantly associated chromosomal regions have been identified on chromosomes 2 [160;296], 6 [296], 15 [160], and 17 [160] by employing various breeding schemes derived from A/J and C57BL/6 mice. Amongst the many regions on these chromosomes, the only candidate gene which has been successfully identified and validated is hemolytic complement (Hc formerly complement factor 5 or C5) which modulates airway responsiveness and is located within the chromosome 2 region identified by Ewart and colleagues [161;165].

The AcB/BcA recombinant congenic strains (RCS) were derived from the A/J and C57BL/6 inbred strains of mice in order to aid in the dissection of complex traits, which makes these mice, due to the parental strains’ drastically differing airway responsiveness phenotypes and the common use of these strains in genetic studies of airway responsiveness, an ideal platform for the dissection of this phenotype. This RCS panel has been used successfully to identify QTLs for numerous traits including amphetamine induced locomotion[297], emotionality and stress response [298;299], alcohol preference [300], and susceptibility to
malaria [301] and Salmonella [302] infection. Each RCS is fully inbred and contains approximately 12.5% of the genome of one parental strain on a background of the other parental strain thus allowing subsets of loci that contribute to the phenotype to be isolated in different inbred lines [167]. Furthermore, the association analysis is simplified by negating dominance effects and allowing multiple measurements of the phenotype to be performed on several genetically identical animals, thereby reducing the phenotypic noise.

The objective of this study was to replicate the identification of loci previously identified in A/J x C57BL/6 crosses, but rarely if ever replicated, and we expected that the RCS would allow the detection of additional chromosomal regions associated with airway responsiveness. Furthermore, the identification of informative RCS, those strains which exhibit the phenotype of their minor genetic donor, immediately allows the localization of airway responsiveness loci enabling rapid identification of causative genes.
Materials and Methods

Recombinant Congenic Strain Panel and Genotyping

Recombinant congenic strains (RCS) of mice were generated at the Montreal General Hospital Research Institute from methacholine challenge resistant, C57BL/6 (B), and susceptible, A/J (A), mice by creating an A/J x C57BL/6 F1 cross followed by two backcrosses to each of the original parental strains (Figure 1) [167]. The resulting progeny (both [(F1 x A) x A]) (referred to as AcB) and [(F1 x B) x B] (referred to as BcA)) were then inbred for more than 30 generations generating 15 distinct AcB strains, containing on average 13.25% of the B genome on an A background (on average 84.4% of the A genome), as well as 22 distinct BcA strains, containing on average 13.24% of the A genome on a B background (on average 85.37% of the B genome). All RCS were previously genotyped with a panel of 625 informative SSLP markers [167]. All markers from this panel which are currently mapped to a single position in the Mouse Genome Informatics (MGI) Mouse Genome Database (www.informatics.jax.org) were selected for use in our analysis. This yielded 603 markers covering the entire genome with an average spacing of 2.7cM. All procedures involving live animals were reviewed and approved by the Montreal General Hospital and McGill University animal care and use committees.

Airway Responsiveness Phenotyping

Airway responsiveness was assessed as the base 2 logarithm of the enhanced pause (Penh) measurement acquired in response to 50μL of a 15mg/mL methacholine aerosol administered over a one minute period using an unrestrained whole-body plethysmograph (Buxco Research Systems). Ten male mice from each parental strain (A/J and C57BL/6) as well as at least 4 male mice from each of the 11 AcB and 22 BcA strains employed in the study were phenotyped at 8 – 12 weeks of age. In all cases, mice were placed in the plethysmograph chamber and allowed to acclimate for 5 minutes following which they were challenged first with a 50μL aerosol of PBS administered over 1
minute and subsequently with similar aerosols containing 5, 10, 15, and 20 mg/mL of methacholine (in PBS) allowing the Penh parameter to return to baseline between each challenge. After each aerosol challenge the average Penh measured during the 5 minutes following exposure was recorded. The Penh in response to the aerosol containing 15mg/mL of methacholine most reliably differentiated the parental strains and was selected for the phenotype. Finally, the base 2 logarithm of the Penh values was used in all analyses in order to normalize the distribution of the phenotype values.

**Identification of Significantly Associated Regions**

We performed a marker-by-marker analysis using a linear model to detect associations between the observed phenotypes and the genotype, or donor strain of origin (DSO), of the marker while correcting for the variance due to the predominant background strain. We used the log transformed median Penh measurement from each RCS as the phenotype and the analysis was performed with the following model $P = \beta_0dso + \beta_1bg + \beta_2dso\times bg + \varepsilon$ where: $P$ is the observed phenotype of the strain (median log$_2$(Penh) at 15mg/mL), $dso$ is the donor strain of origin of the marker and $bg$ is the predominant background (A for AcB strains and B for BcA strains). A permutation analysis was then performed by shuffling the phenotypes across the genotypes (without replacement) to correct for multiple testing. Significance was taken as a p-value which was smaller than the smallest p-value in more than 95% of the permutations ($p \leq 0.05$ of one or more markers with a more significant p-value per whole genome). Regions associated with airway responsiveness were defined as the genomic interval spanning all the sequential markers on a chromosome which were significantly associated with airway responsiveness, as well as that part of the genome which extends from the set of significantly associated markers to the first marker, on each side, which was not significantly associated with airway responsiveness. If two such regions were separated by a single non-significantly associated marker, the interval spanning both regions was considered to form a
single region significantly associated with airway responsiveness. All analysis was performed using the standard R packages (www.R-project.org).

**Databases Used and Gene Characterization**

A gene was considered to be expressed in the lungs if it fulfilled one or more of the following criteria. The MGI Gene Expression Database (GXD)[304] listed the gene as detected in the lungs at any stage of development. The cDNA of the gene was listed in the MGI GXD as isolated from lung tissue. Or, a probeset for the gene was detected in the upper 75th percentile (by signal intensity) of probesets using Affymetrix microarrays of lung mRNA originating from either the A/J or C57BL/6 strain (GEO accession GSE2148). The 75th percentile in signal intensity corresponds roughly to all genes whose signal was equal to, or greater, than the BioB control, considered to be the lower limit of detection on these arrays.

Mutations in or within 2kbp of genes contained within regions identified as significantly associated with airway responsiveness were identified employing the annotations of the MGI MGD. Genes were considered non synonymous or splice site (NS/SS) mutants if they contained any variations which were polymorphic between the A/J and C57BL/6 strains, and were labeled with dbSNP annotations of “Coding-Non Synonymous” or “Splice-Site”. Genes were considered to be synonymous or non-coding mutants if the variations were labeled with any other dbSNP annotation.

The frequency of single nucleotide polymorphisms (SNP) was employed in order to identify intervals which were identical by decent (IBD) within the regions which had been identified as significantly associated with asthma. IBD intervals were defined as any continuous interval, at least 500kbp in length, which contained no more than 10 SNP across any 50kbp. IBD intervals were identified employing all SNPs, polymorphic between A/J and C57BL/6 mice, which could be extracted from the MGI MGD.
Other Statistical Analyses

Comparison of the mean phenotype of the AcB strains to the mean phenotype of the BcA strains was performed with a two-tailed unpaired t-test. Analysis of the phenotypes across all strains was performed with a one-way ANOVA followed by Bonferroni corrected pair-wise comparisons of every AcB strain to the A strain and every BcA strain to the B strain. In all cases significance was set at $p \leq 0.05$. 
Results

Airway Responsiveness

The difference in airway responsiveness between A/J and C57BL/6 mice has previously been well characterized by several methods [160;294] including the Penh measure acquired by whole body plethysmography [296]. As expected the A/J mice in our study exhibited significantly more airway responsiveness than the C57BL/6 mice (Figure 2). Airway responsiveness was then assessed in the 11 AcB and 22 BcA strains which make up the RCS panel and the distribution of phenotypes is illustrated figure 2. A one-way ANOVA was performed and confirmed a very significant strain effect while Bonferroni corrected post-tests comparing all AcB strains to their major genetic donor parental strain, the A/J mice, and similarly, all BcA mice to the C57BL/6 mice, identified two AcB and two BcA strains which were significantly different from the A/J and C57BL/6 mice respectively.

While the empirical Penh parameter is commonly used as a measure of airway responsiveness in mouse studies, caution must be used when interpreting these results [305]. Direct measurements of airway resistance were performed in the A/J, C57BL/6, AcB64, BcA85, and BcA86 strains as well as from several other RCS. We have found perfect concordance between these measurements and the Penh measurements in non-allergic mice among the strains tested indicating that the use of the Penh parameter provides an adequate assessment of lung phenotypes presented in this chapter (data not shown).

Two further characteristics of the phenotype are immediately obvious from a cursory examination of Figure 2. One is that nearly two-thirds of the BcA strains (14 of 22 strains) displayed lower airway responsiveness than the C57BL/6 parental strain indicating that the hyperresponsive A/J strain likely contains alleles which confer resistance to the methacholine challenge, at least in a C57BL/6 background. Also apparent is the effect of the predominant
background on the phenotype of the RCS with most AcB strains showing high
airway responsiveness and most BcA strains low responsiveness. In fact, the
average strain mean for the 22 BcA strains was significantly lower than the
average strain mean of the 11 AcB strains (Figure 3).

**Marker Association**

In order to identify chromosomal regions containing alleles which affect the
airway responsiveness phenotype we performed a test for association at each of
the 603 SSLP markers for which the genotypes of the RCS panel was available.
Because of the strong effect of the predominant genetic background (Figure 3),
the test for association of marker genotypes controlled for the predominant
background strain (as described in the Materials and Methods section). This
analysis led to the identification of 50 markers significantly associated with
airway responsiveness which delimited the 16 chromosomal regions listed in
Table 1.

Of the 16 regions identified as significantly associated with airway
responsiveness, 8 replicate quantitative trait loci (QTL) or regions which have
previously been implicated in the control of airway responsiveness in crosses
involving the A/J strain. These 8 replicated regions are distributed among 4
chromosomes and include the 2 chromosome 2 regions, the 3 chromosome 6
regions, the region on chromosome 15, and the 2 regions on chromosome 17.
Furthermore, the proximal chromosome 17 region was also associated to airway
responsiveness in a BP-2 x BALB/c cross. The remaining 8 regions have, to the
best of our knowledge, never been reported to be associated with airway
responsiveness. These regions are spread across 6 chromosomes and include
one region on chromosomes 1, 5, 7, and 8, as well as 2 regions each on
chromosomes 10 and 12.
Characterization of Associated Regions

The size of most of the regions identified as significantly associated with airway responsiveness results in too large a number of genes for potential candidates to be identified without further analysis (Table 2). In order to narrow the list of potential candidates, we employed several complementary in-silico sequence analysis approaches which employed published sequence data. The genetic variation observed in inbred mice, including the RCS used in our analysis, is almost entirely ancestral (arose before the A/J and C57BL/6 strains split from each other) in origin [306]. This implies that genomic intervals found in regions significantly associated with airway responsiveness, and where common haplotypes are shared in both the susceptible A/J and the resistant C57BL/6 parental strains, are unlikely to contain the polymorphism responsible for the association. Analysis of the regions identified in the present study reveals that, on average, only one third of genes lie in intervals which we consider identical by descent (Table 2) and therefore, by itself, this approach only results in a moderate reduction in the list of potential candidate genes.

Most of the genes affecting airway responsiveness are very likely to be expressed in lung and airway tissues. We employed public databases and published expression data (described in the Materials and Methods section) in order to identify which genes located within the significant regions are expressed in mouse lungs. Analysis of the genes located within the significant regions reveals that there is evidence of pulmonary expression for approximately one third (34%) of genes (Table 2) which allows for a significant reduction of the number of genes which are likely to be responsible for modulation of the phenotype.

Any genes which are responsible for the association of a chromosomal region and airway responsiveness must contain one or more sequence variants that are polymorphic between the A/J and C57BL/6 parental strains. We therefore examined all the genes within the significant regions for known sequence
variations. Nearly half (46%) of the genes in these regions contained known polymorphisms which do not result in amino acid changes while only 12% of the genes contain mutations which result in a difference at the protein level (Table 2).

**Likely Candidate Genes**

While each of the approaches used above only resulted in a moderate reduction in the number of potential candidate genes, the simultaneous use of several approaches has the potential to produce a more manageable list of potential candidates for each region. Requiring that potential candidate genes be expressed in the lungs and that they contain a polymorphism between the A/J and C57BL/6 parental strains which leads to a change in amino acid sequence resulted in a list of 1 to 27 possible candidate genes for each of the chromosomal regions. A literature search was performed for each of the genes in order to identify those involved in processes related to asthma, lung function and development, airway function, or smooth muscle contraction. This resulted in a small number of candidate genes for some of the regions identified as significantly associated with airway responsiveness. These likely candidate genes are listed in Table 3.
Discussion

In this study we employed an RCS panel derived from the airway hyperresponsive A/J strain and hyporesponsive C57BL/6 strain to identify chromosomal regions associated with airway responsiveness to methacholine as assessed by the whole body plethysmography enhanced pause (Penh) method. Consistent with a complex pattern of inheritance involving several loci, the distribution of the airway responsiveness phenotypes of the RCS panel was continuous and extended beyond both parental strains. We observed that the background strain (A/J in AcB mice and C57BL/6 in the BcA mice) had a significant effect on the airway responsiveness phenotype, as is the case with a number of other phenotypes which segregate in the AcB/BcA RCS mice [298;299].

Previous studies in crosses derived from the A/J and C57BL/6 strains have identified 4 QTLs, 1 on distal chromosome 2, and 1 each on chromosomes 6, 15, and 17 [160;296], while crosses derived from the A/J and C3H strains led to the identification of distinct QTL on proximal chromosome 2 and chromosome 7, as well as QTL spanning most of chromosome 6 [161;162;295]. Of these, the QTL on distal chromosome 2 was replicated in at least two independent studies employing crosses derived from A/J and C57BL/6 mice, and a set of partially overlapping QTL on chromosome 6 have been identified in crosses derived from A/J and both C57BL/6 and C3H mice. The analysis performed in the present study independently identified significant association with airway responsiveness for SSLP markers overlapping all these previously identified QTL, with the exception of the QTL on chromosome 7. Given that the chromosome 7 QTL was identified in a cross involving C3H mice, it is possible that the locus responsible for the QTL is simply not polymorphic between the A/J and C57BL/6 strains. Ackerman at al. also reported the existence of significant epistatic interaction between QTL on chromosome 2 and 6 and while we replicated each of the QTL, our study could not effectively test for these effects due to the large number of
chromosomal regions associated with airway responsiveness and the small number of genotypes (33 RCS strains). Overall, given how well the RCS replicate QTL derived from both A/J x C57BL/6 and A/J x C3H crosses, our results indicate that there may be significant overlap of the loci which mediate differential airway responsiveness in A/J mice when compared to both strains.

While the most commonly employed crosses, used in the genetic analysis of airway responsiveness, are derived from A/J mice, other strain combinations have also been analyzed. QTL have been identified on chromosomes 9, 10, 11, and 17 in a (BALB/c x BP2)F<sub>2</sub> cross [163] and a locus on chromosome 11 in a cross derived from BALB/c and HBA mice [307]. While one region identified in the RCS overlapped the QTL on chromosome 17 the remaining QTL were not replicated. This indicates that despite the large number of loci that modulate airway responsiveness in crosses derived from A/J and either C57BL/6 or C3H mice, difference in these crosses may not represent the full breadth of genetic factors which affect the airway responsiveness phenotype.

In addition to replicating several previously described QTL, we have identified 8 novel regions, across 6 chromosomes, which are significantly associated with airway responsiveness but contain no previously reported airway responsiveness QTL. The regions vary in size from a low of 8 Mbp to a high of 28 Mbp and contain between 45 and 357 genes. While both these novel regions, and the regions which replicate previously identified QTL, remain too broad to readily identify relevant candidate genes, we applied several concurrent in-silico approaches in order to narrow the list of potential candidates. Requiring that potential candidate genes be expressed in the lungs and that they contain at least one mutation leading to an amino acid change ensures that the resulting list contains genes which are very likely to result in significant biological effects. The potential candidate gene lists resulting from applying these 2 criteria contained between 1 and 27 genes per region, allowing likely candidate genes to
be identified based on gene functional annotations and a review of the literature.

This approach allowed us to identify several likely candidate genes involved in smooth muscle function including \textit{Tsc2} (tuberous sclerosis 2), located within the proximal chromosome 17 region and involved in the phosphatidylinositol 3-kinase mediated control of airway smooth muscle cell growth and proliferation [308], as well as \textit{Utrn} (utrophin) which is involved in acetylcholine receptor clustering at neuromuscular junctions [309]. Given that airway smooth muscle contraction is the defining feature of airway responsiveness, genes that affect smooth muscle function are likely to be capable of modulating the airway responsiveness phenotype. Other likely candidates include genes that modulate the deposition and maintenance of the extracellular matrix (ECM) which has long been associated with changes in airway function [310]. The discoidin domain receptor family member 2 (\textit{Ddr2}) gene is located within the region identified on chromosome 1. The extracellular domain of the \textit{Ddr2} protein can bind collagen resulting in an inhibition of collagen fibrillogenesis [311] thereby affecting the ECM. Fibrillin 1 (\textit{Fbn1}) was identified as a likely candidate gene residing in the distal chromosome 2 region and is itself an ECM component found in extracellular microfibrils. The severe morphological abnormalities in the lungs of \textit{Fbn1} deficient mice suggest that mutations in this gene could lead to significant changes in pulmonary physiology which is likely to affect airway responsiveness [312]. Two other likely candidate genes are ectonucleotide pyrophosphatase / phosphodiesterase 2 (\textit{Enpp2}) which is located within the chromosome 15 region and has previously been associated to lung function phenotypes [313], and hemolytic complement (\textit{Hc}, formerly C5) which has been demonstrated to explain, at least partially, a QTL identified in a cross derived from A/J and C3H mice which overlaps the region identified on proximal chromosome 2 [165].

The identification of informative strains from the RCS panel provides an important tool for the further dissection of the airway responsiveness trait. Of
the 33 strains which made up the RCS panel, 3 were found to be particularly interesting in this regard. The AcB73, BcA68, and BcA73 strains exhibit a phenotype which is indistinguishable from that of their minor genetic donors while carrying only 12.5% of their genome which includes only 5 or 6 of the regions identified as significantly associated with airway responsiveness. These three informative recombinant congenic strains together contain recombinant segments for 12 of the 16 regions identified in the present study; however, each strain contains a subset of only 5 or 6 regions indicating that the parental phenotypes can be reproduced with more than one set of parental alleles implying that the effects of the loci do not act in a strictly additive manner. Because they significantly simplify the genetic model (5 or 6 loci in each strain compared to the 16 potential loci identified in the RCS panel as a whole) the informative congenic strains now provide an ideal genetic background on which to conduct further studies aimed at reducing the size of the regions in order to identify and confirm candidate genes.
Acknowledgements

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Table 1: Chromosomal regions associated with AR.

Table 1: Chromosomal regions associated with airway hyperresponsiveness in the AcB/BcA RCS panel.

<table>
<thead>
<tr>
<th>Chr</th>
<th>cM range</th>
<th>Mbp range</th>
<th>Significant Markers</th>
<th>Peak p-value</th>
<th>Prior evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>92.3 – 100</td>
<td>171.7 – 179.6</td>
<td>D1Mit113, D1Mit206</td>
<td>7.62x10^{-5}</td>
<td>2000 Ewart [161], Karp [165]</td>
</tr>
<tr>
<td>2</td>
<td>5 – 27.3</td>
<td>9.3 – 40.6</td>
<td>D2Mit80, D2Mit416, D2Mit81, D2Mit293, D2Mit235, D2Mit367, D2Mit133, D2Mit164</td>
<td>3.30x10^{-5}</td>
<td>2000 De Sanctis 1999 [160], Ackerman 2005 [296]</td>
</tr>
<tr>
<td></td>
<td>65 – 68.9</td>
<td>117.9 – 148.7</td>
<td></td>
<td>4.12x10^{-4}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>73 - Telomere</td>
<td>132.6 - Telomere</td>
<td>D5Mit97, D5Mit31, D5Mit99, D5Mit101, D5Mit287</td>
<td>5.45x10^{-5}</td>
<td>1996 Ewart [162], Ackerman 2005 [296]</td>
</tr>
<tr>
<td>4</td>
<td>0.6 – 15.6</td>
<td>5.3 – 34.7</td>
<td>D6Mit264, D6Mit159</td>
<td>4.41x10^{-5}</td>
<td>1996 De Sanctis 1999 [295], Ewart [162]</td>
</tr>
<tr>
<td></td>
<td>38.5 - 43</td>
<td>92.6 – 98.5</td>
<td>D6Mit178</td>
<td>3.19x10^{-4}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td></td>
<td>60.55 – 65.5</td>
<td>125.3 – 136.4</td>
<td>D6Mit290, D6Mit25</td>
<td>2.48x10^{-4}</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>63.5 - Telomere</td>
<td>135.7 - Telomere</td>
<td>D7Mit187, D7Mit259</td>
<td>1.31x10^{-4}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td>8</td>
<td>52 - 67</td>
<td>111.4 – 129.1</td>
<td>D8Mit271, D8Mit200, D8Mit186</td>
<td>3.04x10^{-4}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td>10</td>
<td>3 - 7</td>
<td>8.5 – 18.1</td>
<td>D10Mit80, D10Mit246, D10Mit205, D10Mit103</td>
<td>3.15x10^{-4}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td></td>
<td>67.5 - Telomere</td>
<td>119.4 - Telomere</td>
<td>D10Mit36, D10Mit52, D10Mit14</td>
<td>1.02x10^{-4}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td>12</td>
<td>25 - 38</td>
<td>55.8 – 83.7</td>
<td>D12Mit79, D12Mit133, D12Mit280, D12Mit196, D12Mit8, D12Mit134</td>
<td>8.86x10^{-5}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td></td>
<td>52 - Telomere</td>
<td>109.5 - Telomere</td>
<td>D12Mit79, D12Mit133, D12Mit280, D12Mit196, D12Mit8, D12Mit134</td>
<td>2.70x10^{-6}</td>
<td>1996 Ewart [162]</td>
</tr>
<tr>
<td>15</td>
<td>15.6 – 48.2</td>
<td>17.1 – 74.7</td>
<td>D15Mit111, D15Mit255, D15Mit100, D15Mit115, D15Mit156, D15Mit105</td>
<td>7.63x10^{-5}</td>
<td>1995 De Sanctis 1995 [160]</td>
</tr>
<tr>
<td></td>
<td>24.5 – 44.5</td>
<td>47.9 – 73.9</td>
<td>D17Mit88, D17Mit139, D17Mit7, D17Mit159</td>
<td>1.22x10^{-5}</td>
<td>1995 De Sanctis 1995 [160]</td>
</tr>
</tbody>
</table>

Regions were identified as associated with airway responsiveness based on the analysis of the phenotypes of the RCS (Figure 1) and the 603 known microsatellite polymorphisms of the parental strains [167]. The data was analyzed using a simple generalized linear regression model which controlled for the major genetic donor strain, and significance was assessed based on randomly shuffling the phenotypes 60,000 times across the genotypes. Indicated p-values are not adjusted for multiple testing. †In (BP2 x BALB/c)F2, all other previous evidence employed crosses derived from A/J and C57BL/B6, or A/J and C3H mice.
Table 2: Characteristics of the genes in each of the 16 regions associated with airway responsiveness.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Region (Mb)</th>
<th>Total Genes</th>
<th>A/J vs. C57Bl/6 non-IBD</th>
<th>Genes Expressed in Lungs</th>
<th>NS or SS Mutant Genes</th>
<th>Synonymous and Non-coding Mutant Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>171.8 – 179.6</td>
<td>124</td>
<td>123</td>
<td>56</td>
<td>38</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>9.3 – 40.7</td>
<td>495</td>
<td>234</td>
<td>181</td>
<td>32</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>117.9 – 148.7</td>
<td>419</td>
<td>232</td>
<td>134</td>
<td>64</td>
<td>182</td>
</tr>
<tr>
<td>5</td>
<td>132.6 – Telomere</td>
<td>357</td>
<td>266</td>
<td>137</td>
<td>30</td>
<td>174</td>
</tr>
<tr>
<td>6</td>
<td>5.3 – 34.7</td>
<td>189</td>
<td>84</td>
<td>59</td>
<td>8</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>92.6 – 98.5</td>
<td>25</td>
<td>20</td>
<td>8</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>125.3 – 136.4</td>
<td>183</td>
<td>175</td>
<td>44</td>
<td>51</td>
<td>88</td>
</tr>
<tr>
<td>7</td>
<td>135.7 - Telomere</td>
<td>257</td>
<td>80</td>
<td>99</td>
<td>13</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>111.4 – 129.1</td>
<td>232</td>
<td>132</td>
<td>98</td>
<td>18</td>
<td>111</td>
</tr>
<tr>
<td>10</td>
<td>8.5 – 18.1</td>
<td>45</td>
<td>45</td>
<td>14</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>119.4 - Telomere</td>
<td>158</td>
<td>130</td>
<td>80</td>
<td>11</td>
<td>83</td>
</tr>
<tr>
<td>12</td>
<td>55.8 – 83.7</td>
<td>224</td>
<td>164</td>
<td>83</td>
<td>18</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>109.5 - Telomere</td>
<td>176</td>
<td>176</td>
<td>50</td>
<td>34</td>
<td>71</td>
</tr>
<tr>
<td>15</td>
<td>17.1 – 74.7</td>
<td>280</td>
<td>173</td>
<td>86</td>
<td>23</td>
<td>130</td>
</tr>
<tr>
<td>17</td>
<td>3.9 – 25.0</td>
<td>338</td>
<td>308</td>
<td>81</td>
<td>66</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>47.9 – 73.9</td>
<td>208</td>
<td>135</td>
<td>74</td>
<td>23</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Total:</td>
<td>3710</td>
<td>2477</td>
<td>1284</td>
<td>437</td>
<td>1720</td>
</tr>
</tbody>
</table>

Number of genes, in each region associated with airway responsiveness based on the RCS panel survey, which are expressed in the lungs (based on MXD database and microarray data), which are in regions which are non-identical by decent (non-IBD) between A/J and C57Bl/6 mice (according to SNP density, data from MGI), which contain non-synonymous (NS) or splice-site (SS) mutations (from MGI database), or which contain synonymous mutations or mutations in non-coding regions (from MGI database). Note that these numbers exclude all olfactory receptor genes which are considered extremely unlikely candidates, and include expressed sequence tags, predicted genes, and cDNA clones.
Table 3: Likely candidate genes.

<table>
<thead>
<tr>
<th>Chr. (Mbp)</th>
<th>Symbol</th>
<th>Name</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ddr2</td>
<td>Discoidin domain receptor family, member 2</td>
<td>Expressed in the lungs Contains a non-synonymous SNP May contribute to the regulation of ECM collagen</td>
</tr>
<tr>
<td>2</td>
<td>Hc</td>
<td>Hemolytic complement</td>
<td>Expressed in the lungs Contains several non-synonymous variations Has been shown to modulate AR by Karp et al. (2000)</td>
</tr>
<tr>
<td>2</td>
<td>Fbn1</td>
<td>Fibrillin 1</td>
<td>Expressed in the lungs Contains a non-synonymous SNP Involved in extracellular matrix deposition/maintenance Mutations lead to Marfan syndrome in humans Knock-out mice display several lung morphology phenotypes</td>
</tr>
<tr>
<td>10</td>
<td>Utrn</td>
<td>Utrophin</td>
<td>Expressed in the lungs Contains a non-synonymous SNP It is necessary for the normal clustering of acetylcholine receptors at the neuromuscular synapse</td>
</tr>
<tr>
<td>15</td>
<td>Enpp2</td>
<td>Ectonucleotide pyrophosphatase / phosphodiesterase 2</td>
<td>Expressed in the lungs Contains a non-synonymous SNP Previously identified as a candidate gene for the control of lung function in mice</td>
</tr>
<tr>
<td>17</td>
<td>Tsc2</td>
<td>Tuberous sclerosis 2</td>
<td>Expressed in the lungs Contains a non-synonymous SNP Implicated in the potential regulation of airway smooth muscle growth and differentiation</td>
</tr>
</tbody>
</table>

Likely candidates identified for selected chromosomal regions associated with airway responsiveness. Candidates were identified on the basis of their genomic location, comparative analysis of sequence variants observed in the A/J and C57BL/6 parental strains, known pulmonary expression, and a literature search revealing involvement in processes known, or suspected, to modulate airway responsiveness.
Figure 1: RCS Breeding Scheme

Breeding scheme employed for the generation of AcB/BcA recombinant congenic strains (RCS). Methacholine challenge hyperresponsive A/J (A) and hyporesponsive (B) mice were employed as parental strains. (F1 x A) x A and (F1 x B) x B mice were inbred for at least 20 generations to produce the AcB and BcA, respectively, inbred strains which constitute the RCS panel.
**Figure 2: Airway Responsiveness Phenotypes**

Strain phenotype distribution pattern of the airway responsiveness phenotypes of the AcB and BcA RCS, as well as the A/J and C57BL/6 parental strains. Airway responsiveness was assessed as $\log_2(\text{Penh})$ in response to 50μL of an aerosol containing 15mg/mL of methacholine delivered over 1 minute, in conscious and unrestrained mice, using a whole body plethysmograph. Bars represent the mean value ± SEM of each inbred strain. The AcB and BcA strains are coloured white and black respectively, the A/J and C57BL/6 parental strain are coloured grey.
Figure 3: AcB and BcA Mean Phenotypes.

Mean phenotype of all tested AcB and BcA strains. The major background strain of each RCS has a significant effect on the phenotype. Data presented as the mean ± SEM of the AcB and BcA strain means.
\[ \log_2(Pen h) \]

- AcB
- BcA

\[ p = 0.0036 \]
General Discussion
Given the mutual inhibition of $T_H1$ and $T_H2$ cytokines and the $T_H1$ polarization induced by signalling through a number of TLR; it is no surprise that there has been considerable research into the immunomodulatory potential of TLR agonist for diseases associated with $T_H2$ polarized immune responses, and for diseases in which a $T_H1$ response may be beneficial, such as vaccinations and cancer treatments. Teleologically, because its natural ligands are ssRNA molecules of viral origin, TLR7 agonists would be expected to generate immune responses biased towards the $T_H1$ arm of adaptive immunity as this would promote cellular immunity, thereby conferring resistance to viral pathogens. Indeed, antiviral effects and the induction of $T_H1$ responses have been observed after TLR7 agonist stimulation both in vitro [209;220-222] and in vivo [219;220]. Furthermore, topical formulations of imiquimod, a TLR7/8 agonist in the same family as resiquimod, induce strong $T_H1$ mediated cellular immune responses and are used to treat primary skin tumours and cutaneous metastases [314] and resiquimod itself results in clearance of *Mycobacteria bovis BCG*, an intracellular bacteria, in mice [196].

Atopic allergic asthma, on the other hand, is characterized by a robust $T_H2$ mediated immune reaction to innocuous antigens which results in inflammation and the development of airway pathology. In this context, induction of a $T_H1$ biased immune response has the potential to suppress the ability of the immune system to mount pathological $T_H2$ responses. The data presented in this thesis demonstrates the potential of the TLR7/8 agonist resiquimod to prevent the development of allergic asthma pathology.

In chapters 2 and 3 we demonstrated that resiquimod treatment prevented the development of airway inflammation in mouse, and both acutely and chronically challenged rat, models of ovalbumin induced allergic asthma. Likely as a result of the abrogation of inflammation, resiquimod treatment also prevents the development of airway remodelling, AHR and the increase in plasma IgE levels normally observed following ovalbumin aerosol challenge.
These studies establish that resiquimod treatment is beneficial when administered more or less concurrently with allergen exposure in sensitized mice and rats. While it seems likely that resiquimod treatment can at least reduce the inflammatory response to ongoing airway allergen exposure, an important unanswered question of great importance, if resiquimod is to be used clinically, is whether resiquimod treatment will be able to reverse established asthma pathology.

While the effectiveness of resiquimod treatment of asthma shows promise, before its therapeutic use can be considered, two important obstacles must be overcome; the identification of the best route of administration, and the avoidance of side effects. The data presented in this thesis was generated from animals which were treated with resiquimod by intra-peritoneal injection. For obvious reasons, this would not be practical clinically. Our unpublished data indicates that oral administration of resiquimod can prevent inflammation in mice as effectively as intra-peritoneal administration. Furthermore, oral resiquimod treatment prevented the development of allergen induced AHR in a primate model of allergic asthma (Camateros et al. unpublished data).

A recent small phase 2a clinical trial in patients with chronic hepatitis C virus (HCV) infection evaluated the safety of twice weekly oral administration of resiquimod. The study concluded that resiquimod treatment could produce transient side effects similar to those of IFN-α treatment, the standard therapy for chronic HCV infection. The authors speculated that HCV related liver damage was leading to a decreased tolerability, however the studies performed thus far have been too small to draw reliable conclusions. These results, however, were in contrast to prior experience with healthy subjects [315].

While the data presented in this thesis were generated from mice treated with relatively high doses of resiquimod, our preliminary data indicates that a dose of as little as 1 µg, or one hundredth of the dose used in our studies, is equally effective at
preventing the development of airway inflammation following airway antigen challenge in mice (unpublished data). Furthermore, administration of resiquimod directly into the lungs by tracheal instillation also proved effective in preventing the development of airway inflammation (unpublished data). As inhalers and nebulizers are already commonly used to administer asthma therapy in order to minimize systemic drug exposure, inhalation offers another clinically viable approach for the administration of resiquimod whose effectiveness, safety, and tolerability warrant further investigation.

In chapter 4 we also demonstrated that resiquimod treatment leads to broad and important transcriptional changes. While some of these changes were clearly related to the lack of inflammation and asthma pathology, treatment also induced the expression of many immune system genes. In particular, we saw the up-regulation of genes related to NK cell function, and this was associated with the recruitment of NK cells into the lungs, as well as into the liver. These responses are likely beneficial when treating HCV and other hepatic viral infections [316], and could be beneficial in the treatment of respiratory viral infections, including those leading to asthma exacerbations. However, we also demonstrated that the NK cell recruitment was not necessary in order for resiquimod treatment to prevent the development of asthma pathology further indicating that resiquimod treatment might lead to physiological changes which are unnecessary for effective protection against allergic asthma and which might also be undesirable in the context of asthma treatment. These undesired side effects may nevertheless be useful in the context of other diseases.

Despite the hypothesis that resiquimod treatment exerts its effects through the induction of $T_{H1}$ responses, the role of $T_{H1}$ cells and their cytokines in mediating the treatment effect is not yet entirely clear. Mononuclear cells extracted from the lungs of resiquimod treated ovalbumin sensitized and challenged mice produced more IFN-γ following phorbol-12-myristate-13-actetate and ionomycin stimulation than those from untreated controls [144].
As NK cells are the predominant source of IFN-γ production \textit{in vivo} [317], increased IFN-γ production of lung mononuclear cells after non-specific stimulation is entirely consistent with the increased numbers of NK cells following the pulmonary recruitment described in chapter 4, and not necessarily associated with a broader T\textsubscript{H}1 response. In fact, in both A/J and C57BL/6 mice, as well as Brown Norway rats, resiquimod treatment prevented the induction of both T\textsubscript{H}1 and T\textsubscript{H}2 cytokines in response to airway challenge with ovalbumin. Interestingly, a recent study in BALB/c mice found that resiquimod treatment of allergic asthma was partially effective in IL-12p35 knockout mice, but ineffective in mice also deficient for IL-10 [145]. Studies on human blood derived cells have demonstrated that resiquimod treated myeloid dendritic cells induced the expression of IL-10 in naive CD4\textsuperscript{+} T cells [318]. Furthermore, resiquimod treatment of myeloid dendritic cells allowed these cells to polarize naive CD4\textsuperscript{+} T cells towards a regulatory phenotype with IL-10 mediated anti-inflammatory functions [319]. Taken together, these findings suggest that resiquimod treatment results largely in an anti-inflammatory effect, possibly mediated by myeloid dendritic cells and IL-10 secreting regulatory T cells.

Despite demonstrating similar pathology following allergen challenge in chapter 2 and similar transcriptional alteration following allergen challenge in chapter 4, A/J and C57BL/6 mice differ markedly with respect to airway responsiveness. In chapter 5 we further explored the genetic differences of these mice. Specifically, we employed a panel of recombinant congenic strains of mice in order to identify chromosomal regions responsible for the difference in airway responsiveness displayed by these strains. Our survey identified 16 such chromosomal regions, 8 of which had never been previously associated with airway responsiveness. Furthermore, we have identified 3 informative strains which contain subsets of 5 or 6 regions which are sufficient to reverse the airway responsiveness phenotype. These findings will greatly facilitate further
studies aimed at the identification of genes controlling airway responsiveness thus greatly advancing our knowledge of the etiology of asthma.

Overall, the studies described in chapters 4 and 5 of this thesis provide important information about genetic regions and genes involved in the development of asthma and asthma pathology. Furthermore, the studies described in chapters 2, 3, and 4 clearly demonstrate the ability of resiquimod treatment to prevent the development of allergic asthma, and provide a solid base enabling a better understanding of the molecular and cellular mechanisms affected by this treatment.
Claims to Originality

- The thesis author contributed to demonstrating that resiquimod treatment prevents development of acute allergic asthma pathology (AHR, IgE, and inflammation) in mice.

- The thesis author contributed to demonstrating that resiquimod treatment prevents the development of chronic asthma pathology (goblet cell hyperplasia, ASM mass increase) in rats.

- The thesis author was the first to demonstrate that systemic resiquimod administration results in NK cell recruitment to the lungs and liver.

- The thesis author contributed to the characterization of the transcriptome associated with allergic asthma in mice.

- The thesis author contributed to characterization of the differential regulation of genes related to immune function by resiquimod treatment in the context of allergic asthma.

- The thesis author contributed to the identification of several novel chromosomal regions which contribute to AHR, and by extension, asthma susceptibility.
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Appendix I: Chapter 4 Supplemental Data

Supplemental Tables and Figures are available from the Physiological Genomics web site at
http://physiolgenomics.physiology.org/cgi/content/full/0057.2009/DC1.

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