Genetic dissection of airway responsiveness and its impact on the susceptibility to allergic asthma

By:

Rafael Leopoldo Marino, MSc

Department of Medicine, Division of Experimental Medicine

McGill University, Montreal

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

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List of Abbreviations

A	A/J
ADAM21	A disintegrin and metaloprotease domain 8
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ADRB2	Beta2-adrenergic receptor
AHR	Airway hyperresponsiveness
AR	Airway responsiveness
Arhgap5	Rho GTPase activating protein 5 gene
B	C57BL/6
CIHR	Canadian institutes of health research
Clec14a	C-type lectin domain family 14, member a
CpG	Unmethylated CG DNA
CR	Congo red
Ctage5	Cutaneous T-cell lymphoma-associated antigen 5 gene
CTSLTR	Cysteinyl leukotriene receptors
ECP	Eosinophilic cationic protein
Ers	Respiratory system elastance
FcεR	Fc receptor, IgE
FEV_1	Forced expiratory volume in 1 second
Foxa1	Forkhead box A1
Foxa2	Forkhead box A2
Foxp3	Forkhead box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPRA	G protein-coupled receptor for asthma susceptibility
Gpx2	Glutathione peroxidase 2
GXD	Gene expression database
H&E	Haematoxylin and eosin

HCV	Hepatitis C virus
IBD	Identical by descent
ICS	Inhaled corticosteroids
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KO	Knockout
LPS	Lipopolysaccharides
LTE4	Leukotriene E4
LTRA	Leukotriene receptor antagonists
LOD	Logarithm of the odds
MAPK	Mitogen activated protein kinase
Mapkapk2	Mitogen activated protein kinase activated protein kinase 2
MGI	Mouse Genome Informatics
Mbp	Mega base pairs
MS	Multiple sclerosis
NFκB	Nuclear factor κB
OVA	Ovalbumin
PAS	Periodic acid-Schiff
Penh	Pause enhanced
PBS	Phosphate buffered saline
Prkd1	Protein kinase D1
PRR	Pattern recognition receptor
Rsad2	S-adenosyl methionine domain containing 2
PTGDR	Prostaglandin D2 receptors
QTL	Quantitative trait locus
RANTES	Regulated upon activation, normal T cell expressed and secreted
RCS	Recombinant congenic strain
Rrs	Respiratory system resistance

Rtn1	Reticulon1
RT-PCR	Reverse transcriptase – polymerase chain reaction
S28	Resiquimod
S28463	Resiquimod
Slc11a1	Solute carrier family 11, member 1
SLPI	Secretory leukocyte protease inhibitor
SNP	Single nucleotide polymorphism
SPAR	Sandler program for asthma research
STAT6	Signal transducer and activator of transcription 6
TBXA2R	Thromboxane A2 receptor
TGF	Transforming growth factor
T_H1	Type 1 T helper cell
T_H2	Type 2 T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WGA	Whole-genome association

Abstract

Asthma is a chronic and inflammatory disease of the airways determined by genetic and environmental factors which trigger an uncontrolled immune response. Airway hyperresponsiveness (AHR) is inherited as a complex trait and associated with asthma. We assessed AHR in naïve A/J and C57BL/6J mice using the non-invasive method and demonstrated its correlation with invasive method evaluating AHR in non-sensitized mice. Aditionally, we studied the genetic causes underlying AHR using six F2 backcrosses generated from recombinant congenic strains (RCS) created from A/J and C57BL/6J parental strains. Our genetic study allowed us to narrow down the significant regions previously identified in the RCS, as associated with AHR. Quantitative trait loci analysis of BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses shows 3 significant regions on chromosome 12. These significant regions contain 232 genes, of which 9 genes have been identified as potential candidates. Those genes include the *Arhgap5*, *Foxa1, Clec14a*, *Ctage5*, *Rtn1*, *Gpx2*, *Rsad2*, *Prkd1* and *Adam21*.

Additionally, increased levels of secretory leukocyte protease inhibitor (SLPI), an anti-inflammatory mediator, have been observed in asthmatic patients compared to healthy controls. We investigated the role of SLPI in the development of phenotypes associated with allergic asthma and the effect of resiquimod on SLPI expression. Our results demonstrated that over expression of SLPI leads to a lower degree of airway inflammation and higher lung resistance, whereas the ablation of SLPI has the opposite effect on allergic asthma phenotypes. We also demonstrated that the treatment with resiquimod is independent of SLPI expression.

Overall, the results presented in this thesis demonstrate how the non-invasive method is a reliable tool for the assessment of AHR in naïve A/J and C57Bl/6J mice. Furthermore, loci on mouse chromosome 12 are linked to the susceptibility to develop AHR. Candidate genes have been identified in these loci and remain to be explored. Finally, we demonstrate the immunoregulatory activity of SLPI in the airways by diminishing inflammation and improving

lung function in allergic asthma. Taken together, our results provide a better understanding of the inheritance and molecular mechanisms of phenotypes of asthma as well as provide insights for the design of new therapies.

Résumé

L'asthme est une maladie chronique et inflammatoire des voies respiratoires déterminée par des facteurs génétiques et environnementaux qui déclenchent une réponse immunitaire non contrôlée. L'hyper-responsivité aérienne (HRA) est hérité comme un trait complexe et est associée à l'asthme. Nous avons évalué HRA dans des souris de souches A/J et C57BL/6J suivant la méthode non-invasive et nous avons démontré sa corrélation avec la méthode invasive en évaluant l'HRA dans des souris non-sensibilisés. En outre, nous avons étudié la cause génétique de l'HRB en utilisant six croissement en retour (F2) générer avec des souches de souris cogéniques recombinantes (SCR) et les souches parentales, A/J ou C57BL/6J. Notre étude génétique nous a permis de rétrécisse les régions signifiantes identifier dans les SCR. L'analyse des locus quantitatifs des croisements BcA86xC57BL/6J (F2) et BcA86xC3H/HeJ (F2) prouve trois régions significative sur le chromosome 12. Cette région significative contient 232 gènes dont neuf ont été identifié comme gènes candidats. Ces gènes incluent *Arhgap5*, *Foxa1, Clec14a*, *Ctage5*, *Rtn1*, *Gpx2*, *Rsad2*, *Prkd1* and *Adam21*.

En outre, une augmentation du niveau de l'inhibiteur de la protéase des leucocytes sécrétoire (IPLS), un médiateur anti-inflammatoire, a été observée dans des patients souffrant de l'asthme comparé à des contrôles sains. Nous avons étudié le rôle de l'IPLS dans le développent des phénotypes associe avec l'asthme allergique et l'effet de resiquimod sur l'expression de l'IPLS. Nos résultats démontrent que l'hyper-expression de l'IPLS réduit le degré d'inflammation dans les voies respiratoires et augmente la résistance des poumons, tandis que l'ablation de l'IPLS a l'effet opposé sur les phénotypes associés avec l'asthme allergique. Nous avons aussi démontré que le traitement avec resiquimod est indépendant de l'expression de l'IPLS.

En somme, les résultats présentés dans cette thèse démontrent que la méthode non-invasive est une technique fiable pour l'évaluation de l'HRA dans des souris des souches A/J et C57BL/6J non-sensibilises. De plus, des locus sur le chromosome 12 des souris sont liés à la susceptibilité pour développer l'HRA. Des gènes candidats on été identifié dans ces locus et restent à être

explorés. Finalement, on a démontré la propriété immuno-modulatrice de l'IPLS dans les voies respiratoires, en diminuant l'inflammation et en améliorant la performance des poumons des individus souffrant de l'asthme allergique. En tout, nos résultats fournissent une meilleure compréhension de l'hérédité et le mécanisme moléculaire des phénotypes de l'asthme et aux développements de nouvelles stratégies thérapeutiques.

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, McGill University Professor at the Center of the Study of Host Resistance, Research Institute of the McGill University Health Center.

The thesis author performed the literature search and wrote the bulk of the manuscript described in the Chapter 1. Cynthia Kanagaratham and Gabriella Wojewodka contributed to the editing and drafting.

For the Chapter 2, the thesis author designed and supervised all experimental protocols; performanced all experiments, including airway responsiveness assessment, histology and immune assays; collected and analysed all data; interpretated all results and wrote the manuscript related to this chapter. Dr. Pierre Camateros participated as advisor for the analysis of the data. Jennifer Henri was responsible for technical assistance involving mice. Cynhia Kanagaratham contributed to the editing and drafting.

For the Chapter 3, the thesis author designed and supervised all experimental protocols; performed all phenotyping of F1, F2 and control mice. Additionally, the thesis author designed the genotyping of all mice; collected all data; performed the quantitative trait loci and statistical analyses; interpretated all results and wrote the manuscript related to this chapter. Genotyping using microsatellites technology was performed by Dr. Anny Fortin with technical assistance of Sylwia Wiecek Jancik and Vladimira Palkova. Genotyping using SNPs was designed by the author and performed as outsourcing facility by the McGill University and Genome Quebec Innovation Center and University of Laval. Dr. Pierre Camateros contributed int the phenotyping of recombinant congenic strains. Cynthia Kanagaratham contributed in the analysis of the candidate genes linked to airway responsiveness. J. Henri was responsible for technical assistance involving mice. Dr. JC Loredo-Osti contributed in some quantitative trait

loci analysis. Cynthia Kanagaratham and Gabriella Wojewodka contributed to the editing and drafting of the manuscript.

For Chapter 4, the thesis author designed and supervised all experimental protocols; performanced all experiments, including airway responsiveness assessment, histology and immune assays; collected and analysed all data; interpretated all results and wrote the manuscript related to this chapter. Dr. Thusanth Thuraisingam advised in some experiments using SLPI knock-out and transgenic mice. Dr. Pierre Camateros advised in some statistical analysis. Jennifer Henri was responsible for technical assistance involving mice. Dr. Yong Zhong Xu participated with some protein analysis. Dr. Jingxuan Yang, Dr. Guoan He and Dr. Aihao Ding participated in generation of transgenic mice. Dr Aiaho Ding provided the SLPI knock-out mice. The thesis author wrote the manuscript. All authors contributed editing the manuscript.

For the Appendix 1, the thesis author performed the quantitative trait loci analysis of the recombinant congenic strains and wrote the section related to the genetic analysis. All the authors participated in the experimental design and contributed in editing the manuscript.

At the time of the writing of this thesis, the data presented in Chapter 3 and Chapter 4 has been prepared for submission to peer-reviewed journals. The thesis author designed and performed all the experiments, collected the data, performed statistical analyses, and wrote the manuscripts

General Introduction

Asthma is defined as a chronic, complex and inflammatory disease of the airways generated by genetic and environmental factors which trigger an uncontrolled immune response to a broad variety of allergens.[1] Asthma is one of the most common chronic diseases in developed countries,[2] and remains a major public health issue[3] affecting around 300 million people worldwide and 3 million people in Canada (www.asthma.ca). During the last four decades there has been an increase in the prevalence and severity of diagnosed asthma cases around the world, accompanied by an augmented number of hospitalization and mortality rates.[4-6]

Asthma symptoms include shortness of breath, chest tightness, cough and wheezing. This disease is characterized by both acute and chronic inflammatory responses of the airways leading to activation of immune cells, increase in both T helper 2 (T_H2) cytokines and IgE levels, and eosinophilic infiltration in the lungs.[7] The bronchial inflammation in turn leads to airway hyperresponsiveness in response to various inhaled stimuli (allergens, cold air, acetylcholine, or methacholine), airflow obstruction, goblet cell hyperplasia with excessive mucus production and airway remodelling.[8]

In spite of the broad spectrum of strategies for the treatment of asthma, physicians and patients still need more effective new therapeutic options with fewer side effects. Actual asthma treatments include inhaled corticosteroids (ICS), which decrease the immune response and reduce inflammation but show several side effects.[9] Leukotrienes Receptor Antagonists or anti-leukotrienes, those avoid bronchoconstriction.[10;11] Short- and long-acting β2 agonists, exert their effect as bronchodilators and often used in combination with ICS.[12] Many other alternative therapies for asthma have been developed but with fewer efficacies than the previously mentioned, those include inhaled ipratropium bromide (anticholinergic bronchodilator),[12] inhaled helium – oxygen mixture,[13-15] inhaled anesthetics such as halothane and isoflurane,[16-19] and anti-IgE, which basically blocks immunoglobulin E.[20]

Airway responsiveness (AR) is the ability of the airway to reduce the diameter after exposure to direct or indirect stimuli and airway hyperresponsiveness (AHR) has been defined as the exacerbation of that ability.[21] AR is inherited as a complex trait and it has been associated with the development of allergic asthma. In mouse models, AR can be assessed both before and after the experimental induction[22] of allergic airway inflammation using either invasive[23] or non-invasive methods.[24] Many studies had measured AR after allergen exposure.[24-27] The invasive method has been considered to be the gold standard for the assessment of AR. However, it is technically demanding, requires the use of anaesthesia, tracheotomy, endotracheal intubation, and mechanical ventilation, making this method laborious, time consuming, unsuitable for phenotyping large numbers of animals and it is impossible to perform repeated experiments on the same subject.[27] The non-invasive method using the whole body plethysmograph (WBP) has been used to assess AR because it overcomes some of the limitations of the invasive method. WBP is simple, fast to use, has the ability to measure AHR in conscious mice, and is a non terminal technique.[27;28]

Previous studies have reported correlation between enhanced pause (Penh) measured by WBP, and airway resistance measured using the invasive method. However, other studies demonstrate a limited correlation between these techniques.[25;29] Additionally, preliminary studies performed in our laboratory showed how Penh increases after serial challenges with methacholine (Mch). As described in Chapter 2, we performed many different experiments in order to validate the phenotype method and analysis of the factors that may modify the output parameter. We studied how consecutive challenges with Mch affect Penh assessed using the WBP in A/J and C57BL/6J mice and how this measurement correlates with airway resistance measured using the invasive method. Our results demonstrate that under our experimental conditions, non-sensitized A/J and C57BL/6J mice show a dose dependent response to increasing doses of Mch using both the non-invasive and invasive methods. Additionally, consecutive challenges with Mch increases the Penh values in non-sensitized A/J and C57BL/6J mice using both the non-invasive method and invasive method. Furthermore, to obtain a

reliable reassessment of AHR in mouse models we established of a minimum rest period of 15 days in between exposures to Mch.

Once our phenotyping method was validated, we employed recombinant congenic mice, derived from A/J and C57BL/6J strains of mice and six different F2 backcrosses generated from the recombinant strains, in order to dissect the genetic factor underlying the susceptibility to airway responsiveness and its association to allergic asthma. As presented in Chapter 3, we performed a phenotyping survey of all 35 BcA/AcB congenic strains (previously genotyped using 625 microsatellite markers) and identified 3 of the most informative strains. From there, we generated six different crosses backcrossing with the parental background strain or with a non related strains with similar phenotype as the parental background. We phenotyped and genotyped around 1700 mice and performed a linkage analysis. The quantitative trait loci analysis of the two F2 crosses [F2(BcA86xB) and F2(BcA86xC3H/HeJ)] independently confirmed the association between intrinsic airway hyperresponsiveness and the region on chromosome 12.

In the significant region on chromosome 12, we identified 9 candidate genes expressed in the lungs that might be responsible for changes in airway responsiveness. Those genes include the *Arhgap5* gene related to cell activation, myogenesis, and hepatocellular carcinoma; *Foxa1* associated with inflammation and hematopoiesis; *Clec14a* involved in the process of cell-cell adhesion and immune response to pathogens, and *Ctage5* linked with the pathophysiology of meningioma and glioma. We had also identified the *Rtn1* gene associated with neuroendocrine secretion, intracellular traffic, and susceptibility to ethanol; the *Gpx2* gene related to hepatocarcinogenesis in rats and the protection from allergen-induced airway inflammation in mice, and the *Rsad2* gene located at 27.1 Mbp that has antiviral activity and has been involved in the resistance to fungus infection. Within the significant region, we identified two additional genes, even if they have not exactly fulfilled our gene selection criteria. These are the *Prkd1* associated with activation and TLR9 signalling, and *Adam21*, related to cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis.

Secretory leukocyte protease inhibitor (SLPI) has shown higher levels in asthmatic patients and its anti-inflammatory effect has been documented in chronic obstructive pulmonary disease[20] and cystic fibrosis (reviewed by Weldon *et al*. 2007[21]). But the role of SLPI in the immunomodulation of the response during allergic asthma, however, has not yet been fully elucidated. Previous studies have shown that asthmatic patients showed a significant increase in SLPI levels compared to healthy controls[22] while our group and others have demonstrated the protective effect of resiquimod (S28463), a synthetic TLR7 ligand, against allergic asthma in mice[23] and rats.[24]

As explained on Chapter 4, preliminary experiments performed in our laboratory show a significant increase in SLPI expression at the both messenger RNA and protein levels following in-vitro treatment of macrophages with resiquimod. We postulated that SLPI might play a protective role in the development of allergic asthma and that it might also be involved, directly or indirectly, in mediating the protective effect of resiquimod treatment against allergic asthma. Our results demonstrated that over expression of SLPI leads to a lower degree of airway inflammation and higher lung resistance, whereas the ablation of SLPI has opposite effect against allergic asthma phenotypes. We also showed that the treatment with resiquimod in independent of the SLPI expression. Our results demonstrate that SLPI plays an immunoregulatory role in the respiratory tract reducing the inflammatory process and improving lung physiology in a model of acute allergic asthma.

Overall, the results presented in this thesis demonstrate how the non-invasive method is a reliable tool in the assessment of airway hyperresponsiveness using non-sensitized A/J and C57BL/6J strains of mice. Furthermore, the susceptibility to develop airway hyperresponsiveness is linkaged to loci in the chromosome 12 of the mouse and where several candidate genes have been identified at the genetic and transcriptional level. Finally, SLPI plays an immunoregulatory role in the airways diminishing inflammation and improving lung function in allergic asthma. Taken together, our results contribute to better understanding of the

inheritance of disease-related phenotype and molecular mechanisms underlying asthma resistance or susceptibility as well as provide novel knowledge for the design of new therapeutic alternatives.

Chapter 1 - Literature Review

Abstract

Asthma is a chronic, complex and inflammatory disease affecting both adults and children. The disease is generated by genetic and environmental factors which trigger an uncontrollable immune response to a variety of allergens resulting in symptoms such as shortness of breath, chest tightness, cough, wheezing and phenotypes such as eosinophilia, high IgE levels and increased airway responsiveness. To understand the mechanism underlying the pathogenesis of asthma, it is necessary to identify the factors that trigger, modulate or inhibit the inflammatory response of the airways. Since asthma is an inflammatory disease with several sub-phenotypes, many studies have looked for biomarkers that might predict the development or progression of the disease. These phenotypes should be objective, quantitative, affected by only one set of genetic factors and reproducible in animal models in order to assess them in systematic manner. Despite significant progress in genome-wide association and linkage studies of human populations, numerous genes have been postulated as being responsible for the development and severity of allergic asthma but none of them have fulfilled the criteria of an informative biomarker which could be linked to the development or progression of allergic asthma.

1. - Definition and epidemiology of asthma

1.1. – Definition

Asthma is a complex, multi-factorial and inflammatory disease which includes symptoms such as shortness of breath, chest tightness, cough and wheezing.[1] The disease is characterized by episodes of intermittent reversible bronchoconstriction, airway hyperresponsiveness, airway smooth muscle hyperplasia, excessive mucous production and chronic inflammation of the airways and the infiltration of inflammatory cell into the airways.[30] Asthma is one of the most common chronic diseases affecting children and adolescents in industrialized countries and affecting more than 20 million people around the world.[2] Despite many advances in treatment, asthma has became a major public health issue[3] not only because of its high and increasing worldwide morbidity and mortality,[31] but also due to health care costs it entails.[32] The response to the treatments available for asthma vary from patient to patient due to the heterogeneity of the genetic composition of the disease between individuals.[33] Moreover, the development, progression and severity of asthma is controlled by the expression of several genes which interact with multiple environmental factors. Airway responsiveness (AR) and atopy represent two important genetically-regulated phenotypes that might be the key to understanding the mechanisms underlying this disease.

1.2. - Epidemiology of asthma

Asthma is a worldwide problem affecting around 300 million individuals[34] and according to the World Health Organization an estimate of 255 000 people died of asthma in 2005. Asthma is the most common chronic childhood disease and over 80% of asthma deaths occur in low and lower-middle income countries (source: www.who.int). For years, asthma has not been promptly diagnosed and many patients still remain without any treatment, generating a significant burden in lifestyle and reducing the quality of life of many individuals and families (source: www.who.int). During the last four decades there has been an increase in prevalence

and severity of diagnosed asthma cases around the world, accompanied by an augmented number of hospitalized cases and mortality rates. However, there is still no clear explanation for the expansion of this disease.[4-6]

The prevalence of asthma varies between 1-18% depending on the ethnicity, but this range appears to be closing as the prevalence is increasing at a faster rate in regions such as Africa and South America (ginasthma.com). The general prevalence of asthma has increased in Latin America and parts of Asia[35] and the global prevalence of diagnosed asthma in children increased significantly, probably due to the awareness about this disease and the improvement in the guidelines in the diagnosis of asthma.[36]

Asthma is one of the most prevalent chronic conditions in Canada, representing an economic burden for around $12 billion in 2003. Asthma was reported in 12.2% of youth less than 20 years of age in Canada, based on the data from the 1998-1999 National Population Health Survey (source: www.statcan.gc.ca). Despite the many asthma treatments available, the prevalence of this disease has been increasing over time. It is unclear if this is the result of the effort in a more accurate diagnosis of asthma or there is a real increase in prevalence. Around 485,700 children (15.6%) between 4 to 11 years old have been diagnosed with asthma in Canada and around 2.2 million (8.3%) of Canadians 12 years of age and over have been diagnosed with asthma by a physician. Since 1994-95, the prevalence of physician-diagnosed asthma has been higher in young boys compared to girls, but no significant difference has been observed between both genders in adult population. The prevalence of physician-diagnosed asthma increased by 60% in women from 35 to 44 years old and 80% among women aged from 45-64 years, from 1994 to 2005 (source: www.phac-aspc.gc.ca).

Despite of the decrease in hospitalized asthma cases, the economical burden that this disease entails represents a governmental issue for many industrialized countries around the world. A decline in hospitalization rates in children, young adults and seniors aged 65 and over with asthma in Canada has been reported since 1987; which likely reflects the improvement in

asthma control and the potential downsizing in the hospital sector about the decrease in the availability of beds. Asthma still represents a major cause of hospitalization for children in Canada and is considered as one of the first five diagnoses on the hospitalization record. In 2004, asthma generated 18% of all hospitalization cases in patients from 0 to 14 years old. For the same year, patients 5 years old or younger had the highest hospitalization rates for asthma. Asthma mortality rates have been decreasing in all age groups since 1987 (0.38/100,000 in 1987 to 0.06/100,000 in 2004). Deaths from asthma are uncommon among children and young adults but it shows the highest mortality rate in women over 65 in comparison to men in the same age group, representing 680 and 300 death cases in 2004, respectively (Source: www.phac-aspc.gc.ca).

Finally, social and economic factors are the key point to understanding the complexity of asthma as a disease and its consequences. Asthma represents a significant problem not only for the patients but also for the health care professionals and the individuals that covers the health care expenses. Additional concerns are the social impact represented by work or school day absence by affected individuals or relatives of patients with asthma.

2. - Biomarkers and pathophysiology of asthma

Airway narrowing is a final effect of different mechanisms that generated a variety of symptoms observed in allergic asthma. Theses mechanisms include airway smooth muscle contraction, airway inflammation (cell infiltration and cytokine production), atopy, airway edema, airway membrane thickening and goblet cell hyperplasia.[37] Many changes in sub-phenotypes can be observed in the central and peripheral airways and vary based on the severity of the disease. Although, there are some exceptions such as allergic asthma can be observed without increase in eosinophil migration to the airways.[38]

2.1 - Biomarkers of Asthma

Asthma is a complex disease characterized with the presence of several sub-phenotypes. Diagnosis of asthma is not always easy; it is important to validate the diagnostic tests and the differences between asthmatic patients compared with healthy or asthma like subjects.[39] Biomarkers of asthma are defined as laboratory tests which are associated with the physiology and pathogenesis of the clinical process of the disease. A broad diversity of biomarkers has been associated with asthma, including different measurements of airway physiology, levels of inflammatory mediators (in urine, blood and secretions) and composition of the sputum.[40]

Bronchial biopsy has been considered the gold standard method to demonstrate airway inflammation. However, this is an invasive method, not conducive to repetition and only a limited number of indications justify performing this procedure. Another method to identify biomarkers is through the examination of bronchoalveolar lavage fluid, however this method is also not ideal due to its excessive dilution of the samples and also its invasiveness.[41] Therefore, repeatable and non-invasive methods are needed to assess degree of airway inflammation in asthmatic patients. The measurement of inflammation-related parameters, including mediators of inflammation such as eosinophilic cationic protein (ECP), exhaled nitric oxide (eNO), eosinophils count in sputum, interleukin-5 (IL-5) and eotaxin in bronchial secretions has been proposed for the evaluation of inflammation in the airways.[41]

Eosinophilic cationic protein

Asthma is a complex disease characterized by a chronic airway inflammation and remodelling.[42] Eosinophils are effector cells against parasitic infection and in asthma through the release of inflammatory mediators such as platelet-activating factor, cysteinyl leukotrienes, oxygen radicals, cytokines and ECP.[43] ECP belongs to the superfamily of RNases, and is also known as RNase 3. It is one of several highly basic proteins present in the secretory granules of the eosinophils and mediates its actions through its cytotoxic and fibrogenic functions.[44;45]

ECP or its derivatives has been used as indicators of the inflammatory activation of eosinophils and some authors suggests that eosinophils and their products can be employed as markers of disease activity in asthma.[46] Other studies have shown the role of ECP in the tissue remodelling and development of fibrosis, using both experimental and animal models.[45;47] Furthermore, significantly high levels of ECP have been reported in serum of children with acute asthma than in those with stable asthma or healthy individuals.[48;49] High levels of ECP have been found in blood, bronchoalveolar lavage fluid, saliva and sputum of subjects with allergic asthma.[50]

ECP levels has been also well correlated with peripheral blood eosinophil count and IL-5 levels in the serum of children with acute asthma, suggesting that eosinophil activation during acute asthma is mediated at least in part by IL-5.[48] Additionally, Zagai *et al.* demonstrated how ECP stimulates lung fibroblast migration, suggesting a potential mechanism of airway remodelling leading to airway fibrosis in asthmatic patients.[51] Taken together, all these studies demonstrate a broad variety of functions attributed to ECP and its correlation with the inflammatory role in asthma, suggesting that this protein might be considered as a biomarker of asthma progression and severity making it a therapeutic target in asthma.

Exhaled Nitric Oxide (eNO)

Nitric Oxide (NO) is a product of the molecular oxygen and guanidine nitrogen of L-arginine and generates different nitrogen oxides (i.e. nitrite and peroxynitrite) involve in many biological processes.[52] Method of eNO measurement is a reproducible, non invasive, fast and painless that makes it ideal for the clinical practice.[53] eNO supplemented with eosinophil count in sputum has been used as indirect measurement of inflammation in asthma, aiding in its diagnosis[53] and treatment,[54] by reducing drug dosage and preventing side effects, however, without compromising the control of the disease.[55] Also, eNO has been employed in the differentiation of asthmatics from non asthmatic patients.[56] In the lower airways, eNO reflects inflammation and it has been well adapted for diagnosing children.[57] It proportionally increases

in the presence of wall inflammation, eosinophilia and airway hyperresponsiveness,[55] correlates with disease severity and loss of asthma control.[56]

For the diagnosis and management of asthma, eNO shows increased concentration in patients with asthma[58] and has been correlated to the increase in expression and activity of inducible NO synthase within the airways.[59] Shome *et al.* found significant elevated eNO levels in moderate to severe asthmatic patients compared with controls but eNO was not different in mild asthmatics comparing with the same control group. They also found a correlation between eNO and decrease in IL-4 and IL-13 expression by $CD8^+$ peripheral blood lymphocytes.[52]

eNO has been correlated with many other phenotypes such as breathing patterns, sputum and peripheral eosinophil count, methacholine challenge, airway hyperresponsiveness, ECP and bronchial wall thickness. Nordwall *et al*, found a strong association between eNO and allergic asthma symptoms, while breathing patterns were not, suggesting that eNO is a sensitive marker of asthma.[57] In addition, Lumiere *et al*. did not find correlation comparing eNO and sputum eosinophils counts of subjects with severe and moderated asthma, but they find a good correlation between eNO and mucosal eosinophils counts in biopsies of the same subjects.[60]

Senna *et al.* postulated that eNO detection is a more sensitive method of detecting bronchial inflammation than forced expiratory volume in 1 second (FEV_1).[61] eNO has shown similar diagnostic sensitivity to asthma tested on methacholine challenge.[62] Additionally, it correlates with others markers like increase in sputum and peripheral eosinophil count, airway hyperresponsiveness, ECP and bronchial wall thickness.[53] Ten Broeke *et al*. have shown that both the airway hyperresponsiveness and airway inflammation could be attenuated by the overexpression of the endothelial nitric oxide synthase in a model of allergic asthma, also founding a 46% reduction in the influx of eosinophils into the lungs after ovalbumin challenge.[63]

eNO could be influenced by the airway pH. Acidopnea (acid breath) has been associated with exacerbations of acute asthma. Gaston *et al*. showed that eNO decreases when airway acid is

neutralized using phosphate buffer and this change is greatest in persistent asthma. They concluded, PBS challenge should improve the analysis and interpretation of the eNO as a noninvasive test of asthmatic airway inflammation.[64] Normal level of eNO in absence of sputum eosinophils, could help to exclude mucosal eosinophilia in endo-bronchial biopsy.[54] Overall, these data provide clear evidence that eNO is a valuable test which represents the inflammatory condition of the airway but it has to be complemented with others such as airway responsiveness and sputum analysis.

Total and differential eosinophils count in sputum

Induced sputum cell count is a non invasive, easy, safe and useful procedure that provides assessment of the presence, type and degree of the airway inflammation.[65-67] There is a controversy surrounding eosinophils count as a marker for non-eosinophilic asthma patients.[68] However, quantitative induced sputum cell count has been used to identify non-eosinophilic asthmatic patients, whose are not responsive to corticosteroids treatment. It is clear that patients with asthma present a sputum eosinophilia while normal subjects have an induced sputum rich in macrophages and neutrophils but poor in eosinophils (less than 2.4%), lymphocytes and epithelial cells.[69] In terms of sensitivity and specificity, sputum cell count, methacholine bronchoconstriction response and eNO are the most reliable tests in the differential diagnostic of asthma from asthma-like subjects and the non invasive method to evaluate the severity of asthma and monitoring the steroids therapy.[39]

The reliability of the results generated using non invasive tests (sputum and eNO) compared to invasive tests (bronco alveolar lavage and biopsies) is questioned. Lex *et al*. performed the first pediatric study including twenty children with moderate to severe persistent asthma and compared sputum eosinophils, eNO, bronco alveolar lavage (BAL), bronchoscopy and bronchial biopsy. They found that BAL eosinophilia could be predicted by the presence of elevated sputum eosinophils and elevated eNO levels, and the absence of mucosal eosinophilia could be correlated with the absence of sputum eosinophils and normal eNO. Concluding, there is the

need for better non invasive markers to evaluate anti-inflammatory therapies involving airway wall eosinophilia.[54]

Lemiere *et al*. compared airway inflammation using invasive (bronchial biopsy) and non invasive (induced sputum, exhaled NO) methods in subjects with moderate (35 subjects) and severe (32 subjects) asthma. They found a correlation between high sputum eosinophils counts and asthma exacerbations, but did not find correlation between the sputum eosinophils counts and mucosal eosinophils counts. They also did not find correlation between sputum eosinophils counts and eNO in subjects with moderate and severe asthma. Concluding that sputum eosinophils count is a better marker for current disease activity than bronchial biopsies or eNO.[60] Those results corroborate with previous findings where the sputum eosinophilia, being particularly sensitive and specific, was a valid marker of asthma in adults.[70]

Piacentini *et al.* found significant correlation between induced sputum eosinophil count with eNO and exhaled air temperature in 41 children with allergic mild asthma.[71] Di Lorenzo *et al*. evaluated the validity of diagnostic test of asthma (including induced sputum eosinophils count) in 115 individuals including: 60 patients with mild asthma, 30 patients with gastro-esophageal reflux disease and 25 healthy controls. They established that methacholine exposure causing 20% fall in the forced expiratory volume in 1s (MCh PC_{20}/FEV_1) and induced sputum eosinophil count are the most sensitive and specific markers of mild bronchial asthma and the most clinically useful tests in the diagnosis of asthma from patients with asthma-like. Induced eosinophil count can be a valid marker of bronchial asthma and it is more sensitive and specific than blood eosinophils counts.[39]

The correlation between the eosinophil count and symptoms in asthmatic patients is controversial, mainly when eosinophil counts are low. Some studies reported a low correlation between sputum and peripheral blood parameter that could be explained by the lack of deep analysis of the sputum parameters.[72] Moreover, the heterogeneity of the sputum samples and variability of the distribution of airway inflammation generate large variation in the number of

eosinophils and other components, such as ECP concentration in the same individual.[73] Bacci *et al*. studied sixty-seven symptomatic patients with moderate asthma and treated with corticosteroids for 4 weeks and found that 26% (17 patients) had low sputum eosinophils count despite the presence of symptoms, giving low clinical importance to the sputum eosinophils analysis. In the same study, patients treated with inhaled corticosteroids and expressing high eosinophils count showed larger improvement in bronchial hyperresponsiveness and lung function than patients under the same treatment but low eosinophils count. They concluded that sputum eosinophils counts have some predicted value when assessing corticosteroid treated patients with low baseline eosinophil count.[74]

There are additional factors that may change the results of the total and differential cell count analysis in induced sputum. Those factors include age, gender, quality of the sample, method and time of processing samples, treatments patient has been subjected to, etc. And, inflammatory response in asthma could be more accurately evaluated when sputum markers are detected in valid samples.[75]

The lack of a shared protocol for sputum collection and processing significantly contributes to the observed variation of the total and differential cells counts by different groups. In some studies the analysis was done in the total expectoration, while others analyzed saliva and the rest of the expectoration independently. The total cell count is higher when the sputum is processed and filtered and lower when is not purified.[67] However, Spanevello *et al*. showed no difference in the differential cell count in a selected proportion of sputum and the total expectoration assessed in the same group of healthy volunteers.[69] Considering the age of population in study, Belda *et al*. obtained 81% of valid samples in a total of 118 adults included in the study.[67] Spanavello *et al*. obtained 84% of valid samples from a total of 114 healthy adults,[69] whereas Wilson *et al*. obtained only between 60 – 61% valid sputum samples in 87 children involved in the study.[76]

When analyzing the processing time of the sputum samples, Efthimiadis *et al*. showed that ideally the samples should be processed within two hours of collection, but the samples can still

be analyzed reliably even nine hours post collection if kept at 4°C.[77] Repeated sputum induction in healthy subjects increases the percentage of eosinophils, neutrophils, decreases the number of sputum macrophages and does not modify ECP levels in sputum samples during the first 48 hours, for that reason repeated induced sputum should be done in a period greater than 48 hours.[78] Additionally, Toungoussova *et al*. explored the composition of the sputum at 5, 10 and 15 minutes of induction in healthy subjects and patients with asthma and COPD; and they found that there was no significant difference in the cellular composition between samples.[65]

Gender is another factor influencing the results obtained from the total and differential cells counts. Belda *et al*. found a correlation between atopy and female gender in eosinophils counts possibly due to a hormone effect increasing the number of cell and receptor expression.[67] Those results are not apparently related to the quality, provenience or way of processing samples. We can conclude that induced sputum cell count is a sensitive, easy and non invasive technique which reflects the degree of the airway inflammation in most of the patients with asthma. But specific guidelines should be established in order to provide a standardized procedure to guarantee reproducible and accurate results.

Interleukin 5 (IL-5)

IL-5 is a cytokine that promotes migration, activation and prolongs the survival of eosinophils. Higher levels of IL-5 correlate with higher eosinophils viability in the sputum of asthmatic patients and is associated with higher airway hyperresponsiveness.[79] It is well documented in the literature that IL-5 is an important cytokine expressed by epithelial cells and fibroblasts in the respiratory tract which regulates eosinophil activity in the pathogenesis of asthma and allergic inflammation, and previous studies have shown correlation between markers of eosinophils activation and the number of lymphocyte T.[80;81] Enhanced IL-5 levels seems to coincide not only with an increased eosinophil count and but also with overexpressed IL-3 in allergic patients compared with normal controls.[82]

The increase of eosinophil counts in peripheral blood after IL-5 elevation, suggests that IL-5 is involved in the eosinophils hematopoiesis and release from the bone marrow.[72] IL-5 increases the interaction of epithelial cells with inflammatory cells and there is also well substantiated evidence demonstrating that IL-5 abrogates apoptosis *in vitro* and inhibits apoptosis of eosinophils *in vivo*. Additionally, IL-5 in induced sputum of asthmatic patients shows positive correlation with the number of eosinophils.[83] IL-5 may be used as biomarker of allergic asthma not only for its proven role in the pathogenesis of this disease but also for its correlation with the severity and progression of asthma. It is also convenient that IL-5 can be easily measured using both blood and sputum samples. Although several asthmatic patients show mild symptoms there is a subpopulation who suffer from severe asthma, characterized by eosinophilia in their sputum, which is considered to be a potential diagnostic asthma bio-marker. Since IL-5 is a chemoattractant and activator of eosinophils, this interleukin has become a target in the development of novel therapies. The studies using humanized antibodies directed at IL-5 have demonstrate that neutralization of IL-5 is effective in reducing eosinophilia in patients with refractory asthma subsequently leading to reduction of asthma exacerbation.[84]

Despite the important role of IL-5 in the recruitment and activation of eosinophils, the importance of this interleukin is not exclusive to the regulation of asthma progression and its severity. Increased levels of IL-5 were demonstrated also in viral infections (hepatitis C) and in parasitic infections (e.g. giardia infection).[85;86] IL-5 increase was also demonstrated in gastro-esophageal reflux diseases where no pathogen organism is involved. In this particular disease, an inhalation of gastric fluid and vagal reflex, was shown to lead to the release of neurotransmitter and tachyninis, increasing IL-5 and recruiting eosinophils and polymorphonuclear leukocytes.[87;88] Up to date, a unique biomarker for allergic asthma for disease diagnosis and monitoring its progression has not yet been identified. Therefore, a combination of markers and clinical parameters has to be considered when trying to diagnose allergic asthma and to assess the effectiveness of asthma treatments.

Eotaxin

Airway smooth muscle cells have been described as the responsible cells for triggering airway hyperresponsiveness; this airways ability to reduce their diameter in response to bronchoconstricting stimuli is mediated by the expression of several proteins such as adhesion and costimulatory molecules, and by secreting multiple pro-inflammatory cytokines and chemokines.[89] Chemokines are a subfamily of cytokines that mediate leukocyte migration into tissues in both physiological and pathological states.[90] Eotaxin is a CC chemokine that was first purified from the bronchoalveolar lavage fluid of antigen-sensitized and -challenged guinea pigs.[91]

Several diseases have a characterized eosinophilia, including allergic disorders such as rhinitis, conjunctivitis, systemic vasculitis and asthma. Several inflammatory disease including pneumonia and inflammatory bowel disease, and some neoplasias including T-cell leukemia and Hodgkin's disease are also associated with an increase in the number of eosinophils.[92] Moreover, several published studies investigating the role of eotaxin demonstrated the relationship between an increase in eotaxin and the number of eosinophils. However, there are also reports demonstrating the association of eotaxin with various cellular mechanisms which involve mast cells, basophils, T helper 2 lymphocytes and dendritic cells.[93;94]

Eosinophilia has been associated with an increased eotaxin expression in several diseases. Eotaxin is detected at high levels in the sputum of asthmatic patients; is produced and regulated by cells in the bronchi, appears to be involved in the pathogenesis of asthma and several studies showed a immunoregulatory role in the inflammatory process lead by eosinophils.[95] Previous studies have reported increased levels of eotaxin in the bronchial mucosa and bronchoalveolar lavage fluid from asthmatic patients compared to healthy controls.[96] Moreover, Nakamnura *et al.* suggested that eotaxin concentrations correlate well with the severity of asthma disease.[97] In sputum samples of asthmatic patients, eotaxin levels positively correlate with IL-5 levels and eosinophil counts, suggesting an important role of

eotaxin in chemotaxis of eosinophils into the lungs. Therefore, increased inflammation may affect eotaxin expression, suggesting that this chemokine might also participate as a hematopoietic factor in addition to its role as a chemoattractant explaining, at least in part, its role in the accumulation of eosinophils in the asthmatic airways.[83]

The direct participation airway smooth muscle cells[98] and eotaxin in the chemotaxis of eosinophils has been demonstrated using anti-eotaxin antibodies in mice which substantially reduced eosinophil recruitment following antigen challenge. These results document further functional role of eotaxin in promoting eosinophil migration and in enhancing airway inflammation in asthma.[91] Furthermore, decreased eosinophilia due to the neutralizing effect of antibodies on eotaxin, supports the potential therapeutic value of anti-eotaxin antibodies in allergic diseases.[99]

Antibodies against different molecules involved in the mechanisms associated with inflammation or immune response of asthma have been studied as therapeutic alternatives with different degree of success. Omalizumab (anti-IgE), a recombinant humanized IgG1 monoclonal and the first antibody to be approved for the treatment of moderate to severe persistent allergic asthma,[100] reduces exacerbations and corticosteroid use in patients with severe persistent allergic (IgE-mediated) asthma[3] that remains inadequately controlled despite extensive pharmacological therapies[101] and low incidence of side effects.[102] Furthermore, mepolizumab (anti-IL-5) has recently been studied in a bronchial challenge model[103] and the management of hyper-eosinophilic syndrome.[104] Although some studies demonstrated that tissue eosinophilia was markedly reduced following neutralization of IL-5, no significant changes in intensity of responses to allergen challenge or bronchial hyperactivity were observed. These data suggests that inhibition of IL-5 alone, does not represent a significant mechanism of elimination of eosinophil chemotaxis to the airways which could prevent inflammatory process characterizing allergic asthma.[105] Overall, the results from the previous studies using eotaxin demonstrate that this chemokine represents a potential candidate in the treatment of allergic

asthma; however the effects of such therapy are likely to go far beyond its pathogenic mechanism mediated by eosinophils.

2.2. - Pathophysiology of asthma

Many sub-phenotypes are expressed in the pathogenesis of asthma and include the participation of structural components such as airway epithelium, airway smooth muscle and endothelium; as well as, immunoregulatory molecules such as cytokines, chemokines and antibodies, specifically IgE. However, interaction between environmental and genetic factors is required in order to develop and to express these phenotypes.[106] Asthma is clinically characterized by repeated, variable, episodic attacks of breathlessness, cough, and wheezing as consequences of bronchoconstriction due to airway hyperresponsiveness and excessive mucous production. In the inflammatory cell infiltration that characterizes asthma, it has been demonstrated the presence of eosinophils and T-helper (T_h1 and T_h2) cells, which secrete cytokines such as IL-4, IL-5, and IL-13. These cytokines direct different effects such as recruiting eosinophils into the tissue, stimulating mast cells and leukocytes, triggering the IgE production by B-cell, and also exerting regulatory roles in the airway remodelling of asthma.[106;107]

Cytokines

Asthma is characterized as airway inflammation present with a large numbers of inflammatory cells and mediators, in the lungs of asthmatic patients. Although numerous inflammatory cell types participate in the inflammation in asthma, many studies highlight the roles of eosinophils, mast cells and T cells.[108] T cells are responsible for the allergen sensitization and B cell production of allergen-specific IgE. Cytokines produced by T_h2 cells, particularly IL-4 and IL-13, promote allergen-reactive B cells activation and expansion, and together with IL-4 and IL-13, they trigger the IgE class-switching and up regulation of IgE receptors. Additionally, IL-4 and IL-13 lead to growth of airway epithelia and their transformation into mucus-producing goblet

cells. Furthermore, IL-5 produced by Th2 cells is responsible for the development, differentiation, recruitment, activation and survival of eosinophils.[109]

Finally, in the inflammatory process observed in the airways of allergic patients and animal models, inflammatory cell are activated and generate more inflammation through the release of granules which potentiate the tissue damage. Degranulation of neutrophils, eosinophils and mast cells is trigger by the paracrine, autocrine and endocrine effects of cytokine. Some cytokines activate transcription factors which bind to the promoter region of the genes, initiate the inflammatory response. The list of those transcription factors include nuclear factor-κB, activator protein-1, nuclear factor of activated T cells, cyclic AMP response element binding protein and several transduction-activated transcription factors. These transcription factors encode for chemokines, adhesion molecules and other cytokine which induce and sometime potentiate inflammation, tissue damage, airway narrowing and increased airway responsiveness.[110]

Atopy

Atopy is the predisposition of certain individuals to produce immunoglobulin E (IgE) antibody in response to common and innocuous antigens.[100] IgE is one of the five antibody classes and is responsible of type I immediate hypersensitivity immune response.[108] Previous studies have demonstrated an association between IgE and the severity of asthma, and transforming this phenotype into a marker linked with the risk of developing this disease.[111] Similarly to AR, the presence of IgE is controlled genetically, this phenomenon has been studied in humans and reproduced in animal models.[112;113]

To generate IgE, inhaled antigens are processed by dendritic cells and presented to T and B cells in the lymph nodes and the interaction between these cells is influenced by cytokines (IL-4 and IL-13) and costimulatory molecules (CD40, CD28 and B7). IgE is then synthesized by activated plasma cells, IgE circulate in the blood for a short period of time and bind to mast cells

expressing high- affinity IgE receptors (FcεRI) and low-affinity IgE receptors (FcεRII) on lymphocytes, eosinophils, platelets, macrophages. Antigens bind to FcεRI, resulting in the cross-linking of IgE at the surface of the mast cells. Mast cells are thus activated and release inflammatory mediator such as IL-4, IL-13, histamine and tryptase. Basophils and mast cells can secrete IL-4 and IL-13 but since the release of cytokines depends on the cross-linking of IgE by allergen, these cells amplify the synthesis of IgE. Atopy and AHR are the hallmark of allergic asthma; they have been used as markers to evaluate the risk for developing this disease for their correlation with the severity and progression.[38]

Based in the relevance of IgE in the pathophysiology of allergic asthma, antibodies against this immunoglobulin have been employed as a treatment for patients with non treatable moderate to severe persistent allergic asthma,[100;114] patients with severe non-allergic asthma and allergic asthma. Omalizumab is a monoclonal anti-immunoglobulin E antibody which has been demonstrated as safe and tolerable by patients with severe persistent allergic (IgE-mediated) asthma[3] that remains inadequately controlled despite optimal pharmacological therapy.[101]

One of the mechanisms of action of Omalizumab in severe asthma consists in the removal of IgE from circulation, blocking it from fixing to low and high-affinity receptors in mast cells and basophils, and reducing the infiltration of inflammatory cells, especially eosinophils, into the airways. Omalizumab inhibits the early and late phases of the allergen-induced asthmatic and prevents the development of eosinophilia and bronchial hyperreactivity.[115] The anti-IgE is effective in preventing or minimizing the IgE-mediated severe reaction during immunotherapy. In combination with specific immunotherapy, it is reported to improve bronchial asthma and allergic rhinitis, and also decrease the symptoms of interstitial cystitis.[116]

Airway responsiveness (AR)

AR is the ability of the airway to reduce their diameter after exposure to direct (histamine, acetylcholine, methacholine, prostaglandin D_2 and leukotrienes) or indirect (exercise, cold air, distilled water, pollutants and allergens) stimuli and airway hyperresponsiveness (AHR) is defined as an exaggerated AR.[21] AHR is inherited as a complex trait; it has been associated with development of allergic asthma and shows homology between mouse and human.[22;117] It has been demonstrated that the allergic response leads to early and late responses and airway responsiveness in murine models resembles what is observed in humans.[118]

Repeatable and non-invasive methods are needed to assess lung physiology in asthmatic patients. AR to bronchoconstricting stimulants is one of the most reliable methods, not only for its reproducibility but also for it characteristic measuring the physiology of the airway; which has been employed as a tool in the diagnosis of asthma and as a monitor of treatment effectiveness.[119] Previous studies demonstrate that AHR precedes asthma and it has been considered as a risk factor for the development of this pathology.[120] AHR, eosinophilic inflammation of the airway and lung function impairment are considered as hallmarks of allergic asthma.[23;121] Furthermore, asthmatic patients show much stronger airway response to bronchoconstrictor stimuli than healthy controls.[21] Palmer *et al*. has also demonstrated that AHR in response to histamine is significantly associated with the development of asthma by age 6,[120] it has been reported that children with AHR are more likely to develop asthma than children with low AR.[122]

The mechanisms underlying the development of AHR are unclear but genetic studies showed that AHR is inherited as a complex trait, in both man and mouse[123] and it has been associated with atopy,[124;125] where increased bronchoconstriction response and infiltration of mast cells within the muscle may be involved.[126] Moreover, determinant data has shown that AHR precedes the development of asthma[127] and non-asthmatic parents of asthmatic children express higher levels of AR compared with non-asthmatic adults with no familial history of

asthma.[127;128] In both clinical and research studies, AR is assessed using histamine or methacholine (Mch) as airway constrictor agonists in order to characterize patients with asthma and to evaluate the therapy effectiveness.[21;121] Furthermore, several studies have demonstrated genetic linkage of several specific loci and AHR in response to histamine and methacholine.[124;125] Overall, the results generated from genetic studies show that AHR is genetically determined and is associated with the development of asthma.

However, it is important to remember that AHR is the result of airway narrowing, which is triggered by the combination of genetic and environmental factor. However, the exact mechanisms controlling the development and severity of this phenotype are still unclear. Some of those mechanisms include structural component of the airway, such as sensory nerves that may be sensitized by the inflammatory process and leading to an exacerbated response to bronchoconstricting stimuli.[129] Additionally, excessive contraction of the smooth muscles, due to increased contractibility and size of airway smooth muscle cells.[130] Control loss in the smooth muscle contraction originated by the airway inflammation which leads to excessive narrowing.[131] And finally, airway remodelling, characterized by thickening of the airway walls.[132] Taken together, these data demonstrate how changes in structural component of the airway lead by genetic and environmental factor, are responsible for the airway narrowing and AHR observed in asthma.

Other mechanisms of asthma

Additional mechanisms involved in both triggering and exacerbation of asthma have been proposed, including those observed in acute asthma attacks and exercise induced asthma (EIA). Exercise can trigger an acute asthma attacks or exacerbate the symptoms of asthma in some patients with chronic asthma and reduced lung function at rest. There is another kind of asthma patients which do not have a reduced lung function or any symptoms of asthma in the absence of exercise, showing normal and physical examination, however the exercise elicits asthma episodes and reduces the lung function in these individuals without evidence of chronic

inflammation.[4;133] The pathophysiology of EIA has not been well clarified and some theories have been proposed to explain the mechanisms responsible for this condition, like hyperosmolarity, re-warming and heat loss theories.[134-137]

The hyperosmolarity theory proposes that a high osmolarity condition of the airway caused by water loss during exercise, leading to an hypertonicity of the airway surface liquid and a hypermolar condition.[134] During or after exercise hyperventilation there is evaporation and airway drying. The airway cells become hyperosmolar, provide osmotic stimulus for water to move from nearby resident cells, which originates cell volume loss.[136] The volume decrease generates the release of pro-inflammatory mediators such as histamine, leukotrienes and prostaglandins, which generate smooth muscle contraction and increased mucus production. A feedback mechanism controlled by the inflammation that amplifies the process, increasing the water loss seem to be involved in this process.[134] Additionally, Hogman M *et al*. proposed that the bronchial hyper-reactivity caused by exercise and dry air, mediates the water loss from the fluid covering the tracheal mucosa, creating the environment of a hypertonic salt solution over the mucosa. This condition changes the epithelium permeability by opening the tight junction between cells. This pathophysiologic mechanism might be the key in the understanding of asthma and could be explored therapeutically since it potentially facilitated the movement of some medications through the respiratory epithelium.[137]

Other of the proposed mechanisms is explained by the re-warming theory, whereas the low temperature of inspired air and the increased minute ventilation (V_E) causes the humidification and warming processes to occur deep in the airway. During the exercise, there is a cooling of the surface cells in the airway produced by hyperventilation and after exercise, a re-warming process occurs and result in rebound vascular engorgement (dilatation of the small bronchiolar vessels around the airways); this process originates a hyperemia of the airway lining, fluid exudation from the blood vessels into the submucosa of the airway wall and mediators release and bronchoconstriction.[133;134] This response amplifies the airway narrowing caused by the normal contraction of the bronchial smooth muscle or the presence of mucus.[136] Although cold

dry air is considered a trigger for EIB, the dryness rather than the temperature in the inspired air is the causative factor. However, cold air has low water content regardless of relative humidity, therefore chronic inhalation of high-volume of air causes damage to mucosa, and increase airway hyperresponsiveness.[136]

Finally, the heat loss theory supports the fact that asthma is an inflammatory disease of the airways, characterized by increased vascular and bronchial flood flow. These changes can trigger a reduction in the airway temperature and bronchoconstriction, and then the release of inflammatory mediators, such as histamine, bradikynin, leukotrienes, platelet-activating factor, prostaglandin E_2, adenosine, and nitric oxide, which cause bronchial vascular dilatation and airway narrowing. Paredi P *et al*. found that patients with asthma have faster increase of exhaled breath temperature compared with normal subjects and correlates with exhaled nitric oxide concentration.[138]

3. - Genetics of asthma

Asthma is trigger and determine by the interaction between environmental and genetic factors, however, the mechanisms of how asthma is controlled is not well understood.[30] Previous studies have demonstrated that genetic factors are in part responsible for the development of asthma and most results show how intermediate phenotypes such as total and allergen specific IgE levels, cytokine levels, eosinophil counts and airway responsiveness are correlated with the development and severity of this disease.[139]

Intermediate phenotypes have been used for linkage analyses and gene mapping analyses rather than the diagnosis of asthma, because they are objective, quantitative, can be reproduced in animal models and be affected by genetic factors associated with asthma.[140;141] Association studies on asthma have shown the role of multiple genes in the pathophysiology of the disease and the list of genes vary depending on the ethnic groups used in the study.[142] Several genetic studies have focused their attention on intermediate phenotypes, including

chemokines, cytokines (Th1 and Th2), inflammatory mediators, growth factors, atopy and airway responsiveness.[143;144]

Linkage and case-control association studies in humans have identified potential chromosomal regions associated with asthma susceptibility and bronchial hyperresponsiveness; and potential QTLs are found on chromosomes 2q, 5q, 6p, 11q, 12q and 13q.[145-147] However, the success of human studies has been relative, due to the genetic variability between individuals, the large chromosomal regions and the large number of candidate genes for the phenotype investigated. While, large-scale multicenter whole-genome association studies look promising for the identification of significantly more candidate genes from these regions in human populations,[148] animal models, represents a more practical tool in the identification of candidate genes because genetic and environmental factors are precisely controlled. Other phenotypes studied in the human population using genome-wide analysis have identified other genes linked to asthma susceptibility, including STAT6,[149-151] GATA2[152] and FCER1A,[153] dipeptidyl peptidases cleaving terminal dipeptides from cytokines (e.g. Interlekin-5, -13 and -33)[150] and chemokines, transcription factors (e.g. MYB),[154] and the cytokine receptors (e.g. IL-1R1),[114] WDR26 (a protein co-regulated with IL-2), and IRAK-M.[155]

Additional association studies have identified several other candidate genes linked to the asthma genetic risk loci. Those gene include urokinase-type plasminogen activator which is associated with atopy.[156-159] Furthermore, several other genes which belong to the G-protein coupled receptor class have been associated with atopy and a predisposition to several respiratory diseases including bronchial hyperreactivity. Those genes include cysteinyl leukotriene receptors (CTSLTR1 and CTSLTR2),[100;160-162] prostaglandin D2 receptors (PTGDR and CRTH2),[163-165] thromboxane A2 receptor (TBXA2R),[166;167] beta2-adrenergic receptor (ADRB2),[168] chemokine receptor 5 (CCR5),[169;170] and the G protein-coupled receptor associated with asthma (GPRA).[162;171] Finally, a mechanistic role has recently been identified for a disease-associated genetic variant within the ADAM33 asthma susceptibility gene;[172;173] and asthma progression has been shown to correlate with increased expression of the ADAM33[187] and ADAM8[174-176]

genes, although only a small proportion of asthma cases could be explained by variants in these genes.

Airway hyperresponsiveness (AHR) has been demonstrated to precede the development of asthma and to be controlled by genetic factors in both humans and animal models.[122;127;128] Furthermore, AHR to a variety of bronchoconstrictor stimulants, usually modeled in mice using methacholine or acetylcholine, has shown considerable variability among inbred strains.[177] Despite significant progress in genome-wide association studies of human populations, it has been difficult to dissect the asthmatic phenotype and to identify genes which are responsible for each aspect of the asthma pathophysiology. For these reasons, significant stratification of the sample population has been required in order to discriminate between different sub-phenotypes in asthmatic patients; requiring very large sample populations in order to have reasonable statistical power. As an alternative and complementary approach to whole-genome association studies, genetic analysis using mice can be used to identify candidate genes for targeted human studies and to confirm loci found to be associated with asthma.

To dissect the genetic underlying the development and severity of complex diseases modeled in mice, generation of F2 crosses from 2 strains with opposite phenotype has been employed to identify quantitative trait loci ruling the pathophysiology of those disease. A/J and C57BL/6J or C3H/HeJ strains have been identified as hyperresponsive and hyporesponsive to bronchoconstricting agonist, respectively, and have been used in several studies in the analysis of AHR. Three of the eight studies have employed intercrosses between the A/J and C3H/HeJ strains and the rest used A/J and C57BL/6J. In those previous studies, significant or suggestive QTL for airway responsiveness were identified on chromosomes 2,[136] 6,[136;178] 7,[178] and 17[178] by employing F2 intercrosses generated from A/J and C3H mice and QTL have been identified on chromosomes 2,[178] 6,[179] 15,[178] and 17[178] by employing various breeding schemes derived from A/J and C57BL/6 mice.

To narrow QTLs associated to AHR, additional studies have combined increased genetic diversity with improved analysis. Furthermore, as with many complex diseases, each susceptibility gene, by itself, only accounts for a small percentage of the observed phenotypic variance. What has been lacking in many asthma gene mapping projects is the ability to assess multiple interacting genes, which together are expected to explain a larger portion of the observed phenotypes. The Recombinant Congenic Strain (RCS) panel of mice presents an ideal and unique platform to investigate the genetics of such diseases and it is likely that the proposed study will result in the identification of novel genes controlling asthma susceptibility, progression and severity.

The AcB/BcA RCS were derived from the A/J and C57BL/6J inbred strains of mice in order to aid in the dissection of complex traits. This RCS panel has allowed to identify QTLs for numerous traits, including amphetamine-induced locomotion,[180] emotionality and stress response,[181;182] nicotine effect,[183] opioids dependence,[184] and Salmonella infection.[185] Each RCS is fully inbred and contain 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.[186]

Additional studies using AcB/BcA RCS identified genetic loci underlying the trait of AR in response to methacholine as assessed by whole-body plethysmography. This screen has shown 16 chromosomal regions significantly associated with AHR: 8 are supported by previously and independent published reports while 8 are entirely novel.[22] 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). These novel regions still represent large number of genes in order to identify potential candidates in an efficient manner; around 157 potential candidate genes are included in these 16 chromosomal regions significantly associated with AHR.

Overall, previous studies have shown the invaluable effort in this study of the genetic underlying asthma and the phenotypes associated to this disease, however, new strategies in the analysis of the genetics of asthma are required in order to narrow down the significant intervals and the potential candidate genes associated with each phenotype, which will offer a better understanding of inheritance and molecular mechanisms controlling the pathophysiology of this disease.

Acknowledgements

R. Marino is a recipient of Doctoral Award from the Canadian Institutes of Health Research (CIHR). I would like to acknowledge Cynthia Kanagaratham for reviewing the manuscript.

Chapter 2 - Assessment of airway responsiveness in mice is affected by previous exposure to methacholine

Preface

Airway hyperresponsiveness has been associated with the development of allergic asthma in humans, is inherited as a complex trait and shows homology between mouse and human. Methacholine exposure is performed in the assessment of airway responsiveness using both invasive and non-invasive methods. However, re-exposures to methacholine show modification in airway responsiveness. The goal of the studies presented in this chapter was to validate the assessment of airway responsiveness using the whole body plethysmograph as the phenotyping technique in our study of genetics of asthma and to determine how consecutive challenges with methacholine affect airway responsiveness in non-sensitized C57BL/6J and A/J mice. Our results demonstrate that airway responsiveness can be assessed in non-sensitized C57BL/6J and A/J mice using both the non-invasive and invasive methods. Consecutive challenges with Methacholine increased airway responsiveness if the re-exposure has happened within the first few days after initial methacholine challenge. Based in our results, we concluded that a reliable reassessment of airway responsiveness in the same mouse should be performed following a rest period of at least 15 days between exposures to Mch. The results provide relevant data enabling a better design of experimental protocols and thereby allowing the generation of reliable and reproducible results using mouse models of airway hyperresponsiveness and asthma.

Abstract

Airway responsiveness (AR) is defined as the ability of airways to reduce their diameter after bronchoconstricting stimuli and an association between airway hyperresponsiveness and the development of asthma in humans has been found. Assessment of AR to methacholine (Mch) in mice is performed using invasive or non-invasive methods. The non-invasive method using a whole body plethysmograph (WBP) has overcome some of the limitations of the invasive method and has allowed testing individual animals multiple times. The effect of methacholine has been considered to be very transient but the result of our meticulous study presented in this manuscript aimed at testing this assumption. We evaluated how consecutive challenges with methacholine affect airway responsiveness assessed using the WBP in C57BL/6J and A/J mice and how this measurement correlates with airway resistance measured using an invasive method. Our results demonstrate that C57BL/6J and A/J mice responded in a dose dependent manner to increasing concentrations of Mch using both the non-invasive and invasive methods. C57BL/6J mice showed significantly higher ($p<0.0001$) ED_{200} using Penh compared to A/J mice and similar a relationship between the ED_{200} values was observed when measuring airway resistance ($p<0.0001$). Consecutive challenges with Mch significantly increased Penh ($p<0.05$) and airway resistance ($P<0.05$) in both C57BL/6J and A/J mice, and no inflammatory cell infiltration in the lungs or difference in plasma IgE levels was found. Finally, our data demonstrate a good correlation ($p<0.05$) between Penh and airway resistance only after 15 days rest period during re-exposures to Mch, whereas shorter period of rest seemed to be associated with greater AR. Overall, our results demonstrate that non-sensitized C57BL/6J and A/J mice show a dose dependent response to increasing doses of Mch using both the non-invasive and invasive methods and a significant difference in AR between both strains. Consecutive challenges with Mch increases the Penh values in non-sensitized C57BL/6J and A/J mice using the non-invasive method, as well as airway resistance using the invasive method. Furthermore, to obtain a reliable reassessment of AR in mouse models we established a minimum rest period of 15 days between exposures to Mch. Our results provide relevant data

for improved experimental protocol design, which should allow more reliable and reproducible results in mouse models and more accurate retesting of the same animal to the same stimulus.

Introduction

Airway responsiveness (AR) is the ability of the airway to reduce their diameter after exposure to direct or indirect stimuli and airway hyperresponsiveness (AHR) is defined as an exaggerated AR.[21] AHR is inherited as a complex trait; it has been associated with development of allergic asthma and shows homology between mouse and human.[22;117]

Animal models have become an important tool to study the pathophysiology and genetics associated with the development of AHR.[187-190] In mouse models, AR can be assessed both before and after the experimental induction[22] of allergic airway inflammation using either invasive or non-invasive methods.[23] Many studies have used the BALB/c strain of mice to measure AR after allergen exposure.[24-27] However, since the C57BL/6J and A/J strains of mice have proven to be hypo- and hyper-responsive to Methacholine (Mch), respectively, both before and after allergen exposure, they have been employed in both respiratory diseases studies and studies of the genetics of airway responsiveness.[22;178;179;188] Additionally the C57BL/6J strain is commonly used for genetic manipulation to generate transgenic, knockout, consomic and congenic strains. [22;25;178;179;191]

For the study of respiratory diseases, invasive methods are considered to be the gold standard for the assessment of parameters of AR, such as lung resistance and compliance. However, these methods are comparatively technically demanding, requiring the use of anaesthesia, tracheotomy, endotracheal intubation, and mechanical ventilation, making this method laborious, time consuming, inefficient for phenotyping large numbers of animals, and do not allow for repeated measurements of individual subjects.[27]

Whole body plethysmograph (WBP) has been used to assess AR because it overcomes some of the limitations of the invasive methods. WBP is simple, quick, has the ability to measure AHR in conscious mice, and is a non terminal technique.[27;28] Enhanced pause (Penh) is commonly used to evaluate airway responsiveness in conscious and unrestrained mice; it is calculated based on

the relationship between the pressure signals from inspiration and expiration and the timing of expiration. Penh increases after challenges with bronchoconstrictors such as histamine or methacholine, and it has been described as an empirical parameter which also reflects changes in the respiratory system in addition to changes in airway responsiveness.[24] These changes may occur in the upper airway (nasal cavity, larynx, and pharynx) and represent airflow limitation.[27]

Previous studies have reported correlations between Penh, measured by WBP, with airway resistance, measured using invasive methods, after a constrictor challenge with MCh in OVA sensitized and challenged BALB/c[24] and C57BL/6 mice.[191] However, other studies have demonstrated a limited correlation between these techniques.[25;29] In a mouse model of allergic asthma created by allergen sensitization and challenge, the inflammatory process generated in the upper airways may induce changes in the pattern of breathing, which affects Penh values. Additionally, since WBP assesses AR in conscious and unrestrained mice, changes in stress in addition to changes in the respiratory mechanics might contribute to changes in Penh.[192] We identified discrepancies in consecutive assessment of AR to Mch using WBP in non-sensitized mice which prompted us to determine how consecutive challenges with Mch affect Penh assessed using WBP in both C57BL/6J and A/J mice, and how these measurements correlate with airway resistance using an invasive method. Our results enabled the development of reliable protocols for the repetitive assessment of lung physiology in the same animal following methacholine exposure in mouse models.

Material and Methods

Animals

C57BL/6J and A/J mice were purchased from Charles River Canada. Male mice between 8-10 weeks-old were used in all experiments. No allergen sensitization or challenges were performed. Mice were bred in the pathogen-free facility of the Montreal General Hospital Research Institute at McGill University Health Center (Montreal, Canada). All procedures performed on the animals were approved by the McGill University Animal Care Committee in compliance with the Canadian Council of Animal Care guidelines.

Non-invasive assessment of airway responsiveness

AR was assessed using an unrestrained WBP (Buxco Research Systems) as described previously[22], and expressed as the Penh parameter. Briefly, the system was turned on and warmed up for 20 minutes; four chambers were calibrated and used simultaneously. An unrestrained and spontaneously breathing mouse was placed in each chamber and allowed to acclimate for at least 5 minutes. Following a baseline reading, mice were exposed to nebulized PBS for 1 minute and subsequently to increasing doses of Mch (5, 10, 15, and 20 mg/ml) (Sigma-Aldrich, Oakville, ON), allowing the Penh to return to baseline between each challenge. After each aerosol challenge the average Penh measured during the 5 minutes after Mch exposure was recorded.

Kinetics of airway responsiveness to Mch using non-invasive method

To assess the effect of consecutive challenges with Mch on AR using the non-invasive method, we employed WBP in C57BL/6J and A/J mice as described previously. Three groups of 8 mice of each strain were challenged every 5 days with increasing doses of Mch and the AR was expressed as the Penh parameter.

Invasive assessment of airway hyperresponsiveness

AHR was assessed using an invasive method as described previously[193;194], and expressed as airway resistance (Rrs, cm H_2O*s/ml). Briefly, mice were anaesthetized with Ketamine (70 mg/Kg) and Xylaxine (10 mg/Kg). After the depth of anesthesia was verified, mice were tracheotomized, endotracheally intubated (with an 18 gauge stainless steel cannula), injected intraperitoneally (i.p.) with of 10 µl/g of Pancuronium (1 mg/ml) and finally connected to the MiniVent (type 845) small animal ventilator (Harvard Apparatus, Saint-Laurent, QC). Tidal volume was adjusted to 10 µl/g and a respiratory frequency of 180 strokes/min. Increasing doses of Mch in aerosol (10 µL of 0–80 µg/ml) were administered consecutively and the peak of resistance was measured with the Buxco resistance system (Buxco Electronics, Inc. Wilmington, NC). Mice were euthanized and samples were collected. We used 40 µg/ml of Mch as the most reliable dose to compare Rrs. The dose required to increase airway resistance by 200% (ED_{200}) was also calculated.

Correlation between invasive and non-invasive methods for measuring airway responsiveness to Mch

To correlate AR to Mch using the invasive and non-invasive methods, we measured Penh and airway resistance in C57BL/6J and A/J mice following the following schedule. On day 0, five groups of 8 mice of each strain were challenged with increasing doses of Mch and the Penh parameter was assessed. Then, every group was challenged with Mch every five days and Penh was re-tested on days 0, 5, 10, 15, and 20. At each time point, one group was assessed for airway resistance to Mch using the invasive method, the latter group was euthanized and samples were collected. To determine the optimal rest time between Mch exposures, different groups of mice were assessed on day 0 using the WBP and re-assessed for airway resistance on day 5, 10, 15 or 20 without consecutive exposures to Mch. Correlation between ED_{200} for Penh and airway resistance was calculated for all groups of mice.

Histological analysis of lung inflammation

After AR assessment the mice were euthanized, the lungs were dissected, inflated with 10% buffered formalin by gravity force, kept overnight in a 50ml tube filled with 10% formalin, and paraffin embedded. Finally, the lungs were cut into 5µm sections; deparaffinized and hydrated for further staining with Hematoxylin and Eosin (H&E). Inflammation of the lungs was assessed by histological analysis of H&E stained sections by identifying inflammatory cell infiltration in the perivascular space of the airways.

Plasma immunoglobulin E (IgE) levels

Mice were euthanized after AR to Mch was assessed; blood was collected by cardiac puncture and preserved with 0.05M EDTA. Plasma was isolated from blood by centrifugation and total IgE levels were measured using a commercial ELISA kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Briefly, Immulon 2HB 96-well plates (Thermo Labsystems, Franklin, MA, USA) were coated with anti-mouse IgE monoclonal antibody at 4°C overnight and blocked for 1 h at room temperature (RT) with 10% fetal bovine serum (FBS) (Winsent Inc, Saint-Bruno, QC, Canada) in PBS. Standards and plasma samples were incubated for 2 hours at room temperature and mouse IgE was detected with biotinylated anti-mouse IgE and streptavidin-horseradish peroxidase (HRP)-conjugate using 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt (ABTS) reagent mixture (Roche, Laval, QC, Canada). The plate was read at 450nm in an ELISA plate reader (Biorad, Hercules, CA, USA). Purified mouse IgE was used as a standard.

Statistical Analysis

Time points in the Penh kinetic and IgE plasma levels were analyzed using a non-parametric one-way analysis of variance method followed by Bonferroni multiple comparison post-test and differences were considered significant at $p \leq 0.05$. Comparison of AHR to Mch using the non-

invasive and invasive methods was performed by correlation between Penh and resistance in C57BL6 and A/J mice; correlation coefficient was calculated and significance was considered at $p \leq 0.05$. The concentration of Mch required to increase airway resistance by 200% (ED_{200}) was calculated by linear interpolation. All the analysis were performed using GraphPad Prism 4, version 4.03 (GraphPad Software Inc., San Diego, CA).

Results

Airway responsiveness to methacholine in C57BL/6 and AJ mice

To evaluate the effect of Mch on AR after multiple and consecutives challenges, we first assessed Penh and airway resistance to increasing doses of Mch in C57BL/6J and A/J mice using the non-invasive and invasive methods. A/J mice showed significantly ($p<0.05$) higher Penh compared to C57BL/6J mice with Mch doses higher than 7.5 mg/ml (Figure 1 panel A). The same effect was observed using the invasive method where A/J mice displayed significantly ($p<0.05$) higher airway resistance compared to C57BL/6J mice following their exposure to 20 µg/ml or more of Mch (Figure 1 panel B). As expected, C57BL/6J and A/J mice exhibited dose response effect to increasing concentrations of Mch evaluating Penh and airway resistance.

We calculated the Mch dose required to increase Penh and airway resistance to 200% of baseline (ED_{200}). C57BL/6J mice showed significantly higher ($p<0.0001$) ED_{200} (16.6±1.7 mg/ml) using Penh compared to A/J mice (7.7±1.3 mg/ml) (Figure 1 panel C). A similar relationship for the ED_{200} values using airway resistance were observed where the values for C57BL/6J mice were significantly higher ($p<0.0001$) ED_{200} (34.6±2.1 mg/ml) compared to A/J mice (8.2±1.3 mg/ml) (Figure 1 panel D). These results demonstrate that the hypo- and hyper-responsiveness phenotype to Mch of C57BL/6J and A/J mice, respectively, can be reliably reproduced using both invasive as well as non-invasive methods.

Consecutive challenges with methacholine modify measured Penh in mice

To study the effect of consecutive exposure to Mch on the AR, we challenged C57BL/6J and A/J mice every five days and measured Penh after each Mch exposure. AR to Mch significantly increased ($p<0.05$) on days 10 and 15 compared to baseline or to the previous time point using either the hypo-responsive C57BL/6J mice (Figure 2 panel A) or hyper-responsive A/J mice (Figure 2 panel B). Our data demonstrated that consecutives challenges with Mch transiently

increase Penh as measured by the WBP in both C57BL/6J and A/J mice under our experimental conditions.

Next, we have assessed if airway resistance is also modified by Mch. Five groups of C57BL/6J and A/J mice were challenged with Mch and the Penh parameter was evaluated on day 0. Every group was challenged once more with Mch on either day 5, 10, 15, or 20, at which time Penh and airway resistance were assessed. Penh and airway resistance significantly increased ($p<0.05$) on days 10 and 15 compared to baseline using either C57BL/6J (Figure 2 panel C) or A/J mice (Figure 2 panel D). Overall, our results show that both Penh and airways resistance are affected by consecutive challenges with Mch and to obtain consistent results of Penh vs. resistance following repeated exposure to Mch, it would be most reliable if the measurements are performed in a 2 weeks period.

Inflammatory effect of methacholine

To investigate if the increase in AR observed after consecutive exposures to Mch was caused by changes in the airways due to an inflammatory process, we evaluated the infiltration of inflammatory cells in the perivascular space of the lungs as well as IgE levels in the plasma of C57BL/6J and A/J mice. No inflammatory cell influx was observed in either strain of mice after 5 consecutive challenges with Mch (Figure 3, Panel A). Furthermore, no significant difference was found in the plasma IgE levels in these same animals (Figure 3, Panel B). These results demonstrate that changes in AR after consecutives exposure to Mch did not result from inflammation of the airways or an atopic condition.

Correlation of airway hyperresponsiveness to methacholine using the non-invasive and invasive methods

To investigate if AR is modified by consecutive challenges with Mch in C57BL/6J and A/J mice. We challenged individual animals with Mch and evaluated Penh in all groups on day 0.

Following this, airway resistance was assessed in the same animals on either day 5, 10, 15, or 20. These experiments allowed us to evaluate the effect of a single challenge of Mch and the reliability of Penh for the assessment of AR compared to airway resistance, avoiding the potential effect of conditioned behaviour of the mice which might affect Penh assessed by WBP.

We found no correlation between ED_{200} of Penh and airway resistance in C57BL6J mice, when the airway resistance measurement was performed 5 or 10 days ($p=0.351$ and $p=0.396$ respectively) following the measurement of Penh (and Mch exposure) in the same mouse (Figure 4, panel A and B). When the measurements and proceeding exposure to Mch were separated by 15 or 20 days, we found very good correlation ($p=0.038$ and $p=0.021$ respectively) between the two methods using C57BL6J mice (Figure 4, panel C and D). The same effect was observed using A/J mice. No correlation was found after a 5 or 10 day interval (Figure 5, panel A and B), however the measurements done after 15 and 20 days from both methods correlated significantly (Figure 5, panel C and D).

Taken together, these results demonstrate that re-assessment of lung physiology, which requires consecutive exposure to methacholine, needs to be carefully designed and a period of two to three weeks between assessments should be scheduled to obtain consistent and reliable results.

Discussion

The objective of this study was to evaluate how consecutive exposures to methacholine affect the assessment of AR in C57BL/6J and A/J mice. We evaluated the Penh parameter using WBP, and airway resistance using an invasive method, to assess AR to methacholine in these two strains of mice in order to establish the interval required to obtain the most reliable and consistent results reflecting the lung physiology of the mice under study.

In the study of the pathophysiology and the genetics of the development of AHR, animal models have become a key tool due to their versatility in reproducing the allergic response and relatively easy genetic manipulation[187-190]. In mouse models, AR has been assessed using either invasive[23] or non-invasive[24] methods before and after allergen sensitization and challenge. Most of the previously publish studies have employed sensitized BALB/c mice to assess AHR.[24-27] In our study we used both the C57BL/6J and A/J strains of mice due to their well known hypo- and hyper-responsive phenotypes to Mch challenge, respectively.[22] Also, these two inbred strains have been used for the generation of 35 recombinant congenic strains for the dissection of complex traits[178;195;196] and various respiratory disorders.[22;25;29;178;191]

Responsiveness to Mch is routinely used in mouse models of allergic asthma in order to demonstrate the development of AHR.[24;25;179;193;197] Zhang *et al*.[198] have proposed that Penh values obtained from conscious and spontaneously breathing mice in unrestrained plethysmography includes a contribution from the upper airway. Based on the hypothesis that allergen sensitization may modify the upper and lower airway, using BALB/c mice. We studied AR in non-sensitized mice, avoiding any possible change in the upper airways caused by inflammation that may affects the Penh parameter using the non-invasive method. As expected, our results demonstrated that there is a dose dependent response to Mch which results in increased Penh and airway resistance in C57BL/6 and A/J mice which are hypo- and hyper-responsive to Mch, respectively. These results corroborate previous studies using the non-invasive method to assess AR in non-sensitized animal models.[199;200]

It is technically more efficient and certainly faster to evaluate large numbers of mice simultaneously when employing the non-invasive method compared to the invasive method. Furthermore, as the non-invasive method is not a terminal procedure, the assessment of AR at different time points in the same animal is possible. As far as we know, this is the first study which systematically compares measurements of Penh on the same non-sensitized mice. Our results demonstrate a transient increase in Penh following consecutive exposures to methacholine. This effect appears to be temporary as Penh values returned to baseline following the third challenge. These results may be explained by the fact that the non-invasive method assesses AR in unrestrained and conscious mice and it is possible that stress, neural control, or conditioned behavioural responses are affecting the Penh values which may not reflect changes in the underlying respiratory mechanims.[192] The modulation of Penh following re-exposure to Mch is an important consideration, so the correct protocol design can be applied if repeated measurements of Penh are intended.

Previous studies used allergic animals to study the AR to Mch [24;25;179;193;197] and based on the theory that changes in Penh may be caused by modification of the airways, including inflammation.[198] We examined the lungs of mice following consecutive challenges with Mch, over a 25 day period, in order to determine if any inflammatory reaction might be detected. Our results indicate that there is no inflammatory cell infiltration in the lungs and no difference in plasma IgE levels existed that can be observed even after six consecutive challenges with Mch in either C57BL/6J or A/J mice.

To determine if the increase in Penh after consecutive challenges with Mch was caused by changes in respiratory mechanics, we determined the correlation of ED_{200} for Penh on day 0 with ED_{200} for airway resistance on days 5, 10, 15, and 20 using different groups of C57BL/6 and A/J mice. Our results demonstrate correlation ($p<0.05$) between Penh and airway resistance only after a 15 day rest period after exposure to Mch. These results are consistent with previous reports in C57BL/6J mice where no correlation was found between these two methods when measurements were taken 5 and 9 days apart.[191] There is the possibility that consecutive

exposure to Mch modifies Penh to a greater degree than it does airway resistance. However, since the invasive method is a terminal technique this hypothesis cannot be verified.

Overall, our results demonstrate that non-sensitized C57BL/6J and A/J mice show a dose dependent response to increasing doses of Mch using both the non-invasive and invasive method. Consecutive challenges with Mch increase the Penh values in both non-sensitized C57BL/6J and A/J mice using the non-invasive method, as well as airway resistance using the invasive method. Furthermore, to obtain a reliable reassessment of AR in mouse models we established that a rest period of about 15 days between exposures to Mch is necessary. It is also highly recommended to ascertain the reliability and consistency of results when using mouse models of airway hyperresponsiveness.

Acknowledgements

This work was supported by a grant from the American Asthma Foundation Research Program. R. Marino and P. Camateros are recipients of Doctoral Award from the Canadian Institutes of Health Research (CIHR).

Figure 1. Airway responsiveness to methacholine in C57BL/6J and A/J mice, and ED_{200}.

(A) Penh in response to increasing doses of methacholine. **(B)** Airway resistance (Rrs) in response to increasing doses of methacholine. Dots and triangles represent the mean ± SEM of three independent experiments. n=36 for all groups. *p<0.05, **p<0.01 and ***p<0.001 compare to baseline. **(C)** ED_{200} for Penh. **(D)** ED_{200} for Rrs. Bars represent the mean ± SEM of three independent experiments. n=36 for all groups. A/J mice are hyper-responsive to Mch compare to C57BL/6J mice, as it shows by significant lower ED_{200}. Penh and Rrs show a dose dependent response in both strains of mice.

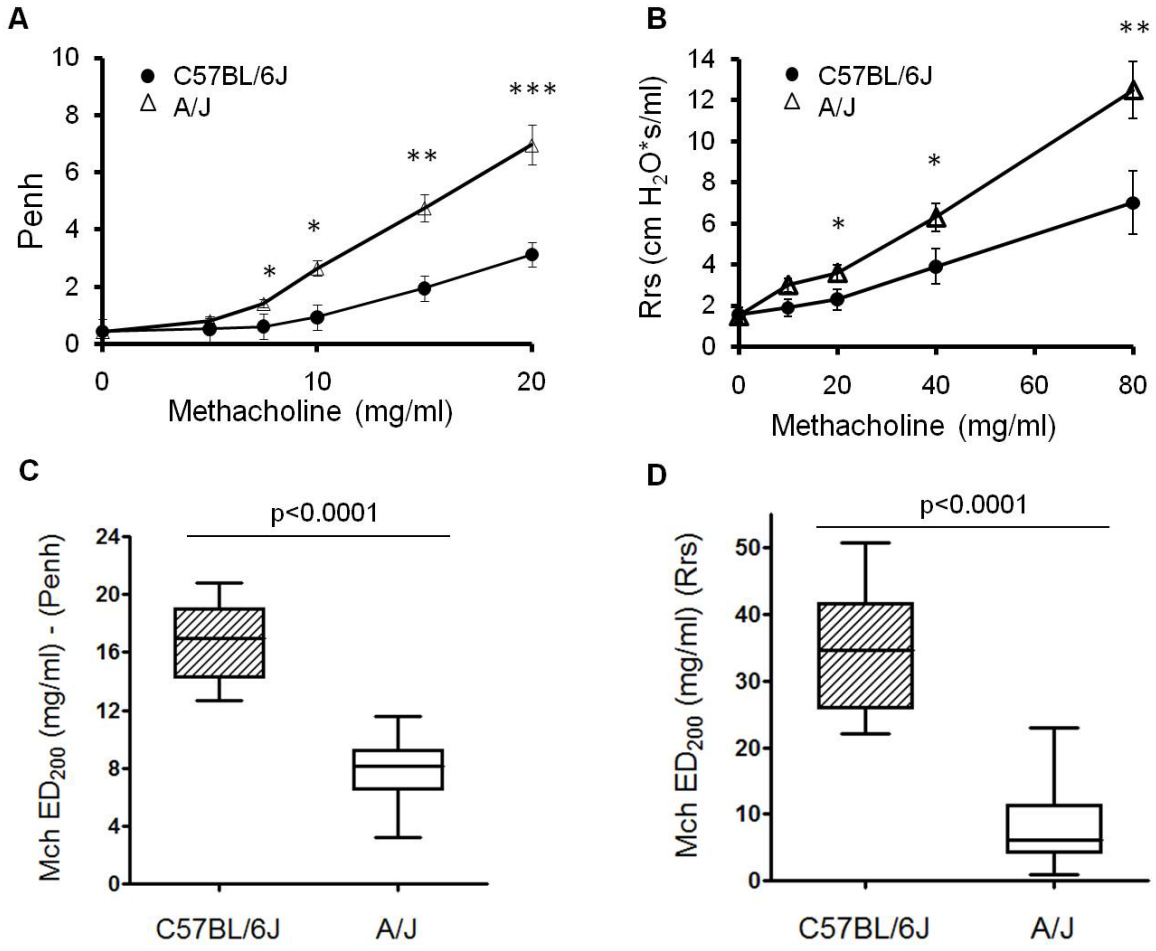

A
B
C
D
C57BL/6J
A/J
Penh
Methacholine (mg/ml)
Rrs (cm H2O*s/ml)
p<0.0001
Mch ED200 (mg/ml) - (Penh)
Mch ED200 (mg/ml) (Rrs)

Figure 2. Airway responsiveness to consecutive challenges with methacholine (Mch) in C57BL/6J and A/J mice.

Penh and airway resistance (Rrs) were measured at 15 mg/ml and 40 mg/ml of MCh, respectively. **(A)** Penh in response to Mch every 5 days C57BL/6J mice. **(B)** Penh in response to Mch every 5 days in A/J mice. A and B, n=24 for all groups, *****$p<0.05$ compared to the previous time point and **$p<0.01$ compared to baseline. **(C)** Penh and Rrs in different time point groups of C57BL/6J mice. **(D)** Penh and Rrs in different time point groups of A/J mice. Dots and triangles represent the mean ± SEM of three independent experiments. C and D, n=8 for all groups. *$p<0.05$ compared to the previous time point and **$p<0.01$ compared to baseline. Penh and Rrs significantly increased after consecutive exposures to Mch in both strain of mice.

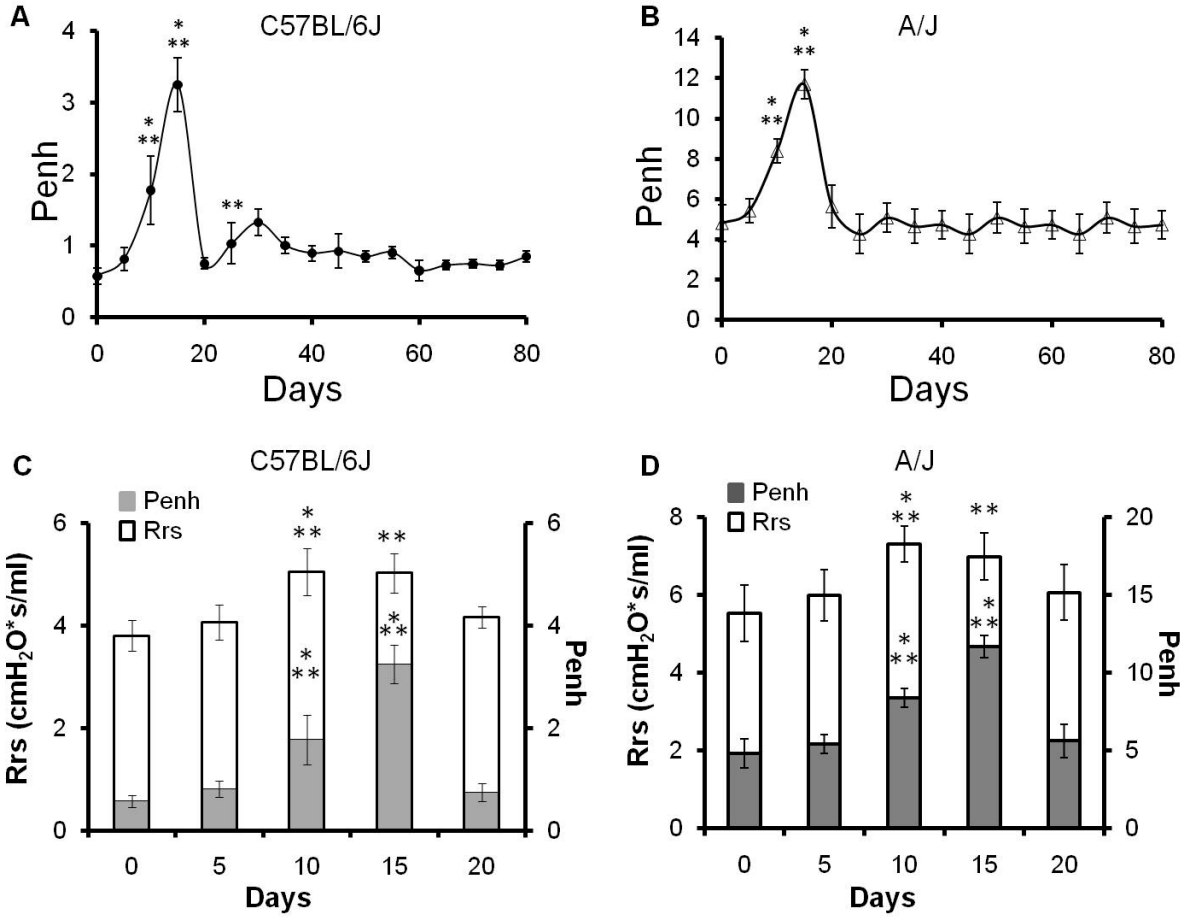

A
C57BL/6J
Penh
Days
B
A/J
Penh
Days
C
C57BL/6J
Penh
Rrs
Rrs (cmH2O*s/ml)
Penh
Days
D
A/J
Penh
Rrs
Rrs (cmH2O*s/ml)
Penh
Days

Figure 3. Lung sections and immunoglobulin E levels in plasma from C57BL/6J and A/J mice. After six consecutive exposures to methacholine every 5 days **(A)** Representative hematoxylin- and eosin-stained lung sections. **(B)** IgE levels in plasma before and after Mch challenges. Bars represent the mean ± SEM of three independent experiment, n=24 animals for all groups. No inflammatory cell infiltration was observed in the lungs. IgE levels in plasma were significantly higher in A/J mice compared to C57BL/6J mice but there was no difference after Mch challenges.

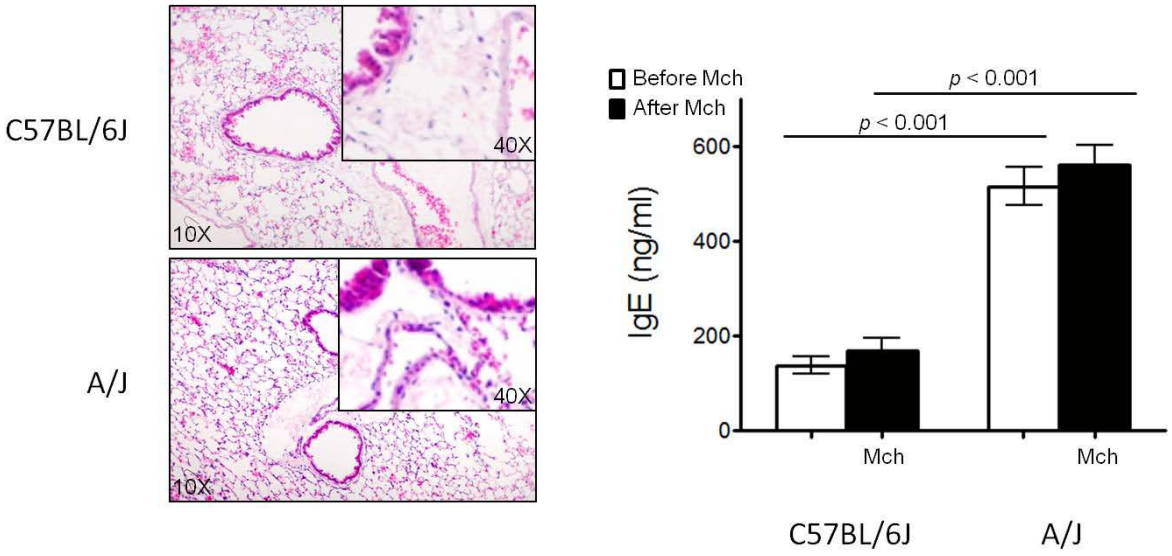

C57BL/6J
A/J
10X
40X
10X
40X
Before Mch
After Mch
p < 0.001
p < 0.001
IgE (ng/ml)
600
400
200
0
Mch
Mch
C57BL/6J
A/J

Figure 4. Correlation between methacholine (Mch) concentrations ED_{200} for airway resistance (Rrs) vs. Penh in C57BL/6J mice at different time points.

(A) 5 days apart **(B)** 10 days apart. **(C)** 15 days apart **(D)** 20 days apart. n=8 for all groups. There was very good correlation between both methods 15 and 20 days apart.

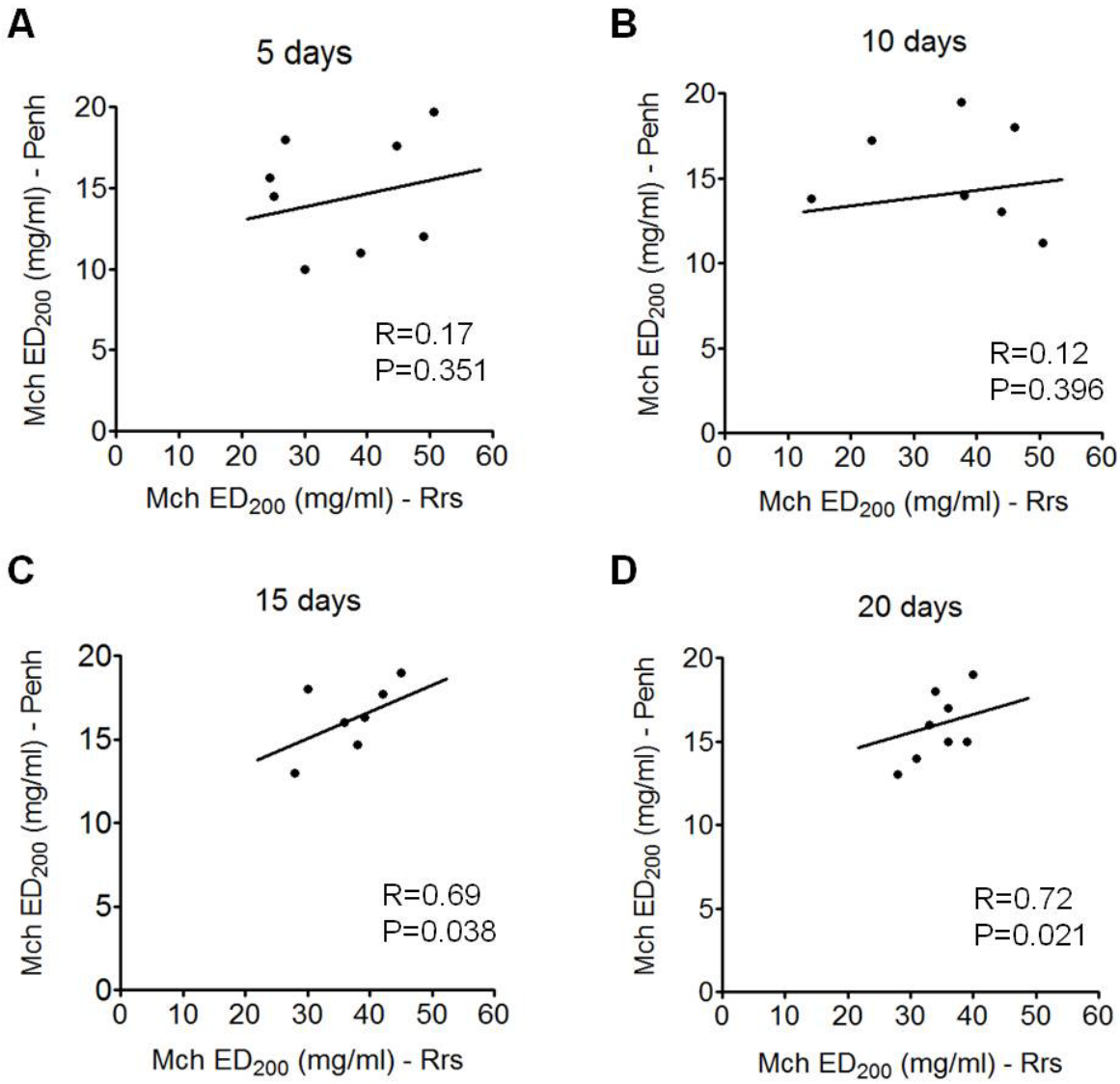
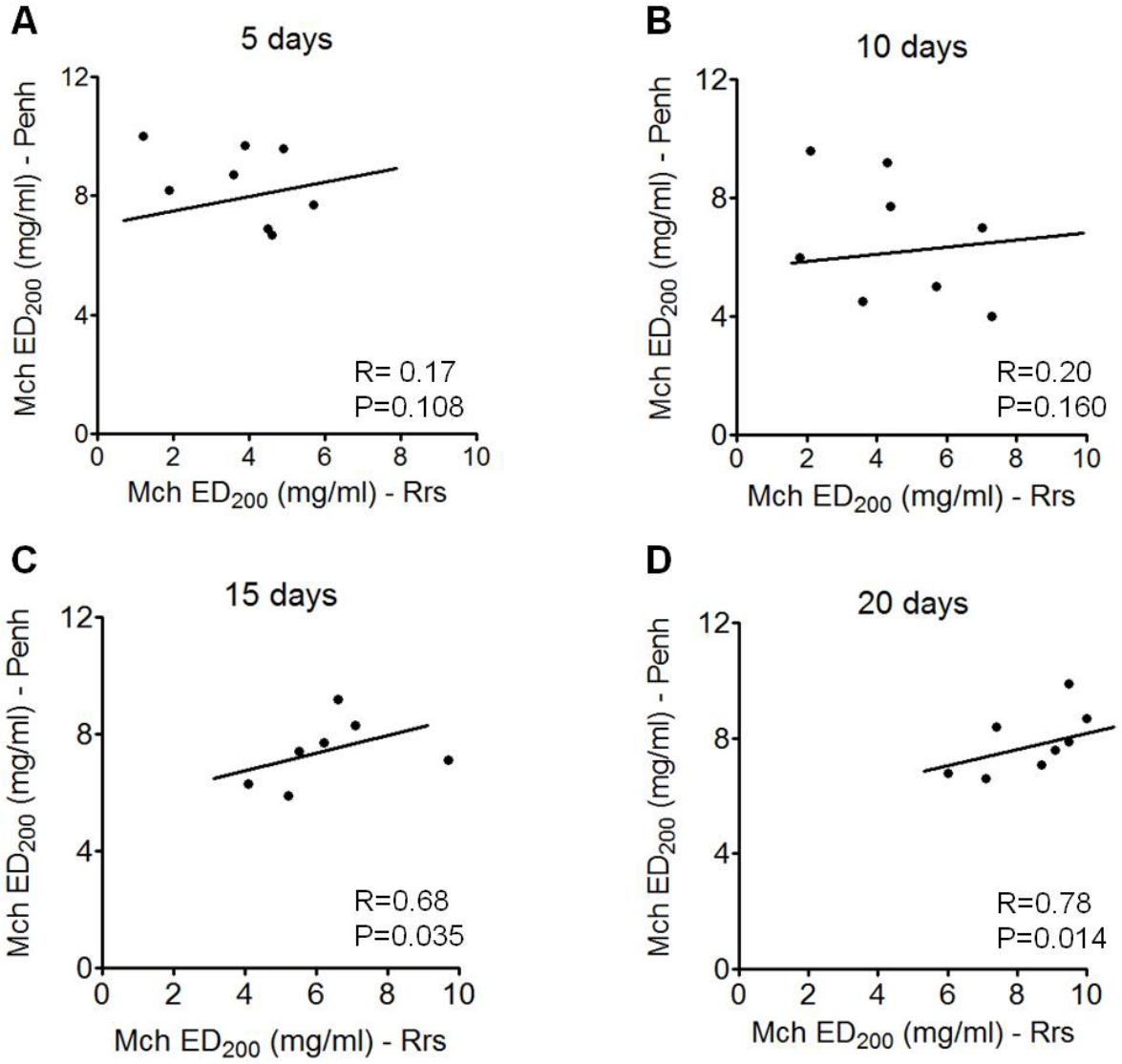

A
5 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.17
P=0.351
B
10 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.12
P=0.396
C
15 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.69
P=0.038
D
20 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.72
P=0.021

Figure 5. Correlation between methacholine (Mch) concentrations ED_{200} for airway resistance (Rrs) vs. Penh in A/J mice at different time points.

(A) 5 days apart. **(B)** 10 days apart. **(C)** 15 days apart. **(D)** 20 days apart. n = 8 for all groups. There was correlation between both methods 15 and 20 days apart.


A
5 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R= 0.17
P=0.108
B
10 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.20
P=0.160
C
15 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.68
P=0.035
D
20 days
Mch ED200 (mg/ml) - Penh
Mch ED200 (mg/ml) - Rrs
R=0.78
P=0.014

Chapter 3 - Genetic dissection of airway responsiveness and its impact on the susceptibility to allergic asthma

Partial fragments of this chapter have been adapted from:

P. Camateros, **R. Marino**, A. Fortin, J. G. Martin, E. Skamene, R. Sladek and D. Radzioch. Identification of novel chromosomal regions associated with airway hyperresponsiveness in recombinant congenic strains of mice. Mamm Genome. 2010 Feb; 21(1-2):28-38.

Preface

Airway hyperresponsiveness has been associated with the development of allergic asthma in humans; it is inherited as a complex trait and has been reproduced in mouse models. In the second chapter our data shows the difference in airway responsiveness in C57BL/6J and A/J mice. The airways of A/J mice, like the airways of asthmatics and individuals at risk of developing asthma, are hyperresponsive to bronchoconstricting stimuli such as methacholine, while the airways of C57BL/6J mice are hyporesponsive. Using recombinant congenic strains to generate F2 strains of mice, the study presented in this chapter described the unique approach in the identification of narrowed chromosomal regions associated to the development of airway hyperresponsiveness and points out a few candidate genes that might be responsible for this phenotype and potentially related to the susceptibility to allergic asthma.

Abstract

Airway responsiveness is the ability of airways to reduce their diameter after bronchoconstricting stimuli. Airway responsiveness is inherited as a complex trait and has been associated with the development and severity of asthma. In mouse models C57BL/6J and A/J mice have shown to be hypo- and hyperresponsive to methacholine, respectively. Using recombinant congenic strains (RCS) of mice derived from the C57BL/6J and A/J strains, we generated six different F2 crosses, in order to identify genetic loci underlying the trait of airway responsiveness in response to methacholine as assessed by whole-body plethysmography. Our strategy allowed us to narrow down the significant regions identified in the RCS and specifically the region on chromosome 12, from 96 Mbp to 34 Mbp. Quantitative trait loci analysis shows 3 significant loci on chromosome 12 (Lod 5.11 and 4.18; $p<0.001$ and $p<0.001$, respectively) in the BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses. This significant region contains 232 genes, 43 of which are expressed in the lung and after excluding those which do not contain non-synonymous or splice-site mutations only 7 genes were identified as potential candidates. Those genes include the *Arhgap5* gene related to cell activation, myogenesis, and hepatocellular carcinoma; *Foxa1* associated with inflammation and hematopoiesis; *Clec14a* involved in the process of cell-cell adhesion and immune response to pathogens, and *Ctage5* linked with the pathophysiology of meningioma and glioma. We had also identified the *Rtn1* gene associated with neuroendocrine secretion, intracellular traffic, and susceptibility to ethanol; the *Gpx2* gene related to hepatocarcinogenesis in rats and the protection from allergen-induced airway inflammation in mice, and the *Rsad2* gene located at 27.1 Mbp which has antiviral activity and has also been involved in the resistance to fungus infection. Within the significant region we identified two additional genes, even if they have not exactly fulfilled our gene selection criteria. These are the *Prkd1* associated with activation and TLR9 signalling, and *Adam21*, related to cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis. Overall, our results demonstrate how the generation of F2 crosses from RCS offers enables identification of genes when dealing with complex trait

diseases and allowed us to identify 9 candidate genes that might be associated with airway responsiveness in mice.

Introduction

Asthma is a chronic inflammatory disease of the airways characterized by episodes of reversible bronchoconstriction. Asthma is considered a complex disease in which genetic and environmental factors interact together to trigger an uncontrolled immune response to a broad variety of allergens.[1;30] In the study of genetic factors that might be responsible for the development of asthma, studies have focused on intermediate phenotypes, one of which is airway responsiveness to bronchoconstricting stimuli.[139]

Airway responsiveness (AR) is the ability of the airway to reduce their diameter after exposure to direct or indirect stimuli and airway hyperresponsiveness (AHR) is defined as the exaggeration of this ability.[21] AHR is considered a hallmark of asthma and it has been associated with the development of this disease.[117;120;127;193] AHR has been proven to be inherited as a complex trait, precedes the development of asthma, and has been efficiently reproduced in mouse models.[120] AR can be assessed both before and after the experimental induction[22] of allergic airway inflammation using either invasive[23] or non-invasive[24] methods in mice.

Although identification of the genes responsible for the susceptibility to the development of AHR in human populations has proven to be difficult, several large human population studies employing genome-wide analysis resulted in the identification of asthma susceptibility loci and some candidate genes.[148] These studies identified significant linkages, such as STAT6, GATA2 and FCεR1A as a novel loci at which common genetic variation influences serum IgE levels.[153] Furthermore, genes such as dipeptidyl peptidases cleaving terminal dipeptides from cytokines and chemokines, cytokines (e.g. IL-33), transcription factors (e.g. MYB), and the cytokine receptors (e.g. IL-1R1), WDR26 (a protein co-regulated with IL-2), and IRAK-M have been linked to AHR.[154;201-208]

Several other candidate genes were also demonstrated to be associated with asthma susceptibility using other phenotypes, including urokinase-type plasminogen activator which is

associated with atopy.[156;157] Furthermore, the chemokine receptor 5 (CCR5), cysteinyl leukotriene receptors (CTSLTR1 and CTSLTR2), prostaglandin D2 receptors (PTGDR and CRTH2), thromboxane A2 receptor (TBXA2R), beta2-adrenergic receptor (ADRB2), and the G protein-coupled receptor (GPRA) genes have been associated with asthma.[125;161;203] A mechanistic role has recently been identified for a disease-associated genetic variant within the ADAM33 asthma susceptibility gene;[209;210] and asthma progression has been shown to correlate with increased expression of the ADAM33 and ADAM8 genes.[176] Using a candidate gene approach, linkage or association studies have been reported for IL4R, FcR1, IL-4, IL-5, IL-9, and CD14 (one of the LPS receptors), although only a small proportion of asthma cases could be explained by variants in these genes.[125;169;172;211-213]

Despite significant progress in genome-wide association studies of human populations, it has been difficult to dissect the asthmatic phenotype and identify genes which are responsible for each aspect of the asthmatic condition in humans. For that reason, significant stratification of the sample population has been required in order to discriminate between different sub-phenotypes in asthmatic patients. In humans, these types of analyses require very large sample populations in order to have reasonable statistical power. As an alternative and complementary approach to whole-genome association studies, the genetic analysis of mice can be used to identify candidate genes for targeted human studies and to confirm loci found by the association studies.

The AcB/BcA recombinant congenic strains (RCS) were derived from the A/J and C57BL/6J inbred strains of mice in order to aid in the dissection of complex traits. This RCS panel has resulted in the identification of QTLs for numerous traits, including amphetamine-induced locomotion,[180] emotionality and stress response,[181;182] nicotine effect,[183] opioid dependence,[184] and Salmonella infection.[185] 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.[186]

As evidenced by the fact that it varies considerably among inbred strains,[214] airway responsiveness is not only a heritable trait in humans but also in mice. The A/J strain is one of the most hyperresponsive of the common laboratory strains, and crosses involving A/J mice and either the C57BL/6J 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,[136] 6,[122;178] 7,[178] and 17[178] by employing F2 intercrosses generated from A/J and C3H mice while QTL or significantly associated chromosomal regions have been identified on chromosomes 2,[178] 6,[179] 15, and 17[178] by employing various breeding schemes derived from A/J and C57BL/6 mice.

In our previous study, we analyzed the AcB/BcA RCS of mice and identified genetic loci underlying the trait of AR in response to methacholine as assessed by whole-body plethysmography.[22] Our screen identified 16 chromosomal regions significantly associated with AHR; 8 are supported by previously and independently published reports while 8 were 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).

The chromosomal regions identified using the RCS of mice are generally too large to select candidate genes in an efficient manner; around 157 potential candidate genes are included in these 16 chromosomal regions significantly associated with AHR. We hypothesized that the generation of F2 crosses from the AcB/BcA RCS as parental strains will allow us to narrow down the significant intervals already identified in the RCS panel, lead to the identification of a few candidate genes associated with AHR, and would offer a better understanding of inheritance and molecular mechanism underlying AR and its relation with allergic asthma.

Material and Methods

Recombinant Congenic Strain Panel and F2 crosses

Recombinant congenic strains (RCS) of mice were generated from reciprocal backcrosses between methacholine challenge resistant, C57BL/6J [B], and susceptible, A/J [A] strains.[215] The RCS were produced by inbreeding independent pairs of mice from the (F1×A)×A backcross to produce the AcB strains, and (F1×B)×B backcross to produce the BcA strains (Figure 1). Then, the resulting mice were inbred for more than 20 generations creating 14 distinct AcB strains, containing on average 13.25% of the B genome on an A background (on average 85.3% 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 86.1% of the B genome).

Following a phenotyping survey of all 36 AcB/BcA strains in the panel,[22;125] we selected the three most informative strains from which F2 crosses were generated. Every F2 cross was obtained from the breeding of the most informative recombinant strains with two strains: the major genetic donor progenitor strain (A or B) and a phenotypically similar but more distantly related strain, C3H/HeJ or AKR. In total, we generated six different F2 crosses identified as BcA86xB, BcA86xC3H/HeJ, BcA85xB, BcA85xC3H/HeJ, AcB64xA and AcB64xAKR. The generation of the F2 crosses was performed in order to narrow down the recombinant regions identified in the RCS which might be associated with airway responsiveness.

The RCS of mice and F2 crosses were generated in the pathogen-free facility of the Montreal General Hospital Research Institute at McGill University Health Center (Montreal, Canada). Male mice between 8-10 weeks-old were used in all experiments. All procedures performed on the animals were approved by the McGill University Animal Care Committee in compliance with the Canadian Council of Animal Care guidelines.

Airway responsiveness phenotyping

Non-invasive method

AHR was assessed using an unrestrained whole body plethysmograph (WBP) (Buxco Research Systems) as described previously,[22] and expressed as the Penh parameter. Briefly, the system was warmed up for 20 minutes; four chambers were calibrated and used simultaneously. An unrestrained and spontaneously breathing mouse was placed in each chamber and allowed to acclimate for at least 5 minutes. Following a baseline reading, mice were exposed to nebulized PBS for over 1 minute and subsequently to increasing doses of Mch (5, 10, 15, and 20 mg/ml) (Sigma-Aldrich, Oakville, ON), allowing the Penh to return to baseline between each challenge. After each aerosol challenge the average Penh measured during the 5 minutes after exposure was recorded.

Invasive Method

AHR was assessed using an invasive method as described previously,[193;194] and expressed as airway resistance (Rrs, cm H_2O*s/ml). Briefly, mice were anaesthetized with 3:1 Ketamine (70 mg/Kg) and Xylaxine (10 mg/Kg). After the depth of anesthesia was verified, mice were tracheotomized, endotracheally intubated (with an 18 gauge stainless steel cannula), injected intraperitoneally (i.p.) with of 10 µl/g of Pancuronium (1 mg/ml) and finally connected to the MiniVent (type 845) small animal ventilator (Harvard Apparatus, Saint-Laurent, QC). Tidal volume was adjusted to 10 µl/g and a respiratory frequency of 180 strokes/min. Increasing doses of Mch in aerosol (10 µL of 0–80 µg/ml) were administered consecutively and the peak of resistance was measured with the Buxco resistance system (Buxco Electronics, Inc. Wilmington, NC). Mice were then euthanized and samples were collected. We used 40 µg/ml of Mch as the most reliable dose to compare Rrs. The dose required to increase airway resistance by 200% (ED_{200}) was also calculated.

Correlation between invasive and non-invasive methods for measuring airway responsiveness to Methacholine

In addition to the experiments described in chapter 2 about the validation of the non-invasive method as the phenotyping technique, we correlated the assessment of AR to methacholine using both the non-invasive and invasive methods in all six F2 crosses. We measured Penh and airway resistance in 15 – 20 mice of each F2 cross at an interval of 15 days and calculated the correlation between both parameters on each mouse. Penh at 15mg/ml and resistance at 40mg/ml of methacholine represented the most reliable concentration of Mch that showed a dose-response effect with a linear relation and significant difference ($p<0.05$) between both strains.

Genotyping

All RCS were previously genotyped using mouse Illumina Chip medium density (md) linkage genome-wide panel of 1449 single nucleotide polymorphisms (SNPs) placed at intervals of 5Mb created through GoldenGate Genotyping Assay (unpublished data). 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.[216] DNA samples from the F2 crosses were extracted from the tail of the experimental mice following an overnight digestion in lysis buffer and proteinase K, followed by phenol-chloroform extraction. DNA concentrations were measured with a Quant-iT DNA Assay Kit (Molecular Probes, Invitrogen, Burlington, Ontario, Canada) and adjusted to 50 ng/ml. The genotype of all F2 crosses was performed using microsatellites every 10 Mbp. Then, a re-genotype was carried out using custom SNPs at 4 Mpb intervals by the Genome Quebec Innovation Center and University of Laval, in the BcA86xB and BcA86xC3H/HeJ F2 crosses. The chosen SNPs were polymorphic between the A strain, and both the B and C3H/HeJ strains, selected from public databases (e.g. phenome.jax.org).

Genetic Analysis

Identification of significantly associated regions in the RCS

As previously described,[22;125] we performed the association study of airway responsiveness in the RCS but this time used the 1,442 SNP panel. Briefly, a marker by marker analysis was performed 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 $P = \beta_0 dso + \beta_1 bg + \beta_2 dso \times bg + \varepsilon$ 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 $a \leq p0.05$ 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).

Quantitative trait loci analysis in the F2 crosses

QTL analysis was performed by computing single marker regression using the Bioconductor packages in R version 2.1.0 (www.R-project.org), employing microsatellites and custom SNPs as

genotype markers. Significance thresholds were established by performing 10,000 permutations, shuffling the phenotypes across the genotypes to correct for multiple testing. Significance was established as a P value smaller than the smallest P value in more than 95% of the permutations. Support intervals were calculated as one LOD above the significant threshold. Detection of epistatic interaction was carried out on the whole genome scan by fitqtl analysis between the highest LOD peaks. Significant QTL by QTL interactions were detected as locus pairs with significance as $P < 0.001$.

Databases and Gene Characterization

To be considered a relevant gene associated with airway responsiveness, expression in the lungs was based on the following: the gene has to be listed in the MGI Gene Expression Database (GXD)[217] as detected in the lungs at any stage of development. The MGI GXD has to list the cDNA gene as isolated from the lungs. Or, a probeset for the gene has to be 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/6J 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.

To identify intervals which were identical by decent (IBD), the frequency of single nucleotide polymorphisms (SNP) was employed within the regions which had been identified as significantly associated with asthma. IBD intervals were defined as any continuous interval, at least 500 kbp 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.

The annotations of the MGI MGD were employed to identify mutations in or within 2 kbp of genes contained within regions identified as significantly associated with airway responsiveness. Non synonymous or splice site (NS/SS) mutant genes were considered those

containing any variations polymorphic between the A/J and C57BL/6J 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.

Other statistical analysis

Comparison of AR to Mch using the non-invasive and invasive methods was performed by correlation between Penh and resistance from each of the F2 crosses; correlation coefficients were calculated and significance was considered at $p \leq 0.05$. 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 $p \leq 0.05$. All the correlation analyses were performed using GraphPad Prism 4, version 4.03 (GraphPad Software Inc., San Diego, CA).

Results

Airway responsiveness

Previous studies have demonstrated the difference in airway responsiveness between the A/J and C57BL/6J strains of mice using invasive measurements,[178;218;219] as well as the non-invasive method of WBP.[179] Additionally, as demonstrated in Chapter 2, we assessed AR in both strains of mice under our laboratory conditions and corroborated that A/J mice exhibited significantly higher airway responsiveness compared to C57BL/6 mice using both the invasive and the non-invasive methods. We also demonstrated that assessment of AR to methacholine using whole body plethysmography can be reliably performed in non-sensitized A/J and C57BL/6J mice.

Airway responsiveness was previously assessed in the 11 AcB and 22 BcA strains of the RCS panel and the distribution of phenotypes is illustrated Figure 2. Confirmation of a strong strain effect was obtained by one-way ANOVA. Additionally, Bonferroni-corrected post-tests comparing all AcB strains to their major genetic donor parental strain, the A/J mice, and all BcA mice to the C57BL/6 mice identified one AcB strains (AcB64) that showed opposite phenotype than the A/J strain and two BcA strains (BcA85 and BcA86) that also demonstrated opposite phenotype compared to the C57BL/6 parental strain.[22]

Inheritance of a genetic component that confers resistance to methacholine challenge, is observed in the distribution of AR phenotypes of the BcA strain, displaying lower airway responsiveness than the C57BL/6 strain in almost two-thirds (14 of 22 strains) of the strains, and demonstrating that the hyperresponsive A/J strain likely contains alleles that confer resistance to the methacholine challenge, at least when they are expressed on a C57BL/6J 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.

Marker association in the RCS

In our previous association study using 603 SSLP markers, we identified 50 markers that are significantly associated with airway responsiveness. These markers delimited 16 chromosomal regions significantly associated with airway hyperresponsiveness (genome-wide $p<0.05$): 8 of the regions have been previously reported by other groups while 8 are entirely novel. The 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 (92–100 cM), chromosome 5 (>73 cM), chromosome 7 (>63 cM), chromosome 8 (52–67 cM), chromosome 10 (3–7 cM and >68 cM), and chromosome 12 (25–38 cM and >52 cM).[22]

In this study we performed a different association study of AR in the RCS using 1442 SNPs as genotype. The objective of the analysis was to replicate the identification of loci that we previously described and to detect additional chromosomal regions associated with airway responsiveness. As expected, we found the same 8 previously reported regions, the 8 additional regions recently reported by our group, and 1 additional region in Chromosome 9 (>113 Mbp).

Generation and phenotyping of the F2 crosses

While the Penh parameter is commonly used as a measure of airway responsiveness in mouse studies, some studies suggested some precaution in the analysis of the results. In Chapter 2, we demonstrated that the use of the Penh is an accurate and sensitive parameter for studying airway responsiveness. Additionally in this study, validation and quality control of the phenotyping method in the F2 crosses was performed by the assessment of airway responsiveness to methacholine using both the non-invasive and invasive methods, in batches of 5 animals during the time frame of the phenotyping. As we expected, we obtained very good correlation ($p<0.05$) between the Penh parameter and airway resistance in all of the six F2 crosses. These results demonstrated that the assessment of airway responsiveness by WBP is a reliable technique for the phenotyping of the F2 crosses (Figure 3).

We selected three of the most informative strains from the phenotyping survey of all AcB/BcA strains and generated six F2 crosses of mice, in order to narrow down the recombinant regions identified in the RCS that might be associated with AR. BcA86, BcA85 and AcB64 RCS were bred with two strains: the major genetic donor progenitor strain (A/J or C57BL/6J) and a phenotypically similar but more distantly related strain, C3H/HeJ or AKR. Around 230 mice from each F2 crosses were produced and identified as BcA86xC57BL/6J, BcA86xC3H/HeJ, BcA85xC57BL/6J, BcA85xC3H/HeJ, AcB64xA/J and AcB64xAKR. All the F2 crosses were assessed for airway responsiveness to increasing doses of methacholine using the non-invasive method.
In Figure 4, the distribution of the Penh values in each of all F2 crosses show the complex genetic control associated with airway responsiveness. Additionally, our results show normal (Figure 5) distribution of the Penh parameter in all the F2 crosses, suggesting differential contribution of the traits associated with the phenotype and the participation of multiple traits associated with AR.

Quantitative trait loci analysis

As described above, we first genotyped all F2 crosses using microsatellites and after preliminary QTL analysis using marker regression, we selected two of the six F2 crosses with the most significant chromosomal regions (higher LOD scores) and more dense genotype. Additional genotypes were then carried out for these crosses using custom SNPs at 4 Mpb intervals in the BcA86xB and BcA86xC3H/HeJ F2 crosses in order to improve the resolution in the analysis and to determine more precisely the edges of the significant regions associated with AR. The chosen SNPs were polymorphic between the A strain, and both the B and C3H/HeJ strains. Quantitative trait locus analysis was performed using a combination of microsatellite and SNP markers across the genome. Single- and two-locus marker regressions were used for the analysis in the BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses employing 200 mice from each strain. Significance thresholds were established by 10,000 permutations.

Using this approach, QTL mapping on 200 male BcA86xC57BL/6J (F2) identified two significant QTL on chromosome 12 at 21.9 cM (Figure 7, panel A) with a peak LOD score of 5.11 (p=0.001, 7.7% of the variance). Looking at LOD score values for each marker on the physical map, we identified 2 significant peaks higher than one LOD above the threshold which correspond to D12Mit285 and D12Mit52. Those regions delimit two segments of chromosome 12 at 44-61 Mbp and 73-85 Mbp, respectively (Figure 7, panel B). Additionally, QTL mapping on 200 male BcA86xC3H/HeJ (F2) mice identified one significant QTL on chromosome 12 at 9.05 cM (Figure 8, panel A) with a peak LOD score of 4.18 (p=0.001, 10.7% of the variance). The peak corresponds to rs13481355 on chromosome 12 and delimits one region of 4 Mpb on the physical map, specifically at 24-28 Mbp (Figure 8, panel A). These results allowed us to identify QTL that are strongly associated with AR to methacholine, and to demonstrate how our genetic approach narrowed down the recombinant region identified on chromosome 12 of the RCS from 96 Mbp to three significant regions with a total of 34 Mbp.

Likely candidate genes

Using the above genetic strategy, this work resulted in a strong reduction of the significant regions identified in the RCS as associated with AR and offers a more manageable list of potential candidates. In our previous study, we identified 3710 genes included in the significant regions of the recombinant congenic strains. The generation of F2 crosses from the RCS allowed us to narrow down these significant regions (Figure 8). As criteria for choosing candidate genes (Table 1), we selected those identified as non-identical by descent (IBD) between A/J and C57BL/6 or C3H/HeJ mice, expressed in the lungs, and labeled with dbSNP annotations of “Coding-Non Synonymous” or “Splice-Site”.

As result, seven candidate genes were identified in chromosome 12 as significantly associated with airway responsiveness. These likely candidate genes include the radical S-adenosyl methionine domain containing 2 (Rsad2) gene located at 27.1 Mbp, codes for an antiviral activity protein which regulates T lymphocyte activation and cytokine production[220] and is

reported to be up-regulated in the lung during *Pneumocystis* pneumonia.[221] Also identified were the Rho GTPase activating protein 5 gene (Arhgap5) located at 53.6 Mbp and related with cell activation, myogenesis, [222] and hepatocellular carcinoma.[223] Forkhead box A1 gene (Foxa1) (58.1 Mbp) associated with inflammation and hematopoiesis; C-type lectin domain family 14 member A gene (Clec14a) at 59.3 Mbp and involved in the process of cell-cell adhesion, immune response to pathogens and apoptosis. Cutaneous T-cell lymphoma-associated antigen 5 gene (Ctage5) linked with the pathophysiology of meningioma and glioma.[224] Additionally, the Reticulon1 gene (Rtn1) located at 73.3 Mbp and associated with neuroendocrine secretion and apoptosis; and Glutathione peroxidase 2 (Gpx2) (77.8 Mpb) related to hepatocarcinogenesis in rats[225] and to the protection from allergen-induced airway inflammation in mice.[226]

Besides our criteria in the selection of candidate genes associated with AR, there are 2 genes included in the significant regions that might deserve special attention due their importance in several molecular mechanisms. One is protein kinase D1 gene (Prkd1) located at 51.4 Mbp and related with TLR9 signalling and A disintegrin and the other one is methaloprotease domain 21 (Adam21) located at 82.6 Mbp and related to cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis.

Discussion

The objective of this study was to narrow down the significant chromosomal regions, previously identified in the RCS,[22] and to identify new candidates genes associated with airway responsiveness to methacholine, employing F2 crosses generated from an AcB/BcA RCS panel which was derived from the airway hyperresponsive A/J strain and the hyporesponsive C57BL/6 strain.[181] Our results demonstrated that enhanced pause (Penh) as assessed by whole body plethysmography is a reliable technique for the assessment of AR in the RCS panel and F2 crosses. Additionally, the airway responsiveness distribution in the F2 crosses shows a complex pattern of inheritance indicating the involvement of several loci. Furthermore, the F2 crosses allowed us to narrow down the recombinant regions described as significantly associated with AR in the RCS panel, and to identify some candidate genes that might be responsible for the susceptibility to develop airway hyperresponsiveness or allergic asthma.

Asthma is a heterogeneous and complex disease. Several association and linkage studies in humans have explored different intermediate phenotypes as biomarkers of allergic asthma such as bronchial hyperresponsiveness, total and allergen-specific IgE levels, eosinophilia, skin-prick tests reactivity to different allergens, cellular receptors and cytokine levels.[145;146;227] Airway hyperresponsiveness has been established as a key component of this pathology; however, the genetic bases of this complex trait and its role in the aetiology of asthma have not been elucidated. It has been shown that airway hyperresponsiveness precedes the development of asthma.[21;154;228] It is inherited as a complex trait and is largely controlled by genetic factors, in both mouse and humans.[124;125;229] Furthermore, airway hyperresponsiveness is an objective phenotype that can be quantified using both invasive or non-invasive methods and varies among inbred strains of mice, offering an ideal phenotype in the genetic dissection of asthma susceptibility.[177]

The A/J and C57BL/6J strains of mice, have been identified as a hyper- and hyporesponsive to methacholine challenge, respectively.[182;183] We previously showed that the A/J and C57BL/6J

background strains had a significant effect on the airway responsiveness in the AcB/BcA RCS panel. Of 33 strains that made up the RCS panel, we selected the three most informative strains (AcB64, BcA85, and BcA86) which exhibit a phenotype that is opposite from that of their minor genetic donors while carrying only 12.5% of their genome. These three informative RCS together contain recombinant segments for 12 of the 16 regions identified in the RCS as associated with airway hyperresponsiveness; however, each strain contains a subset of only five or six regions.[22] This subset of recombinant region in the three most informative strains still represented a large amount of candidate genes, therefore a more precise mapping using F2 crosses was required to narrow down the intervals and make the selection of candidate genes feasible.

We generated two F2 strain from each of the three most informative strains, the first one was generated by crossing to the major genetic donor progenitor strain (A/J or C57BL/6J), and a second was generated with a phenotypically similar but more distantly related strain (AKR or C3H/HeJ).

Previous studies using the A/J, C57BL/6, and C3H strains have identified QTLs associated with airway hyperresponsiveness on chromosome 2, 6, 7, 15, and 17[136;179;230] Our previous study replicated most of the regions already described as associated with AHR and showed 8 novel regions included in chromosome 1, 5, 7, 8, 10 and 12.[22] The analysis performed in the present study show that we effectively narrowed down the recombinant regions previously identified in the RCS, specifically the significant region on the chromosome 12 (from 96 Mbp to 34 Mbp) by generating F2 crosses from the most informative RCS. Additionally, the QTL analysis identified 3 loci located on chromosome 12; the first one at 24-28 Mbp, the second one at 44-61 Mbp and the third one at 73-85 Mbp, overlap with one of the novel regions associated with airway hyperresponsiveness previously described by our group.[22] Once we identified the significant region associated with AR to methacholine in the F2 crosses, we found 7 candidate genes described as non-IBD between A/J and C57BL/6 or C3H/HeJ mice, expressed in the lungs, and labelled as “Coding-Non Synonymous” or “Splice-Site”.

These likely candidate genes include the *Arhgap5* gene related with cell activation, myogenesis,[222] and hepatocellular carcinoma;[223] *Foxa1* associated with inflammation and hematopoiesis; *Clec14a* involved in the process of cell-cell adhesion and immune response to pathogens,[231] and Ctage5 linked with the pathophysiology of meningioma and glioma.[224] Additionally, the *Rtn1* gene associated with neuroendocrine secretion, intracellular traffic, and susceptibility to ethanol;[232] *Gpx2* related to hepatocarcinogenesis in rats[225] and the protection from allergen-induced airway inflammation in mice,[226] and *Rsad2* gene located at 27.1 Mbp which has antiviral activity and has been involved in resistance to infection.[221] Besides our criteria in the selection of candidate genes associated with AR, there are 2 genes included in the significant regions of the F2 which deserve special attention. The first one is the protein kinase D1 gene (*Prkd1*), located at 51.4 Mbp and involved in cell activation, Toll-like receptor 9 pathway, and muscle performance.[233;234] And the second gene, A disintegrin metaloprotease domain 21 (*Adam21*), located at 82.6 Mbp and associated with muscle development, neurogenesis and cell-cell and cell-matrix interactions, including fertilization.[235;236]

Between the genes identified in the BcA86xC57BL/6J we have the *Arhgap5* gene, located at 53.6 Mbp and coding for Rho GTPase-activating protein 5. Enzymatic activity has been attributed to *Arhgap5* and several studies have described its role during in the mechanism that regulates skeletal muscle differentiation, myogenesis,[222] cellular signalling,[237] hepatocellular carcinoma.[223] and hematopoietic stem cells engraftment.[238]

Another gene is *Foxa1*, which is a member of the highly conserved winged helix family of transcription factors, shares structural similarities with *Foxa2* and *Foxa3*, and plays important regulatory functions in the development process. In vertebrates, *Foxa* genes are expressed in the notochord and floor plate during the embryonic development; *Foxa1* is present in the nuclei of lung buds during lung morphogenesis and later in development, nuclear staining for *Foxa1* is detected in tissues originating from the endoderm, ectoderm, and mesoderm.[239-242]

Foxa1 and *Foxa2* are expressed in epithelial cells of the airways and alveolar type II epithelial cells. Furthermore, these two transcription factors are expressed in an overlapping manner, influencing lung morphogenesis and epithelial cell differentiation at distinct times during perinatal development.[243] *Foxa1* mRNA increased after deletion of *Foxa2* in respiratory epithelial cells, giving a clear indication of compensatory mechanism between *Foxa1* and *Foxa2* in the lung development.[244] Moreover, *Foxa1* has been involved in the regulation of smooth muscle differentiation and is necessary for the branching morphogenesis of the peripheral parts of the lungs.[245] Song et al. shows that *Foxa1* plays an important role promoting apoptosis of pulmonary epithelial cells, whereas *Foxa1* deficiency decreases alveolar type II epithelial cell apoptosis induced by hydrogen peroxide.[246] Additionally, *Foxa1* and *Foxa2* are described as transcription factors important for mucus production by goblet cells which regulates endocrine and paracrine mechanisms.[247]

In the same F2 cross, we also identified the *Clec14* gene, a lectin which is part of a superfamily of proteins that contain one or more C-type lectin-like domains (CTLDs), that recognize complex carbohydrates present in cells and tissues and have been described as responsible for several regulatory mechanisms such as cell to cell and cell to matrix interactions.[248] Additionally, genes which belong to this superfamily are involved in the immune response through recognition of endogenous proteins and the detection of virally infected or transformed cells.[231]

The *Ctage5* gene, initially named as meningioma expressed antigen-6 (*Mgea6*), belongs to the Ctage family of genes and was originally identified as an immunogenic antigen in meningioma patients.[224;249] *Ctage* is considered a potential biomarker and suitable therapeutic target in acute myeloid leukaemia due to its over expression and possible role as regulator in this pathology; and also it is detected exclusively in the sera of cancer patients, suggesting it as a highly specific diagnostic markers.[250]

Another gene identified in this F2 cross is *Rtn1*. Reticulons (RTNs) are a group of integral membrane proteins characterized by a C-terminal domain named RHD and originally referred as

neuroendocrine-specific protein (NSP) due to their location on the membrane of smooth endoplasmic reticulum.[66] RTNs are expressed in fungi, plants and vertebrates. Four reticulon genes (*Rtn1, Rtn2, Rtn3,* and *Rtn4*) have been described in mammalian genomes and several genes products attributed to them. Many studies have demonstrated the role of RTNs in many intracellular regulatory mechanisms, endocytosis and exocytosis.[251] Other researchers have demonstrated its participation in the regulation of axonal growth and regeneration,[252] spinal cord injury,[253] and neurodegenerative disorders.[232] Moreover, *Rtn1* has been shown to be one of the genes that contribute to the genetic susceptibility to ethanol and to be associated with nervous system.[254]

Finally, the *Gpx2* gene, located at 77.8 Mbp on chromosome 12, is an enzyme that has been related to hepatocarcinogenesis in rats,[225] and the oxidative stress observed in respiratory diseases.[255] Dittrich *et al.* proposed that glutathione peroxidase-2 (*Gpx2*) might be involved in the allergen-mediated inflammation cascade. The results of their study demonstrate that *Gpx2* is up regulated in allergic airway inflammation and that the absence of this enzyme leads to an increase in the inflammatory process of the airway.[226]

In the F2 cross generated from a non-related strain of mice, we identified *Rsad2* gene as a candidate gene. It is located at 27.1 Mbp on chromosome 12, considered as an interferon-inducible antiviral protein, it has involved in resistance to infection and in the defense induced by type I and type II interferon against human cytomegalovirus, HIV-1 virus, and hepatitis C virus.[256] Cheng *et al*. demonstrated *Rsad2* up-regulation in alveolar macrophages during Pneumocystis pneumonia, and dendritic cells with a Th2-inducible cytokine pattern.[221] Furthermore, other studies have demonstrated the effectiveness of *Rsad2* as an antiviral agent against flavivirus such as hepatitis C virus and dengue.[257;258]

Prkd1 and *Adam21* are two genes that deserve special attention for their role in different cellular and molecular mechanism and their association with the development or susceptibility to asthma. *Prkd1* is a serine/threonine kinase that belongs to the family of calcium/calmodulin-

dependent kinases and regulates oxidative stress, gene expression, cell survival, and vesicle trafficking.[259] *Prkd1* is highly expressed in the lung and skin, and is one of the components of TLR9 signalling working through the MyD88/IRAK1-dependent pathway.[233;234] Previous studies have shown that CpG oligodeoxynucleotides, which are TLR9 ligands, have some potential as a treatment of asthma in animal models and we speculate that *Prkd1* might play a role in the mechanism of action of these compounds.[260;261]

Adam21 is an A disintegrin and metalloprotease transmembrane proteins located at 82.6 Mbp on chromosome 12, and related to cell-cell and cell-matrix interactions, including fertilization, and muscle development.[235;236] Additionally, *Adam21* is associated with neurogenesis and growing axons in rats and mice.[262] *Adam21* is important for cleavage-dependent activation of amyloidal precursor protein and transforming growth factor a, and can bind integrins. *Adam21* has been suggested to play a determinant role in the development of the nervous system, where they regulate proliferation, migration, differentiation of various cells, and myelination.[236] Since other metalloprotease such as ADAM33 has been associated with the susceptibility and pathogenesis of allergic asthma in human and mice;[172;263;264] *Adam21* deserves special attention due its relationship in structure and biological function with others members of the ADAM family, and for its location in the significant region of our F2 crosses associated with airway hyperresponsiveness.

Taken together, our results demonstrate how the use of recombinant congenic strains of mice offer an invaluable tool in the dissection of complex traits such as airway responsiveness to methacholine. Furthermore, our genetic strategy based in the generation of F2 crosses allowed us narrow down the significant recombinant regions described in the RCS and to identify 9 candidate genes that might be associated with airway responsiveness in mice. Those genes include *Arhgap5, Foxa1, Clec14a, Ctage5, Rtn1, Gpx2, Rsad2, Prkd1* and Adam21. Further studies have to be performed in order to elucidate the role of these genes in the mechanisms associated with the development of AHR and the relation with the pathogenesis of allergic asthma.

Acknowledgements

This work was supported by grants from Strategic Program for Asthma Research (SPAR; which now became part of American Asthma Association) and the Canadian Institutes of Health Research (CIHR) awarded to Dr. D. Radzioch and a grant from Genome Quebec/Genome Canada awarded to Dr. E. Skamene. R. Marino and P. Camateros are both recipients of Superior doctoral scholarships from the CIHR. I would also like to acknowledge the invaluable collaboration and guidance of Dr. Silvia Vidal, Dr. James Martin, Dr. Sabah Hussain and Dr. Mario Chevrette. Special thanks to Cynthia Kanagaratham and Pierre Camateros for performing part of the genetic analysis and reviewing the manuscript.

Figure 1. Breeding scheme of AcB/BcA recombinant congenic strains (RCS).

Methacholine-challenge hyperresponsive A/J [A] and hyporesponsive [B] mice were used as parental strains. (F_1 X A) X A and (F_1 X B) X B mice were inbred for at least 20 generations to produce the AcB and BcA strains, respectively, those inbred strains constitute the RCS panel.

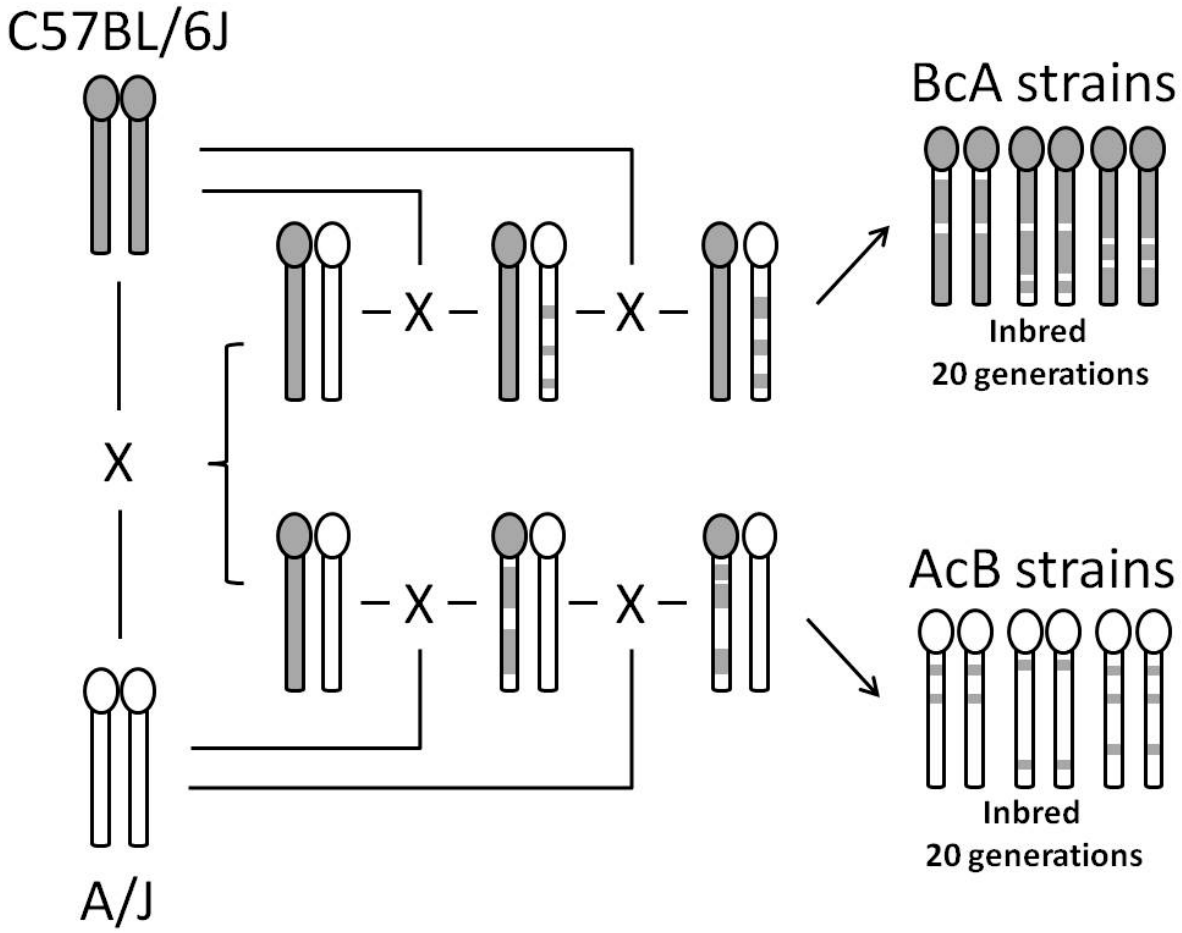

C57BL/6J
X
A/J
– X – – X –
– X – – X –
BcA strains
Inbred
20 generations
AcB strains
Inbred
20 generations

Figure 2. Airway responsiveness phenotype in the AcB/BcA recombinant congenic strains.

Airway responsiveness was assessed using the non-invasive method by the whole-body plethysmograph. Bars represent the mean value ± SEM of Penh (log_2) values in response to 50 µl of methacholine (15 mg/ml) delivered over 1 min. The AcB **(A)** and BcA **(B)** strains are represented by white and black bars, respectively, and the A/J and C57BL/6 parental strains are gray. * $P<0.05$ and ** $P<0.001$ by Bonferroni-corrected ANOVA.

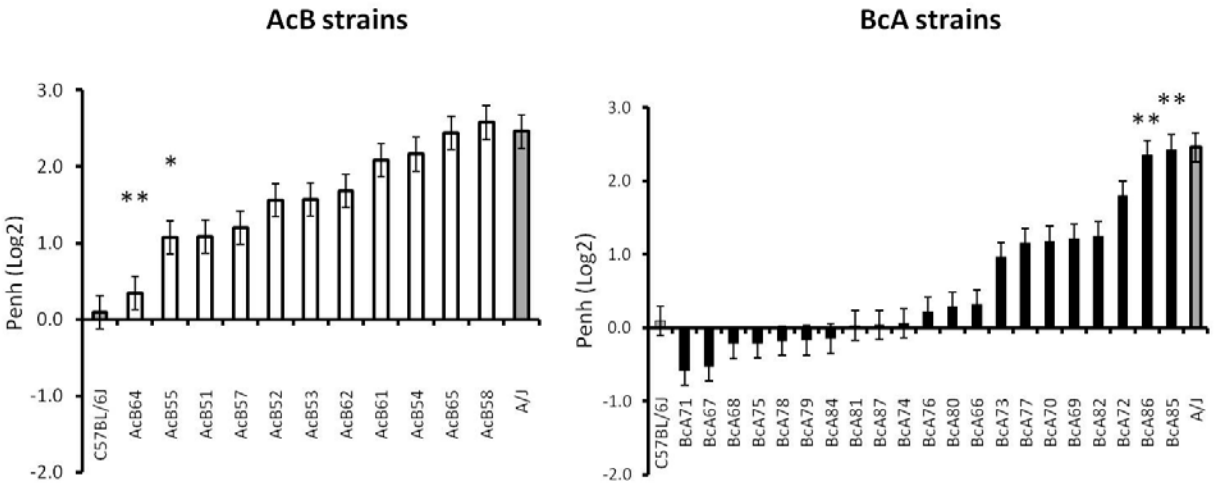

AcB strains
BcA strains
Penh (Log2)
3.0
2.0
1.0
0.0
-1.0
-2.0
**
*
**
**
C57BL/6J
AcB64
AcB55
AcB51
AcB57
AcB52
AcB53
AcB62
AcB61
AcB54
AcB65
AcB58
A/J
C57BL/6J
BcA71
BcA67
BcA68
BcA75
BcA78
BcA79
BcA84
BcA81
BcA87
BcA74
BcA76
BcA80
BcA66
BcA73
BcA77
BcA70
BcA69
BcA82
BcA72
BcA86
BcA85
A/J

Figure 3. Correlation between airway resistance (Rrs) vs. Penh in the F2 crosses.

Penh and Rrs values correspond to 15 mg/ml and 40 mg/ml of Mch, respectively. N=15–20 for each group **(A)** BcA86xB F2 **(B)** BcA86xC3H/HeJ F2 **(C)** BcA85xB F2 **(D)** BcA85xC3H/HeJ F2 **(E)** AcB64xA/J F2 and **(F)** AcB64cAKR F2. Good correlation was found between both methods assessing airway responsiveness to methacholine in all of the F2 crosses.

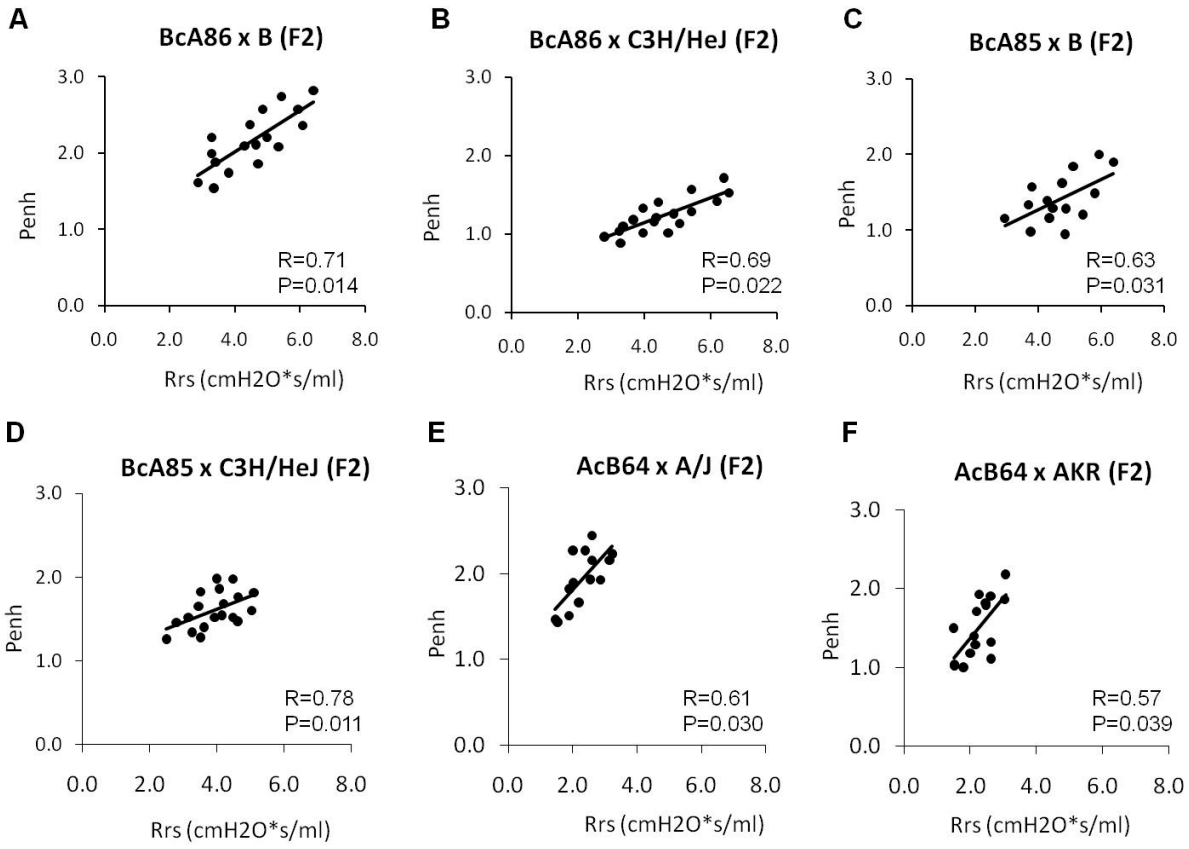

A
BcA86 x B (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.71
P=0.014
B
BcA86 x C3H/HeJ (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.69
P=0.022
C
BcA85 x B (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.63
P=0.031
D
BcA85 x C3H/HeJ (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.78
P=0.011
E
AcB64 x A/J (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.61
P=0.030
F
AcB64 x AKR (F2)
Penh
Rrs (cmH2O*s/ml)
R=0.57
P=0.039

Figure 4. Airway responsiveness distribution (Penh) of the F2 crosses and parental strains.

Dots represent Penh (Log2) of each mouse in response to 15 mg/ml. N=200–230 for each group. Phenotype distribution in each of the F2 crosses show the differential alleles associated with more than one trait with airway responsiveness.

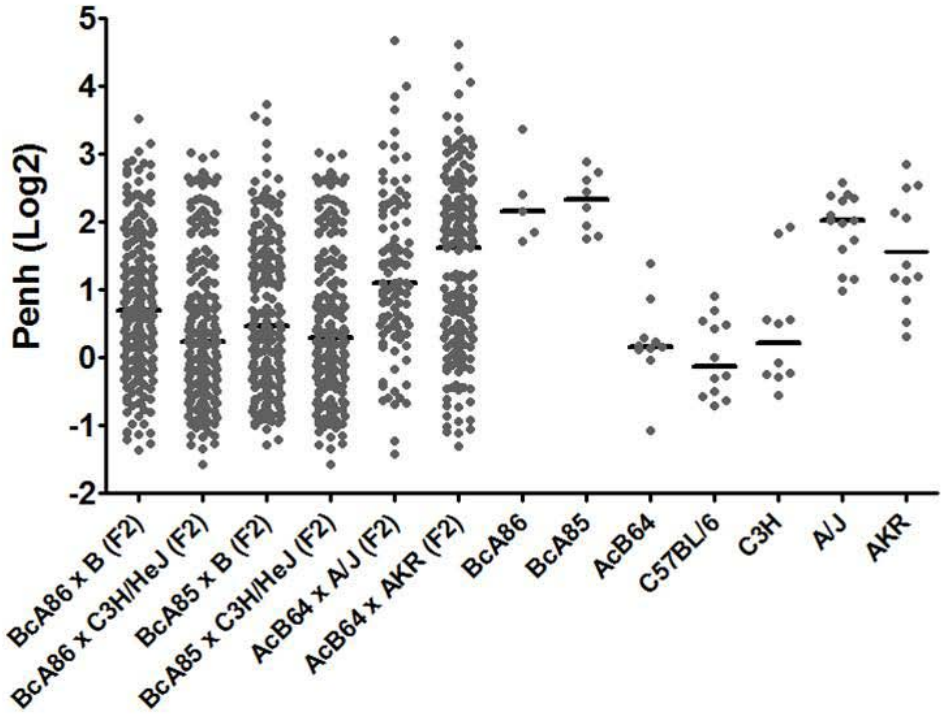

Penh (Log2)
5
4
3
2
1
0
-1
-2
BcA86 x B (F2)
BcA86 x C3H/HeJ (F2)
BcA85 x B (F2)
BcA85 x C3H/HeJ (F2)
AcB64 x A/J (F2)
AcB64 x AKR (F2)
BcA86
BcA85
AcB64
C57BL/6
C3H
A/J
AKR

Figure 5. Frequency distribution of Penh in the F2 crosses.

Bars represent the frequency distribution of the Penh (log2) in response to 15 mg/ml of Mch. n = 200 – 230 for each group. **(A)** BcA86xB F2 **(B)** BcA86xC3H/HeJ F2 **(C)** BcA85xB F2 **(D)** BcA85xC3H/HeJ F2 **(E)** AcB64xA/J F2 and **(F)** AcB64xAKR F2. Phenotype distribution in each of the F2 crosses suggests the association of more than one trait with airway responsiveness.

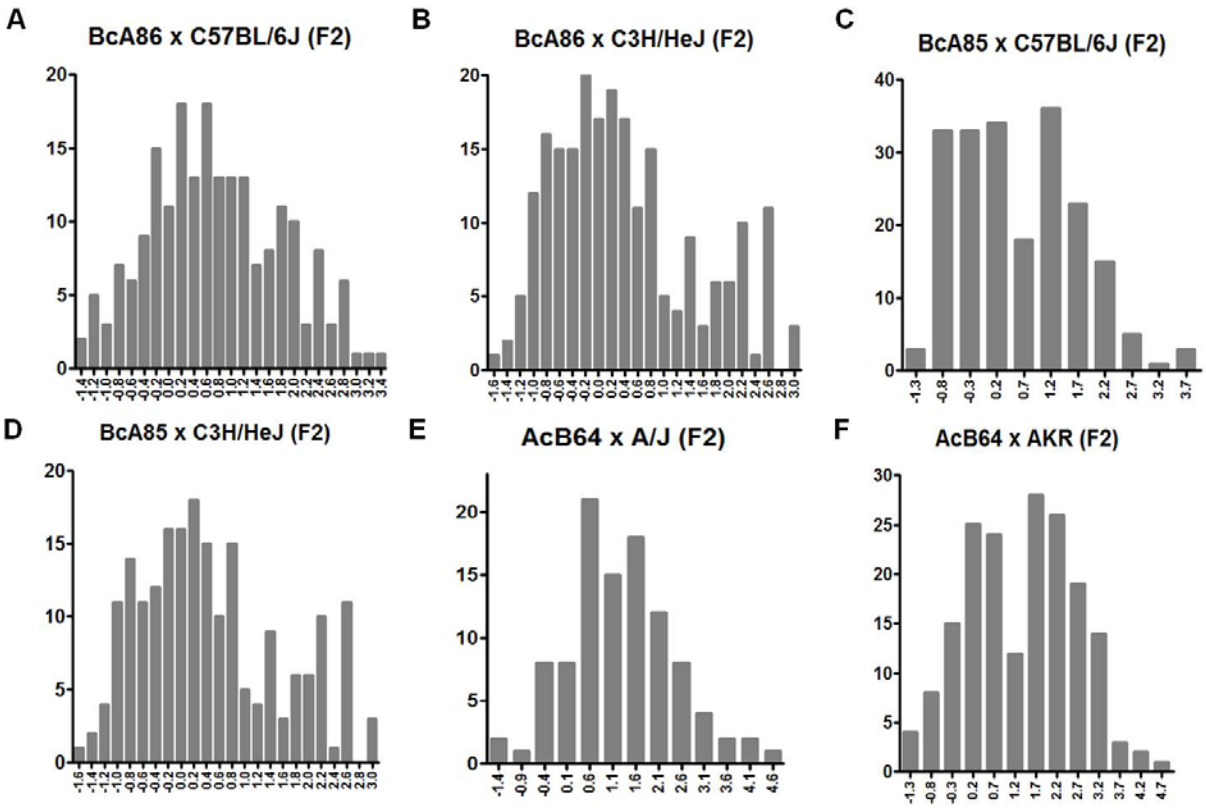
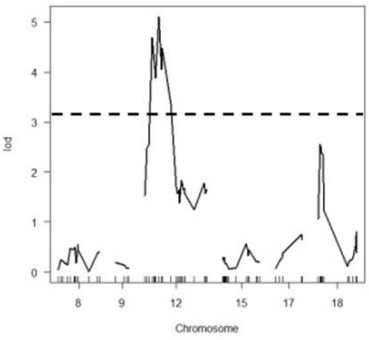
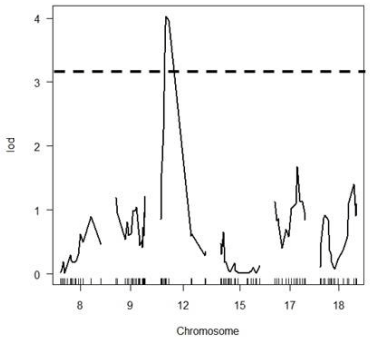

A
BcA86 x C57BL/6J (F2)
B
BcA86 x C3H/HeJ (F2)
C
BcA85 x C57BL/6J (F2)
D
BcA85 x C3H/HeJ (F2)
E
AcB64 x A/J (F2)
F
AcB64 x AKR (F2)

Figure 6. Quantitative trait loci analysis of the F2 crosses.

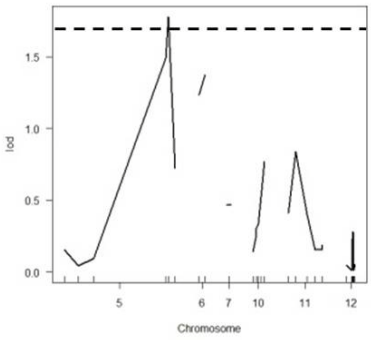
Logarithm of odds (LOD) plots using marker regression scans. N=200 for each group. **(A)** BcA86xB F2 **(B)** BcA86xC3H/HeJ F2 **(C)** BcA85xB F2 **(D)** BcA85xC3H/HeJ F2 **(E)** AcB64xA/J F2 and **(F)** AcB64xAKR F2. Penh (Log2) was used as a quantitative trait. The dashed line represents a genome-wide significance threshold (p<0.05) for each cross, calculated with 10,000 random permutations.



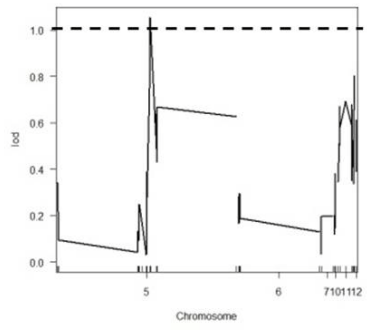
BcA86 x C57BL/6J (F2)



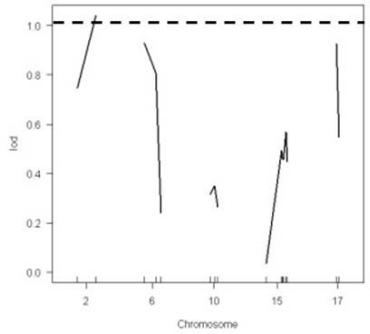
BcA86 x C3H (F2)



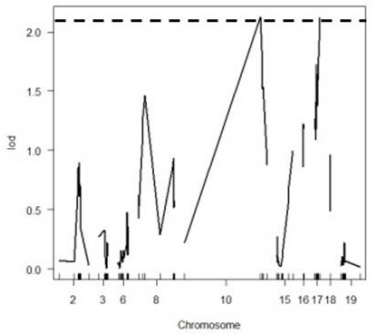
BcA85 x C57BL/6J (F2)



BcA85 x C3H/HeJ (F2)



AcB64 x A/J (F2)



AcB64 x AKR (F2)

Figure 7. Quantitative trait loci analysis of the BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses.

Logarithm of odds (LOD) plots for marker regression scan and QTL genetic location (cM) **(A)** BcA86xC57BL/6J (F2) cross and **(B)** BcA86xC3H/HeJ (F2) cross. The dashed lines represent a genome-wide significance threshold (p<0.05; 10,000 permutations). Penh (Log2) of the 15 mg/ml methacholine dose was used as a quantitative trait and N=200 for each group. One significant peak LOD score was identified on chromosome 12 of each F2 cross.

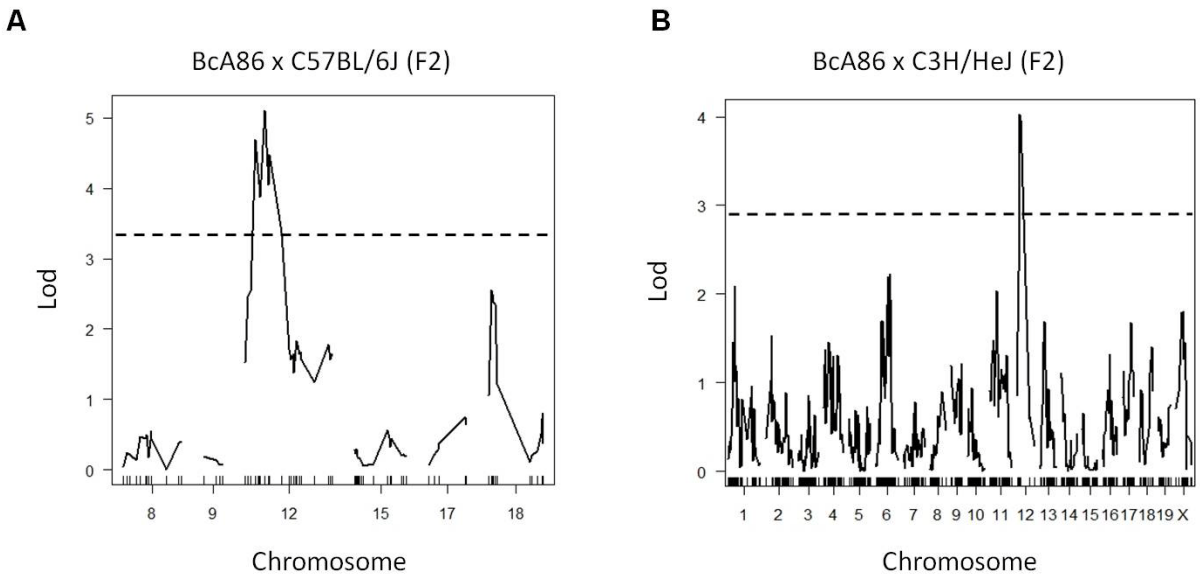
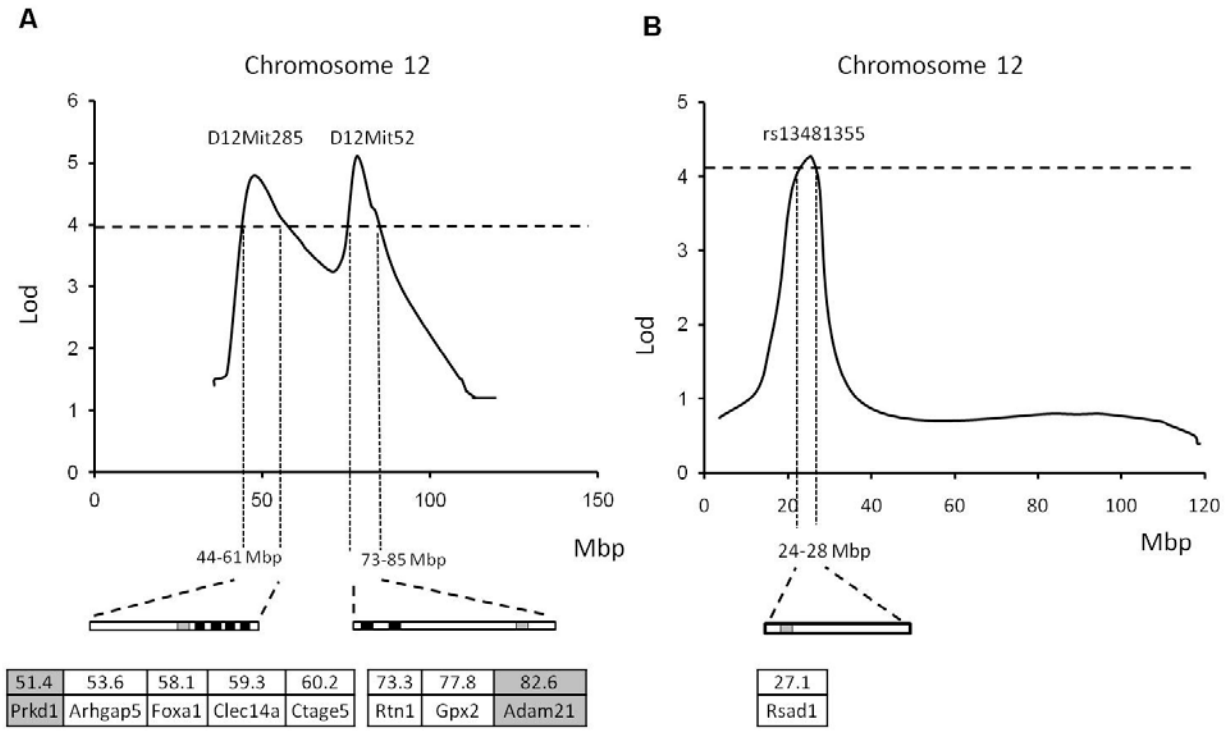

A
BcA86 x C57BL/6J (F2)
Lod
0
1
2
3
4
5
8
9
12
15
17
18
Chromosome
B
BcA86 x C3H/HeJ (F2)
Lod
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
Chromosome

Figure 8. Physical location of candidate genes based on the QTL analysis of the BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses.

Logarithm of odds (LOD) plots for marker regression scans and QTL location calculated in Mbp. **(A)** BcA86xC57BL/6J (F2) cross and **(B)** BcA86xC3H/HeJ (F2) cross. Penh (Log2) was used as a quantitative trait and N=200. The dashed line represents 1 Lod above from the significance threshold. Seven candidate gene expressed in lung was identified as associated with airway responsiveness in this crosses. Two additional genes (Prkd1 and Adam21) were added to the list of seven genes selected for more detailed analysis, based on their chromosomal localization and their importance in the regulation of inflammation or cell activation.



51.4	53.6	58.1	59.3	60.2
Prkd1	Arhgap5	Foxa1	Clec14a	Ctage5

73.3	77.8	82.6
Rtn1	Gpx2	Adam21

27.1
Rsad1

Figure 9. Candidate genes selection in BcA86xC57BL/6J (F2) and BcA86xC3H/HeJ (F2) crosses. Candidate genes were considered those included in the significant regions identified by quantitative trait loci from the F2 crosses, described as non-IBD between A/J and C57BL/6 or C3H/HeJ mice, considered to be synonymous or coding mutants, expressed in the lungs, and labelled with dbSNP annotations of “Coding-Non Synonymous” or “Splice-Site”. The genetic strategy based in the generation of F2 crosses using recombinant congenic strains (RCS) as parental strains, allow to narrow down the significant region associated with airway responsiveness to methacholine and identified only 7 genes linked with this pathology.

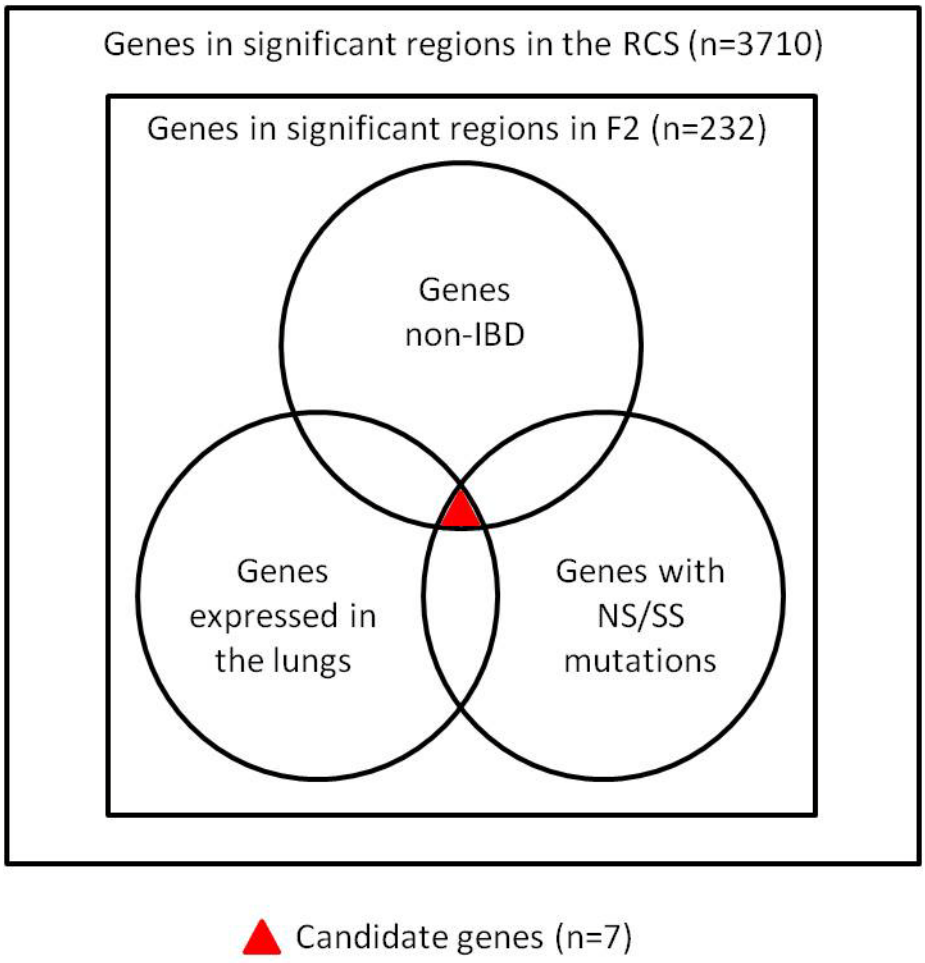

Genes in significant regions in the RCS (n=3710)
Genes in significant regions in F2 (n=232)
Genes non-IBD
Genes expressed in the lungs
Genes with NS/SS mutations
Candidate genes (n=7)

Table 1: Significant regions and candidates genes associated with airway responsiveness in Chromosome 12 of the BcA86xB6 (F2) and BcA86xC3H (F2) crosses.

Table 1: Significant regions and candidates genes associated with airway responsiveness in Chromosome 12 of the BcA86xB6 (F2) and BcA86xC3H (F2) crosses.

F2 cross	Region (Mb)	Total Genes	*A/J vs. C57BL/6J or C3H/HeJ non-IBD*	*Synonymous and Non-coding Mutant Genes*	*Genes Expressed in Lungs*	*NS or SS Mutant Genes*	*Candidate genes*
Region 1 (C3H)	24 – 28	24	*12*	*12*	*5*	*2*	*1*
Region 2 (B6)	44 – 61	89	*71*	*60*	*30*	*9*	*4*
Region 3 (B6)	73 – 85	119	*87*	*75*	*45*	*5*	*2*
	Total:	232	*170*	*147*	*82*	*16*	***7***

Number of genes expressed in the lungs, in each region significant associated with airway responsiveness from the quantitative trait loci analysis in the BcAc86xC57BL/6J (F2) and BcAc86xC3H/HeJ (F2) crosses. Based on MXD database and microarray data, which are in regions which are non-identical by decent (non-IBD) between A/J and C57BL/6J or C3H/HeJ mice (according to SNP density, data from MGI), which contain synonymous mutations or mutations in non-coding regions (from MGI database) or which contain non-synonymous (NS) or splice-site (SS) mutations (from MGI database). The number of genes described in this table excludes all olfactory receptor genes which are considered extremely unlikely candidates, and include expressed sequence tags, predicted genes, and cDNA clones. C57BL/6J = B6 strain and C3H/HeJ = C3H strain.

Chapter 4 - Secretory Leukocyte Protease Inhibitor plays an important role in the regulation of allergic asthma in mice

Adapter from the manuscript submitted for publication:

R. Marino, T. Thuraisingam, P. Camateros, Y. Z. Xu, J. Henri, J. Yang, G. He, A. Ding and D. Radzioch. Secretory Leukocyte Protease Inhibitor plays an important role in the regulation of allergic asthma in mice

Fragments of this chapter have been adapted from:

P. Camateros, M. Tamaoka, M. Hassan, **R. Marino**, J. Moisan, D. Marion, M.C. Guiot, J.G. Martin and D. Radzioch. Chronic asthma-induced airway remodelling is prevented by toll-like receptor-7/8 ligand S28463. Am J Respir Crit Care Med. 2007 Jun 15;175(12):1241-9.

Preface

Secretory leukocyte protease inhibitor (SLPI) is expressed at higher levels in asthmatic patients and it has been shown to have anti-inflammatory effect in respiratory diseases. Additionally, treatment with resiquimod increases the expression of SLPI *in vitro*. The work presented in this chapter shows the results of our investigations designed to assess the role of SLPI in the development of phenotypes associated with allergic asthma and the effect of resiquimod on SLPI. Our results demonstrate that over expression of SLPI leads to a lower degree of airway inflammation and higher lung resistance, whereas the ablation of SLPI has opposite effect against allergic asthma phenotypes. We also show that the treatment with resiquimod is independent of the SLPI expression. Taken together, our results demonstrate that SLPI plays an immunoregulatory role in the respiratory tract reducing the inflammatory process and improving lung physiology in a model of acute allergic asthma.

Abstract

Asthma is a complex and inflammatory disease. Secretory leukocyte protease inhibitor (SLPI) has shown higher levels in asthmatic patients and its function as an anti-inflammatory protein has been documented in respiratory diseases. However, its role in the immunomodulation of the response during allergic asthma has not yet been fully elucidated. The aim of our work was to evaluate the role of SLPI in the development of phenotypes associated with allergic asthma and the effect of resiquimod on SLPI. We assessed airway resistance and inflammatory parameters in the lungs of SLPI transgenic and knock-out mice using ovalbumin induced acute allergic asthma, and treatment with resiquimod. Our results showed that allergic SLPI transgenic mice displayed a significant decrease ($p<0.001$) in lung resistance compared to wild-type mice; the same effect was observed with inflammatory cell infiltration, eosinophil percentage ($p<0.001$), goblet cells ($p<0.001$) in the lungs and plasmatic IgE levels ($p<0.001$). Allergic SLPI knock-out mice showed significantly more severe asthma associated phenotypes compared to wild-type mice. These include lung resistance ($p<0.001$), inflammatory cell influx, eosinophils ($p<0.001$), goblet cells ($p<0.001$), cytokine levels in the lungs ($p<0.05$), and plasma IgE levels ($p<0.001$). Expression of SLPI was associated with a decreased inflammation in the lungs, plasma IgE levels, and lung resistance, whereas the ablation of SLPI had the opposite effect. Treatment with resiquimod increased the expression of SLPI and decreased inflammation in the lungs; however resiquimod was still effective in SLPI knock-out mice. Overall, our results demonstrated that SLPI plays an immunoregulatory role in the respiratory tract reducing the inflammatory process and improving lung physiology in a murine model of acute allergic asthma.

Introduction

Asthma is a complex, multi-factorial,[1] and inflammatory disease whose symptoms include cough, wheezing, and shortness of breath.[7] Asthma pathophysiology is characterized by both acute and chronic inflammatory responses of the airways leading to activation of immune cells,[7] increased plasma IgE levels, and eosinophilic infiltration.[52] The bronchial inflammation in turn leads to airway hyperresponsiveness, airflow obstruction, goblet cell hyperplasia, and airway remodeling.[8] A better understanding of the role of various effector molecules in regulating allergic asthma may lead to the development of new therapy for the symptoms associated with allergic asthma, or to treatments aimed at preventing the initial allergic response.

SLPI, a 11.7 kDa secretory protein, is constitutively expressed in mucosal tissues and cells of the immune system, including monocytes, macrophages and neutrophils[265;266] and can be found in bronchial secretions, seminal fluid, saliva, and breast milk.[267] Besides being a protease inhibitor,[265] it also exhibits anti-inflammatory, antifungal,[268] and antimicrobial functions.[269] SLPI was also demonstrated to modulate the activity of matrix metalloproteinases,[270] improve cutaneous wound healing,[271;272] and prevents HIV-1 infection.[273-275]

The protective effect of SLPI as an anti-inflammatory mediator has been documented in chronic obstructive pulmonary disease[276] and cystic fibrosis (reviewed by Weldon et al. 2007[277]). Wright et al. showed the potential therapeutic effect of recombinant SLPI administrated by aerosol and intratracheal, using Ascaris-sensitized sheep and OVA-sensitized guinea pigs, respectively.[7] The role of SLPI in the immunomodulation of the response during allergic asthma, however, has not yet been fully elucidated.

In spite of the broad spectrum of strategies for the treatment of asthma, physicians and patients still need more effective new therapeutic options with fewer side effects. Previous studies have shown that asthmatic patients showed a significant increase in SLPI levels

compared to healthy controls[278] while our group and others have demonstrated the protective effect of resiquimod (a.k.a S28463, R0848), a synthetic TLR7 ligand, against allergic asthma in mice[279] and rats.[193] A significant increase in SLPI expression at the both messenger RNA and protein levels was observed following *in vitro* treatment of macrophages with resiquimod. We postulated that SLPI might play a protective role in the development of allergic asthma and that it might also be involved, directly or indirectly, in mediating the protective effect of resiquimod treatment against allergic asthma.

We hypothesized that increased expression of SLPI during asthmatic attacks may protect the lungs against allergic asthma induced inflammation and thereby lead to improvements in lung physiology. Overall, our findings documented the importance of SLPI in the regulation of allergic asthma using an acute asthma model of ovalbumin sensitization and challenge in SLPI gene transgenic (TG) and SLPI knock-out (KO) mice.

Material and Methods

Animals

Male mice between 8-10 weeks-old from SLPI KO (generously donated by Dr. Sharon M. Wahl[271]), SLPI KO wild-type (WT^{KO}) littermates, SLPI TG and SLPI TG wild-type (WT^{TG}) littermate mice were used in all experiments. SLPI transgenic mice were generated as previously described.[280] Mice were bred in the pathogen-free facility of the Montreal General Hospital, Research Institute at the McGill University Health Center (Montreal, Canada). All the procedures performed on the animals were approved by the McGill University Animal Care Committee in compliance with the Canadian Council of Animal Care guidelines.

Sensitization and challenge

Sensitization and challenge was performed as previously described.[24] Briefly, mice were injected intraperitoneally with 100 µg of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) adsorbed to 1.5 mg of aluminum hydroxide (Imject® Alum, Pierce, Rockford, IL, USA) in a total volume of 0.2 ml of sterile PBS on days 0, 7 and 14. Mice were then challenged on days 21, 22 and 23 by aerosol exposure to either a 1% OVA solution or to PBS for 30 minutes. One group of mice was injected intraperitoneally with 100 µg of resiquimod on 3 consecutive days starting 1 day before the first ovalbumin challenge.

The animals were organized in ten different groups: SLPI transgenic mice challenged with PBS (TG-PBS) or OVA (TG-OVA); SLPI transgenic mice wild-type littermates challenged with PBS (WT^{TG}-PBS) or OVA (WT^{TG}-OVA); SLPI knock-out mice challenged with PBS (KO-PBS) or OVA (KO-OVA); and SLPI KO wild-type littermates challenged with PBS (WT^{KO}-PBS) or OVA (WT^{KO}-OVA). SLPI knock-out mice treated with resiquimod and challenged with OVA (KO-RES-OVA) and SLPI KO wild-type littermates treated with resiquimod and challenged with OVA (WT^{KO}-RES-OVA).

Assessment of respiratory resistance

Respiratory resistance was evaluated using Buxco resistance system (Buxco Electronics, Inc. Wilmington, NC, USA). Forty-eight hours after the last challenge, mice were anaesthetized, tracheotomized and connected to a MiniVent small animal ventilator (Harvard Apparatus, Saint-Laurent, QC). The peak respiratory system resistance was measured with the Buxco resistance system (Buxco Electronics, Inc. Wilmington, NC, USA) after the administration of increasing doses of aerosolized methacholine (Mch) (10µL of 0–80 µg/mL) (Sigma-Aldrich, Oakville, ON, Canada).

Histological analysis of lung inflammation

Forty-eight hours after the last challenge, the left lung was dissected, gravity inflated with 10% buffered formalin at a pressure of 25 cm H_2O, kept in a 50ml tube filled with 15ml of 10 % formalin overnight, and paraffin embedded. Lungs were cut into 5 µm sections for histological analysis. Deparaffinized and hydrated sections were stained with Hematoxylin and Eosin (H&E), Congo Red (CR) or Periodic Acid-Schiff (PAS).

Inflammation of the lungs was assessed by histological analysis of H&E and CR stained lung sections. Quantification of the infiltrating cells was performed by counting 300 infiltrating cells in the peribronchial space and the percentage of eosinophils was calculated based on the nuclear morphology and the presence of eosin staining in the cytoplasm. The percentage of eosinophils was corroborated with CR stained lung sections. Goblet cell hyperplasia of the airways was assessed by histological analysis of PAS stained sections. Goblet cell hyperplasia was quantified by determining the percentage of PAS+ epithelial cells in at least five different airway cross-sections per slide.

Plasma immunoglobulin E (IgE) levels

Mice were euthanized with CO_2 48 hours after the final challenge, blood was collected by cardiac puncture and 0.05M EDTA was added. Plasma samples were isolated by centrifugation and total IgE levels were measured using a commercial ELISA kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Briefly, 96-well ELISA plates (Thermo Labsystems, Franklin, MA, USA) were coated with anti-mouse IgE monoclonal antibody and blocked with 10% FBS (Winsent Inc, Saint-Bruno, QC, Canada) in PBS. Standards and plasma samples were incubated for 2 hours at RT and mouse IgE was detected with biotinylated anti-mouse IgE and streptavidin-horseradish peroxidase (HRP)-conjugate using ABTS reagent mixture (Roche, Laval, QC, Canada). The plate was read at 450 nm in an ELISA plate reader (Biorad, Hercules, CA, USA). Purified mouse IgE was used as a standard.

RNA extraction

Total RNA was extracted from half lung using TRIzol reagent (Invitrogen, Burlington, ON, Canada) and homogenized with a Mixer Mill type MM 301 (Retsch GmbH & Co. KG, Haan, Germany). RNA quality was tested by electrophoresis in a 2.2 M formaldehyde - 1.2% agarose gel and then with a 2100 Bioanalyser using the RNA 6000 Nano LabChip Kit (Agilent Technologies, Böblingen, Germany). Total RNA was employed for the analysis of gene expression by real-time reverse transcriptase-quantitative PCR.

Quantitative real-time PCR

A DNA-free Kit (Ambion, Austin, TX, USA) was used to digest any residual DNA from 1 μg of RNA obtained from the lungs. A reverse transcription reaction was performed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Subsequently, 2 µl of the 25 µl of the reverse transcriptase reaction was added to 50 µl Brilliant® II SYBR® Green quantitative PCR Master Mix (Stratagene, Cedar Creek, TX, USA),

and the Stratagene MX-4000 apparatus was used to amplify the target cDNA to assess SLPI expression by real-time quantitative PCR. The following primers 5'-CTCAGGCAAGATGTATGATG-3' (sense) and 5'-TTTCCCACATATACCCTCAC-3' (antisense) were used to amplify murine SLPI cDNA. The amount of cDNA was calculated based on the threshold cycle (C_T) value, and was standardized by the amount of the house-keeping gene *Gapdh* using the $2^{-\Delta\Delta CT}$ method:[281]

$$\Delta\Delta C_T = (C_{T,\ Target} - C_{T,GAPDH}) - (C_{T,\ Target} - C_{T,GAPDH})_{(\text{calibrator avg})}$$

Where the "target" represents the gene of interest and the "calibrator" represents the KO-PBS mice. *SLPI* gene expression was standardized against the expression of *GAPDH*. Melting curve analysis and agarose gel electrophoresis was also performed. We confirmed that a single product of the expected length was amplified.

The amplification program consisted of an enzyme activation step at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72° C for 30 s. *GAPDH* was used as the normalizing gene to compensate for potential differences in total cDNA amounts. The primer sequences were all designed from the NCBI GenBank mRNA sequence using the PrimerQuest web-based software, Integrated DNA Technologies (www.idtdna.com/Scitools/Applications/ Primerquest/).

Effect of resiquimod in mRNA expression of SLPI

C57BL/6 mice were injected i.p. with PBS or 0.1 mg of resiquimod (generously provided by Dr. T.C. Meng Graceway Pharmaceuticals LLC, Exton, PA, USA). Three or 6 hours following i.p. injection, the mice were euthanized and their lung RNA was extracted as described above.

Immunoprecipitation and Western Blot

Mice were euthanized 48 hours after the last challenge; the right lung was dissected and homogenized in 500 µl of PBS containing a protease inhibitor cocktail (Complete Inhibitor, Roche Diagnostics, Laval, PQ, Canada). One microgram of rabbit anti-mouse SLPI antibody, generated as previously described[282], was added to 500 µl of the homogenate and incubated at 4°C for 1 hour. 20 µl of protein A/G plus agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were added and the solution was gently mixed at 4°C overnight. Supernatants were removed after centrifugation at 1000 rpm for 5 minutes and washed three times with 500 µl of immunoprecipitation buffer (2% Triton X-100, 100mM and 300 mM NaCl). Samples were concentrated using Microcon YM-3 centrifugal filters (Millipore, Billerica, MA, USA) and total protein concentration was measured using the Bio-Rad protein assay dye (Biorad Laboratories, Hercules, CA, USA).

Protein concentration was adjusted and 30 µg of each sample was mixed with SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol (Fisher, Fairlawn, NJ, USA), heated at 95°C and loaded in a 4–12% Bis–Tris NuPAGE gel (Invitrogen, Burlington, ON, Canada) for acrylamide electrophoresis. Human recombinant SLPI protein was used as a positive control and SeeBlue Plus2® (Invitrogen, Burlington, ON, Canada) molecular weight marker as reference. Next, proteins were transferred onto Immobilon transfer membranes (Millipore, Billerica, MA, USA) by semi-dry transfer. Membranes were blocked with 5% non-fat skimmed milk at 4°C overnight, then incubated with rabbit anti-mouse SLPI[282] at a concentration of 1 µg/ml in 5% non-fat skim milk for 1 hour at RT and subsequently with a solution of anti-mouse IgG HRP-conjugated antibody (1:5000 dilution) for 1 hour at RT. The signal was visualized using Western Lightning™ Plus-ECL reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Cytokine analysis

Mice were euthanized 3 hours after the last challenge, the lungs were dissected and homogenized in 500 µL of PBS containing a protease inhibitor cocktail (Complete Inhibitor, Roche Diagnostics, Laval, PQ, Canada). Using the Microcon YM-3 centrifugal filters (Millipore, Billerica, MA, USA) the samples were concentrated 20-fold and total protein concentration was adjusted using the Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA, USA) for further analysis using a custom Lincoplex mouse cytokine kit (Linco Research, St. Charles, MO, USA) on a Luminex 100 LS apparatus with software version 2.3 (Luminex Corporation, Austin, TX, USA).

Statistical Analysis

Data was analyzed using a non-parametric one-way analysis of variance method followed by Bonferroni multiple comparison post-test and correlation coefficient using confident intervals of 95% (GraphPad Prism 4, version 4.03; GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at $p<0.05$.

Results

SLPI mRNA and protein expression in the lungs of SLPI TG mice

Since transgenic mice frequently show a highly variable transgene expression, we first evaluated both SLPI mRNA and protein levels in lung homogenates. As expected, OVA sensitized SLPI TG mice, compared to similarly treated WT controls, clearly expressed higher levels of SLPI mRNA following both mock (PBS) and allergen (OVA) aerosol challenge (Figure 1, panel A). Furthermore, the expression of SLPI mRNA was induced by allergen challenge in both SLPI transgenic (304±91 in TG-OVA vs. 91±17 in TG-PBS, $p<0.001$) and wild-type (85±7 in WT^{TG}-OVA vs. 12±3 in WT^{TG}-PBS, $p<0.01$) mice. SLPI protein expression, on the other hand, was not detectable in the lungs of either wild-type or transgenic mice following PBS aerosol challenge but was found to be significantly increased in the lungs of transgenic animals, when compared to their wild-type counterparts, following allergen challenge (Figure 1, panel B). These results demonstrated the over-expression of SLPI protein in OVA-sensitized and -challenged TG mice had protective effect against development of asthma-associated phenotypes.

SLPI over-expression improves airway hyperresponsiveness (AHR) and influences IgE levels in plasma

Previous studies suggested that SLPI was able to modify the airway response in a chronic model of allergic asthma in guinea pigs.[2] We evaluated whether the expression of SLPI, was able to influence AHR and levels of plasma IgE in sensitized and challenged SLPI gene transgenic or knock-out mice. [15]

As expected, allergen challenge increased airway resistance in response to methacholine, using SLPI transgenic (6.3±1.1 cm H_2O x s/ml) and wild-type (8.0±2.1 cm H_2O x s/ml) mice. However, while both transgenic (3.6±0.3 cm H_20 x s/ml) and wild-type (4.3±0.5 cm H_20 x s/ml) mice demonstrated similar levels of airway responsiveness in the absence of allergen aerosol

challenge, SLPI overexpression was able to significantly (p<0.001) diminish the increase in responsiveness observed in the wild-type mice (Figure 2, panel A). The same protective effect of SLPI overexpression was observed for plasma IgE levels (Figure 2, panel B), where significantly (p<0.01) lower levels were observed in transgenic (2014.1±309.2 ng/ml) mice than in the similarly treated wild-type (4128.1±609.2 ng/ml) mice, no difference was observed compare with the TG-PBS (925.3±136.4 ng/ml) group. WT^{TG}-PBS (3308±396.3 ng/ml) control group displayed lower (p<0.001) IgE levels than WT^{TG}-OVA mice. These results clearly demonstrate that higher SLPI expression was associated with lower lung resistance and a decreased induction of IgE.

SLPI decreases allergic inflammation in SLPI TG mice

OVA challenged SLPI transgenic mice displayed a lower degree of inflammatory cell infiltration in the lungs compared to WT^{TG} mice under the same conditions (Figure 3). Consistent with allergic inflammation, the inflammatory cells found in the lungs of allergen challenged wild-type mice contained a significant (p<0.05) larger proportion of eosinophils (24±1.1%) compared to PBS (29±2.3) challenged mice (Figure 4, panel A). Almost no eosinophilic infiltration was observed in the control groups which were sensitized with OVA and challenged with PBS (TG-PBS (1±0.2%) and WT^{TG}-PBS (1±0.3%)). Again, SLPI overexpression exerted a protective effect against the development of allergic asthma by significantly reducing the inflammatory cell influx and the proportion of eosinophils into the peribronchial space.

SLPI decreases goblet cell hyperplasia in SLPI TG mice

Another histological change which characterizes allergic asthma is the development of goblet cell hyperplasia and mucus production.[26] As expected, allergen aerosol challenge caused a significant and marked increase (p<0.001) in the number of airway goblet cells using OVA sensitized mice (Fig. 4, panel B). However, we found that transgenic OVA-challenged mice had significant lower (p<0.05) percentage of goblet cells (25±2.4 %) compared to wild type under

the same conditions (36±4.0 %) or transgenic mice (p<0.001) challenged with PBS (6±1.4 %), and correlates with eosinophils percentage (r=0.72). Wild type OVA-challenged mice showed significantly (p<0.001) higher percentage of goblet cells than the control group challenged with PBS (8±1.9 %). These results demonstrated that the expression of SLPI correlated with a protective role against the induction goblet cell hyperplasia in our model of allergic asthma.

SLPI mRNA and protein expression in the lungs of SLPI KO mice

To further corroborate our findings and conclusions regarding the biological importance of SLPI in allergic asthma we employed an SLPI-KO mouse model. As anticipated, SLPI mRNA was not expressed in the lungs of any KO mice (Figure 5, panel A) independently of the presence of allergen challenge. SLPI mRNA levels in the pulmonary tissue from WT^{KO}-OVA mice (73±8.7) demonstrated a significant increase following OVA challenge compared with WT^{KO}-PBS (26±3.1) (p<0.01) and KO-OVA (0±0.0) (p<0.001) animals. Western blot analysis was used to confirm the expression of SLPI at the protein level. We found that SLPI protein expression, as expected, was not detectable in the KO-PBS, KO-OVA or WT^{KO}-PBS strains (Figure 5, panel B) whereas SLPI protein expression was detected in the WT^{KO}-OVA group only. These results demonstrated the absence of SLPI in SLPI KO mice and showed the need for OVA- challenge to observe the presence of SLPI protein expression in WT^{KO} sensitized mice.

SLPI ablation increases airway hyperresponsiveness and IgE levels in plasma

Our results demonstrated that the overexpression of SLPI protein prevented the development of AHR following allergen challenge and prevented the increase of plasma IgE levels. We then hypothesized that the ablation of SLPI would generate the opposite effect.

As illustrated in Figure 6, panel A, allergen challenge led to a significant increase in airway responsiveness in both SLPI KO and wild-type mice. Furthermore, SLPI gene ablation led to an increased severity of AHR following allergen challenge. KO-OVA (10.2±1.6 cm H_20 x s/ml) mice

was significantly higher compared either to the WT^{KO}-OVA (6.6±0.5 cm H_20 x s/ml) ($p < 0.05$) or KO-PBS (3.1±0.5 cm H_20 x s/ml) ($p < 0.001$) groups. WT^{KO}-PBS (2.6±0.2 cm H_20 x s/ml) mice displayed significantly ($p < 0.05$) lower lung resistance than WT^{KO}-OVA (6.6±0.5 cm H_20 x s/ml) mice. Similarly, in both SLPI KO and wild-type mice, allergen challenge led to a marked and significant increase in plasma IgE titters (Figure 6, panel B). Again, ablation of the SLPI gene led to a more severe asthmatic phenotype in the form of higher plasma IgE levels. Plasma IgE levels in KO-OVA (3598±204.7 ng/ml) mice were significantly higher compared either to WT^{KO}-OVA (2763±220.3 ng/ml) ($p<0.01$) mice or KO-PBS (920±125.4 ng/ml) ($p<0.001$) control group. While, WT^{KO}-PBS mice (958±166.8 ng/ml) showed lower ($p<0.001$) IgE plasma levels than the WT^{KO}-OVA mice. Overall, we were able to conclude that the ablation of SLPI enhanced hyperresponsiveness and IgE levels in OVA-sensitized and -challenged mice, whereas higher SLPI expression correlated with an improvement at the levels of lung physiology and was able to limit the atopic condition.

Absence of SLPI promotes inflammation in an acute asthma model

Using SLPI TG mice we observed an anti-inflammatory effect in the lungs of OVA challenged mice. To demonstrate that SLPI is responsible for these results, we sensitized and challenged SLPI KO mice, expecting to observe higher inflammatory cell infiltration in their lungs compared to the WT controls. As expected, KO-OVA mice displayed a greater degree of infiltration than WT^{KO}-OVA mice (Figure 7). All PBS challenged animals showed a minimal inflammatory cell infiltration, as compared to the groups challenged with OVA. These results confirmed that in the absence of SLPI, mice were unable to control the inflammatory process in response to OVA challenge, resulting in much greater influx of inflammatory cells into peribronchial spaces of the lungs in OVA-sensitized and -challenged mice.

SLPI ablation increases eosinophilia and goblet cell hyperplasia in SLPI KO mice

To further corroborate the effect of SLPI gene ablation on lung inflammation, the percentage of eosinophils in the inflammatory infiltrate of the lungs and the degree of goblet cell hyperplasia was evaluated. SLPI KO-OVA mice (36±2.7%) demonstrated a significantly higher percentage of eosinophils when compared either to the WT^{KO}-OVA (29±1.5%) controls (p<0.05) or KO-PBS (0±0.0%) (p<0.001) (Figure 8, panel A)**.** Allergen challenge significantly induced goblet cell hyperplasia in both knock-out and wild-type mice (Figure 8, panel B)**.** Interestingly, ablation of the SLPI gene caused a significant (p<0.05) increase in the frequency of goblet cells in KO-OVA (40±4.1**%**) compared to WT^{KO}-OVA (30±1.4**%**) mice and correlated (r=0.75) with the eosinophil percentage, indicating that the anti-proliferative effect which SLPI exerted on goblet cells was active in the presence of allergic inflammation.

SLPI affects the cytokine levels in an acute asthma model

In the assessment of the cytokine profiles of OVA-sensitized and -challenged mice, we found significantly higher (p<0.001) levels of IL-4, IL-5 and IL-6 in both allergen challenged wild-type and SLPI knock-out mice compared to their respective PBS challenged controls (Figure 9). Consistent with their more severe inflammation and higher plasma IgE levels, SLPI knock-out mice expressed the cytokines at higher levels.

The levels of IFN-γ and IL-2 followed similar patterns of expression indicating that allergen challenge did not uniquely induce TH2 cytokines and that SLPI demonstrates a broadly anti-inflammatory effect in the context of allergic asthma. RANTES and TNF-α were also significantly higher (p<0.05) in KO-OVA mice as compared with KO-PBS mice but no significant difference was found in the assessment of MCP-1 levels.

Our data showed that both TH1 and TH2 cytokines were expressed at higher levels in SLPI KO-OVA mice than in WT control mice as assessed 3 hours following the last allergen challenge.

These results can explain in part both the degree of inflammation in the lungs and the magnitude of lung resistance observed in each group of animals.

The effect of resiquimod treatment on SLPI mRNA and protein expression

Previous studies completed by our laboratory have demonstrated that resiquimod treatment prevents the development of AHR in allergic A/J and C57BL/6 mice, as well as Brown Norway rats[23,24]. We also found higher SLPI expression in macrophages derived from OVA-sensitized and -challenged mice compared to control animals (data not shown). Those results led us to investigate the mRNA expression of SLPI after resiquimod treatment in naïve C57BL/6J mice. We found significantly higher ($p<0.05$) SLPI mRNA expression 3 and 6 hours after resiquimod injection compared with non treated mice.

As expected, SLPI mRNA was absent from the lungs of SLPI KO mice (Figure 10, panel A) independently of PBS or OVA challenge, or resiquimod treatment. In the analysis of SLPI protein expression (Figure 10, panel B), there was no difference between WT^{KO}-OVA mice compared to the WT^{KO}-RES-OVA group. WT^{KO}-PBS and WT^{KO}-RES-PBS displayed no SLPI protein expression in the Western Blots.

Resiquimod treatment prevents OVA-induced AHR independently of SLPI

In order to evaluate the role of SLPI expression in resiquimod treatment of allergic asthma, we treated SLPI knock-out mice with resiquimod prior to allergen aerosol challenge. As we assumed, SLPI gene ablation in KO-OVA (12.3±1.6 cm H_2O*s/mL) mice increased the severity of AHR following allergen challenge compared with WT^{KO}-OVA (8.4±0.8 cm H_2O*s/mL) ($p<0.05$) (Figure 11). However treatment with resiquimod was able to significantly reduce ($p<0.05$) the lung responsiveness in both KO-RES-OVA (5.8±0.9 cm H_2O*s/mL) and WT^{KO}-RES-OVA mice compared to untreated animals. Overall these results showed that treatment with resiquimod prevents AHR and the effectiveness of the drug was not dependent on the expression of SLPI.

Resiquimod treatment prevents inflammation in allergic asthma

To determine whether the inhibition of allergic inflammation mediated by resiquimod is dependent on SLPI expression, we assessed the inflammatory cell infiltration, and the percentage of eosinophils, in resiquimod treated OVA-sensitized and -challenged SLPI KO and WT^{KO} mice.

We found that OVA challenged SLPI KO mice and the group treated with resiquimod (KO-RES-OVA), showed increased numbers of inflammatory cells compared with their respective controls (Figure 12, panel A). Also, the percentage of eosinophils was significantly (p<0.001) higher in KO-OVA mice (29.0±2.2 %) (Figure 12, panel B) compared to the control mice. After treatment with resiquimod, KO-RES-OVA (1.0±0.2%) and WT^{KO}-RES-OVA mice (0.5±0.2%) showed a significantly lower (p<0.001) fraction of eosinophils compared either to KO-OVA or WT^{KO}-OVA groups.

These results confirmed that treatment with resiquimod prevented AHR, diminished inflammatory cell infiltration into allergen-sensitized and -challenged lungs and that the effect of the drug was independent on the induction or even basal expression of SLPI.

Discussion

The objective of this study was to evaluate the role of SLPI in allergic asthma and to assess whether the protective effect of resiquimod treatment depends on the expression of SLPI. We therefore assessed lung physiology and inflammatory responses in a murine model of acute allergic asthma using, for the first time, both SLPI transgenic and knock-out mice. Our results demonstrates that overexpression of SLPI prevented the development of AHR and also decreased the influx of inflammatory cells into the lungs, following airway allergen challenge. Furthermore, ablation of SLPI expression led to the development of more severe AHR and inflammation under similar conditions.

Airway hyperresponsiveness is one of the characteristics of allergic asthma in humans[283-285] and also reproduced in animal models.[24;28;279] Allergic C57BL/6 mice are hyporesponsive to MCh compared to Balb/c and A/J mice.[23;286] In preliminary experiments we found that SLPI KO mice, on a C57BL/6 genetic background, were relatively hyperresponsive to MCh compared to WT C57BL/6 mice. To confirm if this effect was generated by the ablation of expression of SLPI, we assessed airway resistance in knock-out and transgenic mice constitutively expressing different levels of SLPI. Our results showed that allergic SLPI TG mice were hyporesponsive to MCh when compared with WT^{TG} mice, whereas SLPI KO mice were hyperresponsive compared with WT^{KO} mice. Hence, we were able to demonstrate that lung physiology was directly influenced by the differential expression of SLPI.

It has been shown that acute OVA-sensitization and -challenge increases airway reactivity to the allergen, which causes inflammation, narrowing the airways and increasing resistance to expiratory airflow.[23;31;287] SLPI expressed in TG and WT mice may decrease the airway responsiveness to MCh by exerting its anti-inflammatory effect.[277] Therefore, it is likely that ablation of SLPI contributed to the hyperresponsiveness to allergen challenge observed in SLPI KO mice. Considering that asthma is characterized by an important inflammatory component, we evaluated the inflammatory response and the role of SLPI in this process. Previous studies

performed in our laboratory, using acute and chronic allergic asthma models, showed a significant inflammatory process in the lungs characterized by infiltration of the peribronchial space in the lungs.[193;279]

In the present study, we found that OVA-challenged SLPI transgenic mice showed significantly lower inflammatory cell influx, including eosinophils, surrounding the airways compared to wild-type controls, and the opposite phenomenon was observed in KO mice under the same conditions. The presence of eosinophils could be explained by the triggering of an inflammatory response where epithelial cells secrete chemotactic factors that recruit granulocytes, including eosinophils.[288] The milder inflammatory response found in mice where SLPI expression was not ablated, confirming the importance of SLPI in controlling inflammation in this acute asthma model. These results are consistent with the findings of Wright *et al*., who reported, that leukocyte influx into the airway could be inhibited by administration of intratracheal SLPI in guinea pigs after allergen sensitization and challenge.[7]

To further evaluate the degree of inflammation, goblet cell hyperplasia was also assessed. The higher goblet cell hyperplasia found in KO-OVA mice correlated (r=0.72) with the inflammatory cell influx with the percentage of eosinophils in the peribronchial region. The increase of goblet cell activity could be explained by the fact that in asthma there has been an active inflammatory process happening in the lungs, including increased mucus production due to goblet cells hyperplasia. The excess of mucus along with edema and smooth muscle contraction has been reported to result in the closure of distal airways and increased resistance in the airway.[32]

Allergic asthma is an atopic disease characterized by an elevation of blood IgE levels.[279;289 23,36] Westin et al. showed that SLPI regulated allergic reactions by inhibiting IgE.[290] It is tempting to speculate that early inflammatory events in acute allergic asthma, characterized by cell infiltration in the lungs and IgE, are directly modulated by SLPI. We found higher levels of IgE in the plasma of OVA-challenged SLPI KO mice, while animals constitutively expressing SLPI

showed the opposite phenomenon. These findings are consistent with the observation by Nakamura *et al.* of increased B cell proliferation and immunoglobulin production in SLPI KO mice.[291] Our results could be explained by the theories that SLPI plays a role as a negative feedback protein in the production of antibodies by interfering with the NF-κB-dependent transcriptional activation during the immune response[292] or by the lack of control in the production of IgE in SLPI KO mice compared with those who express normal levels of SLPI.[286;289]

During the inflammatory process in the airway, epithelial cells interact with dendritic cells. Subsequently, dendritic cells activate $CD4^+$ T helper cells, initiate a T helper type 2 (T_H2) response with production of IL-4 and IL-10, and finally, the T_H2 cytokines stimulate B cells to produce IgE.[38;288;293] We assessed the cytokine profile of OVA-sensitized and -challenged mice in an attempt to further clarify the mechanism of action of SLPI in the immune response of allergic asthma, and we found significant increases in IL-4, IL-5, IL-6, IFN-γ, IL-2, RANTES, TNF-α and MCP-1 using KO-OVA mice compared either with KO-PBS or WT^{KO}-OVA mice. No specific T_H1 or T_H2 pattern of cytokine/chemokine expression seems to be distinguishing SLPI KO from their WT controls. These results demonstrate an association between the heightened inflammatory responses in SLPI KO mice and their higher levels of IgE in plasma compared to the WT controls.

Previous studies using resiquimod treatment in mice performed in our laboratory showed that resiquimod was able to induce higher level of SLPI expression in macrophages derived from OVA-sensitized and -challenged mice compared with untreated controls. This observation led us to assess the effect of resiquimod on SLPI expression and its role in modulating the development of allergic asthma.

In this study we found that resiquimod increased SLPI mRNA expression in naïve C57BL/6 mice. Additionally, our results showed that resiquimod treatment inhibited the inflammatory process in SLPI KO and TG OVA-sensitized and -challenged mice, which was evidenced by the lower degree of inflammatory cell infiltration in the lungs, the decreased percentage of eosinophils, and the reduction of goblet cell hyperplasia compared with untreated animals. We can

conclude that treatment with resiquimod is effective against allergic asthma independently of the expression of SLPI.

Using an acute model of allergic asthma in SLPI TG and KO mice, our results showed that SLPI was able to participate in the early stages of asthma and demonstrated that its expression directly reduced the inflammatory response in the respiratory tract improving lung function. Due to the anti-inflammatory and antimicrobial functions attributed to SLPI, the lack of genetic polymorphism of this protein and the importance of managing asthma at an early stage, this protein should be considered as a target for novel therapies in allergic asthma.

Acknowledgements

We acknowledge the histology assistance of Dr. Marie-Christine Guiot. This work was supported by a grant from the American Asthma Foundation Research Program. R. Marino and P. Camateros are recipients of the Doctoral Award from the Canadian Institutes of Health Research (CIHR).

Figure 1. Analysis of SLPI mRNA and protein expression in lung homogenates from OVA-sensitized and -challenged SLPI TG and WT^{TG} mice.

(A) SLPI mRNA relative expression (mean ± SEM). *n* = 11 for all groups. **(B)** SLPI and GAPDH protein expression. SLPI Transgenic mice showed higher SLPI mRNA and protein expression compared to wild type mice.

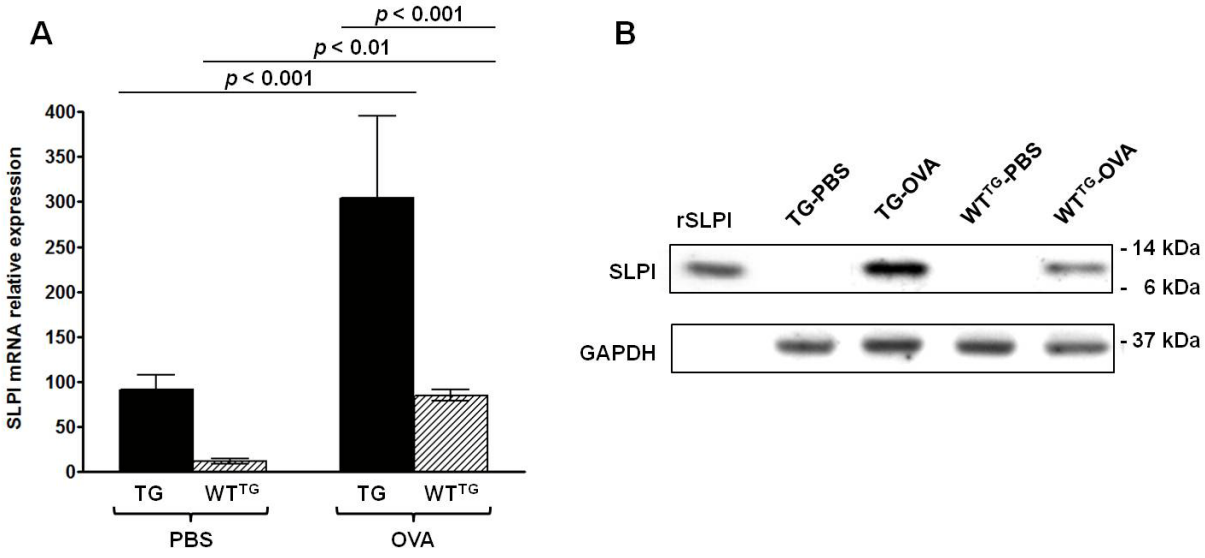

A
p < 0.001
p < 0.01
p < 0.001
SLPI mRNA relative expression
400
350
300
250
200
150
100
50
0
TG
WTTG
TG
WTTG
PBS
OVA
B
rSLPI
TG-PBS
TG-OVA
WTTG.PBS
WTTG.OVA
SLPI
- 14 kDa
- 6 kDa
GAPDH
- 37 kDa

Figure 2. Airway resistance and IgE levels in plasma from SLPI transgenic (TG) and wild-type (WT^{TG}) mice.

Bars represent the mean ± SEM of 5 independent experiments and *n* = 15 for all groups. **(A)** Airway resistance. **(B)** IgE levels in plasma. TG-OVA mice showed significant lower lung resistance and IgE levels in plasma compared to WT^{TG}-OVA or TG-PBS mice.

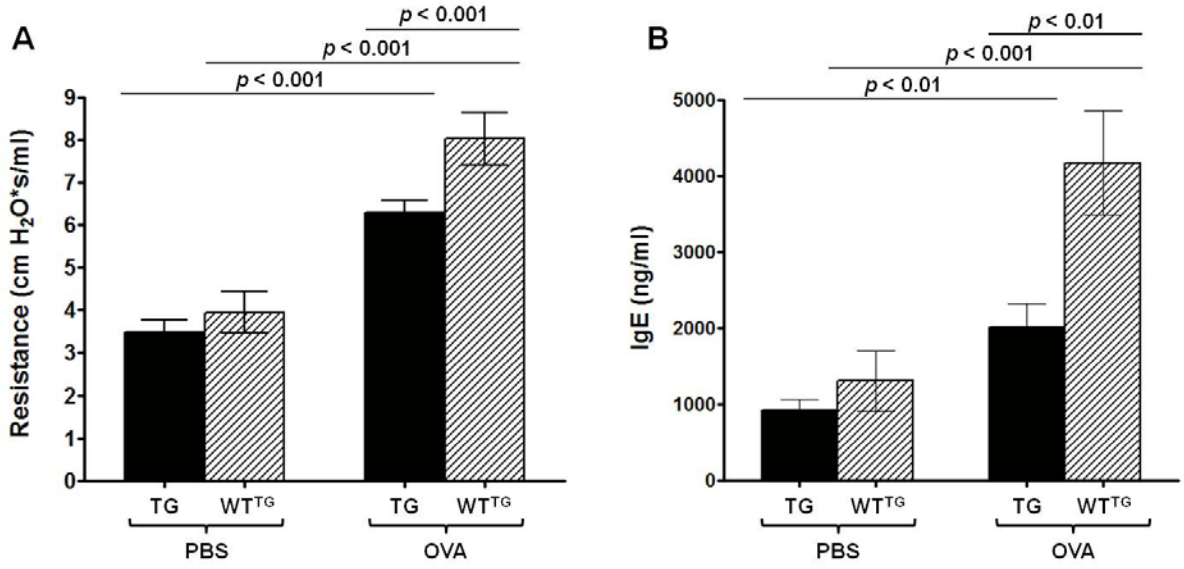

A
p < 0.001
p < 0.001
p < 0.001
Resistance (cm H2O*s/ml)
0
1
2
3
4
5
6
7
8
9
TG
WTTG
TG
WTTG
PBS
OVA
B
p < 0.01
p < 0.001
p < 0.01
IgE (ng/ml)
0
1000
2000
3000
4000
5000
TG
WTTG
TG
WTTG
PBS
OVA

Figure 3. Hematoxylin- and eosin-stained lung sections from SLPI transgenic (TG) and wild-type (WT^{TG}) mice.

Panels **(A)** and **(B)** represent the lungs from TG-PBS and TG-OVA mice respectively. **(C)** Lungs of WT^{TG}-PBS. **(D)** Lungs of WT^{TG}-OVA mice. The lungs from TG-OVA mice showed a decrease in cellular infiltration after OVA challenge compared to the lungs of similarly treated WT^{TG}-OVA mice.

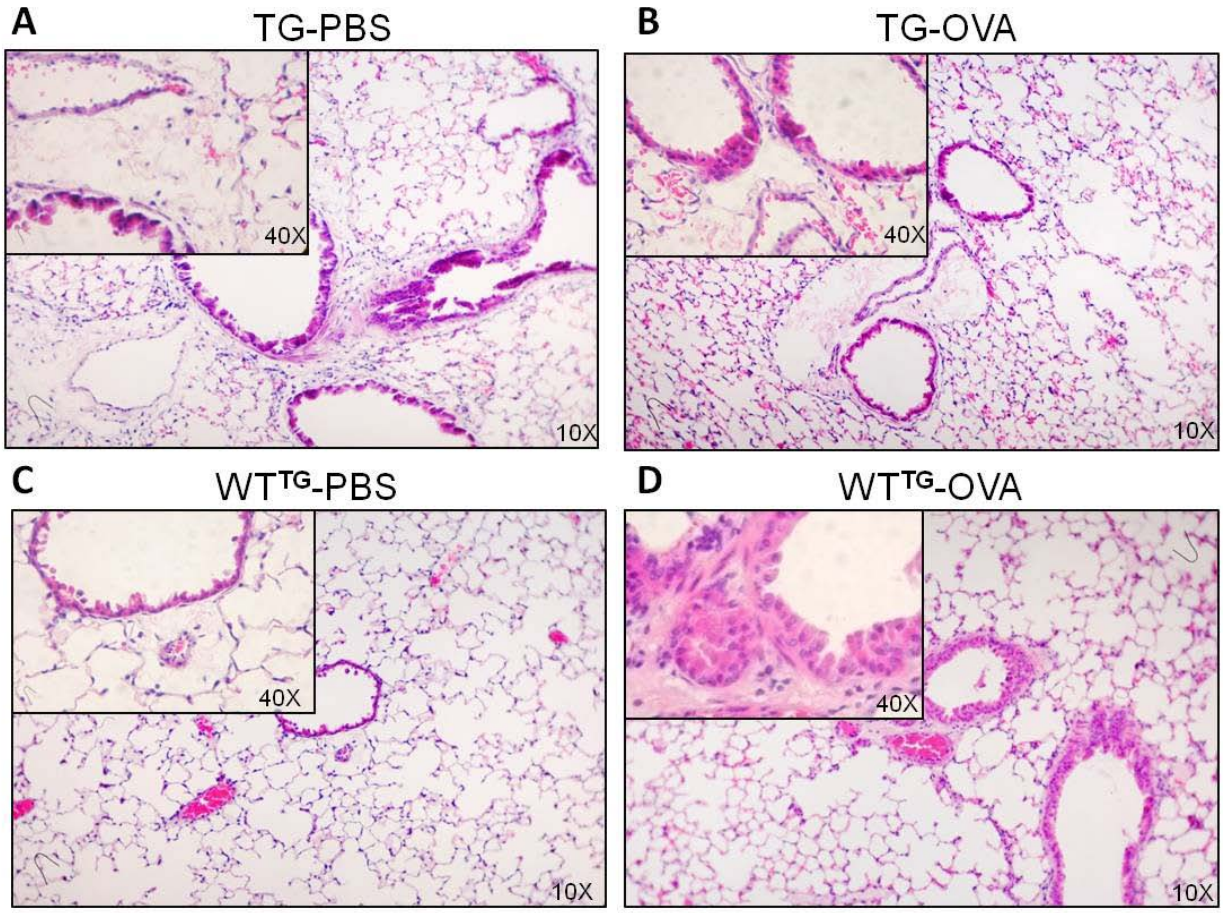

A
TG-PBS
40X
10X
B
TG-OVA
40X
10X
C
WT^TG-PBS
40X
10X
D
WT^TG-OVA
40X
10X

Figure 4. Eosinophils and Goblet cell hyperplasia in lung sections from SLPI TG and WT^{TG} mice. **(A)** Percentage of eosinophils (mean ± SEM). *n* = 14 for all groups. **(B)** Percentage of goblet cells (mean ± SEM). *n* = 14. TG-OVA showed significant lower percentage of eosinophils and goblet cells compared WT^{TG}-OVA.

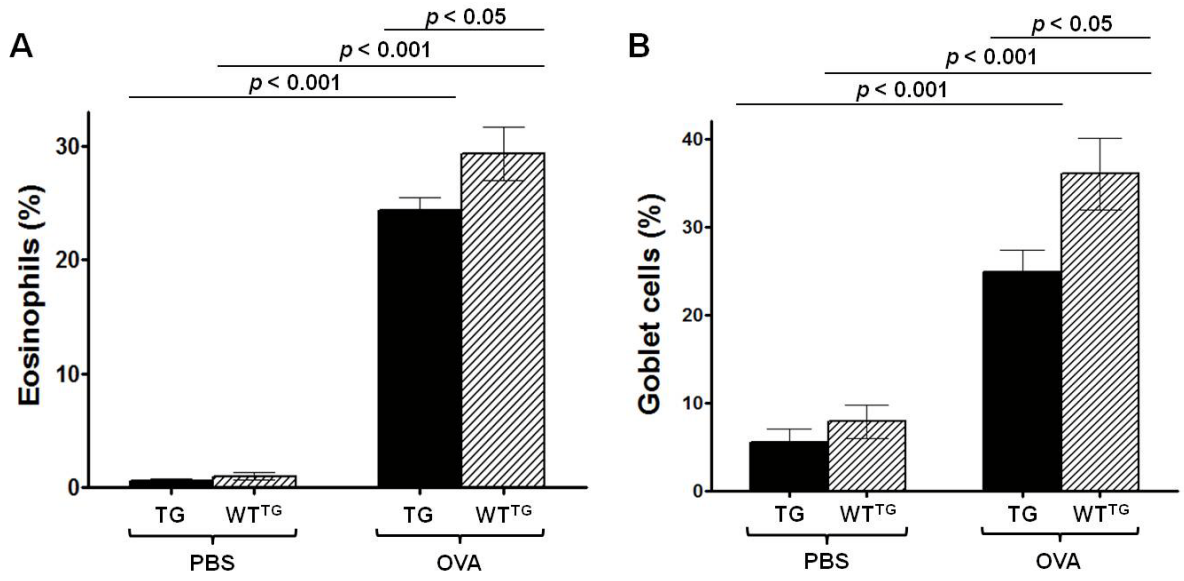

A
p < 0.05
p < 0.001
p < 0.001
Eosinophils (%)
30
20
10
0
TG
WTTG
TG
WTTG
PBS
OVA
B
p < 0.05
p < 0.001
p < 0.001
Goblet cells (%)
40
30
20
10
0
TG
WTTG
TG
WTTG
PBS
OVA

Figure 5. Analysis of SLPI mRNA and protein expression in lung homogenates from KO WT^{KO} mice.

(A) SLPI mRNA relative expression (mean ± SEM), *n*=10 for all. **(B)** SLPI and GAPDH protein expression from SLPI KO and TG mice challenged with PBS or OVA. SLPI KO mice showed neither SLPI mRNA nor protein expression compared to wild type mice.

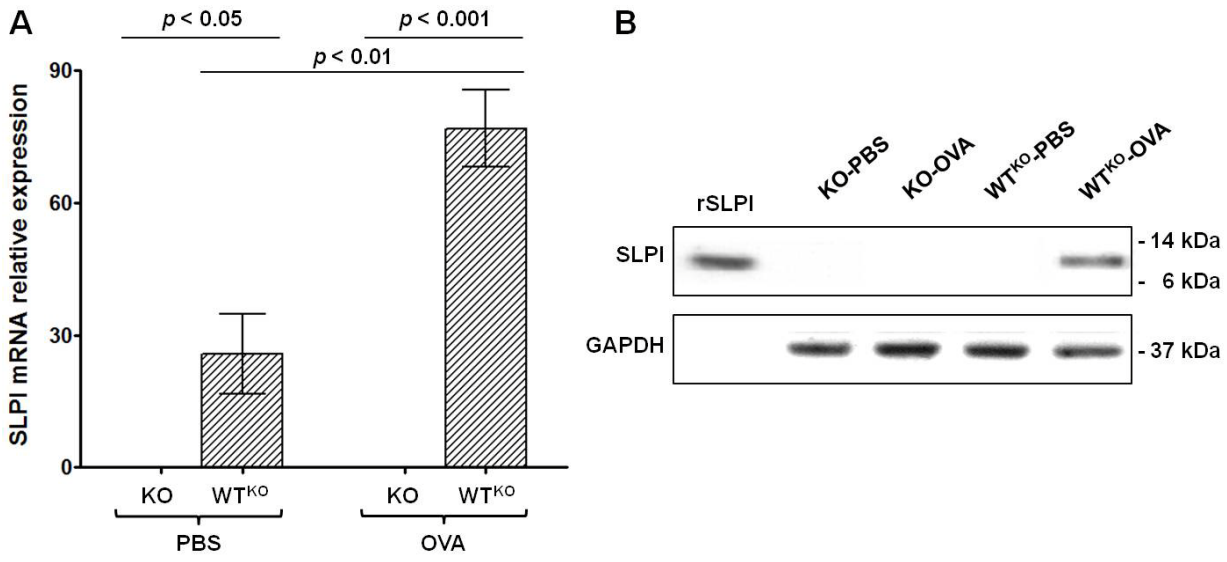

A
p < 0.05
p < 0.001
p < 0.01
SLPI mRNA relative expression
90
60
30
0
KO
WTKO
KO
WTKO
PBS
OVA
B
rSLPI
KO-PBS
KO-OVA
WTKO-PBS
WTKO-OVA
SLPI
GAPDH
- 14 kDa
- 6 kDa
- 37 kDa

Figure 6. Airway resistance and IgE levels in plasma from SLPI knock-out (KO) and wild-type (WTKO) mice.

Bars represent the mean ± SEM from 5 independent experiments and *n*=15 for all groups. **(A)** Airway resistance. **(B)** IgE levels in plasma. KO-OVA mice showed significant higher lung resistance and IgE levels in plasma compared to WT^{KO}-OVA or KO-PBS.

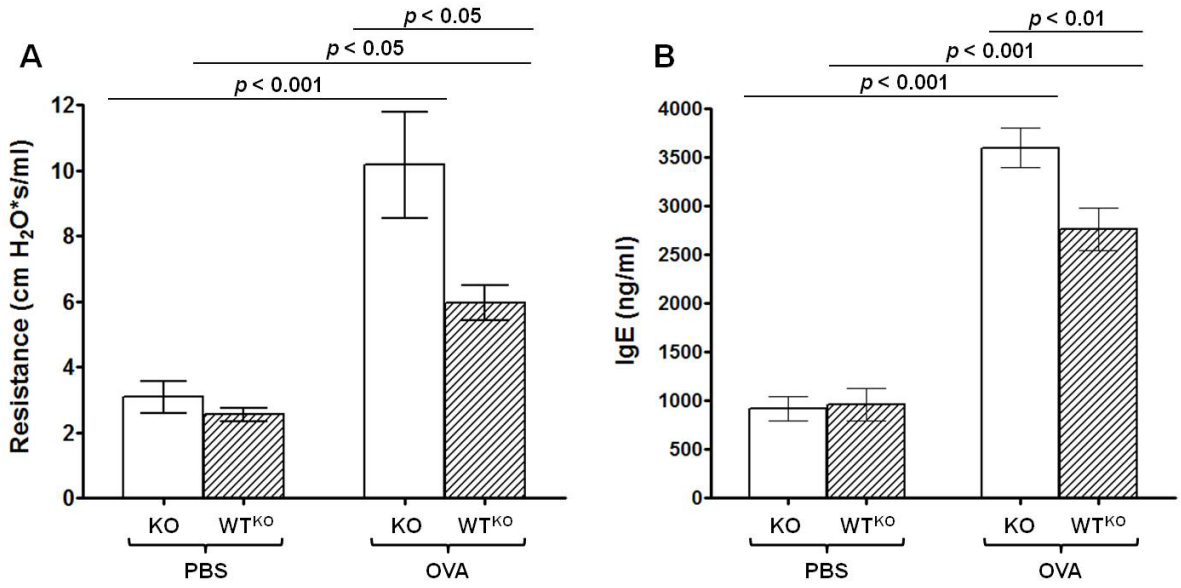

A
p < 0.05
p < 0.05
p < 0.001
Resistance (cm H2O*s/ml)
12
10
8
6
4
2
0
KO
WTKO
KO
WTKO
PBS
OVA
B
p < 0.01
p < 0.001
p < 0.001
IgE (ng/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
KO
WTKO
KO
WTKO
PBS
OVA

Figure 7. Hematoxylin- and eosin-stained lung sections from SLPI knock-out (KO) and wild-type (WTKO) mice.

Panels **(A)** and **(B)** represent the lungs from KO-PBS and KO-OVA mice, respectively. **(C)** Lungs from WT^{KO}-PBS and **(D)** Lungs from WT^{KO}-OVA mice. Lungs from KO-OVA mice showed an increase in cellular infiltration after OVA challenge compared to the lungs of similarly treated WT^{KO}-OVA mice.

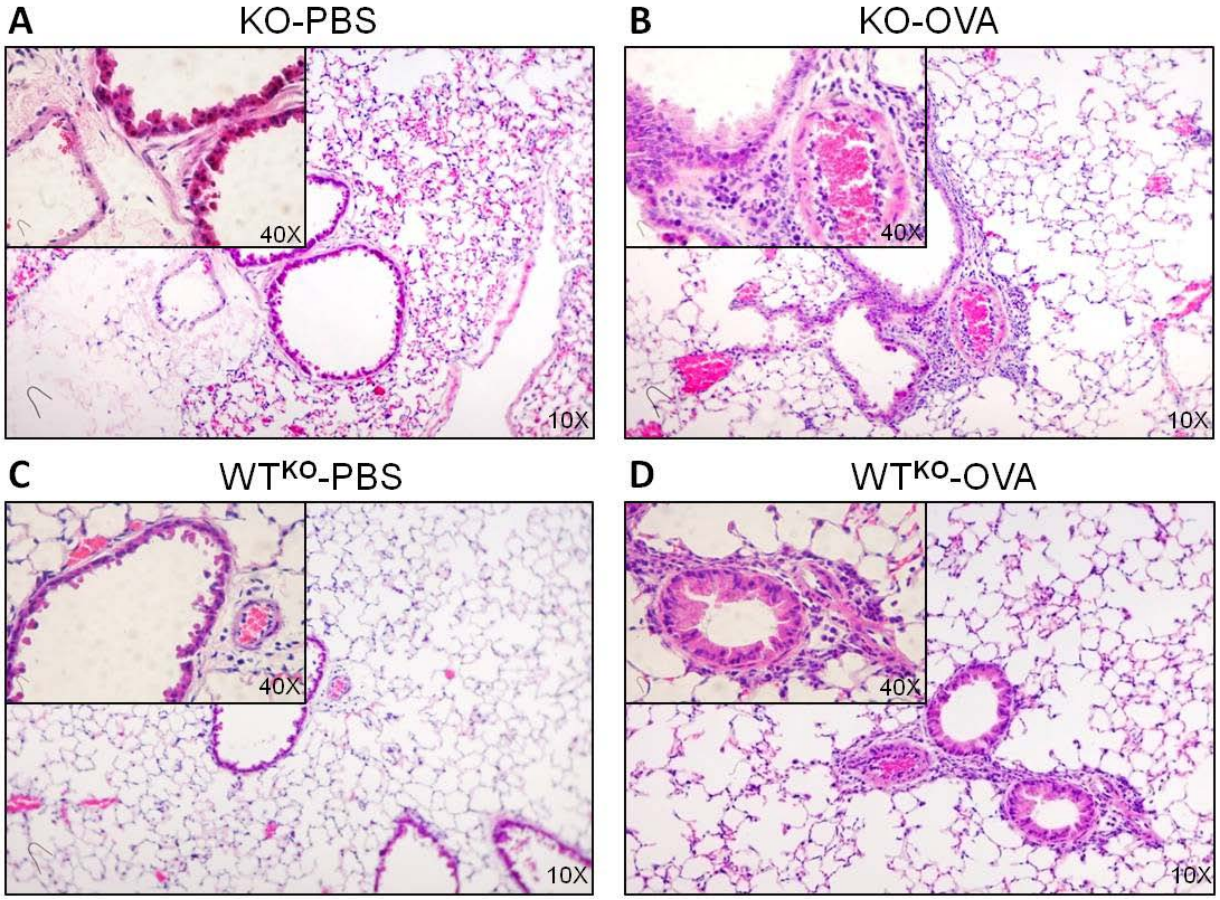

A
KO-PBS
40X
10X
B
KO-OVA
40X
10X
C
WTKO-PBS
40X
10X
D
WTKO-OVA
40X
10X

Figure 8. Eosinophils and goblet cells in lungs from SLPI knock-out (KO) and wild-type (WTKO) mice.

Bars represent the mean ± SEM from 5 independent experiments and n=15 for all groups. **(A)** Percentage of eosinophils. **(B)** Percentage of goblet cell hyperplasia. KO-OVA mice showed a significant higher percentage of eosinophils and goblet cells hyperplasia compared to WT^{KO}-OVA and KO-PBS mice.

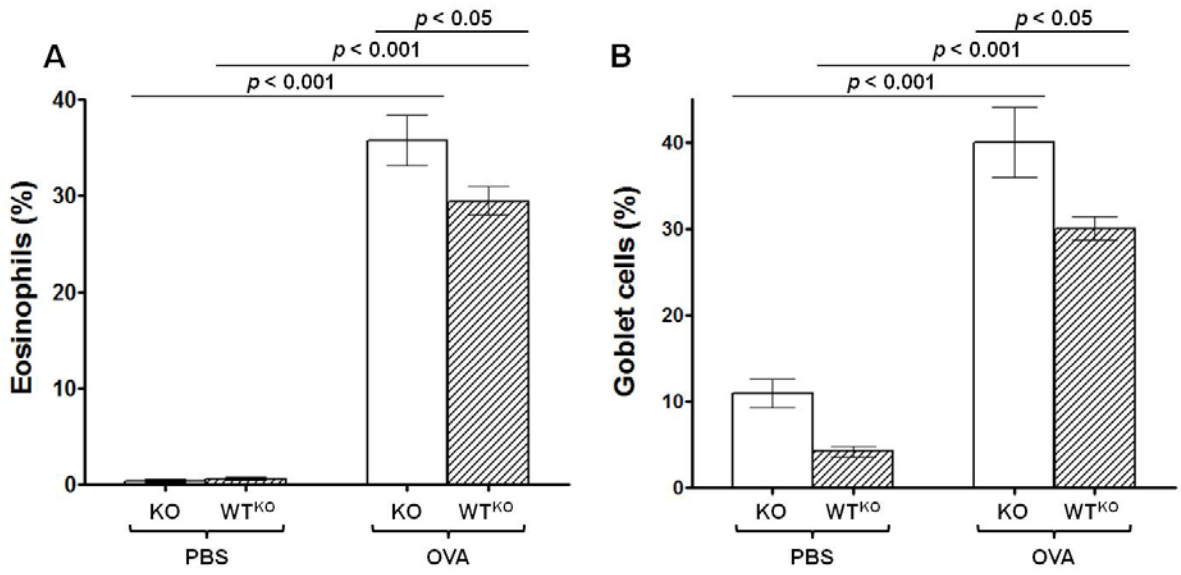

A
p < 0.05
p < 0.001
p < 0.001
Eosinophils (%)
40
30
20
10
0
KO
WTKO
KO
WTKO
PBS
OVA
B
p < 0.05
p < 0.001
p < 0.001
Goblet cells (%)
40
30
20
10
0
KO
WTKO
KO
WTKO
PBS
OVA

Figure 9. Cytokine levels in lungs of SLPI KO and WTKO mice.

The bars represent the mean ± SEM. n = 6 for all groups. For IL-4, IL-5, IL-6, IL-2 and MCP-1; $p<0.001$ in KO-PBS vs. KO-OVA and WT^{KO}-PBS vs. WT^{KO}-OVA mice. For IFN-γ, RANTES and TNF-γ; $p<0.05$ in KO-PBS vs. KO-OVA and WT^{KO}-PBS vs. WT^{KO}-OVA mice.

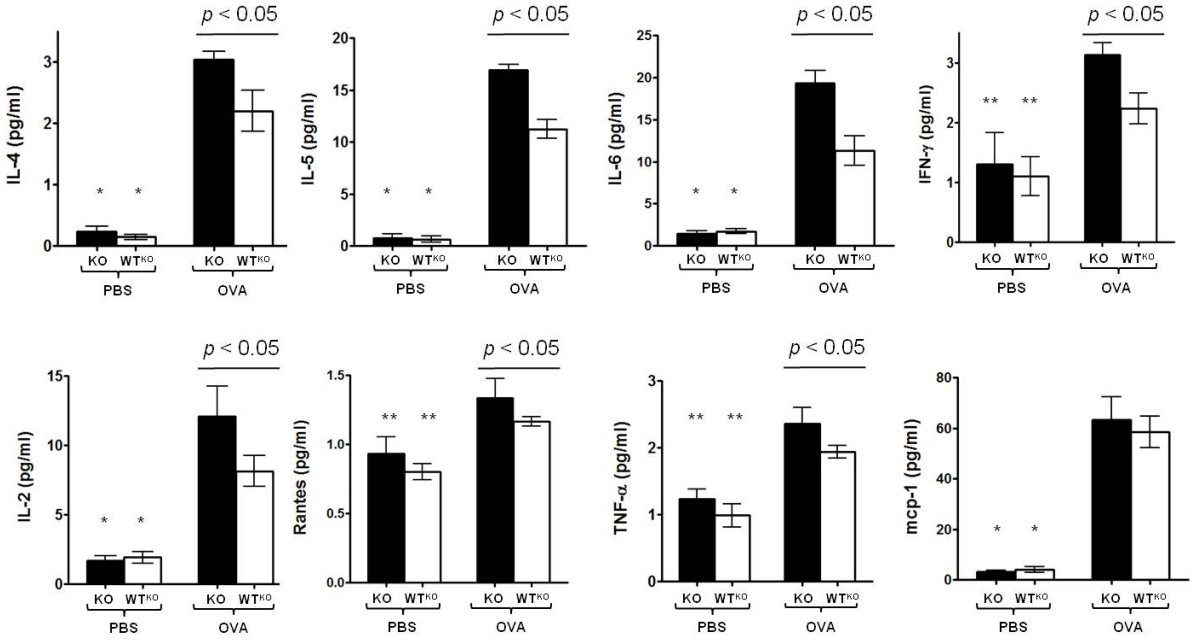

IL-4 (pg/ml)
IL-5 (pg/ml)
IL-6 (pg/ml)
IFN-γ (pg/ml)
IL-2 (pg/ml)
Rantes (pg/ml)
TNF-α (pg/ml)
mcp-1 (pg/ml)
p < 0.05
KO
WTKO
PBS
OVA

Figure 10. SLPI mRNA and protein expression in lung homogenates from SLPI knock-out (KO) and wild-type (WTKO) mice after PBS or OVA challenge, including resiquimod treatment.

(A) SLPI mRNA relative expression (mean ± SEM) and n=10 for all groups. **(B)** SLPI and GAPDH protein expression. SLPI KO mice showed no mRNA and protein expression under any condition. RES = resiquimod.

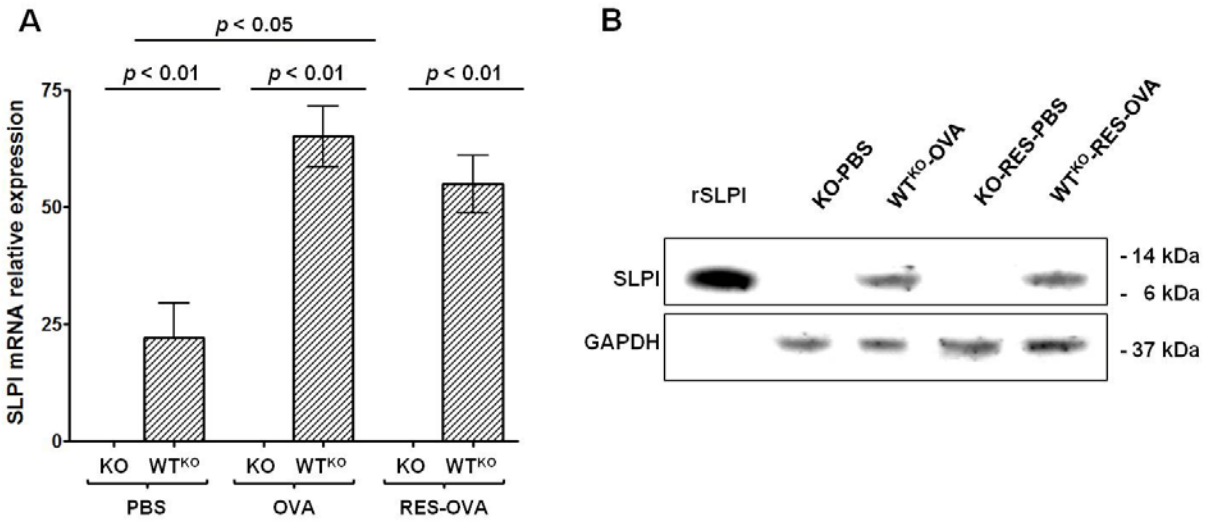

A
p < 0.05
p < 0.01
p < 0.01
p < 0.01
SLPI mRNA relative expression
75
50
25
0
KO
WTKO
KO
WTKO
KO
WTKO
PBS
OVA
RES-OVA
B
rSLPI
KO-PBS
WTKO-OVA
KO-RES-PBS
WTKO-RES-OVA
SLPI
GAPDH
- 14 kDa
- 6 kDa
- 37 kDa

Figure 11. Airway resistance from OVA-sensitized and -challenged SLPI knock-out (KO) and wild-type (WT^{KO}) mice after resiquimod treatment.

Bars represent the mean ± SEM from 3 independent experiments and n=6 for all groups. Resiquimod treatment significantly reduced airway resistance in SLPI KO (KO-RES-OVA) and wild type (WT^{KO}-RES-OVA) compared to untreated mice under the same conditions. RES=Resiquimod (S28463).

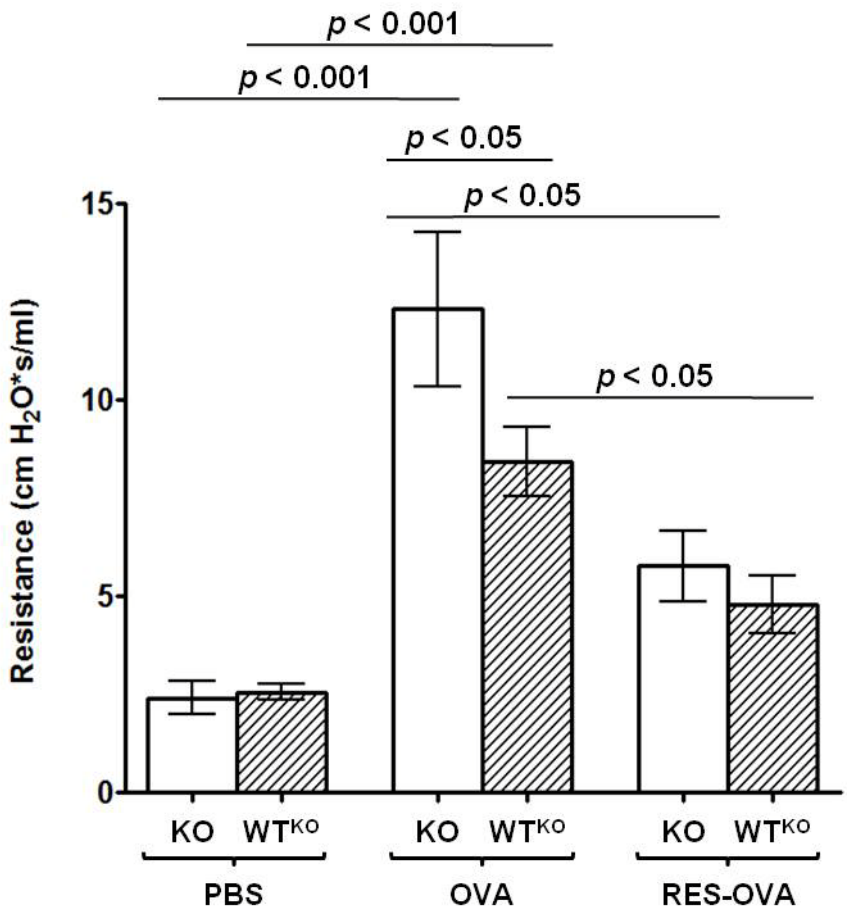
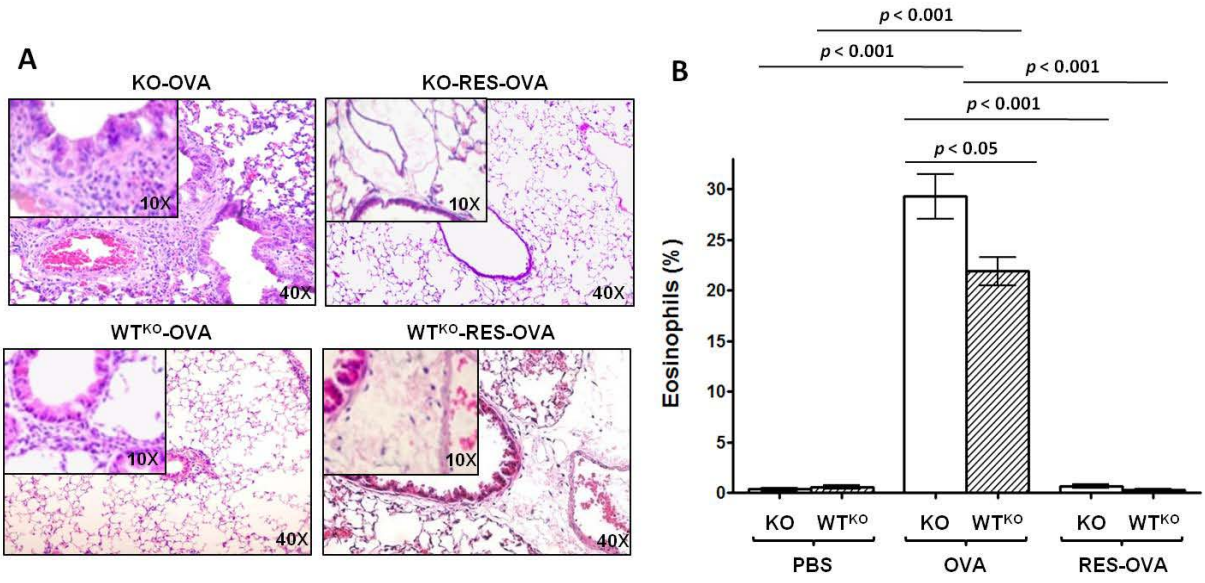

p < 0.001
p < 0.001
p < 0.05
p < 0.05
p < 0.05
Resistance (cm H2O*s/ml)
15
10
5
0
KO
WTKO
KO
WTKO
KO
WTKO
PBS
OVA
RES-OVA

Figure 12. Inflammatory cell infiltration and eosinophils in lungs from SLPI knock-out (KO) and wild-type (WTKO) mice after resiquimod treatment.

(A) Hematoxylin- and eosin-stained lung sections. **(B)** Percentage of eosinophils (mean ± SEM). n=6 for all groups. KO-RES-OVA and WT^{KO}-RES-OVA mice showed lower cellular infiltration and a significant decrease ($p<0.001$) in eosinophil percentage after resiquimod treatment compared to untreated mice. RES=Resiquimod.


A
KO-OVA
KO-RES-OVA
WTKO-OVA
WTKO-RES-OVA
10X
40X
B
p < 0.001
p < 0.001
p < 0.001
p < 0.001
p < 0.05
Eosinophils (%)
0
5
10
15
20
25
30
KO
WTKO
PBS
OVA
RES-OVA

General Discussion

Asthma is a complex, multi-factorial, and inflammatory disease characterized by episodes of intermittent reversible bronchoconstriction, airway hyperresponsiveness, airway smooth muscle hyperplasia, excessive mucous production and chronic inflammation of the airways.[30] Asthma is one of the most common chronic diseases affecting children and adolescents in the industrialized countries, with a total of more than 20 million people around the world.[2] Despite many advances in treatment, asthma has became a major public health issue[3] not only because of its high and increasing worldwide morbility and mortality,[31] but also because of the health care resources it entails.[32] Around 300 million individuals suffer from asthma worldwide, representing 1% to 18% of the global prevalence.[34] Despite to the awareness about this disease and the improvement in the guidelines in the diagnosis of asthma,[36] this disease is considered a major public health and economic problem. Direct and indirect costs represented around US$12 billion in the United States in 2006 (aafa.org), €18 billion in Europe in 2003 and in Canada, the healthcare costs of uncontrolled asthma were estimated in around CAD$ 162 million per year in 2004.[294]

It has been described that asthma is composed of heterogeneous diseases and shows a broad variety of sub-phenotypes.[33] Many of these sub-phenotypes are expressed in the pathogenesis of asthma and include inflammatory cell infiltration in the lung, cytokines and chemokines levels, the presence of antibodies (IgE levels), and lung physiology response including airway responsiveness to bronchoconstricting stimuli.[106] AHR is inherited as a complex trait; it has been associated with development of allergic asthma and shows homology between mouse and human.[22;117] It has been demonstrated that the allergic response in murine models resembles the human asthma, including the early and late response. Moreover, the development, progression and severity of asthma is controlled by the expression of several genes which interact with multiple environmental factors, and whereas airway responsiveness and atopy represent two important genetically-regulated phenotypes that might be the key to understand the mechanisms underlying this disease.[118]

Repeatable and non-invasive methods are needed to assess lung physiology in asthmatic patients.[119] In mouse models, AR can be assessed both before and after the experimental induction of allergic airway inflammation using either invasive or non-invasive methods.[22-24] Invasive methods are considered to be the gold standard for the assessment of parameters of airway responsiveness, however, some of their technical features make them inefficient for phenotyping large numbers of animals, and do not allow for repeated measurements of lung functions in the same individual.[27] Additionally, some previous studies have described that the non-invasive method using the WBP is strain specific and in some cases, this methods does not reflex the physiologic mechanism of the lung.[25;29]

In Chapter 2 we demonstrated that non-sensitized C57BL/6J and A/J strains of mice can be reliable assessed by airway responsiveness to Mch using the WBP; both strains showed a dose dependent response to increasing doses of Mch and correlation between the non-invasive and invasive method. These results are consistent with previous studies assessing AR in non-sensitized animal models.[199;200] Furthermore, our results also show a transient increase in Penh following consecutive exposures to methacholine in both non-sensitized C57BL/6J and A/J mice using the non-invasive method, as well as airway resistance using the invasive method. This effect appears to be temporary as the values returned to baseline following the third challenge. Using Penh, these results may be explained, by the fact that the non-invasive method assesses AR in unrestrained and conscious mice and it is possible that stress, neural control, or conditioned behavioural responses are affecting the Penh values which may not reflect changes in the underlying respiratory mechanims.[192] However, this theory does not explain the changes observed in airway resistance using the invasive method and there is the possibility that consecutive exposure to Mch modifies Penh to a greater degree than it does airway resistance; but since the invasive method is a terminal technique this hypothesis cannot easily be verified.

We also demonstrated that changes in Penh and resistance after consecutive challenges with Mch were not a consequence of the airway inflammation; we did not find inflammatory cell

infiltration in the lungs or increased IgE levels in plasma even after six consecutive challenges. These results prove that the effect of consecutive challenges with Mch on Penh or Resistance, is through a different mechanism than structural modification of the airways, including inflammation.[198] Finally, we conclude that to obtain a reliable reassessment of AR in mouse models there should be a period of 15 days between exposures to Mch. These results support previous reports in C57BL/6J mice where no correlation was found between these two methods when measurements were taken 5 and 9 days apart.[191] As remark in the assessment of AR, we recommend that to ascertain the reliability and consistency of results when using mouse models of airway hyperresponsiveness; every strain of mice has to be assessed using both methods and special attention has to be taken about the technical condition of these type of experiments.

Previous studies have demonstrated that genetic factors are in part responsible for the development of asthma and many intermediate phenotypes such as airway responsiveness have been correlated with the development and severity of this disease, however, the mechanisms whereby they trigger and exacerbate asthma are not well understood.[30;139] AHR is considered as a hallmark of asthma, inherited as a complex trait, modeled in animals and associated with development of this disease.[22;117;117;127;295]

Despite significant progress in genome-wide association studies of human populations, linkage and case-control association studies in humans have identified potential chromosomal regions associated with asthma susceptibility, specifically, bronchial hyperresponsiveness; those regions include potential QTLs on chromosomes 2q, 5q, 6p, 11q, 12q and 13q.[145-147] However, the success of these studies has been difficult, due to the genetic variability between individuals, significant stratification of the sample population, broad amount of potential chromosomal regions and large amount of potential candidate genes associated with the variety of phenotypes. As an alternative and complementary approach to whole-genome association studies, genetic analysis using animal models represent a very useful tool in the

identification of candidate genes, where genetic and environmental factors are precisely controlled.

In our previous study, we analyzed the AcB/BcA RCS of mice and identified 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; 8 are supported by previously and independent 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.[22] The chromosomal regions identified using the RCS of mice were too large to select candidate genes in an efficient manner; around 157 potential candidate genes are included in these 16 chromosomal regions significantly associated with AHR.

Based in our knowledge, the Chapter 3 of the thesis shows the largest study performed in the genetic of AHR. Our results demonstrate how the genetic strategy based in the generation of F2 crosses from the AcB/BcA RCS allowed us to narrow down the significant recombinant regions previous described in the RCS, and to identify 9 candidate genes that might be associated with airway responsiveness in mice. Those genes include *Arhgap5, Foxa1, Clec14a, Ctage5, Rtn1, Gpx2, Rsad2, Prkd1* and *Adam21*. However, further studies have to be performed in order to elucidate the role of these genes in the mechanisms associated with the development of airway hyperresponsivess and the relation with the pathogenesis of allergic asthma.

While we performed the experiments related to genetic dissection of AR and allergic asthma, we also assessed the role SLPI in allergic asthma using SLPI knock-out and transgenic mice, as shown in Chapter 4. Previous studies have shown that asthmatic patients display a significant increase in SLPI levels compared to healthy controls[22] while our group and others have demonstrated the protective effect of resiquimod, a synthetic TLR7 ligand, against allergic asthma in mice[23] and rats.[24] Our preliminary experiment show a significant increase in SLPI expression at the both messenger RNA and protein levels following *in vitro* treatment of

macrophages with resiquimod. We hypothesized that increased expression of SLPI during asthmatic attacks may protect the lungs against allergic asthma induced inflammation and thereby lead to improvements in lung physiology. And using an acute model of allergic asthma in SLPI TG and KO mice, our results have shown that SLPI participates in the early stages of asthma and have demonstrated that its expression directly reduces the inflammatory response in the respiratory tract and improves lung function in this context.

The exact mechanism of SLPI in the prevention of lung inflammation is not clear. Previous studies have shown that SLPI modulates the IgE production.[37] We found higher levels of IgE in the plasma of OVA-challenged SLPI KO mice, while animals constitutively expressing SLPI showed the opposite phenomenon. These findings are consistent with the other studies showing an increase in B cell proliferation and immunoglobulin production in SLPI KO mice.[38] Our data also show that SLPI transgenic mice display lower inflammatory cell infiltration in the lung compared to control mice, and the opposite phenomenon was observed in KO mice under the same conditions. The milder inflammatory response found in mice where SLPI expression was not ablated, confirms the effect of SLPI in controlling inflammation in this acute asthma model. These results support the work of Wright *et al.,* who reported, that leukocyte influx into the airway could be inhibited by administration of intratracheal SLPI in guinea pigs after allergen sensitization and challenge.[2]

Overall, the studies described in the Chapter 2 of this thesis provide important information about a reliable assessment of airway responsiveness in mouse models and the effect in the results after reexposure to methacholine. Furthermore, the studies described in the Chapter 3, show novel data about the genetics underlying AHR and propose some candidate genes associated with this phenotype. Finally, the studies described in the chapter 4 demonstrate the role of SLPI in the in allergic asthma. Taken together, all the studies presented in this thesis offer novel information about the genetics of AHR that might be directly associated with the development and severity of asthma; providing fundamental knowledge in the understanding of the mechanisms of asthma and potential target in the development of new treatments.

Claims to originality

- The thesis author demonstrates that repetitive challenges with methacholine increase Penh and airway resistance values using both naïve C57BL/6J and A/J strains of mice.

- The thesis author demonstrates that there is good correlation between Penh and airway resistance in the assessment of airway responsiveness using both naïve C57BL/6J and A/J strains of mice.

- The thesis author demonstrates that to obtain an accurate reassessment of airway responsiveness to methacholine in mouse models, a rest period of 15 days or more between exposures is highly advisable.

- The thesis author is responsible for the genetic dissection of airway hyperresponsivenes in F2 crosses generated from recombinant congenic strains.

- The thesis author identifiifies 9 novel candidate genes associated with airway hyperresponsivenes, which may also contribute to regulation of allergic asthma susceptibility.

- The thesis author demonstrates for the first time, the protective role of constitutive expression of SLPI in acute allergic asthma model using SLPI knockout and transgenic mice.

Reference List

1. Myers, T. R. 2006. Use of heliox in children. *Respir.Care.* 51:619-631.

2. Blais, L., M. F. Beauchesne, and S. Levesque. 2006. Socioeconomic status and medication prescription patterns in pediatric asthma in Canada. *J.Adolesc.Health.* 38:607-616.

3. Brown, R., F. Turk, P. Dale, and J. Bousquet. 2007. Cost-effectiveness of omalizumab in patients with severe persistent allergic asthma. *Allergy.* 62:149-153.

4. Lucas, S. R. and T. A. Platts-Mills. 2005. Physical activity and exercise in asthma: relevance to etiology and treatment. *J.Allergy Clin.Immunol.* 115:928-934.

5. Toelle, B. G. and G. B. Marks. 2005. The ebb and flow of asthma. *Thorax.* 60:87-88.

6. Burney, P. 2002. The changing prevalence of asthma? *Thorax.* 57 Suppl 2:II36-II39.:II36-II39.

7. Wright, C. D., A. M. Havill, S. C. Middleton, M. A. Kashem, P. A. Lee, D. J. Dripps, T. G. O'Riordan, M. P. Bevilacqua, and W. M. Abraham. 1999. Secretory leukocyte protease inhibitor prevents allergen-induced pulmonary responses in animal models of asthma. *J.Pharmacol.Exp.Ther.* 289:1007-1014.

8. Bandi, V., S. Velamuri, C. Sirgi, J. Wendt, R. Wendt, and K. Guntupalli. 2005. Deposition pattern of heliox-driven bronchodilator aerosol in the airways of stable asthmatics. *J.Asthma.* 42:583-586.

9. Takaku, Y., K. Nakagome, T. Kobayashi, T. Yamaguchi, F. Nishihara, T. Soma, K. Hagiwara, M. Kanazawa, and M. Nagata. 2010. Changes in airway inflammation and hyperresponsiveness after inhaled corticosteroid cessation in allergic asthma. *Int.Arch.Allergy Immunol.* 152 Suppl 1:41-6. Epub;%2010 Jun 4.:41-46.

10. Singh, R. K., S. Gupta, S. Dastidar, and A. Ray. 2010. Cysteinyl Leukotrienes and Their Receptors: Molecular and Functional Characteristics. *Pharmacology.* 85:336-349.

11. Boulay, M. E., E. Duchesneau, E. Jacques, J. Chakir, and L. P. Boulet. 2010. CysLT(1)-R expression following allergen provocation in asthma and allergic rhinitis. *Prostaglandins Leukot.Essent.Fatty Acids.*

12. Edwards, M. R., J. Haas, R. A. Panettieri, Jr., M. Johnson, and S. L. Johnston. 2007. Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. *J.Biol.Chem.* 282:15366-15375.

13. Mistry, N. N., Y. Qi, L. W. Hedlund, and G. A. Johnson. 2010. Ventilation/perfusion imaging in a rat model of airway obstruction. *Magn Reson.Med.* 63:728-735.

14. O'Callaghan, C., J. White, J. Jackson, D. Crosby, B. Dougill, and H. Bland. 2007. The effects of Heliox on the output and particle-size distribution of salbutamol using jet and vibrating mesh nebulizers. *J.Aerosol Med.* 20:434-444.

15. Burns, S. M. 2006. Ventilating patients with acute severe asthma: what do we really know? *AACN.Adv.Crit Care.* 17:186-193.

16. Restrepo, R. D., R. Pettignano, and P. DeMeuse. 2005. Halothane, an effective infrequently used drug, in the treatment of pediatric status asthmaticus: a case report. *J.Asthma.* 42:649-651.

17. Hayashi, M., S. G. Penheiter, T. Nakayama, A. R. Penheiter, D. O. Warner, and K. A. Jones. 2006. Halothane does not inhibit the functional coupling between the beta2-adrenergic receptor and the Galphas heterotrimeric G protein. *Anesthesiology.* 104:754-762.

18. Cooper, M. K. and S. T. Bateman. 2007. Cisatracurium in "weakening doses" assists in weaning from sedation and withdrawal following extended use of inhaled isoflurane. *Pediatr.Crit Care Med.* 8:58-60.

19. Shankar, V., K. B. Churchwell, and J. K. Deshpande. 2006. Isoflurane therapy for severe refractory status asthmaticus in children. *Intensive Care Med.* 32:927-933.

20. Fritscher, L. and K. R. Chapman. 2009. Omalizumab for asthma: pharmacology and clinical profile. *Expert.Rev.Respir.Med.* 3:119-127.

21. O'Byrne, P. M. and M. D. Inman. 2003. Airway hyperresponsiveness. *Chest.* 123:411S-416S.

22. Camateros, P., R. Marino, A. Fortin, J. G. Martin, E. Skamene, R. Sladek, and D. Radzioch. 2009. Identification of novel chromosomal regions associated with airway hyperresponsiveness in recombinant congenic strains of mice. *Mamm.Genome.*

23. Jonasson, S., G. Hedenstierna, H. Hedenstrom, and J. Hjoberg. 2009. Comparisons of effects of intravenous and inhaled methacholine on airway physiology in a murine asthma model. *Respir.Physiol Neurobiol.* 165:229-236.

24. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am.J.Respir.Crit Care Med.* 156:766-775.

25. Adler, A., G. Cieslewicz, and C. G. Irvin. 2004. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J.Appl.Physiol.* 97:286-292.

26. Mehta, A. K., S. N. Gaur, N. Arora, and B. P. Singh. 2007. Effect of choline chloride in allergen-induced mouse model of airway inflammation. *Eur.Respir.J.* 30:662-671.

27. Zhang, Q., K. Lai, J. Xie, G. Chen, and N. Zhong. 2009. Does unrestrained single-chamber plethysmography provide a valid assessment of airway responsiveness in allergic BALB/c mice? *Respir.Res.* 10:61.:61.

28. Hoymann, H. G. 2007. Invasive and noninvasive lung function measurements in rodents. *J.Pharmacol.Toxicol.Methods.* 55:16-26.

29. Petak, F., W. Habre, Y. R. Donati, Z. Hantos, and C. Barazzone-Argiroffo. 2001. Hyperoxia-induced changes in mouse lung mechanics: forced oscillations vs. barometric plethysmography. *J.Appl.Physiol.* 90:2221-2230.

30. Busse, W. W. and R. F. Lemanske, Jr. 2001. Asthma. *N.Engl.J.Med.* 344:350-362.

31. Burburan, S. M., D. G. Xisto, and P. R. Rocco. 2007. Anaesthetic management in asthma. *Minerva Anestesiol.* 73:357-365.

32. Sorkness, R. L., E. R. Bleecker, W. W. Busse, W. J. Calhoun, M. Castro, K. F. Chung, D. Curran-Everett, S. C. Erzurum, B. M. Gaston, E. Israel, N. N. Jarjour, W. C. Moore, S. P. Peters, W. G. Teague, and S. E. Wenzel. 2008. Lung function in adults with stable but severe asthma: air trapping and incomplete reversal of obstruction with bronchodilation. *J.Appl.Physiol.* 104:394-403.

33. Borish, L. and J. A. Culp. 2008. Asthma: a syndrome composed of heterogeneous diseases. *Ann.Allergy Asthma Immunol.* 101:1-8.

34. Cote, J., A. Cartier, P. Robichaud, H. Boutin, J. L. Malo, M. Rouleau, A. Fillion, M. Lavallee, M. Krusky, and L. P. Boulet. 1997. Influence on asthma morbidity of asthma education programs based on self-management plans following treatment optimization. *Am.J.Respir.Crit Care Med.* 155:1509-1514.

35. Dahl, R., B. B. Larsen, and P. Venge. 2002. Effect of long-term treatment with inhaled budesonide or theophylline on lung function, airway reactivity and asthma symptoms. *Respir.Med.* 96:432-438.

36. Van, S. D. 2010. The next generation of public health approaches to asthma in Asia and the Middle East. *Asia Pac.J.Public Health.* 22:229S-235S.

37. Vignola, A. M., P. Chanez, A. M. Campbell, F. Souques, B. Lebel, I. Enander, and J. Bousquet. 1998. Airway inflammation in mild intermittent and in persistent asthma. *Am.J.Respir.Crit Care Med.* 157:403-409.

38. Chtanova, T. and C. R. Mackay. 2001. T cell effector subsets: extending the Th1/Th2 paradigm. *Adv.Immunol.* 78:233-66.:233-266.

39. Di, L. G., P. Mansueto, M. Esposito-Pellitteri, V. Ditta, F. Castello, B. C. Lo, M. S. Leto-Barone, F. G. Di, M. Traverso, G. Rotolo, S. Vigneri, and G. Rini. 2007. The characteristics of different diagnostic tests in adult mild asthmatic patients: comparison with patients with asthma-like symptoms by gastro-oesophageal reflux. *Respir.Med.* 101:1455-1461.

40. Deykin, A. 2006. Biomarker-driven care in asthma: are we there? *J.Allergy Clin.Immunol.* 118:565-568.

41. Tug, T., A. Godekmerdan, N. Sari, F. Karatas, and E. S. Erdem. 2007. Effects of supportive treatment such as antioxidant or leukotriene receptor antagonist drugs on inflammatory and respiratory parameters in asthma patients. *Clin.Pharmacol.Ther.* 81:371-376.

42. Wilson, J. W. and T. L. Bamford. 2001. Assessing the evidence for remodelling of the airway in asthma. *Pulm.Pharmacol.Ther.* 14:229-247.

43. Kato, M., M. Suzuki, Y. Hayashi, and H. Kimura. 2006. Role of eosinophils and their clinical significance in allergic inflammation. *Expert.Rev.Clin.Immunol.* 2:121-133.

44. Venge, P., J. Bystrom, M. Carlson, L. Hakansson, M. Karawacjzyk, C. Peterson, L. Seveus, and A. Trulson. 1999. Eosinophil cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. *Clin.Exp.Allergy.* 29:1172-1186.

45. Zagai, U., C. M. Skold, A. Trulson, P. Venge, and J. Lundahl. 2004. The effect of eosinophils on collagen gel contraction and implications for tissue remodelling. *Clin.Exp.Immunol.* 135:427-433.

46. Ferguson, A. C., R. Vaughan, H. Brown, and C. Curtis. 1995. Evaluation of serum eosinophilic cationic protein as a marker of disease activity in chronic asthma. *J.Allergy Clin.Immunol.* 95:23-28.

47. Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson, and A. B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J.Clin.Invest.* 112:1029-1036.

48. Oymar, K., S. Elsayed, and R. Bjerknes. 1996. Serum eosinophil cationic protein and interleukin-5 in children with bronchial asthma and acute bronchiolitis. *Pediatr.Allergy Immunol.* 7:180-186.

49. Tang, R. B. and S. J. Chen. 2001. Serum levels of eosinophil cationic protein and eosinophils in asthmatic children during a course of prednisolone therapy. *Pediatr.Pulmonol.* 31:121-125.

50. Venge, P. 2004. Monitoring the allergic inflammation. *Allergy.* 59:26-32.

51. Zagai, U., J. Lundahl, J. Klominek, P. Venge, and C. M. Skold. 2009. Eosinophil cationic protein stimulates migration of human lung fibroblasts in vitro. *Scand.J.Immunol.* 69:381-386.

52. Shome, G. P., J. D. Starnes, III, M. Shearer, R. Kennedy, A. Way, A. Arif, and S. Prabhakar. 2006. Exhaled nitric oxide in asthma: variability, relation to asthma severity, and peripheral blood lymphocyte cytokine expression. *J.Asthma.* 43:95-99.

53. Menzies, D., A. Nair, and B. J. Lipworth. 2006. Non-invasive measurement of airway inflammation in asthma. *J.Asthma.* 43:407-415.

54. Lex, C., F. Ferreira, A. Zacharasiewicz, A. G. Nicholson, P. L. Haslam, N. M. Wilson, T. T. Hansel, D. N. Payne, and A. Bush. 2006. Airway eosinophilia in children with severe asthma: predictive values of noninvasive tests. *Am.J.Respir.Crit Care Med.* 174:1286-1291.

55. Smith, A. D., J. O. Cowan, K. P. Brassett, G. P. Herbison, and D. R. Taylor. 2005. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. *N.Engl.J.Med.* 352:2163-2173.

56. Menzies, D., A. Nair, and B. J. Lipworth. 2007. Portable exhaled nitric oxide measurement: Comparison with the "gold standard" technique. *Chest.* 131:410-414.

57. Nordvall, S. L., C. Janson, P. Kalm-Stephens, T. Foucard, K. Toren, and K. Alving. 2005. Exhaled nitric oxide in a population-based study of asthma and allergy in schoolchildren. *Allergy.* 60:469-475.

58. Malik, G. and S. W. Turner. 2007. Is the "instant flow" option on the NIOX analyser needed? *Med.Eng Phys.* 29:72-75.

59. Foley, S. C., N. O. Hopkins, M. X. Fitzgerald, S. C. Donnelly, and P. McLoughlin. 2007. Airway nitric oxide output is reduced in bronchiectasis. *Respir.Med.* ..

60. Lemiere, C., P. Ernst, R. Olivenstein, Y. Yamauchi, K. Govindaraju, M. S. Ludwig, J. G. Martin, and Q. Hamid. 2006. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. *J.Allergy Clin.Immunol.* 118:1033-1039.

61. Senna, G., G. Passalacqua, M. Schiappoli, C. Lombardi, and L. Wilcock. 2007. Correlation among FEV, nitric oxide and asthma control test in newly diagnosed asthma. *Allergy.* 62:207-208.

62. Craig, T. J. 2010. Aeroallergen sensitization in asthma: prevalence and correlation with severity. *Allergy Asthma Proc.* 31:96-102.

63. Ten, B. R., C. R. De, H. R. Van, V. Verweij, T. Leusink-Muis, A. Van, I, C. F. De, F. P. Nijkamp, and G. Folkerts. 2006. Overexpression of endothelial nitric oxide synthase suppresses features of allergic asthma in mice. *Respir.Res.* 7:58.:58.

64. Gaston, B., R. Kelly, P. Urban, L. Liu, E. M. Henderson, A. Doctor, W. G. Teague, A. Fitzpatrick, S. Erzurum, and J. F. Hunt. 2006. Buffering airway acid decreases exhaled nitric oxide in asthma. *J.Allergy Clin.Immunol.* 118:817-822.

65. Toungoussova, O., G. B. Migliori, M. P. Foschino Barbaro, L. M. Esposito, S. Dragonieri, G. E. Carpagnano, F. G. Salerno, M. Neri, and A. Spanevello. 2007. Changes in sputum composition during 15 min of sputum induction in healthy subjects and patients with asthma and chronic obstructive pulmonary disease. *Respir.Med.* 101:1543-1548.

66. van, d., V, A. J. Roebroek, N. H. Senden, F. C. Ramaekers, and d. van, V. 1994. NSP-encoded reticulons, neuroendocrine proteins of a novel gene family associated with membranes of the endoplasmic reticulum. *J.Cell Sci.* 107:2403-2416.

67. Belda, J., R. Leigh, K. Parameswaran, P. M. O'Byrne, M. R. Sears, and F. E. Hargreave. 2000. Induced sputum cell counts in healthy adults. *Am.J.Respir.Crit Care Med.* 161:475-478.

68. Simpson, J. L., R. Scott, M. J. Boyle, and P. G. Gibson. 2006. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology.* 11:54-61.

69. Spanevello, A., M. Confalonieri, F. Sulotto, F. Romano, G. Balzano, G. B. Migliori, A. Bianchi, and G. Michetti. 2000. Induced sputum cellularity. Reference values and distribution in normal volunteers. *Am.J.Respir.Crit Care Med.* 162:1172-1174.

70. Hunter, C. J., C. E. Brightling, G. Woltmann, A. J. Wardlaw, and I. D. Pavord. 2002. A comparison of the validity of different diagnostic tests in adults with asthma. *Chest.* 121:1051-1057.

71. Piacentini, G. L., D. Peroni, E. Crestani, F. Zardini, A. Bodini, S. Costella, and A. L. Boner. 2007. Exhaled air temperature in asthma: methods and relationship with markers of disease. *Clin.Exp.Allergy.* 37:415-419.

72. Koopmans, J. G., R. Lutter, H. M. Jansen, and J. S. van der Zee. 2005. Adding salmeterol to an inhaled corticosteroid reduces allergen-induced serum IL-5 and peripheral blood eosinophils. *J.Allergy Clin.Immunol.* 116:1007-1013.

73. Fahy, J. V., H. A. Boushey, S. C. Lazarus, E. A. Mauger, R. M. Cherniack, V. M. Chinchilli, T. J. Craig, J. M. Drazen, J. G. Ford, J. E. Fish, E. Israel, M. Kraft, R. F. Lemanske, R. J. Martin, D. McLean, S. P. Peters, C. Sorkness, and S. J. Szefler. 2001. Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. *Am.J.Respir.Crit Care Med.* 163:1470-1475.

74. Bacci, E., S. Cianchetti, M. Bartoli, F. L. Dente, F. A. Di, B. Vagaggini, and P. Paggiaro. 2006. Low sputum eosinophils predict the lack of response to beclomethasone in symptomatic asthmatic patients. *Chest.* 129:565-572.

75. Currie, G. P., N. J. Syme-Grant, L. C. McFarlane, F. A. Carey, and B. J. Lipworth. 2003. Effects of low dose fluticasone/salmeterol combination on surrogate inflammatory markers in moderate persistent asthma. *Allergy.* 58:602-607.

76. Wilson, N. M., P. Bridge, A. Spanevello, and M. Silverman. 2000. Induced sputum in children: feasibility, repeatability, and relation of findings to asthma severity. *Thorax.* 55:768-774.

77. Efthimiadis, A., L. Jayaram, S. Weston, S. Carruthers, and F. E. Hargreave. 2002. Induced sputum: time from expectoration to processing. *Eur.Respir.J.* 19:706-708.

78. van, d., V, D. S. Postma, W. Timens, H. F. Kauffman, M. N. Hylkema, and N. H. Ten Hacken. 2006. Repeated sputum inductions induce a transient neutrophilic and eosinophilic response. *Chest.* 130:1157-1164.

79. Adachi, T., S. Motojima, A. Hirata, T. Fukuda, and S. Makino. 1995. Eosinophil viability-enhancing activity in sputum from patients with bronchial asthma, Contributions of interleukin-5 and granulocyte/macrophage colony-stimulating factor. *Am.J.Respir.Crit Care Med.* 151:618-623.

80. Vargaftig, B. B. 1997. Modifications of experimental bronchopulmonary hyperresponsiveness. *Am.J.Respir.Crit Care Med.* 156:S97-102.

81. Kabesch, M., M. Depner, I. Dahmen, S. K. Weiland, C. Vogelberg, B. Niggemann, S. Lau, T. Illig, N. Klopp, U. Wahn, D. Reinhardt, M. E. von, and R. Nickel. 2007. Polymorphisms in eosinophil pathway genes, asthma and atopy. *Allergy.* 62:423-428.

82. Simon, H. U., S. Yousefi, C. Schranz, A. Schapowal, C. Bachert, and K. Blaser. 1997. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J.Immunol.* 158:3902-3908.

83. Xu, J., F. Jiang, F. Nayeri, and O. Zetterstrom. 2007. Apoptotic eosinophils in sputum from asthmatic patients correlate negatively with levels of IL-5 and eotaxin. *Respir.Med.* 101:1447-1454.

84. Ahmad, F. and J. Roman. 2009. Treating refractory asthma with antibodies against IL-5: is it ready for prime time? *Expert.Rev.Respir.Med.* 3:227-230.

85. Justa, S., R. W. Minz, M. Minz, A. Sharma, N. Pasricha, S. Anand, Y. K. Chawla, and V. K. Sakhuja. 2010. Cellular Immune Response and Cytokine Profile Among Hepatitis-C Positive Living Donor Renal Transplant Recipients. *Transplantation.*

86. Suzuki, Y., T. Nakamura, M. Tokoro, T. Togano, M. Ohsaka, M. Kohri, Y. Hirata, K. Miyazaki, M. Danbara, R. Horie, I. Miura, K. Sunakawa, and M. Higashihara. 2010. A case

of giardiasis expressing severe systemic symptoms and marked hypereosinophilia. *Parasitol.Int.*

87. Harding, S. M. 2003. Recent clinical investigations examining the association of asthma and gastroesophageal reflux. *Am.J.Med.* 115 Suppl 3A:39S-44S.:39S-44S.

88. Canning, B. J., D. G. Farmer, and N. Mori. 2006. Mechanistic studies of acid-evoked coughing in anesthetized guinea pigs. *Am.J.Physiol Regul.Integr.Comp Physiol.* 291:R454-R463.

89. Rahman, M. S., A. Yamasaki, J. Yang, L. Shan, A. J. Halayko, and A. S. Gounni. 2006. IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J.Immunol.* 177:4064-4071.

90. Main, S., R. Handy, J. Wilton, S. Smith, L. Williams, L. D. Fou, J. Andrews, L. A. Conroy, R. May, I. Anderson, and T. J. Vaughan. 2006. A potent human anti-eotaxin1 antibody, CAT-213: isolation by phage display and in vitro and in vivo efficacy. *J.Pharmacol.Exp.Ther.* 319:1395-1404.

91. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J.Exp.Med.* 179:881-887.

92. Giembycz, M. A. and M. A. Lindsay. 1999. Pharmacology of the eosinophil. *Pharmacol.Rev.* 51:213-340.

93. Heinemann, A., A. Hartnell, V. E. Stubbs, K. Murakami, D. Soler, G. LaRosa, P. W. Askenase, T. J. Williams, and I. Sabroe. 2000. Basophil responses to chemokines are regulated by both sequential and cooperative receptor signaling. *J.Immunol.* 165:7224-7233.

94. Beaulieu, S., D. F. Robbiani, X. Du, E. Rodrigues, R. Ignatius, Y. Wei, P. Ponath, J. W. Young, M. Pope, R. M. Steinman, and S. Mojsov. 2002. Expression of a functional eotaxin (CC chemokine ligand 11) receptor CCR3 by human dendritic cells. *J.Immunol.* 169:2925-2936.

95. Conroy, D. M. and T. J. Williams. 2001. Eotaxin and the attraction of eosinophils to the asthmatic lung. *Respir.Res.* 2:150-156.

96. Hadjicharalambous, C., G. Dent, R. D. May, R. L. Handy, I. K. Anderson, D. E. Davies, and R. Djukanovic. 2004. Measurement of eotaxin (CCL11) in induced sputum supernatants: validation and detection in asthma. *J.Allergy Clin.Immunol.* 113:657-662.

97. Nakamura, H., S. T. Weiss, E. Israel, A. D. Luster, J. M. Drazen, and C. M. Lilly. 1999. Eotaxin and impaired lung function in asthma. *Am.J.Respir.Crit Care Med.* 160:1952-1956.

98. Laporte, J. C., P. E. Moore, S. Baraldo, M. H. Jouvin, T. L. Church, I. N. Schwartzman, R. A. Panettieri, Jr., J. P. Kinet, and S. A. Shore. 2001. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am.J.Respir.Crit Care Med.* 164:141-148.

99. Gonzalo, J. A., C. M. Lloyd, L. Kremer, E. Finger, A. Martinez, M. H. Siegelman, M. Cybulsky, and J. C. Gutierrez-Ramos. 1996. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J.Clin.Invest.* 98:2332-2345.

100. Hamilton, R. G. 2010. Proficiency survey-based evaluation of clinical total and allergen-specific IgE assay performance. *Arch.Pathol.Lab Med.* 134:975-982.

101. Bousquet, J., K. Rabe, M. Humbert, K. F. Chung, W. Berger, H. Fox, G. Ayre, H. Chen, K. Thomas, M. Blogg, and S. Holgate. 2007. Predicting and evaluating response to omalizumab in patients with severe allergic asthma. *Respir.Med.* 101:1483-1492.

102. Liebhaber, M. and Z. Dyer. 2007. Home therapy with subcutaneous anti-immunoglobulin-E antibody omalizumab in 25 patients with immunoglobulin-E-mediated (allergic) asthma. *J.Asthma.* 44:195-196.

103. Leckie, M. J., B. A. ten, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk, and P. J. Barnes. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet.* 356:2144-2148.

104. Schwartz, L. B., J. Sheikh, and A. Singh. 2010. Current strategies in the management of hypereosinophilic syndrome, including mepolizumab. *Curr.Med.Res.Opin.* 26:1933-1946.

105. Flood-Page, P. T., A. N. Menzies-Gow, A. B. Kay, and D. S. Robinson. 2003. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am.J.Respir.Crit Care Med.* 167:199-204.

106. Murphy, D. M. and P. M. O'Byrne. 2010. Recent advances in the pathophysiology of asthma. *Chest.* 137:1417-1426.

107. Loza, M. J. and B. L. Chang. 2007. Association between Q551R IL4R genetic variants and atopic asthma risk demonstrated by meta-analysis. *J.Allergy Clin.Immunol.* 120:578-585.

108. Finkelman, F. D. and D. Vercelli. 2007. Advances in asthma, allergy mechanisms, and genetics in 2006. *J.Allergy Clin.Immunol.* 120:544-550.

109. Montuschi, P., A. Sala, S. E. Dahlen, and G. Folco. 2007. Pharmacological modulation of the leukotriene pathway in allergic airway disease. *Drug Discov.Today.* 12:404-412.

110. Barnes, P. J. 2006. Transcription factors in airway diseases. *Lab Invest.* 86:867-872.

111. Matsui, E. C., H. A. Sampson, H. T. Bahnson, R. S. Gruchalla, J. A. Pongracic, S. J. Teach, P. J. Gergen, G. R. Bloomberg, J. F. Chmiel, A. H. Liu, M. Kattan, C. A. Sorkness, S. F. Steinbach, R. E. Story, and C. M. Visness. 2010. Allergen-specific IgE as a biomarker of exposure plus sensitization in inner-city adolescents with asthma. *Allergy.*

112. Strachan, D. P., H. J. Wong, and T. D. Spector. 2001. Concordance and interrelationship of atopic diseases and markers of allergic sensitization among adult female twins. *J.Allergy Clin.Immunol.* 108:901-907.

113. Godfrey, S. 2001. Independent inheritance of serum immunoglobulin E concentrations and airway responsiveness. *Am.J.Respir.Crit Care Med.* 163:1030.

114. Lee, J. H., L. C. Wang, H. H. Yu, Y. T. Lin, Y. H. Yang, and B. L. Chiang. 2010. Type I IL-1 Receptor (IL-1RI) as Potential New Therapeutic Target for Bronchial Asthma. *Mediators.Inflamm.* 2010:567351. Epub;%2010 Jul 5.:567351.

115. Sarinho, E. and A. A. Cruz. 2006. Anti-IgE monoclonal antibody for the treatment of the asthma and other manifestations related to allergic diseases. *J.Pediatr.(Rio J.).* 82:S127-S132.

116. Lee, J., R. Doggweiler-Wiygul, S. Kim, B. D. Hill, and T. J. Yoo. 2006. Is interstitial cystitis an allergic disorder?: A case of interstitial cystitis treated successfully with anti-IgE. *Int.J.Urol.* 13:631-634.

117. Lilly, C. M. 2005. Diversity of asthma: evolving concepts of pathophysiology and lessons from genetics. *J.Allergy Clin.Immunol.* 115:S526-S531.

118. Campbell, E. M., S. L. Kunkel, R. M. Strieter, and N. W. Lukacs. 1998. Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. *J.Immunol.* 161:7047-7053.

119. Crapo, R. O., R. Casaburi, A. L. Coates, P. L. Enright, J. L. Hankinson, C. G. Irvin, N. R. MacIntyre, R. T. McKay, J. S. Wanger, S. D. Anderson, D. W. Cockcroft, J. E. Fish, and P. J. Sterk. 2000. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am.J.Respir.Crit Care Med.* 161:309-329.

120. Palmer, L. J., P. J. Rye, N. A. Gibson, P. R. Burton, L. I. Landau, and P. N. Lesouef. 2001. Airway responsiveness in early infancy predicts asthma, lung function, and respiratory symptoms by school age. *Am.J.Respir.Crit Care Med.* 163:37-42.

121. Gronke, L., F. Kanniess, O. Holz, R. A. Jorres, and H. Magnussen. 2002. The relationship between airway hyper-responsiveness, markers of inflammation and lung function depends on the duration of the asthmatic disease. *Clin.Exp.Allergy.* 32:57-63.

122. Carey, V. J., S. T. Weiss, I. B. Tager, S. R. Leeder, and F. E. Speizer. 1996. Airways responsiveness, wheeze onset, and recurrent asthma episodes in young adolescents. The East Boston Childhood Respiratory Disease Cohort. *Am.J.Respir.Crit Care Med.* 153:356-361.

123. Townley, R. G., A. Bewtra, A. F. Wilson, R. J. Hopp, R. C. Elston, N. Nair, and G. D. Watt. 1986. Segregation analysis of bronchial response to methacholine inhalation challenge in families with and without asthma. *J.Allergy Clin.Immunol.* 77:101-107.

124. Postma, D. S., E. R. Bleecker, P. J. Amelung, K. J. Holroyd, J. Xu, C. I. Panhuysen, D. A. Meyers, and R. C. Levitt. 1995. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. *N.Engl.J.Med.* 333:894-900.

125. van, H. L., S. B. Harrap, Z. Y. Wong, M. J. Abramson, J. J. Kutin, A. B. Forbes, J. Raven, A. Lanigan, and E. H. Walters. 1995. Linkage of high-affinity IgE receptor gene with bronchial hyperreactivity, even in absence of atopy. *Lancet.* 346:1262-1265.

126. Slats, A. M., K. Janssen, S. A. van, D. T. van der Plas, R. Schot, J. G. van den Aardweg, J. C. de Jongste, P. S. Hiemstra, T. Mauad, K. F. Rabe, and P. J. Sterk. 2007. Bronchial inflammation and airway responses to deep inspiration in asthma and chronic obstructive pulmonary disease. *Am.J.Respir.Crit Care Med.* 176:121-128.

127. Hopp, R. J., R. G. Townley, R. E. Biven, A. K. Bewtra, and N. M. Nair. 1990. The presence of airway reactivity before the development of asthma. *Am.Rev.Respir.Dis.* 141:2-8.

128. Longo, G., R. Strinati, F. Poli, and F. Fumi. 1987. Genetic factors in nonspecific bronchial hyperreactivity. An epidemiologic study. *Am.J.Dis.Child.* 141:331-334.

129. Tillie-Leblond, I., P. Gosset, and A. B. Tonnel. 2005. Inflammatory events in severe acute asthma. *Allergy.* 60:23-29.

130. Black, J. L. 2004. Asthma--more muscle cells or more muscular cells? *Am.J.Respir.Crit Care Med.* 169:980-981.

131. McParland, B. E., P. T. Macklem, and P. D. Pare. 2003. Airway wall remodeling: friend or foe? *J.Appl.Physiol.* 95:426-434.

132. Wang, L., B. E. McParland, and P. D. Pare. 2003. The functional consequences of structural changes in the airways: implications for airway hyperresponsiveness in asthma. *Chest.* 123:356S-362S.

133. Storms, W. W. 2003. Review of exercise-induced asthma. *Med.Sci.Sports Exerc.* 35:1464-1470.

134. Anderson, S. D. and E. Daviskas. 2000. The mechanism of exercise-induced asthma is .. *J.Allergy Clin.Immunol.* 106:453-459.

135. Anderson, S. D. and K. Holzer. 2000. Exercise-induced asthma: is it the right diagnosis in elite athletes? *J.Allergy Clin.Immunol.* 106:419-428.

136. Ewart, S. L., D. Kuperman, E. Schadt, C. Tankersley, A. Grupe, D. M. Shubitowski, G. Peltz, and M. Wills-Karp. 2000. Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. *Am.J.Respir.Cell Mol.Biol.* 23:537-545.

137. Hogman, M., A. C. Mork, and G. M. Roomans. 2002. Hypertonic saline increases tight junction permeability in airway epithelium. *Eur.Respir.J.* 20:1444-1448.

138. Paredi, P., S. A. Kharitonov, and P. J. Barnes. 2002. Faster rise of exhaled breath temperature in asthma: a novel marker of airway inflammation? *Am.J.Respir.Crit Care Med.* 165:181-184.

139. Wills-Karp, M. and S. L. Ewart. 2004. Time to draw breath: asthma-susceptibility genes are identified. *Nat.Rev.Genet.* 5:376-387.

140. Palmer, L. J., P. R. Burton, J. A. Faux, A. L. James, A. W. Musk, and W. O. Cookson. 2000. Independent inheritance of serum immunoglobulin E concentrations and airway responsiveness. *Am.J.Respir.Crit Care Med.* 161:1836-1843.

141. Zosky, G. R. and P. D. Sly. 2007. Animal models of asthma. *Clin.Exp.Allergy.* 37:973-988.

142. Holloway, J. W., B. Beghe, and S. T. Holgate. 1999. The genetic basis of atopic asthma. *Clin.Exp.Allergy.* 29:1023-1032.

143. Huang, C. H., Y. T. Chu, C. H. Kuo, W. L. Wang, Y. M. Hua, M. S. Lee, and C. H. Hung. 2010. Effect of procaterol on Th2-related chemokines production in human monocyte and bronchial epithelial cells. *Pediatr.Pulmonol.*

144. Chen, L., K. Lai, J. Xie, and N. Zhong. 2010. Establishment of airway eosinophilic bronchitis mouse model without hyperresponsiveness by ovalbumin. *Clin.Exp.Med.*

145. Cookson, W. 1999. The alliance of genes and environment in asthma and allergy. *Nature.* 402:B5-11.

146. Lemiere, C. 2004. The use of sputum eosinophils in the evaluation of occupational asthma. *Curr.Opin.Allergy Clin.Immunol.* 4:81-85.

147. Celedon, J. C., D. K. Milton, C. D. Ramsey, A. A. Litonjua, L. Ryan, T. A. Platts-Mills, and D. R. Gold. 2007. Exposure to dust mite allergen and endotoxin in early life and asthma and atopy in childhood. *J.Allergy Clin.Immunol.* 120:144-149.

148. Moffatt, M. F. 2008. Genes in asthma: new genes and new ways. *Curr.Opin.Allergy Clin.Immunol.* 8:411-417.

149. Elo, L. L., H. Jarvenpaa, S. Tuomela, S. Raghav, H. Ahlfors, K. Laurila, B. Gupta, R. J. Lund, J. Tahvanainen, R. D. Hawkins, M. Oresic, H. Lahdesmaki, O. Rasool, K. V. Rao, T. Aittokallio, and R. Lahesmaa. 2010. Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. *Immunity.* 32:852-862.

150. Anagnostopoulou, P., L. Dai, J. Schatterny, S. Hirtz, J. Duerr, and M. A. Mall. 2010. Allergic airway inflammation induces a prosecretory epithelial ion transport phenotype in mice. *Eur.Respir.J.*

151. Chiba, Y., M. Todoroki, and M. Misawa. 2010. Antigen exposure causes activations of signal transducer and activator of transcription 6 (STAT6) and STAT1, but not STAT3, in lungs of sensitized mice. *Immunopharmacol.Immunotoxicol.*

152. Kim, K., N. Suzuki, K. Ohneda, N. Minegishi, and M. Yamamoto. 2010. Fractionation of mature eosinophils in GATA-reporter transgenic mice. *Tohoku J.Exp.Med.* 220:127-138.

153. Weidinger, S., C. Gieger, E. Rodriguez, H. Baurecht, M. Mempel, N. Klopp, H. Gohlke, S. Wagenpfeil, M. Ollert, J. Ring, H. Behrendt, J. Heinrich, N. Novak, T. Bieber, U. Kramer, D. Berdel, B. A. von, C. P. Bauer, O. Herbarth, S. Koletzko, H. Prokisch, D. Mehta, T. Meitinger, M. Depner, M. E. von, L. Liang, M. Moffatt, W. Cookson, M. Kabesch, H. E. Wichmann, and T. Illig. 2008. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. *PLoS.Genet.* 4:e1000166.

154. Gudbjartsson, D. F., U. S. Bjornsdottir, E. Halapi, A. Helgadottir, P. Sulem, G. M. Jonsdottir, G. Thorleifsson, H. Helgadottir, V. Steinthorsdottir, H. Stefansson, C. Williams, J. Hui, J. Beilby, N. M. Warrington, A. James, L. J. Palmer, G. H. Koppelman, A. Heinzmann, M. Krueger, H. M. Boezen, A. Wheatley, J. Altmuller, H. D. Shin, S. T. Uh, H. S. Cheong, B. Jonsdottir, D. Gislason, C. S. Park, L. M. Rasmussen, C. Porsbjerg, J. W. Hansen, V. Backer, T. Werge, C. Janson, U. B. Jonsson, M. C. Ng, J. Chan, W. Y. So, R. Ma, S. H. Shah, C. B. Granger, A. A. Quyyumi, A. I. Levey, V. Vaccarino, M. P. Reilly, D. J. Rader, M. J. Williams, A. M. van Rij, G. T. Jones, E. Trabetti, G. Malerba, P. F. Pignatti, A. Boner, L. Pescollderungg, D. Girelli, O. Olivieri, N. Martinelli, B. R. Ludviksson, D. Ludviksdottir, G. I. Eyjolfsson, D. Arnar, G. Thorgeirsson, K. Deichmann, P. J. Thompson, M. Wjst, I. P. Hall, D. S. Postma, T. Gislason, J. Gulcher, A. Kong, I. Jonsdottir, U. Thorsteinsdottir, and K. Stefansson. 2009. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat.Genet.* 41:342-347.

155. Balaci, L., M. C. Spada, N. Olla, G. Sole, L. Loddo, F. Anedda, S. Naitza, M. A. Zuncheddu, A. Maschio, D. Altea, M. Uda, S. Pilia, S. Sanna, M. Masala, L. Crisponi, M. Fattori, M. Devoto, S. Doratiotto, S. Rassu, S. Mereu, E. Giua, N. G. Cadeddu, R. Atzeni, U. Pelosi, A. Corrias, R. Perra, P. L. Torrazza, P. Pirina, F. Ginesu, S. Marcias, M. G. Schintu, G. S. Del Giacco, P. E. Manconi, G. Malerba, A. Bisognin, E. Trabetti, A. Boner, L. Pescollderungg, P. F. Pignatti, D. Schlessinger, A. Cao, and G. Pilia. 2007. IRAK-M is involved in the pathogenesis of early-onset persistent asthma. *Am.J.Hum.Genet.* 80:1103-1114.

156. Fukai, H., Y. Ogasawara, O. Migita, M. Koga, K. Ichikawa, M. Shibasaki, T. Arinami, and E. Noguchi. 2004. Association between a polymorphism in cysteinyl leukotriene receptor 2 on chromosome 13q14 and atopic asthma. *Pharmacogenetics.* 14:683-690.

157. Hasegawa, K., M. Tamari, C. Shao, M. Shimizu, N. Takahashi, X. Q. Mao, A. Yamasaki, F. Kamada, S. Doi, H. Fujiwara, A. Miyatake, K. Fujita, G. Tamura, Y. Matsubara, T. Shirakawa, and Y. Suzuki. 2004. Variations in the C3, C3a receptor, and C5 genes affect susceptibility to bronchial asthma. *Hum.Genet.* 115:295-301.

158. Hirota, T., K. Obara, A. Matsuda, M. Akahoshi, K. Nakashima, K. Hasegawa, N. Takahashi, M. Shimizu, H. Sekiguchi, M. Kokubo, S. Doi, H. Fujiwara, A. Miyatake, K. Fujita, T. Enomoto, F. Kishi, Y. Suzuki, H. Saito, Y. Nakamura, T. Shirakawa, and M. Tamari. 2004. Association between genetic variation in the gene for death-associated protein-3 (DAP3) and adult asthma. *J.Hum.Genet.* 49:370-375.

159. Begin, P., K. Tremblay, D. Daley, M. Lemire, S. Claveau, C. Salesse, S. Kacel, A. Montpetit, A. Becker, M. Chan-Yeung, A. L. Kozyrskyj, T. J. Hudson, and C. Laprise. 2007. Association of urokinase-type plasminogen activator with asthma and atopy. *Am.J.Respir.Crit Care Med.* 175:1109-1116.

160. Duroudier, N. P., D. P. Strachan, J. D. Blakey, and I. P. Hall. 2009. Association of the cysteinyl leukotriene receptor 1 gene with atopy in the British 1958 birth cohort. *J.Allergy Clin.Immunol.* 124:566-72, 572.

161. Thompson, M. D., J. Takasaki, V. Capra, G. E. Rovati, K. A. Siminovitch, W. M. Burnham, T. J. Hudson, Y. Bosse, and D. E. Cole. 2006. G-protein-coupled receptors and asthma endophenotypes: the cysteinyl leukotriene system in perspective. *Mol.Diagn.Ther.* 10:353-366.

162. Thompson, M. D., M. E. Percy, B. W. McIntyre, and D. E. Cole. 2008. G protein-coupled receptors disrupted in human genetic disease. *Methods Mol.Biol.* 448:109-37.:109-137.

163. Palikhe, N. S., S. H. Kim, Y. M. Ye, G. Y. Hur, B. Y. Cho, and H. S. Park. 2009. Association of CRTH2 gene polymorphisms with the required dose of antihistamines in patients with chronic urticaria. *Pharmacogenomics.* 10:375-383.

164. Leung, T. F., C. Y. Li, A. P. Kong, I. H. Chan, M. C. Ng, M. H. Chan, W. Y. So, G. W. Wong, C. W. Lam, and J. C. Chan. 2009. PTGDR is not a major candidate gene for asthma and atopy in Chinese children. *Pediatr.Allergy Immunol.* 20:556-562.

165. Maeda, Y., N. Hizawa, D. Takahashi, Y. Fukui, S. Konno, and M. Nishimura. 2007. Genetic impact of functional single nucleotide polymorphisms in the 3'-UTR region of the chemoattractant receptor expressed on Th2 cells (CRTH2) gene on asthma and atopy in a Japanese population. *Int.Arch.Allergy Immunol.* 142:51-58.

166. Suzuki, Y., K. Asano, K. Niimi, J. Miyata, Y. Shiraishi, K. Fukunaga, T. Shiomi, T. Nakajima, T. Oguma, K. Sayama, and A. Ishizaka. 2008. TP receptor-mediated release of eosinophil chemotactic activity from human bronchial smooth muscle cells. *Eur.J.Pharmacol.* 600:133-139.

167. Kim, J. H., S. Y. Lee, H. B. Kim, H. S. Jin, J. H. Yu, B. J. Kim, B. S. Kim, M. J. Kang, S. O. Jang, and S. J. Hong. 2008. TBXA2R gene polymorphism and responsiveness to leukotriene receptor antagonist in children with asthma. *Clin.Exp.Allergy.* 38:51-59.

168. Carroll, C. L., P. Stoltz, C. M. Schramm, and A. R. Zucker. 2009. Beta2-adrenergic receptor polymorphisms affect response to treatment in children with severe asthma exacerbations. *Chest.* 135:1186-1192.

169. Berce, V., K. Repnik, and U. Potocnik. 2008. Association of CCR5-delta32 mutation with reduced risk of nonatopic asthma in Slovenian children. *J.Asthma.* 45:780-784.

170. Suzaki, Y., K. Hamada, T. Nomi, T. Ito, M. Sho, Y. Kai, Y. Nakajima, and H. Kimura. 2008. A small-molecule compound targeting CCR5 and CXCR3 prevents airway hyperresponsiveness and inflammation. *Eur.Respir.J.* 31:783-789.

171. Wu, H., I. Romieu, J. J. Sienra-Monge, B. E. del Rio-Navarro, L. Burdett, J. Yuenger, H. Li, S. J. Chanock, and S. J. London. 2008. Lack of association between genetic variation in G-protein-coupled receptor for asthma susceptibility and childhood asthma and atopy. *Genes Immun.* 9:224-230.

172. Bijanzadeh, M., N. B. Ramachandra, P. A. Mahesh, M. R. Savitha, P. Kumar, B. S. Manjunath, and B. S. Jayaraj. 2010. Association of IL-4 and ADAM33 Gene Polymorphisms with Asthma in an Indian Population. *Lung.*

173. Agrawal, D. K. and Z. Shao. 2010. Pathogenesis of allergic airway inflammation. *Curr.Allergy Asthma Rep.* 10:39-48.

174. Naus, S., M. R. Blanchet, K. Gossens, C. Zaph, J. W. Bartsch, K. M. McNagny, and H. J. Ziltener. 2010. The metalloprotease-disintegrin ADAM8 is essential for the development of experimental asthma. *Am.J.Respir.Crit Care Med.* 181:1318-1328.

175. Knolle, M. D. and C. A. Owen. 2009. ADAM8: a new therapeutic target for asthma. *Expert.Opin.Ther.Targets.* 13:523-540.

176. Foley, S. C., A. K. Mogas, R. Olivenstein, P. O. Fiset, J. Chakir, J. Bourbeau, P. Ernst, C. Lemiere, J. G. Martin, and Q. Hamid. 2007. Increased expression of ADAM33 and ADAM8 with disease progression in asthma. *J.Allergy Clin.Immunol.* 119:863-871.

177. Konno, S., M. Adachi, K. Asano, K. Okamoto, and T. Takahashi. 1993. Anti-allergic activity of roxithromycin: inhibition of interleukin-5 production from mouse T lymphocytes. *Life Sci.* 52:L25-L30.

178. De Sanctis, G. T., J. B. Singer, A. Jiao, C. N. Yandava, Y. H. Lee, T. C. Haynes, E. S. Lander, D. R. Beier, and J. M. Drazen. 1999. Quantitative trait locus mapping of airway responsiveness to chromosomes 6 and 7 in inbred mice. *Am.J.Physiol.* 277:L1118-L1123.

179. Ackerman, K. G., H. Huang, H. Grasemann, C. Puma, J. B. Singer, A. E. Hill, E. Lander, J. H. Nadeau, G. A. Churchill, J. M. Drazen, and D. R. Beier. 2005. Interacting genetic loci cause airway hyperresponsiveness. *Physiol Genomics.* 21:105-111.

180. Torkamanzehi, A., P. Boksa, M. Ayoubi, M. E. Fortier, N. M. Ng Ying Kin, E. Skamene, G. Rouleau, and R. Joober. 2006. Identification of informative strains and provisional QTL mapping of amphetamine (AMPH)-induced locomotion in recombinant congenic strains (RCS) of mice. *Behav.Genet.* 36:903-913.

181. Gill, K. J. and A. E. Boyle. 2005. Quantitative trait loci for novelty/stress-induced locomotor activation in recombinant inbred (RI) and recombinant congenic (RC) strains of mice. *Behav.Brain Res.* 161:113-124.

182. Thifault, S., S. Ondrej, Y. Sun, A. Fortin, E. Skamene, R. Lalonde, J. Tremblay, and P. Hamet. 2008. Genetic determinants of emotionality and stress response in AcB/BcA recombinant congenic mice and in silico evidence of convergence with cardiovascular candidate genes. *Hum.Mol.Genet.* 17:331-344.

183. Gill, K. J. and A. E. Boyle. 2005. Genetic basis for the psychostimulant effects of nicotine: a quantitative trait locus analysis in AcB/BcA recombinant congenic mice. *Genes Brain Behav.* 4:401-411.

184. Kest, B., S. B. Smith, A. Schorscher-Petcu, J. S. Austin, J. Ritchie, G. Klein, G. C. Rossi, A. Fortin, and J. S. Mogil. 2009. Gnao1 (G alphaO protein) is a likely genetic contributor to variation in physical dependence on opioids in mice. *Neuroscience.* 162:1255-1264.

185. Roy, M. F., N. Riendeau, J. C. Loredo-Osti, and D. Malo. 2006. Complexity in the host response to Salmonella Typhimurium infection in AcB and BcA recombinant congenic strains. *Genes Immun.* 7:655-666.

186. Fortin, A., E. Diez, J. E. Henderson, J. S. Mogil, P. Gros, and E. Skamene. 2007. The AcB/BcA recombinant congenic strains of mice: strategies for phenotype dissection, mapping and cloning of quantitative trait genes. *Novartis.Found.Symp.* 281:141-53; discussion 153-5,;%208-9.:141-153.

187. Levitt, R. C., W. Mitzner, and S. R. Kleeberger. 1990. A genetic approach to the study of lung physiology: understanding biological variability in airway responsiveness. *Am.J.Physiol.* 258:L157-L164.

188. Burgio, G., M. Szatanik, J. L. Guenet, M. R. Arnau, J. J. Panthier, and X. Montagutelli. 2007. Interspecific recombinant congenic strains between C57BL/6 and mice of the Mus spretus species: a powerful tool to dissect genetic control of complex traits. *Genetics.* 177:2321-2333.

189. Wills-Karp, M. and S. L. Ewart. 1997. The genetics of allergen-induced airway hyperresponsiveness in mice. *Am.J.Respir.Crit Care Med.* 156:S89-S96.

190. Piavaux, B., P. V. Jeurink, P. C. Groot, G. A. Hofman, P. Demant, and A. J. Van Oosterhout. 2007. Mouse genetic model for antigen-induced airway manifestations of asthma. *Genes Immun.* 8:28-34.

191. Albertine, K. H., L. Wang, S. Watanabe, G. K. Marathe, G. A. Zimmerman, and T. M. McIntyre. 2002. Temporal correlation of measurements of airway hyperresponsiveness in ovalbumin-sensitized mice. *Am.J.Physiol Lung Cell Mol.Physiol.* 283:L219-L233.

192. Oruc, T., M. Terzioglu, G. Sahin, and S. Dursun. 1982. Response of the central respiratory control mechanisms to hyperoxia and hypoxia. *Bull.Eur.Physiopathol.Respir.* 18:439-447.

193. Camateros, P., M. Tamaoka, M. Hassan, R. Marino, J. Moisan, D. Marion, M. C. Guiot, J. G. Martin, and D. Radzioch. 2007. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am.J.Respir.Crit Care Med.* 175:1241-1249.

194. Lukacs, N. W., M. M. Glovsky, and P. A. Ward. 2001. Complement-dependent immune complex-induced bronchial inflammation and hyperreactivity. *Am.J.Physiol Lung Cell Mol.Physiol.* 280:L512-L518.

195. Bauer, A. K., A. M. Malkinson, and S. R. Kleeberger. 2004. Susceptibility to neoplastic and non-neoplastic pulmonary diseases in mice: genetic similarities. *Am.J.Physiol Lung Cell Mol.Physiol.* 287:L685-L703.

196. Prows, D. R., M. J. Daly, H. G. Shertzer, and G. D. Leikauf. 1999. Ozone-induced acute lung injury: genetic analysis of F(2) mice generated from A/J and C57BL/6J strains. *Am.J.Physiol.* 277:L372-L380.

197. Camateros, P., C. Kanagaratham, J. Henri, R. Sladek, T. J. Hudson, and D. Radzioch. 2009. Modulation of the allergic asthma transcriptome following resiquimod treatment. *Physiol Genomics.* 38:303-318.

198. Zhang, Y., J. Lefort, V. Kearsey, Lapa e Silva JR, W. O. Cookson, and B. B. Vargaftig. 1999. A genome-wide screen for asthma-associated quantitative trait loci in a mouse model of allergic asthma. *Hum.Mol.Genet.* 8:601-605.

199. Silbaugh, S. A. and J. L. Mauderly. 1984. Noninvasive detection of airway constriction in awake guinea pigs. *J.Appl.Physiol.* 56:1666-1669.

200. Jacky, J. P. 1978. A plethysmograph for long-term measurements of ventilation in unrestrained animals. *J.Appl.Physiol.* 45:644-647.

201. Allen, M., A. Heinzmann, E. Noguchi, G. Abecasis, J. Broxholme, C. P. Ponting, S. Bhattacharyya, J. Tinsley, Y. Zhang, R. Holt, E. Y. Jones, N. Lench, A. Carey, H. Jones, N. J. Dickens, C. Dimon, R. Nicholls, C. Baker, L. Xue, E. Townsend, M. Kabesch, S. K. Weiland, D. Carr, M. E. von, I. M. Adcock, P. J. Barnes, G. M. Lathrop, M. Edwards, M. F. Moffatt, and W. O. Cookson. 2003. Positional cloning of a novel gene influencing asthma from chromosome 2q14. *Nat.Genet.* 35:258-263.

202. Altmuller, J., L. J. Palmer, G. Fischer, H. Scherb, and M. Wjst. 2001. Genomewide scans of complex human diseases: true linkage is hard to find. *Am.J.Hum.Genet.* 69:936-950.

203. Bosse, Y. and T. J. Hudson. 2007. Toward a comprehensive set of asthma susceptibility genes. *Annu.Rev.Med.* 58:171-84.:171-184.

204. Hoffjan, S. and C. Ober. 2002. Present status on the genetic studies of asthma. *Curr.Opin.Immunol.* 14:709-717.

205. Hui, J., A. Oka, A. James, L. J. Palmer, A. W. Musk, J. Beilby, and H. Inoko. 2008. A genome-wide association scan for asthma in a general Australian population. *Hum.Genet.* 123:297-306.

206. Kauppi, P., T. Laitinen, V. Ollikainen, H. Mannila, L. A. Laitinen, and J. Kere. 2000. The IL9R region contribution in asthma is supported by genetic association in an isolated population. *Eur.J.Hum.Genet.* 8:788-792.

207. Kauppi, P., K. Lindblad-Toh, P. Sevon, H. T. Toivonen, J. D. Rioux, A. Villapakkam, L. A. Laitinen, T. J. Hudson, J. Kere, and T. Laitinen. 2001. A second-generation association study of the 5q31 cytokine gene cluster and the interleukin-4 receptor in asthma. *Genomics.* 77:35-42.

208. Yokouchi, Y., Y. Nukaga, M. Shibasaki, E. Noguchi, K. Kimura, S. Ito, M. Nishihara, K. Yamakawa-Kobayashi, K. Takeda, N. Imoto, K. Ichikawa, A. Matsui, H. Hamaguchi, and T.

Arinami. 2000. Significant evidence for linkage of mite-sensitive childhood asthma to chromosome 5q31-q33 near the interleukin 12 B locus by a genome-wide search in Japanese families. *Genomics.* 66:152-160.

209. Wjst, M. 2007. Public data mining shows extended linkage disequilibrium around ADAM33. *Allergy.* 62:444-446.

210. Sakagami, T., N. Jinnai, T. Nakajima, T. Sekigawa, T. Hasegawa, E. Suzuki, I. Inoue, and F. Gejyo. 2007. ADAM33 polymorphisms are associated with aspirin-intolerant asthma in the Japanese population. *J.Hum.Genet.* 52:66-72.

211. Berce, V. and U. Potocnik. 2010. Association of Q551R polymorphism in the interleukin 4 receptor gene with nonatopic asthma in Slovenian children. *Wien.Klin.Wochenschr.* 122 Suppl 2:11-8.:11-18.

212. Martinati, L. C., E. Trabetti, A. Casartelli, A. L. Boner, and P. F. Pignatti. 1996. Affected sib-pair and mutation analyses of the high affinity IgE receptor beta chain locus in Italian families with atopic asthmatic children. *Am.J.Respir.Crit Care Med.* 153:1682-1685.

213. Cheong, H. S., L. H. Kim, B. L. Park, Y. H. Choi, H. S. Park, S. J. Hong, B. W. Choi, C. S. Park, and H. D. Shin. 2005. Association analysis of interleukin 5 receptor alpha subunit (IL5RA) polymorphisms and asthma. *J.Hum.Genet.* 50:628-634.

214. Levitt, R. C. and W. Mitzner. 1989. Autosomal recessive inheritance of airway hyperreactivity to 5-hydroxytryptamine. *J.Appl.Physiol.* 67:1125-1132.

215. Fortin, A., E. Diez, D. Rochefort, L. Laroche, D. Malo, G. A. Rouleau, P. Gros, and E. Skamene. 2001. Recombinant congenic strains derived from A/J and C57BL/6J: a tool for genetic dissection of complex traits. *Genomics.* 74:21-35.

216. Bult, C. J., J. T. Eppig, J. A. Kadin, J. E. Richardson, and J. A. Blake. 2008. The Mouse Genome Database (MGD): mouse biology and model systems. *Nucleic Acids Res.* 36:D724-D728.

217. Smith, C. M., J. H. Finger, T. F. Hayamizu, I. J. McCright, J. T. Eppig, J. A. Kadin, J. E. Richardson, and M. Ringwald. 2007. The mouse Gene Expression Database (GXD): 2007 update. *Nucleic Acids Res.* 35:D618-D623.

218. De Sanctis, G. T., M. Merchant, D. R. Beier, R. D. Dredge, J. K. Grobholz, T. R. Martin, E. S. Lander, and J. M. Drazen. 1995. Quantitative locus analysis of airway hyperresponsiveness in A/J and C57BL/6J mice. *Nat.Genet.* 11:150-154.

219. Levitt, R. C., S. M. Eleff, L. Y. Zhang, S. R. Kleeberger, and S. L. Ewart. 1995. Linkage homology for bronchial hyperresponsiveness between DNA markers on human chromosome 5q31-q33 and mouse chromosome 13. *Clin.Exp.Allergy.* 25 Suppl 2:61-3.:61-63.

220. Qiu, L. Q., P. Cresswell, and K. C. Chin. 2009. Viperin is required for optimal Th2 responses and T-cell receptor-mediated activation of NF-kappaB and AP-1. *Blood.* 113:3520-3529.

221. Cheng, B. H., Y. Liu, X. Xuei, C. P. Liao, D. Lu, M. E. Lasbury, P. J. Durant, and C. H. Lee. 2010. Microarray studies on effects of Pneumocystis carinii infection on global gene expression in alveolar macrophages. *BMC.Microbiol.* 10:103.:103.

222. Kontaridis, M. I., S. Eminaga, M. Fornaro, C. I. Zito, R. Sordella, J. Settleman, and A. M. Bennett. 2004. SHP-2 positively regulates myogenesis by coupling to the Rho GTPase signaling pathway. *Mol.Cell Biol.* 24:5340-5352.

223. Gen, Y., K. Yasui, K. Zen, T. Nakajima, K. Tsuji, M. Endo, H. Mitsuyoshi, M. Minami, Y. Itoh, S. Tanaka, M. Taniwaki, S. Arii, T. Okanoue, and T. Yoshikawa. 2009. A novel amplification target, ARHGAP5, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells. *Cancer Lett.* 275:27-34.

224. Comtesse, N., I. Niedermayer, B. Glass, D. Heckel, E. Maldener, W. Nastainczyk, W. Feiden, and E. Meese. 2002. MGEA6 is tumor-specific overexpressed and frequently recognized by patient-serum antibodies. *Oncogene.* 21:239-247.

225. Dewa, Y., J. Nishimura, M. Jin, M. Kawai, Y. Saegusa, S. Kenmochi, K. Shimamoto, T. Harada, M. Shibutani, and K. Mitsumori. 2010. Immunohistochemical analyses at the late stage of tumor promotion by oxfendazole in a rat hepatocarcinogenesis model. *Arch.Toxicol.*

226. Dittrich, A. M., H. A. Meyer, M. Krokowski, D. Quarcoo, B. Ahrens, S. M. Kube, M. Witzenrath, R. S. Esworthy, F. F. Chu, and E. Hamelmann. 2010. Glutathione peroxidase-2 protects from allergen-induced airway inflammation in mice. *Eur.Respir.J.* 35:1148-1154.

227. Weidinger, S., M. O'Sullivan, T. Illig, H. Baurecht, M. Depner, E. Rodriguez, A. Ruether, N. Klopp, C. Vogelberg, S. K. Weiland, W. H. McLean, M. E. von, A. D. Irvine, and M. Kabesch. 2008. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J.Allergy Clin.Immunol.* 121:1203-1209.

228. Gauvreau, G. M., M. Jordana, R. M. Watson, D. W. Cockroft, and P. M. O'Byrne. 1997. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am.J.Respir.Crit Care Med.* 156:1738-1745.

229. Van, E. P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, A. Walsh, Z. Liu, B. Hayward, C. Folz, S. P. Manning, A. Bawa, L. Saracino, M. Thackston, Y. Benchekroun, N. Capparell, M. Wang, R. Adair, Y. Feng, J. Dubois, M. G. FitzGerald, H. Huang, R. Gibson, K. M. Allen, A. Pedan, M. R. Danzig, S. P. Umland, R. W. Egan, F. M. Cuss, S. Rorke, J. B. Clough, J. W. Holloway, S. T. Holgate, and T. P. Keith. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature.* 418:426-430.

230. De Sanctis, G. T. and J. M. Drazen. 1997. Genetics of native airway responsiveness in mice. *Am.J.Respir.Crit Care Med.* 156:S82-S88.

231. Yokoyama, W. M. and B. F. Plougastel. 2003. Immune functions encoded by the natural killer gene complex. *Nat.Rev.Immunol.* 3:304-316.

232. Yan, R., Q. Shi, X. Hu, and X. Zhou. 2006. Reticulon proteins: emerging players in neurodegenerative diseases. *Cell Mol.Life Sci.* 63:877-889.

233. Park, J. E., Y. I. Kim, and A. K. Yi. 2008. Protein kinase D1: a new component in TLR9 signaling. *J.Immunol.* 181:2044-2055.

234. Osada, S., K. Mizuno, T. C. Saido, Y. Akita, K. Suzuki, T. Kuroki, and S. Ohno. 1990. A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. *J.Biol.Chem.* 265:22434-22440.

235. Seldin, M. F., S. Hirohata, and S. S. Apte. 2000. Chromosomal mapping of Adam9, Adam15 and Adam21. *Matrix Biol.* 19:185-187.

236. Yang, P., K. A. Baker, and T. Hagg. 2006. The ADAMs family: coordinators of nervous system development, plasticity and repair. *Prog.Neurobiol.* 79:73-94.

237. Heckman, B. M., G. Chakravarty, T. Vargo-Gogola, M. Gonzales-Rimbau, D. L. Hadsell, A. V. Lee, J. Settleman, and J. M. Rosen. 2007. Crosstalk between the p190-B RhoGAP and IGF signaling pathways is required for embryonic mammary bud development. *Dev.Biol.* 309:137-149.

238. Xu, H., S. Eleswarapu, H. Geiger, K. Szczur, D. Daria, Y. Zheng, J. Settleman, E. F. Srour, D. A. Williams, and M. D. Filippi. 2009. Loss of the Rho GTPase activating protein p190-B enhances hematopoietic stem cell engraftment potential. *Blood.* 114:3557-3566.

239. Monaghan, A. P., K. H. Kaestner, E. Grau, and G. Schutz. 1993. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development.* 119:567-578.

240. Altaba, A., C. Cox, T. M. Jessell, and A. Klar. 1993. Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. *Proc.Natl.Acad.Sci.U.S.A.* 90:8268-8272.

241. Zhou, L., C. R. Dey, S. E. Wert, C. Yan, R. H. Costa, and J. A. Whitsett. 1997. Hepatocyte nuclear factor-3beta limits cellular diversity in the developing respiratory epithelium and alters lung morphogenesis in vivo. *Dev.Dyn.* 210:305-314.

242. Zhou, L., L. Lim, R. H. Costa, and J. A. Whitsett. 1996. Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J.Histochem.Cytochem.* 44:1183-1193.

243. Besnard, V., S. E. Wert, W. M. Hull, and J. A. Whitsett. 2004. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. *Gene Expr.Patterns.* 5:193-208.

244. Whitsett, J. A. and Y. Matsuzaki. 2006. Transcriptional regulation of perinatal lung maturation. *Pediatr.Clin.North Am.* 53:873-87, viii.

245. Wan, H., S. Dingle, Y. Xu, V. Besnard, K. H. Kaestner, S. L. Ang, S. Wert, M. T. Stahlman, and J. A. Whitsett. 2005. Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. *J.Biol.Chem.* 280:13809-13816.

246. Song, L., B. Zhang, Y. Feng, X. Luo, X. Wei, and X. Xiao. 2009. A role for forkhead box A1 in acute lung injury. *Inflammation.* 32:322-332.

247. Ye, D. Z. and K. H. Kaestner. 2009. Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. *Gastroenterology.* 137:2052-2062.

248. Sharon, N. and H. Lis. 2004. History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology.* 14:53R-62R.

249. Comtesse, N., K. Reus, and E. Meese. 2001. The MGEA6 multigene family has an active locus on 14q and at least nine pseudogenes on different chromosomes. *Genomics.* 75:43-48.

250. Guinn, B. A., A. F. Gilkes, G. J. Mufti, A. K. Burnett, and K. I. Mills. 2006. The tumour antigens RAGE-1 and MGEA6 are expressed more frequently in the less lineage restricted subgroups of presentation acute myeloid leukaemia. *Br.J.Haematol.* 134:238-239.

251. Oertle, T. and M. E. Schwab. 2003. Nogo and its paRTNers. *Trends Cell Biol.* 13:187-194.

252. Strittmatter, S. M. 2002. Modulation of axonal regeneration in neurodegenerative disease: focus on Nogo. *J.Mol.Neurosci.* 19:117-121.

253. Bandtlow, C. E. and M. E. Schwab. 2000. NI-35/250/nogo-a: a neurite growth inhibitor restricting structural plasticity and regeneration of nerve fibers in the adult vertebrate CNS. *Glia.* 29:175-181.

254. Schafer, G. L., J. C. Crabbe, and K. M. Wiren. 1998. Identification of neuroendocrine-specific protein as an ethanol-regulated gene with mRNA differential display. *Mamm.Genome.* 9:979-982.

255. Goven, D., A. Boutten, V. Lecon-Malas, J. Marchal-Somme, N. Amara, B. Crestani, M. Fournier, G. Leseche, P. Soler, J. Boczkowski, and M. Bonay. 2008. Altered Nrf2/Keap1-Bach1 equilibrium in pulmonary emphysema. *Thorax.* 63:916-924.

256. Duschene, K. S. and J. B. Broderick. 2010. The antiviral protein viperin is a radical SAM enzyme. *FEBS Lett.* %19;584:1263-1267.

257. Jiang, D., H. Guo, C. Xu, J. Chang, B. Gu, L. Wang, T. M. Block, and J. T. Guo. 2008. Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. *J.Virol.* 82:1665-1678.

258. Fink, J., F. Gu, L. Ling, T. Tolfvenstam, F. Olfat, K. C. Chin, P. Aw, J. George, V. A. Kuznetsov, M. Schreiber, S. G. Vasudevan, and M. L. Hibberd. 2007. Host gene expression profiling of dengue virus infection in cell lines and patients. *PLoS.Negl.Trop.Dis.* 1:e86.

259. Manning, G., D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam. 2002. The protein kinase complement of the human genome. *Science.* 298:1912-1934.

260. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, U. J. Van, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J.Immunol.* 161:7054-7062.

261. Banerjee, B., K. J. Kelly, J. N. Fink, J. D. Henderson, Jr., N. K. Bansal, and V. P. Kurup. 2004. Modulation of airway inflammation by immunostimulatory CpG oligodeoxynucleotides in a murine model of allergic aspergillosis. *Infect.Immun.* 72:6087-6094.

262. Yang, P., K. A. Baker, and T. Hagg. 2005. A disintegrin and metalloprotease 21 (ADAM21) is associated with neurogenesis and axonal growth in developing and adult rodent CNS. *J.Comp Neurol.* %19;490:163-179.

263. Jie, Z., M. Jin, Y. Cai, C. Bai, Y. Shen, Z. Yuan, Y. Hu, and S. Holgate. 2009. The effects of Th2 cytokines on the expression of ADAM33 in allergen-induced chronic airway inflammation. *Respir.Physiol Neurobiol.* 168:289-294.

264. Undarmaa, S., Y. Mashimo, S. Hattori, N. Shimojo, K. Fujita, A. Miyatake, S. Doi, Y. Kohno, Y. Okamoto, T. Hirota, M. Tamari, A. Hata, and Y. Suzuki. 2010. Replication of genetic association studies in asthma and related phenotypes. *J.Hum.Genet.* 55:342-349.

265. Eisenberg, S. P., K. K. Hale, P. Heimdal, and R. C. Thompson. 1990. Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J.Biol.Chem.* 265:7976-7981.

266. Odaka, C., T. Mizuochi, J. Yang, and A. Ding. 2003. Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response. *J.Immunol.* 171:1507-1514.

267. Fakioglu, E., S. S. Wilson, P. M. Mesquita, E. Hazrati, N. Cheshenko, J. A. Blaho, and B. C. Herold. 2008. Herpes simplex virus downregulates secretory leukocyte protease inhibitor: a novel immune evasion mechanism. *J.Virol.* 82:9337-9344.

268. Tomee, J. F., P. S. Hiemstra, R. Heinzel-Wieland, and H. F. Kauffman. 1997. Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J.Infect.Dis.* 176:740-747.

269. Sagel, S. D., M. K. Sontag, and F. J. Accurso. 2009. Relationship between antimicrobial proteins and airway inflammation and infection in cystic fibrosis. *Pediatr.Pulmonol.* 44:402-409.

270. Ramadas, R. A., L. Wu, and A. M. LeVine. 2009. Surfactant protein A enhances production of secretory leukoprotease inhibitor and protects it from cleavage by matrix metalloproteinases. *J.Immunol.* 182:1560-1567.

271. Ashcroft, G. S., K. Lei, W. Jin, G. Longenecker, A. B. Kulkarni, T. Greenwell-Wild, H. Hale-Donze, G. McGrady, X. Y. Song, and S. M. Wahl. 2000. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat.Med.* 6:1147-1153.

272. Thuraisingam, T., H. Sam, J. Moisan, Y. Zhang, A. Ding, and D. Radzioch. 2006. Delayed cutaneous wound healing in mice lacking solute carrier 11a1 (formerly Nramp1): correlation with decreased expression of secretory leukocyte protease inhibitor. *J.Invest Dermatol.* 126:890-901.

273. Jana, N. K., L. R. Gray, and D. C. Shugars. 2005. Human immunodeficiency virus type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. *J.Virol.* 79:6432-6440.

274. McNeely, T. B., D. C. Shugars, M. Rosendahl, C. Tucker, S. P. Eisenberg, and S. M. Wahl. 1997. Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. *Blood.* 90:1141-1149.

275. Py, B., S. Basmaciogullari, J. Bouchet, M. Zarka, I. C. Moura, M. Benhamou, R. C. Monteiro, H. Hocini, R. Madrid, and S. Benichou. 2009. The phospholipid scramblases 1 and 4 are cellular receptors for the secretory leukocyte protease inhibitor and interact with CD4 at the plasma membrane. *PLoS.ONE.* 4:e5006.

276. Wang, Z., T. Zheng, Z. Zhu, R. J. Homer, R. J. Riese, H. A. Chapman, Jr., S. D. Shapiro, and J. A. Elias. 2000. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J.Exp.Med.* 192:1587-1600.

277. Weldon, S., N. McGarry, C. C. Taggart, and N. G. McElvaney. 2007. The role of secretory leucoprotease inhibitor in the resolution of inflammatory responses. *Biochem.Soc.Trans.* 35:273-276.

278. Belkowski, S. M., J. D. Boot, M. A. Mascelli, Z. Diamant, G. L. de, B. Hertzog, D. Polkovitch, M. Towers, A. Batheja, and M. R. D'Andrea. 2009. Cleaved secretory leucocyte protease inhibitor as a biomarker of chymase activity in allergic airway disease. *Clin.Exp.Allergy.* 39:1179-1186.

279. Moisan, J., P. Camateros, T. Thuraisingam, D. Marion, H. Koohsari, P. Martin, M. L. Boghdady, A. Ding, M. Gaestel, M. C. Guiot, J. G. Martin, and D. Radzioch. 2006. TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. *Am.J.Physiol Lung Cell Mol.Physiol.* 290:L987-L995.

280. Ghasemlou, N., D. Bouhy, J. Yang, R. Lopez-Vales, M. Haber, T. Thuraisingam, G. He, D. Radzioch, A. Ding, and S. David. 2010. Beneficial effects of secretory leukocyte protease inhibitor after spinal cord injury. *Brain.* 133:126-138.

281. Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25:402-408.

282. Jin, F. Y., C. Nathan, D. Radzioch, and A. Ding. 1997. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell.* 88:417-426.

283. Goodman, J. E., J. K. Chandalia, S. Thakali, and M. Seeley. 2009. Meta-analysis of nitrogen dioxide exposure and airway hyper-responsiveness in asthmatics. *Crit Rev.Toxicol.* 39:719-742.

284. Motomura, C., H. Odajima, J. Tezuka, Y. Murakami, Y. Moriyasu, N. Kando, N. Taba, D. Hayashi, K. Okada, and S. Nishima. 2009. Effect of age on relationship between exhaled nitric oxide and airway hyperresponsiveness in asthmatic children. *Chest.* 136:519-525.

285. Komatsu, Y., K. Fujimoto, M. Yasuo, K. Urushihata, M. Hanaoka, T. Koizumi, and K. Kubo. 2009. Airway hyper-responsiveness in young adults with asthma that remitted either during or before adolescence. *Respirology.* 14:217-223.

286. Fukunaga, J., M. Abe, A. Murai, Y. Akitake, M. Hosokawa, and M. Takahashi. 2007. Comparative study to elucidate the mechanism underlying the difference in airway hyperresponsiveness between two mouse strains. *Int.Immunopharmacol.* %20;7:1852-1861.

287. Lougheed, M. D., T. Fisher, and D. E. O'Donnell. 2006. Dynamic hyperinflation during bronchoconstriction in asthma: implications for symptom perception. *Chest.* 130:1072-1081.

288. Dragon, S., M. K. Takhar, L. Shan, K. T. Hayglass, F. E. Simons, and A. S. Gounni. 2009. T(H)2 cytokines modulate the IL-9R expression on human neutrophils. *Biochem.Biophys.Res.Commun.*

289. Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J.Exp.Med.* 183:109-117.

290. Westin, U., E. Lundberg, and K. Ohlsson. 1998. IgE-mediated histamine release from nasal mucosa is inhibited by SLPI (secretory leukocyte protease inhibitor) to the level of spontaneous release. *Mediators.Inflamm.* 7:217-220.

291. Nakamura, A., Y. Mori, K. Hagiwara, T. Suzuki, T. Sakakibara, T. Kikuchi, T. Igarashi, M. Ebina, T. Abe, J. Miyazaki, T. Takai, and T. Nukiwa. 2003. Increased susceptibility to LPS-induced endotoxin shock in secretory leukoprotease inhibitor (SLPI)-deficient mice. *J.Exp.Med.* 197:669-674.

292. Xu, W., B. He, A. Chiu, A. Chadburn, M. Shan, M. Buldys, A. Ding, D. M. Knowles, P. A. Santini, and A. Cerutti. 2007. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat.Immunol.* 8:294-303.

293. Ding, A., H. Yu, J. Yang, S. Shi, and S. Ehrt. 2005. Induction of macrophage-derived SLPI by Mycobacterium tuberculosis depends on TLR2 but not MyD88. *Immunology.* 116:381-389.

294. Seung, S. J. and N. Mittmann. 2005. Urgent care costs of uncontrolled asthma in Canada, 2004. *Can.Respir.J.* 12:435-436.

295. Wu, X., Y. Li, Q. Chen, F. Chen, P. Cai, L. Wang, and L. Hu. 2010. Association and gene-gene interactions of eight common single-nucleotide polymorphisms with pediatric asthma in middle china. *J.Asthma.* 47:238-244.

296. Saikku, P. 2000. Chlamydia pneumoniae in atherosclerosis. *J.Intern.Med.* 247:391-396.

297. Castellani, L. W., A. Weinreb, J. Bodnar, A. M. Goto, M. Doolittle, M. Mehrabian, P. Demant, and A. J. Lusis. 1998. Mapping a gene for combined hyperlipidaemia in a mutant mouse strain. *Nat.Genet.* 18:374-377.

298. Fijneman, R. J., S. S. de Vries, R. C. Jansen, and P. Demant. 1996. Complex interactions of new quantitative trait loci, Sluc1, Sluc2, Sluc3, and Sluc4, that influence the susceptibility to lung cancer in the mouse. *Nat.Genet.* 14:465-467.

299. Lipoldova, M., M. Svobodova, M. Krulova, H. Havelkova, J. Badalova, E. Nohynkova, V. Holan, A. A. Hart, P. Volf, and P. Demant. 2000. Susceptibility to Leishmania major

infection in mice: multiple loci and heterogeneity of immunopathological phenotypes. *Genes Immun.* 1:200-206.

300. Krulova, M., H. Havelkova, M. Kosarova, V. Holan, A. A. Hart, P. Demant, and M. Lipoldova. 1997. IL-2-induced proliferative response is controlled by loci Cinda1 and Cinda2 on mouse chromosomes 11 and 12: a distinct control of the response induced by different IL-2 concentrations. *Genomics.* 42:11-15.

301. van, W. T., A. P. Stassen, C. J. Moen, A. A. Hart, d. van, V, and P. Demant. 1996. Gene interaction and single gene effects in colon tumour susceptibility in mice. *Nat.Genet.* 14:468-470.

302. Stevenson, M. M., P. A. Kongshavn, and E. Skamene. 1981. Genetic linkage of resistance to Listeria monocytogenes with macrophage inflammatory responses. *J.Immunol.* 127:402-407.

303. Li, D., A. Vaglenov, T. Kim, C. Wang, D. Gao, and B. Kaltenboeck. 2005. High-yield culture and purification of Chlamydiaceae bacteria. *J.Microbiol.Methods.* 61:17-24.

304. Huang, J., M. D. Wang, S. Lenz, D. Gao, and B. Kaltenboeck. 1999. IL-12 administered during Chlamydia psittaci lung infection in mice confers immediate and long-term protection and reduces macrophage inflammatory protein-2 level and neutrophil infiltration in lung tissue. *J.Immunol.* 162:2217-2226.

305. DeGraves, F. J., D. Gao, and B. Kaltenboeck. 2003. High-sensitivity quantitative PCR platform. *Biotechniques.* 34:106-5.

306. Huang, J., F. J. DeGraves, S. D. Lenz, D. Gao, P. Feng, D. Li, T. Schlapp, and B. Kaltenboeck. 2002. The quantity of nitric oxide released by macrophages regulates Chlamydia-induced disease. *Proc.Natl.Acad.Sci.U.S.A.* %19;99:3914-3919.

307. Wang, C., F. W. van Ginkel, T. Kim, D. Li, Y. Li, J. C. Dennis, and B. Kaltenboeck. 2008. Temporal delay of peak T-cell immunity determines Chlamydia pneumoniae pulmonary disease in mice. *Infect.Immun.* 76:4913-4923.

308. Skamene, E., E. Schurr, and P. Gros. 1998. Infection genomics: Nramp1 as a major determinant of natural resistance to intracellular infections. *Annu.Rev.Med.* 49:275-87.:275-287.

309. Bernstein-Hanley, I., Z. R. Balsara, W. Ulmer, J. Coers, M. N. Starnbach, and W. F. Dietrich. 2006. Genetic analysis of susceptibility to Chlamydia trachomatis in mouse. *Genes Immun.* 7:122-129.

310. Miyairi, I., V. R. Tatireddigari, O. S. Mahdi, L. A. Rose, R. J. Belland, L. Lu, R. W. Williams, and G. I. Byrne. 2007. The p47 GTPases Iigp2 and Irgb10 regulate innate immunity and inflammation to murine Chlamydia psittaci infection. *J.Immunol.* 179:1814-1824.

311. Fortin, A., A. Belouchi, M. F. Tam, L. Cardon, E. Skamene, M. M. Stevenson, and P. Gros. 1997. Genetic control of blood parasitaemia in mouse malaria maps to chromosome 8. *Nat.Genet.* 17:382-383.

312. Beebe, A. M., S. Mauze, N. J. Schork, and R. L. Coffman. 1997. Serial backcross mapping of multiple loci associated with resistance to Leishmania major in mice. *Immunity.* 6:551-557.

313. Pasche, B., S. Kalaydjiev, T. J. Franz, E. Kremmer, V. Gailus-Durner, H. Fuchs, A. M. Hrabe de, A. Lengeling, and D. H. Busch. 2005. Sex-dependent susceptibility to Listeria monocytogenes infection is mediated by differential interleukin-10 production. *Infect.Immun.* 73:5952-5960.

314. Min-Oo, G., L. Lindqvist, A. Vaglenov, C. Wang, P. Fortin, Y. Li, B. Kaltenboeck, and P. Gros. 2008. Genetic control of susceptibility to pulmonary infection with Chlamydia pneumoniae in the mouse. *Genes Immun.* 9:383-388.

315. Broman, K. W. 2001. Review of statistical methods for QTL mapping in experimental crosses. *Lab Anim (NY).* 30:44-52.

316. Gill, K. J. and A. E. Boyle. 2003. Confirmation of quantitative trait loci for cocaine-induced activation in the AcB/BcA series of recombinant congenic strains. *Pharmacogenetics.* 13:329-338.

317. Joober, R., J. M. Zarate, G. A. Rouleau, E. Skamene, and P. Boksa. 2002. Provisional mapping of quantitative trait loci modulating the acoustic startle response and prepulse inhibition of acoustic startle. *Neuropsychopharmacology.* 27:765-781.

Appendix 1 - Quantitative trait loci for the susceptibility to *Chlamydia pneumoniae* identified in A/J × C57BL/6J recombinant congenic strains of mice

Adapted from the manuscript submitted for publication:

Yihang Li, **Rafael Marino**, David M. Carpenter, Alexander Vaglenov, Chengming Wang, Sudhir Ahluwalia, Anny Fortin, and Bernhard Kaltenboeck.

Preface

Human infection by the obligate intracellular bacterium *Chlamydia pneumoniae* is a common cause of pneumonia. The susceptibility of mammalian hosts to chlamydial infections is presumably a combined effect of genetic factor. Identification of allelic variations that determine the severity of chlamydial disease may therefore contribute to our understanding of chlamydial infections. Recombinant congenic mouse strains have been used as a powerful tool to study complex traits such as susceptibility to cancer, infections and metabolic diseases. Using a panel of 36 AcB and BcA recombinant congenic strains, we proposed to study the resistance/susceptibility to *C. pneumoniae* infection, by evaluating parameters for chlamydial lung loads and concomitant disease; in order to delineate the effector genes and increase the understanding of chlamydial pathogenesis.

Abstract

Two commonly used lab mouse strains, A/J and C57BL/6J, show different levels of disease after *Chlamydia pneumoniae* infections, and eliminate *C. pneumoniae* by different kinetics. Using thirty-five recombinant congenic strains we proposed to identify genomic regions that encompass potential quantitative trait loci (QTL) associated with resistance / susceptibility to *C. pneumoniae*. All RCS mice, and parental A/J and C57BL/6J controls, were intranasally challenged with 5×10^7 *C. pneumoniae* elementary bodies, and their day-15 post-inoculation lung weights and *C. pneumoniae* lung load were obtained. Multiple logistic regression and general linear model were used to identify markers that are most significantly associated with the phenotypes. We found that some RCS were significantly different compared to their parental strains in lung index (disease), *C. pneumoniae* lung loads, response efficiency index, or disease susceptibility index. Multiple regression analyses identified markers DXMit172, D18Mit186 and D18Mit120 that are highly significantly correlated with *C. pneumoniae* lung loads, D11Mit79 and D11Mit285 with lung index, D11Mit79 and D8Mit45 with response inefficiency index, and D9Mit303 and D2Mit370 with disease susceptibility index. We concluded that using A/J × C57BL/6J recombinant congenic strains of mice, we have identified RCS with extreme phenotypes (hyper-phenotypes), and multiple murine QTL that highly significantly associate with each phenotype.

Introduction

Human infection by the obligate intracellular bacterium *Chlamydia pneumoniae* is a common cause of pneumonia. It is also strongly associates with chronic diseases such as asthma and coronary atherosclerosis.[296] The susceptibility of mammalian hosts to chlamydial infections and disease is presumably a combined effect of allelic variations of a group of host quantitative trait loci (QTL). Identification of these QTL that determine the severity of chlamydial disease may therefore contribute to our understanding of chlamydial infections.

Recombinant congenic mouse strains (RCS) have been used as a powerful tool to study complex traits such as susceptibility to cancer, infections and metabolic diseases.[297-301] A set of 35 RCS has recently become available which are especially designed for genetic analysis of quantitative traits associated with the host response to infectious and inflammatory agents.[215] The parental mice are well characterized and represent extremes in the mouse variation to inflammatory stimuli: B mice (C57BL/6J) have a vigorous inflammatory and macrophage chemotactic response while A mice (A/J) have a weak response.[302] They also show differential susceptibility to numerous infectious diseases.[215] Many of the qualitative and quantitative polymorphisms of these strains, such as susceptibility to atherosclerosis (C57BL/6 susceptible, A/J resistant), represent major issues in human health care. The AcB/BcA set has been genotyped for 625 informative markers (average spacing 2.6 cM) and the position of all congenic fragments has been established.[186;215] Individual resistance/susceptibility loci contributing to a complex trait may have segregated in individual RCS and can be studied in isolation, both for gene mapping/identification experiments but also for elucidating monogenic contributions to the overall phenotype. Importantly, the relatively small size of the congenic segments fixed in individual RCS facilitates the search and testing of candidate genes.

In this investigation, we have used the complete set of available AcB and BcA recombinant congenic mouse strains to study resistance/susceptibility to *C. pneumoniae* infection, by evaluating parameters for chlamydial lung loads and concomitant disease. We report a number

of recombinant congenic strains with hyperphenotypes, in which the extreme traits can be studied further. Multiple regression analyses also identified genetic markers that are highly significantly associated with each phenotype. Further fine mapping can be used to delineate the effector genes and increase the understanding of chlamydial pathogenesis.

Materials and Methods

Experimental design

This study used 13 AcB and 22 BcA recombinant congenic mouse strains to analyze and identify the murine quantitative trait loci (QTLs) that influence susceptibility / resistance to *C. pneumoniae* infection and disease. At 10-16 weeks of age, 1170 female mice (317 parental A/J and C57BL/6J controls, and 853 RCS mice) were inoculated with *C. pneumoniae*, and sacrificed on day 15 post infection. Post-infection lung weight relative to pre-infection body weight and *C. pneumoniae* load in the lung were measured as indicators of the host response. Significant associations of mouse strain, and of non-redundant microsatellite markers of these strains, with these parameters were determined by logistic and multiple regression analyses.

Animals

Inbred A/J (A) and C57BL/6J (B) female mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6-7 weeks of age. Recombinant congenic strain AcB/BcA and parent A and B breeding pairs were provided by Emerillon Genetics, Inc. (Montreal, Quebec, Canada). These mice were bred at Auburn University and female offspring was used for inoculation experiments. Five animals were housed per cage in ventilated cage racks in a temperature-controlled room with a 12-hour light/dark cycle. Mice were fed a 19% protein/1.33% L-arginine standard rodent maintenance diet. All mice were challenge-inoculated at 10-16 weeks of age. Beginning 2 weeks prior to challenge infection, all mice were fed a 24% protein/1.8% L-arginine custom diet (Harlan Teklad (Madison, WI, USA). All animal protocols followed NIH guidelines and were approved by the respective Institutional Animal Care and Use Committees (IACUC).

C. pneumoniae culture and purification

C. pneumoniae strain CDC/CWL-029 (ATCC VR-1310) was propagated and purified as described by.[303] Buffalo Green Monkey Kidney cells (Diagnostic Hybrids, Inc. Athens, OH) were used as host cells, and elementary bodies (EB) in culture medium were concentrated by high-speed sedimentation, re-suspended in low-volume of sucrose-phosphate-glutamate (SPG) buffer followed by low-speed centrifugation for removal of host cell nuclei, and by centrifugation of the supernatant in a 30% RenoCal-76 (Bracco Diagnostics, Princeton, NJ) - 50% sucrose step-gradient. Sediments of purified infectious EBs were suspended in SPG buffer and stored in 250 µl aliquots at -80°C.

C. pneumoniae lung challenge infection

Intranasal inoculation followed the procedure described by.[304] Briefly, mice received a light isoflurane inhalation anesthesia, and a 30 µl inoculum dose containing 5 x 10^7 *C. pneumoniae* organisms was placed on the nostrils and rapidly absorbed by the hyperventilating animals. For time course experiments, mice were sacrificed by CO_2 inhalation 2 hours (day 0), 3, 10 , 13, 16, or 21 days later. Mice in RCS challenge experiments mice were sacrificed on day 15 post inoculation (pi). Lungs were weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Disease in RCS and F2 mice was determined as lung index (square root of 100x g challenge lung weight over g pre-challenge body weight). The lung index as a measure of disease was used because individual RCS and F2 mice differ strongly in body weight and pre-challenge lung weight, making a reliable calculation of percent lung weight increase impossible.

Determination of *C. pneumoniae* lung burden by real-time PCR

Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent (Roche Applied Science, Indianapolis, IN) in disposable tissue grinders (Fisher Scientific, Atlanta, GA) to create a 10% (wt/vol) tissue suspension. This suspension was

used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) as described. The copy number of *C. pneumoniae* genomes was determined in *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) real-time PCRs using extracted nucleic acids corresponding to 0.25 mg lung tissue.[305]

Statistical Analysis

Statistical analyses were performed with the Statistica 7.1 (StatSoft, Inc., Tulsa, OK) and SAS/STAT 9.1® (SAS Institute Inc., Cary, NC) software packages. Data for RCS in different experiments were normalized against control parental A and B strain data of each experiment. Normal distribution of data was confirmed by Shapiro-Wilk's W test, and homogeneity of variances by Levene's test. Results of time course experiments were analyzed by factorial analysis of variance (ANOVA) and two-sided Student's *t*-test. Percent lung weight increase was used as metric of disease and was based on naïve lung weights of 138.4 mg and 133 mg for 10 week-old A/J mice and C57BL/6J mice, respectively. *C. pneumoniae*/mg lung data were logarithmically transformed. Comparisons of means under the assumption of no *a priori* hypothesis were performed by Tukey's honest significant difference (HSD) test. Differences at $P \leq 0.05$ in the Tukey HSD were considered significant.

The relationships between lung index and *C. pneumoniae* load data to RCS and the non-redundant RCS microsatellite markers were analyzed by Logistic Regression and General Linear Regression with SAS®. Redundant markers, i.e. markers with alleles identical to the adjacent marker in all RCS, were excluded from analysis. Because of the semi-quantitative nature of the normalized *C. pneumoniae* genomes lung loads (177 out of 853 observations or 20.7% were zero), this response was dichotomized into a binary response based on high/low responses, with values at or below the median (≤ 673.64 *C. pneumoniae* genomes/mg lung) being assigned a zero and those values above the median (> 673.64) being assigned a one. Logistic regression analysis (using the Logistic procedure in SAS®) was performed on the binary *Chlamydia* load response to find which collection of markers best correlated with *C. pneumoniae* load. Odds

ratio estimates and their 95% confidence intervals were computed and reported for each marker within each model. To assess the overall fit, Hosmer and Lemeshow goodness-of-fit tests were conducted for each model on a binary response of low versus high. Models were only retained if there was no significant lack of fit ($P > 0.05$).

The lung index response was separately analyzed with a general linear model (GLM). First, genetic variability with respect to this measure was defined as the percent variability (partial R^2) in the lung index that was explained by strain (using a one-way ANOVA treating strain as the class variable). This gave a benchmark by which to judge how well a selected subset of markers could account for genetic variability in the lung index. Stepwise selection techniques, as well as standard model building and assessment techniques, were used to determine the model that best explains the genetic variability induced in the lung index measure.

Specifically, four models were built in order to determine which markers best explained the genetic variability with respect to both *C. pneumoniae* lung load and lung index, and the combined host response to the chlamydial infection, both in terms of efficiency of elimination without concomitant disease as well as susceptibility to infection. Model 1: Logistic regression with binary *C. pneumoniae* lung load (high/low) regressed on multiple markers. Model 2: General linear regression of the continuous variable lung index regressed on the markers. Model 3: Response inefficiency index regressed on multiple markers. Response inefficiency index was calculated as standardized lung index plus standardized $\log_{10}$ *C. pneumoniae* per mg lung (zero lung loads were also assumed as zero $\log_{10}$). Lung index and *C. pneumoniae* load were respectively standardized by linear transformation, so that the mean of all groups is 0 and the standard deviation is 1. Model 4: Disease susceptibility index regressed on multiple markers. Disease susceptibility index was calculated as standardized lung index minus $\log_{10}$ *C. pneumoniae* per mg lung. All markers in these models were considered significant at $P < 10^{-4}$.

Quantitative trait loci analysis

Quantitative trait loci analysis was performed computing single marker regression R version 2.1.0 (www.R-project.org), using 605 markers (microsatellites), strain means and variances. Significance thresholds were established by permutation analysis consisting of 10,000 permutations performed by shuffling the phenotypes across the genotypes (without replacement) to correct for multiple testing. Significance was established as a P value smaller than the smallest P value in more than 95% of the permutations. Significant QTL by QTL interactions were detected as locus pairs with significance as *P* value smaller than 0.001.

Results

The kinetics of *C. pneumoniae* elimination from infected lungs differ between A/J and C57BL/6J mice

Initially, A/J and C57BL/6J mice were evaluated in a lung disease model of *C. pneumoniae* infection. These strains were chosen because i) they represent the opposites in inflammatory response to intracellular bacterial pathogens,[302] which are also correlate with susceptibility to chlamydial disease;[304] ii) C57BL/6J mice are well known for their susceptibility in chronic *C. pneumoniae* disease models;[296] and iii) recombinant inbred and congenic mouse strains derived from these parents are available that facilitate genetic analyses.[215]

To establish the course of *C. pneumoniae* lung disease in these parental strains, female, 10-week old mice were intranasally challenged with $5x10^7$ *C. pneumoniae* organisms, half of the challenge dose used in a vaccine challenge model.[303] The course of disease and chlamydial elimination from lungs was evaluated on days 0, 3, 10, 13, 16 and 21 pi (Figure 1). Lung disease, expressed as percent increase in lung weight over that of naïve animals,[304] reached peak intensity in both strains on day 10 pi, and remained at this peak level for next 11 days (figure 1*A*). Disease severity was marginally, but consistently higher in C57BL/6J mice than in A/J mice. The time course of *C. pneumoniae* elimination differed highly significantly between the two strains: early after infection, A/J mice eliminated lung chlamydiae much less efficiently than C57BL/6J mice, which had 2 hours pi ~1.5 $\log_{10}$ lower lung chlamydial loads than A/J mice (Figure 1*B*). After day 10, however, A/J, but not C57BL/6J mice, rapidly reduced the chlamydial lung burden, presumably due to efficient adaptive Th1 cell immunity.[306] Overall, A/J mice highly significantly reduced the early day 0-10 lung chlamydial loads by day 13-21 ($P < 10^{-4}$), while C57BL/6J mice failed to significantly eliminate chlamydiae after day 10. This resulted in similar chlamydial lung loads after day 10 in A/J and C57BL/6J mice, but the strains arrived at this similar end point with different kinetics of the lung burdens, and different disease responses.

A/J×C57BL/6J recombinant congenic strains differ significantly from parental *C. pneumoniae*-responder phenotypes

The differences in chlamydial lung load kinetics and lung disease suggested that different genetic mechanisms in A/J and C57BL/6J lead to indistinguishable day-15 chlamydial load and disease phenotypes. Use of recombinant congenic AcB/BcA strains, which contain ~12.5% homozygous donor DNA on the parental background, is therefore very useful for identification of quantitative trait loci associated with susceptibility/resistance to *C. pneumoniae* infection, a multigenic quantitative trait. To establish a connection between the genotyped strains and their phenotypes, responses of 13 AcB and 22 BcA strains to a *C. pneumoniae* infection were evaluated. All mice were intranasally infected with $5x10^7$ *C. pneumoniae* organisms, and their pre-inoculation body weight, and post-inoculation lung weight and *C. pneumoniae* lung load on day 15 pi were obtained.

The lung index of all RCS as well as the A/J and C57BL/6J parental controls are shown in Figure 2A. Strains with extreme phenotypes emerge from the randomly assorted polymorphic quantitative traits. For instance, strains AcB52, AcB58, AcB57, AcB55, AcB56, BcA83, BcA75 and BcA80 have significantly higher lung index than their respective parental strains, whereas BcA86, BcA73, BcA76 and BcA70 have significantly lower lung index than their parental C57BL/6J strain. The $\log_{10}$ *C. pneumoniae*/mg lung data are shown in Fig 2B. Strains AcB62, AcB57, and BcA81 have significantly higher *C. pneumoniae* lung loads than their respective parental strains, whereas AcB53, BcA66 and BcA85 have significantly lower *C. pneumoniae* lung loads. Thus, re-assortment of polymorphic QTL did result in RCS phenotypes that significantly deviated from the parental phenotypes.

For more in-depth evaluation of the overall response to the *C. pneumoniae* infection, a response inefficiency index was created by adding the standardized lung index and *C. pneumonia* lung load data. As a combined measure, it quantifies the host's elimination of

chlamydiae from the lung, and simultaneously the disease response to the chlamydial stimulus. Therefore, this index is highest in animals that are ineffective at eliminating chlamydiae, but respond to the chlamydiae with a severe disease outcome. It is lower in animals that eliminate chlamydiae effectively, but respond to them with high disease, as well as in animals that respond with low disease to ineffective chlamydial elimination. The index is lowest in animal that both clear the chlamydial infection effectively and respond to the bacteria with low disease. As shown in Figure 2C, strains AcB52, AcB57, AcB58, AcB56, and BcA75 have significantly higher response inefficiency index values than their respective parental strains, whereas BcA86 and BcA66 have significantly lower response inefficiency index values than their parental C57BL/6J strain.

A disease susceptibility index was created by subtracting the standardized *C. pneumoniae* lung load from the standardized lung index. This index indicates disease intensity relative to a given chlamydial stimulus, and therefore is largely independent of the host's efficiency at chlamydial elimination. As shown in Figure 2D, strains AcB52, AcB55, AcB58, BcA80, BcA83 and BcA77 have significantly higher disease susceptibility index than their respective parental strains, whereas BcA73, BcA70, BcA76, BcA86, and BcA72 have significantly lower disease susceptibility index than their parental C57BL/6J strain.

Statistical models identify QTLs that associate with outcomes of *C. pneumoniae* challenge infection

To obtain an initial understanding of the relation between *C. pneumoniae* lung burden and disease intensity in the RCS, we evaluated for all RCS the correlation between *C. pneumoniae* lung load and lung index by non-parametric Spearman's rank test. The overall Spearman's rank correlation coefficient is significantly greater than zero at $\rho = 0.526$ ($P < .001$). The strength of the association for each strains varied between -0.123 and 0.870 ($P < .001$, data not shown). These data indicated that the lung index explained a substantial fraction of the *C. pneumoniae* lung loads. To link the RCS phenotypes with their genotypes, four statistical models were

developed that respectively regress *C. pneumoniae* lung load, lung index, response inefficiency index, and disease susceptibility index on the non-redundant markers.

C. pneumoniae lung burden (Log_{10} *C. pneumoniae*/mg lung)

Initial logistic regression modeling of the dichotomized *C. pneumoniae* lung concentration (Log_{10} *C. pneumoniae*/mg lung) was performed by stepwise regression on all non-redundant genetic markers and the lung index categorized into 4 quartiles, to account for the correlation between *C. pneumoniae* lung load and lung index. This model showed no significant lack-of-fit in the Hosmer and Lemeshow test for lack-of-fit ($P = 0.240$). A classification table indicated 76% sensitivity (percent of high loads being correctly identified as high, i.e., above the median) and 82% specificity (percent of lows correctly identified as low) for this model. These data indicated the optimal modeling results achievable, and served as benchmark for *C. pneumoniae* load modeling exclusively by marker regression.

Table 1 lists the final regression models accepted for each of the 4 phenotypes determined in this study. In Model 1, the dichotomized Log_{10} *C. pneumoniae*/mg lung was regressed by stepwise logistic regression on the non-redundant genetic markers, but not the lung index ($P = .993$; Hosmer and Lemeshow test for lack-of-fit). This model predicted high versus low *C. pneumoniae* lung load with 72% sensitivity and 71% specificity. Marker DXMit172 (45-50.5 cM on the X chromosome) has the strongest explanatory power ($\chi^2 = 118$) for Log_{10} *C. pneumoniae*/mg lung, with an odds ratio estimate of 2.446 that the "a" allele at this position associates with high *C. pneumoniae* lung concentration (Table 1; Figure 3*A*). Two markers on chromosome 18, D18Mit186 (43 cM-end of chromosome) and D18Mit120 (8.7-20 cM) are the two other highly significant markers, with odds ratio estimates of 4.023 and 3.164, respectively (Table 1). Figure 3*B* shows the *C. pneumoniae* lung burden phenotypes of RCS with recombination events in the region of these significant QTL (± five non-redundant flanking markers).

Disease intensity (lung index)

Regression model 2 showed the lung index as continuous variable in a general linear model (GLM) against the genetic markers (Table 1). First, total genetic variability was defined as the percent variability (R^2) in the lung index that is explained by strain in a one-way ANOVA that treated strain as the class variable. Approximately 32.1% of the variance in lung index is explained by strain. This gave a benchmark by which to judge how well a selected subset of markers could account for genetic variability in the lung index. Stepwise selection techniques, as well as standard model building and assessment techniques, were used to determine the model that best explains the genetic variability induced in the lung index measure. The two most significant markers, D11Mit79 (8-11 cM) and D11Mit285 (50-54 cM), account for 56% of the variability (Table 1; Figure 4A and 4B), whereas the full 13-marker model account for 98.1% of the genetic variability (31.5%/32.1%).

Combined *C. pneumoniae* clearance and disease avoidance (response inefficiency index)

Regression model 3 showed the response inefficiency index in GLM against the genetic markers (Table 1) which can explain up to 21.3% of the total variance. Stepwise selection and standard model building techniques determined the model that best explains the genetic variability in the response inefficiency index. The two most significant markers, D11Mit79 (8-11 cM) and D8Mit45 (37-43 cM), account for 59% of the genetic variability (Table 1; Figure 5A and 5B), whereas the full 12-marker model accounts for 97.6% (20.8%/21.3%).

***C. pneumoniae* disease susceptibility (disease susceptibility index)**

Regression model 4 displayed the disease susceptibility index (18.3% explained by genetic variance) by stepwise selection in GLM against the genetic markers (Table 1). The two most significant markers, D9Mit303 (29-38 cM) and D2Mit370 (26.2-30 cM), account for 62% of the

variability (Table 1; Figure 6A and 6B), whereas the full 11-marker model accounts for 97.4% of the genetic variability (17.8%/18.3%).

Discussion

Initially, we evaluated the time course of disease and chlamydial lung burden of female A/J and C57BL/6J mice. C57BL/6J mice reduced the chlamydial lung loads immediately after inoculation approximately 30-fold as compared to A/J mice (Figure 1A). This is consistent with the strong inflammatory response of C57BL/6J mice[302] and suggests more effective innate immune mechanisms than in A/J mice. A consistently enhanced disease response appears to be the corollary of the early resistance of C57BL/6J mice to *C. pneumoniae*.[306;307] Subsequently, both mouse strains reduced *C. pneumoniae* lung loads by approximately 10-fold between days 0 and 10 pi. However, after day 10 A/J mice reduced the chlamydial lung load by an additional approximately 100-fold while C57BL/6J mice did not reduce it significantly (Figure 1B). Thus, A/J mice are highly efficient at eliminating chlamydiae without major lung lesions when the adaptive immunity becomes fully developed at around 10 days after primary exposure to *C. pneumoniae*, compensating for the early permissiveness to *C. pneumoniae*.[307] The net effect of these immune differences between the two mouse strains is a similar chlamydial lung burden 15 days pi, but consistently higher lung disease in C57Bl/6J. These results are also consistent with our data in another study.[307]

The aim of the genetic dissection component of this study was to relate the mouse model to the clinical symptoms of chlamydial disease in which multiple re-infections are required to precipitate severe pathological lesions caused by a delayed-type hypersensitivity response to the chlamydiae.[307] Thus, to obtain clinically relevant genetic mapping data we decided to use mouse strains with different disease outcomes late in the infection (i. e. the healing phase). Thus, phenotyping F2 crosses between A/J and C57BL/6J mice is one approach to this problem.[307] Alternatively, phenotyping the entire set of AcB/BcA RCS for a differential response[308] late in primary infection when both the innate and a peaking adaptive cellular immune response contribute to the phenotype, would achieve the objective of genetic dissection for QTL regulating innate as well as adaptive responses to *C. pneumoniae*.

The RCS challenge results in Figure 2 clearly demonstrate that re-assortment of QTL influencing permissiveness to *C. pneumoniae* results in phenotypes that strongly deviate from those of the parent strains, and thus marker regression will likely be informative in the late phenotype model. Results of the RCS challenge further reveal that genetic control of *C. pneumoniae* disease and elimination of the organisms appear to be mediated by different gene effects, suggesting a complex genetic control of *C. pneumoniae*. The following observations are noteworthy: 1) There is a correlation between lung index and *C. pneumoniae* lung load. 2) RCS on A background tend to be stable for all phenotypes while RCS on B background are highly variable. AcB strains have higher lung index and *C. pneumoniae* lung loads than those on B background. The increased lung index is in part related to the fact that naïve A/J mice have approximately 4% heavier lungs than naïve C57BL/6J of equal body weight. 3) The parental A and B strains do not differ significantly in their disease response, but the A strain has significantly higher chlamydial lung load and response inefficiency, and lower disease susceptibility than the B strain. 4) Congenic B chromosome segments appear to transfer largely an increase in disease and response inefficiency to the A background, resulting in various combinations of disease, *C. pneumoniae* lung load, response inefficiency, and disease susceptibility "hyperphenotypes" at the high end of the outcome spectrum in strains AcB55 - 58, and particularly in strain AcB52 with the highest lung, response inefficiency, and disease susceptibility indices observed among all RCS. 5) Conversely, in B background strains congenic A segments appear to transfer decreases as well as increases in all phenotypes. Therefore, hyperphenotypes appear on both ends of the outcome spectrum of all phenotypes. Particularly interesting are the very low response inefficiency phenotypes (very effective combined disease and chlamydial elimination responses) of strains BcA66 and BcA86, and the low and high extremes of disease susceptibility in strains BcA73 and BcA80, respectively.

Marker regression analyses identified 2-3 putative QTL for each phenotype that either are highly predictive of the phenotype (*C. pneumoniae* lung burden) or account for a large proportion of the phenotypic variance. However, the genomic regions that these QTL encompass are too large (up to 12 cM) to pinpoint potential effector genes, and confirmation

and fine mapping are required.[309] Studied in early innate control of intravenous *Chlamydia trachomatis* infection in C57BL/6J and C3H/HeJ mice by F2 mapping and identified three QTLs on chromosomes 2, 3 and 11, respectively, that associated with differential splenic *C. trachomatis* counts one day after challenge. They confirmed the chromosome 11 QTLs by transferring a portion of C3H/HeJ chromosome 11 (20-44.8cM) to C57BL/6J mice. Miyairi et al. used C57BL/6J × DBA/2J recombinant inbred mouse strains to identify a 1.5-Mbp region at around 31-33cM on chromosome 11 encoding a cluster of three p47 GTPases that associate with resistance to lethal challenge with *Chlamydia psittaci*.[310] Thus, our finding of two QTL on chromosome 11 that associate with different disease intensity (lung index: D11Mit79, 8-11 cM; D11Mit285, 50 - 54 cM) in the healing phase of non-lethal *C. pneumoniae* lung disease may indicate common effector genes on chromosome 11 that modulate both innate resistance as well as disease after chlamydial challenge. Similarly, the QTL on chromosome 2 (D2Mit370, 26.2-30 cM) associated with differential susceptibility to *C. pneumoniae*-induced disease may colocalize to the QTL for *C. trachomatis* resistance identified by Bernstein-Hanley et al. at approximately 40-87 cM map position in chromosome 2.[309] Collectively, these studies indicate that chromosomes 11 and 2 are of particular interest and might harbor multiple genes that associate with differential resistance against chlamydial infection.

This is the first time that loci on the mouse chromosomes 8, 9 and 18 have been associated with susceptibility/resistance to chlamydial infections. However, for another intracellular parasite, *Plasmodium chabauti*, a locus on Chromosome 8, D8Mit41, which is in close proximity to D8Mit45 identified in this study, has been associated with blood parasitemia.[311] The identification of a QTL on Chromosome X modulating chlamydial clearance is also consistent with previous reports. For example, gender-dependent susceptibility has been reported in several pathogens, including the protozoon *Leishmania major* and the obligate intracellular bacterium *Listeria monocytogenes*.[312;313] Also, the use of only female mice in this investigation may have been advantageous for the identification of this X-linked trait. The identification of X-linked chlamydial susceptibility may open new approaches for studying the host-parasite interactions in chlamydial infections, of which particularly in *C. trachomatis* urogenital infection

disproportionally affect women. However, overall male individuals may be more at risk for extra-genital chlamydial disease manifestations since they are by definition homozygous at X-linked QTL.

Interestingly, the QTL on chromosome 17 associated by Min-Oo et al.[314] with regulation of chlamydial lung load and found in the identical *C. pneumoniae* challenge model was not identified in this study in any of the models for RCS phenotypes. Markers D17Mit21 and D17Mit83 in the D17Mit62 region and D17Mit66 showed significantly higher *C. pneumoniae* loads for the "a" allele than for the "b" allele (Log_{10} *C. pneumoniae*/mg lung D17Mit21/83 = 3.212 vs. 2.520, P = 0.0005; D17Mit66 = 3.103 vs. 2.575, P = 0.009). However, these associations obviously did not critically influence the overall model. This inability to reliably detect QTL shows a principal shortcoming of RCS for QTL mapping because the statistical power of RCS, due to non-random distribution of haplotypes, may not be sufficient for robust linkage analysis.[315]

Summarizing the results, these RCS can nevertheless be very valuable if used as parents in F2 linkage mapping in informative crosses with other inbred susceptible and resistant mouse strains.[316;317] Strains with, for example, a B phenotype on an A background may be indicative of a robust gene effect from one parent that has become fixed on the other parental background. Such genetic control can then be explored in subsequent F1 and F2 crosses to test for segregation as simple or complex trait. Therefore, "hyperphenotypes", i.e. strains that exceed the phenotype of either parent, are a very good starting point for F2 mapping by crossing with informative strains. For example, strain BcA66 represents a hyperresistant RCS both in disease and chlamydial load phenotype, while strain AcB52 is hypersusceptible in both phenotypes. Such strains likely correspond to novel mutations that may have arisen and become fixed during breeding of the strains, and they can be explored for accelerated gene mapping and testing.

Acknowledgments

This work was supported by Public Health Service grant AI47202 from the National Institute for Allergy and Infectious Disease to BK, and a Presidential Fellowship from Auburn University to YL. R. Marino is a recipient of doctoral scholarships from the Canadian Institutes of Health Research (CIHR).

Table 1. Murine QTLs associated with resistance/susceptibility to *C. pneumoniae* infections identified by multiple regression analyses in four models.

	QTL[a]	Odds Ratio[b]	95% Wald Confidence Limits	
Model 1: *Chlamydia* lung load	DXMit172	2.446	1.449	4.129
	D18Mit186	4.023	2.468	6.559
	D18Mit120	3.164	1.765	5.674
	QTL	Partial R^{2c}	Relative Partial R^{2d}, %	
Model 2: Lung index (genetic R^2 = 0.3206)	D11Mit79	0.1495	46.6	
	D11Mit285	0.0302	9.4	
Model 3: Response inefficiency index (genetic R^2 = 0.2126)	D11Mit79	0.0773	36.4	
	D8Mit45	0.0243	11.4	
Model 4: Disease susceptibility index (genetic R^2 = 0.1829)	D9Mit303	0.0777	42.5	
	D2Mit370	0.0349	19.1	

[a] All markers that explain more than 9% of the genetic variability (partial R^2/model R^2) at $P < 0.0001$ are listed.

[b] Describes the ratio of the "a" over "b" allele for association with a parameter increase.

[c] Describes the contribution of the marker to the variability in the regression model. The overall contribution of genetic variability was determined by including "strain" in the regression model.

[d] Describes the percent contribution of the marker to the genetic variability.

Figure 1. *C. pneumoniae*-induced lung disease and *C. pneumoniae* lung burdens in A/J and C57BL/6J mice.

Equal numbers of ten-week old female A/J and C57BL/6J mice (n=10-20/group) were intranasally inoculated with $5x10^7$ *C. pneumoniae* EBs and sacrificed 2 hours (day 0) to 21 days post inoculation (pi). Data are shown as means ± 95% confidence interval. *A*, percent lung weight increase is based on naïve lung weights of 138.4 mg for A/J mice and 133 mg for C57BL/6 mice. Lung disease, expressed as lung weight increase, peaks at around day 10 pi and remains approximately constant until day 21 in both mouse strains. Disease at individual time points is not significantly different (Tukey's HSD test), but the average lung weight increase of combined data for all time points in A/J mice is significantly lower than in C57BL/6J mice (20.3% vs. 35.9%; $P = 0.0001$, 2-sided *t*-test). *B*, the copy number of *C. pneumoniae* genomes per mg lung was determined by *C. pneumoniae* 23S FRET real-time PCR. Combined chlamydial lung burdens at the early time points (days 0, 3, 10) are significantly higher in A/J than in C57BL/6J mice (Log_{10} *C. pneumoniae*/mg lung 4.36 vs. 3.14; $P < 0.0001$, 2-sided *t*-test). Combined late (day 13, 16, 21) chlamydial lung burdens do not differ between A/J and C57BL/6J mice (2.52 vs. 2.61; $P = 0.71$, 2-sided *t*-test). A/J mice significantly reduce the chlamydial lung burden from early to late post inoculation time points ($P < 0.0001$) while C57BL/6J mice do not ($P = 0.11$, 2-sided *t*-test).

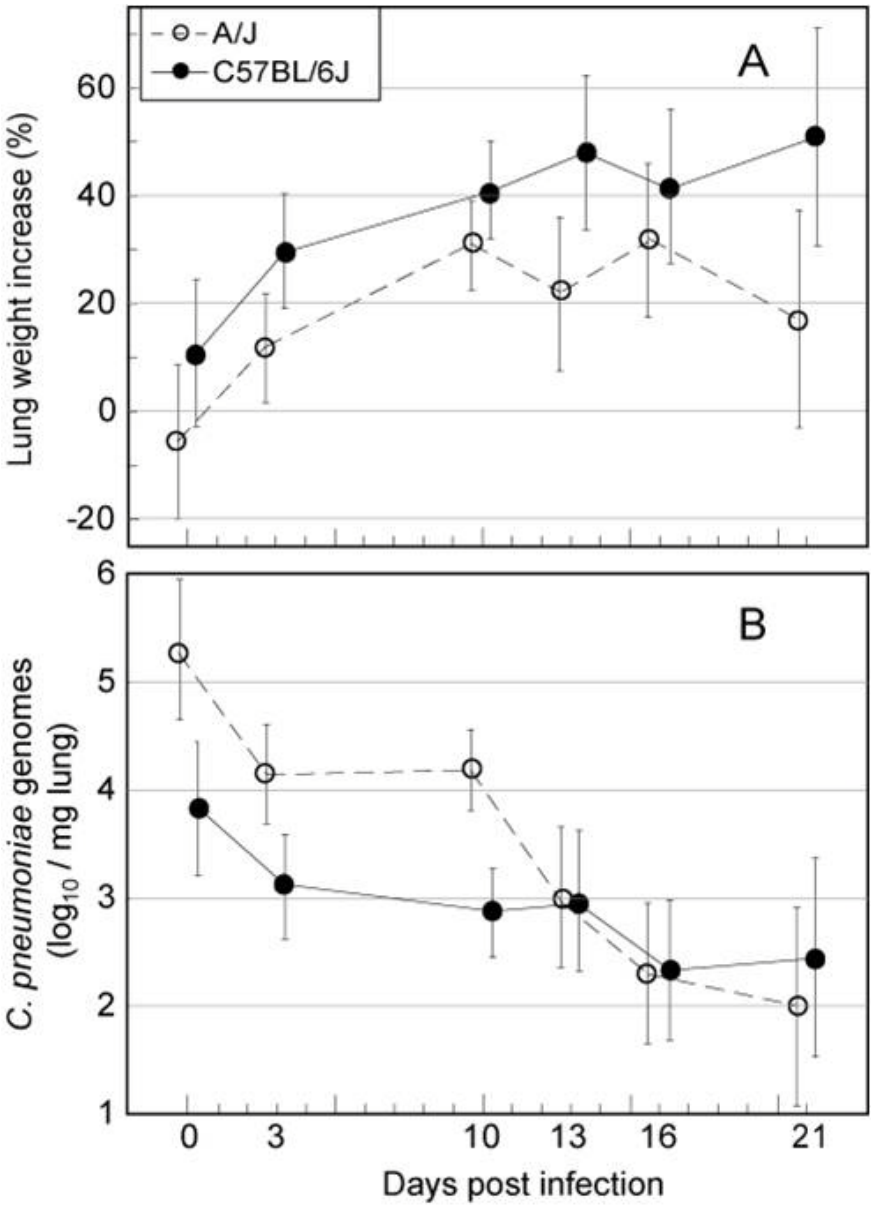

A/J
C57BL/6J
A
B
Lung weight increase (%)
60
40
20
0
-20
C. pneumoniae genomes
(log10 / mg lung)
6
5
4
3
2
1
0
3
10
13
16
21
Days post infection

Figure 2. Phenotype distribution of the AcB/BcA RCS and parental strains.

C. pneumoniae-induced lung disease (lung index), *C. pneumoniae* lung load (Log_{10} *C. pneumoniae*/mg lung) phenotypes, and combined effects lung index and *C. pneumoniae* lung load of A/J, C57BL/6J parental strains and the complete set of 35 AcB/BcA RCS. Eight to 16 week-old mice (n=20/group) were intranasally inoculated with $5x10^7$ *C. pneumoniae* organisms and sacrificed on day 15 after inoculation. Beginning two weeks prior to inoculation, mice were fed a custom diet containing 24% protein and1.8% L-arginine (high protein diet). Data of different experiments were normalized against A and B parental strain data in each experiment (calibration groups). Data are shown as means ± 95% CI computed for covariate means of 15.3 weeks for age and 22.3 g for body weight before challenge. Asterisks indicate strains that differ significantly from the parent strain ($P < 0.05$; Tukey's HSD test). *A*, Lung index values of all recombinant congenic strains and the A/J and C57Bl/6J parental strains are shown. The lung index is calculated as square root of post-challenge lung weight / (10*pre-challenge body weight) for each mouse. *B*, Log_{10} *C. pneumoniae* / mg lung values are shown for all RCSs and both parental strains. AcB62, AcB57, and BcA81 have significantly higher *C. pneumoniae* lung loads than their respective parent strain, whereas BcA66 and BcA85 are significantly lower than their parent controls. *C*, Response inefficiency index values are calculated as standardized lung index plus standardized log_{10} *C. pneumoniae* per mg lung of all RCS and parental strains. Lung index and *C. pneumoniae* load were respectively standardized by linear transformation, so that the mean of all groups is 0 and the standard deviation is 1. Strain AcB52 has the highest response inefficiency index, thus is both unable to efficiently eliminate *C. pneumoniae* and responds to the high *C. pneumoniae* load with high disease. *D*, Disease susceptibility index values are calculated as standardized lung index minus log_{10} *C. pneumoniae* per mg lung of all RCS and parental strains. Strain BcA73 has the lowest score, indicating that this strain develops little disease despite a high *C. pneumoniae* load in the lung, hence is the strain most resistant to develop disease in response to the *C. pneumoniae* infection.

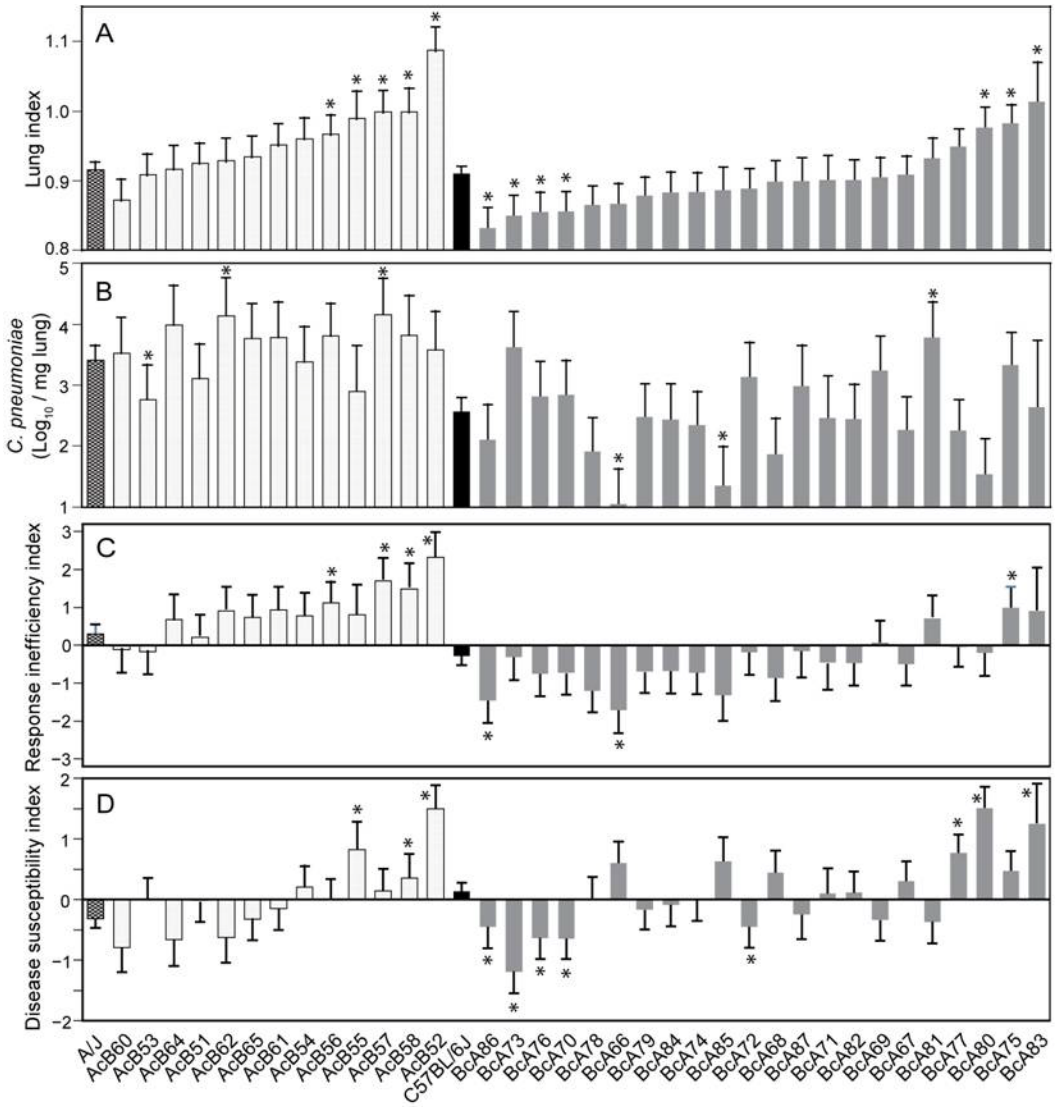

A
Lung index
1.1
1.0
0.9
0.8
B
C. pneumoniae
(Log10 / mg lung)
5
4
3
2
1
C
Response inefficiency index
3
2
1
0
−1
−2
−3
D
Disease susceptibility index
2
1
0
−1
−2
A/J
AcB60
AcB53
AcB64
AcB51
AcB62
AcB65
AcB61
AcB54
AcB56
AcB55
AcB57
AcB58
AcB52
C57BL/6J
BcA86
BcA73
BcA76
BcA70
BcA78
BcA66
BcA79
BcA84
BcA74
BcA85
BcA72
BcA68
BcA87
BcA71
BcA82
BcA69
BcA67
BcA81
BcA77
BcA80
BcA75
BcA83

Figure 3. Significant QTL explaining the genetic contribution to *C. pneumoniae* lung burden.

Lung *C. pneumoniae* loads (means ± 95%CI) and allele distribution of parents and RCS with recombination events in the markers flanking the significant QTL are shown (a = A/J, b = C57BL/6J). A/J mice have significantly higher *C. pneumoniae* lung loads than C57BL/6J mice ($P < 10^{-6}$). Significant microsatellite markers are indicated by the bold print. Lack of flanking markers indicates the end of the chromosome. *A*, RCS with the "a" allele at marker DXMit172 on the X chromosome have highly significantly higher *C. pneumoniae* lung loads than "b" allele RCS (3.747 vs. 2.505, $P < 10^{-16}$, two-sided Student's *t*-test). *B*, All polymorphic markers mapped on chromosome 18 are shown. The allele of strain AcB51 at D18Mit120 is unspecified. RCS with the "a" allele at D18Mit120 or D18Mit186 have highly significantly higher *C. pneumoniae* lung loads than mice with the "b" allele (3.673 vs. 2.580 and 3.518 vs. 2.512, respectively, both $P < 10^{-16}$). Mice with the "a" allele at both markers have approximately a 1.5-log higher *C. pneumoniae* lung burden than mice with "b" allele ($P < 10^{-16}$).

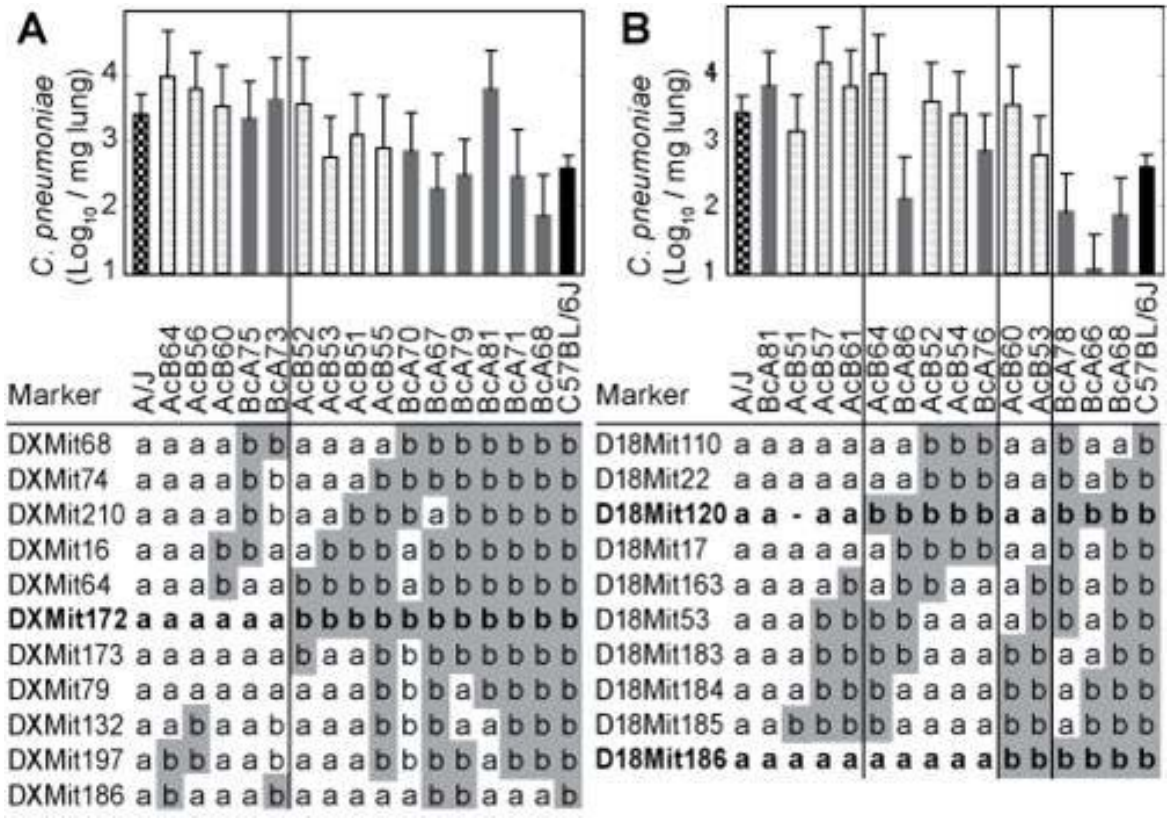

A
B
C. pneumoniae (Log10 / mg lung)
Marker
A/J
AcB64
AcB56
AcB60
BcA75
BcA73
AcB52
AcB53
AcB51
AcB55
BcA70
BcA67
BcA79
BcA81
BcA71
BcA68
C57BL/6J
DXMit68
DXMit74
DXMit210
DXMit16
DXMit64
DXMit172
DXMit173
DXMit79
DXMit132
DXMit197
DXMit186
BcA81
BcA51
AcB57
AcB61
AcB64
BcA86
AcB52
AcB54
BcA76
AcB60
AcB53
BcA78
BcA66
BcA68
D18Mit110
D18Mit22
D18Mit120
D18Mit17
D18Mit163
D18Mit53
D18Mit183
D18Mit184
D18Mit185
D18Mit186

Figure 4. Significant QTL explaining the genetic contribution to lung index.

Lung index values (means ± 95%CI) and allele distribution of parents and RCS with recombination events in the markers flanking the significant QTL are shown (a = A/J, b = C57BL/6J). A/J and C57BL/6J mice have similar lung indices ($P = 0.49$). Significant microsatellite markers are indicated by the bold print. *A*, RCS with the "a" allele at marker D11Mit79 have highly significantly higher lung index values than "b" allele RCS (0.9565 vs. 0.8838, $P < 10^{-16}$, two-sided Student's *t*-test). *B*, RCS with the "a" allele at D11Mit285 have highly significantly higher lung index values than mice with the b allele (0.9545 vs. 0.8927, $P < 10^{-16}$).

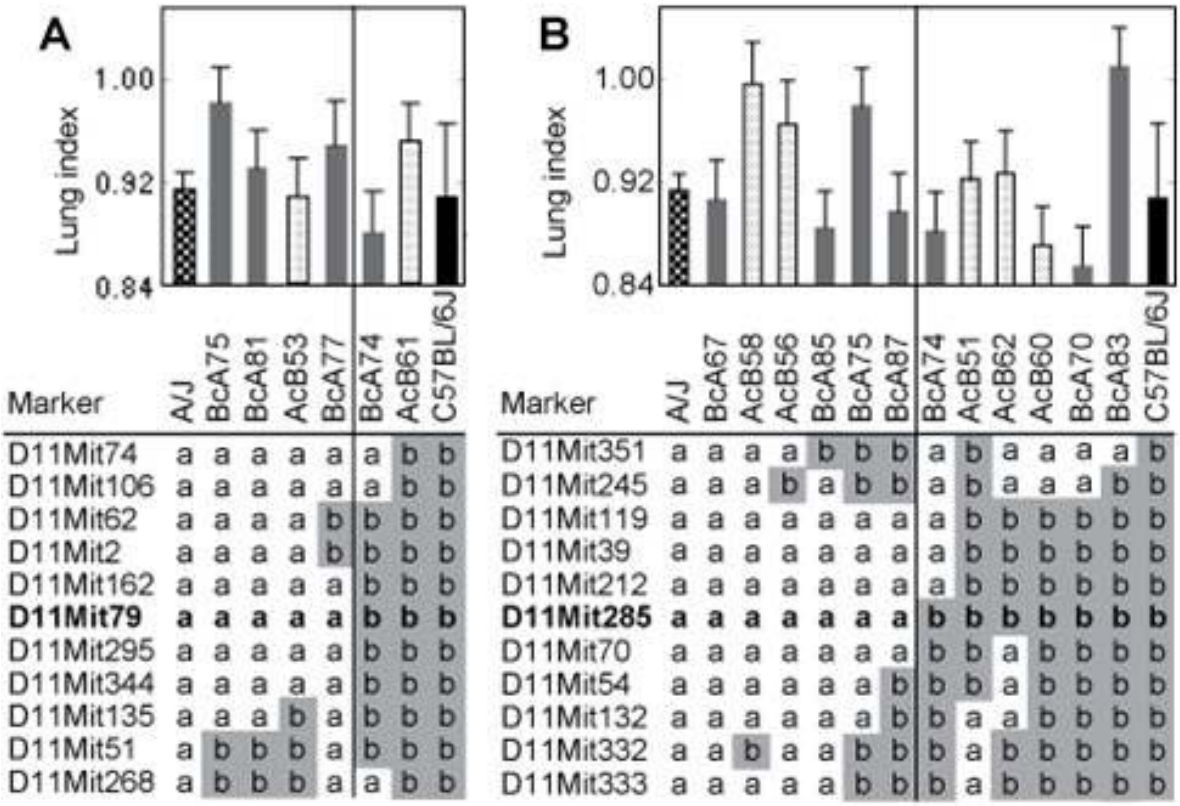

A
Lung index
1.00
0.92
0.84
Marker
A/J
BcA75
BcA81
AcB53
BcA77
BcA74
AcB61
C57BL/6J
D11Mit74 a a a a a a b b
D11Mit106 a a a a a a b b
D11Mit62 a a a a b b b b
D11Mit2 a a a a b b b b
D11Mit162 a a a a a b b b
D11Mit79 a a a a a b b b
D11Mit295 a a a a a b b b
D11Mit344 a a a a a b b b
D11Mit135 a a a b a b b b
D11Mit51 a b b b a b b b
D11Mit268 a b b b a a b b
B
Lung index
1.00
0.92
0.84
Marker
A/J
BcA67
AcB58
AcB56
BcA85
BcA75
BcA87
BcA74
AcB51
AcB62
AcB60
BcA70
BcA83
C57BL/6J
D11Mit351 a a a a b b b a b a a a a b
D11Mit245 a a a b a b b a b a a a b b
D11Mit119 a a a a a a a a b b b b b b
D11Mit39 a a a a a a a a b b b b b b
D11Mit212 a a a a a a a a b b b b b b
D11Mit285 a a a a a a a b b b b b b b
D11Mit70 a a a a a a a b b a b b b b
D11Mit54 a a a a a a b b b a b b b b
D11Mit132 a a a a a a b b a a b b b b
D11Mit332 a a b a a b b b a b b b b b
D11Mit333 a a a a a b b b a b b b b b

Figure 5. Significant QTL explaining the genetic contribution to response inefficiency index.
Response inefficiency index values (means ± 95%CI) and allele distribution of parents and RCS with recombination events in the markers flanking the significant QTL are shown (a = A/J, b = C57BL/6J). A/J mice have a significantly higher response inefficiency index than C57BL/6J mice ($P < 10^{-3}$). Significant microsatellite markers are indicated by the bold print. *A*, RCS with the "a" allele at marker D11Mit79 have highly significantly higher response inefficiency index values than "b" allele RCS (0.2075 vs -0.05845, $P < 10^{-16}$, two-sided Student's *t*-test). *B*, RCS with the "a" allele at D8Mit45 have highly significantly higher response inefficiency index values than mice with the "b" allele (0.4004 vs. -0.1788, $P < 10^{-16}$).

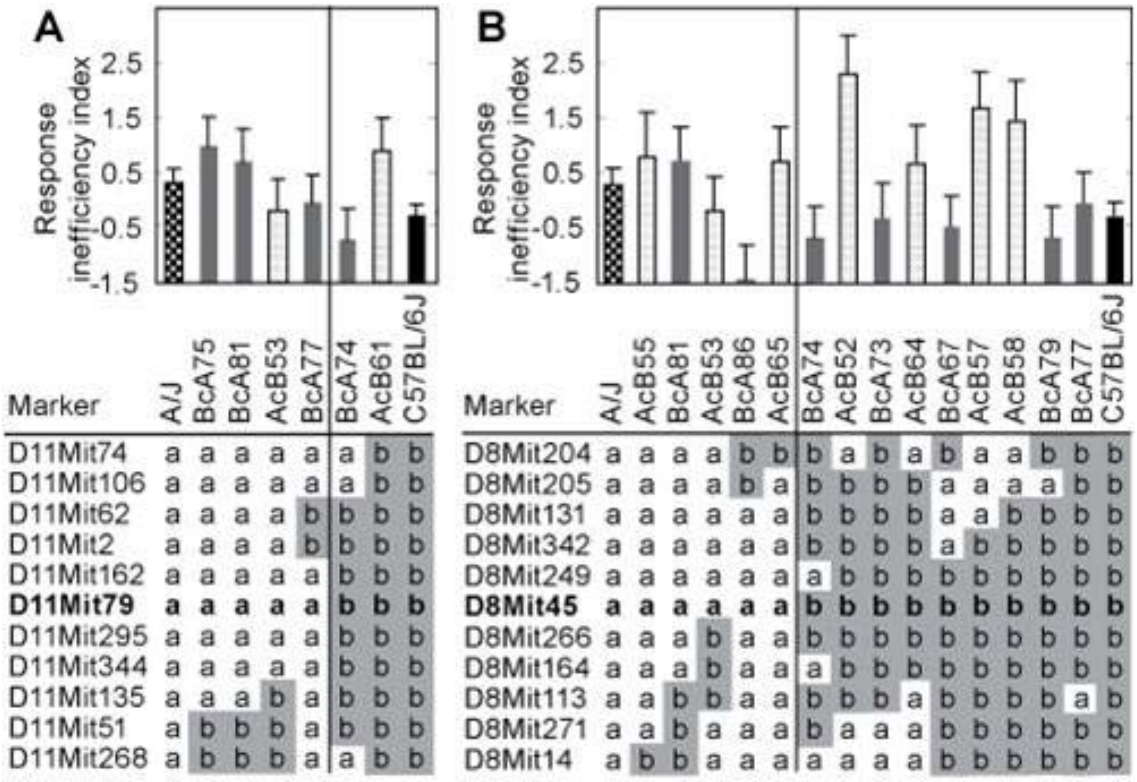
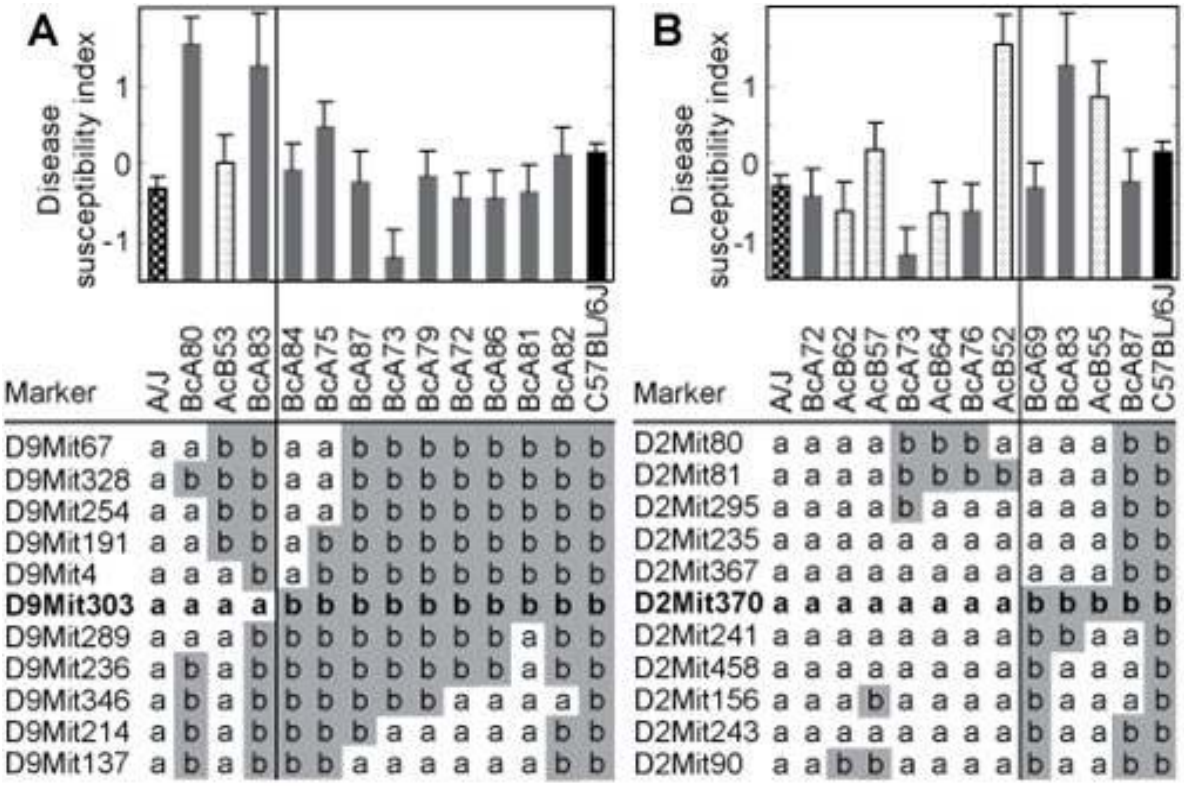

A
Response inefficiency index
2.5
1.5
0.5
-0.5
-1.5
Marker
A/J
BcA75
BcA81
AcB53
BcA77
BcA74
AcB61
C57BL/6J
D11Mit74 a a a a a a b b
D11Mit106 a a a a a a b b
D11Mit62 a a a a b b b b
D11Mit2 a a a a b b b b
D11Mit162 a a a a a b b b
D11Mit79 a a a a a b b b
D11Mit295 a a a a a b b b
D11Mit344 a a a a a b b b
D11Mit135 a a a b a b b b
D11Mit51 a b b b a b b b
D11Mit268 a b b b a a b b
B
Response inefficiency index
2.5
1.5
0.5
-0.5
-1.5
Marker
A/J
AcB55
BcA81
AcB53
BcA86
AcB65
BcA74
AcB52
BcA73
AcB64
BcA67
AcB57
AcB58
BcA79
BcA77
C57BL/6J
D8Mit204 a a a a b b b a b a b a a b b b
D8Mit205 a a a a b a b b b b a a a a b b
D8Mit131 a a a a a a b b b b a a b b b b
D8Mit342 a a a a a a b b b b a b b b b b
D8Mit249 a a a a a a a b b b b b b b b b
D8Mit45 a a a a a a b b b b b b b b b b
D8Mit266 a a a b a a b b b b b b b b b b
D8Mit164 a a a b a a a b b b b b b b b b
D8Mit113 a a b b a a b b b a b b b b a b
D8Mit271 a a b a a a b a a a b b b b b b
D8Mit14 a b b a a a a a a a b b b b b b

Figure 6. Significant QTL explaining the genetic contribution to disease susceptibility index.

Disease susceptibility index values and allele distribution of parents and RCS with recombination events in the markers flanking the significant QTL are shown (a = A/J, b = C57BL/6J). A/J mice have significantly lower disease susceptibility index than C57BL/6J mice ($P < 10^{-4}$). Significant microsatellite markers are indicated by the bold print. *A*, RCS with the "a" allele at marker D9Mit303 have significantly higher disease susceptibility index values than "b" allele RCS (0.13652 vs. -0.1434, $P < .01$, two-sided Student's *t*-test). *B*, RCS with the "a" allele at D2Mit370 have significantly lower disease susceptibility index values than mice with the "b" allele (-0.2236 vs. 0.2339, $P < 10^{-9}$).


A
Disease susceptibility index
1
0
-1
Marker
A/J
BcA80
AcB53
BcA83
BcA84
BcA75
BcA87
BcA73
BcA79
BcA72
BcA86
BcA81
BcA82
C57BL/6J
D9Mit67 a a b b a a b b b b b b b b
D9Mit328 a b b b a a b b b b b b b b
D9Mit254 a a b b a a b b b b b b b b
D9Mit191 a a b b a b b b b b b b b b
D9Mit4 a a a b a b b b b b b b b b
D9Mit303 a a a a b b b b b b b b b b
D9Mit289 a a a b b b b b b b b a b b
D9Mit236 a b a b b b b b b b b a b b
D9Mit346 a b a b b b b b b a a a a b
D9Mit214 a b a b b b b a a a a a b b
D9Mit137 a b a b b b a a a a a a b b
B
Disease susceptibility index
1
0
-1
Marker
A/J
BcA72
AcB62
AcB57
BcA73
AcB64
BcA76
AcB52
BcA69
BcA83
AcB55
BcA87
C57BL/6J
D2Mit80 a a a a b b b a a a a b b
D2Mit81 a a a a b b b b a a a b b
D2Mit295 a a a a b a a a a a a b b
D2Mit235 a a a a a a a a a a a b b
D2Mit367 a a a a a a a a a a a b b
D2Mit370 a a a a a a a a b b b b b
D2Mit241 a a a a a a a a b b a a b
D2Mit458 a a a a a a a a b a a a b
D2Mit156 a a a b a a a a b a a a b
D2Mit243 a a a a a a a a b a a b b
D2Mit90 a a b b a a a a b a a b b