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Mapping of asthma susceptibility in recombinant congenic mouse strains

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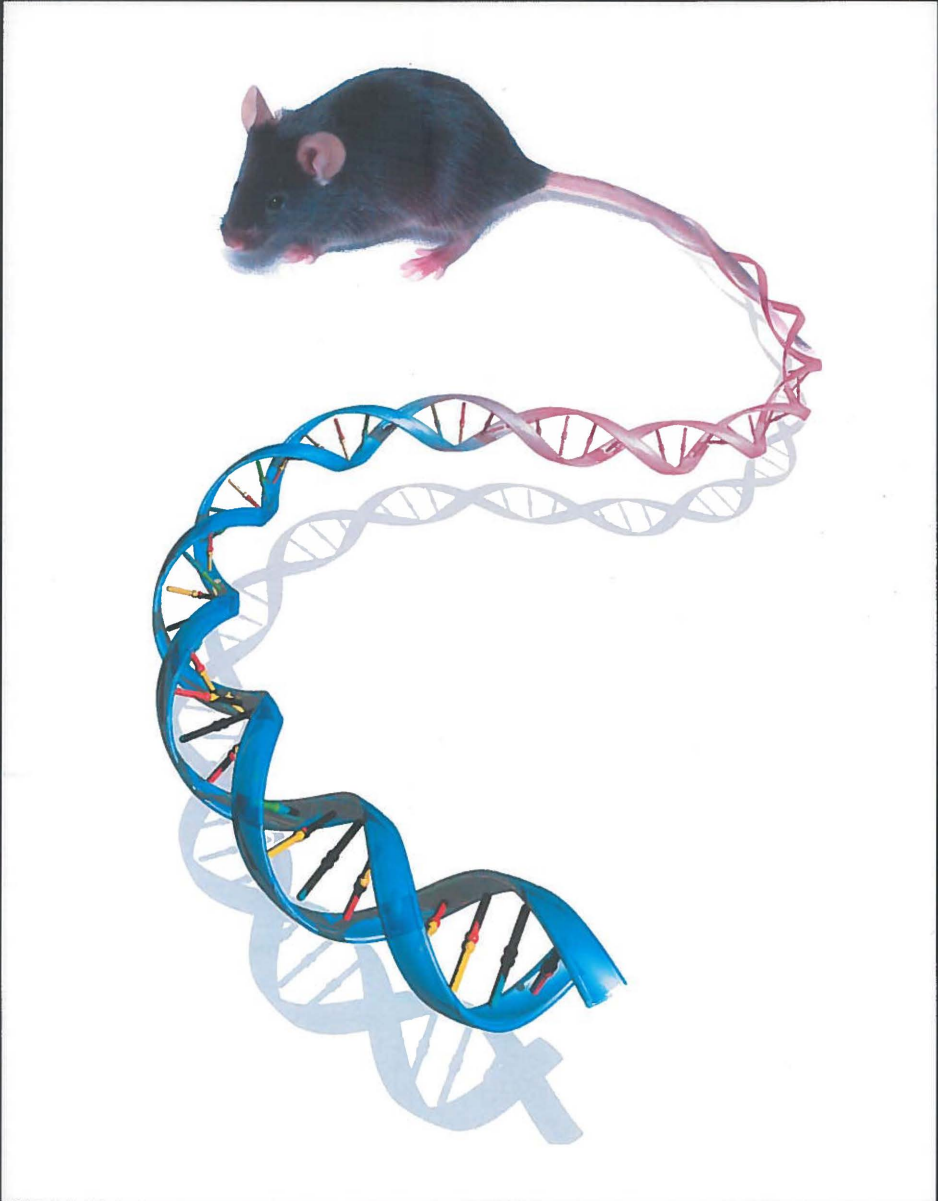
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Mapping of asthma susceptibility in recombinant congenic mouse strains



Benoit J.A. Piavaux

Mapping of asthma susceptibility in recombinant congenic mouse strains

Stellingen

1. Recombinant congenic stammen zijn een goede 'tool' voor lage resolutie mapping van genen in complexe ziektes.
2. Voor hoge resolutie genetische mapping zijn andere methodes, zoals F2 kruisingen of 'expression profiling', beter geschikt dan het gebruik recombinant congenic stammen.
3. In de genetica zien we dat we vaak de keuze hebben tussen hoge resolutie en lage statistische power of lage resolutie en hoge statistische power. Zelfs de nieuwe high throughput SNP technieken hebben hier weinig verandering in gebracht.
4. De beste oplossing voor mappen van genen lijkt dus om een combinatie van technieken te gebruiken waarbij een lage resolutie methode wordt gebruikt voor identificatie van loci waarna hoge resolutie methodes kunnen gebruikt worden voor identificatie van kandidaat genen.
5. Comparative genomics, waarbij genetische data van verschillende diersoorten worden vergeleken, is een erg waardevolle methodiek gebleken in genetisch onderzoek in verschillende complexe ziekten. Voor astma genetisch onderzoek is dit echter niet bruikbaar door gebrek aan genetische data in andere diersoorten.
6. Er zijn reeds veel astma genen geïdentificeerd, maar er is relatief weinig onderzoek gedaan naar de functie en rol van veel van deze genen. Astma genetisch onderzoek zal de transitie naar meer functioneel onderzoek moeten maken.
7. Met de huidige genetische studies hebben we de limiet van de mogelijkheden van de klassieke statistiek bereikt. Tijd voor innovatie?
8. Waarom worden in Nederland zoveel PhDs opgeleid, als er daarna voor de grote meerderheid toch geen werk voor ze is in Nederland? Of is dit een methode om goedkope arbeidskrachten binnen te halen?
9. Waarom moeten er stellingen toegevoegd worden aan een proefschrift als niemand deze gebruikt tijdens de verdediging?
10. De Pyreneeën zijn het ideale gebergte voor solo alpinisten. Alles is er op menselijke maat. Dit betekend echter niet dat de klassieke gevaren van bergsport niet op de loer liggen.

Benoit J.A. Piavaux

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Mapping of asthma susceptibility in recombinant congenic mouse strains

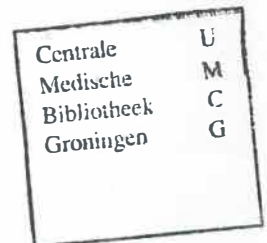
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Mouse models for asthma and mouse asthma genetics

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Introduction

Allergic asthma is a heterogeneous disease which can be characterized by high levels of allergen-specific immunoglobulin (Ig)E in serum, chronic eosinophilic inflammation of the airway tissue and hyperreactivity of the airways (AHR) to bronchospasmogenic stimuli. Genetic susceptibility to asthma appears to be due to multiple genes that interact with each other and the environment. Although genetic linkage and association studies have identified many susceptibility loci for specific asthma traits and identified a number of susceptibility genes, fine-mapping and identification of all the genes involved in asthma-related traits may prove to be extremely difficult not in the least part due to epistatic interactions. Yet, in order to understand the genetic basis of asthma, it is important to identify as many different susceptibility genes as possible and to determine their interactions. Although an animal model of asthma may not exhibit all aspects of human disease, identification of genes involved in certain well-defined characteristics may accelerate further human studies. Given the considerable homology between the human and mouse genome, the mouse may prove to be a useful genetic model organism for complex human diseases. Furthermore, genetic homogeneity and strictly controlled developmental (age) and environmental (infection, food, climate) conditions are major advantages of a mouse model as well as the fact that the mouse “toolbox” (gene-targeted mice, genetic and physical map) is very well developed. The possibility to generate congeneric lines makes it possible to determine if the phenotypic effect of a locus is caused by one gene or by a combination of multiple closely linked genes at the locus, something that can not be done in humans.

An illustration of the relevance of comparative genetics is the quantitative trait loci (QTL) that determine plasma lipid concentrations, risk factors for atherosclerosis, in mice and humans. Most of the human QTLs controlling plasma lipids have concordant QTLs in mice, suggesting that many genes identified in mice may also regulate the same traits in humans¹. Furthermore, if a gene controlling an experimental asthma trait in mice is not genetically linked to asthma in humans, understanding the biological

function of this gene and the pathway leading to the asthma trait in mice may still be relevant for asthma in humans.

In this chapter, we will discuss some of the mouse models for experimental asthma and the genetic tools available to study asthma genetics in the mouse. Furthermore, genetic studies of asthma-related traits in the mouse will be discussed.

Mouse models for asthma

Allergens

Various allergens including ovalbumin (OVA), house dust mite (HDM) and *Aspergillus fumigatus* are being used in the mouse in an attempt to provide insights into the complex immunological and pathophysiological mechanisms of human IgE-mediated allergic diseases.

Ovalbumin

In the majority of current models for allergic airway inflammation, OVA is used as a model allergen, though it is a food- in stead of an aero-allergen. In most protocols, mice are sensitized by an intraperitoneal (i.p.) injection of OVA, often together with a Th2 skewing adjuvant, such as aluminium hydroxide². Sensitization in itself induces production of OVA-specific IgE resembling allergic sensitization in atopic individuals.

Sensitized animals can subsequently be exposed to allergen, administered to the target organ, the lung, via inhalation, intranasal or intratracheal routes. The mouse is not the most suitable model to measure immediate bronchoconstrictive responses upon allergen provocation although a small bronchoconstrictive reaction can be measured which is associated with mast-cell degranulation^{3,4}. Furthermore, the late phase bronchoconstriction is absent or mild compared to the response in patients with allergic asthma^{4,5}.

Without prior systemic sensitization, repeated exposure to aerosolized OVA results in inhalation tolerance and no or very low serum levels of OVA-specific IgE^{6,7}. Following repeated airway allergen challenge, OVA sensitized mice gradually develop AHR and recruit eosinophils, T cells and some neutrophils into the airways which can be monitored by histologic examination of airway tissue, bronchoalveolar lavage (BAL) or single cell suspension after enzymatic digestion of lung tissue⁵. Th2 cytokines such as

IL-4, IL-5 and IL-13 can be measured in BAL fluid^{8,9} or upon antigenic re-stimulation of thoracic lymph node cells¹⁰.

AHR is most often determined in response to the acetylcholine analogue methacholine but other nonspecific bronchospasmogenic agents such as serotonin have also been used¹¹. Different methods to measure airway constriction and AHR in the mouse have been described as reviewed by Drazen and colleagues¹². Whole-body barometric plethysmography that determines the empirical variable "enhance pause" (penh) has become extremely popular during the last decade because of its ease of use, however, criticism regarding this technique has been accumulating¹³. At present, this technique is no longer recommended unless as a preliminary technique for screening purposes. Fortunately, other techniques are available such as the airway pressure time index (APTI), forced oscillations and the classic approach of measuring dynamic lung compliance and resistance¹².

Importantly, most of the OVA mouse models described in the literature show differences with respect to levels of airway inflammation and AHR. This is probably due to the fact that mouse models differ with respect to the genetic background of the mice, the route, dose and frequency of exposure to the allergen and the type of adjuvant being used. In particular, the route of OVA administration has an important impact on the quality of the immune response. Development of allergic airway disease can also be induced by administration of antigen-pulsed dendritic cells into the airways following limited inhaled allergen exposure¹⁴. However, virtually all of the protocols, which avoid systemic sensitization with adjuvant, elicit less prominent airway inflammation when compared to sensitization with adjuvant. Importantly, the percentage of eosinophils in BAL of patients with allergic asthma (approximately 5-20%) is more close to the percentages observed in models without adjuvant (20%) than those with alum as adjuvant (50-80%)^{2,15}. One has to be aware that there are no perfect mouse models that fully reflect all aspects of human asthma.

House dust mite

House dust mites (HDM) are the most common sources of aeroallergens which in genetic susceptible individuals can cause symptoms ranging from atopic dermatitis to bronchial asthma. In Europe, *Dermatophagoides pteronyssinus* (*Der p*) is the most frequently encountered HDM and their faecal particles contain several groups of molecules acting as allergen. The most important is *Der p* 1, a cysteine protease derived from the mite's intestinal tract^{16,17}. A number of studies have demonstrated that *Der p* 1 is capable of cleaving human proteins with potentially immunomodulatory effects including α 1-proteinase inhibitor (A1-Pi)¹⁸, CD23 (the human low-affinity IgE receptor)¹⁹, CD25 (the α subunit of the IL-2 receptor)²⁰ and tight junctions of bronchial epithelium, leading to increased bronchial epithelial permeability²¹. In contrast to the OVA model, in which robust allergic sensitization relies on introduction of OVA in the peritoneum in conjunction with an adjuvant, for HDM it has been shown that intranasal exposure generated acute²² as well as chronic²³ airway inflammation and AHR to methacholine with the characteristic hallmarks of a Th2-type immune-inflammatory response. Interestingly, a recent study showed that HDM facilitates OVA-specific allergic sensitization and airway inflammation²⁴. In order to study the impact of concurrent allergen exposure on the development of allergic responses, mice were exposed to HDM concurrently with OVA for 5 weeks. Subsequently, mice were allowed to rest for 8 weeks, at which time they were then re-exposed to aerosolized OVA alone. This *in vivo* OVA recall elicited a robust airway inflammatory response in the lung but also systemically (OVA-specific IgE). Collectively, these data demonstrate that HDM is able to subvert OVA's intrinsic innocuous nature and to privilege a Th2 inflammatory response over the default tolerogenic bias.

HDM is also used in a protocol in which mice are sensitized with an intraperitoneal injection of HDM/aluminium hydroxide and exposed to HDM via trachea instillation^{25,26}.

Aspergillus fumigatus

Among the allergic fungi, *Aspergillus fumigatus*, a saprophytic mold, distributed widely in the environment is a frequently recognized etiologic agent in a number of allergic conditions. Inhalation of *A. fumigatus* antigens complicates a number of pulmonary diseases ranging from infective (invasive aspergillosis), allergic bronchopulmonary aspergillosis and allergic exacerbation of asthma. Allergic responses to *A. fumigatus* involve a number of immunologic abnormalities including: elevated IgE, enhanced Th2 cytokines such as IL-4, IL-5, and IL-13²⁷, eosinophilic and T-cell inflammation, AHR to methacholine and profound airway remodeling²⁸. Currently over 20 recombinant allergens from *A. fumigatus* have been cloned and expressed. However, in spite of the recombinant antigens available in pure form, soluble, crude *A. fumigatus* antigen extract is still most commonly used for the induction of allergic airway disease by intranasal instillation in animals²⁹. Crude extracts are highly antigenic and contain biologically active substances such as ribotoxin and proteases³⁰. In the models for *A. fumigatus* induced allergic airway inflammation, potent sensitization to the extract occurs in the absence of exogenous adjuvants. Enzymes such as proteases may serve as adjuvants, perhaps by inducing epithelial damage and allowing normally excluded antigens to bypass the mucosal barrier³⁰.

Gender

A striking common feature of many autoimmune and inflammatory diseases is that females are more susceptible to specific immunological disorders than males³¹. In general, females have better B-cell-mediated immunity than age-matched male counterparts. They have higher immunoglobulin levels and stronger antibody responses to various foreign antigens^{32,33}. For asthma it also has been described that, after puberty, asthma occurs more frequently among women who also have a higher risk of more severe asthma^{34,35}. We have used an OVA mouse model of allergic airway inflammation to characterize gender differences in mice with respect to the local immune response in the lung³⁶. We found that female mice are evidently more susceptible to allergic airway inflammation than males. They have higher numbers of

eosinophils, (activated) T cells and B cells in lung tissue. In addition, they have higher levels of IL-4, IL-5 and IL-13 in lung homogenates. Serum levels of total and OVA-specific IgE were also higher in females than in males. Our study is in accordance with Hayashi who also found a clear difference with respect to eosinophilia³⁷ and studies from Seymour *et al.* and Corteling *et al.* who both found elevated IgE levels in female mice sensitized and challenged with OVA as compared to males sensitized and challenged with OVA^{38,39}. Preliminary data from our group indicate that gender differences in mice with respect to allergic airway inflammation also exist in a HDM mouse model of asthma⁴⁰.

Models for chronic airway inflammation and hyperreactivity

Typical features of asthma are recurrent episodes of airway inflammation and airway obstruction, and some patients experience a gradual decline in lung function and fixed obstruction. These fixed alterations have been attributed to structural changes in the airways, which are apparent on histologic sections and have been termed 'airway remodeling'. To create a chronic model with features of airway remodeling, different approaches have been tried, mainly by sustaining airway challenges over weeks to months following the initial acute response. However, there have been relatively few descriptions of satisfactory mouse models of chronic antigenic challenge associated with airway inflammation and remodeling resembling human asthma. Experimental protocols have involved repeated exposure by OVA inhalation^{41,42}, repeated bolus delivery of antigen intratracheally using OVA⁴³ or *A. fumigatus*²⁹ or intranasally using OVA⁴⁴ or HDM²³. In these chronic mouse models, marked inflammatory processes, mucus hypersecretion, epithelial cell hyperplasia and thickening of the basement membrane are found in the bronchi in response to repeated airway challenges with the allergen. However, in most chronic models structural changes in the proximal airways have been described and structural changes in small airways were usually absent. Recently a protocol was developed by the group of Renz that demonstrated chronic allergic inflammation of both proximal and distal airways⁴⁵. Airway inflammation was associated with subepithelial fibrosis, goblet cell hyperplasia and increased mass of α -

smooth muscle actin-positive cells in small airways. Furthermore, the inflammatory and structural changes in this model were associated with stable airflow limitation and increased AHR. Interestingly, compared with acute allergic inflammation, the chronic inflammation was dominated by lymphocytes and not by eosinophils.

Assessment of chronic changes in the airways and lung tissue cannot be quantitated without very careful tissue preparation and morphometry. Given discrepancies in methodological approaches, problems of tolerance induction and issues related to differences among certain strains and allergens, more thorough studies of 'chronic' models are needed to critically judge their applicability and usefulness.

Mouse Asthma Genetics

Mouse toolbox for gene-hunting

For geneticists inbred mouse strains are powerful tools to identify new disease related genes. Each mouse is ‘completely’ homozygous and genetically identical to all other mice of the same strain and the environment can be controlled throughout the experiments. Additionally mice can be crossed at discretion of the investigator and they have a very short generation time, 3 weeks gestation and 8 weeks to sexual maturity.

In human genetics linkage, association and DNA micro-array studies are the only tools available. These tools were also extensively used in mice. However in mice other approaches are possible. For example recombinant inbred (RI) and Recombinant congenic (RC) strains, which are derived from two inbred strains, have shown to be a powerful tool in the identification of QTLs in all kind of multigenic diseases and traits^{46,47}. Another interesting technique is transgenesis with artificial chromosomes by which effects of human loci can be investigated in mice⁴⁸.

As there is almost no genetic nor phenotypic variation within one strain, all genetic and phenotypic variation has to be introduced by outcrossing 2 inbred mouse strains. The selection of these two mouse strains is critical. There are many mouse strains, but only few are regularly used for genetics. These are well characterized strains, from which SNPs and microsatellite markers polymorphisms are known. The best option is to phenotype these strains first. If no phenotypic difference in the trait of interest is found in these well-known strains, other strains can be screened. Choosing strains with extreme phenotypic differences can make phenotyping easier and linkage or association analysis more powerful.

Induced mutagenesis was widely used in the genetics of micro-organisms and *Caenorhabditis elegans* and can also be used in mice. As part of the PGA project of the NHLBI (<http://www.nhlbi.nih.gov/resources/pga/index.htm>) the Jackson Laboratory is

screening ENU-induced mutants for naïve AHR and other heart, lung and blood disease related phenotypes (<http://pga.jax.org/>). In order to allow the detection of recessive mutations, the second generation of offspring is phenotyped. Direct comparison of both, mutant and wild-type, strains is difficult as there is not enough genetic variability between both strains for classical mapping methods (linkage, association, RC and RI strains). Without introducing genetic variability by outcrossing with another inbred strain only micro-array comparisons and sequencing can be used to identify the mutation. By outcrossing both mutant and wild-type strains with the same strain the mutation can be mapped using conventional techniques. As the only difference between both mapping experiments is the presence of a mutation in one of the parental strains, the differences in results can only be due to this mutation and experimental variability. The mutant strain can of course also be used as any other inbred strain in conventional mapping experiments. Despite these mapping difficulties, a big advantage of ENU-induced mutagenesis is that it can lead to new interesting mutations which are not present in any inbred strains. The biggest advantage however, is that mutant strains are a very powerful tool to study the biological implications of the mutation, once it is mapped and identified.

Linkage and association analysis

Linkage and association studies have massively been performed in human and mouse to identify genes involved in multigenic traits. In mice these studies are largely facilitated by the short generation time with large nest sizes and the homozygous genotypes. However, less resolution is obtained due to twice as low recombination frequencies in mice compared to human. The power of the analysis can be increased by selecting strains with extreme phenotypic differences or by the selection of the extreme phenotypes for analysis.

DNA micro-array experiments

DNA micro-array experiments have become a powerful tool to explore the transcriptome. As RNA levels are measured, functional mutations, which do not lead to

altered RNA expression levels can not be detected. Micro-arrays have also been used in combined approaches. The expression QTL strategy is a nice example which is described below⁴⁹.

Recombinant congenic and Recombinant inbred strains

Both RI and RC strains are obtained by outcrossing two inbred strains. In RI strains offspring is intercrossed (heterozygote x heterozygote) a few times and part of the offspring is then inbred in order to obtain a series of strains which contain a random 50% of the donor genome. For RC strains two backcrosses (heterozygote offspring x homozygote parental) are done with one of the parental strains (the background strain) in order to reduce the fraction of genome inherited from the donor strain to 12.5%. (see figure 1). Generally a RC series of strains is composed of approximately 20 strains.

Mapping loci with RC strains is easy and straightforward. The strains from which the genotype is known, and thus also the genomic fragments inherited from the donor strain, are all phenotyped. The loci of interest are the chromosomal fragments inherited from the donor-strain in the strains with the donor's phenotype. Fine-mapping can be done by analysis of the overlapping regions and by backcrossing with the background strain in order to obtain more recombination in the region of interest. RI strains can be used for mapping in the same way as RC strains, but it will require more backcrossing as 50% of the genome is donor inherited compared to 12.5% in RC strains. RI strains are also a very good starting point for association analysis as many intercrosses were already done before inbreeding. For this RI strains can be outcrossed, but generally it is preferred to backcross them, especially when recessive traits are studied. Backcrossing with the parental strain carrying the recessive allele will raise the fraction of genome of this strain and by this the probability to have an affected recessive phenotype.

The Complex Trait Consortium (CTC), a group of research labs working on genetics of multigenic traits, is planning to make a very large panel of RI strains (approx. 1000 strains), derived from 8 common inbred strains. Seen the large number of strains, these

could be used directly for association type studies without the need for additional crosses between the RI strains. As each mouse within a strain is genetically identical, mice would have to be genotyped only once. Phenotyping also becomes easier and more robust as more than one mouse with the same genotype can be tested. For this same reason gene-environment interaction would be also easier to study⁵⁰.

Artificial chromosomes

With artificial chromosomes human loci or parts of it are added to the mouse genome on a separate small chromosome. A major disadvantage of this technique is that no gene-replacement occurs, the mouse loci are still present and unaltered. The fact that human encoded proteins are expressed in a foreign host (mouse) is the second big disadvantage but also the power of this technique. This second disadvantage could be circumvented by using a syntenic mouse locus.

Interesting ‘proof of concept’ experiments were done by Symula et al., who used Yeast artificial chromosomes (YACs) to explore the effect of a human locus in mice⁴⁸. For this a panel of YAC transgenic mice containing 1 Mb of the human locus (5q31) was made. Multiple transgenic lines that showed a lowered IgE response in an OVA model of asthma, had a 180 kb shared region of human chromosome, encoding 5 genes including genes for human IL-4 and IL-13, both cytokines involved in B-cell isotype switching. The presence of this 180kb fragment of the locus appeared to down-regulate mouse IL-4 and IL-13. This down-regulation can explain the lowered amount of IgE as was already demonstrated in mice using gene-inactivation^{51,52} or by using transgenesis^{53,54}. The mechanisms by which both IL-4 and IL-13 are down-regulated were not investigated.

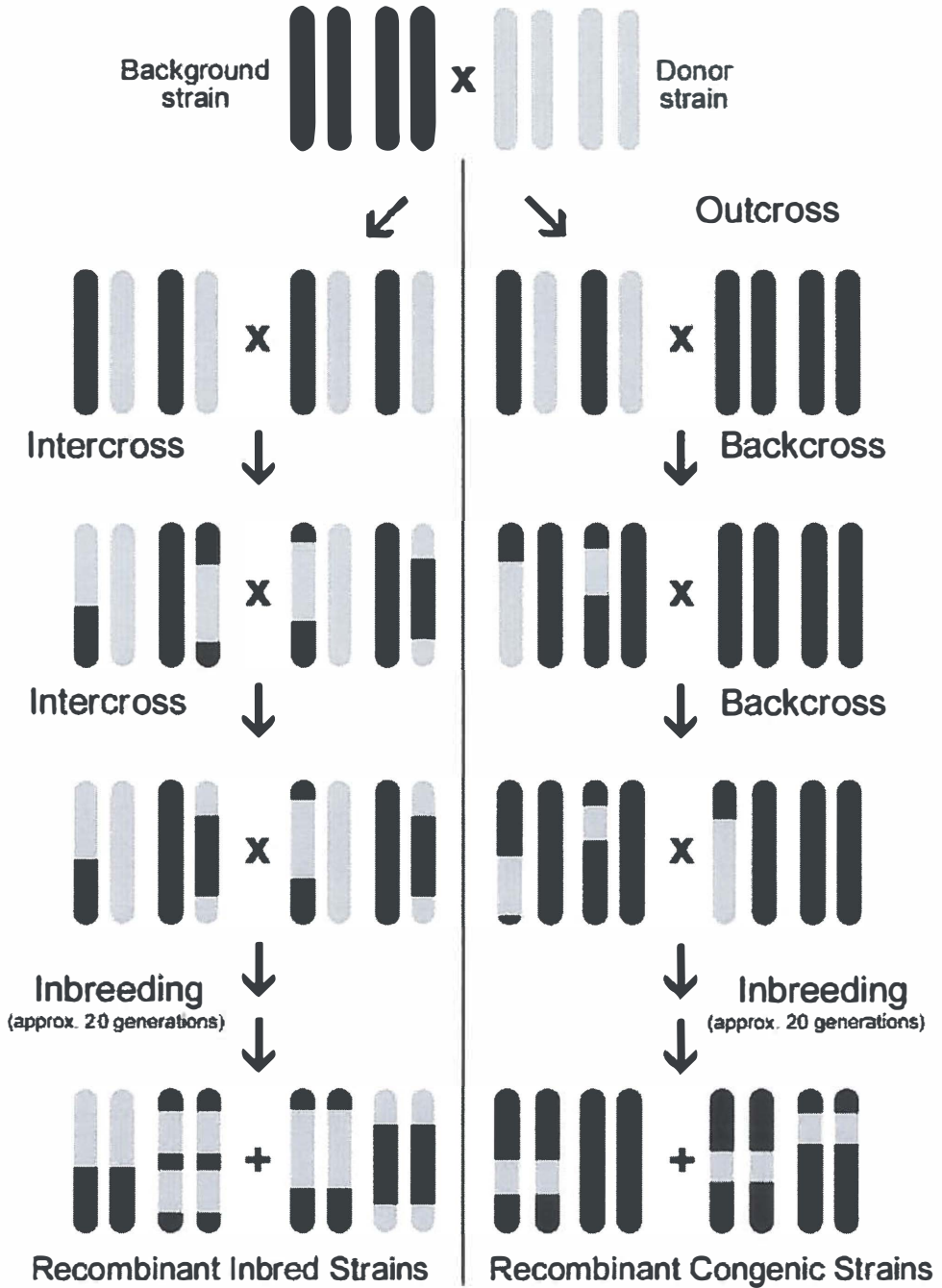


Figure 1: Breeding scheme for RI and RC strains. Only 2 chromosome pairs showed.

Screening of standard inbred strains

Most inbred mouse strains were derived from only a few wild mice⁵⁵. They can therefore be considered as an advanced panel of RI strains and can thus be used to map genes by comparing the phenotypes of interest in the different strains⁵⁶. Due to the high number of generations and crosses preceding the inbreeding of the currently available inbred strains, the resolution of such a screening is significantly higher than the resolution that could be obtained by the screening of the recently developed RI strains. In the original 'proof of concept' paper Pletcher et al. were able to map previously known genes for single locus traits (coat color, albinism) and complex traits (saccharin preference, HDL cholesterol and gallstones)⁵⁶.

Comparative Genomics and other bio-informatic tools

Comparative genomics is an approach in which genetic data of both human and animal models are used to reduce the size of loci of interest and identify candidate genes. The assumption in comparative genomics is that the same genes are involved in the sensitivity to develop the disease in both human and animal models. Under this assumption and if a QTL exists which shows homology in both species, the gene of interest should be in the homologous region and the QTL can be reduced to that homologous region only.

For example a 54 Mbp asthma QTL was mapped on mouse chromosome 7 in 2 different studies which both used crosses between A and C3H^{57,58}. This locus shows synteny to a human asthma QTL on chromosome 19⁵⁹. If we would only consider the homologous region then the mouse QTL could be reduced from 54 to 28.5 Mbp⁶⁰.

Despite being a powerful tool for the reduction of the size of QTLs, there is a possibility that the assumption that the same gene underlies the sensitivity to develop a complex trait in different species is wrong. In this case the gene of interest could be within the discarded part of the locus. However this will become evident when the candidate genes are tested.

As stated above the commonly used inbred mouse strains were all derived from a few wild mice and thus they share regions of DNA that are identical by descent. As 97% of the polymorphism between these mouse strains are ancestral⁵⁵, QTL genes are unlikely to be in regions which are identical by descent^{61,62}. By consequence QTLs can be reduced to regions outside of the ones which are identical by descent. This latter method is called haplotype analysis as the regions of identity by descent are inferred from SNP haplotypes.

For both comparative genomics and haplotype analysis it is important to have as much mouse cross data as possible. Considering the low number of mouse crosses done for asthma, these can not yet be optimally used to reduce asthma QTLs.

Combined approaches

All methods described above, except micro-arrays, can identify QTLs with a maximal resolution of approximately 1 cM. Linkage and association studies are generally faster to map a locus as there is no inbreeding required (typically 20 generations). These techniques are also more able to detect interacting genes, as more different genotypes are analyzed, compared to RC strains. Fine-mapping however works best by backcrossing RC strains as it requires fewer mice. Interesting recombinants can be made homozygous in only 2 generations, which allows fast and efficient testing even when recessive traits are studied.

Combined approaches are therefore most used. In their discovery of the Tim gene family McIntire et al. screened RC strains (BALB/c x DBA/2). Then they used one congenic strain which had BALB/c as background and a chromosomal fragment syntenic to human asthma locus 5q23-q35 from DBA/2 for fine-mapping using linkage analysis⁶³. In another successful gene-hunting study, Karp et al. used linkage analysis in C3H/HeJ x A/J cross as a first step. Then they used micro-arrays to compare mice from a (C3H/HeJ x A/J) x A/J backcross to A/J. Complement factor 5 was significantly

downregulated in A/J mice, the susceptible strain. Sequencing allowed the identification of relevant polymorphisms⁶⁴. Both these studies are discussed in more detail below.

Expression-QTL (eQTL) analysis is a new combined approach in which DNA microarray experiments and QTL-mapping are combined. In eQTL analysis expression levels of genes are used as quantitative traits in a QTL-mapping experiment. First two parental mouse strains with difference in the trait of interest are selected and a micro-array experiment is done to compare the expression levels between both strains. From this experiment the genes for the eQTL-mapping are chosen. The two strains are then intercrossed a few times. The offspring of the last intercross is then used in a QTL (trait of interest) and eQTL (differentially expressed genes) mapping experiment. QTL and eQTL maps can then be compared. Of particular interest are the regions of the chromosome in which both QTL and eQTL maps show a peak in LOD-score especially if the gene of the eQTL maps to that region too. The pool of genes selected this way is enriched for genes with expression levels related to the trait (QTL-peak = eQTL-peak) and that are encoded within the region of the QTL-peak. This eQTL mapping technique is mathematically very complex and still under development. There are still a few statistical issues related to multiple testing to be addressed. However it has already produced some nice results, especially in genetics of obesity⁶⁵ and hematopoietic stem cell function⁶⁶. It has not been used in mouse models of allergic asthma yet.

Mouse asthma genetics

Allergic asthma, in both human and mouse models, is characterized by AHR, eosinophilic inflammation of the lungs and elevated allergen specific serum IgE levels. These 3 important traits in asthma do not always correlate and can even be mapped to different regions of the genome of both human and mice.

A simple but nice illustration of this are the experiments by Ewart et al.⁵⁷. They measured airway responsiveness, OVA-specific serum IgE levels and eosinophil

content of the bronchoalveolar lavage fluid after OVA sensitization and challenge in 5 inbred strains of mice. A/J and AKR/J both show equally high airway reactivity. In A/J both eosinophil and IgE levels were also high compared the other strains, but in AKR/J only low levels of IgE and eosinophils were detected. C57BL/6J and C3H/J are both unresponsive, except for a mild eosinophilia in C3H/J. BALB/cJ showed intermediate phenotype for all 3 traits.

Loci and genes involved in AHR

AHR in both naïve and sensitized animals has been used as quantitative traits for mapping. An overview of AHR loci is given in Table 1. Interestingly the chromosome 6 locus for naïve AHR found in a A/J x C3H/HeJ is not found in the same cross when mice were sensitized with OVA, while in this same cross the suggestive locus on chromosome 7, with synteny to human 19q13, is found in both naïve and OVA sensitized mice⁶⁷. A good candidate gene for this chromosome 7 locus is kallikrein, as bronchial tissue kallikrein is highly correlated to the appearance of histamine and kinin after allergen exposure⁶⁸ and the BAL levels are highly associated with immediate type hypersensitivity events. Additionally kallikrein inhibitors were shown to significantly reduce eosinophilia⁶⁹.

Nicolaidis et al. identified IL-9 as a candidate AHR susceptibility gene in naïve mice using a series of RI strains derived from C57BL/6 and DBA/2. The RI strains were used in a QTL-analysis in order to identify a locus on chromosome 13. Only 2 candidate genes were selected in the region of linkage: CamL and IL-9. The selection was based upon proximity to the marker and the presence of the human homologue in syntenic region 5q31-q33, a region with high LOD-score for AHR and IgE levels in human. The selected RI strains as well as the parental strain C57BL/6 showed significantly lower IL-9 production by splenocytes compared to DBA/2. The IL-9 ORF and the 5' and 3' flanking regions were sequenced in both parental strains, but no polymorphisms in coding neither in regulatory sequences can explain such a drastic loss of IL-9 expression. As no significant polymorphisms are found, as yet, only an undiscovered

cis-acting mechanisms could explain the loss of IL-9 expression on the C57BL/6 allele⁷³.

Position (Chromosome (cM))	Name	Cross	Naïve/ Sensitized	Human synteny**	Ref
2 (10.0)	Abhr1	A/J x C3H/HeJ	OVA-sensitized	10p11-13, 2q12- q14, 9q22-q34	57
2 (30.0)	Abhr2/C5	A/J x C3H/HeJ	OVA-sensitized	9q33-q34, 2q14-q24	57
2 (74.0)	Bhr1	A/J x C57BL6J	Naïve		70
6 (50.5)	Bhr5	A/J x C3H/HeJ	Naïve		58
7 (1.5)*	Abhr3	A/J x C3H/HeJ	OVA-sensitized	19q13	57
7 (16.0)*	Bhr4	A/J x C3H/HeJ	Naïve		58
9 (18)		BP2 x BALB/c	OVA-sensitized	11q23	71
10 (44)	Asthm1	BP2 x BALB/c	OVA-sensitized	12q22-q24	71
11 (7)		BP2 x BALB/c	OVA-sensitized	5q31	71
11 (23.0)	Tapr/Tim1	BALB/c x DBA/2	KLH-sensitized	5q33.2	63
11 (52)	Asthm2	BP2 x BALB/c	OVA-sensitized	17q12-q22	71
13 (38.0)	Bhr6	A/J x C3H/HeJ	Naïve		58
15 (47.1)	Bhr2	A/J x C57BL6J	Naïve		70
17 (7.6 – 55.7)	Sea-1	BALB/c x C57BL/6 RC	OVA-sensitized	6p21	72
17 (10)		BP2 x BALB/c	OVA-sensitized	6p21	71
17 (14.0)	Bhr3	A/J x C57BL6J	Naïve		70

Table 1: Airway hyperreactivity loci identified in mice. * Only suggestive linkage,

** :Corresponding human QTLs.

The group of van Oosterhout used RC strains to map asthma susceptibility loci in an OVA mouse model of asthma. In a first ‘proof of concept’ study CcS/Dem strains, which are RC strains with BALB/c as background and STS as donor, were used⁷⁴. From this study it appears that the different phenotypes related to asthma, i.e. AHR, airway inflammation and elevated IgE levels, can be separately mapped in the mouse using RC strains, as no correlations between these asthma related phenotypes could be found in every strain⁷⁵. CcS/Dem-5, which was previously reported to be resistant to *Leishmania major* infection⁷⁶, appeared to be almost completely resistant to experimental asthma⁷⁵. In a second study the C.lmr RC strains (BALB/c as background and C57BL/6 as donor) were used^{77,78}. The C.lmr.1 strain appears to be resistant to all 3 major experimental asthma phenotypes⁷². Interestingly the congenic fragment inherited from C57BL/6 (D17Mit57 to D17Mit129) maps to the same chromosomal region as the QTL identified by the group of Vargaftig in a BP2 x BALB/c cross⁷¹. Also CcS/Dem-5 inherited a chromosomal fragment (D17Mit19 to D17Mit10) from STS which can be mapped to the same chromosomal region. Currently the group of van Oosterhout is using C.lmr.1 sub-congenics (obtained by backcrossing C.lmr.1 with the background strain BALB/c) to narrow down this chromosome 17 locus.

Ackerman et al. used a backcrossing approach to identify 2 interacting loci involved in naïve AHR. First A/J (susceptible strain) and C57BL/6 (resistant strain) were outcrossed. The most susceptible offspring was then selected for backcrossing with the resistant strain (C57BL/6). Using this backcrossing strategy, the susceptible alleles for the trait of interest are selected while the resistance alleles are lost during breeding. As traits encoded by other genes are not used for selection of offspring to cross the alleles of these genes are still randomly distributed. The trait can now be mapped in the offspring of the last backcross by genotyping and searching for conserved chromosomal regions⁶⁷. Using this strategy Ackerman et al. identified 2 interacting loci, one on chromosome 2 and one on chromosome 6. They confirmed these results with chromosome substitution strains with C57BL/6 as background and A/J as donor strain. Interestingly the chromosome 2 locus corresponds to *Bhr1* and the locus on

chromosome 6 corresponds to Bhr5 which were both identified in a C3H/HeJ x A/J cross (see Table 1).

Adam33, a gene that was previously identified as a susceptibility gene for AHR in humans⁷⁹, could have been a good candidate gene for the chromosome 2 locus. However no polymorphisms between both strains can be found in the sequence of Adam33 and its flanking regions suggesting that, at least in the mouse, another closely linked gene is contributing to AHR⁶⁷.

Loci and genes involved in airway inflammation: a few success stories

Only few groups use mouse-models to study the genetics of asthma and most of the work is done on AHR. Despite this, one gene-family, Tim, and one other gene, C5, related to airway inflammation and AHR were identified using combined approaches as already mentioned above.

The Tim-gene family identified

The first step in the identification of the Tim-gene family was the screening for AHR and Th2 cytokines in the BAL fluid in a BALB/c (sensitive, background) x DBA/2 (resistant, donor) RC series of inbred strains, which were sensitized and challenged with OVA. Only one strain was unresponsive (HBA). Interestingly the DBA/2 inherited fragment is syntenic to human 5q23-35⁶³.

The linkage analysis for fine-mapping was done by backcrossing this unresponsive congenic strain with the susceptible BALB/c background strain. Mice were then genotyped and only mice with interesting recombinations in the DBA/2 inherited locus were phenotyped (IL-4 expression after keyhole limpet hemocyanin immunization). In order to obtain a resolution able to distinguish the locus from other genes nearby, as for example IL-4 and IL-12p40, more than 2000 mice were genotyped. Screening for AHR was done the same way. Both QTLs mapped to the same region on chromosome 11⁶³.

Once this mapping was performed, the search for a candidate gene could start. First the human sequence was aligned to identify the genes present in both human and mouse loci, but none of the known genes identified this way showed coding polymorphisms. Tim-1 the homologue of rat Kim1 and human HAVcr-1 was another possible candidate gene. Coding sequences for Tim-1 from both BALB/c and DBA/2 were compared and major polymorphisms identified. DBA/2 has a 15 bp deletion and a 3 amino-acid polymorphism. Tim-3, identified by EST alignment, does also map to the same locus but shows no polymorphisms between both strains. From these EST databases it also appeared that Tim1 was mainly expressed by T-cells⁶³. Transgenic mice with a T-cell receptor specific to OVA were generated in both BALB/c and DBA background. DBA CD4⁺ T-cells showed significantly less IL-4 and IL-13 production in an in-vitro assay⁸⁰.

In a recent paper the same group reports the role of Tim-1 in the regulation of Th-cell differentiation and Th2-cell proliferation. When Tim-1 is crosslinked on CD4⁺ T-cells using a Tim-1 monoclonal antibody IL-4 and IFN- γ expression is significantly upregulated. Tim-1 expression is maintained only on Th2-cells, where crosslinking results in increased Th2 cytokine production. Crosslinking of Tim-1 by antibody in vivo was able to prevent the induction of tolerance, leading to more severe AHR and airway inflammation compared to untreated mice⁸⁰.

Complement factor 5

Also the group of Wills-Karp used a combined approach in the identification of complement factor 5 (C5) as asthma susceptibility gene. First they performed a QTL analysis for AHR in a A/J (susceptible strain) x C3H/HeJ (resistant strain) cross and found two loci on chromosome 2, Ahr1 and Ahr2 (see table 1)⁵⁷. In the second step micro-arrays were used to compare gene-expression in the lungs of both resistant (C3H/HeJ and (C3H/HeJ x A/J) x C3H/HeJ backcross low responders) and susceptible (A/J and (C3H/HeJ x A/J) x C3H/HeJ backcross high responders) mice. From the 21

differentially expressed genes only one, C5, could be mapped to one of both loci identified in the first step (Ahr2)⁵⁷.

A significant correlation ($p < 0.005$) between AHR and C5 mRNA expression levels is observed in the (C3H/HeJ x A/J) x C3H/HeJ backcross, confirming the role of C5 in the development of AHR in this OVA model of asthma. Additionally the lowered expression levels of C5 significantly correlated with the presence of a 2 bp deletion in the 5' exon of the C5 gene in low and high responders from the backcross and the parental strains but also some other inbred strains⁵⁷.

The group of Wang further studied the role of C5 on the development of airway inflammation and AHR and its effects on the early and late allergic responses using anti-C5 antibodies and C5 deficient mice. Their results show that C5 is involved, but not critical, in the development of airway inflammation, as airway inflammation is inhibited partially when anti-C5 antibodies are administered. In contrast to these observations, there is no difference in airway inflammation in mice carrying the C3H/HeJ resistant allele compared to mice with the A/J allele, suggesting C5 is not involved in asthmatic airway inflammation. AHR however, is largely inhibited by C5 depletion by anti-C5 antibody or in C5 deficient mice. In these C5 deficient mice AHR could be restored by i.v. injection of recombinant C5. The late and early allergic responses are both lower when mice are treated with anti-C5 antibody, indicating a role for C5 in these processes too⁸¹.

Concluding remarks

Several genome-wide screens for asthma-related traits, in particular AHR, have been carried out in mice. These studies have generated many susceptibility loci some of which are concordant with loci that have been linked with the same trait in humans (see: <http://cooke.gsf.de/asthmagen>). Despite the advantages of mice over humans to find susceptibility gene(s) in a locus, so far only two susceptibility genes, C5 and Tim-1, have been identified. Many of the initial linkage studies in mice appear to be discontinued or are not yet completed. One of the reasons may be that genetics takes time and this is a disadvantage in open competition for research grants that are often focused on short-term results. Nevertheless, there is still a strong need for mouse asthma genetics, in particular for finding genes in QTLs that have human homologous counterparts since this is more cost-effective. Once a mouse gene is identified, it can be directly tested in humans. Furthermore, mice can be used to dissect the genetics of different steps in the disease process like allergic sensitization, progression to allergic disease and asthma and severity and persistence of the disease.

One of the alleged advantages of mouse asthma genetics is to find interacting genes, which are difficult to identify in human genetic studies. For instance, using a set of congenic strains it was possible to demonstrate that a locus on mouse chromosome 3, which is linked to the development of autoimmune diabetes in the nonobese diabetic (NOD) mouse, consisted of 3 different loci, Idd3, Idd10 and Idd17, and that Idd10 interacted with both Idd3 and Idd17⁸². Again, the benefits of mouse genetics have not been exploited extensively in asthma research. At present, only one study has described interacting loci involved in AHR⁶⁷. Future research on mouse asthma genetics should therefore focus on gene-gene interactions and on gene-environment interactions. In contrast to human asthma genetics studies, the environment can be well-controlled in mouse studies. However, selecting the relevant environment to carry out these type of mouse asthma genetics studies is rather challenging since the potential environmental variation is tremendous. Therefore, mouse asthma genetic studies involving gene-environment interaction should carefully take into account what is already known regarding the role of the environment in the pathogenesis of asthma like exposure to

endotoxin or cigarette smoke. Furthermore, the environment to which humans are exposed is continuously changing and consequently new genes will be implicated in development of asthma in these new (micro)environments.

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Mouse genetic model for antigen-induced airway manifestations of asthma

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Abstract

Allergic asthma is a genetically complex disease characterized by allergen-specific IgE, eosinophilic inflammation of the lungs and airway hyperresponsiveness (AHR) to bronchospasmogenic stimuli. In the present study, we compared 13 recombinant congenic (RC) mouse strains in an OVA model of allergic asthma.

Different intensities and types of responses are observed throughout the RC strains. Intensities range from resistance to asthma in CcS05, to a very severe bronchoconstrictive reaction upon methacholine challenge for the parental STS strain. All strains show a 'modified' Th2 response except CcS14 which shows a 'true' Th2 response.

When data from all strains are pooled, airway reactivity shows significant correlations with the serum immunoglobulin levels and the levels of IL-4, IL-5 and IL-13 in the broncho-alveolar lavage (BAL), at low dosage of methacholine (below 25 mg/ml) while at high dosage airway reactivity only correlates with BAL neutrophil levels. This indicates that at least two different mechanisms are involved in the airway reactivity to methacholine. None of these correlations can be found in every individual strain, which demonstrates that the asthma traits in this mouse model are genetically dissociated and that the loci can be genetically mapped.

Introduction

Allergic asthma is a heterogeneous disease which can be characterized by allergen-specific IgE levels in serum, reversible airway obstruction, chronic eosinophilic inflammation of the airway tissue and hyperresponsiveness of the airways to non-specific bronchospasmogenic stimuli. Genetic susceptibility to asthma appears to be due to multiple genes that interact with each other and the environment. Genome wide screens for asthma and atopy have been performed in different ethnic populations and resulted in statistical evidence for linkage to several chromosomal regions including 5q, 11q and 12q¹⁻⁶. Some of these regions contain relevant candidate genes that may regulate IgE responses, Th-cell differentiation, inflammatory processes and airway hyperresponsiveness. Although an increasing number of linkages for atopic diseases have been confirmed ⁷, it remains to be demonstrated that all candidate genes are linked to the pathophysiology of asthma.

Fine-mapping and identification of all the genes involved in asthma-related traits has proven to be extremely difficult not in the least part due to epistatic interactions. Until now 5 potential susceptibility genes for atopy or asthma were identified using positional approaches: a desintegrin and metalloproteinase 33 (ADAM33)⁸⁻¹⁰, dipeptidyl peptidase 10 (DPP10)¹¹, plant homeodomain zinc finger protein 11 (PHF11)¹², G-protein coupled receptor for asthma (GPRA)¹³ and serine protease inhibitor Kazal type 5 (SPINK5)¹⁴.

Polymorphisms of both, ADAM33 and SPINK5, were not associated with asthma in some of the tested populations^{15,16}. This could be due to different phenotyping of asthma or epistatic interactions but it can not be excluded that other polymorphisms in close linkage disequilibrium to the polymorphisms of both ADAM33 and SPINK5 are conferring the asthma susceptibility.

Although an animal model of asthma may not exhibit all aspects of the disease, identification of genes involved in certain well-defined characteristics may accelerate

further human studies. Given the considerable homology between the human and mouse genome, the mouse has proven to be a useful genetic model for complex human diseases^{17,18}. Furthermore, genetic homogeneity and strictly controlled environmental conditions are major advantages of mouse models as well as the fact that the mouse “toolbox” (gene-targeted mice, genetic and physical map) is very well developed.

Nowadays a number of genetic tools is available for localization of genes involved in disease development¹⁸. In several diseases the recombinant congenic (RC) strains of mice have proven to be a powerful tool to provide the mapping of genes controlling complex traits^{17,19-27}. A series of RC strains comprises approximately 20 homozygous strains, each of which contains on average 87.5% genes of a common background strain and 12.5% of a common donor strain^{26,28}. In this way, the RC system transforms a multigenic difference into a set of mono- or oligogenic differences and hence offers higher resolution power in mapping the QTLs (quantitative trait loci) and detecting their mutual interactions than the standard genetic methods. The RC system has been used most successfully to map genes in colon tumor susceptibility²⁹⁻³².

One of the RC strains series, CcS/Dem, generated by Demant and colleagues uses BALB/cHeA as background strain and STS/A as donor strain³³. The BALB/c mice produce high serum IgE levels whereas the STS/A shows low serum IgE levels after *Leishmania major* infection¹⁷. This series of RC strains has been used to dissect the genetics of T-cell activation and several models of inflammatory diseases, including *Mycobacterium tuberculosis* and *Leishmania major* infection^{17,19-25,27}.

In the present study, we used the CcS/Dem series to determine the susceptibility of each strain and both parental strains BALB/cHeA and STS/A for antigen-induced airway manifestations of asthma as described by Deurloo *et al.*³⁴. Generated data were used to calculate correlations between the different asthma related characteristics for all individual strains and in the pooled population.

Materials and methods

Mice

Male, 5 to 9 week old BALB/cHeA, STS/A and CcS/Dem²² mice were obtained from the breeding colony of P. Demant. 7 to 11 mice per strain were phenotyped (see table 1). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal experiments. The mice were kept under specified pathogen free conditions according to the guidelines of the Federation of European Laboratory Animal Science Association⁴⁷. They were housed in enriched macrolon cages under filter top or in a laminar flow cabinet with food and water administered *ad libitum*.

Experimental Asthma protocol

On day 0 and 7 the animals were intraperitoneally sensitized with 10 µg OVA in 2 mg Alum adjuvant (Pierce, Rockfort, IL, USA). One week after the second sensitization blood was taken. Subsequently, the animals were challenged for 20 minutes with 10 mg OVA/ml in saline, starting on day 21 and then once per three days for a total of three times. Twenty-four hours after the last challenge airway responsiveness to methacholine was measured. Consecutively, blood and bronchial alveolar lavage fluid were collected.

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice before (day 18) and after (day 28) OVA challenges. Airway reactivity was determined by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl-β-methylcholine chloride, Sigma, St. Louis, MO, USA) at doses of 1.6, 3.1, 6.3, 12.5, 25 and 50 mg/ml using barometric whole-body plethysmography (BUXCO, Wilmington,

NC, USA). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously⁴⁸.

STS mice were not exposed to doses above 12.5 mg/ml methacholine as higher doses induce a very severe bronchoconstrictive reaction.

Ovalbumin-specific immunoglobulin levels in serum

After measurement of in vivo airway reactivity, mice were sacrificed by intraperitoneal injection of 1 ml 10% urethane in pyrogen-free saline (Sigma, St. Louis, MO, USA). Subsequently, mice were bled by cardiac puncture and serum was collected and stored at -70°C until analysis. Levels of OVA-specific IgE, IgG1 and IgG2a in the serum were measured as described previously⁴⁹. A reference standard was used with arbitrary units of each isotype of 1,000 EU/ml. The detection levels of the ELISAs were 0.05 EU/ml for IgG2a, and 0.5 EU/ml for IgE and 0.005 EU/ml for IgG1.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and 2 µg/ml aprotinine (Roche Diagnostics, Basel, Switzerland). Cells were spun down and supernatant was stored at -20°C until measurement of cytokines by ELISA. Subsequently, lungs were lavaged four times with 1 ml aliquots of saline. The BAL cells were washed with PBS (400 × g, 4°C, 5 min) and the pellet was resuspended in 150 µl PBS. Total numbers of BAL cells were counted in a Burker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential BAL cell counts, cytopsin preparations were stained with Diff-Quick (Merz & Dade A.G., Dudingon, Switzerland). After they were coded, all cytopsin preparations were evaluated by one observer. Cells were identified and differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology. Per cytopsin preparation, at least 200 cells were counted.

Cytokine ELISAs

Cell-free supernatants of the first ml of BAL fluid were analyzed for IL-4, IL-5, IL-10, IL-13, and IFN- γ content by sandwich ELISA using antibody pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/ml for IL-4, 32 pg/ml for IL-5, 100 pg/ml for IL-10 and IL-13 and 160 pg/ml for IFN- γ .

Statistical analysis

Results obtained before and after OVA-challenge were compared using the signed rank tests. The different strains were compared to BALB/c using Mann Whitney U-test. Correlations were calculated using Spearman rank correlations. False discovery rate for correlation tests was estimated to be 5% for the 95% and 1% for the 99% confidence intervals by computing correlations in 1000 permuted datasets. Statistical analyses were performed using SAS for Windows version 9.0 (SAS-Institute, Cary, NC, USA).

Results

Airway reactivity

After OVA challenges the parental STS strain shows a very severe bronchoconstrictive reaction at doses of methacholine above 12.5 mg/ml. Therefore STS was not exposed to the complete dose-range and Penh measurements obtained for STS were not used for further statistical analysis.

All tested strains show an increase in airway reactivity after OVA challenge compared to before challenge (data not shown), which is significant at high doses of methacholine (25 and 50 mg/ml) for all strains except CcS03 (only at 50 mg/ml), CcS05, 12, 13 and 15.

After challenge none of the tested strains, except STS, show a consistent and significant deviation in Penh compared to BALB/c at doses of methacholine below 25 mg/ml. At 25 and 50 mg/ml however, significantly lower Penh values are seen in CcS02, 03, 05, 07, 11, 12, 13, 15 and 18. For CcS20 this difference is only significant at 25 mg/ml methacholine. Surprisingly none of the tested RC strains show a significant higher extent of airway reactivity, compared to BALB/c, while the donor strain, STS, shows a much more severe bronchoconstrictive reaction compared to the background strain BALB/c (see Table 1 and Figure 1).

Serum immunoglobulin levels

OVA-specific IgE levels are significantly increased after challenge compared to before challenge in BALB/c, CcS02, 07, 14 and 20. IgG1 and IgG2a levels after challenge are increased in all strains except CcS03 (only IgG2a is increased), 11, 13, 18 and STS (only IgG2a is increased), compared to before challenge.

After challenge, CcS01, 05, 07 and STS have significantly lower OVA specific IgE levels compared to BALB/c, while significantly higher levels are measured in the serum of CcS11, 12 and 14. IgG1 levels after challenge are strongly and significantly lower in CcS05 and CcS13 compared to BALB/c after challenge. Only CcS05 mice show significant lower IgG2a levels compared to BALB/c (see Table 1).

Cellular composition of the BAL fluid

Due to the severe bronchoconstriction in the STS mice, BAL could not be performed reliably. Compared to BALB/c total cell counts are higher in CcS01, CcS02 and CcS03 and significantly lower in all other strains except in CcS07, 14, 15 and 20. Despite this significant lower total cell count, the number of eosinophils is not significantly lower in CcS10, 11, 12 and 13 compared to BALB/c.

Cytokine composition of the BAL fluid

No IFN- γ could be detected in the BAL fluid of any of the tested strains (detection limit 160 pg/ml). Compared to BALB/c, higher IL-4 levels are measured in the BAL fluid of CcS01, 07, 11, 12, 14 and 18. None of the tested strains show significantly lower IL-4 levels compared to BALB/c. IL-5 is higher in the BAL fluid of CcS14 and CcS18 and strongly lower in CcS05 compared to BALB/c. The anti-inflammatory cytokine IL-10 is significantly higher in CcS01, 07, 12, 14, 15 and 18. None of the tested strains show significantly lower IL-10 content of the BAL fluid, compared to BALB/c. IL-13 is significantly lower in CcS05 and significantly higher in CcS14 and 18.

Parameter	% of BALB/c							
	BALB/c (n = 11)	CcS01 (n = 7)	CcS02 (n = 9)	CcS03 (n = 7)	CcS05 (n = 13)	CcS07 (n = 10)	CcS10 (n = 11)	
Penh (1.6 mg/ml)	1,2 ± 0,2	175,1 ± 31,6 *	89,3 ± 6,4	74,2 ± 6,9	83,4 ± 6,8	61,7 ± 5,1 *	104,4 ± 21,0	
Penh (3.1 mg/ml)	2,8 ± 1,0	105,9 ± 13,6	50,0 ± 4,6	43,0 ± 4,5	49,3 ± 3,7	37,0 ± 3,5	68,2 ± 14,4	
Penh (6.2 mg/ml)	3,8 ± 1,2	108,0 ± 20,4	54,5 ± 5,3	42,9 ± 5,4	48,1 ± 3,4	36,2 ± 2,6	84,2 ± 13,0	
Penh (12.5 mg/ml)	7,4 ± 1,4	75,4 ± 14,2	40,5 ± 3,2 *	30,8 ± 5,8 **	23,4 ± 3,6 **	23,7 ± 2,3 **	79,9 ± 7,2	
Penh (25 mg/ml)	10,7 ± 1,6	60,9 ± 10,5	35,4 ± 2,2 **	29,7 ± 4,5 **	27,7 ± 3,1 **	25,7 ± 3,0 **	72,2 ± 2,7	
Penh (50 mg/ml)	11,5 ± 1,7	63,2 ± 7,1 *	37,2 ± 3,4 **	36,9 ± 6,9 **	31,2 ± 4,0 **	32,7 ± 2,9 **	79,1 ± 5,7	
Mononuclear cells	1,8 ± 0,2 10 ⁶ cells	328,0 ± 56,4 **	133,0 ± 12,8	251,6 ± 44,6 *	23,3 ± 2,4 **	88,2 ± 11,0	43,4 ± 6,1 **	
Eosinophils	2,7 ± 1,0 10 ⁶ cells	362,4 ± 70,9 **	201,9 ± 32,0 *	246,7 ± 44,7 *	2,8 ± 1,2 **	106,8 ± 20,1	37,3 ± 7,4	
Neutrophils	3,2 ± 0,8 10 ⁵ cells	630,8 ± 155,8 *	192,5 ± 29,2 *	271,2 ± 90,2	11,2 ± 4,7 *	169,1 ± 28,9	64,0 ± 11,9	
Total cell count	5,4 ± 1,1 10 ⁶ cells	366,5 ± 53,1 **	181,0 ± 23,6 *	251,5 ± 41,3 **	9,9 ± 1,3 **	105,6 ± 17,1	40,9 ± 6,1 *	
IgE	1,1 ± 0,3 10 ⁵ EU/ml	40,4 ± 8,1	117,8 ± 28,0	96,2 ± 32,2	28,5 ± 11,0 *	30,5 ± 5,6 *	64,9 ± 16,8	
IgG1	5,4 ± 1,9 10 ⁹ EU/ml	61,4 ± 11,0	66,3 ± 14,5	69,0 ± 18,3	10,1 ± 2,2 *	41,5 ± 12,9	134,6 ± 52,3	
IgG2a	5,9 ± 3,7 10 ⁵ EU/ml	37,0 ± 14,0	82,4 ± 39,6	62,5 ± 25,5	4,5 ± 1,3	10,6 ± 2,4	34,2 ± 11,8	
IL-4	8,8 ± 1,7 pg/ml	150,2 ± 12,4	97,6 ± 13,0	111,6 ± 24,1	95,9 ± 32,4	255,2 ± 100,9	263,0 ± 87,3	
IL-5	272,5 ± 86,4 pg/ml	204,0 ± 27,0 *	167,2 ± 28,3	152,3 ± 54,5	5,3 ± 2,9 *	97,6 ± 21,7	59,9 ± 19,5	
IL-10	9,0 ± 0,6 pg/ml	1322,1 ± 38,0 **	610,7 ± 46,7 *	1105,8 ± 174,3	112,4 ± 4,8	1109,7 ± 79,4 **	414,4 ± 69,5	
IL-13	50,3 ± 15,6 pg/ml	117,4 ± 18,5	104,8 ± 23,6	150,3 ± 39,1	10,8 ± 2,9 *	63,1 ± 8,0	27,7 ± 8,1	

Table 1a: Mean ± standard error of mean for BALB/c and mean % of BALB/c ± % standard error defined as ((mean for strain ± standard error of mean) / mean for BALB/c) x 100, *: Significant at 95% confidence level, **: Significant at 99% confidence level

Parameter	% of BALB/c						
	CcS11 (n = 7)	CcS12 (n = 11)	CcS13 (n = 7)	CcS14 (n = 7)	CcS15 (n = 7)	CcS18 (n = 8)	CcS20 (n = 8)
Penh (1.6 mg/ml)	85,7 ± 13,0	108,6 ± 5,4	69,8 ± 4,3	89,4 ± 21,3	113,4 ± 16,3	169,8 ± 10,2 **	119,2 ± 17,5
Penh (3.1 mg/ml)	67,9 ± 7,0	56,9 ± 3,9	41,7 ± 5,4	56,0 ± 6,7	66,9 ± 11,6	81,0 ± 4,6	68,0 ± 13,1
Penh (6.2 mg/ml)	59,2 ± 5,1	51,4 ± 2,1	49,4 ± 11,8	66,7 ± 3,5	66,6 ± 9,9	76,7 ± 3,9	61,8 ± 12,8
Penh (12.5 mg/ml)	37,8 ± 2,8 **	37,4 ± 3,2 **	39,7 ± 6,4 *	81,1 ± 14,5	38,2 ± 6,0 *	50,5 ± 3,8 *	49,1 ± 12,5
Penh (25 mg/ml)	29,6 ± 3,0 **	29,1 ± 3,5 **	39,0 ± 7,2 **	73,1 ± 9,7	39,8 ± 7,0 **	39,5 ± 6,0 **	54,5 ± 9,0 *
Penh (50 mg/ml)	31,2 ± 1,9 **	26,1 ± 3,1 **	49,5 ± 8,3 *	84,0 ± 12,3	51,6 ± 8,7 *	48,4 ± 9,3 *	68,8 ± 8,4
Mononuclear cells	28,9 ± 4,9 **	36,0 ± 5,4 **	40,1 ± 9,5 **	52,2 ± 7,1 **	91,1 ± 15,2	13,1 ± 2,5 **	109,3 ± 28,7
Eosinophils	21,9 ± 8,3 *	45,3 ± 10,6	41,4 ± 19,8	43,7 ± 10,1	140,2 ± 22,7	17,5 ± 4,1 *	112,2 ± 33,8
Neutrophils	10,3 ± 7,1 *	4,1 ± 2,1 **	29,5 ± 13,1 *	88,1 ± 32,3	193,7 ± 36,4	29,9 ± 8,4 *	75,5 ± 17,7
Total cell count	23,8 ± 6,7 **	40,7 ± 7,4 *	40,7 ± 16,1	49,1 ± 9,9 *	127,2 ± 18,5	16,6 ± 3,5 **	109,8 ± 30,5
IgE	277,2 ± 52,9 **	257,2 ± 46,0 *	106,6 ± 31,9	841,1 ± 196,8 *	157,1 ± 53,6	232,5 ± 86,0	190,1 ± 38,5
IgG1	94,0 ± 27,0	94,2 ± 17,4	0,1 ± 0,0 *	56,4 ± 16,9	155,7 ± 49,1	68,6 ± 23,8	167,1 ± 38,4
IgG2a	46,0 ± 12,3	16,0 ± 3,3	30,2 ± 21,3	81,7 ± 26,5	208,1 ± 99,3	113,8 ± 95,7	57,9 ± 22,6
IL-4	406,4 ± 115,3 *	461,2 ± 84,1 **	52,2 ± 3,2 *	4013,7 ± 1119,6 *	70,4 ± 4,3	693,2 ± 154,0 **	62,8 ± 5,2
IL-5	127,0 ± 50,3	76,6 ± 24,8	61,3 ± 15,4	339,4 ± 74,3 **	75,2 ± 12,7	262,9 ± 52,5 *	58,7 ± 16,3
IL-10	922,6 ± 71,8 *	571,7 ± 33,4 **	178,6 ± 3,3	6477,1 ± 491,2 *	637,9 ± 22,1 **	1902,7 ± 126,2 **	382,1 ± 15,9 *
IL-13	68,3 ± 81,3	51,2 ± 53,2	26,9 ± 7,0	326,3 ± 47,7 **	49,6 ± 11,0	252,7 ± 27,7	37,9 ± 4,0

Table 1b: Mean ± standard error of mean for BALB/c and mean % of BALB/c ± % standard error defined as ((mean for strain ± standard error of mean) / mean for BALB/c) x 100, *: Significant at 95% confidence level, **: Significant at 99% confidence level

Correlations between asthma related parameters

Remarkably, in the pooled population different correlations between airway reactivity (Penh) and other asthma related parameters are observed at high doses of methacholine (25 and 50 mg/ml) compared to low doses of methacholine (12.5 mg/ml and below). At low doses of methacholine airway reactivity significantly correlates to serum IgE levels, BAL IL-4, -5 and -13 levels while at high doses of methacholine, airway reactivity correlates to neutrophil content of the BAL (at 25 and 50 mg/ml). At 1 mg/ml methacholine Penh also correlates with the eosinophil and IL-10 content of the BAL fluid (see Table 2). Airway reactivity correlates to both IgG1 and IgG2a (except at 3 mg/ml) at all doses of methacholine.

Strong significant correlations are seen between IgE, IgG1 and IgG2a levels. The IgE levels also correlate significantly with all measured Th2 cytokines and the eosinophil content of the BAL. IgG1 and IgG2a serum levels correlate with all cell-types and the IL-4 and IL-5 content of the BAL. Additionally IgG2a serum levels correlate with the IL-13 BAL levels.

Eosinophil, neutrophil, mononuclear and total cell content of the BAL fluid significantly correlate to each other. A significant correlation is also seen between all cell-types and the IL-5, IL-10 and IL-13 levels (no significant correlation between IL-13 and neutrophils), but surprisingly not with IL-4.

Some of these correlations, which are observed in the pooled population, appear in some of the individual strains but none is observed in every single strain. Additionally some strains show significant correlations which are not detected in the pooled population (data not shown).

Penh 3.2 mg/ml	Penh 6.3 mg/ml	Penh 12.5 mg/ml	Penh 25 mg/ml	Penh 50 mg/ml	Mono nuclear cells	Eosinophils	Neutrophils	Total cell count	IgE	IgG1	IgG2a	il4	il5	Il10	il13	Parameter
0,81 **	0,65 **	0,46 **	0,30 **	0,26 **	-0,04	0,18 *	0,03	0,12	0,28 **	0,32 **	0,19 *	0,29 **	0,22 *	0,21 *	0,25 *	Penh 1.6 mg/ml
	0,77 **	0,51 **	0,26 **	0,22 *	-0,06	0,11	0,00	0,06	0,29 **	0,23 *	0,17	0,29 **	0,25 **	0,15	0,30 **	Penh 3.2 mg/ml
		0,68 **	0,49 **	0,45 **	-0,02	0,15	0,06	0,10	0,34 **	0,28 **	0,22 *	0,35 **	0,36 **	0,17	0,38 **	Penh 6.3 mg/ml
			0,78 **	0,70 **	0,08	0,14	0,15	0,13	0,29 **	0,25 **	0,21 *	0,29 **	0,26 **	0,11	0,32 **	Penh 12.5 mg/ml
				0,85 **	0,19 *	0,16	0,23 **	0,17	0,12	0,20 *	0,20 *	0,11	0,13	0,06	0,11	Penh 25 mg/ml
					0,20 *	0,16	0,28 **	0,18 *	0,12	0,23 *	0,26 **	0,07	0,10	0,10	0,11	Penh 50 mg/ml
						0,85 **	0,74 **	0,93 **	0,00	0,30 **	0,41 **	-0,06	0,38 **	0,29 **	0,23 *	Mononuclears
							0,73 **	0,98 **	0,23 *	0,45 **	0,50 **	0,09	0,56 **	0,35 **	0,37 **	Eosinophils
								0,76 **	-0,13	0,22 *	0,35 **	-0,01	0,41 **	0,29 **	0,16	Neutrophils
									0,16	0,42 **	0,48 **	0,04	0,52 **	0,34 **	0,33 **	Total cell count
										0,55 **	0,43 **	0,40 **	0,39 **	0,29 **	0,52 **	IgE
											0,63 **	0,25 **	0,29 **	0,12	0,21	IgG1
												0,21 *	0,36 **	0,08	0,29 **	IgG2a
													0,48 **	0,51 **	0,51 **	il4
														0,74 **	0,79 **	il5
															0,59 **	il10

Table 2: Spearman rank correlation coefficients: *: Significant at 95% confidence level, **: significant at 99% confidence level. The Spearman Rank correlation coefficients for positive correlations range from 0 to 1 (-1 to 0 for negative correlations), the higher the absolute value of the coefficient the tighter the correlation.

Discussion

In this study we clearly demonstrate the influence of the genetic background on the susceptibility to experimental asthma, as a broad range of susceptibilities is observed throughout the RC CcS/Dem strains. Ranging from a severe methacholine-induced bronchoconstriction in the parental STS strain to resistance observed in the CcS05 strain, which only showed limited airway reactivity at the highest dose of methacholine (50 mg/ml), low IgE, IgG1 and IgG2a levels and low levels of IL-5 and IL-10 in the BAL fluid. The group of Demant observed that CcS05 was also highly resistant to *Leishmania Major* infection, another Th2 related disease²³.

Interestingly not only the susceptibility but also the type of response varies from one CcS/Dem strain to another. For example mouse strain CcS14 shows a “true” Th2 response upon OVA challenge, characterised by high IgE levels, low levels of IgG1 and IgG2a and high levels of Th2 cytokines. These high IgE and low IgG levels can be explained by an IL-4 mediated isotype switch, as high amounts of this cytokine can be found in the BAL fluid. Most other strains show a “modified” Th2 response, characterised by lower IgE levels and a higher IgG1/IgE ratio. This ‘modified’ Th2 response has been previously described as a mechanism which induces less severe atopic or asthmatic responses, compared to ‘true’ Th2 response³⁵. In our study, however, the ‘true’ Th2 responder, CcS14, does not show higher airway reactivity nor allergic inflammation, compared to BALB/c.

These differences in asthma related phenotypes are in accordance with Whitehead *et al*, who also showed the importance of genetic background on the development of allergen-induced airway diseases, using nine genetically diverse inbred mouse strains in an OVA-model of asthma³⁶.

Several groups have shown that IL-13 is crucial for the development of asthmatic AHR. IL-13-KO mice, for example, show no AHR despite an extensive eosinophilic inflammation of the lungs³⁷. The groups of Bleecker and Postma showed an association

between a polymorphism in the IL-13 promoter and bronchial hyperreactivity in a Dutch population of asthmatics³⁸. Vladich *et al.* recently demonstrated that the IL-13 2044GA polymorphism, which is associated with allergy and asthma, encodes for a form of IL-13 which is more active and less effectively neutralised³⁹. In our experiments a significant correlation is seen between IL-13 and airway reactivity at low doses of methacholine (below 25 mg/ml), but not at high doses. Interestingly CcS14 has highly increased and CcS10 highly decreased levels of all measured Th2 cytokines but no significant difference in airway reactivity is observed, compared to the parental strain BALB/c. This nicely illustrates the complexity of the development of AHR in our mouse model of asthma.

The group of Van Scott demonstrated the importance of IL-10 in the development of asthma using a ragweed sensitized and challenged C57/BL6 IL-10-KO mouse. These mice only developed AHR when recombinant IL-10 was administered together with the ragweed challenge, but not when IL-10 was given just prior to airway reactivity measurement using methacholine⁴⁰. Also the group of Gelfand used an IL-10-KO mouse, but in an OVA model of asthma. The mice only developed AHR when they were infected with a adenovirus encoding an IL-10 expression cassette prior to challenge⁴¹. Zhang *et al.* found evidence for linkage between AHR and a locus on mouse chromosome 9, which among other genes also encodes the IL-10R⁴². And more recently Chatterjee *et al.* found genetic association between asthma and a polymorphism in the IL-10 promoter sequence in human⁴³. In our experiments IL-10 is only measured in the BAL at the end of the experiment. A correlation with airway reactivity is only seen at 1.6 mg/ml methacholine.

Also IgE was investigated for its role in the development of AHR. Wynn *et al.* suggest that the development of AHR is not dependent on the IgE production⁴⁴. This is supported by our results as the parental STS shows the lowest serum IgE levels but shows the strongest increase in Penh. On the other hand CcS11 has significantly higher

serum IgE levels, compared to BALB/c, but has significantly lower airway reactivity at methacholine doses of 12.5 mg/ml and above. Despite these observations, a significant correlation between airway reactivity and IgE levels is observed in our data at low methacholine doses, below 25 mg/ml, but not at higher doses.

These correlations may not reflect causal relationship but they nicely fit in a model in which the response to methacholine is caused by at least 2 mechanisms. A first one is predominant at low doses of methacholine (below 25 mg/ml) and is related to the Th2 mediated inflammatory state of the lungs and by this correlates to the cytokine and Ig levels and eosinophil count.

The second mechanism becomes predominant at high doses of methacholine and is related to the neutrophil content of the BAL. This second mechanism gives rise to much higher airway responses, compared to the first one, and could therefore be considered as the main mechanism involved in AHR in this mouse model of allergic asthma.

Interestingly none of the correlations described above can be found in every individual strain. This confirms the complex multigenic character of asthma features and highlights the suitability of the CcS/Dem strains to map the genes involved. It also highlights the fact that none of the tested phenotypes are directly linked and that other factors play a role in the development of an asthmatic response in this mouse model.

RC strains are a powerful tool to unravel the genetics of genetically complex processes as it allows mapping of the genes involved. For example Nicolaides *et al.* could identify IL-9 as a quantitative trait locus implicated in the normal airway response to acetylcholine⁴⁵. Also the CcS/Dem strains, which were also used in our experiments, have already been successfully used by different groups. Lipoldova *et al.* for example were able to identify 5 novel Leishmania Major resistance loci using CcS05¹⁷ and

Havelkova *et al.* used CcS09 and CcS11 for the identification of genes involved in T-cell-receptor induced activation⁴⁶.

It can be concluded that the RC system is a useful tool to map and identify asthma related genes, as the same correlations between asthma related characteristics are found in human and mouse model and a broad range of intensity and type of 'asthmatic' responses are observed. Moreover, the different asthma characteristics segregate independently, which makes it possible to map loci implicated in these different characteristics.

Additionally it can be hypothesized that at least 2 mechanisms are involved in the airway reactivity to methacholine in our mouse model of asthma, a first one, predominant at low doses of methacholine, is related to the allergic inflammatory state of the lungs, while the second one is related to BAL neutrophils and is predominant at high doses of methacholine.

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Identification of the *MHCII* region as an asthma susceptibility locus in RC mice

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Abstract

Allergic asthma is a complex disease characterized by airway hyperreactivity (AHR), Th2 driven eosinophilic airway inflammation, high allergen-specific IgE (spIgE) levels in serum and airway remodeling. Since asthma susceptibility has a strong genetic component, we aimed to identify new asthma susceptibility genes in the mouse by analyzing the asthma phenotypes of the *Leishmania major* resistant (*Imr*) recombinant congenic (RC) strains. The *Imr* RC strains are derived from C57BL/6 and BALB/c intercrosses and carry congenic loci on chromosome 17 (*Imr1*) and 9 (*Imr2*) in both backgrounds. Whereas the *Imr2* locus on chromosome 9 only contributes to a small background-specific effect on spIgE and AHR, the *Imr1* locus on chromosome 17 mediates a strong effect on Th2-driven eosinophilic airway inflammation as well as background-specific effects on spIgE and AHR. The *Imr1* locus contains almost 600 polymorphic genes. To narrow down this number of candidate genes, we performed genome-wide transcriptional profiling on lung tissue from C.*Imr1* RC mice and BALB/c controls. We identify a small number of differentially expressed genes located within the congenic fragment, including a number of *Mhc* genes that are polymorphic between BALB/c and C57Bl/6. The analysis of asthma phenotypes in the C.B10-H2b RC strain, carrying the C57Bl/6 haplotype of the *Mhc* locus in a BALB/c genetic background, reveals a strikingly similar asthma phenotype compared to C.*Imr1*, indicating that the differentially expressed genes located within the C.B10-H2b congenic fragment are the most likely candidate genes to contribute to the reduced asthma phenotypes associated with the C57Bl/6 allele of *Imr1*.

Introduction

Allergic asthma is a disease that is mainly characterized by high allergen specific IgE levels in serum, airway hyperreactivity to non-specific bronchospasmogenic stimuli, Th2-driven pulmonary inflammation and airway remodeling. The genetic susceptibility to develop asthma has been shown to be complex and is influenced by many genes. At the end of 2007, polymorphisms in 173 genes have been associated with asthma and related phenotypes¹. However, only 33 of these associations were replicated in 5 or more populations². This discrepancy can partially be explained by differences in phenotype and phenotyping method. In addition, gene-gene and gene-environment interactions have been shown to play a major role in the expression of a genetic susceptibility to develop asthma³. For example, Battle and coworkers recently showed an ethnicity specific epistatic interaction between IL-4R α and IL-13 among African Americans⁴. Based on functional data, which are available for 63 genes out of the 173, asthma susceptibility genes can be subdivided into 4 functional categories: (i) innate immunity and immune modulation, (ii) Th2 cell differentiation, (iii) epithelial integrity and (iv) lung function and airway remodeling².

Inbred mouse strains also differ in their susceptibility to experimentally induced asthma⁵. This genetic variation between inbred strains can be exploited to identify asthma susceptibility genes. One approach to this end is the characterization of recombinant congenic strains, which consist of a series of inbred strains containing fractions of a donor genome on an otherwise homogeneous genetic background⁶. RC strains have been used as a powerful tool for the mapping of genes involved in complex traits^{7,8}. Using a model of experimental allergic asthma in the CcS/Dem series of RC strains⁸, we have previously established the applicability of RC strains for the mapping of asthma related quantitative trait loci (QTLs)⁹. Different intensities and types of responses were observed throughout the series of strains, indicating independent segregation of multiple asthma susceptibility loci, in theory allowing the identification of the genes involved in the different asthma manifestations^{7,9}.

In order to identify novel asthma susceptibility loci in the mouse, we have employed the *lmr* series of RC strains¹⁰. In parallel to the CcS/Dem strains, the *lmr* strains were developed to map genes involved in *Leishmania major* infection. Two *Leishmania major* resistance (*lmr*) loci have been studied into detail, the *lmr1* locus, on chromosome 17, and the *lmr2* locus, on chromosome 9¹⁰. Both loci were previously identified as *Leishmania major* susceptibility loci in a classical F2 cross with C57Bl/6 and BALB/c^{10,11}. An impaired wound healing process was shown to play a major role in the increased susceptibility of BALB/c compared to C57Bl/6, resulting in significantly smaller lesions, less inflammation and faster collagen deposition in C.*lmr1/2*, the *lmr1/2* RC strain in the BALB/c genetic background^{12,13}. In addition, differences in activities of Th cell subsets have been shown to contribute to *Leishmania* susceptibility¹⁴. Since both an impaired wound-healing response at the level of the airway epithelium and a strong allergen-specific Th2 response are thought to contribute to the pathogenesis of allergic asthma¹⁵, we hypothesized that the *lmr* loci might also contribute to asthma susceptibility. Here, we evaluate the asthma manifestations induced by an OVA-driven allergic asthma model in *lmr1/2* RC strains on both BALB/c and C57Bl/6 backgrounds and identify *lmr1* as an asthma susceptibility locus in the mouse. Furthermore, we employ transcriptional profiling on lung tissue to narrow down the *lmr1* locus to polymorphic genes that are differentially expressed between the two genetic backgrounds. We identify a small number of candidate genes, including a number of MHCII genes. Finally, we employ the C.B10-H2b RC strain to directly test the effect of the haplotype of the MHCII locus on the asthma phenotype in the BALB/c genetic background.

Materials and methods

Mice

Animal housing and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (DEC) at the University of Groningen. BALB/cAnBradley, C57BL/6J, B6.lmr1/2, B6.lmr1, B6.lmr2 and C.lmr1/2 were bred and phenotyped at the Walter and Eliza Hall Institute in Melbourne, Australia, and were obtained from the breeding colonies of S. Foote for experiments. BALB/cAnBradley, C.lmr1 and C.lmr2 were obtained as breeding pairs and were bred and phenotyped at the animal facilities of the University of Groningen, The Netherlands. C.B10-H2b/LilMcdJ (BALB.B) and BALB/cJ controls were obtained from the Jackson Laboratory (Bar Harbor, Maine, US). The mice were kept under specified pathogen free conditions and were housed in enriched macrolon cages under filter top kept in a laminar flow or in individually ventilated cages with food and water administered *ad libitum*.

Experimental Asthma protocol

Mice were phenotyped at age 5 to 9 weeks. On day 0 and 7 the male (for lmr1 and lmr2 RC strains and controls) or female (for compound lmr1/2 RC strains and controls) mice were intraperitoneally sensitized with 10 µg OVA in 2.25 mg Alum adjuvant (Pierce, Rockfort, IL, USA). Serum was isolated and airway responses to nebulized methacholine were measured at day 18. On days 21, 24 and 27, the animals were challenged for 20 minutes with 10 mg OVA/ml in saline. On day 28, mice were used for the measurement of airway responsiveness to methacholine, followed by section and collection of blood and broncho-alveolar lavage fluid. In some experiments, mice were used to isolate individual lobes of the lungs for histology and RNA purification without prior lung function measurements.

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice as described previously, and expressed as enhanced pause (Penh)¹⁶. Airway reactivity was determined by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl- β -methylcholine chloride, Sigma, St. Louis, MO) using barometric whole-body plethysmography (BUXCO, Wilmington, NC). For invasive measurement of airway resistance, mice were anesthetised by i.p. injection of urethane (Sigma; 2 g/kg), tracheotomized and cannulated (*vena jugularis*). Mice were then attached to a computer controlled small-animal ventilator (Flexivent; Scireq, Montreal, Canada). Spontaneous breathing was stopped by i.p. administration of d-tubocurarine (Sigma; 1 mg/kg). Mice were ventilated at a breathing frequency of 280 breaths per minute and a tidal volume of 10 ml/kg. Tidal volume was pressure-limited at 300 mmH₂O. Resistance in response to i.v. administered methacholine was calculated from the pressure response to a 2 s pseudo random pressure wave.

Ovalbumin-specific immunoglobulin levels in serum

Serum was collected and stored at -70°C until analysis. Levels of OVA-specific IgE, IgG1 and IgG2a in the serum were measured as described previously¹⁷. A reference standard was used with arbitrary units of each isotype of 1,000 EU/ml. The detection levels of the ELISAs were 0.05 EU/ml for IgG2a, and 0.5 EU/ml for IgE and 0.005 EU/ml for IgG1.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and Complete mini anti-protease cocktail (Roche Diagnostics, Basel, Switzerland). Cells were spun down and supernatant was stored at -20°C. Subsequently, lungs were lavaged four additional times with 1 ml aliquots of saline. Total BAL cell numbers were counted in a Burker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential BAL cell counts, cytopsin preparations were

stained with Diff-Quick (Merz & Dade A.G., Dudingon, Switzerland). Cells were identified and differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology.

Histology

Eosinophils were identified in 4 µm sections of formalin fixed, paraffin embedded lung tissue by staining for cyanide-resistant endogenous peroxidase activity with diaminobenzidine (Sigma Aldrich) and quantified by morphometric analysis using Leica Qwin image analysis software (Leica Microsystems, Rijswijk, The Netherlands) and expressed as volume percentages, as previously described¹⁸.

Cytokine ELISAs

Cell-free supernatants of the first ml of BAL fluid were analyzed for IL-4, IL-5, IL-10, IL-13, TNFα and IFN-γ content by sandwich ELISA using antibody pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/ml for IL-4, 32 pg/ml for IL-5, 100 pg/ml for IL-10 and IL-13 and 160 pg/ml for IFN-γ.

Isolation of RNA, amplification, hybridization and analysis of microarray data

Individual lung lobes were isolated, quickly frozen in liquid nitrogen and stored until further use at -70°C. Total RNA was isolated using TriReagent according to the manufacturer's instructions (MRC, Cincinnati, OH). Total RNA was DNaseI treated for 30' at 37°C, followed by RNA purification using RNeasy columns (Qiagen, Venlo, Netherlands). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Isogen Lifesciences, de Meern, Netherlands). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). The 3'IVT-Express labeling kit

(Affymetrix, Santa Clara, CA) was used to synthesize Biotin-labeled cRNA using 100 ng of RNA as input material, according to manufacturers' instructions. Concentration and quality of cRNA was confirmed to be within required range using Nanodrop and Agilent Bioanalyzer measurements. Hybridization of cRNA to Affymetrix HT Mouse Genome 430 PM Plate Arrays was performed according to the 3'IVT-Express labeling kit instructions (Affymetrix) using 4.5 μ g of cRNA as input material. cRNA synthesis, labeling and hybridization was performed by ServiceXS (Leiden, the Netherlands).

Average signal intensities for each probeset within arrays were calculated by and exported from Affymetrix's Expression Console (Version 1.1) software using the RMA method, which incorporates convolution background correction, summarization based on a multi-array model fit robustly using the median polish algorithm, and sketch-quantile normalization. For this experiment, two pair-wise comparisons were used to statistically resolve gene expression differences between groups using the R/maanova analysis package¹⁹. Differentially expressed genes were detected by using Fs, a modified F-statistic incorporating shrinkage estimates of variance components from within the R/maanova package^{19,20}. Statistical significance levels of the pair-wise comparisons were calculated by permutation analysis (1000 permutations) and adjusted for multiple testing using the false discovery rate (FDR), q-value, method²¹. Differentially expressed genes were declared at an FDR q-value threshold of 0.05.

Mouse SNP and other polymorphism data

Polymorphisms between BALB/cJ and C57Bl/6J (reference strain) were obtained from the Sanger website: <http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl> (SNPs and insertions/deletions (indels)). Genes were assigned polymorphic in the presence of SNPs or indels in one or more of the exons, irrespective of SNP/indel class. Polymorphisms between C57Bl/6J, C57Bl/10J and BALB/c in the *MHC* locus were derived from the mouse phenome database at Jackson laboratory (<http://phenome.jax.org/SNP/>) using the CGD imputed strain set.

Statistical analysis

Comparison of immunoglobulin and cytokine ELISA data obtained before and after OVA-challenge were performed using signed rank tests.

The different strains were compared to their background strains using Mann Whitney U-test. Statistical analyses were performed using SAS for Windows version 9.0 (SAS-Institute, Cary, NC).

Results

The *lmr* series of RC strains were developed to map genes involved in the sensitivity to *Leishmania major* infection. Susceptibility to *Leishmania major* has been associated with a Th2 type response and a delayed wound healing response^{12,14}. Since both the wound healing response and allergen-responsive Th2 cell activity are highly relevant to the pathogenesis of allergic asthma¹⁵, we hypothesized that the previously identified *lmr1* and *lmr2* loci might contain genes that contribute to the genetic predisposition to develop asthma. The *lmr1/2* RC mice carry both loci, either the BALB/c alleles on C57Bl/6 background in case of B6.*lmr1/2*, or the C57Bl/6 alleles on BALB/c background in the C.*lmr1/2* strain. To test our hypothesis, we assessed the asthma phenotypes of the RC strains C.*lmr1/2* and B6.*lmr1/2*, in comparison to that of the respective parental strains, BALB/c and C57Bl/6.

Asthma phenotypes of *lmr1/2* double congenic mice.

First, we characterized OVA-specific immunoglobulin responses in the RC strains and the parental strains. In all four strains, we found that OVA-specific IgE (spIgE) serum levels are significantly increased after OVA inhalation challenge (Figure 1A). We find that B6.*lmr1/2* mice display a strongly elevated spIgE level in comparison to C57Bl/6, both after sensitization and after the OVA-challenges. In contrast, the C.*lmr1/2* strain and the parental BALB/c strain have similar spIgE levels after sensitization. Nevertheless, the C.*lmr1/2* strain displays a reduced spIgE response after OVA challenges as compared to BALB/c mice (Figure 1A).

For OVA-specific IgG1 (spIgG1), B.*lmr1/2* does show a significant increase in spIgG1 serum levels after OVA inhalation challenges, in contrast to the parental C57Bl/6 strain. The opposite is observed in the BALB/c background, where C.*lmr1/2* shows significantly lower spIgG1 levels after challenge compared to the parental strain BALB/c (Figure 1B).

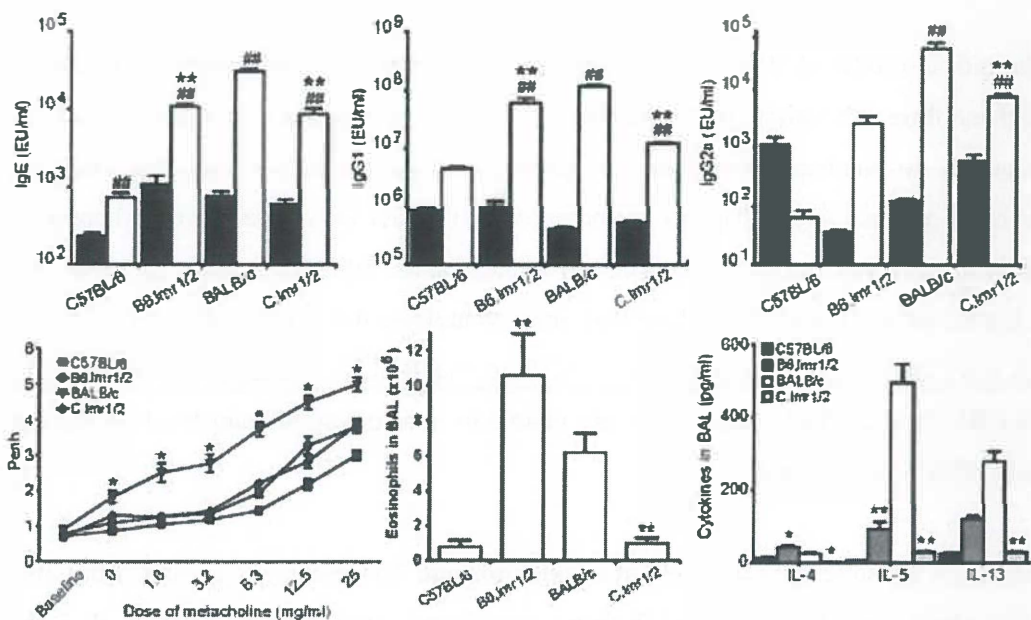


Figure 1: Asthma phenotypes of *lmr1/2* RC strains in both backgrounds. Serum levels of OVA-specific IgE (A), IgG1 (B) and IgG2a (C) before (solid black bars) and after (open bars) OVA inhalation challenges. AHR to increasing doses of methacholine (D). Total numbers of eosinophils in BAL (E) and levels of Th2 cytokines in BAL (F). #: significantly different compared to before challenge with $\alpha \leq 0.05$, ##: with $\alpha \leq 0.01$; *: significantly different from background strain with $\alpha \leq 0.05$, **: with $\alpha \leq 0.01$.

In case of OVA-specific IgG2a (spIgG2a), the C57Bl/6 parental strains shows higher levels after sensitization than the RC B6.*lmr1/2* strain, while the BALB/c parental strain has lower spIgG2a levels in serum compared to C.*lmr1/2*. After challenge the opposite is observed, with higher spIgG2a serum levels in B6.*lmr1/2* and lower in C.*lmr1/2* compared to their respective background strains (Figure 1C). Taken together, these data indicate that the *lmr1/2* loci affect the OVA-specific serum responses of IgE, IgG1 and IgG2a in a consistent fashion, with the C57Bl/6 alleles being associated with an increased spIgG2A response and the BALB/c alleles being associated with increased spIgE and spIgG1 responses.

The mouse model of allergic asthma is also characterized by the induction of AHR to methacholine inhalation. Before challenge all four strains show comparable airway reactivity to methacholine (data not shown). Interestingly, we find that only the BALB/c parental strain displays an increased AHR after OVA challenges, whereas no AHR is observed in the parental C57Bl/6 and the RC B6.*lmr1/2* strain or in the RC C.*lmr1/2* strain (Figure 1D). These data seem to indicate that the C57Bl/6 *lmr1/2* alleles are associated with a suppression of AHR in the BALB/c background, but that the BALB/c *lmr1/2* alleles, in contrast, are unable to induce a significant level of AHR in the C57Bl/6 background.

Finally, we also tested the levels of eosinophils and Th2 cytokines in BAL fluid after OVA challenges. Here, B6.*lmr1/2* shows significantly higher levels of IL-4, IL-5 and higher eosinophil counts in the BAL compared to C57Bl/6, while the C.*lmr1/2* shows significantly lower levels of IL-4, IL-5 and IL-13 and lower eosinophil counts in the BAL than the parental BALB/c strain (Figures 1E and 1F). No Th1 cytokines were detectable in BAL of challenged mice. These data indicate that the *lmr1/2* loci have a consistent effect on Th2 cell activity and eosinophilic airway inflammation, with the C57Bl/6 allele being associated with a suppression of Th2 cell activity and eosinophilia. Taken together, these data indicate that the *lmr1/2* loci present in these RC mice do contribute to susceptibility for allergic asthma phenotypes in the OVA-driven mouse model.

Asthma phenotypes of *lmr1* and *lmr2* single congenic mice.

In order to identify which congenic locus, *lmr2* on chromosome 9 or *lmr1* on chromosome 17, is responsible for the altered asthma phenotypes, we measured the critical asthma parameters in the single congenic strains, carrying either the *lmr1* or the *lmr2* congenic region. Here, the *lmr1* mice carry the chromosome 17 locus, either the BALB/c allele on C57Bl/6 background in case of B6.*lmr1*, or the C57Bl/6 allele on

BALB/c background in the C.lmr1. The B6.lmr2 and C.lmr2 mice carry the congenic chromosome 9 locus in the C57BL/6 and BALB/c background, respectively.

As shown in figure 2A, both RC strains and background strains show similar levels of spIgE before challenge. B6.lmr1 shows significantly more spIgE after challenge compared to C57BL/6 whereas no differences can be observed between C57BL/6 and B6.lmr2 (Figure 2A). In contrast, the C.lmr2 mice show significantly lower spIgE compared to BALB/c mice while no significant differences can be observed between BALB/c and C.lmr1 (Figure 2B). Hence, we do observe divergent and background specific effects for the two *lmr* loci with regard to spIgE responses.

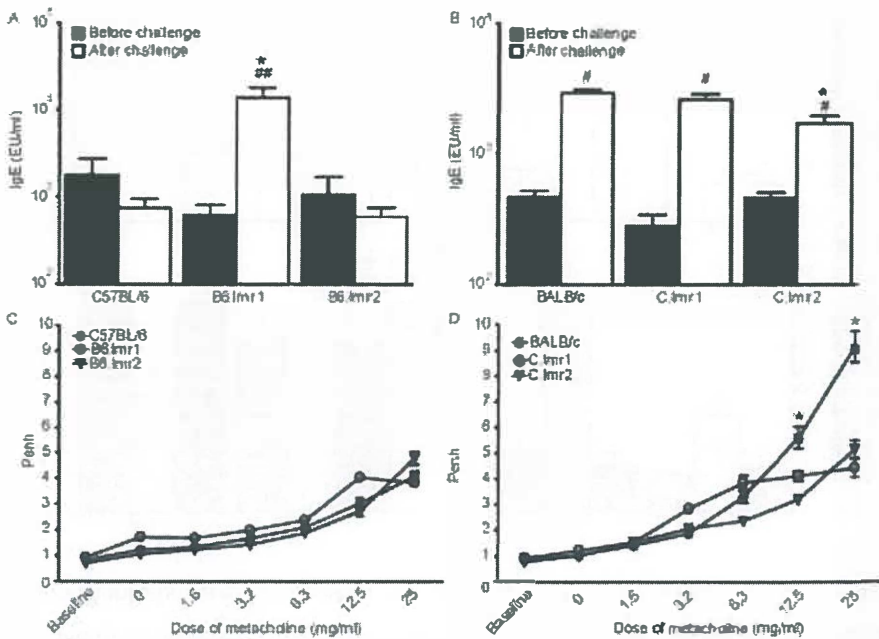


Figure 2: Asthma phenotypes in *lmr1* and *lmr2* RC strains in both backgrounds (1). OVA-specific IgE in single congenic mice on C57BL/6 background (A) and BALB/c background (B) before (solid black bars) and after (open bars) OVA inhalation challenges. AHR to increasing doses of methacholine of single congenic mice on the C57BL/6 background (C) and BALB/c background (D). #: significantly different compared to before challenge with $\alpha \leq 0.05$, ##: with $\alpha \leq 0.01$; *: significantly different from background strain with $\alpha \leq 0.05$, **: with $\alpha \leq 0.01$.

Eosinophil counts as well as IL-4 and IL-5 levels are significantly increased in B6.lmr1 mice compared to the background strain C57Bl/6, whereas no difference can be observed between C57Bl/6 and B6.lmr2 (Figure 3A and 3C). The BALB/c background displays consistent results: the C.lmr1 strain shows significantly lower eosinophil counts as well as IL-4 and IL-5 levels than the BALB/c background strains, whereas the C.lmr2 display comparable eosinophils counts and Th2 cytokine levels in BAL (Figure 3B and 3D). None of the tested strains showed measurable amounts of Th1 cytokines in BAL (data not shown).

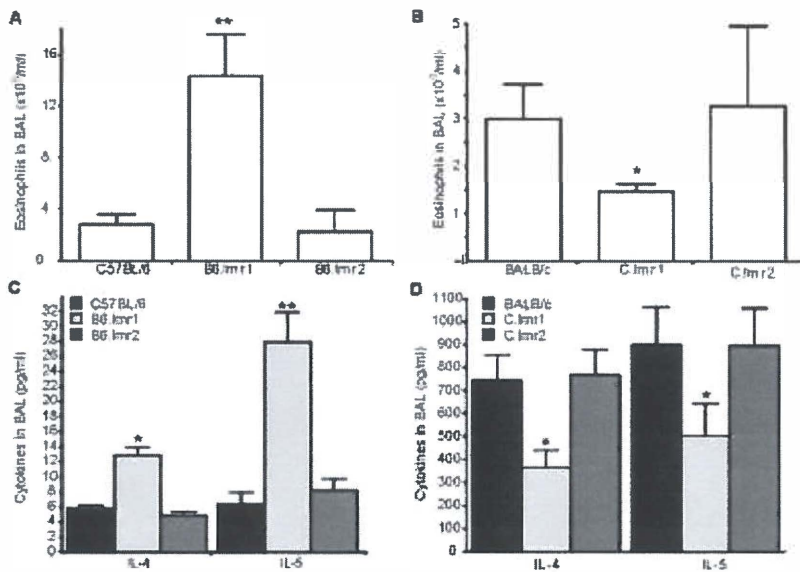


Figure 3: Asthma phenotypes in lmr1 and lmr2 RC strains in both backgrounds (2). Total numbers of eosinophils in BAL of single congenic mice on C57Bl/6 background (A) and BALB/c background (B). Th2 cytokine levels of BAL fluid from single congenic mice on the C57Bl/6 background (C) and BALB/c background (D). *: significantly different from background strain with $\alpha \leq 0.05$, **: with $\alpha \leq 0.01$.

We observed no induction of AHR after OVA challenges in the RC mice on a C57Bl/6 background (Figure 2C). On the BALB/c background, both C.lmr1 and C.lmr2 show a significantly reduced PenH compared to BALB/c at methacholine dosages of 12.5 76

mg/ml and higher (Figure 2D). These data indicate that the C57Bl/6 alleles of both the *lmr1* and *lmr2* loci seem to contribute to the AHR resistant phenotype in the BALB/c background observed in C.lmr1/2. Since we have phenotyped the airway responses in the RC strains using whole-body plethysmography, which is an indirect and not undisputed measure of airway responsiveness²², we aimed to confirm our data by using an invasive method to directly assess airway resistance in the C.lmr1 RC mice and its respective background strain, the BALB/c. To this end, we performed invasive measurement of airway resistance the day after the last OVA challenge. As shown in figure 4, we find that the airway resistance of the C.lmr1 RC strain is strongly suppressed as compared to the parental BALB/c strain, underscoring the suppressive effect of the C57Bl/6 allele on the AHR in the BALB/c background.

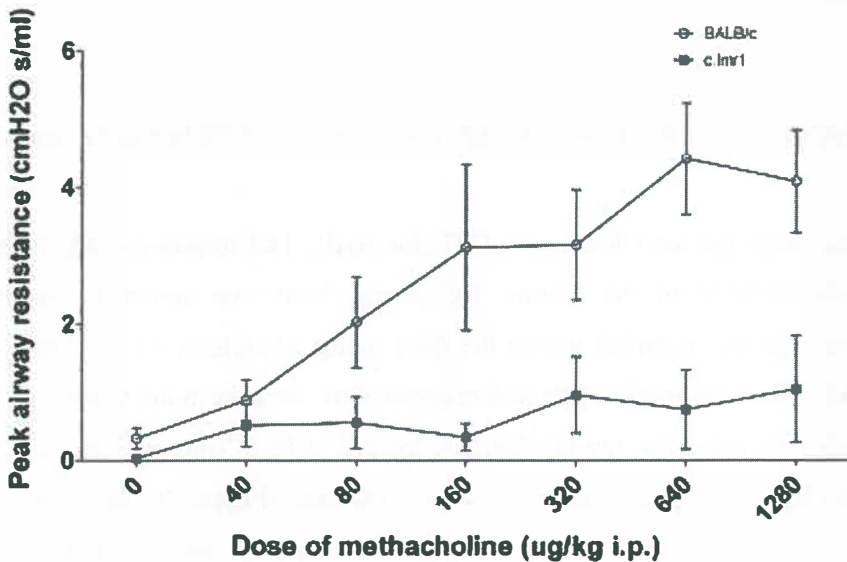


Figure 4: Airway resistance of C.lmr1 and BALB/c mice in response to methacholine. The peak airway resistance (cm H₂O.s/ml) C.lmr1 RC mice and the BALB/c parental strain in response to increasing doses of i.v. administered methacholine.

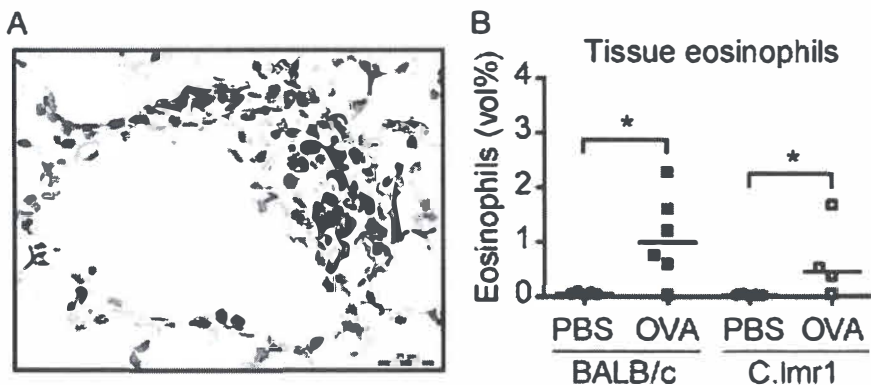


Figure 5: Histological evaluation of eosinophilic airway inflammation. Eosinophilic inflammation in the lungs of OVA challenged C.lmr1 RC mice (A) and BALB/c parental strain (B) was determined by staining for cyanide-resistant endogenous peroxidase activity with diaminobenzidine. Images were obtained and staining intensity was quantified by morphometric analysis and expressed as volume percentages (C).

Fine-mapping the *lmr1* locus by analysis of differentially expressed genes.

Our data identify the *lmr1* locus as a QTL for AHR, Th2 responses and eosinophilic airway inflammation in the C.lmr1 RC strain. Next, we aimed to identify the susceptibility genes contained within the *lmr1* locus. Therefore, we first genotyped a large panel of polymorphic SNPs and microsatellite markers in the C.lmr1 RC strain. This analysis revealed that the D17Mit185, located at 68.8 Mbp, was the first marker with a BALB/c genotype on chromosome 17 in C.lmr1 (Figure 7). Hence, *lmr1* spans the larger part of chromosome 17, and contains close to 600 polymorphic genes, precluding the efficient identification of the relevant susceptibility gene(s). Several approaches can be taken to further fine-map this locus, such as F2 crosses to generate recombinant haplotypes of the *lmr1* locus that can then be phenotyped in the asthma protocol, an approach successfully employed to identify the TIM family of AHR and Th2 inflammation susceptibility genes²³. However, this particular approach requires the breeding and genotyping of very large numbers of mice, and we preferred to take a more straightforward approach to susceptibility gene identification. To this end, we

performed whole-genome transcriptional profiling on lung material derived from C.lmr1 RC mice and BALB/c controls, in order to attempt to identify genes located within the *lmr1* locus that were differentially expressed between the two strains. We hypothesized that combining the gene expression data with the known polymorphisms between C57Bl/6 and BALB/c on the *lmr1* locus would lead us towards identification of the relevant susceptibility genes present in this rather large QTL. A similar approach has previously been applied by Karp *et al.* to identify complement factor 5 as an asthma susceptibility gene located within a known QTL on mouse chromosome 2²⁴.

Hence, we performed OVA inhalation challenges as well as a PBS inhalation control treatment in both C.lmr1 and BALB/c control mice. Individual lobes of the lungs of these mice were used for RNA isolation or histological evaluation to confirm the asthma phenotypes in the mice. In both BALB/c and C.lmr1 backgrounds, OVA challenges readily induced a pronounced eosinophilic airway inflammation (Figure 5) that was absent from PBS challenged mice (not shown), confirming the induction of an asthma phenotype.

To identify genes differentially expressed between C.lmr1 and BALB/c, we compared the transcriptional profiles of the PBS-challenged mice from both genotypes and those of OVA-challenged mice from both genotypes. This analysis revealed the presence of a total of 101 genes that were differentially expressed at a genome-wide level of significance between C.lmr1 and BALB/c in either PBS or OVA challenged mice. As expected, the majority of these genes (60; see table 1) were located within the congenic region, although we also identified a number (41) of additional differentially expressed genes located outside of the *lmr1* locus. Interestingly, more than half of the differentially expressed genes located within the *lmr1* locus were identified in both experimental conditions, whereas nearly all of the genes located outside of the *lmr1* locus were found to be differentially expressed only in either PBS or in OVA-challenged mice.

The identity of the differentially expressed genes located within the *lmr1* locus revealed that a large proportion of these (20 out of 60) belonged to the *Mhc* genes. The *lmr1* locus does encompass the entire *Mhc* region and the two background strains are polymorphic for the *Mhc* genes. We identify differential expression of class I (11), class II (7) and class III (2) *Mhc* genes (table 1). The observation of differential *Mhc* gene usage between C.lmr1 and BALB/c by our approach is a confirmation of the power of this analysis to identify differentially expressed genes in the locus. Moreover, since several *Mhc* genes have been identified as a candidate genes in human asthma^{25,26}, it is of interest to further dissect the contribution of these polymorphisms to the asthma phenotype in our screen.

The asthma phenotype of the C.B10-H2b strain.

In order to test whether the observed differential expression of the polymorphic *Mhc* genes could indeed contribute to the phenotypic differences between C.lmr1 and BALB/c in the mouse asthma model, we made use of the existing C.B10-H2b (BALB.B) strain of mice, which carries the C57Bl/10 haplotype of the *Mhc* locus on a BALB/c genetic background. Since C57Bl/6 and C57Bl/10 share the *b* haplotype of the *Mhc* locus, the BALB.B strain allows the analysis of the effects of the presence of this *Mhc* haplotype in a BALB/c genetic background on the asthma phenotype. Indeed, BALB.B mice showed a strongly reduced induction of AHR to methacholine compared to BALB/c (Figure 6A). Moreover, the number of eosinophils and the level of Th2 cytokines in BAL fluid were significantly reduced in BALB.B mice compared to BALB/c controls (Figure 6B, 6C). In contrast, the level of SpIgE was comparable between the two strains, both before and after the OVA inhalation challenges (Figure 6D). In addition to the Th2 cytokines, we also measured TNF α levels in BAL, since the LTA/TNF α /TAP-1 cluster, a known asthma susceptibility locus in human asthma²⁷⁻³⁰, is part of the *Mhc* region and contained within the BALB.B congenic fragment. TNF α levels in BAL did not differ between the two strains (Figure 6E). Taken together, these data indicate that the Th2 driven eosinophilic airway inflammation and AHR are strongly reduced in BALB.B mice compared to BALB/c. Remarkably, these data largely mirror the phenotype of the C.lmr1 strain in the OVA-induced asthma model.

Alignment	Gene.Symbol	RFC PBS	Qvalue PBS	RFC OVA	Qvalue OVA	MHC class	C.B10- 1/2b
6124229-6124844	Tulp4	2.65	0.00E+00	2.72	0.00E+00		
6306455-6646628	Tmem181	-1.38	3.91E-02	-1.60	1.40E-03		
6808201-6813686	Dynlt1	-1.50	6.95E-03	ND	NS		
8138451-8172470	Rsh12a / Rsh12b	1.41	3.62E-02	1.47	1.20E-02		
8535581-8536945	Prr18	ND	NS	-1.66	4.02E-02		
15115571-15126399	Gm3435	-2.18	0.00E+00	-2.26	0.00E+00		
15842061-15908869	Chd1	ND	NS	1.55	4.88E-02		
15941318-15943205	Rgmb	1.77	3.33E-02	ND	NS		
21672505-21699826	Zfp52	1.92	4.04E-03	2.14	7.80E-04		
21705495-21710871	BC049807	ND	NS	1.56	2.51E-02		
21711279-21800327	3110052M02Rik	-1.86	1.39E-02	ND	NS		
22067092-22069398	3110048L19Rik	3.17	1.72E-03	3.59	4.35E-04		
22335458-22335831	Gm4944	2.87	2.05E-04	3.36	4.58E-05		
22474961-22498355	6330416L07Rik	1.65	3.58E-02	1.65	3.58E-02		
22498492-22514247	Zfp758	1.57	1.62E-02	1.71	2.88E-03		
22998692-23000920	A630033E08Rik	12.4	0.00E+00	14.7	0.00E+00		
22998692-23000920	A630033E08Rik	-2.06	6.96E-03	-1.76	2.98E-02		
23319882-23320523	Zfp40	2.10	3.43E-02	2.22	1.64E-02		
23956463-23961708	Srrm2	ND	NS	-1.41	3.58E-02		
24312377-24342505	Tbc1d24	2.51	2.93E-03	2.51	2.93E-03		
24489014-24547317	Abca3	-1.55	1.41E-02	-1.46	2.70E-02		
24593511-24593945	BC026600	-2.21	2.16E-02	-2.68	2.93E-03		
24830134-24833090	Gfer	ND	NS	1.99	2.35E-03		
25025111-25025904	Eme2	3.56	0.00E+00	3.04	0.00E+00		
26218155-26227246	Decr2	-2.26	1.62E-03	-2.00	3.97E-03		
27700525-27702672	AV039444	ND	NS	1.57	3.91E-03		
29285239-29285925	Rab44	ND	NS	2.58	1.91E-02		
29632605-29633056	Pim1	2.79	1.14E-02	3.44	1.61E-03		
30729805-30749567	Glo1**	-2.31	5.69E-05	-2.08	4.16E-04		
31038821-31073465	Gip1r	4.54	1.63E-03	2.91	1.95E-02		
31060152-31060536	BM233846	-13.95	0.00E+00	-17.12	0.00E+00		
31075243-31077736	2900001G08Rik	4.42	7.92E-04	4.54	4.35E-04		
31981201-31992692	Sik1	1.76	1.90E-02	2.15	1.25E-03		
33823813-33824438	March2	1.94	1.02E-03	2.24	1.31E-04		
34103775-34103873	H2afb3	12.0	0.00E+00	10.6	0.00E+00		
34133107-34137144	H2-DI*	-189	0.00E+00	-299	0.00E+00	II	
34272577-34276028	H2-DMa	ND	NS	2.08	1.87E-02	II	
34375849-34390850	H2-Ob	2.33	2.00E-02	3.93	3.17E-04	II	
34400205-34406290	H2-Ab1	ND	NS	2.08	1.13E-02	II	
34419696-34419924	H2-Aa*	14.7	0.00E+00	36.4	0.00E+00	II	

Alignment	Gene.Symbol	RFC PBS	Qvalue PBS	RFC OVA	Qvalue OVA	MHC class	C.B10- H2b
34476410-34478355	H2-Eb2	ND	NS	2.31	1.11E-02	II	
34478947-34481590	H2-Ea*	-468	0.00E+00	-622	0.00E+00	II	
35162926-35164861	Ng23	-1.98	3.62E-03	-2.03	1.81E-03	III	
35175350-35181956	Msh5	-2.50	2.97E-03	-2.73	9.87E-04	III	
35400049-35404127	H2-D1**	ND	NS	1.62	2.70E-02	I	
35400451-35404439	H2-D1/L	-112	0.00E+00	-129	0.00E+00	I	
35457497-35483615	BE136769	7.52	0.00E+00	8.78	0.00E+00	I	
35495945-36871453	H2-K1	-15.7	0.00E+00	-20.57	0.00E+00	I	
35561795-35566998	H2-Q1	ND	NS	2.41	2.83E-02	I	
35576133-35611504	H2-Q7	ND	NS	3.60	6.53E-04	I	
35607046-35611498	H2-Q10	1.96	2.89E-02	2.52	1.64E-03	I	
36093585-36106708	Abcf1	2.04	2.72E-03	2.13	1.25E-03	I	
36136110-36136696	H2-T24	3.38	1.08E-04	2.42	2.88E-03	I	
36136110-36136696	H2-T24	-1.98	1.41E-02	ND	NS	I	
36167766-36221156	H2-Q5	-2.04	4.65E-03	-1.97	5.59E-03	I	
36245850-36248615	H2-T23	ND	NS	1.63	2.98E-02	I	
43594927-43596506	Gpr116	ND	NS	-2.08	3.84E-02		
44215820-44223061	Enpp5	2.17	1.65E-02	2.21	1.11E-02		
48559273-48560072	Apobec2	2.10	1.45E-02	2.03	1.49E-02		
51896624-51897581	C230085N15Rik	ND	NS	1.81	4.52E-02		
55836391-55851926	Pot1b	1.97	2.38E-02	2.29	3.91E-03		
57219488-57227053	Tubb4	2.04	3.76E-03	ND	NS		
65787125-65788038	Mrps10	2.65	0.00E+00	ND	NS		

Table 1: *Lmr1* genes differentially expressed between C.lmr1 and BALB/c. Gene ID and alignments on chromosome 17 of *Lmr1* genes that are differentially expressed between C.lmr1 and BALB/c in either PBS or OVA-challenged groups, or both. RFC PBS: Relative fold change C.lmr1 over BALB/c in PBS condition. Qvalue.PBS: q-value (False Discovery Rate) in PBS comparison. RFC OVA: Relative fold change C.lmr1 over BALB/c in OVA condition, Qvalue.OVA: q-value (False Discovery Rate) in OVA comparison. MHC Class: annotation of genes contained within the *Mhc* region to either MHC class I, II or III genes according to (40). C.B10-H2b: Genes annotated to the C.B10-H2b congenic fragment are indicated in grey. * indicates a transcript identified by 2 independent probes and ** indicates a transcript identified by 3 independent probes.

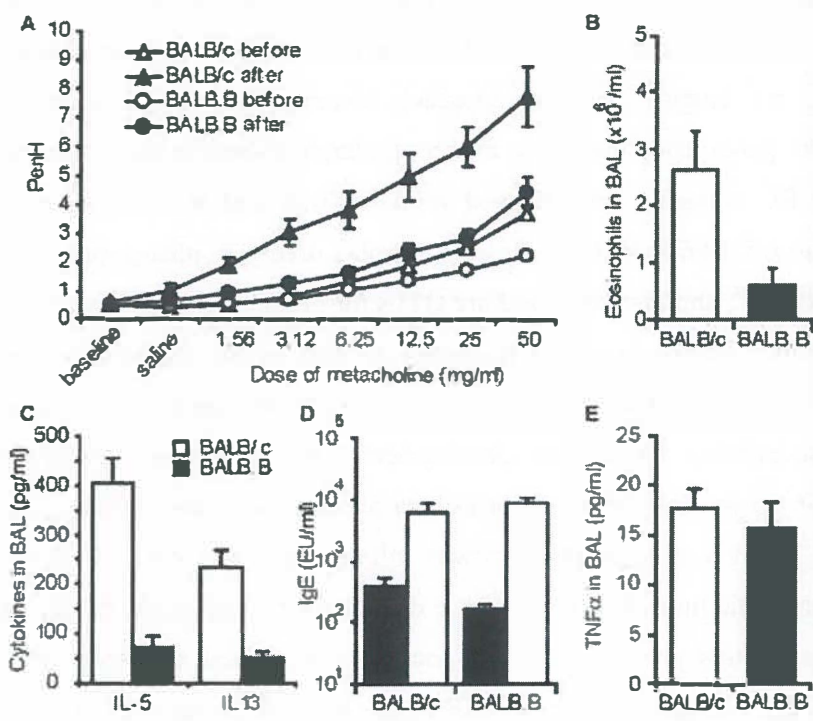


Figure 6: Asthma phenotype of the C.B10-H2b RC strain. AHR to increasing doses of metacholine of C.B10-H2b (BALB.B) and BALB/c mice (A). Total numbers of eosinophils in BAL (B) from BALB/c and BALB.B mice (as indicated), Th2 cytokine levels in BAL fluid (C) from BALB/c and BALB.B mice (as indicated), OVA-specific IgE before (black solid bars) and after (open bars) OVA inhalation challenges of BALB/c and BALB.B mice (D) and levels of TNFα in BAL fluid from BALB/c and BALB.B mice as indicated (E).

Discussion

Asthma susceptibility has a large genetic component, and only a small portion of the heritability of asthma can be explained by the currently known asthma susceptibility loci. Here, we employ a novel approach towards the identification of asthma susceptibility genes using the mouse model of allergic asthma to screen the phenotypes of the *lmr* RC congenic mice derived from C57Bl/6 and BALB/c intercrosses. As BALB/c and C57Bl/6 have been shown to display divergent phenotypes in the mouse asthma model³¹⁻³⁴, and *lmr1* and *lmr2* are QTLs for *Leishmania major* resistance, which is affected by Th2-biased T cell responses as well as an impaired wound healing response^{14,15}, we hypothesized that the *lmr* loci could also contain genes that contribute to the susceptibility for asthma development. Our experiments clearly show an influence of the *lmr* loci on all major asthma phenotypes in this mouse model: SpIgE responses, Th2-driven eosinophilic airway inflammation and AHR. In dissecting the contribution of the individual *lmr* loci, we demonstrate a major role for the *lmr1* locus on the inflammatory phenotype on both backgrounds, with an additional effect on IgE in the C57Bl/6 background and on AHR in the BALB/c background. The *lmr2* locus only shows a small BALB/c background specific effect on IgE and AHR.

The *lmr1* locus was selected for further candidate gene identification, since it exerted dominant effects in both backgrounds. The congenic locus in the C.lmr1 strain spans 68.8 MBp on mouse chromosome 17 from the centromeric region onward (Figure 7). When comparing the C57Bl/6 and BALB/c genomic sequences, we identified almost 600 polymorphic genes located within the congenic fragment. To further fine-map the *lmr1* locus towards the identification of (candidate) susceptibility genes, we choose to use genome-wide transcriptional profiling on lung tissue of both C.lmr1 RC mice and BALB/c controls. Annotation of the genes that are differentially expressed between the two genetic backgrounds to their chromosomal location allows for the identification of the differentially expressed genes located within the *lmr1* locus, which we hypothesized to include the susceptibility genes relevant to the C.lmr1 phenotype.

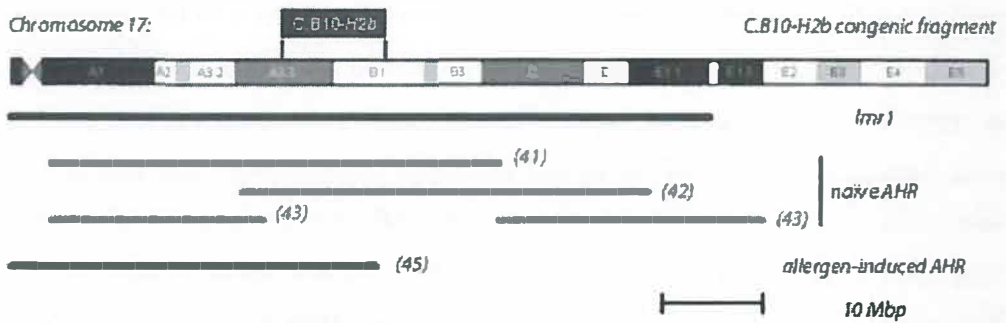


Figure 7: Overview of the *lmr1* locus on mouse chromosome 17. Schematic representation of mouse chromosome 17. Indicated are the relative positions of the *lmr1* locus, as well as the known mouse QTLs for airway reactivity, and the congenic fragment present in the C.B10-H2b RC strain.

A similar approach has previously been applied to identify candidate susceptibility genes for various mouse models of human disorders, including asthma²⁴, pertussis³⁵, type I diabetes³⁶, adiposity³⁷, tuberculosis³⁸ and anxiety³⁹. We found a total number of 101 genes that were differentially expressed between the two strains with genome-wide significance, 60 of which were located within the *lmr1* locus (table 1). Two third of these were found to be differentially expressed independently of experimental condition (40 out of 60), indicating a potential cis-regulation of their expression by polymorphisms between C57Bl/6 and BALB/c.

Our approach to fine-map the *lmr1* asthma susceptibility genes by genome-wide transcriptional profiling has two major drawbacks. First, polymorphisms between the two background strains could be present in the probes used to detect the levels of gene expression in the lung tissue RNA, and might give false-positive outliers due to differences in cRNA annealing efficiency to the array. Such false-positive hits showing differential expression would be expected to occur only within the *lmr1* locus, and independently of experimental condition. In addition, expression arrays are potentially sensitive to false-negative results, since the sensitivity of the array to detect differences in gene expression is dependent on the average expression level of the gene. As such, differences in relatively poorly expressed genes are much harder to identify as a

significant difference between the two strains. Therefore, we have to take into account that we might have missed some differentially expressed genes due to the limitations of our approach. Nevertheless, we expect these effects to be relatively minor, given the strong supportive evidence of the asthma phenotype of the C.B10-H2b (BALB.B) RC strain, which carries the C57Bl/6 allele of the *Mhc* region in a BALB/c genetic background. A large number of these genes map to the *Mhc* locus, located on mouse chromosome 17 approximately between *coll1A2* at 34.1 Mbp and *Zfp57* at 37.1 Mbp⁴⁰, which is entirely contained within the *Imr1* locus. Our analysis of the BALB.B asthma phenotype indicates that the presence of the C57Bl/6 haplotype of the *Mhc* locus in an otherwise homogeneous BALB/c background is sufficient to induce the reduced AHR, Th2 responses and airway eosinophilia in the mouse asthma model, but does not affect the SpIgE response in serum (Figure 6). These results are strikingly similar to our results obtained with the C.lmr1 mice (Figures 3, 4). These data provide experimental proof for the notion that the C57Bl/6 allele of the *Mhc* locus, harboring the most strongly differentially expressed genes, indeed renders the asthma manifestations to be at the level of the C.lmr1 RC strain. The BALB.B congenic region has been mapped to be located between coordinates 26.9 and 37.3 Mbp on mouse chromosome 17⁴¹. This includes not only the *Mhc* region (34.1 to 37.1 Mbp) but also some additional 7 Mbp located on the centromeric side of the *Mhc* locus. This latter region includes several polymorphic genes differentially expressed between the two strains, including *GlyoxalaseI*, *RAB44* and *Pim1* (table 1). Consequently, although the BALB.B data allow us to narrow down the *Imr1* candidate susceptibility genes to a relatively small number, these still include both *Mhc* genes and a small number of unrelated genes.

Both the *Imr1* locus and the BALB.B congenic locus specifically affect the Th2-driven eosinophilic airway inflammation and AHR in the BALB/c genetic background, but have no effect on spIgE. This discrepancy between the individual asthma phenotypes has previously been observed in genetic screens both by us and by others^{9,42,43}. As a result, QTLs or susceptibility genes also differ between the individual asthma phenotypes. Consequently, we would designate the *Imr1* locus as a QTL for eosinophilic airway inflammation and allergen-induced AHR.

The *lmr1* locus overlaps with several previously identified QTLs for naïve airway reactivity on mouse chromosome 17 (Figure 7). Two loci were identified in backcrosses between C57Bl/6 and A/J ((C57Bl/6 x A/J) x C57Bl/6)⁴⁴ and C3H and A/J ((C3H x A/J) x C3H)⁴⁵, both of which also overlap with the BALB/B congenic region. In addition, a single study using RC strains derived from C57Bl/6 and A/J intercrosses reported the presence of two separate QTLs for naïve AHR on mouse chromosome 17, both overlapping with the *lmr1* locus but not with the BALB.B congenic region⁴⁶. Finally, a haplotype association mapping survey involving 36 inbred strains failed to find any association with chromosome 17 haplotypes to naïve AHR⁴⁷. So far, only a single QTL for allergen-induced AHR on chromosome 17 has been identified (Figure 7) and overlaps with *lmr1* and the BALB.B congenic region⁴⁸. In contrast, a screen for allergen-induced AHR in a (C3H x A/J) x A/J backcross did not reveal any significant linkage to chromosome 17⁴³. The human *Mhc* genes are located in the syntenic region on chromosome 6. Genetic association with asthma (questionnaire based asthma, AHR) have been found for both HLA-G²⁵ and MICB²⁶. However, both genes lack mouse homologues⁴⁰. In addition, the LTA, TNF- α and TAP-1 genes, part of the *MhcIII* region, have been shown to be associated with questionnaire based asthma and/or AHR in different populations²⁷⁻²⁹. We do not observe a differential expression of any of the homologous genes in the LTA/TNA/TAP-1 cluster, nor do we observe differences in TNF α levels in BAL obtained after OVA inhalation challenges of sensitized BALB.B and BALB/c mice (Figure 6E). Therefore, we think that the LTA/TNF/TAP-1 cluster genes are not likely candidates for the *lmr1* QTL.

In conclusion, we have identified *lmr1* as an asthma susceptibility locus in the mouse using recombinant congenic strains derived from BALB/c and C57BL/6. Transcriptional profiling of lung tissue from C.lmr1 RC and BALB/c mice has identified a limited number of candidate genes located within the *lmr1* locus that are differentially expressed between the two strains, with a number of *Mhc* genes being strongly differentially expressed. Analysis of the asthma phenotype of the BALB.B RC strain indicates that the presence of the C57Bl/6 haplotype at the *Mhc* locus in the

BALB/c genetic background is sufficient for the suppression of asthma phenotype observed in the C.lmr1 strain, indicating that the small number of polymorphic, differentially expressed genes mapping to the BALB.B congenic fragment likely contain the relevant *lmr1* asthma susceptibility gene.

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Segregation of asthma phenotypes in the *Imr1* locus on mouse chromosome 17

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Abstract

Asthma susceptibility has been shown to be the result of numerous genes which interact with other genes and the environment. We have previously identified the *Lmr1* locus as an asthma susceptibility locus in recombinant congenic mouse strains derived from BALB/c and C57Bl/6, and identified several differentially expressed genes located within or proximal to the *H2* locus as the most likely candidate genes contributing to the asthma phenotypes. However, in literature, several QTLs located within the *lmr1* locus have been described that do not overlap with the identified *H2* region, indicating that additional susceptibility genes might well be contained within the *lmr1* locus. In order to test whether additional asthma susceptibility loci were present within the *lmr1* locus, we evaluated the asthma phenotypes of sub-congenic mouse strains derived from C.lmr1, the recombinant congenic (RC) mouse strain carrying the C57Bl/6 allele of the *lmr1* locus on a BALB/c background and compared these phenotypes to that of the parental BALB/c strain. These subcongenic strains all carry partially overlapping fragments of the *lmr1* locus, and we find that they display a wide range of responses for the different asthma phenotypes, indicating the presence of additional susceptibility genes and potential genetic interactions between them. These results show that the *lmr1* locus contains multiple, separate susceptibility loci for the individual asthma phenotypes. Moreover, these loci are divergent for the different asthma parameters: airway hyperresponsiveness, eosinophilic airway inflammation and serum Sp-IgE (OVA specific IgE) levels. Further fine-mapping is needed to identify candidate genes in these different loci.

Introduction

Allergic asthma is a chronic, inflammatory pulmonary disease in which patients develop airway hyperresponsiveness (AHR) to bronchoconstrictive stimuli and high Sp-IgE serum levels. Eosinophilic inflammation and remodelling of the airways are frequently observed. Allergic asthma is a genetically complex disease and influenced by many genes which interact with each other¹ and the environment^{2,3}.

To map genes involved in asthma two main approaches have been used; the unbiased genome wide studies and the candidate gene/pathway studies. The main advantage of the genome wide approaches is that pathways and genes, not previously associated with asthma susceptibility, can be identified. However, replication of results from these genetic studies in different populations has been shown to be problematic, not in the least part due epistatic- and gene-environment interactions^{4,5}. In a recent study in 2 distinct populations by Hersh and co-workers for example, replication at SNP level was only found for 1 gene out of 5 previously positionally cloned asthma susceptibility genes⁶. Also, the associations found in genome-wide association studies (GWAS) cannot be replicated in all populations¹.

We took a complementary approach to the human genome-wide association studies by using the mouse model to identify susceptibility genes for three important asthma parameters: airway eosinophilic inflammation, airway hyperreactivity (AHR) and serum OVA specific IgE (Sp-IgE) levels. To this end, we performed a mouse model of allergic asthma in recombinant congenic (RC) mouse strains. RC strains have shown to be a powerful tool for identifying loci involved in genetically complex disorders⁷⁻⁹ such as asthma¹⁰. RC strains are generally derived from classical F2 screens and are a series of mouse strains carrying a range of genomic fragments inherited from the donor strain on a common genetic background, with donor and background parental strains differing in susceptibility to the phenotype under scrutiny. Using the *lmr* RC strains, derived

from BALB/c and C57BL/6, we have previously identified the *H2* locus and neighbouring genomic regions on mouse chromosome 17 to contribute to asthma susceptibility. In order to identify candidate genes in this locus we have compared the expression profiles of C.lmr1 to its background strain BALB/c. Most differentially expressed genes are located within the *H2* locus, the equivalent of the human MHC locus. To investigate the role of this locus, we phenotyped the BALB/B mouse which inherited the C57BL/6 haplotype of the *H2* locus on a BALB/c background. This mouse shows AHR and airway inflammation that is comparable to that of BALB/c¹¹. This makes the *H2* locus an interesting candidate locus to explain the differences seen in the lmr series of mice. However other loci for both naive and allergen induced AHR have been identified in mouse crosses (see figure 6)¹²⁻¹⁵. One of these loci does not overlap with the *H2* locus¹⁵. To investigate the role of those different loci in our mice we submitted subcongenic mice derived from C.lmr1 to our asthma protocol and phenotyped them. This clearly shows that different loci are involved in the different asthma parameters.

Materials and methods

Experimental design

The subcongenic mouse strains derived from C.lmr1 were submitted to an OVA induced allergic asthma protocol. AHR to metacholine and serum Sp-IgE levels were measured before and after the OVA inhalation challenges. The eosinophil content of the BAL was evaluated after challenge only. In a first set of experiments, each subcongenic strain was phenotyped separately together with BALB/c as control. As this approach is sensitive to inter-experiment variation of the asthma phenotypes of the reference strain BALB/c, we carried out an additional set of experiments, using all strains in a direct side-by-side comparison. Data from all experiments were pooled for analysis.

Mice

Breeding pairs of the sub-congenic strains and the parental BALB/cAnBradleyWEHI were obtained from the breeding colonies of S. Foote, Australia. Animal care and use were performed in accordance with the guidelines of and after ethical review by the institutional animal care and use committee at the university of Groningen (IACUC-RUG). The mice were kept under specified pathogen free conditions according to the guidelines of the Federation of European Laboratory Animal Science Association¹⁶. They were housed in individually ventilated cages with food (RMH-B, Abdiets, Woerden, The Netherlands) and water administered *ad libitum*.

Experimental Asthma protocol

For all experiments, male mice of 6 to 8 weeks old were used. On day 0 and 7 the animals were intraperitoneally sensitized with 10 µg OVA (Ovalbumin fraction V, Sigma, St. Louis, MO, USA) in 2 mg Alum adjuvant (Pierce, Rockford, IL, USA). One

week after the second sensitization blood was taken. Subsequently, the animals were challenged for 20 minutes by inhalation of aerosols of 10 mg OVA/ml in saline on day 21, 24 and 27, as described previously¹⁷. Twenty-four hours after the last challenge, airway responsiveness to metacholine was measured, followed by bleeding the mice (Vena cava) and broncho-alveolar lavage (BAL).

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice before (day 18), and after (day 28) a series of three OVA inhalation challenges. Airway reactivity was determined by recording respiratory pressure curves in response to inhaled nebulized metacholine (acetyl- β -methylcholine chloride, Sigma, St. Louis, MO, USA) at doses of 1.6, 3.1, 6.3, 12.5, 25 and 50 mg/ml using barometric whole-body plethysmography (BUXCO, Wilmington, NC, USA). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously¹⁷.

Ovalbumin-specific immunoglobulin levels in serum

After measurement of *in vivo* airway reactivity, mice were anesthetized with isoflurane/oxygen and blood was collected through the orbital sinus (before challenge) or through the Vena cava (after challenge). Serum was collected and stored at -80°C until analysis. Levels of Sp-IgE in the serum were measured as described previously¹⁸. A reference standard was used with arbitrary units of 1,000 EU/ml. The detection level of the ELISA was 0.5 EU/ml.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 5 times 1 ml saline at 37°C. The BAL cells were spun down (400 × g, 4°C, 5 min), washed with PBS, after which the pellet was resuspended in 150 μ l PBS. Total numbers

of BAL cells were counted using a coulter counter (Z1, Beckman-Coulter, Brea, CA, USA), and cytopsin preparations were made (450g, 5 minutes on a Cytospin 3, Thermo Fisher Scientific Inc, Waltham, MA, USA). For differential BAL cell counts, cytopsin preparations were stained with Diff-Quick (Merz & Dade A.G., Dudingon, Switzerland). Cells were identified and differentiated into mononuclear cells, neutrophils, and eosinophils by standard morphology. Per cytopsin preparation, at least 200 cells were counted.

Genotyping

Genotyping was performed using microsatellite markers and SNP assays on genomic DNA extracted from tail snips or spleen. Microsatellite markers were first evaluated by comparing C.lmr1 and BALB/c mice. Fragment sizes were analyzed on 4% Methaphor high resolution agarose gel (Cambrex, East Rutherford, NJ, USA) electrophoresis. The SNP assays were ordered from Applied Biosystems (Taqman SNP genotyping assays, Applied Biosystems, Foster City, CA, USA) and performed according to the manufacturer instructions using a 7900 HT real-time PCR system (Applied Biosystems, Foster City, CA, USA).

***In silico* analysis**

Data on polymorphic SNPs between C57Bl/6J and BALB/cJ were extracted from the 'Mouse Genomes' SNP database (release REL-0912) at the Sanger Institute (<http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl>). Ambiguous sequences were discarded. Data about location and expression of genes were extracted from the Mouse Genome Informatics database from The Jackson Laboratory (<http://www.informatics.jax.org>). Datasets were merged and analysed using SAS v9.1

for Windows (SAS institute, Cary, NC, USA). Inferred polymorphic SNPs from the database were not confirmed in our strains.

Statistics

Statistical analysis was performed using Prism 5 for Windows (GraphPad Software, La Jolla, CA, USA). Data from Sp-IgE and BAL cells were compared to BALB/c using the Man-Whitney U test and compared to each other using the Kruskal Wallis test. Metacholine dose response curves were compared using the Wilcoxon test.

Results

The parental C.lmr1 RC strain displayed reduced allergen-induced AHR and airway eosinophilia upon OVA sensitization and OVA inhalation challenges in comparison to BALB/c¹¹. This indicates that the C57Bl/6 allele of *lmr1* contributed to lower asthma susceptibility. Here, we evaluated the same asthma parameters together with the Sp-IgE serum levels in the subcongenic strains in order to narrow down the *lmr1* genomic locus.

Genotyping

We first performed high-density genotyping on the five subcongenic strains, to carefully map the borders of the subcongenic fragments. These strains cover the first 71 Mbp of chromosome 17 and subdivide the parental *lmr1* locus into 4 congenic regions. The first region, *lmr1A*, spanning the first 47.7 Mbp is present in both C.lmr1.2 and C.lmr1.3. The second congenic region, *lmr1B*, spans the region on chromosome 17 from the end of *lmr1A* to 52 Mbp and is present in both C.lmr1.3 and C.lmr1.4. The third region, *lmr1C* is present in C.lmr1.4 and C.lmr1.5 and is located between 52 and 60.5 Mbp. Finally the last congenic region *lmr1D* spanning chromosome 17 between 60.5 and 71 Mbp is present in C.lmr1.4, C.lmr1.5 and C.lmr1.7 (Table 1, figure 6).

Strain	Last BALB/c marker before congenic fragment		First BALB/c marker after congenic fragment	
	Marker	Pos (Mbp)	Marker	Pos (Mbp)
C.lmr1	NA		D17Mit39	74.7
C.lmr1.2	NA		D17Mit68	47.7
C.lmr1.3	NA		Rsl3483027	52.0
C.lmr1.4	D17Mit49	45.4	D17Mit160	71.0
C.lmr1.5	rs13483027	52.0	D17Mit160	71.0
C.lmr1.7	rs13483055	60.5	D17Mit160	71.0

Table 1: Overview of the congenic borders of C.lmr1 and its subcongenic strains. NA: not applicable, centromeric part of chromosome 17 inherited from C57BL/6

Asthma phenotypes

All strains show comparable Sp-IgE levels before challenge (data not shown), which are significantly increased after OVA inhalation challenges. C.lmr1.2 and C.lmr1.3 both show comparable Sp-IgE levels after challenge, which are significantly lower compared to their genetic background strain BALB/c. In contrast, the strains carrying a congenic region at the telomeric side of the *lmr1* locus, *i.e.* C.lmr1.4, C.lmr1.5 and C.lmr1.7, show Sp-IgE serum levels after challenge that are not significantly different from BALB/c (figure 1).

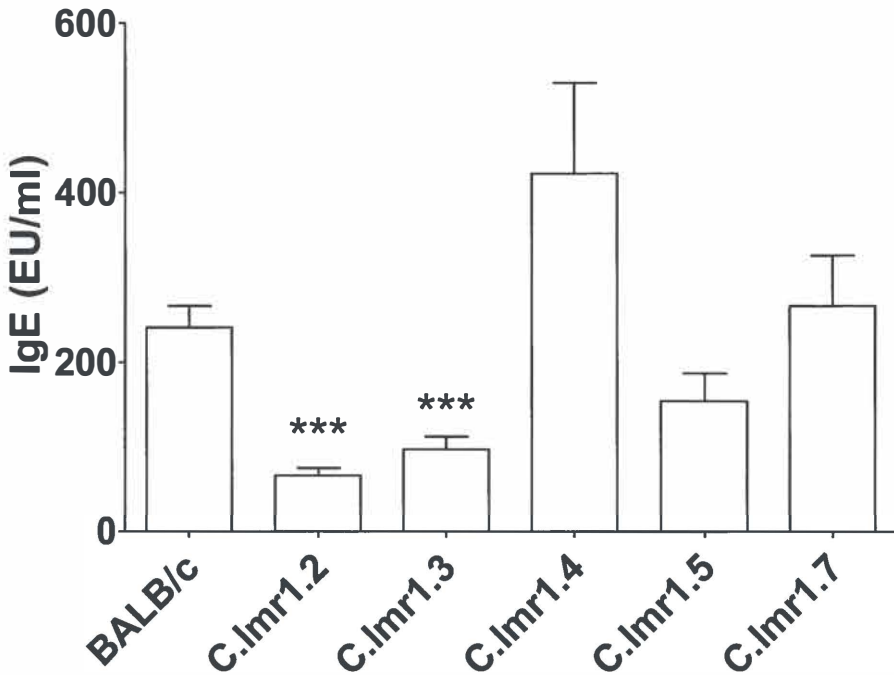


Figure 1: Sp-IgE serum levels after OVA challenge in the different C.lmr1 derived subcongenic strains; ***: Significantly different ($p \leq 0.001$) compared to its BALB/c control.

We measured airway responsiveness to metacholine before and after OVA inhalation challenges. Baseline AHR (before the OVA challenges) is significantly higher in

C.lmr1.3 and C.lmr1.4 and significantly lower in C.lmr1.7 compared to BALB/c. C.lmr1.2 and C.lmr1.5 show results comparable to BALB/c (figure 2). After challenge C.lmr1.3 and C.lmr1.5 show significantly lower AHR and C.lmr1.4 significantly higher AHR, compared to BALB/c. For C.lmr1.4 the relative increase in penh before and after challenge (after minus before challenge) is comparable to that of BALB/c, indicating that the increased AHR after challenge can be entirely attributed to the increased naïve AHR in this subcongenic strain (figure 3).

In addition to Sp-IgE and AHR responses, we evaluated the airway inflammation by analyzing total cell counts and numbers of eosinophils, neutrophils and mononuclear cells in BAL. Total cell count in the BAL is increased in C.lmr1.4 compared to its BALB/c control (figure 4, panel A). This is due to an increase in all cell types, as eosinophils (figure 4, panel B), neutrophils (data not shown) and mononuclear cells (data not shown) are significantly higher in this strain compared to BALB/c. All other strains show cell counts that are comparable to BALB/c (figure 4).

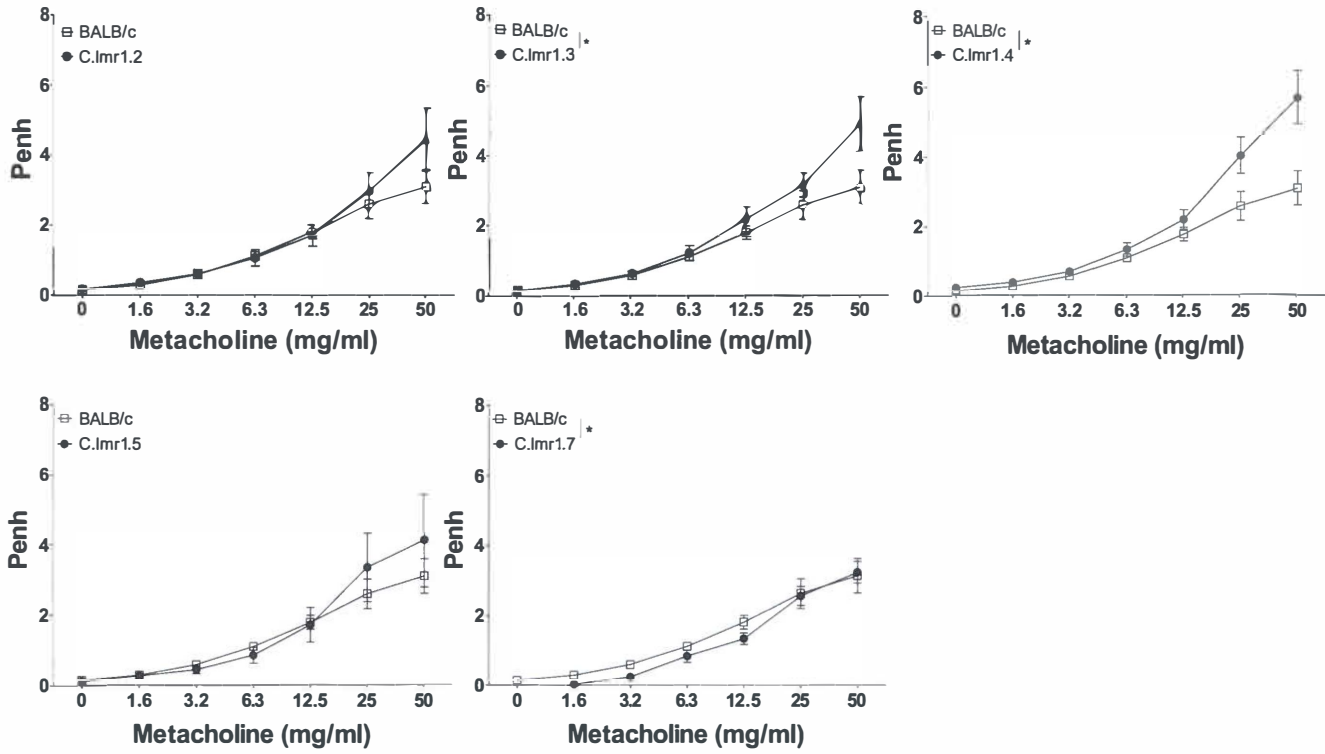


Figure 2 : Penh (baseline corrected) response to metacholine before challenge for the different C.Imr1 derived subcongenic strains*: Significantly ($p \leq 0.05$) different compared to BALB/c.

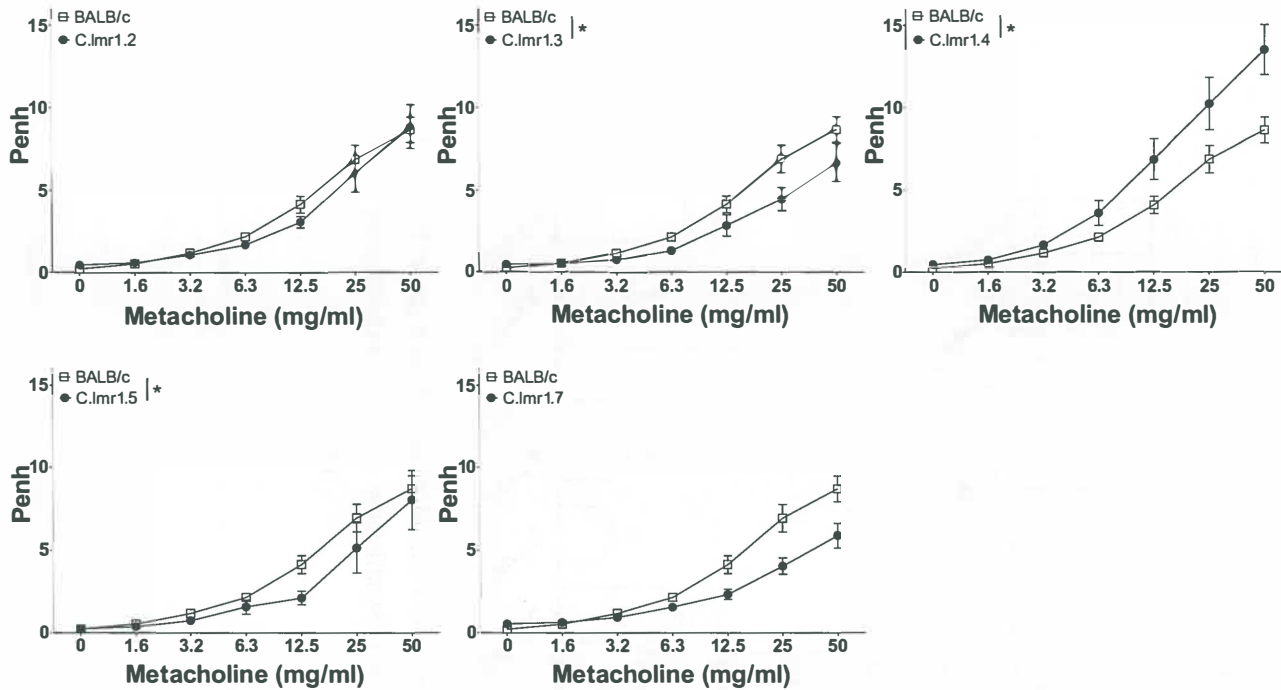


Figure 3: Penh (baseline corrected) response to metacholine after challenge for the different C.lmr1 derived subcongenic strains*: Significantly different ($p \leq 0.05$) compared to BALB/c.

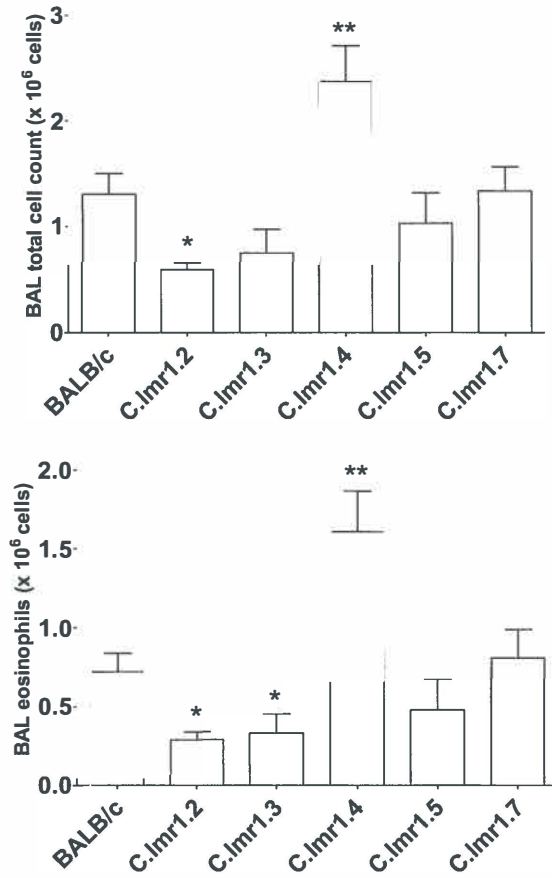


Figure 4: Total cell count (panel A) and eosinophil count (panel B) in BAL for the C.lmr1 derived subcongenic strains; *: Significantly ($p \leq 0.05$) different compared to BALB/c, **: ($p \leq 0.01$).

Discussion

Using the *lmr* series of recombinant congenic strains, we previously showed that the *lmr1* locus, which is located on mouse chromosome 17, suppresses both AHR and the eosinophilic inflammation of the lungs. To identify candidate genes in this locus we performed transcriptional profiling of C.*lmr1*, which inherited the *lmr1* locus from C57BL/6 on a BALB/c background. Most differentially expressed genes are located within the *H2* locus. To test whether this locus can explain the differences in AHR and inflammation we phenotyped the BALB/B strain in our mouse model of allergic asthma. This strain inherited the *H2* locus from C57BL/10, which has the same haplotype as C57BL/6. BALB/B shows comparable inflammation and AHR to C.*lmr1*, which makes the *H2* locus a prime candidate locus for both these asthma traits¹¹. Other groups have also identified QTLs for both naive and allergen induced AHR which show overlap with *lmr1* locus. 3 out of 4 of these QTLs include the *H2* locus (see figure 6)¹²⁻¹⁵. In order to assess whether additional susceptibility loci could be identified in the *lmr1* congenic fragment, sub-congenic strains derived from C.*lmr1*, were submitted to our standard asthma protocol and phenotyped. The individual C.*lmr1* sub-congenic strains show a wide range of responses for the different asthma phenotypes (table 2). This indicates the presence of multiple independent susceptibility loci within the *lmr1* locus each exerting a different effect on the individual asthma parameters we measure in these experiments. Based on the genotypes of the subcongenic strains we can discriminate 4 sub-loci within the *lmr1* locus (figure 6).

The Sp-IgE levels are influenced by *lmr1A*, as both strains that carry the C57BL/6 allele of this locus, C.*lmr1.2* and C.*lmr1.3*, show significantly reduced Sp-IgE serum levels after challenge compared to BALB/c. The other subcongenic strains, carrying the BALB/c allele of *lmr1A*, display Sp-IgE responses comparable to the parental BALB/c strain. C.*lmr1*, which carries the C57BL/6 allele of *lmr1*, shows Sp-IgE levels comparable to BALB/c¹¹. This could be due to the presence of a second Sp-IgE locus

between the end of *lmr1D* and the end of the congenic fragment of C.lmr1. But it could also be due to a lack of power to detect differences in Sp-IgE serum levels between C.lmr1 and BALB/c, as B6.lmr1, which inherited the BALB/c *lmr1* locus on a C57BL/6 background, does show significantly higher Sp-IgE serum levels compared to C57BL/6. BALB/B doesn't show any difference compared to BALB/c¹¹, which indicates that the *H2* locus is not involved in the differences in Sp-IgE levels. If we exclude the BALB/B inherited locus from *lmr1A*, 20 genes that are polymorphic are also differentially expressed between BALB/c and C.lmr1 (see table 3). None of these genes have previously been found associated with IgE serum levels (total or specific). Another interesting polymorphic gene is serum-PAFAH. Polymorphisms in this gene have been found associated with atopy, asthma and AHR in human¹⁹. Kruse *et al.* showed that this was due to a loss of function mutation²⁰. There are coding non-synonymous SNP which differ between BALB/c and C57BL/6 in the sequence of the mouse homolog of this gene, which makes serum-PAFAH a good candidate gene to explain the differences in Sp-IgE levels observed.

Strain	Genotype of locus				Phenotype compared to BALB/c			
	<i>lmr1A</i>	<i>lmr1B</i>	<i>lmr1C</i>	<i>lmr1D</i>	Sp-IgE after	AHR before	AHR after	EOS
C.lmr1.2	B6	C	C	C	LOW	Ns	Ns	Low
C.lmr1.3	B6	B6	C	C	LOW	HIGH	LOW	Low
C.lmr1.4	C	B6	B6	B6	Ns	HIGH	HIGH	HIGH
C.lmr1.5	C	C	B6	B6	Ns	Ns	LOW	Ns
C.lmr1.7	C	C	C	B6	Ns	LOW	Ns	Ns

Table 2: Genotype-phenotypes summary. C: BALB/c; B6: C57BL/6; Ns: no significant difference compared to BALB/c

For naive AHR, we observe an effect for the *lmr1B* locus, as both strains with the C57BL/6 allele of this locus, C.lmr1.3 and C.lmr1.4 have significantly higher AHR before challenge compared to BALB/c. 2 loci for naive AHR which show overlap with the *lmr1B* locus were previously identified in backcross experiments involving A/J and C57BL/6¹² or A/J and C3H/J¹³. The only polymorphic and differentially expressed gene

in *lmr1B* is *Apobec2* (see table 3). The protein encoded by this gene is a member of the Apobec family of mRNA editing enzymes^{21,22}. Anant *et al.* showed that it functions as inhibitor of the ApoB C-toU mRNA editing enzyme complex, which plays a critical role in isotype class switching in B-cells^{23,24}. Interestingly, the human ortholog was recently found associated to airway function in a genetic screen for COPD (M. ten Berge, personal communication). The lower AHR before challenge in C.lmr1.7 but not C.lmr1.5 strongly suggest a genetic interaction in which the effect of the *lmr1D* locus is dependent on the haplotype of the *lmr1C* locus. Both loci show overlap with the naive AHR loci from an F2 cross between DBA and C57BL/6¹⁴ and the previously mentioned backcross between A/J and C3H/J¹³ (see figure 6).

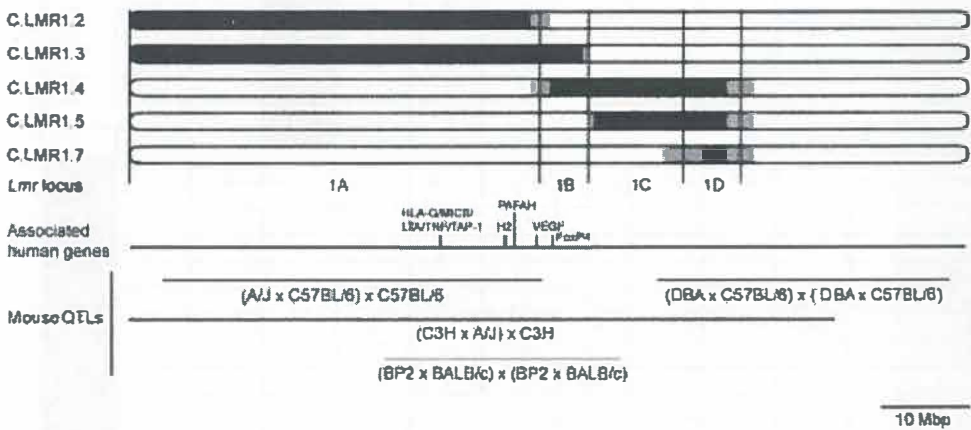


Figure 6: Overview of the C.lmr1 derived subcongenic strains, black bars represent C57BL/6 genotype, white bars represent BALB/c genotype and unknown genotype is represented by grey bars. Mouse QTLs represented in black show loci for naïve airway responsiveness¹²⁻¹⁴, the one in grey represents an antigen-induced AHR locus¹⁵.

Gene	Intronic	Essential splice site	Stop codon gained	3Prime UTR	Synonymous coding	Non-synonymous coding	SPRIME UTR	Sp-IgE	Naive AR	Allergen induced AHR	Airway inflammation
<i>Tulp4</i>	132			4	1						
<i>Tmem181a</i>	65			3	1	1					
<i>Tmem181b</i>				1							
<i>Rshl2b</i>	60				12	7	3				
<i>Rshl2a</i>	4										
<i>Prr18</i>				5	3	2	1				
<i>Chd1</i>	203				6		1				
<i>Rgmb</i>	6			4							
<i>Zfp52</i>	99			14	23	15	2				
<i>BC049807</i>	99				6	4	3				
<i>6330416L07Rik</i>	63			5	2	1	5				
<i>Zfp758</i>	30			3	1		2				
<i>A630033E08Rik</i>	44			32	24	15					
<i>Zfp40</i>	30			2	1	1	3				
<i>Srrm2</i>	43			12	15	8	1				
<i>Tbcd24</i>	215			47	2		1				
<i>Abca3</i>	697	1		14	32	6	6				
<i>Gfer</i>	10			1	2	1					
<i>Eme2</i>	9			5	3	3					
<i>Decr2</i>	18			5	1						
<i>Pim1</i>	4				3						
<i>Glo1</i>	34			3	1						
<i>Gplr</i>	78				1						
<i>H2-K1</i>	96			14	43	32	3				
<i>H2-DMa</i>	22				4	2					
<i>H2-Ob</i>	230			12	12	6	1				
<i>H2-Ea</i>	156			9	32	20	1				
<i>H2-Eb2</i>	152			22	11	9					
<i>Ng23</i>	12				1	1					
<i>Msh5</i>	62			6	8	2					
<i>H2-D1</i>	39		1	6	22	22	1				
<i>H2-Q1</i>	3			4	5	4	1				
<i>H2-Q10</i>	4	1		4	3	2	1				
<i>Abcf1</i>	6			2							
<i>H2-T24</i>	3										
<i>H2-T23</i>	7				3	1					
<i>Gpr116</i>	160			41			1				
<i>Enpp5</i>	8			10	2	1					
<i>Mrps10</i>	65			4	6	2	1				
<i>Apobec2</i>	116				3						
<i>Pot1b</i>	524			10	11	1					
<i>Tubb4</i>	13										

Table 3: Overview of SNP polymorphisms between BALB/c and C57BL/6 in genes found to be differentially expressed between BALB/c and C.lmr1. In the phenotype columns black cells indicate that this gene is located in a locus that influence the phenotype. Grey cells indicate that the genes are located in interacting loci.

After challenge C.lmr1.4 shows significantly higher AHR compared to BALB/c. However the increase in response after challenge compared to before challenge is comparable to that of BALB/c. The observed effect on AHR after challenge in C.lmr1.4 is thus not caused by an increased allergen induced AHR but by higher naive AHR. C.lmr1.3, but not C.lmr1.2 shows significantly lower AHR compared to BALB/c, which indicates an effect of the *lmr1B* locus. Interestingly this effect is opposite to the effect of *lmr1B* on the naive AHR observed in C.lmr1.3 and C.lmr1.4. Also C.lmr1.5 but not C.lmr1.7 has a significantly decreased AHR after challenge compared to BALB/c, which also implicates the *lmr1C* locus for allergen induced AHR. Both *lmr1B* and *lmr1C* show overlap with the locus for OVA induced AHR found in a F2 cross of BP2 and BALB/c¹⁵. Apart from *Apobec-2* no other genes were reported to be associated to asthma in both loci *lmr1B* and *lmr1C*. The latter contains 2 polymorphic genes which show difference in their expression level between BALB/c and C57BL/6, i.e. *Pot1b* and *Tubb4* (see table 3). *Pot1b*, is a protein that protects the telomeres from degradation and homolog recombination between telomeres²⁵. *Pot1b* KO mice show increased senescence in rapidly dividing cells including cells of the immune system²⁶, which makes it an interesting candidate gene. No lung phenotype was described in these KO mice, however lungfunction was not assessed. *Tubb4* encodes for a member of the tubulin family of cytoskeletal proteins. It is implicated in cell-adhesion and mobility^{27,28}. It could exert a role on AHR by influencing the epithelial integrity or the ability of immune cells to penetrate into the lung tissue.

As for the Sp-IgE serum levels, the eosinophilic inflammation is lower in C.lmr1.2 and C.lmr1.3 compared to BALB/c. *Lmr1A* is thus also encoding genes that have an influence on the sensitivity to develop airway inflammation. This is consistent with the previous observation made in C.lmr1, which showed lower airway inflammation compared to BALB/c. Also BALB/B showed lower eosinophilic inflammation compared to BALB/c, which make the *H2* locus the prime candidate to explain the

differences in airway inflammation caused by the *lmr1A* locus. The high number of eosinophils in the BAL of C.lmr1.4 but not C.lmr1.3 or C.lmr1.5 suggests that *lmr1B* is also involved in genetic interactions with *lmr1A*. Alternatively, the absence of an increased airway eosinophilia in C.lmr1.3 as we see it in C.lmr1.4 is not the result of a genetic interaction at all. In this scenario, it is not the genetic susceptibility for eosinophilia which causes the increased airway inflammation in C.lmr1.4, but the increased susceptibility to develop AHR. In literature, some indications can be found that in fact AHR is an independent risk factor for other asthma traits such as eosinophilia²⁹⁻³². Given the increased AHR that is unique to the C.lmr1.4 substrain, the increased eosinophilia we observe after OVA challenges might be direct consequence of the increased AHR itself rather than a consequence of an increased susceptibility to airway inflammation per se.

In summary, 4 new asthma susceptibility loci were identified using subcongenic strains derived from C.lmr1. The first locus, *lmr1A*, spans the first 47.7 Mbp of chromosome 17. It has an influence on Sp-IgE serum levels and the numbers of eosinophils in the BAL. The second locus, *lmr1B*, is located between the 45.4 Mbp to 52.0 Mbp and has an influence on both naive and allergen induced AHR and interacts with *lmr1A* on the number of eosinophils. Finally the third locus, *lmr1C*, is located between 52.0 and 60.5 Mbp has an effect on allergen induced AHR and interacts with *lmr1D*, located between 60.5 and 71.0 Mbp, on naive AHR. To identify the genes involved in the different asthma phenotypes, further fine-mapping is necessary. This could be done by performing an F2 cross or by comparison of the mouse data with data from human genome wide association studies.

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Fine-mapping the IgE QTL within the *Imr1* by an adapted F2 screen

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Abstract

Allergic asthma is a pulmonary disease characterized by airway hyperreactivity to bronchospasmogenic stimuli, eosinophilic airway inflammation and high allergen specific IgE (sp-IgE) levels in serum. Allergic asthma is a complex disease, and the result of an interaction between the genetic make-up and environmental factors. We have previously identified an asthma susceptibility locus on mouse chromosome 17 affecting airway eosinophilia, airway hyperresponsiveness (AHR) and sp-IgE in recombinant congenic (RC) mice. Here, we performed a classical F2 cross using the RC mice carrying the QTL for sp-IgE to finemap this locus. The recombinants were selected by rough genotyping and subsequently submitted to an OVA-induced model of allergic asthma. These mice were then further genotyped and phenotyped.

One small QTL for sp-IgE serum levels was identified together with a second putative locus. *Pla2g7* is proposed as the relevant asthma susceptibility gene for the first locus, as it was previously identified as an asthma susceptibility gene in human. Based upon differential expression between BALB/c and C.lmr1, *Gpr116* and *Enpp5* are also interesting candidate genes.

The second locus contains both the *H2* locus and the *TNF*, *LTA-1*, *TAP-1* cluster, which were both previously associated with sp-IgE levels in both human and mice. Considering the fact that all differentially expressed genes with polymorphisms in their sequence map to the *H2* locus, this locus is the prime candidate to explain the effects of the secondary QTL on serum sp-IgE.

Introduction

Identification of new asthma susceptibility genes should lead us to new insights in the complex pathophysiology of asthma, which is a key step in the development of new therapeutic strategies. Until now more than 150 asthma susceptibility genes have been identified, from which most were identified by candidate gene approaches¹. However it has shown to be difficult to replicate these observations^{2,3}. Only 8 genes, *DPP10*, *CYFIP2*, *HLAG*, *GPRA*, *SFRS8*, *PHF11*, *ADAM33* and *PCDH-1* were identified by positional cloning. All of these genes have been replicated in some but not in all studies they were tested in^{1,4,5}, which nicely illustrates the genetic complexity of asthma in which genes interact with one another and the environment. Using the recently developed high-throughput SNP genotyping platforms, 13 new asthma susceptibility genes were identified in only 7 studies⁶⁻¹². It remains to be determined how well these results can be replicated.

We have used an alternative approach and have started mapping asthma susceptibility in recombinant congenic (RC) mouse strains. Using the *lmr* series of RC strains¹³ and sub-congenic strains we have previously identified the *lmr1* locus (chapter 4), on mouse chromosome 17, as an asthma susceptibility locus. It contains several QTLs for the different asthma phenotypes. On a BALB/c background, mice with the C57Bl/6 allele of the *lmr1A* sublocus, spanning the first 45.4 cM of chromosome 17, have significantly lower sp-IgE levels in serum compared to mice with the BALB/c allele (chapter 4).

In the current study we aim to fine map this sp-IgE QTL by a classical F2 phenotyping approach using an intercross with BALB/c and the two C.*lmr1* derived sub-congenic strains that carry the C57Bl/6 allele of this locus. We find that the susceptibility to a reduced sp-IgE response is conferred by a small genomic region proximal to, but not containing the H2 locus. The most prominent candidate gene within the locus is the *Pla2g7* gene which is polymorphic between the two background strains, and which has been identified as a susceptibility gene for atopy and asthma in human as well. Gpr116

and *Enpp5*, which are both differentially expressed between BALB/c and C.lmr1, are also interesting candidate genes. We also find a secondary putative locus, which does include the H2 locus and the TAP-1/TNF- α /LTA-1 cluster. All genes which are differentially expressed after OVA sensitisation and challenge between BALB/c and C.lmr1¹³ and have polymorphisms in their sequence are located within the *H2* locus, making this locus the prime candidate to explain the differences in sp-IgE encoded by this secondary putative locus.

Materials and methods

Mice

Colonies of BALB/cAnBradley, C.lmr1, C.lmr1.2 and C.lmr1.3 were bred at the animal facilities (CDL) of the University Medical Centre Groningen, The Netherlands. Animal care and use were performed in accordance with the guidelines of the Federation of European Laboratory Animal Science Association¹⁴. All animal experiments were reviewed by and approved of by the institutional animal care and use committee at the University of Groningen. The mice were kept under specified pathogen free conditions and were housed in individually ventilated cages with food (RMH-B, Abdiets, Woerden, The Netherlands) and water administered *ad libitum*.

Experimental Asthma protocol

On day 0 and 7 male mice were sensitized by intraperitoneal injection with 10 µg OVA (Seikagaku Corp, Tokyo, Japan) in 2 mg Alum adjuvant (Pierce, Rockfort, IL, USA). One week after the second sensitization blood was taken for serum quantification of allergen-specific immunoglobulins. Subsequently, the animals were challenged by aerosol exposure for 20 minutes with 10 mg OVA/ml in saline, starting on day 21 and then once every three days for a total of three times as described previously¹⁵. Twenty-four hours after the last OVA inhalation challenge, airway responsiveness to metacholine was measured. Subsequently, blood and bronchial alveolar lavage fluid were collected.

Ovalbumin-specific immunoglobulin levels in serum

For euthanasia, mice were anesthetized with isoflurane and bled through the vena cava. Serum was collected and stored at -70°C until analysis. Levels of OVA-specific IgE in the serum were measured as described previously¹⁶. A reference standard was used with

arbitrary units of 1,000 EU/ml. The detection level of the ELISA was 0.5 EU/ml.

Genotyping

Genotyping was performed using microsatellite markers and SNP assays on genomic DNA extracted from tail snips or spleen. Microsatellite PCR primers and conditions were extracted from the MGI database (<http://www.informatics.jax.org>), and product sizes were analyzed on 4% Methaphor high resolution agarose gel (Cambrex, East Rutherford, New Jersey, USA) electrophoresis. The SNP assays were ordered from Applied Biosystems (Taqman SNP genotyping assays, Applied Biosystems, Foster City, CA, USA) and performed according to the manufacturer instructions using a 7900 HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). An overview of the markers used and their position on chromosome 17 is shown in supplementary table 1.

Genetic analysis

Genetic analysis was performed using J/QTL version 1.2.1 (The Jackson Laboratory, Bar Harbor, Maine, USA) with R version 2.8.1. For the single QTL analysis genotypes were estimated at a 0.3 cM interval with a genotyping error rate of 0.005. Significance was estimated by 50 000 permutations. Markers positions in cM were estimated using the Mouse Map Converter (<http://cgd.jax.org/mousemapconverter>) based upon their position in basepairs as reported on the MGI database (<http://www.informatics.jax.org>). After the QTL scan, the position in basepairs was estimated with the same tool. To identify the polymorphisms present in the identified QTL between the two background strains, data was extracted from the Sanger database (<http://www.sanger.ac.uk/cgi-bin/modelsorgs/mousegenomes/snps.pl>). Data was analysed using SAS for Windows version 9.0 (SAS Institute, Cary, NC, USA). All data was mapped on NCBI build 37 of the mouse genome.

Results

In order to fine-map the *lmr1A* sp-IgE QTL present in the C.lmr1.2 and C.lmr1.3 subcongenic strains (figure 1), a classical F2 cross was performed with the BALB/c background strain. To this end, the two subcongenic strains were first backcrossed to BALB/c and the offspring (F1) was intercrossed to obtain an F2 generation.



Figure 1: Overview of the genotypes of C.lmr1 derived sub-congenic strains and the position of the *lmr1A* locus together with the loci identified in these experiments. For genotypes, black represents C57Bl/6, white BALB/c and grey is unknown genotype.

In order to enrich for mice with an informative genotype, the male F2 progeny was genotyped using an array of polymorphic SNP markers spaced roughly 6 cM apart. 63 mice carrying novel recombinations within the *lmr1A* locus were used for phenotyping and further high-density genotyping (overview of the markers given in supplementary data). Five BALB/c mice were included as control.

We analyzed the association of sp-IgE levels after OVA challenge by single QTL analysis using J/QTL version 1.2.1 (The Jackson Laboratory, Bar Harbor, Maine, USA). For the post-challenge sp-IgE serum levels a significant genetic association peak can be observed between 31.7 Mbp and 44.8 Mbp with a LOD score above 3 (black curve figure 3). The strongest association is observed between 43.4 Mbp and 44.5 Mbp with a LOD score higher than 4. This locus is further denoted *lmr1A1*. From Figure 3 it appears that the association peak might in fact harbour 2 separate association signals, as a broad shoulder appears on the centromeric side of the main association peak (see figure 3). To further investigate the presence of a second independent association

signal, we re-analyzed the data using the genotype of SNP rs3656008 (genotyping SNP in the main association peak, LOD = 4.137) as covariate (red curve in Figure 3). This covariate analysis indicates that a second association signal might be present with a LOD score just over 3.0 for sp-IgE levels, which is located centromeric to the main association peak between 31.7 and 36.1 Mbp. This second locus is further called *lmr1A2*.

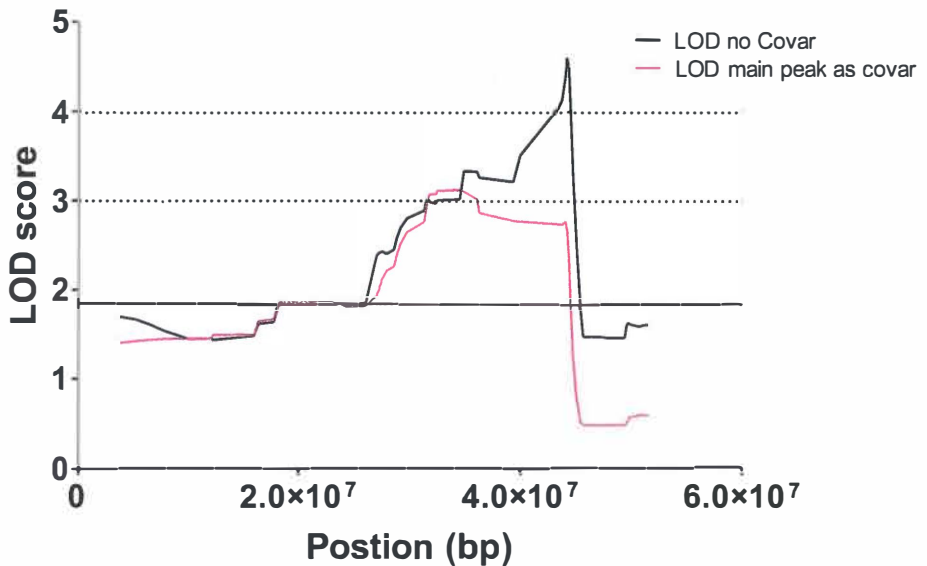


Figure 3: LOD-score for association with post-challenge sp-IgE levels versus position on chromosome 17 (black curve) and with SNP rs3656008, located in the main peak, as covariate. Horizontal black line is the 95% significance level for the analysis without covariate.

In order to evaluate the number and identity of the remaining candidate genes present within *lmr1A1*, we analyzed the presence of genes within this genomic region carrying polymorphisms at the SNP level between the two parental strains. According to the publicly available Sanger SNP database (<http://www.sanger.ac.uk/cgi-bin/modelsorgs/mousegenomes/snps.pl>) the genomic region within the main association peak (LOD > 4) harbors 4328 SNPs that are polymorphic between BALB/cJ and

C57Bl/6NJ. From these, 2664 SNPs are located within the sequence of a gene, an overview of the polymorphic genes identified by this analysis can be found in table 1. We previously performed genome-wide transcriptional profiling to identify genes differentially expressed between C.lmr1 and BALB/c in lung tissue of sensitized and PBS or OVA challenged mice. Two genes located within the *lmr1A1* locus were found to be differentially expressed in this experiment: *Gpr116* and *Enpp5*¹³.

SNP class	Gene										
	Gpr110	Gpr116	Mep1a	Pla2g7	Tdrd6	Slc25a27	Cyp39a1	Rcan2	Enpp5	Enpp4	Clic5
Coding non-synonymous	4	0	1	1	9	0	2	4	1	0	0
Coding synonymous	1	0	0	2	21	0	5	2	1	0	0
Intronic	236	160	3	111	46	14	722	759	8	21	441
UTR	4	42	2	2	0	0	4	0	10	23	1
Stop codon gained	0	0	0	0	0	0	0	1	0	0	0
Associated to asthma or atopy in human				Yes							
Differentially expressed between BALB/c and C.lmr.1		Yes							Yes		

Table 1: Number of SNPs between BALB/c and C57Bl/6 in the main sp-IgE QTL peak (LOD \geq 4) classified per gene and per type of SNP. Association of the human analogue with asthma and differential expression in lung tissue between BALB/c and C.lmr.1 (see chapter 3) is shown in the last 2 rows.

The second putative locus (LOD \geq 3), *lmr1A2*, located between 31.7 and 36.1 Mbp contains 147 genes with polymorphisms between BALB/cJ and C57Bl/6NJ. Of these 147 genes, 10 were differentially expressed between BALB/c and C.lmr1. Interestingly all these genes are annotated as MHC genes (Table 2).

SNP class	Gene									
	H2-DMa	H2-Ob	H2-Eb2	H2-Ea	Msh5	H2-D1	H2-K1	H2Q1	H2-Q10	Abcf1
Coding non-synonymous	2	6	9	20	1	22	31	3	1	
Coding synonymous	2	6	2	12	1		11	1	1	
Intronic	22	216	152	156	61	38	77	2	3	1
UTR		13	22	1	6	7	17	5	5	2
Essential splice site									1	
Stop codon gained						1				
Differentially expressed after PBS challenge	ND	Yes	ND	Yes	Yes	Yes	Yes	ND	Yes	Yes
Differentially expressed after OVA challenge	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 2: Number of SNPs between BALB/c and C57Bl/6 in *Imr1A2* (LOD \geq 3) classified per gene and per type of SNP. ND: Not detected.

Discussion

Here, we present an F2 genetic screen using the previously characterized C.lmr1 subcongenic strains C.lmr1.2 and C.lmr1.3 (Chapter 4) to fine-map the sp-IgE susceptibility present within the *lmr1A* locus on chromosome 17 (figure 1). By this approach we identify an association peak for post-challenge sp-IgE serum levels in the mouse model of OVA-induced allergic asthma, from 43.4 Mbp to 44.5 Mbp (*lmr1A1*, LOD \geq 4). An additional QTL was found centromeric to the main association peak, from 31.7 Mbp to 36.1 Mbp (*lmr1A2*, LOD \geq 3).

Both QTLs show synteny to human 6p21, which is the most replicated locus in genetic screen for asthma in human. Despite the solid genetic association of 6p21, it has been difficult to identify the relevant asthma susceptibility genes in this region¹⁷. Our data indicate that there might be multiple independent signals for sp-IgE present in this locus, which is concordant to the human situation, in which *HLA-G*¹⁸, *PLA2G7*^{19,20}, the *HLA*²¹ locus and the *LTA-1*²²/*TAP1*²³/*TNF α* ²⁴ cluster have found associated to atopy.

Polymorphisms in genes can lead to differential expression of genes or alteration of the function of the proteins they encode. The *lmr1A1* locus contains 2 genes, G-protein coupled receptor 116 (*Gpr116*) and Ectonucleotide pyrophosphatase/phosphodiesterase 5 (*Enpp5*), which are differentially expressed in lung tissue between BALB/c, one of the parental strains, and C.lmr1, from which the other parental strains C.lmr1.2 and C.lmr1.3 are derived (Chapter 3). No functional data is yet available for these genes. The *lmr1A1* locus also contains 6 genes with non-synonymous mutations that might contribute to altered protein function. One of these genes, serum platelet-activating factor acetyl hydrolase (serum *PAF-AH*; *pla2g7*), has previously been associated with asthma and atopy in human^{19,20}. The groups of Deichmann and Arinami were able to identify loss of function polymorphisms within the sequence of the human *pla2g7* gene which leads to increased risk of atopy and asthma and increased total IgE serum levels^{25,26}. The coding non-synonymous polymorphism between BALB/c and C57Bl/6

is not located within the conserved region of the PAF-AH type II subfamily of acetylhydrolases, which comprises the catalytic domain (<http://vega.sanger.ac.uk>), so it remains to be determined if the polymorphism has any effect on the activity of *Pla2g7*.

Platelet activating factor (PAF) is a pro-inflammatory lipid mediator released by a wide variety of cells, including eosinophils, endothelial cells, neutrophils, monocytes and macrophages. It modulates inflammation directly as a chemotactic factor and indirectly by stimulating the release of other inflammatory agents. PAF is continuously synthesized at low levels by constitutively expressed lysophosphatidylcholine acetyltransferase (LPCAT). The serum levels of PAF are controlled by the activity of *pla2g7*. During inflammation PAF synthesis rates are increased by inducible LPCAT and oxidative mechanisms²⁷⁻²⁹. Jancar *et al* investigated the role of PAF in the development of high serum sp-IgE levels in sensitized but unchallenged mice using WEB2086, a PAF receptor antagonist. No differences in sp-IgE serum levels were observed in mice treated with WEB2086 compared to untreated mice³⁰, which suggests that PAF does not exert its modulatory role on sp-IgE serum levels during sensitization.

In our experiments with the C.lmr1 subcongenic strains that were used in this F2 cross, a significantly lower AHR is observed in C.lmr1.2 and C.lmr1.3. Administration of PAF has been shown to induce AHR in animal models^{31,32} and human³³. In the current F2 cross, however, we did not find a QTL peak for AHR within the *lmr1A* locus. This could be due to a lack of power as the effect of the AHR QTL is smaller (36 % suppression of AHR for C.lmr1.3 at 25 mg/ml metacholine compared to BALB/c) than the effect of the sp-IgE QTL (72% reduced serum levels for C.lmr1.2 compared to BALB/c). This indicates that the effect size of *pla2g7* polymorphisms might be smaller on AHR than on sp-IgE, and therefore requiring a larger number of mice to be measured before we can detect any signal on AHR. Moreover, we found strong evidence for genetic interactions between multiple loci present within the *lmr1* locus (chapter 4), precluding a straightforward QTL identification in a classical F2 approach

as we employed here. Finally, it remains a possibility that *pla2g7* is not the (only) relevant polymorphism for AHR present within the *lmr1A1* locus.

Stratification of the data for the genotype on SNP rs3656008, located within the main *lmr1A1* locus, reveals the presence of a second weak (max LOD score of 3.1) but significant, independent QTL ranging from position 31.7 Mbp to position 36.1 Mbp on chromosome 17. Within this *Lmr1A2* locus, 2 clusters of genes are present that have previously been associated with asthma and atopy. The first cluster contains the *TNF α* ²⁴, *LTA-1*²² and *TAP-1*²³ genes. These 3 genes have been found associated to asthma and atopy. However, due to their close proximity and strong linkage disequilibrium (LD), it remains to be determined which of these genes contributes to the susceptibility to asthma. Both TNF α and LTA-1 are Th1 pro-inflammatory cytokines and can thereby directly modulate the inflammatory reaction. TAP-1 is involved in antigen processing and presentation and could have an influence on the initiation of the allergic response and thus on the sp-IgE levels.

The *H2* locus, which encodes for the major histocompatibility complex is also contained within this second putative QTL peak for sp-IgE. All polymorphic and differentially expressed genes between BALB/c and C57BL/6 in *Lmr1A2*, are located within this locus. This together with the fact that the mouse *H2* locus or the human homologue *MHC* locus have previously been found associated to sp-IgE levels^{34,35}, makes the *H2* locus a strong candidate locus to explain the differences in sp-IgE levels. However, this is not supported by our previous findings in which BALB/B, which carries the same *H2* haplotype as C57BL/6 on a BALB/c background, does not show differences in serum sp-IgE levels compared to BALB/c¹³. Therefore we cannot exclude that the effect of the *Lmr1A2* locus is due to polymorphisms located proximal to the *H2* locus, outside of the congenic fragment of BALB/B.

As for *Pla2g7*, the mouse *H2* locus or its human homologue, the *MHC* locus have been found associated to asthma severity and AHR^{9,13,18}. In the current experiment we were unable to replicate these observations. As mentioned above this is probably due to the small effect of the *Lmr1A* locus on AHR and the limited number of animals that were included in the experiments.

Future experiments will need to positively identify the gene contributing to the divergent sp-IgE response between C.lmr1.2/1.3 mice and the BALB/c background strain. In order to confirm that *Pla2g7* is the relevant gene for the regulation of sp-IgE responses, it would be interesting to measure the PAF levels or directly measure *Pla2g7* activity in the sera of the subcongenic strains or the F2 progeny used in the genetic analysis. This could reveal whether the C57Bl/6 and BALB/c alleles of the *pla2g7* gene are functionally different in PAF metabolism. Further functional studies could then be performed to assess whether this difference in serum PAF-AH activity is instrumental in the altered sp-IgE response between the two strains.

Supplementary data

Marker	Position (bp)	Position (cM)	Marker	Position (bp)	Position (cM)
<i>D17mit164</i>	3924615	1.9580	<i>Rs33512005</i>	10051462	3.1588
<i>D17mit57</i>	10055276	3.1609	<i>D17mit113</i>	12172308	3.9104
<i>Rs13482891</i>	15612924	4.3370	<i>D17mit213</i>	16752157	5.0500
<i>Rs13482915</i>	21145938	6.472	<i>D17mit133</i>	24994554	7.062
<i>D17mit46</i>	25502885	7.062	<i>Rs8252771</i>	26642535	7.226
<i>D17mit80</i>	26859833	7.226	<i>D17mit198</i>	27796090	7.85
<i>Rs13482948</i>	32078992	10.767	<i>D17mit16</i>	33737692	11.058
<i>D17mit62</i>	33874679	11.058	<i>D17mit34</i>	34880998	11.2502
<i>D17mit24</i>	37619109	11.997	<i>Rs33213800</i>	38052154	11.997
<i>Rs3656008</i>	43752288	12.5173	<i>D17mit49</i>	45448429	14.5599
<i>D17mit35</i>	45679392	14.7605	<i>D17mit115</i>	47711568	15.9057
<i>D17mit68</i>	47707105	15.8869	<i>D17mit177</i>	48698276	16.557
<i>D17mit202</i>	47992431	16.557	<i>D17mit178</i>	48845637	16.557
<i>Rs13483014</i>	49071704	16.557	<i>D17mit108</i>	49744546	17.4697
<i>D17mit106</i>	49999780	17.601	<i>D17mit117</i>	50270475	17.601
<i>D17mit180</i>	51571276	17.9346			

Supplementary table 1: Markers used for genetic analysis. Position in bp as reported by MGI database (<http://www.informatics.jax.org>). Position in cM were calculated using the Mouse Map Converter (<http://cgd.jax.org/mousemapconverter>) based upon their position in basepairs.

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Discussion and conclusions

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Discussion

Asthma is a chronic inflammatory pulmonary disease in which patients develop shortness of breath and wheezing. The pathophysiology of asthma is complex. The most common cause is an allergic response in the lungs¹, but it can also be caused by pulmonary infection, long term exposure to noxious gasses or particulate matter suspended in the air. It is characterized by reversible airway narrowing in response to physical or chemical stimuli. Concomitant airway inflammation, airway remodelling and high serum levels of allergen specific IgE (sp-IgE, only in allergic asthma) are usually observed.

Asthma susceptibility is partially determined by genetics, with heritability estimated between 35 and 75%². In human, genes were mapped using both candidate gene and genome wide approaches in family based and case-control designs. These studies have shown that asthma is influenced by many genes that are involved in many different processes, like Th cell differentiation, epithelial barrier function, airway remodelling, cytokine signalling, and others³. Replication of the results from these genetic studies, especially the candidate gene studies, have shown to be difficult, which is at least partially due to epistatic⁴ and gene-environment interactions⁵. The most replicated asthma locus is located in the 6p21 cytogenetic band³ which is syntenic to mouse chromosome 17. It encodes the MHC locus and the TAP1/TNF- α /LTA-1 cluster, both of which were previously found associated to asthma. The MHC locus is a complex locus that contains many genes involved in antigen presentation and immune signalling. HLA-G⁶ and more recently HLA-DR/DQ⁷ have been identified as candidate genes. Other genes in the MHC locus were identified based upon their differential expression in lung biopsies from asthmatics versus healthy controls⁸⁻¹¹. The 3 genes contained in the TAP1/TNF- α /LTA-1 cluster were all found to be associated with asthma¹²⁻¹⁴. Considering the strong LD in this region it remains to be determined which genes are true susceptibility genes for asthma.

Mouse models also allow for genetic screens to identify loci or genes contributing to a complex disease. In mice genes can be mapped using linkage or association like in human. The genetic variability, however, needs to be introduced by crossing inbred mouse strains, as minimal genetic variability exist within an inbred strain. Due to the ability to breed mice at will and the short generation time of 12 weeks, several genetic screening techniques have become available which are reviewed in chapter 1.

The most relevant genetic screen for this manuscript is the use of recombinant congenic (RC) and recombinant inbred (RI) strains. To generate these strains, offspring of second or later generation crosses are inbred and homozygous strains are thereby generated. RI strains are derived from the offspring of intercrosses and have thus inherited half of their genome from both parental strains. RC strains on the other hand are bred from offspring of backcrosses and have thus inherited less of their genome from the donor strain than from the background strain. RC strains can be both randomly generated, like the CcS/Dem strains used in chapter 2, or bred to contain specific congenic fragments, like the *Imr* strains used in chapter 3, 4 and 5. Randomly generated RC strains and RI strains are used for genome wide mapping of traits by association analysis, with the additional advantage of being able to test more individual mice with the same genotype and thereby increase the statistical power to detect the phenotypic effect of individual loci. However, resolution of such a screen is lower than for second generation crosses, due to the limited number of available RC strains and the fact that for the same number of mice less genotypes can be tested. Therefore, RC and RI strains are usually used for primary screening at high power and low resolution, whilst follow-up using a different approach is required to positively identify the relevant candidate genes. These methods can be genetic, like screening of sub-congenic strains (chapter 4) and/or an F2 cross on the relevant (sub-)congenic strains (chapter 5), but other methods, like transcriptional profiling (chapter 3), can also be used¹⁵. We decided to use RC strains in our experiments as the increase in power should allow the detection of asthma genes with a

relatively small influence on the genotype, which is typically observed in genetically complex diseases as asthma.

As asthma does not naturally occur in laboratory mice, an OVA-driven mouse model of allergic asthma was used in our experiments. This mouse model closely mimics the human disease and allows the quantification of major asthma phenotypes, like airway hyperreactivity (AHR), allergen specific IgE (sp-IgE) levels in serum, airway eosinophilia and a strong Th2 biased pulmonary inflammation. Despite the many similarities between the human and mouse disease there are also important differences. These are due to differences in anatomy of the lungs (less branching and a reduced number of airway smooth muscle cells in the mouse), and differences in cellular responses (less degranulation of eosinophils, differences in mast cell mediators released upon IgE crosslinking and differences in response to mediators such as Th2 cytokines)¹⁶⁻¹⁸.

RC strains have been used successfully to map genes involved in complex traits as cancer^{19,20}, tuberculosis²¹ and *Leishmania major* infection^{22,23}, but were never previously used to map asthma related traits. Therefore, we first investigated the power of this approach for mapping of asthma related traits using the OVA-driven model of allergic asthma in the CcS/Dem strains, a panel of random RC strains derived from STS/A and BALB/cHeA²⁴. These strains show a range of quantitative outcomes for the different asthma phenotypes tested. For AHR after OVA inhalation challenge, for example, the responses ranged from a severe bronchoconstriction in the parental STS/A strain (lethal at doses of 25 mg/ml metacholine) to no response in the CcS05 strain (no significant increase in airway responsiveness after OVA inhalation challenges compared to before challenge). This shows that RC strains can be used efficiently to map asthma susceptibility genes in mice. Interestingly, we also observed different types of immunological response throughout the CcS/Dem strains. The CcS14 strain shows a 'true' Th2 response with high IgE and Th2 cytokine levels and low IgG1 while the other strains all showed a modified Th2 response, characterised by lower IgE levels and

a higher IgG1/IgE ratio. Although such a modified Th2 response was originally described as a tolerance mechanism²⁵, our data show that the true Th2 responder, CcS14, does not show increased AHR or pulmonary inflammation in comparison to the strains showing a modified Th2 response. This already indicates the presence of divergent mechanisms governing the individual asthma traits, and thereby different genetic predispositions for the individual parameters in the screen. To formally test this, we analysed the correlations between the different asthma phenotypes in the pooled dataset of all strains. We observed that the individual asthma parameters did not correlate with each other to a large extent. This indicates that, in mice, the larger part of the genetic susceptibility is not shared between the individual phenotypes, which is comparable to what is observed in human²⁶.

Further fine-mapping of asthma susceptibility in the CcS/Dem strains would require a major effort in backcrossing, genome-wide genotyping and phenotyping. Therefore, we decided to use the lmr RC strains instead for further phenotypic analyses. As these strains only have 1 or 2 congenic fragments, less genotyping and screening would be necessary. Part of the *lmr1* locus, including the mouse equivalent of the *MHC* locus: the *H2* locus, shows synteny to the most replicated human asthma locus on 6p21³. This was one of the main arguments to use the lmr series of recombinant congenic (RC) mouse strains in our experiments. The C.lmr series of RC mice have inherited the C57Bl/6-derived allele of *lmr1*, on chromosome 17, and/or *lmr2*, on chromosome 9, on a BALB/c genetic background. The B.lmr strains have the reciprocal genotype with the BALB/c allele of *lmr1* and *lmr2* on a C57Bl/6 background^{27,28}. Both, *lmr1* and *lmr2* were originally identified as *Leishmania major* infection susceptibility loci in an F2 cross between C57Bl/6 and BALB/c²⁹. The strong Th2 biased inflammatory response in both asthma and severe *Leishmania major* infection suggested a common genetic origin for the susceptibility to develop either disease. This was the other main argument in favour of the use of the lmr series of RC mice in our experiments.

The data from the single congenic strains show that the *lmr1* locus makes the largest contribution to the asthma susceptibility, as this locus modulates both the Th2 cytokine response and the eosinophilic inflammation, on both backgrounds, with an additional background specific effect on AHR on the BALB/c background and on IgE serum levels on the C57Bl/6 background. The *lmr2* locus on the other hand only shows a BALB/c background specific effect on AHR and sp-IgE. In order to identify candidate genes in the *lmr1* locus, we have chosen to compare the genome-wide expression profiles of lung tissue from C.lmr1 and BALB/c, challenged with either OVA or PBS in OVA-sensitized mice. This approach was previously used for the mapping of genes involved in complex traits^{30,31} including asthma³². We find 101 genes to be differentially expressed between our two strains, 63 of which are located within the *lmr1* locus. Interestingly, 21 of these 63 differentially expressed genes are located within the *H2* locus, the mouse ortholog of the human MHC locus.

To further investigate the involvement of the *H2* locus, we phenotyped the C.B10-H2b (BALB.B) strain, which is a BALB/c derived RC strain that inherited the *H2* locus from C57Bl/10. This latter strain has the same *H2^b* haplotype of the *H2* locus as C57Bl/6 (and C.lmr1) and the phenotypic information from this strain should therefore give us insight into the role of the C57Bl/6 *H2^b* allele on the sensitivity to develop asthma. The experimental data suggest that the presence of the *H2^b* haplotype is sufficient to induce the reduced AHR and the airway eosinophilic inflammation seen in C.lmr1. However the RC fragment in BALB.B (from 26.9 to 37.3 Mbp)³³ is slightly larger than the *H2* locus (from 34.1 to 37.1 Mbp) alone and does contain an additional 3 genes, *Pim1*, *Glyoxalase1* and *Rab44* that are both polymorphic and differentially expressed between BALB/c and C.lmr1. These 3 genes, together with the *H2* locus, are therefore good candidate genes to explain the differences in sensitivity to develop AHR and high number eosinophils in BAL between BALB/c and C57Bl/6.

The human *MHC* locus and its mouse equivalent, the *H2* locus, are complex loci of approximately 3 Mbp, which encode for more than 100 highly variable genes related to

immunity. These genes can be subdivided into 3 classes of genes, i.e. Class I to III. The Class I genes are expressed on most nucleated cells and are involved in processing and presentation of cytosolic antigens to cytotoxic T lymphocytes. The Class II genes are also involved in anti-gen processing and presentation of extra-cellular antigens on the surface of professional antigen-presenting cells (APCs). These APC present the antigen-peptides to T helper lymphocytes. Class III contains genes which are not directly involved in antigen processing and presentation but which are implicated in signalling of infection, like proteins of the complement cascade, cytokines and heat shock proteins. Most differentially expressed genes are from Class I, but the strongest differences in expression levels between BALB/c and C.lmr1 are observed in genes of Class II, i.e. *H2-D1* and *H2-Ea*. This finding is consistent with findings in human, in which differences in expression of genes from both Class I⁸ and Class II^{7,9-11} were found associated to asthma. Despite the fact that these genes and their function in the immune response is rather well characterised it remains to be determined how their differences in expression influences the susceptibility to develop asthma. Our data shows that mouse models could be used to further investigate this using recombinant congenic mice with different *H2* haplotypes on a common genetic background like the BALB/c and BALB/B strains used in our experiments.

The *lmr1* locus spans the larger part of mouse chromosome 17 (71 Mbp) and overlaps with chromosomal regions away from the *H2* locus that were previously identified as QTLs for naïve AHR (figure 1). To further investigate the contribution of the different regions contained within the *lmr1* locus, we phenotyped subcongenic strains derived from C.lmr1 in our OVA driven mouse model of allergic asthma. These subcongenic strains divide the *lmr1* locus into 4 different subloci, *lmr1A* to *lmr1D* (figure 1). In these experiments we measured OVA-specific IgE levels in serum (sp-IgE), the numbers of eosinophils in BAL fluid, the naïve airway responsiveness (AR) and the allergen induced airway hyperresponsiveness (AHR) to methacholine. Given the divergence of

the genetic contribution to the individual asthma traits, we discuss the three phenotypes separately.

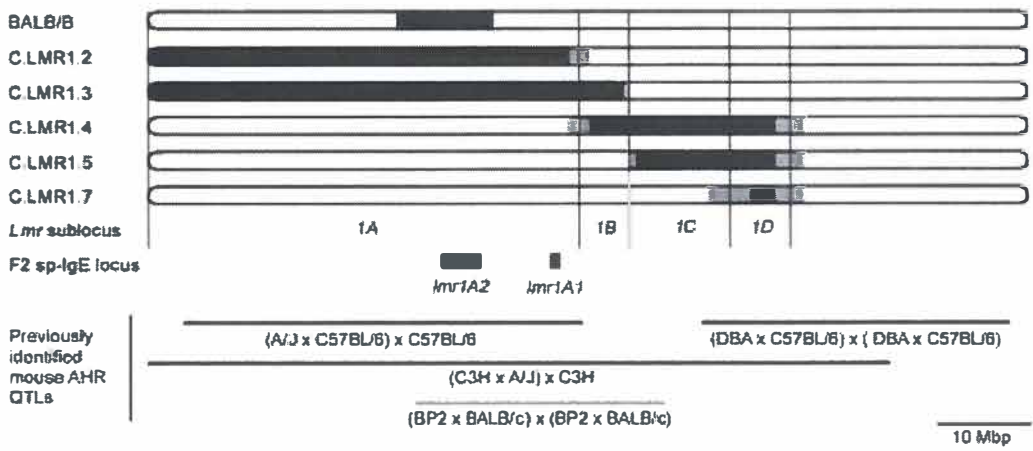


Figure 1: Overview of chromosome 17 genotypes of the C.lmr1 derived congenic strains. Black represents C57Bl/6 (or C57Bl/10 for BALB/B) genotype, white is BALB/c genotype and grey is unknown. Interesting candidate genes and previously identified AHR QTLs are shown in lower part of picture.

Sp-IgE

C.lmr1.2 and C.lmr1.3 do both show significantly lower sp-IgE compared to their background strain BALB/c. This clearly indicates that the C57Bl/6 allele of the *lmr1A* locus, which is present in both these strains, confers resistance towards the development of high sp-IgE serum levels. Interestingly, C.lmr1 did not show significantly altered sp-IgE levels compared to BALB/c, but B.lmr1 did. This suggests a genetic interaction between *lmr1A* and another unknown sublocus within the *lmr1* locus which counteracts the effect of the *lmr1A* locus or could be due to a lack of statistical power to detect differences in the C.lmr1 versus BALB/c experiments.

To gain insight in this issue and to further fine-map this locus for sp-IgE we performed a F2 cross between BALB/c and both strains containing the *lmr1A* locus, i.e. C.lmr1.2 and C.lmr1.3. A primary genotyping screen was added before the phenotyping to select mice with recombinations within the *lmr1A* locus. With these experiments we identified 2 QTL peaks, one major peak with LOD score above 4, together with a second putative peak with LOD score over 3. The main peak is located telomeric to the *H2* locus, whilst the secondary putative peak includes the *H2* locus.

The main peak contains 11 genes carrying polymorphisms between BALB/c and C57Bl/6. Interestingly, one of these genes, serum platelet activating factor acetyl hydrolase (*Pla2g7* encoding for sPAF-AH) was previously found to be associated with asthma, atopy and IgE serum levels in both human³⁴ and animal models^{35,36}, making this gene the prime candidate gene for this QTL. PAF is a lipid mediator of inflammation which is degraded by PAF-AH. To confirm the role of the PAF-AH polymorphisms in the development of high serum IgE levels it would be interesting to measure the serum levels of PAF from mice which were phenotyped and test for correlations between these levels and the serum IgE levels measured in those mice. However, even a tight correlation between those 2 parameters would not proof the importance of PAF-AH in our mouse model of asthma. To provide proof for this, intervention studies with administration PAF receptor agonists and antagonists should be performed. To avoid the influence of the secondary QTL in these experiments a C.lmr1 derived sub-congenic mouse with a congenic fragment which only contains the main QTL should be generated. Two other genes in the main QTL were found to be differentially expressed between BALB/c and C.lmr1 on the micro-array experiments: *Gpr116* and *Enpp5*. However, these results could not be confirmed by qRT-PCR.

All genes differentially expressed between C.lmr1 and BALB/c in *lmr1A2* are located in the *H2* locus, which makes this complex locus the prime candidate to explain the

effects of *lmr1A2*. This is consistent with observations in both animals^{37,38} and human³⁹ in which association between the MHC/*H2* locus and sp-IgE serum levels was shown.

Naive AR

Beside sp-IgE levels we also measured naive AR in the *C.lmr1* subcongenic strains. Naive AR has shown to be risk factor for asthma in human⁴⁰. Genetic factors contributing to naive AR could therefore be relevant to asthma susceptibility in our model. It is influenced by *lmr1B* as both strains with the C57Bl/6 allele of this locus, C.lmr1.3 and C.lmr1.4 show significantly increased naive AR compared to BALB/c, while the other congenic strains show naive AR which is either comparable (C.lmr1.2, C.lmr1.5 and BALB.B) or lower (C.lmr1.7) than the naive AR observed in BALB/c. The *lmr1B* locus is included in the large naive AR QTL found by De Sanctis *et al.* in a (C3H x C57Bl/6) x C3H backcross⁴¹ and partially overlaps with the locus identified by the same group in a (A/J x C57Bl/6) x C57Bl/6 backcross⁴² (figure 1). Given the availability of the genome-wide expression data from lungs of BALB/c versus C.lmr1, both under naïve (PBS-challenged) and OVA-challenged conditions, we analyzed which differentially expressed genes would map to the *lmr1B* locus, and could therefore be considered putative candidate genes for the effects on naïve AHR. Only 1 gene in *lmr1B* is polymorphic and differentially expressed between BALB/c and C.lmr1 after sham PBS challenge, i.e. *Apobec-2*, an RNA editing enzyme which plays a critical role in the isotype switching of B-cells^{43,44}. In human a correlation between the *Apobec-2* expression in bronchial biopsies⁴⁵ and lung-function decline has been observed in the ‘GLUCOLD’ genetic screen for COPD⁴⁶ (M. ten Berge, personal communication), which supports our evidence for an effect of *Apobec-2* on lung-function. It was not found associated in any GWAS for asthma^{47,48} or COPD⁴⁹. No GWAS on lungfunction in asthma has been published to date but they are ongoing. No associations between SNPs in *Apobec-2* and AR or AHR were found in the GWAS done by the genetics group of Dirkje Postma in Groningen (G.H. Koppelman, personal communication).

The lower naive AR in C.lmr1.7 but not C.lmr1.5 suggests that *lmr1C* and *lmr1D* are implicated in a genetic interaction in which the protective effect on naive AR is only expressed with the BALB/c allele of *lmr1C* and the C57Bl/6 allele of *lmr1D*. *Lmr1C* contains 2 genes which are differentially expressed after PBS challenge, i.e. *Pot1b* and *Tubb4*. The first one protects telomeres from degradation and recombination⁵⁰ and the second is involved cell adhesion and mobility^{51,52}. Both are interesting candidate genes which require further analysis. *Mrps10* is the only differentially expressed gene in the *lmr1D* locus. Based on sequence homologies it is annotated as a mitochondrial and ribosomal protein. Its function is currently unknown (<http://www.informatics.jax.org/>).

Allergen-induced AHR

The data for the allergen-induced AHR is difficult to interpret. This is probably at least partially due to lack of power as an important variability is observed in penh in the different subcongenic strains. The results however suggest that at least 3 loci have an influence on the allergen induced AHR, i.e. *lmr1A*, *lmr1B* and *lmr1C*. These 3 loci show at least partial overlap with the allergen-induced AHR QTL found by Zhang *et al* in an intercross with BP2 and BALB/c³⁸ (figure 1). Additionally the *lmr1A* locus also contains the *H2* locus which we previously found associated to allergen-induced AHR⁵³ (chapter 3). *Lmr1A* to *lmr1C* are all 3 also modulating naive AR.

Airway eosinophilic inflammation

Lmr1A, which is involved in sp-IgE levels, is also implicated in the number of eosinophils in the BAL, as both C.lmr1.2 and C.lmr1.3 show significantly lower eosinophil counts than BALB/c, while the other strains show comparable (C.lmr1.5 and C.lmr1.7) or higher (C.lmr1.4) eosinophil counts. The congenic fragment of BALB.B is the prime candidate to explain these differences as BALB.B also shows lower airway eosinophilia compared to BALB/c. This congenic fragment inherited from C57Bl/10 contains the *H2* locus, *Pim1*, *Glyoxalase1* and *Rab44*, which are polymorphic and differentially expressed between C.lmr1 and BALB/c. For *Pim1* an anti-apoptotic effect

on eosinophils in the lungs was previously shown⁵⁴. *Pim1* is a serine/threonine kinase which in eosinophils is activated through stimulation with IL-5 or GM-CSF. The exact mechanism for its anti-apoptotic effect in eosinophils remains unknown^{55,56}.

The high number of eosinophils in the BAL of C.lmr1.4 but not C.lmr1.3 suggests a role for *lmr1B* which is counteracted in the C.lmr1.3 strain by the decreasing effect on eosinophilia of the *lmr1A* locus. *Apobec-2* is the only gene in *lmr1B* which is differentially expressed between BALB/c and C.lmr1 after OVA challenge. There is no direct evidence in literature for a role of *Apobec-2* on eosinophil numbers. However, considering its potential role in naïve and allergen-induced AHR it is an interesting candidate gene for asthma which requires further investigation.

The combined genetic approach used in this manuscript, in which we combined the phenotyping of RC strains, expression profiling and a classic F2 intercross, has shown to be powerful. We were able to identify candidate genes for the 4 phenotypes we studied, i.e. sp-IgE, naïve AR, allergen-induced AHR and airway inflammation. Despite its power the approach we have taken has some limitations.

A first series of limitations arise from the use of microarrays on lung tissue for the expression profiling. In micro-array experiments, differentially expressed genes with low expression levels are rarely identified as such and would thus not be found in our screen. This issue could be avoided by the use of next generation sequencing. However, this was not an option considering the high costs of such an experiment. In our experiments we only analysed RNA from the lung, which is certainly the most relevant organ affected by asthma, but not the only relevant one as critical immune processes take place in other locations. Genes which play a critical role in these processes would not be identified if they are not (differentially) expressed in the lungs. Another concern is that a difference in expression for a gene could be due solely to the infiltration of inflammatory cells.

To avoid these issues we have also considered all the polymorphic genes between BALB/cJ and C57Bl/6NJ, as identified by the presence of SNPs, insertions or deletions according to the 'Mouse genomes database' at the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl>). This database is based upon data of complete genome sequencing of standard Jackson Laboratory mouse strains. These strains are from a different sub-strain than the mice used to generate the *lmr* series of RC strains (WEHI substrains) and we therefore cannot exclude that new mutations could influence the different asthma phenotypes. However, considering the estimated mutation rate of approximately 1.1×10^{-8} mutations / bp per generation⁵⁷ in a genome of approximately 2.7 Gbp this should not influence this analysis to a significant extent, but care should be taken to sequence the relevant genes before further analysis.

In our experiments we used *penh* from whole body plethysmography as an indirect measurement of AHR. We have chosen to use this method as it is a high-throughput technique that is non-invasive and allows for measurement of both naive AR and allergen-induced AHR in the same animal. A recent paper by the group of Svenson showed that in some strains *penh* can be different from the more direct measurements of lung-function, as resistance and compliance, which are obtained in anaesthetised mice using invasive methods like the Flexivent⁵⁸. We did confirm the allergen-induced AHR results using this method in *C.lmr1* vs BALB/c experiments. Additionally we showed a significant correlation between the 2 types of AHR measurements in an experiment in which we measured AHR by both methods in the same mice in both BALB/c and *C.lmr1* (data not shown). This correlation between the 2 methods strongly suggests that, at least for the *lmr1* locus, the *penh* is an acceptable approximation of AHR.

The experiments with the subcongenic mice have generated significant amounts of data that had to be analyzed. The way to analyse these data has been a point of concern. The aim of these experiments was to determine which *Lmr1* sublocus was encoding the differences in asthma phenotypes observed between BALB/c and *C.lmr1*. For logistic

reasons we have chosen to analyze each subcongenic strain together with BALB/c in separate experiments. This would allow us to evaluate the effect of each individual *Lmr1* sublocus on the different asthma phenotypes. However this approach is sensitive to inter-experiment variability and thereby does not allow for direct comparison of the different subcongenic strains. To avoid those issues we have done a series of experiments in which the different subcongenic strains were phenotyped side-by-side. Data from these experiments have been pooled with the data of the first series of phenotyping experiments and were analysed using the Kruskal-Wallis test, a non-parametric equivalent of the ANOVA, or with the Friedman test, a non-parametric equivalent of the repeated measures ANOVA, for the AHR dose response curves. These tests show that the *Lmr1* subloci have an influence on both naive and allergen-induced AHR and airway inflammation, which confirms our previous findings in the C.lmr1 and BALB/B vs BALB/c experiments. Additionally we also observed a significant effect of the *Lmr1* subloci on sp-IgE. This effect of *Lmr1* on sp-IgE was also observed in B.lmr1 vs C57Bl/6 but not in C.lmr1 vs BALB/c, which is indicative for a lack of power in some of our experiments. To determine which sublocus has an influence on the different asthma phenotypes we have chosen to compare all the subcongenic strains to their background strain, BALB/c, using a Mann-Whitney U test (or paired equivalent for the AHR data) as post-hoc test. This approach was chosen as the Dunn's post-hoc test failed to point out which strain differed from which in some experiments, which again indicates a lack of power in some of our experiments. The used method should have more power than the Dunn's post-hoc test as data from other strains do not influence the ranking in the statistical tests. Additionally BALB/c was phenotyped in every experiment and is thus very well characterised (n=49) in our mouse model of allergic asthma. This approach has shown to be simple and efficient to identify loci that are relevant to the 4 asthma phenotypes under investigation. It has also shown to produce reproducible results as we were able to identify 2 small loci for sp-IgE in the *Lmr1A* sublocus (identified by the above method) by another independent method (F2 cross).

An important issue when using animal models of human diseases, is how relevant the data obtained in the model is for the human disease. Some of the loci identified in our experiments contain genes that were previously identified as asthma susceptibility genes/loci, *i.e.* *Pla2g7* in *lmr1A1* and the *H2* locus in *lmr1A2*, or genes involved in lung-function decline in COPD for *Apobec-2* in *lmr1B*. This is a strong indication that these genes could be true asthma susceptibility genes. But how to define true susceptibility genes? Dr B. Paigan (Jackson Laboratory, Bar Harbour, Maine, USA) proposes to define true susceptibility genes as genes for which at least 3 lines of evidence, genetic and functional, exist whereby replication in 1 or more animal models has to be considered as 1 line of evidence only (B Paigan, oral presentation). This implies that there can be only 2 lines of genetic evidence, *i.e.* identification and replication in relevant species or model species, and that at least one of the lines of evidence has to be functional. This is the case for both the *H2* locus and *Pla2g7*. Others have also identified true asthma susceptibility genes in mice, like IL-9^{59,60} and complement factor C5^{32,61} (chapter 1). The fact that we and others were able to identify these loci and genes shows that mouse genetic experiments can generate data that is relevant to the human asthma.

Is it, considering the availability of GWAS in human, still useful to use animal models for genetic studies? A serious issue with the current GWAS is the lack of statistical power caused by multiple testing issues (1 test per SNP, 600 000 or more SNPs) and the huge number of predictor variables (SNPs) compared to the number of subjects⁶². One way to reduce the number of SNPs to include in the analysis and thus reduce the correction factors for multiple testing is to use comparative genomics, in which loci syntenic to susceptibility loci identified in animal models are selected for analysis (see chapter 1). Our data clearly shows that this could be a good approach for asthma genetics as we were able to identify candidate genes and loci that were previously identified in human. However, there are only few asthma genetic studies in mice with only few inbred strains involved. A major effort should be undertaken to increase these

numbers. An additional advantage of using mouse models and RC strains in particular for genetic mapping is that once genes or loci have been identified a model is relatively easily established to explore the role of this candidate as we did in our experiments with BALB/B to explore the role of the *H2* locus.

Conclusions

With the experiments presented in this manuscript we have clearly demonstrated that RC strains can be a powerful tool to map asthma susceptibility in the mouse. This was first done in a ‘proof of concept’ experiment in the CcS/Dem series of randomly generated RC strains which showed different types and intensities of asthmatic responses. This experiment was followed by a series of ‘proof in practice’ experiments in which we actually mapped loci for sp-IgE, AHR and airway eosinophilic inflammation.

We have identified the *H2* locus as a major contributor in the differences in asthma susceptibility between C.lmr1 and BALB/c. C.lmr1 is a BALB/c derived RC strain which inherited the *lmr1* locus on chromosome 17 from C57Bl/6. The *H2* locus, located within *lmr1*, was found to have a significant effect on allergen-induced AHR and airway eosinophilic inflammation in experiments using BALB/B, an RC strain derived from BALB/c and with the same *H2^b* haplotype as C57Bl/6. *H2* was also found linked to sp-IgE serum levels in a F2 cross between subcongenic strains derived from C.lmr1 and BALB/c. The human ortholog of *H2*, the *MHC*, locus has also been found associated to asthma and related traits in many different studies, including the recent GWAS studies for asthma. This shows that our mouse models in RC strains can identify genes which are relevant to human asthma. It also shows that RC strains can be good models to further investigate the role of the *MHC/H2* locus on asthma susceptibility.

In the F2 cross we also identified the *lmr1A1* locus as a locus that influence sp-IgE serum levels. The locus is located from 43.4 Mbp to 44.5 Mbp on chromosome 17 and contains 11 genes with polymorphisms between BALB/c and C67Bl/6. From these genes 1, *Pla2g7*, was previously found associated to sp-IgE levels in human and animal models. Additionally loss of function mutations in this gene have been associated to high sp-IgE serum levels in human. The fact that we were able to identify this gene again validates our method for asthma gene-hunting in the mouse.

A third promising locus, *lmr1B*, located on chromosome 17 from 47.7 Mbp to 52.0 Mbp, was found associated to naive AR using subcongenic mice derived from C.lmr1. *Apobec-2* is the only gene in this locus which was found differentially expressed in the lungs between BALB/c and C.lmr1 after PBS (sham) challenge. It was also found overexpressed in lung biopsies of COPD patients versus healthy controls but was never previously identified as asthma susceptibility gene. It remains an interesting candidate gene that could be further investigated in mouse strains derived from C.lmr1.3 or C.lmr1.4.

Lmr1C and *Lmr1D* also appear to have an effect on some of the asthma phenotypes, but the evidence is less strong than for the previously mentioned loci. Additional experiments should be undertaken to characterize the role of these loci and identify candidate genes.

In summary, we have identified 3 strong asthma susceptibility loci using RC strains and a F2 cross between RC strains and their background strain. Based upon the literature, in silico SNP analysis and expression profiling we were able to identify candidate genes in 2 of these loci: *Pla2g7* in *lmr1A1* and *Apobec-2* in *lmr1B*. For the *H2* locus different genes of both Class I and Class II are found to be overexpressed and polymorphic between the parental strains. Our models should be a valuable tool to investigate this complex locus which was also found associated to human asthma in many studies including the recent GWAS.

Future perspectives

The loci we have identified are rather small and further fine-mapping with F2 crosses or other genetic approaches would require a lot of breeding to obtain enough recombinants in the loci of interest. Most loci contain only few candidate genes which are differentially expressed and/or polymorphic between BALB/c and C.lmr1. Human orthologs have also been associated to asthma for *Pla2g7* and *Apobec-2*, making these the strongest candidate genes for *lmr1A1* and *lmr1B* respectively.

For *Pla2g7* it would be interesting to measure serum PAF and PAFAH activity in C.lmr1.3 and BALB/c. If these experiments show a difference in PAF levels and PAFAH activity, PAFAH inhibitors could be used in a mouse model of asthma to prove the involvement of *Pla2g7* on reduced sp-IgE serum levels in the C.lmr1.3 strain. As a LoxP flanked *Pla2g7* mouse and many tissue or cell-type specific Cre recombinant mice already exist it would be possible to knock *Pla2g7* out in the relevant tissue. This KO mouse would be a powerful tool to explore the role of *Pla2g7* in sp-IgE serum levels, but considering the extensive breeding needed, only to be undertaken if previous experiments did clearly show role for *Pla2g7*.

Apobec-2 is less well characterized but from our experiments we know that it is expressed in the lungs. A first series of experiments would be to determine which cell-type express *Apobec-2* at both RNA and protein level. Inducible tissue specific mice can be made as an *Apobec-2* LoxP flanked mouse exists. This mouse strain could be used for both asthma and COPD related experiments.

The other candidate genes, which have not previously been associated with asthma, could be investigated by a similar approach, however less tools and functional data are available to investigate these genes.

The *H2* locus and its human equivalent *MHC* locus encodes genes that are essential for antigen presentation and other immune functions. Many of these processes are relevant in the development of allergic asthma. Our data shows that mouse models could be a powerful tool to gain a better understanding of the role of the H2/MHC locus in asthma.

The data we have generated are also a valuable tool for human geneticists who can use our data to select syntenic regions to be analysed, for association or interactions, in their GWAS data. This would hugely reduce the number of statistical tests and thus the correction factors for multiple testing. This should significantly increase the power to detect relevant genes in the human GWAS data. On the other hand, we could use the GWAS data to test our candidate genes and to explore the genetic interactions we observe. Considering the differences in inflammatory response and anatomy of the lungs between human and mouse, we do not expect to be able to replicate all our findings in human studies. Our findings should however lead to a better understanding of asthma in the mouse and with careful extrapolation in human too.

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List of abbreviations

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
BSA:	Bovine serum albumin
cM:	Centi Morgan (unit of genetic distance)
ELISA:	Enzyme linked immunosorbent assay
ENU:	N-ethyl-N-nitrosourea
eQTL:	Expression quantitative trait locus
EST:	Expression sequence tag
FDR:	False discovery rate
GWAS:	Genome wide association studies
HDM:	House dust mite
KO:	Knock-out
Lmr:	<i>Leishmania major</i> resistance locus
MHC:	Major histocompatibility complex
ORF:	Open reading frame
OVA:	Ovalbumin
PBS:	Phosphate buffered saline
Penh:	Enhanced pause
QTL:	Quantitative trait locus
RC:	Recombinant congenic
RI:	Recombinant inbred
sp-IgE:	OVA specific IgE

Nederlandse samenvatting

Astma wordt veelal veroorzaakt door een allergische reactie in de longen. Deze reactie is gericht tegen bepaalde stoffen uit de omgeving, de zogenaamde allergenen, en wordt gekenmerkt door de aanmaak van IgE antilichamen. Deze IgE antilichamen zijn erg specifiek en herkennen maar 1 allergeen. Ze worden geproduceerd door de B-cel, een type witte bloedcel, en worden verspreid in het bloed. Als deze IgE antilichamen een zogenaamde mestcel tegenkomen in het weefsel, zullen ze aan de oppervlakte van deze cel binden. Bij hernieuwde blootstelling aan het allergeen kan dit door opname via bijvoorbeeld de longen binden aan dit IgE op de mestcel. Deze mestcel wordt hierdoor geactiveerd en zal plots verschillende signaalstoffen zoals histamine vrijgeven. Deze stoffen veroorzaken de typisch allergische reacties als niezen, snotteren, enz. Bij astma patiënten vindt deze reactie plaats in de longen. Het histamine zorgt dan voor het samentrekken van de spieren rond de luchtwegen, waardoor benauwdheid en piepende ademhaling ontstaat. Ook vindt er een ontstekingsreactie plaats, die enkele uren na de eerste symptomen van de allergische reactie optreedt. Door het herhaaldelijk inademen van allergenen en de allergische reacties en ontsteking die dit veroorzaakt, kunnen er specifieke klachten gaan optreden zoals chronische luchtwegontsteking, verhoogde slijmproductie en niet-specifieke hyperreactiviteit van de luchtwegen. Bij niet-specifieke hyperreactiviteit is er een buitensporig samentrekken van de spieren in de luchtwegen als reactie op andere prikkels dan het allergeen, zoals koude lucht of prikkelende stoffen in de lucht.

Aanleg om astma te ontwikkelen is voor een groot deel erfelijk bepaald. Ons erfelijk materiaal is het DNA dat in de kern van elke cel zit. Het DNA is een lange streng die in een aantal chromosomen is geordend. Van elke chromosoom heb je er 2, één van elke ouder. Genen zijn de stukjes DNA die de bouwplannen bevatten voor alle eiwitten die in ons lichaam aanwezig zijn. Deze bouwplannen zijn gecodeerd in de DNA sequentie in een opeenvolging van de letters A, T, G en C. Genen liggen niet allemaal strak achter elkaar aan, sommige stukken DNA coderen voor niets en andere stukken, de

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zogenaamde regulerende stukken DNA, bepalen hoeveel van een bepaald eiwit moet aangemaakt worden en waar en wanneer dat dient te gebeuren. Een klein verschil in DNA sequentie kan dus geen direct verschil uitmaken voor de structuur van een eiwit als het in het niet-coderend stuk DNA tussen de genen ligt. Als het verschil in DNA sequentie echter in het gen zelf ligt kan dit leiden tot een verkeerd eiwit. Als het verschil in de regelende sequenties is kan dit een invloed hebben op de hoeveelheid eiwit die aangemaakt wordt, dit wordt ook invloed op de expressie van een gen genoemd.

In de zoektocht naar genen die astma beïnvloeden werden er al heel wat genen gevonden. Ook zijn er genvarianten gevonden die enkel een effect hebben dat afhankelijk is van de aanwezigheid van een ander genvariant. Dit heet gen-gen-interactie. Er zijn ook gen-omgevings-interacties, waarbij een genvariant enkel een effect heeft bij aanwezigheid van bepaalde omgevingsfactoren, zoals de hoeveelheid allergenen, fijn stof of ozon in de lucht. Deze complexiteit maakt dat er nog veel genetisch en medisch biologisch onderzoek moet gebeuren om astma goed te kunnen begrijpen. Deze kennis is essentieel om deze ziekte optimaal te kunnen behandelen.

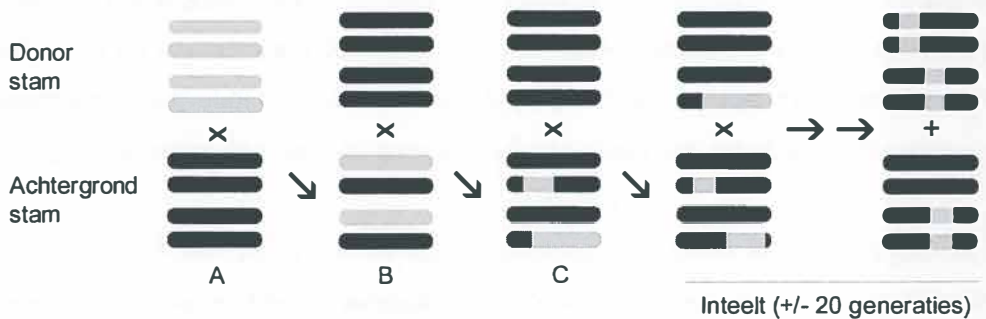
Veel onderzoek naar astma gebeurt in proefdieren en dan vooral in muizen. Dit laat toe om experimenten te doen waarbij je de ontwikkeling van astma kan proberen te beïnvloeden. Astma komt in de muis van nature niet voor en moet dus opgewekt worden. Dit gebeurt net zoals in de mens in 2 fasen, de sensitisatie en de provocatie. Tijdens de sensitisatie wordt het allergeen toegediend zodat het afweer systeem hier IgE voor gaat aanmaken. Daarna volgt de provocatie waarbij de muis het allergeen in de longen krijgt toegediend via aerosol. In deze fase ontstaat de longontsteking en ontwikkelt zich de luchtweg hyperreactiviteit. Het voordeel van het kunstmatig opwekken van astma is dat je de omstandigheden en tijd waarin astma ontstaat zelf kan bepalen. Het nadeel is dat je observaties gedaan zijn in een kunstmatig systeem en niet in

de mens. Enige voorzichtigheid is dus geboden in de interpretatie en extrapolatie van de experimenten naar de mens.

Ook voor genetisch onderzoek worden vaak muizen gebruikt. Er zijn verschillende inteelt stammen bij de labmuizen. Deze zijn ontstaan door kruisingen met muizen die uit het wild gevangen zijn rond het begin van de 20^{ste} eeuw. Door deze lange periode van inteelt zijn muizen stammen ontstaan waarin alle muizen genetisch identiek zijn aan elkaar. Tussen de stammen zijn er natuurlijk wel genetische verschillen en ook verschillen in gevoeligheid voor astma. Deze genetische verschillen kunnen we bepalen, dit heet genotyperen. Door muizen stammen een paar keer met elkaar te kruisen en dus het genetisch materiaal van beide ouderstammen te mengen, ontstaan er nieuwe combinaties van genen die we kunnen genotyperen. Door de sterkte van astma ontwikkeling te volgen in deze muizen (na opwekken) en dit te vergelijken met de informatie over welke genvarianten er dan aanwezig zijn kan men astma genen identificeren in de muis.

In dit proefschrift hebben we een alternatief op deze techniek gebruikt. We hebben gebruik gemaakt van recombinant congenic stammen. Deze zijn ontstaan door eerst 2 muizenstammen, de achtergrond stam en de donor stam, met elkaar te kruisen (figuur 1, stap A). De muizen uit deze eerste generatie worden dan nog eens gekruist met de achtergrond stam (figuur 1, stap B) en de tweede generatie ook (figuur 1, stap C). Uit de muizen van de derde generatie worden dan fokpaartjes samengesteld voor inteelt waardoor muizenstammen ontstaan die allemaal verschillende stukken DNA van de donorstam hebben overgeërfd. Alle andere DNA hebben ze dan van de achtergrondstam (zie figuur 1). Als je nu in één van deze stammen een verschil in sterkte van astma ten opzichte van de achtergrondstam observeert, moet dit verschil wel te wijten zijn aan de fragmenten van de donor stam. Dit hebben we getest door in een reeks RC stammen astma op te wekken en dan te testen op astma gevoeligheid. In deze experimenten vertoonden de RC stammen duidelijke verschillen ten opzichte van de achtergrond en donor stammen. Omdat de muizen allemaal onder dezelfde omstandigheden zijn getest

kunnen we hieruit besluiten dat de genetisch verschillen tussen een RC stam en zijn achtergrond stam ook meetbare verschillen in astma gevoeligheid veroorzaken. Hiermee is aangetoond dat het gebruik van RC stammen in astma genetisch onderzoek een goede en waardevolle methode is. Ook zagen we de waarde van de ene astma parameter niets zegt over de waarde van een andere parameters. Zo kunnen muizenstammen met hoge luchtweg hyperreactiviteit zowel hoge als lage IgE bloedspiegels hebben en ernstige of milde longontsteking. Dit is vergelijkbaar met wat er in de mens wordt geobserveerd.



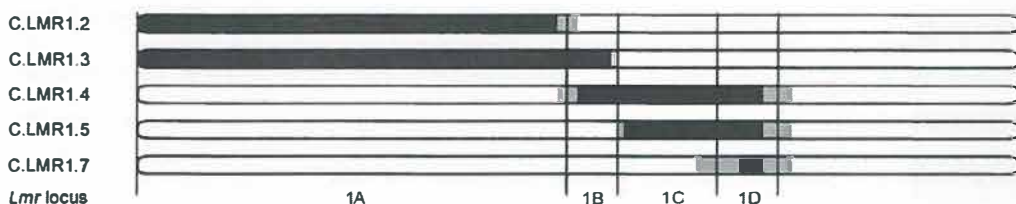
Figuur 1: Kruisingsschema voor het maken van recombinant congenic stammen. Van elke stam of generatie worden 2 chromosoom paren getoond. A: kruising van de ouder stammen, B: Eerste kruising met achtergrond stam, C: tweede kruising met achtergrond stam.

Daarna zijn we astma genen gaan zoeken in de *lmr* serie van recombinant congenic muizenstammen. Deze muizenstammen hebben een congeen fragment op chromosoom 9 en/of 17. De muizen met beide fragmenten of fragmenten op chromosoom 17 tonen een duidelijk afwijking in astma gevoeligheid vergeleken met hun achtergrond stammen. Hieruit leiden we dus af dat het chromosoom 17 congeen fragment inderdaad genen bevat die een invloed hebben op astma, dit chromosomaal fragment of kortweg locus heet *lmr1*. Dit locus is echter vrij groot en bevat vele honderden genen. We zullen dit congeen fragment door kruising nog verkleinen om minder kandidaat genen over te houden. Maar eerst hebben we gekeken welke genen in dit *lmr1* locus een verschil in

expressie (hoeveelheid eiwit dat aangemaakt wordt) in de longen hebben tussen de congene stam en de achtergrondstam. Ongeveer 1/3 van de genen met een sterk verschil in expressie liggen dicht bij elkaar in het *MHC* locus. Dit is een groep genen die allemaal deel uitmaken van het afweer systeem. Om te testen of het echt het *MHC* locus is dat een invloed heeft op astma in de muis, hebben we een andere recombinant congene stam getest die enkel het *MHC* locus heeft als congeen fragment. Ook deze muizen vertonen een verminderde astma respons ten opzichte van hun achtergrond stam. Hieruit kunnen we concluderen dat genen in het *MHC* locus in de muis een invloed hebben op astma. Dit is een erg interessante bevinding omdat ook in de mens deze genen in vele astma genetische studies werden gevonden. Deze genen zijn in de mens erg moeilijk te bestuderen, maar onze experimenten tonen aan dat dit in de muis wel onderzocht kan worden. Ook bevestigt het dat onze methode met recombinant congene muizen resultaten kan genereren die ook voor de mens van toepassing zijn.

Uit de literatuur bleek echter dat op chromosoom 17 niet alleen het *MHC* locus een invloed heeft op astma. Om dit te verder te onderzoeken hebben we in subcongene stammen astma geïnduceerd en getest. Deze muizen verdelen het *lmr1* locus in kleinere stukken doordat ze allemaal maar een deel van het fragment van de *lmr1* muis hebben (figuur 2). Bij deze muizen zagen we dat de verschillende astma parameters die we meten in onze experimenten, nl: IgE, long ontstekingsparameters en luchtweg hyperreactiviteit, door verschillende delen van het oorspronkelijke *lmr1* locus worden beïnvloed. Een eerste locus, *lmr1A*, die ook het *MHC* locus bevat heeft een invloed op alle gemeten parameters. Dit locus is nog steeds te groot om kandidaat genen te kunnen identificeren en zal dus nog verder moeten worden verkleind door kruising zoals hieronder beschreven. De *lmr1B* locus, die een invloed heeft op luchtweg hyperreactiviteit, heeft maar 1 gen die verschil in expressie veroorzaakt, nl *Apobec-2*. Dit gen werd in de mens ook in relatie gebracht met luchtweg hyperreactiviteit in COPD. De functie van dit gen is niet goed gekend maar het zou waarschijnlijk een effect hebben op welk type antilichaam er door de B cel aangemaakt wordt. Hoe het

Apobec-2 gen invloed kan uitoefenen op luchtweg hyperreactiviteit in astma en COPD is niet duidelijk. Verder onderzoek zal dit moeten uitwijzen.



Figuur 2: Genetische samenstelling van chromosoom 17 van de verschillende subcongenen en de overeenkomstige *lmr* loci. Als een fragment van de donor komt is dit wit voorgesteld, zwart voor de donor stam. Er wordt maar 1 chromosoom voorgesteld, de andere is identiek (inteeft).

Door muizen met het *lmr1A* locus te kruisen met hun achtergrond stam om deze eerste generatie vervolgens onderling te kruisen zijn er muizen ontstaan met een verkleinde *lmr1A* locus. In deze muizen werd astma opgewekt waarna we de muizen hebben getest op astma gevoeligheid. Hierdoor konden we binnen het *lmr1A* fragment 2 kleine fragmentjes identificeren die bepalend zijn voor de hoeveelheid IgE in het bloed. Een van deze loci hadden we al vroeger geïdentificeerd, nl het *MHC* locus. De andere locus, *lmr1A1*, is erg klein en bevat slechts enkele genen waarvan er 1, *Pla2g7*, ook in de mens in verband gebracht werd met astma. Het eiwit waarvoor dit gen codeert is betrokken bij de afbraak van het ontstekingsbevorderend eiwit, PAF.

Samengevat, hebben we gebruik makend van recombinant congene muizen, astma gevoeligheid op muis chromosoom 17 in kaart gebracht. Hierbij hebben we 3 interessante loci kunnen identificeren. 2 van deze loci zijn nu zo klein, dat we een sterk kandidaat gen hebben kunnen identificeren, nl *Pla2g7* in *lmr1A1* en *Apobec-2* in *lmr1B*. Het *MHC* locus is nog te groot om echte kandidaten te kunnen aanwijzen vooral omdat hier veel genen dicht bij elkaar liggen. Het feit dat dit locus en *Pla2g7* eerder al in de mens in verband werden gebracht met astma en IgE bloedspiegels, toont duidelijk aan dat genetisch onderzoek naar astma gevoeligheid in de muis weldegelijk resultaten

kan produceren die ook voor de mens relevant zijn. *Apobec-2* werd in de mens niet geïdentificeerd als astma gen, maar wel gerelateerd aan luchtweg hyperreactiviteit in COPD (longemfyseem). Dit is een duidelijke aanwijzing dat dit gen betrokken is bij luchtweg hyperreactiviteit. De exacte rol van deze genen in astma gevoeligheid kan niet door onze klassieke genetische methodes achterhaald worden en verdere proeven zullen noodzakelijk zijn om dit te bepalen. Wel kunnen de muizen en muismodellen die in dit proefschrift gebruikt werden hiervoor gebruikt worden.



Dankwoord

Aha, het boekje is af. Niet zonder enige moeite, maar “Here it is”. Nu blijft me nog een laatste taak over, voor we naar de drukker kunnen, nl het danken van alle mensen die hebben bijgedragen tot het ontstaan van dit boekje en de daaraan verbonden promotie.

Voor mij begon dit verhaal eind 2004 toen ik met mijn vers diploma op zak op zoek ging naar een nieuwe uitdaging. En toen zag ik op internet een vacature voor een PhD student in Groningen, een project met genetica in muizen, erg interessant... Reageren dus... En met succes, want ik mocht op sollicitatie gesprek komen. Ik dus, pak aan en vroeg op de trein (7 uur reistijd vanuit Gent). En dan een gesprek met Prof Dr Antoon van Oosterhout, Dr Irene Heijink en Mevrouw Jacobien Noordhoek (sorry Jacobien, zo stond het nu eenmaal op de brief). Dat gesprek ging erg lekker en ontspannen, daarna nog een theetje en dan een rondleiding door het lab met Marco en weer naar huis. Op de terugweg, een hel om thuis te geraken door voetbal supporters op het spoor met de typische spoorweg chaos als gevolg. Maar onderweg een telefoontje om mij te vertellen dat ik de baan had. Eindelijk was ik rond 3h00 thuis, moe maar heel erg blij.

En dit brengt ons dus bij mijn promotie traject, met Antoon als promotor en het astma fonds als sponsor gaan we astma genetica doen in de muis. En dus mijn oprechte dank aan het astma fonds en aan Antoon om dit mogelijk te maken. Maar Antoon is natuurlijk niet alleen de grote baas, hij is ook de collega die samen met het lab lekker mee ‘een biertje gaat drinken’/’pintje gaat pakken’. Vooral in het begin van het project hebben we nauw samengewerkt, samen met Renee Gras en Pieter Klok, om de muismodellen en luchtwegfunctie metingen met de Flexivent snel en efficiënt op te zetten. Daarna was de samenwerking minder noodzakelijk maar bleef Antoon toch altijd beschikbaar voor een snelle vraag of een goede discussie. Antoon, ik vond het heerlijk om voor jou te werken, dank je daarvoor.

Chronologisch past het niet echt in het verhaaltje nu, maar toch wil ik het even hebben over mijn co-promotor, Dr Martijn Mawijn. Martijn kwam iets later op het lab, maar desondanks dat heeft hij een enorme bijdrage geleverd aan dit proefschrift. Toen hij op het lab toekwam konden mijn project en ikzelf wel een boost gebruiken. Martijn heeft alles weer lekker op de rails gezet. Ook heeft hij in het nakijken van de manuscripten heel veel uren gestoken. Martijn, dank je.

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Goed dan gaan we ons verhaaltje maar verder chronologisch voeren. Zoals hierboven al vermeld was de eerste taak om de muismodellen en de Flexivent metingen draaiende te krijgen. Eerst met Pieter Klok, die de eerste proeven samen met mij heeft gedaan en ook heel veel intubaties van muizen voor de Flexivent. Ook stond hij altijd klaar om mij te adviseren. Maar we hebben meer gedaan dan alleen werken, zo hebben we heerlijke discussies gevoerd over bijna elk mogelijk onderwerp en heeft Pieter mij een dagje meegenomen door het Groningse polder gebied. Pieter heel erg bedank voor dit alles. In die periode van optimalisaties van Flexivent metingen kwan Renee als analist bij ons op het lab werken. Ze leerde snel de nodige technieken van Pieter en ontwikkelde zich snel tot een onmisbaar persoon voor heel veel proefdier werk van het lab. Ik heb veel en graag met Renee samengewerkt, bedankt daarvoor. Ook nog de 2 mensen die voor mijn muizen hebben gezorgd bedanken. Dank je, Silvia Kiewit en Natsha Broersma.

Maar natuurlijk hebben we niet heel de tijd op het dierenlab doorgebracht maar ook heel veel tijd op de werkkamer en op het lab. Ja, werkkamers... heb er wel een hele boel gehad, en was altijd wel gezellig, zal proberen niemand te vergeten, Jelena, Mirjan, Andrea, Inge, Wijnand, Martin, Judith, Anne, Andre, Alex, Roland, Jan, Corry-Anke, Dries, Hadi, Soheila en Henk. Het was leuk samen op de kamer. Ook op lab was het altijd fun, met Marjan, de moederfiguur op het lab en altijd in voor een gezellig babbeltje, Harold, een fijne kerel waar je echt wat aan hebt, Renee (zie hierboven), Jacobien, altijd klaar om een handje te helpen en een eindejaarsborrel bij haar thuis, altijd leuk, Marco, altijd super discussies, geen onderwerp te gek of te goor, Alex, als je maar van mijn labtafel wegbleef was het heerlijk om af en toe eens Frans te kunnen spreken, Irene, ga niet met haar borrelen want je weet niet wat je overkomt, Brigitte, je was er bijna altijd als ik je nodig had, Janneke, altijd gezellig, Ibolya en Hessel stille water hebben diepe gronden, Sicco, als je eens goed doordacht advies nodig hebt is hij de man, Marcel, grapjas..., Simone, altijd in voor een babbel en voor de actuele bui voorspellingen. En dan nog een speciale vermelding voor Lisette en Uilke die beide behoorlijk wat werk gehad hebben aan het F2 experiment zowel voor de genotypering als voor de fenotypering. Heel erg bedankt daarvoor. En dan hebben we op het lab ook nog een aantal studenten gehad, Josien, zo gek als een deur, heerlijk, enjoy it down-under, Tanja, met hoge hakken door het lab is geen probleem, kookplaten darentegen ... en last but not least mijn eigen studente Marjan, harde werkster, geen uitdaging haar te groot, accuraat en precies, zo zouden alle studenten moeten zijn ...

De eerste paper die er kwam was met data die door Prescilla was vergaard. Het was geen makkelijke opgave om al deze stammen te testen. Ook een aantal lmr dubbel en enkel congenen werden door Prescilla gedaan.

Buiten het werken was er natuurlijk ook de heerlijke trekkings in de Pyreneeën, skiën in de Alpen en het klimmen bij Bjoeks. Daarom een speciale vermelding voor mijn

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En als we het nu toch over vrienden hebben, zo'n 3.5 jaar na de start van mijn promotietraject kwam ik Freddy tegen, op 16 mei 2008 om precies te zijn. Fred heeft mij door de moeilijke periodes heen geholpen, mij opgevangen nadat ik terugkwam uit Belfast, samen hebben we super vakanties gehad zowel in de zomer als in de winter, maar ook gewoon lekker samen op de bank gehangen, geluierd in de zon... Zonder hem was het allemaal veel moeilijker geweest. Daarom een megaknuffel en een welgemeende DANK JE!

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Zo dan zijn we aanbeland bij het einde van het traject en dus dit boekje. De laatste die ik nog moet danken zijn de sponsors van het drukwerk: Rijksuniversiteit Groningen, GUIDE, Nederlands astma fonds, Stichting astma bestrijding, Novartis en Scireq.