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STUDIES OF INTERLEUKIN-1 RECEPTOR ANTAGONIST AS A POSITIONAL CANDIDATE GENE IN ALLERGIC ASTHMA

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STUDIES OF INTERLEUKIN-1 RECEPTOR ANTAGONIST AS A POSITIONAL CANDIDATE GENE IN ALLERGIC ASTHMA

By

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ABSTRACT

STUDIES OF INTERLEUKIN-1 RECEPTOR ANTAGONIST AS A POSITIONAL CANDIDATE GENE IN ALLERGIC ASTHMA

By

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Asthma is a chronic airway inflammatory disease due to inappropriate immune responses to common environmental factors. It is characterized by AHR, eosinophilic infiltration, airway obstruction, increased mucus production and increased serum IgE levels. Asthma is controlled by genetic and environmental factors, and their interactions. IL-1 receptor antagonist plays a protective role in asthma by inhibiting the pro-inflammatory cytokine IL-1.

Our mouse model for allergic asthma is comprised of airway hyperreponsive (A/J) and hyporesponsive (C3H/HeJ) strains. Genetic linkage analyses performed in A/J backcross mice ((A/J x C3H/HeJ) F1 x A/J) identified two quantitative trait loci for AHR (*Abhr1* and *Abhr2*) on mouse chromosome 2. The murine IL-1 receptor antagonist gene (*Il1rn*) is located within *Abhr1*. We hypothesized that genetic polymorphisms in *Il1rn* were responsible for the difference in AHR manifestation between A/J and C3H/HeJ strains. Hence, based on the genetic evidences, we investigated *Il1rn* as a positional candidate gene for allergic asthma in our mouse model at DNA, mRNA and protein levels.

We sequenced the *Il1rn* gene (~16 kb) in A/J and C3H/HeJ mice, but did not find polymorphisms that could explain the differences in AHR manifestation between A/J and C3H/HeJ strains. A time course of allergen induced mRNA and protein levels of IL-1 receptor antagonist was performed by real-time RT-PCR and ELISA. The mRNA

expression of IL-1 receptor antagonist was increased due to ovalbumin treatment, and this increase was significantly higher in A/J mice at the earlier timepoints. The protein production of IL-1 receptor antagonist was increased due to ovalbumin treatment only in the A/J strain, and not in the C3H/HeJ strain. These results indicate that IL-1 receptor antagonist plays an important role in allergic asthma, but the absence of qualitative differences at the DNA level indicates that it might not be the quantitative trait gene for the QTL *Abhr1*.

We also have access to a human birth cohort characterized for asthma phenotypes over 10 years. We tested for the association of the human IL-1 receptor antagonist gene polymorphisms with asthma phenotypes in our birth cohort, to comparatively investigate the effect of IL-1 receptor antagonist in humans. We hypothesized that polymorphisms in the human *IL1RN* was associated with asthma and related phenotypes.

We tested three *IL1RN* SNPs for associations with asthma, chest infections, BHR and FEV1/FVC ratios. At the single SNP level, we found the SNPs to be associated with asthma at age 2 and chest infections at age 2. Haplotype pair analysis confirmed that the haplotype pair containing the minor alleles at all loci (GCT/GCT) conferred increased risk of asthma and chest infection in the children tested. Then, we tested for the effect of environmental tobacco smoke exposure on this association. We also found that maternal smoking during pregnancy coupled with postnatal tobacco smoke exposure caused several-fold increase in the risk of getting asthma and chest infection in children possessing the GCT/GCT haplotype pair. Taken together, our results suggest a major role of *IL1RN* in asthma and chest infections in this population.

DEDICATION

This dissertation is dedicated to my parents, sister, friends and Jorge Luis Borges.

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LIST OF ABBREVIATIONS

Abhr	allergen induced bronchial hyperresponsiveness
Ach	acetylcholine
AHR	airway hyperresponsiveness
AP-1	activating protein-1
BAL	bronchoalveolar lavage
BHR	bronchial hyperresponsiveness
C.I	confidence intervals
CT	cycle threshold
ECRHS	European community respiratory health survey
ELISA	enzyme linked immunosorbant assay
FcεR1	immunoglobulin crystallizable fraction epsilon receptor 1
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
GM-CSF	granulocyte macrophage – colony stimulating factor
IgE	immunoglobulin E
IL	interleukin
IL1A	interleukin-1 alpha (human gene)
Il1a	interleukin-1 alpha (mouse gene)
IL1B	interleukin-1 beta (human gene)
Il1b	interleukin-1 beta (mouse gene)
IL-1Ra	interleukin-1 receptor antagonist (human protein)
IL-1ra	interleukin-1 receptor antagonist (mouse protein)
IL1RN	interleukin-1 receptor antagonist (human gene)
Il1rn	interleukin-1 receptor antagonist (mouse gene)
IL-1α	interleukin-1 alpha (protein)
IL-1β	interleukin-1 beta (protein)
LD	linkage disequilibrium
MCP	macrophage chemoattractant protein
MIT	Massachusetts Institute of Technology
NF-kB	nuclear factor of kappa light chain gene enhancer in B-cells
OR	odds ratio
OVA	ovalbumin
PBS	phosphate buffered saline
QTG	quantitative trait gene
QTL	quantitative trait locus
SNP	single nucleotide polymorphism
Th	T helper cell
VNTR	variable number of tandem repeat

Chapter One: Background and significance

A. Asthma

B. Genetics of asthma using mouse models

C. Genetics of asthma in human populations

D. IL-1 receptor antagonist in asthma

E. Summary

A. ASTHMA

Phenotype description:

Asthma is a multifactorial chronic respiratory disorder occurring in genetically susceptible individuals due to inappropriate immune responses. The cardinal pathophysiological features of asthma are airway hyperresponsiveness (AHR), airway inflammation and elevated serum immunoglobulin (Ig) E levels[1]. It manifests as a chronic syndrome of the airway with recurrent wheezing, coughing, chest tightness and shortness of breath. The expression of the asthma phenotype is a result of interplay between multiple genetic and environmental factors. Gene products that contribute to the asthmatic phenotype can be derived from a variety of physiological pathways[2-4], and exert their effect based on variations in their sequence and/or expression. Environmental factors such as allergens and smoke from various sources, exercise and season also substantially contribute to the asthmatic phenotype. The impact of genes on asthma is modified by the layers of environmental influence over the individual in a complex disease like asthma[5]. The definition and classification of the phenotypes in complex diseases such as asthma can vary based on the etiology, manifestation and associated symptoms[6]. In a random mating population like humans with a high degree of genetic diversity, this interaction between genes and environment is a critical factor in explaining the disease idiosyncrasies observed among individuals or population subgroups. Not all the mechanisms and genetic reasons for asthma are clear and remain to be elucidated.

Epidemiology

The prevalence, morbidity and mortality of asthma have dramatically increased in recent years. Asthma ranked 25th in the worldwide list of Disability-Adjusted Life Years lost due to common disorders[7]. It affects about 300 million people worldwide, and the prevalence of asthma has been shown to be higher in industrialized countries with a modern lifestyle compared to countries with traditional lifestyles[8]. This asthma incidence gradient has also been shown to be proportional to the level of urbanization. An additional 100 million people are estimated to be affected with asthma as urbanization in the world population is projected to increase from 45% to 59% by 2025[9]. In the United States alone, about 31 million people have been diagnosed with asthma at least once in their lifetime[10]. Non-Hispanic blacks and American Indians had current asthma prevalence 30% higher than the non-Hispanic whites. Females had a 30% higher prevalence compared to males, and this pattern was reversed among children[10]. It is the most common chronic condition of childhood in the United States affecting about 4.8 million children, most of whom are diagnosed with asthma by 6 years of age[11]. Another alarming trend in the United States is the increasing rate of asthma mortality after 1970, in contrast to the declining asthma mortality rate in other western countries[12].

Various forms of asthma:

Asthma is a multifactorial chronic inflammatory disease of the lung characterized by symptoms of recurrent episodes of coughing, wheezing and breathlessness. The manifestation of the clinical signs of asthma depends on the subject's genetic makeup, environmental conditions and biological statuses such as age and gender. The interplay of

these factors results in a multitude of features characteristic of asthma. A subject can display a few but not necessarily all of these phenotypes, and yet be clinically diagnosed as an asthmatic. Overlapping phenotypes and the lack of consensus on a combination of objective and subjective parameters necessary to draw a clinically definitive border around asthma is a major problem in airway disease taxonomy[6].

Based on the severity of the disease, asthma has been classified into four groups - mild intermittent, mild persistent, moderate persistent and severe persistent[13]. Asthma is also classified based on the pattern of clinical presentation or on the suggested etiology (extrinsic vs intrinsic, occupational, aspirin induced)[14,15]. It can also be classified as atopic and non-atopic asthma based on the levels of IgE-mediated response. Atopy is the hereditary predisposition to develop certain hypersensitivity reactions on exposure to specific antigens. Atopic allergic asthma, the most common form of asthma, is an inflammatory disorder arising as a result of inappropriate immune responses to common environmental antigens in genetically susceptible individuals[16]. It is characterized by the cardinal features of airway hyperresponsiveness (AHR) to a variety of stimuli, pulmonary eosinophilia, increased mucus production and elevated serum IgE levels.

Diagnosis:

Physician-based subjective observations and pulmonary function test-based objective parameters are used for asthma diagnosis. Forced expiratory volume in 1 second (FEV1), forced expiratory vital capacity (FVC) and expiratory peak flow (PEF) are the important indices measured using pulmonary function tests. The ratio between forced expiratory volume in one second to forced vital capacity (FEV1/FVC) is decreased

in asthmatic patients. National Institutes of Health (NIH) expert panel report on Guidelines for the diagnosis and treatment of asthma classifies the severity of asthma based on a combination of symptoms, nighttime symptoms and pulmonary function test results[13]. Serum IgE levels measured by Enzyme-linked Immunosorbant Assay (ELISA) and number of eosinophils in the bronchoalveolar lavage (BAL) fluid and lung tissues are also used as indicators of asthma. Airway hyperresponsiveness, the ability of the airways to constrict when exposed to small concentrations of bronchoconstrictor agents[17], is a key phenotype used to model human asthma in mice.

Therapy:

Therapy for asthma is based on the severity of symptoms. The drugs used against asthma function through two main mechanisms. Bronchodilators aim at relaxing the airways to ensure ease in breathing, and anti-inflammatory therapies aim at reducing the inflammation that is responsible for this airway constriction.

The major bronchodilators used are β_2 adrenergic receptor agonists (salbutamol, terbutaline, salmeterol and formoterol), inhaled anticholinergics (ipatropium bromide and tiotropium bromide) and slow-release preparations of the drugs theophylline and aminophylline. β_2 adrenergic receptors exist in an active form and an inactive form in vivo, and are located in a wide variety of cells such as airway smooth muscle cells, epithelial and endothelial cells of the lung and mast cells. When the receptor is in the active form, β_2 adrenergic agonists bind to the receptor, increase the production of cyclic AMP (cAMP), and result in airway smooth muscle relaxation through mechanisms not fully understood[18]. The efficacy and duration of action of these drugs depend on tissue

stochastics such the number of receptors available, and the potential functional antagonism of other bronchoconstrictors acting simultaneously on the airways. Selectivity of the agonists that bind to β_2 adrenergic receptors, compared to β_1 and β_3 receptors, and potential side effects due to their non-specific binding on cardiac β adrenergic receptors are important factors taken into consideration for devising and usage of this class of drugs[19].

There are a variety of drugs that target the anti-inflammatory mechanisms involved in asthma. The major classes of drugs that are currently used to treat the inflammatory component of asthma are inhaled corticosteroids (budesonide, fluticasone propionate, beclomethasone dipropionate and mometasone), antileukotrienes (monteleukast, pranleukast and zafirleukast), 5-lipoxygenase inhibitors (zileuton), cromones (sodium cromoglycate and nedocromil sodium) and anti-IgE (omalizumab).

Corticosteroids have been shown to inhibit histone acetylation and promote histone deacetylation in the chromatin, resulting in the transcriptional suppression of many pro-inflammatory transcription factors such as AP-1 and NF-κB[20-22]. Leukotriene inhibitors and 5-lipoxygenase inhibitors help to reduce the asthma-like pathophysiological responses resulting from leukotrienes and other products from the 5-lipoxygenase pathway. This class of drugs might have particular advantages over the other drugs in the treatment of exercise-induced asthma and aspirin-induced asthma[23]. The exact anti-allergic mechanisms of cromones are not known with certainty[24]. The recently developed anti-IgE drug omalizumab binds to the crystallizable fraction (Fc) of IgE and prevents its binding to the high affinity immunoglobulin crystallizable fraction epsilon receptor 1 (FcεR1) present on the mast cells. This prevents the degranulation of

the mast cells and the type I hypersensitivity reactions that results from the inflammatory mediators secreted from the degranulated mast cells[25].

Only a few new asthma treatment drugs have reached the clinic in the past few decades. A combination of inhalable corticosteroids and long-acting β_2 agonists seems to be the treatment of choice at present and in the near future, although several other molecules in other pathophysiological pathways are being investigated[26].

B. GENETICS OF ASTHMA

It is well established that asthma is under the control of both genetic and environmental influences. The relative contribution of these two influences might vary between populations. In humans, family history, twin studies and segregation analyses are used for the focused investigation of the genetic component of asthma[27,28].

Major genetic approaches used for asthma gene discovery:

Candidate gene and genome-wide screening approaches have been used in animal models and human studies to determine the genes responsible for asthma (Table 1). Candidate genes are selected based on their functional relevance to asthma, and hence the process depends on selecting and characterizing one or several genes whose role has been implicated in a functional pathway leading to the asthma phenotype. Genome screens, unlike the candidate gene approach, do not need a priori knowledge about genes or their functional relevance to the disease under investigation. In humans, genetic markers throughout the genome are genotyped in family members to identify chromosomal regions that are co-inherited ('linked') with specific phenotypes such as asthma,

bronchial hyperresponsiveness (BHR) or a positive skin prick test (SPT)[4]. In animal models of asthma, phenotypes such as airway hyperresponsiveness (AHR), the rodent corollary to BHR, can be treated as quantifiable continuous traits. Genome screens performed in segregating backcrosses of inbred strains of mice for a number of genetic markers equally spaced across the genome identifies chromosomal regions on the genome that are responsible for regulating such quantitative traits, and the loci thus identified are termed quantitative trait loci (QTLs). These QTLs, whose statistically defined boundaries cover several megabases of genomic DNA, contain tens to hundreds of genes. This is then followed up with fine-mapping of the linked regions, followed by positional cloning or positional candidate cloning to identify specific genetic variations in one or more genes within the fine-mapped region that are responsible for genetic susceptibility.

Recent advances in asthma gene discovery approaches:

While the discovery of broad genomic regions that control susceptibility to asthma and other complex diseases has been relatively easy, progression from those regions to specific gene(s) controlling the phenotype has been difficult. A variety of genetic, molecular and bioinformatics approaches have been suggested, and are being used to determine quantitative trait genes (QTGs) in animal models[29]. These approaches use the flexibility animal models offer in terms of breeding, availability of genetic information in the public domain and amenability to phenotype studies.

Genetic studies in humans are accelerating faster than ever, and the current research largely makes use of the genetic variability available in humans in the form of

single nucleotide polymorphisms (SNPs). Any two human genomes differ from each other by 0.1% of the nucleotide sequences (on an average of 1 variant per 1000 basepairs of DNA in the genome)[30-32]. The most common variation in the human and other mammalian genomes sequenced are SNPs, and gene-based analysis of SNPs and their associations with disease phenotypes, such as asthma, have been at the forefront of genetic studies in asthma[33-37]. Hapmap, a global effort to catalogue and classify these polymorphisms from populations around the world[38-40], has opened the way for genome-wide association studies. Genome-wide association studies aim at genotyping millions of SNPs spread over the entire genome in individuals who have the disease and those who don't have the disease[41-43]. This is a logical extension of single gene association studies, with the difference being that a majority of the genes in the genome are simultaneously investigated instead of a single gene or a few genes at a time. Though at present the costs are prohibitive, it is considered to be a strategy for the future, at least for the next decade.

Types of association:

Association studies begin with genetic analysis of samples from a population where incidence of the investigated disease is reasonably common. The individuals in the population are then genotyped for several polymorphisms in the candidate genes, and the frequency of the alleles, genotypes and haplotypes are determined. Association studies rely on the detection of such polymorphisms in candidate genes and on the demonstration that particular polymorphisms are associated with one or more phenotypic traits[34]. The first possibility with a positive association is that an allele might directly influence the phenotype by causing a functional change at the genomic, mRNA or protein level. The

other possibility is that an allele associated with the phenotype in the study might have been co-inherited (linked) with another allele in the vicinity or in another locus, which is the real functional regulator of the disease phenotype. When two alleles are indirectly related in this manner, they are said to be in 'linkage disequibrium', which is a widely used approach to determine allelic associations.

The success of an association study depends on the population investigated and also the statistical and genetic techniques used for analysis. The population tested should be a random-mating population, which can be tested by the conformity of the selected SNPs to Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium states that in a random-mating population, the gene and genotype frequencies remain constant from generation to generation in the absence of migration, mutation and selection. The frequencies of the disease causing alleles might be different in different populations, and hence replication of results in different populations is difficult. Moreover, the associations could also be confounded by population stratification, in which the investigated population consists of a mixture of two or more subpopulations that have different allele frequencies and disease risks[44,45]. Presence of multiple disease-causing alleles in a gene (allele heterogeneity) and presence of multiple disease-causing genes or loci in a disease (genetic or locus heterogeneity) also influence the power of the reported associations[46,47]. The major statistical constraint faced in genetic association studies are inflated type I error rates due to multiple hypothesis testing. Type I errors, which occur when a null hypothesis is rejected when it is true, usually result in false positive associations. Such false positive results are compounded by the problem of multiple hypotheses testing in association studies, which involve large number of subjects, SNPs

and phenotypes. To reduce false positives resulting from this problem, the resulting significance values are adjusted for Type I errors due to multiple testing using conventional tests such as Bonferroni correction or by Bayesian approaches[44,48]. Apart from these, errors in genotyping could result in another major constraint in determining accurate genotype data for association studies[49]. These problems can be circumvented by carefully selecting the population, stringent definition of phenotypes investigated, efficient genotyping methods and applying suitable statistical tests to reduce type I error rates.

Types of populations used in genetic association studies:

Genetic association studies in human populations are performed either as cross-sectional case-control studies or longitudinal cohort-based studies. In the former approach, allele, genotype or haplotype frequencies in a population are compared between a set of people affected with the disease (cases) and a set of people not affected with the disease (controls). This approach provides a snapshot of the gene-phenotype association at a particular age in which the disease was diagnosed in the population investigated. However, this approach has a marked disadvantage because, being a chronic disease, asthma manifests as a combination of phenotypes over the various stages of life of an individual. Therefore investigation of the effect of the candidate genes on the disease at various ages of an individual would provide valuable information about the trajectory of the disease and its severity, and shed light on the most suitable points of therapeutic intervention. Longitudinal cohort-based genetic studies satisfy this need by investigating if a gene is associated with a phenotype over a period of time or at a

specific period during the progression of the disease. This helps to develop preventive strategies by elucidating the earliest stage in which gene-specific therapeutic intervention is possible in relation to a specific asthma pathway. Results from the data accumulated in this manner will be of tremendous importance in asthma-specific gene therapies in the future.

Associations reported so far:

While candidate gene studies provide definitive evidence for the role of a particular gene in asthma, the genome screen approach offers the additional possibility that novel genes, whose roles haven't been previously implicated in the asthmatic process can also be identified. Several asthma regulatory loci have been identified on the human and mouse genomes so far, especially on human chromosomes 5 (containing IL-4, IL-5, IL-13 and GM-CSF genes), 6 (the MHC gene cluster) and 11 (FcεRI-β, the β chain of the high affinity receptor for IgE)[50,51], all of which are important in the pathophysiology of asthma. These genetic approaches have recently been used to identify several asthma-influencing genes (Table 1, reviewed in[1,5]). More than 60 candidate genes have been investigated using this method[4], and this approach can be used to identify and confirm the validity of candidate genes directly in human populations, and also to confirm the results from animal studies. One such recent confirmation across species is a polymorphism in the myostatin gene[52-54], which is responsible for downregulating the muscle mass formation. A polymorphism in the myostatin gene resulted in a splice site disruption, preventing the formation of myostatin protein and resulting in increased muscle mass in mice and humans, and double-muscling in cattle. Such polymorphic asthma-influencing

genes, if identified in mice and confirmed in humans or directly identified in humans, may prove to be excellent therapeutic targets to counter the disease process of asthma.

C. ENVIRONMENTAL INFLUENCES ON ASTHMA:

Environmental influence is an important dimension in the conceptual scaffold of asthma[55]. While animal models can be investigated under controlled environmental conditions ranging from specific pathogen free environments to selective environmental exposures, the same is not possible in humans. Depending on personal and community lifestyle and geographical location, humans are influenced by a variety of indoor and outdoor environmental factors that lead to their susceptibility or resistance to asthma. Environmental influences on asthma are studied under the framework of several hypotheses[56].

Probably the most widely investigated of these is the hygiene hypothesis proposed by David Strachan in 1989[57]. It suggested that infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally from a mother infected by contact with her older children could prevent the development of allergic symptoms. This view has been supported by studies that reported a decreased incidence of asthma and atopy in children living under farming conditions[58-60], where chances of being exposed to such protective influences are high. Von Mutius et al., reported that children in former West Germany had more asthma and atopy prevalence than children from former East Gemany[61], showing that level of industrialization is an important factor in asthma prevalence.

Environmental exposure to a variety of allergens[62-65], or pollutants like ozone, sulphur dioxide, nitrogen dioxide and diesel exhaust particles can strongly incite or accentuate asthma-like symptoms[66-73]. Climate changes have also been shown to influence asthma symptoms, such as bronchoconstriction due to inhalation of cold air. Period of thunderstorms have also been shown to be associated with increased incidence of asthma attacks, possibly due to bursting of pollen and the release of paucimicronic allergenic particles in the atmosphere[74-77]. It has been shown that the prevalence of asthma is much higher during childhood in males and conversely higher post-puberty in females. The *in utero* and postnatal influences such as maternal smoking and breastfeeding are also being extensively investigated as important factors in asthma susceptibility[78-82].

D. GENE-ENVIRONMENT INTERACTIONS:

While underlying genetic factors play an important role in asthma, they also interact with one or more environmental factors to influence the outcome of the final phenotype. While this gene-environment interaction plays a very important role in asthma studies in human populations, this effect of environment can be controlled to a major degree in animal models, such as rodent models of asthma. By housing the experimental and control animals under the same environmental conditions, the phenotypes observed in the animal models can be largely attributable to genetic factors. The concept of gene-environment interactions is used to explain the situations where a particular gene is associated with the disease in some populations, but not others.

The essence of such gene-environment interaction effects have been captured most successfully by the hygiene hypothesis[83]. The hygiene hypothesis is supported by the fact that asthma incidence has increased severalfold over the last few decades[84] especially in urbanized industrial lifestyles. This poses some interesting questions and provides new perspectives about the etiology of asthma. While it is possible that misdiagnosis and underreporting of cases in the previous decades could be a factor in this surge, it is less likely that such factors would significantly change the observed increasing trends. Moreover, it is also less likely that this surge in asthma incidence over the last few decades could be solely by genetic factors, because the amount of causal genetic variation required to bring about such an increase in incidence could not have been introduced in such a short interval of time in a random mating population like humans[85-87]. A paradigm that explains this temporal variation that integrates the effects of genes and environment is epigenetic variation. Heritable short term alterations not involving changes in the nucleotide sequence resulting in disease phenotypes are classified as 'epigenetic' changes[88]. Epigenetic changes can occur due to several factors such aging and diets that supply methyl groups for metabolic enzyme activities[89-92]. The most extensively investigated epigenetic changes are methylation of nucleotides in the DNA sequence[93], and modification of histone proteins[94] that surround the DNA sequence to form the chromatin structure. Both these modifications have been shown to influence asthma by modulating transcription factors like NF-kB, which play a major role in asthma pathophysiology[95-99].

As asthma is a complex disease driven by multiple genes, interaction between the genes influencing asthma is also gaining importance. In these lines, interactions between

interleukin-13 (*IL13*) and interleukin-4 receptor alpha chain (*IL4RA*) genes have been shown to be associated with asthma[100,101]. Thus, asthma and associated phenotype manifestations result from genes, environment and epigenetic factors, which interact within and between themselves in multiple combinations.

E. INTERLEUKIN-1 RECEPTOR ANTAGONIST AND ITS ROLE IN ASTHMA:

This dissertation research is based on the results obtained from an asthma linkage study performed by Ewart et al[16] in a murine model of allergic asthma with A/J (asthma hyperresponsive) and C3H/HeJ (asthma hyporesponsive) mouse strains. This study identified two QTLs on mouse chromosome 2, which control allergen induced bronchial hyperresponsiveness (*Abhr1* and *Abhr2*). Positional candidate genes within each of these regions were chosen for further investigation to determine if those genes are responsible for the difference in airway hyperresponsiveness between these two strains. While complement factor 5 (*C5*) has been shown as the susceptibility gene for the locus *Abhr2*[102], such a gene has not been identified for the locus *Abhr1*. The murine IL-1 receptor antagonist gene (*Il1rn*) is located within the *Abhr1* QTL, and based on its functional relevance in asthma as explained in the subsequent sections, it was chosen as the positional candidate gene for investigation of the QTL *Abhr1*.

The interleukin-1 (IL-1) gene complex consists of two agonists, IL-1α and IL-1β. Both these agonists have similar functions, and the IL-1β gene (*Il1b*) is hypothesized to be a reverse-transcriptase mediated duplication product of the gene for IL-1α (*Il1a*)[103]. Apart from the agonists, the complex consists of genes for the functional receptor (IL-1 receptor type I – IL-1RI), a decoy receptor (IL-1 receptor type II – IL-1RII), and an

antagonist (IL-1 receptor antagonist – IL-1ra). In mice, the genes for these products are indicated by the symbols *Il1r1*, *Il1r2* and *Il1rn* respectively. Recently, several new members (*Il1f5 – Il1f10*) have also been identified and added to the IL-1 gene complex. In the humans, all the genes encoding these proteins (*IL1A, IL1B, IL1RN, IL1R1, IL1R2* and *IL1F5 – IL1F10*) are located in the long arm of chromosome 2. In the mice, *Il1r1* and *Il1r2* are located in chromosome 1, while all the other genes are located in chromosome 2 (Figure 1).

Interleukin-1 receptor antagonist is a major anti-inflammatory cytokine in the IL-1 cascade involved in a variety of chronic diseases like asthma, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease[104,105]. The functional significance of IL-1ra in asthma pathophysiology can be viewed better in the context of other members of the IL-1 complex, especially the agonists IL-1α and IL-1β. IL-1ra abrogates the pro-inflammatory effects of IL-1, and hence the mechanistic perspectives on IL-1 receptor antagonist function have always coexisted with those on IL-1.

The mechanism of IL-1 receptor antagonist activity:

Human *IL1RN* gives rise to two different isoforms, an intracellular (ic) and an extracellular (ec) isoform. They are created by the alternative splicing of different first exons; the first exon of the intracellular isoform is located ~9.4 kb upstream from the first exon for the extracellular isoform[106]. The intracellular isoform lacks a functional leader signal peptide, and remains in the cytoplasm[104]. Two additional intracellular isoforms have also been described[107,108]. The longest transcript in the human IL-1 receptor

antagonist gene (NCBI Refseq mRNA: NM_173841) contains six exons, and the longest transcript in the murine gene contains five exons (NCBI Refseq mRNA: NM_031167).

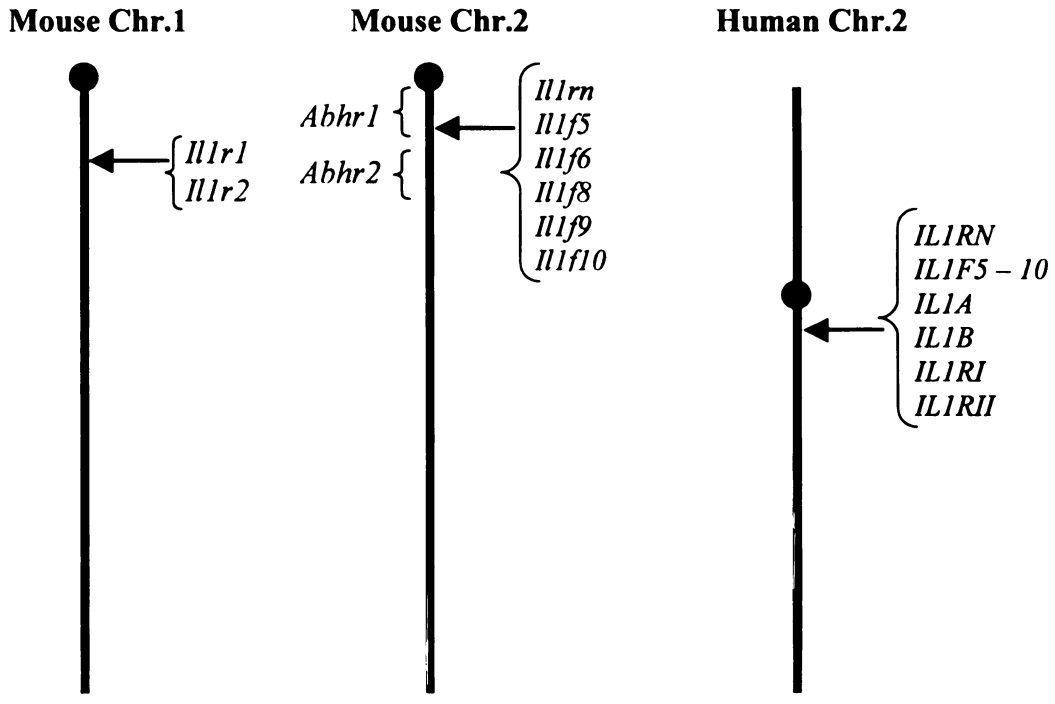

Figure 1. Location of IL-1 complex genes on mouse and human chromosomes. *Abhr* – Allergen induced bronchial hyperresponsiveness. ● – centromere.

The IL-1RI binds with the IL-1 receptor accessory protein (IL-RAcP) to form a dimer on the cell surface, which acts as the functional receptor complex and transduces signals on agonist ligand (IL-1α or IL-1β) binding. On the contrary, when IL-1ra binds to IL1-RI, it does not elicit any downstream signal transduction. It has been shown that IL-1RAcP is also a critical factor for IL-1 mediated signal transduction[109-111]. The interactions between these proteins and the signal transduction mechanisms have been

elucidated by studies of their crystal structures. IL-1RI consists of an intracellular Toll IL-1 receptor (TIR) domain and three extracellular immunoglobulin (Ig) domains. Crystal structures of IL-1RI bound to IL-1β[112] and IL-1Ra[113] have shown that the first two Ig domains in the receptor are tightly linked, whereas the third domain was separate and connected to the first two domains by a flexible linker. When IL-1β binds to IL-1RI, it binds to all the three Ig domains. The receptor then wraps around IL-1β and this is thought to result in the dimerization of the receptor with IL-1RAcP, resulting in signal transduction. On the contrary, IL-1 receptor antagonist binds only to the first two Ig domains and not to the flexible third domain, hence the receptor could not wrap around the ligand and this could be the reason for the lack of signal transduction in this situation (reviewed in[114]).

Another mechanism of IL-1 antagonizing is mediated through the decoy receptor (IL-1RII), which has three extracellular Ig domains, but lacks the cytoplasmic TIR domain critical for signal transduction. IL-1RII is released from the cells, binds to IL-1 and limits the binding of IL-1 to the functional receptor IL-1RI[115] and limits the availability of IL-1RAcP[116]. Moreover, IL-1RII has only a very weak affinity to IL-1 receptor antagonist[117], and hence it does not hinder the anti-inflammatory properties exerted by the actions of IL-1 receptor antagonist. Thus, both IL-1 receptor antagonist and IL-1RII act as independent anti-inflammatory mechanisms. IL-1 receptor antagonist was chosen for this study because it was located within the region of genetic linkage observed in our mouse study[16], and the other major members of the IL-1 complex were not chosen because they were not located inside the region of genetic linkage.

The role of IL-1 receptor antagonist in asthma

As mentioned previously, the role of IL-1 receptor antagonist in asthma can be most efficiently explained from the perspective of its counter-regulatory capacity on the pro-inflammatory effects of IL-1. Interleukin-1 is directly involved in both the major stages of disease progress – airway hyperresponsiveness and inflammation. Although asthma typically involves reversible airway obstruction, in some cases it becomes irreversible due to airway remodeling[118]. Accumulation of inflammatory mediators and growth factors burden the airways with additional workload, and this might lead to these irreversible changes in the airways that hamper the normal breathing capacity. IL-1 receptor antagonist serves to endogenously counter the pro-inflammatory effects of IL-1, and is also a suitable molecule for the therapeutic management of asthma[119,120]. Being a pleiotrophic cytokine, IL-1 seems to exert its effect at various stages of asthma pathophysiology. Hence its role is not in essence restricted to one specific pathway that leads all the way from IL-1 to asthma, and it has been shown to be involved in many pathways that lead to asthma. The anti-inflammatory role of IL-1 receptor antagonist also should be viewed from a similar perspective.

- **IL-1 receptor antagonist in asthma – Functional evidences:**

IL-1, on binding to the functional receptor, IL-1RI, stimulates the expression of a large number of proinflammatory proteins[114]. IL-1 is one of the first wave cytokines, along with TNF-α and IL-6, that may be released on exposure to inhaled allergens via FcεRII receptors[2]. Studies by Nakae et al. utilizing IL-1α^{-}/β^{-} or IL-1ra deficient mice in an ovalbumin (OVA) exposure model demonstrated that allergen-induced AHR, OVA-specific T-cell proliferative responses and the levels of Th2 cytokines IL-4 and IL-5 were

significantly decreased in IL-1α/β$^{-/-}$ mice, but were significantly increased in IL-1ra$^{-/-}$ mice compared to the wild-type mice[121]. This study showed that IL-1 and IL-1Ra have a direct effect on AHR, the major phenotype tested in animal asthma models, and also on T helper 2 (Th2) cell cytokine expression. On similar lines, in a guinea pig model of pulmonary anaphylaxis, IL-1ra has been shown to inhibit bronchoalveolar lavage fluid inflammatory leukocyte influx and antigen-induced airway hyperreactivity to intravenous substance P in a time dependent manner[122]. IL-1ra pretreatment reduced the generation of late asthmatic responses in terms of pulmonary resistance and reduced the presence of hypodense eosinophils in the bronchoalveolar lavage fluid in another guinea pig model, where *Ascaris* antigen was used for sensitization[123]. In a toluene-diisocyanate model of murine allergic asthma, blocking IL-1 activity attenuated AHR and inflammation[124]. Similarly, IL-1 receptor antagonist has also been shown to attenuate AHR following exposure to ozone[125]. Thus, IL-1Ra is able to decrease AHR induced by a variety of antigens in both allergic and non allergic animal models of asthma. In humans, the levels of IL-1β in BAL fluid from patients with asthma were found to be increased compared with those of non-asthmatic volunteers[126] and increased levels of both IL-1β and IL-1ra have been identified in asthmatic bronchial epithelium[127]. As IL-1β and IL-1Ra are co-regulated, it has been suggested that the ratio between them could also be an important factor in inflammation[128]. These studies have either demonstrated the effect of IL-1 receptor antagonist on AHR, or provided a snapshot of IL-1 receptor antagonist in asthmatic conditions.

The molecular mechanisms behind these results have also been examined, mostly using *in vitro* studies. The allergic component of asthma is characterized by the IgE

dependent triggering of the mast cells and the subsequent release of inflammatory mediators. IL-1 has been shown to induce a variety of pro-inflammatory cytokines such as IL-5, IL-6 and IL-9 from the murine mast cells[129]. Activated mast cells have also been shown to express IL-13[130], a central cytokine mediator of asthma[131,132]. IL-1 treatment increased the expression of IL-13 through a NF-κB dependent mechanism, and increased IL-13 promoter activity and mRNA stability in human mast cells[133]. IL-1 results in the production of a NF-κB[134], and NF-κB has been shown to be critical for the expression of the Th2 cell specific transcription factor GATA-3[135]. GATA-3 binds to the promoter regions of the Th2 cytokines IL-4, IL-5 and IL-13, induces their expression and increases allergic inflammation[136-139]. These results establish the functionally relevant role of IL-1 receptor antagonist in the inflammatory component of asthma.

Changes due to asthma also include changes in airway smooth muscles, inflammatory mediators, airway epithelial and subepithelial damage. Contrary to the notion that airway obstruction in asthma is reversible, it is now beginning to be accepted that in certain asthmatic conditions the obstruction may be irreversible[140]. Airway smooth muscle cell hyperplasia, subepithelial fibrosis, bronchial neovascularization and pro-inflammatory exudates from the smooth muscles are some major factors that drive this airway remodeling[118]. In the smooth muscles, IL-1 has been shown to increase the expression of granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP) -1, MCP-2, MCP-3, RANTES and eotaxin[141]. IL-1β has been shown to reduce the airway smooth muscle response to bronchodilator agonists operating through the β_2-adrenergic receptors[142], and cause airway thickening, subepithelial fibrosis and mucus cell metaplasia[143]. Extracellular regulated kinase (ERK)

as well as p38 mitogen associated protein kinase (MAPK) and Jun n-terminal kinase (JNK) pathways have been identified as major regulators of IL-1β induced aiway smooth muscle constriction and proliferation[144]. IL-1α has been shown to induce the activation of the p38 MAPK, and result in the inhibition of glucocorticoid receptor function. IL-1 mediated contractile responses to acetylcholine was ablated on pretreatment with IL-1 receptor antagonist in human atopic asthmatic smooth muscle cells[145,146].

- **IL-1 receptor antagonist in asthma - Genetic evidences:**

Only a few genetic studies have investigated the role of human *IL1RN* polymorphisms in asthma and related phenotypes. Gohlke et al., found significant association of *IL1RN* polymorphisms with asthma in a German population, and the results were also confirmed in an independent Italian population[147]. This study was performed in collections of father-mother-affected child trios from Germany, Sweden and Italy where one or neither of the parents had confirmed clinical asthma. The association was later reconfirmed in another German population[148] consisting of adult individuals participating in the follow-up of the European Community Respiratory Health Survey (ECRHS). The second intron of *IL1RN* contains variable numbers of an 86-bp tandem repeat (VNTR)[149], and five alleles (alleles 1-5) have been described for this polymorphism. *IL1RN**2 allele has primarily been associated with diseases of epithelial cells[104]. The *IL1RN**2 allele is associated with non-atopic asthma, while asthmatics and non-asthmatics possessing the *IL1RN**2 allele had significantly lower serum IL-1ra levels in a Japanese population[128]. The genotype combination containing homozygotes of *IL1A*1* (IL1A SNP+4845; GG genotype in a G/T polymorphism in exon 5), *IL1B**1 (SNP +3954; CC genotype in a C/T polymorphism) and *IL1RN**2 was associated with the

highest risk of skin prick test positivity[150]. A homozygous genotype for the G allele in the IL1A +4845 (G/T) polymorphism was associated with nasal polyposis, a chronic inflammatory disease often found coexisting with asthma[151]. *IL1A*, *IL1B* and *IL1RN* are located on chromosome 2 in both mice and humans (Fig. 1). Due to the complex biology of the IL-1 signaling system, it is possible that individual or a distinct combination of alleles from *IL1A, IL1B* and *IL1RN* might determine the susceptibility or resistance of an individual to asthma directly or by regulation of other cytokines involved in the inflammatory process.

Table 1. Asthma or atopy genes identified using genetic approaches (adapted from[5])

Gene	Location	Name
Genes identified by positional cloning following linkage studies		
ADAM33	20p13	A disintegrin and metalloproteinase-33
PHF11	13q14	Plant homeodomain zinc finger protein 11
DPP10	2q14	Dipeptidyl peptidase 10
GPRA	7p15-p14	G-protein-related receptor for asthma
HLA-G	6p21	Human leukocyte antigen G
CYFIP2	5q33	Cytoplasmic fragile X mental retardation protein interacting protein 2
Genes identified by candidate gene studies and replicated in ≥5 samples		
IL4	5q31	IL-4
IL13	5q31	IL-13
ADRB2	5q32-q34	Adrenergic receptor β 2
TNF	6p21	TNF
LTA	6p21	Lymphotoxin α
HLA-DRB1	6p21	HLA-DR
FCERB1	11q13	Beta chain of the high-affinity Fc receptor for IgE
IL4RA	16p12-p11	IL-4 receptor α chain
Genes identified by candidate gene studies and replicated in 2–4 samples		
IL10	1q31-q32	IL-10
CTLA4	2q33	Cytotoxic T lymphocyte antigen 4
CCR5	3p21	CC chemokine receptor 5
CD14	5q31	Cluster of differentiation antigen 14
LTC4S	5q35	Leukotriene C4 synthase
NOS3	7q36	Nitric oxide synthetase 3
CC10	11q12-q13	Clara cell secretory 10 kD protein
STAT6	12q13	Signal transducer and activator of transcription 6
IFNG	12q14	IFN-γ
NOS1	12q24	Nitric oxide synthetase 1
CARD15	16q12	Caspase-recruitment domain containing protein 15
RANTES	17q11-q12	Regulated on activation, normal T cell expressed and secreted
SCYA11	17q21	Small inducible cytokine A11
Genes identified by candidate gene studies and replicated in 2–4 samples since 2003		
TLR10	4p14	TLR10
SPINK5	5q32	Serine protease inhibitor Kazal type 5
IL12B	5q31-q33	IL-12B
TIM1	5q33	T cell immunoglobulin- and mucin-domain-containing molecule 1
TLR4	9q32-q33	TLR4
IL18	11q22	IL-18
CYSLTR2	13q14	Cysteinyl-leukotriene receptor 2
PTGDR	14q22	Prostanoid DP receptor
ITGB3	17q21	Integrin β3
TGFB1	19q13	TGF-β1

SUMMARY

Asthma is a chronic airway inflammatory disease due to inappropriate immune responses to common environmental factors. It is characterized by AHR, eosinophilic infiltration, airway obstruction, increased mucus production and increased serum IgE levels. Asthma is controlled by genetic and environmental factors, and their interactions. IL-1 receptor antagonist plays a protective role in asthma by inhibiting the pro-inflammatory cytokine IL-1. Our mouse model for allergic asthma is comprised of airway hyperreponsive (A/J) and hyporesponsive (C3H/HeJ) strains. Genetic linkage analyses performed in A/J backcross mice ((A/J x C3H/HeJ) F1 x A/J) identified two quantitative trait loci for AHR (*Abhr1* and *Abhr2*) on mouse chromosome 2. The murine IL-1 receptor antagonist gene is located within *Abhr1*. We hypothesized that genetic polymorphisms in *Il1rn* were responsible for the difference in AHR manifestation between A/J and C3H/HeJ strains. Hence, based on the genetic and functional evidences, we investigated *Il1rn* as a positional candidate for allergic asthma in our mouse model at DNA, mRNA and protein levels.

We also have access to a human birth cohort characterized for asthma phenotypes over 10 years. We tested for the association of the human IL-1 receptor antagonist gene polymorphisms with asthma phenotypes in our birth cohort, to comparatively investigate the effect of IL-1 receptor antagonist in humans. We hypothesized that polymorphisms in the human *IL1RN* was associated with asthma and related phenotypes.

Chapter 2. IL-1 receptor antagonist – Mouse studies

A. Mouse model of allergic asthma

B. Sequencing of mouse IL-1 receptor antagonist gene

C. Transcript and protein studies in IL-1 receptor antagonist and related genes

D. Summary of the mouse study

A. MOUSE MODEL OF ALLERGIC ASTHMA

- **Animal models of asthma**

Animal models of asthma are primarily used to investigate the pathophysiologic mechanisms in human asthma. Investigation of complex genetic traits such as asthma in humans is difficult because of various factors like genetic heterogeneity, phenocopies and incomplete penetrance[152]. Mice, rats, guinea pigs, rabbits, ferrets, cats and horses are some of the existing animal models of asthma[153], of which the mouse has been the most widely used for investigation of asthma pathophysiology. The mouse model has several advantages, the most important of which are the availability of the complete sequence of the murine genome and the availability of several inbred strains. Inbred strains of mice differ markedly in their susceptibility to asthma, which can be effectively used to identify novel genes that contribute to the different susceptibilities. All the individuals in any single inbred strain are genetically identical (homozygous alleles at all loci), and this reduces the problem of genetic complexity in an outbred population like humans. Inbred mice have a short life span (1.5-2.5 years), early sexual maturity, short generation intervals and good litter size[154]. Moreover, availability of knock-out mice and reagents required for immunological investigation facilitate the rapid investigation of asthmatic phenotypes. Ethical considerations limit the type of experiments that could be done in humans, and the techniques like bronchial biopsies and BAL fluid analyses are not free from risk for the patient. Moreover, the ease of taking samples from organs like bronchioles or pulmonary parenchyma and the ability to conduct temporal studies also make animal models better suited for investigation of asthma than humans[153]. For example, the causal link between systemic IL-5 and eosinophil recruitment to airways[155],

the central role of the pro-asthmatic cytokine IL-13[131] and the transcription factor T-bet[156] were first established in mice. Mice are also used to study the relationship between the inflammatory processes in the upper and lower airways, facilitating the investigation of the so-called 'united airway disease' concept (reviewed in[157]). Despite all these advantages in the mouse models, it has been suggested that *in vivo* models can only be used to model one or more traits of human asthma, and the possibility of having an overall model of asthma should be treated with caution[158]. However, information obtained from inbred mouse models is invaluable to reveal the common pathways of human diseases, and identify novel therapeutic targets for the management of asthma.

- **A/J and C3H/HeJ inbred mouse model**

The animal model used in this study consists of two strains of mice – A/J and C3H/HeJ, modeled for the traits AHR, IgE levels and eosinophilic infiltration. The QTL controlling airway responsiveness to acetylcholine (Ach) without allergen sensitization or challenge was mapped to chromosome 6 in this model by Ewart et al[159]. The same model was subjected to the following allergen challenge protocol. Both the strains of mice were sensitized with an intraperitoneal injection of OVA or phosphate buffered saline (PBS) at day 0, and a tracheopharyngeal instillation of OVA or PBS was used to challenge the sensitized mice on day 14. A/J mice showed increased levels of AHR, pulmonary eosinophilia and serum IgE levels compared to C3H/HeJ mice in the OVA treated group on day 17[16]. Of those phenotypes, AHR was pursued for the subsequent linkage analyses because it is an outcome that most closely mimics the clinical manifestations of asthma. The time-integrated rise in peak inspiratory pressure subsequent to intravenous

acetylcholine challenge was calculated and reported as airway pressure time index (APTI)[160].

Without OVA treatment, A/J mice had higher APTI than that of C3H/HeJ mice. Following OVA treatment, the APTI of A/J mice increased sharply relative to that of PBS treated A/J mice. Contrary to this observation, no significant changes occurred in the C3H/HeJ mice following OVA treatment. The APTI of the F1 mice (A/J x C3H/HeJ or C3H/HeJ x A/J) was intermediate to the parental strains and no changes occurred following OVA treatment[16]. The APTI distribution in A/J backcross mice was broad, covering those of A/J and C3H/HeJ mice; therefore they were suitable for linkage analysis. To determine the chromosomal locations that control the allergen-induced AHR, a genome wide linkage analysis was performed in A/J backcross mice by using microsatellite markers spaced at approximately 10 cM intervals. Two QTLs were found on chromosome 2: *Abhr1* (between *D2Mit359* and *D2Mit416*, Lod score = 4.2) and *Abhr2* (between *D2Mit238* and *D2Mit298*, Lod score = 3.7). The QTL *Abhr2* has been resolved to a quantitative trait gene (QTG), which is the complement factor 5 (*C5*) by Karp et al[102]. The gene encoding IL-1 receptor antagonist (*Il1rn*) maps within the *Abhr1* region. It was chosen as the positional candidate gene for *Abhr1* based on the genetic evidence and its functional relevance in the asthmatic pathway.

B. SEQUENCING OF THE MOUSE IL-1 RECEPTOR ANTAGONIST GENE:

- **Introduction**

According to National Center for Biotechnological Information (NCBI), the murine IL-1 receptor antagonist gene has been mapped to 10.0 cM on chromosome 2. Refined linkage mapping done in our laboratory (Li et al – unpublished results) showed that the sequence-tagged site (STS) marker *D2Mit60*, located in *Il1rn*, was mapped to the QTL *Abhr1*. The physical map available from Ensembl (http://www.ensembl.org/) showed that *Il1rn* is located from 24,269,046 bp to 24,283,646 bp on mouse chromosome 2. As *Il1rn* has been shown to have a significant role to play in the pathophysiology of asthma (Chapter 1, section E), it was chosen as a positional candidate for allergen-induced AHR in our mouse model of asthma.

To investigate the role of *Il1rn* in allergen-induced AHR, the first step was to compare its gene sequences between A/J and C3H/HeJ mice to determine whether DNA polymorphisms were present. Polymorphisms could be of several kinds – repeats of a short stretch of nucleotides (microsatellites), repeats of a long stretch of nucleotides (minisatellites or VNTR), or single nucleotide polymorphisms (substitution, insertion and deletion). These polymorphisms could be present in the introns, exons or the regulatory regions of the gene.

Murine *Il1rn* spans across a region of ~14.5 kb on chromosome 2. Prior studies in humans have demonstrated that alternative splicing of two different first exons of the *Il1rn* mRNA produces a secretory protein containing a leader sequence (sIL-1ra) and an intracellular isoform (icIL-1ra), which lacks the leader sequence and remains intracellular [104]. The first exon of the intracellular isoform lies ~9.4 kb upstream from the first exon of

the extracellular isoform[106], and both the isoforms have been shown to be regulated by separate upstream regulatory elements[161,162]. The most proximal regulatory region in human *IL1RN* is the promoter for the intracellular isoform of *IL1RN*. A 1.8 kb region upstream of the first exon of intracellular *IL1RN* has been shown to be critical for its promoter activity and has binding sites for several transcription factors[106]. Similarly in the mice, the most proximal regulatory sequences for the intracellular isoform of murine *Il1rn* have been shown to be located in the -598 and -288 bp region upstream of the transcription start site[163].

- **Sequencing of *Il1rn* in A/J and C3H/HeJ mice**

Primers were designed to sequence introns, exons and regulatory regions of both the intracellular and extracellular isoforms, spanning a total length of ~16 kb (Figure 2). Genomic sequence of *Il1rn* (mCG4837) from Celera (www.celeradiscoverysystem.com) was used to design the primers (Table 2). The Celera *Il1rn* sequence was a consensus sequence from 129x1/SvJ, 129S1/SvImJ, DBA/2J and A/J mouse strains. Primers designed covered the most proximal regulatory regions described in the literature so far, and the regulatory region for the secreted isoform was included in the intron between the first exons of the intracellular and the extracellular isoforms. Genomic DNA isolated from the kidneys of A/J and C3H/HeJ mice were used for sequencing. Sequencing was performed at the MSU Research Technology Support facility (http://genomics.msu.edu) using fluorescence-labeled dideoxy sequencing method. A total of 16 kb of genomic DNA encompassing *Il1rn* was sequenced in both the strains, and the sequences were submitted to NCBI. The Genbank accession numbers for the submitted sequences are DQ383807 (A/J) and DQ383808 (C3H/HeJ).

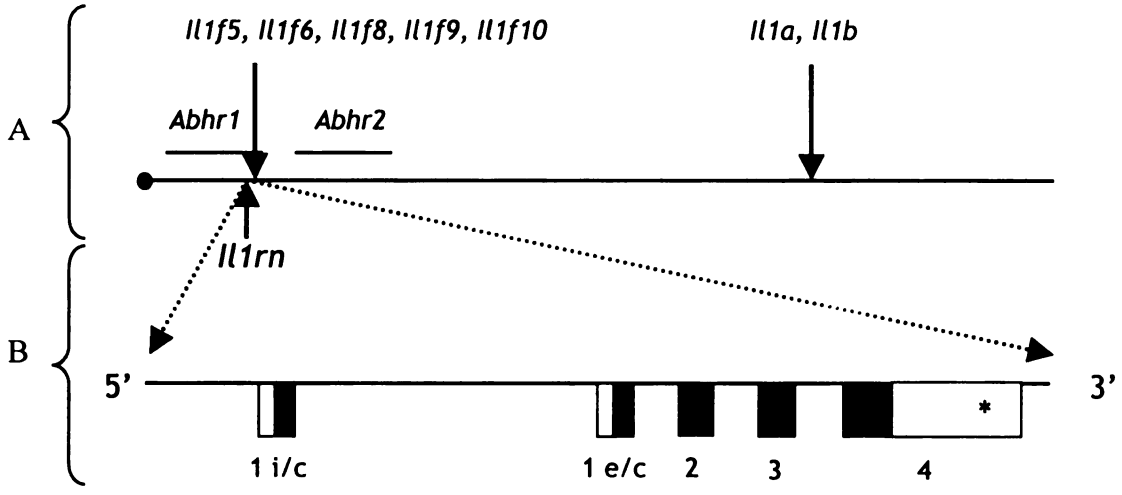

Figure 2. Sequencing of the murine *Il1rn* gene. (A) Several IL-1 family genes map to murine chromosome 2. (B) *Il1rn* contains 4 exons, including alternative exon 1 for intracellular (i/c) and extracellular (e/c) isoforms.

☐ Untranslated regions in exons; ■ coding regions in exons.

* Dinucleotide repeat polymorphism.

Table 2. Primers used to sequence *Il1rn* in A/J and C3H/HeJ mice

No.	Direction		Target	Primer sequence (5' – 3')	Primer – Lab ID	[1]Position
1	F		5'upstream region	TGACAAGAGCCCGATCT	icIl1rn prom 1F	[2]Start…
		R	5'upstream region	CAAGTCTGTCTCTGCGGATG	icIl1rn prom 1R	548
2	F		5'upstream region	GAGAGACCTTGGGAATGAGT	icIl1rn prom 2F	433
		R	5'upstream region	CCCCATTTTCCAAGTATG	icIl1rn prom 2R	1030
3	F		5'upstream region	GAAGCCAGAGCAAGTTGT	icIl1rn prom 3F	880
		R	5'upstream region	AAACCCAGCTGTTATGAGAC	icIl1rn prom 3R	1451
4	F		5'upstream region	TTTGTTTGGCTTGCAACC	icIl1rn prom 4F	1333
		R	Intron 1	ACCTGGCATTGAGCATTGAG	icIl1rn prom 4R	1900
5	F		Intron 1	ATAGACACTGCCTGGGTGCT	icIl1rn Prom 5F	1716
		R	Intron 1	CACACTTCAACCTTGGACACA	icIl1rn Prom 5R	2151
6	F		Intron 1	CAAAATCCCAGGAAACTACC	Il1rn Intl PF1	1947
		R	Intron 1	ACATGCTTAACTTCTCCGTT	Il1rn Intl PR1	2486
7	F		Intron 1	AGCTTCCAAATGTGACCAAG	Il1rn Intl PF2	2428
		R	Intron 1	GCCACAAATTGCAAACAC	Il1rn Intl PR2	2991
8	F		Intron 1	CGTACCTTTGCCAATAATAA	Il1rn Intl PF3	2918
		R	Intron 1	AGCAAGGCTGCATCTCCTA	Il1rn Intl PR3	3498
9	F		Intron 1	CATTCCCATCATGAACGTGT	Il1rn Intl PF4a	3443
		R	Intron 1	GGAGACAGGGCATAAAC	Il1rn Intl PR4a	4063
10	F		Intron 1	CCATCTAGGAAGACTTAAC	Il1rn Intl PF5a	3945
		R	Intron 1	TATATTTCCAGAAAACTTTA	Il1rn Intl PR5a	4595
11	F		Intron 1	CTCACAAATGGTGCTTAGTG	Il1rn Intl PF6	4508
		R	Intron 1	GGGTTGTTATGATTTGCAT	Il1rn Intl PR6	5061
12	F		Intron 1	CATAAAATTGCCCAGCTTCTG	Il1rn Intl PF7e	4772
		R	Intron 1	GAGGGACAACTGGGTTCTTC	Il1rn Intl PR7e	5374

[1] Position in basepairs based on our Genbank entry DQ383807 (A/J *Il1rn*)
[2] First primer sequence – present upstream of the beginning of the reference sequence used here (DQ383807 - A/J *Il1rn*) & hence the location cannot be denoted in basepairs in relation to this reference sequence

No.	Direction		Target	Primer sequence (5' – 3')	Primer – Lab ID	Position
13	F		Intron 1	TTTGAGGTGCCTCAGAAAAT	Il1rn Int1 PF7f	5204
		R	Intron 1	GCCTTACGGTCAGTCTCTGC	Il1rn Int1 PR7f	5721
14	F		Intron 1	AGAAGGGGAAGCAAAACACC	Il1rn Int1 PF7g	5651
		R	Intron 1	TACCCACTCCCAATTCCTGA	Il1rn Int1 PR7g	6179
15	F		Intron 1	TATGAACTAACCAGTACCCT	Il1rn Int1 PF9	5980
		R	Intron 1	CCCAGACTCTCTAGATCTC	Il1rn Int1 PR9	6572
16	F		Intron 1	TTCTCATAGCAACCCACATC	Il1rn Int1 PF10	6493
		R	Intron 1	GTGGCTACTGATATAGCG	Il1rn Int1 PR10	7051
17	F		Intron 1	GTCTGAGGGCAGCTATGGAG	Il1rn Int1 PF10a	6856
		R	Intron 1	TGCATTCATAGGAAGACAAAAA	Il1rn Int1 PR10a	7314
18	F		Intron 1	CAAAGTCTCGCCAGT	Il1rn Int1 PF11	6958
		R	Intron 1	CACAGATCTAATGTATGCCA	Il1rn Int1 PR11	7634
19	F		Intron 1	TTGCAAGTTTCAGTGTTTAG	Il1rn Int1 PF12	7541
		R	Intron 1	ATCCAGAAACTGTTGAATTA	Il1rn Int1 PR12	8238
20	F		Intron 1	CAGCAGCTGTGATAGCAACA	ecIl1rn prom 1F	8150
		R	Intron 1	TTTTGAGACAGGGTTGCTC	ecIl1rn prom 1R	8708
21	F		Intron 1	TTGGGTTGAAATAAGGTCAG	ecIl1rn prom 2F	8543
		R	Intron 1	TGGCTTTCTTGGAGGTAGTA	ecIl1rn prom 2R	9091
22	F		Intron 1	AATTGTGTGAAGGGAACTTA	ecIl1rn prom 3F	9004
		R	Intron 1	TTGCTGACCTCTGCCCTAGA	ecIl1rn prom 3R	9591
23	F		Intron 1	GGAATGTAAATAAATAAGAT	ecIl1rn prom 4F	9461
		R	Intron 1	CCCAAAATTAAGTTCT	ecIl1rn prom 4R	10043
24	F		Intron 1	CAGCAAATAGACTCGGAGTA	ecIl1rn prom 5F	9936
		R	Intron 2	GGTCCTTGGTCTATGCAATC	ecIl1rn prom 5R	10441
25	F		Intron 1	ATATTTACCATCCGGTTCTG	IL1RN Intron1A F	9745
		R	Intron 2	TTGCCTTGGGATAGATGTAT	IL1RN INT1a2 R	11316
26	F		Intron 2	TTGTTGGTGCATATGATCTGT	IL1RN INT1a2 F	11174
		R	Intron 3	AGGGCAAAGGACTCTATGT	IL1RN Intron1A R	12436
27	F		Exon 2	GGGTACTTACAAGGACCAAA	IL1RN Intron 2F	12350
		R	Exon 3	CCCAAGAACACACTATGAAG	IL1RN Intron 2R	13390

No.	Direction		Target	Primer sequence (5' – 3')	Primer – Lab ID	Position
28	F		Exon 3	TAGACATGGTGCCTATTGAC	IL1RN Intron 3F	13351
		R	Exon 4	CGGATGAAGGTAAAGC	IL1RN Intron 3R	14305
29	F		Intron 4	ATAGCCACAAGCATGAGTTT	IL1RN 3'UTR F	14137
		R	3'UTR + downstream sequence	TCATTGTGTGGCATTGAGT	IL1RN 3'UTR R	[3]End...
30	F		3'UTR	AGGAGCTGGGGATTAGATGCT	IL1RN 3'UTR NF 2	15120
		R	3'UTR + downstream sequence	TCATTGTGTGGCATTGAGT	IL1RN 3'UTR R	End...

[3] Last primer sequence – present downstream of the end of the reference sequence used here (DQ383807 - A/J *Il1rn*) & hence the location cannot be denoted in basepairs in relation to this reference sequence

- ***Il1rn* gene sequence of A/J strain – NCBI Genbank entry**

LOCUS DQ383807 15993 bp DNA linear ROD 26-FEB-2006

DEFINITION Mus musculus strain A/J IL-1 receptor antagonist (Il1rn) gene, complete cds, alternatively spliced.

ACCESSION DQ383807

VERSION DQ383807.1 GI:88595941

KEYWORDS .

SOURCE Mus musculus (house mouse)

ORGANISM Mus musculus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Mus.

REFERENCE 1 (bases 1 to 15993)

AUTHORS Ramadas,R.A., Li,X., Shubitowski,D.M. and Ewart,S.L.

TITLE Direct Submission

JOURNAL Submitted (30-JAN-2006) Large Animal Clinical Sciences, Michigan State University, 242 National Food Safety and Toxicology Center, East Lansing, MI 48824, USA

FEATURES Location/Qualifiers

source 1..15993

/organism="Mus musculus"

/mol_type="genomic DNA"

/strain="A/J"

/db_xref="taxon:10090"

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/gene="Il1rn"

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13345..13457,14244..>14459)

/gene="Il1rn"

/product="IL-1 receptor antagonist isoform"

/note="transcript for possible intracellular isoform;

alternatively spliced"

CDS join(1812..1821,10349..10400,12295..12383,13345..13457,

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/note="possible intracellular isoform; similar to

NM_031167; alternatively spliced"

/codon_start=1

/product="IL-1 receptor antagonist isoform"

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DQ"

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14244..>14459)
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/product="IL-1 receptor antagonist isoform"
/note="transcript for possible extracellular isoform;
alternatively spliced"

CDS join(10282..10400,12295..12383,13345..13457,14244..14459)
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Accession Number M64404; alternatively spliced"
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121 tagacaggct cccccaacaa cttccagact tccctcccag agcagcttcc caggtttgca

181 aagctggagt ttgtagagtt tccaggtttg caaagctagt tcctgattct tcacaaacat

241 ttctctacaa aagctccttt ctaattccta gttaaggctg aaagacctag acagtctagc

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421 cagtcccaat agagagacct tgggaatgag tccagatgaa caaacagcta gaggaaaagc

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901 gagctgcaga ggaagaggag tcagagaatt gagaacttcc agagaaagtt gagagagtgc

961 atctggcctt ctggagtcag tcttgacagt ggcgcattgg tgaaatgcaa gcatacttgg

5' upstream region

1021 aaaatggggt aaacctctaa gtgatgctgt gatcacatag tagtagtagt agtagtagtt

1081 agcataatac tcagatcatt aaatatccca tcccaagtca tcttccttgc tgtttcctgt

1141 ggttgtaaca ttctttccct tccccagctc aaatgccacc attctcaaag aggcttccct

1201 ggacaccttc gaagatatct gcctgggtac ctcatagaca tttgatttct cctgtgacct

1261 ttacccccat agagaattag cttattttat tctttccctc tccactttct ctcattagaa

1321 cacaagtggc ctttgtttgg cttgcaaccg gtgtttgtga gagcacttgt aagtaccaca

1381 ggcaggacta agtcagaatg tgttttaaac aaatttatgc acacataatg gtctcataac

1441 agctgggttt ggggtgatac aaaggtccca cctcataaat ccagctcctt tcccacatgt

1501 gacctccagg tttacatata taagaaccag tttggcttct gctagactga gtcacgcctc

1561 tggaagctgg gcctggcttg gcttcagtca tcagcaacca acctcctagg gctgactggc

1621 ctgtgtagga gtatgggagt ggccatctcc ccccttttat tctgcttagg tagctgggaa

1681 ggaggggcag ttccaccctg ggaaggtctg tgccatagac actgcctggg tgctccttta

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2101 gtaatgaaaa ctgttgatat aaagtcatct gtgtccaagg ttgaagtgtg atagagaaag

2161 atagagtctg cagaggagta acagattgac acttaggcaa catgtttgcc atagagaata

2221 taggctcagg tggacattct taaagggaaa gccaaatatt atctgtgcta ctacctggta

2281 cttccaaaac cagacagaat cagacacatt aatgttacat gctggtgagc atgtattggg

2341 ttgaaccagt gcaagcaagc acaatataga caccacagtc tctgaaagaa ggaatcagaa

5' upstream region

Start site for i/c isoform

Intron 1

[4] Start codon of the intracellular (i/c) IL-1 receptor antagonist isoform

2401 acagcatcaa aatccaaaga gatgggagct tccaaatgtg accaagcaga aaccatggct

2461 ctaagaacgg agaagttaag catgtgactt gatggggggt ggggcttgca gagtaaggac

2521 aggagcaagg gtgtaccctg tgcttccctg ggactttact gtctctctcc atggagtctg

2581 gcttcttgcc tcacagatga tgcttgtaaa gaagggaggg agaaagagaa ggagaaagca

2641 atggtaatgg gaagtataaa tcctgtttag gacccgcatc tgaagatagt ctaagaatgc

2701 tagacactaa cattgtctta agataaatgg cttaacttgg aatgaatcaa tggtggatgt

2761 gaccattctt ttatttacta tggtctcttc ctgggtgaga agttcatttt tggcttttgt

2821 atatatttca gttatctatt tgtctgtctg tctgtcttat ttataatcta tgaatttgtc

2881 tatcatccaa actgcatacc atctatccaa atggatcgta cctttgccaa taataacaag

2941 ctaaattagc aagtgacagt cattaagtca ttgtgtttgc aatttgtggc accatccacc

3001 aggattcaca atttaggttt tcaaagactg aggaggtcac tgaagtatag aatgttcagt

3061 aatgaagcta aaatagtctt tgacaaacca aggcagtttg ccatcctagt ccataaccaa

3121 gtaaaatggc tttcattata gcctatacaa aggttttaaa ttattttcct tacattccac

3181 atgcttttcc atatttccaa gcagttatga actaaatagt tactccatag gataatgagc

3241 cagagccaaa catttaaatc ataagtaaag gtcttgctct tgttgacctt acaatgaagc

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3421 tttctgcaag ctatccaggt tcattcccat catgaacgtg tgttctatgc ccggttggtg

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3541 accgtttaac acctcagtcc acttccagtt catatctgct ttgtgtctgt ggttgaaaat

3601 gtgaactccc aactgcctgc tccagccacc atgctttccc tgcaatgtag actcgtatcc

3661 cttcttgagc catataaact ctttttttt ctataagttt cttcaaatta tggtgtttta

3721 tcactgcagc ataaaagtag tgaatgtgaa aacagaggtg ggcacagcaa atgacagcac

Intron 1

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3901 gttcagacca atacatatca cccgcacacc atggaattaa aagccatcta ggaagactta

3961 acctcagaaa ggattcaggg aaggtttcct atctggatct gggaagaaat tgagcctctt

4021 gaaaagcact gatccacaat tgttggttta tgccctgtct cctgaatgac ctttgctagc

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Intron 1

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5341 agccatacac tgggaagaac ccagttgtcc ctcaacagag gaatggatac agaaaatgtg

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5461 aggcaaatgg atggatctgg aggatatcat cctgagtgag gtaacccaat cacaaaagaa

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5641 attcttctta gaaggggaag caaaacaccc atggaaagag ttacagagac aaagtgtgga

5701 gcagagactg accgtaaggc catccagaga ctgcccccac ctggggatcc atcccatata

5761 taatcaacca aacccagaca cttttgtgga tgccaacaac tgcttgctga caggagcctg

5821 atatagctgt ctcctgaaag gctctgccag gacctgacaa ataaagatgt ggatgctcac

5881 acctatccat ggaactgagc acagggtccc caatgaagga gctagagaaa gtacccaagg

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Intron 1

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7381 caccataatc aggctaatta acacatcttc ctcctcacat agatcccatt ttctttctcc

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7801 ctacacatct ctggttcctg atgttgttgg ctcaaagcca gacaggctgt cctttctggg

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Intron 1

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9241 aaaggactcc aacttcacag aggaccacca caataggctc ttgtctgcaa acataacaga

Intron 1

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9421 caaattttga accaaaagtt ttatagtttt gtgtgcattt ggaatgtaaa taaataagat

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10381 ***tgcaagatgc aagccttcag*** gtaagtcttc caaagacaca ggattgcata gaccaaggac

10441 cagagacaca tgccatatgt ccagagcata tgcaggaata ggagatatat atacatgtat

10501 aatatatata atgcgtgtgt gtatgtgtgt atacacatat gtatgtatgt atgtgtatat

10561 atatatatat atatatatat atataatgtg tgtgtataca catatgtatg tatgtgtata

Intron 1

Exon 1

Intron 2

[5] Start codon of the extracellular (e/c) IL-1 receptor antagonist isoform

[6] Alternate splice site where the start codon and subsequent sequences of intracellular (i/c) isoform joins to form the first exon of the i/c IL-1 receptor antagonist isoform.

10621 tatatatata tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgtatc cttgattcaa

10681 gaacagcatg ctaaatgcag tctttaagtc ttatgtttta aaatattcca tgcatggaca

10741 acaagacagt taactgtgct cactttctca gacacctaga tgttcagtaa gtgatggaca

10801 ggcatccggg aataatgcta gctttgggat cgagcaaaga ggaatacttc agcaggacac

10861 agtcaaaggc tcagaccaac agtctacact ctgtatctgt gttgacttgg aagatatctc

10921 tcgttggagt ccccagtttc cttatctgta acatgatact gctctgatga taacccccttg

10981 tgtgccttac agggtgaaca ctaaatacat gagtgatact gtaaccatgt tctgagacct

11041 atgctctgag aactgtaaag tgcctgaaaa ataacctgag ttttaaaaat tggatcaaaa

11101 gccttgggag atgccatcaa ccttatagta aaaatggcag gcctcgattt tgattttaaa

11161 atgaataaag agattgttgg tgcatatgat ctgttcttga tccttcctga gagtgaagtc

11221 tgtgttgagt cacttcccct ttgaccctgt ctgctttgga tccacagctg gaggctggga

11281 ctctaactgt gattctatac atctatccca aggcaagtct gtcccacaga tccagtaact

11341 gcttcgtgag atttaccatc atcacatcct cttagcagcc tcaagagagg tccctggagt

11401 cctgttagca agactattga gtcccttgag tttgaagctc accagagata tagacaccag

11461 tcacaaaggc acaaatactc tttcacgtgc agagtacttg gtttgtcctc cacccatccc

11521 tgagctccta ggctgctcca agctactcaa aaagtcctgt cagctctgct gaccaggtaa

11581 agagataagg gacagatcca aggtcatatc atcaggcctc ttaccacacc tcacaggtgc

11641 ctgcctctct ggaagccaga gggcctttca ccaagaagtc agagagtaac aaacaggccc

11701 tggctgagct agacaggaag ctgacttatt tccaaggaca gctgtccctg tcaggcccag

11761 agcagatggt cccacaagag gttttagttg tagacttgca ggtctaagta gagtagcttg

11821 aggtaggagt agtggagcca gactagcttg gctacaatac attctaaccc ttgaacctgt

11881 aacactatga tgtggtggcc acgagctaca agtggccatc taaatttaca cataaacgca

11941 tgaaagcaga agaaagtcct gtacctggca actctattta gtggagtgac tataggatgt

Intron 2

12001 gcttgcatcg cctaagtttc tatcagatgc tgacgctcta tagaaaattc tgctaaagtc

12061 atggatgtcc atgctgggat tctgaggtga ggaacaagaa aaagaggttt tctgttcacc

12121 agatgtgaga gatgggctca tttcttacat ggtatttgct taaatcttcc catttgtgtt

12181 atgaacttgg taagtacgac acttccagca agtctagatg taaattaggt gactctgagg

12241 aagctggaaa gggctctgta ctgcctactc cagctaggcc attttgcttt tcag***aatctg***

12301 ***ggatactaac cagaagacct tttacctgag aaacaaccag ctcattgctg ggtacttaca***

12361 ***aggaccaaat atcaaactag aag***gtgagtg gataacaggg aagctggtgt aatatggaca

12421 tagagtcctt tgccctgctc ctctgcctgg aggtgggatg tcctcatttc tgttgagttg

12481 gaaatgagag atttgaccac caggggacat atgggagtgg cctcaagaga gcagaaaaga

12541 taaagactgg gtcacaatgc tccagggaca cagctgagag gaacagaggc cagaaggcac

12601 ctgggcacct ccttagtcct tctgtgctgg tagtccacta taccccagtg ttattcgaac

12661 tctacccttg ccctaggcta atataacatg tatgtgggct gggtagcatt tttactgtgg

12721 acaccaccct catcatgtac cctctaaact aggacaaagc cacatgaact tggaggagca

12781 ttacccacag attcttcagt ttttctttta gaaaaaatga gggcacttag ttgacagaat

12841 ctctgtttgt gagggaacga agcattactt gtatctcctc aggatccccc aagccttctg

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12961 catgaacaca ctcaagcata gaaggtgctg gctttctatt gctacccagt aacaggatgg

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13081 aacagcaaat ctgttgactt ggaggtctgg ttcactgtag aaagtaaagg aaagttggga

13141 gcagtgtaga atctaggaag ctggtcctta catagagtgt gctcatttgg atcttttgct

13201 tggaggcaga ctagaaagat agagccttct tgaccttctt gaccttctag ttttataaaa

13261 aggaagacag aaaatacaca cagacgctcc cctacccttg cctcctcttc tctctttctg

13321 acaccatcct ctactcttct ccag***aaaaga tagacatggt gcctattgac cttcatagtg***

Intron 2

Exon 2

Intron 3

Exon 3

13381 ***tgttcttggg catccacggg ggcaagctgt gcctgtcttg tgccaagtct ggagatgata***

13441 ***tcaagctcca gctggag***gta agaatctggt ttagctatca aatccttcta aaacccaatg

13501 gttatgacaa cctcaggtgt ttctcataac cctgagcatg caaagatgag ggaggctttt

13561 ccttcttcac agagtactat tttgaggtca ctccttaagc agtttccaca atgttcttgg

13621 ttgatattgg gtgtccaagg tggtttctca ttctctcaac tacccтttac gtaacttctt

13681 tgcattcagt caacactctg agcttcctta agcgtggtga ccaactttta tgagagattg

13741 ttccagaaag atgagcctca atgtgaaagt gcttattaag cttgggctta tgtaagtcta

13801 ttggcagaag cctgtgacgt ggttgatatg gactcattgt agaaaggtac tgcacaagga

13861 tctaaacttt aggaggagac atggtcatta gaggagcacg acctgaacca ccatgggtct

13921 tgtgcctcct aaaccagttg agcctacctt cttctagcaa ggtcaattct caagactata

13981 cactcccaag catcatctat gctatttatt atctacgctc ctaatttaca tcccacacag

14041 acctgtgtca cttactcctt tacctagtca gtagtaatgg gctgttcaaa cattatcttg

14101 agggattagc tggacaaact tttaatccaa ctgcaaatag ccacaagcat gagtttgttg

14161 ataactctta ccaatggaca ggaacacctt ttagaggact ttctcagccc tcggcaatta

14221 cctgaccatt tcttgacttc ag***gaagtta acatcactga tctgagcaag aacaaagaag***

14281 ***aagacaagcg ctttaccttc atccgctctg agaaaggccc caccaccagc tttgagtcag***

14341 ***ctgcctgtcc aggatggttc ctctgcacaa cactagaggc tgaccgtcct gtgagcctca***

14401 ***ccaacacacc ggaagagccc cttatagtca cgaagttcta cttccaggaa gaccaa***[7]***tag***t

14461 actgccgagg cctgtaataa tcaccaactg cctgatcact ctggccatca ttggggcctg

14521 aggaacaact tttgcagggt gtatgtacag tagaaggaga cagaagagtt ctgatgatag

14581 atctctgcct cagtctgttg gctggcctaa tccccatgat gattccagaa taatcttgca

14641 aattggatca tggcaggtgc ttgttcaaag ccctttcttg ttgcctctgc catctgggtg

14701 aagtctagac cacttgcttg gcctaggtgt cttctgctct accacccacc ctacccctgc

Exon 3

Intron 4

Exon 4

3' UTR

[7] Stop codon for both the intracellular and extracellular IL-1 receptor antagonist isoforms.

14761 cacaaacaca cacttttttt gtttttgttt tttccattgt tctgcacttc cacagtccag

14821 accaatcaag tcacttgaca atatgcccca agtgactccc ttaccctgtt ttataaacct

14881 gtgcctgtct atggagaagg ttttaattct ccttgttatt cattttgggc tttttgatga

14941 aaccaccagg gcatcacata tactaagcat gtgctctacc atcatgctat gcttccagct

15001 caggggggca cttttaagga tctagaaaac agaaattaag gatctcatag ttatttttatt

15061 aggccagcct tattccatgt cggcaagagg tttcttgtgg aaattatgtc ctttctgaga

15121 ggagctgggg attagatgct cctgcatttg tgaaatggtt ataagcatag aaaaataggt

15181 ggtaagcttt ccttctttcc ttattttgtg tgatgcctta aactgaaaag ttaaaaattg

15241 atggattgta gcattcccat aatctccccc ttctttttttt ttcctttgga aatgtccaat

15301 agtctatatt cctctgtccc gcccaaacac catcttcact ccaagcctac cacagatgcc

15361 tgaagaagtt cctcactatc tgcaaatgtg gctctcaggc ccttcctgat gtgatgaatg

15421 aatctactaa tcatttcttg accattcatt ttatcacttc taaccttgaa acatgtggaa

15481 gtagctatgt tcctgactgt ttcctctgcc agacaatgaa ctctggagat cagggagctt

15541 **cgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg t**gcgtgcgcg cgcgcgtgcg

15601 cacgcacgtg catgcacatg ctatgtattg ggtccctcca aggatgaacc ctctctttgg

15661 cttagaaggc actcagagaa tatgtgttat tcgtgctcac ggaaagtttc ttactcatcc

15721 ctgtgacttt ggctttattt tacaataaaa cactgaaaat gtccactttg ttagttgtga

15781 acatgagccc aggcctaagg tgctgggaaa cagaaagggc gggagatttt tctttattct

15841 atggctagaa aatagttacc tcctctctga aagtcttctt cctcatttct gggtaacaga

15901 atatcaaaca ccttgcttat aagttataaa gtagtgttgt ccaccatgaa cccaccaagt

15961 aaaaacaacc caaataccta tcatggatga ata

3' UTR + downstream sequence

$(GT)_{20}$

- ***Il1rn* gene sequence of C3H/HeJ strain – NCBI Genbank entry**

LOCUS DQ383808 15997 bp DNA linear ROD 26-FEB-2006

DEFINITION Mus musculus strain C3H/HeJ IL-1 receptor antagonist (Il1rn) gene, complete cds, alternatively spliced.

ACCESSION DQ383808

VERSION DQ383808.1 GI:88595944

KEYWORDS.

SOURCE Mus musculus (house mouse)

ORGANISM Mus musculus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;

Sciurognathi; Muroidea; Muridae; Murinae; Mus.

REFERENCE 1 (bases 1 to 15997)

AUTHORS Ramadas,R.A., Li,X., Shubitowski,D.M. and Ewart,S.L.

TITLE Direct Submission

JOURNAL Submitted (30-JAN-2006) Large Animal Clinical Sciences, Michigan State University, 242 National Food Safety and Toxicology Center,

East Lansing, MI 48824, USA

FEATURES Location/Qualifiers

source 1..15997

/organism="Mus musculus"

/mol_type="genomic DNA"

/strain="C3H/HeJ"

/db_xref="taxon:10090"

gene <1813..>14460

/gene="Il1rn"

mRNA join(<1813..1822,10350..10401,12296..12384,

13346..13458,14245..>14460)

/gene="Il1rn"

/product="IL-1 receptor antagonist isoform"

/note="transcript for possible intracellular isoform;

alternatively spliced"

CDS join(1813..1822,10350..10401,12296..12384,13346..13458,

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/note="possible intracellular isoform; similar to

NM_031167; alternatively spliced"

/codon_start=1

/product="IL-1 receptor antagonist isoform"

/protein_id="ABD43199.1"

/db_xref="GI:88595946"

/translation="MASEAACRPSGKRPCKMQAFRIWDTNQKTFYLRNNQLIAGYLQG

PNIKLEEKIDMVPIDLHSVFLGIHGGKLCLSCAKSGDDIKLQLEEVNITDLSKNKE

EDKRFTFIRSEKGPTTSFESAACPGWFLCTTLEADRPVSLTNTPEEPLIVTKFYFQE

DQ"

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14245..>14460)

/gene="Il1rn"

/product="IL-1 receptor antagonist isoform"

/note="transcript for possible extracellular isoform;

alternatively spliced"

CDS join(10283..10401,12296..12384,13346..13458,14245..14460)

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/note="possible extracellular isoform; similar to GenBank

Accession Number M64404; alternatively spliced"

/codon_start=1

/product="IL-1 receptor antagonist isoform"

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/db_xref="GI:88604776"

/translation="MEICWGPYSHLISLLLILLFHSEAACRPSGKRPCKMQAFRIWDT

NQKTFYLRNNQLIAGYLQGPNIKLEEKIDMVPIDLHSVFLGIHGGKLCLSCAKSG

DDIKLQLEEVNITDLSKNKEEDKRFTFIRSEKGPTTSFESAACPGWFLCTTLEADR

PVSLTNTPEEPLIVTKFYFQEDQ"

repeat_region 15543..15584

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/rpt_type=tandem

/rpt_unit_seq="gt"

ORIGIN

1 ttcattcaac agatgtttcc aaagtcacca aaatacagtt tacaaagctg atatttgaag

61 ctctgaatgc cagaccttgc agtaccacat gacctcctct tgtcatatca gatatgcaca

121 ctagacaggc tcccccaaca acttccagac ttccctccca gagcagcttc ccaggtttgc

181 aaagctggag tttgtagagt ttccaggttt gcaaagctag ttcctgattc ttcacaaaca

241 tttctctaca aaagctcctt tctaattcct agttaaggct gaaagaccta gacagtctag

301 cttggtaagc ctaaactaac aagagcagga agttagggcc caatgggaag tccctgcggt

361 tcagagtgcc aggcagggat cctttctcac gtcagggcct cagtgcctcc cacacacatg

421 tcagtcccaa tagagagacc ttgggaatga gtccagatga acaaacagct agaggaaaag

481 caggggactt tggccaggtc actcaagtga gggtgagctg tcctgtccca tccgcagaga

541 cagacttgca ggtggcaaga ttacaggcag cattatgtct gcctgccttc tccttcatct

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661 ttttatggtc tgcatatcag tctaaggctg ggcagggagt gtcaggttgt ttttgcttcc

721 attaaagtaa ggcctagagg ggaaaatgac atgcccaggc tctctcaaag agttactgtc

781 agcccaaggg atcacaggtt gctgttaccc acctatctgc ctaatggttg ttaagcacac

841 aagagttgtc cactccttgg tgggagggca cagaggcagg aagccagagc aagttgtttc

901 tgagctgcag aggaagagga gtcagagaat tgagaacttc cagagaaagt tgagagagtg

961 catctggcct tctggagtca gtcttgacag tggcgcattg gtgaaatgca agcatacttg

5' upstream region

1021 gaaaatgggg taaacctcta agtgatgctg tgatcacata gtagtagtag tagtagtagt

1081 tagcataata ctcagatcat taaatatccc atcccaagtc atcttccttg ctgtttcctg

1141 tggttgtaac attctttccc ttccccagct caaatgccac cattctcaaa gaggcttccc

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1261 tttaccccca tagagaatta gcttatttta ttctttccct ctccactttc tctcattaga

1321 acacaagtgg cctttgtttg gcttgcaacc ggtgtttgtg agagcacttg taagtaccac

1381 aggcaggact aagtcagaat gtgttttaaa caaatttatg cacacataat ggtctcataa

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1861 ttgtccaccg ctagtgttga ctcaatgctc aatgccaggt gcatggagca ctaggctatc

1921 aggaaaatct ggccaatggg aagccacaaa atcccaggaa actacctaga tatgctgggg

1981 cagagagggt atgcccgata tgagaaaagt catcattgaa tggtgttggc cattctccac

2041 tgtttgtgaa aacaagaaat agatgaaatg tctctgtaag atggtgaaaa gaagtatgtg

2101 tgtaatgaaa actgttgata taaagtcatc tgtgtccaag gttgaagtgt gatagagaaa

2161 gatagagtct gcagaggagt aacagattga cacttaggca acatgtttgc catagagaat

2221 ataggctcag gtggacattc ttaaagggaa agccaaatat tatctgtgct actacctggt

2281 acttccaaaa ccagacagaa tcagacacat taatgttaca tgctggtgag catgtattgg

2341 gttgaaccag tgcaagcaag cacaatatag acaccacagt ctctgaaaga aggaatcaga

5' upstream region

Start site for i/c isoform

Intron 1

[8] Start codon for the intracellular (i/c) IL-1 receptor antagonist isoform

2401 aacagcatca aaatccaaag agatgggagc ttccaaatgt gaccaagcag aaaccatggc

2461 tctaagaacg gagaagttaa gcatgtgact tgatgggggg tggggcttgc agagtaagga

2521 caggagcaag ggtgtaccct gtgcttccct gggactttac tgtctctctc catggagtct

2581 ggcttcttgc ctcacagatg atgcttgtaa agaagggagg gagaaagaga aggagaaagc

2641 aatggtaatg ggaagtataa atcctgttta ggacccgcat ctgaagatag tctaagaatg

2701 ctagacacta acattgtctt aagataaatg gcttaacttg gaatgaatca atggtggatg

2761 tgaccattct tttatttact atggtctctt cctgggtgag aagttcattt ttggcttttg

2821 tatatatttc agttatctat ttgtctgtct gtctgtctta tttataatct atgaatttgt

2881 ctatcatcca aactgcatac catctatcca aatggatcgt acctttgcca ataataacaa

2941 gctaaattag caagtgacag tcattaagtc attgtgtttg caatttgtgg caccatccac

3001 caggattcac aatttaggtt ttcaaagact gaggaggtca ctgaagtata gaatgttcag

3061 taatgaagct aaaatagtct ttgacaaacc aaggcagttt gccatcctag tccataacca

3121 agtaaaatgg ctttcattat agcctataca aaggttttaa attattttcc ttacattcca

3181 catgcttttc catatttcca agcagttatg aactaaatag ttactccata ggataatgag

3241 ccagagccaa acatttaaat cataagtaaa ggtcttgctc ttgttgacct tacaatgaag

3301 caaagaaggt aaacacctgt agcacaactg tatgttgtat gggaaagaac aaaaaccaga

3361 atttaaacct tgccttagac agcgtctaaa agaaatgtag tctttctggg ccttgagttt

3421 ttttctgcaa gctatccagg ttcattccca tcatgaacgt gtgttctatg cccggttggt

3481 gggctgtctg gggaatttag gagatgcagc cttgcttgag gagagcatta cagtgagtag

3541 gaccgtttaa cacctcagtc cacttccagt tcatatctgc tttgtgtctg tggttgaaaa

3601 tgtgaactcc caactgcctg ctccagccac catgctttcc ctgcaatgta gactcgtatc

3661 ccttcttgag ccatataaac tctttttttt tctataagtt tcttcaaatt atggtgtttt

3721 atcactgcag cataaaagta gtgaatgtga aaacagaggt gggcacagca aatgacagca

Intron 1

3781 cagctgtgcc ctctctcaga attcctgccc atctccaaat tctcttgcct tatgaagtta

3841 accatcagag aaaagagtca gcatgtacct ctgaaaatgt aataactata aaaacaatga

3901 cgttcagacc aatacatatc acccgcacac catggaatta aaagccatct aggaagactt

3961 aacctcagaa aggattcagg gaaggtttcc tatctggatc tgggaagaaa ttgagcctct

4021 tgaaaagcac tgatccacaa ttgttggttt atgccctgtc tcctgaatga cctttgctag

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4441 gggctacgag gtgattttgt tacaacttag ggtgcatatt tattcaccca aggccaaatg

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4681 agaaacttga acatatgggc acagtggaaa ttttcctgaa cagaatacca atggcttgtg

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5041 aaatgcaaat cataacaacc ctgagattcc acctcacacc agtctgaatg gctaagataa

5101 aaaactcagg tgacagcaga tgctggcgaa gatgtggaga aagaggaaca ctcctccatt

Intron 1

5161 gttggtggga ttgcaagctg gtacaaccac tctggaaatc agttttgagg tgcctcagaa

5221 aattggacat agtacgatcg gaggatccag caatacctct cctgggtata cacccaggag

5281 atgctccaac atgtaataag gacacatgtt ccactatgtt catagtagtc ttatttataa

5341 tagccataca ctgggaagaa cccagttgtc cctcaacaga ggaatggata cagaaaatgt

5401 ggtacatttt cacaatggag tactactcag ctattacaaa caatgaattt atgaaattct

5461 taggcaaatg gatggatctg gaggatatca tcctgagtga ggtaacccaa tcacaaaaga

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5701 agcagagact gaccgtaagg ccatccagag actgccccca cctggggatc catcccatat

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5941 gagttaaagg ggtttgcagc cccataggag gaacaacaat atgaactaac cagtaccctc

6001 agagctccca gggaataaac caccaaccaa agagtacaca tcgtgggact catggatcca

6061 gctgcatatg tagcagagga tggcctaatt gacatcaatg ggagaagagg cccttggtcc

6121 tgtgaaggct ctatacccca gtgtagggga atgctagggt caggaattgg gagtgggtag

6181 attggtgagc agggaggagg gggaatggga tggggagaca gggttttgga gggaaatga

6241 gcaaagggga taacatttta aatgtaaata aagaaaatat ccaataaagt caaagaagga

6301 ggaggagaag gattgtggat cagtagcagg gagaggaaga gttgtacaag ctgcacatgt

6361 ggaccaagac tgtaggggtg gtagacactg tgactcatcc ctcccatttt gcctgaagta

6421 aggaactatg cacaatcact actgtttgag taattcccac gtgccagtac atcatctcag

6481 agtcttactg aattctcata gcaacccaca tcatggtttc cagtattaaa cctggttaat

Intron 1

6541 gggtaatgaa actgagatct agagagtctg ggcaccctgt ctgaggcagt gatgtttcag

6601 aatgatgtaa gaggattgaa acgtgaggtc agcccaagaa ctcctttcct gcacactcgg

6661 aaagaacata ctgctggcct ttgtggaggt gagagtggtc atgggaagaa ggcacagacg

6721 aggggagtct gggagcccaa tctgtccaac ctctaggaat agtgtcttcc catctgaatt

6781 tgggctgaga tcagagaaac attactcatc agctatcatg tgggggcagg ggggaaggat

6841 ttcacttgga tagctgtctg agggcagcta tggagcctcg ccattcacct gccaagtcac

6901 atctcttcca cagtcttcaa gaatgtttga gatgatgata ctgatcagat gttctagcaa

6961 agtctcgcca gtttgctaaa gtttggctcc tttagacact tatcctcatt aagaatctct

7021 agttttcatt caccgctata tcagtagcca ccaaaacttc ggttttcaaa aaaacaacat

7081 tctataaaaa tctgaaaatg gaatgaaaag aataacaaac cctaatatgg taaattacat

7141 attaagaaac tgaaagaatg tataacttaa tgacattctt gtatcctgca tatagaaatc

7201 acatcacttg taaatatcat aatcacagga caatgtgata taaataattt gtgcttcact

7261 tcaactatat tgtcttgttt ttcttgtgtt aatttttgtc ttcctatgaa tgcataatta

7321 ataagaagtt gaatatattt aaggcatagg cttaacatcg tgttataagt tgcagaatat

7381 tcaccataat caggctaatt aacacatctt cctcctcaca tagatcccat tttctttctc

7441 cttccttttt cctccctccc tttccccttc tgtccctctc attttccttc tcctttcctt

7501 ttagagggga gaggtagtga aggctactca gcaaccactc ttgcaagttt cagtgtttag

7561 taaacagtac tatcactgtc ataagatcct cagataacgc tcaccttagc aacttggcat

7621 acattagatc tgtgaagtgg cacagaggca gaagagagac aggcgtctgc ccttcaaccg

7681 atggggtagc ctctctccct cagagaactg gatggtctac caggaggcaa agatactgca

7741 atcctttata gggatggggt ggggagtttg ggaacgagga aattgttcct gctgtagtgg

7801 cctacacatc tctggttcct gatgttgttg gctcaaagcc agacaggctg tcctttctgg

7861 ggcaactggt aaccgttgag caaagtcacc tgcactctct taggaatcct gctacacata

Intron 1

7921 caactgaggc tccaatcctt gcatttaact ctatgcattc cttgtcatct cctgcctctg

7981 gcaattatca acctattgtc tacttctgaa ttttaatgtg aattttattg atatgcaaaa

8041 tgtagcattt gtacaaataa ataagtaata agagagtcca gagtggcttc aaaagcacat

8101 taaacaagca taagagcttc tgggagcagg atggcagctg cttctccttt cagcagctgt

8161 gatagcaaca gtttgtacct gatgactaat aaacctttca gaaacttgaa agcttaatta

8221 attcaacagt ttctggatat tgtaaataac tagacaagtt tatgcacatt ccctctttca

8281 gccagcttca caaaagattt tcaaaacaag aaatgagcaa atagaatagt cccttgactg

8341 tcacaagtag atatagcatt tttctacagt cactcaagaa agatatggac ttgccatttt

8401 gactctcaaa aattatcatc cagcattgta gtagcagaca tgtcccattt tgtggggtgg

8461 gggacagaag aacttcttaa agacttgata gttctttagc ttttccatga aaggatttaa

8521 ctctttgaag atactctggt ttcttgggtt gaaataaggt cagatgcatc cattgagcag

8581 tggtgggcac acctttaatc ccagcatgag ggaagcagag acaggaggat ctctgagttc

8641 aaagtcagcc tggtctacaa agaatgttcc aggacaccca gggctataca gagcaaccct

8701 gtctcaaaaa caaaaagaaa aaagaaaatg aggtcaaatt catcaagatc aatgagtgcc

8761 ataagaactg gggtttgatc ctctgccatt aacaatctct acaacttttc ctaatgtctc

8821 tttctacatc tctttaaagt agtgagaggt tgagataagg tgaccactat gctccaaccc

8881 agttctgaaa atcctctgcc tgtgctcctg acagcactct cccttgttgg aacacacaag

8941 gactctcttc acctttgat aacataaact aggaagagcc ttacctttcc ccaactgtcc

9001 agaaaattgt gtgaagggaa cttaatgttt tttaatgctc acttgggatc aaaatttaac

9061 tcccttcttt cctactacct ccaagaaagc catgatcctc ctcattctga gaataaagaa

9121 gcagagacac aaataaaaga ttttttcaag gtcacacaga tgatagtgac aagcagcaaa

9181 gaccggttgc tgcccacaca cttaatccac ttttccatga tttgggaatg aagtcacctc

9241 taaaggactc caacttcaca gaggaccacc acaataggct cttgtctgca aacataacag

Intron 1

9301 agtatcatta atgatgtcag ggattgatgg ttgcccatgg gatggtctca agttgggcca

9361 attattggtt ggccattcct tcagtctctg ttccatagtt gttctgcatt tttaggggag

9421 acaaattttg aaccaaaagt tttatagttt tgtgtgcatt tggaatgtaa ataaataaga

9481 taattaattt tacaaaagaa acttcaactt tccagatgca gaattgggaa aagatggcca

9541 attttaacac acctcttggg aggacttatt tctctagggc agaggtcagc aaacttctac

9601 actacgggtg acctttcctg ttttgtaaac aaggcatgtt ggagcagagt tgacttttct

9661 ggcttacaaa ttgcttgtga gcgcttttgc accatgacag ccgagctgag tattatgtta

9721 gattggatag ttcacaaaat accaaatatt taccatccgg ttctggacag aatgtttatg

9781 tctagccaac tgtcccttct ccaaagaaca caataacagt gacatgacac tgtcctttgt

9841 tcaccaggcc ctattgcttg ccttcaaatg aaaaggggac atttctattc agatctggtt

9901 ttttttgggg ggggaggggg atcgggacag ggggatcagc aaatagactc ggagtacctg

9961 tcatgcaaat gagggagtct ggttttcatt gtgctcttct tcccaggaac accatgaagg

10021 ggaaacagag aacttaattt tggggaaatt acacagggta agggggagga gatcagttac

10081 aacacaccat tgcgacactt tcagggttga cagcgacagc agtaaaggtt tctcttttg

10141 gaaatatgag ggtttttccg cttctgacag tggaacggaa tgacagcagc acaggctggt

10201 gaatgactac tttctttata agcaaccacc ttgagcctga aatggcagtc gctagtctct

10261 attgccttgc tgtggcctcg gg[9]***atggaaat ctgctgggga ccctacagtc acctaatctc***

10321 ***tctccttctc atccttctgt ttcattcaga*** [10]***ggcagcctgc cgcccttctg ggaaaagacc***

10381 ***ctgcaagatg caagccttca g***gtaagtctt ccaaagacac aggattgcat agaccaagga

10441 ccagagacac atgccatatg tccagagcat atgcaggaat aggagatata tatacatgta

10501 taatatatat aatgcgtgtg tgtatgtgtg tatacacata tgtatgtatg tatgtgtata

10561 tatatatata tatatatata tatataatgt gtgtgtatac acatatgtat gtatgtgtat

Intron 1

Exon 1

Intron 2

[9] Start codon for the extracellular (e/c) IL-1 receptor antagonist isoform

[10] Alternate splice site where the start codon and subsequent sequences of intracellular (i/c) isoform joins to form the first exon of the i/c IL-1 receptor antagonist isoform.

10621 atatatatat atgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtat ccttgattca

10681 agaacagcat gctaaatgca gtctttaagt cttatgtttt aaaatattcc atgcatggac

10741 aacaagacag ttaactgtgc tcactttctc agacacctag atgttcagta agtgatggac

10801 aggcatccgg gaataatgct agctttggga tcgagcaaag aggaatactt cagcaggaca

10861 cagtcaaagg ctcagaccaa cagtctacac tctgtatctg tgttgacttg gaagatatct

10921 ctcgttggag tccccagttt ccttatctgt aacatgatac tgctctgatg ataaccccct

10981 gtgtgcctta cagggtgaac actaaataca tgagtgatac tgtaaccatg ttctgagacc

11041 tatgctctga gaactgtaaa gtgcctgaaa aataacctga gttttaaaaa ttggatcaaa

11101 agccttggga gatgccatca accttatagt aaaaatggca ggcctcgatt ttgattttaa

11161 aatgaataaa gagattgttg gtgcatatga tctgttcttg atccttcctg agagtgaagt

11221 ctgtgttgag tcacttcccc tttgaccctg tctgctttgg atccacagct ggaggctggg

11281 actctaactg tgattctata catctatccc aaggcaagtc tgtcccacag atccagtaac

11341 tgcttcgtga gatttaccat catcacatcc tcttagcagc ctcaagagag gtccctggag

11401 tcctgttagc aagactattg agtcccttga gtttgaagct caccagagat atagacacca

11461 gtcacaaagg cacaaatact ctttcacgtg cagagtactt ggtttgtcct ccacccatcc

11521 ctgagctcct aggctgctcc aagctactca aaaagtcctg tcagctctgc tgaccaggta

11581 aagagataag ggacagatcc aaggtcatat catcaggcct cttaccacac ctcacaggtg

11641 cctgcctctc tggaagccag agggcctttc accaagaagt cagagagtaa caaacaggcc

11701 ctggctgagc tagacaggaa gctgacttat ttccaaggac agctgtccct gtcaggccca

11761 gagcagatgg tcccacaaga ggttttagtt gtagacttgc aggtctaagt agagtagctt

11821 gaggtaggag tagtggagcc agactagctt ggctacaata cattctaacc cttgaacctg

11881 taacactatg atgtggtggc cacgagctac aagtggccat ctaaatttac acataaacgc

11941 atgaaagcag aagaaagtcc tgtacctggc aactctattt agtggagtga ctataggatg

Intron 2

12001 tgcttgcatc gcctaagttt ctatcagatg ctgacgctct atagaaaatt ctgctaaagt

12061 catggatgtc catgctggga ttctgaggtg aggaacaaga aaaagaggtt ttctgttcac

12121 cagatgtgag agatgggctc atttcttaca tggtatttgc ttaaatcttc ccatttgtgt

12181 tatgaacttg gtaagtacga cacttccagc aagtctagat gtaaattagg tgactctgag

12241 gaagctggaa agggctctgt actgcctact ccagctaggc cattttgctt ttcag***gaatct***

12301 ***gggatactaa ccagaagacc ttttacctga gaaacaacca gctcattgct gggtacttac***

12361 ***aaggaccaaa tatcaaacta gaag***gtgagt ggataacagg gaagctggtg taatatggac

12421 atagagtcct ttgccctgct cctctgcctg gaggtgggat gtcctcattt ctgttgagtt

12481 ggaaatgaga gatttgacca ccaggggaca tatgggagtg gcctcaagag agcagaaaag

12541 ataaagactg ggtcacaatg ctccagggac acagctgaga ggaacagagg ccagaaggca

12601 cctgggcacc tccttagtcc ttctgtgctg gtagtccact ataccccagt gttattcgaa

12661 ctctaccctt gccctaggct aatataacat gtatgtgggc tgggtagcat ttttactgtg

12721 gacaccaccc tcatcatgta ccctctaaac taggacaaag ccacatgaac ttggaggagc

12781 attacccaca gattcttcag ttttttcttt agaaaaaatg agggcactta gttgacagaa

12841 tctctgtttg tgagggaacg aagcattact tgtatctcct caggatcccc caagccttct

12901 gctttcctgt atcactcagc agttatgcaa ctggcttttc ctgtctttct agtaattctc

12961 ccatgaacac actcaagcat agaaggtgct ggctttctat tgctacccag taacaggatg

13021 gaaaggtgaa ctgtgtggaa cctattcatg ggccttgtga gcttttgtgc ctctgtctac

13081 taacagcaaa tctgttgact tggaggtctg gttcactgta gaaagtaaag gaaagttggg

13141 agcagtgtag aatctaggaa gctggtcctt acatagagtg tgctcatttg gatcttttgc

13201 ttggaggcag actagaaaga tagagccttc ttgaccttct tgaccttcta gttttataaa

13261 aaggaagaca gaaaatacac acagacgctc ccctaccctt gcctcctctt ctctctttct

13321 gacaccatcc tctactcttc tccag***gaaaag atagacatgg tgcctattga ccttcatagt***

Intron 2

Exon 2

Intron 3

Exon 3

13381 ***gtgttcttgg gcatccacgg gggcaagctg tgcctgtctt gtgccaagtc tggagatgat***

13441 ***atcaagctcc agctggag***gt aagaatctgg tttagctatc aaatccttct aaaacccaat

13501 ggttatgaca acctcaggtg tttctcataa ccctgagcat gcaaagatga gggaggcttt

13561 tccttcttca cagagtacta ttttgaggtc actccttaag cagtttccac aatgttcttg

13621 gttgatattg ggtgtccaag gtggtttctc attctctcaa ctacccttta cgtaacttct

13681 ttgcattcag tcaacactct gagcttcctt aagcgtggtg accaactttt atgagagatt

13741 gttccagaaa gatgagcctc aatgtgaaag tgcttattaa gcttgggctt atgtaagtct

13801 attggcagaa gcctgtgacg tggttgatat ggactcattg tagaaaggta ctgcacaagg

13861 atctaaactt taggaggaga catggtcatt agaggagcac gacctgaacc accatgggtc

13921 ttgtgcctcc taaaccagtt gagcctacct tcttctagca aggtcaattc tcaagactat

13981 acactcccaa gcatcatcta tgctatttat tatctacgct cctaatttac atcccacaca

14041 gacctgtgtc acttactcct ttacctagtc agtagtaatg ggctgttcaa acattatctt

14101 gagggattag ctggacaaac ttttaatcca actgcaaata gccacaagca tgagtttgtt

14161 gataactctt accaatggac aggaacacct tttagaggac tttctcagcc ctcggcaatt

14221 acctgaccat ttcttgactt ccag***gaagtt aacatcactg atctgagcaa gaacaaagaa***

14281 ***gaagacaagc gctttacctt catccgctct gagaaaggcc ccaccaccag ctttgagtca***

14341 ***gctgcctgtc caggatggtt cctctgcaca acactagagg ctgaccgtcc tgtgagcctc***

14401 ***accaacacac cggaagagcc ccttatagtc acgaagttct acttccagga agaccaa***[11]***tag***

14461 tactgccgag gcctgtaata atcaccaact gcctgatcac tctggccatc attggggcct

14521 gaggaacaac ttttgcaggg tgtatgtaca gtagaaggag acagaagagt tctgatgata

14581 gatctctgcc tcagtctgtt ggctggccta atccccatga tgattccaga ataatcttgc

14641 aaattggatc atggcaggtg cttgttcaaa gccctttctt gttgcctctg ccatctgggt

14701 gaagtctaga ccacttgctt ggcctaggtg tcttctgctc taccacccac cctacccctg

Exon 3

Exon 4

3' UTR

[11] Stop codon for both intracellular and extracellular IL-1 receptor antagonist isoforms

14761 ccacaaacac acacttttttt tgtttttgtt ttttccattg ttctgcactt ccacagtcca

14821 gaccaatcaa gtcacttgac aatatgcccc aagtgactcc cttaccctgt tttataaacc

14881 tgtgcctgtc tatggagaag gttttaattc tccttgttat tcattttggg ctttttgatg

14941 aaaccaccag ggcatcacat atactaagca tgtgctctac catcatgcta tgcttccagc

15001 tcaggggggc acttttaagg atctagaaaa cagaaattaa ggatctcata gttatttttat

15061 taggccagcc ttattccatg tcggcaagag gtttcttgtg gaaattatgt cctttctgag

15121 aggagctggg gattagatgc tcctgcattt gtgaaatggt tataagcata gaaaaatagg

15181 tggtaagctt tccttctttc cttattttgt gtgatgcctt aaactgaaaa gttaaaaatt

15241 gatggattgt agcattccca taatctcccc cttctttttt tttcctttgg aaatgtccaa

15301 tagtctatat tcctctgtcc cgcccaaaca ccatcttcac tccaagccta ccacagatgc

15361 ctgaagaagt tcctcactat ctgcaaatgt ggctctcagg cccttcctga tgtgatgaat

15421 gaatctacta atcatttctt gaccattcat tttatcactt ctaaccttga aacatgtgga

15481 agtagctatg ttcctgactg tttcctctgc cagacaatga actctggaga tcagggagct

15541 tc**gtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgt**gcgtgc gcgcgcgcgt

15601 gcgcacgcac gtgcatgcac atgctatgta ttgggtccct ccaaggatga accctctctt

15661 tggcttagaa ggcactcaga gaatatgtgt tattcgtgct cacggaaagt ttcttactca

15721 tccctgtgac tttggcttta ttttacaata aaacactgaa aatgtccact ttgttagttg

15781 tgaacatgag cccaggccta aggtgctggg aaacagaaag ggcgggagat ttttctttat

15841 tctatggcta gaaaatagtt acctcctctc tgaaagtctt cttcctcatt tctgggtaac

15901 agaatatcaa acaccttgct tataagttat aaagtagtgt tgtccaccat gaacccacca

15961 agtaaaaaca acccaaatac ctatcatgga tgaataa

3' UTR + downstream sequence

$(GT)_{21}$

- **Results from sequencing**

We optimized PCR conditions for all sequencing primer sets, and performed bidirectional sequencing on all the regions to ensure sequence accuracy, hence we are confident that our results are accurate and the sequences are of high quality. We aligned the A/J and C3H/HeJ *Il1rn* sequences with the Celera *Il1rn* sequence (mCG4837) and the sequence from the NCBI contig AL732528, which contains the murine *Il1rn* gene to check our sequence results. A previously identified microsatellite was confirmed in the 3' untranslated region (UTR) in the last exon of *Il1rn* gene. The microsatellite is a GT dinucleotide repeat, with 21 copies in the C3H/HeJ mice [$(GT)_{21}$] and 20 copies in the A/J mice [$(GT)_{20}$]. As this polymorphism exists in the non-coding region, it is less likely to contribute to a major functional difference. Apart from this, no other polymorphisms were observed between A/J and C3H/HeJ strains. We found only one other difference between the four *Il1rn* sequences we thus compared. A guanine (G) nucleotide was deleted in the Celera *Il1rn* sequence at position 15578 (position number based on Genbank Accession No. DQ383807). The missing G nucleotide was present in the NCBI contig AL732528, and was also present in both A/J and C3H/HeJ strains in our sequence. We conclude that the deletion in the Celera *Il1rn* sequence is probably due to a sequencing error. We have sequenced all the exons and introns of *Il1rn*, and have sequenced all the regulatory regions reported in the literature that are important for transcriptional regulation. This low level of genetic variation in ~16 kb of examined sequence extends a greater distance than the commonly reported single nucleotide polymorphism rate of 1/1,000 bp as a theoretical possibility across the genome[164]. However, the mosaic structure of the mouse genome, as recently described by Wade and

colleagues, results in long segments of DNA with extremely high (~40 SNPs/10 kb) or extremely low (~0.5 SNPs/10 kb) polymorphisms rates[165]. Thus, the genomic region containing *Il1rn* appears to reside in a low SNP block. The lack of genetic variation in coding sequence may further indicate strong conservation pressure on this gene and underscores the importance of *Il1rn*.

C. TRANSCRIPT AND PROTEIN STUDIES IN IL-1 RECEPTOR ANTAGONIST AND RELATED GENES

- **Introduction:**

Along with the sequencing of *Il1rn* described previously (Chapter 2, Section B), we also tested for the mRNA expression and protein production levels in our mouse model of allergic asthma. IL-1 receptor antagonist is a major component of the IL-1 complex of genes consisting of IL-1 agonists, antagonists, receptors and accessory genes required for signal transduction. With the IL-1 receptor antagonist operating from within such a complex, its effects on asthma can be better interpreted in conjunction with the other genes. The IL-1 family genes present within the QTL *Abhr1* include *Il1rn, Il1f5, Il1f6, Il1f8, Il1f9* and *Il1f10*. Of these, *Il1rn* has been shown to be involved in a variety of inflammatory disorders[104,105], while all the other genes have been discovered and mapped fairly recently[166]. The roles of the genes *Il1f5 – Il1f10* in asthma haven't been clearly elucidated. Previous studies in the same mouse model have shown that there is a clear temporal pattern in Th1 and Th2 cytokines in both the strains of mice (Li et al – manuscript in review). In a similar manner, we mapped the expression profiles of the

genes in the IL-1 complex, with an emphasis on our positional candidate gene *Il1rn*. This will help to expand our understanding temporality of the cytokine patterns in this model, and most importantly, elucidate the role of *Il1rn* and related genes in the pathophysiological mechanisms. IL-1 complex is being actively investigated as a therapeutic target to devise asthma intervention strategies[119,120]. Elucidation of the temporal patterns of *Il1rn* and related genes would shed light on their role in airway inflammation and airway obstruction and assist such processes. The maximal AHR in our mouse model was observed at 72 h after allergenic challenge[16], hence we decided to measure *Il1rn* mRNA and protein levels at various timepoints (6, 12, 24, 48 and 72 h) after allergen challenge.

- **Experimental time line**

Age-matched, virus-free, A/J and C3H/HeJ male mice obtained from the Jackson Laboratory (Bar Harbor, ME) at 4 wk of age were allowed to acclimatize for 1-2 wk before experimentation. They were housed under HEPA filtered laminar flow hoods in an environmentally controlled facility and allowed free access to ovalbumin-free rodent chow and water. All animals were maintained and treated in accordance with the specific guidelines provided by the All University Committee on Animal Use and Care of Michigan State University.

A/J and C3H/HeJ mice (n = 6/group) were sensitized with 10 μg chicken egg ovalbumin (crude grade IV; Sigma, St. Louis, MO) in 0.2 ml calcium and magnesium-free phosphate-buffered saline (PBS) or an equivalent amount of PBS alone on day 0. On day 14 mice were anesthetized (ketamine, 45 mg/kg, intraperitoneally and xylazine, 8

mg/kg, intraperitoneally), challenged by pharyngeo-tracheal instillation of 1.5% ovalbumin in 45 µl PBS, or PBS alone. The mice were sacrificed and the lungs, tracheobronchial lymph nodes and spleens were collected 6, 12, 24, 48 and 72 h post challenge (Figure 3). Lungs were collected because they are the pertinent foci of inflammation in the asthmatic process. Spleens were collected because of their importance as a peripheral component of the immune system containing T and B cells which play a major role in inflammation.

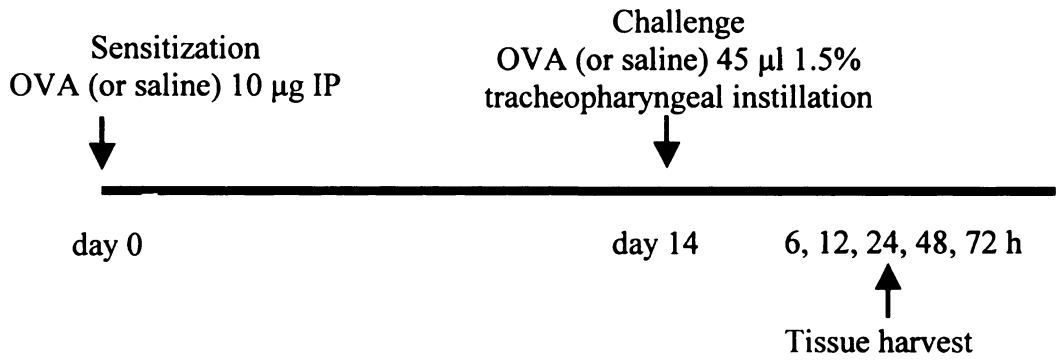

Figure 3. Experimental time line of in vivo allergen exposure

- **Real-time RT-PCR**

A variety of techniques are available to detect mRNA, such as the classical Northern blot hybridization and the recent quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Northern blot hybridization is only semi-quantitative, and requires radioactive labeled DNA or RNA probes, while quantitative real-time PCR is much more sensitive than Northern blots. There are two major types of real-time PCR chemistries – SYBR Green based assays and 5' nuclease assays (commercially known as TaqMan® assays). These are the most widely used assays for

detecting mRNA expression at present, and possess numerous advantages over the conventional RNA detection methods[167]. They are very sensitive in detecting RNA transcripts in very low copy numbers, have very high sequence-specificity, require very minimal post-amplification processing and are amenable to high-throughput analyses. Moreover, availability of efficient primer design software, experimental design and data analysis protocols developed for these assays also helps to minimize the causes of intra- and inter-assay variations and enhance the accuracy of quantitative results.

SYBR Green real-time PCR technique uses a PCR buffer containing the fluorescent dye SYBR Green, which binds to double stranded DNA products that are produced during the PCR process. As the PCR proceeds, fluorescence is produced proportional to the amount of double stranded products formed, and is quantified by a laser-assisted fluorescence detection system. It is a powerful technique, but the only downside is that it detects all double-stranded products, and hence careful primer design is required for accurate quantification. Non-specific amplifications can be easily detected by performing a dissociation curve run subsequent to the real-time PCR run. The non-specific products with smaller sizes dissociate at lower temperatures while the products of the correct size (usually between 80-150 bp) dissociate at higher temperatures.

Compared with SYBR Green techniques, TaqMan real-time RT-PCR assays are more specific, and these involve a forward primer, a reverse primer and a probe that is located between the two primers. The primers and probe are sequence specific, and they span a gene-specific location of about 80-100 bp. The probe is labeled with 5' reporter dye (FAM or VIC) and 3' quencher (TAMRA or non fluorescent) dyes. During the PCR process, the 5'-3' nucleolytic activity of the AmpliTaq Gold polymerase enzyme cleaves

the probe when it is bound to the target. The reporter dye is released from the cleaved probe and emits the fluorescent signal, which is then detected and quantified. The PCR reaction proceeds usually for 40 cycles, during which there is an exponential amplification of the PCR product. The results obtained are in terms of cycle threshold (C_T) values, which is the cycle number at which a statistically significant signal over the background signal is detected. The C_T value is used as the index of the original copy number of target mRNA (Figure 4). The higher the original copy number of target mRNA, the lower the C_T value obtained. One C_T unit difference is equal to a two-fold difference in target mRNA difference.

Since we were more interested in the relative gene expression between A/J and C3H/HeJ mice and between PBS- and OVA-treated mice, rather than the absolute gene copy numbers, a relative quantification of gene expression method was used. We used 18S rRNA as an endogenous reference (internal control). Equal quantities of total RNA from lung tissues were reverse transcribed and diluted five fold with nuclease-free water. This product was used as the template for real-time PCR reactions (more details available in 'Materials and Methods' section). Real-time PCR was performed on all the samples in duplicate and the average of the two duplicates was used for further data analysis.

If the difference in C_T value between the duplicates of a sample was more than 1.0, data from that particular sample was excluded from the analysis. When the real-time PCR amplification efficiency is 100%, a C_T difference of 1.0 translated to two-fold differences in mRNA expression. When one of the duplicates did not work, data from such samples were also excluded from the analyses.

A standard series was used with each run and data were obtained using the standard curve-separate tubes method[168]. A series dilution of 20, 10, 5, 2, 1 and 0.5 ng of total RNA of a single sample was used as the standard curve.

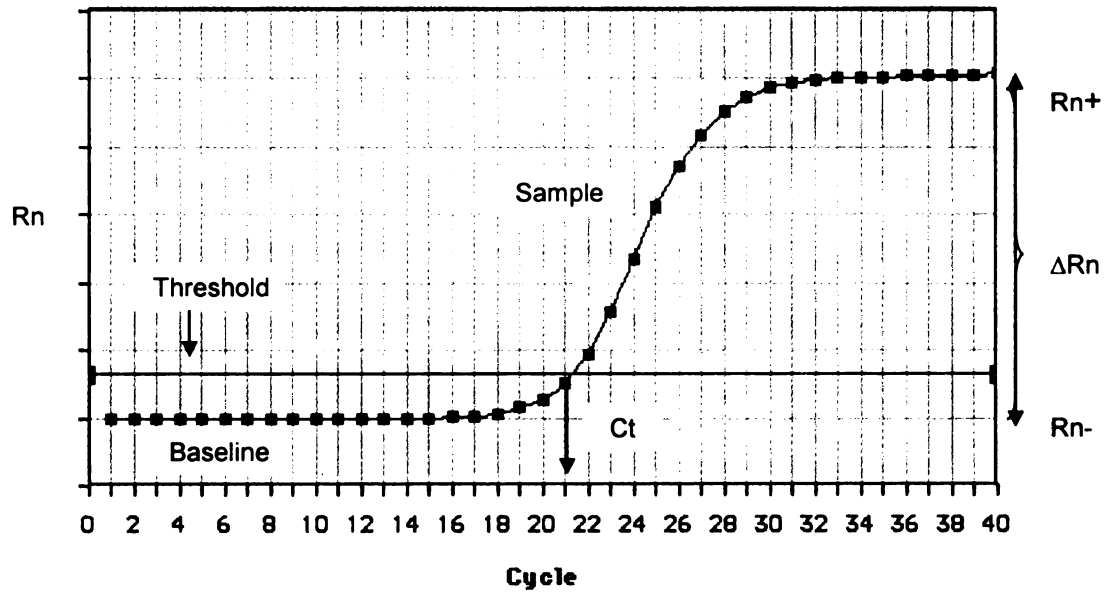

Figure 4. The threshold cycle (C_T) of TaqMan real-time RT-PCR. Rn is the ratio of the emission intensity of the reporter dye to the passive reference (ROX). Rn- is the Rn value of the unreacted sample or early cycles without signal increases (baseline). Rn+ is the Rn value of a reaction sample. ΔRn is the difference between Rn+ and Rn-, which shows the signal increases due to PCR amplification. The threshold cycle (C_T) is the cycle at which a statistically significant signal increase in ΔRn is first detected.

All the samples in a single timepoint were assayed in a single plate with standard curves. The same standard curve was used for samples assayed from all time points for any single target gene, facilitating comparison across time points. The amplification efficiency of the standard curve was used to quantify the expression levels in the samples.

The expression levels in each strain/treatment/time group were averaged, and the averages and standard deviations were used to calculate the relative expression of target genes after normalizing the values to $18S^{[168]}$. Normalized gene/18S value of the 6 h–PBS–C3H/HeJ group was used as the calibrator, and the expression of strain/treatment groups within all the timepoints were calculated in relation to this value.

- **Data analysis**

The ratio of the RNA value of the target gene to the RNA value of 18S rRNA for each sample (ratio) was used as the input data set for statistical analysis. The data was analyzed using two or three-way ANOVA tests using PROC MIXED model in SAS v 9.1. Model significance levels were tested for the factors time, strain, treatment and the interactions time*strain, time*treatment, strain*treatment and time*strain*treatments. Differences of least-square means were then calculated for these factors and interactions. All the p values obtained from the differences in least square means were adjusted for multiple comparisons using Tukey (for balanced datasets) and Tukey-Kramer tests (for unbalanced datasets). A residual plot of predicted values vs residuals was analyzed, along with normal probability plots and stem and leaf plots, and if trends are observed in residuals due to lack of normality or unequal variances, the input data (ratio) was log transformed and the analysis was repeated using the transformed data. All the significance values indicated in figures 4-7 are Tukey-adjusted significance values ($p < 0.05$). These values were obtained by comparing the least square means between time*strain*treatment interaction groups in the three-way ANOVA approach. In certain situations when the entire dataset was used for analysis, adjustment for multiple

comparisions led to the inflation of Type II error rates due to the presence of too many unplanned comparisons. In such situations, we used a two-way ANOVA model instead of a three-way ANOVA model, and investigated the strain*treatment interactions within each time. We followed this approach because the most important effects that we wanted to identify from these analyses were effects due to treatment, strain and time, in that order. Using the three-way ANOVA, we were able to calculate significance values for the effects of treatment and strain, and the effect of time on treatment*strain combinations across all timepoints. The two-way ANOVA approach was able to calculate the significance values for the effects of treatment and strain, but could not capture the information about the effect of time on treatment*strain combinations across all timepoints. This was because the third factor 'time' was used to physically slice the dataset so that maximum information about the most important factors – treatment and strain, could be obtained from the model without inflating type II error rates and losing actual significance values. Two-way ANOVA approach was used for analysis of *Il1rn* mRNA and protein data, and three-way ANOVA approach was used for the analysis of mRNA and protein data of all other genes.

- **Transcript expression profiles for *Il1rn* and genes from the IL-1 complex**

IL-1 receptor antagonist

We observed both treatment-induced and strain-specific increases in *Il1rn* mRNA expression in the lungs of A/J mice at 6, 12 and 24 h subsequent to ovalbumin challenge (Figure 5A). The level of *Il1rn* mRNA in A/J mice returned to baseline at the 72 h time point. Expression of *Il1rn* was also increased in C3H/HeJ mice at 6 and 12 h, but it was to a significantly lesser extent than in the A/J strain and returned to baseline by 24 h. At

48 h, there was only a treatment induced increase in expression in both A/J and C3H/HeJ mice, but at much lesser levels compared to the earlier time points. In the spleens, no statistically significant changes in *Il1rn* mRNA expression were observed due to the effects of either treatment or strain (Figure 9A).

IL-1 agonists

The expression of IL-1a and IL-1b was examined in an effort to get a more complete assessment of the IL-1 related role in our model. While little is known about the mechanisms of the newly-identified IL-1 family members, *Il1f5, Il1f6, Il1f8, Il1f9* and *Il1f10,* they were also examined as they map near *Il1rn* within the *Abhr1* locus. The active agonist, *Il1b*, showed a similar expression pattern as *Il1rn* with significant treatment-related increases in expression in the lungs of ovalbumin-treated A/J mice at 6, 12 and 24 h time points (Figure 6A). Strain-related increase in expression in A/J mice compared to C3H/HeJ mice was observed only at 12 h time point (Figure 6A). In the C3H/HeJ mice, there was a treatment-specific increase in expression of *Il1b* at 6 and 12 h time points, but not at 24 h timepoint. In the spleens, no statistically significant changes in *Il1b* mRNA expression were observed due to the effects of either treatment or strain (Figure 9B).

Transcript levels of *Il1a* also showed significant treatment-induced increases at the 6 h time point in both the strains, but no strain-specific differences were observed (Figure 7A). Both treatment- and strain-specific increases were observed in the new IL-1 family member, *Il1f9*, at 6 h (Figure 7B), with the expression levels in the A/J mice being higher than in C3H/HeJ mice. The levels of all IL-1 agonists detected returned to (or

below) baseline levels by 72 h. We were not able to quantify the expression of *Il1f5*, *Il1f6*, *Il1f8* or *Il1f10* in treated or control lung tissues using the same template concentrations and real-time PCR conditions that were used to detect and quantify the other RNAs. These RNAs were not quantifiable despite using three different SYBR Green primer pairs.

IL-1 receptors

The active receptor, *Il1r1*, was not induced by allergen exposure in either strain (Figure 8A), but its expression was decreased in both the strains at 72 h. In contrast, the decoy receptor, *Il1r2*, showed an increased expression in the lungs of A/J mice due to ovalbumin treatment at 6 h (Figure 8B), but there were no significant strain-specific differences. The expression levels of these two receptors returned to the baseline level at 72 h after ovalbumin challenge.

- **ELISA assays for IL-1ra and IL-1β**

Of the cellular products, proteins are the most important drivers of biological functions. So, we tested for differences in protein production between A/J and C3H/HeJ strains under allergen challenge. Although we found no major genetic polymorphisms between A/J and C3H/HeJ strains, mRNA studies showed that there was a difference in the expression of *Il1rn* and IL-1 complex genes between the two mouse strains due to OVA treatment. To confirm these findings, we decided to measure the protein production levels of the positional candidate gene *Il1rn*, and its major agonist *Il1b* in a subset of time points.

Protein quantification can be performed by two major methods – western blot and enzyme-linked immunosorbant assay (ELISA). Based on the principle of antigen-antibody interaction, ELISA is a quantitative approach that is suited to our aims of measuring treatment-dependent and strain-dependent changes in protein production with greater accuracy. The ELISA process involves the following steps. A primary antibody to the analyte (IL-1ra or IL-1β) tested for is coated on an ELISA plate. Then, the samples are added to the plate, and the analytes are bound by the primary antibody. To this, a secondary antibody (linked with an enzyme) is added, and it binds to a different epitope on the analyte. A substrate is added to this analyte-antibody complex, which is enzymatically converted by the enzyme linked to the secondary antibody to produce a color reaction or light proportional to the amount of analyte bound in the complex. A dilution series is prepared from purified recombinant protein (IL-1ra or IL-1β), and is used as a standard with each assay for the corresponding protein. This colorimetric reaction can be measured in an ELISA plate reader to quantify the protein present in the samples based on the colorimetric quantification obtained from the standard series.

ELISA kits are commercially available, but only a limited number of assays could be performed with each kit, and the kits are expensive. So we tried to develop our own ELISA assays, and succeeded in developing an assay for IL-1β, which was used to measure IL-1β protein levels in our experimental samples. As we were unable to develop a similar assay for IL-1ra, therefore we purchased commercial IL-1ra ELISA kits and used them to measure the protein levels in our experimental samples.

Mouse lungs from the experiments (Figure 3) were homogenized in 1xPBS-Tween20 buffer, centrifuged, and the supernatants containing the proteins were aliquoted

and used for analysis using ELISA (more details available in 'Materials and methods'). The time points used for the protein study were 6, 24 and 48 h post allergen challenge.

- **Protein production profiles of IL-1ra and IL-1β**

IL-1ra

IL-1ra protein was significantly induced by ovalbumin treatment in A/J mice at all time points examined (Figure 5B). While some increase in IL-1ra protein levels were observed in the C3H/HeJ strain these changes were not significant. No strain-dependent increases in IL-1ra expression were observed in any of the time points examined.

IL-1β

IL-1β protein production was significantly increased due to OVA in both strains of mice at the 6 h time point, and declined to baseline at 24 and 48 h (Figure 6B). The protein production at 6 h was not significantly different between the strains.

There are some minor differences between the transcript and the protein levels in these genes at individual time points, but the overall trend remains the same. In the mRNA studies, we have shown that all the genes in the IL-1 complex, including *Il1rn* show an increased expression at the earlier time points and decline with time. This trend was also observed in the protein studies, but to a lesser degree in IL-1ra protein. This is probably due to the fact that its stable expression over time is required to counteract the pro-inflammatory properties of IL-1. Maximal IL-1ra protein production was observed at 24 h, contrary to 6 h in mRNA studies. This is also understandable because the protein production follows mRNA production, and the mRNA and protein production could peak

at different timepoints. No other studies have measured the *in vivo* temporal pattern of IL-1 receptor antagonist mRNA and protein productions in an allergic asthma model so far. The studies that have measured IL-1 receptor antagonist production in allergic asthma models have only done so at a single timepoint, usually when AHR measurements were done[121]. In other cases, temporality has been measured in cell cultures from lung or airway tissues[146]. Our measurements show the *in vivo* temporal variation in our mouse model, and we believe this is a more accurate representation of the biological levels of the gene products. Thus, our results have provided a profile of the entire temporal variation pattern in mRNA and protein expression preceding the AHR measurement.

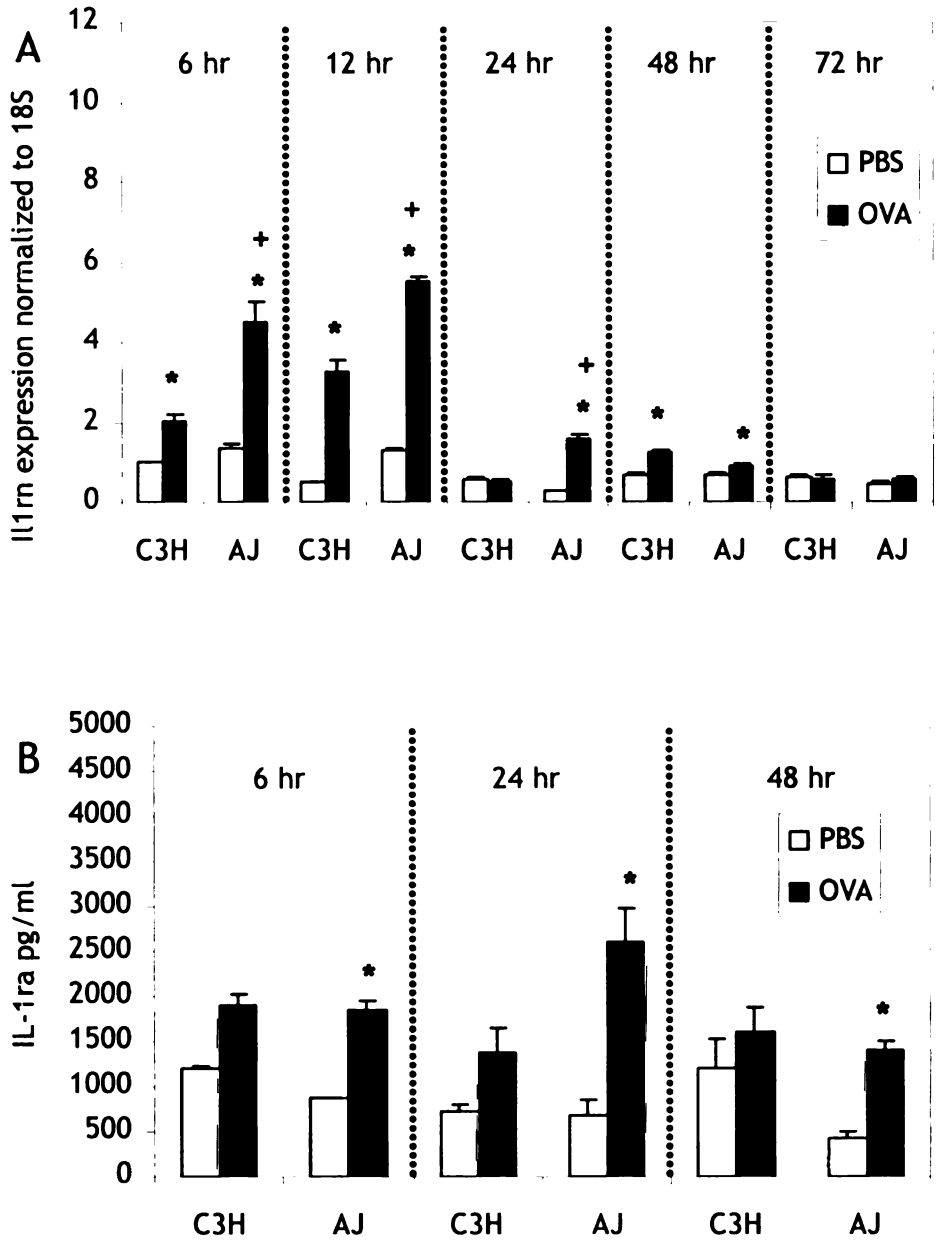

Figure 5. Lung homogenates were assayed for *Il1rn* (A) mRNA (n = 6/group) using TaqMan assay and (B) protein (n = 5/group) using ELISA. IL-1 receptor antagonist message and protein were increased in OVA-treated A/J mice. Samples were assayed in duplicate and values reported as mean ±SEM. Significance differences ($p < 0.05$) due to: * treatment; + strain.

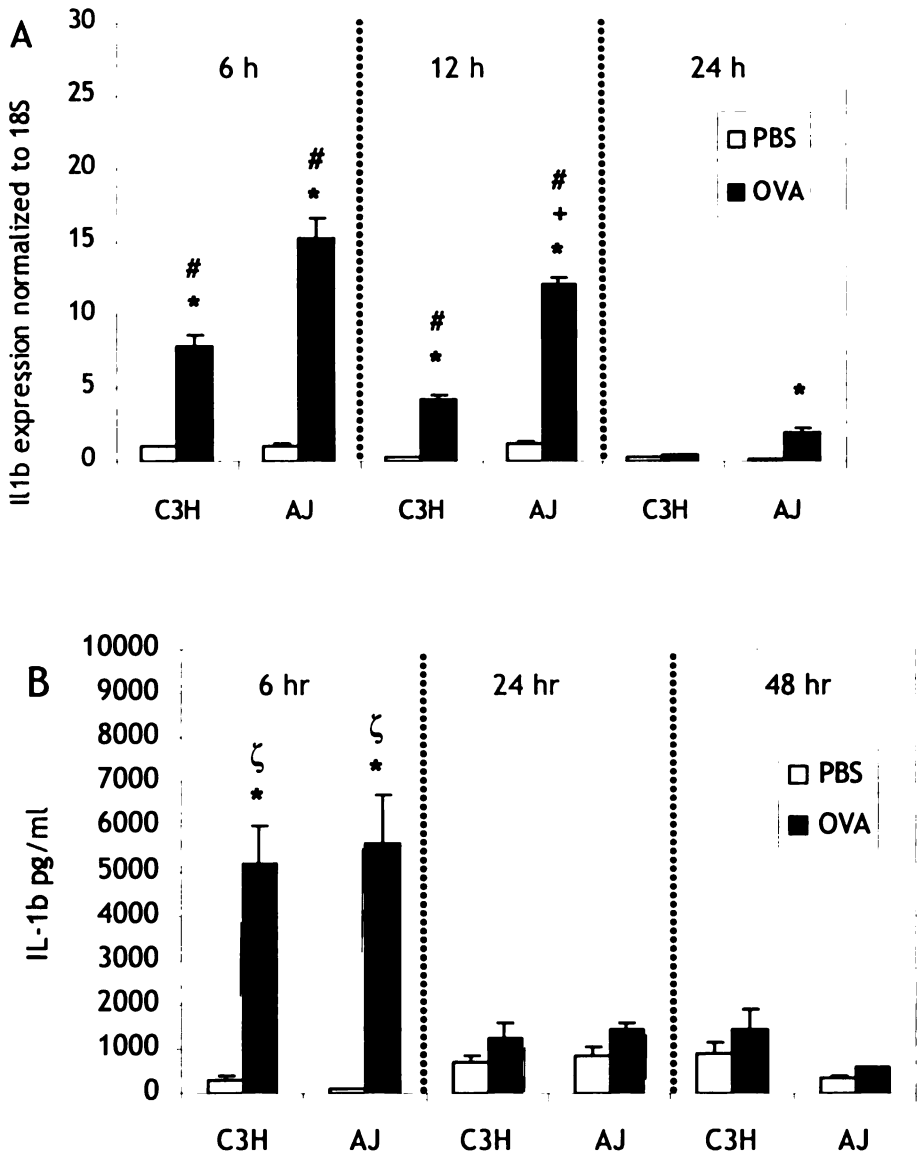

Figure 6. Lung homogenates were assayed for *Il1b* (A) mRNA (n = 6/group) using assays-on demand, and (B) protein (n = 5/group) using ELISA. IL-1 beta message and protein were increased in OVA-treated A/J mice. Samples were assayed in duplicates and values are reported as mean ±SEM. Significant differences ($p < 0.05$) due to: * treatment; + strain; # different from 24 h time point; and ζ different from all other time points.

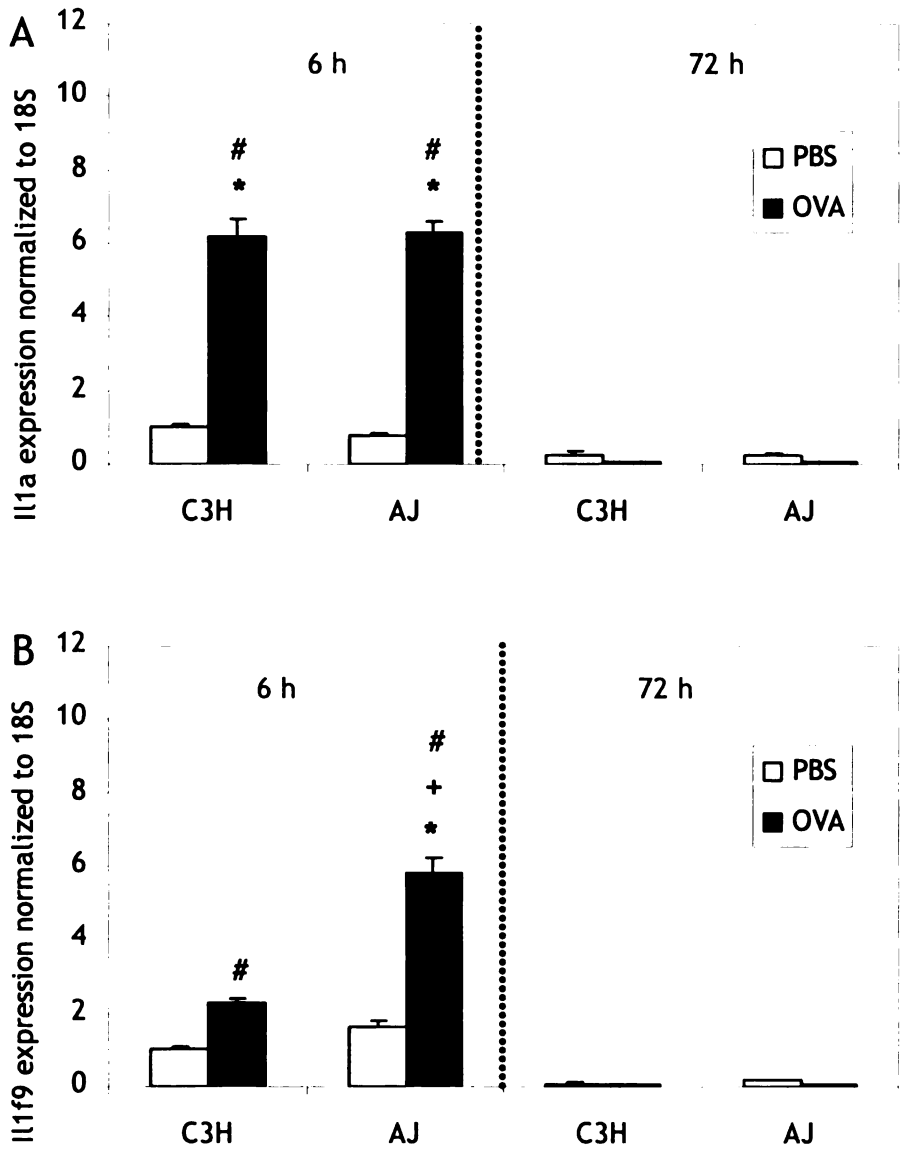

Figure 7. Lung homogenate (n = 6/group) transcript expression profiles of (A) *Il1a* showed treatment- and time-specific differences, but no strain difference, and (B) *Il1f9* was increased in OVA-treated A/J mice at the 6 hr time point. SYBR Green assays were used to measure mRNA levels. Samples were assayed in duplicate and values are reported as mean ±SEM. Significant differences ($p < 0.05$) due to: * treatment; + strain; and # time.

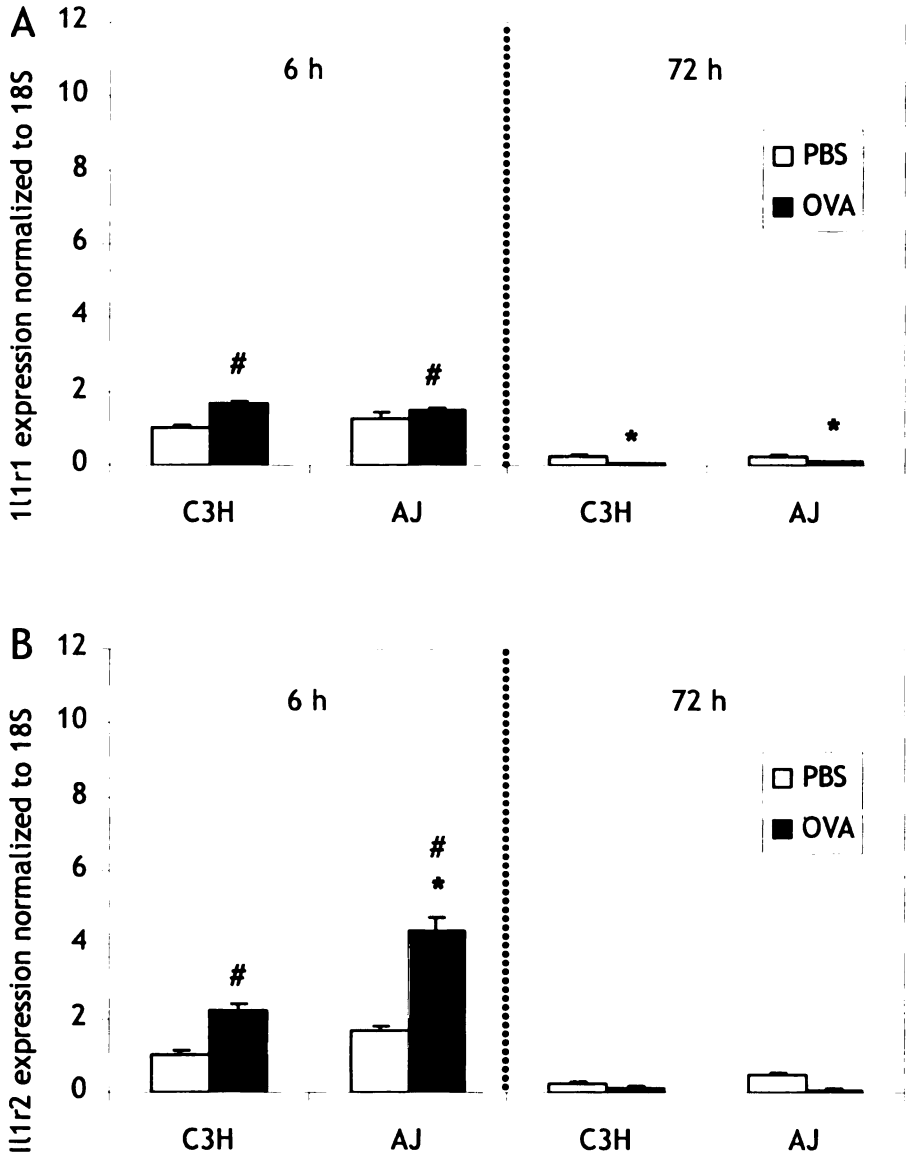

Figure 8. Lung homogenates (n = 6/group) were assayed for (A) *Il1r1* and (B) *Il1r2* message expression. *Il1r2* was upregulated in OVA-treated A/J mice at the 6 h time point, in contrast no change was detected in *Il1r1* in any group. SYBR Green assays were used to measure mRNA levels. All the samples were assayed in duplicate and values reported as mean ±SEM. Significant differences ($p < 0.05$) due to: * treatment; and # time.

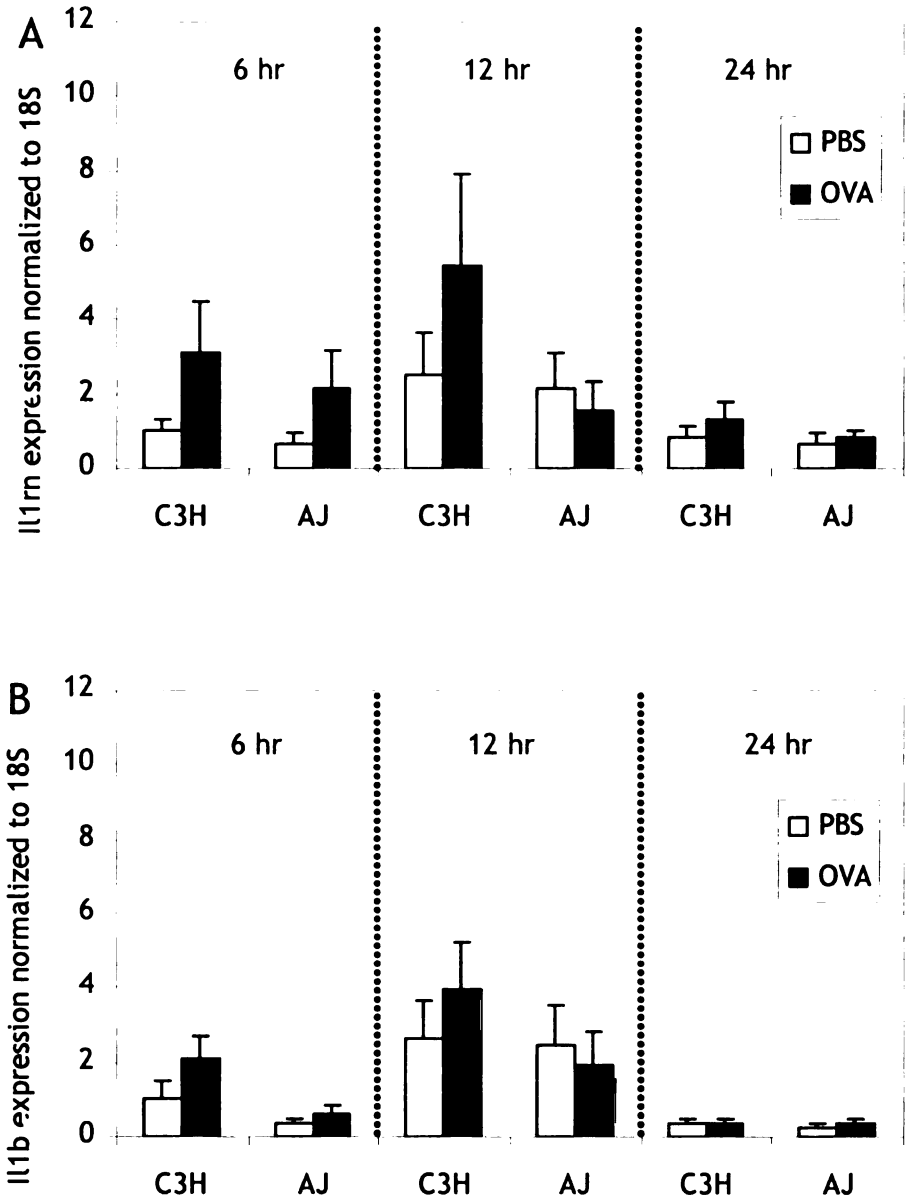

Figure 9. Spleen homogenates (n = 6/group) were assayed for (A) *Il1rn* and (B) *Il1b* message expression. No significant treatment- or strain-specific differences were observed in *Il1rn* and *Il1b* expression. Taqman and assay-on demand were used to measure mRNA levels of *Il1rn* and *Il1b* respectively. All the samples were assayed in duplicate and values reported as mean ±SEM.

D. SUMMARY OF THE ROLE OF IL-1 RECEPTOR ANTAGONIST IN THE MOUSE MODEL

We investigated the role of IL-1 receptor antagonist as a positional candidate gene for the QTL *Abhr1* in our murine model of allergic asthma. We hypothesized that genetic polymorphisms in IL-1 receptor antagonist were responsible for the difference in AHR manifestation between A/J and C3H/HeJ strains. We sequenced the murine IL-1 receptor antagonist gene (*Il1rn*) in A/J and C3H/HeJ strains of mice to identify polymorphisms in IL-1 receptor antagonist that might make A/J an airway hyperresponsive strain and C3H/HeJ an airway hyporesponsive strain. In the same model, we simultaneously examined the mRNA and protein production profiles of IL-1 receptor antagonist and its major agonist IL-1beta. We also tested the mRNA expression profiles of genes in the IL-1 complex, to determine the role of the IL-1 complex in this model of allergic asthma, with the focus on IL-1 receptor antagonist as a positional candidate gene.

We sequenced a region of ~16 kb of genomic DNA from A/J and C3H/HeJ strains containing *Il1rn*, but found no functional or regulatory polymorphisms. The only polymorphism we found was a previously established microsatellite in the 3'UTR. Being a microsatellite, it is less likely to contribute to the differences in transcript expression or protein production. There are some evidences to suggest that it could influence mRNA stability and play a role in intron splicing[169-171], investigation of a similar role was beyond the scope of this study. Moreover, the positional candidate gene for *Abhr2* (*C5*) had a deletion mutation in the A/J strain, resulting in a premature stop codon that abrogated the production of C5 protein in that strain. The lack of C5 protein in A/J strain has been shown to be responsible for insufficient IL-12 production, resulting in decreased

interferon-gamma (IFN γ) production, and increased airway inflammation and AHR[102]. Our investigation at this level involved the conductance of a set of studies aimed at detecting a gene with similar effects, an approach widely followed in refining QTLs to QTGs based on positional candidate gene candidate gene approaches[172]. We found transcript and protein level increases in IL-1 receptor antagonist production in the airway hyperresponsive A/J strain compared to the hyporesponsive C3H/HeJ strain. This increase was co-regulated with the increase in the major agonist (IL-1β) levels and that of other genes in the IL-1 complex. While we cannot totally exclude the role of the microsatellite repeat difference between these strains, investigation of its role at this point is beyond the scope of this study, and will have to be pursued in the future.

In summary, we found limited *Il1rn* genetic polymorphisms between A/J and C3H/HeJ strains, but found that mRNA and protein levels were increased in the A/J strain. A similar pattern was observed with the mRNA expression of other IL-1 complex genes. While we conclude that only these changes are not sufficient to produce increased AHR in A/J mice compared to C3H/HeJ mice, it is nevertheless plays an important role in preparing the milieu for the other gene products to take over the inflammatory process. Other potentital candidate genes within *Abhr1* should also be investigated, that might have a major effect on AHR in our mouse model.

Chapter Three: IL-1 receptor antagonist – Human studies

A. Isle of Wight birth cohort

B. IL-1 receptor antagonist SNP association studies

C. IL-1 receptor antagonist haplotype pair association studies

D. Summary of the human study

A. ISLE OF WIGHT BIRTH COHORT

- **Introduction**

Asthma is a multifactorial disorder resulting from multiple genes, environmental factors and their interactions (Chapter 1, Sections B & C). In a multifactorial disease like asthma, patients manifest a dynamic combination of multiple phenotypes at various stages of disease during the course of their lifetime[6]. Genetic association studies test the association of polymorphisms in a candidate gene (or locus) with various disease phenotypes. Of the approaches used in genetic association studies, the case-control study design tests for such associations at a single point of time, in a set of people affected (cases) and not affected (controls) with the disease. On the other hand, population-based longitudinal cohort studies investigate such associations in a recruited cohort of people over various points of life. These are usually prospective studies, which follow the recruits at various stages of their lives to record and investigate the appearance, morbidity patterns and the trajectory of the disease over time. In a disease like asthma, which is driven by both genetic and environmental factors and exhibits age and gender specific trajectories, longitudinal studies are probably the best tools available at present to dissect the pathophysiology of asthma.

This study is an extension of our linkage studies in mice[16], and the investigation of murine *Il1rn*, as described in Chapter 2. Mouse and human IL-1 receptor antagonist genes are syntenic (present in the same chromosome in both the species – Chromosome 2). Moreover, human *IL1RN* is located (human chromosome 2q14) very close to the recent positionally cloned novel asthma gene *DPP10*[173]. Based on these evidences, we

hypothesized that human *IL1RN* gene is associated with asthma and related phenotypes, and polymorphisms in *IL1RN* have an important role to play in asthma pathophysiology.

Most of the studies that have examined the relationship between *IL1RN* polymorphisms and asthma or related phenotypes[147,150,151,174] have been performed on cross-sectional adult case-control populations. Longitudinal genetic association studies that examine the dynamics of both objective and subjective asthma phenotypes over different ages provide additional valuable information for preventive and age-specific asthma management strategies[175,176]. A few existing and concluded longitudinal birth cohort studies have effectively investigated asthma and relative phenotypes using this approach[177-181]. The primary objective of our study was to establish the effect of the *IL1RN* gene on asthma in a longitudinal cohort of children who were evaluated for asthma and related phenotypes at ages 1, 2, 4 and 10 years. Apart from asthma, the additional phenotypes we tested for association with *IL1RN* polymorphisms were recurrent chest infections, bronchial hyperresponsiveness (BHR) and FEV1/FVC ratios.

- **Isle of Wight birth cohort – population characteristics**

Between January 1989 and February 1990 children born on the Isle of Wight, U.K. were recruited to participate in a longitudinal study (n = 1,456). The study was approved by the Local Research Ethics Committee and informed written parental consent was obtained for all the participants. The population is largely Caucasian (99%), living in a semi-rural environment with no heavy industry.

At birth, data from birth records and extensive questionnaires were collected, including information on asthma and allergy family history, as well as maternal smoking

habits. Maternal and cord sera were collected and assayed for IgE. At ages 1 and 2 years, the questionnaire-based data collection was repeated, physical examinations were performed on the children by a study physician and symptoms of asthma and allergic diseases were recorded. At age 4, questionnaires and physical examinations were repeated and skin prick tests to common aeroallergens and food allergens were performed[182]. At age 10, a subset of the population underwent pulmonary function testing (Table 1). Along with the physical examinations and questionnaire information update at age 10, anticoagulated blood samples were collected and stored frozen for subsequent DNA analysis (n = 921). Additionally, International Study of Asthma and Allergy in Childhood (ISAAC) written questionnaires were used to assess respiratory, nasal and dermatological symptoms[183]. The characteristics of the study population are shown in Table 3.

- **Outcomes tested for genetic association in the Isle of Wight birth cohort**

We investigated the following four outcomes, which were assessed by the study physician based on questionnaire data, clinical diagnosis and pulmonary function tests as applicable to the various ages in which the phenotypes were measured. The analyses were carried out on a representative unselected subset of 921 individuals from the Isle of Wight birth cohort, whose DNA was available for genetic studies.

❖ **Asthma:**

At ages 1, 2 and 4, asthma was defined as having three or more episodes of wheezing, each lasting for more than three days in the past twelve months based on questionnaire data. At age 10 asthma was defined as ever having a physician diagnosis of

asthma in addition to wheeze in the past twelve months.

❖ **Recurrent chest infections:**

Recurrent chest infection was defined as parental report of two or more episodes of productive cough lasting for five or more days in the past year. The presence of wheeze and antibiotic usage were not prerequisites for the diagnosis of chest infection. This phenotype was measured at ages 1 and 2.

❖ **Bronchial Hyperresponsiveness (BHR):**

BHR was defined as being present when the PC_{20} was < 4.0 mg/ml of methacholine during pulmonary function tests. This phenotype was measured at age 10.

❖ **FEV_1/FVC1 ratio:**

The ratio of forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) was calculated from the forced expiratory maneuver during spirometry. This phenotype was measured at age 10.

- **Risk factors evaluated for the outcomes tested:**

❖ **Genetic risk factors:**

Genotypes in the single SNP analyses and haplotype pairs in the multilocus analyses were evaluated as the risk factors that could explain the effect of *IL1RN* gene on the outcomes investigated. Maternal smoking during pregnancy, environmental tobacco smoke exposure, low birth weight (< 2,500 g), male gender and breastfeeding status at least until 3 months were used as confounders in these analyses.

❖ **Environmental risk factors:**

The modifying effect of the various degrees of exposure to environmental tobacco smoke (ETS) on the genetic risk that the children would have for asthma, chest infections, BHR and reduced FEV_1/FVC ratios was evaluated. Other relevant environmental factors, such as low birth weight (< 2,500 g), male gender and breastfeeding for at least 3 months, were used as confounders in these analyses. The levels of ETS exposure on children were classified into the following three groups. When mothers did not smoke during pregnancy and there was no exposure to household ETS in children up to the age of 10, children were categorized under the group "ETS-0" (n = 431). When mothers did not smoke during pregnancy, but household members (including mothers) smoked within the home at some point up to the children's age of 10 years, the exposure status was categorized as "ETS-1" (n =194). When mothers smoked during pregnancy and the children were also exposed to household ETS at some point up to the age of 10, the exposure was categorized as "ETS-2" (n = 293). No children had mothers who smoked during pregnancy but no exposure to household tobacco smoke after birth.

TABLE 3. POPULATION CHARACTERISTICS OF ISLE OF WIGHT BIRTH COHORT

Variable		Initial sample (%) n = 1,491	No. used in analysis (%) n = 921
Asthma at age 1	Yes	133 (8.9)	95 (10.3)
	No	1,241 (83.2)	776 (84.3)
	Missing	117 (7.9)	50 (5.4)
Asthma at age 2	Yes	132 (8.9)	105 (11.4)
	No	1,099 (73.7)	707 (76.8)
	Missing	260 (17.4)	109 (11.8)
Asthma at age 4	Yes	181 (12.1)	133 (14.4)
	No	1,033 (69.3)	698 (75.8)
	Missing	277 (18.6)	90 (9.8)
Asthma at age 10	Yes	178 (11.9)	134 (14.6)
	No	1,192 (80.0)	786 (85.3)
	Missing	121 (8.1)	1 (0.1)
Chest infections at age 1	Yes	101 (6.8)	71 (7.7)
	No	1,273 (85.4)	800 (86.9)
	Missing	117 (7.8)	50 (5.4)
Chest infections at age 2	Yes	157 (10.5)	118 (12.8)
	No	1,074 (72.0)	694 (75.4)
	Missing	260 (17.4)	109 (11.8)
BHR	Yes	169 (11.3)	157 (17.0)
	No	614 (41.2)	542 (58.8)
	Missing	708 (47.5)	222 (24.1)
FEV1/FVC	5^{th}-95^{th} percentile	1033 (69.3)	912 (99)
	Missing	458 (30.7)	9 (1.0)
Smoke exposure	ETS-0	647 (43.4)	431 (46.8)
	ETS-1	464 (31.1)	194 (21.1)
	ETS-2	370 (24.8)	293 (31.8)
	Missing	10 (0.7)	3 (0.3)

Definition of abbreviations: ETS-0, mothers did not smoke during pregnancy and children not exposed to environmental tobacco smoke in the household; ETS-1, mothers did not smoke during pregnancy, but children were exposed to environmental tobacco smoke in the household; ETS-2, mothers smoked during pregnancy and children were exposed to environmental tobacco smoke in the household.

B. IL-1 RECEPTOR ANTAGONIST SNP ASSOCIATION STUDIES

- **Polymorphism selection and genotyping:**

We checked SNPper (http://snpper.chip.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) databases for SNPs in the *IL1RN* gene. None of the reported SNPs found in the databases resulted in an amino acid change. We analyzed the *IL1RN* SNP information available from Hapmap (http://hapmap.org/) and found that majority of the SNPs within the gene were in strong linkage disequilibrium (LD). LD is a statistical measure of the strength of association between two alleles at different markers[38]. Previous reports from Gohlke et al also reported that the SNPs they tested covering the *IL1RN* gene were also in strong LD[147]. As our population is primarily Caucasian, similar to the population used by Gohlke et al, and as the SNPs within the genes are in strong LD, we chose to investigate the three SNPs that were associated with asthma in the Golke study (rs2234678, rs878972 and rs454078). All these SNPs had minor allele frequencies greater than 10 per cent (Table 4).

TABLE 4. *IL1RN* SINGLE NUCLEOTIDE POLYMORPHISMS TESTED

SNP	Alleles	Allele frequency	Genotype	Genotype frequency (n)
rs2234678	A/G	0.75/0.25	AA/GA/GG	0.56/0.37/0.07 (921)
rs878972	A/C	0.75/0.25	AA/AC/CC	0.56/0.38/0.06 (921)
rs454078	A/T	0.74/0.26	AA/AT/TT	0.55/0.38/0.07 (918)

Definition of abbreviations: n = Number of individuals for whom genotype information is available. (Total number of individuals genotyped = 921)

Genomic DNA was isolated from blood samples using QIAamp DNA Blood Kits (Qiagen, Valencia CA) or the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). DNA yields were quantified by spectrophotometry (NanoDrop technologies, Wilmington DE) or by measuring the quantity of the single copy gene, *RNAseP*, using 5'nuclease fluorescent chemistry PCR normalized to known genomic DNA standards by cycle threshold. Genotyping was performed by Pyrosequencing®[184]. To avoid background signals, a blocking primer was used as required for individual SNPs[185]. Primers were designed using pyrosequencing primer design resources (http://primerdesign.pyrosequencing.com/jsp/TemplateInput.jsp, http://biodev.hgen.pitt.edu/sop3/index.php). The SNPs selected were genotyped in all children with available DNA (n = 918 - 921).

- **Pyrosequencing:**

Pyrosequencing is a non-electrophoretic method for DNA sequencing, and the technique works in the following way. Primers are designed for a region of ~150 bp around the SNP to be genotyped. One of the primers is biotinylated at one end. The PCR product formed contains two strands of DNA – a non-biotinylated strand and a biotinylated strand. The biotinylated strand is immobilized on a Pyrosequencing reaction plate, and 10 – 15 nucleotides around the SNP are sequenced by addition of each of A, G, T and C nucleotides in a predetermined order, as inferred from the sequence around the SNP. With the incorporation of each nucleotide, a pyrophosphate is released, and it undergoes an enzymatic reaction to give light (Figure 10), which is captured by a laser-assisted camera. The resulting sequences, called pyrograms (Figure 11), are used to determine the genotype of an individual.

$$(NA)_n + \text{Nucleotide} \xrightarrow{\text{Polymerase}} (NA)_{n+1} + PPi$$

$$PPi + APS \xrightarrow{\text{ATP sulfurylase}} ATP + SO_4^{2-}$$

$$ATP + \text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} AMP + PPi + \text{Oxyluciferin} + CO_2 + \text{Light}$$

Figure 10. Schematic representation of the progress of the enzyme reaction in pyrosequencing. PPi indicates pyrophosphate. (Figure and legend adapted from[184]).

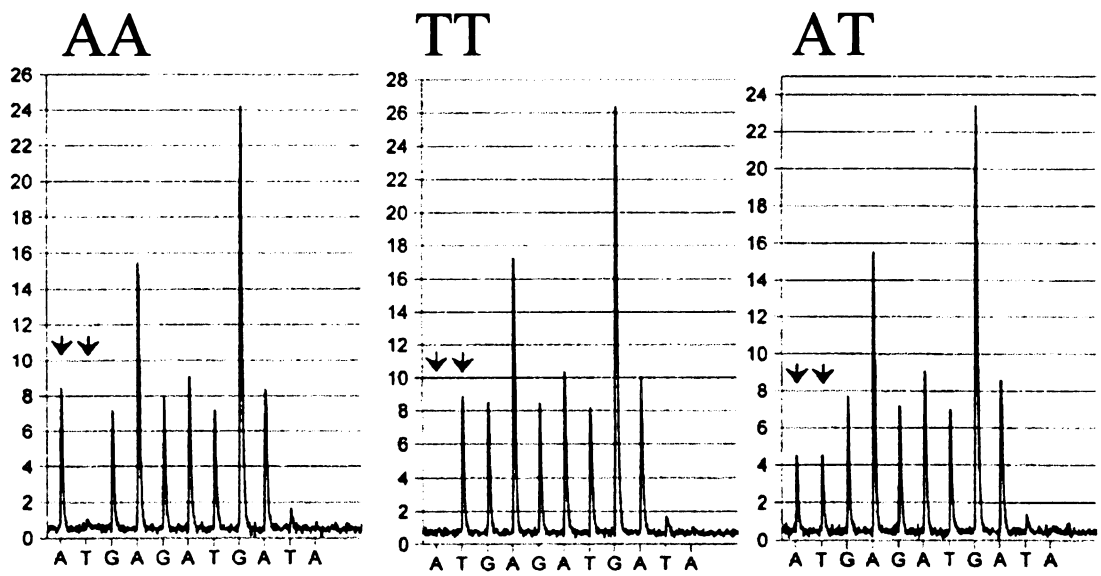

Figure 11. Pyrograms of the investigated sequence (A/T)GAAGATGGGA The SNP is in parentheses; A and T are the alleles. AA and TT are homozygote genotypes and AT is a heterozygote genotype (Figure adapted from[186]), as visualized in the pyrograms. AA genotype has a single peak at the A position, and no peak at the G position. GG genotype has a single peak at the G position, and no peak at the A position. AG genotype has one peak at the A position and one peak at the G position, and both these peaks are half the height of either the A or G peaks in AA or GG homozygotes.

- **Statistical analysis:**

The SNPs were tested for Hardy-Weinberg equilibrium and linkage disequilibrium using Haploview 3.2 (http://www.broad.mit.edu/mpg/haploview/) software. Using the genotype data from SNPs rs2234678, rs878972 and rs454078, chi-square tests were used to test for associations of *IL1RN* SNPs with asthma, recurrent chest infection, BHR and FEV/FVC1 ratio. Permutation tests were performed using R software v2.1.1 (http://cran.r-project.org) to confirm that the associations between *IL1RN* SNPs and the outcomes tested were not spurious. The statistical significance threshold was $p < 0.05$.

- **Results**

All SNPs were in Hardy-Weinberg equilibrium, and were in high LD with each other (Table 5) based on D' and pairwise r^2 values calculated by the default algorithm in Haploview software[187]. Of the children genotyped, 54.25% of children were homozygous for the major allele in all three loci (n = 498), 35.51% were heterozygous at all three loci (n = 326), and 5.88% were homozygous for the minor allele at all three loci (n = 54).

Table 5. Pairwise comparison of linkage disequilibrium for *IL1RN* SNPs

SNP ID	rs2234678	rs878972	rs454078
rs2234678	1	0.988 (0.968)	0.970 (0.899)
rs878972		1	0.976 (0.902)
rs454078			1

Values indicated in the cells: pairwise LD estimates - D'(r^2)

The SNPs rs2234678 and rs878972 were significantly associated with asthma at age 2, as well as with recurrent chest infection at age 2 (Table 6). Similarly, permutation

tests based on 50,000 Monte Carlo simulations identified significant associations of the SNPs rs2234678 and rs878972 with asthma and recurrent chest infection ($p < 0.05$), limited to age 2. We did not find any associations with BHR or the FEV_1/FVC ratio. The significance values for the associations of the individual SNPs (Table 6) were adjusted for false discovery rates using the Benjamini-Hochberg algorithm[188].

TABLE 6. ASSOCIATION OF *IL1RN* SNPS, ASTHMA AND CHEST INFECTIONS

Outcome	Number of children		rs2234678		rs878972		rs454078	
	Affected	Not affected	p value	FDR adjusted	p value	FDR adjusted	p value	FDR adjusted
Asthma at age 1	95	776	0.74	0.91	0.68	0.78	0.93	0.93
Asthma at age 2	105	707	**0.01**	**0.04**	**0.007**	**0.028**	0.07	0.28
Asthma at age 4	133	698	0.67	0.91	0.39	0.62	0.64	0.85
Asthma at age 10	134	786	0.8	0.91	0.68	0.78	0.64	0.85
Chest infections at age 1	71	800	0.15	0.40	0.1	0.27	0.15	0.40
Chest infections at age 2	118	694	**0.003**	**0.024**	**0.002**	**0.016**	**0.02**	0.16
BHR	157	542	0.97	0.97	0.96	0.96	0.83	0.93
FEV1/FVC *		912	0.31	0.62	0.24	0.48	0.35	0.70

* As FEV1/FVC ratio is a continuous outcome variable, Wilcoxon test was used to get the significance values. Chi-square tests were used to obtain p values for all other outcomes. False discovery rate (FDR) adjusted = p values adjusted for false discovery rates using Benjamini - Hochberg algorithm. Significant p values indicated in boldface.

C. IL-1 RECEPTOR ANTAGONIST HAPLOTYPE PAIR ASSOCIATION STUDIES

- **Haplotype frequency estimation and haplotype pair construction**

Apart from the single SNP analyses, haplotype analysis is also important in genetic association studies. A new allele created in the genome would be surrounded by a set of pre-existing alleles during its creation. Thus, at the time of creation, a unique grouping of alleles (called haplotype) is established. If this group of alleles is inherited together in the subsequent generations, they are said to be inherited as a 'haplotype block'. The strength of non-random association between two alleles at different markers is called linkage disequilibrium (LD)[38], and the alleles within a haplotype block are usually in high LD with each other. The lengths of such blocks are dependent on a variety of factors such as the genomic location, allele frequencies, recombination rates and population history[187,189-193].

Although all SNPs examined were in linkage disequilibrium and in a single haplotype block, we still chose to construct haplotype pairs and test for associations with the outcomes we investigated. This is because haplotype blocks are unique to different populations, and their nebulous boundaries depend largely on the allele frequencies of the SNPs selected for analysis and a host of other factors[194,195]. Genetic association studies use both haplotype and haplotype pairs for analysis, but currently there is no consensus as to the superiority of one approach over another, and different research groups employ different strategies[196,197]. We chose haplotype pairs for our analyses because they better represent the allelic combinations present in the diploid human genome. Using the SNPs we genotyped for the single marker analyses (rs2234678, rs878972 and rs454078),

population haplotype frequencies were estimated (Table 7) using PHASE Version 2 (http://www.stat.washington.edu/stephens/software.html). Haplotypes with frequencies over 5 per cent were used to determine haplotype pairs (Table 7).

TABLE 7. *IL1RN* HAPLOTYPES AND HAPLOTYPE PAIRS

Haplotypes	**Frequencies* (n = 921)**	**SE**
AAA	0.7343	0.0004
GCT	0.2431	0.0002
AAT	0.0132	0.0004
GCA	0.0033	0.0002
GAA	0.0022	0.0001
GAT	0.0016	0.0001
ACA	0.0012	0.0003
ACT	0.001	0.0003

Haplotype pairs	**Frequencies** (n = 921)**
AAA/AAA	0.54
GCT/AAA	0.36
GCT/GCT	0.06
Other haplotype pairs	0.04

n refers to number of children analyzed
*Haplotype frequencies estimated by PHASE v.2
**Haplotype pair frequencies used for analysis using SAS v.8.2
SNP order is rs2234678 (A/G) - rs878972 (A/C) - rs454078 (A/T)

Two major haplotypes (AAA and GCT) were present in about 97% of the total samples genotyped (n=921). Three haplotype pairs (AAA/AAA, GCT/AAA and GCT/GCT) were present in 96% of the total samples genotyped. All the other haplotype pairs put together, were present only in about 4% of the samples we genotyped. So, for further statistical analyses, we used only the three major haplotype pairs.

- **Statistical analysis**

For the analyses using haplotype pairs, we excluded pairs with rare combinations

(total 4%, Table 7). We used logistic regression analysis to obtain adjusted and unadjusted effect estimates of *IL1RN* haplotype pairs on the risk of getting asthma at ages 1, 2, 4 and 10, and chest infections at ages 1 and 2. We stratified our analyses based on ETS exposure levels, to determine the influence of ETS exposure levels on the increase or decrease in genetic risk associated with the phenotypes that are associated with the SNPs at the single marker level. We used repeated measures methodology (generalized estimating equation (GEE), GENMOD procedure, SAS v9.1) to test for associations with *IL1RN* haplotype pairs with asthma at ages 1, 2, 4 and 10, when we stratified the asthma data by ETS exposure levels. The rationale for using GEE analysis was that the dichotomous outcome variable (asthma) was repeatedly measured over time and the goal was to estimate marginal probabilities[198].

The statistical significance threshold was $p < 0.05$. Unlike the chi-square tests used for single marker analyses (Table 6), the logistic regression and repeated measurement models used correlated outcomes (asthma at 1, 2, 4, and 10 years and recurrent chest infection at 1 and 2 years). Hence the p values obtained were not adjusted for multiple hypothesis testing[100].

- **Results**

The haplotype pair AAA/AAA (n = 500), comprised of major alleles at all loci, was used as the reference. In the individual logistic regression models for asthma at the four ages of data collection (ages 1, 2, 4 and 10), the haplotype pair 'GCT/GCT' was associated with asthma only at age 2 (Table 8). We then evaluated the effect of ETS exposure on this association with asthma, using a repeated measurement model. If the children had the GCT/GCT haplotype pair and were exposed to maternal smoking during

pregnancy and postnatal ETS exposure, their risk of getting asthma was four-fold higher compared to the children with the reference haplotype pair (Table 9).

In the individual logistic regression models for chest infection at the two ages of data collection (ages 1 and 2), the haplotype pair GCT/GCT (n = 54) containing minor alleles at each locus was associated with recurrent chest infection at age 1 and age 2 (Table 8). We then evaluated the effect of ETS exposure on this association by stratifying the sample based on ETS exposure levels and used individual logistic regression for analysis. When children were exposed to maternal tobacco smoke prenatally and household ETS during childhood (group ETS-2), the risk of getting recurrent chest infection at ages 1 and 2 was approximately seven-fold increased in children with the GCT/GCT haplotype pair compared to the reference haplotype pair (Table 9). The corresponding odds ratio in children exposed to tobacco smoke in the household during childhood but whose mothers did not smoke during pregnancy (group ETS-1) was also elevated, but did not attain statistical significance (Table 9).

We then examined the interactions between *IL1RN* haplotype pairs, asthma and chest infection. Because *IL1RN* SNPs and haplotype pairs were associated with asthma and recurrent chest infection specifically at age 2 (Tables 4 and 5), we examined the haplotype pair frequencies (Figure 12), evaluating chest infection as an intervening variable for asthma. The percentage incidence of chest infection was the highest in children with the haplotype pair GCT/GCT. The percentage incidence of asthma was increased in children with chest infection irrespective of the haplotype pairs, but this increase was also the highest in children with the haplotype pair GCT/GCT (Figure 12).

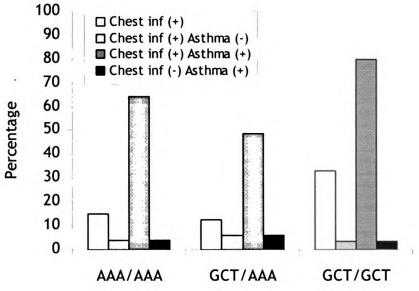

Figure 12. Percentage incidence of asthma and chest infection in children with specific haplotype pairs. Percentages indicated are the ratios of affected children to unaffected children within each haplotype pair.

Testing *IL1RN* haplotype pairs as a risk factor for asthma using a repeated measurement approach revealed only a moderate, non-significant effect (OR = 1.58, p = 0.10). When we included recurrent chest infection (age 1 and 2 combined) in the repeated measurement asthma model, chest infection showed a 6.45 fold increased odds ratio for having asthma (data not shown). However, when the repeated measurement model of asthma was stratified based on chest infection status to test for risk-conferring abilities of haplotype pairs, none of the haplotype pairs displayed a significantly increased risk compared to the others (Table 10). This indicates that recurrent chest infection probably acts as an intervening variable between *IL1RN* and asthma (*IL1RN* → recurrent chest infection → asthma).

TABLE 8. HAPLOTYPE PAIR ANALYSIS FOR ASTHMA AND RELATED PHENOTYPES

Risk factors	AAA/AAA vs AAA'/AAA			GCT/AAA vs AAA/AAA			GCT/GCT vs AAA/AAA		
Phenotypes	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
Asthma at age 1	1.00	-	-	1.07	0.63 - 1.80	0.81	1.42	0.54 - 3.69	0.48
Asthma at age 2	1.00	-	-	0.93	0.57 - 1.52	0.77	**3.07**	**1.42 - 6.63**	**0.0043**
Asthma at age 4	1.00	-	-	0.87	0.57 - 1.35	0.53	1.54	0.71 - 3.32	0.27
Asthma at age 10	1.00	-	-	0.89	0.58 - 1.37	0.59	1.00	0.42 - 2.38	0.99
Chest infection at age 1	1.00	-	-	1.02	0.55 - 1.90	0.96	**3.32**	**1.35 - 8.16**	**0.0090**
Chest infection at age 2	1.00	-	-	0.90	0.55 - 1.47	0.67	**3.39**	**1.59 - 7.24**	**0.0016**

Confounders used in the analysis were gender, environmental tobacco smoke exposure, breastfeeding until 3 months, low birth weight (< 2500 g) and birth order. Significant ($p < 0.05$) associations indicated in boldface.

TABLE 9. EFFECT OF SMOKE EXPOSURE ON ASTHMA AND RECURRENT CHEST INFECTION - HAPLOTYPE PAIR ANALYSES

Repeated measurement of asthma at ages 1, 2, 4 and 10

	ETS-0 (1087 observations, 381 subjects)			ETS-1 (923 observations, 244 subjects)			ETS-2 (572 observations, 151 subjects)		
Risk factors	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
AAA/AAA vs AAA/AAA	1.00	-	-	1.00	-	-	1.00	-	-
GCT/AAA vs AAA/AAA	0.91	0.56 - 1.49	0.71	0.88	0.52 - 1.47	0.62	1.19	0.59 - 2.38	0.63
GCT/GCT vs AAA/AAA	1.62	0.53 - 4.94	0.40	0.67	0.22 - 2.00	0.47	**4.12**	**1.68 - 10.08**	**0.0019**

Recurrent chest infection age 1

	ETS-0 (n = 358)			ETS-1 (n = 225)			ETS-2 (n = 141)		
Risk factors	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
AAA/AAA vs AAA/AAA	1.00	-	-	1.00	-	-	1.00	-	-
GCT/AAA vs AAA/AAA	0.83	0.32 - 2.17	0.71	1.41	0.5 - 3.99	0.51	0.98	0.28 - 3.43	0.98
GCT/GCT vs AAA/AAA	1.18	0.14 - 10.11	0.88	2.27	0.42 - 12.36	0.34	**6.97**	**1.67 - 29.12**	**0.0077**

Recurrent chest infection age 2

	ETS-0 (n = 341)			ETS-1 (n = 216)			ETS-2 (n = 132)		
Risk factors	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
AAA/AAA vs AAA/AAA	1.00	-	-	1.00	-	-	1.00	-	-
GCT/AAA vs AAA/AAA	1.20	0.58 - 2.48	0.62	0.69	0.31 - 1.56	0.38	0.81	0.25 - 2.57	0.71
GCT/GCT vs AAA/AAA	2.36	0.58 - 9.60	0.23	3.08	0.96 - 9.81	0.058	**6.96**	**1.50 - 32.28**	**0.0132**

Confounders used in the analysis were gender, environmental tobacco smoke exposure, breastfeeding until 3 months, low birth weight (< 2500 g) and birth order. Significant associations indicated in boldface.

ETS-0, mothers did not smoke during pregnancy and children not exposed to household ETS

ETS-1, mothers did not smoke during pregnancy, but children were exposed to household ETS

ETS-2, mothers smoked during pregnancy and children were exposed to household ETS

TABLE 10. EFFECT OF *IL1RN* HAPLOTYPE PAIRS ON ASTHMA
(REPEATED MEASUREMENTS : AGES 1, 2, 4, 10) STRATIFIED FOR RECURRENT CHEST INFECTION

	Recurrent chest infection (-) 2,460 observations, 647 subjects			**Recurrent chest infection (+) 490 observations, 129 subjects**		
Risk factors	**OR**	**95% CI**		**OR**	**95% CI**	
AAA/AAA vs AAA/AAA	1	-	-	1	-	-
GCT/AAA vs AAA/AAA	0.93	0.63	1.37	0.86	0.51	1.46
GCT/GCT vs AAA/AAA	1.13	0.42	3.01	0.79	0.39	1.61

Confounders used in the analysis were gender, environmental tobacco smoke exposure, breastfeeding until 3 months, low birth weight (< 2500 g) and birth order

D. SUMMARY OF THE ROLE OF IL-1 RECEPTOR ANTAGONIST IN THE ISLE OF WIGHT BIRTH COHORT

We investigated the role of IL-1 receptor antagonist polymorphisms in the Isle of Wight birth cohort. We tested three *IL1RN* SNPs for associations with asthma, chest infections, BHR and FEV1/FVC ratios. At the single SNP level, we found the SNPs to be associated with asthma at age 2 and chest infections at age 2. Haplotype pair analysis confirmed that the haplotype pair containing the minor alleles at all loci (GCT/GCT) conferred increased risk of asthma and chest infection in the children tested. Thus, after confirming the association of SNPs and haplotype pairs with asthma and chest infections, we tested for the effect of environmental tobacco smoke exposure on this association. We also found that maternal smoking during pregnancy coupled with postnatal tobacco smoke exposure caused several-fold increase in the risk of getting asthma and chest infection in children possessing the GCT/GCT haplotype pair. Taken together, our results suggest a major role of *IL1RN* in asthma and chest infections. We have also shown that depending on environmental exposure conditions, children with specific genetic makeup can have increased risk of getting asthma and chest infections.

Chapter Four: Discussion of mouse and human *IL1RN* studies

A. Role of IL-1 Receptor antagonist in A/J and C3H/HeJ mice

B. Role of IL-1 Receptor antagonist in the Isle of Wight birth cohort

The goal of this project was to investigate the role of IL-1 receptor antagonist in asthma using a comparative study in mice and humans. This was supported by the fact that in both these species, the IL-1 receptor antagonist gene is located on chromosome 2.

A. ROLE OF IL-1 RECEPTOR ANTAGONIST IN A/J AND C3H/HeJ MICE

To make the transition from the *Abhr1* QTL to a specific gene, we considered candidate genes within the *Abhr1* linkage region based on their functional relevance to the pathophysiology of allergic asthma. *Il1rn* presented itself as a strong positional candidate gene for *Abhr1* based on its critical role in the IL-1 signal transduction cascade. In this study we conducted a comprehensive comparative sequence analysis that effectively excluded the *Il1rn* gene as the quantitative trait gene underlying *Abhr1*, however, our results suggested an important role for IL-1ra in the early stages of the allergic airway phenotype.

Prior studies have demonstrated the *Il1rn* gene structure, which includes alternative splicing of two different first exons of the *Il1rn* mRNA producing a secretory protein containing a leader sequence (sIL-1ra) and an intracellular isoform (icIL-1ra) that differs from the secreted IL-1ra by an additional seven amino acids at the amino terminus, the lack of a hydrophobic leader sequence, the absence of glycosylation and remains intracellular[104,105,199]. The first exon of intracellular *Il1rn* is located about 8 kb upstream of the first exon of the extracellular isoform. Transcriptional regulation studies in mice have shown that the region spanning from -598 to -288 bp in the intracellular isoform is critical for promoter activity[163]. Studies of the human *IL1RN* gene indicate that important transcriptional elements are present within the region 2 kb upstream of the extracellular

isoform[162]. Our sequence analysis examined these critical regions along with the complete coding and intronic sequences (Figure 1).

Sequence homology within and flanking the *Il1rn* gene was high with a microsatellite, *D2Mit60*, being the only variant we observed in ~16 kb of sequence. The 86 bp VNTR variant that has been reported in the human *IL1RN* gene[149] was not detected in the comparable location within the *Il1rn* gene of either mouse strain that we sequenced. As the only identified polymorphism was a 3' UTR dinucleotide repeat, the probability of it being responsible for a functional difference in *Il1rn* underlying allergic airway phenotypes in our murine model is minimal. Not only did we observe limited polymorphism between our two strains of interest, A/J and C3H/HeJ, but we also found no further variation between these strains and five additional strains represented in the databases that we compared. This low level of genetic variation in ~16 kb of examined sequence extends a greater distance than the commonly reported single nucleotide polymorphism rate of 1/1,000 bp as a theoretical possibility across the genome[164]. However, the mosaic structure of the mouse genome, as recently described by Wade and colleagues, results in long segments of DNA with extremely high (~40 SNPs/10 kb) or extremely low (~0.5 SNPs/10 kb) polymorphisms rates[165]. Thus, the genomic region containing *Il1rn* appears to reside in a low SNP block.

Apart from its general role in as an anti-inflammatory cytokine, IL-1ra has been specifically associated with the down regulation of BHR in asthma[121,125]. IL-1ra has also been shown to prevent the development of BHR, bronchoalveolar lavage fluid neutrophilia and the degradation of airway epithelial cells following ozone exposure[125]. Moreover, transgenic mice with lung-specific overexpression of human IL-1ra showed

decreased neutrophilic inflammation and macrophage inflammatory proteins[200]. Conversely, studies by Nakae et al. utilizing mice deficient in IL-1α/β or IL-1ra in an ovalbumin-exposure model demonstrated that allergen-induced BHR, ovalbumin-specific T-cell proliferative responses and the levels of Th2 cytokines IL-4 and IL-5 were significantly increased in IL-1ra$^{-/-}$ mice compared to the wild-type mice, while these responses were significantly decreased in IL-1α/β$^{-/-}$ mice[121]. These studies provide evidence for the anti-inflammatory properties of IL-1ra in asthma, directed against the inflammatory effects of IL-1. Based on these observations one would anticipate that in our model functional IL-1ra would be higher in asthma-protected C3H/HeJ mice as compared to the A/J strain, which demonstrates both greater BHR and airway inflammation subsequent to allergen exposure. However, our results were not consistent with this projection as *Il1rn* mRNA and protein levels were consistently higher in the lungs of A/J mice. This indicates that this increased expression of *Il1rn* is a consequence of increased inflammation in the A/J mice, rather than the cause that determines the difference in AHR between A/J and C3H/HeJ mice. In addition, we did not find treatment- or strain-specific differences of *Il1rn* mRNA in spleen tissues.

Despite not finding the predicted relationship between A/J and C3H/HeJ mice with regards to *Il1rn* mRNA and protein levels, we conducted further studies on additional IL-1 family members because examining *Il1rn* in isolation may not give a complete picture. The IL-1 family of genes contains *Il1a, Il1b*, and *Il1rn* along with several newly-identified family members; *Il1f5, Il1f6, Il1f8, Il1f9* and *Il1f10*. IL-1ra competitively binds to the IL-1RI receptor but does not elicit a downstream signal transduction cascade. In this manner IL-1ra is one part of a unique system of negative

feedback in the IL-1 family that also involves an inert receptor (IL-1RII), a low activity agonist (IL-1a) and carefully balanced agonist-receptor affinities. This complex feedback mechanism is needed to keep in check the potent pro-inflammatory response induced by the binding of the active agonist, IL-1β, and the functional receptor, IL-1RI. Of the IL-1 family members, it appears that the relationship between IL-1Ra and IL-1β is of particular importance as this ratio affects disease outcomes. As an example, in human patients with status asthmaticus, both IL-1Ra and IL-1β were increased, however, the IL-1Ra levels did not appear to be high enough to block IL-1 biological activity[201] as a marked excess of IL-1Ra is required to alter the effects of IL-1β[202]. As measured by both message and protein, IL-1β was in excess of IL-1ra in our model, which supports the pro-inflammatory outcome that we observe in A/J mice. This trend was consistent in the mRNA and protein levels indicating these two genes may be co-regulated. We suggest that these increased *Il1rn* mRNA and protein levels in the asthma-susceptible A/J mice are in response to allergic inflammation, but not the cause of AHR in our model.

The new IL-1 family members were of particular interest to us for several reasons. First, *Il1f5, Il1f6, Il1f8, Il1f9* and *Il1f10* genes are located near *Il1rn* within the *Abhr1* locus (NCBI *Mus musculus* Build 35.1). Additionally, some of the new members like *Il1f5* have both high sequence homology and functional similarity to *Il1rn*[203,204]. *Il1f5* is a highly specific antagonist of the *Il1r6*-mediated response to *Il1f9*, and it has been suggested that *Il1f9*, *Il1f5* and *Il1r6* constitute an independent signaling system analogous to *Il1a/b*, *Il1ra* and *Il1r1*, respectively[204]. We could not detect quantifiable levels of *Il1f5* expression in lungs in our mouse model, but it was interesting to find a treatment-induced and strain-specific increase in expression of *Il1f9* (UniGene Mm.249379) gene. This gene

(*Il1f9*) has been shown to increase the production of NF-κB through an orphan receptor, IL-1 receptor (IL-1R)-related protein 2 (IL-1Rrp2) [204,205]. IL-1 has also been shown to induce the production of NF-κB [134], which is critical for the expression of the Th2 cell specific transcription factor GATA-3 [135], which in turn is necessary for the expression of pro-asthmatic cytokines IL-4, IL-5 and IL-13. In this regard, further investigation focused on *Il1f9* is warranted.

Of the receptors, we did not find differential expression of *Il1r1* due to treatment or due to strain, but the expression of *Il1r2* was increased in response to ovalbumin treatment. This increase in *Il1r2* expression was significantly higher in the A/J mice compared to C3H/HeJ mice, and was very similar to *Il1rn*, suggesting that this increased expression of both these counter-regulatory genes could serve to balance the increased expression of the pro-inflammatory *Il1a* and *Il1b* genes.

The increase we observed in *Il1rn* mRNA and protein levels in response to ovalbumin treatment was more pronounced in the hyperresponsive A/J strain as compared to the hyporesponsive C3H/HeJ strain, yet induction of IL-1ra was to some degree a common response in both strains as elicited by an allergenic stimulus, and thus it may not be the exclusive phenomenon that adequately explains the strain differences in A/J and C3H/HeJ susceptibilities to asthma. Our results further suggest that in this model of allergic asthma, endogenous mRNA expression of *Il1rn* is co-regulated with that of *Il1b*, which is increased in the early phase and gradually declines over time. The fact that these transcripts are at their lowest expression levels at 72 h after antigenic challenge, the time at which maximum BHR to acetylcholine was observed, indicates that their direct role is restricted to the early stages of inflammation. The lack of functional genetic

polymorphisms identified between the A/J and C3H/HeJ strains of mice indicates that although *Il1rn* plays an important role in allergic asthma, as evidenced from the mRNA and protein expression studies, it is not the primary source of genetic susceptibility. There is a possibility that its regulation might occur through a genetic variant operating from outside the *Il1rn* gene and its regulatory regions, but lying within the *Abhr1* QTL. Future studies to discern the *Abhr1* gene should focus on identifying such potential regulatory polymorphisms, as well as investigate other positional candidate genes within the *Abhr1* QTL. Specifically, studies that further examine the other IL-1 family members that map within *Abhr1*, in particular *Il1f5* and *Il1f9*, would be useful further to expand our knowledge of these potentially relevant cytokines.

B. ROLE OF IL-1 RECEPTOR ANTAGONIST IN THE ISLE OF WIGHT BIRTH COHORT

We found that *IL1RN* was associated with asthma and chest infections in Isle of Wight children. Both these associations were strengthened at the haplotype level when children were exposed to tobacco smoke during gestation and childhood. In all analyses, recurrent chest infection and asthma (tables 6, 8, 9) as well as the repeated measurement analysis, the adverse effect was due to the haplotype pair with the minor alleles at each locus (GCT/GCT). The relative risk of the heterozygous haplotype pair (GCT/AAA) was not different from the haplotype pair with the major alleles. These findings suggest a recessive model.

Previous evidence, while limited, supports a role for genes in the IL-1 cascade in determining the susceptibility or resistance to chronic inflammatory diseases, yet only a

few studies have tested *IL1RN* for associations with asthma phenotypes. Gohlke and colleagues found significant association of *IL1RN* polymorphisms with asthma in a German population, they confirmed their results in an independent Italian population and replicated the association of *IL1RN* with asthma in another German population[147,174]. The polymorphisms that reached significance for asthma in the original study by Gohlke et al. were examined in the current study. The two SNPs that were associated with asthma in both the German and Italian populations[147] (rs2234678 and rs878072) were also associated with asthma and chest infection in our study (Table 6). While confirming the associations reported in the Gohlke study, our results are unique in that we have extended the genetic association by showing that there is an environmental component to this association, as the risk conferred by specific haplotype pairs to the incidence of asthma and chest infection is accentuated by exposure to maternal smoking during pregnancy and ETS exposure after birth (Tables 8 and 9). This could be due to insufficient production of IL-1Ra in those susceptible individuals possessing specific haplotype pairs, a view supported by previous reports that specific *IL1RN* alleles are associated with lower serum IL-1Ra levels in asthmatics[128]. The association of maternal smoking (during and after pregnancy) on the development of asthma at age 1 and 2 years has been previously reported in the Isle of Wight birth cohort[206,207]. Thus, it was not surprising that smoke exposure during gestation and early childhood was an important driver for both asthma and chest infection in this study, as maternal smoking has been shown to be associated with increased risk of asthma and chest infection in the offspring of mothers who smoke[183,208-214].

IL-1, on binding to the functional receptor, IL-1RI, stimulates the expression of a large number of proinflammatory proteins[215]. This proinflammatory response is important in host defense, however, as an antagonist, IL-1Ra serves an equally important role to moderate the IL-1 driven proinflammatory cascade and prevent its untoward consequences such as (septic) shock. As such, IL-1Ra is a critical modulator in many inflammatory conditions. Thus, we also evaluated the relationship between *IL1RN* and recurrent chest infection. This is a novel approach as in the past chest infection has been, at most, considered a risk factor, but not an outcome influenced by genetic susceptibility. However, in light of the growing body of literature on genetic susceptibility to pathogen resistance[216], and the role of IL-1 and IL-1Ra in host defense, the examination of the *IL1RN* gene influence on an infectious outcome that relates to asthma seemed prudent. Lower respiratory tract infections in childhood are primarily of viral origin, typically caused by influenza, parainfluenza and respiratory syncytial viruses (RSV). While we did not make definitive diagnoses of chest infections based on specific pathogens in our study, the phenotype measured was clearly of lower respiratory tract origin, and was differentially diagnosed from upper respiratory tract infections. Despite lack of pathogen specificity, the phenotype of recurrent chest infection has been shown previously to be a strong risk factor for persistence of early childhood wheeze up to age 10 years and current wheeze and asthma at age 10 in the same population[217,218].

Our indidvidual SNP and haplotype pair analyses showed that *IL1RN* was significantly associated with asthma at age 2, but not at ages 1, 4 or 10 using individual logistic regression (Tables 6 and 8) and with repeated measurement analysis of asthma stratified for ETS exposure levels (Table 9). To understand why the association was

confined to a specific age group, we need to consider the natural history of asthma. In our cohort, at each period (for example, from 4 to 10 years) approximately half the children lose asthma symptoms, but nearly equal numbers of children develop new onset or recurrent asthma and report symptoms at the ensuing follow-up (unpublished observations). Therefore, only a subset of the individuals with asthma diagnosis at age 2 also had an asthma diagnosis at ages 4 or 10. This longitudinal variation expressed as instability of asthma diagnosis during the childhood period reflects the nature of this disease, which follows a pattern of remission and relapse not only in childhood but also in adult life[181,219]. Since there is only partial overlap (~50%) in the individuals within the asthma positive group at each age it is reasonable that the genetic association results at each age are unique. This leads us to carefully consider the other factors unique to each age group.

Another reason that the *IL1RN* - asthma association was focused mainly on a single age may relate to the co-occurrence of persistent wheeze (diagnosed as asthma) and chest infection in the early childhood period. As the *IL1RN*-related genetic susceptibility to asthma appears to occur, at least in part, through recurrent chest infection, the effect on asthma may be limited to the early childhood time period during which chest infection is most common. However, Martinez et al. have previously shown that wheeze occurring in the first year of life is largely due to small airway caliber with resultant reduced conductance, that can be demonstrated before any viral respiratory infection[220]. Young et al. confirmed that this lung function abnormality usually resolves by 12 months of age (termed transient infantile wheeze) and more persistent wheeze or asthma develops during the second year of life when it may be related to recurrent viral

respiratory infections[221]. This may explain the lack of a demonstrable association between *IL1RN* polymorphisms and asthma at age 1 in our cohort, coupled with strong linkage of *IL1RN* polymorphisms to asthma at age 2. Furthermore, a feature distinguishing early and late childhood asthmatics is that in later childhood, asthma is more likely allergic. In agreement with the report by Mao et al. in which *IL1RN* was associated with non-atopic asthma[128], a change in asthma characteristics may explain why there was no association of *IL1RN* with asthma at age 10. This is also confirmed by the lack of association of *IL1RN* polymorphisms to two objective asthma outcomes measured at age 10 (BHR and FEV_1/FVC ratios) in our study.

One limitation in this study is that our results were based on the subset of children who donated blood for DNA collection during the 10 year follow-up. The percentage of children who had asthma and those who had ETS-0 was higher in the samples used for analyses compared to the original sample (Table 3). However, tests for associations between ETS and repeated measurements of asthma in the whole population and in the subset of samples used in the analyses yielded nearly identical odds ratios (data not shown).

In this report, we focused on SNPs and haplotype pairs, but not haplotypes. We pursued the haplotype pair approach, since it simultaneously takes both haplotypes into account and reduces the number of genetic combinations and thus the number of statistical tests without losing information. Currently, there is no consensus as to the superiority of one approach over another and different research groups employ different strategies[196,197]. Given the strong linkage disequilibrium, our SNP and haplotype pair

analyses revealed identical results. Thus, the findings are not attributable to the applied analytical strategy.

In conclusion, our results contribute to the evidence supporting the role of *IL1RN* in asthma and show that this association is likely to be mediated through recurrent chest infection. The observation that *IL1RN* is related to asthma via recurrent chest infection needs to be pursued to determine whether chest infection is serving as an infectious cause of asthma via this gene, in particular when children are exposed to tobacco smoke. The effect of *IL1RN* in asthma and chest infection seems to dissipate as the children grow older, but it may reappear in adolescence. This can be determined by additional follow-up of the cohort children during adolescence. Additionally, treatment of chest infection may impact asthma outcomes. Furthermore, the interaction of chest infection and smoking on asthma extends the general no-smoking recommendation, such that adolescents and adults with a history of early life chest infection in particular should be discouraged from smoking. Thus, these findings may have public health significance, therapeutic value as well as value to asthmatic patients and their families.

- **Future directions**

In the mouse model, other potential candidate genes can be investigated. Mouse Phenome Database (http://phenome.jax.org/pubcgi/phenome/mpdcgi?rtn=docs/home), has a collection of phenotypes and SNP information available for several mouse strains, aggregated from several resources. Polymorphisms between A/J and C3H/HeJ mice within *Abhr1* interval could be collected for the genes that have been already sequenced, and interesting candidate genes could be investigated further. Genes that possess non-

synonymous coding SNPs can be prioritized, followed by SNPs in the coding, regulatory or the intronic regions, in that order. Microarray analysis of genes only within the *Abhr1* region could be performed to detect genes that are differentially regulated. As suggested in a recent review[29], SNP information from other mouse strains that are susceptible and resistant to asthma can be compared with our susceptible (A/J) and resistant (C3H/HeJ) strains to look for specific haplotype patterns within the *Abhr1* region. If such a haplotype pattern unique to the susceptible and different strains was observed within *Abhr1*, genes in such locations could be further investigated. Such genes should be tested comprehensively at the physiological and molecular levels in A/J and C3H/HeJ mice, and also in the congenic (C3H/HeJ.A/J-*Abhr1* and C3H/HeJ.A/J-*Abhr2*) strains of mice developed in Dr. Susan Ewart's lab.

In the human study, the SNP information available from the Hapmap project (http://hapmap.org) indicated that a majority of the SNPs in *IL1RN* are in a single haplotype block. Interestingly, all the major genes in the IL-1 complex (*IL1RI, IL1RII, IL1A, IL1B, IL1RN, IL1F5-10*) are located adjacent to each other on human chromosome 2, in an interval of 12 Mbp. If the present results from *IL1RN* polymorphisms are to be investigated further, it might be useful to investigate other genes in the IL-1 complex, due to their physical proximity and functional relatedness. To locate one or more specific loci that are unique to this Isle of Wight birth cohort which control the major phenotypes like asthma, atopy or BHR, a genome-wide SNP association study is the method of choice at present[42,43]. It also has to be borne in mind that the process of identifying one or more specific causative genes from within such locus/loci faces the same kind of challenges that are involved in any linkage study. Confirmation of findings from the association

study in a different population is also is a valuable methodology. Causative genes thus identified can be tested in our mouse models using knock-out or gene over-expression strategies to determine the role of the identified gene in asthma and related phenotypes.

Chapter Five: Materials and methods

A) Ovalbumin sensitization and challenge

B) DNA sequencing

C) TaqMan/SYBR Green real-time RT-PCR

D) Statistical anlysis of mRNA expression data

E) Protein collection from lung tissues

F) Enzyme-linked immunosorbant assay (ELISA)

G) Pyrosequencing

A. Ovalbumin sensitization and challenge

A/J and C3H/HeJ male mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age and allowed to acclimatize for one week before the experiment. Animals were housed 3-5/cage, under high-efficiency particulate absolute (HEPA) flow hoods and allowed free access to ovalbumin (OVA)-free rodent chow and water.

On day 0, mice (n=6/group) were sensitized with an intraperitoneal injection of 10μg chicken egg OVA (crude grade IV; Sigma, St. Louis, MO) in 200 μl phosphate-buffered saline (PBS) or PBS alone. On day 14, mice were anesthetized (ketamine, 45mg/kg and xylazine 8mg/kg, intraperitoneally). The anesthetized mice were placed on a 45° dorsal recumbency, and their tongues were gently retracted out. PBS or 1.5% OVA in 45 μl PBS was placed on the base of the tongue with a sterile pipette tip. A pedal reflex was induced by applying gentle force on the lower limbs with a blunt forceps, resulting in the aspiration of the fluid from the base of the tongue into the trachea. The mice were recovered from anesthesia, and the lungs, tracheobronchial lymphnodes and spleens were harvested at 6, 12, 24, 48 and 72 h after challenge.

B. DNA SEQUENCING

Primer pairs were constructed from Celera (www.celeradiscoverysystem.com) sequence of murine *Il1rn* (mCG4837) using the program Oligo Primer Analysis software v6.24 (Molecular Biology Insights, Inc., Cascade, CO). To sequence a LINE element in the first intron of *Il1rn*, primers designed by Primer 3 from the Whitehead institute (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were used, which takes into

account a rodent mispriming library and avoids designing primers in the repeat regions. All the primers were designed to amplify between 600 – 700 bp of genomic DNA. The length of the primers was between 15 – 25 nucleotides, and the GC content was approximately 50-60%. Care was taken to avoid choosing primers that could form primer dimer formation or hairpin structures.

Genomic DNA samples isolated from the kidneys of A/J and C3H/HeJ mice available from the lab DNA stock were used as the template. Polymerase chain reactions (PCR) were performed in 30 µl volumes containing: 3.0 µl of 10X PCR buffer, 2.4 µl 1 mM dNTPs, 0.9 µl 50 mM $MgCl_2$, 0.15 µl Taq DNA polymerase (all from Invitrogen, Carlsbad, CA), 2 µl 10 µM forward primer, 2 µl of 10 µM reverse primer (both from IDT, Coralville, IA), 6 µl 10 ng/ml genomic DNA and 13.55 µl double distilled water. For each primer pair, A/J and C3H/HeJ DNA were used as templates, and to test for possible contaminations, a non-template control PCR reaction was also performed. In the non-template control, 6 µl double distilled water was added instead of A/J or C3H/HeJ genomic DNA, while all the other ingredients remained constant. PCR reactions were performed in MJ Research Peltier PTC-200 or PTC-225 thermal cyclers (MJ Research, Watertown, MA).

PCR reactions were performed as follows: 94°C (denaturing, 4 min), followed by 40 cycles of 94°C (denaturing, 30 sec), [12]55°C (annealing, 1 min) and 72°C (extension, 1 min). After cycling, a final extension was performed (72°C, 10 min) followed by an indefinite hold at 4°C. PCR products were separated on a 2% agarose gel stained with ethidium bromide, with an appropriate ladder as the reference.

[12] Annealing temperatures were dependent on the base pair content of the primer pairs, normally ranging from 50°C to 60°C.

Target DNA bands were extracted using QIAquick gel extraction kits (QIAGEN Inc, Valencia, CA). The DNA fragments were excised from the agarose gels, and 500 µl Buffer QG was added to the excised slices. The excised slice in the QG buffer was then incubated at 50°C for 10 min in a water bath, until the gel slice has completely dissolved. To help dissolve the gel, the tubes were vortexed every 2-3 minutes. The desired color of the mixture was yellow, indicating that the pH of the solution was ≤ 7.5, which was required for efficient DNA extraction. After the gel had completely dissolved, 100 µl of isopropanol were added to the tube if the PCR product size was < 500 bp or > 4 kb. If the product size was 500 bp – 4 kb, addition of isopropanol has no effect on DNA yield. Then, QIAquick spin columns were placed in the provided 2 ml collection tube. The solution in the tube was transferred to the spin columns, and centrifuged at 14,000 rpm for 1 min. The flow-through was discarded and the spin columns were placed again in the collection tube. 0.5 ml of Buffer QG was added to the QIAquick columns, and centrifuged again at 14,000 rpm for 1 min. The flow-through was discarded again, the QIAquick columns were placed again in the collection tubes. 0.75 ml of Buffer PE was added to the column, allowed to stand for 5 minutes and centrifuged at 14,000 rpm for 1 min. The flow-through was discarded, the QIAquick column placed again inside the collection tube and centrifuged under the same conditions one more time. Then, The QIAquick column was removed and placed inside a clean 1.5 ml microcentrifuge tube. 30 µl of Buffer EB was added to the center of the QIAquick membrane in the column, and allowed to stand for 1 minute. The fresh collection tube with the column was centrifuged again at 14,000 rpm for 1 minute. About 28 µl of eluate containing the amplified DNA fragments was obtained, and was stored at 4°C for further use. A mixture of 9 µl eluate

and 3 μl 10 μM forward primer, and another mixture containing 9 μl eluate and 3 μl 10 μM reverse primer was submitted for fluorescent automated sequencing in Research Technology Support Facility (http://genomics.msu.edu) in Michigan State University. Sequencing was done using BigDye™ terminator Cycle Sequencing Ready reaction DNA sequencing kits (Applied Biosystems, Foster City, CA) in an ABI Prism 377 DNA sequencer. Sequences were analyzed and aligned using Chromas v1.45 (Technelysium Pty Ltd, Australia) and DNAssist v1.02 (Bellville, S. A.) software packages. Multiple alignments of Genbank, Celera, A/J and C3H/HeJ *Il1rn* sequences were performed with ClustalW option in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

C. TaqMan/SYBR Green real-time RT-PCR

Total RNA was extracted from lungs using TRIzol reagent (Invitrogen, Carlsbad, CA). Tissues were homogenized in 2 ml of TRIzol reagent in 10 ml tissue dounces at room temperature using about 10 strokes. The pestle was rinsed with 1 ml TRIzol reagent. This 1.5 ml mixture was poured into a sterile microfuge tube. The sample was incubated at room temperature for 5 min to allow complete dissociation of cells. Subsequently, 200 μl chloroform were added, tubes were capped securely and shaken vigorously by hand for 15 sec, then incubated at room temperature for an additional 3 min. Samples were centrifuged at 12,000 rpm at 4°C for 15 min. The upper, aqueous phase containing the RNA was transferred to a new, sterile 1.5 ml microfuge tube and RNA precipitated by adding 500 μl isopropyl alcohol. This mixture was incubated at room temperature for 10 min. Samples were centrifuged at 12,000 rpm at 4°C for 15 min. The RNA precipitate formed a gel-like paste on the bottom of the tube. The supernatants were carefully

removed and the pellets washed with 1 ml of 75% ethanol. The samples were vortexed and centrifuged at 9,500 rpm for 5 min at 4°C. The supernatants were carefully removed and air-dried for 15-30 min being careful not to contaminate the samples by aerosol or over dry. RNA pellets were dissolved in 300 µl 0.2% DEPC-treated water and stored at -70°C.

Genomic DNA was removed by DNAseI treatment. Total RNA (300 µl) was combined with 20 µl 1M Tris (pH = 7.5), 4 µl 1M $MgCl_2$, 2 µl 10 mg/ml BSA, 4 µl DNAseI (RNAse free), and 70 µl DEPC-H_2O to make 400 µl total volume. This was incubated at 37°C for 30 min and then precipitated with 40 µl 3M sodium acetate (pH = 5.2). The tubes were filled with ice-cold 95-100% ethanol and incubated at -20°C for 30 min, then centrifuged at 12,000 rpm for 5 min at room temperature. The supernatants were carefully removed and 1 ml 75% ethanol was added, the samples were vortexed, and re-spun under the same conditions. The pellets were air dried for 5-10 min and resuspended in 300 µl DEPC-H_2O.

Complementary DNA (cDNA) was reverse transcribed from total RNA using TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). According to the manufacturer's instructions, 2 µl 10X RT buffer was combined with 4.4 µl 25 mM $MgCl_2$, 4 µl deoxy NTPs mixture, 1 µl random hexamers, 0.4 µl RNAse inhibitor, 1.25 µl Multiscribe reverse transcriptase, 400 ng total RNA and a suitable amount of DEPC-H_2O to make 20 µl total volume. The reagents were capped and centrifuged at 2000 rpm for 2 minutes. Thermal cycling was conducted as follows: 25°C (10 min), 37°C (60 min), 95°C (5 min), followed by an indefinite hold period at 4°C. Samples were diluted to 4 ng cDNA/ µl in double distilled H_2O.

Three different types of mRNA expression assays were used. *Il1rn* was assayed using a self-designed primer set and a TaqMan probe. *Il1b* mRNA levels were measured using assay-on demand gene expression assays (Applied Biosystems, Foster City, CA). The other genes were measured using SYBR Green assays using self-designed primers. For the Taqman assays, 12.5 µl 2X TaqMan mastermix, 1.25 µl 20X *Il1rn* probe, 2.25 µl 10 µM forward primer, 2.25 µl 10 µM reverse primer, 5.75 µl RNase free H_2O and 1 µl of cDNA from the corresponding samples were added to the Applied Biosystems optical plates and sealed with optical caps. The plate was centrifuged at 2,000 rpm for 2 min in an Eppendorf 5810R centrifuge (Eppendorf, Westbury, NY). For assay-on demand, 12,5 µl 2X TaqMan Universal PCR mastermix, 1.25 µl 20X assay-on demand Gene expression assay mix, 10.25 µl RNAse free water and 1.0 µl cDNA from corresponding samples were added to the Applied Biosystems optical plates and sealed with optical caps. The plate was centrifuged at 2,000 rpm for 2 min in an Eppendorf 5810R centrifuge (Eppendorf, Westbury, NY). Real-time PCR was performed for these TaqMan or assay-on demand assay plates in an ABI7700 sequence detection system (Applied Biosystems, Foster City, CA) with the following conditions: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 sec) and 60°C (1 min). Data were collected during all the steps in the PCR reaction.

For SYBR Green assays, 2.5 µl 10X SYBR Green buffer, 0.25 µl of 10 µM forward primer, 0.25 µl of 10 µM reverse primer, 3.0 µl 25 mM $MgCl_2$, 2.0 µl 12.5 mM dNTPs, 0.15 µl 5U/µl AmpliTaq Gold, 0.25 µl Uracil N-glycosylase, 15.6 µl double distilled H_2O and 1 µl of cDNA from corresponding samples were added to the Applied Biosystems optical plate, sealed with optical caps and centrifuged at 2,000 rpm for 2 min.

in an Eppendorf 5810R centrifuge (Eppendorf, Westbury, NY). Real-time PCR was performed for these TaqMan or assay-on demand assay plates in an ABI7700 sequence detection system (Applied Biosystems, Foster City, CA) with the following conditions: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 sec), 60°C (1 min) and 72°C (15 sec). Data were collected at 72°C. Primers and probes used for real-time RT-PCR are listed in Table 11.

Table 11. PRIMERS AND PROBES USED FOR REAL-TIME RT-PCR ASSAYS

*Primers & probe	Sequence (5' – 3')	Assay based on
	Il1rn	GenBank M64404
Forward primer	AGTACTGCCGAGGCCTGTAATAA	
Reverse primer	TTGTTCCTCAGGCCCCAAT	
Probe	ACCAACTGCCTGATCACTCTGGCCAT	
	Il1a	GenBank NM_010554
Forward primer	CAGGGCAGAGAGGGAGTCAAC	
Reverse primer	CAGGAACTTTGGCCATCTTGAT	
	Il1f9	GenBank NM_153511
Forward primer	CCCTTGTGACAGTTCCACGAA	
Reverse primer	GGGTACTTGCATGGGAGGATAG	
	Il1rl	GenBank NM_008362
Forward primer	CGGCGCATGTGCAGTTAATA	
Reverse primer	TGTAGCCGTGAGGATGATAAAGC	
	Il1r2	GenBank NM_010555
Forward primer	AGTGCAGCAAGACTCTGGTACCTA	
Reverse primer	AGTTCCACAGACATTTGCTCACA	
	18S rRNA	
Forward primer	CGGCTACCACATCCAAGGAA	
Reverse primer	GCTGGAATTACCGCGGCT	

* Primers & probe designed using Primer Express® software

D. Statistical analysis of mRNA expression data

Complementary DNA from a single time point (n=24, 6 samples/group) as well as a standard curve were assayed in 96 well optical plates in duplicates. The CT values of duplicates were averaged, and the relative amounts of target RNA were calculated by referring to the standard curve. The averages and standard deviations of target RNA and 18S RNA from each strain/treatment/time group (n = 6 samples) were calculated. These

averages were compared to show the effects of strain, treatment and time using the manufacturer's protocol[168].

The data of the relative ratio of target gene/18S rRNA were analyzed using SAS v 9.1 (SAS Institute, Cary, NC) software. The data was analyzed by two- or three-factor ANOVA mixed model, and the *P* values were adjusted for multiple comparisons using Tukey or Tukey-Kramer tests. The results were considered statistically significant when *P* values were < 0.05. When the residual plots in SAS v 9.1 showed trends indicating unequal variances within the groups, the data was analyzed after subjecting it to a logarithmic transformation.

E. PROTEIN COLLECTION FROM LUNG TISSUES

A/J and C3H/HeJ mice were sensitized and challenged as described above (Materials and methods – Section A). 1X PBS-Tween 20 buffer required for protein collection was prepared as follows. A 10X PBS stock buffer was prepared by adding 1.25 g $NaH_2PO4.H_2O$, 7.1 g Na_2HPO_4 and 43.85 g NaCl to a suitable quantity of nanopure double distilled H_2O to make a volume of 500 ml. 1X PBS-Tween 20 buffer was prepared as follows: To 100 ml of 10X PBS, 900 ml of nanopure ddH_2O was added, and the pH was adjusted to 7.2. To this, 1 ml of Tween-20 was added, mixed well with a stirrer, autoclaved and stored at 4°C.

Mice were sacrificed and lungs and spleens were collected at 6, 24 and 48 hrs post-challenge. Right lungs and spleens were collected and placed in sterile 5 ml tubes (USA Scientific, Ocala, FL) on ice. After all the right lung lobes and spleens were collected in a single time point, the tissues were weighed using a Mettler Toledo AX504

balance (Mettler Toledo, Columbus, OH), and 20 µl 1X PBS-Tween 20 buffer was added to each mg of tissue. The tissues in the buffer were then homogenized using a Pro 200 homogenizer with a 5mm x 75mm generator (Pro Scientific Inc, Oxford, CT). The homogenized tissues were centrifuged at 5000 rpm for 5 min at 4°C. The supernatants containing the proteins were aliquoted and stored at -80°C.

F. ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

- **ELISA for IL-1ra**

IL-1ra protein levels in the lungs were determined by an IL-1ra/IL-1F3 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). All the reagents were reconstituted and the recombinant standard for the IL-1ra assay was prepared as per the manufacturer's protocol (http://www.rndsystems.com/pdf/mra00.pdf). A microplate coated with a polyclonal antibody specific for mouse IL-1ra is available with the kit. The plate layout was recorded prior to the assay. 50 µl of assay diluent RD1W was added to each well, followed by the addition of 50 µl of standard, control or sample per well. The wells were covered with the adhesive strip provided. They were incubated for 2 h at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. This was followed by washing the plate five times with 400 µl wash buffer. After the last wash, the plate was inverted and blotted against clean paper towels. 100 µl of mouse IL-1ra conjugate was added to each well, the plate was covered with new adhesive cover strips and incubated for 2 h at room temperature on the shaker. After incubation, the plate was washed five times using 400 µl washing buffer, and blotted on clean paper towels after the last wash. 100 µl of Substrate Soultion was added to each well. The plate was

incubated for 30 minutes at room temperature at the benchtop, protected from light. After the incubation, 100 µl stop solution was added to the wells and gently tapped to ensure even mixing of the solutions. The optical densities of the samples were read in an ELISA reader (Microplate ELISA Reader, SoftMax program, Molecular Devices, Sunnyvale, CA) at 450 nm, with the correction set to 570 nm. The concentrations of IL-1ra in the samples (pg/ml) were calculated based on the standard curve. The sensitivity of the assay was 4-13 pg/ml.

- **ELISA for IL-1β**

Anti-mouse IL-1β antibody, biotinylated anti mouse IL-1β antibody and recombinant mouse IL-1β were purchased from R&D Systems (Minneapolis, MN). The following materials were purchased from sources as indicated in parenthesis. p-nitro-phenyl phosphate (PNPP) (Sigma, St Louis, MO, USA); Streptavidin alkaline hosphatase (Jackson ImmunoResearch, West Grove, PA); ELISA plates (Costar, Corning Inc., Corning, NY).

ELISA plates (96-well EIA/RIA plate, 96-well easy wash™, high binding, Corning, NY) were coated with anti-mouse IL-1β antibody (purified and unlabeled; 1µg/ml) diluted in carbonate buffer (0.005 M, pH 9.6) and incubated overnight at 4°C. Unbound antibody was discarded and the plates were blocked (0.17% BSA/PBS) at 37°C for 3 h. After washing (0.05% Tween 20 in PBS) recombinant IL-1β protein (standard) in two-fold dilutions and samples at appropriate dilutions (1 in 5 dilutions for 6 h and 1 in 2.5 diiutions for 24 and 48 h) in dilution buffer (0.085% BSA, 0.05% Tween 20 in PBS), were added to the plates and incubated overnight at 4°C. Following incubation, plates were washed four times and biotin-labeled IL-1β was added (0.1 µg/ml) and incubated at

37°C for 90 min. After incubation, plates were washed four times and streptavidin alkaline phosphatase (SAP) conjugate was added at 1:4000 (in dilution buffer). Subsequently, plates were washed again and p-nitro phenyl phosphate (PNPP) substrate added (1 tablet per 5 ml substrate buffer, according to manufacturer's instructions). Reactions were allowed to develop at room temperature in the dark and absorbance was measured in a microplate reader with dual mode of wavelength at 405 nm (peak) minus 690 nm (background) using KC4 software program (Synergy HT Multifunction Reader, Bio-Tek, Winooski, VT). According to the manufacturer's instructions, dual mode provides relatively better measurements since it adjusts the reading for background interference. All reagents were used at a final volume of 50 µl/well except for blocking buffer that was used at 75 µl/well. The sensitivity of the assay was 13 pg/ml.

G. PYROSEQUENCING

- **Polymerase chain reaction for Pyrosequencing**

The sequences surrounding the SNPs tested were obtained from dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Primers were designed using pyrosequencing resource (http://primerdesign.pyrosequencing.com/jsp/TemplateInput.jsp, http://biodev.hgen.pitt.edu/sop3/index.php) available on internet. The primers used for pyrosequencing are listed in Table 12.

Forward and reverse primers were obtained first, and PCR reactions were performed with the primer pairs in a subset of samples as follows. 2.5 µl 10X PCR buffer, 2.0 µl 25 mM $MgCl_2$, 0.3125 µl 10mM dNTPs, 0.15 µl 5U/µl AmpliTaq Gold DNA polymerase (all from Applied Biosystems, Foster City, CA) 0.5 µl 10 µM forward primer,

0.5 µl 10 µM reverse primer (both from IDT, Coralville, IA), 14.0375 µl double distilled H_2O and 5.0 µl genomic DNA (2 ng/µl) were added to PCR reaction tubes or plates, capped and centrifuged at 2,000 rpm for 2 minutes.

TABLE 12. PRIMERS USED FOR GENOTYPING BY PYROSEQUENCING

Primers	Primer sequences (5' – 3')
	dbSNP ID: rs2234678
Forward Primer	TGCTACTTTATGGGCAGCAG
Reverse Primer*	/5' Bio/TGAGAGTGGAAGGAGCTTACC
Sequencing Primer	TTGAGTTAGAGTCTGGAAGA
Blocking primer	TGCTACTTTATGGGCAGddC
	dbSNP ID: rs878972
Forward Primer	TCCCACCACTTCCCTTACAG
Reverse Primer*	/5' Bio/GCCTAAAATTGTTTTCAAACTTGG
Sequencing Primer	TGCTGACTCAAAGGGTA
Blocking primer	TGGAGGAGGAGGAGAAGGTGAAGAddC
	dbSNP ID: rs454078
Forward Primer	CAGTGGCTTGAAACAACCAA
Reverse Primer*	/5'Bio/TGAATGCAGCTTCCAAAGTG
Sequencing Primer	TTGAAACAACCAA
Blocking primer	None

* Biotinylated Primer

PCR reactions were performed in MJ Research Peltier PTC-200 or PTC-225 thermal cyclers (MJ Research, Watertown, MA) with the following conditions: 95°C (5 min), followed by 45 cycles of 95°C (15 sec), 60°C (30 sec), 72°C (15 sec), and a final extension and hold temperatures of 72C° (5 min) and 4°C (forever), respectively. The PCR products were separated on 2% agarose gels, and if the PCR reaction produced a clean, robust product, biotinylated primers were obtained. PCR products obtained from the reactions using one ordinary and one biotinylated primer were used for

pyrosequencing. All PCR reactions were performed on DNase/RNase free non-skirted 96 well PCR plates (Dot Scientific Inc, Burton, MI).

- **Sample preparation using the vacuum prep tool**

Four reagents were required for sample preparation using the vacuum prep tool for pyrosequencing – binding buffer, annealing buffer, denaturing reagent and washing buffer.

In the same PCR plate, 25 µl of each biotinylated PCR product was mixed with 3 µl Streptavidin-Sepharose high performance beads (Amersham Biosciences, Uppsala, Sweden), 12 µl of nanopure H_2O and 40 µl of binding buffer (10 mM Tris, 2 M NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA) and 1 ml Tween-20/liter of buffer; pH 7.6). The plate containing the biotinylated PCR product and the binding buffer was shaken at 1400 rpm for 10 minutes at room temperature.

While the plate was shaking, 0.2 µl 100 µM sequencing primer and 40 µl 1X annealing buffer (20 mM Tris, 2mM Magnesium acetate tetrahydrate; pH 7.6) were added to all the wells in a PSQ™ plate (Biotage, Uppsala, Sweden). A master mix was prepared for the number of reactions for every assay, and was added to the plate using a multi-channel pipette.

Four troughs supplied with the Pyrosequencing vacuum prep tool were filled with approximately 180 ml of high purity water, 70% ethanol, denaturing solution (0.2 M Sodium hydroxide) and washing buffer (10 mM Tris; pH 7.6). These troughs were refilled whenever needed. The probes in the vacuum tool were primed by applying vacuum and lowering the tool into the trough with high purity water for approximately 30

seconds to wash the filter probes. The PCR plate containing the biotinylated PCR product and the binding reaction was removed from the shaker, and the sepharose beads containing the immobilized biotinylated DNA strand were immediately captured by slowly lowering the vacuum prep tool with the probes into the PCR plate. The probes captured the streptavidin beads containing the biotinylated DNA strand. The PCR plate and the vacuum prep tool were carefully lifted together to check if all the beads had been captured on the probes.

Without touching the sides of the wells in the PCR plates, the vacuum prep tool was lifted from the PCR plate and washed for 5 seconds in the troughs with 70% ethanol, denaturing solution and the washing buffer. Then, the vacuum connection was removed from the vacuum prep tool, and the beads in the probes were released into the PSQ™ plate containing the sequencing primer and the annealing buffer. Release into the PSQ™ plate was facilitated by gently rubbing the filter probes in small circles against the bottom of the wells. After the beads were released, the vacuum prep tool with the filter probe was placed in nanopure water to clean the probes for subsequent use.

The PSQ™ plate containing the beads with biotinylated DNA strand, sequencing primer and the annealing buffer was heated at 80°C for 2 min using the PSQ 96 HS Sample Prep Thermoplate Kit. The plates were removed and cooled at room temperature for approximately 10 min, and the sequencing reaction was done in a PSQ™ 96MA pyrosequencer in the following way.

The PSQ™ program was started on the computer connected to the PSQ™ 96MA pyrosequencer, and later PSQ™ 96MA pyrosequencer was turned on, and allowed to warm up for 15 minutes. Information about the SNPs and the plates assayed was filled in

the necessary places in the program, and the program calculated the amount of reagents required for pyrosequencing each 96 well plate depending on the sequence composition. The cooled PSQ™ plate was then placed in the assigned slot in the PSQ™ 96MA pyrosequencer. The slots in the cartridge that dispensed the A, G, C, T nucleotides, the enzyme and substrate were filled with appropriate amounts of respective reagents (Pyrosequencing PSQ™ 96MA reagent kit, Biotage, Uppsala, Sweden) and placed in the cartridge slot. Pyrosequencing reactions were initiated, and the results were exported for data analyses and pyrograms were printed and saved for lab records. The exported results were later imported into the Isle of Wight SNP genotyping database in Dr. Susan Ewart's laboratory.

***Table 13.** *Il1rn* mRNA expression in lungs

			Il1rn Taqman - Lung 6Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rn RNA(ng)**	**SD**	**18S RNA (ng)**	**SD**
79	6Hr-PBS-C3H	25.580	28.954	-3.374	27.267	~~3.539~~		~~3.117~~	
80	6Hr-PBS-C3H	28.425	28.980	-0.555	28.703	1.881		3.495	
81	6Hr-PBS-C3H	29.109	29.091	0.018	29.100	1.579		2.980	
82	6Hr-PBS-C3H	29.063	29.729	-0.666	29.396	1.386		3.001	
83	6Hr-PBS-C3H	29.106	29.325	-0.219	29.216	1.501		2.651	
84	6Hr-PBS-C3H	29.290	28.912	0.378	29.101	1.578		3.093	
					Avg RNA	**1.585**	**0.183**	**3.044**	**0.303**
85	6Hr-PBS-A/J	30.170	29.833	0.337	30.002	1.062		2.152	
86	6Hr-PBS-A/J	27.350	26.793	0.557	27.072	3.857		4.315	
87	6Hr-PBS-A/J	28.483	28.420	0.063	28.452	2.101		2.996	
88	6Hr-PBS-A/J	27.958	27.711	0.247	27.835	2.756		3.517	
89	6Hr-PBS-A/J	28.606	28.654	-0.048	28.630	1.942		3.125	
90	6Hr-PBS-A/J	29.985	29.689	0.296	29.837	1.142		2.752	
					Avg RNA	**2.143**	**1.051**	**3.143**	**0.731**
91	6Hr-OVA-C3H	27.463	27.065	0.398	27.264	3.543		2.333	
92	6Hr-OVA-C3H	26.771	27.250	-0.479	27.011	3.961		2.765	
93	6Hr-OVA-C3H	25.811	24.787	1.024	25.299	~~8.414~~		~~3.173~~	
94	6Hr-OVA-C3H	29.391	29.321	0.070	29.356	1.411		1.808	
95	6Hr-OVA-C3H	28.104	27.210	0.894	27.657	2.980		3.683	
96	6Hr-OVA-C3H	27.121	26.557	0.564	26.839	4.272		2.391	
					Avg RNA	**3.234**	**1.128**	**2.596**	**0.697**
97	6Hr-OVA-A/J	26.321	26.788	-0.467	26.555	4.842		1.791	
98	6Hr-OVA-A/J	26.737	26.162	0.575	26.450	5.071		1.833	
99	6Hr-OVA-A/J	28.117	28.151	-0.034	28.134	2.416		2.133	
100	6Hr-OVA-A/J	25.156	25.615	-0.459	25.386	8.100		2.683	
101	6Hr-OVA-A/J	25.647	25.323	0.324	25.485	7.753		2.554	
102	6Hr-OVA-A/J	24.089	23.919	0.170	24.004	14.879		3.685	
					Avg RNA	**7.177**	**4.314**	**2.447**	**0.708**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 13 (cont'd…)

			Il1rn Taqman Lung-12 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
103	12Hr-PBS-C3H	32.836	33.680	-0.844	33.258	0.675		5.468	
104	12Hr-PBS-C3H	32.371	40.000	-7.629	32.371	~~1.050~~		~~5.207~~	
105	12Hr-PBS-C3H	26.780	32.903	-6.123	29.842	~~3.701~~		~~1.592~~	
106	12Hr-PBS-C3H	32.392	32.880	-0.488	32.636	0.920		4.031	
107	12Hr-PBS-C3H	32.911	32.636	0.275	32.774	0.859		3.899	
108	12Hr-PBS-C3H	32.899	32.772	0.127	32.836	0.833		4.865	
					Avg RNA	**0.821**	**0.104**	**4.566**	**0.738**
109	12Hr-PBS-A/J	32.359	32.327	0.032	32.343	1.064		5.185	
110	12Hr-PBS-A/J	29.688	29.512	0.176	29.600	4.174		4.368	
111	12Hr-PBS-A/J	29.362	29.807	-0.445	29.585	4.206		5.808	
112	12Hr-PBS-A/J	32.374	32.212	0.162	32.293	1.091		5.650	
113	12Hr-PBS-A/J	32.424	32.351	0.073	32.388	1.041		3.645	
114	12Hr-PBS-A/J	33.564	33.963	-0.399	33.764	0.524		1.965	
					Avg RNA	**2.017**	**1.697**	**4.437**	**1.460**
115	12Hr-OVA-C3H	30.474	30.063	0.411	30.269	2.992		4.209	
116	12Hr-OVA-C3H	31.007	30.596	0.411	30.802	2.294		3.563	
117	12Hr-OVA-C3H	29.852	28.619	1.233	29.236	~~5.005~~		~~5.781~~	
118	12Hr-OVA-C3H	27.297	27.284	0.013	27.291	13.190		7.360	
119	12Hr-OVA-C3H	30.622	30.251	0.371	30.437	2.751		3.707	
120	12Hr-OVA-C3H	29.699	29.139	0.560	29.419	4.568		6.658	
					Avg RNA	**5.159**	**4.570**	**5.099**	**1.777**
121	12Hr-OVA-A/J	28.754	28.609	0.145	28.682	6.596		4.902	
122	12Hr-OVA-A/J	28.351	28.104	0.247	28.228	8.270		3.604	
123	12Hr-OVA-A/J	28.103	28.169	-0.066	28.136	8.656		3.815	
124	12Hr-OVA-A/J	28.621	28.744	-0.123	28.683	6.593		4.374	
125	12Hr-OVA-A/J	27.495	27.600	-0.105	27.548	11.605		4.720	
126	12Hr-OVA-A/J	27.824	27.588	0.236	27.706	10.724		5.015	
					Avg RNA	**8.741**	**2.077**	**4.405**	**0.585**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 13 (cont'd…)

			Il1rn Taqman Lung 24 Hrs						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
1	24Hr-PBS-C3H	30.491	30.951	-0.460	30.721	0.735		4.007	
2	24Hr-PBS-C3H	31.141	31.219	-0.078	31.180	0.588		5.325	
3	24Hr-PBS-C3H	30.291	30.467	-0.176	30.379	0.868		9.160	
4	24Hr-PBS-C3H	40.000	40.000	0.000	40.000	x		x	
5	24Hr-PBS-C3H	30.037	30.357	-0.320	30.197	0.948		3.540	
6	24Hr-PBS-C3H	29.221	29.280	-0.059	29.251	1.503		2.813	
					Avg RNA	**0.928**	**0.349**	**4.969**	**2.515**
7	24Hr-PBS-A/J	32.270	32.494	-0.224	32.382	0.328		2.458	
8	24Hr-PBS-A/J	31.568	32.775	-1.207	32.172	~~0.363~~		~~3.366~~	
9	24Hr-PBS-A/J	32.115	31.183	0.932	31.649	0.468		2.969	
10	24Hr-PBS-A/J	31.302	31.423	-0.121	31.363	0.538		4.346	
11	24Hr-PBS-A/J	31.089	31.348	-0.259	31.219	0.577		3.946	
12	24Hr-PBS-A/J	32.091	32.325	-0.234	32.208	0.357		2.986	
					Avg RNA	**0.453**	**0.109**	**3.341**	**0.778**
13	24Hr-OVA-C3H	31.736	32.173	-0.437	31.955	0.403		2.621	
14	24Hr-OVA-C3H	30.524	30.374	0.150	30.449	0.839		4.320	
15	24Hr-OVA-C3H	29.631	29.462	0.169	29.547	1.301		4.811	
16	24Hr-OVA-C3H	30.055	29.706	0.349	29.881	1.106		4.095	
17	24Hr-OVA-C3H	30.698	30.886	-0.188	30.792	0.710		3.459	
18	24Hr-OVA-C3H	31.580	31.042	0.538	31.311	0.552		3.442	
					Avg RNA	**0.819**	**0.338**	**3.791**	**0.776**
19	24Hr-OVA-A/J	28.732	28.841	-0.109	28.787	1.884		3.041	
20	24Hr-OVA-A/J	27.577	27.684	-0.107	27.631	3.305		6.918	
21	24Hr-OVA-A/J	29.053	29.219	-0.166	29.136	1.589		5.749	
22	24Hr-OVA-A/J	29.100	29.127	-0.027	29.114	1.607		3.778	
23	24Hr-OVA-A/J	29.103	29.117	-0.014	29.110	1.609		4.556	
24	24Hr-OVA-A/J	26.730	26.818	-0.088	26.774	5.014		3.672	
					Avg RNA	**2.501**	**1.398**	**4.619**	**1.460**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 13 (cont'd...)

			Il1rn Taqman Lung 48Hrs						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
25	48Hr- PBS- C3H	31.192	31.630	-0.438	31.411	0.846		4.134	
26	48Hr- PBS- C3H	30.277	30.361	-0.084	30.319	1.448		6.347	
27	48Hr- PBS- C3H	30.853	31.053	-0.200	30.953	1.060		3.862	
28	48Hr- PBS- C3H	31.099	31.012	0.087	31.056	1.008		4.360	
29	48Hr- PBS- C3H	30.817	31.271	-0.454	31.044	1.013		3.266	
30	48Hr- PBS- C3H	30.806	30.642	0.164	30.724	1.186		5.976	
					Avg RNA	**1.094**	**0.205**	**4.658**	**1.227**
31	48Hr- PBS- A/J	30.909	31.062	-0.153	30.986	1.043		4.798	
32	48Hr- PBS- A/J	30.744	30.700	0.044	30.722	1.188		5.241	
33	48Hr- PBS- A/J	31.009	31.041	-0.032	31.025	1.023		3.710	
34	48Hr- PBS- A/J	31.517	31.061	0.456	31.289	0.898		3.412	
35	48Hr- PBS- A/J	30.528	30.690	-0.162	30.609	1.256		4.570	
36	48Hr- PBS- A/J	30.687	30.428	0.259	30.558	1.288		5.180	
					Avg RNA	**1.116**	**0.152**	**4.485**	**0.763**
37	48Hr- OVA- C3H	29.043	29.047	-0.004	29.045	2.713		5.780	
38	48Hr- OVA- C3H	29.854	30.081	-0.227	29.968	1.722		3.020	
39	48Hr- OVA- C3H	30.529	30.188	0.341	30.359	1.420		2.866	
40	48Hr-OVA-C3H	40.000	38.275	1.725	39.138	~~0.019~~		~~0.017~~	
41	48Hr- OVA- C3H	29.641	29.566	0.075	29.604	2.060		6.284	
42	48Hr- OVA- C3H	29.602	30.009	-0.407	29.806	1.865		4.089	
					Avg RNA	**1.956**	**0.483**	**4.408**	**1.566**
43	48Hr- OVA- A/J	29.615	29.427	0.188	29.521	2.146		5.856	
44	48Hr- OVA- A/J	31.775	32.244	-0.469	32.010	0.630		0.646	
45	48Hr- OVA- A/J	30.221	30.304	-0.083	30.263	1.489		2.812	
46	48Hr- OVA- A/J	30.010	30.043	-0.033	30.027	1.673		3.157	
47	48Hr- OVA- A/J	30.369	30.523	-0.154	30.446	1.361		3.580	
48	48Hr- OVA- A/J	30.344	30.681	-0.337	30.513	1.317		3.658	
					Avg RNA	**1.436**	**0.496**	**3.285**	**1.676**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 13 (cont'd…)

			Il1rn Lung 72 Hr Taqman						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
49	72Hr- PBS- C3H	30.887	31.054	-0.167	30.971	0.810		1.897	
50	72Hr- PBS- C3H	31.483	31.295	0.188	31.389	0.651		1.390	
51	72Hr- PBS- C3H	30.383	30.521	-0.138	30.452	1.060		2.727	
52	72Hr- PBS- C3H	31.151	31.614	-0.463	31.383	0.654		2.220	
53	72Hr- PBS- C3H	30.770	31.423	-0.653	31.097	0.758		1.678	
54	72Hr- PBS- C3H	29.408	29.290	0.118	29.349	1.880		1.884	
					Avg RNA	**0.969**	**0.471**	**1.966**	**0.463**
55	72Hr- PBS- A/J	31.074	31.075	-0.001	31.075	0.767		2.585	
56	72Hr- PBS- A/J	32.073	31.357	0.716	31.715	0.550		1.769	
57	72Hr- PBS- A/J	30.444	30.480	-0.036	30.462	1.054		2.838	
58	72Hr- PBS- A/J	31.059	30.317	0.742	30.688	0.938		2.717	
59	72Hr- PBS- A/J	32.105	31.676	0.429	31.891	0.502		2.104	
60	72Hr- PBS- A/J	32.500	32.481	0.019	32.491	0.368		1.073	
					Avg RNA	**0.696**	**0.268**	**2.181**	**0.676**
61	72Hr- OVA- C3H	30.291	30.278	0.013	30.285	1.156		3.110	
62	72Hr- OVA- C3H	32.071	31.720	0.351	31.896	0.501		2.413	
63	72Hr- OVA- C3H	33.834	32.523	1.311	33.179	~~0.257~~		~~1.211~~	
64	72Hr- OVA- C3H	30.901	31.308	-0.407	31.105	0.755		1.867	
65	72Hr- OVA- C3H	30.441	30.504	-0.063	30.473	1.049		1.118	
66	72Hr- OVA- C3H	30.576	30.627	-0.051	30.602	0.981		0.454	
					Avg RNA	**0.888**	**0.262**	**1.793**	**1.046**
67	72Hr- OVA- A/J	30.077	30.144	-0.067	30.111	1.266		2.964	
68	72Hr- OVA- A/J	32.103	31.504	0.599	31.804	0.525		2.140	
69	72Hr- OVA- A/J	32.290	31.377	0.913	31.834	0.517		1.395	
70	72Hr- OVA- A/J	29.860	30.388	-0.528	30.124	1.257		1.553	
71	72Hr- OVA- A/J	30.298	30.279	0.019	30.289	1.154		1.618	
72	72Hr- OVA- A/J	31.882	32.050	-0.168	31.966	0.483		0.854	
					Avg RNA	**0.867**	**0.395**	**1.754**	**0.723**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 14. *Il1b* mRNA expression in lungs

		Il1beta Assay on Demand Lung-6 Hr							
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1b RNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
79	6Hr-PBS-C3H	28.285	28.471	-0.186	28.378	0.148		3.117	
80	6Hr-PBS-C3H	28.884	28.858	0.026	28.871	0.105		3.495	
81	6Hr-PBS-C3H	29.015	28.199	0.816	28.607	0.127		2.980	
82	6Hr-PBS-C3H	28.343	28.347	-0.004	28.345	0.152		3.001	
83	6Hr-PBS-C3H	28.451	28.983	-0.532	28.717	0.117		2.651	
84	6Hr-PBS-C3H	27.102	27.097	0.005	27.100	0.361		3.093	
					Average	**0.168**	**0.096**	**3.060**	**0.270**
85	6Hr-PBS-A/J	30.444	29.972	0.472	30.208	0.042		2.152	
86	6Hr-PBS-A/J	27.209	27.045	0.164	27.127	0.354		4.315	
87	6Hr-PBS-A/J	29.374	30.012	-0.638	29.693	0.060		2.996	
88	6Hr-PBS-A/J	27.146	26.742	0.404	26.944	0.402		3.517	
89	6Hr-PBS-A/J	29.044	28.609	0.435	28.827	0.109		3.125	
90	6Hr-PBS-A/J	28.001	28.832	-0.831	28.417	0.145		2.752	
					Average	**0.185**	**0.154**	**3.143**	**0.731**
91	6Hr-OVA-C3H	25.408	25.499	-0.091	25.454	1.135		2.333	
92	6Hr-OVA-C3H	25.232	25.510	-0.278	25.371	1.201		2.765	
93	6Hr-OVA-C3H	24.522	24.116	0.406	24.319	2.497		3.173	
94	6Hr-OVA-C3H	28.242	27.996	0.246	28.119	0.178		1.808	
95	6Hr-OVA-C3H	25.507	25.404	0.103	25.456	1.133		3.683	
96	6Hr-OVA-C3H	24.845	24.673	0.172	24.759	1.839		2.391	
					Average	**1.330**	**0.780**	**2.692**	**0.666**
97	6Hr-OVA-A/J	24.828	24.029	0.799	24.828	#~~1.753~~		#~~1.791~~	
98	6Hr-OVA-A/J	25.202	25.290	-0.088	25.246	1.311		1.833	
99	6Hr-OVA-A/J	24.814	25.168	-0.354	24.991	1.565		2.133	
100	6Hr-OVA-A/J	24.355	24.567	-0.212	24.461	2.262		2.683	
101	6Hr-OVA-A/J	23.909	23.699	0.210	23.804	3.572		2.554	
102	6Hr-OVA-A/J	23.716	23.482	0.234	23.599	4.119		3.685	
					Average	**2.566**	**1.234**	**2.578**	**0.705**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0; # sample excluded due to pipetting error

Table 14 (cont'd…)

			Il1beta Assay on Demand Lung-12 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1bRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
103	12Hr-PBS-C3H	36.069	36.014	0.055	36.042	0.030		5.468	
104	12Hr-PBS-C3H	35.158	35.015	0.143	35.087	0.056		5.207	
105	12Hr-PBS-C3H	35.685	36.144	-0.459	35.915	0.032		1.592	
106	12Hr-PBS-C3H	36.372	36.114	0.258	36.243	0.026		4.031	
107	12Hr-PBS-C3H	35.419	35.972	-0.553	35.696	0.037		3.899	
108	12Hr-PBS-C3H	34.602	34.592	0.010	34.597	0.077		4.865	
					Average	**0.043**	**0.019**	**3.919**	**1.413**
109	12Hr-PBS-A/J	34.631	34.546	0.085	34.589	0.077		5.185	
110	12Hr-PBS-A/J	31.394	31.450	-0.056	31.422	0.611		4.368	
111	12Hr-PBS-A/J	32.160	32.126	0.034	32.143	0.381		5.808	
112	12Hr-PBS-A/J	36.048	35.620	0.428	35.834	0.034		5.650	
113	12Hr-PBS-A/J	36.017	36.202	-0.185	36.110	0.028		3.645	
114	12Hr-PBS-A/J	36.359	35.667	0.692	36.013	0.030		1.965	
					Average	**0.194**	**0.246**	**4.437**	**1.460**
115	12Hr-OVA-C3H	31.800	31.754	0.046	31.777	0.485		4.209	
116	12Hr-OVA-C3H	32.823	32.952	-0.129	32.888	0.234		3.563	
117	12Hr-OVA-C3H	32.132	31.845	0.287	31.989	0.422		5.781	
118	12Hr-OVA-C3H	29.399	29.158	0.241	29.279	2.486		7.360	
119	12Hr-OVA-C3H	34.526	34.221	0.305	34.374	0.089		3.707	
120	12Hr-OVA-C3H	31.829	31.565	0.264	31.697	0.511		6.658	
					Average	**0.704**	**0.888**	**5.213**	**1.613**
121	12Hr-OVA-A/J	29.643	29.520	0.123	29.582	2.039		4.902	
122	12Hr-OVA-A/J	30.470	30.015	0.455	30.243	1.323		3.604	
123	12Hr-OVA-A/J	30.145	30.152	-0.007	30.149	1.407		3.815	
124	12Hr-OVA-A/J	30.018	29.741	0.277	29.880	1.678		4.374	
125	12Hr-OVA-A/J	28.950	28.871	0.079	28.911	3.163		4.720	
126	12Hr-OVA-A/J	29.234	29.163	0.071	29.199	2.620		5.015	
					Average	**2.038**	**0.727**	**4.405**	**0.585**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 14 (cont'd...)

			Il1beta Assay on Demand Lung-24 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1bRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
1	24Hr-PBS-C3H	31.101	31.302	-0.201	31.202	0.066		4.007	
2	24Hr-PBS-C3H	33.189	33.242	-0.053	33.216	0.021		5.325	
3	24Hr-PBS-C3H	31.859	31.875	-0.016	31.867	0.045		9.160	
4	24Hr-PBS-C3H	40.000	40.000	0.000	40.000	x		x	
5	24Hr-PBS-C3H	31.556	31.461	0.095	31.509	0.055		3.540	
6	24Hr-PBS-C3H	31.045	30.749	0.296	30.897	0.078		2.813	
					Average	**0.053**	**0.022**	**4.969**	**2.515**
7	24Hr-PBS-A/J	34.475	35.449	-0.974	34.962	0.008		2.458	
8	24Hr-PBS-A/J	34.031	33.729	0.302	33.880	0.014		3.366	
9	24Hr-PBS-A/J	33.129	33.019	0.110	33.074	0.023		2.969	
10	24Hr-PBS-A/J	33.575	33.455	0.120	33.515	0.018		4.346	
11	24Hr-PBS-A/J	32.273	32.493	-0.220	32.383	0.033		3.946	
12	24Hr-PBS-A/J	33.480	33.200	0.280	33.340	0.019		2.986	
					Average	**0.019**	**0.009**	**3.345**	**0.696**
13	24Hr-OVA-C3H	32.660	34.045	-1.385	32.660	~~0.029~~		~~2.621~~	
14	24Hr-OVA-C3H	31.432	31.385	0.047	31.409	0.058		4.320	
15	24Hr-OVA-C3H	30.147	30.332	-0.185	30.240	0.114		4.811	
16	24Hr-OVA-C3H	31.113	30.976	0.137	31.045	0.072		4.095	
17	24Hr-OVA-C3H	31.421	29.687	1.734	30.554	~~0.095~~		~~3.459~~	
18	24Hr-OVA-C3H	32.374	32.281	0.093	32.328	0.035		3.442	
					Average	**0.070**	**0.033**	**4.167**	**0.568**
19	24Hr-OVA-A/J	29.919	29.594	0.325	29.757	0.150		3.041	
20	24Hr-OVA-A/J	28.599	28.647	-0.048	28.623	0.288		6.918	
21	24Hr-OVA-A/J	30.225	30.526	-0.301	30.376	0.106		5.749	
22	24Hr-OVA-A/J	29.259	29.079	0.180	29.169	0.210		3.778	
23	24Hr-OVA-A/J	28.306	28.440	-0.134	28.373	0.332		4.556	
24	24Hr-OVA-A/J	26.558	26.480	0.078	26.519	0.959		3.672	
					Average	**0.341**	**0.314**	**4.619**	**1.460**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 15. *Il1a* mRNA expression in lungs

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1a RNA(ng)	SD	18SRNA (ng)	SD
79	6 hrs	C3H PBS	31.84	31.74	0.100	31.79	0.35		3.24	
80	6 hrs	C3H PBS	33.27	32.46	0.810	32.87	0.19		2.72	
81	6 hrs	C3H PBS	31.71	32.49	-0.780	32.10	0.30		2.66	
82	6 hrs	C3H PBS	31.02	30.42	0.600	30.72	0.67		3.01	
83	6 hrs	C3H PBS	31.06	31.05	0.010	31.06	0.55		4.79	
84	6 hrs	C3H PBS	30.53	29.87	0.660	30.20	0.90		6.63	
						Average	**0.49**	**0.27**	**3.84**	**1.57**
85	6 hrs	AJ PBS	34.68	33.65	1.030	34.17	~~0.09~~		~~3.32~~	
86	6 hrs	AJ PBS	32.66	32.56	0.100	32.61	0.22		2.02	
87	6 hrs	AJ PBS	31.37	31.25	0.120	31.31	0.47		6.33	
88	6 hrs	AJ PBS	29.62	28.49	1.130	29.06	~~1.77~~		~~5.01~~	
89	6 hrs	AJ PBS	31.42	31.29	0.130	31.36	0.46		3.12	
90	6 hrs	AJ PBS	30.14	28.52	1.620	29.33	~~1.51~~		~~3.36~~	
						Average	**0.38**	**0.14**	**3.82**	**2.24**
91	6 hrs	C3H OVA	29.22	29.13	0.090	29.18	1.65		6.18	
92	6 hrs	C3H OVA	27.66	27.53	0.130	27.60	4.19		5.02	
93	6 hrs	C3H OVA	27.02	26.83	0.190	26.93	6.22		5.52	
94	6 hrs	C3H OVA	30.62	30.56	0.060	30.59	†~~0.72~~		†~~0.08~~	
95	6 hrs	C3H OVA	28.81	28.62	0.190	28.72	2.17		1.90	
96	6 hrs	C3H OVA	31.1	29.03	2.070	30.07	~~0.98~~		~~5.67~~	
						Average	**3.04**	**2.14**	**4.86**	**1.70**
97	6 hrs	AJ OVA	36.35	36.25	0.100	36.30	†~~0.02~~		†~~0.00~~	
98	6 hrs	AJ OVA	29.07	28.51	0.560	28.79	2.07		2.61	
99	6 hrs	AJ OVA	28.55	28.41	0.140	28.48	2.49		3.84	
100	6 hrs	AJ OVA	28.26	27.54	0.720	27.90	3.50		3.31	
101	6 hrs	AJ OVA	27.4	27.61	-0.210	27.51	4.42		4.65	
102	6 hrs	AJ OVA	27.87	28.49	-0.620	28.18	2.97		3.08	
						Average	**3.09**	**0.91**	**3.50**	**0.78**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0; † Excluded due to consistent poor performance across SYBR Green assays for all the genes tested in that batch of reverse-transcribed lung samples

Table 15 (cont'd...)

Sample	Time	Strain & Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1a RNA(ng)	SD	18SRNA (ng)	SD
49	72 hrs	C3H PBS	33.01	33.56	-0.550	33.29	0.08		1.90	
50	72 hrs	C3H PBS	34.71	35.42	-0.710	35.07	0.03		1.39	
51	72 hrs	C3H PBS	34.26	35.89	-1.630	35.08	~~0.03~~		~~2.73~~	
52	72 hrs	C3H PBS	33.46	35.46	-2.000	34.46	~~0.05~~		~~2.22~~	
53	72 hrs	C3H PBS	34.01	35.77	-1.760	34.89	~~0.04~~		~~1.68~~	
54	72 hrs	C3H PBS	31.42	30.81	0.610	31.12	0.25		1.88	
						Average	**0.12**	**0.11**	**1.72**	**0.29**
55	72 hrs	AJ PBS	35.02	35.52	-0.500	35.27	0.03		2.59	
56	72 hrs	AJ PBS	34.67	36.53	-1.860	35.60	~~0.03~~		~~1.77~~	
57	72 hrs	AJ PBS	33.08	32.62	0.460	32.85	0.11		2.84	
58	72 hrs	AJ PBS	29.97	30.58	-0.610	30.28	0.39		2.72	
59	72 hrs	AJ PBS	35.30	35.28	0.020	35.29	0.03		2.10	
60	72 hrs	AJ PBS	34.99	34.91	0.080	34.95	0.04		1.07	
						Average	**0.12**	**0.15**	**2.26**	**0.72**
61	72 hrs	C3H OVA	34.63	34.08	0.550	34.36	0.05		3.11	
62	72 hrs	C3H OVA	34.05	34.31	-0.260	34.18	0.05		2.41	
63	72 hrs	C3H OVA	37.71	37.58	0.130	37.65	0.01		1.21	
64	72 hrs	C3H OVA	34.47	35.24	-0.770	34.86	0.04		1.87	
65	72 hrs	C3H OVA	35.42	34.71	0.710	35.07	0.03		1.12	
66	72 hrs	C3H OVA	37.76	35.69	2.070	36.73	~~0.01~~		~~0.45~~	
						Average	**0.03**	**0.02**	**1.70**	**0.96**
67	72 hrs	AJ OVA	34.60	34.15	0.450	34.38	0.05		2.96	
68	72 hrs	AJ OVA	37.81	36.26	1.550	37.04	~~0.01~~		~~2.14~~	
69	72 hrs	AJ OVA	40.00	40.00	0.000	40.00	x		1.40	
70	72 hrs	AJ OVA	40.00	37.10	2.900	38.55	~~0.01~~	-	~~1.55~~	
71	72 hrs	AJ OVA	37.75	36.60	1.150	37.18	~~0.01~~	-	~~1.62~~	
72	72 hrs	AJ OVA	40.00	37.49	2.510	38.75	~~0.01~~	-	~~0.85~~	
						Average	**0.02**	**0.02**	**1.83**	**0.78**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 16. *Il1f9* mRNA expression in lungs

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1f9 RNA(ng)	SD	18SRNA (ng)	SD
79	6 hrs	C3H PBS	31.49	31.35	0.14	31.42	0.21		3.24	
80	6 hrs	C3H PBS	31.61	31.54	0.07	31.58	0.20		2.72	
81	6 hrs	C3H PBS	32.28	32.24	0.04	32.26	0.13		2.66	
82	6 hrs	C3H PBS	30.38	30.38	0.00	30.38	0.38		3.01	
83	6 hrs	C3H PBS	30.38	30.44	-0.06	30.41	0.38		4.79	
84	6 hrs	C3H PBS	29.49	29.80	-0.31	29.65	0.58		6.63	
						Average	**0.31**	**0.17**	**3.84**	**1.57**
85	6 hrs	AJ PBS	31.25	31.27	-0.02	31.26	0.23		3.32	
86	6 hrs	AJ PBS	29.83	29.78	0.05	29.81	0.53		2.02	
87	6 hrs	AJ PBS	30.23	30.38	-0.15	30.31	0.40		6.33	
88	6 hrs	AJ PBS	28.88	29.07	-0.19	28.98	0.85		5.01	
89	6 hrs	AJ PBS	30.32	30.55	-0.23	30.44	0.37		3.12	
90	6 hrs	AJ PBS	29.21	29.33	-0.12	29.27	0.72		3.36	
						Average	**0.52**	**0.23**	**3.82**	**2.24**
91	6 hrs	C3H OVA	30.00	30.06	-0.06	30.03	0.47		6.18	
92	6 hrs	C3H OVA	29.00	28.62	0.38	28.81	0.93		5.02	
93	6 hrs	C3H OVA	28.30	28.29	0.01	28.30	1.25		5.52	
94	6 hrs	C3H OVA	32.94	33.13	-0.19	33.04	†~~0.09~~		†~~0.08~~	
95	6 hrs	C3H OVA	30.60	30.89	-0.29	30.75	0.31		1.90	
96	6 hrs	C3H OVA	29.48	29.49	-0.01	29.49	0.64		5.67	
						Average	**0.72**	**0.37**	**4.86**	**1.70**
97	6 hrs	AJ OVA	35.21	35.47	-0.26	35.34	†~~0.02~~		†~~0.00~~	
98	6 hrs	AJ OVA	29.46	29.22	0.24	29.34	0.69		2.61	
99	6 hrs	AJ OVA	28.47	28.46	0.01	28.47	1.13		3.84	
100	6 hrs	AJ OVA	27.70	27.67	0.03	27.69	1.76		3.31	
101	6 hrs	AJ OVA	26.55	26.59	-0.04	26.57	3.30		4.65	
102	6 hrs	AJ OVA	27.39	27.12	0.27	27.26	2.24		3.08	
						Average	**1.83**	**1.02**	**3.50**	**0.78**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0; † Excluded due to consistent poor performance across SYBR Green assays in that batch of reverse-transcribed lung samples

Table 16 (cont'd…)

Sample	Time	Strain & Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1f9 RNA(ng)	SD	18SRNA (ng)	SD
49	72 hrs	C3H PBS	35.06	36.17	-1.11	35.62	~~0.02~~		~~1.90~~	
50	72 hrs	C3H PBS	35.59	33.71	1.88	34.65	~~0.03~~		~~1.39~~	
51	72 hrs	C3H PBS	33.70	34.18	-0.48	33.94	0.04		2.73	
52	72 hrs	C3H PBS	36.10	35.31	0.79	35.71	0.02		2.22	
53	72 hrs	C3H PBS	36.39	40.00	-3.61	38.20	~~0.01~~		~~1.68~~	
54	72 hrs	C3H PBS	32.72	40.00	-7.28	36.36	~~0.01~~		~~1.88~~	
						Average	**0.03**	**0.01**	**2.47**	**0.36**
55	72 hrs	AJ PBS	35.23	35.47	-0.24	35.35	0.02	-	2.59	
56	72 hrs	AJ PBS	34.90	35.79	-0.89	35.35	0.02		1.77	
57	72 hrs	AJ PBS	33.73	34.05	-0.32	33.89	0.04		2.84	
58	72 hrs	AJ PBS	31.00	30.99	0.01	31.00	0.15		2.72	
59	72 hrs	AJ PBS	35.82	36.65	-0.83	36.24	0.01		2.10	
60	72 hrs	AJ PBS	34.96	37.45	-2.49	36.21	~~0.01~~		~~1.07~~	
						Average	**0.05**	**0.06**	**2.40**	**0.45**
61	72 hrs	C3H OVA	35.71	34.73	0.98	35.22	0.02		3.11	
62	72 hrs	C3H OVA	34.11	33.96	0.15	34.04	0.04		2.41	
63	72 hrs	C3H OVA	36.03	35.65	0.38	35.84	0.02		1.21	
64	72 hrs	C3H OVA	34.99	37.34	-2.35	36.17	~~0.01~~		~~1.87~~	
65	72 hrs	C3H OVA	40.00	36.49	3.51	38.25	~~0.01~~		~~1.12~~	
66	72 hrs	C3H OVA	36.01	34.33	1.68	35.17	~~0.02~~		~~0.45~~	
						Average	**0.02**	**0.01**	**2.24**	**0.96**
67	72 hrs	AJ OVA	35.67	35.67	0.00	35.67	0.01		2.96	
68	72 hrs	AJ OVA	37.42	37.46	-0.04	37.44	0.01		2.14	
69	72 hrs	AJ OVA	35.77	35.33	0.44	35.55	0.01		1.40	
70	72 hrs	AJ OVA	37.36	37.27	0.09	37.32	0.01		1.55	
71	72 hrs	AJ OVA	38.42	36.58	1.84	37.50	~~0.01~~		~~1.62~~	
72	72 hrs	AJ OVA	36.77	35.44	1.33	36.11	~~0.01~~		~~0.85~~	
						Average	**0.01**	**0.00**	**2.01**	**0.71**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 17. *Il1r1* mRNA expression in lungs

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1r1 RNA(ng)	SD	18SRNA (ng)	SD
79	6 hrs	C3H PBS	26.94	27.15	-0.210	27.05	3.02		3.24	
80	6 hrs	C3H PBS	27.99	27.71	0.280	27.85	2.05		2.72	
81	6 hrs	C3H PBS	28.33	27.99	0.340	28.16	1.76		2.66	
82	6 hrs	C3H PBS	28.02	27.84	0.180	27.93	1.97		3.01	
83	6 hrs	C3H PBS	27.27	26.97	0.300	27.12	2.91		4.79	
84	6 hrs	C3H PBS	26.51	26.14	0.370	26.33	4.27		6.63	
						Average	**2.66**	**0.94**	**3.84**	**1.57**
85	6 hrs	AJ PBS	28.54	28.47	0.070	28.51	1.50		3.32	
86	6 hrs	AJ PBS	27.01	26.67	0.340	26.84	3.36		2.02	
87	6 hrs	AJ PBS	27.68	28.07	-0.390	27.88	2.03		6.33	
88	6 hrs	AJ PBS	24.79	25.42	-0.630	25.11	7.79		5.01	
89	6 hrs	AJ PBS	27.35	27.55	-0.200	27.45	2.50		3.12	
90	6 hrs	AJ PBS	27.88	26.17	1.710	27.03	~~3.07~~		~~3.36~~	
						Average	**3.43**	**2.53**	**3.82**	**2.24**
91	6 hrs	C3H OVA	26.32	26.99	-0.670	26.66	3.67		6.18	
92	6 hrs	C3H OVA	25.39	25.39	0.000	25.39	6.78		5.02	
93	6 hrs	C3H OVA	25.58	25.72	-0.140	25.65	5.98		5.52	
~~94~~	6 hrs	C3H OVA	29.45	30.49	-1.040	29.97	~~†0.73~~		~~†0.08~~	
95	6 hrs	C3H OVA	27.89	27.71	0.180	27.80	2.11		1.90	
96	6 hrs	C3H OVA	26.42	26.79	-0.370	26.61	3.76		5.67	
						Average	**4.46**	**1.89**	**4.86**	**1.70**
97	6 hrs	AJ OVA	37.52	38.4	-0.880	37.96	~~†0.02~~		~~†0.00~~	
98	6 hrs	AJ OVA	27.08	26.8	0.280	26.94	3.17		2.61	
99	6 hrs	AJ OVA	26.06	26.48	-0.420	26.27	4.38		3.84	
100	6 hrs	AJ OVA	26.38	25.55	0.830	25.97	5.08		3.31	
101	6 hrs	AJ OVA	26.88	27.18	-0.300	27.03	3.04		4.65	
102	6 hrs	AJ OVA	26.29	26.22	0.070	26.26	4.42		3.08	
						Average	**4.02**	**0.88**	**3.50**	**0.78**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0; † Excluded due to consistent poor performance across SYBR Green assays in that batch of reverse-transcribed lung samples

Table 17 (cont'd...)

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1r1 RNA(ng)	SD	18SRNA (ng)	SD
49	72 hrs	C3H PBS	30.09	30.62	-0.530	30.36	0.43		1.90	
50	72 hrs	C3H PBS	29.89	30.51	-0.620	30.20	0.47		1.39	
51	72 hrs	C3H PBS	28.92	29.03	-0.110	28.98	0.86		2.73	
52	72 hrs	C3H PBS	28.4	28.86	-0.460	28.63	1.02		2.22	
53	72 hrs	C3H PBS	29.87	29.92	-0.050	29.90	0.54		1.68	
54	72 hrs	C3H PBS	40	40	0.000	40.00	0.00		1.88	
						Average	**0.66**	**0.26**	**1.98**	**0.52**
55	72 hrs	AJ PBS	29.35	29.39	-0.040	29.37	0.70		2.59	
56	72 hrs	AJ PBS	29.83	29.96	-0.130	29.90	0.54		1.77	
57	72 hrs	AJ PBS	28.85	29.04	-0.190	28.95	0.87		2.84	
58	72 hrs	AJ PBS	28.12	28.23	-0.110	28.18	1.28		2.72	
59	72 hrs	AJ PBS	31.2	31.4	-0.200	31.30	0.27		2.10	
60	72 hrs	AJ PBS	30.23	30.52	-0.290	30.38	0.43		1.07	
						Average	**0.68**	**0.36**	**2.18**	**0.68**
61	72 hrs	C3H OVA	30.8	30.78	0.020	30.79	0.35		3.11	
62	72 hrs	C3H OVA	31.17	30.97	0.200	31.07	0.30		2.41	
63	72 hrs	C3H OVA	33.14	32.19	0.950	32.67	0.14		1.21	
64	72 hrs	C3H OVA	32.27	32.77	-0.500	32.52	0.15		1.87	
65	72 hrs	C3H OVA	34.12	34.24	-0.120	34.18	0.06		1.12	
66	72 hrs	C3H OVA	33.07	33.55	-0.480	33.31	0.10		0.45	
						Average	**0.18**	**0.11**	**1.70**	**0.96**
67	72 hrs	AJ OVA	29.67	29.51	0.160	29.59	0.63		2.96	
68	72 hrs	AJ OVA	31.9	31.68	0.220	31.79	0.21		2.14	
69	72 hrs	AJ OVA	31.48	31.9	-0.420	31.69	0.22		1.40	
70	72 hrs	AJ OVA	32.77	33.52	-0.750	33.15	0.11		1.55	
71	72 hrs	AJ OVA	32.2	32.58	-0.380	32.39	0.16		1.62	
72	72 hrs	AJ OVA	32.32	32.41	-0.090	32.37	0.16		0.85	
						Average	**0.25**	**0.19**	**1.75**	**0.72**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 18. *Il1r2* mRNA expression in lungs

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1r2 RNA(ng)	SD	18SRNA (ng)	SD
79	6 hrs	C3H PBS	36.24	36.02	0.220	36.13	0.23		3.24	
80	6 hrs	C3H PBS	37.74	37.52	0.220	37.63	0.12		2.72	
81	6 hrs	C3H PBS	36.12	36.14	-0.020	36.13	0.23		2.66	
82	6 hrs	C3H PBS	35.59	35.12	0.470	35.36	0.32		3.01	
83	6 hrs	C3H PBS	34.47	34.22	0.250	34.35	0.50		4.79	
84	6 hrs	C3H PBS	31.91	32.18	-0.270	32.05	1.35		6.63	
						Average	**0.46**	**0.46**	**3.84**	**1.57**
85	6 hrs	AJ PBS	36.46	36.28	0.180	36.37	0.21		3.32	
86	6 hrs	AJ PBS	36.18	36.28	-0.100	36.23	0.22		2.02	
87	6 hrs	AJ PBS	34	33.53	0.470	33.77	0.64		6.33	
88	6 hrs	AJ PBS	31.49	32.12	-0.630	31.81	1.50		5.01	
89	6 hrs	AJ PBS	33.58	33.74	-0.160	33.66	0.67		3.12	
90	6 hrs	AJ PBS	32.01	32.18	-0.170	32.10	1.32		3.36	
						Average	**0.76**	**0.55**	**3.82**	**2.24**
91	6 hrs	C3H OVA	32.69	30.7	1.990	31.70	~~1.58~~		~~6.18~~	
92	6 hrs	C3H OVA	31.61	40	-8.390	31.61	~~1.64~~		~~5.02~~	
93	6 hrs	C3H OVA	31.69	31.07	0.620	31.38	1.81		5.52	
94	6 hrs	C3H OVA	38.13	38.21	-0.080	38.17	†~~0.09~~		†~~0.08~~	
95	6 hrs	C3H OVA	34.9	35.07	-0.170	34.99	0.38		1.90	
96	6 hrs	C3H OVA	33.17	33.06	0.110	33.12	0.85		5.67	
						Average	**1.01**	**0.73**	**4.86**	**1.70**
97	6 hrs	AJ OVA	38.77	39.48	-0.710	39.13	†~~0.06~~		†~~0.00~~	
98	6 hrs	AJ OVA	31.49	31.56	-0.070	31.53	1.70		2.61	
99	6 hrs	AJ OVA	31.18	31.17	0.010	31.18	1.98		3.84	
100	6 hrs	AJ OVA	31.1	30.53	0.570	30.82	2.31		3.31	
101	6 hrs	AJ OVA	30.17	30.07	0.100	30.12	3.13		4.65	
102	6 hrs	AJ OVA	33.16	32.39	0.770	32.78	0.99		3.08	
						Average	**2.02**	**1.07**	**3.50**	**0.78**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0; † Excluded due to consistent poor performance across SYBR Green assays in that batch of reverse-transcribed lung samples

Table 18 (cont'd...)

Sample	Time	Strain &Rx	Ct1	Ct2	Ct1-Ct2	Average	Il1r2 RNA(ng)	SD	18SRNA (ng)	SD
49	72 hrs	C3H PBS	38.26	35.4	2.860	35.40	~~0.09~~		~~1.90~~	
50	72 hrs	C3H PBS	34.11	34.57	-0.460	34.34	0.13		1.39	
51	72 hrs	C3H PBS	36.54	35.87	0.670	36.21	0.07		2.73	
52	72 hrs	C3H PBS	33.38	33.84	-0.460	33.61	0.17		2.22	
53	72 hrs	C3H PBS	35.29	36.23	-0.940	35.76	0.08		1.68	
54	72 hrs	C3H PBS	40	40.00	0.000	40.00	x		x	
						Average	**0.11**	**0.05**	**2.00**	**0.59**
55	72 hrs	AJ PBS	34.86	36.23	-1.370	35.55	~~0.09~~		~~2.59~~	
56	72 hrs	AJ PBS	36.26	35.66	0.600	35.96	0.08		1.77	
57	72 hrs	AJ PBS	31.6	32.04	-0.440	31.82	0.33		2.84	
58	72 hrs	AJ PBS	31.2	31.38	-0.180	31.29	0.40		2.72	
59	72 hrs	AJ PBS	37.61	38.31	-0.700	37.96	0.04		2.10	
60	72 hrs	AJ PBS	34.68	33.62	1.060	34.15	~~0.14~~		~~1.07~~	
						Average	**0.21**	**0.18**	**2.36**	**0.51**
61	72 hrs	C3H OVA	36.67	35.73	0.940	36.20	0.07		3.11	
62	72 hrs	C3H OVA	35.97	35.01	0.960	35.49	0.09		2.41	
63	72 hrs	C3H OVA	37.46	38.47	-1.010	37.97	~~0.04~~		~~1.21~~	
64	72 hrs	C3H OVA	38.64	36.4	2.240	37.52	~~0.04~~		~~1.87~~	
65	72 hrs	C3H OVA	37.26	37.5	-0.240	37.38	0.05		1.12	
66	72 hrs	C3H OVA	38.66	37.11	1.550	37.89	~~0.04~~		~~0.45~~	
						Average	**0.07**	**0.02**	**2.21**	**1.01**
67	72 hrs	AJ OVA	37.18	35.74	1.440	36.46	~~0.06~~		~~2.96~~	
68	72 hrs	AJ OVA	37.43	38.3	-0.870	37.87	0.04		2.14	
69	72 hrs	AJ OVA	37.46	37.68	-0.220	37.57	0.04		1.40	
70	72 hrs	AJ OVA	38.07	40	-1.930	39.04	~~0.03~~		~~1.55~~	
71	72 hrs	AJ OVA	39.24	38.28	0.960	38.76	0.03		1.62	
72	72 hrs	AJ OVA	37.17	38.12	-0.950	37.65	0.04		0.85	
						Average	**0.04**	**0.01**	**1.50**	**0.53**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 19. IL-1ra protein production in lungs

		IL-1ra ELISA Lungs - 6 hr				
Sample	**Time**	**Strain & Rx**	**IL-1ra Protein - 1**	**IL-1ra Protein - 2**	**IL-1ra protein average**	**S.D**
127	6 hr	C3H PBS	1091.2	1152.7	1122.0	
128	6 hr	C3H PBS	1294.5	1407.7	1351.1	
129	6 hr	C3H PBS	1081.8	1178.3	1130.1	
130	6 hr	C3H PBS	1214.6	1154.1	1184.4	
131	6 hr	C3H PBS	1127.3	1181	1154.2	
				Average	**1188.3**	**94.2**
132	6 hr	C3H OVA	1644.2	1774.5	1709.4	
133	6 hr	C3H OVA	2024.2	1903.1	1963.7	
134	6 hr	C3H OVA	2195.3	1884.6	2040.0	
135	6 hr	C3H OVA	1473.7	1444.8	1459.3	
136	6 hr	C3H OVA	2320.1	2246	2283.1	
				Average	**1891.1**	**316.5**
137	6 hr	AJ PBS	891.56	877.18	884.4	
138	6 hr	AJ PBS	871.95	938.8	905.4	
139	6 hr	AJ PBS	847.16	899.42	873.3	
140	6 hr	AJ PBS	845.86	913.84	879.9	
141	6 hr	AJ PBS	784.85	783.55	784.2	
				Average	**865.4**	**47.0**
142	6 hr	AJ OVA	1616.3	1756.2	1686.3	
143	6 hr	AJ OVA	2002.7	1811.1	1906.9	
144	6 hr	AJ OVA	2027	1924.4	1975.7	
145	6 hr	AJ OVA	2204	2114.6	2159.3	
146	6 hr	AJ OVA	1559.4	1512.3	1535.9	
				Average	**1852.8**	**245.0**

Table 19 (cont'd…)

		IL-1ra ELISA Lungs - 24 hr				
Sample	**Time**	**Strain & Rx**	**IL-1ra Protein - 1**	**IL-1ra Protein - 2**	**IL-1ra protein average**	**S.D**
271	24 hr	C3H PBS	569.69	531.57	550.6	
272	24 hr	C3H PBS	640.31	980.3	810.3	
~~273~~	~~24 hr~~	~~C3H PBS~~	~~5188.1~~	~~6335.3~~	~~5761.7~~	
274	24 hr	C3H PBS	565.46	650.21	607.8	
275	24 hr	C3H PBS	845.36	1006.7	926.0	
				Average	**723.7**	**174.9**
276	24 hr	C3H OVA	738.13	783.74	760.9	
277	24 hr	C3H OVA	992.04	1247	1119.5	
278	24 hr	C3H OVA	1073.3	1333.8	1203.6	
279	24 hr	C3H OVA	1061.4	1575.8	1318.6	
280	24 hr	C3H OVA	2194.6	2684.3	2439.5	
				Average	**1368.4**	**634.0**
281	24 hr	AJ PBS	1263.9	1377.7	1320.8	
282	24 hr	AJ PBS	401.05	406.76	403.9	
283	24 hr	AJ PBS	423.87	527.33	475.6	
284	24 hr	AJ PBS	671.44	779.45	725.4	
285	24 hr	AJ PBS	393.9	521.68	457.8	
				Average	**676.7**	**380.8**
286	24 hr	AJ OVA	2002.9	2104.2	2053.6	
287	24 hr	AJ OVA	1479.7	1735.1	1607.4	
288	24 hr	AJ OVA	2269.7	2771.3	2520.5	
289	24 hr	AJ OVA	2997.3	3775.8	3386.6	
290	24 hr	AJ OVA	2885	4006.6	3445.8	
				Average	**2602.8**	**810.0**

* Values struck through were discarded, as the supernatant collected from sample No. 273 was not a clear solution like the other 19 samples in the time point, and hence could not be representative of the group.

Table 19 (cont'd...)

		IL-1ra ELISA Lungs - 48 hr				
Sample	**Time**	**Strain & Rx**	**IL-1ra Protein - 1**	**IL-1ra Protein - 2**	**IL-1ra protein average**	**S.D**
251	48 hr	C3H PBS	1340	1346.3	1343.2	
252	48 hr	C3H PBS	558.4	671.44	614.9	
253	48 hr	C3H PBS	1999.2	2630.9	2315.1	
254	48 hr	C3H PBS	1059.9	1322.8	1191.4	
255	48 hr	C3H PBS	433.83	575.34	504.6	
				Average	**1193.8**	**722.8**
256	48 hr	C3H OVA	2571.9	2673.6	2622.8	
257	48 hr	C3H OVA	1329.1	1368.3	1348.7	
258	48 hr	C3H OVA	1251.6	1433.2	1342.4	
259	48 hr	C3H OVA	1296.4	1539.8	1418.1	
260	48 hr	C3H OVA	1216.4	1423.7	1320.1	
				Average	**1610.4**	**567.1**
261	48 hr	AJ PBS	443.79	436.68	440.2	
262	48 hr	AJ PBS	358.02	439.52	398.8	
263	48 hr	AJ PBS	316.03	373.84	344.9	
264	48 hr	AJ PBS	320.39	419.6	370.0	
265	48 hr	AJ PBS	660.11	606.4	633.3	
				Average	**437.4**	**115.1**
266	48 hr	AJ OVA	1363.6	1098.6	1231.1	
267	48 hr	AJ OVA	1219.5	1406.2	1312.9	
268	48 hr	AJ OVA	1733.4	1747.1	1740.3	
269	48 hr	AJ OVA	1201.2	1489.4	1345.3	
270	48 hr	AJ OVA	1326	1510.5	1418.3	
				Average	**1409.6**	**196.7**

Table 20. IL-1β protein production in the lungs

		IL-1β ELISA Lungs - 6hr					
Sample	**Time**	**Strain & Rx**	**IL-1β Protein-1**	**IL-1β Protein-2**	**IL-1β Protein-3**	**IL-1β protein avg**	**IL-1β protein SD**
127	6hrs	C3H PBS	164.19	151.28	124.89	146.79	
128	6hrs	C3H PBS	183.26	238.72	176.94	199.64	
129	6hrs	C3H PBS	280.44	268.63	280.44	276.50	
130	6hrs	C3H PBS	183.26	164.19	no sample	173.73	
131	6hrs	C3H PBS	673.35	724.86	719.73	705.98	
					Average	**300.53**	**231.77**
132	6hrs	C3H OVA	4014.90	4010.20	4028.90	4018.00	
133	6hrs	C3H OVA	6621.30	6631.40	7170.10	6807.60	
134	6hrs	C3H OVA	5308.20	5890.20	5424.70	5541.03	
135	6hrs	C3H OVA	2275.70	2570.80	2764.30	2536.93	
136	6hrs	C3H OVA	7040.80	6717.90	6953.30	6904.00	
					Average	**5161.51**	**1876.56**
137	6hrs	AJ PBS	138.19	144.76	124.89	135.95	
138	6hrs	AJ PBS	29.53	37.88	45.92	37.78	
139	6hrs	AJ PBS	124.89	104.48	76.17	101.85	
140	6hrs	AJ PBS	111.35	68.83	68.83	83.00	
141	6hrs	AJ PBS	20.76	no value	61.36	27.37	
					Average	**89.64**	**40.94**
142	6hrs	AJ OVA	7399.20	7572.50	7398.80	7456.83	
143	6hrs	AJ OVA	8067.50	8392.40	8062.20	8174.03	
144	6hrs	AJ OVA	6223.40	6353.70	6153.40	6243.50	
145	6hrs	AJ OVA	2141.60	2169.40	2095.30	2135.43	
146	6hrs	AJ OVA	3721.00	4254.10	3963.40	3979.50	
					Average	**5597.86**	**2505.83**

Table 20 (cont'd…)

		IL-1β ELISA Lungs - 24 hr				
Sample	**Time**	**Strain & Rx**	**IL-1β Protein-1**	**IL-1β Protein-2**	**IL-1β protein avg**	**IL-1β protein SD**
271	24 hr	C3H PBS	408.49	403.65	406.07	
272	24 hr	C3H PBS	778.29	774.49	776.39	
~~273~~	~~24 hr~~	~~C3H PBS~~	~~3216.30~~	~~3376.30~~	~~3296.30~~	
274	24 hr	C3H PBS	496.62	418.11	457.37	
275	24 hr	C3H PBS	958.57	1279.10	1118.84	
				Average	**689.67**	**329.70**
276	24 hr	C3H OVA	446.44	427.64	437.04	
277	24 hr	C3H OVA	724.44	736.09	730.27	
278	24 hr	C3H OVA	1520.20	1552.90	1536.55	
279	24 hr	C3H OVA	972.85	1025.90	999.38	
280	24 hr	C3H OVA	2280.40	2572.00	2426.20	
				Average	**1225.89**	**783.66**
281	24 hr	AJ PBS	1679.70	1763.90	1721.80	
282	24 hr	AJ PBS	599.91	566.28	583.10	
283	24 hr	AJ PBS	649.10	732.21	690.66	
284	24 hr	AJ PBS	641.00	685.15	663.08	
285	24 hr	AJ PBS	496.62	636.93	566.78	
				Average	**845.08**	**492.87**
286	24 hr	AJ OVA	1208.40	1365.60	1287.00	
287	24 hr	AJ OVA	1126.60	1272.40	1199.50	
288	24 hr	AJ OVA	1428.40	1510.40	1469.40	
289	24 hr	AJ OVA	1825.40	1984.00	1904.70	
290	24 hr	AJ OVA	1359.00	1530.00	1444.50	
				Average	**1461.02**	**271.97**

* Values struck through were discarded, as the supernatant collected from sample No. 273 was not a clear solution like the other 19 samples in the time point, and hence could not be representative of the group.

Table 20 (cont'd…)

		IL-1β ELISA Lungs - 48 hr				
Sample	**Time**	**Strain & Rx**	**IL-1β Protein-1**	**IL-1β Protein-2**	**IL-1β protein avg**	**IL-1β protein SD**
251	48 hr	C3H PBS	1533.30	1618.00	1575.65	
252	48 hr	C3H PBS	867.98	937.06	902.52	
253	48 hr	C3H PBS	5279.00	5425.30	5352.15	
254	48 hr	C3H PBS	987.00	864.30	925.65	
255	48 hr	C3H PBS	205.79	218.61	212.20	
				Average	**904.01**	**556.83**
256	48 hr	C3H OVA	2916.10	3227.10	3071.60	
257	48 hr	C3H OVA	1171.10	1248.90	1210.00	
258	48 hr	C3H OVA	1657.00	1670.00	1663.50	
259	48 hr	C3H OVA	720.54	778.29	749.42	
260	48 hr	C3H OVA	369.03	469.51	419.27	
				Average	**1422.76**	**1034.68**
261	48 hr	AJ PBS	118.73	101.46	110.10	
262	48 hr	AJ PBS	327.72	369.03	348.38	
263	48 hr	AJ PBS	353.78	289.66	321.72	
264	48 hr	AJ PBS		451.09	451.09	
265	48 hr	AJ PBS	393.89	413.31	403.60	
				Average	**326.98**	**131.15**
266	48 hr	AJ OVA	661.19	464.93	563.06	
267	48 hr	AJ OVA	437.08	432.37	434.73	
268	48 hr	AJ OVA	641.00	632.86	636.93	
269	48 hr	AJ OVA	612.34	562.02	587.18	
270	48 hr	AJ OVA	628.77	661.19	644.98	
				Average	**573.38**	**84.69**

Table 21. *Il1rn* mRNA expression in spleen

			Il1rn Taqman - Spleen 6Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
79	6Hr-PBS-C3H	29.834	30.014	-0.180	29.924	9.796		7.078	
80	6Hr-PBS-C3H	33.351	33.242	0.109	33.297	1.540		6.363	
81	6Hr-PBS-C3H	31.509	31.538	-0.029	31.524	4.073		6.154	
82	6Hr-PBS-C3H	32.013	32.466	-0.453	32.240	2.750		4.170	
83	6Hr-PBS-C3H	31.569	31.455	0.114	31.512	4.099		9.619	
84	6Hr-PBS-C3H	29.494	29.637	-0.143	29.566	11.926		12.668	
					Average	**5.697**	**4.166**	**7.675**	**3.013**
85	6Hr-PBS-A/J	33.953	34.209	-0.256	34.081	1.001		9.867	
86	6Hr-PBS-A/J	39.158	33.029	6.129	33.029	1.783		6.558	
87	6Hr-PBS-A/J	34.576	34.745	-0.169	34.661	0.728		1.983	
88	6Hr-PBS-A/J	30.385	30.374	0.011	30.380	7.630		5.629	
89	6Hr-PBS-A/J	32.753	32.280	0.473	32.517	2.362		4.355	
90	6Hr-PBS-A/J	32.314	32.614	-0.300	32.464	2.431		3.156	
					Average	**2.656**	**2.534**	**5.258**	**2.794**
91	6Hr-OVA-C3H	27.578	27.796	-0.218	27.687	33.429		7.886	
92	6Hr-OVA-C3H	30.422	30.407	0.015	30.415	7.485		5.035	
93	6Hr-OVA-C3H	30.627	30.246	0.381	30.437	7.395		4.661	
94	6Hr-OVA-C3H	32.087	32.336	-0.249	32.212	2.792		3.711	
95	6Hr-OVA-C3H	29.752	29.990	-0.238	29.871	10.085		4.901	
96	6Hr-OVA-C3H	31.038	30.627	0.411	30.833	5.951		2.685	
					Average	**11.190**	**11.153**	**4.813**	**1.747**
97	6Hr-OVA-A/J	33.537	33.313	0.224	33.425	1.435		3.708	
98	6Hr-OVA-A/J	31.629	31.329	0.300	31.479	4.174		4.994	
99	6Hr-OVA-A/J	36.326	37.539	-1.213	36.933	~~0.209~~	-	~~2.643~~	-
100	6Hr-OVA-A/J	31.781	32.079	-0.298	31.930	3.259		3.425	
101	6Hr-OVA-A/J	31.005	30.854	0.151	30.930	5.642		4.888	
102	6Hr-OVA-A/J	29.028	29.169	-0.141	29.099	15.409		1.434	
					Average	**5.984**	**5.485**	**3.690**	**1.440**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 21 (cont'd…)

			Il1rn Taqman - Spleen 12 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
103	12Hr-PBS-C3H	30.401	30.596	-0.195	30.499	10.807		12.348	
104	12Hr-PBS-C3H	27.855	28.312	-0.457	28.084	60.865		5.135	
105	12Hr-PBS-C3H	31.040	31.072	-0.032	31.056	7.251		8.228	
106	12Hr-PBS-C3H	30.078	30.843	-0.765	30.461	11.105		21.535	
107	12Hr-PBS-C3H	29.922	29.916	0.006	29.919	16.362		22.958	
108	12Hr-PBS-C3H	27.702	27.922	-0.220	27.812	73.920		25.591	
					Average	**30.052**	**29.362**	**15.966**	**8.518**
109	12Hr-PBS-A/J	30.450	30.817	-0.367	30.634	9.811		4.670	
110	12Hr-PBS-A/J	28.672	29.077	-0.405	28.875	34.554		7.272	
111	12Hr-PBS-A/J	32.276	32.260	0.016	32.268	3.046		6.272	
112	12Hr-PBS-A/J	30.864	30.406	0.458	30.635	9.801		11.501	
113	12Hr-PBS-A/J	32.254	32.132	0.122	32.193	3.214		5.675	
114	12Hr-PBS-A/J	30.714	30.674	0.040	30.694	9.396		8.166	
					Average	**11.637**	**11.676**	**7.259**	**2.409**
115	12Hr-OVA-C3H	29.190	29.443	-0.253	29.317	25.183		15.173	
116	12Hr-OVA-C3H	31.624	31.819	-0.195	31.722	4.503		10.150	
117	12Hr-OVA-C3H	27.261	27.550	-0.289	27.406	98.883		10.315	
118	12Hr-OVA-C3H	27.168	27.278	-0.110	27.223	112.680		9.885	
119	12Hr-OVA-C3H	29.204	29.399	-0.195	29.302	25.455		15.561	
120	12Hr-OVA-C3H	32.442	32.093	0.349	32.268	3.047		5.681	
					Average	**44.958**	**48.288**	**11.127**	**3.711**
121	12Hr-OVA-A/J	32.918	33.221	-0.303	33.070	1.716		4.643	
122	12Hr-OVA-A/J	29.211	29.199	0.012	29.205	27.275		22.001	
123	12Hr-OVA-A/J	31.417	31.427	-0.010	31.422	5.580		12.323	
124	12Hr-OVA-A/J	29.236	29.734	-0.498	29.485	22.322		9.707	
125	12Hr-OVA-A/J	30.884	31.296	-0.412	31.090	7.077		4.707	
126	12Hr-OVA-A/J	31.599	31.922	-0.323	31.761	4.379		5.018	
					Average	**11.392**	**10.648**	**9.733**	**6.791**

Table 21 (Cont'd…)

				Il1rn Taqman - Spleen 24Hr					
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
1	24Hr-PBS-C3H	30.252	30.516	-0.264	30.384	7.957		17.373	
2	24Hr-PBS-C3H	30.278	30.460	-0.182	30.369	8.045		14.723	
3	24Hr-PBS-C3H	30.056	30.095	-0.039	30.076	9.983		17.427	
4	24Hr-PBS-C3H	40.000	40.000	0.000	40.000	~~0.007~~	-	~~0.000~~	-
5	24Hr-PBS-C3H	28.913	29.071	-0.158	28.992	22.148		20.874	
6	24Hr-PBS-C3H	31.123	30.879	0.244	31.001	5.054		12.198	
					Average	**10.637**	**6.671**	**16.519**	**3.257**
7	24Hr-PBS-A/J	29.753	30.009	-0.256	29.881	11.518		4.766	
8	24Hr-PBS-A/J	30.232	30.200	0.032	30.216	9.003		15.920	
9	24Hr-PBS-A/J	32.613	32.935	-0.322	32.774	1.372		16.320	
10	24Hr-PBS-A/J	32.488	32.722	-0.234	32.605	1.554		6.588	
11	24Hr-PBS-A/J	32.693	33.472	-0.779	33.083	1.093		7.709	
12	24Hr-PBS-A/J	32.064	33.185	-1.121	32.625	~~1.531~~	-	~~7.800~~	-
					Average	**4.908**	**4.969**	**10.261**	**5.453**
13	24Hr-OVA-C3H	30.022	30.033	-0.011	30.028	10.342		26.350	
14	24Hr-OVA-C3H	29.530	29.356	0.174	29.443	15.896		24.609	
15	24Hr-OVA-C3H	27.843	28.082	-0.239	27.963	47.225		8.823	
16	24Hr-OVA-C3H	29.363	29.354	0.009	29.359	16.915		16.141	
17	24Hr-OVA-C3H	30.546	30.144	0.402	30.345	8.188		8.609	
18	24Hr-OVA-C3H	30.342	30.384	-0.042	30.363	8.080		25.305	
					Average	**17.774**	**14.918**	**18.306**	**8.271**
19	24Hr-OVA-A/J	31.347	31.261	0.086	31.304	4.045		11.483	
20	24Hr-OVA-A/J	31.440	31.498	-0.058	31.469	3.582		9.317	
21	24Hr-OVA-A/J	29.623	29.557	0.066	29.590	14.267		21.597	
22	24Hr-OVA-A/J	30.207	29.956	0.251	30.082	9.939		6.253	
23	24Hr-OVA-A/J	29.640	30.058	-0.418	29.849	11.793		16.285	
24	24Hr-OVA-A/J	30.542	30.554	-0.012	30.548	7.053		18.884	
					Average	**8.446**	**4.296**	**13.970**	**5.917**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 22. *Il1b* mRNA expression in spleen

			Il1b Taqman - Spleen 6Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rnRNA(ng)**	**SD**	**18SRNA (ng)**	**SD**
79	6Hr-PBS-C3H	27.361	27.451	-0.090	27.406	10.503		7.078	
80	6Hr-PBS-C3H	29.926	29.729	0.197	29.828	2.156		6.363	
81	6Hr-PBS-C3H	27.877	27.994	-0.117	27.936	7.429		6.154	
82	6Hr-PBS-C3H	29.748	30.052	-0.304	29.900	2.056		4.170	
83	6Hr-PBS-C3H	28.182	27.511	0.671	27.847	7.874		9.619	
84	6Hr-PBS-C3H	25.543	25.503	0.040	25.523	35.986		12.668	
					Average	**11.001**	**12.691**	**7.675**	**3.013**
85	6Hr-PBS-A/J	31.068	31.370	-0.302	31.219	0.868		9.867	
86	6Hr-PBS-A/J	30.708	30.459	0.249	30.584	1.315		6.558	
87	6Hr-PBS-A/J	31.112	30.285	0.827	30.699	1.220		1.983	
88	6Hr-PBS-A/J	27.974	27.771	0.203	27.873	7.742		5.629	
89	6Hr-PBS-A/J	29.827	29.992	-0.165	29.910	2.043		4.355	
90	6Hr-PBS-A/J	30.081	29.803	0.278	29.942	2.000		3.156	
					Average	**2.531**	**2.594**	**5.258**	**2.794**
91	6Hr-OVA-C3H	25.701	25.887	-0.186	25.794	30.142		7.886	
92	6Hr-OVA-C3H	27.630	27.593	0.037	27.612	9.183		5.035	
93	6Hr-OVA-C3H	26.596	26.655	-0.059	26.626	17.499		4.661	
94	6Hr-OVA-C3H	27.595	27.240	0.355	27.418	10.425		3.711	
95	6Hr-OVA-C3H	28.287	28.247	0.040	28.267	5.981		4.901	
96	6Hr-OVA-C3H	27.074	26.928	0.146	27.001	13.689		2.685	
					Average	**14.486**	**8.623**	**4.813**	**1.747**
97	6Hr-OVA-A/J	30.067	30.353	-0.286	30.210	1.679		3.708	
98	6Hr-OVA-A/J	29.915	29.337	0.578	29.626	2.459		4.994	
99	6Hr-OVA-A/J	32.350	32.013	0.337	32.182	0.462		2.643	
100	6Hr-OVA-A/J	30.383	30.120	0.263	30.252	1.634		3.425	
101	6Hr-OVA-A/J	29.283	29.071	0.212	29.177	3.299		4.888	
102	6Hr-OVA-A/J	27.825	27.789	0.036	27.807	8.081		1.434	
					Average	**2.936**	**2.691**	**3.516**	**1.357**

Table 22 (Cont'd…)

			Il1b Taqman - Spleen 12 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rn RNA(ng)**	**SD**	**18S RNA (ng)**	**SD**
103	12Hr-PBS-C3H	28.251	28.253	-0.002	28.252	31.987		12.348	
104	12Hr-PBS-C3H	27.618	27.851	-0.233	27.735	43.186		5.135	
105	12Hr-PBS-C3H	28.299	28.621	-0.322	28.460	28.351		8.228	
106	12Hr-PBS-C3H	27.909	28.103	-0.194	28.006	36.893		21.535	
107	12Hr-PBS-C3H	26.833	27.215	-0.382	27.024	65.213		22.958	
108	12Hr-PBS-C3H	25.315	25.713	-0.398	25.514	156.579		25.591	
					Average	**60.368**	**48.902**	**15.966**	**8.518**
109	12Hr-PBS-A/J	30.000	30.577	-0.577	30.289	9.816		4.670	
110	12Hr-PBS-A/J	26.579	26.827	-0.248	26.703	78.560		7.272	
111	12Hr-PBS-A/J	29.946	29.608	0.338	29.777	13.207		6.272	
112	12Hr-PBS-A/J	29.394	29.321	0.073	29.358	16.845		11.501	
113	12Hr-PBS-A/J	30.571	30.437	0.134	30.504	8.663		5.675	
114	12Hr-PBS-A/J	28.873	28.459	0.414	28.666	25.158		8.166	
					Average	**25.375**	**26.724**	**7.259**	**2.409**
115	12Hr-OVA-C3H	27.710	27.666	0.044	27.688	44.367		15.173	
116	12Hr-OVA-C3H	28.946	29.009	-0.063	28.978	20.999		10.150	
117	12Hr-OVA-C3H	25.835	26.012	-0.177	25.924	123.473		10.315	
118	12Hr-OVA-C3H	26.330	26.480	-0.150	26.405	93.384		9.885	
119	12Hr-OVA-C3H	26.562	26.587	-0.025	26.575	84.639		15.561	
120	12Hr-OVA-C3H	30.049	29.965	0.084	30.007	11.557		5.681	
					Average	**63.070**	**44.286**	**11.127**	**3.711**
121	12Hr-OVA-A/J	29.624	29.407	0.217	29.516	15.370		4.643	
122	12Hr-OVA-A/J	26.994	27.057	-0.063	27.026	65.156		22.001	
123	12Hr-OVA-A/J	29.739	29.566	0.173	29.653	14.196		12.323	
124	12Hr-OVA-A/J	27.787	28.118	-0.331	27.953	38.056		9.707	
125	12Hr-OVA-A/J	40.000	40.000	0.000	40.000	~~0.035~~	-	~~4.707~~	-
126	12Hr-OVA-A/J	40.000	40.000	0.000	40.000	~~0.035~~	-	~~5.018~~	-
					Average	**33.195**	**23.971**	**12.168**	**7.289**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0, or were failed reactions

Table 22 (Cont'd…)

			Il1b Taqman - Spleen 24 Hr						
Sample	**Time & Rx**	**Ct1**	**Ct2**	**Ct1-Ct2**	**Average**	**Il1rn RNA(ng)**	**SD**	**18S RNA (ng)**	**SD**
1	24Hr-PBS-C3H	29.328	29.497	-0.169	29.413	2.843		17.373	
2	24Hr-PBS-C3H	27.672	27.692	-0.020	27.682	8.410		14.723	
3	24Hr-PBS-C3H	27.047	27.190	-0.143	27.119	11.972		17.427	
4	24Hr-PBS-C3H	40.000	40.000	0.000	40.000	~~0.000~~	-	~~0.000~~	-
5	24Hr-PBS-C3H	26.574	27.051	-0.477	26.813	14.503		20.874	
6	24Hr-PBS-C3H	28.216	27.957	0.259	28.087	6.527		12.198	
					Average	**8.851**	**4.565**	**16.519**	**3.257**
7	24Hr-PBS-A/J	30.501	29.465	1.036	29.983	1.989		4.766	
8	24Hr-PBS-A/J	28.182	28.812	-0.630	28.497	5.047		15.920	
9	24Hr-PBS-A/J	27.870	27.491	0.379	27.681	8.418		16.320	
10	24Hr-PBS-A/J	29.364	29.705	-0.341	29.535	2.634		6.588	
11	24Hr-PBS-A/J	29.406	30.151	-0.745	29.779	2.261		7.709	
12	24Hr-PBS-A/J	30.022	30.014	0.008	30.018	1.946		7.800	
					Average	**3.716**	**2.580**	**9.851**	**4.980**
13	24Hr-OVA-C3H	27.172	26.896	0.276	27.034	12.623		26.350	
14	24Hr-OVA-C3H	27.516	27.609	-0.093	27.563	9.064		24.609	
15	24Hr-OVA-C3H	26.301	26.656	-0.355	26.479	17.879		8.823	
16	24Hr-OVA-C3H	28.079	27.858	0.221	27.969	7.028		16.141	
17	24Hr-OVA-C3H	29.205	28.846	0.359	29.026	3.624		8.609	
18	24Hr-OVA-C3H	27.314	27.654	-0.340	27.484	9.521		25.305	
					Average	**9.957**	**4.890**	**18.306**	**8.271**
19	24Hr-OVA-A/J	29.906	30.142	-0.236	30.024	1.938		11.483	
20	24Hr-OVA-A/J	29.362	29.093	0.269	29.228	3.193		9.317	
21	24Hr-OVA-A/J	28.808	28.111	0.697	28.460	5.167		21.597	
22	24Hr-OVA-A/J	27.586	27.999	-0.413	27.793	7.847		6.253	
23	24Hr-OVA-A/J	27.270	27.100	0.170	27.185	11.483		16.285	
24	24Hr-OVA-A/J	27.001	26.779	0.222	26.890	13.815		18.884	
					Average	**7.241**	**4.699**	**13.970**	**5.917**

* Ct1 and Ct2 are replicates. Values struck through did not meet the inclusion criteria of [Ct1 – Ct2] < 1.0

Table 23. Isle of Wight genotypes for the SNPs rs2234678, rs878972 and rs454078

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1	G	A	C	A	T	A	31	-	-	-	-	-	-	61	-	-	-	-	-	-
2	A	A	A	A	A	A	32	A	A	A	A	A	A	62	A	A	A	A	A	A
3	G	A	C	A	T	A	33	-	-	-	-	-	-	63	A	A	A	A	A	A
4	G	G	C	C	T	T	34	G	A	C	A	T	A	64	G	G	C	C	T	T
5	G	A	C	A	T	A	35	-	-	-	-	-	-	65	A	A	A	A	A	A
6	-	-	-	-	-	-	36	A	A	A	A	A	A	66	A	A	A	A	A	A
7	-	-	-	-	-	-	37	G	A	C	A	T	A	67	G	G	C	C	T	T
8	A	A	A	A	A	A	38	-	-	-	-	-	-	68	A	A	A	A	A	A
9	G	A	C	A	T	A	39	G	A	C	A	T	A	69	A	A	A	A	A	A
10	A	A	A	A	A	A	40	G	A	A	A	A	A	70	G	A	C	A	T	A
11	G	A	C	A	T	A	41	-	-	-	-	-	-	71	G	A	C	A	T	A
12	A	A	A	A	A	A	42	-	-	-	-	-	-	72	A	A	A	A	A	A
13	-	-	-	-	-	-	43	G	A	C	A	T	A	73	-	-	-	-	-	-
14	G	A	C	A	T	A	44	G	A	C	A	T	A	74	-	-	-	-	-	-
15	-	-	-	-	-	-	45	-	-	-	-	-	-	75	G	A	C	A	T	A
16	G	A	C	A	T	A	46	G	A	C	A	T	A	76	-	-	-	-	-	-
17	-	-	-	-	-	-	47	-	-	-	-	-	-	77	G	A	C	A	T	A
18	-	-	-	-	-	-	48	-	-	-	-	-	-	78	A	A	A	A	A	A
19	-	-	-	-	-	-	49	-	-	-	-	-	-	79	-	-	-	-	-	-
20	A	A	A	A	A	A	50	-	-	-	-	-	-	80	A	A	A	A	A	A
21	A	A	A	A	A	A	51	-	-	-	-	-	-	81	-	-	-	-	-	-
22	-	-	-	-	-	-	52	G	A	C	A	T	A	82	G	G	C	C	T	T
23	G	A	C	A	T	A	53	A	A	A	A	A	A	83	A	A	A	A	A	A
24	-	-	-	-	-	-	54	G	A	C	A	T	A	84	G	A	C	A	T	A
25	G	A	C	A	T	A	55	-	-	-	-	-	-	85	G	A	C	A	T	A
26	-	-	-	-	-	-	56	G	A	C	A	T	A	86	A	A	A	A	A	A
27	-	-	-	-	-	-	57	-	-	-	-	-	-	87	-	-	-	-	-	-
28	-	-	-	-	-	-	58	-	-	-	-	-	-	88	A	A	A	A	A	A
29	A	A	A	A	A	A	59	A	A	A	A	A	A	89	G	A	C	A	T	A
30	A	A	A	A	A	A	60	A	A	A	A	A	A	90	G	A	C	A	T	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
91	-	-	-	-	-	-	121	-	-	-	-	-	-	151	G	A	C	A	T	A
92	-	-	-	-	-	-	122	-	-	-	-	-	-	152	-	-	-	-	-	-
93	-	-	-	-	-	-	123	A	A	A	A	A	A	153	-	-	-	-	-	-
94	-	-	-	-	-	-	124	-	-	-	-	-	-	154	G	A	C	A	T	A
95	-	-	-	-	-	-	125	-	-	-	-	-	-	155	A	A	A	A	A	A
96	A	A	A	A	A	A	126	-	-	-	-	-	-	156	-	-	-	-	-	-
97	-	-	-	-	-	-	127	-	-	-	-	-	-	157	-	-	-	-	-	-
98	-	-	-	-	-	-	128	A	A	A	A	A	A	158	-	-	-	-	-	-
99	A	A	A	A	A	A	129	-	-	-	-	-	-	159	A	A	A	A	A	A
100	-	-	-	-	-	-	130	G	A	C	A	T	A	160	A	A	A	A	A	A
101	-	-	-	-	-	-	131	A	A	A	A	A	A	161	A	A	A	A	A	A
102	-	-	-	-	-	-	132	-	-	-	-	-	-	162	G	A	C	A	T	A
103	-	-	-	-	-	-	133	G	A	C	A	T	A	163	A	A	A	A	A	A
104	G	A	C	A	T	A	134	A	A	A	A	A	A	164	A	A	A	A	A	A
105	-	-	-	-	-	-	135	G	A	C	A	T	A	165	A	A	A	A	A	A
106	G	A	C	A	T	A	136	A	A	A	A	A	A	166	-	-	-	-	-	-
107	A	A	A	A	A	A	137	G	A	C	A	T	A	167	A	A	A	A	A	A
108	G	A	C	A	T	A	138	A	A	A	A	A	A	168	-	-	-	-	-	-
109	-	-	-	-	-	-	139	A	A	A	A	A	A	169	G	A	C	A	T	A
110	G	A	C	A	T	A	140	-	-	-	-	-	-	170	-	-	-	-	-	-
111	G	A	C	A	T	A	141	-	-	-	-	-	-	171	G	A	C	A	T	A
112	-	-	-	-	-	-	142	G	A	C	A	T	A	172	-	-	-	-	-	-
113	-	-	-	-	-	-	143	-	-	-	-	-	-	173	G	A	C	A	T	A
114	A	A	A	A	T	A	144	G	A	C	A	T	A	174	-	-	-	-	-	-
115	A	A	A	A	A	A	145	A	A	A	A	A	A	175	-	-	-	-	-	-
116	A	A	A	A	A	A	146	-	-	-	-	-	-	176	-	-	-	-	-	-
117	-	-	-	-	-	-	147	-	-	-	-	-	-	177	G	A	C	A	T	A
118	-	-	-	-	-	-	148	-	-	-	-	-	-	178	A	A	A	A	A	A
119	A	A	A	A	A	A	149	A	A	A	A	A	A	179	-	-	-	-	-	-
120	G	A	C	A	T	A	150	A	A	A	A	A	A	180	A	A	A	A	A	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
181	G	A	C	A	T	A	211	A	A	A	A	A	A	241	G	G	C	C	T	T
182	A	A	A	A	A	A	212	-	-	-	-	-	-	242	G	G	C	C	T	T
183	A	A	A	A	A	A	213	-	-	-	-	-	-	243	A	A	A	A	A	A
184	-	-	-	-	-	-	214	G	A	C	A	T	A	244	G	A	C	A	T	A
185	A	A	A	A	A	A	215	A	A	A	A	A	A	245	A	A	A	A	A	A
186	A	A	A	A	A	A	216	A	A	A	A	A	A	246	A	A	A	A	A	A
187	G	A	C	A	T	A	217	-	-	-	-	-	-	247	A	A	A	A	A	A
188	-	-	-	-	-	-	218	G	A	C	A	T	A	248	G	A	C	A	T	A
189	-	-	-	-	-	-	219	A	A	A	A	A	A	249	A	A	A	A	A	A
190	G	A	C	A	T	A	220	A	A	A	A	A	A	250	G	A	C	A	T	A
191	A	A	A	A	A	A	221	-	-	-	-	-	-	251	A	A	A	A	A	A
192	G	A	C	A	T	A	222	-	-	-	-	-	-	252	-	-	-	-	-	-
193	-	-	-	-	-	-	223	-	-	-	-	-	-	253	G	A	C	A	T	A
194	-	-	-	-	-	-	224	-	-	-	-	-	-	254	A	A	A	A	A	A
195	-	-	-	-	-	-	225	-	-	-	-	-	-	255	G	A	C	A	T	A
196	G	A	C	A	T	A	226	G	A	C	A	T	A	256	G	A	C	A	T	A
197	-	-	-	-	-	-	227	A	A	A	A	A	A	257	G	A	C	A	T	A
198	-	-	-	-	-	-	228	G	A	C	A	T	T	258	-	-	-	-	-	-
199	A	A	A	A	A	A	229	A	A	A	A	A	A	259	G	A	C	A	T	A
200	G	A	C	A	T	A	230	A	A	A	A	A	A	260	A	A	A	A	A	A
201	G	A	C	A	T	A	231	G	A	C	A	T	A	261	A	A	A	A	T	A
202	-	-	-	-	-	-	232	-	-	-	-	-	-	262	-	-	-	-	-	-
203	A	A	A	A	A	A	233	G	A	C	A	T	A	263	A	A	A	A	A	A
204	G	A	C	A	T	A	234	A	A	A	A	A	A	264	A	A	A	A	A	A
205	G	A	C	A	T	A	235	A	A	A	A	A	A	265	-	-	-	-	-	-
206	-	-	-	-	-	-	236	A	A	A	A	A	A	266	-	-	-	-	-	-
207	-	-	-	-	-	-	237	A	A	A	A	A	A	267	A	A	A	A	A	A
208	A	A	A	A	A	A	238	G	A	C	A	T	A	268	G	A	C	A	T	A
209	A	A	A	A	A	A	239	-	-	-	-	-	-	269	A	A	A	A	A	A
210	-	-	-	-	-	-	240	-	-	-	-	-	-	270	A	A	A	A	A	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
271	-	-	-	-	-	-	301	G	A	C	A	T	A	331	A	A	C	A	A	A
272	-	-	-	-	-	-	302	G	A	C	A	T	A	332	G	A	C	A	T	A
273	-	-	-	-	-	-	303	-	-	-	-	-	-	333	-	-	-	-	-	-
274	A	A	A	A	A	A	304	-	-	-	-	-	-	334	A	A	A	A	A	A
275	-	-	-	-	-	-	305	-	-	-	-	-	-	335	-	-	-	-	-	-
276	G	A	C	A	T	A	306	-	-	-	-	-	-	336	G	G	C	C	T	A
277	G	A	C	A	T	A	307	A	A	A	A	A	A	337	-	-	-	-	-	-
278	A	A	A	A	A	A	308	-	-	-	-	-	-	338	A	A	A	A	A	A
279	A	A	A	A	A	A	309	A	A	A	A	A	A	339	A	A	A	A	A	A
280	-	-	-	-	-	-	310	G	A	C	A	T	A	340	-	-	-	-	-	-
281	-	-	-	-	-	-	311	G	G	C	C	T	T	341	-	-	-	-	-	-
282	-	-	-	-	-	-	312	G	A	C	A	T	A	342	-	-	-	-	-	-
283	-	-	-	-	-	-	313	-	-	-	-	-	-	343	-	-	-	-	-	-
284	-	-	-	-	-	-	314	A	A	A	A	A	A	344	A	A	A	A	A	A
285	-	-	-	-	-	-	315	G	A	C	A	T	A	345	-	-	-	-	-	-
286	-	-	-	-	-	-	316	-	-	-	-	-	-	346	G	A	A	A	T	A
287	-	-	-	-	-	-	317	-	-	-	-	-	-	347	G	A	C	A	T	A
288	-	-	-	-	-	-	318	-	-	-	-	-	-	348	-	-	-	-	-	-
289	-	-	-	-	-	-	319	-	-	-	-	-	-	349	A	A	A	A	A	A
290	-	-	-	-	-	-	320	-	-	-	-	-	-	350	-	-	-	-	-	-
291	G	A	C	A	T	A	321	G	A	C	A	T	A	351	A	A	A	A	A	A
292	A	A	A	A	A	A	322	-	-	-	-	-	-	352	-	-	-	-	-	-
293	-	-	-	-	-	-	323	-	-	-	-	-	-	353	A	A	A	A	A	A
294	-	-	-	-	-	-	324	G	G	C	C	T	T	354	A	A	A	A	A	A
295	A	A	A	A	A	A	325	-	-	-	-	-	-	355	A	A	A	A	A	A
296	G	A	C	A	T	A	326	-	-	-	-	-	-	356	A	A	A	A	A	A
297	A	A	C	A	A	A	327	A	A	A	A	A	A	357	-	-	-	-	-	-
298	-	-	-	-	-	-	328	G	A	C	A	T	A	358	-	-	-	-	-	-
299	G	A	C	A	T	A	329	A	A	A	A	A	A	359	A	A	A	A	A	A
300	-	-	-	-	-	-	330	A	A	A	A	A	A	360	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
361	A	A	A	A	A	A	391	G	A	C	A	T	A	421	G	A	C	A	T	A
362	A	A	A	A	A	A	392	A	A	A	A	A	A	422	-	-	-	-	-	-
363	G	G	A	A	T	T	393	G	A	C	A	A	A	423	G	A	C	A	T	A
364	G	A	C	A	T	A	394	A	A	A	A	A	A	424	-	-	-	-	-	-
365	A	A	A	A	A	A	395	-	-	-	-	-	-	425	G	A	C	A	T	A
366	-	-	-	-	-	-	396	A	A	A	A	A	A	426	-	-	-	-	-	-
367	-	-	-	-	-	-	397	A	A	A	A	A	A	427	A	A	A	A	A	A
368	-	-	-	-	-	-	398	G	A	C	A	T	A	428	A	A	A	A	A	A
369	G	A	C	A	-	-	399	A	A	A	A	A	A	429	-	-	-	-	-	-
370	G	A	C	A	T	A	400	G	A	C	A	T	A	430	-	-	-	-	-	-
371	-	-	-	-	-	-	401	-	-	-	-	-	-	431	A	A	A	A	A	A
372	A	A	A	A	A	A	402	A	A	A	A	A	A	432	A	A	A	A	A	A
373	A	A	A	A	A	A	403	G	A	C	A	T	A	433	A	A	A	A	A	A
374	A	A	A	A	A	A	404	G	A	C	A	T	A	434	-	-	-	-	-	-
375	A	A	A	A	A	A	405	-	-	-	-	-	-	435	G	A	C	A	T	A
376	A	A	A	A	A	A	406	G	A	C	A	T	A	436	A	A	A	A	A	A
377	G	A	C	A	T	A	407	-	-	-	-	-	-	437	-	-	-	-	-	-
378	-	-	-	-	-	-	408	G	A	C	A	T	A	438	A	A	A	A	A	A
379	-	-	-	-	-	-	409	-	-	-	-	-	-	439	A	A	A	A	A	A
380	G	A	C	A	T	A	410	A	A	A	A	A	A	440	A	A	A	A	A	A
381	G	A	C	A	T	A	411	-	-	-	-	-	-	441	G	A	C	A	T	A
382	-	-	-	-	-	-	412	G	A	C	A	T	A	442	-	-	-	-	-	-
383	-	-	-	-	-	-	413	-	-	-	-	-	-	443	-	-	-	-	-	-
384	-	-	-	-	-	-	414	G	A	C	A	T	A	444	A	A	A	A	A	A
385	-	-	-	-	-	-	415	A	A	A	A	A	A	445	-	-	-	-	-	-
386	-	-	-	-	-	-	416	G	G	C	C	T	T	446	-	-	-	-	-	-
387	A	A	A	A	A	A	417	-	-	-	-	-	-	447	G	A	C	A	T	A
388	-	-	-	-	-	-	418	A	A	A	A	A	A	448	-	-	-	-	-	-
389	-	-	-	-	-	-	419	A	A	A	A	A	A	449	G	A	C	A	T	A
390	A	A	A	A	A	A	420	G	A	C	A	T	A	450	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
451	G	A	C	A	T	A	481	G	G	C	C	T	T	511	A	A	A	A	A	A
452	G	A	C	A	T	A	482	A	A	C	A	T	A	512	-	-	-	-	-	-
453	A	A	A	A	A	A	483	G	A	C	A	T	A	513	-	-	-	-	-	-
454	A	A	A	A	A	A	484	A	A	A	A	A	A	514	-	-	-	-	-	-
455	G	A	C	A	T	A	485	-	-	-	-	-	-	515	A	A	A	A	A	A
456	-	-	-	-	-	-	486	-	-	-	-	-	-	516	G	G	C	C	T	T
457	A	A	A	A	A	A	487	G	G	C	C	T	T	517	-	-	-	-	-	-
458	A	A	A	A	A	A	488	-	-	-	-	-	-	518	-	-	-	-	-	-
459	-	-	-	-	-	-	489	-	-	-	-	-	-	519	-	-	-	-	-	-
460	-	-	-	-	-	-	490	G	G	C	C	T	T	520	-	-	-	-	-	-
461	G	A	C	A	T	A	491	-	-	-	-	-	-	521	A	A	A	A	A	A
462	G	A	C	A	T	A	492	-	-	-	-	-	-	522	A	A	A	A	A	A
463	G	A	C	A	T	A	493	-	-	-	-	-	-	523	-	-	-	-	-	-
464	G	A	C	A	T	A	494	-	-	-	-	-	-	524	G	G	C	C	T	T
465	-	-	-	-	-	-	495	-	-	-	-	-	-	525	A	A	A	A	A	A
466	G	A	C	A	T	A	496	-	-	-	-	-	-	526	A	A	A	A	A	A
467	-	-	-	-	-	-	497	-	-	-	-	-	-	527	A	A	A	A	A	A
468	-	-	-	-	-	-	498	-	-	-	-	-	-	528	G	A	C	A	A	A
469	-	-	-	-	-	-	499	A	A	A	A	A	A	529	A	A	A	A	A	A
470	-	-	-	-	-	-	500	G	A	C	A	T	A	530	A	A	A	A	A	A
471	G	A	A	A	A	A	501	-	-	-	-	-	-	531	A	A	A	A	A	A
472	A	A	A	A	A	A	502	A	A	A	A	A	A	532	A	A	A	A	A	A
473	-	-	-	-	-	-	503	G	A	C	A	T	A	533	A	A	A	A	A	A
474	G	A	C	A	T	A	504	A	A	A	A	A	A	534	-	-	-	-	-	-
475	G	A	C	A	T	A	505	A	A	A	A	A	A	535	A	A	A	A	A	A
476	A	A	A	A	A	A	506	-	-	-	-	-	-	536	-	-	-	-	-	-
477	A	A	A	A	A	A	507	A	A	A	A	A	A	537	G	A	C	A	T	A
478	G	A	C	A	T	A	508	A	A	A	A	A	A	538	A	A	A	A	A	A
479	G	A	C	A	T	A	509	A	A	A	A	A	A	539	-	-	-	-	-	-
480	A	A	A	A	A	A	510	G	A	C	A	T	A	540	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
541	A	A	A	A	A	A	571	G	A	C	A	T	A	601	-	-	-	-	-	-
542	-	-	-	-	-	-	572	-	-	-	-	-	-	602	A	A	A	A	A	A
543	A	A	A	A	A	A	573	-	-	-	-	-	-	603	G	A	C	A	T	A
544	G	A	C	A	T	A	574	A	A	A	A	A	A	604	-	-	-	-	-	-
545	-	-	-	-	-	-	575	-	-	-	-	-	-	605	G	A	C	A	T	A
546	-	-	-	-	-	-	576	-	-	-	-	-	-	606	A	A	A	A	A	A
547	A	A	A	A	A	A	577	-	-	-	-	-	-	607	A	A	A	A	A	A
548	-	-	-	-	-	-	578	G	G	C	C	T	A	608	-	-	-	-	-	-
549	G	A	C	A	T	A	579	G	G	C	C	T	T	609	-	-	-	-	-	-
550	A	A	A	A	A	A	580	G	A	C	A	T	A	610	G	G	C	C	T	T
551	A	A	A	A	A	A	581	-	-	-	-	-	-	611	A	A	A	A	A	A
552	-	-	-	-	-	-	582	A	A	A	A	A	A	612	A	A	A	A	A	A
553	A	A	A	A	A	A	583	G	A	C	A	T	A	613	A	A	A	A	A	A
554	-	-	-	-	-	-	584	-	-	-	-	-	-	614	A	A	A	A	A	A
555	G	G	C	C	T	T	585	-	-	-	-	-	-	615	G	A	C	A	T	T
556	-	-	-	-	-	-	586	-	-	-	-	-	-	616	-	-	-	-	-	-
557	G	G	C	C	T	T	587	A	A	A	A	A	A	617	A	A	A	A	A	A
558	-	-	-	-	-	-	588	-	-	-	-	-	-	618	-	-	-	-	-	-
559	A	A	A	A	A	A	589	-	-	-	-	-	-	619	-	-	-	-	-	-
560	G	A	C	A	T	A	590	G	G	C	C	T	T	620	G	A	C	A	T	A
561	G	A	C	A	T	A	591	G	A	C	A	T	A	621	G	A	C	A	T	A
562	A	A	A	A	A	A	592	A	A	A	A	A	A	622	-	-	-	-	-	-
563	G	A	C	A	T	A	593	G	A	C	A	T	A	623	-	-	-	-	-	-
564	-	-	-	-	-	-	594	G	A	C	A	T	T	624	A	A	A	A	A	A
565	-	-	-	-	-	-	595	-	-	-	-	-	-	625	G	A	C	A	T	A
566	G	A	C	A	T	A	596	-	-	-	-	-	-	626	-	-	-	-	-	-
567	-	-	-	-	-	-	597	G	G	C	C	T	T	627	-	-	-	-	-	-
568	G	A	C	A	T	A	598	-	-	-	-	-	-	628	A	A	A	A	A	A
569	-	-	-	-	-	-	599	A	A	A	A	A	A	629	G	A	C	A	T	A
570	G	G	C	C	T	T	600	A	A	A	A	A	A	630	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
631	A	A	A	A	A	A	661	G	A	C	A	T	A	691	A	A	A	A	A	A
632	A	A	A	A	A	A	662	A	A	A	A	A	A	692	A	A	A	A	A	A
633	-	-	-	-	-	-	663	-	-	-	-	-	-	693	G	A	C	A	T	A
634	A	A	A	A	A	A	664	G	A	C	A	T	A	694	G	A	C	A	T	A
635	G	G	C	C	T	T	665	-	-	-	-	-	-	695	-	-	-	-	-	-
636	A	A	A	A	A	A	666	-	-	-	-	-	-	696	A	A	A	A	A	A
637	-	-	-	-	-	-	667	G	A	C	A	T	A	697	A	A	A	A	T	A
638	A	A	A	A	A	A	668	A	A	A	A	A	A	698	A	A	A	A	A	A
639	G	A	C	A	T	A	669	G	A	C	A	T	A	699	-	-	-	-	-	-
640	G	A	C	A	T	A	670	A	A	A	A	A	A	700	-	-	-	-	-	-
641	-	-	-	-	-	-	671	A	A	A	A	A	A	701	A	A	A	A	A	A
642	G	A	C	A	T	A	672	G	G	C	C	T	T	702	-	-	-	-	-	-
643	A	A	A	A	A	A	673	G	A	C	A	T	A	703	A	A	A	A	A	A
644	A	A	A	A	A	A	674	A	A	A	A	T	A	704	A	A	A	A	A	A
645	G	A	C	A	T	A	675	-	-	-	-	-	-	705	A	A	A	A	A	A
646	G	A	C	A	T	A	676	A	A	A	A	A	A	706	-	-	-	-	-	-
647	-	-	-	-	-	-	677	-	-	-	-	-	-	707	-	-	-	-	-	-
648	-	-	-	-	-	-	678	A	A	A	A	A	A	708	-	-	-	-	-	-
649	-	-	-	-	-	-	679	A	A	A	A	A	A	709	-	-	-	-	-	-
650	-	-	-	-	-	-	680	A	A	A	A	A	A	710	-	-	-	-	-	-
651	-	-	-	-	-	-	681	G	A	C	A	T	A	711	A	A	A	A	A	A
652	A	A	A	A	A	A	682	G	G	C	C	T	T	712	A	A	A	A	A	A
653	-	-	-	-	-	-	683	G	A	C	A	T	A	713	-	-	-	-	-	-
654	A	A	A	A	A	A	684	-	-	-	-	-	-	714	-	-	-	-	-	-
655	A	A	A	A	A	A	685	A	A	A	A	A	A	715	-	-	-	-	-	-
656	-	-	-	-	-	-	686	A	A	A	A	A	A	716	-	-	-	-	-	-
657	A	A	A	A	A	A	687	-	-	-	-	-	-	717	-	-	-	-	-	-
658	G	G	C	C	T	T	688	G	A	C	A	T	A	718	G	G	C	C	T	T
659	A	A	A	A	A	A	689	G	A	C	A	T	A	719	A	A	A	A	A	A
660	A	A	A	A	A	A	690	A	A	A	A	A	A	720	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
721	G	A	C	A	T	A	751	G	A	C	A	T	A	781	G	A	C	A	T	A
722	A	A	A	A	A	A	752	-	-	-	-	-	-	782	A	A	A	A	A	A
723	A	A	A	A	A	A	753	-	-	-	-	-	-	783	-	-	-	-	-	-
724	A	A	A	A	A	A	754	-	-	-	-	-	-	784	A	A	A	A	A	A
725	-	-	-	-	-	-	755	-	-	-	-	-	-	785	-	-	-	-	-	-
726	-	-	-	-	-	-	756	A	A	A	A	A	A	786	-	-	-	-	-	-
727	A	A	A	A	T	A	757	A	A	A	A	A	A	787	A	A	A	A	A	A
728	A	A	A	A	A	A	758	G	A	C	A	T	A	788	G	A	C	A	T	T
729	A	A	A	A	A	A	759	G	A	C	A	T	A	789	A	A	A	A	A	A
730	-	-	-	-	-	-	760	-	-	-	-	-	-	790	G	A	C	A	T	A
731	-	-	-	-	-	-	761	A	A	A	A	A	A	791	-	-	-	-	-	-
732	-	-	-	-	-	-	762	G	A	C	A	T	A	792	-	-	-	-	-	-
733	A	A	A	A	A	A	763	-	-	-	-	-	-	793	G	A	C	A	T	A
734	-	-	-	-	-	-	764	-	-	-	-	-	-	794	A	A	A	A	-	-
735	G	A	C	A	T	A	765	-	-	-	-	-	-	795	-	-	-	-	-	-
736	A	A	A	A	A	A	766	G	A	C	A	T	A	796	A	A	A	A	A	A
737	A	A	A	A	A	A	767	A	A	A	A	T	A	797	-	-	-	-	-	-
738	G	A	C	A	T	A	768	-	-	-	-	-	-	798	G	G	C	C	T	T
739	-	-	-	-	-	-	769	-	-	-	-	-	-	799	A	A	A	A	A	A
740	-	-	-	-	-	-	770	A	A	A	A	A	A	800	-	-	-	-	-	-
741	G	A	C	A	T	A	771	-	-	-	-	-	-	801	A	A	A	A	A	A
742	A	A	A	A	A	A	772	-	-	-	-	-	-	802	A	A	A	A	A	A
743	A	A	A	A	T	A	773	A	A	A	A	A	A	803	-	-	-	-	-	-
744	A	A	A	A	A	A	774	G	A	C	A	T	A	804	-	-	-	-	-	-
745	A	A	A	A	A	A	775	-	-	-	-	-	-	805	-	-	-	-	-	-
746	A	A	A	A	A	A	776	-	-	-	-	-	-	806	G	G	C	C	T	T
747	G	A	C	A	T	A	777	-	-	-	-	-	-	807	A	A	A	A	A	A
748	A	A	A	A	A	A	778	-	-	-	-	-	-	808	-	-	-	-	-	-
749	A	A	A	A	A	A	779	-	-	-	-	-	-	809	G	A	C	A	T	A
750	A	A	A	A	A	A	780	G	A	C	A	T	A	810	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
811	G	A	C	A	T	A	841	-	-	-	-	-	-	871	G	A	C	A	T	A
812	G	G	C	C	T	T	842	A	A	A	A	A	A	872	-	-	-	-	-	-
813	G	G	C	C	T	T	843	-	-	-	-	-	-	873	-	-	-	-	-	-
814	-	-	-	-	-	-	844	-	-	-	-	-	-	874	-	-	-	-	-	-
815	G	A	C	A	T	A	845	G	A	C	A	T	A	875	A	A	A	A	A	A
816	-	-	-	-	-	-	846	G	A	C	A	T	A	876	-	-	-	-	-	-
817	-	-	-	-	-	-	847	A	A	A	A	A	A	877	G	A	C	A	T	A
818	A	A	A	A	A	A	848	A	A	A	A	A	A	878	A	A	A	A	A	A
819	-	-	-	-	-	-	849	G	A	C	A	T	A	879	-	-	-	-	-	-
820	-	-	-	-	-	-	850	G	A	C	A	T	A	880	-	-	-	-	-	-
821	A	A	A	A	A	A	851	A	A	A	A	A	A	881	-	-	-	-	-	-
822	A	A	A	A	A	A	852	A	A	A	A	A	A	882	G	A	C	A	T	A
823	-	-	-	-	-	-	853	-	-	-	-	-	-	883	A	A	A	A	A	A
824	-	-	-	-	-	-	854	G	A	C	A	T	T	884	G	G	C	C	T	T
825	-	-	-	-	-	-	855	-	-	-	-	-	-	885	A	A	A	A	A	A
826	-	-	-	-	-	-	856	G	A	C	A	T	A	886	-	-	-	-	-	-
827	-	-	-	-	-	-	857	G	G	C	C	T	T	887	-	-	-	-	-	-
828	G	A	C	A	T	A	858	-	-	-	-	-	-	888	A	A	A	A	A	A
829	G	A	C	A	T	A	859	A	A	A	A	A	A	889	-	-	-	-	-	-
830	-	-	-	-	-	-	860	A	A	A	A	A	A	890	G	A	C	A	T	A
831	G	G	C	C	T	T	861	A	A	A	A	A	A	891	-	-	-	-	-	-
832	-	-	-	-	-	-	862	G	A	C	A	T	A	892	G	A	C	A	T	T
833	A	A	A	A	A	A	863	-	-	-	-	-	-	893	G	A	C	A	T	A
834	G	A	C	A	T	A	864	-	-	-	-	-	-	894	-	-	-	-	-	-
835	G	A	C	A	T	A	865	G	A	C	A	T	A	895	G	G	C	A	T	A
836	A	A	A	A	A	A	866	G	A	C	A	T	A	896	A	A	A	A	A	A
837	A	A	A	A	A	A	867	-	-	-	-	-	-	897	-	-	-	-	-	-
838	A	A	A	A	A	A	868	G	A	C	A	T	A	898	-	-	-	-	-	-
839	G	G	C	C	T	T	869	A	A	A	A	A	A	899	A	A	A	A	A	A
840	A	A	A	A	A	A	870	G	A	C	A	T	A	900	A	A	A	A	A	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
901	-	-	-	-	-	-	931	G	A	C	A	T	A	961	G	A	C	A	T	A
902	A	A	A	A	A	A	932	A	A	A	A	A	A	962	G	A	C	A	T	A
903	G	G	C	C	T	T	933	A	A	A	A	A	A	963	-	-	-	-	-	-
904	-	-	-	-	-	-	934	A	A	A	A	A	A	964	A	A	A	A	A	A
905	-	-	-	-	-	-	935	A	A	A	A	T	A	965	-	-	-	-	-	-
906	-	-	-	-	-	-	936	-	-	-	-	-	-	966	-	-	-	-	-	-
907	-	-	-	-	-	-	937	A	A	A	A	A	A	967	A	A	A	A	A	A
908	-	-	-	-	-	-	938	-	-	-	-	-	-	968	A	A	A	A	A	A
909	G	A	C	A	T	A	939	-	-	-	-	-	-	969	-	-	-	-	-	-
910	-	-	-	-	-	-	940	G	A	C	A	T	A	970	G	A	C	A	T	A
911	-	-	-	-	-	-	941	A	A	A	A	A	A	971	G	A	C	A	T	A
912	A	A	A	A	A	A	942	-	-	-	-	-	-	972	-	-	-	-	-	-
913	-	-	-	-	-	-	943	-	-	-	-	-	-	973	G	A	C	A	T	A
914	G	A	C	A	T	A	944	A	A	A	A	A	A	974	-	-	-	-	-	-
915	G	A	C	A	T	A	945	-	-	-	-	-	-	975	G	A	C	A	T	A
916	-	-	-	-	-	-	946	A	A	A	A	A	A	976	-	-	-	-	-	-
917	A	A	A	A	A	A	947	A	A	A	A	A	A	977	G	A	C	A	T	A
918	A	A	A	A	A	A	948	A	A	A	A	A	A	978	-	-	-	-	-	-
919	A	A	A	A	A	A	949	-	-	-	-	-	-	979	-	-	-	-	-	-
920	G	A	C	A	T	A	950	-	-	-	-	-	-	980	G	A	C	A	T	A
921	-	-	-	-	-	-	951	-	-	-	-	-	-	981	A	A	A	A	A	A
922	-	-	-	-	-	-	952	-	-	-	-	-	-	982	G	A	C	A	T	A
923	G	G	C	C	T	T	953	A	A	A	A	A	A	983	A	A	A	A	A	A
924	A	A	A	A	A	A	954	A	A	A	A	A	A	984	A	A	A	A	A	A
925	-	-	-	-	-	-	955	A	A	A	A	A	A	985	-	-	-	-	-	-
926	-	-	-	-	-	-	956	A	A	A	A	A	A	986	G	A	C	A	T	A
927	-	-	-	-	-	-	957	G	A	C	A	T	A	987	A	A	A	A	T	A
928	G	A	C	A	T	A	958	A	A	A	A	A	A	988	A	A	A	A	A	A
929	-	-	-	-	-	-	959	A	A	A	A	A	A	989	G	A	C	A	T	A
930	-	-	-	-	-	-	960	G	A	C	A	T	A	990	G	A	C	A	T	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1021	-	-	-	-	-	-	1021	-	-	-	-	-	-	1051	-	-	-	-	-	-
1022	G	A	C	A	T	A	1022	G	A	C	A	T	A	1052	-	-	-	-	-	-
1023	A	A	A	A	A	A	1023	A	A	A	A	A	A	1053	-	-	-	-	-	-
1024	-	-	-	-	-	-	1024	-	-	-	-	-	-	1054	-	-	-	-	-	-
1025	G	A	C	A	T	A	1025	G	A	C	A	T	A	1055	A	A	A	A	T	A
1026	-	-	-	-	-	-	1026	-	-	-	-	-	-	1056	A	A	A	A	A	A
1027	G	A	C	A	T	A	1027	G	A	C	A	T	A	1057	G	A	C	A	T	A
1028	A	A	A	A	A	A	1028	A	A	A	A	A	A	1058	-	-	-	-	-	-
1029	A	A	A	A	A	A	1029	A	A	A	A	A	A	1059	A	A	A	A	A	A
1030	G	A	C	A	T	A	1030	G	A	C	A	T	A	1060	-	-	-	-	-	-
1031	A	A	A	A	A	A	1031	A	A	A	A	A	A	1061	-	-	-	-	-	-
1032	-	-	-	-	-	-	1032	-	-	-	-	-	-	1062	-	-	-	-	-	-
1033	A	A	A	A	A	A	1033	A	A	A	A	A	A	1063	-	-	-	-	-	-
1034	-	-	-	-	-	-	1034	-	-	-	-	-	-	1064	-	-	-	-	-	-
1035	G	A	C	A	T	A	1035	G	A	C	A	T	A	1065	-	-	-	-	-	-
1036	G	A	C	A	T	A	1036	G	A	C	A	T	A	1066	-	-	-	-	-	-
1037	-	-	-	-	-	-	1037	-	-	-	-	-	-	1067	G	A	C	A	T	A
1038	-	-	-	-	-	-	1038	-	-	-	-	-	-	1068	G	G	C	C	T	T
1039	G	A	C	A	T	A	1039	G	A	C	A	T	A	1069	G	A	C	A	T	A
1040	A	A	A	A	A	A	1040	A	A	A	A	A	A	1070	A	A	A	A	A	A
1041	A	A	A	A	A	A	1041	A	A	A	A	A	A	1071	-	-	-	-	-	-
1042	A	A	A	A	A	A	1042	A	A	A	A	A	A	1072	-	-	-	-	-	-
1043	A	A	A	A	A	A	1043	A	A	A	A	A	A	1073	A	A	A	A	A	A
1044	A	A	A	A	A	A	1044	A	A	A	A	A	A	1074	A	A	A	A	A	A
1045	A	A	A	A	A	A	1045	A	A	A	A	A	A	1075	-	-	-	-	-	-
1046	A	A	A	A	A	A	1046	A	A	A	A	A	A	1076	G	A	C	A	T	A
1047	A	A	A	A	A	A	1047	A	A	A	A	A	A	1077	A	A	A	A	A	A
1048	G	A	C	A	T	A	1048	G	A	C	A	T	A	1078	-	-	-	-	-	-
1049	A	A	A	A	A	A	1049	A	A	A	A	A	A	1079	-	-	-	-	-	-
1050	A	A	A	A	A	A	1050	A	A	A	A	A	A	1080	G	A	C	A	T	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1081	-	-	-	-	-	-	1111	A	A	A	A	A	A	1141	G	A	C	A	T	A
1082	G	A	C	A	T	A	1112	A	A	A	A	A	A	1142	-	-	-	-	-	-
1083	-	-	-	-	-	-	1113	G	A	C	A	T	A	1143	A	A	A	A	A	A
1084	-	-	-	-	-	-	1114	-	-	-	-	-	-	1144	-	-	-	-	-	-
1085	-	-	-	-	-	-	1115	G	A	C	A	T	A	1145	-	-	-	-	-	-
1086	-	-	-	-	-	-	1116	A	A	A	A	A	A	1146	-	-	-	-	-	-
1087	G	A	C	A	T	A	1117	A	A	A	A	A	A	1147	-	-	-	-	-	-
1088	G	A	C	A	T	A	1118	-	-	-	-	-	-	1148	A	A	A	A	A	A
1089	-	-	-	-	-	-	1119	-	-	-	-	-	-	1149	-	-	-	-	-	-
1090	-	-	-	-	-	-	1120	G	G	C	C	T	T	1150	G	A	C	A	T	A
1091	G	A	C	A	T	A	1121	A	A	A	A	A	A	1151	-	-	-	-	-	-
1092	G	A	C	A	A	A	1122	A	A	A	A	A	A	1152	A	A	A	A	A	A
1093	A	A	A	A	A	A	1123	-	-	-	-	-	-	1153	-	-	-	-	-	-
1094	-	-	-	-	-	-	1124	-	-	-	-	-	-	1154	-	-	-	-	-	-
1095	A	A	A	A	A	A	1125	G	A	C	A	T	A	1155	A	A	A	A	A	A
1096	A	A	A	A	T	A	1126	A	A	A	A	A	A	1156	-	-	-	-	-	-
1097	-	-	-	-	-	-	1127	G	G	C	C	T	T	1157	A	A	A	A	A	A
1098	A	A	A	A	A	A	1128	G	A	C	A	T	A	1158	A	A	A	A	A	A
1099	-	-	-	-	-	-	1129	G	A	C	A	T	A	1159	-	-	-	-	-	-
1100	G	A	C	A	T	A	1130	-	-	-	-	-	-	1160	A	A	A	A	A	A
1101	-	-	-	-	-	-	1131	A	A	A	A	A	A	1161	-	-	-	-	-	-
1102	G	A	C	A	T	A	1132	A	A	A	A	A	A	1162	G	A	C	A	T	A
1103	-	-	-	-	-	-	1133	G	A	C	A	T	A	1163	G	A	C	A	T	A
1104	A	A	A	A	A	A	1134	A	A	A	A	A	A	1164	A	A	A	A	A	A
1105	-	-	-	-	-	-	1135	G	G	C	C	T	T	1165	A	A	A	A	A	A
1106	-	-	-	-	-	-	1136	-	-	-	-	-	-	1166	A	A	A	A	A	A
1107	-	-	-	-	-	-	1137	A	A	A	A	A	A	1167	-	-	-	-	-	-
1108	A	A	A	A	A	A	1138	A	A	A	A	A	A	1168	A	A	A	A	A	A
1109	G	A	C	A	T	A	1139	A	A	A	A	A	A	1169	-	-	-	-	-	-
1110	-	-	-	-	-	-	1140	-	-	-	-	-	-	1170	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1171	-	-	-	-	-	-	1201	A	A	A	A	A	A	1231	-	-	-	-	-	-
1172	-	-	-	-	-	-	1202	-	-	-	-	-	-	1232	-	-	-	-	-	-
1173	G	A	C	A	T	A	1203	G	A	C	A	T	A	1233	-	-	-	-	-	-
1174	A	A	A	A	A	A	1204	G	A	C	A	T	A	1234	A	A	A	A	A	A
1175	-	-	-	-	-	-	1205	G	A	C	A	T	A	1235	-	-	-	-	-	-
1176	-	-	-	-	-	-	1206	G	A	C	A	T	A	1236	-	-	-	-	-	-
1177	-	-	-	-	-	-	1207	-	-	-	-	-	-	1237	A	A	A	A	A	A
1178	-	-	-	-	-	-	1208	-	-	-	-	-	-	1238	-	-	-	-	-	-
1179	A	A	A	A	A	A	1209	-	-	-	-	-	-	1239	-	-	-	-	-	-
1180	G	G	C	C	T	T	1210	-	-	-	-	-	-	1240	-	-	-	-	-	-
1181	A	A	A	A	A	A	1211	G	A	C	A	T	A	1241	-	-	-	-	-	-
1182	-	-	-	-	-	-	1212	A	A	A	A	A	A	1242	A	A	A	A	A	A
1183	A	A	A	A	A	A	1213	-	-	-	-	-	-	1243	-	-	-	-	-	-
1184	G	A	C	A	T	A	1214	A	A	A	A	A	A	1244	-	-	-	-	-	-
1185	A	A	A	A	A	A	1215	-	-	-	-	-	-	1245	-	-	-	-	-	-
1186	A	A	A	A	A	A	1216	-	-	-	-	-	-	1246	-	-	-	-	-	-
1187	G	A	C	A	T	A	1217	A	A	A	A	A	A	1247	G	G	C	C	T	T
1188	-	-	-	-	-	-	1218	-	-	-	-	-	-	1248	G	A	C	A	T	A
1189	-	-	-	-	-	-	1219	G	A	C	A	T	A	1249	-	-	-	-	-	-
1190	-	-	-	-	-	-	1220	A	A	A	A	A	A	1250	A	A	A	A	A	A
1191	A	A	A	A	A	A	1221	-	-	-	-	-	-	1251	A	A	A	A	A	A
1192	-	-	-	-	-	-	1222	A	A	A	A	A	A	1252	-	-	-	-	-	-
1193	-	-	-	-	-	-	1223	A	A	C	A	T	A	1253	G	A	C	A	T	A
1194	G	G	C	C	T	T	1224	G	A	C	A	T	A	1254	G	A	C	A	T	A
1195	-	-	-	-	-	-	1225	-	-	-	-	-	-	1255	A	A	A	A	A	A
1196	G	A	C	A	T	A	1226	-	-	-	-	-	-	1256	G	G	C	C	T	T
1197	-	-	-	-	-	-	1227	G	A	C	A	T	A	1257	-	-	-	-	-	-
1198	-	-	-	-	-	-	1228	A	A	A	A	A	A	1258	G	A	C	A	T	A
1199	A	A	A	A	A	A	1229	G	A	C	A	T	A	1259	A	A	A	A	T	A
1200	G	A	C	A	T	A	1230	A	A	A	A	A	A	1260	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1261	A	A	A	A	A	A	1291	A	A	A	A	A	A	1321	A	A	A	A	T	A
1262	-	-	-	-	-	-	1292	A	A	A	A	A	A	1322	G	A	C	A	T	A
1263	A	A	A	A	A	A	1293	A	A	A	A	A	A	1323	-	-	-	-	-	-
1264	A	A	A	A	A	A	1294	-	-	-	-	-	-	1324	G	A	C	A	A	A
1265	G	A	C	A	T	A	1295	-	-	-	-	-	-	1325	A	A	A	A	A	A
1266	G	A	C	A	T	A	1296	A	A	A	A	A	A	1326	-	-	-	-	-	-
1267	G	G	C	C	T	T	1297	A	A	A	A	A	A	1327	A	A	A	A	A	A
1268	A	A	A	A	A	A	1298	G	G	C	A	T	A	1328	A	A	A	A	A	A
1269	A	A	A	A	A	A	1299	G	A	C	A	T	A	1329	-	-	-	-	-	-
1270	G	A	C	A	T	A	1300	A	A	A	A	A	A	1330	A	A	A	A	A	A
1271	-	-	-	-	-	-	1301	G	A	C	A	T	A	1331	A	A	A	A	A	A
1272	A	A	A	A	A	A	1302	-	-	-	-	-	-	1332	-	-	-	-	-	-
1273	-	-	-	-	-	-	1303	A	A	A	A	A	A	1333	-	-	-	-	-	-
1274	A	A	A	A	A	A	1304	A	A	A	A	A	A	1334	A	A	A	A	A	A
1275	A	A	A	A	A	A	1305	A	A	A	A	A	A	1335	-	-	-	-	-	-
1276	A	A	A	A	A	A	1306	A	A	A	A	A	A	1336	G	A	C	A	T	A
1277	A	A	A	A	A	A	1307	-	-	-	-	-	-	1337	-	-	-	-	-	-
1278	-	-	-	-	-	-	1308	A	A	A	A	A	A	1338	-	-	-	-	-	-
1279	G	A	C	A	T	A	1309	-	-	-	-	-	-	1339	G	A	C	A	T	A
1280	A	A	A	A	A	A	1310	-	-	-	-	-	-	1340	A	A	A	A	A	A
1281	-	-	-	-	-	-	1311	A	A	A	A	A	A	1341	-	-	-	-	-	-
1282	G	A	C	A	T	A	1312	G	A	C	A	T	A	1342	-	-	-	-	-	-
1283	A	A	A	A	A	A	1313	A	A	A	A	A	A	1343	A	A	A	A	A	A
1284	G	G	C	C	T	T	1314	A	A	A	A	A	A	1344	A	A	A	A	A	A
1285	-	-	-	-	-	-	1315	A	A	A	A	A	A	1345	-	-	-	-	-	-
1286	A	A	A	A	A	A	1316	-	-	-	-	-	-	1346	-	-	-	-	-	-
1287	-	-	-	-	-	-	1317	A	A	A	A	A	A	1347	A	A	A	A	A	A
1288	-	-	-	-	-	-	1318	-	-	-	-	-	-	1348	-	-	-	-	-	-
1289	A	A	A	A	A	A	1319	G	G	C	C	T	T	1349	A	A	A	A	A	A
1290	A	A	A	A	A	A	1320	A	A	A	A	A	A	1350	G	A	C	A	T	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1351	-	-	-	-	-	-	1381	A	A	A	A	A	A	1411	G	A	C	A	T	A
1352	-	-	-	-	-	-	1382	-	-	-	-	-	-	1412	-	-	-	-	-	-
1353	A	A	A	A	A	A	1383	G	G	C	C	T	T	1413	A	A	A	A	A	A
1354	A	A	A	A	A	A	1384	-	-	-	-	-	-	1414	-	-	-	-	-	-
1355	G	A	C	A	T	A	1385	A	A	A	A	A	A	1415	-	-	-	-	-	-
1356	G	A	C	A	T	A	1386	A	A	A	A	A	A	1416	-	-	-	-	-	-
1357	-	-	-	-	-	-	1387	G	G	C	C	T	T	1417	A	A	A	A	A	A
1358	A	A	A	A	A	A	1388	A	A	A	A	A	A	1418	G	A	C	A	T	A
1359	-	-	-	-	-	-	1389	A	A	A	A	A	A	1419	-	-	-	-	-	-
1360	G	A	C	A	T	A	1390	A	A	A	A	A	A	1420	A	A	A	A	A	A
1361	A	A	A	A	A	A	1391	A	A	A	A	A	A	1421	A	A	A	A	A	A
1362	A	A	A	A	A	A	1392	-	-	-	-	-	-	1422	-	-	-	-	-	-
1363	G	A	C	A	T	T	1393	G	A	C	A	T	A	1423	-	-	-	-	-	-
1364	G	A	C	A	T	A	1394	G	A	C	A	T	A	1424	A	A	A	A	A	A
1365	A	A	A	A	A	A	1395	G	A	C	A	T	A	1425	G	G	C	C	T	T
1366	A	A	A	A	A	A	1396	G	A	C	A	T	A	1426	A	A	A	A	A	A
1367	A	A	A	A	A	A	1397	A	A	A	A	A	A	1427	G	A	C	A	T	A
1368	A	A	A	A	A	A	1398	-	-	-	-	-	-	1428	-	-	-	-	-	-
1369	A	A	A	A	A	A	1399	A	A	A	A	A	A	1429	A	A	A	A	A	A
1370	A	A	A	A	T	A	1400	G	A	C	A	T	A	1430	A	A	A	A	A	A
1371	-	-	-	-	-	-	1401	G	A	C	A	T	A	1431	G	A	C	A	T	A
1372	-	-	-	-	-	-	1402	A	A	A	A	A	A	1432	-	-	-	-	-	-
1373	G	A	C	A	T	A	1403	-	-	-	-	-	-	1433	-	-	-	-	-	-
1374	A	A	A	A	A	A	1404	G	A	C	A	T	A	1434	G	A	C	A	T	A
1375	A	A	A	A	A	A	1405	A	A	A	A	A	A	1435	-	-	-	-	-	-
1376	A	A	A	A	A	A	1406	-	-	-	-	-	-	1436	-	-	-	-	-	-
1377	A	A	A	A	A	A	1407	G	A	C	A	T	A	1437	-	-	-	-	-	-
1378	G	A	C	A	T	A	1408	G	A	C	A	T	A	1438	-	-	-	-	-	-
1379	G	A	C	A	T	A	1409	G	A	C	A	T	A	1439	-	-	-	-	-	-
1380	A	A	A	A	A	A	1410	A	A	A	A	A	A	1440	-	-	-	-	-	-

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078		ID	rs2234678		rs878972		rs454078	
1441	-	-	-	-	-	-	1471	-	-	-	-	-	-	1531	-	-	-	-	-	-
1442	A	A	A	A	A	A	1472	A	A	A	A	A	A	1532	A	A	A	A	A	A
1443	G	A	C	A	T	A	1473	G	A	C	A	T	A	1533	-	-	-	-	-	-
1444	A	A	A	A	A	A	1474	-	-	-	-	-	-	1534	-	-	-	-	-	-
1445	A	A	A	A	A	A	1475	A	A	A	A	A	A	1535	G	A	C	A	T	A
1446	G	A	C	A	T	A	1476	-	-	-	-	-	-	1536	G	A	C	A	T	A
1447	G	A	C	A	T	T	1477	-	-	-	-	-	-	1531	-	-	-	-	-	-
1448	-	-	-	-	-	-	1478	-	-	-	-	-	-	1532	A	A	A	A	A	A
1449	-	-	-	-	-	-	1479	-	-	-	-	-	-	1533	-	-	-	-	-	-
1450	G	A	C	A	T	A	1480	A	A	A	A	A	A	1534	-	-	-	-	-	-
1451	-	-	-	-	-	-	1481	-	-	-	-	-	-	1535	G	A	C	A	T	A
1452	-	-	-	-	-	-	1482	-	-	-	-	-	-	1536	G	A	C	A	T	A
1453	A	A	A	A	A	A	1483	G	A	C	A	T	A	1531	-	-	-	-	-	-
1454	-	-	-	-	-	-	1484	G	A	C	A	T	A	1532	A	A	A	A	A	A
1455	-	-	-	-	-	-	1485	-	-	-	-	-	-	1533	-	-	-	-	-	-
1456	-	-	-	-	-	-	1486	G	A	C	A	T	A	1534	-	-	-	-	-	-
1457	G	A	C	A	T	A	1487	-	-	-	-	-	-	1535	G	A	C	A	T	A
1458	A	A	A	A	A	A	1488	-	-	-	-	-	-	1536	G	A	C	A	T	A
1459	G	A	C	A	T	A	1489	A	A	A	A	A	A	1531	-	-	-	-	-	-
1460	G	A	C	A	T	T	1490	A	A	A	A	A	A	1532	A	A	A	A	A	A
1461	G	A	C	A	T	A	1491	G	A	C	A	T	A	1533	-	-	-	-	-	-
1462	-	-	-	-	-	-	1492	-	-	-	-	-	-	1534	-	-	-	-	-	-
1463	-	-	-	-	-	-	1493	A	A	A	A	A	A	1535	G	A	C	A	T	A
1464	-	-	-	-	-	-	1494	A	A	A	A	A	A	1536	G	A	C	A	T	A
1465	-	-	-	-	-	-	1495	-	-	-	-	-	-	1531	-	-	-	-	-	-
1466	-	-	-	-	-	-	1496	-	-	-	-	-	-	1532	A	A	A	A	A	A
1467	G	A	C	A	T	A	1497	G	A	C	A	T	A	1533	-	-	-	-	-	-
1468	A	A	A	A	A	A	1498	G	A	C	A	T	A	1534	-	-	-	-	-	-
1469	G	G	C	C	T	T	1499	G	A	C	A	T	A	1535	G	A	C	A	T	A
1470	-	-	-	-	-	-	1500	A	A	A	A	A	A	1536	G	A	C	A	T	A

Table 23 (cont'd...)

ID	rs2234678		rs878972		rs454078	
1531	-	-	-	-	-	-
1532	A	A	A	A	A	A
1533	-	-	-	-	-	-
1534	-	-	-	-	-	-
1535	G	A	C	A	T	A
1536	G	A	C	A	T	A

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