Institut für Experimentelle Genetik,
GSF-Forschungszentrum für Umwelt und Gesundheit, Neuherberg

Dr. von Haunersches Kinderspital

TOLL-LIKE RECEPTOR HETERODIMER VARIANTS INFLUENCE TH1/TH2 SIGNALING AND PROTECT FROM THE DEVELOPMENT OF CHILDHOOD ASTHMA

Michael Sebastian Daniel Kormann

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Vorsitzender: Univ.-Prof. Dr. rer. nat. Alfons Gierl

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1. apl. Prof. Dr. rer. nat. Dr. rer. biol. hum. habil. Jerzy Adamski
2. Priv.-Doz. Dr. med. Michael Kabesch, Ludwig-Maximilians-Universität München
3. Univ.-Prof. Dr. med. Dr. h. c. Hermann Wagner, Ph. D. (Melbourne)

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"To stay human is to break a limitation."

[from ***The beauty of the husband: a fictional assay in 29 tangos*** (Ann Carson)

and as quoted in ***Oryx & Crake*** (Margaret Atwood)]

ABBREVIATIONS

AA	amino acid
APC	antigen presenting cell
BHR	bronchial hyperresponsiveness
bp	base pair
CARD	caspase recruitment domains
CD	cluster of differentiation
CDS	coding sequence
(d)d-	(di)desoxy-
DC	dendritic cell
DNA	desoxyribonucleic acid
FITC	fluorescein isothiocyanate
g	gravitation force
HET	heterozygous/heterozygote
hME	homogeneous mass extension
HPLC	high performance liquid chromatography
HWE	Hardy Weinberg Equilibrium
IFN	interferone
Ig	immunoglobuline
IL	interleukine
IQR	interquartiles
ISAAC	international study of asthma and allergies in childhood
LASER	light amplification of stimulated emission of radiation
LD	linkage disequilibrium
LOD	logarithm of odds
LTA	lipoteichoic acid
MAF	minor allele frequency
MALDI-TOF MS	matrix associated LASER desorption/ionization time-of-flight mass spectometry
MDA	multible displacement amplification
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger RNA
MUT	mutant (minor allele)
MyD88	myeloid differentiation primary response gene 88
NOD	nucleotide-binding oligomerization domain containing
NTP	nucleotide triphosphate
OR	odds ratio
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reaction
PE	phycoerythrin
PRR	pattern recognition receptor
qRT RT PCR	quantitative real time reverse transcriptase PCR
RNA	ribonucleic acid
RT	room temperature
SAP	shrimp alkaline phosphatase
SNP	single nucleotide polymorphism
SPOLA	solid-phase oligonucleotide ligation assay
SPT	skin prick test
TCR	T cell receptor
TF	transcription factor
Th1 (2)	T helper cell type 1 (2)
TIR	Toll/IL-1 receptor
TIRAP	TIR containing adaptor protein
TLR	toll like receptor
TRAM	TRIF related adaptor molecule
TRIF	Toll/IL-1 domain containing adaptor inducing IFN
WT	wild type
X	any amino acid
Y	hydrophobic amino acid

ABSTRACT

Toll-like receptors (TLRs 1-10) represent a major group of receptors for the specific recognition of pathogen-associated molecular patterns capable of activating innate and adaptive immunity. Adaptive immunity is T-cell dependent and may result in the production of allergen specific IgE in the case of atopic sensitisation, which is a common feature of childhood asthma. Intriguingly, early exposure to microbes reduces the risk for asthma and a number of polymorphisms in TLR genes may be involved. However, previous publications dealt mostly with single SNPs in individual TLRs, they were often afflicted with small sample size and did not offer a model explaining the observed effects. Therefore, gradually the following aims were formulated:

- a comprehensive genetic analysis to evaluate the effects of variations and haplotypes in the human TLR system on childhood asthma
- testing reproducibility of associations in several independent populations
- applying interaction models based on functional clustering and putative heterodimerisation of TLRs
- investigating the influence of associated SNPs on TLR mRNA as well as protein expression, and – after stimulation – on TLR signaling and cytokine production *ex vivo*
- proposing a model how asthma susceptibility could be modulated in a beneficial way

Implementing different *in silico* approaches, 21 polymorphisms in all 10 human TLRs were systematically selected for putative function. Genotyping was performed in three German populations (N=1,872) utilizing MALDI-TOF MS, SPOLA and sequencing. Protective association with lower incidence of atopic asthma were identified for SNPs in TLR1 (P=0.002), TLR6 (P=0.003) and TLR10 (P=0.006), all capable of forming heterodimers with TLR2. The risk to develop atopic asthma was thereby reduced by nearly 50%. Analysis of epistasis highlighted that interactions between SNPs in heterodimerizing TLRs significantly influenced the development of asthma in children. Quantitative RealTime RT-PCR revealed that carriers of the minor alleles in *TLR1*, *TLR6* and *TLR10* (mediating protection) showed an increased expression of the respective TLR mRNAs compared to wildtype carriers, which was subsequently confirmed on the protein level with flow cytometry. Additionally, stimulation of PBMCs of minor allele carriers with ligands specific for TLR2/1 and TLR2/6 heterodimers lead to an augmented inflammatory response, elevated Th1 cytokine expression and reduced Th2 related IL-4 production compared to wildtype carriers.

These results suggest TLR1 and TLR6 as intriguing new targets for asthma research as they have not been recognised to be involved in atopic disease mechanisms so far. Stimulating certain TLR heterodimers, rather than arbitrary microbial exposure, may protect from the development of childhood asthma. These findings may help to identify protective agents (acting through TLR1 and TLR6 activation) for a beneficial and specific modification of the TLR system to reduce asthma susceptibility.

ZUSAMMENFASSUNG

Toll-like Rezeptoren (TLRs 1-10) repräsentieren eine wichtige Gruppe von Rezeptoren, welche spezifisch molekulare Strukturen von Pathogenen erkennen und das angeborene sowie adaptive Immunsystem aktivieren können. Das adaptive Immunsystem ist T-Zell-abhängig und kann - im Falle einer atopischen Sensibilisierung - zur Ausschüttung von Allergen spezifischem IgE führen, was ein häufiges Merkmal von Asthma darstellt. Interessanterweise reduziert der frühe Kontakt zu Mikroben das Risiko Asthma zu entwickeln. Eine Reihe von Polymorphismen in TLR Genen könnten dabei eine Rolle spielen. In vorangegangenen Publikationen wurden jedoch hauptsächlich SNPs getrennt in einzelnen TLRs untersucht - in jeweils kleinen Populationen und ohne ein erklärendes Modell vorzuschlagen. Daher wurden für die Dissertation folgende Ziele gewählt:

- eine übergreifende genetische Analyse um die Effekte von Variationen und Haplotypen im humanen TLR System auf Asthma im Kindesalter zu beschreiben
- Test der Reproduzierbarkeit der Assoziationen in unabhängigen Populationen
- Berechnung von Interaktionsmodellen speziell vor dem Hintergrund der Heterodimerisierung bestimmter TLRs
- Analyse des Einflusses der assoziierten SNPs auf TLR mRNA und Proteinexpression, und auf Signalweiterleitung und Zytokinproduktion nach Stimulation *ex vivo*
- Erstellung eines Modells, wie Asthmaanfälligkeit verringert werden könnte

Mit Hilfe verschiedener *in silico* Verfahren wurden aus allen 10 humanen TLRs 21 Polymorphismen ausgewählt. Die Genotypisierung erfolgte in drei deutschen Populationen (N=1.872) via MALDI-TOF MS, SPOLA und Sequenzierung. SNPs in TLR1 (P=0,002), TLR6 (P=0,003) und TLR10 (P=0,006) (TLRs, welche mit TLR2 Heterodimere formen können) senkten das Risiko der Entstehung von atopischem Asthma um beinahe 50%. Gen-Gen-Interaktionen zwischen den heterodimerisierenden TLRs beeinflussten dabei signifikant die Entstehung von Asthma bei Kindern. Quantitative RT-PCR zeigte, dass Träger der selteneren, protektiven Allele in *TLR1*, *TLR6* und *TLR10* erhöhte mRNA Level der entsprechenden TLRs aufwiesen. Dies wurde mit Durchflusszytometrie auf Proteinebene bestätigt. Außerdem führte die Stimulation von PBMCs - gewonnen aus Trägern der selteneren Allele - mit spezifischen TLR2/1 und TLR2/6 Liganden *ex vivo* zu einer erhöhten inflammatorischen Antwort sowie zu erhöhter Th1 Zytokinexpression und verminderter Th2 Antwort, verglichen mit Trägern der Wildtypallele.

Diese Ergebnisse präsentieren TLR1 und TLR6 als neue Kandidaten der Asthmaforschung. Nicht jeder mikrobielle Kontakt, aber die Stimulation bestimmter TLR Heterodimere könnte vor der Entstehung von Asthma in der Kindheit schützen. Diese Erkenntnisse könnten dabei helfen protektive Liganden zu finden, die eine vorteilhafte Modifikation des TLR Systems erlauben um damit die Asthmaanfälligkeit zu senken.

1 INTRODUCTION

1.1 ASTHMA AND ATOPY

1.1.1 PATHOLOGY

Atopy (greek for „out of place“) comprises a tendency to develop the classical allergic diseases: atopic dermatitis, allergic rhinitis and asthma. It involves the capacity to produce IgE antibodies in response to common environmental allergens (e.g. pollen, dander, food and insect venoms) (KAY 2001). Although atopy has various definitions, most consistently it is defined by the presence of elevated levels of total and allergen-specific IgE in the serum, leading to positive skin-prick tests to common allergens.

Asthma is a chronic disease of the respiratory system in which the airway occasionally constricts, shows inflammation, and is lined with excessive amounts of mucus (FIG. 1.1). Acute episodes may be triggered by exposure to environmental stimulants (allergens), cold air, exercise or emotional stress. In children, viral illnesses are the most common triggers (ZHAO *et al.* 2002).

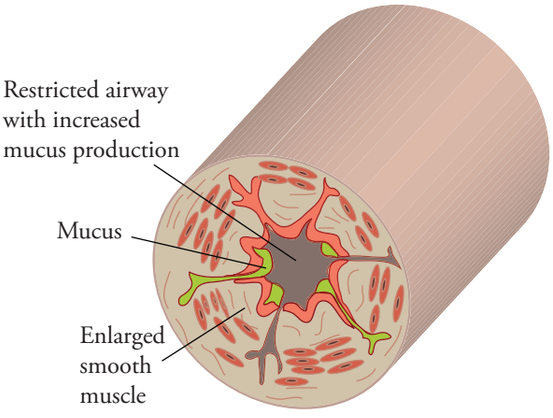


FIG. 1.1 *Inflamed airways with swelling of the bronchial mucous membrane (mucosa) and secretion of excessive thick mucus that is difficult to expel. The smooth muscles are enlarged and occasionally lead to contraction or spasm of the airways, making it difficult to breath.*

Asthma and atopy show a complex interrelation. Preexisting atopy increases the risk of developing asthma by 10-20 fold, depending on both the amount and type of sensitizations (HOLGATE 1999). Studies have shown that initial sensitization to airborne environmental allergens occurs typically in early childhood, while subsequent progression to persistent atopic asthma, which may not manifest for several years, is restricted to only a subset of atopics (HOLT *et al.* 1999).

1.1.1.1 Atopic and non-atopic asthma

Asthma has long been recognized as a heterogeneous disease. In the early twentieth century, Rackemann proposed classifying asthma into "extrinsic" (atopic) and "intrinsic" (non-atopic) asthma (Rackemann 1921). Even today, based on clinical correlates, most conceptual models of asthma differentiate into asthma due to an atopic pathogenesis or non-atopic pathogenesis of airway inflammation. In contrast to the atopic model, non-atopic asthma implies an absence of sensitisation, with IgE thought to be less important in the inflammation pathogenesis (Amin *et al.* 2000). To a variable extent, both models incorporate the same effector cells and the same soluble mediators. In a key study the cellular variability and overlap of both models were exemplified (Woolley *et al.* 1996). It was shown that the sputum content of eosinophils varied nearly 100-fold among the patients at different time points and that even "low" levels of airway mucosal eosinophilia may have clinical consequences in some patients.

1.1.1.2 Eosinophilic asthma

Early bronchoscopy studies examining the immunopathology of patients with mild asthma suggested that eosinophilic airway inflammation was the characteristic abnormality (Wardlaw *et al.* 1988; Broide *et al.* 1991). This was thought to be the case in both atopic and non-atopic asthma (Humbert *et al.* 1996; Humbert *et al.* 1997). On the whole, these studies reported a broad correlation between clinical asthma severity and the degree of airway eosinophilia, particularly when eosinophils appear to be activated (Wardlaw *et al.* 1988; Bousquet *et al.* 1990). Studies comparing the correlation between sputum eosinophilia and lung function (Pin *et al.* 1992; Crimi *et al.* 1998) have been inconsistent and airway hyperresponsiveness and eosinophilia appear to be independent components of the asthma phenotype (Brightling *et al.* 2002). In contrast, eosinophilic airway inflammation appears to be much more closely related to the risk of severe asthma exacerbations (Jayaram *et al.* 2006).

1.1.1.3 Noneosinophilic asthma

The use of induced sputum (see Brightling 2006; Simpson *et al.* 2006), along with small bronchoscopy studies of patients with severe asthma (Wenzel *et al.* 1999), have clearly demonstrated that eosinophilic inflammation is not universally present. Instead, previous studies have noted neutrophilic inflammation in some patients with severe asthma (Jatakanon *et al.* 1999; Wenzel *et al.* 1999) and in those who die suddenly of asthma (Sur *et al.* 1993). However, it has to be considered that effects of (strong) corticosteroid treatment may be confounding which makes it unclear whether the neutrophilic asthma phenotype is particularly associated with more severe disease.

1.1.2 EPIDEMIOLOGY

The prevalence of asthma in western societies has increased substantially in recent decades (BEASLEY 2002), following similar trends observed for other allergic and autoimmune disorders (BACH 2002).

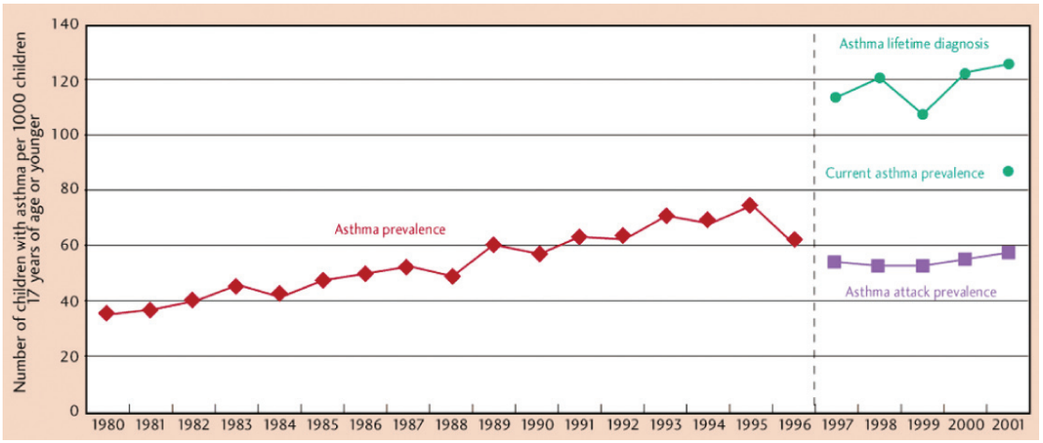


FIG. 1.2 *Asthma prevalence in the US (1980-1996) as well as asthma lifetime diagnosis, current asthma, and asthma attack prevalence (1997-2001), in children.*

Clearly, prevalence rates depend on the phenotypic definitions that were applied at the time of the survey. A good example could be observed in an asthma survey in the UK coordinated by the National Health Intelligence Service (NHIS). In contrast to the previous NHIS asthma question that produced asthma period prevalence estimates, the redesigned questions in the 1997-2000 NHIS produced an estimate of asthma attack prevalence (**FIG. 1.2**). To be included as a "case", a respondent had to report to have been diagnosed with asthma by a health professional and had an attack or episode of asthma in the past 12 months (AKINBAMI *et al.* 2003). Since this case definition is more specific, asthma attack prevalence estimates are lower than pre-1997, that were based on respondents' reporting that they had asthma in the previous 12 months.

Evaluating literature on an inter- and multinational level, the diversity of findings in asthma prevalence rates, including its changes in time, is remarkable (see EDER *et al.* 2006A), reflecting the complex nature of the illness. In developed and industrialized countries the asthma prevalence is generally rising for the last years, while in some areas of the western world, the prevalence may have plateaued, with - depending on the actual study - large differences in the actual prevalence values (especially in UK populations). The rise in asthma is paralleled by a rise in atopy, as measured by a positive skin prick test, over the last decades, but only a part of the rise in asthma could be explained by atopy (PEARCE *et al.* 1999). Triggers for both atopy and asthma include smoking, exposure to

allergens and air pollution, while living on a farm, especially during early childhood, seems to be protective (LEYNAERT *et al.* 2001; BRAUN-FAHRLANDER *et al.* 2002). Effects of other factors like infant breast feeding, infections in early life and diet remain controversially discussed (WEISS 1997).

In contrast, the change to a modern, western life style with high hygiene standards and reduced contact to microbes increases the risk for these diseases (STRACHAN 1989; MATRICARDI *et al.* 2000; (SHERRIFF & GOLDING 2002). Nowadays, in the more affluent, industrialized world asthma is the most common chronic illness of children. One possible explanation for this phenomenon has been summarized in what is now known as the hygiene hypothesis.

1.1.3 ASTHMA IMMUNOLOGY / TH1 AND TH2 RESPONSES

The hygiene hypothesis was originally proposed in 1989 by David Strachan. He studied a national sample of 17,414 British children born during one week in March 1958 and followed them up for 23 years. His observations revealed that hay fever incidence was inversely related to the number of children in the household, and eczema in the first year of life was related to the number of older children in the household. He stated that "allergic diseases could be prevented by infections in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally" (STRACHAN 1989). It was later translated into immunological terms by Martinez and Holt (see MARTINEZ & HOLT 1999). While Strachan based his hypothesis on the epidemiological evidence of an association[1] of atopic disease, particularly hay fever and eczema, with small family size and birth order, the suggestion that infection or a more microbe-rich environment may be beneficial had been raised previously (GODFREY 1975; GERRARD *et al.* 1976).

Already in studies decades ago a substantial rise in the cumulative incidence of atopic diseases in children has been documented in cross-sectional studies from several North European countries (ARKWRITE & DAVID 2004). Thus there has been a true and significant rise in the prevalence of atopic dermatitis over the past three decades in the western industrialized society, with a concomitant rise in hygiene standards. Another study involving 812 twin pairs in Denmark revealed that the cumulative incidence rate of atopic dermatitis (up to seven years) increased from 0.06 for birth cohort 1965-1969 to 0.12 for the birth cohort 1975-1979 (SCHULTZ 1993). In this study, the concordance rate in monozygotic twins was 0.72 and in dizygotic twins was 0.23. This twin study provided a convincing evidence of a rapidly increasing disease frequency due to the influence of exogenous factors. Also, the incidence of atopic disease is shown to be related to the age of entry into the nursery in a large study conducted by Kramer *et al.* (KRAMER *et al.* 1999) in Germany. Those children from small families who enter the nursery at a later age (and are less exposed to infections from other children) have a higher chance of getting atopic disorder than those who enter the nursery at a younger age. This effect is not seen in children from larger families as they are exposed to other children in the house itself.

[1] in statistics, an association comes from two variables that are related

According to the hygiene hypothesis, insufficient early exposure to microorganisms in an increasingly sterile environment in industrialized western countries may be a key factor for the steady increase in atopy and asthma prevalence rates (VON MUTIUS *et al.* 1999). Although the immunological mechanisms behind the hypothesis remained unclear it is intriguing to speculate that exposure to the overall load of infectious/microbial agents encountered early in life is an important factor that influences the maturation of the adaptive immune system, especially with regard to certain T helper cell responses (MARTINEZ & HOLT 1999; ILLI *et al.* 2001).

With the discovery of T cell-derived cytokines that regulate IgE antibody production by B cells, type 1 (Th1) and type 2 (Th2) T helper cells have been described in mice (Mosmann *et al.* 1986; (MOSMANN & COFFMAN 1989) and humans (DEL PRETE *et al.* 1991; ROMAGNANI 1994). Representing a sub-group of lymphocytes, Th cells play an important role in establishing and maximising the capabilities of the immune system as well as in activating and directing other immune cells. Typically, Th1 cells produce the cytokines interferon-gamma (IFNγ) and tumor necrosis factor beta (TNFβ), thereby increasing the killing efficacy of macrophages and cytotoxic T cells. According to the current model, Th2-type T cell production seems to be the major reponse in allergic reactions to common environmental allergens. Th2 cells trigger the recruitment and the involvement of other cell types and soluble factors that result in an inflammatory cascade of unequaled complexity. Type 2 cells can both directly recognize allergen peptides (via the T cell receptor TCR) and account for the joint involvement of IgE-producing B cells (via interleukins IL-4, IL-13), mast cells (IL-4, IL-10) and eosinophils (IL-5). The link between Th2 type responses and allergic diseases was also supported by observations in the murine model. The transfer of Th2-cells into recipient mice induced airway eosinophilia, mucus hypersecretion, and airway hyperresponsiveness (COHN *et al.* 1997; COHN *et al.* 1998). The same symptoms could be observed in transgenic mice that overexpressed Th2 cytokines in airway epithelium (RANKIN *et al.* 1996; LEE *et al.* 1997). In contrast, allergy and asthma were not inducible in animal models displaying a deficiency in Th2 responses (CORRY *et al.* 1998; MIYATA *et al.* 1999). In humans, it could be shown that allergens evoke Th2 responses in atopic subjects (WIERENGA *et al.* 1990; PARRONCHI *et al.* 1991) with Th2 cells accumulating in target organs of atopic subjects (HAMID *et al.* 1991; VAN DER HEIJDEN *et al.* 1991). Furthermore, it could be demonstrated that successful immunotherapy shifts allergen-specific responses from Th2 to Th1 (SECRIST *et al.* 1993; MCHUGH *et al.* 1995).

However, the development and/or deviation of the immune system towards a type 1 or type 2 response may occur within a short timeframe in late pregnancy / early neonatal period. Most developmental stages in the fetus and newborn infant are subjected to a narrow time scale. It is speculated that the immune system differentiation is also timed to occur over a short period, although without an intervention study, the timing and duration of this window remains uncertain. However, one critical event determining the atopic state of an individual seems to be the omitted or deprived switch from a predominant Th2 profile observed in newborns to an immune state characterized by the prevalence of regulatory and/or Th1 cells and cytokine responses (PRESCOTT *et al.* 1999, KONING *et al.* 1996, HOLT *et al.* 1997). Although controversially discussed, it is speculated that the Th2 immune environment during pregnancy may be necessary in minimising the possibility of rejec-

tion of the growing fetus *in utero* (GUPTA 1998). Maternal exposure to pathogens might be able to alter the development: pathogen exposure may result in marination of the fetus by interleukin 12 in the amniotic fluid, stimulating development of Th1 cells. Normal development involves activation of the Th1 pathway to provide the appropriate balance: Th1 responses develop gradually in response to the immunological challenges of life, such as infections, while fetal primed Th2 immunity tends to persist also in the later life periods and boosts Th2 responses to encountered allergens.

Asthma, like many immunologic diseases, is considered to be driven by a dysregulation of adaptive immune responses, and has been unambiguously linked to an imbalance of T cell responses (COHN *et al.* 1998; KUPERMAN *et al.* 1998). CD4+ T cells are increased in broncho-alveolar lavage (BAL) fluid and bronchial biopsy specimens from patients with asthma (AZZAWI *et al.* 1990, ROBINSON *et al.* 1993A). These activated CD4+ T cells correlate with eosinophil numbers and activation (ROBINSON *et al.* 1993B). In addition to atopic asthma, CD4+ T cells are also activated in occupational (BENTLEY *et al.* 1992A) and non-atopic (BENTLEY *et al.* 1992B) asthma. Several authors have demonstrated an increase in expression of both mRNA and protein products of IL-4 and IL-5 in asthmatic tissue and BAL (WALKER *et al.* 1992, ROBINSON *et al.* 1992). Using the technique of double immunohistochemistry / *in situ* hybridization, T cells appear to be the major source of mRNA for these cytokines in both BAL and bronchial biopsy specimens (YING *et al.* 1995). Notably, IL-4 and IL-5 mRNA colocalizes predominantly to CD4+ cells, but was also noted to be present in CD8+ cells (YING *et al.* 1997). In addition to IL-5 protein (WALKER *et al.* 1992), expression of IL-5 mRNA has been shown to correlate with disease severity in asthma using both *in situ* hybridization and also semiquantitative reverse-transcriptase polymerase chain reaction (ROBINSON *et al.* 1993B, HUMBERT *et al.* 1997).

However, an increase in Th2 responses alone is unlikely to explain the recent rise in atopic disorders. A set of epidemiologic studies has shown that populations with high endemic levels of helminth infections (a potent natural stimuli for Th2 responses) appear to be protected against atopy (see YAZDANBAKHSH & WAHYUNI 2005). Babies born to mothers with parasitic infection/exposure produce specific IgE to gastrointestinal helminth parasites, as demonstrated by studies of cord blood samples, indicating that the exposure has stimulated production of IgE by fetal lymphocytes during pregnancy (KING *et al.* 1998). Such infants do not acquire infection in early life with the strain of parasite carried by the mother. Paradoxically, this sensitisation appears to tune the immune system away from inappropriate allergic responses later in life (COOKSON & MOFFATT 1997).

Another important advance in our understanding of immunological processes in atopy is the identification of regulatory suppressive T cells (Treg) which can prevent activation of self-reactive or pathological T cells in Th1 autoimmune disease models (SAKAGUCHI 2000; MALOY & POWRIE 2001). For many years allergen injection immunotherapy has been used to control allergic diseases including rhinitis and seasonal asthma (DURHAM *et al.* 1999; WALKER *et al.* 2001). Allergen immunotherapy also induces a predominant IL-10 response to allergen, and this may be associated with development of regulatory T cells which were CD4+CD25+ (FRANCIS *et al.* 2003; JUTEL *et al.* 2003).

However, although alternate hypotheses do exist that may better fit some aspects of asthmatic

inflammation or protection against it, the clearest link to asthma pathology remains the activation of allergen-specific Th2 cells.

1.1.4 INNATE IMMUNE RECOGNITION MODULATES ADAPTIVE IMMUNITY

Th2 development from a naïve CD4+ T cell requires both priming and differentiation. One model that elucidates the process of T cell priming is the two-signal hypothesis of lymphocyte activation (SCHWARTZ 1990). In this model the T cell activation occurs by engagement of both the major histocompatibility complex (MHC) peptide complexes and CD28 ("clusters of differentiation") ligation by costimulatory molecules. Those signals can be provided by mature dendritic cells (DCs). DCs are phagocytic cells present in tissues that are in contact with the external environment, mainly the skin (Langerhans cells), the respiratory and gastrointestinal tract and intestines. Their main function is to constantly sample the surroundings for pathogens, process antigen material and present it on their surface to other cells of the immune system. Once activated, they migrate to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the immune response. Therefore, the transition from immature to mature DCs represents a key event in the initiation of adaptive immune responses. Those are triggered and supposedly tightly regulated by innate immune system signals, which represent an ancient form of immunity with dual roles in host defense, providing a direct and immediate response against microbial invaders and an instructive role, influencing the maturation of adaptive immunity. Recognition of the critical role of innate immunity came with the identification of pattern recognition receptors (PRRs) and, in particular, Toll-like receptors (TLRs) (MEDZHITOV *et al.* 1997; see COUTINHO & POLTORACK 2003).

1.2 TOLL-LIKE RECEPTORS (TLRs)

1.2.1 DISCOVERY OF THE TLRs

Their name derives from homology to a family of molecules in the fruit fly *Drosophila melanogaster*, the prototypic member of which was called Toll. In fruit flies, Toll was first identified as a gene important in embryogenesis in establishing the dorsal-ventral axis. In 1996, Toll was found by Jules Hoffmann and his colleagues to have a role in the fly's immune response to fungal infection (LEMAITRE *et al.* 1996).

1.2.2 TLR MEMBERS

Most mammalian species express ten to fifteen types of Toll-like receptors. Thirteen TLRs (named simply TLR1 to TLR13) have been identified in humans and mice together, and equivalent forms of many of these have been found in other mammalian species (CHUANG & ULEVITCH 2000; DU *et al.* 2000; TABETA *et al.* 2004). However, equivalents of certain TLRs found in humans are not present in all mammals. For example, a gene coding for a protein analogous to TLR10 in humans is present in mice, but appears to have been damaged at some point in the past by a retrovirus (HASAN *et al.* 2005). On the other hand, mice express TLRs 11, 12, and 13, none of which is represented in humans. Other mammals may express TLRs which are not found in humans, hampering the efforts of using experimental animals as models of human innate immunity.

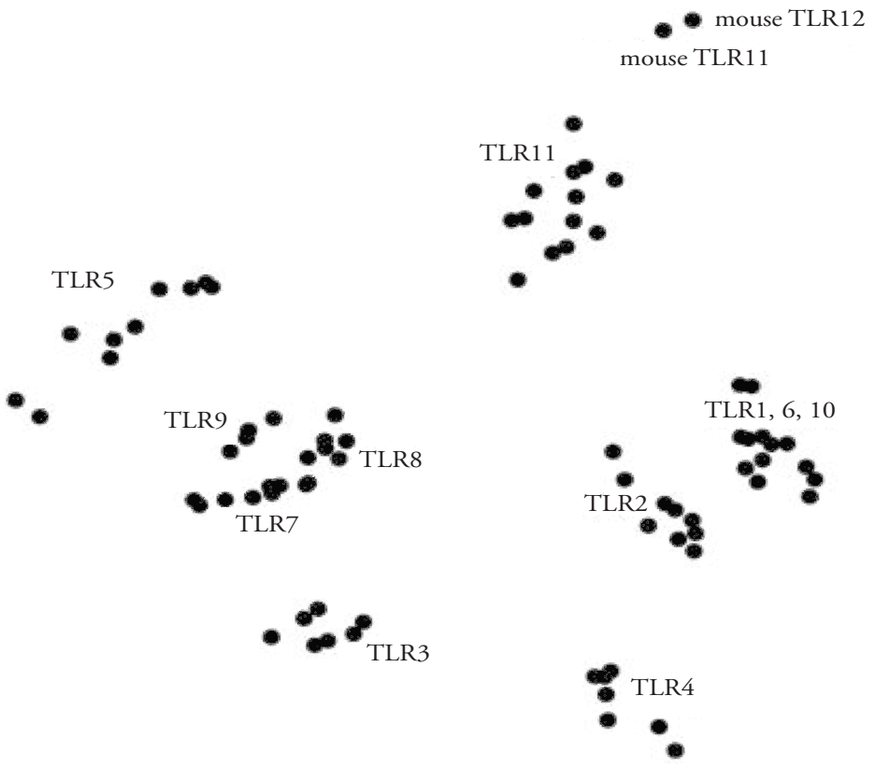


FIG. 1.3 *Multidimensional scaling (MDS) of the molecular distances between TLRs (an MDS algorithm starts with a matrix of item-item similarities, then assigns a location of each item in a low-dimensional space). The distance between the gene families compared with the distances within the gene families is so great that portraying this information as a molecular tree could be misleading. Note that, like geographical maps of intercity distances, MDS representations have no axes. Orientation and scale are completely arbitrary. Not all TLRs are shown. Graphic adapted from* ROACH *et al.* 2005.

Multigene families like the TLRs often evolve in ways that violate assumptions necessary for simple and objective gene phylogeny estimation. If genes of some members take on a significantly novel function – thus encountering significantly different selective pressure – they may evolve at much faster rates compared to the other multigene family members. However, by comparing the molecular distance of pairs of paralogs present in the same species, a sense of the amount of within-species coincidental evolution can be gained. Even when orthology for members of multigene families between species of different subphyla shall be assigned, molecular distance between such sequences may provide hints to relationships (FIG. 1.3) (ROACH *et al.* 2005).

1.2.3 TLR STRUCTURE AND LOCALISATIONS

TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs) . However, their extracellular regions differ markedly: TLRs have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR), whereas IL-1Rs have three immunoglobulin (Ig)-like domains. TLRs and IL-1Rs have a conserved cytoplasmic domain, that is known as the ~200 amino acids long Toll/IL-1R (TIR) domain (SLACK *et al.* 2000).

The TIR domain is characterized by the presence of three highly homologous regions ("boxes"). Amino acid sequence conservation among the TIR domains is generally 20-30%, and these domains vary in size. In part, crystal structures of the TIR domains have been obtained and analysed. Human TLR1 and TLR2 contain a central five-stranded parallel ß-sheet, surrounded by five α-helices on each side (XU *et al.* 2000). The interaction with adaptor molecules is believed to depend mostly on the conserved boxes 1 and 2 and the BB loop connecting the strand ß-B and the helix α-B.

The extracellular domain of TLRs contains 19-25 tandem copies of the LRR motif. Each repeat consists of 24-29 amino acids and contains the leucine-rich sequence XLXXLXLXX, and another conserved sequence $XYXXYX_4FXXLX$ (BELL *et al.* 2003), where X denotes any amino acid and Y a hydrophobic amino acid.

Remarkably, despite the conservation among LRR domains, different TLRs can recognize several structurally unrelated ligands (see JANEWAY & MEDZHITOV 2002 and AKIRA *et al.* 2001; MEDZHITOV 2001), and the subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. For example, TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation, while TLR3, TLR7, TLR8 and TLR9 – all of which are involved in the recognition of nucleic-acid like structures, are expressed intracellularly (AHMAD-NEJAD *et al.* 2002; HEIL *et al.* 2003; MATSUMOTO *et al.* 2003).

1.2.4 TLRs IN THE GENERATION OF TH2 IMMUNITY

TLRs are expressed on professional antigen-presenting cells (APCs), such as DCs, but have also been characterized on CD4+ T cells (CARAMALHO *et al.* 2003; GELMAN *et al.* 2004). The TLR-mediated innate immune system has a bow-tie architecture (see CSETE & DOYLE 2004) in which a variety of pathogens and their molecules are represented by a much smaller number of ligands.

TABLE 1.1 *TLRs, their ligands and downstream adaptors.*

TLR	Adaptor molecules	Ligand		References
		Exogenous	Endogenous	
TLR1, TLR2	see TLR2	Triacyl lipopeptides		(TAKEUCHI *et al.* 2002)
		Lipoarabinomannan		(TAPPING & TOBIAS 2003)
TLR2	MyD88, TIRAP	Lipopeptide/lipoprotein		(ALIPRANTIS *et al.* 1999)
		Peptidoglycan		(SCHWANDNER *et al.* 1999)
		Lipopolysaccharide		(WERTS *et al.* 2001)
		HSP 60		(GOBERT *et al.* 2004)
			HSP 70	(ASEA *et al.* 2002)
			HMGB1	(PARK *et al.* 2004)
TLR6, TLR2	see TLR2	Diacyl lipopeptides		(TAKEUCHI *et al.* 2001)
		Lipoteichoic acid		(HENNEKE *et al.* 2005)
		Zymosan		(OZINSKY *et al.* 2000)
TLR3	TRIF	dsRNA		(ALEXOPOULOU *et al.* 2001)
		siRNA		(KARIKO *et al.* 2004A)
			mRNA	(KARIKO *et al.* 2004B)
TLR4	MyD88, TIRAP TRAM, TRIF	Lipopolysaccharide		(POLTORAK *et al.* 1998)
		Peptidoglycan		(UEHORI *et al.* 2005)
		Heat-shock protein 60		(BULUT *et al.* 2002)
			HSP 60	(OHASHI *et al.* 2000)
			HSP 70	(VABULAS *et al.* 2002)
			ß-Defensin 2	(BIRAGYN *et al.* 2002)
			Fibrinogen	(SMILEY *et al.* 2001)
			Hyaluronic acid	(TERMEER *et al.* 2002)
			SP-A	(GUILLOT *et al.* 2002)
TLR5	MyD88	Flagellin		(HAYASHI *et al.* 2001)
TLR7	MyD88	ssRNA		(HEIL *et al.* 2004)
		Imidazoquinoline		(HEMMI *et al.* 2002)
TLR8	MyD88?	ssRNA		(HEIL *et al.* 2004)
		Imidazoquinoline		(JURK *et al.* 2002)
TLR9	MyD88	CpG DNA		(HEMMI *et al.* 2000) (KRUG *et al.* 2004)
			Chromatin complex	(LEADBETTER *et al.* 2002)
TLR10	MyD88?	not determined		
TLR11	MyD88?	Profilin-like molecule		(YAROVINSKY *et al.* 2005)

They recognize a specific repertoire of conserved motifs on bacteria, viruses, parasites, or fungi (BASU & FENTON 2004; DIEBOLD *et al.* 2004; HEIL *et al.* 2004; ZHANG *et al.* 2004). These motifs have been termed pathogen-associated molecular patterns (PAMPs) (**TABLE 1.1**).

In general, TLR stimulation results in cytokine production and a critical change in APC cell surface expression of migratory molecules and immune stimulatory molecules. Immature DCs located beneath epithelial barriers, once exposed to antigen, are induced by TLR signaling to mature into effective APCs that migrate to the draining lymph node and provide the required repertoire of signals for T cell priming. Furthermore, DCs are thought to provide signals that influence CD4+ T cell differentiation (KALINSKI *et al.* 1999). For example, DCs readily produce IL-12, the main Th1 differentiation factor, in response to many infectious stimuli following TLR engagement. However, it seems that induction of diverse adaptive immune responses by different TLRs relates to the differential use of intracellular adaptor molecules following TLR ligation. A number of signaling pathways used by different TLRs have been found (see AKIRA *et al.* 2003). Subsequent studies suggested the possibility that Th differentiation in part depends on the adaptor molecules in TLR signaling (DIDIERLAURENT *et al.* 2006; SCHNARE *et al.* 2006). **FIG. 1.4** illustrates the complex relationship between TLR pattern recognition, intracellular signaling, T cell activation, Th2 cell development and subsequent IgE production.

1.3 ASTHMA GENETICS

The previous chapter emphasised that changes in the pathogen recognition system, like certain modifications of proteins or differences in the TLR expression profiles, might lead to profound changes in downstream and Th1/Th2 signaling, implicating that the risk to develop atopic diseases like asthma could be modified. As protein structure and protein expression patterns are determined by their respective genes and gene regulatory regions, an influence of the genetic make-up on atopic diseases should be traceable. However, it is of crucial importance to be aware that genetic research is hampered a) by the lack of a gold standard to diagnose asthma, and b) by the fact that genes may interact with the environment in the development of the phenotype.

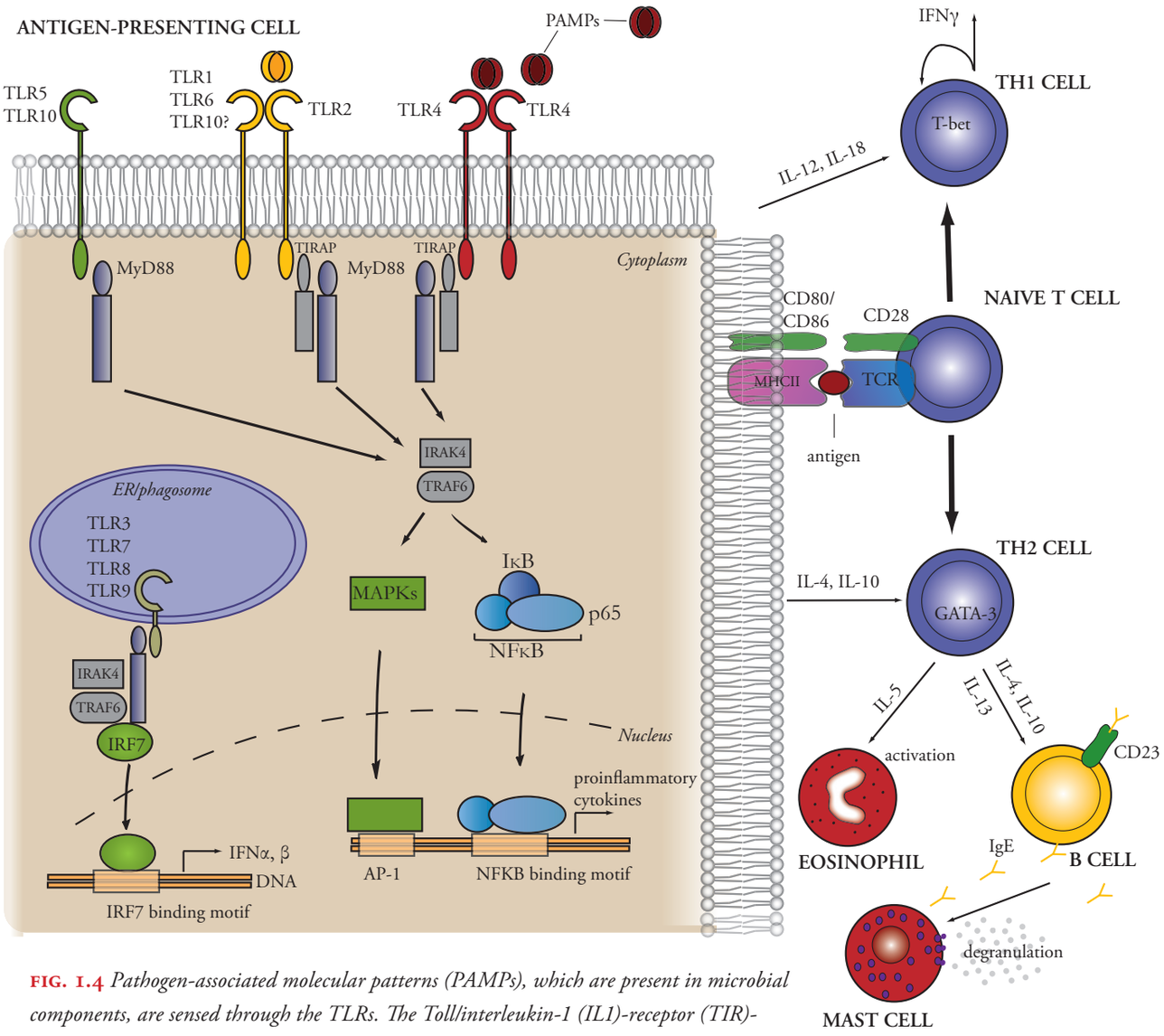


FIG. 1.4 *Pathogen-associated molecular patterns (PAMPs), which are present in microbial components, are sensed through the TLRs. The Toll/interleukin-1 (IL1)-receptor (TIR)-domain-containing adaptor molecule MyD88 (myeloid differentiation primary-response protein 88) mediates the TLR signaling pathway that activates IRAKs (IL-1-receptor-associated kinases) and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6), and leads to the activation of the IKK complex (inhibitor of nuclear factor-κB (IκB)- kinase complex). This pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 and releases NF-κB from its in hibitor so that it translocates to the nucleus and induces expression of inflammatory cytokines, while MAPKs (mitogen-activated protein kinases) are able to directly activate AP-1 (activator protein 1) dependent genes. TIRAP (TIR-domain-containing adaptor protein inducing IFNβ) is involved in the MyD88-dependent signaling pathway through TLR1, TLR2, TLR4, TLR6 and probably TLR10. In asthmatic individuals, antigen presentation and the transcription factor GATA-binding protein-3 (GATA-3) is thought to result in the polarization of T cell differentiation towards a Th2 pattern, wheras T cells from non-atopic, non-asthmatic individuals show the opposing Th1 (IFNγ) pattern of cytokine secretion, maintained by T-box expressed in T cells (T-bet). Activated Th2 cells secrete a panel of cytokines, such as IL-4 (essential for T-cell survival and expansion), IL-5, IL-10 and IL-13, which promote eosinophil activation and the IgE production of B cells with subsequent mast cell degranulation.*

1.3.1 ASTHMA SUSCEPTIBILITY GENES

Although in general, genetic complex diseases show no single relation of genotype and phenotype, indeed early epidemiology studies have suggested that asthma is in part attributable to genetic factors. Findings include (a) a four- to fivefold increase in asthma prevalence in first-order relatives of an affected individual, (b) greater concordance of the disease in monozygotic twin pairs compared to dizygotic twins, and (c) heritability estimates in the range of 40-60% (see MANIAN 1997). Many research efforts have investigated genetic variants that may predispose to asthma, and after several years of investigation, a number of candidate genes have been identified. Although different approaches can be taken to discover asthma-susceptibility genes, the two basic methods are the genome-wide search and the candidate gene approach, with each having advantages and disadvantages and different (necessary) downstream proceedings (FIG. 1.5).

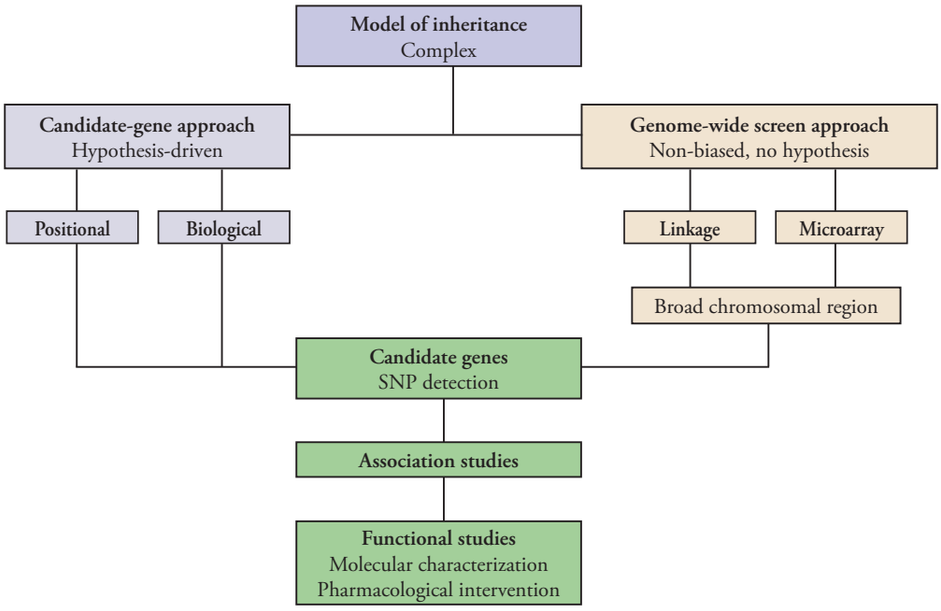


FIG. 1.5 *Overview of strategies to search for genes and their variants that underlie complex diseases.*

At the end, causality can only be established through further functional characterization of the gene and its variants, through gene targeting experiments in animal models or *ex/in vivo* or *in vitro* studies.

In genome-wide screens linkage[1] analysis of markers that span the human genome is used to identify all loci with detectable effects on the phenotype of interest without *a priori* hypothesis (see WILLS-KARP & EWART 2004). Although it more reliably identifies susceptibility genes, even if current concepts would not support a role of those genes, genome-wide screens are costly and can suffer from lack of statistical power. Indeed, only few of the reported linkages have met the accepted criteria for significant genome-wide linkage (that is, a LOD score of 3.7 and $P < 2x10^{-5}$) (LANDER & KRUGLYAK 1995). In contrast, the "candidate gene" approach is hypothesis-driven. Because there is a multitude of potential candidate genes for a complex disease as asthma, genes that most likely might be associated with the disease have to be selected. Immunological pathways that lead to the asthmatic response are quite complex and many genes might indeed be required for the regulation of the immune response to allergens. Most of the genes investigated so far were identified by a candidate gene strategy. Often, in this approach, genes are selected based on their known functions. As a result of current concepts of asthma pathogenesis, geneticists have studied genes involved in inflammation, immunoregulation, bronchoconstriction, airway remodeling and mucus secretion. TABLE 1.2 provides an overview of innate immunity gene variants currently known to be associated with asthma or asthma-related phenotypes (HOFFJAN & OBER 2002; HOFFJAN *et al.* 2003; OBER & HOFFJAN 2006).

TABLE 1.2 *Innate immunity genes and their association with atopic phenotypes.*

Gene	Location	Phenotype	Literature
CARD4	Cytoplasm	IgE, asthma, atopic eczema	(HYSI *et al.* 2005; WEIDINGER *et al.* 2005)
CARD15	Cytoplasm	IgE, atopy, allergic rhinitis	(KABESCH *et al.* 2003)
TLR2	Plasmamembrane	asthma	(EDER *et al.* 2004; REDECKE *et al.* 2004)
TLR4	Plasmamembrane	severity of atopy in asthmatics, endotoxin effects on asthma	(WERNER *et al.* 2003; YANG *et al.* 2004)
TLR6	Plasmamembrane	asthma	(TANTISIRA *et al.* 2004)
TLR9	Plasmamembrane	asthma	(LAZARUS *et al.* 2003)
TLR10	Plasmamembrane	asthma	(LAZARUS *et al.* 2004)
CD14	Plasmamembrane	IgE, asthma, atopy	(BALDINI *et al.* 1999; GAO *et al.* 1999)
C3AR1	Plasmamembrane	asthma	(HASEGAWA *et al.* 2004)
DEFB1	Extracellular	asthma	(LEVY *et al.* 2005)
MIF	Extracellular	atopy, asthma	(HIZAWA *et al.* 2004; MIZUE *et al.* 2005)
C3	Extracellular	asthma	(HASEGAWA *et al.* 2004)
C5	Extracellular	asthma	(BOURGAIN *et al.* 2003; HASEGAWA *et al.* 2004)

[1]in this context linkage is defined as the co-occurence of certain chromosomal markers with a condition/disease/trait

If taking all asthma susceptibility genes into account, it is apparent that most of the genes associated with asthma to date produce proteins that are located in either the plasma membrane or secrete inflammatory molecules (see BOSSE & HUDSON 2007). New disease-causing mechanisms are also emerging from genetic studies. An elegant example was recently provided by PALMER *et al.* 2006. They showed that DNA variants in the filaggrin (FLG) gene disrupted the skin barrier, allowing allergen sensitization and subsequently promoting the development of asthma (HUDSON 2006).

However, the validated genetic factors involved in asthma appear to have rather modest effect sizes, suggesting the genetic component of the disease is more likely to be polygenic (many genetic variants, each with a small effect), making it difficult to establish a straightforward genotype-phenotype relationship.

A yet unknown number of genes influences the development of asthma, with each gene effect depending on an unknown number of alleles or groups of alleles (haplotypes). If combinations of alleles - not necessarily on the same chromosome - occur more or less frequently in a population as would be expected from a random formation of haplotypes (based on their frequencies), this is referred to as linkage disequilibrium (LD). High LD between two or more single nucleotide polymorphisms (SNPs) makes it possible to investigate associations of only one of those SNPs without losing information about the others. In addition, changes in LD may indicate selection processes in finite populations and are therefore important to consider.

Different genes may affect different populations having different genetic backgrounds or environmental exposures. In some circumstances, a gene with a small individual effect may make a substantial contribution to the manifestation of asthma by interacting with a second gene (epistasis) or an environmental factor (gene-environment interaction). Gene-gene and gene-environment interactions are currently difficult to test but are likely to be important in the context of asthma and other complex traits (KABESCH 2006; OBER *et al.* 2006).

1.3.2 *TLR* POLYMORPHISMS AND ASTHMA

TLRs have emerged as a key mediator of the innate immune reponse. As they effect the interface between the environment and adaptive immune system regulation, genetic changes in those receptors may have a significant and observable impact on the development of atopic diseases. Furthermore, altered recognition of microbes due to TLR variations may influence the risk to develop disease in the general population largely independent of levels of microbial exposure. Indeed, polymorphisms in genes coding for microbial pattern recognition receptors (KABESCH *et al.* 2003; LAZARUS *et al.* 2004; TANTISIRA *et al.* 2004; EDER *et al.* 2006B), receptor components (BALDINI *et al.* 1999) and components of the complement system (HASEGAWA *et al.* 2004) have been associated with susceptibility to allergies

and asthma, indicating a role of the innate immune system in allergic diseases (TABLE 1.2). An effect of *TLR* SNPs in the development of asthma phenotypes could be found in multiple populations ranging from European farmers (*TLR2*), UK Caucasians (*TLR4*) to European Americans (*TLR9*, *TLR10*). Taking the important role of TLRs in pathogen recognition and T cell differentiation into account (with their immanent close relationship to the development of asthma, see 1.1.3 and 1.2.4) it is not surprising that variations in TLRs, that might lead to a dysregulated function of particular TLRs, may alter the risk to develop asthma. This concept is even more strongly supported by experimental data showing that, in MyD88-deficient mice, the lack of TLR signaling resulted in increased Th2 responses with overwhelmed IgE production (SCHNARE *et al.* 2001).

1.4 THE AIMS OF THE PROJECT

Previous publications associating *TLR* polymorphisms with asthma dealed mostly with single SNPs in individual TLRs, they were often afflicted with small sample size and did not offer a model explaining the observed effects. Therefore in this thesis the following aims were gradually formulated: (a) a comprehensive genetic analysis of the role of genetic variations and haplotypes in all TLRs 1-10 on asthma and atopy in three independent populations of children of German origin, (b) applying interaction models based on functional clustering and putative heterodimerisation of TLRs, (c) investigating the influence of associated SNPs on TLR mRNA as well as protein expression, and – after specific stimulation – on TLR signaling and Th1/Th2 cytokine production *ex vivo*, ending up with (d) proposing a model how asthma susceptibility could be modulated.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 REAGENTS AND CHEMICALS

2'-deoxyadenosine 5'-triphosphate (dATP)	www.peqlab.de
2'-deoxycytidine 5'-triphosphate (dCTP)	www.peqlab.de
2'-deoxyguanosine 5'-triphosphate (dGTP)	www.peqlab.de
2'-deoxythymidine 5'-triphosphat (dTTP)	www.peqlab.de
2'-deoxynucleotide 5'-triphosphate (dNTP)	www.peqlab.de
Agarose	www.biozym.com
Ammonium chloride	www.sigmaaldrich.com
Antibiotics	www.invitrogen.com
Betaine (Trimethylglycine)	www.sigmaaldrich.com
Boric acid	www.sigmaaldrich.com
Bromo phenol blue	www.carl-roth.de
DNA ladder 100 bp	www.neb.com
Dulbecco's modified Eagle's medium	www.invitrogen.com
Ethanol	www.merck.de
Ethidiumbromide	www.bio-rad.com
Ethylenediaminetetracetic acid (EDTA)	www.sigmaaldrich.com
FastBreak cell lysis reagent	www.promega.com
Fetal calf serum	www.roche-applied-science.com
Glycerol	www.merck.de
H2O bidistilled	www.h-kerndl.de
H2O molecular biology grade	www.eppendorf.de
H2O LiChrosolv	www.merck.de
Isopropanol	www.merck.de
Magnesium chloride	www.promega.com
Oligonucleotides	www.metabion.com
pFLAG-CMV	www.sigmaaldrich.com
Polymer POP-7™	www.appliedbiosystems.com
Potassium bicarbonate	www.merck.de
RPMI	www.invitrogen.com
Sodium chloride	www.sigmaaldrich.com
Sodium hydroxide	www.merck.de
Sodium lauryl sulfate	www.serva.de
SpectroCLEAN™ Resin	www.sequenom.com
Tris(hydroxymethyl)methylamine (TRIS)	www.sigmaaldrich.com
TRIS chloride	www.sigmaaldrich.com
Xylencyanole	www.merck.de

2.1.2 ENZYMES

Taq-Polymerase [5u/µl]	in-house production
AccuPrime™ GC-Rich DNA Polymerase	www.invitrogen.com
HotStar Taq® Polymerase	www.qiagen.com
Platinum® Taq DNA Polymerase High Fidelity	www.invitrogen.com
Proteinase-K	www.sigmaaldrich.com
Restriction Enzymes	www.neb.com
Shrimp Alkaline Phosphatase	www5.amershambiosciences.com

2.1.3 SOLUTIONS AND BUFFERS

Buffer Y	200 mM TRIS-HCl (pH 8.55) 160 mM (NH4)2SO4 20 mM MgCl2
Erythrocyte lysis buffer	155mM NH4Cl 10 mM KHCO3 0.1 mM EDTA pH 7.4, adjust with HCl or NaOH autoclave
Loading Dye – solution	0.05% (w/v) bromo phenol blue 0.05% (w/v) xylencyanole 50% (w/v) glycerol
Proteinase-K buffer	10 mM TRIS-Cl (pH 7.5) 25 mM EDTA (pH 8.0) 100 mM NaCl autoclave
TBE-buffer [5x]	450 mM TRIS 450 mM boric acid 10 mM EDTA pH 8.0
TE buffer	10 mM TRIS-Cl (pH 7.5) 1mM EDTA autoclave

2.1.4 ANTIBODIES

TLR1-PE mouse IgG1	www.ebioscience.com
TLR2-PE mouse IgG2α	www.ebioscience.com
TLR6-biotin rat IgG2α	www.ebioscience.com
TLR10 mouse IgG1	www.imgenex.com
anti-mouse PE goat IgG	www.imgenex.com
mouse IgG1-PE	www.bdbiosciences.com
anti-human CD19 mouse IgG1 FITC	www.bdbiosciences.com

2.1.5 TLR STIMULANTS

IL-1ß	www.peprotech.com
LTA	www.sigmaaldrich.com
Zymosan A	www.sigmaaldrich.com
PGN	www.invivogen.com
Pam_3CSK_4	www.microcollections.de

2.1.6 REAGENT KITS

BigDye® Terminator v3.1 Cycle Sequencing	www.appliedbiosystems.com
ELISA kits	www.rndbiosciences.com
FlexiGene DNA	www.qiagen.com
GenomiPhi DNA Amplification	www6.amershambiosciences.com
HotStar Taq® Plus	www.qiagen.com
iPLEX™	www.sequenom.com
iQ™ SYBR® Green Supermix	www.bio-rad.com
iScript™ cDNA Synthesis	www.bio-rad.com
QuikChange	www.stratagene.com
SAP	www.usbcorporation.com
VARIOM	www.variom.com
Versagene™ Total RNA Purification	www.gentra.com

2.1.7 CONSUMABLES

96-well clusterplate	www.qiagen.com
96-well PCR plate	www.4ti.co.uk
96-well sequencing plate low profile	www.peqlab.de
384-well mikrotiter plate	www.abgene.com
384-well silicon chip (SpectroCHIP™)	www.sequenom.com
Clear Seal	www.4ti.co.uk
Peel-it lite thermo sealing-foil	www.eppendorf.de
S-Monovette	www.sarstedt.com

2.1.8 EQUIPMENT

ABI PRISM® 3700 Genetic Analyzer	www.appliedbiosystems.com
BioPhotometer	www.eppendorf.de
Digital camera Kodak DC 290 zoom	www.kodak.de
FACSCalibur	www.bd.com
Gel electrophoresis units	www.bio-rad.com www.peqlab.de www.owlsci.com
Gene Pulser II system	www.bio-rad.com
Heat sealing machine	www.eppendorf.de
iCycler	www.bio-rad.com
MassARRAY™ MALDI-TOF MS	www.sequenom.com
Nanoliterspotter Spectro Point Nanoliter Pipetting System™	www.sequenom.com
Precision cuvette 10 mm	www.hellma-worldwide.de
Robot Genesis RSP 150 Workstation	www.tecan.de
Robot Multimek 96 Automated 96-Channel	www.beckmancoulter.com

2.1.9 FREE ONLINE DATABASES/SOFTWARE

IIPGA Genetic Data	http://innateimmunity.net/
AliBaba 2.1	http://darwin.nmsu.edu/
BioEdit Sequence Alignment Editor	http://www.mbio.ncsu.edu/BioEdit
FastSNP	http://fastsnp.ibms.sinica.edu.tw
GOLD 1.0 package	http://www.well.ox.ac.uk/asthma/GOLD
Haploview	http://www.broad.mit.edu/mpg/haploview/
MatInspector	http://www.genomatix.de/
NCBI	http://www.ncbi.nlm.nih.gov
NetPrimer	https://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html
PupaSNP	http://pupasnp.bioinfo.ochoa.fib.es/
SNPper	http://snpper.chip.org/bio/snpper-enter-gene
Vista Genome Browser	http://pipeline.lbl.gov/cgi-bin/gateway2

2.1.10 COMMERCIAL SOFTWARE

Adobe Photoshop	www.adobe.de
Cell Quest 3.1f	www.bd.com
iCycler iQ software 3.1	www.bio-rad.com
Magellan	www.tecan.de
Microsoft Office	www.microsoft.com
SAS software (Version 9.1.3.)	www.sas.com
Vector NTI 9.0	www.invitrogen.com

2.2 METHODS

2.2.1 POPULATIONS

2.2.1.1 CROSS-SECTIONAL POPULATION (N = 3,099)

Between 1995 and 1996, cross sectional studies were conducted in Munich and Dresden as part of the International Study of Asthma and Allergies in Childhood phase II (ISAAC II) to assess the prevalence of asthma and allergies in 5,629 schoolchildren age 9 to 11 years (WEILAND *et al.* 1999) (TABLE 2.1A).

TABLE 2.1A *ISAAC II population characteristics. SPT, skin prick test.*

	Munich (N = 2,612)		Dresden (N = 3,017)	
	n	%	n	%
Diagnosis ever				
Asthma	264	10.3	235	7.9
Current asthma	132	5.1	119	4.0
Bronchitis	575	24.4	1041	36.8
Hay fever	235	9.3	289	9.8
Atopic dermatitis	441	17.5	482	16.6
SPT positive*	425	23.5	576	25.7

*a positive reaction (≥3 mm wheal after subtraction to the negative control) to at least one tested allergen

Of those, only German children from the cities Munich and Dresden who had both DNA and IgE data available (N = 1,159 and N = 1,940, respectively) were included in the cross-sectional association analyses (TABLE 2.1B).

TABLE 2.1B *ISAAC II population characteristics (both DNA and IgE data available). SPT, skin prick test.*

		Munich		Dresden	
		n	%	n	%
Age	9	543	50.5	672	34.6
	10	507	47.2	1149	59.3
	11	25	2.3	118	6.1
Sex	male	529	49.1	994	51.2
Asthma		122	11.4	142	7.5
SPT positive		262	24.6	493	26.5

The exact phenotyping methods have been described elsewhere (WEILAND *et al.* 2004; WEILAND *et al.* 1999). Parental questionnaires for self-completion according to ISAAC standards were sent through the schools to the families of all 4th graders (age 9-11)(WEILAND *et al.* 1999) while slightly different questionnaires were used for the Leipzig population (VON MUTIUS *et al.* 1998). All children whose parents reported that a doctor diagnosed "asthma" at least once or "asthmatic, spastic or obstructive bronchitis" more than once were defined as having asthma. Atopy was defined as positive skin prick test (≥3mm difference in weal size compared to negative control) to at least one of the following aeroallergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and tree pollen.

Informed written consent was obtained from all parents of children included in the study and all study methods were approved by the local ethics committees.

2.2.1.2 CASE-CONTROL POPULATION (N = 1,872)

In a nested case control approach, all children who had a doctor's diagnosis of asthma and/or showed bronchial hyperresponsiveness (N = 671, Munich n = 264, Dresden n = 276, Leipzig n = 131) were selected from the total study population (including children from Leipzig (VON MUTIUS *et al.* 1998)) as previously reported (KORMANN *et al.* 2005). These children were matched at a 1:2 ratio with a random selection of healthy, non-asthmatic, non-atopic children without a diagnosis of BHR (N = 1,342, Munich n = 528, Dresden n = 552, Leipzig n = 262), and finally only children of German origin who had both DNA and IgE data available were included (N = 1,872, Munich n = 690, Dresden n = 789, Leipzig n = 393). Slightly different questionnaires were used for the Leipzig population (VON MUTIUS *et al.* 1998). Lung function was measured by MasterScope Version 4.1 (Jäger, Würzburg, Germany) according to ISAAC phase II protocols. A minimum of two baseline spirograms was recorded and the highest of two reproducible (within 5%) measurements of forced expiratory volume in 1 s (FEV_1) was recorded as baseline FEV_1. In Munich and Dresden bronchial reactivity was assessed by changes in FEV_1 after inhalation of nebulized, hyperosmolar (4.5%) saline for increasing periods of time using ultrasound nebulizers (DeVilbiss Sunrise Medical, Langen, Germany). In Leipzig, BHR was measured by cold air (-15°C) hyperventilation for 4 minutes (VON MUTIUS *et al.* 1998). All children with a drop in FEV_1 of 15% or more from baseline after hypertonine saline challenge or a 9% drop after cold air challenge were classified as having bronchial hyperresponsiveness (BHR).

2.2.1.3 ADULTS POPULATION (N = 40)

EDTA-blood was taken from 40 unrelated adult volunteers, representing an easy-to-access resource of random sample. Plasma was collected and stored at 4°C. DNA was isolated utilizing an inorganic salting-out method (MILLER *et al.* 1988) or the FlexiGene Kit and stored at -20°C. RNA

was extracted using the Versagene Kit according to manufacturers' protocol and stored at -80°C. Concentration and purity were determined by analyzing A260/A280 and A260/A230 absorbance ratios.

2.2.2 SNP SELECTION

All SNP data was based on the results of the National Institute of Health mutation screening program of innate immunity genes performed in a standard set of immortalised human samples from the Coriell Institute of Medical Research, Camden, New Jersey (ccr.coriell.org). To determine SNP induced changes in putative transcription factor binding sites in promoter and intronic regulatory sites *in silico*, FastSNP was used. With VectorNTI Advance 9.0 amino acid changes were determined, sequences were organized and alignments were performed.

2.2.3 STANDARD POLYMERASE CHAIN REACTION (PCR)

PCR is a molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. Like amplification using living organisms, the technique allows a small amount of the DNA molecule to be amplified exponentially.

2.2.3.1 OPTIMISING PCR REACTIONS

The DNA fragment to be amplified is determined by the selected primers. Primers are short, artificial DNA strands — often not more than 50 and usually only 18 to 27 base pairs long — that are complementary to the beginning or the end of the DNA fragment to be amplified. They anneal by adhering to the DNA template at these starting and ending points, where the DNA polymerase binds and begins the synthesis of the new DNA strand.

The following parameters were considered to enhance likelihood of efficient and specific amplification of the desired sequence by the designed primers (in part according to www.biocrawler.com/encyclopedia/PCR):

- GC-content should be between 40-60%
- Calculated T_m for both primers used in reaction should not differ > 5°C and T_m of the amplification product should not differ from primers by > 10°C
- Annealing temperature usually is 5°C below the calculated lower T_m; however it should be chosen empirically for individual conditions

- Inner self-complementary hairpins of > 4 and of dimers > 8 should be avoided
- Primer 3' terminus design is critical to PCR success since the primer extends from the 3' end; the 3' end should not be complementary over greater than 3-4 bases to any region of the other primer (or even the same primer) used in the reaction and must provide correct base matching to template
- BlastSearch hits < 100 enhance specificity of the primer (via VectorNTI Advance 9.0)
- Predicted deltaG > –5 kcal/mol for hairpins, dimer and cross dimer formation reduces self-annealing of primers (via NetPrimer)

Different betain concentrations were applied using the following 15 µl reaction protocol:

Reagent	Volume
Buffer Y	1.5 µl
Primer (each fwd/rev) 25 µM	0.15 µl
dNTPs 10 mM	0.3 µl
Betain 5 M	0 / 0.6 / 1.5 / 3 µl
H_2O	11.85 / 11.25 / 10.35 / 8.85 µl
Taq-Polymerase (5 u/µl)	0.05 µl
DNA (20 ng/µl)	1 µl

The PCRs were set up using the following gradient cycler program:

Step	Temp	Time
1	94°C	1’
2	94°C	20”
3	Tm*	20”
4	72°C	20”
5	Goto step 2, repeat 34x	
6	72°C	5’
end	20°C	inf

**gradient Tm +/– 7°C*

Subsequently, the reactions were analysed on an agarose gel (see 2.2.8).

2.2.3.2 PCR PRECEDING THE SEQUENCING REACTION

Using the optimized conditions (2.2.3.1; one specific single product band at the expected range, no or weak primer dimer bands), 50 µl PCR reactions were set-up.

2.2.4 PRIMER EXTENSION PREAMPLIFICATION (PEP) PCR

Whole genome amplification methods began with the introduction of PEP PCR in the early 1990s, with the goal of generating a complete and unbiased representation of the entire genome (ZHANG *et al.* 1992). PEP PCR is based on the frequent annealing of a totally degenerate 15-mer primer.

The following 50 µl reactions were used with the appropriate gradient cycler program:

Step	Temp	Time
1	94°C	3'
2	94°C	1'
3	37°C (= T_n)	2'
4	T_n	1''
5	$T_n = T_n + 0.2$°C	
6	Goto step 4	Repeat 90
7	55°C	4'
8	Goto step 2, repeat 49x	
9	72°C	5'
end	20°C	inf

Reagent	Volume
Buffer Y 10x	5 µl
MgCl2 25 mM	5 µl
dNTPs 2 mM	5 µl
N_{15}-Primer 200 µM	10 µl
H_2O	19 µl
DNA (1 ng/µl)	5 µl
Taq-Polymerase (5 u/µl)	1 µl

2.2.5 MULTIPLE DISPLACEMENT AMPLIFICATION (MDA)

As a further variant for direct amplification of entire genomic DNA a Phi29 DNA polymerase-based amplification (isothermal MDA) was used (DEAN *et al.* 2002). Phi29 DNA polymerase is a highly processive enzyme that incorporates at least 70,000 nucleotides in one binding event and performs efficient strand displacement synthesis at a rate of 25 to 50 nucleotides/second with an error rate of 3×10^{-6}, which allows for high fidelity replication of input DNA template. These two properties, strand displacement and processivity, allow phi29 DNA polymerase to be used in novel isothermal amplification strategies to amplify nanograms of genomic DNA into several micrograms.

Here, the GenomiPhi Kit was used to amplify 5 ng DNA to a final amount of about 4-6 µg in 20 µl reactions. Subsequently, the amplified DNA was diluted in H_2O to a final concentration of 20 ng/µl.

2.2.6 SEQUENCING

To exclude genotyping errors in the case of HWE violation a part of the genotypes assessed with MALDI-TOF were validated by resequencing and comparing PCR fragments. Sequencing offered

the most accurate and detailed analysis of given fragments.

2.2.6.1 PCR CLEANUP

To remove excess primers, dNTPs and tagged ddNTPs the reaction products were purified with Millipore MultiScreen plates, which incorporate size-exclusion technology.

PCR products were mixed with 250 µl H_2O, transferred into Multiscreen™ PCR plates and centrifuged at 3,200 g for 7 min. Subsequently, 30 µl HPLC-H_2O were added and the plate was shaken at low frequency. The DNA containing H_2O was transferred into a reaction tube.

2.2.6.2 CYCLE SEQUENCING

Basically, cycle sequencing reactions by Sanger's dideoxy terminator method were applied, in which only one strand can be sequenced in one reaction (SANGER *et al.* 1977). If the PCR fragment was larger than 550 to 600 bp, both forward and reverse primer were used in two seperate reactions to assure a complete sequence.

The following protocol was used to set up 5 µl cycle reactions:

Reagent	Volume
Sequencing Buffer 5x	1 µl
Primer (fwd OR reverse) 10 µM	0.5 µl
BigDye 3.1	1 µl
H_2O	0.5 µl
PCR (~ 20 ng/µl)	1 µl

Step	Temp	Time
1	94°C	30"
2	50°C	15"
3	60°C	4'
4	Goto step 1, repeat 34x	
end	4°C	inf

2.2.6.3 POST-SEQUENCING REACTION CLEANUP

To remove excess primers, dNTPs and tagged ddNTPs, the reaction products were purified by precipitation. 100 µl 70% EtOH (v/v) were added, incubated 15 min at RT, followed by centrifugation at 3,000 g for 30 min. The supernatant was discarded. The plate was turned upside down and centrifuged at 300 g for 5 s to remove most of the ethanol. Then 150 µl 70% EtOH (v/v) were added, followed by centrifugation at 3,000 g for 10 min. After discarding the supernatant the plate was again spinned upside-down at 300 g for 1 min. The cleaned DNA pellet was dried for an additional 5 min at RT and stored at –20°C for no longer than 5 days.

2.2.6.4 SEQUENCING / CAPILLARY GEL ELECTROPHORESIS

Capillary gel electrophoresis is the adaptation of traditional gel electrophoresis to a capillary using polymers in solution to create a molecular sieve also known as replaceable physical gel. This allows analytes having similar charge-to-mass ratios to be resolved by size. This technique is commonly employed in the sizing of applications of DNA sequencing and genotyping.

The DNA pellet was resolved in 50 µl HPLC-H_2O, of which 25 µl were put onto low profile sequencing plates. Data was generated on an ABI PRISM® 3700 Genetic Analyzer with POP-7 polymer and buffer according to manufacturer's specification.

2.2.6.5 ANALYSIS OF SEQUENCE DATA

Sequence data in .ab1 format was compared and analyzed using the VectorNTI ContigExpress plug in. Frequent occurrence of polymorphisms was additionally marked utilizing a probability highlighting of the respective columns.

FIG 2.1 illustrates the three possible allelic states of a regular SNP (disregarding deletions, insertions and large-scale mutations in chromosomal structure). The signal amplitudes are nearly reduced by half in case of a heterozygote state.

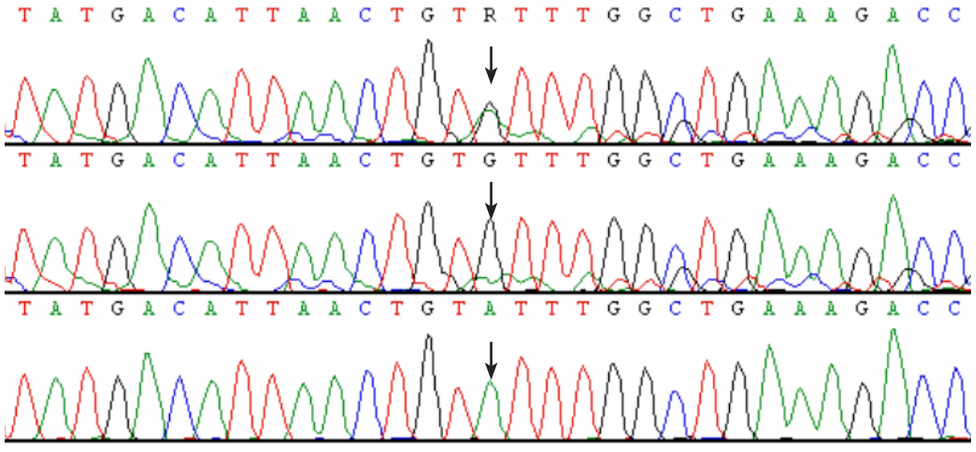


FIG. 2.1 ***Electropherogram printout from an automated sequencer showing part of the TLR1 DNA sequence.*** *The arrows indicate different allelic states at one position.*

2.2.7 GEL ELECTROPHORESIS

Gel electrophoresis is a group of techniques used to separate molecules based on physical characteristics such as size, shape, or isoelectric point. In this thesis it was performed to analyze optimising PCR, qRT RT PCR and templates for DNA sequencing and mass extension reactions, but may also be used as a preparative technique to partially purify molecules prior to use of other methods such as DNA cloning.

At least 50-100 ng PCR were applied together with 10% loading dye (v/v) on an agarose gel (usually 1.5%, depending on fragment sizes), stained with 0.5 µg/ml ethidiumbromide. Electrophoresis was performed with 100-120 V at 400 mA for 30-40 min. Subsequently, the gel was photo documented under UV light, the contrasts optimized using Photoshop and digitally filesaved.

2.2.8 MEASURING DNA/RNA CONCENTRATION

In different reactions it was necessary to use a specific amount of DNA or RNA. The concentration or a nucleic acid containing solution was determined using a photometer. In optics, the Beer-Lambert-Bouguer law is an empirical relationship that relates the absorption of light to the properties of the material through which the light is travelling (FIG 2.2).

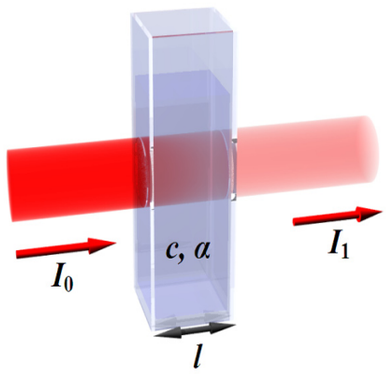


FIG. 2.2 *I_0, intensity of the incident light; I_1, intensity of the light after passing through the material; l, path length; c, concentration of absorbing species in the material; a, absorption coefficient; absorbance = $-\log_{10}(I_1/I_0)$.*

The absorbence of DNA samples was measured at 280 nm as well as 260 nm in a quartz glass cuvette. Both proteins and nucleic acids absorb light at 280 nm. The A260 of DNA with the concentration of 50 mg/ml is 1 OD unit. To be accurate the A260 readings should be between 0.1 and 1.

By obtaining the ratio of A260/A280 one may get an idea of the purity of the DNA sample. For good quality DNA with low protein contamination this ratio should be between 1.8 and 2. The purity of RNA was additionally determined using the A260/230 ratio, which should be higher than 2.

2.2.9 MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

MALDI-TOF is a soft ionization technique used in mass spectrometry, allowing the ionization of biomolecules (such as proteins, peptides and DNA) which tend to be more fragile and quickly lose structure when ionized by more conventional ionization methods. The ionization is triggered by a LASER beam, while a certain matrix is used to protect the biomolecule from being destroyed during this procedure.

In this thesis both homogenous MassEXTEND (hME) assay and iPLEX assay were used for genotyping. Assay specific "tagged" primers are shown in TABLE 2.2.

First the amplified DNA was pipetted into 96 deep well plates (1 ng/µl) applying the following scheme (NC = negative control = H_2O):

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			NC									
C												
D					NC							
E							NC					
F												
G											NC	
H												

Four 96 deep well plates ("mother plates") were prepared for one 384 well plate ("daughter plates"). 5 µl DNA from the 96 deep well plates were subsequently pipetted onto 384 well plates using a Tecan Genesis Workstation.

The DNA was dried overnight (to minimize subsequent reaction volumes) at RT.

TABLE 2.2 *PCR and extension primer used in MALDI-TOF MS.*

Gene	rs number	PCR Primer	Extension Primer
TLR1	rs5743594	1st ACGTTGGATGGCTAATACCATCACCTTGGG	TGGATGTTATAGCTTGAATGTTT
		2nd ACGTTGGATGCACACAAGGAGCAATATTTC	
	rs5743595	n.a.	n.a.
	rs4833095	1st ACGTTGGATGCTGGAGGATCCTAATGAAAG	TCAAACAAATCCAAAGTTATCAA
		2nd ACGTTGGATGCCTAAGTATTCTGGCGAAAC	
TLR2	rs4696480	1st ACGTTGGATGAGTCCAAGATTGAAGGGCTG	GTAGCCAGATGACCCTC
		2nd ACGTTGGATGCTCACCATGTGATGCTTTCC	
	rs1898830	1st ACGTTGGATGCCTTAAAAACTGGAAAAGGA	CTTATATTATTATTTCCCCTGTTC
		2nd ACGTTGGATGCCCCTATTTTCTAGCACATT	
	rs3804099	1st ACGTTGGATGGATCTACAGAGCTATGAGCC	TGAAGGATCAGATGACTTAC
		2nd ACGTTGGATGGCTGCTTCATATGAAGGATC	
TLR3	rs3775291	1st ACGTTGGATGTATCACTTGCTCATTCTCCC	AGATTTTATTCTTGGTTAGGTTGA
		2nd ACGTTGGATGCCCAACCAAGAGAAAGCATC	
TLR4	rs6478317	1st ACGTTGGATGCTCCTCTACCTGGCTTTTAC	GATGATTAGGGCTGAATAAC
		2nd ACGTTGGATGCCTGGACCTGTGATGATTAG	
	rs10759932	1st ACGTTGGATGAAATGCAAGCTTCTGCTATG	TTTCACATCTTCACCAAC
		2nd ACGTTGGATGCAGGAGTTCTCATTTTTTCAC	
	rs4986791	1st ACGTTGGATGACACCATTGAAGCTCAGATC	TCAAAGTGATTTTGGGACAA
		2nd ACGTTGGATGAGGTTGCTGTTCTCAAAGTG	
TLR5	rs5744168	n.a.	n.a.
	rs2072493	1st ACGTTGGATGATATATGTCTGCAGGAGGCC	ATGTGAACTTAGCACTTTTATCA
		2nd ACGTTGGATGTGTGAATGTGAACTTAGCAC	
	rs5744174	1st ACGTTGGATGCCAATGTCACTATAGCTGGG	GAGGGAAACCCCAGAGA
		2nd ACGTTGGATGTCCGTGGAAAGAGAGAAGAG	
TLR6	rs5743789	1st ACGTTGGATGACACTGTTAAGTTGGACTTC	TCTTTTTCACTCTCTTGCAG
		2nd ACGTTGGATGGGTTTGTCTTTTTCACTCTC	
	rs5743810	1st ACGTTGGATGTCGTTTCTATGTGGTTGAGG	TTTTATCAGAACTCACCAGAGGT
		2nd ACGTTGGATGGAATGATGACAACTGTCAAG	
TLR7	rs179008	1st ACGTTGGATGTTAGGAAACCATCTAGCCCC	ATGTGGACACTGAAGAGAC
		2nd ACGTTGGATGCAGGTGTTTCCAATGTGGAC	
TLR8	rs3761624	1st ACGTTGGATGCCCTGGCCACAAGAATAAAG	GTGTAAGGCAAGATGAAACAT
		2nd ACGTTGGATGTTGGTTTTCTCCCACTCCTG	
TLR9	rs187084	1st ACGTTGGATGTTACTATGTGCTGGGCACTG	CTGCTGGAATGTCAGCTTCTT
		2nd ACGTTGGATGTATTCCCCTGCTGGAATGTC	
	rs5743836	1st ACGTTGGATGAGCAGAGACATAATGGAGGC	GCTGTTCCCTCTGCCTG
		2nd ACGTTGGATGTTGGGATGTGCTGTTCCCTC	
TLR10	rs11096956	1st ACGTTGGATGTAAACAACTCGTCTGTTAAG	ATGCCACACATGCTTTTCCC
		2nd ACGTTGGATGTGCACAAATGCCACACATGC	
	rs4129009	1st ACGTTGGATGGGCGTAAATGTGGGCTTTTC	GGTGGCTAATACATTAACATTAA
		2nd ACGTTGGATGTCATACATTTCTCTGGTGGC	

All mastermix set-ups and reactions on plates were centrifuged prior to automated pipetting or thermocycler processing.

First, a small area surrounding each SNP was amplified using the HotStar Taq plus Kit (FIG. 2.3).

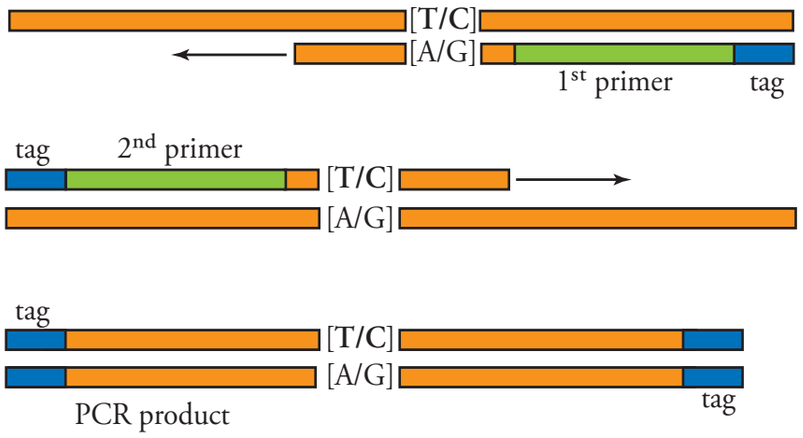


FIG. 2.3 *Amplification of an area surrounding the SNP using tagged primers.*

Combinations of primer pairs ensured simultaneous amplification of up to eight different fragments (multiplex PCR). In this case, the amount of Hotstar Taq was increased:

2- & 3-plex: 0.04 µl Taq; 4- & 5-plex: 0.05 µl Taq; 6-, 7- & 8-plex: 0.06 µl Taq.

The following modified protocol was used:

Reagent	Volume
Hotstar Buffer (15 mM $MgCl_2$)	0.6 µl
dNTPs (2 mM)	0.6 µl
$MgCl_2$ (25 mM)	0.24 µl
Primer fwd (100 µM)	0.01 µl
Primer rev (100 µM)	0.01 µl
Tag-Polymerase (5 u/µl)	0.02 µl
H_2O	4.52 µl

Step	Temp	Time
1	95°C	15’
2	95°C	20”
3	56°C	30”
4	72°C	1’
5	Goto step 2, repeat 44x	
6	72°C	10’
end	20°C	inf

Random checks of the PCR reactions were performed on an agarose gel (2%) to verify successful amplification.

Shrimp alkaline phosphatase (SAP) allowed the dephosphorylation of dNTPs for efficient removal of unincorporated nucleotides prior to further SNP analysis. 2 µl each were pipetted on the 384 well plates utilizing a Multimek 96, followed by the reaction parameters:

Reagent	Volume
H_2O	1.53 µl
hME buffer	0.17 µl
SAP	0.30 µl

Step	Temp	Time
1	37°C	20’
2	85°C	10’
3	20°C	inf

An extension primer binds directly adjacent to the respective SNP and three didesoxynucleotides and one desoxynucleotide ensure that – depending on the choice of the two kinds of nucleotides – the extension primer will be elongated by one or two bases (Sanger’s dideoxy termination). Depending on the allelic state, DNA fragments of different size will occur (FIG. 2.4).

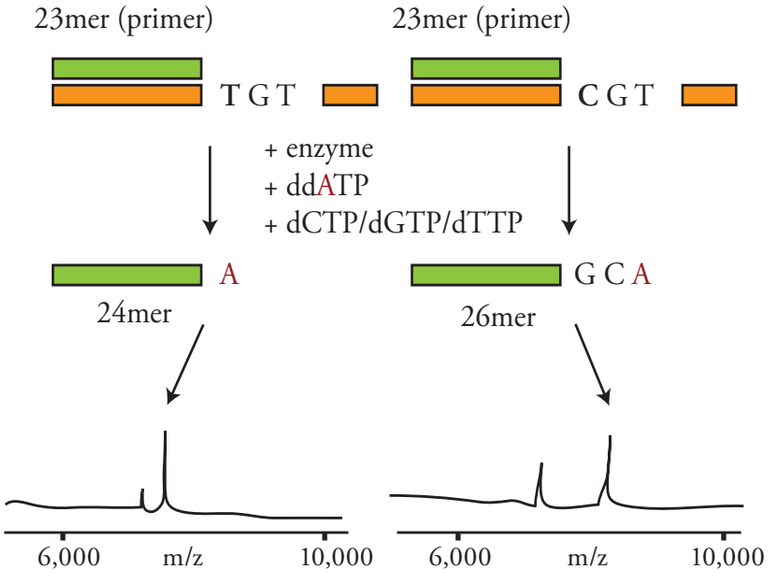


FIG. 2.4 *The respective extension primer binds adjacent to the SNP; depending on the incorporated bases the products will differ in mass and size; m/z, mass-to-charge ratio.*

The extension reaction (2 µl each were pipetted on the 384 well plates utilizing a Multimek 96) was set up as follows:

Reagent	Volume
H_2O	1.728 µl
hME Mix (buffer / d/ddNTPs)	0.200 µl
hME primers	0.054 µl
Thermosequenase	0.018 µl

Step	Temp	Time
1	95°C	2’
2	95°C	5”
3	52°C	5”
4	72°C	10”
5	Goto step 2, repeat 54x	
end	20°C	inf

The finished hME reaction was then desalted by the addition of 6 mg SpectroClean resin per well, followed by the addition of 16 µl HPLC-H_2O, 10 min slow rotation (ensuring the liquid was properly intermingled with the cation exchange resin) and 5 min centrifugation at 3,000 rcf.

Utilizing a Nanoliterspotter, 8-12 nl (depending on the liquid's viscosity) of the purified hME products were then spotted onto a silica SpectroCHIP containing matrix pads (3-hydroxypicolinic acid). Additionally, a calibrant (oligonucleotide) with an exactly defined mass was spotted onto the chip at ten different positions. The chips were individually analyzed using a Bruker Autoflex MALDI-TOF MS. The LASER-mediated desorption and ionization of the extended oligonucleotide products resulted in acceleration of those nucleotides towards a detector. The velocity of the sample is directly correlated to the oligonucleotide length, i.e. the time from desorption/ionization to detector signaling (= time of flight) is proportional to the oligonucleotide's mass (FIG. 2.5).

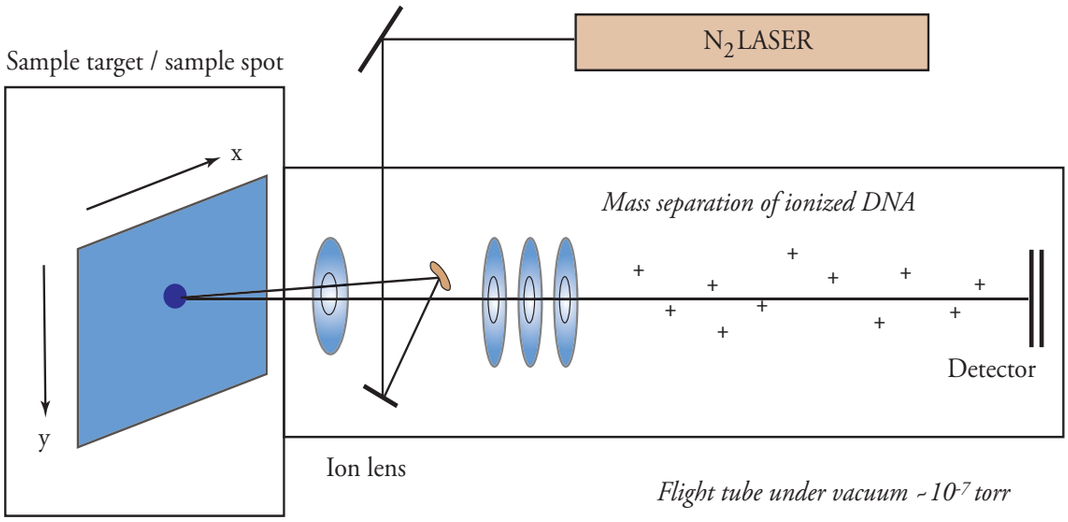


FIG. 2.5 *The LASER is fired at the crystals in the sample spot. The spot absorbs the LASER energy, primarily ionizing the matrix. The matrix transfers part of its charge to the analyte molecules (e.g. DNA), thus ionizing them while still protecting them from the disruptive energy of the LASER. Different masses are then distinguished by the different time-of-flight (TOF) until the molecules reach the detector.*

The resulting spectra were converted to genotype data using the SpectroTYPER-RT software, which interprets the spectral output based on information for expected allele-specific masses.

2.2.9.2 iPLEX ASSAY

All mastermix set-ups and reactions on plates were centrifuged prior to automated pipetting or thermocycler processing.

First, a small area surrounding each SNP was amplified using the HotStar Taq plus Kit using the following 5 µl reaction set-up:

Reagent	Volume
Hotstar Buffer 10x	0.625 µl
H_2O	2.850 µl
$MgCl_2$ (25 mM)	0.325 µl
dNTPs (25 mM)	0.100 µl
Primermix (0.5 µM)	1.000 µl
Hotstar Taq (5 u/µl)	0.100 µl

Step	Temp	Time
1	95°C	15'
2	95°C	20''
3	56°C	30''
4	72°C	1'
5	Goto step 2, repeat 44x	
6	72°C	3'
end	20°C	inf

Random checks of the PCR reactions were performed on an agarose gel (2%).

The SAP reaction was set up as described in the hME protocol.

Compared to the hME reaction principle (Sanger termination) iPLEX allows higher primer multiplexing. As the mass differences would be more subtle and less easy or impossible to differentiate with Sanger termination, mass modified terminators assure that the mass difference between the extension products is higher than 15 Da:

Terminator	A	C	G	T
A	0	-24	16	55.9
C	24	0	40	79.9
G	-16	-40	0	39.9
T	-55.9	-79.9	-39.9	0

The extension reaction and cycler program were set up as follows:

Reagent	Volume
H_2O	0.755 µl
iPlex buffer 10x	0.200 µl
iPlex termination mix	0.200 µl
Primermix	0.804 µl
iPlex enzyme	0.041 µl

Step	Temp	Time
1	94°C	30''
2	94°C	5''
3	52°C	5''
4	80°C	5''
5	Goto step 3, repeat 4x	
6	Goto step 2, repeat 39x	
7	72°C	3'
end	20°C	inf

Subsequent MALDI-TOF probe processing was performed as described for the hME assay.

2.2.10 SOLID-PHASE OLIGONUCLEOTIDE LIGATION ASSAY (SPOLA)

For genotyping *TLR1*_b and *TLR5*_a in the case-control population, a SPOLA was performed according to manufacturers' protocol (VARIOM assay). Detection oligonucleotides were photochemically attached to polystyrene carrier materials, providing the surface on which the planar assay takes place. The relevant gene fragment was amplified by PCR. A mixture of hybridization buffer, labelled signal probes and the PCR product were added to the sample well of the detection plate that was coated with wild type and minor allele specific detection probes. During the incubation, a hybridization complex forms, in which the detection and signal probes were hybridized directly adjacent to one another on one strand of the PCR fragment (FIG. 2.6). The variable nucleotide in the PCR fragment was situated at the junction of the probes. The variable nucleotide in the PCR fragment was determined by a mismatch-sensitive enzymatic ligation of detection and signal probes. The probes were joined into a single molecule only when the variable nucleotide in the PCR fragment was complementary to the detection probe. Depending on which genotypes were present in the PCR fragments,

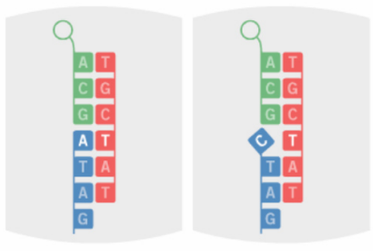


FIG. 2.6 ***SPOLA hybridisation.*** *PCR fragments (blue), labeled signal probes (green) and specific detection probes (red).*

either exclusively the wild type allele-specific detection probe (homozygous wild type genotype), both allele-specific detection probes (heterozygous genotype), or exclusively the minor allele-specific detection probe (homozygous minor allele genotype) could be joined with the signal probe.

Non-ligated signal probes and PCR fragments were removed by washing. Detection of ligation occured through a streptavidin-peroxidase enzymatic conjugate bound to the remaining ligated signal probes. After another washing step, specifically-bound conjugate was detected by the addition of a colorimetric substrate. A green signal is generated only in those wells where a complementary variable nucleotide and its detection probe had been present. The signal was detected with a Wallac Victor^2.

2.2.11 FUNCTIONAL ANALYSES — TLR1, TLR6 & TLR10

2.2.11.1 QUANTITATIVE REAL-TIME PCR (QRT-PCR)

QRT-PCR represents a modification of PCR, which allows to simultaneously quantify and amplify a specific part of a given DNA molecule.

RNA ISOLATION

Total RNA was extracted from 5 ml whole blood utilizing the Versagene RNA blood Kit, which included DNase I treatment. The recommended steps were performed consistently and with a minimum of interruption to minimize variation between the RNA preps. Isolated RNA was stored at –80°C. Concentration and purity were determined by analyzing A260/A280 and A260/A230 absorbance ratios.

REVERSE TRANSCRIPTION (RT)

1 µg RNA each was reverse transcribed using the iScript Kit (20 µl reaction volume). Due to a combination of random primers and Oligo(dT)primers the iScript Kit transcribes mRNA as well as other kind of RNA (e.g. rRNA) into cDNA.

REALTIME PCR

cDNA was amplified by iCycler in duplicates, using *18S* as a reference gene. Each 30 µl reaction-contained 15 µl 2x SybrGreen supermix, 0.16 µM of each primer (TABLE 2.3) and 1 µl of template. Ct values were obtained using iCycler iQ software 3.1 which automatically calculated baseline cycles and threshold positions.

TABLE 2.3 ***Primers used for RealTime PCR.*** *ß2-mic, ß2-microglobulin.*

Gene	Primer	
	Fwd 5'-3'	**Rev 5'-3'**
TLR1	GAAGAAATCAGGATAACAAAGGC	TTCTTCAGATAATTGTATTCTGATC
TLR2	ATTGTGCCCATTGCTCTTTCACTGC	TGAGGGAATGGAGTTTAAAGATCC
TLR6	TTCATGTTCCAAAAGACCTACCG	GAAACTCACAATAGGATGGCAGG
TLR10	GAATCCTGACTTACCTCAACAAC	CTCTGGAGCATCACCCTCTG
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
ß2-mic	ATGTCTCGCTCCGTGGC	AATGTCGGATGGATGAAACC

Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid (GIVAN 2001). It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and electronic detection apparatus (FIG. 2.7). In the presented work it was used to measure differences in surface expression of TLR protein.

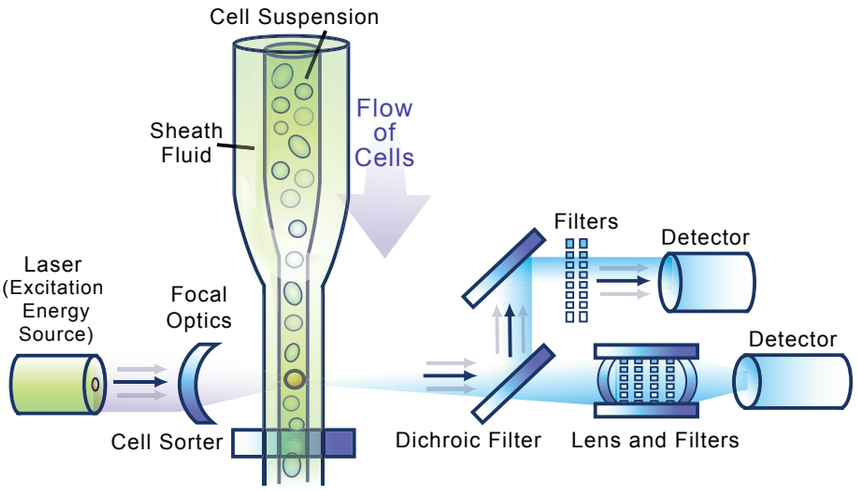


FIG. 2.7 ***Scheme of a flow cytometer.*** *A beam of light of a single wavelength is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (forward scatter or FSC) and several prependicular to it (side scatter or SSC) and one or more fluorescent detectors. The pattern of light scattering is dependent on cell size and shape, giving relative measures of these cellular characteristics as cells flow through the beam.*

Fluorescence-based detection depends on the absorption of light by the cell and the subsequent re-emission of this light at a different frequency. Flow cytometers make use of this technology by employing filters to block the original light source from reaching the detector, while the fluorescence emission is allowed through for detection, which allows only a very low background of stray light to reach the detector. In flow cytometry experiments, fluorescence achieved by the deliberate labeling of a cellular component using a fluorescent marker, usually a type of dye that is attached to appropriate antibodies. In an experiment determining the presence or absence of a particular cell marker or a relative increase or decrease of a marker after experimental treatment, a histogram shows the shift in the fluorescence intensity of the sampled cells (FIG. 2.8).

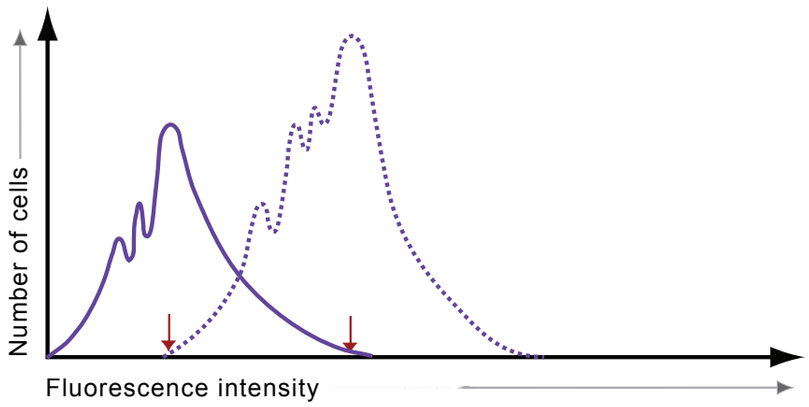


FIG. 2.8 ***Histogram charting the numer of cells counted and the fluorescence intensity.*** *The red arrows mark the peak fluorescence intensities; the shift shows a relative increase in the average cell fluorescence (dotted line).*

To analyse TLR protein expression, the following antibodies were used: TLR1-phycoerythrine (PE) mouse IgG1, TLR2-PE mouse IgG2a, TLR6-biotin rat IgG2a, Streptavidin-PE, TLR10 mouse IgG1, anti-mouse-PE goat IgG, mouse IgG1-PE and anti-human CD19 mouse IgG1 fluorescein isothiocyanate (FITC). Both FITC and PE show a maximum absorption at 488 nm, with FITC emitting at 520 nm and PE at 575 nm. Mouse IgG1-PE, mouse IgG1-FITC, streptavidin-PE and anti-mouse-PE goat IgG were used to determine non-specific binding; these values were subtracted from the respective specific antibody staining. Cellular fixation and antibody staining were performed using the Intraprep Kit according to the manufacturer´s instructions, optimized to avoid non-specific staining. Whole blood (2 ml) anticoagulated with EDTA was obtained from five subjects bearing homozygous minor alleles for *TLR1*_b/*TLR6*_a/*TLR10*_b and nine subjects bearing homozygous h alleles for this SNP block. For TLR1 and TLR6, only surface staining was performed. For TLR10, surface staining and intracellular staining was performed. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation, where the sample is layered onto a Ficoll-sodium metrizoate gradient, and incubated for 30 minutes with anti-human TLR1-PE, TLR6-biotin or TLR10 antibodies. Afterwards, the cells were washed and the streptavidin-PE or the anti-mouse-PE antibodies were added for further 30 minutes. After washing, the TLR1 and TLR6 stained aliquots underwent red-cell lysis (see below), whereas the TLR10 stained cells were permeabilized with saponin and intracellular staining was performed with anti-TLR10 for 30 minutes and anti-mouse-PE antibodies for further 30 minutes. After washing, erythrocytes were lysed in ice-cold NH_4Cl-buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA at pH 7.4) prior to the analysis. Leukocytes (including lymphocytes, monocytes and neutrophils) were gated due to their forward/side-scatter characteristics. At least 10.000 cells/sample were analyzed on a FACSCalibur. Results are reported as mean fluorescence intensity (MFI).

2.2.11.3 PBMC STIMULATION

Freshly isolated PBMCs were seeded in 96 well plates at 3 x 10^5 cells/200µl/well in RPMI supplemented with 10% FCS and stimulated for 24h with the following TLR1/2/6 ligands: Pam_3CSK_4 (10 µg/ml; an artificial triacylated lipopeptide; TLR1/2 agonist), PGN (1 µg/ml; TLR2/NOD2 agonist), LTA (10 µg/ml; TLR2 agonist), Zymosan (10 µg/ml; TLR2/6 agonist) and with IL1-β (50 ng/ml) according to the methods previously described (RENNER *et al.* 2005).

2.2.11.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

After the incubation period, the PBMCs were washed twice and levels of IL-6, TNFα, IL-12, IFNγ, IL-10 and IL-4 were analyzed in duplicates by sandwich ELISA according to the manufacturer´s instructions. In brief, samples were pipetted into antibody precoated wells and detected by a horseradish peroxidase-linked polyclonal antibody specific for the respective chemokine. The optical density values were read in a microplate reader (MRX II, Life Sciences, Eglsbach, Germany) at 450/540nm. The concentrations were calculated from standard curves. The detection limits were: IL-6 (minimum 3 pg/ml - maximum 40000 pg/ml), TNFα (5-8000 pg/ml), IL-12 (5-9000 pg/ml), IFNγ (8-2000 pg/ml), IL-10 (5-3000 pg/ml) and IL-4 (10-5000 pg/ml).

2.2.12 STATISTICS

All statistics were performed with the SAS 9.1.3 / SAS genetics module if not indicated otherwise. Statistical significance was generally defined as $P < 0.05$.

2.2.12.1 LINKAGE DISEQUILIBRIUM (LD)

Pairwise LD was assessed between single SNPs using the R^2-statistic with Haploview (BARRETT *et al.* 2005). The GOLD 1.0 package was used to visualize the interchromosomal disequilibrium graph.

2.2.12.2 HARDY-WEINBERG EQUILIBIUM (HWE)

HWE states that, under certain conditions (see **DISCUSSION 4.3.1**), after one generation of random mating, the genotype frequencies at a single gene locus will become fixed at a particular equilibrium value. It also specifies that those equilibrium frequencies can be represented as a simple function of the allele frequencies at that locus (**FIG 2.9**).

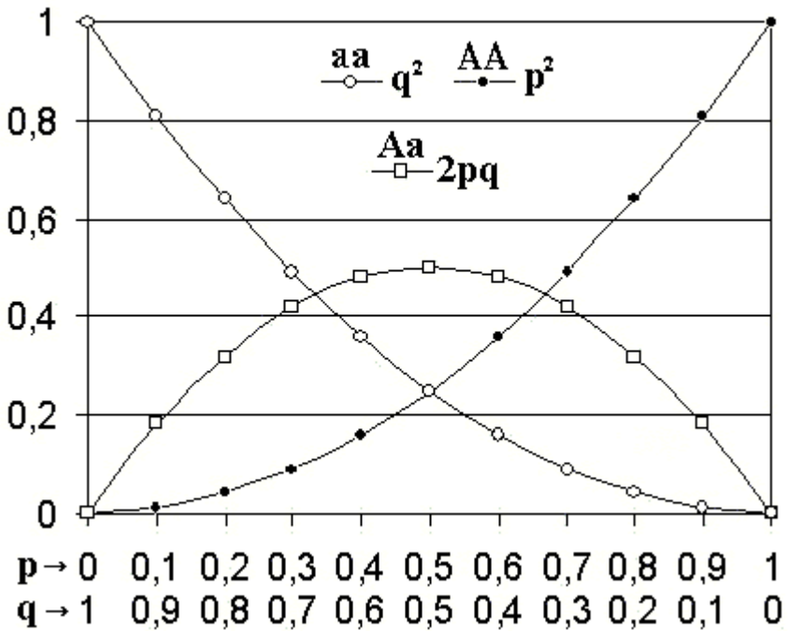


FIG. 2.9 ***Hardy–Weinberg principle for two alleles.*** *The horizontal axis shows the two allele frequencies p and q, the vertical axis shows the genotype frequencies, and the three possible genotypes are represented by the different glyphs.*

Deviations of HWE might indicate genotyping errors and were therefore investigated using the χ^2-statistic, with expected frequencies derived from allele frequencies.

2.2.12.3 ASSOCIATION BETWEEN SINGLE SNPS AND QUALITATIVE OUTCOMES

Associations between SNPs and qualitative outcomes were tested using χ^2-tests (SASIENI *et al.* 1997). χ^2-tests are hypothesis tests in which the test statistic shows a χ^2 distribution when the null hypothesis is true, or any in which the probability distribution of the test statistic (assuming the null hypothesis is true) can be made to approximate a χ^2 distribution as closely as desired by making the sample size large enough.

2.2.12.4 Association between single SNPs and quantitative outcomes

To detect significant associations between single SNPs and total serum IgE level as a continuous variable, *t*-tests were performed, in which the test statistic has a Student's *t* distribution, assuming the null hypothesis is true. Here, as the values were not normally distributed, the test was preceded by transforming the data to natural log, with subsequent confirmation of normality.

2.2.12.5 Association between haplotypes and qualitative outcomes

Haplotype frequencies were estimated using the EM (expectation-maximisation) algorithm (Excoffier & Slatkin 1995). A haplotype is a set of alleles on a single chromatid. To a certain probability, the identification of a few alleles of a haplotype block can unambiguously identify all other polymorphic sites in its region. To evaluate associations between traits and haplotypes, haplotype trend regressions (HTR) were performed, where the estimated probabilities of the haplotypes are modeled in a logistic regression as independent variables (Zaykin *et al.* 2002).

2.2.12.6 Odds ratios (ORs)

For each locus ORs and 95% confidence intervals are reported for the *dominant* model, in which effects of the pooled heterozygote and homozygote variants are compared to the wild type configuration. The OR is a measure of effect size. It is defined as the ratio of the odds of being exposed (in this study to the minor allele) in the group with the outcome (e.g. asthma) to the odds of being exposed in the group without the outcome, or to sample-based estimates of that ratio. If the probabilities of the event in each of the groups are p and q, then the OR is:

$$\frac{p/(1-p)}{q/(1-q)} = \frac{p/(1-q)}{q/(1-p)}$$

An OR of 1 indicates that the condition or event under study is equally likely in both groups. An odds ratio greater than 1 indicates that the condition or event is more likely in the first group. And an odds ratio less than 1 indicates that the condition or event is less likely in the first group.

The 95% confidence interval of the OR is the interval in which the actual OR will be situated in 95% of the cases of repeated random sampling. In modern applied practice, most confidence intervals are stated at the 95% level.

2.2.12.7 GENE-GENE INTERACTION

According to heterodimer structures, pair wise interactions of dominant SNP effects in *TLR1*, *TLR6* and *TLR10* with *TLR2* were analysed in logistic regression models. Epistasis and genetic interaction refer to the same phenomenon; however, epistasis is widely used in population genetics and refers especially to the statistical properties of the phenomenon. A correlation of zero is expected under a multiplicative penetrance model, negative correlations provide evidence for a genetic heterogeneity model, and positive correlations suggest the presence of positive epistasis in the sense of departure from a multiplicative model. Tests for pair wise interaction of SNPs were performed using the interaction term of the dominant effects of two SNPs in different genes in a logistic regression model, in which the interaction term is the product of both variables (CORDELL *et al.* 2002, COX *et al.* 1999).

2.2.12.8 FUNCTIONAL STUDIES

Differences in mRNA expression between groups were analysed by a pair wise fixed reallocation randomisation test as implemented in the REST 2005 software (PFAFFL *et al.* 2002). The exact version of the Wilcoxon-Mann-Whitney test was used for the *ex vivo* data gathered from TLR expression and cytokine analyses due to small numbers in the respective groups. Graph pad prism 4.0 was used for the figures of TLR expressions and TLR ligand stimulations.

2.2.13 BIOINFORMATICS

2.2.13.1 FASTSNP TO DETERMINE PUTATIVE FUNCTION

FASTSNP (Function Analysis and Selection Tool for Single Nucleotide Polymorphisms) was used to efficiently identify SNPs that were most likely to have functional effects.

It prioritizes SNPs according to twelve phenotypic risks and putative functional effects, such as changes to the transcriptional level, pre-mRNA splicing, premature translation termination, protein structure and others. A unique feature of FASTSNP is that the prediction of functional effects is always based on the most up-to-date database information, which FASTSNP extracts from eleven external Web servers (TABLE 2.4) at query time, using a team of re-configurable Web wrapper agents. These Web wrapper agents automate Web browsing and data extraction and can be easily configured and maintained with a tool that uses a machine learning algorithm.

TABLE 2.4 *External Web servers accessed by FASTSNP.*

Name	URL	Usage
NCBI dbSNP	http://www.ncbi.nlm.nih.gov/SNP	Provides the location of a SNP in a gene and its alleles, allele frequency, and context sequence.
Ensembl	http://www.ensembl.org/	Provides a cross-reference/alternative data source for dbSNP.
TFSearch	http://www.cbrc.jp/research/db/TFSEARCH.html	Predicts if a non-coding SNP alters the transcription factor binding site of a gene.
PolyPhen	http://www.bork.embl-heidelberg.de/PolyPhen	Predicts if a non-synonymous SNP alters an amino acid in a protein resulting in structural changes (damaged or benign) in a protein.
ESEfinder	http://rulai.cshl.edu/ESE	Predicts if a synonymous SNP is located in a exonic splicing enhancer motif, which would diminish the motif with a different allele.
Rescue-ESE	http://genes.mit.edu/burgelab/rescue-ese	Provides a cross-reference/alternative data source for ESEfinder.
NCBI GeneBank	http://www.ncbi.nlm.nih.gov/Genbank	Provides all spliced form mRNAs and their translated proteins of the gene sequence.
SwissProt	http://us.expasy.org/sprot	Provides the information about protein domains to determine if a SNP causes an alternative splicing that leads to a protein domain being abolished.
UCSC Golden Path	http://genome.ucsc.edu/	Provides information about the final draft assembly of the genome sequence (i.e., Golden Path) for quality control of candidate SNPs.
NCBI Blast	http://www.ncbi.nlm.nih.gov/BLAST	Sequence comparison and search tool for quality control of candidate SNPs
HapMap	http://www.hapmap.org/	Provides information about the haplotype and linkage disequilibrium around a SNP.
FAS-ESS	http://genes.mit.edu/fas-ess/	Predicts whether a coding SNP will abolish exonic splicing silencer motifs

2.2.13.2 HAPLOVIEW TO DETERMINE TAGGING SNPS

Haploview was used to perform LD and haplotype block analysis and especially to determine tagging SNPs by use of the implemented *Tagger* tag SNP selection algorithm (DE BAKKER *et al.* 2005).

First, TLR1-10 genotype data from Innate Immunity PGA was converted into a compatible Excel format. Then the raw format was imported into Haploview together with marker position data. Pairwise tagging was performed with an r^2 threshold of 0.8.

3. RESULTS

First, for being able to effectively investigate the role of genetic variations in TLR genes, common *TLR* variations were evaluated for their capacity to alter gene regulation or function of the protein *in silico* while avoiding redundancy by considering the LD between respective SNPs.

3.1 TLR1-10 SNP SELECTION

The Innate Immunity PGA, NHLBI Program for Genomic Applications, describes a total of 682 variations in *TLR1-10*, found by sequencing a set of Coriell samples (24 African American samples and 23 CEPH samples (Utah residents with ancestry from Northern and Western Europe)). A comprehensive algorithm was applied to select the most informative variations. Only SNPs found in the European American samples were considered (N = 259). FIG. 3.1 summarizes the selection process.

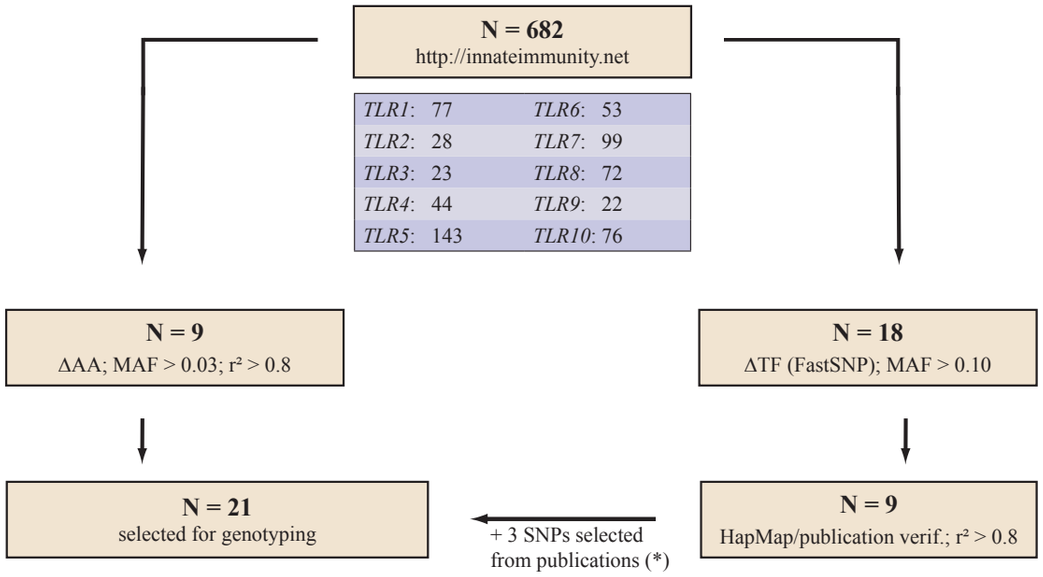


FIG. 3.1 ***Scheme of the selection algorithm used to select 21 putative functional SNPs in TLR1-10 for genotyping.*** ΔAA, *leads to amino acid change;* ΔTF, *affects transcription factor binding site;* r^2, *correlation coefficient;* (*), *previously reported to be directly or indirectly associated with the development of asthma.*

In a first step all polymorphisms with a MAF > 0.03 leading to amino acid changes were determined. The respective mRNA contigs were imported into VectorNTI 9.0. The direct strands of the wild type and mutant sequences were translated and comparison revealed changes of amino acids (TABLE 3.1).

TABLE 3.1 ***Non-synonymous TLR SNPs.*** **functional changes of the receptor variation have been described previously; [a]associated with asthma in a previous publication; selected SNPs are marked in blue.*

Gene	mRNA contig	Amino acid changes (MAF > 0.03, European American)
TLR1	NM_003263.3	N248S (rs4833095), S602I (rs5743618)
TLR2	NM_003264.3	n.a.
TLR3	NM_003265.2	L412F (rs3775291)
TLR4	NM_138554.2	D259G (rs4986790), T359I (rs4986791)
TLR5	NM_003268.3	R392X* (rs5744168), S592N (rs2072493), L616F* (rs5744174)
TLR6	NM_006068.2	P249S[a] (rs5743810)
TLR7	NM_016562.3	L11Q (rs179008)
TLR8	NM_016610.2	n.a.
TLR9	NM_017442.2	n.a.
TLR10	NM_030956.2	H241N (rs11096957), L369I (rs11096955), I473T (rs11466657), V775I[a] (rs4129009)

In total, 9 non-synonymous SNPs were selected. Other polymorphisms tagged by the selected SNPs were not considered for genotyping (data not shown).

In a next step all SNPs with a MAF > 0.1 (n = 180) were tested for their capacity to change putative transcription factor binding sites, including intronic enhancers using FastSNP. Six SNPs in *TLR1*, one in *TLR2*, five in *TLR4*, two in *TLR5*, one in *TLR6* and *TLR8* as well as two SNPs in *TLR9* matched these criteria (TABLE 3.2 A-F).

TABLE 3.2 A-F ***Putative transcription factor binding evaluated with FastSNP.*** *+ / -, indicates the occurrence / loss of a respective binding matrix in rare allele configuration; rel. ATG, position relative to ATG.*

A *TLR1*

rel. ATG	Location	rs number	FastSNP
-5748	intron	rs5743560	-CDX1
-5565	intron	rs5743563	-CDX1
-2609	intron	rs5743592	+Statx
-2299	intron	rs5743594	+CDX1
-2192	intron	rs5743595	+SRY
-833	intron	rs5743604	-CREB

B *TLR2*

rel. ATG	Location	rs number	FastSNP
-15607	promoter	rs1898830	+SP1

C *TLR4*

rel. ATG	Location	rs number	FastSNP
-6721	promoter	rs10759931	+GATA-2
-6143	promoter	rs1927914	+C/EBP, +Oct1
-5724	promoter	rs10759932	-Nkx2
686	intron	rs11536878	+HNF-3b, +XFD-1, +CdxA, +Nkx2
3331	intron	rs2149356	+CdxA

D *TLR5*

rel. ATG	Location	rs number	FastSNP
-25599	intron	rs2096142	+C/EPB, +SRY
-25204	intron	rs2192617	-USF

F *TLR8*

rel. ATG	Location	rs number	FastSNP
-4824	promoter	rs3761624	-SRY, -GATA2, -GATA1

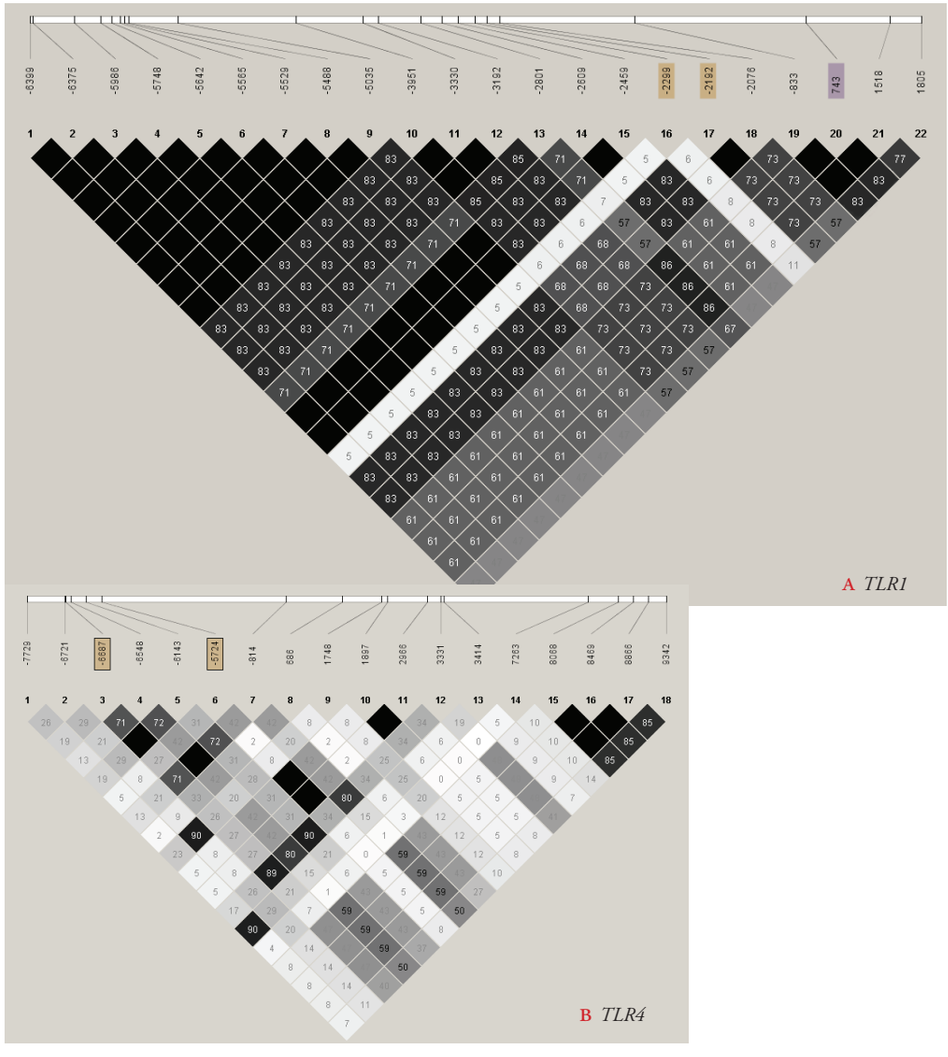
E *TLR6*

rel. ATG	Location	rs number	FastSNP
-1401	promoter	rs5743795	+SRY

G *TLR9*

rel. ATG	Location	rs number	FastSNP
-2871	promoter	rs187084	+SP1
-2622	promoter	rs5743836	+NFkB, +c-Rel

Again, in the presence of tagging blocks ($r^2 > 0.8$) only one SNP representing the block was chosen to avoid redundancy. Of those only the ones that were verified by HapMap and/or by previous publication were selected (n = 9) (FIG. 3.2 A-F).



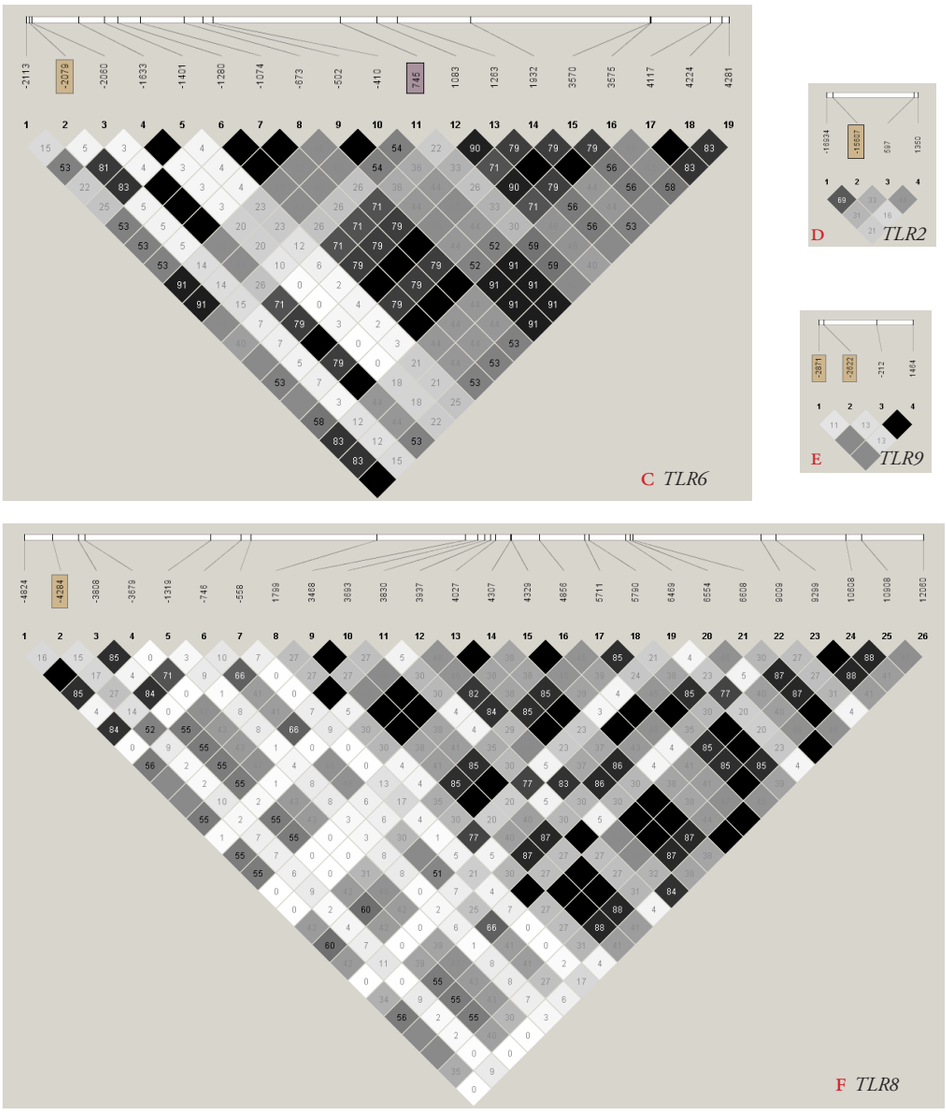


FIGURE 3.2 A-F ***LD correlation coefficients (r^2) for TLR SNPs with a MAF > 0.1.*** *Pure white fields represent $r^2 = 0$, shades of grey $0 < r^2 < 1$ and black fields indicate total LD ($r^2 = 1$); SNPs selected due to possible occurrence of transcription factor binding changes (determined by FastSNP) are marked in ochre; SNPs selected independent of FastSNP results (amino acid changes) in the respective gene are marked in purple.*

LD maps are shown only for TLRs in which SNPs altered transcription factor binding and when those SNPs or tagged SNPs could be verified by HapMap.

Finally, three SNPs that were previously described as being directly or indirectly associated with asthma (rs4696480, rs3804099, rs11096956) were added, resulting in a total of 21 SNPs. TABLE 3.3 shows all selected SNPs and their characteristics.

TABLE 3.3 ***Characteristics of the analyzed TLR1-10 SNPs.*** *ΔAA, non synonymous SNP; ΔTF, SNP or tagged SNP changes matrix of putative transcription factor binding site(s); IE, affects intronic enhancer; rel. ATG, position relative to ATG; [1]based on March 2006 human reference sequence (NCBI build 36.1).*

Gene	SNP	rs number	rel. ATG[1]	Alleles	Location	ΔAA	ΔTF	IE	MAF
TLR1	a	rs5743594	-2299	C/T	intron			✓	0.19
	b	rs5743595	-2192	T/C	intron			✓	0.17
	c	rs4833095	743	A/G	CDS	✓			0.23
TLR2	a	rs4696480	-16934	T/A	promoter				0.50
	b	rs1898830	-15607	A/G	promoter		✓		0.35
	c	rs3804099	597	T/C	CDS				0.43
TLR3	a	rs3775291	6301	C/T	CDS	✓			0.29
TLR4	a	rs6478317	-6687	A/G	promoter		✓		0.32
	b	rs10759932	-5724	T/C	promoter		✓		0.13
	c	rs4986791	4735	C/T	CDS	✓			0.06
TLR5	a	rs5744168	1174	C/T	CDS	✓			0.05
	b	rs2072493	1775	A/G	CDS	✓			0.14
	c	rs5744174	1846	T/C	CDS	✓			0.43
TLR6	a	rs5743789	-2079	T/A	promoter		✓		0.18
	b	rs5743810	745	C/T	CDS	✓			0.40
TLR7	a	rs179008	17962	A/T	CDS	✓			0.23
TLR8	a	rs3761624	-4824	A/G	promoter		✓		0.25
TLR9	a	rs187084	-2871	T/C	promoter		✓		0.42
	b	rs5743836	-2622	T/C	promoter		✓		0.15
TLR10	a	rs11096956	1032	G/T	CDS				0.19
	b	rs4129009	2323	A/G	CDS	✓			0.16

3.2 GENOTYPING

3.2.1 QUALITY CONTROL

All genotyping assays were performed with sufficient call rates, i.e. more than approx. 90% of all possible genotypes were successfully called, either automatically by the MassARRAY RT software or manually after eye inspection of the respective mass spectrums.

The acquired genotype frequencies were compared to predicted frequencies under the HWE assumption as an accepted test for genotyping error. Some SNPs indeed deviated significantly from

the expected allelic frequencies in the control population as shown in TABLE 3.4. Mostly, a lack of heterozygotes and – consequentially – an excess of homozygotes was observed. To determine if those

TABLE 3.4 ***TLR SNPs that showed significant deviation from HWE in either the case-control or the cross-sectional population.*** *obs, total observed genotype numbers; exp, expected genotype numbers (under HWE assumption); sc, super-controls.*

		case-control sc			cross-sectional		
		obs	exp	deviation	obs	exp	deviation
*TLR1*_b	CC	852	844	$P = 0.1698$	2113	2095	$P = 0.025$
	CT	335	350		800	835	
	TT	44	36		101	83	
*TLR1*_c	AA	727	723	$P = 0.6753$	1817	1795	$P = 0.0235$
	AG	419	427		1012	1056	
	GG	67	63		177	155	
*TLR5*_b	AA	878	871	$P = 0.0465$	n.a.		
	AG	239	254				
	GG	26	19				
*TLR6*_a	TT	795	789	$P = 0.2315$	2072	2052	$P = 0.0097$
	TA	334	346		826	867	
	AA	44	38		112	92	
*TLR8*_a	AA	375	361	$P = 0.0020$	n.a.		
	AG	207	236				
	GG	53	39				

deviations were due to a methodological problem (heterozygotes are generally more difficult to detect with MALDI-TOF MS as the mass peaks are diminished by a factor of 2) or due to a true deviation on the population level (random or as a biological effect), the MALDI-TOF MS genotyping was checked by sequencing. This was first performed for a part of the samples homozygote for the minor alleles as they made the biggest contribution to the χ^2 leading to significant deviation. Second, missing samples were sequenced as they could have included an excessive amount of uncalled heterozygotes. Some SNPs were later re-genotyped in the cross-sectional population, in which samples partly overlapped with the case-control ones. *TLR1*_c and *TLR6*_a deviated significantly from HWE in the cross-sectional population, and were therefore also included in the quality analysis by sequencing. On the other hand, *TLR2*_b and *TLR10*_b did no longer show significant HWE deviation in the cross-sectional population ($P = 0.191$ and $P = 0.119$, respectively) and as the genotypes of overlapping samples matched it was assumed that the deviations observed in the super-controls resulted from an accumulation of certain alleles due to the case-control design.

Overall, the homozygote genotypes were verified by sequencing (FIG. 3.3) and integration of the missing genotypes confirmed the originally found HWE deviations (TABLE 3.5), supporting a true population based deviation.

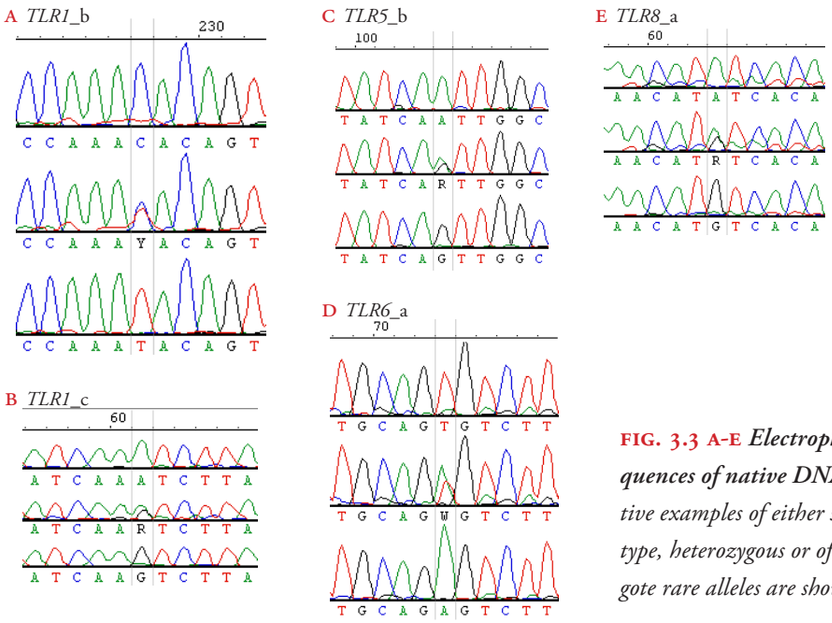


FIG. 3.3 A-E ***Electropherograms showing sequences of native DNA samples.*** *Representative examples of either samples homozygote wild type, heterozygous or of carriers with homozygote rare alleles are shown for each SNP.*

TABLE 3.5 ***Calculation of HWE deviation and call rates.*** *Observed/expected genotype numbers and frequencies are given. For TLR1_b, TLR1_c, TLR5_b, TLR6_a and TLR8_a the corrected values are shown (re-sequencing).*

SNP	observed			expected			missings	chi square	*P* value	call rate (%)
*TLR1*_a	CC	CT	TT	CC	CT	TT				
MDL	1206	541	82				43			97.70
	0.659	0.296	0.045							
MDL_sc	795	374	59	785	393	49				
	0.647	0.305	0.048	0.640	0.320	0.040		2.999	0.083	
*TLR1*_b	TT	TC	CC	TT	TC	CC				
MDL	1301	496	71				4			99.79
	0.697	0.266	0.038							
MDL_sc	854	344	49	844	364	39				
	0.685	0.276	0.039	0.677	0.292	0.031		3.647	0.056	
*TLR1*_c	AA	AG	GG	AA	AG	GG				
MDL	1123	620	110				19			98.99
	0.606	0.335	0.059							
MDL_sc	739	437	72	735	446	68				
	0.592	0.350	0.058	0.589	0.357	0.054		0.482	0.488	
*TLR2*_a	TT	TA	AA	TT	TA	AA				
MDL	466	905	448				53			97.17
	0.256	0.498	0.246							
MDL_sc	321	598	293	317	606	289				
	0.265	0.493	0.242	0.262	0.500	0.239		0.195	0.659	
*TLR2*_b	AA	AG	GG	AA	AG	GG				
MDL	790	777	243				62			96.69
	0.437	0.429	0.134							
MDL_sc	524	517	165	508	550	149				
	0.435	0.429	0.137	0.421	0.456	0.123		4.235	0.040	

TABLE 3.5 (CONTINUED)

*TLR2*_c	TT	TC	CC	TT	TC	CC				
MDL	613	877	341				41			97.81
	0.335	0.479	0.186							
MDL_sc	396	586	237	389	599	230				
	0.325	0.481	0.194	0.319	0.491	0.189		0.585	0.444	
*TLR3*_a	CC	CT	TT	CC	CT	TT				
MDL	882	641	163				186			90.06
	0.523	0.380	0.097							
MDL_sc	566	447	105	558	464	97				
	0.506	0.400	0.094	0.499	0.415	0.086		1.493	0.222	
*TLR4*_a	AA	AG	GG	AA	AG	GG				
MDL	822	736	188				126			93.27
	0.471	0.422	0.108							
MDL_sc	561	474	128	548	501	115				
	0.482	0.408	0.110	0.471	0.431	0.098		3.353	0.067	
*TLR4*_b	TT	TC	CC	TT	TC	CC				
MDL	1285	391	34				162			91.35
	0.752	0.229	0.020							
MDL_sc	861	261	24	858	267	21				
	0.751	0.228	0.021	0.749	0.233	0.018		0.645	0.422	
*TLR4*_c	CC	CT	TT	CC	CT	TT				
MDL	1616	201	5				50			97.33
	0.887	0.110	0.003							
MDL_sc	1075	140	2	1077	136	4				
	0.883	0.115	0.002	0.885	0.111	0.004		1.353	0.245	
*TLR5*_a	CC	CT	TT	CC	CT	TT				
MDL	1609	184	6				73			96.10
	0.894	0.102	0.003							
MDL_sc	1080	114	5	1078	118	3				
	0.901	0.095	0.004	0.899	0.098	0.003		1.117	0.291	
*TLR5*_b	AA	AG	GG	AA	AG	GG				
MDL	1355	429	44				44			97.65
	0.741	0.235	0.024							
MDL_sc	915	280	35	905	300	25				
	0.744	0.228	0.029	0.736	0.244	0.020		5.569	0.018	
*TLR5*_c	TT	TC	CC	TT	TC	CC				
MDL	588	874	344				66			96.47
	0.326	0.484	0.191							
MDL_sc	381	593	229	382	592	230				
	0.317	0.493	0.190	0.317	0.492	0.191		0.004	0.948	
*TLR6*_a	TT	TA	AA	TT	TA	AA				
MDL	1261	530	79				2			99.89
	0.674	0.283	0.042							
MDL_sc	826	366	55	816	385	45				
	0.662	0.294	0.044	0.655	0.309	0.036		3.082	0.079	
*TLR6*_b	CC	CT	TT	CC	CT	TT				
MDL	649	839	283				101			94.60
	0.367	0.474	0.160							
MDL_sc	446	562	172	448	558	174				
	0.378	0.476	0.146	0.380	0.473	0.147		0.055	0.815	

TABLE 3.5 (CONTINUED)

*TLR7*_a	AA	AT	TT	AA	AT	TT				
MDL	1204	313	240				115			93.86
	0.685	0.178	0.137							
MDL_sc	381	231	30	394	225	33				
(girls only)	0.593	0.360	0.047	0.604	0.345	0.051		0.862	0.503	
*TLR8*_a	AA	AG	GG	AA	AG	GG				
MDL	1192	300	296				84			95.51
	0.667	0.168	0.166							
MDL_sc	397	219	54	383	247	40				
(girls only)	0.593	0.327	0.081	0.572	0.369	0.060		8.586	0.003	
*TLR9*_a	TT	TC	CC	TT	TC	CC				
MDL	615	878	326				53			97.17
	0.338	0.483	0.179							
MDL_sc	423	570	216	415	587	208				
	0.350	0.472	0.179	0.343	0.485	0.172		0.989	0.320	
*TLR9*_b	TT	TC	CC	TT	TC	CC				
MDL	1208	418	31				215			88.51
	0.729	0.252	0.019							
MDL_sc	807	267	26	804	273	23				
	0.734	0.243	0.024	0.731	0.248	0.021		0.488	0.485	
*TLR10*_a	GG	GT	TT	GG	GT	TT				
MDL	1167	515	77				113			93.96
	0.663	0.293	0.044							
MDL_sc	767	347	55	757	368	45				
	0.656	0.297	0.047	0.647	0.315	0.038		3.694	0.055	
*TLR10*_b	AA	AG	GG	AA	AG	GG				
MDL	1319	478	58				17			99.09
	0.711	0.258	0.031							
MDL_sc	860	334	42	853	347	35				
	0.696	0.270	0.034	0.690	0.281	0.029		1.817	0.178	

3.2.2 LINKAGE DISEQUILIBRIUM (LD)

Pairwise LD between the *TLR1-10* SNPs was assessed between single SNPs using the r^2-statistic and visualized as an interchromosomal GOLD plot (FIG. 3.4).

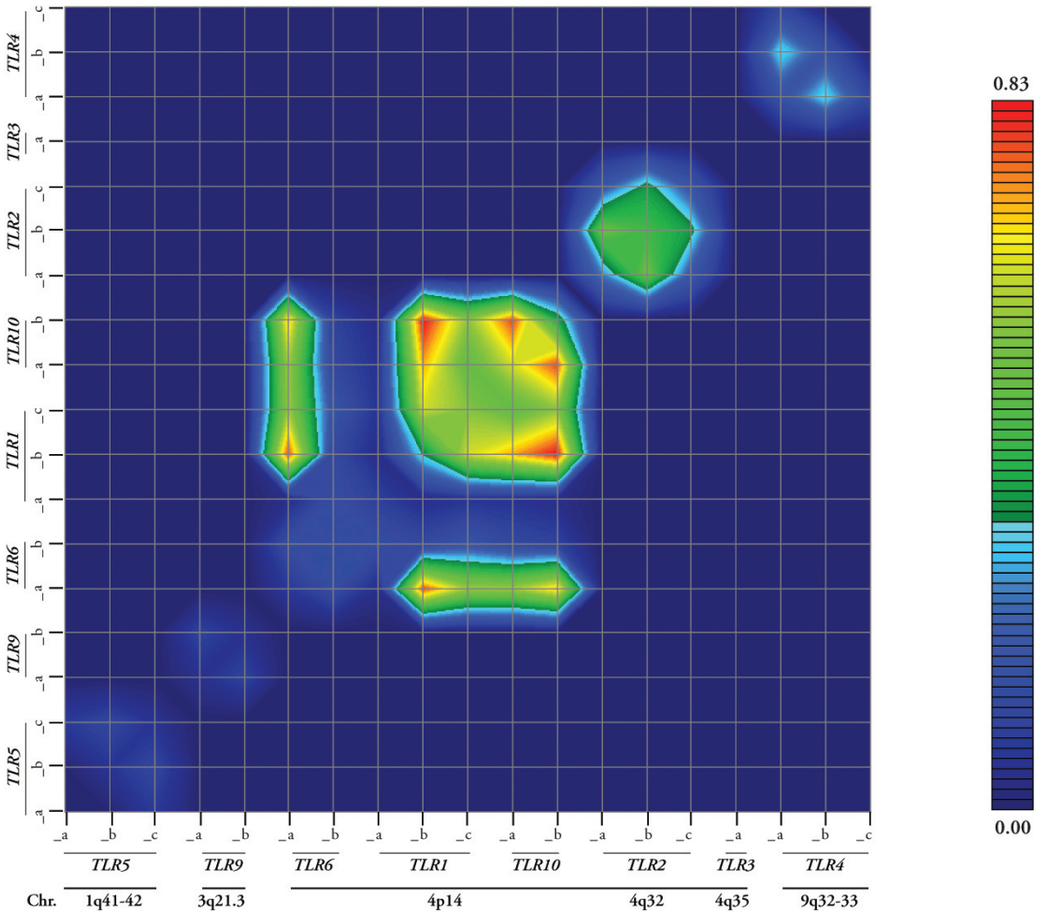


FIG. 3.4 ***GOLD interchromosomal linkage disequilibrium map of the TLR SNPs applying the correlation coefficient r².*** *The genetic regions run from left to right on the x axis and from bottom to top on the y axis, respectively. Pairwise r² values for LD are colour-coded and plotted at the SNP locations after completion by interpolation using the GOLD program. Bright red and dark blue are opposite ends of the scale, with bright red indicating the most significant LD. TLR7 and TLR8 were not considered in the analysis due to their x-chromosomal location.*

Strong LD was observed not only between certain SNPs within the same genes, but also for SNPs clustered in close proximity on the same chromosome, specifically for SNPs in *TLR1*, *TLR6* and *TLR10* mapping to chromosome 4p14.

3.3 ASSOCIATION STUDIES

3.3.1 ASSOCIATION OF SINGLE SNPS WITH ASTHMA PHENOTYPES

For each locus odds ratios and 95% confidence intervals are reported for the dominant model, in which the wild-type is compared to pooled heterozygote and homozygote variants. Only modest effects of *TLR* SNPs were found for unstratified asthma. Carriers of the *TLR4*_a and *TLR9*_a minor alleles showed an increased risk to develop asthma in the dominant model. In a next step, associations with atopic asthma and non-atopic asthma were determined separately. When stratified for atopy SNPs *TLR1*_b, *TLR1*_c, *TLR6*_a and *TLR10*_b were significantly associated with a protective effect on atopic asthma, reducing the risk by almost half, and *TLR6*_b showed an increased risk to develop atopic asthma (FIG. 3.5). *TLR3, TLR5, TLR7* and *TLR8* polymorphisms showed no significant effect on the analyzed asthma phenotypes in the case-control-population (TABLE 3.6).

TABLE. 3.6 ***Association of TLR SNPs with asthma phenotypes in the case-control population.*** *Odds ratios (and 95% confidence intervals) for dominant effects. [f]effect in females, [m]effect in males (codominant model), [1]number of cases; actual genotyped numbers depend on call rates (88-99%).*

Gene	SNP	Asthma (n = 369[1])		Atopic Asthma (n = 171[1])		Non-Atopic Asthma (n = 171[1])	
TLR1	a	0.84 (0.65-1.08)	0.184	0.99 (0.70-1.39)	0.935	0.78 (0.55-1.11)	0.174
	b	0.81 (0.63-1.05)	0.119	**0.54** (0.37-0.81)	0.002	1.07 (0.76-1.50)	0.708
	c	0.82 (0.64-1.05)	0.110	**0.59** (0.41-0.83)	0.003	1.03 (0.74-1.43)	0.848
TLR2	a	1.10 (0.84-1.44)	0.504	1.31 (0.89-1.94)	0.173	0.84 (0.59-1.19)	0.322
	b	1.04 (0.82-1.32)	0.750	0.87 (0.63-1.20)	0.389	1.27 (0.91-1.77)	0.161
	c	0.83 (0.65-1.06)	0.133	0.98 (0.70-1.38)	0.906	**0.69** (0.50-0.96)	0.027
TLR3	a	0.85 (0.67-1.09)	0.201	0.87 (0.62-1.21)	0.410	0.80 (0.57-1.12)	0.199
TLR4	a	**1.32** (1.04-1.68)	0.025	1.25 (0.89-1.74)	0.195	**1.41** (1.01-1.96)	0.045
	b	1.07 (0.81-1.42)	0.621	1.12 (0.76-1.64)	0.574	1.15 (0.79-1.67)	0.467
	c	0.85 (0.58-1.25)	0.397	0.65 (0.36-1.18)	0.154	0.97 (0.58-1.62)	0.913
TLR5	a	1.11 (0.75-1.62)	0.605	0.89 (0.51-1.56)	0.684	1.18 (0.71-1.98)	0.525
	b	1.08 (0.82-1.41)	0.588	0.99 (0.68-1.44)	0.966	1.07 (0.74-1.54)	0.737
	c	0.79 (0.62-1.01)	0.061	0.85 (0.60-1.19)	0.342	0.75 (0.53-1.04)	0.085
TLR6	a	0.78 (0.61-1.01)	0.060	**0.54** (0.37-0.79)	0.002	1.02 (0.72-1.42)	0.927
	b	1.20 (0.93-1.53)	0.154	**1.79** (1.24-2.58)	0.002	0.83 (0.60-1.16)	0.277
TLR7	a	[f]0.82 (0.55-1.20)	0.299	1.01 (0.56-1.82)	0.982	0.74 (0.45-1.22)	0.244
		[m]0.99 (0.68-1.46)	0.976	0.92 (0.56-1.51)	0.751	1.40 (0.84-2.35)	0.200
TLR8	a	[f]0.94 (0.64-1.37)	0.748	0.92 (0.51-1.67)	0.786	0.96 (0.59-1.55)	0.865
		[m]1.16 (0.81-1.66)	0.421	0.99 (0.62-1.58)	0.962	1.33 (0.80-2.22)	0.269
TLR9	a	**1.33** (1.03-1.71)	0.030	1.35 (0.94-1.92)	0.101	1.18 (0.83-1.66)	0.353
	b	1.08 (0.82-1.42)	0.600	1.04 (0.71-1.52)	0.831	1.16 (0.80-1.68)	0.440
TLR10	a	0.90 (0.70-1.16)	0.408	0.75 (0.53-1.08)	0.121	1.07 (0.76-1.52)	0.682
	b	0.79 (0.61-1.03)	0.080	**0.58** (0.39-0.86)	0.006	1.01 (0.71-1.43)	0.965

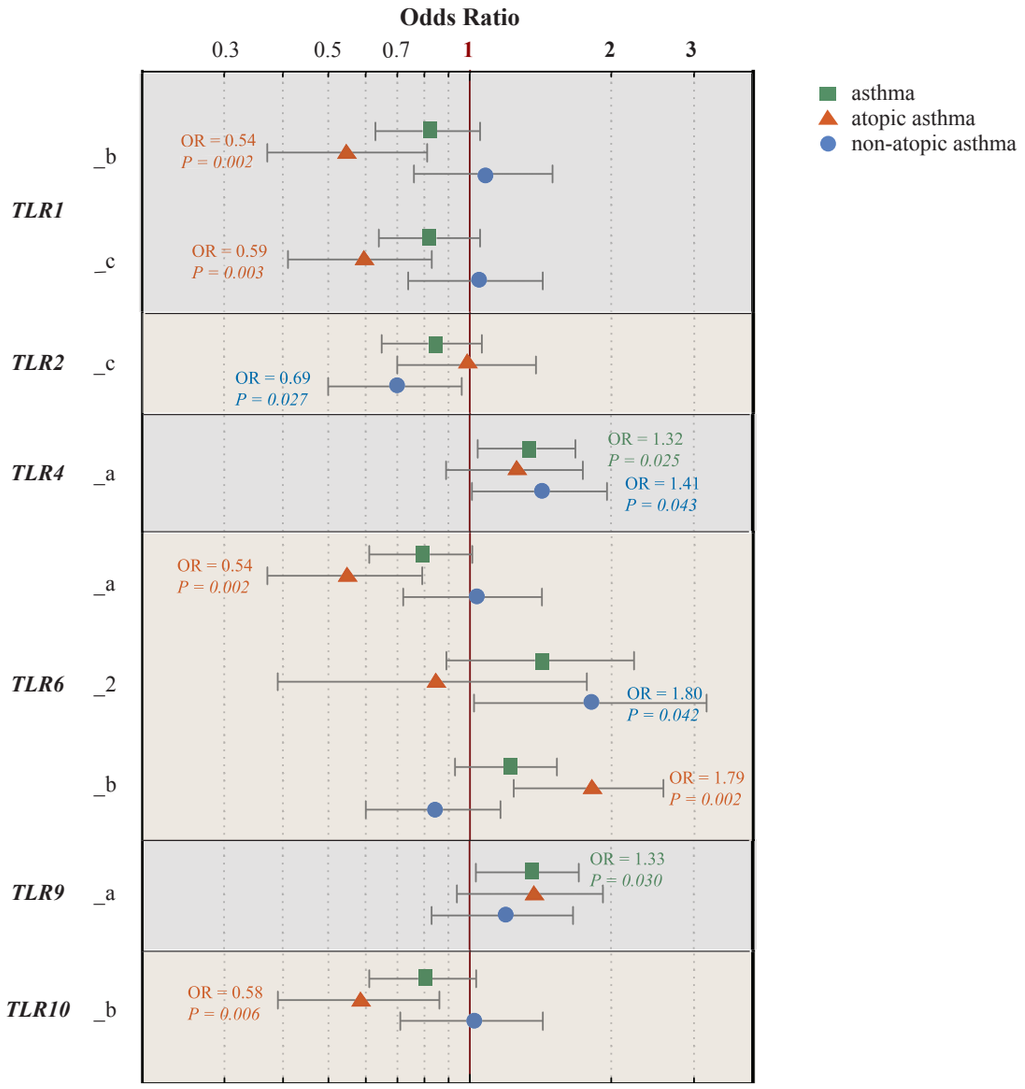


FIG. 3.5 ***Association of TLR single SNPs with asthma phenotypes.*** *Odds ratios (and 95% confidence intervals) for a dominant model in a case-control population of German children (N = 1,872). Here, only SNPs significantly associated (P < 0.05) with at least one asthma phenotype are shown while the complete analysis is presented in* **TABLE 3.6**.

To assess reproducibility of the observed results, an analysis of homogeneity was performed confirming consistency of the effects on atopic asthma of the associated *TLR1*, *TLR6* and *TLR10* SNPs in all three study centres separately (Munich, Dresden and Leipzig) (TABLE 3.7).

TABLE 3.7 ***Association between TLR1, TLR6 and TLR10 SNPs and atopic asthma in homogeneity analysis.*** *Odds ratios (and 95% confidence intervals) for dominant effects in Munich (M), Dresden (D), Leipzig (L) and in the pooled case-control population. [1]number of cases (vs. 1,248 controls); actual genotyped numbers depend on call rates (94-98%).*

SNP	Pop	Asthma (n = 369[1])		Atopic Asthma (n = 171[1])		Non-Atopic Asthma (n = 171[1])	
		Odds Ratio (CI)	p-value	Odds Ratio (CI)	p-value	Odds Ratio (CI)	p-value
*TLR1*_b	M	0.91 (0.60-1.36)	0.6325	0.56 (0.30-1.06)	0.0703	1.18 (0.69-2.02)	0.5377
	D	0.94 (0.62-1.42)	0.7714	0.64 (0.34-1.20)	0.1622	1.23 (0.72-2.12)	0.4426
	L	**0.50** (0.28-0.90)	0.0199	**0.38** (0.16-0.89)	0.0218	0.64 (0.29-1.44)	0.2786
*TLR1*_c	M	0.83 (0.56-1.22)	0.3467	0.60 (0.34-1.05)	0.0707	1.05 (0.62-1.76)	0.8611
	D	0.96 (0.66-1.41)	0.8508	0.64 (0.36-1.13)	0.1234	1.19 (0.71-1.98)	0.5063
	L	0.57 (0.33-0.99)	0.0453	**0.47** (0.22-1.01)	0.0489	0.73 (0.33-1.57)	0.4152
*TLR2*_c	M	0.87 (0.58-1.31)	0.5156	1.04 (0.59-1.86)	0.8846	0.72 (0.42-1.23)	0.2289
	D	0.73 (0.51-1.06)	0.1003	0.91 (0.54-1.54)	0.7285	**0.56** (0.34-0.92)	0.0218
	L	0.98 (0.57-1.67)	0.9303	1.01 (0.50-2.07)	0.9701	1.02 (0.47-2.19)	0.9584
*TLR4*_a	M	1.39 (0.93-2.09)	0.1099	1.02 (0.58-1.80)	0.9465	1.71 (0.98-3.01)	0.0579
	D	1.33 (0.92-1.95)	0.1330	1.65 (0.96-2.86)	0.0703	1.21 (0.73-2.01)	0.4571
	L	1.21 (0.73-2.02)	0.4660	1.12 (0.57-2.18)	0.7420	1.34 (0.65-2.78)	0.4273
*TLR6*_a	M	0.92 (0.62-1.36)	0.6618	0.67 (0.38-1.20)	0.1777	1.10 (0.64-1.86)	0.7355
	D	0.90 (0.59-1.35)	0.6023	0.55 (0.29-1.06)	0.0701	1.26 (0.74-2.14)	0.4013
	L	**0.44** (0.24-0.79)	0.0053	**0.33** (0.14-0.78)	0.0086	0.56 (0.25-1.26)	0.1580
*TLR6*_b	M	1.22 (0.82-1.81)	0.3325	**1.93** (1.06-3.51)	0.0300	0.91 (0.54-1.53)	0.7119
	D	1.08 (0.74-1.58)	0.6959	1.45 (0.84-2.56)	0.1817	0.80 (0.48-1.33)	0.3919
	L	1.44 (0.82-2.51)	0.2002	**2.30** (1.02-5.20)	0.0410	0.78 (0.37-1.63)	0.5085
*TLR9*_a	M	1.39 (0.91-2.12)	0.1311	1.74 (0.93-3.26)	0.0819	1.01 (0.58-1.75)	0.9709
	D	1.35 (0.91-2.01)	0.1335	1.28 (0.74-2.23)	0.3771	1.36 (0.79-2.36)	0.2676
	L	1.19 (0.69-2.05)	0.5207	1.05 (0.52-2.11)	0.8995	1.18 (0.55-2.53)	0.6744
TLR10 _b	M	0.84 (0.55-1.28)	0.4203	**0.50** (0.26-0.97)	0.0372	1.10 (0.64-1.89)	0.7434
	D	0.91 (0.60-1.37)	0.6420	0.70 (0.38-1.30)	0.2523	1.15 (0.67-1.99)	0.6107
	L	**0.53** (0.29-0.97)	0.0373	0.51 (0.23-1.16)	0.1028	0.63 (0.27-1.45)	0.2734

Indications of an association of non-atopic asthma with *TLR2*_c were not confirmed in homogeneity analysis. The same was true for *TLR4*_a and *TLR9*_a, as the confidence intervals made it difficult to prove a consistent effect on non-atopic asthma and asthma, respectively, although the ORs showed the same directions. On the other hand, the effect of *TLR1*_b, *TLR1*_c, *TLR6*_a, *TLR6*_b and *TLR10*_b on atopic asthma remained consistent. Those effects were significant or showed a clear trend for significance ($P < 0.1$), even with respect to the smaller numbers of asthmatics in each city separately.

To further differentiate if *TLR1*_b, *TLR1*_c, *TLR6*_a, *TLR6*_b and *TLR10*_b were associated with atopic sensitisation and the subsequent development of asthma or with atopy independently of asthma, the SNPs were re-genotyped in an unselected cross-sectional population from Munich and Dresden (N = 3,099). Associations with atopic asthma were confirmed and observed effects were clearly stronger with atopic asthma than with atopy or atopy without asthma (TABLE 3.8).

TABLE 3.8 ***Association of TLR1, TLR6 and TLR10 SNPs with asthma phenotypes in the cross-sectional analysis (N = 3,099).*** *Odds ratios (and 95% confidence intervals) for dominant effects; [1]number of respective cases in the cross sectional study sample from Munich and Dresden.*

SNP	Asthma (n = 272[1])	Atopic Asthma (n = 124[1])	Atopy (n = 777[1])	Atopy without Asthma (n = 645[1])
*TLR1*_b	0.92 (0.70-1.21)	**0.60** (0.38-0.93)	**0.78** (0.65-0.94)	0.84 (0.68-1.02)
	0.561	0.021	0.009	0.075
*TLR1*_c	0.94 (0.73-1.21)	**0.61** (0.41-0.91)	**0.75** (0.63-0.89)	**0.79** (0.66-0.95)
	0.635	0.014	0.001	0.012
*TLR6*_a	0.94 (0.72-1.24)	**0.64** (0.42-0.98)	**0.81** (0.67-0.97)	0.84 (0.69-1.03)
	0.664	0.038	0.022	0.087
*TLR6*_b	1.11 (0.85-1.44)	**1.76** (1.16-2.67)	**1.22** (1.02-1.45)	1.13 (0.94-1.36)
	0.457	0.007	0.028	0.202
*TLR10*_b	0.90 (0.68-1.19)	**0.60** (0.38-0.95)	**0.75** (0.62-0.91)	**0.79** (0.64-0.96)
	0.467	0.026	0.003	0.019

3.3.2 ASSOCIATION OF HAPLOTYPES WITH ASTHMA PHENOTYPES

In TLR genes in which more than one SNP was genotyped, haplotypes were estimated. Again, in haplotype trend regressions the most consistent effects could be observed with atopic asthma.

Haplotypes *TLR1*_Hc, *TLR6*_Hc and *TLR10*_Hc decreased the risk to develop atopic asthma by approx. 60%, while *TLR1*_Ha, *TLR2*_Hb and *TLR6*_Hb increased the risk (FIG. 3.6). Non-atopic asthma showed only weak indication for being associated with haplotypes (only *TLR2*_Hb significantly decreased the risk). An effect on asthma itself was mostly depending on the associations of subphenotypes (*TLR1*_Ha, *TLR2*_Hc, *TLR6*_Hb, *TLR9*_Ha and *TLR9*_Hb).

In contrast, *TLR3*, *TLR4*, *TLR5*, *TLR7* and *TLR8* haplotypes showed no significant effects (TABLE 3.9). These results were in accordance with the single SNP analyses. By large, effects observed by single gene analyses were confirmed and extended by haplotype results.

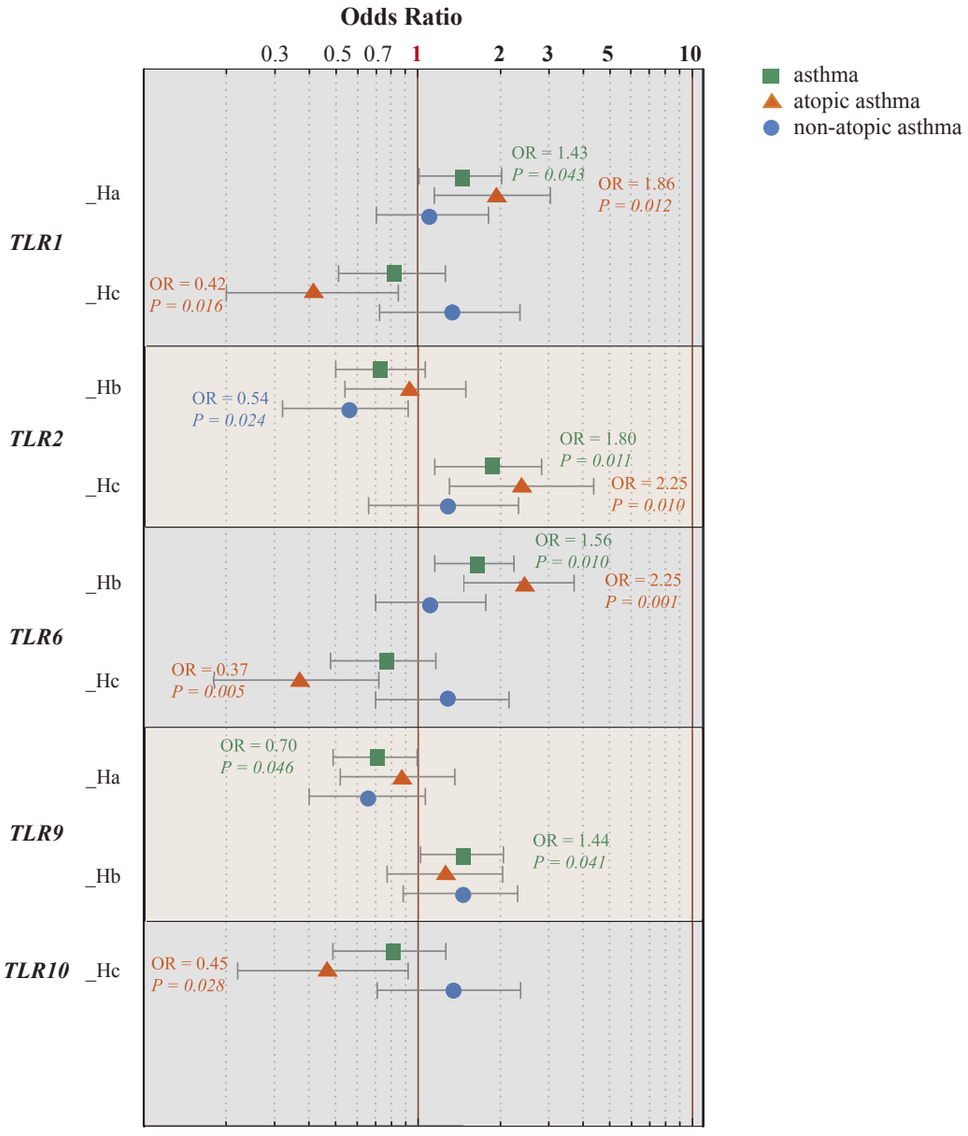


FIG. 3.6 ***Association of TLR haplotypes with asthma, atopic asthma and non-atopic asthma.*** *Odds Ratios ± 95% confidence intervals on logarithmic scale; only frequent haplotypes with significant association to at least one phenotype are shown while the complete analysis is presented in* **TABLE 3.9**.

TABLE 3.9 ***Association of TLR haplotypes with asthma phenotypes in the case-control population.*** *Odds ratios (and 95% confidence intervals) for haplotype trend regression. feffect in females, meffect in males; [1]frequencies estimated from asthmatics and controls; [2]only samples with complete phase information were considered.*

Haplotype	Alleles	Freq[1]	Asthma OR (CI)	Atopic Asthma OR (CI)	Non-Atopic Asthma OR (CI)
TLR1			n = 347/1227	n = 162/1227	n = 159/1227
Ha	C-T-A	0.58	**1.43** (1.01-2.02) 0.043	**1.86** (1.15-3.03) 0.012	1.13 (0.70-1.81) 0.626
Hb	T-T-A	0.19	0.76 (0.49-1.17) 0.207	0.99 (0.56-1.76) 0.974	0.64 (0.35-1.20) 0.164
Hc	C-C-G	0.16	0.80 (0.51-1.26) 0.333	**0.42** (0.20-0.85) 0.016	1.30 (0.72-2.35) 0.387
Hd	C-T-G	0.06	0.98 (0.48-2.00) 0.945	0.67 (0.23-1.94) 0.455	1.11 (0.42-2.93) 0.832
TLR2			n = 354/1184	n = 163/1184	n = 164/1184
Ha	T-G-T	0.35	1.03 (0.73-1.45) 0.884	0.71 (0.44-1.16) 0.176	1.54 (0.97-2.43) 0.066
Hb	A-A-C	0.31	0.72 (0.50-1.06) 0.093	0.90 (0.54-1.49) 0.672	**0.54** (0.32-0.92) 0.024
Hc	A-A-T	0.17	**1.80** (1.15-2.82) 0.010	**2.39** (1.30-4.38) 0.005	1.24 (0.66-2.32) 0.513
Hd	T-A-C	0.11	0.70 (0.38-1.28) 0.248	0.69 (0.30-1.61) 0.395	0.71 (0.31-1.64) 0.423
He	T-A-T	0.06	1.34 (0.60-3.04) 0.477	1.52 (0.51-4.53) 0.450	1.40 (0.46-4.21) 0.551
TLR4			n = 316/110	n = 139/1110	n = 153/1110
Ha	A-T-C	0.68	0.84 (0.58-1.22) 0.365	0.95 (0.56-1.62) 0.862	0.72 (0.44-1.18) 0.185
Hb	G-C-C	0.13	1.13 (0.68-1.87) 0.650	1.28 (0.64-2.58) 0.481	1.21 (0.62-2.38) 0.579
Hc	G-T-C	0.13	1.47 (0.89-2.43) 0.133	1.20 (0.58-2.47) 0.624	1.59 (0.81-3.12) 0.175
Hd	G-T-T	0.05	0.58 (0.24-1.38) 0.218	0.40 (0.11-1.52) 0.180	0.78 (0.26-2.40) 0.668
TLR5			n = 344/1160	n = 158/1160	n = 161/1160
Ha	C-A-T	0.44	1.22 (0.86-1.72) 0.261	1.45 (0.90-2.33) 0.126	1.19 (0.74-1.90) 0.477
Hb	C-A-C	0.37	0.77 (0.54-1.09) 0.133	0.74 (0.45-1.20) 0.221	0.80 (0.50-1.29) 0.362
./Hc	C-G-T	0.14	1.09 (0.67-1.77) 0.729	1.01 (0.51-1.97) 0.986	0.96 (0.49-1.90) 0.915
Hd	T-A-C	0.02	1.09 (0.52-2.31) 0.818	0.69 (0.23-2.12) 0.518	1.25 (0.46-3.37) 0.659

TLR6			n = 363/1218	n = 167/1218	n = 167/1218
Ha	T-C	0.42	0.75 (0.54-1.05) 0.094	0.75 (0.47-1.19) 0.216	0.80 (0.50-1.27) 0.340
Hb	T-T	0.40	**1.61** (1.15-2.24) 0.005	**2.34** (1.47-3.71) <0.001	1.11 (0.70-1.77) 0.656
Hc	A-C	0.18	0.75 (0.48-1.16) 0.192	**0.36** (0.18-0.72) 0.004	1.23 (0.70-2.15) 0.477
TLR9			n = 329/1086	n = 151/1086	n = 152/1086
Ha	T-T	0.44	**0.70** (0.49-0.99) 0.046	0.84 (0.52-1.36) 0.474	0.65 (0.40-1.06) 0.081
Hb	C-T	0.42	**1.44** (1.02-2.05) 0.041	1.26 (0.77-2.04) 0.356	1.43 (0.88-2.31) 0.146
Hc	T-C	0.14	0.98 (0.59-1.64) 0.943	0.90 (0.44-1.83) 0.762	1.15 (0.58-2.29) 0.683
TLR10			n = 351/1159	n = 165/1159	n = 160/1159
Ha	G-A	0.81	1.22 (0.80-1.87) 0.357	1.69 (0.91-3.12) 0.097	0.90 (0.51-1.58) 0.712
Hb	T-G	0.16	1.03 (0.41-2.62) 0.945	1.61 (0.51-5.12) 0.419	0.55 (0.13-2.37) 0.420
Hc	T-A	0.03	0.79 (0.49-1.26) 0.320	**0.45** (0.22-0.92) 0.028	1.29 (0.71-2.37) 0.406

3.3.3 GENE-GENE INTERACTIONS

As TLRs interact physically with each other to form homo- and heterodimers specific for the recognition of different microbial compounds, it was investigated if SNPs in these TLRs would show gene by gene interactions according to heterodimerization patterns. Indeed, significant pair wise interactions were observed between SNPs in *TLR2* and SNPs in *TLR1, TLR6* or *TLR10* (TABLE 3.10).

Stronger dominant protective effects were found for *TLR2* SNPs when stratified for the minor alleles of *TLR1*, *TLR6* and *TLR10* SNPs (FIG. 3.7). For atopic asthma an effect modification of *TLR2_a* was seen with the atopic asthma associated SNP cluster (*TLR1*_b, *TLR1*_c, *TLR6*_a, *TLR10*_b). For non-atopic asthma such modification was found for *TLR2*_c by *TLR1*_c and *TLR10*_b, respectively. For asthma an additional significant interaction could be observed (*TLR1*_a with *TLR2*_b).

TABLE 3.10 *Pair wise interaction effects of SNPs in heterodimerizing TLRs.*

		P value (interaction)		
SNP1	SNP2	Asthma	Atopic Asthma	Non-Atop. Asthma
*TLR1*_a	*TLR2*_a	0.3063	0.4177	0.3416
*TLR1*_a	*TLR2*_b	0.0204	0.1053	0.1110
*TLR1*_a	*TLR2*_c	0.4386	0.6305	0.3465
*TLR1*_b	*TLR2*_a	0.2102	0.0231	0.8759
*TLR1*_b	*TLR2*_b	0.0805	0.4444	0.2793
*TLR1*_b	*TLR2*_c	0.0529	0.6375	0.0505
*TLR1*_c	*TLR2*_a	0.6217	0.0682	0.6295
*TLR1*_c	*TLR2*_b	0.1467	0.5643	0.4337
*TLR1*_c	*TLR2*_c	0.0223	0.9383	0.0044
*TLR6*_a	*TLR2*_a	0.4424	0.0465	0.7557
*TLR6*_a	*TLR2*_b	0.2956	0.5312	0.8510
*TLR6*_a	*TLR2*_c	0.0745	0.5994	0.0910
*TLR6*_b	*TLR2*_a	0.4295	0.1759	0.9723
*TLR6*_b	*TLR2*_b	0.5877	0.8785	0.2768
*TLR6*_b	*TLR2*_c	0.1437	0.5881	0.0590
*TLR10*_a	*TLR2*_a	0.6756	0.1041	0.5146
*TLR10*_a	*TLR2*_b	0.4847	0.8350	0.9096
*TLR10*_a	*TLR2*_c	0.0748	0.8890	0.0644
*TLR10*_b	*TLR2*_a	0.3806	0.0554	0.8292
*TLR10*_b	*TLR2*_b	0.0750	0.3161	0.5381
*TLR10*_b	*TLR2*_c	0.0327	0.7741	0.0327

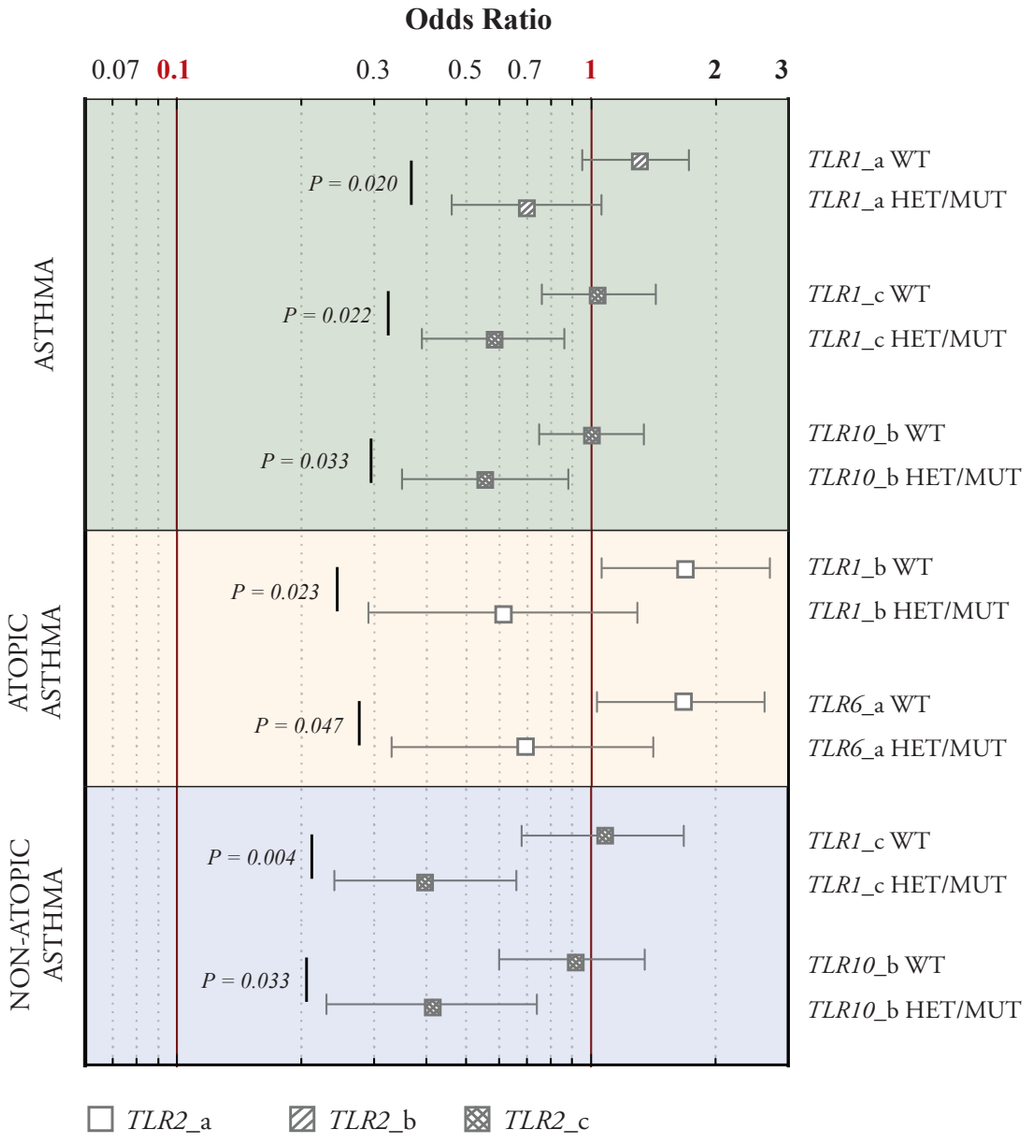


FIG. 3.7 ***Dominant effects of TLR2 SNPs stratified for the genotype of another SNP (right side).*** *Only pairs with significant interaction are shown; WT, wild type; HET/MUT, heterozygotes and homozygous carriers of the rare allele.*

3.4 FUNCTIONAL STUDIES

To test if the selected SNPs in the TLR2 heterodimer system, *TLR1_b*, *TLR6_a* and *TLR10_b*, were not only associated with atopic asthma but would lead to more immediate biological phenotypes on the cellular level, functional studies were performed. As *TLR1*_b might influence an intronic enhancer element and *TLR6*_a was situated in the promoter region, with a tagged SNP putatively changing transcription factor binding (TABLE 3.2E; FIG. 3.2C), it was speculated that transcription regulation might be altered. *TLR10*_b lead primarily to an amino acid change; however, it was in tight LD with three other SNPs in the promoter region of the gene ($r^2 > 0.8$) (FIG. 4.1). As shown in FIG. 3.8, *TLR1*_b, *TLR6*_a and *TLR10*_b are inherited *en bloc*.

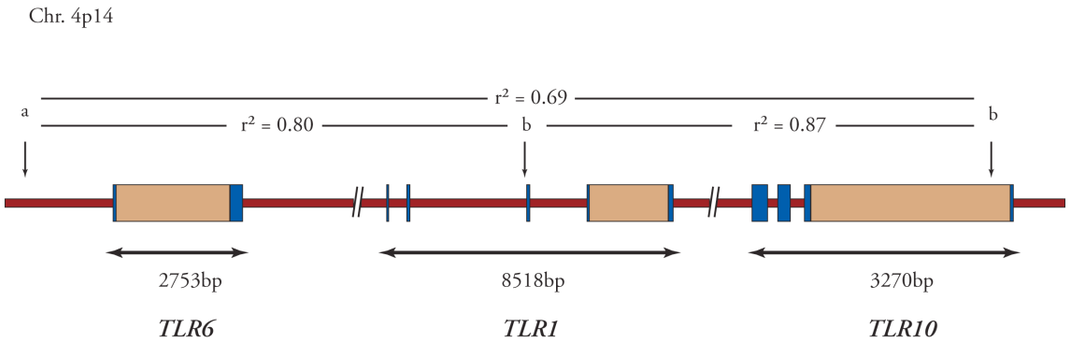


FIG. 3.8 *Interchromosomal linkage of TLR1_b, TLR6_a and TLR10_b.*

The *TLR1*_b variant shows linkage of $r^2 = 0.799$ with *TLR6*_a, and $r^2 = 0.817$ with *TLR10*_b, while *TLR6*_a and *TLR10*_b are linked with a r^2 of 0.699. 40 adult volunteers, who had given blood for immunological experiments, were genotyped for the respective SNPs. Confirming LD analysis, most people carrying two *TLR1*_b wild type alleles were also wild type for *TLR6*_a and *TLR10*_b. The same was true for homozygote rare allele carriers. Hence, blood samples from people wild type for the SNP block (n = 9) and carriers of the homozygote rare alleles (n = 5) were collected. Within both groups, allergy and allergic diseases were equally distributed as assessed by questionnaire (TABLE 3.11). None of the volunteers reported a current infection.

TABLE 3.11 *Occurence of allergies and current infections in the functional analyses groups.*

	*TLR1*_b/*TLR6*_a/*TLR10*_b	
	homozygous wild type	homozygous for the rare alleles
known allergies	4/9 (44%)	2/5 (40%)
current infection	0/9 (0%)	0/5 (0%)

3.4.1 mRNA EXPRESSION TLRs 1/2/6/10

First, it was investigated if people carrying the rare alleles for the *TLR1*_b/*TLR6*_a/*TLR10*_b SNP block differed in mRNA expression of the respective *TLRs* compared to the wild type group. RNA was isolated from nine people wild type for the SNP block and five people bearing the rare alleles. Relative expression levels were quantified with qRT RT PCR. 18S was determined as the housekeeping gene with the most stable and similar amplification curve between the two sample groups compared to ß2-microglobulin (FIG. 3.9 A,B). Additionally, melting curves and agarose gels were analysed to exclude unspecific PCR products and primer dimers.

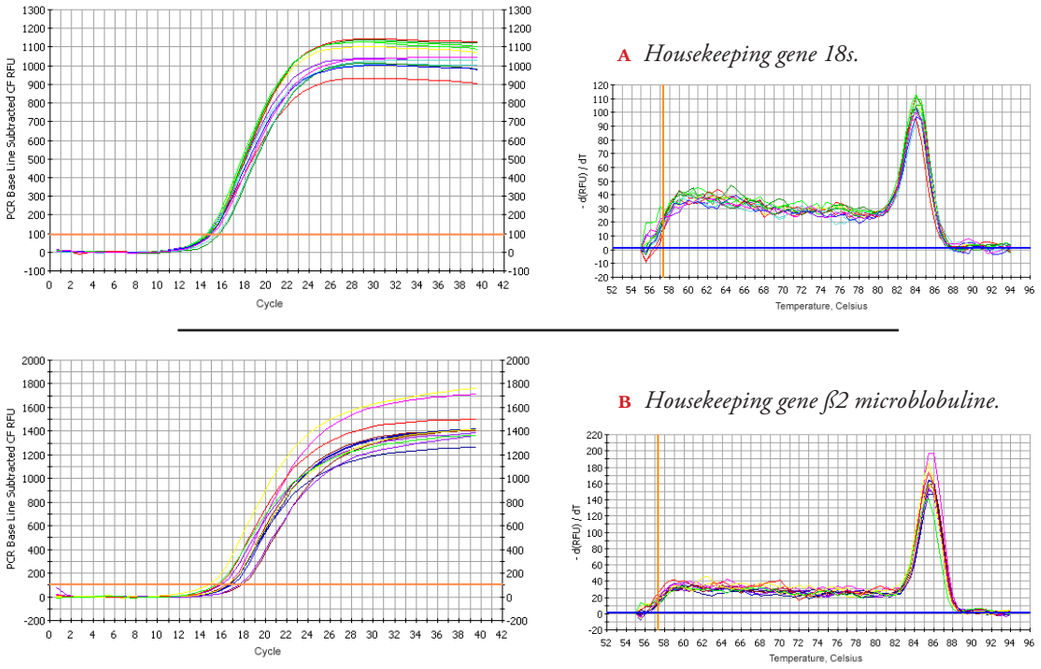


FIG. 3.9 *Housekeeping cDNA amplification curves of ten samples (n=5 of each group) and melting curve analysis.*

Subsequently, specific primers for TLR1, TLR2, TLR6 and TLR10 cDNA amplification were optimized (FIG. 3.10 A-D).

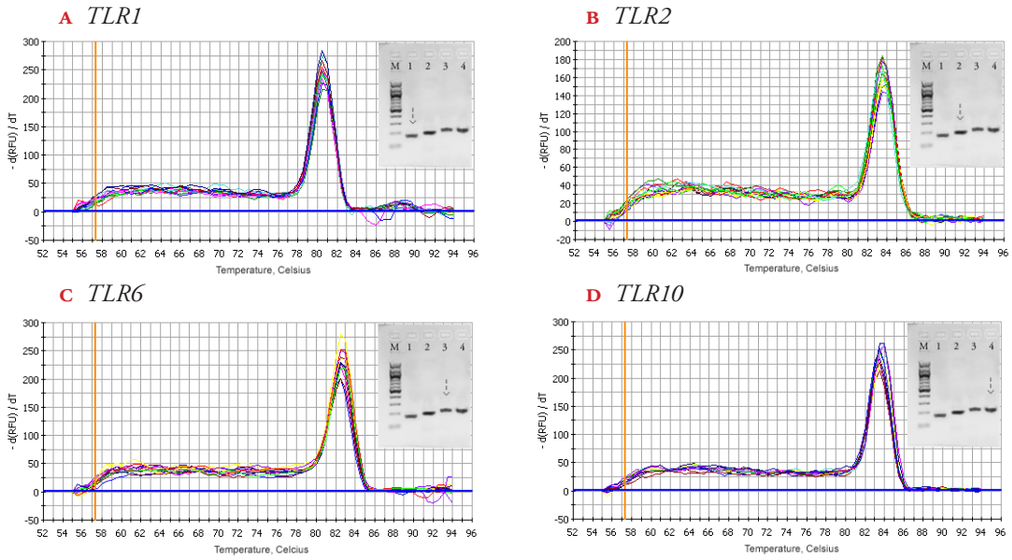


FIG. 3.10 *TLR cDNA melting curve analyses of ten samples (n=5 of each group) and gelelectrophoresis.*

Using *18S* as the reference gene, TLR mRNA levels in individuals homozygote for all three SNPs in *TLR1*_b, *TLR6*_a and *TLR10*_b were compared to subjects homozygote for the wild type alleles. Increased TLR1, TLR6 and TLR10 mRNA expression was found in the group carrying the rare alleles, whereas *TLR1*, *TLR6* and *TLR10* polymorphisms did not influence TLR2 levels (FIG. 3.11).

At a later time point again blood samples were taken from some of the same adults, RNA was extracted and subsequently expression levels were compared. As shown in TABLE 3.12 the results from the first experiment were reproducible and the expression differences seem stable over time.

TABLE 3.12 ***mRNA expression at a 2nd time point.*** *Relative expression of the respective TLR mRNA (isolated from whole blood) of individuals homozygote polymorphic (n=4) for TLR1_b, TLR6_a and TLR10_b is shown in relation to TLR expression in wild type individuals (n=4). Standard error and 95% CI are given.*

Gene	Expression	Standard Error	95% CI	P(H1)
TLR1	2.04	1.31 – 5.42	1.13 – 6.25	0.253
TLR2	0.68	0.54 – 0.82	0.50 – 0.97	0.205
TLR6	2.09	1.43 – 3.01	1.16 – 4.41	0.171
TLR10	1.90	1.55 – 2.59	1.34 – 2.92	0.191
18S	1.00	0.72 – 1.27	0.67 – 1.36	1.000

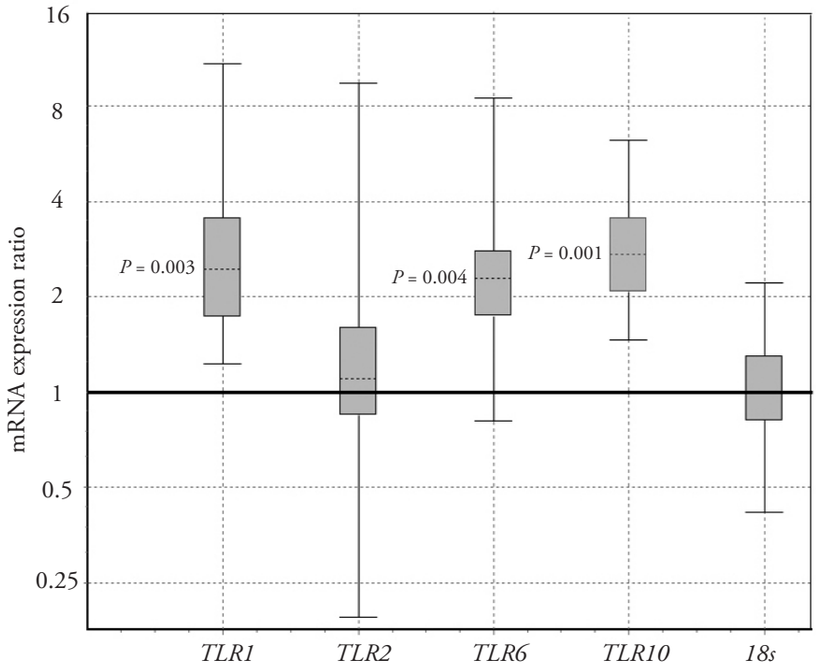


FIG. 3.11 ***Relative expression of the respective TLR mRNA*** *(isolated from whole blood) of individuals homozygote polymorphic (n=5) for TLR1_b, TLR6_a and TLR10_b is shown in relation to baseline (expression ratio 1) as determined by TLR expression in wild type individuals (n=9). Boxes represent medians (dotted line) ± IQR. Whiskers represent the minimum and maximum observations. Statistical analysis was performed using a pair wise fixed reallocation randomisation test. P values are given for significant effects (P<0.05).*

3.4.2 PROTEIN EXPRESSION TLRS 1/2/6/10

Subsequently, it was investigated if the mRNA expression differences could be confirmed on the protein level. The same five homozygote rare allele carriers of *TLR1*_b/*TLR6*_a/*TLR10*_b SNPs and nine wild type controls were selected for whole blood flow cytometry. TLR1 expression levels were significantly higher on peripheral blood mononuclear cells (PBMCs) of the minor allele group, showing an increase of about 50% ($P = 0.009$) (FIG. 3.12). Similar results were observed for TLR6 expression, which was also significantly elevated ($P = 0.003$), while TLR10 levels were only moderately elevated and TLR2 expression did not differ significantly between both groups (FIG. 3.12). TLR1 and TLR6 were expressed on lymphocytes (TLR1: MFI 45 [IQR 41-52]; TLR6: 40 [32-47]) and monocytes (TLR1: 80 [72-89]; TLR6: 60 [52-68]), while TLR10 was exclusively expressed on B cells.

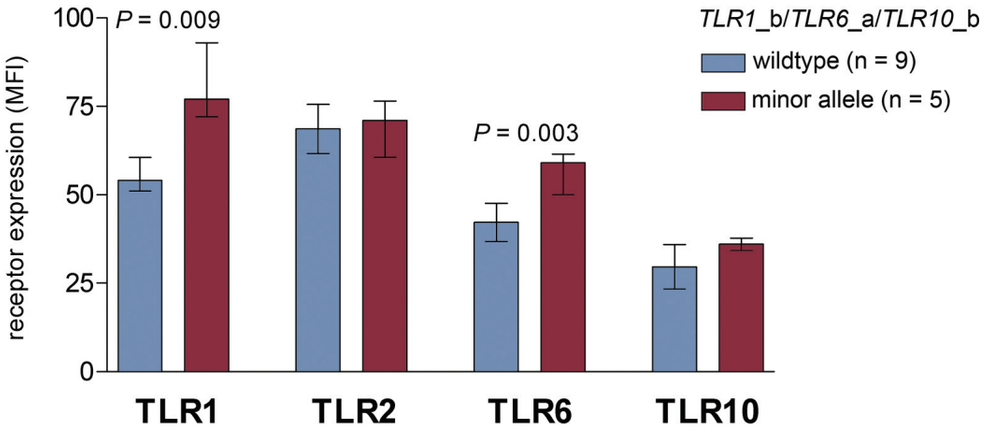


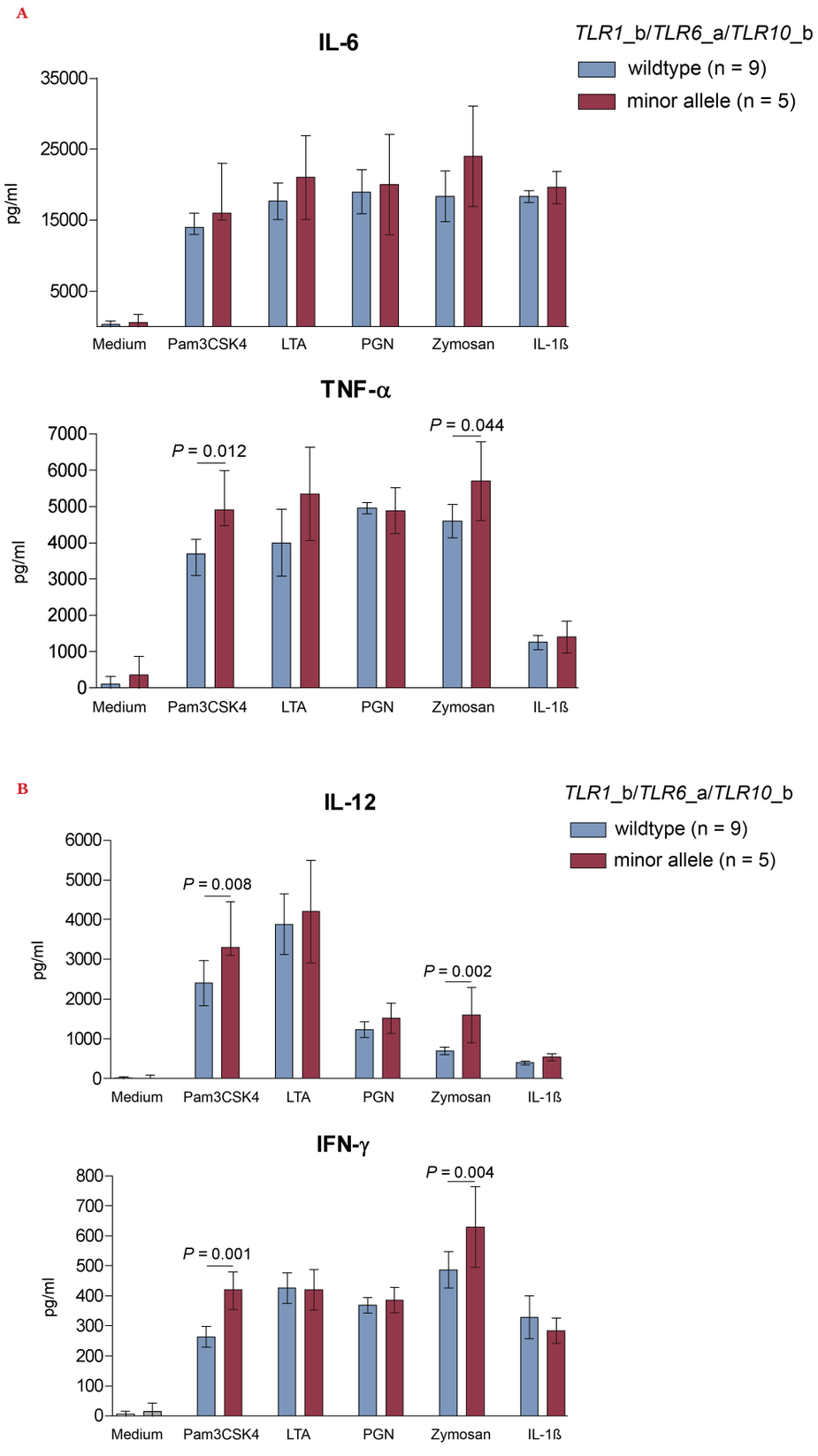
FIG. 3.12 ***TLR1, TLR2, TLR6 and TLR10 protein expression*** *was analyzed using unstimulated PBMCs of adult individuals wild type (n=9; blue bars) or homozygote polymorphic (n=5; red bars) for TLR1_b, TLR6_a and TLR10_b. TLR expression was analyzed by flow cytometry as described in the methods section. MFI: mean fluorescence intensity. Bars represent medians ± IQR of different donors. Statistical analysis was performed using exact Wilcoxon tests. P values are given for significant effects (P<0.05).*

3.4.3 STIMULATION AND CYTOKINE EXPRESSION

In a next step, the influence of genetic changes in TLR1/TLR6/TLR10 on specific immune responses was studied. PBMCs from the same individuals as before were stimulated with TLR2 specific ligands (LTA and PGN) and ligands specific for TLR2/TLR1 (Pam_3CSK_4, a tri-pamitoylated hexapeptide) and TLR2/TLR6 (Zymosan). Then, the subsequent production of pro-inflammatory cytokines as well as Th1/Th2 associated cytokines was analyzed. PBMCs from individuals homozygote for *TLR1*_b, *TLR6*_a and *TLR10*_b SNPs expressed significantly higher levels of the proinflammatory cytokine TNFα upon stimulation with TLR2/TLR1 and TLR2/TLR6 ligands compared to wild type individuals (**FIG. 3.13A**). A same trend could be observed for IL-6.

The Th1 related cytokines IL-12 and IFNγ were also significantly upregulated in PBMCs homozygote for *TLR1*_b, *TLR6*_a and *TLR10*_b after stimulation with the heterodimer ligands Pam_3CSK_4 and Zymosan (**FIG. 3.13B**). Stimulation with LTA and PGN on the other hand resulted in IL-12 and IFNγ levels that did not differ between both groups.

In contrast, levels of the Th2 signature cytokines IL-10 and IL-4 were lower in homozygote carriers of the rare *TLR1*/*TLR6*/*TLR10* alleles after stimulation with TLR2/TLR1 and TLR2/TLR6


A
IL-6
TLR1_b/TLR6_a/TLR10_b
wildtype (n = 9)
minor allele (n = 5)
pg/ml
35000
25000
15000
5000
Medium
Pam3CSK4
LTA
PGN
Zymosan
IL-1ß
TNF-α
P = 0.012
P = 0.044
7000
6000
5000
4000
3000
2000
1000
0
B
IL-12
P = 0.008
P = 0.002
IFN-γ
P = 0.001
P = 0.004
800
700
600
500
400
300
200
100

C

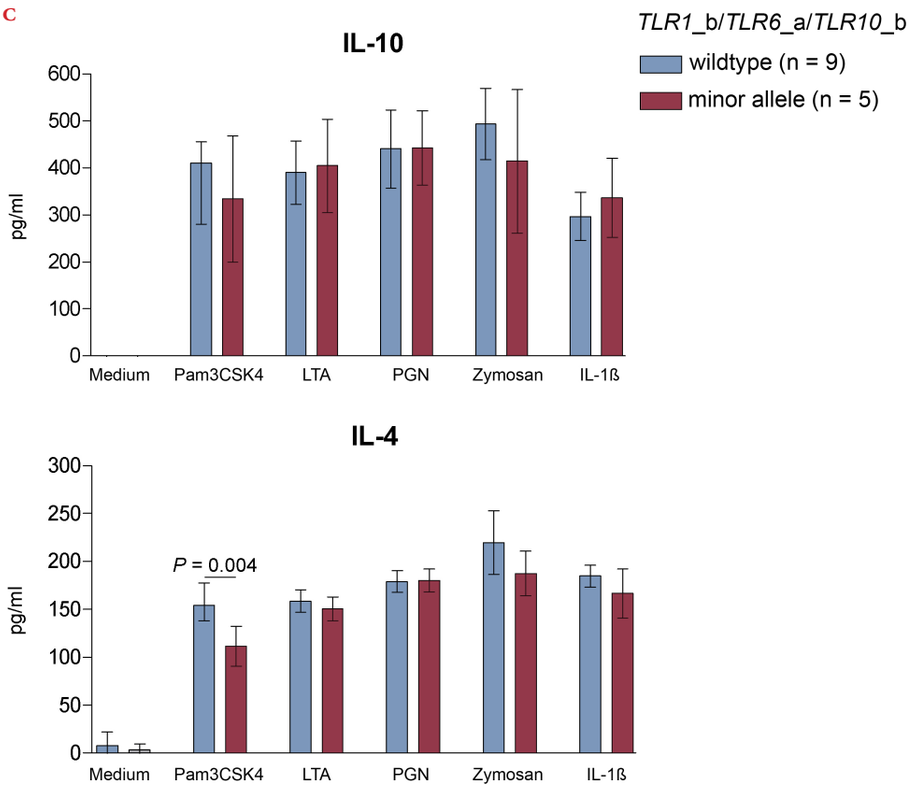


FIG. 3.13 A *Pro-inflammatory,* B *Th1 and* C *Th2 associated cytokine levels after TLR2/1 and TLR2/6 stimulation.* *PBMCs from subjects wild type (n=9; blue bars) or homozygote polymorphic (n=5; red bars) for TLR1_b, TLR6_a and TLR10_b were cultured for 24h in medium alone (medium) or stimulated with Pam_3CSK_4 (10 µg/ml; an artificial triacylated lipopeptide; TLR1/2 agonist), PGN (1 µg/ml; TLR2/NOD2 agonist), LTA (10 µg/ml; TLR2 agonist), zymosan (10 µg/ml; TLR2/6 agonist) and with IL-1β (50 ng/ml, non-TLR ligand control). The secretion of* A *IL6 and TNFa,* B *IL-12 and IFNγ and* C *IL-10 and IL-4 was measured in culture supernatants by ELISA in triplicates. Bars represent medians ± IQR between different donors. Statistical analysis was performed using exact Wilcoxon tests. P values are given for significant effects (P<0.05).*

3.5 ASSOCIATION OF RARE TLR2 SNPs WITH ATOPIC PHENOTYPES

In the previous chapters it could be demonstrated that the TLR2 associated TLRs 1, 6 and 10 seem to play an important role in atopy and atopic asthma susceptibility, however, no significant effects of common TLR2 variants could be found on the investigated phenotypes. Therefore, the role of rare but functionally relevant amino acid changes in TLR2 was assessed. Data mining was performed (www.innateimmunity.net) and sequence analyses revealed seven infrequent TLR2 amino acid changes (MAF 1-3%).

These data were then passed on to Dr. Carsten Kirschning and co-workers (Institute of Medical Microbiology, Immunology and Hygiene, TUM), who determined that all rare TLR2 variants but TLR2_715Stop were expressed to an equal extent as compared to wild-type TLR2 construct in HEK293 cells in a CMV promoter driven fashion (FIG. 3.14). Activities of Y715Stop, R677W, Y715N, and R753Q mirrored acquisition of inhibitory polymorphisms by TLR2 with either NFkB driven reporter gene activation or IL-8 as parameters of cell activation (FIG. 3.14). No Y715Stop lipopetide recognition function was detectable. NFkB driven reporter gene activity was reduced by approximately 80% in the case of Y715N and 50% for R753Q throughout the entire range of different concentrations at which lipopeptide as TLR2 agonist was applied. Notably, IL-8 release upon low dose lipopeptide challenge was not detectable, while the intermediate amount of lipopeptide was recognized by cells expressing the R753Q variant but not by cells expressing the Y715N variant. Upon challenge with lipopeptide at the high dose, however, cells expressing each TLR2 variant released IL-8 to an extent that differed not significantly from IL-8 release from cells expressing wild-type TLR2.

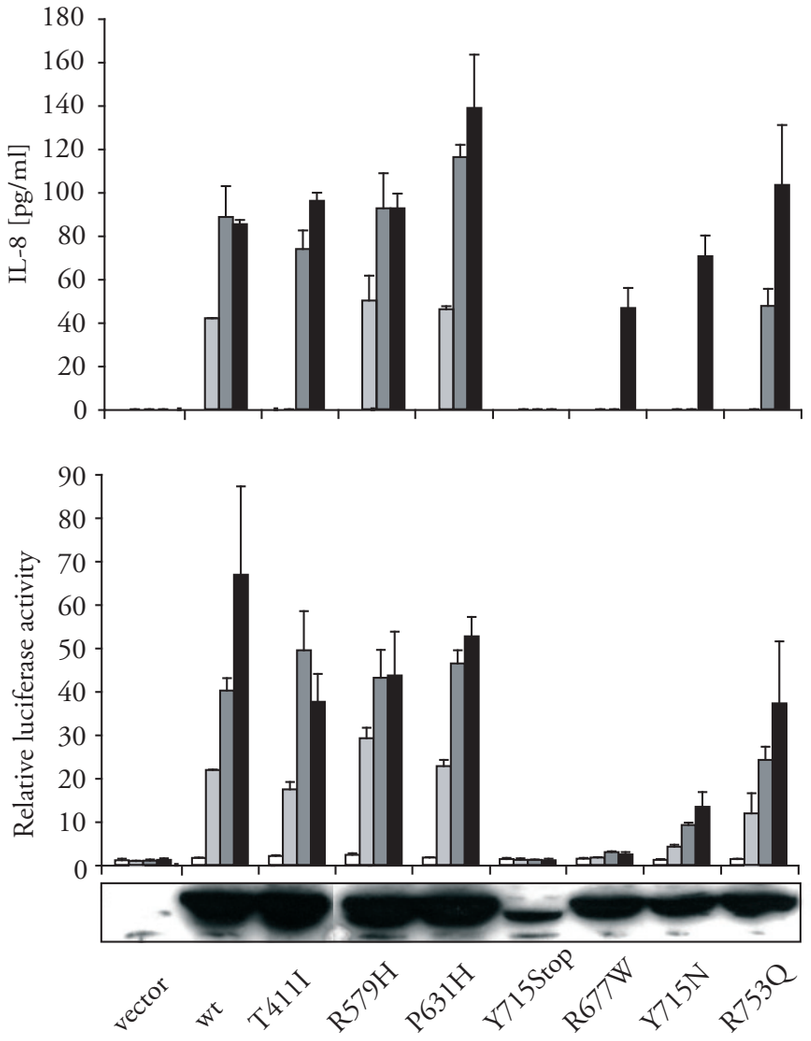


FIG. 3.14 ***Comparative analysis of wild-type and specific polymorphic TLR2 constructs.*** *HEK293 cells were transfected with DNA plasmids driving expression of TLR2 constructs indicated under control of the CMV promotor. Subsequently, cells were challenged with Pam_3CSK_4 at successively increasing concentrations (white columns, unstimulated; bright grey columns, 10 ng/ml Pam_3CSK_4; dark grey columns, 100 ng/ml Pam_3CSK_4; black columns, 1 µg/ml Pam_3CSK_4). After 16 h, IL-8 concentrations in supernatants were determined by ELISA (upper panel) while NFkB-driven luciferase activity was determined upon lysis of cells (middle panel). Alternatively, lysates were applied to immuno blot analysis to determine expression of flag-tagged TLR2 variants (lower panel; wt, wild type). Graphics and data kindly provided by Dr. C. Kirschning.*

Subsequently, I evaluated the presence of these seven mutations in German individuals (n=386) randomly selected from the ISAAC population in Munich. Interestingly, out of the originally described seven mutations four could not be found in our subpopulation while one (TLR2_Y715N) could not be verified due to close proximity to TLR2_Y715Stop (TABLE 3.10). However, two of the nonsynonymous changes could be confirmed (TLR2_P631H and TLR2_R753Q).

TABLE 3.10 ***Frequency of the investigated TLR2 protein changes.*** *[1] the mutation was not detected in a European population as reported by Lee et al. (2006).*

TLR2 SNP	rs number	Alleles	Minor Allele Carrier
T411I	rs5743699	C/T	0/368
I556T	rs5743702	T/C	0/368
R579H	rs5743703	G/A	0/368
P631H	rs5743704	C/A	29/368
Y715N	rs5743705	T/G	0[1]
Y715Stop	rs5743707	T/A	0/368
R753Q	rs5743708	G/A	22/368

In a next step, I studied the effects of both verified mutations on atopic phenotypes. TLR2_753Q (which occured with a frequency of 0.03) significantly increased the risk for atopy in the cross-sectional population from Munich and Dresden (N=3,099) (TABLE 3.11). Additionally, carriers of this protein variant showed elevated geometric means of total serum IgE. TLR2_631H (with a frequency of 0.04) had no significant influence on the investigated atopic phenotypes, whether in the pooled population nor in any of both subpopulations. None of the protein changes were associated with the development of asthma (data not shown).

TABLE 3.11 ***Association of rare TLR2 amino acid changes with total IgE levels and atopy in the cross-sectional population.*** *OR (CI), Odds ratios (and 95% confidence intervals) for dominant effects are given.*

			Total IgE geom. mean *P* value	Atopy OR (CI) *P* value
M/D pooled	**P631H**	PP	69.2 (65.6-73)	0.85 (0.63-1.17)
		PH/HH	62.0 (52.3-73.5)	0.318
			0.247	
	R753Q	RR	**68.8** (65.3-72.6)	**1.52** (1.06-2.17)
		RQ/QQ	**87.9** (70.4-109.8)	0.021
			0.040	

As described in 3.3.3, when regarding frequent *TLR2* SNPs, significant gene-gene-interactions were found between *TLR2* with *TLR1*, *TLR6* and *TLR10*. Therefore pair wise interaction effects were calculated also for the infrequent TLR2 mutations. Surprisingly, no significant interaction effects could be found between the two rare TLR2 amino acid changes and common SNPs in *TLR1*, *TLR6* and *TLR10* (TABLE 3.12).

TABLE 3.12 *Pair wise interaction effects between rare SNPs in TLR2 and common SNPs in TLR2 heterodimerizing TLRs.*

		P value (interaction)		
SNP1	SNP2	Asthma	Atopic Asthma	Non-Atop. Asthma
TLR2_R753Q	*TLR1*_b	0.7812	0.9807	0.9785
TLR2_R753Q	*TLR1*_c	0.3876	0.9850	0.9703
TLR2_R753Q	*TLR6*_a	0.8210	0.7246	0.9682
TLR2_R753Q	*TLR6*_b	0.8218	0.9126	0.9816
TLR2_R753Q	*TLR10*_b	0.8455	0.9811	0.9785
TLR2_P631H	*TLR1*_b	0.4023	0.9424	0.6700
TLR2_P631H	*TLR1*_c	0.3943	0.8625	0.5974
TLR2_P631H	*TLR6*_a	0.5618	0.8237	0.8274
TLR2_P631H	*TLR6*_b	0.8653	0.6021	0.4028
TLR2_P631H	*TLR10*_b	0.6766	0.9003	0.4863

4 DISCUSSION

4.1 GENERAL REMARKS

Chronic complex disorders like cancer, psychiatric diseases, cardiovascular disease, Alzheimer's disease and asthma become more and more important in public health. But despite massive investments in research time and funding, the underlying mechanisms behind those diseases are still largely unknown. It may be argued that pure statistical empirism - «black-box epidemiology» - is simply not working and that facing the enormous economical disbursements that would be required for such «blind approaches» we need to turn to a more determined consideration of the underlying biological mechanism and causation of disease (WEISS & TERWILLIGER 2000; TERWILLIGER & WEISS 2003).

This thesis focuses on studying the association of selected polymorphisms in human TLR genes with the development of childhood asthma. The results mark TLR1 and TLR6 as intriguing new targets for asthma research as they have not been recognised to be involved in atopic disease mechanisms so far. Functional investigation suggests that stimulating certain TLR heterodimers, rather than arbitrary microbial exposure, may protect from the development of childhood asthma. This conclusion shall be discussed in the context of the SNP selection algorithm, the population characteristics and the functional studies that have been performed.

4.2 SELECTING SNPs FOR GENOTYPING

The definition of a SNP is not completely standardized. On the Human Genome Project information page (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml) a SNP is considered to be a genetic variation at a single base pair locus with a minor allele frequency of ≥ 0.01 (the ratio of chromosomes in a population carrying the less common variant to those with the more common variant). Others use a MAF of 0.03 as cut-off, depicting all variations less than that as a mutation.

Associating SNPs with a phenotype and simultaneously with function at the cellular level, is a modern non-invasive way to study the genetic architecture of humans. In parallel to knock-out and knock-in mice, variations in the DNA sequence of humans may affect the phenotype (at many different levels), disease development and response to pathogens or drugs. While in mouse experiments the changes are introduced deliberately by researchers to reveal the function of a gene, in humans we have to rely on natural occuring polymorphisms. In general, natural occuring SNPs often do not

seem to have obvious function on a rough phenotypic level. However, the more detailed a SNP is characterized at the basic level regarding e.g. its effect on transcription chemistry, the more clear a function can be assigned. In contrast to the rather crude method of knocking out a whole gene (and with it its translated protein) completely, SNPs occuring approx. every 100 to 300 bp allow a more subtle differentiation of function of different parts of a gene. Of course, in this case we are limited to variations that already occur in nature, without control of their locus or the severity of impact.

Facing the often large number of SNPs in a gene, selecting particular SNPs for genotyping may present an effective option. Thereby, it is of importance how the occurrence of SNPs was determined in a respective population. Many SNPs described in the official SNP data base dbSNP are not validated and might occasionally represent sequencing errors or rare mutations. Hence, re-sequencing a small sample population is necessary to provide reliable information of naturally occuring SNPs. The Innate Immunity PGA sequenced the chromosomes of 23 European Americans using immortalized cell cultures (Coriell samples). However, to detect SNPs with a MAF > 0.03 with a probability of 95%, 49-50 samples would be needed ((0.97)x = 0.05; x = number of necessary alleles = 99). Consequently, in the Innate Immunity PGA analysis of 23 samples likely SNPs with a MAF > 0.03 have been missed (P = 0.9746 = 24.6%). On the other hand, if one wants to efficiently detect variations with a MAF < 0.03, larger sample numbers for screening would be needed, but more importantly, for subsequent association analyses only very large populations would provide the respective power to significantly detect associations with multigenetic diseases.

In general, SNPs are selected and filtered from a larger group of known SNPs with the aim of expedite, less expensive genotyping and the need of less DNA material without losing important SNP information. Two types of selection algorithms may be generally considered to achieve that goal: a) tagging SNPs and b) functional selection. a) usually implicates any kind of selection algorithm based on a specific MAF as cut-off and consideration of linkage disequilibrium between SNPs (with a specific r^2) to "cover" as many existing SNPs as possible with the selection, while avoiding redundancy. b) accounts for the putative function of SNPs using different *in silico* (or *in vitro*/*vivo* were applicable) approaches and gathers SNPs that most likely will influence transcriptional regulation or the chemical attributes of the protein. Compared to an extensive approach including all known SNPs using a) functional SNPs might in part be only indirectly associated with a phenotype (depending on the r^2) or missed due to too restrictive MAF cut-offs. Using b) it cannot be warranted that actually functional SNPs are missed (as *in silico* and *in vitro* analyses might only predict, neither assure nor exclude function). In this thesis both methods were combined. Putative functional SNPs were selected using *in silico* approaches (transcription factor binding and amino acid changes) while redundancy was avoided by considering tagging SNPs. Although this combination of both strategies might present a very efficient method of selecting SNPs with high probability of function, it cannot be excluded that SNPs with effects on the disease but not matching the respective selection criteria may have been missed.

4.2.1 *IN SILICO* TRANSCRIPTION FACTOR BINDING PREDICTION

In this thesis one central *in silico* approach to characterize putative SNP function was to determine a SNPs' capability to alter regulatory binding regions. Several programs are available on the internet which connect to data bases and — upon entering sequences — suggest putative transcription factor binding sites. Such sites are often differentiated into a "core sequence" (the highest conserved positions of the binding matrix) and a "matrix sequence" (less conserved regions, mostly flanking the core sequence). Depending on how strict the parameters are chosen more or less proteins are suggested to bind to a certain sequence. Binding is predicted based on publications on similar sequences with *in vitro* functional studies like DNase I footprinting, gel shift competition or supershift (antibody binding). However, even if those results are confirmed *in vivo* and a definite binding is suggested by respective methods like chromatin immunoprecipiation (ChIP), a consequence on transcription and translation would have still left to be shown.

Another issue in using bioinformatics is that different prediction tools would offer different results, depending on their algorithm and on the data base they refer to. As an example regulatory regions of *TLR1* were analyzed with three different online programs: FastSNP, MatInspector and AliBaba (TABLE 4.1).

TABLE 4.1 *Putative transcription factor binding events evaluated with different online programs. + / -, indicates the occurrence / loss of a respective binding matrix in rare allele configuration; rel. ATG ,position relative to ATG.*

rel. ATG	Location	FastSNP	MatInsp	AliBaba
-6399	promoter		-MyT1, -GAGA-Box	
-6375	promoter		-Spi-B, -PAX2, +Blimp-1, +IRF1	+ICSBP
-5986	promoter		-Brn-2, -Brn-3, +HNF6, +PBX_HOXA9	-C/EBPalpha
-5748	intron	-CDX1		
-5642	intron		-XBP, +HELT, +WHN	
-5565	intron	-CDX1	-NGN1/3, -SMARCA3, +IRF7	
-5035	intron			-MIG1, -Sp1
-3951	intron		-NRF1, -EGR3	+SP1
-3330	intron		-AHR, +TEAD	-SP1, -Oct1, +TEC1
-3192	intron		+CHREBP	+NFkB, SP1
-2801	intron			
-2609	intron	+Statx	+COMP1, +STAT, +E4F	
-2459	intron		-OCT1, +PRDM1, +MIT, +MYC-MAX	-Oct1, +NF1
-2299	intron	+CDX1	+PAX2, +FREAC7	-AP2, -HNF3
-2192	intron	+SRY	-PLZF, +NFY	-HNF1
-833	intron	-CREB	-RORA	-COUP, +CRE-BP1

Very different results were obtained depending on the program used, with surprisingly few overlaps. In part specific proteins even seem to be restricted to one software (e.g. SP1 to AliBaba, CDX1 to FastSNP; based on additional analyses). However, this seems to be mainly due to the different data bases and threshold parameters used by the software, although other algorithm inconsistencies between the programs leading to those different outcomes cannot be excluded.

In this work FastSNP was used as primary bioinformatical tool to determine putative changes in transcription factor binding. It extracts data from a variety of external bioinformatics web servers using automated web browsing, which ensures updated data. With TFSearch a conservative binding predictor is used (default threshold score: 85.0). Altogether, FastSNP offers a flexible and concise tool, which automatically uses the latest data bases.

4.2.2 TAGGING SNPs

SNPs in high linkage disequilibrium may "tag" each other, i.e. that the allelic state of one SNP is correlated with the allelic state of another one. Extended to several tagging SNPs and regarding each chromosome separately one refers to as "haplotype tagging SNPs". One aspect to consider when selecting tagging SNPs based on the Innate Immunity PGA data is that the original LD information was taken from a small screening population, without knowing about the exact LD's in the large population. However, data comparisons suggest that high LD recognized in a small population is mostly preserved in a large population of the same ethnical origin (HILL & ROBERTSON 1968). A second aspect is that if high LD is observed between two or more SNPs, an association with a penotype cannot be causally assigned to one specific SNP of the group without differentiating functional analyses. Additionally, several modified methods for tagging SNP selection have been proposed (LIU *et al.* 2007; SHAM *et al.* 2007). While in this thesis Haploview was used as it combines data with convenient LD plots, recent suggestions have been made using Bayesian networks to maximize the prediction accuracy (LEE & SHATKAY 2006).

4.3 GENOTYPING QUALITY CONTROL

4.3.1 DOES HWE DEVIATION DETECT GENOTYPING ERROR?

HWE – named after G.H. Hardy and W. Weinberg, states that, under certain conditions the genotype frequencies at a single gene locus will become fixed at a particular equilibrium value. Those frequencies can be presented as a simple function of the allele frequencies at that locus (see FIG. 2.8).

For the mathematics to be applicable, the organism under consideration has to be diploid and sexually reproducing with discrete generations, which is the case for the human species. However, the population under consideration is idealised, i.e. random mating within a single population and infinite population size (to minimize effects of genetic drift) with no selection, no mutation and no migration (gene flow). Obviously, no population under investigation in epidemiological studies is able to perfectly match all these assumptions. However, it is a model which fits surprisingly well with the data found when genotyping populations. Nevertheless, significant deviations from HWE are regularly detected. If the deviation is due to the assumptions (see above) that are violated, two incidents are most common: first, inbreeding, which prohibits random mating. In a subtle way this happens in all crowded areas (cities) where many people live together. Inbreeding increases homozygosity for all genes (which means a relative decrease in heterozygosity). Unfortunately, MALDI-TOF MS tends to fail to call all heterozygotes, which — in this case — makes it nearly impossible to differ between a methodological problem or a real population based deviation if HWE departure with lack of heterozygotes occures. Second, an allele might lead to a selection bias (directional selection with the loss of unfavored alleles or balancing selection).

In general, if departure from HWE occurs without significant influence of any of those population traits a sampling error including genotyping error might be likely (HOSKING *et al.* 2004). Indeed, some of the investigated SNPs showed significant deviation from HWE in either the case-control or the cross-sectional population (TABLE 3.4). In this case, the homozygote rare allele carriers attributed the highest χ^2 to the deviation. They were re-genotyped with an independent method, the gold standard of sequencing. Furthermore, missings were sequenced to check whether incorporating those values would establish HWE. However, the respective deviations remained (TABLE 3.5), suggesting a population based inbalance in genotypes.

Vice versa it is important to question if testing departures from HWE is sensitive enough to detect genotyping errors. Cox and Kraft modeled the power of HWE deviation as a test to detect genotyping error and quantified the effect of genotyping error on disease risk estimates (COX & KRAFT 2006). They concluded that the test has relatively low sensitivity. In the context of screening small datasets as well as large case-control studies, passing a test of HWE does not guarantee a lack of genotyping error. Furthermore, a non-significant test on HWE deviation does not imply that the genotype frequencies are significantly *in* HWE (see WELLEK & SCHUMANN 2004), which substantiates the shortcomings of the HWE test. Although those shortcomings have to be considered and more robust methods of genotype error detection need to be delevoped, at the moment testing HWE deviation is the only feasible and economic method in high-throughout approaches.

4.3.2 UNCERTAINTY IN HAPLOTYPES?

Haplotypes are combinations of alleles within a certain section of one chromosome. Analysing haplotypes in genetic association studies is often more efficient than studying the SNPs separately. One disadvantage of haplotypes is that they regard only allele combinations on the same chromosome (*cis* effects). Another disadvantage in this context is the statistical problem of reconstructing the phase: genotyping SNPs determines the alleles of an individual at one particular locus of the DNA, but does not reveal which allele is located on which one of the two chromosomes. Although stastistical approaches exist to identify the most likely two haplotypes of an individual (given the genotypes), a certain error in prognosis is unavoidable. Additionally, genotyping errors may accumulate and induce further uncertainty in the haplotype. Of course, those uncertainties get stronger by each added SNP, which might obscure the haplotype frequency differences between cases and controls. However, the haplotype model using combination of alleles reflects more the real genetic architecture than does a single SNP focus. This explains why disease associations of haplotypes may be stronger compared to single SNPs. If a haplotype consists of e.g. three SNPs and the allelic states of each of the SNPs confer an independent risk for a disease, the accumulated effect will be stronger than a single SNP effect (as the allelic states from the remaining two SNPs are variable and would blur the effect).

4.4 ASTHMA EVALUATION

Not only genotyping has to be critically evaluated. The question may arise if a doctor's diagnosis of asthma, particularly when it may include "asthmatic, spastic, or obstructive bronchitis" (as implemented in the ISAAC II core questions) is sufficient to reliably identify the asthma phenotype. In the last decades public awareness of rising trends in the prevalence of allergic diseases increased, which could have influenced both doctors' diagnosis and parental reports. As in Germany the terms spastic and obstructive bronchitis have been both used to describe asthma they had to be included in the questionnaire to prevent excessive underestimation of asthma cases in this population.

It is well known that a large number of the children diagnosed by doctors as asthmatics show a positive skin prick test and bronchial hyperresponsiveness (HOLGATE 1999). The ISAAC II questionnaire data were supported by objective measurements of lung function, BHR and SPT. Those were highly correlated with asthma or hay fever frequencies derived from the parents' questionnaires, validating the utilized broadened asthma definition. Additionally, two previous studies described the significance of BHR testing by the method used in ISAAC II and the assessement of asthma by questionnaire (NICOLAI *et al.* 1993, RIEDLER *et al.* 1994).

4.5 ELABORATING ON TLR SNP EFFECTS ON ASTHMA PHENOTYPES

Several groups have studied the effects of selected *TLR* SNPs on asthma but with several limitations. Small sample sizes, the lack of examining haplotypes or investigating interaction effects and performing appropriate *ex vivo* experiments made it difficult to interpret the often contradictory results. The amino acid change D299G in TLR4, the receptor for LPS, is an exemplifying case. Exposure to LPS was associated with the severity of asthma. In at least one study of people sensitive to house dust mite allergen, the severity of their asthma correlated more closely with levels of LPS than with those of the allergen itself (MICHEL *et al.* 1996). People with allergic asthma were also more sensitive to the bronchoconstrictive effects of inhaled endotoxin than were non-asthmatics (MICHEL *et al.* 1989). Thus, LPS seems to exacerbate asthma, probably by increasing the extent of airway inflammation. However, in contrast to its exacerbating effect on asthma, exposure to LPS and other TLR ligands in early childhood may, paradoxically, decrease the incidence of asthma later in life (BRAUN-FAHRLANDER *et al.* 2002; GEHRING *et al.* 2002), which may also in part reflect the manifestation of different asthma aetiologies. This protection from asthma may be related to a dampening of Th2 reponses or an increase in number or activity of regulatory T cells whose function is to down-regulate exaggerated immune responses. Regardless of the mechanism, it is clear that LPS can either exacerbate or diminish the severity of asthma, supposably depending on the timing of exposure and on the genetic make-up of the respective person. Given the opposing effects of LPS, it is perhaps not unexpected that some studies have not demonstrated an effect of the D299G polymorphism on the overall incidence of asthma (RABY *et al.* 2002; WERNER *et al.* 2003). However, a study of asthma specifically associated with LPS in house dust showed that people with the 299G variant had a decreased risk of bronchoreactivity (WERNER *et al.* 2003). Another study showed that asthmatic people with TLR4 299G have an increased severity of atopy (YANG *et al.* 2004).

It is obvious that a full understanding of specific TLR effects on atopy and asthma requires a comprehensive approach, regarding the TLRs as a complex, interacting system, supplemented by distinctive functional characterization of respective variations. In the following chapters some new concepts how TLR variations may influence the development of asthma – with focus on the TLR heterodimer system – will be discussed.

4.5.1 HETERODIMER SYSTEM TLR1/2/6/10

4.5.1.1 SINGLE SNP AND HAPLOTYPE ASSOCIATIONS ON ASTHMA PHENOTYPES

TLR1, TLR2, TLR6 and TLR10 form heterodimers to augment the variety of detectable patterns. TLR1 is most closely related to TLR6 and TLR10 with 68% and 48% overall amino acid sequence identity, respectively, which reflects their phylogenetic conservation (see FIG. 1.3). TLR1 interacts with TLR2 to recognize the lipid configuration of native lipoproteins from bacteria and mycobacteria (TAKEUCHI *et al.* 2002). In our case-control population minor alleles of *TLR1*_b, *TLR1*_c, *TLR6*_a and *TLR10*_b protected from atopic asthma. As suggested by FastSNP the C allele of *TLR1*_b determines a sequence matrix that makes it more likely to bind the sex-determining SRY, which shares a conserved DNA-binding domain (the high mobility group I (HMG I)). The mouse homologue Sry was found to act as a transcriptional activator (DUBIN & OSTRER 1994). As mentioned in 4.2.1 other TF binding prediction programs may provide alternative scenarios: according to MatInspector the C allele makes it more unlikely to bind a TF which might act as a repressor (PLZF, promyelocytic leukemia zink finger) (BARNA *et al.* 2002) but gives rise to a target motif of a heterotrimeric TF (NFY) which might play a role in transcriptional activation (DE SILVIO *et al.* 1999; ROMIER *et al.* 2003). Indeed, it could be demonstrated that TLR1 mRNA and protein is higher expressed in homozygote carriers of the minor allele compared to wild type (see FIG. 3.11 and 3.12). As the specific stimulation of TLR2/1 heterodimers on minor allele carrier PBMCs resulted in a stronger pro-inflammatory response and relatively higher levels of Th1 cytokines with relatively lower levels of Th2 cytokines (see FIG. 3.13), the enhanced expression of TLR1 might indeed underlie the protective effect on atopic asthma.

On the other hand, *TLR1*_b is in a moderate LD with *TLR1*_c (r^2 = 0.73; see FIG. 3.2A), which also confers protection against atopic asthma. *TLR1*_c constitutes an amino acid change in the LRR domain region of the receptor, possibly leading to modified ligand binding and subsequent signal transduction. Unfortunately, the LD between *TLR1*_c and *TLR1*_b was strong enough to make it impossible to select two groups of samples of appropriate size for functional studies that would separate both SNPs. Therefore the actual influences of *TLR1*_b (via upregulation of receptor expression) and of *TLR1*_c on downstream signaling are difficult to estimate independent of each other. Furthermore, *TLR1*_c is in LD (r^2 = 0.83) with another nonsynonymous *TLR1* SNP (*TLR1*_1805; see FIG 3.2A) that leads to an amino acid change from Asn to Ser. Although both hydrophilic acids are structurally similar this change might again lead to altered ligand binding or signaling, which may contribute to the observed associations.

The function of TLR6 instead has not been fully characterized yet, although like TLR1 it is thought to specify or enhance the PAMP sensitivity of TLR2 and contribute to its signaling capabilities through heterodimerization (OZINSKY *et al.* 2000). In our population carriers of the TLR6_a minor allele were strongly protected against the development of atopic asthma. *TLR6*_a is in LD (r^2 = 0.83) with another SNP in the promoter region (*TLR6*_-1401; see FIG 3.2C), again possibly affect-

ing a SRY binding site. MatInspector instead predicts a transcriptional enhancer (C/EBP) to have higher affinity to the mutant state matrix of *TLR6*_a itself. Hence, the same assumptions as made for *TLR1*_b might be true. Consistent with the TLR1 functional results, TLR6 mRNA and protein expression were significantly elevated in people homozygous for the *TLR6*_a minor allele. Additionally, the same upregulated Th1 and diminished Th2 responses could be observed for PBMCs of minor allele carriers when stimulated with the TLR2/6 ligand Zymosan (OZINSKY *et al.* 2000).

Tantisira *et al.* showed an aminoacid change (Ser249Pro = *TLR6*_b) to be associated with asthma in an African American case-control population (N = 149) (TANTISIRA *et al.* 2004), although the direction of the effect was not described. *TLR6*_b conferred a significant risk to develop atopic asthma in our case-control population, supporting a role of TLR6 in the development of atopic disease. Similar to *TLR1*_c, the amino acid change takes place in the LRR region of the protein, potentially affecting ligand binding. At the moment it may only be speculated that the protective *TLR1*_c would lead to an increased downstream signaling and stronger Th1 response upon stimulation, while the risk conferring *TLR6*_b could diminish receptor signaling. As one option to further investigate the actual effects of those amino acid changes, transgenic cell models with constructs carrying the gene coding for the respective protein variant may be generated (as discussed for rare *TLR2* SNPs in 4.5.1.3.).

TLR10, the third TLR which is able to heterodimerize with TLR2 and also with TLR1 (HASAN *et al.* 2005) remains an orphan member among the human TLRs. As it seems to lack a rodent homologue, efforts to identify natural or synthetic ligands are hampered. An association between two SNPs in the coding region of the gene and physician-diagnosed asthma was already observed in a case-control study of European American subjects (N = 1,036) (LAZARUS *et al.* 2004). We found a protective effect of one of those SNPs (*TLR10*_b) on atopic asthma. As the nonsynonymous SNP leads to an Ile to Val transition at amino acid position 775, which is located in the TIR domain, the change might affect intracellular signaling, but rather unlikely ligand recognition. Furthermore, *TLR10*_b is in strong LD ($r^2 = 0.85$) with a SNP in the promoter region of the gene (*TLR10*_-3260; see FIG 4.1). As no specific ligand is known for TLR10, the promoter SNP in LD made it possible to test functional hypotheses to at least some extent. MatInspector predicts that the respective G allele creates a binding site for C/EBP, which might elevate transcription of TLR10. Indeed, the minor allele was associated with a trend to enhance TLR10 expression compared to wild type (FIG. 3.12). Therefore both influences, protein change and higher expression, may account for the observed protective effect.

As expected, when calculating haplotypes for *TLR1*, *TLR6* and *TLR10* SNPs, the protective effects on atopic asthma were even more incisive, culminating in strong protective ORs of 0.42 (*TLR1*_Hc), 0.34 (*TLR6*_Hc) and 0.45 (*TLR10*_Hc). Basically, the respective protective alleles are combined in those haplotypes with their single effects accumulating. *TLR1*_Hc and *TLR6*_Hc occur with 16% and 18%, respectively, therefore having a remarkable impact on the population level. For protective effects the preventable fraction may be calculated (1 – OR). For the frequent *TLR1*_Hc and *TLR6*_Hc the preventable fraction is 1 – 0.42 = 58% and 1 – 0.34 = 66%, respectively. This illustrates that the occurence of the *TLR1*_Hc and *TLR6*_Hc protective allelic states prevents 58% and

66% of all atopic asthma cases in the case-control population; i.e. both protective haplotypes account for the majority of non-atopic non-asthmatics in the population.

Interestingly, frequent polymorphisms in *TLR2* as the connecting link in the TLR heterodimer system, showed only weak evidence for single SNP associations with asthma phenotypes. Originally, *TLR2*_a has been shown to be associated with asthma in children of European farmers (N = 229) (EDER *et al.* 2004). In our population *TLR2*_a showed no significant effects and the protective effect of *TLR2*_c on non-atopic asthma would not withstand the homogeneity analysis, which revealed that the effect was only seen in Dresden, not in Munich or Leipzig (see TABLE 3.7). However, recently it was shown that a TLR2 agonist promotes Th1 response while ameliorating allergic airway response in mice (PATEL *et al.* 2005), backing up a possible role of TLR2 in asthma phenotypes. In line with this assumption, a strong risk effect on atopic asthma could be found for the haplotype *TLR2*_Hc (see FIG 3.6).

4.5.1.2 MULTIPLE TESTING

Due to the large number of SNPs investigated the problem of multiple testing has to be discussed. The more SNPs are investigated and tested for an association in the same set up the more likely a significant association will occur by chance. Several statistical possibilities of correcting for extended multiple testing exist. In general, either more stringent tests are applied (which lower the overall likelihood of significance) or the *P* value threshold of statistical significance is adjusted more conservatively (e.g. $P < 0.01$). However, disadvantages are that true existing effects might be missed and that correcting *P* values may distort the evaluation of significance of a certain association. Reducing the type I error for null associations increases the type II error for those associations that are not null. In this thesis the initially found associations could not only be verified in haplotype and homogenity analyses but they were also strengthend by the functional implications that were evaluated subsequently. Hence, in regard to the shortcomings described above a general correction for multiple testing was not performed on all the data. However, as the associations of *TLR1*, *TLR6* and *TLR10* SNPs are so centrally involved, those effects were checked and would withstand a respective correction (data not shown).

4.5.1.3 TLR2 RARE AMINO ACID SUBSTITUTIONS – CHALLENGING THE COMMON DISEASE / COMMON VARIANT HYPOTHESIS?

All *TLR* polymorphisms so far studied were common and the observations go in line with the classic hypothesis in population genetics that common diseases are caused by common gene variants (LANDER 1996; CHAKRAVARTI 1999; REICH & LANDER 2001).

However, this hypothesis may be challenged in asthma and allergy, which is thought to develop due to multifactorial gene environment interactions. Even common variants may only be effective under certain environmental conditions in a subgroup of the population as shown by Eder *et al.*, when a common TLR2 promoter polymorphism influenced asthma risk only in a setting of high microbial exposure in children living on farms (EDER *et al.* 2004). Thus, it was hypothesized that in complex diseases with multiple genetic factors involved, rare genetic changes with strong functional implication might also exert significant effects on a disease detectable at the population level.

Seven rare TLR2 amino acid changes (MAF 1-3%) had previously been described in the Innate Immunity PGA (www.innateimmunity.net). However, in an initial screening performed in a part of the Munich population (n=368) only two amino acid changes could be confirmed (TLR2_P631H and TLR2_R753Q), which was in concordance with a previous publication dealing with rare TLR2 variations (LEE *et al.* 2006).

To clarify the function of both TLR2 amino acid changes on TLR2 downstream signaling, Dr. C. Kirschning and co-workers determined NFkB (via Luciferase reporter) and IL-8 expression utilizing transfected human HEK 293 cells. Cells bearing the TLR2_753Q vector showed diminished NFkB and IL-8 level after stimulation with TLR2/1 ligand Pam_3CSK_4 compared to the wild type consruct (**FIG. 3.14**), while cells transfected with the TLR2_631H vector showed no drop in downstream signaling. This finding goes well in line with the observed association that TLR2_753Q conferred a risk for a higher geometric mean of total IgE and atopy (**TABLE 3.11**). The TLR2_753Q mutation was initially associated with staphylococcal infections and atopic eczema (11.5% of patients with atopic eczema were identified as heterozygous carriers (AHMAD-NEJAD *et al.* 2004)). This patient subgroup had a 55% risk to cutaneous *S. aureus* colonization and was defined by increased disease severity and highest IgE titers. A more recent study could not confirm an association between TLR2_753Q and atopic eczema (WEIDINGER *et al.* 2006). An association with atopy and elevated serum IgE levels was found more strongly in the Dresden subpopulation. Before unification in 1990, Dresden was a city in former East Germany. Especially day care attendance was characteristic of the former East German lifestyle, which also implicated more frequent early infections. Krämer *et al.* showed in a large cross-sectional survey that children from East Germany that were entering day nursery in the 1st year of life, were at significantly lower risk of developing hay fever and a positive SPT than children attending day care after their 2nd birthday (KRÄMER *et al.* 1999). A prospective study from the USA corroborated these findings (BALL *et al.* 2000). This seems to support the hypothesis that *TLR2* polymorphisms may only influence atopic phenotypes under certain environmental conditions of high microbial exposure. It is also in concordance with the fact that *TLR1*, *TLR6* and *TLR10* rare alleles, that lead to higher expression and signaling, were associated with a reduced risk to develop atopy and atopic asthma.

In general, such effects, that are traceable on a molecular background, may be modulated by environmental factors. While a diminished function of TLR2 may be counterbalanced by a microbe rich environment, a coincidance of both high expression and high exposure might lead to adverse effects. Yet another aspect that may modify single SNP effects and highlights the importance of considering

a genes' *cellular* environment are gene-gene-interactions, as discussed in the following chapter.

4.5.1.4 TLR2 GENE-GENE INTERACTIONS

TLR2 haplotypes offered initial evidence that investigating *TLR2* SNP effects in a more complex and more realistic model revealed a clearer picture of the respective gene variations. Of course, as SNPs do neither occur isolated in one gene nor isolated with respect to other genes, the intergenetic interactions between *TLR2* with *TLR1*, *TLR6* and *TLR10* were determined, and strong effect modifications of *TLR2* SNPs could be specified on all three asthma phenotypes. This phenomenon, also called epistasis, takes place when the action of one gene is modified by one or more others that assort independently. Originally, epistastis described the effect that a gene's phenotype is expressed (the gene is said to be "epistatic"), while another gene's phenotype altered or suppressed is said to be hypostatic. "Fitness epistasis" (where the affected trait is fitness) is one cause of linkage disequilibrium. The original definition was introduced by William Bateson in the year of 1909, while nowadays epistasis mostly describes the more general effects of gene-gene interaction. Mathematically, an interaction occurs when the effect of one single SNP is modified by another SNP in a non-multiplicative way (i.e. SNP A: OR = 1; SNP B: OR = 0.5; SNP A stratified for SNP B: OR =]0.5[) (DIZIER & CLERGET-DARPOUX 1986; MORRIS & WHITTAKER 1999). However, others have assumed that epistasis between loci refers to departure from additivity on the penetrance scale (RISCH *et al.* 1993; TIWARI & ELSTON 1998).

Strikingly, not only significant effect modifications were found for *TLR2*_c on non-atopic asthma, but the respective *TLR2*_c effects became significant and strongly protective by stratification for *TLR1*_c and *TLR10*_b (OR = 0.39 (95% CI: 0.24-0.66) and OR = 0.41 (95% CI: 0.23-0.74), respectively). This emphasises the fact that especially in complex diseases like asthma, single SNP analyses may not be sufficient to evaluate the contribution of one gene to a specified disease in detail. Preliminary negative results may mislead interpretations and gene implications in a disease may be missed due to insufficient complexity of the investigated model. In this regard sex stratification should be mentioned, which recently came into focus. Although the idea of sex stratification is not new, only in 2006 sex-specific heritability and genome-wide linkages for some quantitative traits have been evaluated in large-scale (WEISS *et al.* 2006). Of course, sex-specific effects are mainly based on the genotypic differences between women and men (including epigenetics and imprinting (see WOOD & OAKEY 2006) and ultimately represent another kind of epistasis.

Basically, genes may interact with the environment via their translated protein, although vice versa the environment may itself directly or indirectly affect gene expression (see JAENISCH & BIRD 2003), which would be an example of gene-environment interaction. In nearly scale-free protein-protein interaction networks, or "interactome" networks (JEONG *et al.* 2001) a small but significant proportion of proteins exist, the "hubs", which interact with many partners. Both biological and non-

biological scale-free networks are particularly resistant to random node removal but are extremely sensitive to the targeted removal of hubs. One TLR may not have many TLR partners to interact with, but especially the different TLR combinations in the heterodimer system provide a variable fundament to recognise an exceptional variety of molecular patterns from the environment. These complex environment <> TLR interactions, which are dynamically regulated both in time and place (TRIANTAFILOU *et al.* 2006) present TLRs as the interface between the environment and subsequent immune responses, very similar to the above mentioned hubs. TLRs funnel all different ligand binding to very few adaptors (e.g. MyD88; see ODA & KITANO 2006) which are crucial for subsequent signal transduction that again becomes more and more branched further downstream (reflecting the bow-tie architecture mentioned in 1.2.4). Hence, while central adaptor molecules like MyD88 seem to present true hubs, one may speculate that TLRs - acting in close proximity - present at least "feeders" of such hubs. Indeed, the TLR heterodimer variations contributed a remarkable impact on protein expression and signaling in a way that even in the general population (with supposedly low levels of microbe exposition) a strong protection from atopic asthma was observed.

4.5.1.5 INTERGENETIC LINKAGE

It has to be noted that the polymorphisms studied in this analysis do not act in isolation but that linkage disequilibrium with other SNPs in the respective genes and in between *TLR1*, *TLR6* and *TLR10* exists, as all three genes are located within close vicinity on chromosome 1p14 (**FIG. 3.8**). Inter-gene LD may influence the observed associations on a population level, but it can not explain the distinct and independent *TLR1* and *TLR6* SNP effects in cytokine expression observed after TLR1 and TLR6 specific heterodimer stimulation. However, effects of highly correlated SNPs *within the same gene* are less easy to distinguish. Regarding only SNPs with a MAF > 0.1, *TLR1*_b is in strong LD ($r^2 > 0.8$) with a total of 12 SNPs known in the TLR1 gene (**FIG. 3.2A**). Of these, three may alter transcription factor binding in regulatory regions of the gene (**TABLE 3.2A**). At this point, it is not possible to dissect the contribution of every single SNP tagged by *TLR1*_b on the effects associated with the analyzed tagging SNP *TLR1*_b, but as these SNPs seem to be inherited *en bloc*, a combination of SNP effects may exactly resemble the naturally occurring biological situation. It may be speculated that some (or all) of the three SNPs of putative regulatory function in this block may influence transcription factor binding resulting in differential expression of TLR1 on the mRNA and protein level. *TLR1*_c — leading to an amino acid change — is in a certain LD with *TLR1*_b ($r^2 = 0.73$) and may contribute an effect on cytokine expression after TLR stimulation. Similar arguments are valid for *TLR6*, where the studied SNP (in a regulatory region) is linked to another putative

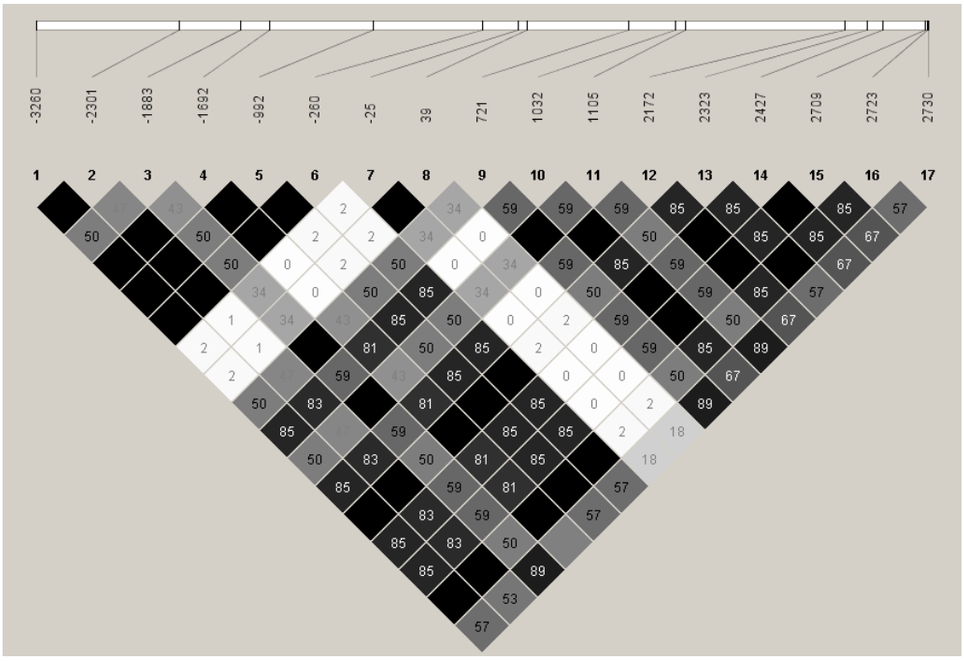


FIG. 4.1 *LD correlation coefficients (r^2) for TLR10 SNPs with a MAF > 0.1. Pure white fields represent $r^2 = 0$, shades of grey $0 < r^2 < 1$ and black fields indicate total LD ($r^2 = 1$).*

functional SNP in the gene, and *TLR10*, where the associated SNP *TLR10*_b is linked with ten other SNPs (**FIG. 4.1**), though FastSNP did not predict significant TF binding changes.

4.5.2 THE ROLE OF VIRAL RECOGNITION

Heterodimers like TLR1/2 and TLR6/2 recognize bacterial components. However, a group of intracellular TLRs (TLR3, TLR7, TLR8 and TLR9) is known to play an important role in viral recognition. TLR3 was the first identified antiviral TLR. It forms homodimers, recognizes dsRNA and has been proposed to play a role in host defense against viruses (ALEXOPOULOU *et al.* 2001), although recent studies questioned this (EDELMANN *et al.* 2004). Consistent with literature no association of *TLR3* variations with asthma was found.

Apart from synthetic compounds TLR7 and TLR8 recognize single-stranded RNA from viruses (e.g. HIV) (HEIL *et al.* 2004) and they are highly homologous to each other. As their genes are located on the X chromosome, the association analysis was performed separately for girls and boys (that are hemizygous). No significant effects could be found on asthma when applying the dominant model

for girls, and the codominant model for boys. Hence, concerning TLR3, TLR7 and TLR8 (that recognize especially viral RNA) it seems as if genetic variations cannot readily be associated with atopic diseases. It may be speculated that the only occasionally occuring viral infections during early childhood (in part due to increased hygiene standards) may be an insufficient simulus (independent of the genetic make-up of the respective TLRs) to confer a general protective effect on atopic diseases. However, this does not imply that specific activation of those TLRs may not be beneficial to prevent the development of asthma (as discussed in 4.6.1.3.).

TLR9 binds unmethylated CpG containing DNA from both bacterial and viral origin. This binding has been shown to result in strong immunomodulatory and anti-allergic activities (BAUER *et al.* 2001; see HORNER & RAZ 2003). An association of *TLR9*_b with asthma, although suggested among European Americans (N = 219) (LAZARUS *et al.* 2003), could not be replicated in our case-control population. Instead we found that promoter SNP *TLR9*_a and a frequent haplotype (*TLR9*_Hb) were associated with moderately increased risk to delevop asthma. In *in silico* prediction the minor allele seems to change promoter matrices in a way that might activate transcription. Hence, insufficient TLR9 expression might account for a higher risk to develop asthma. CpG motifs are able to induce an innate response dominated by type 1 interferons (IFNγ) as well as IL-12, which counterbalance the allergic Th2 responses. Accordingly, pretreating mice with immunostimulatory sequences of DNA containing CpG motifs elicited a strong anti-Th2 response (IKEDA *et al.* 2003) including inhibition of Th2 cell-derived mast cell growth factors in the lung. Recently it was recognized that such immunostimulatory DNA sequences (ISS) inhibit Th2 driven experimental asthma by inhibiting T cell reactivity and inducing a Treg cell response (CHISHOLM *et al.* 2004), suggesting selective TLR9 agonists for therapeutic purposes (ZUANY-AMORIM *et al.* 2002). Together with the association data presented in this thesis it supports TLR9 as a possible target for asthma intervention.

4.6 TLRs: TARGETS FOR ASTHMA PREVENTION?

4.6.1 EXISTING APPROACHES

Unfortunately, despite the advanced understanding of the immune mechanisms at the cellular level (with Th2 cytokines predominating and orchestrating the asthma phenotype), no effective causal or preventive treatment strategies are available so far. However, pioneer studies gave a first general idea that house dust endotoxin levels were lower in homes of allergen-sensitized versus non-sensitized enfants, providing first clues that indoor endotoxin exposure in early life may lower allergen sensitization later-on (GEREDA *et al.* 2000A; GEREDA *et al.* 2000B). Further support was offered by Braun-Fahrlander *et al.* (2002) who identified livestock as a main source of endotoxin in the homes of farmers and who confirmed inverse association between endotoxin exposure and atopy rates in the farmers' family members. However, contradictory effects of controlled endotoxin exposure were

found in animal models (PURKERSON & ISAKSON 1994; GERHOLD *et al.* 2002; GERHOLD *et al.* 2003). Therefore, it is necessary to identify specific microorganic components and their specific recognition pathways to be able to consequently ensure a protective effect on atopic phenotypes.

Although the use of TLR ligands in treating allergic diseases should be approached with caution (AGRAWAL *et al.* 2003; EISENBARTH *et al.* 2004; REDECKE *et al.* 2004), there is no doubt in the great potential offered by microbial products in allergy prevention and therapy, which are believed to target the TLR network.

4.6.1.1 TARGETING TLR4

Nonsynonymous SNPs in TLR4, leading to amino acid changes in the receptor of lipopolysaccharide (HOSHINO *et al.* 1999), may predispose people to develop septic shock with gram-negative microorganisms (LORENZ *et al.* 2002). Intriguingly, a molecular mimic of a portion of the endotoxin molecule has recently been successfully tested as TLR-blocking compound against sepsis. The mentioned amino acid changes in TLR4 (Asp299Gly and Thr399Ile) have been implicated in COPD as well, but could not yet be directly associated with asthma itself (RABY *et al.* 2002). However, they modified endotoxin effects on asthma (WERNER *et al.* 2003) and the severity of atopy in asthmatics (YANG *et al.* 2004). In our case-control population no effect could be found for Asp299Gly (*TLR4*_c) which is in high LD with TLR4_Thr399Ile. Though promoter SNP *TLR4*_a showed a significant risk effect on asthma, an accumulation of risk alleles in the heterozygote cases and not in the homozygotes mainly accounted for the observed odds ratio. Additionally none of the haplotypes showed significant results, suggesting that a direct role of *TLR4* SNPs in asthma susceptibility remains questionable.

However, TLR4 came in focus of possible therapeutic use as in CD14, which forms complexes with LPS (thereby initiating TLR4 signaling), a certain promoter polymorphism could be associated with increased IgE levels in atopic patients (BALDINI *et al.* 1999). Consequently, several TLR4 interacting compounds have been developed recently. In this context, aminoacyl glucosaminide phosphates (AGPs) represent well established Th1 inducers, which may effectively dampen Th2 driven immune responses in atopic or asthmatic individuals. Additionally it has been proven that TLR4 signaling associates with increased production of IL-10, an immunosuppressive cytokine. In spite of this, the natural ligands of TLR4 (LPS and respiratory syncytial virus) are known to exacerbate established asthmatic airway disease (KURT-JONES *et al.* 2000). Thus, antagonists of TLR4 seem to be more beneficial in particular to prevent or treat episodes of asthma exacerbations.

4.6.1.2 TARGETING TLR5

Akira determined that TLR5 is activated by flagellin, a protein in the flagella of bacteria. A STOP codon polymorphism (*TLR5*_a) that abolishes the function of the receptor has been associated with susceptibility to Legionnaires' disease (HAWN *et al.* 2003). In 2006 it was reported that this nonworking variant was about half as common in lupus (a chronic autoimmune disease) patients as it was in their unaffected relatives, suggesting that inactivating TLR5 may protect people against the disorder. Merx *et al.* found *TLR5*_a to be functionally relevant in transiently transfected CHO-K1 cells which could not be shown for the nonsynonymous *TLR5*_b and *TLR5*_c (MERX *et al.* 2006). Until now changes in *TLR5* had not been implicated in lung diseases and neither did we find an influence of *TLR5* SNPs or *TLR5* haplotypes on asthma.

4.6.1.3 TARGETING TLR7 AND TLR8

Recently it was demonstrated that the synthetic TLR7 ligand imidazoquinoline, an antiviral compound and the first identified TLR7 ligand, prevents allergen-induced airway hyperresponsiveness and eosinophilia in a murine model of allergic asthma (MOISAN *et al.* 2006). Furthermore, TLR7/8 binding 3M003 (a more recent analogue of imiquimod and resiquimod) had immune modulating properties as they suppressed established IgE titres (JOHANSEN *et al.* 2005). Taking into account the role of viral infections in disease exacerbations of asthma, application of these agonists might present interesting therapeutic alternatives in therapy of these conditions. A recent report demonstrated in mice that the stimulation of a predominantly Th1 cytokine profile by these compounds might also serve to counterbalance the dominant Th2 response in allergic asthma (QUARCOO *et al.* 2004) and appropriate clinical studies are now anticipated.

4.6.2 NEW APPROACHES PROPOSED BY THIS THESIS

It is surprising that the TLR2 heterodimer system has not been implicated with asthma and atopy so far. One reason might be that paradoxical functional effects have been shown for TLR2 alone. *In vitro* stimulation of human monocyte-derived DC with TLR2 ligands failed to produce IL-12 p70 and interferon-inducible protein (IP)-10 but resulted in the release of the IL-12 inhibitory p40 homodimer, producing conditions that are predicted to favour Th2 development (RE & STROMINGER 2001). Indeed in a mouse model of ovalbumin (OVA) sensitization, TLR2 synthetic ligand Pam_3Cys, given at the time of sensitization, increases Th2 responses and leads to aggravation of the asthma phenotype. In parallel, Pam_3Cys increased bone marrow-derived DC maturation and their production of Th2-associated cytokines like IL-13, GM-CSF and IL-1 (REDECKE *et al.* 2004). Similarly, exposure to ovalbumin associated with peptidoglycan leads to airway hypersensitivity responses (CHISHOLM *et*

al. 2004), although the involvement of TLR2 in the effect of this PAMP is now discussed. However, when administered before sensitization, TLR2 agonists (peptidoglycan from *S. aureus* and PamCys) were recently shown to decrease additional allergen-induced parameters of inflammation in mice (VELASCO *et al.* 2005).

Hence it seems as if the downstream effects of TLR stimulation do in particular depend on already small changes in ligand structure (even if they are basically ligands for the same TLR). The fact that TLR2 heterodimerization has not been considered might be one reason why contradictory results have been found for "general TLR2 stimulation", which obviously has to be differentiated. Diacyl lipopeptides have been described to require TLR2 and TLR6 for signaling, whereas triacylated synthetic compounds like Pam_3CSK_4 are able to activate immunocompetent cells independent of TLR6 and mainly through TLR2/TLR1 heterodimers (TAKEUCHI *et al.* 2001; ALEXOPOULOU *et al.* 2002; MORR *et al.* 2002; TAKEUCHI *et al.* 2002; AKIRA 2003). In this thesis significant modulations of Th1/Th2 downstream signaling could be observed with Pam_3CSK_4 and Zymosan stimulation. While the specificity of Pam_3CSK_4 for TLR2/TLR1 heterodimers is well described, the role of Zymosan in affecting the immune system is far more complex. Originally, it was suggested that TLR2 and TLR6 together coordinate macrophage activation by Zymosan (OZINSKY *et al.* 2000). However, other sources suggested Dectin-1 as the main receptor for Zymosan (BROWN & GORDON 2001), and recently Card9 could be identified to control Dectin-1 mediated myeloid cell activation upon fungal stimulation (GROSS *et al.* 2006). It is therefore surprising that nearly the same relative expression differences were found with Zymosan stimulation depending on the respective *TLR1*/*TLR6*/*TLR10* cluster genotype. Though Dectin-1 and TLR2 signaling seem to function together (YADAV & SCHOREY 2006), it has to be assumed that the described TLR2 heterodimer variations obviously have an independent influence sufficient to exert a remarkable effect on downstream cytokine expression levels.

Intriguingly, the genotype dependent protective effects on atopic asthma and changes in downstream signaling seemed to be independent of general environmental factors. In particular, ORs in literature demonstrating the protective effect of farming environments (OR 0.59 (95% CI: 0.37-0.95) (ERNST & CORMIER 2000) and OR 0.65 (95% CI: 0.39-1.09) (VON EHRENSTEIN *et al.* 2000)) fit the range of genotype effects on atopic asthma observed in this thesis surprisingly well, probably based on the higher TLR heterodimer expression that lead to a general enhanced sensitivity to PAMP recognition from early life on. The possible therapeutical approach would follow an inverse strategy: stimulation of TLR heterodimers during early childhood or even during embryogenesis (i.e. changing the environment) could simulate the protective allelic states and modify the adaptive immune system in a beneficial way (by dampening Th2 and supporting Th1 responses), independent of the respective *TLR1*/*TLR6*/*TLR10* cluster genotype.

In this regard it is very helpful that more and more highly specific artificial TLR ligands are synthesized. Artur Ulmer and colleagues investigated the influence of different structural elements of lipopeptides with respect to their ability to induce TNFα release via activation of TLR2 and TLR2 heterodimers (BUWITT-BECKMANN *et al.* 2006). Especially MALP2 (TLR2/TLR6 dependent), $PamOct_2C$-$(VPGVG)_4VPGKG$-NH_2 (TLR2/TLR1 dependent) and Pam_2CSK_4 (TLR2 de-

pendent but TLR1- and TLR6 independent) and combinations of those could offer new exciting opportunities to stimulate the heterodimer system with clearly enhanced specificity. This could e.g. be tested in an OVA mouse model (KUNG *et al.* 1994; BLYTH *et al.* 1996; XISTO *et al.* 2005) in which different TLR patterns would be stimulated at different time points with a subsequent assessment of *in vivo* and *in vitro* respiratory mechanics (BATES *et al.* 1985), airway responsiveness (LIMA *et al.* 2002) and morphometric analysis (WEIBEL *et al.* 2007).

However, it may be important to consider TLR genetics in order to optimise a respective TLR stimulation approach. One example are *TLR2* gene-gene interactions which confer strong effect modifications of *TLR2* polymorphisms on the development of different asthma phenotypes. TLR2 stimulation may have to be adapted — depending on allelic states in *TLR1*, *TLR6* and *TLR10* — to confer the most beneficial effect on the development of asthma. Of course the cross-talk of downstream signaling is far more complex (see ODA & KITANO 2006), but those network interaction effects are yet largely unknown.

Combination of the aspect of specific TLR pattern stimulation, gene-gene interactions and gene-environment interactions may lead to the inconsistent observations concerning general protective effects of farming. While protection from the development of allergies could be frequently shown, some large studies found no association between farming and asthma (BRAUN-FAHRLANDER *et al.* 1999; REMES *et al.* 2002). Apart from the fact that very different parameters underlie the general term of "farming" (e.g. child's farm activities, mother's farm exposure during pregnancy, consumption of farm milk, stable or barn visits, contact to different animal species etc.), those parameters interact with the immune system via the TLR interface, and may even control TLR expression levels to a certain degree (EGE *et al.* 2006). These interactions may strongly depend on specific time frames or "critical periods of development", in which environmental stimuli — including nutrition — can induce persistent changes in gene expression and metabolism (WATERLAND & GARZA 1999; MCMILLEN & ROBINSON 2005), referred to as epigenetics[1].

4.6.3 FUTURE PROSPECTS

This complex interplay also emphasises that larger studies with more participants and a more detailed differentiation of environmental parameters will probably be insufficient to define the individual risk of a person or provide final clues for a specific therapy or preventive intervention. Rather it should now be the focus to understand how an individual person's genetic architecture reacts to a certain environment. Detailed genetic mapping using microarrays containing SNP, transcriptome and proteome libraries combined with detailed phenotyping may link certain patterns to specific environmental conditions. In the long run protective patterns may emerge which could be connected with a variety of environments and would therefore be quite flexibel. Understanding the dynamics of

[1]Epigenetics is the study of mitotically (and potentially meiotically) heritable alterations in gene expression or regulation by modification of DNA and chromatin.

molcular interactions, determining when, where, and how complexes form and network components interact, will reveal its functional consequences.

Whatever predisposes for asthma genetically, due to the significant improvements in medical supply in the last decades disease conferring allelic states will accumulate in our gene pool. From an evolutionary point of view this is a two sided story. On one hand genetic heterogeneity provides greater developmental stability and the basis for evolutionary adaptation. On the other hand we have to decide if we want to carry the burden of more and more diseases that we want to or have to treat day-to-day while keeping the blueprint of life untouched. In the case of severe diseases manifesting already during embryogenesis it may become even necessary to intervene at a much earlier step. This might also be true for the prevention of asthma, as the underlying immunological courses are set very likely already *in utero*. If exogenous stimulation of TLR patterns succeeds or if someday the manipulation of TLR expression levels may provide balanced signaling and appropriate Th1/Th2 priming from the very beginning of a new life, remains to be seen. However, in this thesis new targets were identified of substantial value for both of these approaches. With this new understanding of the molecular mechanisms protecting from atopic diseases the possibilities of adaptation to different environmental conditions will further increase.

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The following figures and illustrations were taken or adapted from:

Fig. 2.2: www.wikipedia.de

Fig. 2.6: www.variom.de

Fig. 2.7/2.8: www.scq.ubc.ca

Fig. 2.9: www.wikipedia.de

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PUBLICATIONS

Depner M, **Kormann MSD**, Klopp N, Illig T, Vogelberg C, Weiland SK, von Mutius E, Combadière C, Kabesch M (2007). "CX3CR1 polymorphisms are associated with atopy but not asthma in German children. " Int Arch Allergy Immunol **144**(1): 91-94.

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ABSTRACTS NATIONAL/INTERNATIONAL

Kormann MSD, Depner M, Hartl D, Klopp N, Illig T, Adamski J, Vogelberg C, Weiland SK, von Mutius E, Kabesch M "Polymorphismen in Toll-like Rezeptor 2 Komplexen schützen vor Asthma im Kindesalter und beeinflussen die T-Zell vermittelte Immunantwort"

Gesellschaft für Pädiatrische Pneumologie - München, 2007; Poster Prize (500 Euro)

Kormann MSD, Depner M, Klopp N, Illig T, Fritzsch C, Weiland S, Leupold W, von Mutius E, Kabesch M "Protective effect of a CX3CR1 polymorphism on the development of bronchitis"

Pinto LA, Cameron L, **Kormann MSD**, Depner M, Weiland SK, von Mutius E, Lohoff M, Kabesch M "A complete mutation screening of IRF1, an asthma candidate gene"

American Thoracic Society - San Diego, 2006 (Speaker)

Kormann MSD, Depner M, Klopp N, Illig T, Vogelberg C, Fritzsch C, Weiland SK, von Mutius E, Kabesch M "A comprehensive analysis of Toll-like receptor 1-10 polymorphisms and their role in childhood asthma"

Lenz A, **Kormann MSD**, Schedel M, Depner M, Klopp N, Illig T, Fritzsch C, Vogelberg C, Weiland SK, von Mutius E, Kabesch M "Genetic alteration in the mammalian chloride channel CLNS1A influence lung function in children"

Araujo-Pinto L, Depner M, Steudemann L, **Kormann MSD**, Klopp N, Illig T, Vogelberg C, Weiland SK, von Mutius E, Kabesch M "Lack of association between IL15 polymorphisms and atopic phenotypes in a large sample of German children"

European Respiratory Society, München 2006

Kormann MSD, Carr D, Fritzsch C, Weiland S, Leupold W, von Mutius E, Kabesch M "Effects of genetic variations in TLR 1 and TLR 5 on the development of atopy and asthma"

World Allergy Congress - München, 2005

Kormann MSD, Carr D, Fritzsch C, Weiland S, Leupold W, von Mutius E, Kabesch M "Die Effekte von Toll-like Rezeptor (TLR) Varianten auf Asthma und Atopie"

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Nationales Genomforschungsnetz - Berlin, 2004

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Nationales Genomforschungsnetz - Berlin, 2004

Curriculum Vitae

PERSONAL DATA

Name:	Michael Sebastian Daniel Kormann
Date and place of birth:	13th of April 1978 in Munich, Germany
Address:	Fred-Hartmann-Weg 21, D-85435 Erding, Germany
Phone number:	home +49-81222275268, mob +49-1795313561
email:	michael.kormann@med.uni-muenchen.de
Nationality:	German

EDUCATION

1997	Abitur, *Anne-Frank-Gymnasium*, Erding
11/1997 - 11/2002	Study of Biology at the *Technical University of Munich* (TUM), Germany Specializations: Genetics, Biochemistry & Virology Concluded with M.Sc. Degree ("Diplom-Biol. Univ."), grade: "very good"
10/2000 - 07/2001	Seminar on Patent law under the supervision of Prof. Dr. Werner Schmitt-Fumian (Beetz & Partner, Patent lawyers) with certification
01-10/2002	Graduation research project at the *Max-Planck-Institute of Psychiatry*, Munich, Germany; supervisor: Prof. Dr. W. Wurst Thesis: "Effects of the conditional knockout of *corticotropin-releasing-hormone-rezeptor 1* on basal and stress-induced mRNA Expression in the limbic System"
03/2003 - 02/2007	PhD thesis under the supervision of PD Dr. med. Michael Kabesch at the KUBUS Research Center, University Children's Hospital, *Ludwig-Maximilians-University*, Munich. Title: "Toll-like receptor heterodimer variants influence Th1/Th2 signaling and protect from the development of childhood asthma."
02/2007 - now	Postdoc in the research group "Molecular Pneumology & Experimental therapy" at the KUBUS Research Center.

OTHER

Language skills:	German, mother tongue; English, fluent
Computer/Software:	Experienced in Win 2000/XP, Word 2000/2003, Excel 2000/2003, Adobe Photoshop, Adobe Illustrator, Adobe InDesign, VectorNTI, SigmaPlot
Hobbies:	Playing the piano, singing, movies, diving, chess