Available online at www.sciencedirect.com
ScienceDirect
Vaccine 25 (2007) 306–313
Vaccine
www.elsevier.com/locate/vaccine

Impact of genetic variants in IL-4, IL-4 RA and IL-13 on the anti-pneumococcal antibody response

Selma P. Wiertsema [a,b,1], Gareth Baynam [b,1], Siew-Kim Khoo [b,1], Reinier H. Veenhoven [c], Niels van Heerbeek [d], Guicheng Zhang [b], Ingrid A. Laing [b], Ger T. Rijkers [a,e], Jack Goldblatt [b,f], Elisabeth A.M. Sanders [c], Peter N. Le Souëf [b,*]

[a] *Department of Paediatric Immunology, Wilhelmina Children's Hospital, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands*
[b] *School of Paediatrics and Child Health, University of Western Australia, Perth, 6008 WA, Australia*
[c] *Department of Paediatrics, Spaarne Hospital, Hoofddorp, The Netherlands*
[d] *Department of Otorhinolaryngology, Radboud University Nijmegen Medical Centre, The Netherlands*
[e] *Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, The Netherlands*
[f] *Genetic Services of Western Australia, Perth, 6008 WA, Australia*

Received 21 December 2005; received in revised form 30 June 2006; accepted 20 July 2006
Available online 2 August 2006

Abstract

Background: Significant differences in immune responses upon vaccination have been described, suggesting genetics are important in determining the magnitude of vaccine responses. The interleukin (IL)-4 pathway, including IL-4, IL-13 and the IL-4 receptor α chain (IL-4 Rα), is central to humoral responses and therefore could have an impact on vaccine responsiveness.
Objective: To investigate whether single nucleotide polymorphisms (SNPs) in the IL-4, IL-13 and IL-4 RA genes influence pneumococcal serotype-specific IgG antibody responses.
Methods: SNPs in the IL-4 gene (C −589T, G2979T), the IL-13 gene (G −1112A, Arg130Gln) and in the IL-4 RA gene (Ile50Val, Gln551Arg) were investigated in isolation and in combination, for their influence on serotype-specific IgG antibody responses upon combined pneumococcal conjugate and polysaccharide vaccinations in children with a history of recurrent otitis media.
Results: Lower antibody responses were observed for alleles previously associated with atopy, IL-4 −589T, IL-4 2979T and IL-4 Rα 551Gln. Effects were stronger in gene haplotype combinations or in multiple haplotype combination analyses.
Conclusion: This study highlights the importance of host genetic factors in vaccine responses. Furthermore, it supports the approach of studying the effect of combinations of multiple alleles, in haplotypes or in combinations of haplotypes, on complex phenotypes within a biological pathway.

Keywords: Single nucleotide polymorphisms; Haplotypes; Pneumococcal vaccine responsiveness

1. Introduction

Streptococcus pneumoniae may cause severe invasive infections including sepsis, and meningitis or lower respiratory tract infections, like pneumonia. It is also the most commonly reported bacterial cause of upper respiratory tract infections such as otitis media [1,2]. Host defense against *S. pneumoniae* depends largely on opsonization by antibodies

* Corresponding author at: University of Western Australia, School of Pediatrics, GPO Box D184, WA 6840, Perth, Australia.
Tel.: +61 8 9340 8173; fax: +61 8 9388 2097.
E-mail address: plesouef@paed.uwa.edu.au (P.N. Le Souëf).
[1] These authors contributed equally.

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.07.024

and complement [3], followed by phagocytosis and intracellular killing by leukocytes and macrophages [4,5]. Prevention by vaccination is the favoured strategy against pneumococcal infection because of the persisting mortality and morbidity associated with pneumococcal diseases despite antibiotic treatment, and the rapid emergence of multi-drug resistant *S. pneumoniae* [6]. Current pneumococcal vaccines contain capsular polysaccharides from the most prevalent pneumococci and aim to induce protective anti-capsular serotype-specific opsonizing IgG antibodies.

The optimal design of vaccines requires understanding of the factors controlling disease and immune pathways. Significant differences in immune responses upon vaccination between individuals and different ethnic groups have been described, suggesting genetic influences are important in determining the magnitude of vaccine responses [7,8]. The relative contributions of genetic and environmental factors to vaccine responses are yet to be determined, but associations between specific genes and vaccine antibody responses have been described [8,9]. To date, research on genetic influences on vaccine responses has mainly focused on human leukocyte antigen (HLA) alleles [10,11] and the immunoglobulin allotype genes [12,13]. Clearly, many additional genes may be involved and therefore a systematic approach is needed to prioritise which genes to examine. We investigated genetic variation in interleukin 4 (IL-4), IL-13 and the IL-4 receptor α chain (IL-4 Rα), as they are components of the pathway regulating antibody responses by B cells. IL-4 and IL-13 are pleiotropic cytokines produced by mast-cells, basophils, and T cells [14]. They are T helper 2 cytokines that trigger isotype switching from IgM to IgE in B cells [15]. Furthermore, they enhance the expression of surface molecules, such as the IL-4 Rα chain, the low affinity receptor for IgE (FcεRII, CD23) and MHC class II, and down-regulate the IgG type I receptor (FcγRI). Moreover, IL-4 is necessary for the promotion of its own production [16]. IL-13 shares several biological functions with IL-4 [17,18], but also has unique roles in mediating immune responses [19,20]. The receptors for IL-4 and IL-13 share a common α chain: IL-4 Rα. IL-4 Rα dimerizes with the common γ chain in the IL-4 receptor, which is expressed on T and B cells, and with the IL-13 Rα1 chain in the IL-13 receptor, which is expressed on B cells only [14,21]. Polymorphisms in genes coding for these cytokines and their shared receptor, including IL-4 C −589T [22], IL-4 G2979T [23], IL-4 Rα Ile50Val [24], IL-4 Rα Gln551Arg [25], IL-13 G −1112A [26], and IL-13 Arg130Gln [27] have been associated with atopy and asthma phenotypes. Furthermore, atopy has been associated with altered kinetics of the maturation of vaccine responses [28] and atopic eczema has been associated with delayed maturation of the antibody response to pneumococcal vaccine [29]. Therefore, this study investigated the hypothesis that single nucleotide polymorphisms (SNPs) in IL-4, IL-4 RA and IL-13, genes previously associated with atopy, in isolation and in combination, would be associated with impaired antibody responses upon combined pneumococcal conjugate and polysaccharide vaccinations.

2. Materials and methods

2.1. Patients and vaccinations

Serum and DNA was available from 121 randomly selected children with recurrent otitis media participating in one of two randomised controlled pneumococcal vaccination trials investigating prevention of recurrence of otitis media in The Netherlands [30,31]. Both studies were approved by the ethical committees of participating hospitals and institutions. Written informed parental consent was obtained from all subjects.

Pneumococcal polysaccharide-specific IgG antibody responses against the seven conjugate vaccine capsular polysaccharide 4, 6B, 9V, 14, 18C, 19F, and 23F were ascertained.

The first trial group consisted of 89 children aged 1–7 years who had a history of two or more physician diagnosed episodes of acute otitis media (AOM) in the previous 12 months. These children received the seven-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth Pharmaceuticals, Philadelphia, PA, PCV7). Children below 24 months of age received a second dose of PCV7 4 weeks later. After pneumococcal conjugate vaccination all children received a 23-valent pneumococcal polysaccharide booster vaccination 6 months later (Pneumune®, Wyeth Pharmaceuticals, Philadelphia, PA, PPV23).

The second trial group consisted of 32 children aged 2–7 years with a history of at least two prolonged periods of bilateral otitis media with effusion (OME), each lasting 3 months or longer and documented by an ENT specialist. These children were vaccinated with PCV7 once followed by a PPV23 booster vaccination 4 months later.

Since we have previously shown that both groups of children display similar antibody responses after vaccination, these groups were pooled for data analysis [32].

PCV7 consists of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to mutant non-toxic diphtheria toxin (CRM_{197}). PPV23 consists of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

2.2. Genotyping and haplotyping

Genomic DNA was extracted from whole blood, collected at study entry, using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Patients were genotyped for SNPs in IL-4 (C −589T, G2979T), IL-4 Rα (Gln551Arg) and IL-13 (G −1112A) using polymerase chain reaction (PCR) and restriction enzyme digestion. The IL-4 Rα Ile50Val and IL-13 Arg130Gln SNPs were determined using denaturing high-performance liquid chromatography (dHPLC) using an automated Varian HPLC system (Varian Helix-

Table 1
Investigated SNPs, amplification primers and restriction digestion enzymes

SNP[a]	dbSNP identifier	Primer	Restriction enzyme
IL-4 C −589T	rs2243250	F: ACTAGGCCTCACCTGATACG; R: GTTGTAATGCAGTCCTCCTG	*Bsm*FI
IL-4 G2979T	rs2227284	F: TAGGTCCTGGGCTTCACAG; R: TTAGCTCTCTTTGGTAAATAGGGAA	*Hin*fI
IL-4 Rα Ile50Val	rs1805010	F: GCAAGAGAGGCAACCCTA; R: GCCTCCGTTGTTCTCAG	dHPLC
IL-4 Rα Gln551Arg	rs1801275	F: GCCCCCGTCTCGGCCCCCACCAGTGGCTACC; R: GCCCCAAACCCACATTTCTCTGG	*Msp*I
IL-13 G −1112A	rs1800925	F: CGAGGACAGGACGGAGGGAGCCT; R: GTCGCCTTTTCCTGCTCTTCCCG	*Bst*UI
IL-13 Arg130Gln	rs20541	F: CTTCCGTGAGGACTGAATGAGACAGTC; R: GCAAATAATGATGCTTTCGAAGTTTCAGTGGA	dHPLC

Underlined base in primers indicate a base change to create restriction enzyme site.
[a] Amino acids numbered from the beginning of the mature protein.

System®) [33]. See Table 1 for SNP, primer and restriction enzyme details. Haplotypes were inferred using PHASE2.1 (http://www.stat.washington.edu/stephens/software.html) [34,35].

2.3. Pneumococcal antibody responses

Blood samples for determination of pneumococcal antibodies were obtained 4 weeks after the pneumococcal polysaccharide booster vaccination. Serum was isolated and stored at −20 °C until analysis. Post vaccination IgG antibody levels to all PCV7 serotypes, 4, 6B, 9V, 14, 18C, 19F and 23F, were measured by ELISA as described previously [36]. Minimal detection levels for these pneumococcal serotypes in our assay were 0.03, 0.09, 0.12, 0.61, 0.05, 0.11, and 0.08 μg/ml, respectively. All sera were pre-incubated overnight at 4 °C with pneumococcal cell wall polysaccharide (CPS) in diluting buffer for blocking of non-specific anti-CPS antibodies (50 μg/ml; Statens Serum Institute, Copenhagen, Denmark) [37]. The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardisation [38].

2.4. Statistical analyses

The IgG antibody levels appeared to be positively skewed. Consequently, their geometric means (GM) were calculated after applying a logarithmic transformation. A general linear model was used to compare the geometric means between the genotypes and haplotypes, adjusted for the covariates of interest, namely age, gender, type of ear disease, number of PCV vaccinations and total IgE levels. When one of the homozygous genotype frequencies was less than 10%, this genotype was pooled with the heterozygotes before testing. Multiple comparisons were not adjusted for as the analyses were based on an *a priori* hypothesis, but were dealt with by describing all statistical analyses carried out [39,40]. *p*-Values less than 0.05 were considered statistically significant. SPSS 12.0.1 for windows (SPSS Inc., Chicago, IL) was used for all statistical analyses.

3. Results

Six SNPs in three genes in a pathway known to influence antibody production by B cells were studied for their influence on antibody production (Table 1). All genotype frequencies were in Hardy–Weinberg equilibrium. As expected, given their co-location on chromosome 5q31, significant linkage was observed between SNPs in the IL-4 and IL-13 genes (Table 2).

3.1. Analyses by genotype

Pneumococcal-specific IgG antibody responses were analysed for each of the six individual SNPs (Table 3). For the IL-4 C −589T and IL-4 G2979T SNPs, antibody levels against all seven pneumococcal serotypes were lowest in those carrying a CT/TT and GT/TT genotype, respectively. For the IL-4 C −589T SNP these differences were significant when using a model recessive for the C allele for antibodies against serotypes 4 and 23F ($p = 0.002$ and 0.05). For the G2979T SNP a trend was observed for antibodies against these same serotypes when using a model recessive for the G allele ($p = 0.07$ and 0.06). For the IL-4 Rα Gln551Arg SNP, antibodies against all seven serotypes were highest in carriers of Gln/Arg and Arg/Arg genotypes compared to common Gln/Gln homozygotes. These differences were significant for antibodies against serotypes 4, 18C and 14 ($p = 0.001$, 0.02

Table 2
D' scores indicating linkage between SNPs on the same chromosome

	IL-4 G2979T	IL-13 G −1112A	IL-13 Arg130Gln	IL-4 Rα Gln551Arg
IL-4 C −589T	0.89	0.30	0.46	
IL-4 G2979T		0.49	0.76	
IL-13 G −1112A			0.62	
IL-4 Rα Ile50Val				0.36*

$p < 0.0001$, except for * $p = 0.004$.

Table 3
Geometric mean pneumococcal conjugate vaccine serotype-specific IgG antibody concentrations (μg/ml) according to the six investigated SNPs

	IL-4 C −589T			IL-4 G2979T			IL-4 Rα Ile50Val				IL-4 Rα Gln551Arg			IL-13 G −1112A			IL-13 Arg130Gln		
	CC	CT/TT	*p*	GG	GT/TT	*p*	Ile/Ile	Ile/Val	Val/Val	*p*	Gln/Gln	Gln/Arg; Arg/Arg	*p*	GG	GA/AA	*p*	Arg/Arg	Arg/Gln; Gln/Gln	*p*
N	87	31/2		70	41/6		35	64	22		77	36/3		80	35/5		86	30/4	
4	4.4	2.8	0.002	4.5	3.2	0.07	4.1	4.2	2.7	0.1	3.2	5.8	0.001	4.3	3.1	0.09	4.1	3.5	0.5
6B	1.3	1.0	0.5	1.3	1.1	0.5	1.0	1.4	1.0	0.6	1.1	1.5	0.3	1.4	0.9	0.2	1.2	1.1	0.7
9V	27.0	17.8	0.1	26.2	21.4	0.4	19.0	27.3	25.3	0.4	22.4	30.5	0.2	26.6	19.9	0.3	25.4	21.6	0.6
14	73.5	65.8	0.7	71.1	70.1	0.9	73.1	69.0	72.8	0.9	59.5	99.6	0.04	69.6	71.0	0.9	65.4	87.8	0.3
18C	9.1	8.5	0.7	9.4	8.3	0.5	8.2	9.9	7.5	0.4	7.7	12.1	0.02	9.4	8.1	0.5	9.1	8.5	0.7
19F	10.4	10.3	0.9	10.2	10.2	0.9	11.0	9.9	11.0	0.9	9.9	12.1	0.5	10.9	9.3	0.6	10.2	10.8	0.9
23F	4.8	3.0	0.05	4.9	3.2	0.06	3.3	4.6	4.8	0.3	4.0	5.0	0.3	4.2	4.2	0.9	4.2	4.3	0.9

When one of the genotype frequencies is <10%, data for heterozygotes and least common homozygotes were pooled.

and 0.04, respectively). For these three SNPs showing significant differences, a percentage difference in the pneumococcal serotype-specific IgG antibody response between the polymorphic genotypes and the common genotype was calculated (Fig. 1).

For the IL-13 G −1112A SNP, lower antibody levels were found in those with polymorphic GA/AA genotypes compared to common GG homozygotes, except for antibodies against serotype 23F. Differences did not reach significance. For the IL-13 Arg130Gln and IL-4 Rα Il50Val SNPs, no significant differences in antibody levels were observed.

3.2. Analyses by haplotype

Geometric mean (GM) antibody levels according to haplotypes of each gene were investigated in a linear model (Table 4). Given the results of single SNP analyses, it was predicted that those haplotypes composed of two alleles associated with high antibody responses would be associated with the greatest antibody responses. Similarly, it was predicted that haplotypes composed of two alleles associated with low antibody responses, would be associated with lowest antibody responses. For the purposes of this study these were designated as "high responder" and "low responder" haplotypes, respectively.

In accordance with this the predicted IL-4 C·G high responder haplotype, composed of the IL-4 C −589 and IL-4 G2979 alleles, was indeed associated with the highest antibody responses against all seven pneumococcal serotypes compared with the T·T low responder haplotype. These differences were significant for antibodies against serotypes 4 and 23F ($p = 0.04$ and 0.05, respectively) (Table 4).

For the IL-4 Rα haplotypes, composed of the IL-4 Rα Ile50Val and IL-4 Rα Gln551Arg SNPs, the predicted high responder haplotype was Ile·Arg, whereas the predicted low responder haplotype was Val·Gln. Antibody levels against all pneumococcal serotypes indeed were highest in those with an Ile·Arg haplotype, except for antibodies against serotype 23F. Differences in antibody levels between those with Ile·Arg and Val·Gln haplotypes were significant for antibodies against serotypes 4 and 18C ($p = <0.001$ and 0.03, respectively) and a trend was observed for serotype 14 ($p = 0.07$).

For the IL-13 Arg130Gln SNP no clear high or low responder genotype was observed, so no clear high or low responder haplotype could be predicted. No consistently higher antibody levels against the seven pneumococcal serotypes were observed with any individual haplotype.

Since significant differences with both the IL-4 and the IL-4 Rα haplotypes were observed for antibodies against pneumococcal serotype 4, we investigated combinations of these two haplotypes. Carriers of both low responder haplotypes IL-4 T·T and IL-4 Rα Val·Gln showed lower antibody levels (GM 2.6 μg/ml) than when only the two individual haplotypes were investigated (GM of 2.9 μg/ml in IL-4 T·T and 3.2 μg/ml IL-4 Rα Val·Gln carriers; Fig. 2). Thus, a syn-

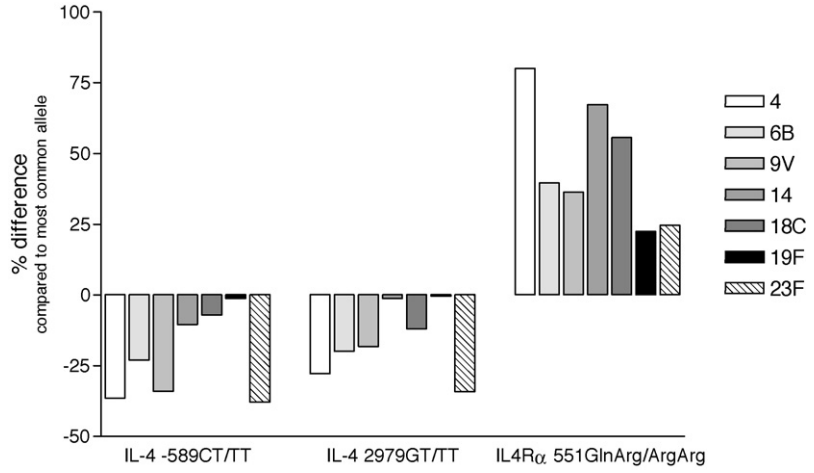

Fig. 1. Percentage difference in mean serotype-specific pneumococcal IgG antibody levels between most common genotype and polymorphic variants. Most common genotype, IL-4 −589CC, IL-4 2979GG and IL-4 Rα 551GlnGln, respectively, is set at 100%.

Table 4
Geometric mean pneumococcal conjugate vaccine serotype-specific IgG antibody concentrations (μg/ml) according to IL-4, IL-4 Rα and IL-13 haplotypes

	IL-4 C −589T·G2979T			IL-4 Rα Ile50Val·Gln551Arg			IL-13 G −1112A·Arg130Gln		
	C·G	T·T	*p*	Ile·Arg	Val·Gln	*p*	G·Arg	A·Gln	*p*
N	185	33		15	82		185	27	
4	4.1	2.9	0.04	7.9	3.2	<0.001	4.0	3.5	0.8
6B	1.3	1.0	0.4	1.5	1.2	0.5	1.2	1.0	0.5
9V	25.3	20.2	0.4	32.3	25.5	0.5	25.1	22.0	0.6
14	71.4	66.0	0.8	127.4	66.1	0.07	69.7	84.4	0.5
18C	9.1	8.1	0.5	14.4	8.1	0.03	9.1	8.0	0.5
19F	10.5	10.3	0.9	12.9	10.3	0.6	10.8	13.0	0.5
23F	4.4	2.9	0.05	4.0	4.6	0.6	4.2	4.5	0.7

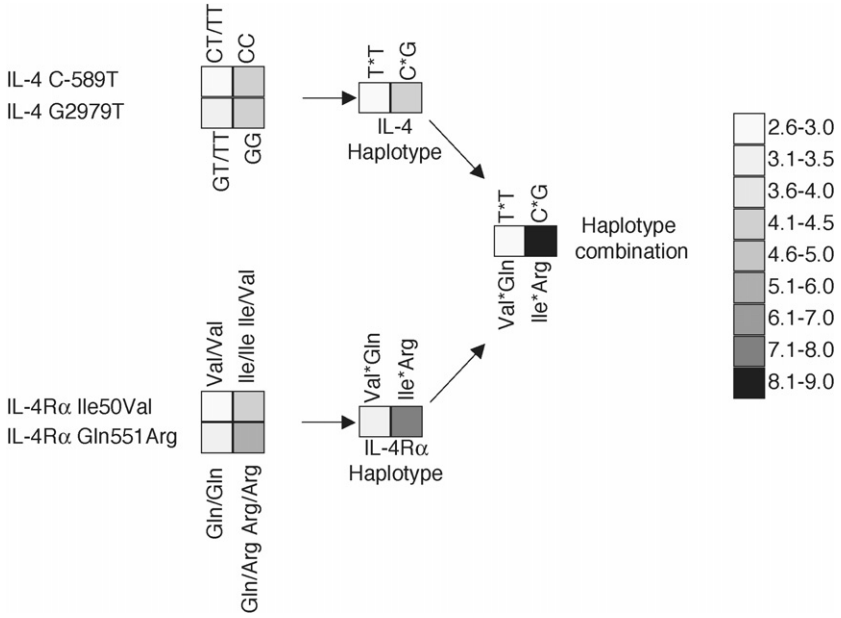

Fig. 2. Schematic overview of geometric mean antibody level against pneumococcal serotype 4 according to IL-4 and IL-4 Rα genotype, haplotype and haplotype combination. Synergistic effect and thus a greater discriminating power of individual haplotypes vs. single loci and of haplotype combination vs. individual haplotypes.

ergistic effect was observed, resulting in significantly lower antibody levels against pneumococcal serotype 4 in those with both low responder haplotypes compared to those with both high responder haplotypes ($p = 0.004$).

4. Discussion

This study demonstrates associations of individual SNPs and haplotypes in IL-4 and IL-4 RA with pneumococcal vaccine antibody responses. The IL-4 −589T, IL-4 2979T and IL-4 Rα 551 Gln alleles were associated with significantly lower pneumococcal antibody levels in single SNP analyses. Several lines of evidence suggest that the IL-4 −589T allele may be associated with dys-regulated immune responses. The IL-4 −589T allele has previously been associated with increased IgE levels [22,41,42], an increased frequency and rate of progression of disease in HIV-infected patients [43] and increased susceptibility to Kawasaki's disease [44]. Furthermore, it was shown that amongst children with severe malaria, total IgE levels were significantly elevated in those carrying the IL-4 −589T allele, suggesting the possibility that there is a relationship between susceptibility to severe malaria, IgE production and genetic variation in IL-4 [45].

IL-4 induces B cell activation and modifies humoral B cell responses to both T cell-dependent and T cell-independent stimuli [46]. The IL-4 −589T allele enhances IL-4 transcription *in vitro* [47]. Therefore, prima facie, the T allele might be expected to be associated with increased humoral responses to vaccines. However, Vos et al. showed T cell-independent humoral responses to be enhanced by short-term exposure to IL-4, in the absence of IFNγ, but suppressed by persistent IL-4 exposure [48]. Thus, humoral responses may be dependent upon the cytokine microenvironment and the length of exposure to this microenvironment. Therefore, under certain conditions an IL-4 allele associated with increased IL-4 transcription might be associated with decreased humoral responses. These effects may be further modified by the coexistence of genetically mediated alterations of IL-4 Rα function. Furthermore a gene–environment interaction between day-care attendance in the first 6 months of life and the IL-4 Rα Ile50Val locus on lipopolysaccharide induced IFNγ production has been shown [49]. Therefore, relationships between individual alleles or haplotypes and vaccination responses may be further dependent on microenvironmental, macroenvironmental and developmental influences.

As for the IL-4 −589T allele, the IL-4 2979T allele has been associated with asthma [23]. However, no functional studies on this SNP have been reported. Functional data on the IL-4 Rα Arg551Gln SNP is conflicting [50,51]. However, the 551Gln allele has been associated with asthma and atopic phenotypes [52–54]. That the IL-4 and IL-4 RA alleles that have been associated with atopy show a diminished antibody response in our study is consistent with the association of atopy with altered kinetics of the maturation of vaccine responses and of atopic eczema with delayed maturation of the antibody response to pneumococcal vaccine [28,29]. This might suggest a relationship between atopy, genotype and the responses upon pneumococcal vaccination, possibly brought about by an altered cytokine milieu secondary to an altered Th1/Th2 balance.

Significant associations with genotype or haplotype were not demonstrated for antibody responses to all serotypes and of the studied pneumococcal serotypes, serotype 4 was most consistently associated with genotype. Potential reasons for lack of consistent replication across all serotypes in this study include: true variation in the underlying association between genotype and outcome (effect heterogeneity), type 1 error or lack of power [55].

Possible biological explanations for variation in the underlying association of genotype with individual serotypes include differing immunogenicities between serotypes, related to variations in the chemical structure of polysaccharide capsules, and/or variations in the magnitude of polysaccharide-specific response dependent on the prevalence of specific serotypes, and thus the presence or absence of natural priming. It is possible that the effect of the studied SNPs might be insufficient to significantly augment IgG antibody responses against poorly immunogenic and/or low prevalent serotypes. Conversely, with high immunogenic and/or highly prevalent serotypes the relative contribution of genetic variation may be small. Thus an association would be most likely to be revealed in serotypes of intermediate immunogenicity and/or prevalence.

Studying haplotypes of multiple polymorphisms in one gene has been described to be more informative than analysis of single SNPs in genetic association studies of complex diseases [56]. Accordingly, haplotypes composed of IL-4 and IL-4 RA SNPs, showed a higher discriminative power in vaccine responsiveness compared to SNP analyses, indicating a synergistic influence on antibody responses of haplotypes over individual SNPs. The importance of investigating gene-gene interactions in complex biological pathways has also been demonstrated [54]. In our study comparing combinations of the IL-4 and IL-4 Rα genes revealed the greatest magnitude of difference in antibody responses, specifically carriers of two poor responding haplotypes had the lowest antibody levels. However, replication of our data in an independent cohort would be valuable to draw general conclusions.

In this study, multiple comparisons were performed to study the associations between IgG antibody responses and selected genotypes, previously associated with atopy. We have not corrected for multiple testing since (i) the analyses were based on an *a priori* hypothesis and (ii) this method can be overly conservative. Instead we have chosen to describe all statistical analyses carried out and to discuss the results accordingly [39,40].

The additive effect of haplotype and haplotype–haplotype combination analyses, compared to single SNP and single haplotype analyses respectively, reported herein supports the approach of studying the effect of combinations of multiple

loci, both within and between genes, on complex phenotypes. Furthermore, the findings of this study provide additional support for the importance of host genetic factors in vaccine responses. Understanding these influences is likely to be important for the development of improved and novel vaccines.

Acknowledgements

The work described in this paper was financially supported by The Netherlands Organisation for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), The Netherlands Health Insurance Company Zilveren Kruis-Achmea, an Australia–Europe Scholarship 2004 funded by the Australian Government through the Department of Education, Science and Training and promoted by AEI, the Australian Government International Education Network, and the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences.

References

[1] Luotonen J, Herva E, Karma P, Timonen M, Leinonen M, Makela PH. The bacteriology of acute otitis media in children with special reference to *Streptococcus pneumoniae* as studied by bacteriological and antigen detection methods. Scand J Infect Dis 1981;13(3):177–83.

[2] Fedson DS, Scott JA. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 1999;17(Suppl 1):S11–8.

[3] Vitharsson G, Jonsdottir I, Jonsson S, Valdimarsson H. Opsonization and antibodies to capsular and cell wall polysaccharides of *Streptococcus pneumoniae*. J Infect Dis 1994;170(3):592–9.

[4] Tuomanen EI, Austrian R, Masure HR. Pathogenesis of pneumococcal infection. N Engl J Med 1995;332(19):1280–4.

[5] Watson DA, Musher DM, Verhoef J. Pneumococcal virulence factors and host immune responses to them. Eur J Clin Microbiol Infect Dis 1995;14(6):479–90.

[6] Harwell JI, Brown RB. The drug-resistant *Pneumococcus*: clinical relevance, therapy, and prevention. Chest 2000;117(2):530–41.

[7] Le Souef PN, Goldblatt J, Lynch NR. Evolutionary adaptation of inflammatory immune responses in human beings. Lancet 2000;356(9225):242–4.

[8] Poland GA, Jacobson RM. The genetic basis for variation in antibody response to vaccines. Curr Opin Pediatr 1998;10(2):208–15.

[9] Newport MJ, Goetghebuer T, Weiss HA, Whittle H, Siegrist CA, Marchant A. Genetic regulation of immune responses to vaccines in early life. Genes Immun 2004;5(2):122–9.

[10] Kruskall MS, Alper CA, Awdeh Z, Yunis EJ, Marcus-Bagley D. The immune response to hepatitis B vaccine in humans: inheritance patterns in families. J Exp Med 1992;175(2):495–502.

[11] Poland GA, Jacobson RM, Schaid D, Moore SB, Jacobsen SJ. The association between HLA class I alleles and measles vaccine-induced antibody response: evidence of a significant association. Vaccine 1998;16(19):1869–71.

[12] Allansmith M, McClellan B, Butterworth M. The influence of heredity and environment on human immunoglobulin levels. J Immunol 1969;102(6):1504–10.

[13] Pandey JP, Zollinger WD, Fudenberg HH, Loadholt CB. Immunoglobulin allotypes and immune response to meningococcal group B polysaccharide. J Clin Invest 1981;68(5):1378–80.

[14] Shirakawa I, Deichmann KA, Izuhara I, Mao I, Adra CN, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol Today 2000;21(2):60–4.

[15] Punnonen J, Yssel H, de Vries JE. The relative contribution of IL-4 and IL-13 to human IgE synthesis induced by activated CD4+ or CD8+ T cells. J Allergy Clin Immunol 1997;100(6 Pt 1):792–801.

[16] Kabesch M, Tzotcheva I, Carr D, Hofler C, Weiland SK, Fritzsch C, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J Allergy Clin Immunol 2003;112(5):893–8.

[17] Izuhara K, Shirakawa T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. Int J Mol Med 1999;3(1):3–10.

[18] Shirakawa I, Deichmann KA, Izuhara I, Mao I, Adra CN, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol Today 2000;21(2):60–4.

[19] McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Curr Biol 1998;8(6):339–42.

[20] Bancroft AJ, McKenzie AN, Grencis RK. A critical role for IL-13 in resistance to intestinal nematode infection. J Immunol 1998;160(7):3453–61.

[21] Murata T, Obiri NI, Puri RK. Structure of and signal transduction through interleukin-4 and interleukin-13 receptors (review). Int J Mol Med 1998;1(3):551–7.

[22] Rosenwasser LJ, Klemm DJ, Dresback JK, Inamura H, Mascali JJ, Klinnert M, et al. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin Exp Allergy 1995;25(Suppl 2):74–8.

[23] Basehore MJ, Howard TD, Lange LA, Moore WC, Hawkins GA, Marshik PL, et al. A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. J Allergy Clin Immunol 2004;114(1):80–7.

[24] Risma KA, Wang N, Andrews RP, Cunningham CM, Ericksen MB, Bernstein JA, et al. V75R576 IL-4 receptor alpha is associated with allergic asthma and enhanced IL-4 receptor function. J Immunol 2002;169(3):1604–10.

[25] Rosa-Rosa L, Zimmermann N, Bernstein JA, Rothenberg ME, Khurana Hershey GK. The R576 IL-4 receptor alpha allele correlates with asthma severity. J Allergy Clin Immunol 1999;104(5):1008–14.

[26] Hummelshoj T, Bodtger U, Datta P, Malling HJ, Oturai A, Poulsen LK, et al. Association between an interleukin-13 promoter polymorphism and atopy. Eur J Immunogenet 2003;30(5):355–9.

[27] Liu X, Nickel R, Beyer K, Wahn U, Ehrlich E, Freidhoff LR, et al. An IL13 coding region variant is associated with a high total serum IgE level and atopic dermatitis in the German multicenter atopy study (MAS-90). J Allergy Clin Immunol 2000;106(1 Pt 1):167–70.

[28] Holt PG, Rudin A, Macaubas C, Holt BJ, Rowe J, Loh R, et al. Development of immunologic memory against tetanus toxoid and pertactin antigens from the diphtheria–tetanus–pertussis vaccine in atopic versus nonatopic children. J Allergy Clin Immunol 2000;105(6 Pt 1):1117–22.

[29] Arkwright PD, Patel L, Moran A, Haeney MR, Ewing CI, David TJ. Atopic eczema is associated with delayed maturation of the antibody response to pneumococcal vaccine. Clin Exp Immunol 2000;122(1):16–9.

[30] Veenhoven R, Bogaert D, Uiterwaal C, Brouwer C, Kiezebrink H, Bruin J, et al. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 2003;361(9376):2189–95.

[31] Straetemans M, van Heerbeek N, Tonnaer E, Ingels KJ, Rijkers GT, Zielhuis GA. A comprehensive model for the aetiology of otitis media with effusion. Med Hypotheses 2001;57(6):784–91.

[32] Wiertsema SP, Sanders EA, Veenhoven RH, van Heerbeek N, van Den HS, Berbers GA, et al. Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media. J Clin Immunol 2004;24(4):354–60.

[33] Oefner P, Underhill P, Dracopoli NC, Haines JL, Korf BR, Moir DT, et al., editors. Current protocols in human genetics. New York: Wiley and Sons; 2005 [DNA mutation detection used denaturating high-performance liquid chromatography (DHPLC), p. 7.10.1–12].
[34] Stephens M, Donnelly P. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003;73(5):1162–9.
[35] Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001;68(4):978–89.
[36] Sanders LA, Rijkers GT, Tenbergen-Meekes AM, Voorhorst-Ogink MM, Zegers BJ. Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr Res 1995;37(6): 812–9.
[37] Musher DM, Luchi MJ, Watson DA, Hamilton R, Baughn RE. Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. J Infect Dis 1990;161(4):728–35.
[38] Quataert SA, Kirch CS, Wiedl LJ, Phipps DC, Strohmeyer S, Cimino CO, et al. Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin Diagn Lab Immunol 1995;2(5):590–7.
[39] Rothman K. Modern epidemiology. Boston: Little, Brown & Company; 1986. p. 147–50.
[40] Perneger TV. What's wrong with Bonferroni adjustments. BMJ 1998;316(7139):1236–8.
[41] Rosenwasser LJ, Borish L. Genetics of atopy and asthma: the rationale behind promoter-based candidate gene studies (IL-4 and IL-10). Am J Respir Crit Care Med 1997;156(4 Pt 2):S152–5.
[42] Noguchi E, Shibasaki M, Arinami T, Takeda K, Maki T, Miyamoto T, et al. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31–q33 in a Japanese population. Am J Respir Crit Care Med 1997;156(5):1390–3.
[43] Smolnikova MV, Konenkov VI. Association of IL2, TNFA, IL4 and IL10 promoter gene polymorphisms with the rate of progression of the HIV infection. Russ J Immunol 2002;7(4):349–56.
[44] Burns JC, Shimizu C, Shike H, Newburger JW, Sundel RP, Baker AL, et al. Family-based association analysis implicates IL-4 in susceptibility to Kawasaki disease. Genes Immun 2005.
[45] Verra F, Luoni G, Calissano C, Troye-Blomberg M, Perlmann P, Perlmann H, et al. IL4-589C/T polymorphism and IgE levels in severe malaria. Acta Trop 2004;90(2):205–9.
[46] Jordan MB, Mills DM, Kappler J, Marrack P, Cambier JC. Promotion of B cell immune responses via an alum-induced myeloid cell population. Science 2004;304(5678):1808–10.
[47] Rosenwasser LJ. Structure/function variants as candidate genes in asthma: linkage vs. association for relevance. Clin Exp Allergy 1998;28(Suppl 1):90–2.
[48] Vos Q, Snapper CM, Mond JJ. T(h)1 versus T(h)2 cytokine profile determines the modulation of in vitro T cell-independent type 2 responses by IL-4. Int Immunol 2000;12(9):1337–45.
[49] Hoffjan S, Nicolae D, Ostrovnaya I, Roberg K, Evans M, Mirel DB, et al. Gene–environment interaction effects on the development of immune responses in the 1st year of life. Am J Hum Genet 2005;76(4):696–704.
[50] Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. N Engl J Med 1997; 337(24):1720–5.
[51] Wang HY, Shelburne CP, Zamorano J, Kelly AE, Ryan JJ, Keegan AD. Cutting edge: effects of an allergy-associated mutation in the human IL-4R alpha (Q576R) on human IL-4-induced signal transduction. J Immunol 1999;162(8):4385–9.
[52] Kruse S, Japha T, Tedner M, Sparholt SH, Forster J, Kuehr J, et al. The polymorphisms S503P and Q576R in the interleukin-4 receptor alpha gene are associated with atopy and influence the signal transduction. Immunology 1999;96(3):365–71.
[53] Ober C, Leavitt SA, Tsalenko A, Howard TD, Hoki DM, Daniel R, et al. Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations. Am J Hum Genet 2000;66(2):517–26.
[54] Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA, et al. Gene–gene interaction in asthma: IL4Rα and IL13 in a Dutch population with asthma. Am J Hum Genet 2002;70(1):230–6.
[55] Colhoun HM, McKeigue PM, Davey SG. Problems of reporting genetic associations with complex outcomes. Lancet 2003; 361(9360):865–72.
[56] Lee JE, Choi JH, Lee JH, Lee MG. Gene SNPs and mutations in clinical genetic testing: haplotype-based testing and analysis. Mutat Res 2005;573(1–2):195–204.