Gene-gene interaction in regulatory T–cell function in atopy and asthma development in childhood

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Background: Regulatory T–cell dysfunction is associated with development of the complex genetic conditions atopy and asthma. Therefore, we hypothesized that single nucleotide polymorphisms in genes involved in the development and function of regulatory T cells are associated with atopy and asthma development.

Objective: To evaluate main effects and gene-gene interactions of haplotype tagging single nucleotide polymorphisms of genes involved in regulatory T–cell function—*IL6*, *IL6R*, *IL10*, heme-oxygenase 1 *(HMOX1)*, *IL2*, Toll-like receptor 2 *(TLR2)*, *TGFB1*, TGF-β receptor *(TGFBR)–1*, *TGFBR2*, *IL2RA*, and forkhead box protein 3 *(FOXP3)*—in relation to atopy and asthma.

Methods: Single-locus and multilocus associations with total IgE (3rd vs 1st tertile); specific IgE to egg, milk, and indoor allergens; and asthma were evaluated by χ^2 tests and the multifactor dimensionality-reduction method in 3 birth cohorts (Allergenic study).

Results: Multiple statistically significant multilocus associations existed. *IL2RA* rs4749926 and *TLR2* rs4696480 associated with IgE in both age groups tested (1-2 and 6-8 years). *TGFBR2* polymorphisms associated with total and specific IgE in both age groups and with asthma. *TGFBR2* rs9831477 associated with specific IgE for milk at age 1 to 2 years and indoor allergens at age 6 to 8 years. For milk-specific IgE, interaction between *TGFBR2* and *FOXP3* polymorphisms was confirmed by logistic regression and consistent in 2 birth cohorts and when stratified for sex, supplying internal replications.

Conclusion: Genes involved in the development and function of regulatory T cells, specifically *IL2RA*, *TLR2*, *TGFBR2*, and *FOXP3*, associate with atopy and asthma by gene-gene interaction. Modeling of multiple gene-gene interactions is important to unravel further the genetic susceptibility to atopy and asthma. (J Allergy Clin Immunol 2010;126:338-46.)

Key words: *T regulatory cells, atopy, IgE, asthma, MDR, polymorphism, interaction, birth cohort*

Atopy and asthma have a complex genetic background, and it is likely that multiple genes contribute to their development through main effects and through gene-gene interactions. Gene-gene interactions can be investigated by multifactor dimensionality reduction (MDR), a method designed to translate high-dimensional genetic data into a single dimension.[1] MDR selects single nucleotide polymorphisms (SNPs) in an unbiased way; therefore, it is possible to analyze genes in a biologically plausible pathway relevant for the development of atopy and asthma, yet without knowledge beforehand which genes will be important.

In recent years, regulatory T (Treg) cells have been identified to play a key role in balancing immune responses to maintain or acquire tolerance against allergens. Treg cells contribute to the control of allergen-specific immune responses in several ways—for example, through suppression of effector T cells or through suppression of dendritic cells that support the generation of effector T cells. Compromised numbers or function of Treg cells may therefore contribute to development and persistence of allergic disease,[2-5] a concept for which evidence is now accumulating from models of allergic inflammation in mice and various studies in human subjects, and from genetic association studies.

In genetic association studies, variations in genes that encode proteins involved in the development and function of Treg cells, such as Toll-like receptor (TLR)–2, *TGFB1*, *IL10*, and heme-oxygenase 1 *(HMOX1)*, have been reported to associate with atopy and asthma phenotypes.[6-18]

In an ovalbumin-induced mouse model of allergic airway inflammation, Xia et al[19] described that induction of HMOX1 induces forkhead box protein 3 (FOXP3) expression and production of IL10 and membrane-bound TGFB1, thereby enhancing activity of Treg cells. These immunologic alterations correlate with a decrease of serum ovalbumin-specific IgE and eosinophil infiltration in bronchial alveolar lavage fluid, suggesting a protective

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Abbreviations used
FDR: False discovery rate
FOXP3: Forkhead box protein 3
KOALA: Dutch acronym for Child, Parent and Health: Lifestyle and Genetic Constitution
HMOX1: Heme-oxygenase 1
MDR: Multifactor dimensionality reduction
OR: Odds ratio
PIAMA: Prevention and Incidence of Asthma and Mite Allergy
PREVASC: PREvention of ASthma in Children
sIgE: Specific IgE
SNP: Single nucleotide polymorphism
TGFBR: TGF-β receptor
TLR: Toll-like receptor
Treg: Regulatory T

role for these proteins in asthma development. Another experiment in an ovalbumin-induced mouse model showed that TGF-β receptor 1 (TGFBR1) is upregulated in lung tissue with allergic airway inflammation.[20]

A contributing role of Treg cells in allergic diseases was further suggested by the observed increase of Treg cells in peripheral blood during the pollen season in children with allergy.[21] Another study demonstrated that the presence of increased numbers of peripheral blood Treg cells associated with spontaneous remission of cow's milk allergy.[22] Furthermore, a quantitative and functional impairment of pulmonary Treg cells has been reported in children with asthma.[23]

Given the important role of the Treg pathway in the development of atopy and asthma, we hypothesize that multiple genes involved in the induction of and suppression by Treg cells are associated with atopy and asthma phenotypes through their main effects, gene-gene interaction, or both. We investigated this by evaluating 11 genes (Fig 1) in relation to atopy and asthma phenotypes in 3062 children participating to the Allergenic study, a prospective birth cohort study composed of 3 Dutch cohorts.[24]

METHODS

Study populations

The Allergenic study includes 3 prospective Dutch birth cohorts: Prevention and Incidence of Asthma and Mite Allergy (PIAMA),[25] PREvention of ASthma in Children (PREVASC),[26,27] and KOALA (Dutch acronym for Child, Parent and Health: Lifestyle and Genetic Constitution).[28] A brief description of the 3 cohorts and their eligibility criteria is included in the Online Repository. Genetic studies were approved by local medical ethics committees of participating institutes. All parents provided written informed consent.

IgE measurements

Total IgE levels were determined in capillary or venous blood collected at ages 1 and 8 years in PIAMA; ages 1, 2, and 6 years in PREVASC; and ages 1 and 2 years in KOALA (Sanquin Research, Amsterdam, The Netherlands). Total IgE levels were measured by radioimmunoassay as described previously.[29-31] Total IgE measurements were clustered at ages 1 to 2 years and 6 to 8 years and analyzed in tertiles (Table I; see this article's Online Repository "Total IgE tertiles and specific IgE measurements" section at www.jacionline.org, including Table E1). Specific IgE (sIgE) to various allergens was tested at different ages in the PIAMA, KOALA, and PREVASC cohorts. To be able to pool data of the separate cohorts, we studied sensitization (defined as sIgE ≥0.35 IU/mL) to food allergens milk and egg clustered at ages 1 to 2 years, and to indoor allergens (to cat, dog, or house dust mite) clustered at ages 6 to 8 years (Table I; see the Online Repository at www.jacionline.org).

Asthma definition

Asthma was clustered at ages 6 and 8 years (PREVASC at age 6 years; PIAMA at age 8 years) and defined as at least 1 episode of wheeze or dyspnea in the last year and/or the use of inhaled steroids.

SNP selection and genotyping

Haplotype tagging SNPs (98) of 11 genes *IL6R*, *IL10*, *HMOX1*, *TGFBR2*, *IL2*, *TLR2*, *IL6*, *TGFBR1*, *IL2RA*, *TGFB1*, and *FOXP3* were selected from the HapMap database[32] or from the Innate Immunity web site[33] on the basis of the largest number of SNPs with a minor allele frequency >0.1 available in each database. In addition, the biomedical literature was screened for SNPs within the candidate genes known to have functional impact or to be associated with atopy or asthma. Genomic DNA was extracted from buccal swabs or blood by using standard methods.[34] DNA was amplified by using REPLI-g UltraFast technology (Qiagen). Genotyping was performed by Competitive Allele-Specific PCR using KASPar genotyping chemistry, performed under contract by K-Biosciences (Hoddesdon Herts, UK). Quality of genotype data was guaranteed by standards of K-Biosciences and verified by comparing the genotyping results in genomic versus amplified DNA in a subset of children.

Statistical methods

All SNPs were analyzed for Hardy-Weinberg equilibrium by using the χ^2 statistic ($P > .01$). We used χ^2 tests to analyze whether genotypes in this pathway are associated with elevated serum IgE levels at 1 to 2 years and at 6 to 8 years (highest vs lowest tertile), sIgE for milk and egg (ages 1-2 years), sIgE for indoor allergens (ages 6-8 years), and the presence of asthma (ages 6-8 years) by using a codominant model. For SNPs with a $P < .10$, the Akaike information criterion was evaluated to determine the best fitting genetic model (additive, dominant, or recessive). Odds ratios (ORs) and 95% CIs were calculated by logistic regression analysis. *FOXP3* is located on the X-chromosome and was therefore analyzed for boys and girls separately. Calculations were performed by using SPSS 14.0 statistical software (Chicago, Ill) and considered significant if $P < .05$ (2-sided). Because the haplotypes-tagging SNP selection uses multimarker predictors to capture all information of the gene (ie, aggressive tagging), we also analyzed SNPs that were captured by multiple SNPs by constructing haplotypes. Single SNP analyses were corrected for multiple testing by using the false discovery rate (FDR) as described by Benjamini and Hochberg[35] with a P value of .05 as the cutoff value. For each gene, we corrected for the number of genetic tests (*ie*, the number of SNPs per gene) as well as the number of phenotypes (n = 6). Single SNP analyses on *FOXP3* and sensitization were reported in a separate article.[36]

Gene-gene interactions were analyzed by using MDR (version 1.0.0). The MDR approach has been described previously[37-39] and applied in this cohort.[40-42] The *TLR2* gene has been studied in relation to other TLRs and atopy development by MDR (Reijmerink et al, unpublished data, May 2010). A detailed description of the MDR method is included in the Online Repository at www.jacionline.org, "MDR" section. The significance of the average prediction error was calculated by using the MDR permutation test, and a P value <.05 was considered significant. Logistic regression analyses were performed to confirm significant 2-way interaction results from MDR analyses; if the interaction term was significant (in a multiplicative model), the best fitting genetic model—for example, dominant or recessive—was analyzed.

RESULTS

Study population

A total of 3062 children were genotyped, and 2927 Dutch children were selected for genetic analyses after exclusion of non-Dutch children (5.7%) to avoid effects resulting from population stratification. Characteristics of the children participating in the genetic study are presented in Table I.

Single SNP and haplotype analysis

Minor allele frequencies and level of linkage disequilibrium per gene are presented in this article's Tables E2 and E3,

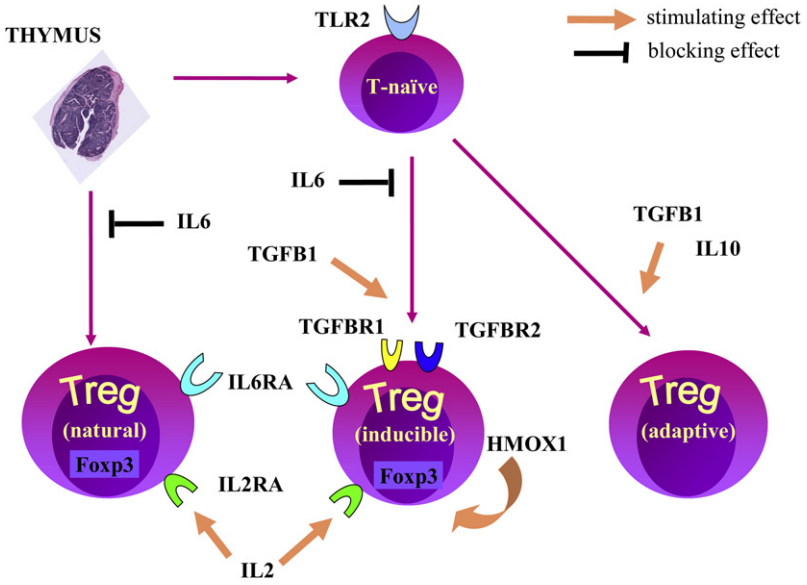

FIG 1. Hypothetical scheme of genes involved in the Treg pathway. Subsets of Treg cells with distinct phenotypes and mechanisms of action include the naturally occurring, thymus-selected Treg cells and the inducible and adaptive Treg cells. *T-naive,* Undifferentiated T lymphocyte.

respectively, in the Online Repository at www.jacionline.org. The mean failure rate for assays was 0.04 (range, 0.02-0.12). One SNP (rs4601580, *IL6R*) failed amplification; 1 SNP was monomorphic (rs13447445, *IL6*). Genotypes of 1 SNP, rs706781 (*IL2RA*), deviated from Hardy-Weinberg equilibrium in controls (at 1-2 years and/or at 6-8 years of age) and was not considered for further analyses, leaving 95 SNPs for single SNP and MDR analysis.

Associations of single SNPs are presented in this article's Tables E4 to E6 in the Online Repository at www.jacionline.org. Several associations of these SNPs were identified with atopy and asthma (ORs varying between 0.3 and 2.3; *P* values ranging from .05 to .001) and did not withstand correction for multiple testing using FDR (all corrected *P* values >.05). We constructed haplotypes that captured nongenotyped SNPs. None of these haplotypes was associated with any of the phenotypes (data not shown).

MDR analyses

Multifactor dimensionality reduction analyses showed that several combinations of SNPs of the Treg genes were significantly associated with allergy and asthma phenotypes (Table II). The *IL2RA* SNP rs4749926 and the *TLR2* SNP rs4696480 associated with serum IgE levels in both age groups 1 to 2 and 6 to 8 years. *TGFBR2* rs9831477 associated with both sIgE to milk allergens (at 1-2 years) and indoor allergens (at 6-8 years). *TGFBR2* SNPs associated with total and sIgE in both age groups and with asthma, although with various SNPs.

Multifactor dimensionality reduction identified several significant 2-way SNP interactions that showed effects of variable direction when evaluated by logistic regression (Table II; Fig 2). The interaction of *TGFBR2* (rs2276767) with *FOXP3* (rs6609857) for sIgE to milk at 1 to 2 years appeared to be negative or redundant—that is, information about case-control status can be obtained from only 1 of the genotypes, and the other genotype does not provide further information (Fig 2, *A*). Because *FOXP3* is located on the X-chromosome, interaction between *TGFBR2* and *FOXP3* was tested in boys and girls separately, and significant effects were shown in the same direction in both sexes ($P = .038$ and .001 in boys and girls, respectively; Fig 3, *A and B*). Interaction of *TGFBR2* with *FOXP3* SNPs and specific IgE to milk was also consistent and significant in the PIAMA and KOALA cohorts separately (Fig 3, *C and D*), supplying internal replication. A similar interaction was observed for *TLR2* (rs4696480) and *TGFBR2* (rs9790292), which showed a decreased risk for elevated total IgE at 6 to 8 years in children carrying 1 or 2 minor alleles of either SNP. In contrast, this decreased risk was not observed in children having 1 or 2 minor alleles of both SNPs, as indicated by a statistically significant interaction (*P* value for interaction, .023; data not shown). The interaction between *IL6* and *TGFBR2* with respect to sIgE to egg allergens showed an increased risk for sensitization when the *IL6* AA genotype was combined with the *TGFBR2* TT genotype (Fig 2, *B*). *TGFB1* and *IL6R* showed a decreased risk for sIgE to indoor allergens when both minor alleles were present; however, the interaction was not significant (Fig 2, *C*).

DISCUSSION

This study shows that interacting genes involved in the development and function of Treg cells are associated with atopy and asthma phenotypes. The results suggest an important influence of *TGFBR2*, because its genetic variation shows multiple associations with atopy phenotypes in both evaluated age groups 1 to 2 and 6 to 8 years. SNPs in *IL2RA* and *TLR2* associated with atopy phenotypes across different age groups, which strengthens the findings of this study by showing internal replication. When testing interaction between SNPs in *FOXP3* and *TGFBR2* and milk sIgE stratified for sex because *FOXP3* is located on the X-chromosome, we found interaction in the same direction in boys and girls separately. Findings were consistent and significant in both the PIAMA and KOALA cohorts, which can also be considered as an internal replication.

Of interest, the genes under study that associated with atopy phenotypes by MDR analysis had no main effects after multiple

TABLE I. Characteristics of participating children in the Allergenic birth cohort

Characteristic	PIAMA	PREVASC	KOALA	P*
Participants in genetic study (N)	1037	374	1651	—†
DNA available (% from total cohort)	25.0	49.8	58.1	—
Ethnicity (% Dutch origin)	95.1	95.7	95.2	—
Boys (%)	51.2	49.2	50.6	.80
Family history (%)				
Atopy in mother	66.5	51.4	33.3	**<.001**
Asthma in mother	16.7	31.4	8.8	**<.001**
Atopy in father	31.6	47.7	36.4	**<.001**
Asthma in father	7.4	21.9	10.0	**<.001**
Phenotypes				
Total serum IgE (IU/mL)‡ age 1 y	7.1 (2.0-17.0) N§ = 369	8.6 (3.5-19.4) N = 226	6.0 (2.6-12.5) N = 699	**.002**
Total serum IgE (IU/mL)‡ age 2 y	NA	11.7 (4.2-28.7) N = 358	12.0 (3.7-38.0) N = 704	.80
Total serum IgE (IU/mL)‡ age 6 y	NA	22.5 (7.6-67.0) N = 218	NA	—
Total serum IgE (IU/mL)‡ age 8 y	64.9 (23.0-240.0) N=748	NA	NA	—
sIgE‖ to milk (%) age 1-2 y	36.3 N = 338	NA	29.2 N = 522	.20
sIgE‖ to egg (%) age 1-2 y	6.6 N = 338	19.2 N=155	10.7 N = 497	**<.01**
sIgE‖ to indoor allergens¶ (%) age 6-8 y	36.9 N = 713	32.0 N = 132	NA	.30
Asthma# (%) age 6-8 y	20.7 N = 921	27.0 N = 207	NA	.16
Allergic asthma**	9.7 N = 723	9.6 N = 125	NA	.98
Doctor's diagnosis asthma††	5.1 N = 934	17.9 N = 229	NA	**<.01**

Boldface indicates *P* values <.05.
NA, Not available.
**P* value for comparison between cohorts by χ^2 test or ANOVA where appropriate.
†Not tested.
‡Geometric mean (interquartile range).
§*N,* Number of individuals available for this phenotype in this specific age group.
‖*sIgE,* sIgE ≥0.35 IU/L to egg, milk, and indoor allergens.
¶*Indoor allergens,* House dust mite, cat, and/or dog allergens.
#*Asthma,* At least 1 episode of wheeze or dyspnea and/or the use of inhaled corticosteroids in the last year.
***Allergic asthma,* At least 1 episode of wheeze or dyspnea and/or the use of inhaled corticosteroids in the last year and sensitization to indoor allergens at age 6 or 8 years.
††Diagnosis of asthma confirmed by a physician.

testing correction. However, main effects were corrected for the number of tests performed by using FDR,[35] which may be an overcorrection because both genotypes and phenotypes are correlated. In contrast, the results from MDR showed several genetic interactions that were significant after permutation testing. Logistic regression of the 2-locus associations identified by MDR showed a variable nature of the gene-gene interactions, revealing the complex nature of gene-gene interactions. SNPs, for example in the genes *TLR2* and *TGFBR2*, showed a main effect that was modified by the alleles of another SNP.

Our findings appear to be of importance and relevant, because we replicated previously described associations with the same SNPs in *TGFB1*[8-11] and *TLR2*[6,7] with atopy and asthma, and in *IL10* with asthma.[12-18] Rs1800469 (*TGFB1* C-509T) in this study showed a main effect, and an epistatic effect with *IL6R* and *TGFBR2* for sensitization to indoor allergens. Rs4696480 and rs1898830 and (*TLR2* -15607A/G and -16934A/T) were associated with sensitization and asthma, respectively. *IL10* SNPs (rs1800871 and rs1800872, -819C/T and -571 or -592C/A) were associated with asthma. Although we observed multiple associations with *TGFBR2*, no genetic association of *TGFBR2* has been described for atopy or asthma previously. However, a study showing decreased expression of TGFBR2 protein in the subepithelium of bronchial biopsies of children with asthma[43] suggests a functional role in asthma.

The strengths of this study are its large sample size and prospective follow-up, which enabled us to evaluate 2 age groups that represent different stages of the developing immune system in early childhood and primary school age. The use of MDR more closely resembles the complex nature of atopic disease by evaluating multiple SNPs in an unbiased way. Furthermore, the selection of haplotype tagging SNPs has made it very unlikely that we have missed important signals from genes with common alleles.

Potential limitations of this study also have to be considered. Because of the use of a combination of 3 birth cohorts, we cannot fully rule out that population stratification may have contributed to some of the results. However, we have previously published consistent genetic effects and gene-environment interactions in childhood atopy development in our Allergenic cohort,[24] showing that major genetic effects and gene-environment interaction can be replicated in this cohort. The combination of these cohorts can also be considered a strength of the study, because similar findings in different age groups replicate the findings within the cohorts. Finally, we did not assess environmental influences, and it is clear that these can be of great importance in the development of atopy; thus, we recommend considering these in future research.

Another limitation of our study is that too few children were tested for bronchial hyperresponsiveness and lung function to investigate gene-gene interactions in Treg genes. We suggest that future studies, including meta-analyses, should be performed to investigate these objective markers of asthma. In addition, we suggest investigating these genes in relation to different wheezing phenotypes of preschool children (ie, late-onset wheeze and persistent wheeze), because these phenotypes may reflect the onset of asthma and the potential role of Treg genes more closely.

It is important to note that several genes were associated with atopic phenotypes at 1 to 2 as well as 6 to 8 years, internally replicating our results. Some associations were found to associate with multiple phenotypes, but in opposite directions—for example, *FOXP3* rs4824747 associated with sIgE to egg at age 1 to 2 years and to indoor allergens at age 6 to 8 years in 2 directions, either a risk or a protection. Associations of genetic polymorphisms that appear to have different effects are frequently observed in genetic studies. This may have several interpretations. Either the results represent false-negative results, because after FDR correction none of the results appeared significant; or the gene may have distinct influences on both phenotypes. It is clear that sIgE to egg has different clinical implications than sIgE to indoor allergens, and thus their pathogenic mechanism may be truly different. Furthermore, and especially because these findings were in different age groups, environmental factors may play a role. For example, from the gene *CD14*, multiple studies have

TABLE II. Summary of the results of MDR analyses of total IgE, sensitization, and asthma

Loci*	Best model: Gene	Best model: SNP	Mean CV consistency	Mean testing balanced accuracy (%)	*P*†
		Total IgE‡ 1-2 y			
1	*IL2RA*	rs2076846	2.6	0.53	.18
2	*TLR2 TGFBR2*	rs4696480 rs304839	3.2	0.55	.06
3	*TGFBR2 TGFBR2 IL2RA*	rs6770038 rs9843942 rs4749926	5.8	0.60	**<.001**
		Total IgE‡ 6-8 y			
1	*TLR2*	rs4696480	6.4	0.55	.09
2	*TLR2 TGFBR2*	rs4696480 rs9790292	5.6	0.57	**.01**
3	*TLR2 IL6RA IL2RA*	rs4696480 rs4845617 rs4749926	3.6	0.58	**.02**
		sIgE§ to milk at 1-2 y			
1	*TGFBR2*	rs2276767	2.4	0.55	.12
2	*TGFBR2 FOXP3*	rs2276767 rs6609857	7.0	0.58	**.02**
3	*IL2RA TGFBR2 TGFBR2*	rs2076846 rs3773663 rs9831477	2.6	0.58	**.02**
		sIgE§ to egg at 1-2 y			
1	*IL6*	rs2069845	7.0	0.57	.08
2	*IL6 TGFBR2*	rs2069845 rs3773634	6.0	0.62	**.02**
3	*HMOX1 FOXP3 IL2RA*	rs2071749 rs3761548 rs706779	2.2	0.60	**.02**
		sIgE§ to indoor allergens at 6-8 y			
1	*IL6R*	rs7514452	7.0	0.57	**.04**
2	*TGFB1 IL6R*	rs1800469 rs7514452	2.8	0.57	**.05**
3	*TGFB1 IL6R TGFBR2*	rs1800469 rs7514452 rs9831477	3.8	0.61	**.02**
		Asthma‖ at 6-8 y			
1	*TLR2*	rs1898830	3.4	0.56	.08
2	*TLR2 FOXP3*	rs1898830 rs3761548	2.0	0.55	.09
3	*TLR2 IL2RA TGFBR2*	rs1898830 rs3118470 rs3773636	1.4	0.62	**.001**
		Diagnosed asthma¶ at 6-8 y			
1	*IL2RA*	rs2386841	3.0	0.57	.06
2	*IL2RA TGFBR2*	rs7910961 rs9831477	1.4	0.59	.06
3	*IL2RA FOXP3 IL2RA*	rs2386841 rs3761548 rs4749926	1.8	0.64	**.008**

CV, Cross-validation.
*Number of loci considered.
†Significance of prediction error (empirical *P* value based on 1000 permutations).
‡Highest vs lowest tertile.
§Specific IgE ≥0.35 IU/mL.
‖At least 1 episode of wheeze or dyspnea in the last year and/or the use of inhaled steroids.
¶Diagnosis of asthma confirmed by a physician.

indicated that dependent on environmental circumstances such as endotoxin or pet exposure, 1 allele can be a risk factor in case of exposure and a protective factor in case of nonexposure.[24,44-46] The effects of various other SNPs observed early in life were not confirmed at age 6 to 8 years for asthma. This suggests that the distinct phenotypes have distinct pathomechanisms. However, we should acknowledge that the results may represent false-negative results.

It has been pointed out that individual genetic effects of complex diseases are mostly moderate or small, even when interactions are taken into account.[47] The strongest genetic effect we found represented a relative risk on the order of 4.0 (Fig 2; prevalence of 20% vs 5% for *TGFBR2* TT vs A in subjects with *IL6* AA). However, the impact in the population is small because the genotype combination with the highest risk is rare in our cohorts (24/926; 2.6%). Other interactions were smaller in terms of relative risk (on the order of 2.0; Fig 2, *A and C*) but affected a larger segment of the population and thus may have a higher population impact.

This study is the first to evaluate multiple novel functionally related genes in Treg cells by using MDR. Therefore, further studies will have to replicate our findings. Nevertheless, because we found association in 2 age groups, in which there was little overlap between the children investigated, our findings have already been replicated within our Allergenic cohort. MDR is a new technique that can be considered an unbiased data-mining approach. Our study can therefore be viewed as hypothesis-generating. Results can guide future studies to determine whether these genetic effects can also be replicated in other cohorts and ultimately in functional studies.

Some of the genes investigated in our study are exclusively expressed by Treg cells—for example, *FOXP3*—whereas other

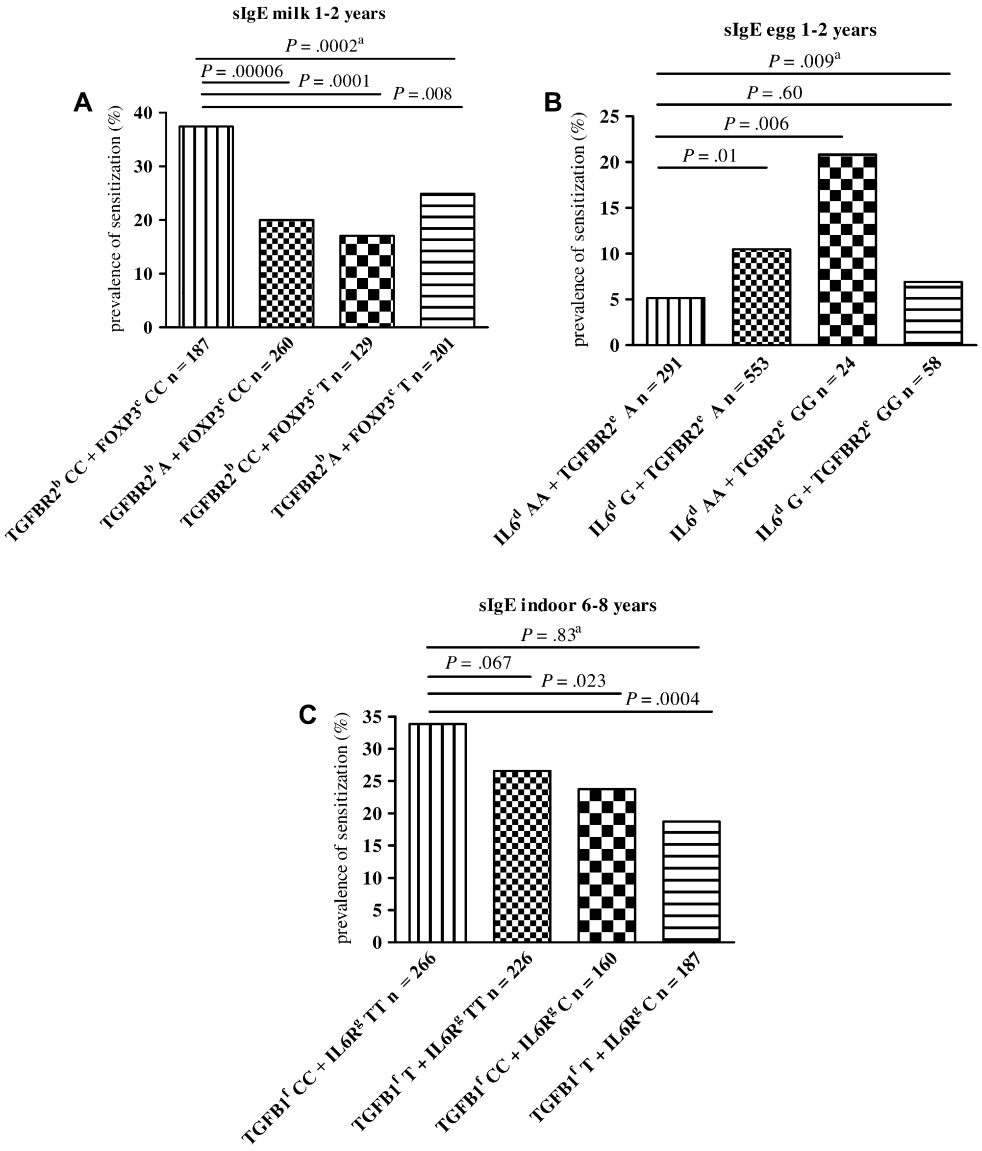

FIG 2. Logistic regression of 2 locus models identified by MDR. [a]*P* value for interaction. [b]*TGFBR2* rs2276767, A = genotypes CA and AA. [c]*FOXP3* rs6609857, T = genotypes CT and TT. [d]*IL6* rs2069845, G = genotypes AG and GG. [e]*TGFBR2* rs3773634, A = genotypes AA and AG. [f]*TGFB1* rs1800469, T = genotypes CT and TT. [g]*IL6R* rs7514452, C = genotypes CT and CC.

genes are also expressed by other cell types relevant for asthma development—for example, *TLR2* and airway epithelium. However, the combined effects of the genes in our study suggest that these genes contribute to allergic disease through their involvement in Treg cells.

TLR2 combined with *TGFBR2* and *IL2RA* SNPs shows association with both total IgE and asthma. *TLR2* has previously been found to associate with atopy and asthma susceptibility in farmers' children[6] and with asthma in an adult population.[7] Because of our results, we propose that the association of *TLR2* with asthma is complex and involves additional genes that contribute to Treg development. Interestingly, activation of innate immune responses through *TLR2* ligands has been shown to induce Treg-associated gene expression in cord blood mononuclear cells from neonates.[48] We speculate that genetic variations in *TGFBR2* and *IL2RA* alter the response from Treg cells to immune stimulation—for example, by lower TGFBR2 and IL2RA protein expression—and skew the response toward a T_H2-mediated reaction depending on the alleles an individual carries for *TLR2* SNPs rs4696480 and rs1898830. Unfortunately, the design of our study does not allow us to evaluate whether interaction between a farming environment and *TLR2* in combination with *TGFBR2* and *IL2RA* exists because we do not have complete information on farming exposure, but we recommend evaluating this in future studies.

TGFBR2 genetic variation showed interaction with *FOXP3* for sensitization to milk allergens, as observed in 2 separate cohorts and in both sexes. Interestingly, the prevalence of asthma and a doctor's diagnosis of asthma at age 6 to 8 years showed a remarkably similar pattern for these 2 SNPs (see this article's Fig E1 in the Online Repository at www.jacionline.org), and the interaction was confirmed by using logistic regression for doctor's diagnosis of asthma ($P = .006$), strongly suggesting true biological

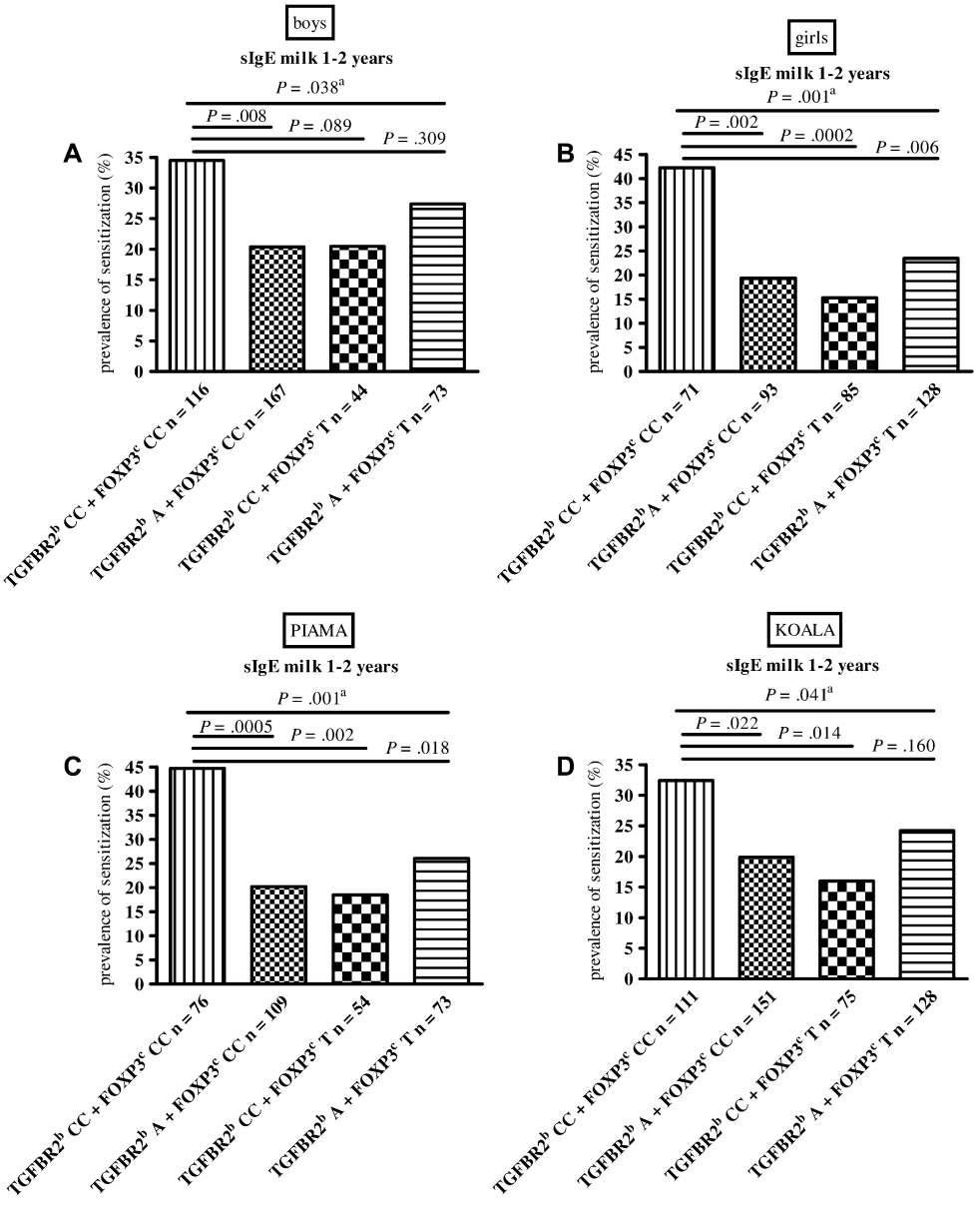

FIG 3. Consistent direction and significance of interaction between *TGFBR2* and *FOXP3* in boys and girls separately, and in each cohort. [a]*P* value for interaction. [b]*TGFBR2* rs2276767, A = genotypes CA and AA. [c]*FOXP3* rs6609857, T = genotypes CT and TT.

relevance of this finding. The mechanism of this interaction cannot be deduced from our study and can only be speculated on. TGFBR2 mediates the numerous activities of TGFB1 in, for example, differentiation of Treg cells. Mice lacking TGFBR2 specifically in T cells were found to have reduced peripheral Foxp3-positive Treg cell numbers.[49] Our data suggest that, dependent on genetic variation in *TGFBR2* and *FOXP3*, altered signaling through TGFBR2 may have distinct effects on activation of *FOXP3* and Treg development.

Intriguingly, this study demonstrates association of *TGFB1*, *TGFBR2*, *IL6*, and *IL6R* SNPs with atopic sensitization. These genes recently appeared to be involved in maintaining a balance between Treg cells and T_H17 cells. Differentiation of Treg cells and T_H17 cells appears to be cross-regulated by TGFB and IL6.[50] TGFB by itself stimulates development of Treg cells, whereas TGFB in combination with IL6 stimulates T_H17 development. T_H17 cells are a recently described subset of IL17 producing effector T cells that have been implicated in the pathogenesis of allergy and allergic airway inflammation.[51] To date, the exact role of T_H17 cells in allergic disease has not been fully elucidated. T_H17 cells are suggested to downregulate the T_H2 response, resulting in a diminished IgE response. However, mice deficient for IL17 showed impaired ovalbumin sensitization and reduced T_H2-type allergic airway inflammation, and thus T_H17 cells may also contribute to the development of allergic sensitization.[52] From the associations found in our study, we hypothesize that a genetically regulated disturbed balance in development of Treg cells and T_H17 cells contributes to allergic sensitization.

Detection of genetic association with main effects of *FOXP3* polymorphisms on sensitization were observed in girls. This

contrasts with our expectations, because the X-linked immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) expresses itself in hemizygous boys but rarely in girls.[53] The pathogenetic or methodologic explanation of our observations is unknown but can be speculated on. First, Wildin and Freitas[53] have proposed several explanatory mechanisms for *FOXP3* to be involved in susceptibility to immunologic disease in females. They observed variable expression of disease in *FOXP3* heterozygous female mice that were also heterozygous to a mutation of another X-chromosomal gene, γC. This suggests that genes on the alternative X-chromosome can interact with the *FOXP3* locus through influence on X-inactivation, thereby modifying disease outcome. Further explanations proposed by these authors[53] included other epigenetic mechanisms such as (1) sex-specific hormonal influences or (2) imprinting, in which allelic expression is dependent on the sex of the parent from whom the specific allele was inherited. Interestingly, it has recently been shown that Y-chromosome–linked polymorphisms may differentially regulate the expression of X-linked and autosomal genes.[54] Thus, hypothetically, *FOXP3* gene expression may be differentially regulated in males and females through suppressive or activation mechanisms regulated by the Y-chromosome. In addition to these pathogenic mechanisms, an alternative explanation for not finding a comparable effect in boys could be less statistical power to detect an effect that increases with the number of alleles, because girls have twice the number of alleles as boys.

In conclusion, this pathway study shows importance of multiple genetic variants in genes involved in the development and function of Treg cells in childhood atopy and asthma development, by combined genetic effects, as indicated by genetic association of a.o. *IL2RA*, *TLR2*, *TGFBR2*, and *FOXP3* replicated across age groups and cohorts. Furthermore, this study adds to recently emerging evidence for a role of the balance between Treg cells and T_H17 cells in atopic disease by showing association of *TGFB1*, *TGFBR2*, *IL6*, and *IL6R* SNPs with atopic sensitization. Finally, we have shown that genetic effects may be missed when gene-gene interaction is not considered, and we recommend future studies to evaluate main as well as combined genetic effects.

We thank the children and parents of the PIAMA, PREVASC, and KOALA studies for their participation. In addition, we acknowledge the field workers, secretaries, and scientific collaborators dedicated to the PIAMA, PREVASC, and KOALA cohorts, and Marcel Bruinenberg for his advice on DNA isolation, processing, and genotyping. We thank Jason H. Moore for his helpful comments on our questions with respect to MDR analyses.

Key messages

- ***TGFBR2*, *IL2RA*, *FOXP3*, and *TLR2* SNPs associate with atopy phenotypes by gene-gene interaction.**
- **Multilocus associations of biologically related genes are common in the complex development of atopic disease, and modeling of multiple gene-gene interactions is important in genetic association studies.**

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DESCRIPTION OF THE PREVASC, PIAMA, AND KOALA COHORTS

The 3 cohorts recruited children during pregnancy. The PREVASC[E1,E2] study investigates the primary prevention of asthma by implementing a multifaceted prenatally started intervention strategy in high-risk infants. The study further includes a separate group of low-risk children followed without intervention for the natural history of asthma and atopy. The PIAMA[E3] study also includes an intervention part—a double-blind placebo-controlled study on the primary preventive effect of the use of mattress covers—and a natural history part. The KOALA[E4] study recruited participating children among pregnant women who were invited for a prospective cohort study on pregnancy-related pelvic girdle pain, and a group of children has been recruited among pregnant women with alternative lifestyles through organic food shops, anthropologic doctors and midwives, Steiner schools, and magazines.

PREVASC

The objective of the PREVASC study is to investigate whether a multifaceted prenatally started intervention strategy in high-risk infants prevents asthma development[E1,E2] High risk for asthma and atopy was defined as at least 1 first-degree family member affected with asthma as registered by their general practitioner. At baseline, 476 children were recruited by primary caregivers (general practitioners and midwives) and advertisements and randomized to either (1) a control group (n = 234) receiving usual care, or (2) an intervention group (n = 242) in which families followed the primary prevention program. The latter included house dust mite–impermeable bed coverings, education on breast and hypoallergenic feeding, timing of solid food introduction, and smoking cessation. In addition, a separate group of 317 children at low risk for asthma were followed without intervention for the natural history of asthma and atopy. Low risk was defined as the absence of asthma in first-degree family members of the children. Children participating in the intervention study were born between July 1997 and July 2000, and the low-risk children were born between March 1999 and October 2002.

PIAMA

The primary aim of the PIAMA study is to investigate the incidence and risk factors and prevention of asthma and inhalant atopy.[E3] At baseline, 4146 children were included, 1327 children of mothers with allergy and 2819 children of mothers without allergy. Recruitment took place during the first trimester of pregnancy with a validated short screening questionnaire[E5] and was conducted by 52 midwife practices in 3 different regions in the Netherlands: north (Groningen and surroundings), central (Bilthoven and Wageningen and surroundings), and southwest (Rotterdam and surroundings). Women reporting any of the following self-reported symptoms were defined as having allergy: a history of asthma, current hay fever, and current atopy to dust house dust mite or pets. Their children were defined as high-risk. Women reporting none of these symptoms were defined as not having allergy, and their children were defined as low-risk. Four hundred seventy-two high-risk children and 2819 low-risk children were included in the natural history part of the study. Eight hundred fifty-five high-risk children were included in the intervention part of the study. The participating children were born between May 1996 and December 1997.

KOALA

The primary aim of the KOALA Birth Cohort Study[E4] is to identify factors that influence the clinical expression of atopic disease. The main focus is lifestyle, dietary habits and infections during the first year of life, and gene-environment interaction. At baseline, 2343 children were recruited among pregnant women who were invited for a prospective cohort study on pregnancy-related pelvic girdle pain.[E6] In addition, a group of 491 children was recruited through organic food shops, anthropologic doctors and midwives, Steiner schools, and magazines among pregnant women who had an alternative lifestyle with regard to child rearing practices, dietary habits, vaccination schemes, and use of antibiotics. All children were enrolled between 14 and 18 weeks of gestation and were born between February 2001 and August 2003.

TOTAL IgE TERTILES

Cases and controls were defined as children with a serum IgE level in, respectively, the highest and lowest tertiles as estimated at each age in boys and girls separately. We clustered measurements from ages 1 and 2 years, and 6 and 8 years. In the subgroup of children with IgE measurements available at both 1 and 2 years, the highest and lowest tertiles were determined from the mean of the 2 measurements (Table E1).

SPECIFIC IgE MEASUREMENTS

Positive specific IgE was defined as (1) specific IgE ≥0.35 IU/mL for milk at age 1 and 2 years (PIAMA at age 1 year; KOALA at age 1 or 2 years), (2) specific IgE ≥0.35 IU/mL for egg at age 1 and 2 years (PIAMA at age 1 year; PREVASC and KOALA at age 1 or 2 years), and (3) specific IgE ≥0.35 IU/mL for house dust mite, dog, or cat (indoor allergens) at ages 6 and 8 years (PREVASC at age 6 years; PIAMA at age 8 years).

MDR

Multifactor dimensionality reduction (version 1.0.0) reduces the dimensionality of multifactor information to 1 dimension: high-risk or low-risk. First, the data are divided into a training set (9/10 of the data) and an independent testing set (1/10 of the data). The model with the best classification error is selected from the training set, and the prediction error of that model is estimated by using the testing set. This procedure is repeated 10 times, and the model with the combination of loci that maximizes the cross-validation consistency and minimizes the prediction error is selected. Cross-validation consistency is a measure of the number of times an MDR model is identified in each possible group of nine tenths of the subjects. Because missing data are not allowed in MDR, missing SNPs were imputed by using the Multivariate Imputation by Chained Equations (Mice) procedure that runs under the statistical program R version 2.4.1. After 100 iterations, convergence was achieved resulting in 5 imputed datasets. MDR analyses were performed separately by using each imputed dataset, and subsequently, average testing accuracies and cross-validation consistencies were calculated.

The model with the highest average cross-validation consistency and lowest average prediction error was selected as the best model. Average cross-validation consistency is the number of times the model was selected as the best model after 10-fold cross-validation runs. Average testing balanced accuracy is the accuracy of classification of cases and controls in the testing

dataset (one tenth of the data) calculated as (sensitivity + specificity)/2. To examine the significance of testing accuracies, we determined 1000 MDR analyses for each test after permutation (200 of each imputation file). The null hypothesis was rejected when the 1-sided *P* value, as estimated by 1000 permutations in Monte Carlo simulation, was <5%. In that case, the best model predicts the status of cases and controls better than chance without the need to correct for multiple testing. To exclude the possibility that low cross-validation consistency was caused by SNPs that were highly correlated with SNPs present in the best model, MDR was performed a second time, and SNPs that showed strong linkage disequilibrium (D' > 0.8) with SNPs in the best model were excluded from analyses.

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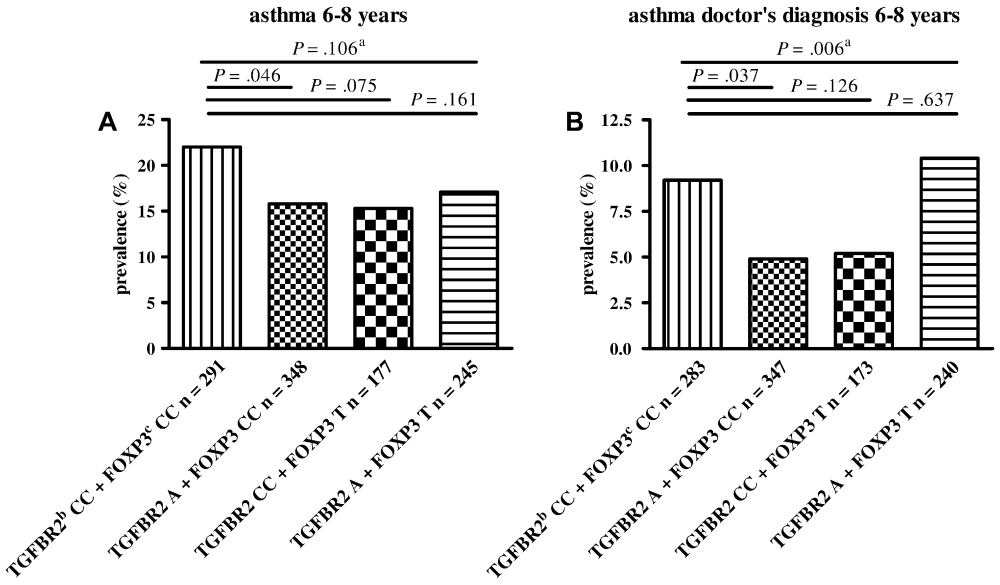

FIG E1. Interaction between TGFBR2 rs2276767 and FOXP3 rs6609857 in asthma **(A)** and doctor's diagnosis of asthma **(B)**. [a]*P* value for interaction. [b]*TGFBR2* rs2276767, A = genotypes CA and AA. [c]*FOXP3* rs6609857, T = genotypes CT and TT.

TABLE E1. Cutoff values of serum IgE levels used to determine highest tertiles of serum IgE in girls and boys separately

	Cutoff values of serum IgE levels in cases and control children (IU/mL)			
	Cases (3rd tertile)		Controls (1st tertile)	
Age (y)	Girls (N)	Boys (N)	Girls (N)	Boys (N)
1 y	9.00 (91)	13.0 (87)	3.0 (101)	4.0 (97)
1-2 y*	12.9 (115)	19.7 (121)	3.9 (114)	5.9 (121)
2 y	18.0 (36)	28.0 (53)	4.4 (49)	7.1 (58)
6 y	46.0 (37)	40.0 (34)	11.0 (38)	12.0 (34)
8 y	93.0 (115)	140.0 (121)	28.0 (116)	34.0 (120)

*Tertiles calculated as the mean of 2 measurements at age 1 and 2 years. Numbers do not add up to 540 controls at age 1 to 2 years because information on sex was missing for 1 individual in the lowest tertile.

TABLE E2. Candidate genes and SNPs evaluated in this study

Gene	Rs no.	MAF	HW equilibrium*	Synonym	Source	Chromosomal location
IL6R	rs1386821	0.20	>0.05		HapMap	1q21
IL6R	rs4075015	0.43	>0.05		HapMap	1q21
IL6R	rs4329505	0.17	>0.05		HapMap	1q21
IL6R	rs4341355	0.25	>0.05		HapMap	1q21
IL6R	rs4453032	0.39	>0.05		HapMap	1q21
IL6R	rs4601580	Failed	NA		HapMap	1q21
IL6R	rs4845617	0.38	>0.05	-183 G/A	HapMap, Galicia et al[E7]	1q21
IL6R	rs7514452	0.20	>0.05		HapMap	1q21
IL6R	rs8192284	0.38	>0.05	D358A	Galicia et al[E7]	1q21
IL10	rs1518111	0.21	>0.05		Ii†	1q31-q32
IL10	rs1554286	0.18	>0.05		Ii†	1q31-q32
IL10	rs1800871	0.23	>0.05	-819C/T	Ii,† Lyon et al,[E8] Karjalainen et al,[E9] Chatterjee et al[E10]	1q31-q32
IL10	rs1800872	0.23	>0.05	-571 or -592C/A	Lyon et al,[E8] Karjalainen et al,[E9] Chatterjee et al,[E10] Hobbs et al[E11]	1q31-q32
IL10	rs1800896	0.49	>0.05	-1082A/G	Ii,† Lyon et al,[E8] Karjalainen et al,[E9] Chatterjee et al[E10]	1q31-q32
IL10	rs3024498	0.27	>0.05		Ii†	1q31-q32
IL10	rs3024505	0.16	>0.05		Ii†	1q31-q32
HMOX1	rs2071746	0.42	>0.05	-413 T/A	Ono et al,[E12] Exner et al[E13]	22q13.1
HMOX1	rs2071748	0.36	>0.05		HapMap	22q13.1
HMOX1	rs2071749	0.48	>0.05		HapMap	22q13.1
HMOX1	rs5995098	0.31	>0.05		HapMap	22q13.1
TGFBR2	rs1036095	0.24	>0.05		HapMap	3p22
TGFBR2	rs11129420	0.49	>0.05		HapMap	3p22
TGFBR2	rs11466500	0.12	>0.05		HapMap	3p22
TGFBR2	rs11924422	0.41	>0.05		HapMap	3p22
TGFBR2	rs12495646	0.34	>0.05		HapMap	3p22
TGFBR2	rs13083813	0.35	>0.05		HapMap	3p22
TGFBR2	rs13086588	0.33	>0.05		HapMap	3p22
TGFBR2	rs1346907	0.44	>0.05		HapMap	3p22
TGFBR2	rs1431131	0.35	>0.05		HapMap	3p22
TGFBR2	rs17025785	0.37	>0.05		HapMap	3p22
TGFBR2	rs2043136	0.25	>0.05		HapMap	3p22
TGFBR2	rs2082224	0.24	>0.05		HapMap	3p22
TGFBR2	rs2116142	0.30	>0.05		HapMap	3p22
TGFBR2	rs2276767	0.34	>0.05		HapMap	3p22
TGFBR2	rs2276768	0.11	>0.05		HapMap	3p22
TGFBR2	rs2372212	0.20	>0.05		HapMap	3p22
TGFBR2	rs304839	0.16	>0.05		HapMap	3p22
TGFBR2	rs3773634	0.29	>0.05		HapMap	3p22
TGFBR2	rs3773636	0.26	>0.05		HapMap	3p22
TGFBR2	rs3773652	0.45	>0.05		HapMap	3p22
TGFBR2	rs3773663	0.45	>0.05		HapMap	3p22
TGFBR2	rs4522809	0.46	>0.05		HapMap	3p22
TGFBR2	rs4583693	0.18	>0.05		HapMap	3p22
TGFBR2	rs4955189	0.38	>0.05		HapMap	3p22
TGFBR2	rs6770038	0.20	>0.05		HapMap	3p22
TGFBR2	rs6792117	0.47	>0.05		HapMap	3p22
TGFBR2	rs876687	0.12	>0.05		HapMap	3p22
TGFBR2	rs934328	0.30	>0.05		HapMap	3p22
TGFBR2	rs9790292	0.44	>0.05		HapMap	3p22
TGFBR2	rs9831477	0.41	>0.05		HapMap	3p22
TGFBR2	rs9843143	0.50	>0.05		HapMap	3p22
TGFBR2	rs9843942	0.34	>0.05		HapMap	3p22
TGFBR2	rs9850060	0.23	>0.05		HapMap	3p22
IL2	rs2069762	0.27	>0.05	384 G/T; -330 G/T	Matesanz et al[E14]	4q26-q27
IL2	rs2069772	0.30	>0.05		HapMap	4q26-q27
IL2	rs2069778	0.16	>0.05		HapMap	4q26-q27
TLR2	rs1898830	0.34	>0.05	-15607A/G	Ii,† Eder et al[E15]	4q32
TLR2	rs3804099	0.44	>0.05	596C/T	Ii†	4q32

(Continued)

TABLE E2. *(Continued)*

Gene	Rs no.	MAF	HW equilibrium*	Synonym	Source	Chromosomal location
TLR2	rs3804100	0.07	>0.05	1349C/T	Ii,† Eder et al[E15]	4q32
TLR2	rs4696480	0.49	>0.05	-16934A/T	Ii,† Eder et al[E15]	4q32
IL6	rs13447445	0	NA		Fishman et al[E16]	7p21
IL6	rs2069840	0.38	>0.05		HapMap	7p21
IL6	rs2069845	0.42	>0.05		HapMap	7p21
TGFBR1	rs10733710	0.20	>0.05		HapMap	9q22
TGFBR1	rs10739778	0.36	>0.05		HapMap	9q22
TGFBR1	rs334356	0.18	>0.05		HapMap	9q22
IL2RA	rs11256456	0.20	>0.05		HapMap	10p15-p14
IL2RA	rs11256497	0.36	>0.05		HapMap	10p15-p14
IL2RA	rs12722486	0.05	>0.05		HapMap	10p15-p14
IL2RA	rs12722561	0.16	>0.05		HapMap	10p15-p14
IL2RA	rs12722588	0.18	>0.05		HapMap	10p15-p14
IL2RA	rs12722596	0.11	>0.05		HapMap	10p15-p14
IL2RA	rs2076846	0.34	>0.05		HapMap	10p15-p14
IL2RA	rs2386841	0.17	>0.05		HapMap	10p15-p14
IL2RA	rs2476491	0.29	>0.05		HapMap	10p15-p14
IL2RA	rs3118470	0.32	>0.05		HapMap	10p15-p14
IL2RA	rs4749926	0.39	>0.05		HapMap	10p15-p14
IL2RA	rs6602392	0.10	>0.05		HapMap	10p15-p14
IL2RA	rs706778	0.40	>0.05		HapMap	10p15-p14
IL2RA	rs706779	0.46	>0.05		HapMap	10p15-p14
IL2RA	rs706781	0.26	0.004‡		HapMap	10p15-p14
IL2RA	rs7910961	0.34	>0.05		HapMap	10p15-p14
IL2RA	rs791587	0.48	>0.05		HapMap	10p15-p14
IL2RA	rs791589	0.14	>0.05		HapMap	10p15-p14
IL2RA	rs9663421	0.29	>0.05		HapMap	10p15-p14
TGFB1	rs11466344	0.20	>0.05		Ii†	19q13.1
TGFB1	rs1800468	0.09	>0.05		Ii†	19q13.1
TGFB1	rs1800469	0.29	>0.05	C-509T	Ii,† Silverman et al,[E17] Hoffjan et al[E18]	19q13.1
TGFB1	rs1800471	0.07	>0.05	915 G/C	Ii,† Awad et al[E19]	19q13.1
TGFB1	rs1982073	0.38	>0.05		Ii†	19q13.1
TGFB1	rs8105161	0.15	>0.05		Ii†	19q13.1
FOXP3	rs2294019	0.29	>0.05§		SNPper‖	xp11.23-q13.3
FOXP3	rs2294021	0.45	>0.05§		SNPper‖	xp11.23-q13.3
FOXP3	rs3761548	0.42	>0.05§		HapMap	xp11.23-q13.3
FOXP3	rs3761549	0.13	>0.05§		HapMap	xp11.23-q13.3
FOXP3	rs4824747	0.13	>0.05§		SNPper‖	xp11.23-q13.3
FOXP3	rs5906761	0.44	>0.05§		SNPper‖	xp11.23-q13.3
FOXP3	rs6609857	0.29	>0.05§		SNPper‖	xp11.23-q13.3

MAF, Minor allele frequency; *NA*, not applicable.

*P value derived from χ^2.

†Innate Immunity Website (http://www.innateimmunity.net/data/homology).

‡Significant deviation from Hardy-Weinberg equilibrium in control individuals for total IgE at age 6 to 8 years ($P < .01$).

§Tested separately in girls because of location on X-chromosome.

‖For this gene, additional SNPs with a MAF >0.1 in white subjects were selected from SNPper database (http://snpper.chip.org/bio/find-gene).

TABLE E3. Level of linkage disequilibrium (LD) per gene

Gene	Mean LD (r^2)	Range (r^2)
IL6R	0.14	0.00-0.91
IL10	0.35	0.04-1.00
HMOX1	0.69	0.61-0.79
TGFBR2	0.08	0.00-0.88
IL2	0.12	0.08-0.16
TLR2	0.22	0.07-0.38
IL6	0.43	0.43-0.43
TGFBR1	0.07	0.00-0.68
IL2RA	0.12	0.00-0.74
TGFB1	0.07	0.00-0.68
FOXP3	0.35	0.06-0.99

TABLE E4. SNPs significantly associated with increased serum IgE at allele and genotype levels ($P < .05$)

		Association at allele level					Association at genotype level			
Gene	Rs no.	MAF* (controls/cases)	Allele	OR†	(95% CI)	P‡	Genotype	OR†	(95% CI)	P§
				Total serum IgE at age 1-2 y						
IL2RA	791589	0.12/0.16	G	1.3	1.0-1.8	**.04**	—	—	—	—
TGFBR2	3773663	0.44/0.48	A	1.2	1.0-1.4	**.04**	G:G/G:A	1.0		
		—	—	—	—	—	A:A	1.4	1.0-1.9	**.04**
TGFBR2	3773652	0.44/0.48	G	—	—	—	A:A	1.0		
		—	—	—	—	—	A:G/G:G	1.3	1.0-1.8	**.03**
				Total serum IgE at age 6-8 y						
IL6R	4453032	0.33/0.40	G	1.4	1.1-1.7	**.01**	—	—	—	—
IL6R	8192284	0.32/0.38	C	1.3	1.0-1.7	**.02**	—	—	—	—
IL10	3024498	0.32/0.26	G	0.7	0.6-0.9	**.01**	A:A	1.0		.05
		—	—	—	—	—	A:G	0.7	0.5-1.0	
		—	—	—	—	—	G:G	0.6	0.3-1.0	
FOXP3‖	2294019	0.26/0.36	A	1.6	1.1-2.2	**.01**	T:T	1.0		**.01**
		—	—	—	—	—	T:A/A:A	1.8	1.2-2.9	
FOXP3‖	6609857	0.27/0.35	T	1.5	1.0-2.1	**.03**	C:C	1.0		**.02**
		—	—	—	—	—	C:T/T:T	1.7	1.1-2.7	

*Minor allele frequency, controls/cases.
†OR calculated by logistic regression.
‡P value determined by $\chi 2$ test (1 df).
§P value determined by logistic regression.
‖Associations were tested in boys and girls separately because of location on X-chromosome, and were associated in girls only.

TABLE E5. SNPs significantly associated with specific serum IgE to egg or milk at 1 to 2 years, and to indoor allergens at 6 to 8 years, at allele and genotype levels ($P < .05$)

		Association at allele level					Association at genotype level			
Gene	**Rs no.**	**MAF***	**Allele**	**OR†**	**(95% CI)**	***P*‡**	**Genotype**	**OR†**	**(95% CI)**	***P*§**
					sIgE to egg at age 1-2 y					
FOXP3‖	3761548	0.41/0.26	A	0.5	0.3-0.9	**.009**	C:C	—	—	—
		—	—	—	—	**—**	C:A/A:A	0.5	0.3-1.0	**.04**
FOXP3‖	3761549	0.11/0.21	T	2.3	1.2-4.2	**.007**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	2.4	1.2-4.9	**.02**
FOXP3‖	4824747	0.11/0.20	T	2.1	1.1-3.8	**.02**	G:G	—	—	—
		—	—	—	—	**—**	G:T/T:T	2.1	1.0-4.2	**.05**
FOXP3‖	2294019	—	—	—	—	**—**	T:T/T:A	—	—	**—**
		—	—	—	—	**—**	A:A	3.0	1.3-6.7	**.009**
TGFBR2	13083813	—	—	—	—	**—**	T:T/T:A	—	—	**—**
		—	—	—	—	**—**	A:A	1.8	1.0-3.3	**.04**
IL6	2069845	—	—	—	—	**—**	A:A	—	—	**—**
		—	—	—	—	**—**	A:G/G:G	1.7	1.0-2.8	**0.04**
					sIgE to milk at age 1-2 y					
TGFBR2	12495646	0.35/0.29	A	0.8	0.6-1.0	**.02**	C:C/C:A	—	—	**—**
		—	—	—	—	**—**	A:A	0.5	0.3-0.9	**.01**
TGFBR2	1431131	0.36/0.30	A	0.8	0.6-1.0	**.03**	T:T/T:A	—	—	—
		—	—	—	—	**—**	A:A	0.4	0.2-0.8	**.006**
TGFBR2	9790292	0.46/0.39	T	0.8	0.6-1.0	**.03**	C:C/C:T	—	—	—
		—	—	—	—	**—**	T:T	0.6	0.4-0.9	**.02**
TGFBR2	17025785	—	—	—	—	**—**	T:T/T:C	—	—	**—**
		—	—	—	—	**—**	C:C	0.6	0.4-1.0	**.05**
TGFBR2	2276767	—	—	—	—	**—**	C:C	—	—	**—**
		—	—	—	—	**—**	C:A/A:A	0.7	0.5-1.0	**.03**
TGFBR2	6792117	—	—	—	—	**—**	G:G	—	—	**—**
		—	—	—	—	**—**	G:A	1.4	1.0-2.0	**—**
		—	—	—	—	**—**	A:A	0.9	0.5-1.4	**.05**
IL6R	1386821	0.22/0.17	C	0.7	0.6-1.0	**.04**	—	—	—	—
		—	—	—	—	**—**	—	—	—	**—**
IL2	2069772	0.33/0.27	G	0.8	0.6-1.0	**.03**	A:A/A:G	—	—	**—**
		—	—	—	—	**—**	G:G	0.5	0.3-1.0	**.04**
IL2RA	706778	0.42/0.36	A	0.8	0.6-1.0	**.04**	G:G	—	—	**—**
		—	—	—	—	**—**	G:A/A:A	0.7	0.5-0.9	**.02**
IL6	13447445	—	—	—	—	**—**	G:G	—	—	**—**
		—	—	—	—	**—**	G:C/C:C	6.2	1.1-33.9	**.04**
TGFB1	1800468	—	—	—	—	**—**	G:G	—	—	**—**
		—	—	—	—	**—**	G:A/A:A	1.6	1.1-2.4	**.02**
FOXP3‖	6609857	—	—	—	—	**—**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	0.6	0.4-1.0	**.03**
					sIgE to indoor allergens at age 6-8 y					
IL6R	4341355	0.29/0.24	C	0.8	0.6-1.0	**.03**	—	—	—	**—**
		—	—	—	—	**—**	—	—	—	**—**
Il6R	7514452	0.25/0.17	C	0.6	0.5-0.8	**.001**	T:T	—	—	**—**
		—	—	—	—	**—**	T:C/C:C	0.6	0.4-0.8	**.001**
FOXP3‖	4824747	0.15/0.12	T	0.7	0.5-1.0	**.03**	G:G	—	—	**—**
		—	—	—	—	**—**	G:T/T:T	0.5	0.3-1.0	**.04**
FOXP3‖	6609857	0.30/0.36	T	1.4	1.0-1.9	**.10**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	1.7	1.1-2.8	**.03**
TGFB1	1800469	0.30/0.25	T	0.8	0.6-1.0	**.06**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	0.7	0.5-0.9	**.01**

*Minor allele frequency, controls/cases.
†OR calculated by logistic regression.
‡*P* value determined by χ^2 test (1 *df*).
§*P* value determined by logistic regression.
‖Associations were tested in boys and girls separately because of location on X-chromosome, and were associated in girls only.

TABLE E6. SNPs significantly associated with asthma at 6 to 8 years at allele and genotype levels ($P < .05$)

		Association at allele level					Association at genotype level			
Gene	**Rs number**	**MAF***	**Allele**	**OR†**	**(95% CI)**	***P*** ‡	**Genotype**	**OR†**	**(95% CI)**	***P*** §
FOXP3‖	3761548	—	—	—	—	**—**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	2.1	1.2-3.7	**.01**
FOXP3‖	2294021	—	—	—	—	**—**	C:C/C:T	—	—	**—**
		—	—	—	—	**—**	T:T	0.5	0.2-1.0	**.04**
FOXP3‖	5906761	—	—	—	—	**—**	T :T/T:C	—	—	**—**
		—	—	—	—	**—**	C:C	0.4	0.2-0.9	**.02**
IL10	1800871	—	—	—	—	**—**	C:C/C:T	—	—	**—**
		—	—	—	—	**—**	T:T	0.4	0.1-1.0	**.04**
IL10	1800872	—	—	—	—	**—**	C:C/C:A	—	—	**—**
		—	—	—	—	**—**	A:A	0.3	0.1-0.8	**.02**
TGFBR1	334356	—	—	—	—	**—**	C:C	—	—	**—**
		—	—	—	—	**—**	C:T/T:T	1.4	1.0-2.0	**.03**
IL2	2069772	0.29/0.35	G	1.3	1.1-1.7	**.02**	A:A/A:G	—	—	**—**
		—	—	—	—	**—**	G:G	1.8	1.1-3.1	**.02**
IL2RA	3118470	0.34/0.27	C	0.7	0.5-0.9	**.01**	T:T	—	—	**—**
		—	—	—	—	**—**	T:C/C:C	0.7	0.5-0.9	**.01**
IL2RA	706779	—	—	—	—	**—**	A:A	—	—	**—**
		—	—	—	—	**—**	A:G/G:G	1.5	1.0-2.2	**.03**

*Minor allele frequency, controls/cases.
†OR calculated by logistic regression.
‡*P* value determined by χ^2 test (1 *df*).
§*P* value determined by logistic regression.
‖Associations were tested in boys and girls separately because of location on X-chromosome, and were associated in girls only.