

Original article

Polymorphisms in interleukin-1 receptor-associated kinase 4 are associated with total serum IgE

Background: Serum immunoglobulin E (IgE) level is recognized to be under strong genetic control, but the causal and susceptibility genes remain to be identified. We sought to investigate the association between single nucleotide polymorphisms (SNPs) in the Toll-like receptor (TLR) signaling pathway and total serum IgE level.

Methods: A population of 206 patients with severe chronic rhinosinusitis (CRS) was used. Precise phenotyping of patients was accomplished by means of a questionnaire and clinical examination. Blood was drawn for measurement of total serum IgE, as well as DNA extraction. A maximally informative set of SNPs in the *TLR1*, 2, 3, 4, 6, 9, 10, *CD14*, *MD2*, *MyD88*, *IRAK4*, and *TRAF6* genes were selected and genotyped. Significant findings were replicated in a second independent population of 956 subjects from 227 families with asthma.

Results: A total of 97 out of 104 SNPs were successfully genotyped. Three SNPs in *IRAK4* – rs1461567, rs4251513, and rs4251559 – were associated with total serum IgE levels ($P < 0.004$). In the replication sample, the same SNPs as well as the same orientation of the risk allele were associated with IgE levels ($P < 0.031$).

Conclusions: These results demonstrate a clear association between polymorphisms in the *IRAK4* gene and serum IgE levels in patients with CRS and asthma. *IRAK4* may be important in the regulation of IgE levels in patients with inflammatory diseases of the airways.

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Diseases associated with elevated serum immunoglobulin E (IgE), such as atopy, asthma, and chronic rhinosinusitis (CRS), are on the rise worldwide (1). The exact role of IgE in these diseases remains unclear. The increased levels could reflect an underlying systemic pathology causing susceptible patients to develop atopy and related diseases,

or they could be the result of inflammation related to the disease load itself. Total serum IgE levels have been found to correlate with allergy and bronchial hyperresponsiveness in asthma, as well as to predict the development of asthma independently of other allergic factors (2).

Chronic rhinosinusitis is viewed as an inflammatory disease of the nose and sinuses from the interaction of sinonasal mucosa with bacteria and/or fungi. Blood IgE levels have been shown to be raised in patients with CRS and nasal polyposis compared to controls (3). There is also correlation between serum IgE levels and radiological severity of CRS (4). Site-specific expression of IgE has also been demonstrated in the sinus mucin of disease

Abbreviations: ASA, acetylsalicylic acid; CRS, chronic rhinosinusitis; ESS, endoscopic sinus surgery; *IRAK4*, interleukin-1 receptor-associated kinase 4; *MyD88*, myeloid differentiation factor 88; *NF-κB*, nuclear factor-kappa B; LD, linkage disequilibrium; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular patterns; SNP, single nucleotide polymorphism; *TRAF6*, TNF receptor-associated factor 6; TLR, toll-like receptor.

patients. A study of nasal secretions in polyposis patients showed no significant decrease in local IgE 1 year after sinus surgery and nasal steroid use (5).

Accurate comprehension of the genetic mechanisms regulating IgE expression is necessary to elucidate the hereditary components of atopic diseases. Twin and family studies have demonstrated that serum IgE is under strong genetic control, with heritability on the order of 44–60% (6). More recent linkage and genome-wide association studies have suggested the presence of important genes on chromosome 4, 5q, 6p, 7q, 7p, 9q, 11q, 12q, 13q, and 16q (7). Previous studies have identified polymorphisms in the promoter region of the *CD14* gene associated with IgE level, including the position -2984 (8), as well as -159 (9) or -260 (10). *CD14* is a pattern recognition receptor that binds to lipopolysaccharide (LPS), and is necessary in the activation of toll-like receptor 4 (TLR4) in response to LPS. Toll-like receptor 4 itself, as well as TLR2 have been shown decrease allergic inflammation in murine models, and have been implicated in the hygiene hypothesis of allergy development (11). These are an example of innate immune effectors modulating the adaptive immune response.

Components of innate immunity are also increasingly thought to play a role in the pathogenesis of CRS (12). The TLRs are important in the recognition of distinct pathogen-associated molecular patterns (PAMPs). They were originally discovered due to their similarity to *Drosophila* Toll protein, and they were also found to possess significant homology to the IL-1 receptor family (13). They constitute an ancient host defense mechanism expressed in nearly all multicellular organisms, from plants to mammals (14). There are currently 11 known TLRs in humans, and they are expressed on macrophages, dendritic cells, endothelial cells, and epithelial cells (12). Activation of the receptors leads to a downstream signaling cascade which results in the activation of multiple pro-inflammatory genes – such as *TNF α* , *IFN*, *IL-1*, and *IL-6* (15) – via the action of the transcription factors NF- κ B (16) and/or AP-1 (17) within the nucleus. The signaling pathway for many of the TLRs is initiated by the recruitment of myeloid differentiation primary response gene 88 (MyD88) to the cytoplasmic surface of the receptor, leading to the activation of interleukin-1 receptor-associated kinase 4 (IRAK4) and TNF receptor-associated factor 6 (TRAF6).

We sought to investigate whether polymorphisms in the genes encoding key TLR signaling molecules might be associated with total serum IgE levels. In order to investigate this, we employed an already established population consisting of 206 patients with severe CRS. By genotyping these individuals for a series of representative tagging single nucleotide polymorphisms (tag SNPs) within TLR signaling pathway genes, we were able to detect whether any of the variability found within these genes is associated IgE levels. We subsequently utilized a large asthma family-based study to replicate the findings.

Methods

Study design and population

A cross-sectional study was undertaken. The study was approved by the McGill University Health Centre surgical ethics committee, and informed consent was obtained from all participants. An established population of 206 prospectively recruited individuals with severe CRS (with or without polyposis, or recurrent acute sinusitis) was used. This population has previous been used to study candidate genes in the cysteinyl leukotriene synthesis pathway (18). Patients were recruited according to either of the following criteria: (1) persistent signs/symptoms of CRS despite previous endoscopic sinus surgery (ESS), or (2) a history of more than one ESS for CRS regardless of outcome.

A standardized questionnaire assessing age, sex, ethnic origin, and family history was administered. Smoking, seasonal and perennial allergies, physician diagnosed asthma and acetylsalicylic acid (ASA) intolerance were also assessed. Information on disease related factors including age at diagnosis, age at first surgery, number of previous surgeries, medications required for management of the disease, and an assessment of whether disease was controlled with medication were recorded. Initial diagnoses of CRS with or without nasal polyposis, or recurrent acute sinusitis, were taken from patient records, and classified according to 2004 American Academy of Otolaryngology-Head and Neck Surgery guidelines (19).

The presence or absence of allergy was assessed according to history of previous epicutaneous testing for airborne seasonal and perennial allergens. Repeat allergy testing was not performed. Sensitivity to ASA was based on a history of previous respiratory symptoms of wheezing and breathlessness or urticaria following ASA ingestion. Acetylsalicylic acid challenge was not performed to verify this.

Patients with cystic fibrosis were excluded from this study. In order to rule out a possibility of cystic fibrosis remaining undiagnosed at the adult age, all patients with either documented nasal polyposis or a history of nasal polyps requiring removal prior to the age of 21 underwent sweat chloride testing.

Blood was drawn for DNA extraction and measurement of total serum IgE. The blood was collected in BD Vacutainer Serum Separator Tubes (BD Diagnostics, Franklin Lakes, NJ, USA), and IgE measurements were performed using a DPC Immulite System (Diagnostic Products Corporation – Siemens, Los Angeles, CA, USA).

DNA extraction

DNA from patients was isolated from peripheral blood leukocytes. Blood was collected in citrate-treated tubes, and DNA was extracted using the Puregene DNA isolation kit (Gentra System, <http://www.gentra.com>), following the High Throughput Protocol for 10 ml Whole Blood provided with this kit. Isolated DNA was stored at -80°C prior to use.

SNP selection

Using the CEU genotype data from the International HapMap project (20), a maximally informative set of SNPs was selected for the *TLR1*, 2, 3, 4, 6, 9, 10, *CD14*, *MD2*, *MyD88*, *IRAK4*, and *TRAF6* genes, including 10 kb up- and downstream, using a pairwise tagging algorithm implemented in Haploview version 3.32 (21). Minor allele frequency and r^2 thresholds were set at 0.05 and 0.8, respectively.

Genotyping

Single nucleotide polymorphisms were assessed using MALDI-TOF mass array spectrometer (Sequenom, San Diego, CA, USA). Primers were designed using the Sequenom SNP Assay Design software version 3.0 for iPLEX reactions. The protocol and reaction conditions were done in accordance with the manufacturer's specifications (22). The SNPs rs2563310 (*CD14*) and rs4251545 (*IRAK4*) were of particular interest, since the former had been shown to be associated with IgE levels in a population of European children (10), and the latter was a coding nonsynonymous SNP located in exon 11 of the *IRAK4* gene. These were assayed using the fluorescence polarization SNP technique (23) due to suboptimal genotyping (below 90% call rate) by the MALDI-TOF mass array spectrometer method.

External validation

Positive associations were tested in a second independent population from Saguenay-Lac-Saint-Jean (SLSJ), an area of Northeastern Quebec (Canada). This asthma family-based cohort has been described in detail elsewhere (24). Single nucleotide polymorphisms were successfully genotyped in 956 individuals using the Illumina GoldenGate assay (25) as part of a larger SNP genotyping panel provided by the Allergy, Genes and Environment Network (Allergen).

Sequencing

The coding region of *IRAK4* was entirely sequenced in unrelated individuals from the CRS population, divided into two groups homozygous for either the major or minor alleles for the *IRAK4* SNP rs4251513. In each group, we chose a subset of five or six patients with high IgE and another subset with low IgE, for an overall total of 23 patients. The products of the sequencing reaction were run on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). This was performed in order to detect the presence of unknown nonsynonymous SNP in linkage disequilibrium (LD) with intronic SNPs showing significant association with IgE levels. Polymerase chain reaction primers to amplify the 11 coding exons are provided in Appendix S1 of the online supplementary materials.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was estimated using a chi square (χ^2) test; a value of 6.63 ($P = 0.01$ for 1 degree of freedom) was used as the cutoff value for passing the HWE test. Associations between genotype and IgE levels for all patients were assessed using an ANOVA test, which was performed in the *R* statistics software version 2.3.1. In the SLSJ population, single SNP associations were tested using the Family Based Association Test (FBAT) program (26). Tests were performed with the additive model using the empirical variance-covariance estimator. Immunoglobulin E levels were log transformed and adjusted for age and gender in both populations, and outliers (*Z*-score greater than 4) were removed. Significant associations between *IRAK4* polymorphisms and IgE levels were also analyzed for confounding factors such as smoking, atopic dermatitis, allergies and age of onset. Adjustment for multiple testing was performed using the method described by Nyholt, which adjusted for the number of SNPs genotyped within a gene by taking into consideration the levels of LD between them (27). The adjusted *P*-value threshold was calculated with the matSpD tool, available at <http://gump.qimr.edu.au/general/daleN/matSpD/>. The

Matrix Spectral Decomposition (matSpD) results for multiple testing adjustments in the *IRAK4* gene are illustrated in Appendix S3 of the online supplementary materials.

Results

Patient characteristics are shown in Table 1. The initial diagnosis was CRS with polyposis in 74.8%, CRS without polyposis in 13.1%, and recurrent rhinosinusitis in 12.1%. The mean total serum IgE measurements for these three groups were 161.31 ± 225.27 , 123.96 ± 167.12 , and 171.84 ± 206.26 IU/ml, respectively. All patients reported a history of previous epicutaneous testing for airborne seasonal and perennial allergens with a reported rate of respiratory allergies of 65.5%. Asthma requiring medication was noted in 53.9%. A history of sensitivity to NSAID's was reported by 33% of the patients. No patients had cystic fibrosis.

A total of 97 out of 104 SNPs were successfully genotyped. Five SNPs were monomorphic, and three failed HWE testing, and thus were not included in the analyses. Detailed information regarding all SNPs tested

Table 1. Patient characteristics

	Initial diagnosis		
	CRS with NP	CRS w/o NP	RAS
Gender			
<i>n</i>	154	27	25
Male	87	10	11
Female	67	17	14
Age at first ESS			
<i>n</i>	154	27	25
Mean	37.34	40.19	40.48
SD	14.67	16.51	16.44
Min	1	6	7
Max	75	76	67
Number of operations			
<i>n</i>	154	27	25
Mean	3.1	3	3.64
SD	2.76	2.45	3.91
Min	1	1	1
Max	20	13	20
Serum eosinophilia (%)			
<i>n</i>	151	27	25
Mean	4.58	3.71	4.27
SD	3.53	3.24	3.26
Min	0	0	0
Max	25	15	13.6
Total IgE level (IU/ml)			
<i>n</i>	150	27	25
Mean	161.31	123.96	171.84
Median	89.5	41	91
SD	225.27	167.12	206.26
Min	2	2	2
Max	1460	798	800

CRS, chronic rhinosinusitis; NP, nasal polyposis; RAS, recurrent acute sinusitis; ESS, endoscopic sinus surgery.

Table 2. Single nucleotide polymorphisms in the *IRAK4* gene selected for genotyping and results of ANOVA testing

Gene (accession)	SNP (rs#_alleles)	MAF†	HWE χ^2 ‡	P-value
<i>IRAK4</i> (NM_016123)	rs11182250_GC	0.063	14.04	—
	rs4251459_CT	0.139	0.00	0.7078
	rs1461567_TC	0.272	2.83	0.0035 *
	620-1delAC_-/AC	—	Failed	—
	rs4251487_TG	—	Failed	—
	821delT_-/T	—	Monomorphic	—
	T877C_CT	—	Monomorphic	—
	rs4251513_CG	0.474	0.35	0.0006 *
	rs4251580_TC	0.132	0.12	0.3409
	rs4251520_CT	0.112	0.16	0.4978
	rs4251583_GA	—	Monomorphic	—
	A1188 + 520G_GA	—	Monomorphic	—
	G1189-1T_TG	—	Failed	—
	rs4251545_AG	0.118	0.01	0.6377
	rs4251559_AG	0.480	0.22	0.0011 *
	rs17121283_TC	0.051	61.96	—
	rs6582484_CT	0.171	0.00	0.3870

The accession number from NCBI Entrez Gene, and SNP identification numbers are from the NCBI Build 35. SNP identification numbers are followed by an underscore, minor and major alleles, respectively. Note: rs4251559 from *IRAK4* has changed to rs1141168 in NCBI Build 36.

*Three SNPs achieving significance following the Nyholt correction for multiple testing, calculated to be at $P = 0.0073$ for the *IRAK4* gene.

†MAF refers to the minor allele frequency.

‡HWE χ^2 refers to the chi square test to verify that the genotyped alleles are in Hardy–Weinberg equilibrium.

are presented in Appendix S2 of the online supplementary materials. Three SNPs in the *IRAK4* gene – rs1461567, rs4251513, and rs4251559 (also known as rs1141168) – were found to be associated with total serum IgE levels. A nominal P -value less than 0.0073 was considered significant at this locus, based on the effective number of independent SNPs estimated at 7 using the multiple testing correction method described above. These SNPs are located in intron 2, intron 8 and in the downstream region of *IRAK4*, respectively. Table 2 illustrates the SNP names, major and minor alleles, the HWE chi square value, the minor allele frequency, as well as the unadjusted P -value of the ANOVA test comparing IgE levels for each genotype of *IRAK4*. The mean IgE levels for each genotype of these three SNPs are plotted in Fig. 1. The significant associations observed between these three SNPs and IgE levels were not influenced by confounding factors such as smoking, atopic dermatitis, allergies and age of onset.

The LD plot surrounding the *IRAK4* gene in our population, as generated by Haploview v3.32, is displayed in Fig. 2. The SNPs rs1461567, rs4251513, and rs4251559 were found to be in moderate to strong LD, which may suggest a common genetic signal.

All three of these SNPs are in noncoding regions of the *IRAK4* gene. In order to determine whether these were simply in LD with another coding SNP, which may be responsible for an alteration of function, we performed

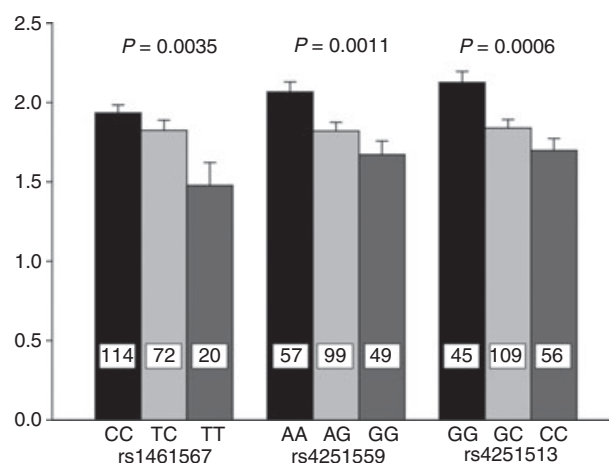


Figure 1. Log 10 scale representation of mean total serum IgE level according to *IRAK4* genotypes. Bar chart illustrating the mean total serum IgE transformed on the log10 scale on the Y-axis and the genotype for SNPs rs1461567, rs4251559, and rs4251513 on the X-axis. Error bars represent the standard error for each group, and the P -values from ANOVA tests are displayed above the bars. The number of subjects is indicated within each bar. The significance threshold required to keep the Type I error rate at 0.05 following the Nyholt correction for multiple testing was calculated to be at $P = 0.0073$.

sequencing for the complete coding region of this gene. This was performed in 23 homozygotes (approximately half for common and half for rare alleles) for the SNP rs4251513, since it was the most strongly associated with serum IgE. Three known polymorphisms were identified, including rs4251500 in intron 7, rs4251529 in intron 9, and rs4251545 in exon 11. In addition, a previously unknown C-to-T SNP was found in one patient at chromosomal position 42457650 (intron 6). Overall, the sequencing failed to identify any possible causal variants explaining the strong genetic association signal obtained with the intronic SNPs.

In order to confirm the association between IgE levels and *IRAK4* polymorphisms, a second independent population was assessed. The characteristics of SNPs in this replication sample are shown in Table 3. The association results are presented in Table 4. The SNPs rs1461567 and rs4251513 were included in the analysis, and their association with elevated total serum IgE levels remained significant after excluding all asthmatic individuals from the analyses ($P = 0.037$ and 0.003 , respectively).

Discussion

Our study suggests that total serum IgE level is associated with polymorphisms in the *IRAK4* gene. Specifically, the C allele of the C/T polymorphism rs1461567 is associated with elevated IgE, as is the G allele of the G/C polymorphism rs4251513, and the A allele of the A/G

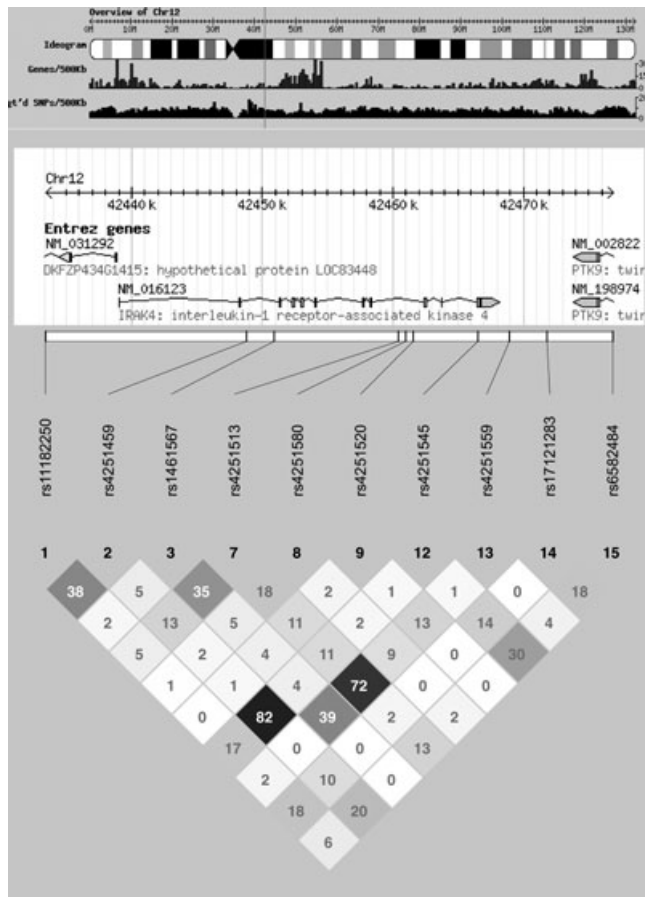


Figure 2. Linkage disequilibrium (LD) plot surrounding the *IRAK4* gene. The chromosomal location and exonic structure of *IRAK4*, including 10 kb up- and downstream, as illustrated by the HapMap genome browser, are displayed at the top of the figure. The *IRAK4* gene is located on chromosome 12. The LD plot surrounding the gene in our population, as generated by Haploview version 3.32, is displayed at the bottom of the figure. The genotyped SNPs are also illustrated with a scale of the physical distance between them. The colors of the squares illustrate the magnitude of pairwise r^2 values on a black and white scale where black indicates perfect LD ($r^2 = 1.00$) and white indicates perfect equilibrium ($r^2 = 0$); the numbers within the squares represent the actual r^2 values in our population.

Table 3. Characteristics of SNPs in the *IRAK4* gene genotyped in the asthma family-based study from Saguenay-Lac-Saint-Jean

SNPs	Call rate (%)	H-W <i>P</i> -value	MAF
rs10880565	100.0	0.231	0.11
rs4251431	99.8	0.022	0.09
rs1461567	100.0	0.762	0.24
rs4251513	100.0	0.755	0.45
rs4251565	100.0	0.387	0.04
rs6582484	99.9	0.197	0.11

H-W, Hardy-Weinberg equilibrium; MAF, minor allele frequencies.

polymorphism rs4251559. For the latter two SNPs, there appears to be a dose effect to this association, as is illustrated in Fig. 1. There is strong LD between these

Table 4. Association between *IRAK4* and IgE levels in an independent population from Saguenay-Lac-Saint-Jean

SNP	Alleles	AF†	Z	<i>P</i> -value*
rs10880565	A	0.887	0.308	0.378982
rs4251431	T	0.091	0.371	0.355427
rs1461567	C	0.763	1.865	0.031073
rs4251513	G	0.549	2.111	0.017389
rs6582484	C	0.124	1.549	0.060708

This table illustrates the results of analyses using the Family Based Association Test (FBAT) program (27), showing the alleles associated with elevated IgE. Five markers from 227 families (956 subjects) were evaluated.

**P*-values are for one-tailed tests.

†AF refers to allelic frequency.

two SNPs within our population ($r^2 = 0.72$), and moderate LD of each of these with rs1461567 ($r^2 = 0.35$ and 0.39). Finally, the associations observed for rs1461567 and rs4251513 were replicated in an independent population with asthma. The fact that the same SNPs with the same risk alleles are associated with a particular phenotype in an independent sample constitutes a solid replication (28). The SNP rs4251559 was not genotyped in the SLSJ cohort. Given its strong LD with rs4251513, we judged it to be adequately represented (or tagged) in the analysis.

In the current study, the strength of the association is lower in the family-based replication sample compared to the original case-control study. Both bias and genuine differences in the two sample sets can explain the effect size variation. However, it should be emphasized that the magnitude of effects observed in genetic studies are regularly found to be smaller in replication samples compared to the initial study examined (29). In fact, the estimate of effect declines as replication studies are pursued, which is a phenomenon known as the 'winner's curse' (30). Accordingly, the lower association in the replication sample observed in the current study is not surprising. It is likely that the association finding made in the case-control series of patients with CRS overestimated the predisposition conferred by the genetic polymorphisms and the true effect size may be best appreciated in the asthma family-based study.

Another possible explanation that is more specific to the current study may come from the IgE measurement variability. The mean and standard deviation of IgE levels are 157.6 ± 215.1 and 178.1 ± 618.5 IU/ml for the case-control CRS cohort and the asthma family-based study, respectively. From the standard deviation, we can see that the spread of IgE values are considerably higher in the asthma family-based study. Accordingly, despite a higher sample size, the high variability observed in the IgE values in the later population is predicted to negatively influence the power. Obviously, further studies are warranted to quantify the genetic effect conferred by *IRAK4* genetic variants on IgE levels.

The mechanism by which these polymorphisms may be exerting their effect is unclear at present. The results of *IRAK4* sequencing failed to identify genetic variants in the coding sequence, which would lead to an alteration in protein function. If there were a coding nonsynonymous SNP responsible for the observed phenotype, we would have expected to find it within the sequenced region of our subset of patients, associated with the G allele of rs4251513 in patients with elevated IgE, or conversely with the C allele in patients with low IgE. Additional studies are required to identify the causal variant(s) in *IRAK4* that conferred genetic susceptibility to serum IgE levels.

The role of *IRAK4* in TLR signaling has been shown to be a dominant one. Suzuki et al. (31) demonstrated that *IRAK4*-deficient mice have been unable to mount an innate immune response to infected bacteria, are resistant to LPS-induced septic shock, and produce no response to IL-1, IL-18, nor to most of the TLRs. Its ability to transmit signals following IL-1 receptor stimulation has been shown to be, at least in part, independent of its kinase activity (32).

Several distinct mutations in *IRAK4* have been described in humans, with loss of function associated to dramatic and recurrent bacterial infections, as well as poor inflammatory response. Medvedev et al. (33) described a patient expressing a compound heterozygous genotype for *IRAK4*, consisting of a point mutation (C877T in cDNA) in one allele, and a two-nucleotide deletion (620–621delAC in cDNA) in the other. Both of these mutations resulted in truncated forms of *IRAK4* lacking kinase activity which, when over expressed in a HEK293T cell line, prevented the normal inflammatory response to LPS and IL-1. Picard et al. (34) described three unrelated children with recurrent pyogenic infection, principally caused by *Streptococcus pneumoniae* and *Staphylococcus aureus*. Two of the children were homozygous for the C877T mutation, and the other child was homozygous for a T deletion at position 821 in cDNA. Another report describes a child recurrent invasive pneumococcal disease expressing a compound heterozygous genotype for *IRAK4* consisting of a G-to-T and an A-to-G substitution at positions 1189–1 and 1188 + 520, respectively (35). None of the above mutations could be detected in our population, leading us to believe that these variants are uncommon. No studies in the literature to date have reported an association between *IRAK4* variants and serum IgE levels, nor has any of the above studies provided information regarding IgE measurements in *IRAK4*-deficient mice or humans. This data would be very useful to help elucidate a functional mechanism for this effect.

We were unable to replicate the association of SNP rs2563310 (*CD14*) with IgE serum levels as reported in a previous study. Our inability to replicate an association with IgE, may be related to difference in the study populations. Baldini et al. (9) studied a cross-

sectional general population sample as part of the Tucson Children's Respiratory Study, whereas we focussed strictly on patients with CRS or asthma. Additionally, other factors such as differential levels of endotoxin exposure between the study populations will undoubtedly affect the serum IgE levels of study subjects (36).

Here we describe for the first time polymorphisms in the *IRAK4* gene, which are associated with total serum IgE in patients with CRS and asthma. Although the link between the innate and adaptive immune systems is well established, importance of *IRAK4* in this respect remains to be fully understood. It may be hypothesized that either an up- or a down-regulated *IRAK4* will affect the signaling following TLR activation. Future studies are needed to characterize the effect of these polymorphisms on the cellular expression of *IRAK4*, and to dissect out the mechanism by which downstream signaling affects IgE production.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1. Sequences of forward and reverse primers used to sequence the coding exons of the *IRAK4* gene.

Appendix S2. Single Nucleotide Polymorphisms in the TLR Signaling Pathway Genes Selecting for Genotyping.

Appendix S3. Matrix Spectral Decomposition (matSpD) results for multiple testing adjustments in the *IRAK4* gene.

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ONLINE REPOSITORY

Polymorphisms in Interleukin-1 Receptor-Associated Kinase-4 are Associated with Total Serum IgE

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Appendix 1. Sequences of forward and reverse primers used to sequence the coding exons of the *IRAK4* gene.

Oligo Name	Sequence
IRAK4E02F	GACTTTGAATGTTTCTTTATTATGG
IRAK4E02R	TGCCACATTAGACAGTACTTATACCA
IRAK4E03F	TGGTATTCTATTATTGCAGCACAA
IRAK4E03R	GCTGAAAGAAAGGGACTCTCAA
IRAK4E04F	TGGAATGATATTAAATGAACCAAG
IRAK4E04R	GCTGCACAAACAAAAGAGCA
IRAK4E05F	TCTTCTCTCATCTTGTCTAATTCAAAA
IRAK4E05R	GGGCACACTATGAAGCAACTC
IRAK4E06F	TGTTTTAAAGAAAGGGAGATGAGC
IRAK4E06R	CAAACATCACTAGTTCATGGCATA
IRAK4E07F	TGGCTGAAAAGAGAAGTATTTGC
IRAK4E07R	AATTTTCATAGAGCAGGAAAAATATG
IRAK4E08F	TCATCTTCAGTTGTTGCCTAGAA
IRAK4E08R	AGACCTGCATATGAATCGTGAA
IRAK4E09F	CGTACATCTAGCTAAGGAACTGTTTG
IRAK4E09R	AAGAGAGATAAACAAGATGGGTGT
IRAK4E10F	TCCCAAAGTGCTGGGATTAC
IRAK4E10R	TGCCAATAGAAAATGTTATTTTGA
IRAK4E10.2F	TCAAATTTTGTAAGCAAGATTCAT
IRAK4E10.2R	TGGAAAGATTGCCAATAGAAAA
IRAK4E1112F	AAAATGAGAGCACATGTTATTACAAA
IRAK4E1112R	CAGCCTATGCCTTGTTAAAGGT

Appendix 2. Single Nucleotide Polymorphisms in the TLR Signaling Pathway Genes

Selected for Genotyping

Gene (Accession)	SNP (rs#_alleles)	MAF*	HWE χ^2 **	P-value
TLR1 (NM_003263)	rs4286521_CT	0.153	0.41	0.8808
	rs4833095_TC	0.308	0.22	0.3068
	rs5743611_GC	0.087	1.55	0.7049
	rs5743594_CT	0.151	0.51	0.8283
	rs4833103_CA	0.471	0.03	0.6257
TLR2 (NM_003264)	rs13150331_AG	0.420	0.04	0.6338
	rs4696480_TA	0.480	0.68	0.9962
	rs1898830_AG	0.353	1.68	0.7779
	rs4696483_CT	0.122	0.33	0.6320
	rs3804100_TC	0.057	0.16	0.6409
	rs5743704_CA	0.026	0.15	0.3901
	rs5743708_GA	0.012	0.03	0.6892
	rs7656411_TG	-	failed	-
	rs1339_AG	0.208	0.00	0.6841
	rs17030340_GA	0.199	2.67	0.9556
	rs2289318_CG	0.187	1.54	0.9351
	rs7695605_CG	0.209	2.68	0.8704
TLR3 (NM_003265)	rs956239_CT	0.183	0.80	0.2244
	rs4861699_GA	0.124	0.60	0.5151
	rs5743305_TA	0.438	1.93	0.3041
	rs7657186_GA	0.243	0.05	0.9556
	rs6552950_GA	-	failed	-
	rs3775296_GT	0.189	0.44	0.2192
	rs35140061_GA	-	monomorphic	-
	rs7668666_CA	0.288	0.67	0.1685
	rs3775292_CG	0.262	0.05	0.4172
	rs35311343_GC	0.005	0.00	0.0507
	rs5743317_TG	0.002	0.00	0.9505
	rs3775291_GA	0.252	3.87	0.1477
	rs5743318_TA	0.002	0.00	0.7671
	rs10025405_AG	0.421	1.32	0.6720
	rs4862633_CT	0.303	0.40	0.8563
	rs4608848_TC	0.385	0.00	0.8604
	rs6857595_CT	0.338	2.49	0.3760
	rs1519309_AG02	0.423	0.51	0.1223
TLR4 (NM_003266)	rs10983754_CT	0.056	0.72	0.2961
	rs10759930_CT	0.346	1.03	0.2380
	rs10759932_TC	0.186	0.28	0.1103
	rs2149356_CA	0.349	0.21	0.9369
	rs4986790_AG	0.053	0.65	0.2973

Gene (Accession)	SNP (rs#_alleles)	MAF*	HWE χ^2 **	P-value
	rs4986791_CT	0.050	0.58	0.6024
	rs11536889_GC	0.155	0.27	0.6846
	rs11536898_CA	0.134	0.02	0.1454
	rs1554973_TC	-	failed	-
	rs7860896_AG	0.086	0.23	0.5855
	rs7037225_CT	0.147	0.09	0.8506
	rs2183016_TG	0.162	0.09	0.2952
TLR6 (NM_006068)	rs5743810_CT	0.370	0.38	0.6775
	rs5743808_TC	0.002	0.00	0.7955
	rs5743794_GA	0.234	0.76	0.8569
	rs5743788_CG	0.481	0.16	0.9118
	rs6833914_CT	0.279	0.07	0.4876
TLR9 (NM_017442)	rs352162_CT	0.485	4.64	0.1393
	rs352140_TC	0.483	3.75	0.1255
	rs5743836_TC	0.127	0.01	0.0439
	rs187084_TC	0.417	3.05	0.1405
	rs352143_AG	0.170	0.27	0.2118
	rs11717574_TC	0.119	0.52	0.3361
TLR10 (NM_030956)	rs4513579_AG	0.232	0.24	0.6587
	rs11466657_TC	0.033	0.25	0.9098
	rs11096955_AC	0.381	0.20	0.6293
	rs11466652_AG	0.148	0.05	0.9288
	rs10856839_AC	0.187	0.11	0.6786
	rs7653908_GC	0.233	0.31	0.5801
CD14 (NM_000591)	rs7721577_TC	0.194	0.14	0.4261
	rs4914_CG	0.129	0.09	0.4733
	rs2569190_GA	0.498	1.73	0.0816
	rs2569193_GA	0.296	0.72	0.5486
	rs2563310_GA	0.495	1.93	0.1125
MD-2/LY96 (NM_015364)	rs1905045_TC	0.229	0.56	0.4714
	rs16938755_TC	0.136	1.54	0.5806
	rs11786591_CT	0.281	0.69	0.7890
	rs6472812_GA	0.045	0.47	0.8189
	rs10504554_TC	0.160	0.04	0.5161
	rs17226566_TC	0.236	1.04	0.0026
	rs12544736_TG	0.167	0.01	0.7649
	rs16938766_GC	-	failed	-
MyD88 (NM_002468)	rs2239621_CT	0.241	1.35	0.7817
	rs4988453_CA	0.046	0.79	0.0889
	rs7744_AG	0.143	0.02	0.8862
	rs6767684_GA	0.146	0.83	0.5498
	rs6796045_AT	0.092	0.39	0.7528
IRAK4 (NM_016123)	rs11182250_CG	0.063	14.04	-
	rs1461567_CT	0.272	2.83	0.0035***

Gene (Accession)	SNP (rs#_alleles)	MAF*	HWE χ^2 **	P-value
	rs4251580_CT	0.132	0.12	0.3409
	rs4251520_TC	0.112	0.16	0.4978
	rs4251559_AG	0.480	0.22	0.0011***
	rs17121283_CT	0.051	61.96	-
	rs6582484_TC	0.171	0.00	0.3870
	rs4251459_TC	0.139	0.00	0.7078
	620-1delAC_AC/-	-	failed	-
	rs4251487_TG	-	failed	-
	821delT_T/-	-	monomorphic	-
	T877C_CT	-	monomorphic	-
	rs4251513_CG	0.474	0.35	0.0006***
	rs4251583_AG	-	monomorphic	-
	A1188+520G_AG	-	monomorphic	-
	G1189-1T_GT	-	failed	-
	rs4251545_GA	0.118	0.01	0.6377
TRAF-6 (NM_145803)	rs3740961_AG	0.133	0.03	0.3689
	rs5030437_CT	0.136	1.21	0.3068
	rs5030416_CA	0.144	0.54	0.5100
	rs5030411_TC	0.410	0.12	0.4844
	rs331455_TC	0.254	7.39	-

The accession number from NCBI Entrez Gene, and SNP identification numbers are from the NCBI Build 35. SNP identification numbers are followed by an underscore, minor and major alleles, respectively. Note: rs4251559 from *IRAK4* has changed to rs1141168 in NCBI Build 36.

*MAF refers to the minor allele frequency.

**HWE χ^2 refers to the chi square test to verify that the genotyped alleles are in Hardy-Weinberg equilibrium.

***Three SNPs achieving significance following the Nyholt correction for multiple testing, calculated to be at $P = 0.0073$ for the *IRAK4* gene.

Appendix 3. Matrix Spectral Decomposition (matSpD) results for multiple testing adjustments in the *IRAK4* gene.

Your Uploaded Correlation Matrix (absolute values to 4 decimal places):

	1	2	3	4	5	6	7	8
1	1.0000	0.0600	0.1450	0.0240	0.0200	0.8380	0.0010	0.2560
2	0.0600	1.0000	0.3350	0.0580	0.0470	0.0500	0.3890	0.0000
3	0.1450	0.3350	1.0000	0.1700	0.1130	0.1190	0.7200	0.1340
4	0.0240	0.0580	0.1700	1.0000	0.0190	0.0200	0.0800	0.0310
5	0.0200	0.0470	0.1130	0.0190	1.0000	0.0170	0.1370	0.0000
6	0.8380	0.0500	0.1190	0.0200	0.0170	1.0000	0.0120	0.3320
7	0.0010	0.3890	0.7200	0.0800	0.1370	0.0120	1.0000	0.0320
8	0.2560	0.0000	0.1340	0.0310	0.0000	0.3320	0.0320	1.0000

Original (total) number of variables (V) after removing redundant (collinear) variables:
8

For factor 1 to V, original eigenvalues associated with the correlation matrix:

1	2.2276
2	1.8452
3	0.9879
4	0.9680
5	0.8505
6	0.7034
7	0.2636
8	0.1537

Variance of the observed eigenvalues:
0.5131

Effective number of independent variables [Veff]:
7.5511

Significance threshold required to keep Type I error rate at 5% (0.05/Veff):
0.00662158668380377

F THE Veff [VeffLi] PROPOSED BY LI AND JI (2005):

Effective Number of Independent Variables [VeffLi] (using Equation 5 of Li and Ji 2005):
7

Experiment-wide Significance Threshold Required to Keep Type I Error Rate at 5%:
0.00730083197901465

NB: I recommend using the Li and Ji (2005) approach only if VeffLi < Veff.