IRAK-M Is Involved in the Pathogenesis of Early-Onset Persistent Asthma

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Asthma is a multifactorial disease influenced by genetic and environmental factors. In the past decade, several loci and >100 genes have been found to be associated with the disease in at least one population. Among these loci, region 12q13-24 has been implicated in asthma etiology in multiple populations, suggesting that it harbors one or more asthma susceptibility genes. We performed linkage and association analyses by transmission/disequilibrium test and case-control analysis in the candidate region 12q13-24, using the Sardinian founder population, in which limited heterogeneity of pathogenetic alleles for monogenic and complex disorders as well as of environmental conditions should facilitate the study of multifactorial traits. We analyzed our cohort, using a cutoff age of 13 years at asthma onset, and detected significant linkage to a portion of 12q13-24. We identified *IRAK-M* as the gene contributing to the linkage and showed that it is associated with early-onset persistent asthma. We defined protective and predisposing SNP haplotypes and replicated associations in an outbred Italian population. Sequence analysis in patients found mutations, including inactivating lesions, in the *IRAK-M* coding region. Immunohistochemistry of lung biopsies showed that IRAK-M is highly expressed in epithelial cells. We report that *IRAK-M* is involved in the pathogenesis of early-onset persistent asthma. *IRAK-M,* a negative regulator of the Toll-like receptor/IL-1R pathways, is a master regulator of NF-κB and inflammation. Our data suggest a mechanistic link between hyperactivation of the innate immune system and chronic airway inflammation and indicate IRAK-M as a potential target for therapeutic intervention against asthma.

Asthma (MIM #600807) is a chronic inflammatory disease of bronchial epithelium and submucosa that leads to irreversible anatomical changes in bronchi and permanent impairment of lung function. Its prevalence in Western industrialized societies is now 5% and growing, with increasing associated mortality.[1,2] Interest in finding etiologic factors has correspondingly intensified.

Whereas the role of the immune system and of specific subsets of T-helper (Th) cells in the pathophysiology of asthma has been clearly established, the genes implicated in this disease are just beginning to be identified. Multiple genetic loci and several gene variants have been recently detected and inferred to contribute to allergic asthma.[3] For most of these genes, however, their relationship to the pathophysiology of asthma remains conjectural, and none appears to be directly involved in the activation of airway inflammatory processes or allergy. Replication of studies has also been difficult because of the genetic heterogeneity of asthma, the extreme variability in disease expression, the presence of phenocopies, and a marked variety of environmental influences.

One approach to reducing heterogeneity in studies of

multifactorial traits focuses on founder populations, which have grown from a few initial members to large modern populations without appreciable in-migration and of which Sardinia provides one of the most promising.[4,5] For example, monogenic disorders such as β-thalassemia, Wilson's disease, and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome, as well as complex disorders like diabetes type I, show limited heterogeneity of pathogenetic alleles.[6–9] The population of the island shares much the same environment, reducing nongenetic sources of variation as well.

To elucidate the genetic components of asthma, we studied affected Sardinian families by linkage and association analysis at chromosome 12q13-24, a region previously implicated in asthma etiology in different populations.[10,11] Here, we report that variants in the *interleukin-1 receptor associated kinase-M* (*IRAK-M*; HUGO nomenclature *IRAK3* [MIM *604459]) gene are associated with early-onset persistent asthma and indicate IRAK-M as a potential new target for therapeutic intervention against asthma and atopic diseases.

Material and Methods
Sample Collection

Patients were recruited by the recovery of information from archives and ongoing clinical activities from all the four provinces of Sardinia, in proportions representative of the local population. Atopic asthmatic sibling pairs (sibs) and trios were collected over a period of 4 years, mainly from pediatric and pneumologic centers. To avoid phenocopies, all patients fulfilled the following criteria: Sardinian origin for at least 3 generations and age at visit >6 years. At the recruitment sessions, each subject was interviewed, disease status was ascertained by physical examination, permission was asked to access personal health records, and blood samples were collected. Each participant signed an informed consent form. All study methods have been approved by the local ethics committee (Azienda Sanitaria Locale number 8 protocol 24/Comitato Etico/02, authorization number 4737).

Asthma was diagnosed by a pulmonary physician, in accordance with American Thoracic Society criteria.[12] Pulmonary function was evaluated by spirometry: forced expiratory volume at the 1st s (FEV1) was expressed in liters/minute. A physician administered a questionnaire collecting clinical history and classi-

fying asthma severity in four levels according to the World Health Organization guidelines (Global Initiative for Asthma). The use of asthma drugs and any other medication was recorded. Atopy was detected by positive skin testing to common inhalant allergens by standard methods. Patients with early onset were interviewed by a physician about persistency of asthma symptoms after the completion of puberty (18 years).

The replication sample was composed of 345 unrelated individuals (67 cases and 278 healthy controls) selected from a cohort of 211 asthmatic families that has been described elsewhere.[10] In particular, since information about age at disease onset was not available for this sample, we selected as cases all atopic individuals older than 18 years with persistency of asthma symptoms (persistent asthmatic cases). All families were ascertained at the Pediatric Clinic of the University of Verona and at the Bolzano Hospital. Phenotyping included interview of the individuals with a modified American Thoracic Society questionnaire, an asthma physician's diagnosis, measurement of serum immunoglobulin E (IgE) levels, skin testing against a panel of allergens, and bronchial hyperresponsiveness testing with methacholine.

Genotyping

Genomic DNA isolated from peripheral blood leukocytes was used for genotyping with both microsatellite and SNP markers. Microsatellite markers, including di-, tri-, and tetranucleotide repeats, were chosen from the Marshfield Center for Medical Genetics, The GDB Human Genome Database, and the Ensembl Genome Browser. All microsatellites were analyzed using the MegaBACE 1000 fluorescence-based genotyping methodology. Genotypes were scored using MegaBACE Genetic Profiler Software v1.5 (Amersham Biosciences). Two DNA standards, consisting of the CEPH control individual number 1347.02 (Applied Biosystems) and an internal DNA control, were incorporated in all the runs to verify accuracy of typing. SNP markers were selected from dbSNP, The SNP Consortium, and Ensembl Genome Browser. SNP-based genotyping was performed after dot-blot preparation of amplified DNA with use of sequence-specific oligonucleotide probes.

All markers were PCR amplified and genotyped a second time when failures occurred during the first round of amplification. Data quality of microsatellite and SNP genotypes was established by three methods: reproducibility of control DNA samples, expected Mendelian inheritance of alleles within a family, and tests of Hardy-Weinberg equilibrium. These last analyses were performed with the PEDSTATS program with the use of unrelated

Table 1. Sardinian Families with Asthma Studied by Multipoint Linkage Analysis and TDT

	Linkage Analysis			TDT	
Characteristic	Total Sample	Age Onset ≤13 Years[a]	Age Onset >13 Years[b]	Total Sample[c]	Early-Onset Persistent Asthma[c]
Subjects (n)	410	243	167	1,100	453
No. of families[d] (no. of sibs)	100 (121)	60 (66)	40 (55)	294	139
Males (%)	61	69	43	56	63
Age at asthma onset (years)[e]	10.69 ± 10.76	5.13 ± 3.61	18.41 ± 12.51	10.15 ± 10.82	4.79 ± 3.59
Age (years)[e]	21.86 ± 12.37	15.51 ± 6.47	30.76 ± 13.18	20.39 ± 12.10	15.95 ± 8.22

[a] Sib pairs in the early-onset group (≤13 years) are concordant for age at asthma onset.

[b] The group with age at onset >13 years includes sibs both concordant and discordant for onset.

[c] Only one affected sibling (the proband) is included in TDT analysis.

[d] Families were selected from all four provinces of Sardinia, in proportions representative of the local population.

[e] Data are reported as means ± SDs.

individuals ($P > .05$).[13] Overall, we obtained a rate of genotyping efficiency >96% in the first step and reached 99.8% in the second.

Mutation and Sequence Analysis

Mutation analysis of all exons and intron/exon boundaries was performed bidirectionally by direct sequencing of PCR products. In brief, for each gene, we first sequenced a subgroup of affected individuals by selecting one affected individual per informative family (the proband). Every mutation was first confirmed in the patient by resequencing, and then its presence was ascertained in the remaining family members (sibs and both parents). Mutations were also checked to verify compatibility with Mendelian inheritance. Confirmed mutations were then analyzed in the entire sample of affected families (294 families) and in 200 healthy controls by dot-blot analysis with the use of allele-specific oligonucleotide probes.

Each sample was amplified using the GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) in accordance with the manufacturer's conditions. Sequencing reactions were performed using the ABI PRISM BigDye chemistry BigDye Terminator v3.1 Cycle Sequencing Kit and the automated sequencer ABI PRISM 3100 (Applied Biosystems), in accordance with the manufacturer's recommendations, and were visualized with the DNA Genetic Analyzer software (ABI PRISM 3100 Genetic Analyzer Data Collection Software [Applied Biosystems]). Sequences were aligned and compared with consensus from the human genome databases (National Center for Biotechnology Information and UCSC Genome Browser).

Statistical Analyses

Multipoint linkage analyses were calculated by maximum-likelihood estimate of identical-by-descent (IBD) sharing for affected sib pairs with use of the GENEHUNTER program v2.1.[14] LOD scores were computed using the possible triangle method, and no assumption was made about mode of inheritance. Sibships containing more than one affected sib pair (nine families) were considered as "all independent pairs." For the analysis on the stratified sample, multipoint linkage analysis was conducted on two subgroups of affected sib pairs stratified for age at asthma onset. The early-onset subgroup contains sib pairs concordant for age at asthma onset ($\leqslant 13$ years), whereas the group with age at onset >13 years includes sibs both concordant and discordant for onset. The order of microsatellites and the genetic intermarker distances were derived using CRIMAP v2.4,[15] after physical localization data was checked with the Ensembl Genome Browser. To obtain a 95% CI in localizing susceptibility genes on 12q, the GENEFINDER program was used.[16] It applies generalized estimating equations to estimate the location of a susceptibility gene on the basis of IBD sharing of multiple markers by affected sib pairs and can incorporate covariate information on sib pairs, such as age at onset. To assess the effect of the risk haplotype on linkage, we also used the genotype-IBD sharing test (GIST),[17] which assigns family-specific weights on the basis of the genotype of the affected family members and the model of interest (dominant, recessive, or additive) and tests for correlations between these weights and family-based IBD sharing (NPL score).

Association analyses of SNPs with asthma were performed by both the transmission/disequilibrium test (TDT) and case-control analysis. For the TDT, only trios consisting of father, mother, and one affected son were included in the analysis. To assess significance of the TDT results, we derived empirical P values by a permutation procedure that used the same genotype data as our sample. Each permuted data set was formed by randomly reassigning alleles as transmitted or untransmitted. Haploview was used to investigate the linkage disequilibrium (LD) block structure and to identify tag SNPs and distribution of haplotypes across the *IRAK-M* gene and the flanking genomic regions.[18] To determine haplotype-transmission rates from parents to affected siblings, we used the UNPHASED program.[19]

The case-control study of the Sardinian sample was performed by comparing allele and genotype distribution of one affected persistent case per family with those of 460 healthy subjects. Logistic-regression analyses were used to calculate odds ratios (ORs) with 95% CI and corresponding P values for all analyzed SNPs, with age and sex controlled for as covariates. P values were adjusted for multiple testing by Bonferroni correction, to maintain an overall error rate of 0.05. We also performed an analysis with the THESIAS program,[20] to test covariate-adjusted haplotype effects on disease. THESIAS was also used to test for deviation from additivity (on a log scale) of haplotype effects by a likelihood-ratio test.

For the Italian subjects, the difference of distributions between cases and controls of genotypic and allele frequencies was assessed by the Fisher exact test. In this sample, the presence of population stratification was excluded by the method proposed by Pritchard and colleagues and was implemented in the program STRUCTURE.[21] In brief, 400 unrelated individuals selected from the sample of asthmatic families were studied using 53 unlinked markers. Several runs of the program were performed under the hypothesis of one, two, three, four, or five clusters in the population. Results showed that the model including only one cluster is much more likely than any other model, indicating that the individuals studied are genetically homogeneous.

Immunohistochemistry

Lung biopsies were fixed in 10% formalin, were embedded in paraffin, were serially sectioned at 5 μm, and were processed for immunohistochemistry by standard methods with the following antibodies: rabbit polyclonal anti-IRAK-M (Cell Signaling), mouse monoclonal anti–thyroid transcription factor-1 (TTF1) (Dako), and mouse monoclonal anti-phospho-NF-κB p65 (Cell Signaling). Immunoperoxidase staining was performed with the biotin/strep-tavidin–based LSAB2 system (Dako). Nuclei were counterstained with hematoxylin. Photographs were taken using a Leica DMR microscope, with use of the program Leica IM50 Image Manager v1.2 (Leica Microsystems).

Results

Linkage Analysis at Chromosome 12q13-24

Our initial analysis of asthma susceptibility genes in the candidate region of chromosome 12q13-24 was conducted with 121 affected sib pairs selected from 100 families coming from all four provinces of Sardinia (table 1). Multipoint nonparametric affected sib pair analysis in this cohort showed suggestive evidence of linkage at marker *D12S75* (fig. 1*A*, and see table A1 for the markers used). To reduce possible sources of variation and to increase the power to detect linkage, we repeated the analysis after stratification of our sample population by age at asthma onset. We ar-
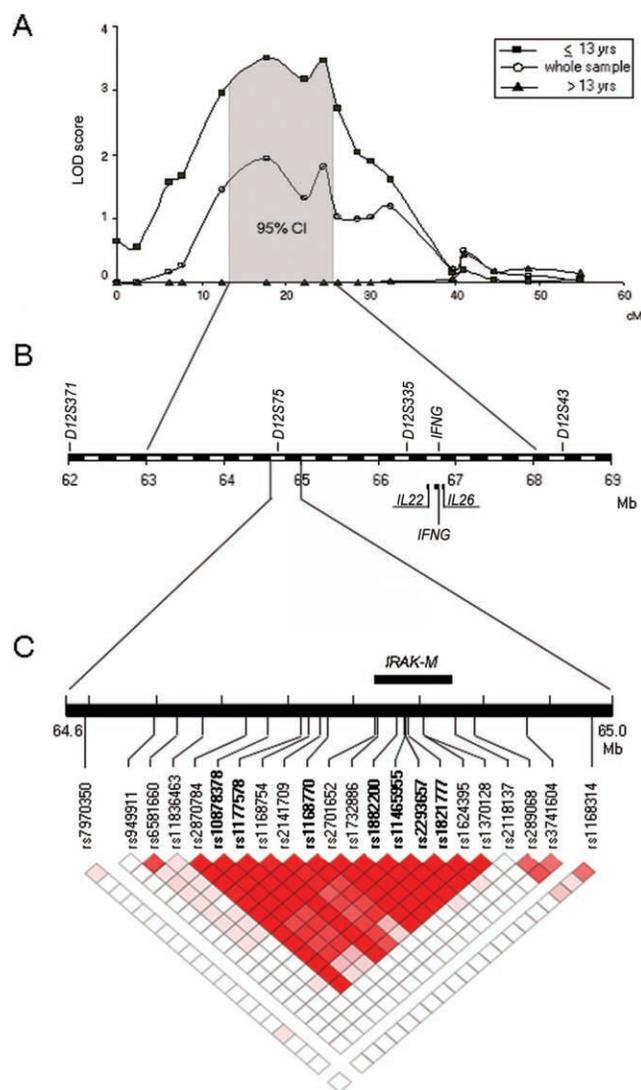
Figure 1. Identification of *IRAK-M* as an asthma susceptibility gene. *A,* Multipoint linkage analysis plots of chromosome 12q13-24 for Sardinian asthmatic sib pairs. LOD scores are shown for the whole sample (*circles*), for affected sibs concordant for early age at onset (≤13 years) (*squares*), and for affected sibs with at least one patient with age at onset >13 years (*triangles*). *B,* Genomic region of the 95% CI for gene location estimate. Asthma candidate genes for which mutation analysis was performed are indicated, along with microsatellite markers used in linkage analysis. *C,* LD in a 400-kb region containing *IRAK-M* (*black bar*). *D'* values for pairwise LD between each marker are shown according to LD strength, from *D'* > 0.8 (*red*) to *D'* < 0.3 (*white*). SNPs used in TDT analysis are also indicated; seven SNPs in bold were significant after multiple test correction (detailed results in table A2).

bitrarily selected a pubertal cutoff age of 13 years, on the basis of clinical observations pointing to the existence of phenotypical heterogeneity in early- versus late-onset forms of the disease (also see the "Discussion" section). Linkage analysis revealed that the 12q13-24 region is significantly linked to asthma in a subgroup of 60 families (66 sibs) with exclusively early-onset cases, yielding a multipoint LOD score of 3.56 ($P = 5.2 \times 10^{-5}$) between markers *D12S75* and *D12S335*. By contrast, no evidence of linkage was detected in the families with at least one patient with age at asthma onset >13 years (fig. 1*A* and table 1). No bias for geographic origin within Sardinia was observed

between the two subgroups. Analysis with the GENEFIN-DER program[16] in the whole sample, with age at onset incorporated as a covariate, showed that the most likely location for one or more genes predisposing an individual to asthma lay within a 95% CI of 10.5 cM centered 2.7 cM distal to *D12S75* ($P = .001$) (fig. 1*A*).

IRAK-M *as the Candidate Gene in the Region of Maximum LOD Score*

The 10.5-cM region of the 95% CI for estimated gene location contains the cytokine genes *interferon gamma*

Table 2. Case-Control Analysis Using 22 SNPs in the Genomic Region Spanning the *IRAK-M* Gene

Marker	Allele 1/ Allele 2	Allele 2 Frequency		Allele 2 versus Allele 1 Analysis		Genotype Analysis				
		Case	Control	P^a	OR (95% CI)^b	Case^c	Control^c	P^a	OR Hom (95% CI)^d	OR Het (95% CI)^d
rs7970350	T/C	.336	.381	.2315	.834 (.620–1.122)	62/54/18	182/197/74	.4748	.714 (.379–1.343)	.804 (.513–1.260)
rs949911	C/A	.137	.148	.1921	.755 (.495–1.151)	103/34/2	335/112/12	.4040	.420 (.083–2.135)	.796 (.493–1.286)
rs6581660	T/G	.291	.280	.4831	1.124 (.811–1.559)	66/58/10	239/179/38	.7821	1.260 (.566–2.807)	1.126 (.731–1.734)
rs11836463	C/A	.241	.281	.1689	.788 (.561–1.106)	78/52/7	242/176/41	.3887	.616 (.253–1.499)	.791 (.512–1.224)
rs2870784	G/T	.187	.195	.3707	.837 (.567–1.235)	85/48/1	296/144/17	.1790	1.100 (.708–1.708)	.155 (.020–1.225)
rs10878378	T/A	.342	.413	.0297	1.399 (1.033–1.894)	59/61/16	160/211/81	.0942	1.960 (1.020–3.764)	1.402 (.735–2.672)
rs1177578	G/C	.338	.416	.0145	1.458 (1.077–1.972)	61/62/16	159/210/83	.0500	2.113 (1.103–4.050)	1.439 (.756–2.739)
rs1168754	G/C	.180	.193	.3361	.826 (.560–1.219)	88/47/1	295/138/18	.7040	1.089 (.702–1.687)	.167 (.022–1.305)
rs2141709	G/A	.263	.322	.0514	.724 (.523–1.002)	75/55/9	213/191/51	.1497	.493 (.220–1.106)	.752 (.489–1.155)
rs1168770	A/G	.335	.415	.0089	1.506 (1.108–2.046)	60/61/15	162/212/84	.0330	2.298 (1.180–4.471)	1.553 (.804–2.300)
rs2701652	G/C	.167	.209	.1847	.751 (.492–1.146)	91/43/1	289/148/22	.1937	.146 (.018–1.177)	.934 (.573–1.524)
rs1732886	A/G	.159	.210	.1063	.704 (.460–1.077)	95/42/1	288/148/22	.1614	.139 (.017–1.118)	.863 (.528–1.409)
rs1882200	C/T	.460	.341	**.0012**	1.721 (1.240–2.390)	39/72/28	207/190/61	**.0022**	2.676 (1.360–5.268)	2.189 (1.312–3.650)
rs11465955	C/T	.460	.354	**.0005**	1.680 (1.256–2.248)	39/71/28	198/197/64	**.0010**	2.599 (1.413–4.780)	2.240 (1.393–3.603)
rs2293657	A/T	.460	.341	**.0013**	1.708 (1.232–2.368)	39/71/28	209/186/63	**.0022**	2.636 (1.343–5.173)	2.179 (1.307–3.631)
rs1821777	T/A	.464	.359	**.0006**	1.673 (1.249–2.240)	38/71/28	195/198/66	**.0011**	2.587 (1.405–4.763)	2.237 (1.387–3.609)
rs1624395	G/A	.511	.417	**.0015**	1.632 (1.206–2.209)	27/81/30	154/220/79	**.0016**	2.626 (1.391–4.958)	2.469 (1.464–4.163)
rs1370128	C/T	.536	0431	**.0005**	1.709 (1.264–2.311)	26/75/36	147/220/85	**.0017**	2.964 (1.593–5.514)	2.204 (1.295–3.751)
rs2118137	C/G	.290	.320	.4341	.879 (.637–1.214)	67/62/9	210/204/45	.6161	.666 (.296–1.499)	.962 (.629–1.470)
rs289068	A/G	.213	.229	.3360	.823 (.554–1.223)	83/48/5	271/166/22	.6246	.638 (.203–2.007)	.840 (.519–1.359)
rs3741604	A/G	.269	.336	.0226	1.475 (1.056–2.060)	72/52/10	195/214/46	.0481	1.781 (.816–3.889)	1.065 (.482–2.354)
rs1168314	T/C	.293	.328	.4325	.879 (.636–1.214)	67/61/10	204/210/46	.7142	.728 (.331–1.599)	.912 (.595–1.397)

^{a} *P* value by logistic regression. Values in bold were still significant after Bonferroni correction (threshold .0022).

^{b} Sex- and age-adjusted OR (95% CI).

^{c} The three values indicate the number of homozygotes for the major allele and the number of heterozygotes and homozygotes for the minor allele in the control sample.

^{d} OR (95% CI) for homozygous and heterozygous risk-allele carriers.

(*IFNG*), *interleukin 22* (*IL22*), and *interleukin 26* (*IL26*), which were previously implicated in asthma (see fig. 1*B*). Sequence analysis of all the exons as well as the intron/exon boundaries of these genes in the patients with asthma chosen as the most informative for linkage revealed no associated variation (data not shown).

On the basis of its function and possible relevance to asthma, we turned to *IRAK-M,* the other well-known gene located within the linkage peak. IRAK-M is one of the four IRAK proteins that mediate signal transduction of the Toll-like receptor (TLR)/IL-1R family in host defense and inflammatory responses, acting as a negative regulator.[22–25] TLRs are key participants in lung host defense and in the regulation of the Th1/Th2 balance and are thus thought to have a major impact on Th2-biased allergic diseases like asthma.[26,27]

To look for association, we performed a TDT, using 22 SNPs distributed across a region of 387 kb spanning the *IRAK-M* gene in an extended sample of 294 families with asthma (100 from the initial linkage analysis and 194 additional families recruited later) (see table 1). We conducted the analysis by stratifying the families according to the age at asthma onset, as described above. Strong evidence of association was detected only in the subgroup including subjects with early-onset persistent asthma (139 families). We identified seven SNPs with significant *P* values even after correction for multiple testing (fig. 1*C* and table A2). Four SNPs mapped inside the *IRAK-M* gene

(*rs1882200, rs11465955, rs2293657,* and *rs1821777*), whereas the remaining three (*rs10878378, rs1177578,* and *rs1168770*) were several kilobases upstream. Unlike those in the *IRAK-M* gene, these SNPs showed no replicated significance in a second population or in case-control studies (see below), and their apparent association with asthma is likely a consequence of LD. Indeed, a disequilibrium estimate of $D' \geqslant 0.70$ defines a single 138-kb haplotype block containing the entire *IRAK-M* gene (fig. 1*C*). In this interval, we identified four common haplotypes tagged by six SNPs, which captured most of the genetic variation in this area of the genome. The most frequent haplotypes (GGGTAT and GCACGC) were significantly over- and undertransmitted, respectively, to early-onset persistent asthmatic patients (empirical *P* = .0011 and *P* = .0282, respectively) (see table A2).

To characterize better the association of *IRAK-M* with asthma in our affected population, we performed a case-control study, using a subgroup of 139 patients with early-onset persistent asthma as cases (one affected case per family) and 460 healthy subjects as controls (table A3). This analysis confirmed the association and showed that six SNPs spanning the *IRAK-M* gene, including the previously described four intragenic SNPs, maintained significant values after Bonferroni correction (.0005 < *P* < .0015; corrected values .011 < *P* < .033; ORs 1.63–1.72) (table 2). Analysis based on genotype counts revealed that the SNP-associated risk within the *IRAK-M* gene ranged

from 2.18 to 2.47 for heterozygous carriers and from 2.59 to 2.96 for homozygous individuals. Three tag SNPs within *IRAK-M* (*rs11465955*, *rs1624395*, and *rs1370128*) were sufficient to identify protective and predisposing SNP haplotypes (table 3). The risk haplotype (TAT) was associated with early-onset persistent asthma (46.0% cases vs. 35.6% controls), whereas the wild-type haplotype (CGC) was less frequent in cases than in controls (46.4% versus 56.8%). Furthermore, analysis with the THESIAS program[20] estimated sex- and age-adjusted haplotype effects and showed that the OR of the risk haplotype was 1.73 (95% CI 1.24–2.40), with a multiplicative effect on disease (table 3).

To assess whether the genetic linkage peak at 12q13-24 could be explained by the presence of the risk haplotype inside the *IRAK-M* gene, we conducted a linkage analysis with the initial sample of 100 families, using microsatellite markers located within the linkage peak. When 64 affected sibs who shared the risk haplotype defined within *IRAK-M* were excluded from this sample, the maximum LOD score (MLS) value at marker *D12S75* dropped dramatically, from 1.93 to 0.30 (fig. 2). Notably, the LOD score values relative to the 64 sibs sharing the risk haplotype (MLS 1.70) were similar to those observed for the whole sample. Furthermore, the GIST confirmed that the risk haplotype accounted for a significant fraction of linkage evidence ($P = .027$), with the recessive model better explaining the linkage signal ($P = .013$). These analyses thus support the relation of the linkage signal to the *IRAK-M* risk haplotype.

Replication in an Independent Italian Cohort

To confirm the association of *IRAK-M* with asthma, we performed a case-control study in a cohort from mainland Italy that had previously shown evidence of linkage on chromosome 12q (see table A3).[10] This population, like other European populations, is genetically distant from Sardinians.[28] We evaluated the six tag SNPs that had defined predisposing and protective haplotypes in the TDT analysis of the Sardinian population (*rs2870784, rs1177578, rs2141709, rs11465955, rs1624395,* and *rs1370128*). The results revealed a significant association for two SNPs within *IRAK-M*—*rs1624395* and *rs1370128* ($P = .004$ and $P = .002$, respectively)—which was maintained even after Bonferroni correction (corrected $P = .024$ and .012, respectively) (table 4). *rs11465955* also showed a positive
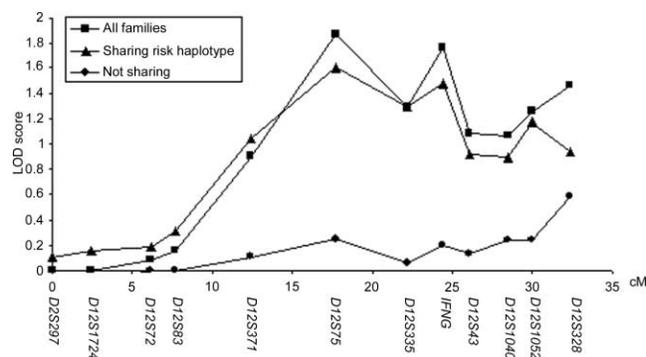

Figure 2. Linkage analysis according to the presence of the risk haplotype. With the use of markers from 12q13.3 to 12q21.1 to analyze the initial sample of 100 families (121 sib pairs), the LOD score values relative to 64 sib pairs sharing the risk haplotype (*triangles*) are similar (MLS 1.70) to those of the whole sample (*squares*) (MLS 1.93), whereas the sample of siblings not sharing the risk haplotype (*circles*) shows a much lower value (MLS 0.30) for the same microsatellite marker, *D12S75*.

trend for association ($P = .029$). Genotype analysis validated these results. In contrast with the haplotype risk model of the case-control analysis in the Sardinian population, the best risk model associated with markers inside *IRAK-M* for the mainland Italian population is recessive, by a model-free approach. Notably, the Sardinian and mainland Italian populations presented the same *IRAK-M*-associated alleles and similar frequency of the most common haplotypes (table A4).

Mutation Analysis of the IRAK-M *Gene*

The *IRAK-M* gene consists of 12 exons spanning a region of ~60 kb in 12q14.3 and encodes a protein of 596 aa. Like the other members of the IRAK family, IRAK-M contains an N-terminal death domain (DD) followed by a central kinase-like domain and a C-terminal unique stretch of amino acids.[22] The mechanism by which IRAK-M acts as a negative regulator of the TLR/IL-1R–signaling pathways is still speculative, but it may prevent phosphorylation and dissociation of IRAK-1 from the receptor-adaptor complex, resulting in the interruption of downstream signaling.[25]

Table 3. Case-Control Analysis Haplotypes Inside the *IRAK-M* Gene in Sardinians

| | Frequency | | | | |
Haplotype[a]	Cases ($N = 139$)	Controls ($N = 460$)	P[b]	Empirical P (10^5 Permutations)	OR (95% CI)
CGC	.464	.568	.0022	.0101	Reference
TAT	.460	.356	.0018	.0081	1.73 (1.24–2.40)
CAT	.051	.058	.5982	.9759	1.13 (.35 –3.60)
CGT	.026	.017	.3454	.3454	2.31 (.83–6.45)

Note.—Likelihood-ratio test for global haplotype effect: $\chi^2 = 14.26$ (df = 4); $P = .0065$.
[a] Haplotypes are defined by three tag SNPs: *rs11465955, rs1624395,* and *rs1370128*.
[b] P value by likelihood-ratio test.

Table 4. Case-Control Analysis in an Italian Sample with Use of the Six Tag SNPs Identified in the Sardinian Sample and Used in TDT Analysis

SNP	Allele 1/ Allele 2	Allele 2 Frequency		Allele 2 versus Allele 1 Analysis		Genotype Analysis			
		Case	Control	P[a]	OR (95% CI)	Case[b]	Control[b]	P[a]	OR[c] (95% CI)
rs2870784	G/T	.189	.251	.079	1.44 (.89–2.32)	45/17/4	155/106/17	.624	.99 (.32–3.05)
rs1177578	G/C	.371	.415	.203	1.20 (.81–1.78)	27/29/10	98/129/51	.341	.79 (.38–1.66)
rs2141709	G/A	.227	.331	.012	1.68 (1.08–2.62)	40/22/4	127/118/33	.122	.48 (.16–1.40)
rs11465955	C/T	.432	.339	.029	1.48 (1.01-2.18)	23/29/14	122/117/34	.055	1.89 (.95–3.78)
rs1624395	G/A	.529	.401	**.004**	1.68 (1.15–2.46)	18/27/22	98/137/43	**.002**	2.67 (1.46–4.89)
rs1370128	C/T	.553	.412	**.002**	1.76 (1.21–2.58)	15/30/22	95/136/46	**.003**	2.45 (1.35–4.47)

[a] P value by Fisher exact test. Values in bold were still significant after Bonferroni correction (threshold .0083).

[b] The three values indicate the number of homozygotes for the major allele and the number of heterozygotes and homozygotes for the minor allele in the control sample.

[c] OR for genotype 22 versus 12 and 11.

To see if any mutations in the *IRAK-M* coding sequence were implicated in asthma in the Sardinian population, we sequenced all the exons as well as the intron/exon boundaries of the *IRAK-M* gene in 100 probands randomly selected from all the families in this study. We identified seven nucleotide-sequence variations present in 10 different families (table 5). Two interesting mutations were detected in families A and B. In family A, a stop codon in the conserved tryptophan within the DD (W76X) could result in nonsense-mediated mRNA decay (NMD) or could generate a protein lacking the N-terminal part, as a consequence of translation initiation at an alternative downstream ATG.[29] In family B, a G→T substitution at position +1 of the donor splice site of exon 3 is expected to severely affect splicing of the *IRAK-M* gene and could give rise to a truncated protein containing only the DD. Both mutations were coinherited with the predisposing risk haplotype (TAT) and were associated with early-onset asthma. In addition, five nucleotide changes give rise to amino acid substitutions in the IRAK-M protein sequence. Among these, amino acid changes P22L in the DD and L400V and R429Q in the kinase domain of the IRAK-M protein are predicted to modify the domain structure, although it remains unclear if they are functionally relevant. As might be expected for a multifactorial disease, we did not observe a correlation between the severity of the asthma phenotype in patients and the presence of these mutations in the *IRAK-M* gene. Dot-blot analysis with the use of allele-specific probes in the entire sample of affected families and in 200 healthy controls did not detect these mutations.

Expression Studies

Previous studies have shown that the *IRAK-M* gene is highly expressed in monocytic cells, compared with low-level expression in other tissues.[24] We extended these studies to lung biopsies from healthy donors. Immunohistochemistry with an antibody directed against IRAK-M showed that this molecule is expressed by macrophages as well as by alveolar and bronchial epithelial cells in the lung (fig. 3*A*). In particular, staining with an antibody directed against TTF1, a marker of type II pneumocytes, revealed that IRAK-M is expressed by that cell type (fig. 3*B*). Staining of biopsies with an antibody against the NF-κB component p65 revealed that type II epithelial cells also express this molecule (not shown). Consistent with previous reports, we observed a high level of NF-κB activation in airway epithelial cells in bronchi and alveoli (fig. 3*C*).[30] The expression of IRAK-M in type II pneumocytes makes it a candidate for more-extensive involvement in pulmonary function.

Discussion

In searches for genes, age has rarely been used to discriminate genetic subclasses of asthma. Yet, several studies indicate the existence of relevant clinical peculiarities in early- versus late-onset disease. Incidence of severe disease, high IgE levels, and lung inflammation, for instance, are more pronounced in children than in adults.[31–33] Furthermore, asthmatic children may go into clinical remission after puberty, along with the shift in steroid hormone levels and developmental changes in lung structure. On the basis of these observations, we selected a cutoff age of 13 years at asthma onset, to stratify the sample. At this age, puberty is expected to have begun in all children. Using this criterion, we detected significant linkage to a portion of 12q13-24 and identified *IRAK-M* as the gene in the candidate region implicated in early-onset persistent asthma.

In particular, a predisposing three-SNP haplotype (TAT) located within the *IRAK-M* gene accounted for practically all the linkage in this region and was associated with early-onset persistent asthma. Also, SNPs mapping within the *IRAK-M* gene was associated with asthma in an independent outbred population. In this population, we were able to evaluate only cases with persistent asthma, since information about disease onset was not available. We believe that association of *IRAK-M* SNPs with asthma in this sample may be due to the contribution of subjects with early disease onset. Indeed, early onset is often associated with persistency of asthma symptoms to adulthood and is the most severe form of the disease. Finally, preliminary

Table 5. Mutations in the Coding Region of the *IRAK-M* Gene in Sardinian Asthmatic Patients

Family and Sibling	Mutation Type	Exon	Amino Acid Change	Nucleotide Position	Mutation Presence in Sibs[a]	Predisposing Haplotype[b]	Age at Asthma Onset (years)
A:	Nonsense	2	p.W76X	c.227G→A			
1					+	+/+	≤13
2					+	+/+	≤13
B:	Splicing site	IVS3	Aberrant splicing	IVS3+1G→T			
1					+	+/+	≤13
2					+	+/+	≤13
3					−	+/+	≤13
C:	Missense	1	p.P22L	c.65C→T			
1					+	−/−	≤13
2					−	−/−	≤13
D:	Missense	1	p.P22L	c.65C→T			
1					+	−/−	>13
2					+	−/−	>13
3					−	+/−	>13
E:	Missense	3	p.P111A	c.331C→G			
1					+	+/+	>13
2					+	+/+	>13
3					−	+/−	>13
4					−	+/−	>13
F:	Missense	4	p.V134M	c.400G→A			
1					+	−/−	≤13
2					+	−/−	≤13
G:	Missense	11	p.L400V	c.1198C→G			
1					+	−/−	≤13
2					+	−/−	≤13
H:	Missense						
1		11	p.L400V	c.1198C→G	+	+/−	>13
I:	Missense	11	p.L400V	c.1198C→G			
1					+	+/−	≤13
2					+	+/−	≤13
L:	Missense	11	p.R429Q	c.1286G→A			
1					+	−/−	>13
2					−	−/−	>13

[a] Plus sign (+) denotes presence and minus sign (−) denotes absence of mutation in siblings.

[b] +/+ Denotes homozygous-predisposing haplotype (TAT); +/− denotes heterozygous-predisposing haplotype; −/− denotes absence of predisposing haplotype.

work has detected rare nonsense, splicing, and missense mutations within the *IRAK-M* gene in a subgroup of family members with asthma but not in healthy controls, suggesting that insufficiency of this protein may predispose individuals to asthma. A more comprehensive sequence-based study of the spectrum of rare variants at this locus may reveal a clustering of such rare variants in subjects with asthma.[34] It is not surprising that the risk haplotype and/or the mutations of the *IRAK-M* gene were not detected in all subjects affected by early-onset asthma, because defective *IRAK-M* function presumably acts in concert with other genes and environmental factors. Consistent with complex causation, SNPs within the *IRAK-M* gene were not found to be associated with asthma in an urban Japanese cohort.[35]

How might *IRAK-M* variants be involved in the development of atopic asthma? *IRAK-M* is induced upon TLR stimulation and negatively regulates TLR signaling and inflammation.[24,25,36] The family of TLRs is crucial in the activation of the adaptive immune response to patho-

gens and lung disease.[23,27] TLR stimulation has been considered primarily linked to the activation of a Th1 response, which could protect against asthma sensitization and initiation but could also trigger asthma symptoms and increase bronchial hyperreactivity once allergic disease is present.[37] Recent studies have suggested that TLR activation can also induce Th2 cytokines and the development of experimental asthma.[38,39] Also, the exposure to lipopolysaccharide doses similar to the levels in the environment of asthmatic children triggers a Th2 response.[40] Several studies have found positive associations of SNPs in different TLRs with asthma and atopy.[41]

Malfunction of the TLR pathway in innate immunity is likely to be involved in atopic disease as well as asthma exacerbation, and our results suggest a critical role for IRAK-M, a major regulator of this pathway. The pathogenetic mechanism(s) remain unclear, but a first hint comes from the observation that IRAK-M is expressed in type II pneumocytes. These cells play key roles in lung function and innate immune defense and may be in-
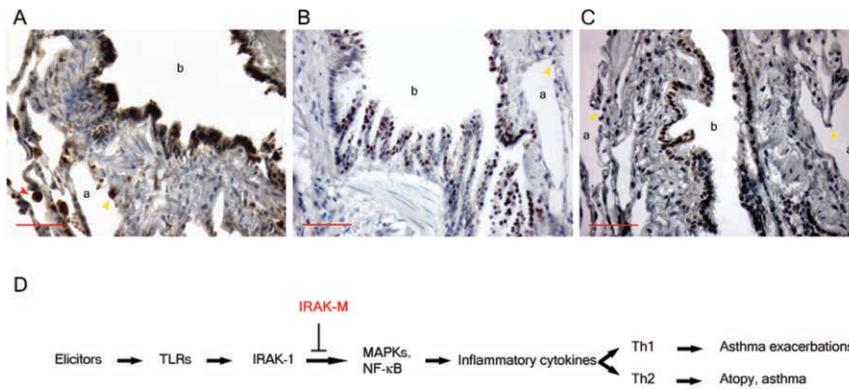
Figure 3. *A, B,* and *C,* Immunohistochemical localization of IRAK-M in normal lung biopsy samples from healthy donors. Expression of IRAK-M (*A*) was detected in the cytoplasm of alveolar macrophages (*red arrowhead*) and type II epithelial cells, particularly at the level of bronchi (b) and alveoli (a [*yellow arrowheads*]). Nuclei of epithelial type II cells in bronchi and alveoli are stained by the anti-TTF1 antibody (*B*) and by the anti-phospho-p65 antibody (*C*) that recognizes the activated form of the NF-κB subunit. Tissues were counterstained with hematoxylin. Results are representative of at least six independent experiments. a = alveolus; b = bronchus. Yellow arrowhead indicates alveolar type II pneumocytes. Scale bar = 50 μm. *D,* IRAK-M as a brake on inflammatory processes involved in asthma. Recognition of different elicitors by TLRs on alveolar macrophages and lung epithelial cells triggers the formation of a receptor/adaptor complex including IRAK-1, IRAK-4, and MyD88. Release of phosphorylated IRAK-1 from the signaling complex leads to activation of MAPKs and NF-κB. This in turn results in the production of inflammatory cytokines that can influence T-cell activation and differentiation of Th precursors into Th1 and Th2 subtypes. Once the immune response is mounted, IRAK-M inhibits TLR signaling by interrupting IRAK-1 downstream signal transduction, thus restoring homeostasis. When IRAK-M function is hampered, excessive TLR stimulation may lead to continuous activation of Th1 and Th2 cells in the lung and to the development of atopic asthma and/or asthma exacerbation in response to infections/allergens.

volved in allergen-induced airway changes. Recent work has suggested a specific role of IRAK-M in lung innate immunity.[42] In particular, it has been reported that lungs from *IRAK-M*–deficient septic mice responded to bacterial challenge by producing elevated levels of inflammatory cytokines and chemokines, which cause the recruitment of increased numbers of neutrophils in the airways. Notably, this cell type is involved in asthma exacerbation. On the basis of our results and previous observations, we propose the following etiological pathway toward asthma (fig. 3*D*). IRAK-M intervenes critically to modulate the activation of NF-κB and downstream inflammatory responses. When its function is diminished or compromised, continuous overproduction of inflammatory cytokines in the lung in response to infection/allergens may lead to a Th2-mediated allergic response and/or Th1-dependent exacerbation of asthma symptoms. If so, modulating IRAK-M function may provide a way to moderate the development of allergic asthma. Finally, our data may provide an entry point to identifying other genetic factors that predispose individuals to early- and late-onset asthma.

Appendix A

Table A1. Microsatellite Markers Genotyped for Multipoint Linkage Analysis on Chromosome 12q13-24

Marker	Sex-Averaged Map (Kosambi cM)	Fluorophore	Primer (3′–5′) Forward	Reverse
D12S297	.0	HEX	GTTTGGTATTGGAGTTTTCAG	AAATCATCAGTGGAGTTAGCA
D12S1724	2.4	TET	CTCTGGAGGCTGAGGTGG	ATCCGTGCTGGTTCTATCTGTGTA
D12S72	6.2	TET	CATCATCCCATGGTCGAAG	GAGAGTAGGTTCCTTATCCTGGG
D12S83	7.7	TET	TTTTTGGAAGTCTATCAATTTGA	TAGCAGAGAAAGCCAATTCA
D12S371	12.3	FAM	AAACCACACAAAGCCTCCAG	TGATGACAGGCTCAAGCG
D12S75	17.6	FAM	GTGGCTCTAAAGCATGACCA	ATTTCTTCCACCTGCATGAT
D12S335	22.2	NED	TCATCCAGGCTTCACC	GTTTCTTTGGCAAGGACAGACACA
IFNG	24.3	HEX	GCTGTTATAATTATAGCTGTC	GTTTCTTCTACTGTGCCTTCCTGTAG
D12S43	26.0	FAM	AATGTCCTTGTACTTAGGAT	CACTTAATATCTCAATGTATAC
D12S1040	28.4	TET	TATGACAGGATGAACAAAAACG	AAATTGAATTTGATTTCTTCATAGC
D12S1052	29.9	HEX	ATAGACAGGCTGGATAGATAGACG	AGTGTGATATGAATAATGAGCTGC
D12S326	32.3	HEX	CCCAGCAGTGCTAGTGTTGA	GTTTCTTGGGCTAGGGTGGAGAATCAA
D12S1064	39.6	TET	ACTACTCCAAGGTTCCAGCC	AATATTGACTTTCTCTTGCTACCC
D12S311	40.9	HEX	CCAAACATTAACTGTTCCC	GTTTCTTGTGCCCTGAGCAACTG
D12S1300	44.6	HEX	CCTCACACAATGTTGTAAGGG	TGTAACATCCGTGATTAAAATAGC
PAH	48.6	TET	GCCAGAACAACTGCTGGTTC	AATCATAAGTGTTCCCAGAC
D12S78	54.7	FAM	CTTTGCAGCACCATGTATTT	ACTGCTGGCTTTAACAGAAA

Table A2. Results of Association Analysis by TDT in Trios with Early-Onset Persistent Asthma, with Use of 22 SNPs in the Genomic Region Spanning *IRAK-M*

SNP or Haplotype[a]	Frequency[b]	T:U[c]	P[d]	Empirical P (10[5] Permutations)
rs7970350	.398	30:42	.1572	.8416
rs949911	.142	25:27	.7815	1.0000
rs6581660	.273	48:34	.1220	.7359
rs11836463	.241	42:34	.3587	.9904
rs2870784	.171	30:24	.4142	.9984
rs10878378	.413	25:59	**.0002**	**.0018**
rs1177578	.413	27:66	**.0000**	**.0003**
rs1168754	.167	27:23	.5716	1.0000
rs2141709	.317	23:45	**.0076**	.0726
rs1168770	.414	27:62	**.0002**	**.0017**
rs2701652	.180	25:23	.7728	1.0000
rs1732886	.181	26:26	1.0000	1.0000
rs1882200	.396	60:30	**.0007**	**.0094**
rs11465955	.391	58:28	**.0012**	**.0113**
rs2293657	.392	58:27	**.0007**	**.0063**
rs1821777	.398	55:29	**.0045**	**.0294**
rs1624395	.451	47:29	**.0389**	.3453
rs1370128	.471	49:32	**.0489**	.4524
rs2118137	.324	46:50	.6830	1.0000
rs289068	.232	34:34	1.0000	1.0000
rs3741604	.338	38:46	.3827	.9945
rs1168314	.350	38:52	.1400	.7785
GGGTAT	.385	61:28	**.0005**	**.0011**
GCACGC	.277	23:46	**.0057**	**.0282**
TGGCGC	.165	31:22	.2163	.8403
GCGCAT	.057	7:16	.0587	.3274

[a] SNPs highlighted in bold italics are within the *IRAK-M* gene. Four haplotypes identified by six tag SNPs (*rs2870784, rs1177578, rs2141709, rs11465955, rs1624395,* and *rs1370128*) are shown at the bottom. Only haplotypes with frequency >0.05 are shown.

[b] Frequency of minor alleles of SNPs and haplotypes.

[c] T:U denotes number of transmitted versus untransmitted minor alleles.

[d] *P* value in bold indicates *P* < .05.

Table A3. Characteristics of Sardinian and Mainland Italian Subjects Used in Case-Control Studies

Characteristic	Sardinians		Mainland Italians	
	Cases[a]	Controls	Cases	Controls
No. of subjects	139	460	67	278
Age (years)[b]	22.37 ± 8.61	30.12 ± 10.09	38.32 ± 9.69	39.01 ± 6.32
Males (%)	55.40	41.30	46.96	48.56

[a] Sardinian cases are subjects with early-onset persistent asthma.
[b] Data are reported as means $\pm$ SDs.

Table A4. Haplotypes Identified by the Six Tag SNPs Showing an Estimated Frequency >5% in the Italian Case-Control Sample

Frequency	rs2870784	rs1177578	rs2141709	rs11465955	rs1624395	rs1370128
.33950	G	G	G	T	A	T
.28680	G	C	A	C	G	C
.22996	T	G	G	C	G	C
.05300	G	C	G	C	A	T

Web Resources

Accession numbers and URLs for data presented herein are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://research.marshfieldclinic.org/genetics/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Ensembl Genome Browser, http://www.ensembl.org/
The GDB Human Genome Database, http://gdbwww.gdb.org/
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for asthma and *IRAK-M*)
The SNP Consortium, http://snp.cshl.org/
UCSC Genome Browser, http://genome.cse.ucsc.edu/

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