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Report

Fine Mapping and Positional Candidate Studies Identify *HLA-G* as an Asthma Susceptibility Gene on Chromosome 6p21

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Asthma affects nearly 14 million people worldwide and has been steadily increasing in frequency for the past 50 years. Although environmental factors clearly influence the onset, progression, and severity of this disease, family and twin studies indicate that genetic variation also influences susceptibility. Linkage of asthma and related phenotypes to chromosome 6p21 has been reported in seven genome screens, making it the most replicated region of the genome. However, because many genes with individually small effects are likely to contribute to risk, identification of asthma susceptibility loci has been challenging. In this study, we present evidence from four independent samples in support of *HLA-G* as a novel asthma and bronchial hyperresponsiveness susceptibility gene in the human leukocyte antigen region on chromosome 6p21, and we speculate that this gene might contribute to risk for other inflammatory diseases that show linkage to this region.

We conducted a genomewide screen of families who participated in the Collaborative Study on the Genetics of Asthma (CSGA) (Collaborative Study on the Genetics of Asthma 1997; Xu et al. 2001). The strongest linkage signal in 129 white families was on chromosome 6p21 at marker *D6S1281* (LOD = 1.91; $P = .003$), which is 2.5 cM telomeric to the human leukocyte antigen (HLA) complex. All the evidence of linkage to asthma was found in the 35 white families ascertained in Chicago (LOD = 3.6) (fig. 1*A*). We focused our subsequent studies on these families, as well as on 46 white child-parent trios with asthma (MIM 600807) also ascertained in Chicago and two other populations that had previously shown evidence of linkage of asthma-associated phenotypes to markers in this region (table 1) (Ober et al. 2000; Koppelman et al. 2002). Details of the statistical methods are described in appendix A (online only).

To further narrow the linked region, we genotyped the Chicago families for five additional STRPs—two that reside within the HLA region (*DQ.CAR* and *TNFa*) and three flanking markers (*D6S258*, *MOGc*, and *D6S1680*) (fig. 1*A*). The LOD score increased to 3.8, peaking at *MOGc*. The information content (Nicolae and Kong 2004) in this region was 95%, indicating that we could not increase the LOD score or improve the resolution by adding more markers. Furthermore, the *MOGc* 136-bp allele was overtransmitted to asthmatic children in the families (42 transmissions [TR]:23 nontransmissions [NT]; *P* [corrected for relatedness and number of alleles] = .06). In the trios, the *MOGc* 134-bp allele was overtransmitted to asthmatic children (17 TR:6 NT). Because of the extensive linkage disequilibrium (LD) in the HLA region (Begovich et al. 1992), the disease locus could have been located at a far distance, despite the evi-

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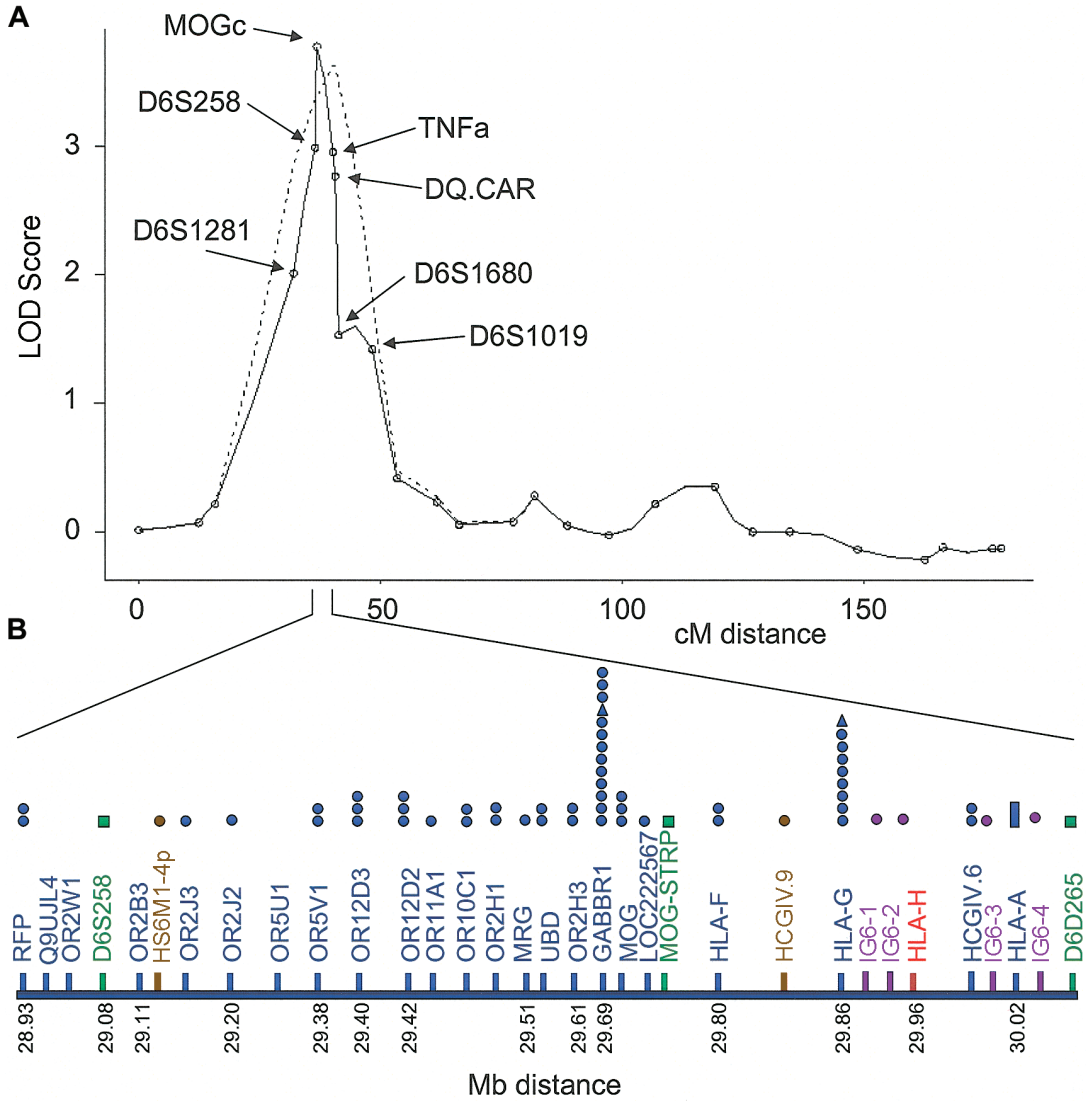

Figure 1 Linkage to 6p21 in the Chicago families. *A*, The dashed line shows the results of the initial genome screen with framework markers. The solid line shows the results of five additional STRPs between framework markers *D6S1281* and *D6S1019*. *B*, 1-Mb region from *D6S258* to *D6S265* with positional candidate genes. (All known genes [*blue*]—but not all pseudogenes [*brown*], STRPs [*green*], and intragenic SNPs [*purple*]—are included.) Circles = SNPs; triangles = in/dels; squares = STRPs; rectangle = HLA-A genotype, which is comprised of multiple SNPs. See appendix A (online only) for allele frequencies and results of association studies.

dence of association between *MOGc* alleles and asthma in two independent samples. To determine whether the haplotype shared among affected individuals extended into the HLA region, we genotyped the families at the *HLA-A* gene and at six STRPs spanning the HLA class I region between *TNFa* and *HLA-A*. There was no evidence of association with *HLA-A* or with markers proximal to it (data not shown).

Chromosome 6p21 is one of the best characterized regions in the human genome, and the nucleotide sequence between *D6S258* and *D6S265* is known (Mungall et al. 2003; Stewart et al. 2004). This region is gene rich, with 20 known or predicted genes and at least 30 pseudogenes in the 1-Mb region from *HLA-A* to *OR2B3* (fig. 1*B*). To localize the susceptibility-associated variation, we genotyped the Chicago families and trios for an additional 59 polymorphisms in 19 genes, two pseudogenes, and the intergenic regions flanking the *HLA-A* and *HLA-G* loci (fig. 1*B*; tables A1 and A4 [online only]). Our strategy was to genotype one common SNP every 10–20 kb across each gene between *D6S258* and *D6S265*, as well as all nonsynonomous SNPs, when possible. SNPs were selected from dbSNP or were discovered in our laboratory. We previously extensively characterized the haplotype struc-

Table 1

Clinical Characteristics of Patient Samples

Characteristic	Chicago Families[a,b] (n = 138)	Chicago Trios[a,c] (n = 46)	Hutterite Families[d] (n = 156)	Dutch Families[e] (n = 200)	Descendents of Dutch Families[e] (n = 327)
Sex (% male)	59.4	60.6	53.2	62.0	42.3
Asthma (%)	100	100	48.0	100	40.7
BHR (%)	100	100	100	100	100
Mean % predicted FEV1 (SD)	88.64 (17.12)	80.13 (16.83)	95.58 (15.71)	69.62 (24.68)	89.49 (12.10)
Mean FEV1/FVC (SD)	.82 (.09)	.77 (.10)	.79 (.10)	.597[f] (.149)	.80[f] (.086)
Mean log IgE (SD) IU	2.11 (.73)	2.19 (.71)	1.60 (.77)	1.95 (.69)	1.95 (.76)
Atopy[g] (%)	67.2	79.8	51.0	81.9	59.1

NOTE.—These studies were approved by the institutional review boards at each institution. Not all individuals were genotyped at all marker loci.

[a] CSGA families were ascertained through two siblings with asthma and were extended to include other affected relatives, never skipping more than one unaffected relative (Lester et al. 2001). Asthma was diagnosed as follows: (1) either a fall in baseline FEV1 by ⩾20% at ⩽25 mg/ml methacholine (BHR) or an increase of ⩾15% in baseline FEV1 after bronchodilator use; (2) two of the following symptoms: cough, wheeze, or shortness of breath (dyspnea); and (3) current medication use or doctor's diagnosis of asthma. All participating relatives of the siblings were studied.

[b] Family size ranged from 4 to 18 members (mean family size, 7 members).

[c] Trios were individuals meeting the same criteria as the CSGA patients with asthma and their parents, who were not evaluated (n = 46).

[d] Hutterites (n = 693), who are related to each other in a 13-generation, 1,623-member pedigree, were evaluated using a modified CSGA protocol (Ober et al. 2000). BHR in this group was defined as a fall in baseline FEV1 by ⩾20% at ⩽25 mg/ml methacholine.

[e] The 200 Dutch families were recruited ~30 years ago through a proband who was diagnosed as having asthma; they were reevaluated in the 1990s, along with their spouses, children, and grandchildren (Panhuysen et al. 1995). The families included 1,183 individuals. BHR in this group was defined as a fall in baseline FEV1 by ⩾20% at ⩽32 mg/ml histamine (30′ protocol).

[f] FEV1/VC.

[g] Defined as a positive skin-prick test to airborne allergens.

ture of the *HLA-G* gene (MIM 142871) in the Hutterites (Ober et al. 1996, 2003). In this gene, we selected SNPs that identify clusters of variants either that are in perfect or near-perfect LD or that uniquely define all of the common *HLA-G* alleles, as in our previous study (Ober et al. 2003). SNPs in intergenic regions were selected to characterize the LD pattern in the proximal end of the region.

To investigate whether any of these variants explained some or all of the original evidence of linkage in the families, we conditioned on the genotype at each polymorphic site and reexamined the evidence of linkage. Multipoint analysis conditional on the genotypes of one variant in the *HLA-G* gene (1489C/T, His93His) yielded a LOD score of 0.9, which is substantially lower than the maximum LOD score (3.8) in this region. Analyses conditional on the other variants led to LOD scores >0.9. Thus, variation in the *HLA-G* gene accounted for most of the linkage in the families; the remaining evidence of linkage could be due to chance sharing among affected family members (Sun et al. 2002) or to the presence of a second susceptibility locus in the linked region. Nonetheless, it is notable that conditioning on a single SNP could result in a reduction in the LOD score of this magnitude (Sun et al. 2002).

We next examined the pattern of LD across this region in unrelated individuals from the Chicago families and trios and identified five LD blocks (Zhang et al. 2002*a*) (fig. 2*A*). To determine which LD block contains variation that contributes to asthma susceptibility, we examined pairwise combinations of SNPs within each block by the transmission/disequilibrium test (TDT), conditional on the evidence of linkage at each position. Only pairwise combinations of SNPs in block 2 showed significant nonrandom transmission of haplotypes ($P < .001$) in both the families and the trios (fig. 2*B*; table A2 [online only]).

Analyses of the individual variants revealed that polymorphisms in *HLA-G* (block 2) were associated with asthma in both the families and the trios; SNPs in two genes in block 4 (*OR12D2* and *OR10C1*) and one gene each in block 3 (*GABBR1*) and block 5 (*OR5V*) were associated with asthma in the families only (table A3 [online only]). Thus, the *HLA-G* gene in block 2 was the only gene that showed evidence of association with asthma in both the families and the trios. However, in the families, the association was with a haplotype carrying the −964G allele (43 TR:25 NT), whereas the association in the trios was with a haplotype carrying the −964A allele (23 TR:12 NT).

To further localize and characterize the susceptibility locus, we genotyped selected markers in two populations that previously showed linkage of asthma-related phenotypes to 6p21: (1) the Hutterites, a founder population of European descent, and (2) Dutch families (Ober et al. 2000; Koppelman et al. 2002). Because of the different

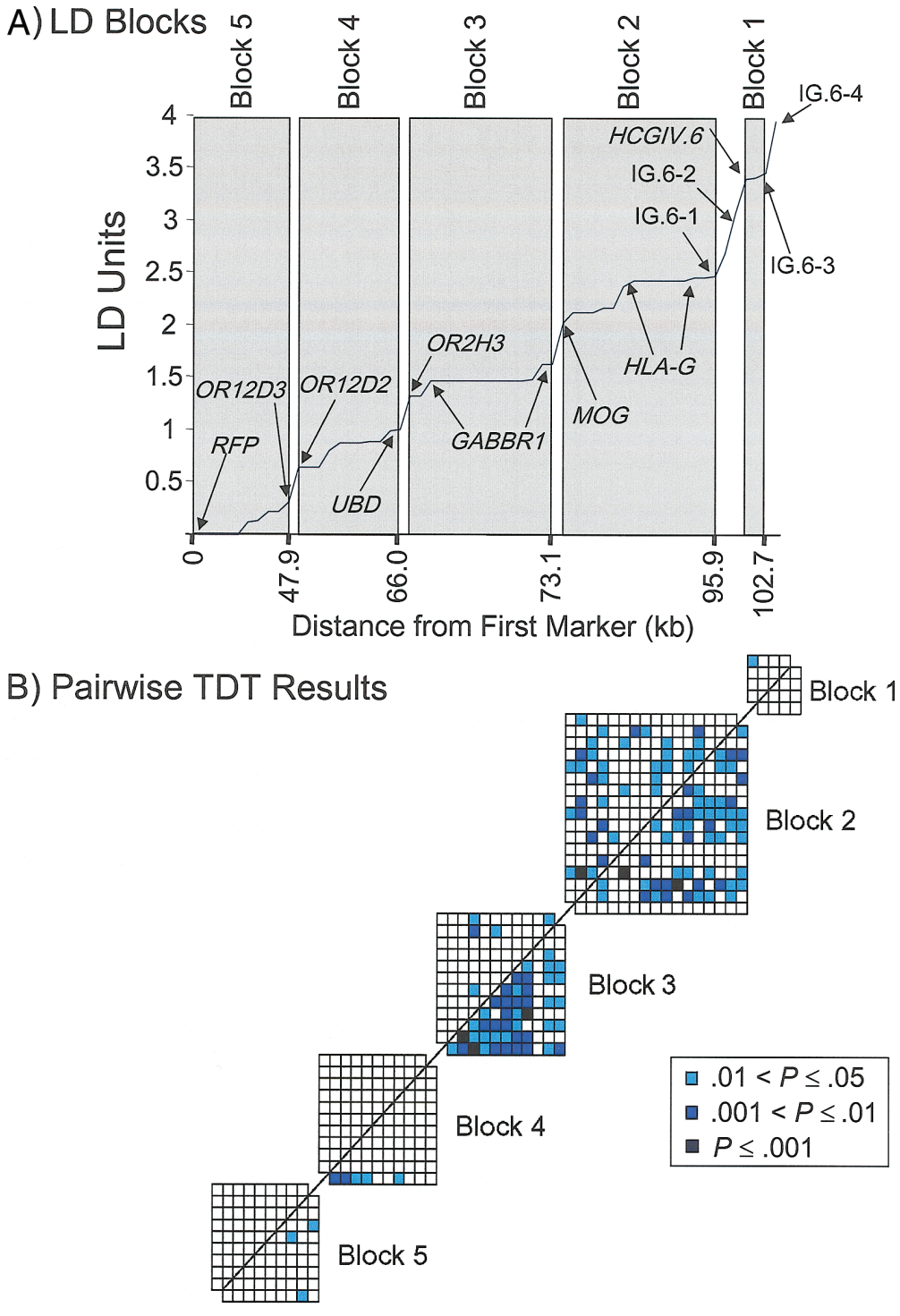

Figure 2 LD block structure in the extended class I region. *A*, Graph of LD map, showing LDUs on the *Y*-axis and distance on the *X*-axis (Zhang et al. 2002*a*). Shaded boxes show the five blocks in this region. *B*, Pairwise TDT of variants within each block. Results for Chicago families are shown in the lower half, and results for Chicago trios are in the upper half. *P* values were derived by simulations that were conditioned on the evidence of linkage but were not corrected for multiple comparisons.

Table 2

Number of Dutch Children with BHR and Atopy, by Child's *HLA-G* −964 Genotype and Mother's Affection Status

Child's Genotype and Mother's Affection Status	No. of Children with Affected Status of			
	BHR+	BHR−	Atopy+	Atopy−
AA:				
BHR+	22	28	33	18
BHR−	27	30	32	25
AG:				
BHR+	57	63	62	59
BHR−	56	68	60	67
GG:				
BHR+	57	45	44	61
BHR−	18	50	28	44

Note.—The prevalence of BHR in children is influenced by both the child's genotype and the mother's affection status. Among GG children, the prevalence of BHR is 56% if the mother has BHR and 26% if the mother does not have BHR ($P = .001$). The prevalence of atopy in children is influenced by the child's genotype but not the mother's affection status ($P = .006$ for differences between genotypes).

ascertainment schemes, we used different approaches in each sample. Ascertainment in the Hutterites was population based; individuals in this single, large pedigree were not selected on the basis of any particular phenotype. As a result, we used a test of association that was designed for large, multigenerational pedigrees (Bourgain et al. 2003). Here, we defined "cases" as individuals with bronchial hyperresponsiveness (BHR [MIM 600807]) ($n = 156$) and "controls" as individuals with a negative history of asthma symptoms and without BHR ($n = 434$). We note that all of the affected individuals in the Chicago families and trios also had BHR (table 1). Only 3 SNPs in *HLA-G* (of 21 SNPs genotyped) showed evidence of association with BHR in the Hutterites ($P < .05$) in whom the −964G allele was associated, similar to the results in the Chicago families.

The 200 Dutch families were ascertained through a parent in whom asthma was diagnosed ~30 years ago (Panhuysen et al. 1995). Therefore, we first examined the prevalence of BHR and atopy (MIM 147050) in the descendents of each proband. The prevalence of atopy differed significantly by genotype at *HLA-G* −964 (60% of AA children, 49% of AG children, and 41% of GG children were atopic; $P = .006$); there was no association with BHR (table 2). However, because of the method of ascertainment used in the Dutch families, because *HLA-G* is an important immunomodulatory molecule during pregnancy (Hunt et al. 2000; Le Bouteiller et al. 2003), and because maternal asthma is a significant risk factor for asthma (Martinez 1997), we next analyzed these data stratified by mothers' and fathers' affection status (BHR+ or BHR−). The −964A allele was overtransmitted to children with BHR if the mother was unaffected (57 TR:27 NT; $P = .004$), whereas the −964G allele was overtransmitted to children with BHR if the mother was affected (61 TR:45 NT; $P = .15$). The differences in transmission patterns of alleles to children with BHR from mothers with and without BHR was highly significant ($P = .0008$). Similar analyses stratified by father's asthma status did not show as significant a trend. Furthermore, the prevalences of BHR are not different if maternal status is ignored, but the prevalence of BHR among children with the GG genotype is significantly influenced by maternal status (table 2). Among children whose mothers have BHR, 56% of GG children have BHR; among children of mothers without BHR, 26% of GG children have BHR ($P = .001$). No such relationship was observed for AA or AG children. Thus, in the Dutch families, GG children are less likely to be atopic but are more likely to have BHR if their mother also has BHR. None of the other five markers typed in the Dutch families showed associations with either BHR or atopy (table A3 [online only]).

To further examine interactions between mother's affection status and child's genotype, we reexamined the Chicago families, stratifying the sample by maternal BHR status (table 3). Genotype distributions in the children with asthma differed by mother's BHR status for the nine polymorphisms in the *HLA-G* gene (data not shown), with three being significant at $P < .01$. Similar to the Dutch families, the −964GG genotype was associated with asthma among children of mothers with a positive BHR affection status, whereas the −964AA genotype was associated with asthma among children of mothers with a negative affection status. Other variants in *HLA-G* that were not genotyped in the Dutch families showed more striking differences, suggesting that variation in the gene in addition to the promoter region may contribute to risk. A similar trend was observed in the Hutterites: the frequency of the −964G allele among children with BHR whose mothers also had BHR was higher (0.69) than among children with BHR whose mothers did not have BHR (0.60), although this difference was not significant ($P = .16$).

Thus, in the Chicago families and trios and in the Hutterite and Dutch families, variants in the *HLA-G* gene were associated with asthma or BHR. Although no other variation that we examined in this region showed associations in all four populations, we cannot exclude the possibility that unidentified variation in *HLA-G* or in other genes in block 2 also contributes to susceptibility. Furthermore, because we observed different alleles and haplotypes associated in the different populations and also when stratified by maternal affection status, susceptibility at this locus is complex, influenced by maternal factors, and associated with multiple related phenotypes.

HLA-G is a novel HLA gene that has limited polymorphisms in the coding region and a restricted tissue

Table 3

Number of Children with Asthma in the Chicago Families, by Child's Genotype and Mother's Affection Status

Mother's Affection Status	No. of Children with Genotype of					
	HLA-G −964			*HLA-G* 1489		
	GG	AG	AA	CC	CT	TT
BHR+	12	17	4	18	15	0
BHR−	9	19	18	9	30	12

Note.—The genotype distribution in the children differs by mother's affection status. The −964GG genotype is more common among children with asthma whose mothers have BHR, whereas the −964AA genotype is more common among children with asthma whose mothers do not have BHR, although these differences do not reach statistical significance. Genotype differences are more striking for *HLA-G* 1489 (difference between children with asthma of BHR+ and BHR− mothers, $P = .009$), the same SNP that explained the evidence of linkage.

distribution (Ober and Aldrich 1997). This gene is the most highly expressed HLA gene in placental cells at the maternal-fetal interface, where it plays important immunoregulatory roles, including the inhibition of maternal NK and T cells and the promotion maternal tolerance of the allogeneic fetus (Hunt et al. 2000; Le Bouteiller et al. 2003). Recently, it has been demonstrated that HLA-G is expressed in adult macrophages, dendritic cells, and myoblasts in response to inflammation (Yang et al. 1996; Khosrotehrani et al. 2001; Wiendl et al. 2003), in intestinal biopsies in patients with Crohn disease (Torres et al. 2004), and in malignant and nonmalignant lung diseases (Pangault et al. 2002). Furthermore, in biopsied myocardial cells from transplanted hearts, expression of HLA-G is correlated with prolonged graft survival and transplantation success (Rouas-Freiss et al. 2003). In this context, HLA-G is thought to inhibit Th1-mediated inflammation, perhaps in a concentration-dependent manner (Kapasi et al. 2000). The association of *HLA-G* variants with asthma is particularly intriguing, because asthma, like pregnancy, is characterized by a predominance of Th2 cytokines (Lin et al. 1993). Furthermore, the interaction between maternal phenotype and the *HLA-G* genotype in children in the Dutch and Chicago families is notable, given the important role that this gene plays in pregnancy and the fact that maternal asthma is a well-established risk factor for asthma (Martinez 1997).

To evaluate whether the *HLA-G* gene could contribute toward the immunologic milieu in the asthmatic lung, we studied the expression pattern of HLA-G in lung tissues from two individuals with asthma, one individual without asthma, and one individual without asthma but with a history of cigarette smoking. We demonstrated by immunohistochemistry the expression of HLA-G in bronchial epithelial cells (fig. 3). Expression in the lung was limited to the soluble isoform, HLA-G5 (also called "soluble G1") (Fujii et al. 1994). Neither the transmembrane G1 and G2 isoforms nor the soluble G6 isoform were identified in these tissues. Thus, expression of HLA-G in the lung might contribute toward the aberrant immunologic response to inhaled allergens in genetically susceptible individuals.

Although the LD pattern in this region makes it impossible to rule out the possibility that variation in other genes in block 2 contributes to susceptibility, several lines of evidence suggest that *HLA-G* is an asthma susceptibility gene. First, a SNP in *HLA-G* accounted for nearly all the linkage in this region. For other variation to be causal, it would have to be in nearly perfect LD with and at a similar frequency to the *HLA-G* 1489C/T SNP (Sun et al. 2002) and not in LD with the other SNPs tested. Moreover, if the effect size of our initial linkage signal was biased upwards because of chance sharing in the families (Sun et al. 2002), then *HLA-G* may be the sole asthma susceptibility gene in this region. Even if the signal was not biased, additional variation in *HLA-G* that we did not sample could also contribute to risk. Second, among the variants that were surveyed across the linked region, only SNPs in *HLA-G* were associated with asthma or BHR in four different populations, which were ascertained using different strategies. Third, we demonstrated by immunohistochemistry that the soluble HLA-G isoform, G5, was highly expressed in bronchial epithelial cells in asthmatic lungs. The presence of soluble HLA-G protein in bronchial epithelial cells indicates that it could participate in a local inflammatory response to airborne allergens or other agents. On the basis of these combined data, we propose that *HLA-G* is an asthma susceptibility gene on chromosome 6p21. The differential association of alleles (or haplotypes) with childhood disease on the basis of maternal affection status is intriguing. Although the contribution of prenatal events to metabolic diseases such as diabetes and obesity is well established (Osmond and Barker 2000), the fetal origins of immune-mediated diseases have received less attention (Jones et al. 2000; Adams and Nelson 2004). Nonetheless, the programming of the fetal immune system likely begins in utero, and our data suggest that the maternal immunologic milieu (captured in this study by the mother's disease status) may influence the child's subsequent response to inhaled allergens in a genotype-specific manner. One potential mechanism is through differential methylation, which has been previously implicated in regulating the expression levels of *HLA-G* (Onno et al. 1997; Moreau et al. 2003; Ober et al. 2003), a hypothesis that we are currently investigating.

Finally, many other inflammatory diseases—such as multiple sclerosis (GAMES and Transatlantic Multiple Sclerosis Genetics Cooperative 2003), psoriasis (Zhang et al. 2002*b*), atopic dermatitis (Soderhall et al. 2001), in-

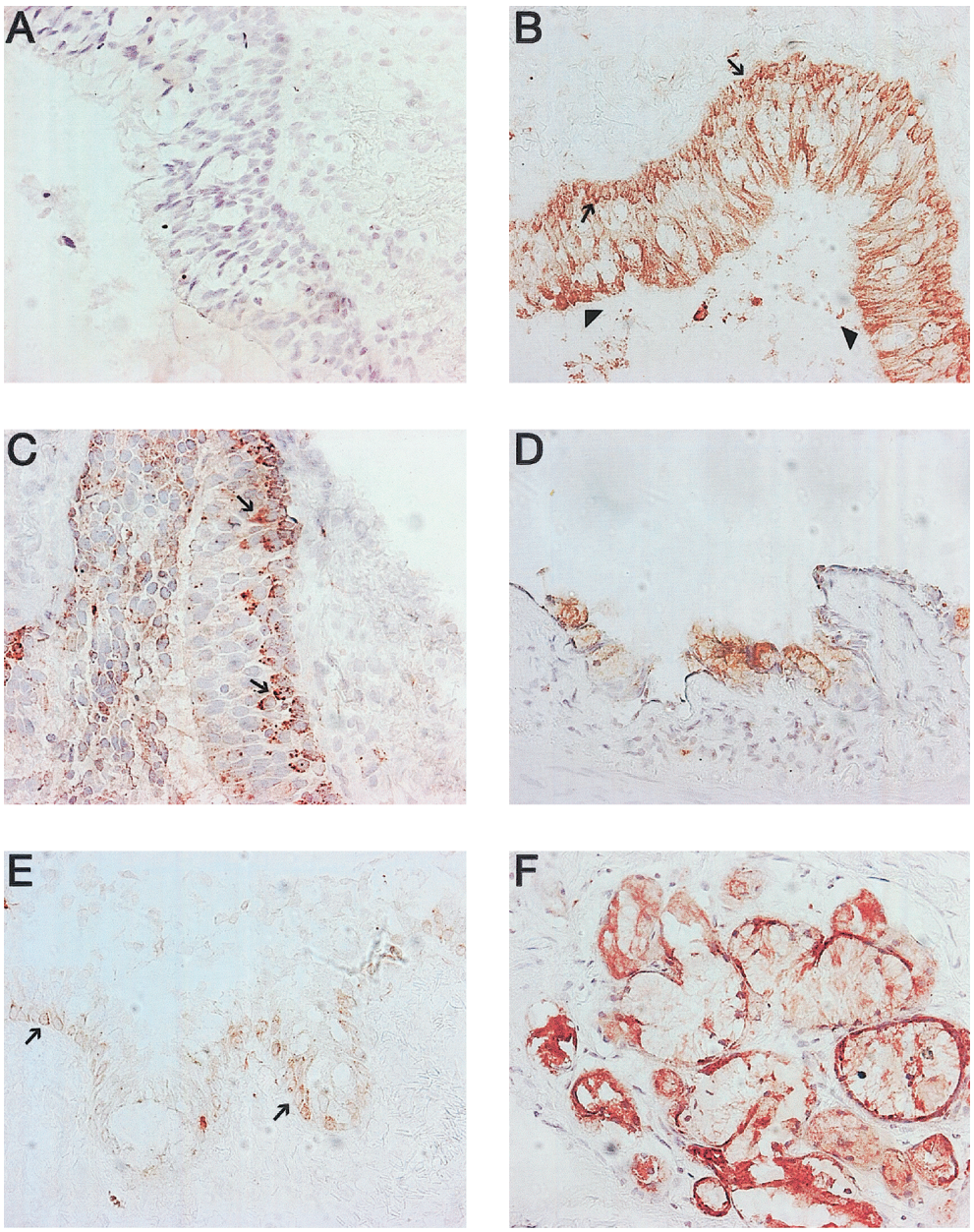

Figure 3 *A,* Control using an irrelevant IgG antibody. *B–F,* Sections labeled with the anti-HLA-G5 antibody. Epithelial cell labeling may be increased in basal epithelial cells immediately above the basement membrane (*arrows* in panels B, C, and E) but also may label columnar cells (*arrowheads* in panel B). In areas of damage and focal denudation (*D*), labeling may be found in remaining epithelial cells. Labeling is also seen in mucosal and submucosal gland cells (*F*) in the airway, which are of epithelial origin. Panels A and E are from individuals without asthma; panel C is from an individual without asthma but with a 30-year smoking history; panels B, D, and F are from individuals with asthma. mAB 1-2C3 detects soluble HLA-G5 in human bronchial epithelial cells. Human donor lungs that could not be used for transplantation were obtained under an institutional review board–approved protocol from the Regional Organ Bank of Illinois. Diagnoses were extracted from medical records. Bronchi were dissected and frozen in OCT. Five-micron sections were stained for antibodies against HLA-G1, HLA-G2, HLA-G5, and HLA-G6 isoforms, as described elsewhere (Morales et al. 2003). Original magnification was ×400 for all images. Antibodies specific for HLA-G1, HLA-G2, and HLA-G6 were negative in lung sections from patients with asthma as well as those without asthma (not shown).

flammatory bowel disease (Mathew and Lewis 2004), and schizophrenia (Wright et al. 2001)—have been linked to the HLA region. To date, all of the variation underlying these linkages has not been identified. It is tempting to speculate that variation in the *HLA-G* gene may contribute to susceptibility to a wide range of inflammatory diseases, including asthma. Thus, this gene that likely evolved to promote tolerance in pregnancy may contribute to risk

for many common diseases, suggesting that novel therapeutic strategies could have broad relevance to these immune-mediated diseases.

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Electronic-Database Information

The URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/
DNAPrint Genomics, http://www.dnaprint.com/genotyping.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for asthma, *HLA-G*, BHR, and atopy)

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