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ARTICLE

Polymorphisms in the sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8) gene are associated with susceptibility to asthma

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Sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8) promotes the apoptosis of eosinophils and inhibits FcεRI-dependent mediator release from mast cells. We investigated the genetic association between sequence variants in Siglec-8 and diagnosis of asthma, total levels of serum IgE (tIgE), and diagnosis of eosinophilic esophagitis (EE) in diverse populations. The effect of sequence variants on Siglec-8 glycan ligand-binding activity was also examined. Significant association with asthma was observed for SNP rs36498 (odds ratios (OR), 0.69, $P=8.8 \times 10^{-5}$) among African Americans and for SNP rs10409962 (Ser/Pro) in the Japanese population (OR, 0.69, $P=0.019$). Supporting this finding, we observed association between SNP rs36498 and current asthma among Brazilian families ($P=0.013$). Significant association with tIgE was observed for SNP rs6509541 among African Americans ($P=0.016$), and replicated among the Brazilian families ($P=0.02$). In contrast, no association was observed with EE in Caucasians. By using a synthetic polymer decorated with 6′-sulfo-sLe^x, a known Siglec-8 glycan ligand, we did not find any differences between the ligand-binding activity of HEK293 cells stably transfected with the rs10409962 risk allele or the WT allele. However, our association results suggest that the Siglec8 gene may be a susceptibility locus for asthma.
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INTRODUCTION

We and others have recently identified sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8), a member of the CD33 subfamily of Siglecs.[1] Siglec-8 is a type-I transmembrane protein expressed on human eosinophils, mast cells, and basophils.[2,3] This transmembrane protein has an extracellular binding domain that recognizes specific complex carbohydrates (glycans such as 6′-sulfated sialyl Lewis X (6′-sulfo-sLe^x)),[4] and intracellular immunoreceptor tyrosine-based inhibition motifs (ITIMs) that facilitate receptor binding into inhibitory signals. We have reported that incubation of human eosinophils with specific Siglec-8 monoclonal antibodies (mAbs) causes rapid caspase- and ROS-dependent apoptosis[5–7] This was further supported by studies using mouse Siglec-F, the closest functional paralog of human Siglec-8, showing that Siglec-F engagement on murine eosinophils by antibodies induces their apoptosis in vitro, and administration of Siglec-F antibodies to hyper-eosinophilic mice reduces circulating and tissue eosinophils.[8,9] Furthermore, mice deficient in mouse Siglec-F have exaggerated allergic lung eosinophilic responses.[10] These findings underscore the importance of Siglec-F/8 in eosinophil apoptosis and human eosinophil-mediated disorders like asthma.

Eosinophils are involved in defense against parasites, but are also known for their role as effector cells involved in the pathobiology of a number of allergic diseases, including asthma and eosinophilic eso-phagitis (EE).[11–13] It is well-recognized that the total eosinophil count in induced sputum reflects asthmatic activity and is useful for adjusting steroid dosage and for early detection of exacerbations.[14,15] The involvement of Siglec-8 in eosinophil apoptosis supports the hypothesis that Siglec-8 may have a role in eosinophil-mediated asthma, allergic inflammation, and EE. Thus, abnormalities in Siglec-8 function due to genetic variations could be associated with the pathogenesis of asthma and other eosinophil-associated diseases. Genetic linkage studies have previously identified chromosomal regions that contain genes involved in the development of asthma and related phenotypes, including 19q13.33–q13.41, where Siglec-8 is located.[16–18]

Here, we explore the role of genetic polymorphisms in the Siglec-8-encoding gene in asthma and other eosinophil-associated diseases. We selected tagging SNPs and functional SNPs encompassing Siglec-8 and conducted genetic association studies of healthy controls and a population of African Americans selected for asthma. The findings were replicated in Brazilian families characterized for current asthma

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and Japanese asthmatic patients and healthy controls. To determine whether the genetic association was applicable to other allergic diseases, we tested for association between Siglec-8 SNPs and EE in the Caucasian population. The effect of the non-synonymous coding SNP rs10409962 on Siglec-8 glycan ligand-binding activity was also examined.

MATERIALS AND METHODS
Study population and phenotypes
African American population. GRAAD (Genomic Research on Asthma in the African Diaspora) comprises NIH-funded studies of asthma in pediatric and adult African American populations and one study of healthy African Americans.[19] A diagnosis of asthma was based on both a reported history of asthma and a documented history of physician-diagnosed asthma. For each of the asthma studies, a standardized questionnaire based on either the American Thoracic Society[20] or The International Study of Asthma & Allergy in Childhood (ISAAC) standards was administrated by a clinical coordinator. All controls were recruited separately and had no history of asthma. In the case–control design, 464 asthma cases (45.5% male, aged 23.8 ± 17.9 years) and 471 control subjects (41.4% male, aged 35.2 ± 16.5 years) were included in the final analysis. Detailed clinical characteristics of these subjects are presented in Table 1. Total serum IgE levels were measured using the UniCap 250 system (Pharmacia and Upjohn, Kalamazoo, MI, USA) at the Johns Hopkins DACI Laboratory (www.hopkinsmedicine.org/allergy/daci).[21]

Brazilian population. A total of 822 individuals among 356 nuclear families (44.2% male, mean age 27 years) from five communities in the Conde District of Bahia, Brazil, were enrolled in the study (Table 1). Detailed information on these subjects has been described previously.[22] The ISAAC questionnaire was administered to determine the prevalence and risk factors associated with asthma and allergic diseases. According to international ISAAC standards, current asthma was defined as a combination of wheezing in the past 12 months and lifetime asthma. Stool samples of the subjects were examined by the Kato-Katz method[23] for the presence and parasite load of *Ascaris lumbricoides*, *Trichuris trichiura*, and Hookworm eggs. Total serum levels of IgE were measured by chemiluminescence (ADVIA Center Bayer Corporation, Tarrytown, NY, USA). Detailed demographic and phenotype summary data of these individuals are presented in Table 1.

Japanese population. A total of 468 subjects with asthma (male, 47.0%, aged 45.9 ± 16.8 years) and 457 healthy control subjects (male, 64.6%, aged 38.8 ± 13.6 years) were recruited from the pulmonary clinic at the First Department of Medicine, Hokkaido University Hospital, as described previously.[24] The criteria for asthma in the Japanese population are presented in the Online Supplementary Information, and detailed clinical characteristics of these subjects are presented in Table 1.

Caucasians with EE. EE patients were recruited through the Cincinnati Center for Eosinophilic Disorders. EE was defined as 24 eosinophils/h.p.f. in at least one high-power field (×400) of an esophageal biopsy as previously described.[25] As shown in Table 1, a total of 298 Caucasians were included for the final analyses, including 166 EE patients from the proband of each family and 132 race/ethnicity-matched unrelated healthy individuals. DNA was isolated from saliva, peripheral blood leukocytes, or buccal swab samples.

The study protocols were approved by the Institutional Review Boards at the Johns Hopkins University School of Medicine, Cincinnati Children's Hospital Medical Center, the Federal University of Bahia, Brazil, and by the Ethics Committee, Hokkaido University, Japan. Informed consent was obtained from each of the study subjects.

Genotyping and quality control
A total of 14 SNPs were genotyped in the African American subjects from GRAAD. These SNPs included seven SNPs in the Illumina HumanHap650Y panel, additional tagging SNPs for the African American population encompassing Siglec-8, and two coding SNPs (rs3829659 Arg388Gly and rs10409962 Ser170Pro). Details for each SNP and minor-allele frequencies (MAFs) are presented in Supplementary Table S1, and the strategy for SNP selection is described in the Online Supplementary Information. Seven SNPs included in Illumina HumanHap650Y version 1 and 3 BeadChips (rs5409541, rs10518263, rs39711, rs10408249, rs36496, rs36498, rs11672925) were genotyped using the Illumina's BeadArray at the Center for Inherited Disease Research (CIDR) as described previously.[19] The rest of the SNPs were genotyped using custom-designed primers and probes for the TaqMan Allelic Discrimination assays using the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).[26]

Six SNPs, including four SNPs in the regulatory regions (rs6509541, rs39711, rs10420357, rs36498) and two (rs3829659 Arg388Gly and rs10409962 Ser170-Pro) in the coding regions of the Siglec-8 gene, were genotyped among 356 nuclear Brazilian families using TaqMan assays. Additionally, findings from both African American case–control and Brazilian family-based studies (rs36498 and rs10409962), together with the additional coding SNP rs3829659, were replicated in the Japanese population. Among them, the SNPs rs10409962 and rs3829659 were genotyped using TaqMan assay, and SNP rs36498 was genotyped using an assay combining kinetic PCR with allele-specific amplification, as described previously.[27] Additionally, eight SNPs, including those associated with asthma, coding SNPs, and tagging SNPs across the Siglec-8 gene for the Caucasians from the International HapMap project, were genotyped in a total of 298 Caucasians consisting of 166 EE patients and 132 controls using TaqMan assays. Quality control for genotyping was described previously.[19,28] Briefly, quality-control measures included a genotyping call rate of >95%, with no discordances on repeat genotyping of

Table 1 Clinical characteristics of the study populations

| Panel A | African American | | | Japanese | | |
	Total	Asthma	Controls	Total	Asthma	Controls
Subjects (n)	935	464	471	925	468	457
Males, n (%)	406 (43.4%)	211 (45.5%)	195 (41.4%)	515 (55.7%)	220 (47.0%)	295 (64.6%)
Age; mean (SD)	29.55 (18.10)	23.78 (17.85)	35.23 (16.51)	42.4 (15.7)	45.9 (16.8)	38.8 (13.6)
Total IgE; mean[a] (95% CI)	213.7 (191.5–238.4)	315.6 (270.4–368.3)	143.3 (123.8–165.8)	132.6 (119.6–147.0)	242.7 (211.0–279.1)	71.8 (63.1–81.7)
Atopy, n (%)	641 (75.2%)	369 (85.2%)	272 (64.9%)	587 (63.5%)	339 (72.4)	248 (54.3)

| Panel B | Brazilian families | | | Caucasian | | |
	Total	Wheeze	Control	Total	EE	Control
Subjects (n)	822	244	550	298	166	132
Males (%)	363 (44.2%)	114 (46.7%)	233 (42.4%)	166 (55.7%)	102 (61.4%)	64 (48.5%)
Age; mean (SD)	26.9 (18.75)	26.6 (18.87)	27.0 (18.70)	—	—	—
Total IgE; mean[a] (95% CI)	2505.6 (2296.1–2734.3)	2631.2 (2292.9–3019.5)	2450.9 (2194.3–2737.4)	—	—	—

[a]Geometric mean serum levels of total IgE.

a random 5% of the sample, no significant departures from Hardy–Weinberg Equilibrium within each study group (exact P-value > 0.01), MAF > 5%, and Mendelian errors in 5% or more of the marker. Population admixture among African Americans was evaluated by genotyping 416 SNPs identified as ancestry-informative markers (AIMs) as described previously.[19]

Plasmid constructs

The wild-type (WT) 246(pFlag–Neo–Siglec-8L) construct containing full-length Siglec-8 was provided by Dr Oksoon H Choi (Johns Hopkins Asthma & Allergy Center, Baltimore, MD, USA). The plasmid construct containing the SNP rs10409962-mutant allele was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), by making a point mutation at 1160 from T to C in the double-stranded DNA from 246(pFlag–Neo–Siglec-8L) (Figure 2a). The mutated plasmid was confirmed by direct sequencing using an ABI 3700 DNA Analyzer.

Generation of stable Siglec-8 transfectants and Siglec-8 ligand binding assays

HEK293 human embryonic kidney cells were cultured in MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (High Clone Laboratories, Logan, UT, USA), and 100 U/ml penicillin-G. The plasmid DNA (1 µg) coding for WT or rs10409962-mutant Siglec-8 was transfected into HEK293 cells using the FuGENE-6 reagent (Roche, Indianapolis, IN, USA). The cells were harvested 24 h after transfection. Siglec-8 mAb 2C4 (mouse IgG1)[2] was used to detect cell-surface expression and a 2000-kDa polyacrylamide biotinylated polymer decorated with 6′-sulfo-sLe[x] (provided by Dr N Bovin, Russian Academy of Sciences, Moscow, Russia) was used in flow cytometric ligand binding assays as previously described.[29] Briefly, cultured cells were washed once in PBS and incubated with either mAb 2C4 or an IgG1 isotype control antibody (2.5 µg/ml, 30 min, 4 °C), washed, and then incubated with a fluorochrome-conjugated anti-mouse IgG F(ab′)2 secondary antibody (Invitrogen, CA, USA). Alternatively, cells were incubated with saturating concentrations (10 µg/ml) of biotinylated a 2000-kDa 6′-sulfo-sLe[x]-containing polyacrylamide polymer or a control 2000-kDa LacNac polymer,[29] washed, and then incubated with saturating concentrations of FITC–strepavidin (R&D Systems, Minneapolis, MN, USA). The cells were then analyzed by flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA).

Statistical analysis

The Cochran–Armitage trend test was used to examine associations between each individual marker (under an additive model) and disease status among African Americans and Caucasians using the PLINK software (http://pngu.mgh.harford.edu/~purcell/plink/to).[30] We tested for associations between SNPs and total serum levels of log-transformed IgE by a linear regression analysis under an additive model adjusted for confounding variables (age and gender). Departures from Hardy–Weinberg equilibrium at each locus were tested by χ^2-test separately for cases and controls, and for founders among Brazilian families. Haplotype analyses were performed with PLINK, using sliding windows of 2–5 SNPs, where empiric P-values for haplotype frequency differences were generated over 10 000 permutations. For the Brazilian family-based analyses, Mendelian inconsistencies were identified with Sib-Pair (version 1.00b). Association between markers in Siglec-8 and wheezing, log[tIgE], and log[Schistosoma mansoni egg count] were tested by using FBAT (version 1.5.5).[31] All analyses with FBAT were first performed under an additive and then by a recessive model. Statistical analysis of the Japanese population used logistic regression analysis to estimate the odds ratio (OR) for asthma using an additive model adjusted and un-adjusted for gender, age, and smoking status using SYSTAT (version 10.2; Systat Inc., Chicago, IL, USA).[24] Statistical power for the case–control study was estimated using QUANTO (version 1.2)125.[32]

RESULTS

Clinical features, SNP information, and the LD structure

Clinical characteristics of the study subjects are presented in Table 1. The African American population is the primary focus and study population; both Brazilian and Japanese populations were used for replication of any significant findings in the primary data analysis. The chromosomal location and MAFs for the 14 Siglec-8 SNPs spanning a 16-kb region on chromosome 19q13.33–q13.41 for healthy controls from our study populations (African American, Brazilian, Japanese, Caucasian) and from the International HapMap project (http://www.hapmap.org/) are presented in Supplementary Table S1. The genotype frequencies for all SNPs agreed with expectations under Hardy–Weinberg equilibrium. Power calculation for each population is presented in Supplementary Figure S1.

Association between Siglec-8 variants and asthma and total serum levels of IgE (tIgE)

We observed two linkage disequilibrium (LD) blocks, comprising five markers in block-1 (rs36485, rs36487, rs36489, rs10518263, rs39711) and two markers in block-2 (rs3829659 and rs10408249), for Siglec-8 among African Americans following the definition of Gabriel et al.[33] (Supplementary Figure S2a). In primary analysis, we observed an association for the promoter SNP rs36498 (−4354C/T), where the T allele was significantly associated with reduced risk of asthma (OR, 0.69, 95% CI, 0.57–0.83, P=8.8×10^{−5}; Table 2). A similar trend toward reduced risk of asthma was also seen for a coding SNP

Table 2 Allelic association of Siglec-8 SNPs with asthma in both African American and Japanese (n=957) populations

| SNP | Minor allele | African American (n=935) | | | | Japanese (n=957) | | | |
		Asthma	Control	OR (95% CI)	P	Asthma	Control	OR (95% CI)	P[a]
rS11672925	C	0.220	0.194	1.17 (0.92–1.48)	0.195				
rs36498	T	0.406	0.499	0.69 (0.57–0.83)	**8.8×10⁻⁵**	0.340	0.330	1.01 (0.83–1.24)	0.899
rs10420357	T	0.269	0.250	1.10 (0.87–1.40)	0.414				
rs36496	C	0.048	0.043	1.13 (0.71–1.81)	0.594				
rs10409962	G (Ser/Pro)	0.446	0.497	0.82 (0.65–1.02)	**0.070**	0.100	0.132	0.69 (0.50–0.94)	**0.019**
rs36495	A	0.376	0.361	1.04 (0.85–1.27)	0.549				
rs10408249	C	0.482	0.469	1.05 (0.87–1.27)	0.571				
rs3829659	G (Arg/Gln)	0.484	0.457	1.12 (0.89–1.40)	0.321	0.327	0.360	0.93 (0.75–1.15)	0.515
rs39711	T	0.478	0.448	1.13 (0.93–1.36)	0.216				
rs10518263	T	0.100	0.116	0.84 (0.62–1.15)	0.273				
rs36489	G	0.151	0.167	0.89 (0.67–1.19)	0.410				
rs36487	A	0.135	0.149	0.89 (0.66–1.19)	0.423				
rs36485	T	0.325	0.292	1.17 (0.93–1.47)	0.171				
rs6509541	T	0.499	0.498	1.01 (0.83–1.22)	0.926				

[a]Adjusted for age, sex, smoking status, and atopic status. Bold P-values are statistically significant.
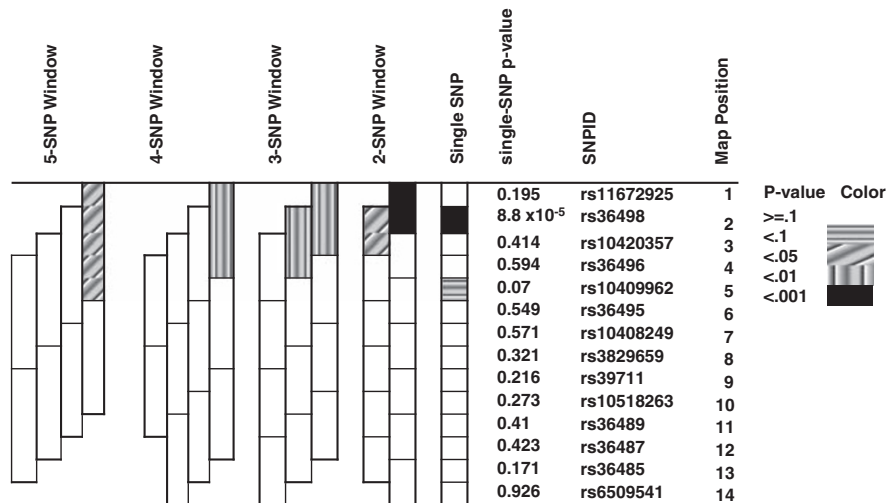
Figure 1 Summary of sliding-window analysis of Siglec-8 haplotypes and asthma in the African American population. Haplotype results show global *P*-values constructed across sliding windows of sizes 2–5 for 14 SNPs and asthma. The color key for *P*-values is also indicated.

Table 3 Association of the two-SNP haplotype (rs36498 and rs11672925) in the promoter region of Siglec-8 and asthma among African Americans

| rs36498 | rs11672925 | Minor allele frequencies | | OR (95% CI) | P-value |
		Asthma	Control		
T	C	0.105	0.097	1.09 (0.70–1.71)	0.576
C	C	0.112	0.094	1.22 (0.78–1.92)	0.191
T	A	0.304	0.401	0.65 (0.49–0.86)	**1.42×10⁻⁵**
C	A	0.479	0.408	1.33 (1.02–1.74)	**0.002**

Global *P*-value=0.00026. Bold *P*-values are statistically significant.

Table 4 Association of Siglec-8 haplotypes with total serum IgE among the African Americans (*n*=935)

Single-SNP	rs6509541	rs36485	rs36487	rs36489	rs10518263		
Risk allele	T	C	C	A	G		
P-value	0.016	0.961	0.896	0.935	0.476	β	P
Two-SNP	T	C				−0.07	0.122
Three-SNP	T	C	C			−0.271	**0.004**
Four-SNP	T	C	C	A		−0.275	**0.005**
Five-SNP	T	C	C	A	G	−0.254	**0.01**

β, regression coefficient. Bold *P*-values are statistically significant.

rs10409962 on exon-2, which causes a Ser–Pro substitution at amino acid 170 (S170P) in domain-2 (OR, 0.82, 95% CI, 0.65-1.02, *P*=0.072). Sliding-window haplotype analysis across all 14 SNPs revealed significant associations for the 2–5 SNP haplotypes and risk of asthma (global *P*-values, 0.008–0.00026) (Figure 1), all of which included SNP rs36498, with the strongest association for a two-SNP haplotype (TA) comprising two promoter SNPs of Siglec-8 (rs36498 and rs11672925) (asthma versus control, 30.4% versus 40.1%; OR, 0.65; *P*=1.42×10⁻⁵; global *P*-value=0.00026) (Table 3), suggesting enhanced association for the haplotype analysis in the promoter region of Siglec-8.

To determine whether Siglec-8 SNPs are associated with tIgE, we performed linear regression analysis using additive models adjusted for confounding variables (age and gender). Among all tested SNPs, SNP rs6509541 in the 3′-untranslated region (3′-UTR) showed significant association with tIgE (*P*=0.016) (Table 4). This association was further enhanced by haplotype analysis: multiple haplotypes of 2–5 SNPs were significantly associated with tIgE (*P*-values, 0.048–0.004) in the African American population, all of which included SNP rs6509541, with the strongest association for a three-SNP haplotype (TCC) consisting of SNPs in the 3′-UTR and downstream from Siglec-8 (rs6509541, rs36485, rs36487; *P*=0.004).

Replication of the primary findings in both Brazilian and Japanese populations
To further confirm these findings, we selected both rs36498 and rs10409962 SNPs that were associated with asthma among the African American subjects and five additional SNPs in the regulatory regions of Siglec-8 with even distribution and performed a family-based association study of 356 Brazilian nuclear families.[22] Detailed location and allele frequencies for all the genotyped SNPs among the Brazilian families are presented in the Supplementary Table S1. Of interest, significant evidence of linkage to current asthma was provided for rs36498 (*P*=0.013) and suggestive evidence was found for the marker rs10409962 (*P*=0.085) under the recessive model (Supplementary Table S2). In addition, association with tIgE was also observed for SNP rs6509541 among the Brazilian families (*P*=0.02, data not shown), which was the same SNP downstream from Siglec-8 that was associated with tIgE in the African Americans. Haplotype analyses did not provide any further evidence for this association for either current asthma or tIgE. Given the association of rs36498 and rs10409962 with asthma in both African American and Brazilian subjects, we further replicated two associated SNPs rs36498 and rs10409962 and one additional coding SNP rs3829659 in the Japanese population comprising 468 asthmatics and 457 healthy control subjects. The association with reduced risk of asthma was replicated for rs10409962 (OR, 0.73; 95% CI, 0.54–0.98; *P*=0.032) but not for SNP rs36498 (OR, 1.04; 95% CI, 0.86–1.27; *P*=0.67) and rs3829659 (OR, 0.86; 95% CI, 0.71–0.98; *P*=0.088) (data not shown). As there
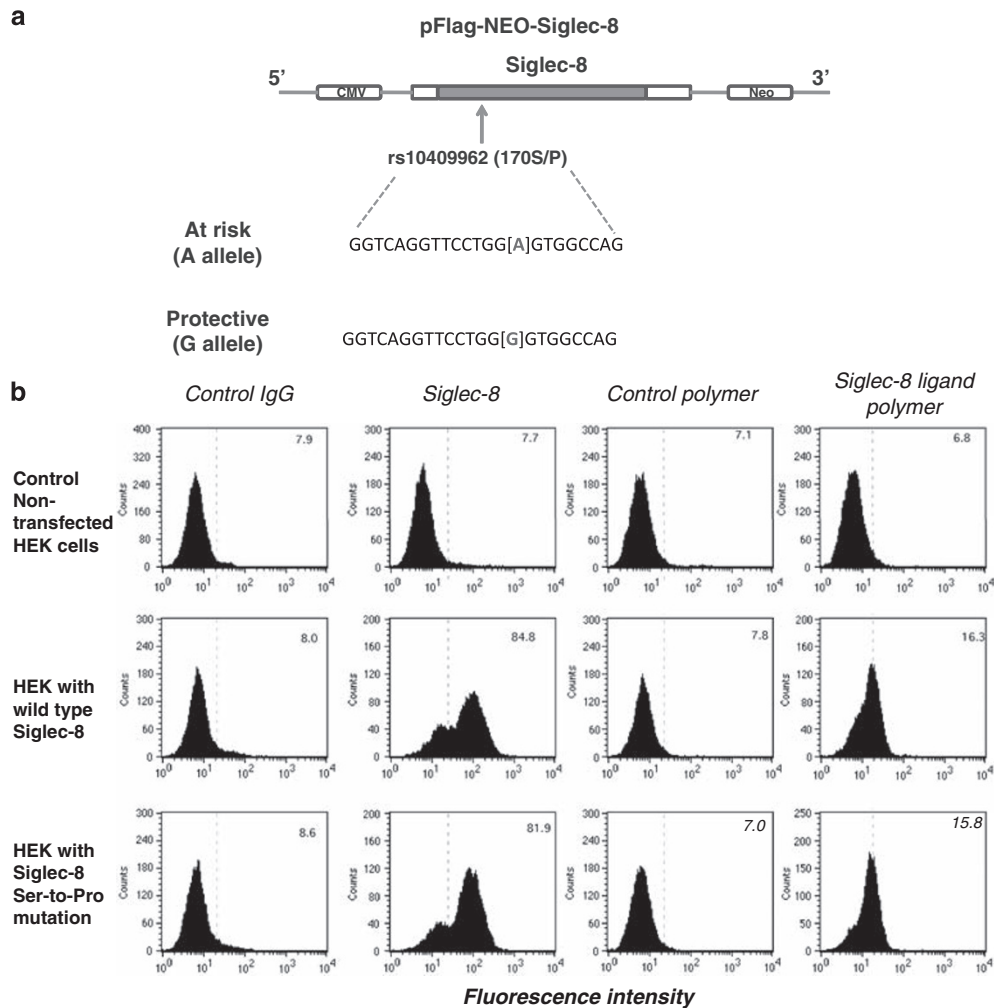
Figure 2 The pFlag–Neo–Siglec-8 plasmid construct and Siglec-8 expression on Siglec-8-transfected HEK293 cells. (**a**) The pFlag–Neo–Siglec-8 plasmid constructs contains either the SNP rs10409962 risk (A allele) or protective allele (G allele). (**b**) Detection of Siglec-8 expression on Siglec-8-transfected HEK293 cells by means of flow cytometry. Cells were analyzed for specific staining with isotype (mouse IgG), Siglec-8 antibody (2C4), and Siglec-8 ligand (6′-sulfo-sLex polymer) in HEK293 cells non-transfected (top), transfected with Siglec-8 WT (middle), and transfected with the Siglec-8 Ser-to-Pro mutation (bottom). The data represent one of three experiments. Values in the upper right corner of each histogram represent percent positive.

were significant differences in male/female, age distribution, atopy, and smoking status between the Japanese cases and controls ($P < 0.001$ for all comparisons),[24] further analysis was performed by adjusting for age, sex, smoking, and atopic status, and we found that the association for SNP rs10409962 still remained significant (OR, 0.69; 95% CI, 0.50–0.94; $P = 0.019$; Table 2). None of the Siglec-8 SNPs were associated with tIgE in the Japanese population (data not shown).

No association between Siglec-8 SNPs and EE
Given the biological relationship between Siglec-8 and eosinophil apoptosis, we tested for association between eight Siglec-8 SNPs and EE, a disorder frequently associated with atopy and characterized by aberrant and localized accumulation of eosinophils in the esophagus.[34] These SNPs, including those associated with asthma, were genotyped using TaqMan assays. We observed two LD blocks comprising six SNPs in block-1 (rs36485, rs36487, rs36489, rs39711, rs3829659, rs36495) and two SNPs in block-2 (rs10409962 and rs10420357) (Supplementary Figure S2B). Genotype frequencies for all SNPs were in Hardy–Weinberg equilibrium in both EE patients and control individuals without EE. However, none of the SNPs were significantly associated with EE, even after adjusting for gender (Supplementary Table S3).

HEK293 cells carrying a Ser–Pro substitution in exon-2 at position 170 of Siglec-8 exhibit normal ligand-binding activity
The coding SNP rs10409962 is located not far from the putative carbohydrate-binding domain of Siglec-8. To investigate whether SNP rs10409962 can lead to a functional change in Siglec-8, we examined the glycan ligand-binding activity of Siglec-8. HEK cells were stably transfected with either WT or the mutant allele, and selected for clones with surface levels of Siglec-8 similar to that on eosinophils (Figure 2b). When tested for their ability to bind the 2000-kDa 6′-sulfo-sLe[x]-containing polyacrylamide polymer ligand, there was no difference in ligand-binding activity between cells transfected with SNP rs10409962 risk allele and those transfected with the WT allele (84.8 versus 81.9% positive), suggesting that this point mutation does not significantly alter the ligand-binding activity of Siglec-8.

DISCUSSION
Siglec-8 is expressed exclusively on human eosinophils, mast cells, and basophils.[2] It has been demonstrated that Siglec-8 engagement induces eosinophil apoptosis *in vitro* by triggering the 'intrinsic' stress-mediated apoptotic pathway through sequential ROS production, mitochondrial dysfunction, and caspase cleavage.[3] Of interest, murine

studies targeting Siglec-F, the closest functional paralog to Siglec-8, also demonstrated a significant role in regulating the pathogenesis of eosinophil-mediated disorders.[8–10] As eosinophils and their many biologically active mediators are associated with allergic diseases and other chronic inflammatory disorders, this suggests that Siglec-8 could be a candidate gene for human eosinophilic disorders like asthma and EE. In this study, we tested the hypotheses that genetic polymorphisms in Siglec-8 are associated with asthma and EE, and that mutations in the glycan-binding extracellular domain of Siglec-8 could disrupt the affinity of Siglec-8 for its ligand, which might then lead to loss of ligand-induced apoptosis in vivo, resulting in exaggerated eosinophilic inflammation.

We took advantage of genome-wide association studies (GWAS) originally performed to identify susceptibility genes for asthma in a total of 935 African Americans.[19] Seven Siglec-8 SNPs were included in the GWAS final data analysis after all the filtering processes were performed for quality control. In parallel with this analysis, an additional seven SNPs were genotyped. Of these, two common non-synonymous variants (rs3829659 Arg388Gly and rs10409962 Ser170-Pro) were located along the functional regions of the Siglec-8 gene in the glycan-binding extracellular domain (Ser170Pro) and the cytoplasmic negative signaling domain (Arg388Gly). Among all the SNPs genotyped, the strongest evidence for association with asthma was observed for the promoter SNP rs36498 (-4354C/T), and suggestive evidence for the non-synonymous coding SNP rs10409962 (Ser170Pro) on exon-2, where both mutant alleles were associated with reduced risk of asthma. When replicating these findings in two additional independent populations, similar associations were found, namely SNP rs36498 was significantly, and rs10409962 was suggestively, associated with current asthma as documented by a combination of wheezing in the past 12 months and lifetime asthma among Brazilian families. In contrast, SNP rs10409962, but not rs36498, was significantly associated with asthma in the Japanese case–control analysis. These findings suggest that genetic variants in the Siglec-8 gene may be associated with asthma. Although we did not observe SNP-for-SNP replication of findings in these populations, this was not unexpected. As highlighted in Supplementary Table S1, the allele frequencies of all the SNPs genotyped differed across ethnic groups, suggesting there was considerable genetic heterogeneity among these populations. It is possible that variants other than those tested here are causal and directly alter Siglec-8's function, and the observed statistical associations in this study are merely due to strong LD between markers and these unobserved causal variants. Heterogeneity of the phenotype is another commonly cited explanation for failure to replicate positive associations across independent populations. However, it is important to note that SNPs in each population showed associations with asthma, providing a convincing basis for further investigation of the role of Siglec-8 in the pathogenesis of asthma. In addition, although SNP rs10409962 failed to pass the correction for Bonferroni Multiple testing, the most stringent test of all, the associated SNP, rs10409962, is located two amino acids away from a putative carbohydrate-binding domain. It is still possible that this SNP results in aberrations in Siglec-8 structure and ligand binding, and subsequently leads to alterations in Siglec-8's function. As a result, we investigated the effect of this coding SNP on Siglec-8 ligand-binding activity using a synthetic polymer decorated with 6′-sulfo-sLe[x] when tested on HEK293 cells stably transfected with the rs10409962 risk allele or the WT allele. We found no difference in binding to 6′-sulfo-sLe[x] ligand-containing polymers between transfectants with or without the risk allele, suggesting that this point mutation does not alter its ligand-binding activity. However, it is possible that the interaction between Siglec-8

and its natural ligand is different and not adequately reflected by the artificial polymer ligand. Alternatively, the assay used for testing interactions might not be sensitive enough to detect the potential differences between Siglec-8 molecules with 170S versus 170P. However, this is the best way to currently explore this issue because the natural Siglec-8 ligand remains to be determined. Another possibility is that it could alter the levels of Siglec-8 surface expression on eosinophils. Further studies are clearly needed to elucidate the mechanisms underlying the role of rs10409962 in the pathogenesis of asthma.

We recognized that, after Bonferroni Multiple testing correction, the SNP rs36498 was the only SNP significantly associated with asthma in the African American case–control group (adjusted $P=6.29\times10^{-4}$). Bonferroni correction offers the most conservative approach to control for false positives, and we therefore believe that the association with asthma for SNP rs36498 is more likely to be true among African Americans. Interestingly, the haplotype centered on rs36948 showed enhanced association with asthma and in particular, a haplotype (TA) carrying two promoter SNPs of Siglec-8 (rs36498 and rs11672925) demonstrated increased association when compared with either SNP individually. These findings suggest that SNP rs36498 and others in the promoter region of Siglec-8 may be critical in conferring susceptibility to asthma. Hence, more SNPs with comprehensive coverage within the promoter region of Siglec-8 are clearly needed to be genotyped, with the aim of identifying the variant(s) influencing transcriptional regulation. Also, we observed a significant association for SNP rs36498 and haplotypes carrying rs36948 among the Brazilian families, but the global P-value from the strongest haplotype is not as impressive as the single SNP rs36498. It can be speculated that the LD-genotyped SNPs are not great in coverage (2.6 kb/SNP) and the signal flagged by the single promoter SNP is washed out by the SNPs surrounding it. An additional association was observed for SNP rs6509541 as an individual or in combination with other SNPs in the 3′-UTR and downstream from Siglec-8 and tIgE in the African American population, and replicated in the Brazilian population. This finding suggests a role for SNP rs6509541 via Siglec-8 in allergic diseases because tIgE is a useful marker for assessing allergic diseases.[35]

Given that Siglec-8 is a key molecule involved in facilitating eosinophil apoptosis and that mutations in Siglec-8 may also be involved in other allergic and eosinophilic disorders, we investigated whether Siglec-8 SNPs are associated with other human eosinophilic disorders like EE. We selected eight SNPs encompassing the Siglec-8 gene with a coverage of 2 kb/SNP, including those associated with asthma. However, none of the SNPs were significantly associated with EE among the Caucasian population. Of these, four SNPs (rs10406692, rs3829659, rs39711, rs10420357) were further replicated among 68 EE families, but no significant association was observed (data not shown). The results suggest that Siglec-8 does not associate with susceptibility to EE at least in Caucasians. However, further studies may be needed to get a comprehensive coverage of genetic variants in and around the Siglec-8 gene in order to fully exclude Siglec-8 as a candidate gene for EE.

In summary, we tested for associations between Siglec-8 SNPs and asthma, tIgE, and EE in diverse populations. We are able, for the first time, to provide evidence of association between Siglec-8 SNPs and asthma and tIgE in different racial populations. The strongest association was seen for SNP rs36498 in the promoter region of Siglec-8, even after correction for multiple tests, suggesting that the Siglec-8 gene may be associated with susceptibility to asthma. The finding that Siglec-8 SNPs were not associated with EE suggests that the associa-

tion is not simply due to atopy, which is found in similar percentages in these otherwise unrelated disorders. Further studies are needed to identify additional genetic variants, with a particular focus on the promoter region of the Siglec-8 gene. As interaction assay failed to show any changes in ligand-binding activity on HEK293 cells stably transfected with the rs10409962 risk allele or the WT allele, additional studies are needed to determine exactly how this SNP might affect the biology of Siglec-8 and contribute to the pathophysiology of asthma.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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