CTLA-4 polymorphisms in allergy and asthma and the T_H1/T_H2 paradigm

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Background: Several genomic regions are reported to be associated with the development of asthma and allergy, including chromosome 2q33. This region harbors the candidate gene cytotoxic T-lymphocyte antigen 4 *(CTLA-4)*, an important regulator of T-cell activation and differentiation.
Objective: We sought to explore possible associations between CTLA-4 polymorphisms and allergy and asthma.
Methods: Seven single nucleotide polymorphisms (SNPs; MH30, −1147CT, +49AG, CT60, JO31, JO30, JO27_1) in *CTLA-4* were analyzed for associations with total serum IgE, allergic sensitization (positive skin prick test to common allergens), bronchial hyperresponsiveness (BHR) to methacholine, asthma, and lung function (FEV_1% of predicted) in 364 asthmatic families from 3 European countries.
Results: Transmission disequilibrium test analysis showed that several SNPs were significantly associated with serum IgE levels, allergy, asthma, and FEV_1% predicted below 80%, but not with BHR, and *CTLA-4* polymorphisms of potentially direct pathogenic significance in atopic disorders were identified.
Conclusion: We identified associations between 4 newly discovered SNPs in the *CTLA-4* gene and serum IgE levels, allergy, asthma, and reduced lung function, but not BHR, suggesting an important role for *CTLA-4* in atopy and reduced lung function in asthmatic subjects rather than asthma per se. The particular SNP alleles found positively associated with our phenotypes were recently shown to be associated negatively with autoimmune disorders. Although a skewing toward a T_H1 reactivity pattern is believed to characterize autoimmune diseases, atopic diseases are considered T_H2-mediated. Hence, our data suggest a role for *CTLA-4* polymorphisms in determining the T_H1/T_H2 balance and identify CTLA-4 signaling as a potential therapeutic target in atopic disease.
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Key words: *Asthma, allergy, atopy, IgE, bronchial hyperresponsiveness, FEV_1, genetic analysis, CTLA-4 polymorphisms, association study*

Allergy and asthma are complex common conditions caused by combinations of environmental and genetic factors. Although allergy, asthma, and atopy (predisposition for producing IgE[1]) frequently coexist and are commonly characterized as T_H2-mediated conditions, there are important differences in clinical disease expression, reflecting different immunologic and genetic mechanisms.[2]

The search for genetic contributions to complex conditions such as allergy and asthma is hampered by incomplete penetrance, genetic heterogeneity, and varying environment-gene interactions. An increasing number of chromosomal regions have shown evidence of linkage to atopic traits. One such region is on chromosome 2q32-33, harboring the candidate gene cytotoxic T-lymphocyte antigen 4 (*CTLA-4*).[3,4] Genetic associations among IgE levels, atopic traits, and polymorphisms in the *CTLA-4* gene have been studied, but whereas some show associations with asthma and bronchial hyperresponsiveness (BHR),[5,6] others show only associations with IgE levels,[7,8] and some do not show associations at all.[9]

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Drs Carlsen and Undlien contributed equally to the planning of the study.

The GAIN study was designed by principal investigators in all study sites. For the current study, the participating centers were Oslo, Groningen, Sheffield, and Aberdeen. Drs Munthe-Kaas, K. H. Carlsen, K. C. Lødrup Carlsen, and Undlien designed the study and wrote the report. Dr Munthe-Kaas performed the main genetics analyses and subsequently related the results to the clinical phenotypes. Dr K. C. Lødrup Carlsen and Drs K. H. Carlsen, Gerritsen, Whyte, and Helms were the principal clinical investigators in Oslo, Groningen, Sheffield, and Aberdeen, and clinical data were also collected also by Drs Feijen (Groningen) and Kwong (Sheffield). Dr Munthe-Kaas and Ms Skinningsrud performed the genotyping, and Ms Main organized the Aberdeen genetic samples. Drs Munthe-Kaas, Lie, Undlien, and K. C. Lødrup Carlsen were involved in the statistical analysis. All authors contributed to the discussion of the study and to the article. The submitted article was approved by all authors.

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Abbreviations used
BHR: Bronchial hyperresponsiveness
CTLA-4: Cytotoxic T-lymphocyte antigen 4
flCTLA-4: Full-length cytotoxic T-lymphocyte antigen 4
GAIN: Genetics of Asthma International Network
sCTLA-4: Soluble cytotoxic T-lymphocyte antigen 4
SNP: Single nucleotide polymorphism
SPT: Skin prick test
TDT: Transmission disequilibrium test

The CTLA-4 molecule is a member of the immunoglobulin superfamily and a surface molecule on activated T cells. It has a central role as a negative costimulator in T-cell regulation, and together with the surface molecule CD28 modifies both activation and differentiation of T cells when stimulated by antigen presenting cells. Although CD28 costimulation promotes T_H2 activation and differentiation, CTLA-4 is believed to weaken this positive costimulation by competing with CD28 for extra cellular ligands (B7) on the antigen presenting cell by interfering with the intracellular signaling of CD28 (they have similar cytoplasmic domains), and finally directly by hampering the T-cell receptor/CD28 complex during an immune response.[10-14] As such, *CTLA-4* is a biologically plausible candidate gene for immune-mediated diseases, and its importance has been demonstrated *in vivo* by the fatal lymphoproliferation of CTLA-4−deficient mice.[15] Significant associations have been demonstrated between *CTLA-4* and autoimmune diseases such as type 1 diabetes, autoimmune hypothyroidism, and Graves disease.[16] Ueda et al[17] recently resequenced 330 kb of chromosome 2q33, including the putative candidate genes *CD28, ICOS*, and *CTLA-4,* to identify all common polymorphisms. From an original 108 single nucleotide polymorphisms (SNPs), the strongest associations with clinical disease (type 1 diabetes, autoimmune hypothyroidism, and Graves disease) were to the newly detected polymorphisms designated MH30, CT60, JO31, JO30, and JO27_1. These are located in the *CTLA-4* gene, suggesting that this region harbors the primary determinants of disease susceptibility (Fig 1).

A meta-analysis of the clinical association between childhood type 1 diabetes and atopic diseases has recently showed an inverse relationship between atopic traits and type 1 diabetes.[18] This is explained in part by the T_H1/T_H2 balance, in which type 1 diabetes represents a T_H1-mediated response and atopic diseases a T_H2-mediated response. As a regulator of T-cell differentiation, *CTLA-4* is a plausible candidate for providing a genetic explanation for this observation.

The main aim of the current study was to explore possible associations among recently identified *CTLA-4* polymorphisms[17] and serum IgE levels, allergy, and asthma. A secondary aim was to explore the hypothesis that *CTLA-4* polymorphisms can provide a genetic explanation for the apparent negative association between T_H1-mediated diseases and atopic diseases.

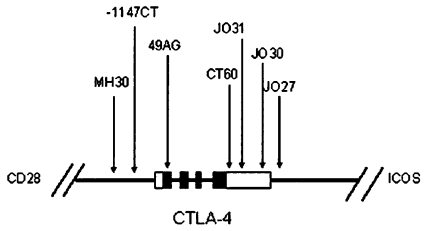

FIG 1. Approximate map location of the SNPs in the current study in relation to the *CTLA-4* gene. *Boxes* indicate the locations of exons, and the *black shaded areas* indicate the translated regions.

METHODS

Study design

Families were recruited at 4 European centers as part of a multinational genetics study of families with asthma, the Genetics of Asthma International Network (GAIN). Each site recruited approximately 100 families, identified through a sibling pair with asthma, who were subject to identical investigations. Entry criteria for families were a minimum of 2 siblings (age 7-35 years) with physician-diagnosed asthma, bronchial obstruction, and/or use of β_2-agonists during the last year for the proband and asthma symptoms after 6 years of age for the sibling, and both biological parents available for participation. Additional siblings (affected and unaffected) were included when available. Families were excluded if the proband or parents had nonobstructive serious chronic pulmonary disease, or if any parent or sibling had disease (including myocardial infarction or heart failure within the last 1 or 3 years, respectively) or treatment (β-blockers) that would contradict methacholine challenge.

All subjects underwent clinical investigation, structured interview, baseline spirometry, skin prick test (SPT), methacholine challenge test, and blood sampling for DNA extraction and serum analysis of IgE. Human subjects protocols were approved at each site by the local or regional Institutional Review Board or Medical Ethics Committee.

Subjects

Genomic DNA was obtained from 378 families from Oslo, Groningen, Sheffield, and Aberdeen. Fourteen families and 9 individuals were excluded because of technical difficulties or apparent misinheritances discovered during genetic analysis. Demographic and clinical data based on the 364 families included in the current study are given in Table I. None of the subjects had received long acting β_2-agonist, whereas 61% of the subjects received inhaled corticosteroids.

Outcomes and phenotyping

Phenotypic outcome measures were defined as total serum IgE, allergy (allergic sensitization to common inhalant allergens), allergic asthma, asthma, lung function (given by FEV_1 above or below 80% of predicted), and BHR.

Asthma was defined as a physician's diagnosis of asthma by the family physician or principal investigator (physician). Allergic asthma was defined as asthma in the presence of allergic sensitization.

Serum was analyzed for total IgE by using the UniCAP fluoroenzyme immunoassay according to the manufacturer's instructions (Pharmacia Upjohn, Uppsala, Sweden) and categorized for analyses as normal (equal to or below) or high (above) with a cutoff level of 119 kU/L (upper reference value for adults given by the manufacturer).

Allergy (allergic sensitization) was defined as the presence of at least 1 positive SPT. SPT was performed according to European guidelines, with a positive SPT defined as mean wheal diameter of at

TABLE I. Demographics of subjects in the TDT analysis*

	Oslo	Groningen	Sheffield	Aberdeen
Family numbers	102	75	95	92
Probands				
Age (y) ± SD	14.6 ± 6.1	13.2 ± 4.9	13.7 ± 5.3	14.6 ± 4.3
Sex, % male	57%	57%	58%	60%
Sensitized, %§	52%	77%	74%	90%
PC_{20} < 8 mg/mL, %‡	35%	44%	49%	70%
IgE > 119 kU/L, %†	49%	69%	58%	70%
Serum IgE geometric mean ± SD†	118.0 ± 5.2	235.3 ± 4.9	186.9 ± 5.6	232.1 ± 5.2
FEV_1% < 80%§	5%	7%	17%	21%
Siblings				
No. = 1	100	75	93	87
No. = 2	26	33	28	22
No. >2	1	20	6	5
Age (y) ± SD	13.6 ± 5.7	14.0 ± 5.6	14.1 ± 5.6	15.4 ± 4.84
Sex, % male	53%	52%	62%	56%
Sensitized, %§	51%	68%	58%	76%
Asthma, %§	91%	66%	84%	86%
Allergic asthma, %‡	43%	55%	55%	70%
PC_{20} < 8 mg/mL, %§	29%	38%	44%	72%
IgE > 119 kU/L, %	45%	56%	46%	59%
Serum IgE geometric mean ± SD	93.5 ± 4.4	163.2 ± 4.8	112.0 ± 6.1	128.4 ± 5.8
FEV_1% < 80%	5%	9%	11%	9%
Parents				
Age (y) ± SD	43.4 ± 6.5	43.7 ± 6.6	43.0 ± 7.0	43.7 ± 5.5
Sensitized, %	60%	47%	54%	53%
Asthma, %§	32%	23%	34%	26%
Allergic asthma, %	21%	19%	23%	21%
PC_{20} < 8 mg/mL, %§	12%	12%	26%	38%
IgE > 119 kU/L, %	25%	25%	31%	27%
Serum IgE geometric mean ± SD	48.0 ± 4.2	47.4 ± 4.2	58.4 ± 3.7	47.8 ± 4.6
FEV_1% < 80%§	7%	7%	23%	12%

*Continuous variables were analyzed by the 1-way ANOVA test. Dichotomous variables were analyzed by Pearson χ^2 test.
†$P < .05$ (significant difference between the populations).
‡$P < .01$.
§$P < .001$.

least 3 mm (larger than the negative control), read after 15 minutes.[19] Antihistamines were suspended for at least 72 hours, and systemic prednisolone doses exceeding 10 mg/day were suspended 24 hours before testing. The following standardized extracts from ALK (Copenhagen, Denmark) were used: histamine 10 mg/mL (positive control), saline (negative control), *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass mix, cat and dog dander, *Alternaria alternata*, *Cladosporium herbarum*, and German cockroach. In addition, the regional specific allergens silver birch and mugwort (Norway) and *Aspergillus fumigatus* and tree mixes (The Netherlands) were included.

Lung function was measured by spirometry according to American Thoracic Society standards[20-22] after ensuring that the following medication was not taken for the given time before investigation: short-acting bronchodilator for 8 hours, long-acting bronchodilator within 48 hours, theophylline or leukotriene receptor antagonist for 48 hours, long-acting antihistamine for 7 days, systemic steroids for a month, and antibiotics for respiratory infection within the preceding month.

Bronchial hyperresponsiveness was assessed with a methacholine challenge test, and the PC_{20} was identified. Individuals with PC_{20} value of ≥8.0 mg/mL were defined as exhibiting BHR,[23] and those that did not fall 20% by 8.0 mg/mL methacholine were defined as not having BHR. Groningen used methacholine-bromide concentrations ranging from 0.038 to 19.6 mg/mL instead of the 0.03 to 16.0 mg/mL used in the other centers. The concentrations of methacholine-bromide were comparable with the concentrations used in the other centers.

Genotyping

DNA extraction was performed at Duke University, Durham, NC, by using the PureGene system (Gentra, Leicestershire, United Kingdom). The following SNPs were analyzed (centromeric to telomeric): MH30, −1147CT, +49AG, CT60, JO31, JO30, and JO27_1 (Fig 1). Details of these SNPs are found elsewhere.[6,17] SNP genotyping was performed by TaqMan allelic discrimination assays (see http://home.appliedbiosystems.com for details) on an ABI 7700 DNA Analyzer (Applied Biosystems, Foster City, Calif) for the −1147CT, +49AG, JO31, JO30, and JO27_1 SNPs. CT60 was analyzed by PCR-RFLP (restriction enzyme HpyCH4 IV) by using a fluorescently labeled forward primer and a modified reverse primer that introduced an additional control cutting site in the PCR product and visualized on an ABI 3730 Genetic Analyzer (Applied Biosystems). A new single nucleotide repeat polymorphism in complete linkage disequilibrium with MH30, 32 bp upstream of the

TABLE II. Results of the TDT analysis for different disease phenotypes

Serum IgE level	Oslo (102 families)	Groningen (75 families)	Sheffield (95 families)	Aberdeen (92 families)	Total (364 families)	
SNP/ allele	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	P_{tdt}
MH30/C	44 (59%)	34 (64%)*	28 (47%)	37 (60%)	143 (57%)	.019
−1147CT/C	16 (57%)	21 (62%)	13 (46%)	17 (63%)	67 (57%)	.115
+49AG/A	36 (58%)	25 (58%)	30 (50%)	32 (53%)	123 (55%)	.161
CT60/A	47 (58%)	33 (67%)*	32 (53%)	41 (62%)	153 (60%)	.002
JO31/T	46 (60%)	35 (66%)*	27 (47%)	32 (56%)	140 (57%)	.021
JO30/A	43 (60%)	35 (66%)*	27 (47%)	33 (58%)	138 (58%)	.020
JO27_1/C	45 (60%)	32 (65%)*	29 (49%)	32 (62%)	138 (59%)	.007

P_{tdt}, *P* value in the TDT analysis comparing the observed transmission rate with the expected 50% under the null hypothesis of no association as a $\chi 2$ test (1 *df*).
*$P < .05$ for specific result in the chart.

TABLE III. Results of the TDT analysis for different disease phenotypes

Allergy	Oslo (102 families)	Groningen (75 families)	Sheffield (95 families)	Aberdeen (92 families)	Total (364 families)	
SNP/ allele	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	P_{tdt}
MH30/C	42 (53%)	37 (62%)	34 (50%)	35 (54%)	148 (54%)	.145
−1147CT/C	19 (51%)	20 (53%)	13 (38%)	18 (60%)	70 (50%)	.932
+49AG/A	39 (57%)	31 (65%)*	39 (57%)	32 (48%)	141 (56%)	.058
CT60/A	47 (54%)	33 (61%)	36 (52%)	39 (55%)	155 (55%)	.073
JO31/T	45 (54%)	38 (64%)*	36 (52%)	32 (52%)	151 (55%)	.069
JO30/A	45 (59%)	38 (64%)*	34 (50%)	34 (53%)	151 (57%)	.032
JO27_1/C	46 (56%)	34 (63%)	37 (52%)	33 (54%)	150 (56%)	.050

P_{tdt}, *P* value in the TDT analysis comparing the observed transmission rate with the expected 50% under the null hypothesis of no association as a $\chi 2$ test (1 *df*).
*$P < .05$ for specific result in the chart.

SNP, was also analyzed on ABI 3730. Details on primer and probe sequences can be found elsewhere[6,17] or on request.

Statistical analysis

Possible differences in demographic data between populations were determined by an ANOVA test and considered statistically significant at the 5% level. Outcome variables were compared between populations by using the ANOVA test for total serum IgE (geometric mean) and with a Pearson χ^2 analysis for dichotomous outcomes by using Statistical Package for Social Sciences version 11 (SPSS Inc, Chicago, Ill).

Pedigree files were checked for incompatibilities by using the program PedCheck[24] and SHOWHAPLO (http://www.-gene.cimr.cam.ac.uk) on the GLUE software (http://menu.hgmp.mrc.ac.uk/~fdubrid/software/). Tests for genetic associations in the families were performed by the transmission disequilibrium test (TDT)[25] by using the GLUE software. Both individual alleles and haplotypes were analyzed. Similar analysis was also performed by using the pedigree disequilibrium test (including siblings)[26] and quantitative TDT for serum IgE levels, neither of which gave any further information. Haplotypes were constructed and analyzed by using the program Unphased in the GLUE interphase. SNPs in linkage disequilibrium with each other are not independent, and it is therefore generally considered inappropriate to use a Bonferroni correction for multiple testing. We have chosen to present the uncorrected *P* values in this article.

RESULTS

The mean age of the probands was 14.1 years, 72% were sensitized to 1 of the assessed allergens, 61% had increased serum IgE levels, and 46% exhibited BHR (Table I). Although age and gender of the probands were not significantly different between the sites, probands in Oslo were significantly less often allergic, exhibited less BHR, exhibited less reduced lung function, and had significantly lower serum IgE levels than probands from the other sites. Among the probands, 86% of those with reduced lung function (<80% predicted FEV_1) were sensitized compared with 70% of those with FEV_1% above the 80% of predicted. There were significant differences between the sites regarding the occurrence of asthma and BHR among parents and siblings, and allergic sensitization and allergic asthma among siblings only (Table I).

Results of the TDT analysis are shown in Tables II to VI and Fig 2. Increased serum IgE levels were significantly associated with 5 of the SNPs (MH30/C, CT60/A, JO31/T, JO30/A, and JO27_1/C). Allergy was significantly associated with JO30/A and showed borderline significance for JO27_1/C. Asthma was significantly associated with JO31/T, JO30/A, and JO27_1/C. Allergic asthma was significantly associated with JO30/A and JO27_1/C, with borderline significance for +49AG/A, CT60/A, and JO31/T. Reduced lung function was significantly associated with −1147CT/C, CT60/A, JO31/T, JO30/A, and JO27_1/C. There was no evidence for association with BHR.

The analyzed markers were in strong linkage disequilibrium (data not shown) with 3 common haplotypes that constituted the vast majority of haplotypes in all of the populations. Other haplotypes were all rare (<3% of

TABLE IV. Results of the TDT analysis for different disease phenotypes

Asthma	Oslo (102 families)	Groningen (75 families)	Sheffield (95 families)	Aberdeen (92 families)	Total (364 families)	
SNP/ allele	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	P_{tdt}
MH30/C	52 (51%)	46 (67%)*	43 (49%)	37 (54%)	178 (54%)	.122
−1147CT/C	25 (51%)	27 (62%)	21 (44%)	18 (58%)	91 (53%)	.400
+49AG/A	47 (52%)	34 (63%)	43 (52%)	40 (52%)	164 (54%)	.151
CT60/A	58 (51%)	43 (67%)†	47 (51%)	44 (56%)	192 (55%)	.061
JO31/T	55 (52%)	49 (70%)‡	46 (51%)	34 (52%)	184 (56%)	.036
JO30/A	53 (53%)	49 (70%)†	41 (48%)	38 (54%)	181 (55%)	.046
JO27_1/C	53 (52%)	45 (70%)†	45 (51%)	37 (54%)	180 (56%)	.039

P_{tdt}, *P* value in the TDT analysis comparing the observed transmission rate with the expected 50% under the null hypothesis of no association as a $\chi 2$ test (1 *df*).
*$P < .05$ for specific result in the chart.
†$P < .01$ for specific result in the chart.
‡$P < .001$ for specific result in the chart.

TABLE V. Results of the TDT analysis for different disease phenotypes

Allergic asthma	Oslo (102 families)	Groningen (75 families)	Sheffield (95 families)	Aberdeen (92 families)	Total (364 families)	
SNP/ allele	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	P_{tdt}
MH30/C	42 (54%)	37 (62%)	34 (50%)	35 (54%)	148 (55%)	.113
−1147CT/C	18 (51%)	20 (53%)	13 (38%)	18 (60%)	69 (50%)	.932
+49AG/A	38 (57%)	31 (64%)	39 (57%)	32 (48%)	140 (56%)	.057
CT60/A	46 (56%)	33 (61%)	36 (52%)	39 (55%)	154 (56%)	.054
JO31/T	44 (56%)	38 (64%)*	36 (52%)	32 (52%)	150 (56%)	.050
JO30/A	44 (60%)	38 (64%)*	34 (50%)	34 (53%)	150 (57%)	.026
JO27_1/C	45 (57%)	34 (63%)	37 (52%)	33 (54%)	149 (56%)	.042

P_{tdt}, *P* value in the TDT analysis comparing the observed transmission rate with the expected 50% under the null hypothesis of no association as a $\chi 2$ test (1 *df*).
*$P < .05$ for specific result in the chart.

TABLE VI. Results of the TDT analysis for different disease phenotypes

BHR	Oslo (102 families)	Groningen (75 families)	Sheffield (95 families)	Aberdeen (92 families)	Total (364 families)	
SNP/ allele	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	Transmitted (%)	P_{tdt}
MH30/C	26 (43%)	29 (55%)	31 (48%)	32 (52%)	114 (52%)	.543
−1147CT/C	16 (53%)	13 (45%)	18 (51%)	14 (58%)	53 (52%)	.692
+49AG/A	22 (42%)	28 (62%)	30 (49%)	31 (46%)	105 (51%)	.674
CT60/A	28 (43%)	27 (53%)	33 (50%)	35 (50%)	115 (50%)	.894
JO31/T	27 (44%)	33 (63%)	30 (45%)	27 (48%)	112 (52%)	.494
JO30/A	24 (44%)	33 (63%)	29 (45%)	32 (52%)	114 (54%)	.214
JO27_1/C	27 (45%)	30 (61%)	33 (50%)	29 (48%)	111 (53%)	.368

BHR, Bronchial hyperresponsiveness to methacholine defined by PC_{20} value ≤8 mg/mL;
P_{tdt}, *P* value in the TDT analysis comparing the observed transmission rate with the expected 50% under the null hypothesis of no association as a $\chi 2$ test (1 *df*).

haplotypes). The most common haplotype (MH30/C, +49AG/A, CT60/A, JO31/T, JO30/A, and JO27_1/C) was positively associated with serum IgE levels, allergy, allergic asthma, and reduced lung function, but not with asthma (Fig 3). This haplotype had a transmission rate >50% in all 4 populations when studied separately, albeit not at levels that reached statistical significance.

DISCUSSION

The current study provides evidence for associations between newly identified SNPs in the *CTLA-4* gene (MH30, CT60, JO31, JO30, and JO27_1) and serum IgE production, allergy, allergic asthma, asthma, and reduced lung function in asthmatics. The associations were strongest for serum IgE levels and reduced lung function, followed by allergic asthma, allergy, and asthma, with no associations with BHR.

The findings of association in the current study are supported by linkage with atopy to this region on chromosome 2q32-33 (harboring *CTLA-4*).[1,2] However, to our knowledge, the markers that showed significant evidence for association in our study (MH30, CT60, JO31, JO30, and JO27_1) have not been previously analyzed for association with asthma, lung function, or atopy. The failure to demonstrate an association with BHR suggested that the main contribution of the polymorphisms was to

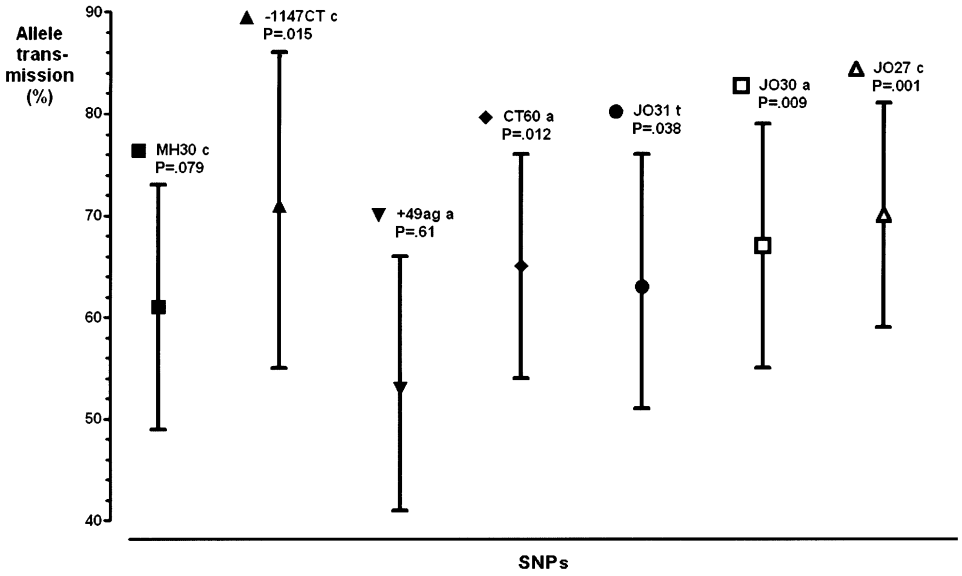

FIG 2. *CTLA-4* SNPs and association with reduced lung function (FEV_1 < 80% predicted). The number of subjects with reduced lung function was too low to assess association within each population separately, and the association is therefore presented as TDT scores with 95% CIs and *P* values for the whole group.

Parental haplotypes:	n	457	140	449	106
	%	40%	12%	39%	9%
Most common haplotypes		Haplo 1	Haplo 2	Haplo 3	Remaining
MH30		C	G	G	
-1147CT		***C***	***T***	***C***	
49AG		***A***	***A***	***G***	
CT60		A	G	G	
JO31		T	G	G	
JO30		A	G	G	
JO27		C	T	T	
Se IgE levels	T	59%	44%	43%	48%
	P_{tdt}	**.028**	.325	.123	.806
Allergy	T	59%	47%	41%	47%
	P_{tdt}	**.015**	.651	**.034**	.633
All. Asthma	T	59%	49%	41%	49%
	P_{tdt}	**.021**	.819	**.031**	.811
FEV % pred.	T	71%	24%	33%	67%
	P_{tdt}	**.016**	**.029**	.070	.200

FIG 3. The 3 most common haplotypes in the populations are presented. The transmission rates demonstrate that the most common haplotype is inherited more often than the expected 50% in the described phenotypes. Because they differ only in +49AG and −1147CT *(italics)*, it is unlikely that these represent the etiologic polymorphism. Significance levels shown are for associations in TDT analysis (χ^2 with 1 *df*). P_{tdt}, *P* value of the TDT test; *T,* transmission rate of haplotype. *Se,* Serum; *All.,* allergic; *pred,* predicted.

atopy rather than to asthma per se, although the association to lung function may also indicate a contribution independent of atopy. However, in our population, the majority of probands with asthma and reduced lung function were also allergic (72% and 86%), and consequently, the association with nonallergic asthma could not be ascertained. Those markers (+49AG and −1147CT) that have been associated with asthma or atopy in earlier studies[5,6,9] did not reach statistical significance in the current study.

Our results suggest that 1 or more of MH30, CT60, JO31, JO30, and JO27_1 are more likely than +49AG or −1147CT to be primarily involved in disease pathogenesis. The −1147CT polymorphism is unlikely to be involved in light of the complete lack of association in all but lung function. The +49AG polymorphism shows some

evidence for association, albeit not statistically significant association. Also, our haplotype analysis provides evidence that both these polymorphisms are unlikely to be involved because identical alleles are found on both predisposing (transmitted >50% in TDT) and protective haplotypes (transmitted < 50% in TDT; Fig 3). Markers in the vicinity of etiologic polymorphisms can display different levels of linkage disequilibrium with the primarily involved polymorphisms, leading to varying ability to detect associations, providing 1 explanation of why there have been inconsistent results for +49AG and atopy in previous studies. Also, to our knowledge, the current study is the largest performed to date on *CTLA-4* polymorphisms in asthma and atopy. Thus, the findings of the current study suggest that the polymorphisms likely to be of pathogenic significance are among MH30, CT60, JO31, JO30, and JO27_1. This is in agreement with the study performed on *CTLA-4* in autoimmune diseases by Ueda et al.[17] We cannot exclude the possibility that there are polymorphisms that we have not analyzed or even other hitherto unidentified polymorphisms, such as etiologic polymorphisms in the promoter region. However, based on the extensive analysis of this gene region by Ueda et al[17] and the identification of these particular SNPs as the best functional candidates in autoimmune diseases, we consider this possibility less likely.

In the current study, the associated alleles of all studied SNPs were the reverse of those found associated with type 1 diabetes,[17] and for the haplotypes most commonly found in type 1 diabetes (haplo 2 and 3 in Fig 3), significant negative associations were found with allergy, reduced lung function, and allergic asthma. This inverse relationship is reflected in a recent meta-analysis of the clinical association between atopic disease and type 1 diabetes, in which negative associations between atopic traits and type 1 diabetes were found.[18] A reason previous epidemiologic studies on this relationship have been so discrepant[27-29] may be the existence of common environmental factors that influence the development of both type 1 diabetes and atopic disorders[29] despite inherent propensities on individual levels toward either T_H1-dominated or T_H2-dominated immune responses.

The strong association with serum IgE levels in the current study suggests that the effects of *CTLA-4* polymorphisms are linked to atopy. On the other hand, the association with reduced lung function in asthmatics may suggest that other mechanisms are also involved through CTLA-4. However, only 13% of our probands had reduced lung function (as defined by FEV_1 <80% predicted), and 71% of these also had increased IgE levels. This makes it difficult to ascertain which parameters are primarily involved, although we may speculate that the association with reduced lung function could be explained by allergic inflammation and remodeling of airways, or could be a result of a subpopulation with more severe asthma. In either case, as a regulator of T-cell differentiation,[12,30] CTLA-4 may stimulate a T_H2 cytokine profile and serum IgE response to allergens and thereby may be associated with allergic diseases such as asthma, eczema, and rhinitis to varying degrees. This possibility is supported by the fact that CTLA-4 fusion protein has been found to block allergen-induced cytokine responses in airway explants from asthmatic subjects.[31] Our hypothesis that *CTLA-4* polymorphisms can provide a genetic explanation for the apparent negative association between T_H1 diseases and T_H2 diseases is thus supported. Simultaneously, the association with reduced lung function, the lack of association with BHR, and the weaker association with asthma are in line with the notion that asthma is not invariably an atopic and IgE-mediated disease.

All of the SNPs analyzed in the current study except +49AG, which is in a coding sequence, are situated in regulatory sequences. This suggests that regulation of CTLA-4 synthesis, rather than the amino acid sequence, is responsible for biological variation. CTLA-4 has 2 alternative splice forms: a full-length protein (flCTLA-4) with a transmembrane domain, and a soluble protein (sCTLA-4) without the transmembrane domain.[32] CTLA4-IgG has been shown to reverse asthma manifestations in murine models[33] and was recently found to be effective in treating rheumatoid arthritis in human beings.[34] The immunosuppressive action of soluble CTLA4-IgG suggests that sCTLA-4 suppresses activation by blocking the positive costimulatory molecule CD28 (both interact with the same receptors on the antigen presenting cell[10]). In the study by Ueda et al,[17] the disease protective genotype for autoimmune diseases was associated with a lower flCTLA-4/sCTLA-4 ratio, suggesting a biological pathway for genetic influence on T-cell activation through differential splicing of *CTLA-4*.[17] This might identify CTLA-4 signaling as a potential therapeutic target in atopic disease, also.

Possible limitations when using populations from different geographical sites in genetic association studies are risks of ethnic admixture, genetic heterogeneity, and different environmental stimuli confounding the results. The current study, however, was conducted with identical clinical procedures and methods, as well as standardized interpretation of results within the GAIN framework. Objective measures such as serum IgE level and allergic sensitization (at least 1 positive SPT) limit the effect of genetic heterogeneity by narrowing outcome traits. The TDT analysis (transmission rates within families) reduces the effect of ethnic admixture. Whereas age and sex of probands, parents, and siblings were similar among all sites, the discrepancies of the outcomes in the different populations in the current study are in accordance with previous reports for other polymorphisms.[35] The finding that total transmission rates were significantly higher than the expected 50% despite these discrepancies strengthens their validity. Indeed, combining populations to attain large sample sizes with clearly defined clinical phenotypes is probably necessary for analyzing complex traits.

In conclusion, the current study demonstrates significant associations between *CTLA-4* polymorphisms and serum IgE levels, allergy, allergic asthma, asthma, and

reduced lung function, but not BHR, suggesting that a major contribution of the polymorphisms is to atopy. Haplotype analysis suggests that 1 or more of the newly identified polymorphisms MH30, CT60, JO31, JO30, and JO27_1 could be involved in the development of allergic disease. All associated alleles were opposite those alleles associated with autoimmune diseases, providing suggestive evidence for a genetic control site for T_H1-mediated versus T_H2-mediated immunity within the *CTLA-4* gene.

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