

Fine mapping of an IgE-controlling gene on chromosome 2q: Analysis of *CTLA4* and *CD28*

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Background: Several genomic regions have been identified that might contain genes contributing to the development of asthma and atopy. These include chromosome 2q33, where we have observed evidence for linkage for variation in total serum IgE levels in a Dutch asthma population. Two candidate genes, *CTLA4* and *CD28*, important homeostatic regulators of T-cell activation and subsequent IgE production, map within this candidate region.

Objective: We sought to fine-map the chromosome 2q33 region and evaluate *CTLA4* and *CD28* as candidate genes for the regulation of total serum IgE levels and related phenotypes.

Methods: The coding regions of *CTLA4* and *CD28* were resequenced in 96 individuals; 4 novel SNPs in *CTLA4* and 10 in *CD28* were identified. Polymorphisms in both genes were analyzed in 200 asthmatic probands and their spouses ($n = 201$). **Results:** Subsequent fine-mapping in this region has resulted in an increased log of the odds (lod) score (1.96 to 3.16) for total serum IgE levels. For *CTLA4*, the +49 A/G single nucleotide polymorphism (SNP) in exon 1 and the 3' untranslated region microsatellite were significantly associated with total serum IgE levels ($P = .0005$ and $.006$, respectively). For the combined +49 A/G and 3' untranslated region genotypes, individuals homozygous for the risk allele for both polymorphisms (AA and 86/86) had the highest total serum IgE values (87.1 IU/mL), whereas those individuals with the GG and XX/XX genotypes (anything but the 86-bp allele) had the lowest IgE values (29.3 IU/mL). Significant association was also observed for the *CTLA4* -1147 C/T SNP with bronchial hyperresponsiveness (BHR) and asthma ($P = .008$ and $.012$, respec-

tively), but not for allergy-related phenotypes. Promoter luciferase assays examining the -1147 polymorphism suggested that the T allele, which was associated with increased BHR susceptibility, was expressed at half the level of the C allele. Individuals with the risk genotypes for both BHR (-1147 CT or TT) and elevated IgE levels (+49 AA) were 4.5 times more likely to have asthma than individuals with both nonrisk genotypes ($P = .0009$). No significant associations were observed for SNPs in *CD28*.

Conclusion: These data suggest that the costimulatory pathway, specifically *CTLA4*, is important in the development of atopy and asthma. (J Allergy Clin Immunol 2002;110:743-51.)

Key words: *CTLA4*, *CD28*, chromosome 2, IgE, asthma, atopy, bronchial hyperresponsiveness

Genetic studies have identified several chromosomal regions that might contain genes that contribute to the development of asthma and atopy. Bronchial hyperresponsiveness (BHR) and increased total serum IgE levels are phenotypes that appear to predispose individuals to the development of allergy and asthma.¹⁻³ A consistent region showing evidence for linkage to total serum IgE or other allergy phenotypes is chromosome 2q32-q33, which has been identified in our Dutch asthma family study^{4,5} and also in other populations.⁶⁻⁸

The genes encoding cytotoxic T lymphocyte-associated 4 (*CTLA4*) and *CD28* are candidate genes in the 2q32-q33 chromosomal region that might be important in IgE regulation and T-cell activation. A control point for IgE synthesis and regulation is the necessity for costimulation and activation of T cells. T cells recognize antigen-presenting cells by the antigen bound to MHC class II molecules, but this binding alone is insufficient for T-cell activation. Costimulation by other receptor-ligand complexes facilitates activation of T cells. Two of the main costimulation complexes are the B7-1 (CD80) and B7-2 (CD86) ligands with CD28 and CTLA-4 receptors. CD28 is constitutively expressed on T cells and acts as a positive costimulator of T-cell activation. CTLA-4 is only expressed on activated T cells and acts as a negative feedback regulator of T-cell activation.

Because of these important functions, alteration of the costimulatory pathway could result in susceptibility to immunologic diseases. Three *CTLA4* polymorphisms have been studied in immune disorders: a C/T polymor-

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Abbreviations used

BHR:	Bronchial hyperresponsiveness
CDP:	Human CCAAT displacement protein
CTLA-4:	Cytotoxic T lymphocyte-associated 4
IDDM:	Insulin-dependent diabetes mellitus
SNP:	Single-nucleotide polymorphism
SRF:	Serum response factor
UTR:	Untranslated region

TABLE I. Clinical characteristics of probands and spouses in a Dutch population

	Probands	Spouses
Sex ratio (M:F)	124:76	76:125
Age, (mean $\pm$ SD)	52.1 $\pm$ 8.4	51.0 $\pm$ 9.2
IgE, IU/mL		
Geometric mean	93.0	26.2
Range	1-2880	0.5-1940
≥ 1 positive skin test response, %	81.9	31.0
FEV ₁ percent predicted		
Before medication (mean)	69.6	98.4
After medication (mean)	82.4	103.9
BHR†		
PC ₂₀ ≤ 32 mg/mL, %	88.2	25.6

*Total sample population consisted of 200 probands and 201 spouses (one of the probands married twice). Different numbers for the SNPs in the following tables are due to missing genotype data.

†Thirty probands were not retested because each had an FEV₁ value that was too low to be tested safely (FEV₁ $\leq 40\%$ predicted). All probands were hyperresponsive to histamine when initially tested.

phism at position -319 (previously referred to as -318 but located 319 bases from the ATG start site in our sequence data) of the promoter,⁹ an A/G change at position +49 of exon 1 (encodes for an amino acid change of Thr to Ala),¹⁰ and a microsatellite AT repeat in the 3' untranslated region (UTR).¹¹ One or more of these polymorphisms has been shown to be associated with type 1 diabetes,¹²⁻¹⁵ autoimmune thyroid disease,¹² celiac disease,¹⁶ Grave's disease,¹⁷ and multiple sclerosis.^{18,19}

Polymorphisms in both *CTLA4* and *CD28* have also been examined in asthma and allergy phenotypes. No association was observed in a sample of German individuals ascertained randomly or through an individual with atopy²⁰ or in a Japanese group of atopic asthmatic subjects, in which the -319, +49, and 3' UTR polymorphisms were examined.²¹ In a separate case-control study comparing patients with asthma with healthy control subjects, no association with the -319 or +49 single-nucleotide polymorphisms (SNPs) in *CTLA4* was observed with asthma, but an association with -319 and total serum IgE levels was reported in patients with asthma.²²

In 200 Dutch families originally ascertained through a parent with asthma, we performed fine mapping on chromosome 2q, where evidence for linkage to increased IgE levels was previously observed,⁴ and analyzed *CTLA4* and *CD28* as candidate genes primarily for increased total serum IgE levels and then for other asthma and allergic phenotypes. In addition to studying previously described polymorphisms, we have identified novel SNPs in both *CTLA4* and *CD28*.

METHODS

This population has been described in detail previously (Table I).^{4,23,24} Families were ascertained through a proband with clinical asthma initially characterized between 1962 and 1975. Between 1990 and 1998, 200 probands with asthma (together with their spouses, children, and available grandchildren) were studied by using a standardized protocol. All individuals underwent spirometry, bronchodilator reversibility to 800 μ g of albuterol, and bronchial responsiveness testing to histamine.^{24,25} All adult subjects underwent intracutaneous skin testing with 16 common aeroallergens, and total serum IgE levels were measured.⁴ The families were ascertained for linkage analysis, whereas the probands and spouses were chosen for association studies because they are unrelated and similar in age and environmental exposures. This study was approved by the Medical Ethics Committee at the University of Groningen and the Institutional Review Board at Wake Forest University School of Medicine. Written informed consent for adults and written parental assent for children was obtained from all participants.

For fine mapping, 9 additional dinucleotide microsatellite markers from the Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/Map_Markers/maps/) were genotyped by using fluorescently labeled oligonucleotide primers. CRIMAP²⁶ was run to determine the order and length of the chromosomal map and to detect double recombinants. Variance component linkage analysis was performed by using the computer program package Sequential and Oligogenic Linkage Analysis Routines (SOLAR).^{4,27}

CTLA4 and *CD28* were sequenced in 96 unrelated individuals from Dutch and US white, African-American, and Hispanic populations by using BigDye terminator chemistry (ABI) and an ABI 3700 DNA Analyzer (ABI). Each group consisted of 16 asthmatic patients and 8 unaffected control subjects. All 4 exons and 1200 bp of the 5' putative promoter region were sequenced by using primers (see table at www.wfubmc.edu/genomics) designed from available genomic sequences. Consensus 5' promoter binding sites were identified with MatInspector from Genomatix (genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl). Because the *CD28* SNPs -1132, -1046, and IVS1 +438 were nearly in complete linkage disequilibrium in the sequenced Dutch samples, only one of these (-1046 A/G) was genotyped in the entire proband-spouse population. SNPs were genotyped by using PCR, followed by RFLP analysis or with the homogeneous MassEXTEND primer extension reaction (Sequenom, Inc). All polymorphisms except -658 C/T were in Hardy-Weinberg equilibrium ($P = .0018$); this SNP was in Hardy-Weinberg equilibrium in the BHR-negative spouses ($P = .23$) but not in the probands ($P = .0014$). Linkage disequilibrium testing between SNPs was performed by using an exact test assuming multinomial probability of the multilocus genotype conditional on the single-locus genotype.²⁸

Because our linkage results provided evidence for an IgE-regulating gene on chromosome 2q33, our primary hypothesis was that polymorphisms in a gene in this region would be directly involved in IgE regulation. Total serum IgE was log transformed to approximate a normal distribution and analyzed as a quantitative trait. Differences between groups were tested with ANOVA, t tests, and multiple regression. As secondary hypotheses, we tested for associations with skin test responsiveness to common allergens and other related phenotypes, such as asthma and BHR. Individuals were considered responsive to an allergen skin test if one or more test results showed a mean wheal diameter of 5 mm or greater. For asthma, probands were compared with BHR-negative (PC₂₀ > 32 mg/mL histamine) spouses. For the BHR phenotype, probands and BHR-positive spouses (PC₂₀ ≤ 32 mg/mL histamine²⁴) were compared with BHR-negative spouses. Each of the biallelic polymorphisms was analyzed

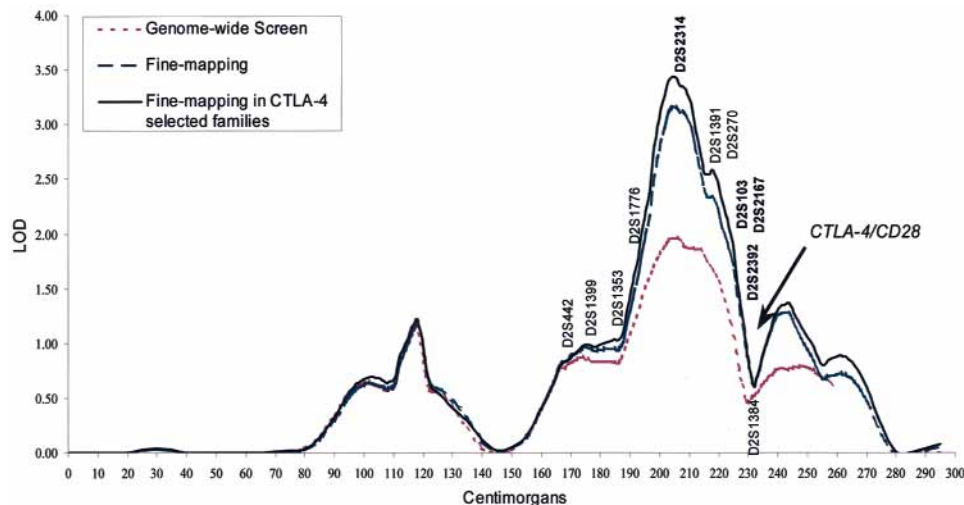


FIG 1. Linkage analysis of total serum IgE levels on chromosome 2. The *short-dashed line* represents the results from the genome-wide screen,⁴ the *longer-dashed line* represents results from additional fine mapping, and the *solid line* represents fine-mapping markers after selection for families with either parent carrying at least one risk allele for the *CTLA4* +49 SNP (ie, the A allele). Genetic markers in bold are fine-mapping markers. *CTLA4* and *CD28* are located between the genetic markers D2S2392 and D2S1384.

by comparing differences in genotype frequencies between the 2 groups. χ^2 Tests assuming a dominant model were performed in instances in which only a small number of homozygotes existed for the rare allele. The 3' UTR microsatellite was analyzed as a biallelic marker by comparing the 86-bp allele, which occurred at a frequency of 0.52, with all other alleles combined (referred to as allele XX).

For the luciferase assays, PCR inserts were prepared from genomic DNA samples of individuals homozygous for the desired haplotype. Linkers were added to the primers that contained restriction enzyme sites for *KpnI* (5' portion of the region) or *BglII* (3' portion of the region). PCR products were digested, gel purified (Promega Wizard PCR Prep Purification Kit), and subcloned into the promoterless pGL3 Enhancer vector (Promega), which had been previously cut with the same restriction enzymes. A549 lung carcinoma cells were maintained in F-12K Nutrient Mixture (Kaighn's Modification, Gibco) and supplemented with 10% FCS and 2 mmol/L L-glutamine. Cells were plated at 0.25×10^6 cells per dish the day before the transfection. Transfectam (Promega) was used to transfect the A549 cells with the pGL3 plasmids along with a positive control, the pGL3 control vector containing the SV40 promoter (Promega), and a negative control that contained no DNA. Total cell extracts were prepared after 48 hours of incubation at 37°C with 1× reagent lysis buffer (Promega), as described in the manufacturer's instructions. Activity was measured by means of luminometry with the luciferase assay system (Promega). Each plasmid was assayed in triplicate in at least 3 separate experiments.

RESULTS

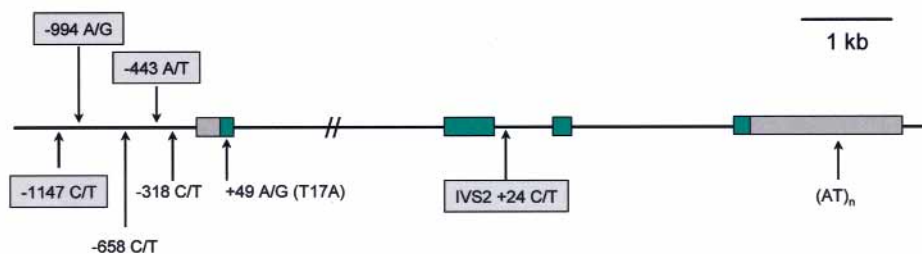
The genome screen reported in this population revealed evidence for linkage of total serum IgE levels to several chromosomal regions, including 2q31-q33.⁴ Genotyping 9 additional markers increased the lod score from 1.96 to 3.16 (Fig 1). The estimate of the total variance in total serum IgE levels accounted for by this linkage is 36%. When only families with either parent heterozygous or homozygous for the *CTLA4* +49 risk allele (ie, the A allele) were included, the lod score increased to 3.43 (Fig

1). It is difficult to interpret this result because the majority of the families have at least one parent heterozygous for *CTLA4* +49 because of high heterozygosity. However, it is interesting that the lod score increased when limiting the analysis to *CTLA4*-informative families.

CTLA4 and *CD28* are located between the genetic markers D2S2392 and D2S1384 on the basis of The Human Genome Project Working Draft (genome.ucsc.edu), placing them on the distal shoulder of the chromosome 2q linkage peak (Fig 1). Sequencing of the immediate putative 5' promoter regions and all 4 exons of both genes revealed 4 novel SNPs in *CTLA4* and 10 in *CD28* (Table II and Fig 2).^{9,14,21,29} The promoter SNPs are identified relative to the translation start site (+1). SNPs found at a greater frequency than 0.05 in the Dutch portion of the sequenced individuals were genotyped in the entire Dutch proband-spouse group. Three of the novel SNPs in *CD28* (2 in the 5' putative promoter region [−1332 C/T and −1046 A/G] and one in intron 1 [IVS1 +438]) were in strong linkage disequilibrium in the sequenced Dutch individuals, and therefore only the −1046 A/G and IVS3 +17 C/T SNPs were genotyped in the complete cohort.

The probands and spouses from the 200 Dutch families were analyzed for the 5 SNPs and the 3' UTR microsatellite in *CTLA4* and 2 SNPs in *CD28*. For *CTLA4*, total serum IgE levels were significantly associated with the +49 A/G and 3' UTR polymorphisms ($P = .0005$ and $.006$, respectively; Table III). With the +49 A/G SNP, AA homozygotes had the highest total serum IgE levels (69.2 IU/mL), GG homozygotes had the lowest levels (28.8 IU/mL), and AG homozygotes had levels intermediate between the two (44.7 IU/mL). For the 3' UTR microsatellite, individuals homozygous for the allele with the fewest repeats (86 bp) had the highest total serum IgE levels (104.9 IU/mL), whereas individuals with at least

CTLA-4



CD28

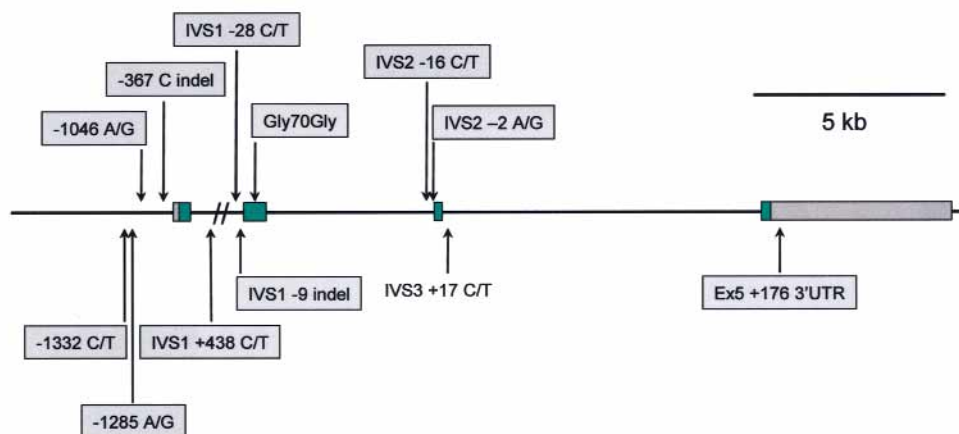


FIG 2. Location of SNPs within the genomic structure of *CTLA4* and *CD28*. Boxes indicate the locations of exons, and shaded portions indicate the translated regions. Novel SNPs are in shaded boxes.

one other allele had significantly lower total serum IgE levels (50.2–58.5 IU/mL). A significant association was observed for the –1147 C/T SNP with asthma and BHR ($P = .012$ and $.008$, respectively) but not with the allergy-related phenotypes (Table IV). The +49 A/G SNP in exon 1 was significantly associated with all 4 phenotypes examined in this population, mainly because of individuals who were homozygous for the A (Thr) allele (recessive effect). Forty-six percent of individuals with total serum IgE levels of 100 IU/mL or greater were AA homozygotes compared with 31% of individuals with total serum IgE levels of less than 100 IU/mL ($P = .007$). In addition, 41% of individuals with at least one positive skin test response, compared with 30% of those with none, were AA homozygotes ($P = .026$). A similar association was observed with asthma and BHR, where 41% of the probands were AA homozygotes compared with only 30% of their unaffected spouses ($P = .042$). No associations were observed with any of these same phenotypes for the 2 *CD28* SNPs tested (Tables II and III).

Significant linkage disequilibrium was observed between all genotyped SNPs, those within each gene and those between *CTLA4* and *CD28* ($P < .035$ for all), except for *CTLA4* –658. Some haplotypes were not observed; for instance, the G allele of the +49 SNP occurred only once on the same chromosome as the 86-bp allele of the 3' UTR microsatellite. Because both the +49 A/G and the 3' UTR microsatellite were strongly associated with total serum IgE levels, we characterized this association in relation to the observed linkage disequilibrium. Total serum IgE levels of the 9 possible genotype combinations of the +49 A/G and 3' UTR polymorphisms were analyzed, and individuals homozygous for the risk alleles (AA and 86/86) for both polymorphisms had the highest total serum IgE values (87.1 IU/mL). The lowest IgE values (29.3 IU/mL) were observed in individuals homozygous for the nonrisk allele (GG and XX) for both polymorphisms. The remaining combinations (in groups with >5 individuals) demonstrated intermediate IgE values.

TABLE II. SNPs and rare allele frequency in 4 asthma populations on the basis of 24 individuals in each group

	Rare allele frequency				Reference
	White	African-American	Hispanic	Dutch	
<i>CTLA4</i>					
−1147 C/T	0.22	0.23	0.11	0.08	This report
−994 A/G	0.00	0.02	0.00	0.00	This report
−658 C/T	0.14	0.00	0.07	0.10	Johnson et al ²⁹
−443 A/T	0.00	0.13	0.00	0.00	This report
−319 C/T	0.05	0.02	0.08	0.07	Deichmann et al ⁵
+49 A/G	0.29	0.43	0.44	0.24	Marron et al ¹⁴
IVS2 +24 C/T	0.03	0.00	0.00	0.00	This report
<i>CD28</i>					
−1332 C/T	0.36	0.24	0.31	0.48	This report
−1285 A/G	0.02	0.26	0.10	0.05	This report
−1046 A/G	0.39	0.23	0.33	0.43	This report
−367 C indel	0.05	0.04	0.00	0.00	This report
IVS1 +437 C/T	0.39	0.20	0.29	0.44	This report
IVS1 −28 C/T	0.00	0.02	0.00	0.00	This report
IVS1 −9 T indel	0.00	0.04	0.02	0.00	This report
Gly70Gly (A/G)	0.02	0.00	0.00	0.02	This report
IVS2 −16 C/T	0.00	0.02	0.00	0.00	This report
IVS2 −2 A/G	0.02	0.00	0.00	0.02	This report
IVS3 +17 C/T	0.23	0.04	0.17	0.14	Nakao et al ²¹
Exon 4 +176 3' UTR	0.00	0.05	0.00	0.00	This report

SNPs that were genotyped in the Dutch asthma population are boxed.

TABLE III. Analysis of total serum IgE levels with SNPs in *CTLA4* and *CD28*

Polymorphisms	N	Geometric mean (IU/mL)	Log (mean ± SD)	P value*
<i>CTLA4</i>				
−1147 C/T				NS
CC	241	47.9	1.68 ± 0.70	
CT	80	58.9	1.77 ± 0.74	
TT	5	24.6	1.39 ± 1.10	
−658 C/T				NS
CC	311	47.8	1.68 ± 0.71	
CT	41	65.9	1.82 ± 0.71	
TT	6	39.6	1.60 ± 0.80	
−319 C/T				NS
CC	305	50.1	1.70 ± 0.73	
CT	51	51.3	1.71 ± 0.66	
TT	3	12.6	1.10 ± 0.54	
+49 A/G (T17A)				.0005
AA	128	69.2	1.84 ± 0.77	
AG	177	44.7	1.65 ± 0.68	
GG	54	28.8	1.46 ± 0.66	
3' (AT)n				.006
86 bp/86 bp	76	104.9	2.02 ± 0.74	
86 bp/>86 bp	147	58.5	1.77 ± 0.66	
>86 bp/>86 bp	95	50.2	1.70 ± 0.76	
<i>CD28</i>				
−1046 A/G				NS
AA	54	56.2	1.75 ± 0.72	
AG	145	56.2	1.75 ± 0.74	
GG	149	40.7	1.61 ± 0.70	
IVS3 +17				NS
TT	223	50.1	1.70 ± 0.75	
CT	94	43.7	1.64 ± 0.68	
CC	14	46.8	1.67 ± 0.41	

NS, Not significant.

*P values were determined by using the appropriate genetic model (dominant, recessive, or codominant) and were adjusted for sex and age.

TABLE IV. Frequency of *CTLA4* and *CD28* genotypes within asthma and allergy phenotypes

SNPs	Asthma		BHR		Skin test	
	Probands	Unaffected spouses	PC ₂₀ ≤32 mg/mL	PC ₂₀ >32 mg/mL	≥1	0
CTLA4						
–1147 C/T	n = 156	n = 117	n = 201	n = 117	n = 175	n = 143
CC	0.69	0.83	0.69	0.83	0.72	0.76
CT	0.30	0.15	0.30	0.15	0.27	0.22
TT	0.01	0.02	0.01	0.02	0.01	0.02
	P = .012		P = .008		P = NS	
–658 C/T	n = 178	n = 131	n = 226	n = 131	n = 202	n = 155
CC	0.87	0.86	0.87	0.86	0.87	0.86
CT	0.11	0.12	0.11	0.12	0.12	0.12
TT	0.02	0.02	0.02	0.02	0.01	0.02
	P = NS		P = NS		P = NS	
–319 C/T	n = 176	n = 131	n = 176	n = 148	n = 195	n = 158
CC	0.82	0.88	0.85	0.86	0.85	0.85
CT	0.17	0.11	0.14	0.12	0.15	0.13
TT	0.01	0.02	0.01	0.01	0.00	0.02
	P = NS		P = NS		P = NS	
+49 A/G (T17A)	n = 177	n = 134	n = 179	n = 151	n = 197	n = 162
AA	0.43	0.29	0.41	0.30	0.41	0.30
AG	0.46	0.54	0.44	0.55	0.47	0.51
GG	0.11	0.17	0.15	0.15	0.12	0.19
	P = .012		P = .042		P = .026	
3' UTR*	n = 156	n = 115	n = 202	n = 115	n = 182	n = 136
86 bp/86 bp	0.28	0.22	0.25	0.22	0.29	0.17
86 bp/>86 bp	0.45	0.48	0.45	0.48	0.44	0.49
>86 bp/>86 bp	0.27	0.30	0.30	0.30	0.27	0.34
	P = NS		P = NS		P = .017	
CD28						
–1046 A/G	n = 173	n = 129	n = 218	n = 129	n = 191	n = 156
AA	0.15	0.16	0.15	0.16	0.17	0.14
AG	0.43	0.41	0.42	0.41	0.43	0.39
GG	0.42	0.43	0.43	0.43	0.40	0.47
	P = NS		P = NS		P = NS	
IVS3 +17 C/T	n = 165	n = 124	n = 208	n = 124	n = 184	n = 146
TT	0.64	0.73	0.64	0.73	0.67	0.68
CT	0.31	0.27	0.29	0.27	0.29	0.27
CC	0.05	0.01	0.06	0.01	0.04	0.05
	P = NS		P = NS		P = NS	

NS, Not significant.

*Analyzed as a recessive trait (86 bp allele is recessive).

Because significant associations to both BHR (–1147 C/T) and total serum IgE levels (+49 A/G and 3' UTR microsatellite) were observed, we examined the risk of asthma on the basis of the combined effect of having the risk genotypes for BHR (–1147 CT or TT) and increased total serum IgE levels (+49 AA). Individuals with both risk genotypes were 4.5 times more likely (95% CI, 1.75–11.83) to have asthma than those with both nonrisk genotypes. Individuals with only one of the 2 risk genotypes were 1.6 (95% CI, 0.9–2.9) and 1.8 (95% CI, 0.8–3.6) times more likely to have asthma.

Sequence analysis of the promoter region suggested that substitution of C for T at –1147 creates consensus binding sites for serum response factor (SRF) and the human CCAAT displacement protein (CDP). Luciferase assays were performed to determine the level of expression of each of the –1147 alternative alleles. The T allele

showed 0.50 times the level of luciferase activity than the C allele, indicating a lower level of transcription (Fig 3). This appeared to be independent of the genotype at the –319 locus. Relative to the T-C haplotype for –1147 and –319, respectively, the T-T haplotype had 1.16 times the expression, whereas the C-C haplotype had twice the expression. These results suggest that individuals with the –1147 T allele have half the expression of *CTLA4* than those with the C allele.

DISCUSSION

We have fine mapped a region on chromosome 2q33 with evidence for linkage to total serum IgE levels and have shown an increase in lod score from 1.9 to 3.16. Evidence for linkage to this region has been observed to related phenotypes in other populations.^{6–8,30,31} Two can-

didate genes for IgE regulation, *CTLA4* and *CD28*, key homeostatic regulators of T-cell activation, map to the outer edge of this region. Because of the genetic complexity of common diseases, it is likely that the putative gene might not map to the highest lod peak. Four novel SNPs were identified in *CTLA4*, and 10 novel SNPs were identified in *CD28*. Association studies with a subset of these and previously reported polymorphisms with the phenotypes IgE, BHR, skin test reactivity, and asthma demonstrate that variations in *CTLA4* are involved in the regulation of IgE levels and the presence of asthma in this population. Polymorphisms in *CD28* were not associated with any of the 4 phenotypes tested. By examining IgE levels of the 9 possible genotype combinations of the +49 A/G and 3' UTR microsatellite polymorphisms, we found that individuals homozygous for the risk allele for both polymorphisms (AA and 86/86) had the highest total serum IgE values (87.1 IU/mL), whereas homozygotes for the nonrisk allele for both polymorphisms (GG and XX) had the lowest IgE values (29.3 IU/mL). We also noted that individuals with the risk genotypes for BHR (–1147 CT or TT) and increased IgE levels (+49 AA) were 4.5 times more likely to have asthma than individuals with both nonrisk genotypes. These data suggest that the costimulatory pathway, specifically *CTLA4*, is important in the development of atopy and asthma-related phenotypes.

The strong linkage disequilibrium between the polymorphisms in *CTLA4* makes it difficult to determine which alleles or combinations of alleles are directly responsible for the observed association. Increased total serum IgE levels are associated with both the A allele of the +49 SNP and the 86-bp allele of the 3' UTR microsatellite, but these 2 alleles almost always occur on the same haplotype. It has been suggested that the length of the 3' UTR microsatellite is a mechanism for transcript stability, so that the longer alleles would be less stable.³² The +49 A/G SNP in exon 1 has been reported to affect the function of *CTLA4*, potentially contributing to immunologic diseases.³³ Therefore the combination of these 2 effects might lead to increased levels of total serum IgE. This same type of combination effect might be responsible for the increased risk of development of asthma in individuals carrying both risk genotypes for the –1147 and +49 SNPs because all but 2 individuals with a T allele at –1147 contain an A allele at +49. This haplotype might confer an effect that leads to asthma susceptibility that is independent of the IgE effect of the +49 A allele.

The observed association of *CTLA4* with insulin-dependent diabetes mellitus (IDDM) is interesting because of the differences between IDDM and atopic diseases (see Marron et al¹⁴). IDDM is caused by a T_H1-mediated immune response, whereas atopic diseases are mediated mostly by a T_H2 response. Most of the previous IDDM studies report overtransmission or association of the *CTLA4* +49 G (Ala) allele with the disease phenotype and not the A (Thr) allele, as observed in this study. Association with the A (Thr) allele has also been

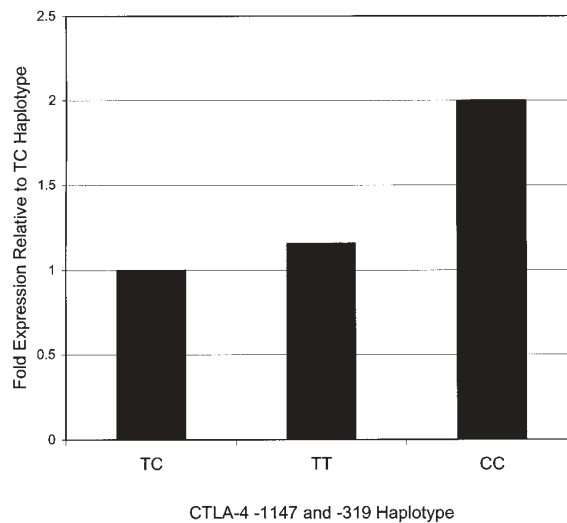


FIG 3. Promoter activity of *CTLA4* on the basis of Luciferase assay. Plasmid constructs containing 3 *CTLA4* promoter haplotypes on the basis of the –1147 and –319 SNPs (TC, TT, and CC) were used to compare the ability of each promoter to initiate transcription. The expression levels of the TT and CC haplotypes are shown relative to the TC haplotype.

observed in celiac disease³⁴ but not with other immune disorders. Possibly, a nearby SNP in linkage disequilibrium with the +49 A/G is the causative variant of the reported associations. Another possibility, however, is that celiac disease and atopic diseases share similar pathophysiologic features that differ from those of autoimmune diseases. Atopic diseases and celiac disease are both triggered by environmental antigens, whereas autoimmune diseases might be caused by intrinsic, organ-specific autoantibodies.

There are important biologic implications of polymorphisms in *CTLA4*. The –1147 substitution of T for C creates a consensus binding site for the SRF and the CDP/*cut*-like protein. SRF is one component of a complex that leads to transactivation of the promoter.³⁵ An additional protein in this complex is one or more Ets family members. Interestingly, 2 genes recently described for asthma susceptibility in Tristan da Cunha and Toronto asthma families were also Ets-related family members (ASTH1I and ASTH1J, US patent no. 6,087,485). These data suggest that the SRF complex might be involved in the regulation of asthma- or allergy-related genes. A second potential binding site that is created by the C to T substitution is for CDP/*cut*, the human homologue of *Drosophila cut*,³⁶ which has been shown to be a repressor of specific MHC class I genes.³⁷ If the potential CDP/*cut*-binding site in the *CTLA4* 5' region is an active site, then binding of CDP/*cut* might decrease transcription of *CTLA4*. Downregulation of *CTLA4* would lead to decreased repression of T-cell activation because *CTLA4* is a negative regulator of this process. Therefore T cells in allergen-exposed individuals would remain in the activated state for longer periods of time, potentially leading to increased expression of allergy or asthma phenotypes.

This is consistent with our data, in which the T allele (which creates the CDP/*cut* site) is more common in individuals with BHR (Table IV) and is associated with the presence of asthma in conjunction with the +49 AA genotype. Furthermore, the luciferase assays suggest that the -1147 T allele confers a lower level of transcription. It is interesting to note that the CDP/*cut* gene is located on chromosome 7q22, the region with the highest lod score for total serum IgE levels from the genome screen in this population.⁴ It is possible that polymorphisms in this gene might also contribute to allergy or asthma phenotypes, either independently or by interacting with variations in other genes, such as *CTLA4*.

In summary, we have further refined a region on chromosome 2q33 that contains one or more susceptibility genes for allergic responses, asthma, or both. Fine mapping with microsatellite markers in Dutch families ascertained on the basis of a proband with asthma has shown increased evidence for linkage and localized this region to an interval containing multiple candidate genes, including *CTLA4* and *CD28*. By resequencing these 2 genes in individuals from diverse ethnic groups, we have identified 14 new SNPs (4 in *CTLA4* and 10 in *CD28*) and evaluated a subset of the SNPs for association with asthma and allergic phenotypes. Significant associations were observed with polymorphisms in *CTLA4* that might regulate its expression levels, the function of the CTLA-4 protein, or both. Additionally, combinations of polymorphism genotypes within *CTLA4* increase the risk for increased total serum IgE levels (with +49 and 3' UTR AT repeat) or asthma (with -1147 and +49) in this population. Further analysis of this gene and its functions are necessary to determine its role in susceptibility to asthma and atopy.

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