

A novel promoter polymorphism in the gene encoding complement component 5 receptor 1 on chromosome 19q13.3 is not associated with asthma and atopy in three independent populations

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Summary

Background The inflammatory functions of complement component 5 (C5) are mediated by its receptor, C5R1, which is expressed on bronchial, epithelial, vascular endothelial and smooth muscle cells. A susceptibility locus for murine allergen-induced airway hyper-responsiveness was identified in a region syntenic to human chromosome 19q13, where linkage to asthma has been demonstrated and where the gene encoding C5R1 is localized.

Objective The aim of this study was to screen for novel polymorphisms in the C5R1 gene and to determine whether any identified polymorphisms are associated with asthma and/or atopy and whether they are functional.

Methods Single-nucleotide polymorphism (SNP) detection in the gene encoding C5R1 was performed by direct sequencing. Genotyping was performed in three populations characterized for asthma and/or atopy: (1) 823 German children from The Multicenter Allergy Study; (2) 146 individuals from Tangier Island, Virginia, a Caucasian isolate; and (3) asthma case–parent trios selected from 134 families ($N = 783$) in Barbados. Functional studies were performed to evaluate differences between the wild-type and the variant alleles.

Results We identified a novel SNP in the promoter region of C5R1 at position –245 (T/C). Frequency of the –245C allele was similar in the German (31.5%) and Tangier Island (36.3%) populations, but higher in the Afro-Caribbean population (53.0%; $P = 0.0039$ to <0.0001). We observed no significant associations between the –245 polymorphism and asthma or atopy phenotypes. Upon examination of the functional consequences of the –245T/C polymorphism, we did not observe any change in promoter activity.

Conclusion This new marker may provide a valuable tool to assess the risk for C5a-associated disorders, but it does not appear to be associated with asthma and/or atopy.

Keywords association, asthma, atopy, complement component 5 receptor 1 (C5R1), single-nucleotide polymorphism (SNP)

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Introduction

Complement is a major effector of innate (natural) immunity, the most ancient arm of anti-microbial host defence [1]. The more recently evolved acquired (adaptive) immune response, which relies upon antigen-specific responses of lymphocytes, is dependent upon signals provided by the innate immune system regarding the origin of the antigen (e.g., self vs. non-self) and the type of response necessary [2–5]. A specific

example of this dependency is the role of the complement system in differentiating between innocuous and harmful antigens via the central molecule in the complement cascade, C3 [6].

Anaphylatoxins are small peptides that are released following proteolysis of complement components such as C3 and C5. Their potent pro-inflammatory effects, particularly those of C3a and C5a, are consistent with the hallmark features of bronchial asthma, including leucocyte chemotaxis, mucus secretion and smooth muscle contraction [7]. Through a combination of genetic and microarray approaches Karp et al. [8] identified C5 as a susceptibility locus for allergen-induced airway hyper-responsiveness in a mouse model of

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asthma. Furthermore, they demonstrated that C5a, in addition to its traditional role as a pro-inflammatory molecule, may also serve as a bridge between the innate and adaptive immune response by regulating production of the cytokine, IL-12. In support of a role for C5 in human asthma, Krug et al. [9] reported an increased production of C5a in the bronchoalveolar lavage fluid of asthmatics 24 h following allergen challenge. Further evidence for the role of anaphylatoxins in allergic disease comes from the recent demonstration that C5a augments IgG-dependent histamine release from basophils of patients with chronic urticaria [10].

The inflammatory functions of C5 are mediated by a specific receptor (C5R1), which is a member of the rhodopsin family of G protein-coupled, seven-transmembrane receptors [11]. C5R1 is expressed on both myeloid cells (macrophages, neutrophils, eosinophils, basophils and mast cells) [11–13] and non-myeloid cells [14–17] and also bronchial, epithelial, vascular endothelial and smooth muscle cells in the lung [15, 18, 19]. These expression patterns implicate the importance of C5R1 in pulmonary inflammation and immunological responses.

The gene encoding C5R1 is clustered with several other human chemotaxis receptor genes in 19q13.3–q13.4 [20, 21], a locus for which positional cloning studies have demonstrated evidence of linkage to asthma [22–25]. C5R1 is a two exon structure separated by a 1 kb intron, with a promoter region that spans more than 300 bp upstream from exon 1 [21]. We describe here a novel polymorphism in the promoter region of C5R1 and the allelic frequencies of this change in three independent populations selected for allergic disease.

Materials and methods

Single-nucleotide polymorphism (SNP) detection

The detection of changes (SNPs) in the gene encoding C5R1 from the sequence originally published by Gerard et al. [21] (GenBank: S56556 and X57250, respectively) was performed by direct sequencing at the DNA Analysis Facility, Johns Hopkins University. PCR products were sequenced using the fluorescent dideoxy terminator method of Cycle Sequencing on an Applied Biosystems 3700 automated DNA Sequencer

(Foster City, CA, USA) per recommended protocols [26, 27] with sets of primers covering the full gene (see primers in Table 1). All sets of primers used to amplify the human C5R1 gene were designed with the software Oligo 4.0 (Molecular Biology Insights, Cascade, CO, USA). PCR were carried out in 25 µL reactions containing 100 ng DNA, 10 × PCR buffer, 2.5 mM of dNTP, 25 mM of MgCl₂, 10 µM of each primer, and 0.15 µL of DNA Taq polymerase (Promega, Madison, WI, USA). DNA samples were amplified with the following conditions: 95 °C × 5 min, 35 cycles of 94 °C for 45 s and 72 °C for 10 min. Annealing temperatures changed slightly for each primer (see Table 1). Electrophoresis was performed at 80 mA using 2% agarose gel for 45 min. Bands were excised from the gel and PCR products were purified from the gel using Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). After purification, direct sequencing was performed on the PCR products. Sequences were analysed using Sequencher 4.0 (GeneCodes Corp., Ann Arbor, MI, USA).

Sequencing was done on genomic DNA samples collected from five Caucasian subjects with asthma (T1–T5) and five Caucasian healthy controls (CT1–CT5) and three additional healthy unrelated subjects of Asian, African, and Caucasian ancestry. All subjects gave written informed consent as approved by The Johns Hopkins University Institutional Review Board.

Patient population and genotyping of the –245T/C polymorphism

Using the software Sequencher 3.0 (GeneCodes Corp.), it was determined that the transition –245T/C creates a *Bpu*1102I restriction site. Reactions were performed in a total volume of 20 µL containing 10 µL of PCR product, 0.4 µL of enzyme (4 U/µL), 2 µL of restriction enzyme buffer (20 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, 60 mM NaCl, 1 mM DTT), 2 µL of bovine serum albumin and 5.6 µL of water under conditions described by the manufacturer (Amersham Biosciences Corp., Piscataway, NJ, USA). Digestion was performed for 16 h at 37 °C. Digested products were separated on a NuSieve-agarose gel at 100 V. Digestion of the PCR fragment yielded fragments of 301 and 280 bp representing the heterozygous CT genotype, one fragment of 280 bp

Table 1. Primers used in the analysis of the C5R1 gene

Primer sets	Primer sequences 5'–3' (forward and reverse)	Annealing temperature (°C)	Size (bp)	Position on the reference sequence
Promoter, exon 1 (1)	AGAGATGGCCCCAAATAG TCGAGACTCACCATGTTC	53	301	–287 –4
Exon 2 (2)	ACTCATCCTCTCTGCTCT AGGAAGGAGGGTATGGTC	50	549	501 31
Exon 2 (3)	CCGTGGCTTGGGGTTTAG CTGTGGAGCGCTGAATG	64	546	473 1001
Exon 2 (4)	AACGTGTTGACTGAAGAG CATGGACTTCTGTTTTGT	50	540	961 1483
Exon 2 (5)	TATTTATGGCAAGTTGG GGGTGAAGGGTACACAAG	53	553	1430 1965
Exon 2, 3'UTR (6)	AAAACAAAACAAAACA AAGCTCAAAACAGTTAC	49	394	1843 2019

C5R1, complement component 5 receptor 1.



Fig. 1. Restriction analysis of primer set 1 PCR products by *Bpu1102I*. The -245 transition creates a novel *Bpu1102I* restriction site. The search for restriction sites was conducted using the program Sequencher 3.0 (GenesCodes Corp.). Reactions were performed in a total volume of 20 μ L containing 10 μ L of PCR product, 0.4 μ L of enzyme (4 U/ μ L), 2 μ L of restriction enzyme buffer (20 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, 60 mM NaCl, 1 mM DTT), 2 μ L of bovine serum albumin and 5.6 μ L of water under conditions described by the manufacturer (Amersham Pharmacia). Digestion was performed for 16 h at 37°C. Digested products were separated on a NuSieve-agarose gel at 100 V. Digestion of the PCR fragment yielded fragments of 301 and 280 bp representing the heterozygous CT genotype, one fragment of 280 bp representing the homozygous TT genotype, and an uncut fragment of 301 bp representing the homozygous CC genotype.

representing the homozygous TT genotype, and an uncut fragment of 301 bp representing the homozygous CC genotype. Restriction site analysis of PCR products with primer set 1 confirmed this polymorphism (Fig. 1).

The frequency of the C5R1 promoter polymorphism was analysed in three independent populations. The primary population for our association studies was a subgroup of 712 children from The Multicenter Allergy Study (MAS), a Caucasian birth cohort that has been described previously [28–30]. Briefly, an initial cohort of 1314 children from five German cities and who were born in 1990 were followed from birth to 10 years of age. There were 499 potential ‘high-risk’ infants with cord blood IgE = 0.9 kU/L and/or at least two atopic family members and 815 randomly selected ‘controls’ in the original study. Annual follow-up visits on the cohort infants included standardized interviews, physical examinations, pulmonary function tests and histamine provocations, and the collection of blood samples for the determination of total and specific IgE against common inhalant and food allergens (CAP-RAST-FEIA, Pharmacia, Uppsala, Sweden) and DNA extraction. Parents gave their written informed consent. The study was approved by the local Ethics Committee. The phenotypes tested for association in this study were the following: (1) current asthma at the age of 7 years, defined as ≥ 1 wheezing episode during the previous 12 months; (2) allergic rhinitis at the age of 7 years, defined as sneeze attacks, runny/blocked/itchy nose in the absence of a common cold during the previous 12 months plus sensitization against birch or grass pollen; (3) atopic dermatitis, using modified criteria of Hanifin and Rajka [31]; (4) polysensitization, defined as specific IgE ≥ 0.7 kU/L against ≥ 3

allergens at age 7 years; (5) ‘high’ total IgE, defined as total IgE above the 85th cohort percentile at ≥ 2 time-points [32]; and (6) ‘low’ total IgE, defined as total IgE below the 15th cohort percentile at ≥ 2 time-points. A healthy control group ($N = 104$) was defined as having no symptoms of asthma, allergic rhinitis, or atopic dermatitis ever, and IgE against any tested allergen < 0.35 kU/L at the ages of 7 and 10 years.

For replication, two additional populations were screened. A Caucasian isolate ($N = 458$) from Tangier Island, VA, USA [33] was recruited (1995–2001) and previously phenotyped. For this study, 146 individuals who were screened for asthma and atopy and who provided blood for genetic analysis were selected (52 cases and 94 controls). Asthma was defined as both: (1) A reported history of asthma using a standardized questionnaire [34] and (2) a history of physician-diagnosed asthma (past/current). Allergic rhinitis was diagnosed as both (1) symptoms (stuffy nose, runny nose, itchy nose/eyes/ears, tearing eyes, red eyes, sneezing, post-nasal drip, loss of smell, or sinus/facial pressure) on a chronic, seasonal or episodic pattern in the absence of a cold or flu; and (2) at least one positive skin prick test (SPT) to 32 common aeroallergens.

The second replicate population included case–parent trios from 783 Afro-Caribbean subjects from 134 families recruited in Barbados as previously described [35]. Briefly, probands were selected systematically from either the Accident and Emergency Department at the Queen Elizabeth Hospital or from private practice clinics. Asthma was defined as described above (Tangier Island population protocol). IgE concentrations were measured as described previously [35] by using the chemiluminometric Magic Lite immunoassay (Magic Lite; ALK, Copenhagen, Denmark/CIBA_Corning, Medfield, MA, USA) kindly provided by Dr Carsten Schou. Both the Tangier Island and Barbados populations were given verbal and written explanations of the study and all participants gave written informed consent. Protocols for the two replicate population studies were approved by the Johns Hopkins University Institutional Review Board.

Statistical analyses

Differences in genotype frequencies between cases and controls within each of the German and Tangier datasets were determined using Pearson’s χ^2 test. Linear regression analysis was used to test for association between C5R1 (T–145C) genotypes and quantitative traits. For the case–parent trios in the Barbados dataset, the transmission disequilibrium test (TDT) was performed according to Spielman et al. [36] using the SIB-PAIR version 0.99 program.

Cell culture

Human monocytic U937 cells (ATCC, Rockville, MD, USA) were maintained in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco BRL), 100 μ M penicillin and 100 μ g/mL streptomycin (Bio-Whittaker), and 2 mM L-glutamine (Gibco BRL). The cells were incubated at 37°C in a 5% CO₂ incubator.

C5R1 promoter fragment reporter gene constructs

The 2711 bp C5R1 promoter fragment was PCR amplified from human genomic leucocyte DNA using the following primers: forward: 5′-GGAGATGGTTGTGGCTTGTGT

AG-3' and reverse: 5'-AAGAATCAGAGAGTCCCGTTT GC-3'. The PCR reaction mix consisted of Taq Polymerase (Invitrogen, Carlsbad, CA, USA), PCR buffer (Invitrogen), 1.0 µM forward primer, 1.0 µM reverse primer, 0.1 mM dNTPs (Invitrogen), and 100 ng DNA to a final volume of 50 µL with double distilled water. PCR was done as follows: one cycle of 96 °C for 5 min, 35 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 2 min, 30 s, and one cycle of 72 °C for 7 min, followed by an infinite hold at 4 °C. The 2711 bp C5R1 promoter PCR fragment was cloned into pCR2.1 as per the manufacturer's instructions (Invitrogen). The C5R1 promoter fragment was excised from pCR2.1 by double digestion with *HindIII/XhoI* (New England Biolabs, Beverly, MA, USA), purified and subcloned into the *HindIII/XhoI* site of the pGL3.basic vector (Promega). The pGL3.basic/C5R1 promoter construct was confirmed by sequencing analysis.

Site-directed mutagenesis of the C5R1 promoter fragment

Site-directed mutagenesis was performed as per the manufacturer's instructions using the Quickchange mutagenesis kit from Stratagene (La Jolla, CA, USA). In order to create a promoter construct with the T/C substitution at -245, mutagenesis was performed using the following primers: forward: 5'-AGAGGGGCGGAGCCTGCACAGGAGCTT CCTCG-3' and its reverse complement 5'-CGAGGAAGCT CCTGTGCAGGCTCGCCCCCTCT-3'. The presence of the mutation in the pGL3. basic/C5R1 promoter mutant construct was confirmed by sequencing.

Transient transfection of U937 cells and measurement of reporter gene expression

U937 cells were simultaneously transfected with 0.792 µg pGL3.basic/C5R1 wild-type promoter or pGL3.basic/C5R1 mutant promoter and using Effectene (Qiagen) according to the manufacturer's instructions and lysed in 100 µL of 1 × Passive Lysis Buffer (Promega) 24–25 h post-transfection. The pGL3.SV40 (Promega) and the promoterless pGL3.basic (Promega) vectors were used as positive and negative controls, respectively. Transfection efficiency was normalized by co-transfecting with 0.008 µg the renilla luciferase vector pRL.SV40 (Promega). The firefly and renilla luciferase activities were determined 24 h later according to the manufacturer's instructions (Dual Luciferase Reporter Assay System, Promega) using 20 µL of the cell lysate and the data were recorded with a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI, USA) as relative light units (RLUs). Data were normalized by dividing luciferase RLU by renilla RLU.

Results

SNP detection, C5R1(-245)T/C frequency and association analyses

PCR products obtained from the primers including the promoter region (primer set 1) exhibited 11 changes from the original report by Gerard et al. [21], including eight insertions (positions -241, -147, -128, -69, -62, -38, -37 and

-34) and three substitutions (-245, -211 and -210, the last two being inversions). The only consistent SNP, however, was a T/C substitution at position -245 (Fig. 2a), present in two asthmatics, three controls, and the three additional healthy subjects (60% of subjects). Figures 2b (chromatogram) and c (sequence alignment) illustrate the T to C transition at position -245. Another polymorphism in exon 2 at bp 1286 was also detected. This substitution (transversion) of A/C was observed in two asthmatics and two healthy controls. We did not observe a substitution at position +450 (C/T) that results in a silent substitution in the Ala¹⁵⁰ codon (GCC>GCT) as previously reported in a population of unrelated healthy volunteers from the Québec City area [37].

The C5R1 -245T/C base substitution (transition) potentially affects transcription because, as detected with MatInspector V2.2 [38], it eliminates an AP-4 motif that is present in the complementary chain of the originally published sequence (T allele). We sought to determine the potential association of this promoter polymorphism in asthma and allergic disease.

The frequency of the C5R1(-245)C polymorphism was similar in the German (31.0%) and Tangier Island (36.3%) populations. Among the German children, 353 (43.3%) and 80 (9.8%) individuals presented the -245T/C and C/C genotypes, respectively. In the Tangier Island population, 74 (50.7%) and 16 (10.9%) individuals presented the -245T/C and C/C genotypes, respectively. Allele frequencies in the Afro-Caribbean population were markedly different: the C variant was more frequent in the Barbados population (53.0%) compared to the German (31.5%, $P < 0.0001$) and the Tangier Islanders (36.3%, $P = 0.0039$). Table 2 summarizes the breakdown in frequencies according to disease status.

In each population, analyses were performed to determine whether the C5R1(-245)T/C polymorphism was associated with asthma or any of the atopy phenotypes. Case-control analyses were performed on the German and Tangier Island populations using regression-based techniques. No associations were observed for any of the phenotypes (Table 3). Among the asthmatics, there was no difference in allelic frequency according to pulmonary function status (data not shown). For the Barbados population, the TDT analysis did not show excessive transmission of either allele to offspring with asthma, total IgE concentrations, or specific IgE antibodies as measured qualitatively by the SPT or quantitatively by the multi-allergen RAST (Table 4).

Isolation and characterization of the wild-type and -245T/C human C5R1 promoter

We amplified a 2711 bp sequence immediately upstream from the 5'UTR of C5R1 and subcloned this fragment into a luciferase reporter plasmid. The T to C substitution was introduced at position -245 by site-directed mutagenesis. These fragments were cloned into the reporter construct, pGL3.basic, which lacks any enhancer or promoter sequences and the promoter activities of the wild-type and -245T/C variant promoter were assessed by transfection of these constructs into human monocytic U937 cells. As shown in Fig. 3, the wild-type C5R1 2711 bp fragment contained promoter activity. In contrast, a plasmid lacking a promoter

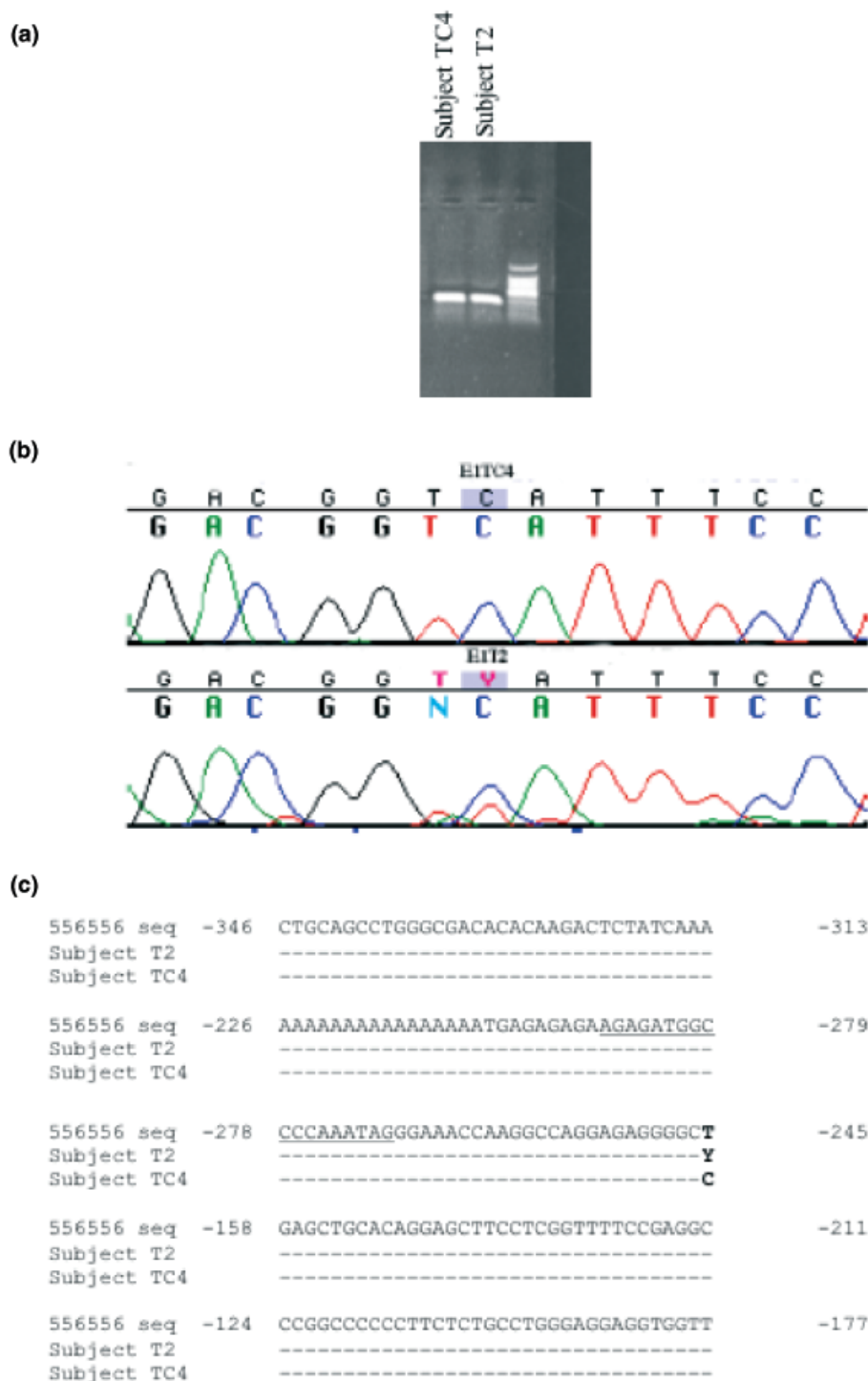


Fig. 2. Sequence analysis of the point mutation at position -245. (a) Analysis of PCR products from primer set 1 obtained from two subjects (TC4 and T2). (b) Chromatogram illustrating T/C transition at position -245. Control subject TC4 was found to be homozygous (C/C) whereas asthma subject T2 was heterozygous (T/C). (c) The PCR reaction products obtained from two subjects (TC4 and T2) were sequenced with primer set 1 (3' primer not shown).

(pGL3.basic) did not result in any luciferase activity, while a control plasmid containing the SV40 promoter and enhancer sequences resulted in strong promoter activity. As an internal control for transfection efficiency, in all experiments the cells were cotransfected with a plasmid containing the Renilla

form of luciferase and the SV40 promoter and enhancer elements. The activity of the Renilla luciferase was similar in all cases validating the comparisons. There was no significant difference between the wild-type (-245T) and the variant (-245C) promoters.

Table 2. Allelic frequencies of the –245T/C polymorphism in two Caucasian and one Afro-Caribbean populations

	German children (N = 712)		Tangier Island (N = 146)		Barbados (N = 769)	
<i>Genotype</i>						
T/T	337 (47.3%)		56 (38.4%)		184 (23.9%)	
T/C	308 (43.3%)		74 (50.7%)		386 (50.2%)	
C/C	67 (9.4%)		16 (10.9%)		199 (25.9%)	
<i>Allele</i>	<i>Asthmatics</i>	<i>Non-asthmatics</i>	<i>Asthmatics</i>	<i>Non-asthmatics</i>	<i>Asthmatics</i>	<i>Non-asthmatics</i>
T	71.7	68.6	64.4	62.5	48.5	51.2
C	28.3	31.4	35.6	37.5	51.5	48.8

Table 3. C5R1 –245T/C frequencies in atopy-related phenotypes and non-atopic controls

Phenotype	German children (N = 815)				Tangier Island, VA, USA (N = 146)			
	N	TT	TC	CC	N	TT	TC	CC
Asthma	83	0.53	0.37	0.10	52	0.40	0.45	0.15
Allergic rhinitis	76	0.47	0.44	0.09	40	0.45	0.43	0.12
Atopic dermatitis	177	0.46	0.42	0.12	ND	–	–	–
Polysensitization at 7 years	73	0.43	0.45	0.12	ND	–	–	–
Polysensitization at 10 years	85	0.49	0.40	0.11	ND	–	–	–
High total IgE	73	0.58	0.31	0.11	ND	–	–	–
Low total IgE	165	0.44	0.51	0.05	ND	–	–	–
Non-atopic controls	104	0.43	0.48	0.09	94	0.37	0.54	0.08

No significant associations were observed in two Caucasian populations. Analyses were performed using the Pearson's χ^2 test comparing each atopic group to the non-atopic controls within each population. C5R1, complement component 5 receptor 1.

Table 4. TDTs for the C5R1 (–245)T/C polymorphism in 134 Afro-Caribbean families (N = 783)

Phenotype	C5R1 (–245)T > C		P-value
	Transmitted	Not transmitted	
Asthma	116	99	0.2463
Total IgE*	–	–	0.3571
+SPT	33	49	0.0772
Specific IgE†	69	67	0.8638

*Quantitative trait. †Measured qualitatively by the multiallergen RAST. TDT, transmission disequilibrium tests; C5R1, complement component 5 receptor 1; SPT, skin prick test.

Discussion

We report here a novel polymorphism (T–245C) in the C5R1 promoter. To date there has only been one other report of a polymorphism in C5R1 (C450T) [26], which was rare in a Caucasian population (<3%) and for which no association was observed with end-stage renal failure patients. The role of the C450T polymorphism was not examined for asthma or allergic disease in our three datasets, and we did not observe the polymorphism following the sequencing of C5R1 in 13 individuals. We detected a second, novel polymorphism in exon 2 at bp 1286 (A>C); however, we were unable to develop an assay for genotyping the >1700 DNA samples from these three populations. Our future plans include the development of such an assay.

Our findings indicate that the –245T/C polymorphism is not associated with asthma or atopy phenotypes in three

independent populations (two Caucasian, one Afro-Caribbean). One possibility for a failure to detect an association with this variant and the trait is insufficient power. We estimated that we were sufficiently powered at 80% to detect: (1) a 30% difference in allele frequencies in the German dataset; (2) a 35% difference in allele frequencies in the Tangier dataset; and (3) a 2-fold increased risk under a log-additive model with the allele frequencies that we observed with 100 informative trios, less than what was actually available in the Barbados family-based dataset. Importantly, a negative association was observed in three independent populations of three different study designs. Finally, our functional studies did not provide evidence for biologic significance of the T–245C promoter polymorphism.

We observed significant differences in allelic frequency of this promoter polymorphism according to ethnicity, wherein –245C is more common in the Afro-Caribbean (Barbados) population (53%) compared to two Caucasian populations (31–36%). Recent studies in asthma and allergic disease focusing on receptors and mediators of innate immunity have demonstrated a high level of genetic diversity, especially among African Americans. For example, Lazarus et al. [39] reported 705 polymorphisms in 16 innate immunity genes (but observed limited associations with asthma or allergic disease; e.g., TLR9 (T–1237C), $P = 0.042$), observing that the mean number of SNPs per gene was highest in the African Americans. It is possible that the differences observed in allele frequencies are representative of disease status rather than ethnicity *per se*. The Barbados population was specifically enriched for asthma and has previously been characterized for very high total IgE [35] and a high prevalence of allergen exposure [28]. Consequently, it is possible that the higher

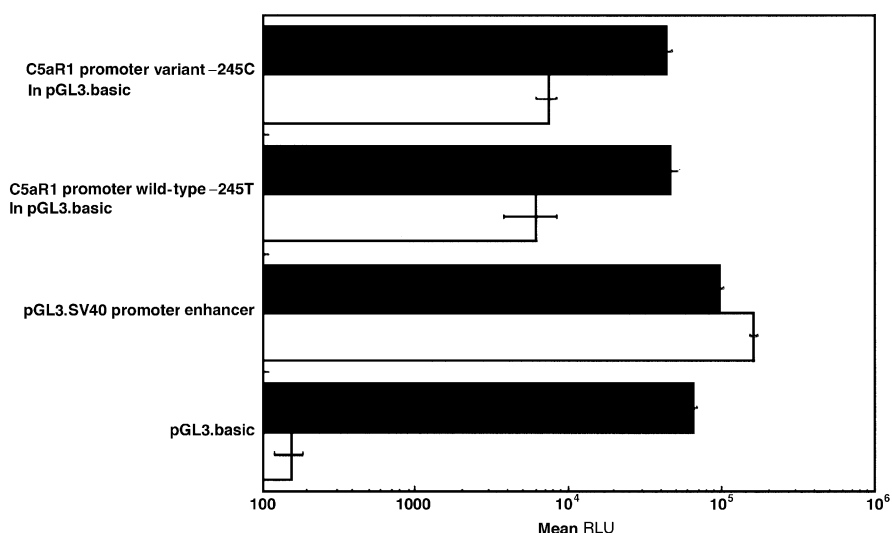


Fig. 3. U937 cells were transfected with the indicated promoter constructs and firefly luciferase activity was assessed (open bars). Transfection efficiency was normalized by co-transfecting with the renilla luciferase vector pRL.SV40 and renilla luciferase activity was assessed (filled bars). Means and standard deviations of three separate experiments are shown.

frequency of the -245C variant observed among the Barbados subjects is reflective of the allergic diathesis, although there was no significant association of this polymorphism for either asthma or high total IgE. However, the German population was similarly enriched for allergic disease, although it was not selected for asthma, to the extent that the subcohort had a strong family history of atopy. Recently, the MAS '90 group compared the baseline characteristics of the studied subcohort of 823 children to the original cohort with at least one German parent ($N = 5569$), and found that there were a substantially higher proportion of children with more than two atopic family members and high cord blood IgE, thus suggesting that this subgroup reflected enrichment for elevated serum IgE levels [40]. Thus, the differences in frequency of the -245T>C polymorphism that we observed are possibly not due to population selection bias for disease.

We examined the functional consequences of this polymorphism; however, the -245T/C polymorphism did not result in any change in promoter activity. The vector that we utilized did not contain an enhancer. It remains possible that a difference may become evident with a vector that contains enhancer elements along with the promoter construct in lieu of the basic vector. The positive control vector did contain enhancer sequences. Furthermore, differences between the wild-type and variant may also become evident with a longer promoter fragment. However, given our data finding no genetic correlation coupled with the functional assays that show no change in function, it is unlikely that there will be a significant difference between the wild-type and variant promoters.

Several possibilities other than insufficient power or a lack of functional consequences of this promoter polymorphism might explain the negative associations that we observed in these three populations. Although the results of our study did not demonstrate an association between the C5R1(-245)C polymorphism and asthma outcomes, we cannot rule out the contribution of alterations in other genes in the complement system in asthma. For example, other variants in C5R1 such as the polymorphism in exon 2 at bp 1286 that we did not assess in our study may be associated with asthma-related traits. Another potential candidate is the newly described

second receptor for C5a referred to as C5L2 [41]. Other members of the C5 pathway such as C5 itself or the other C5 activation fragment C5b may also be candidates for disease genes. Alternatively, genes upstream of C5 such as C3 may be candidates. In support of this contention, we and others have shown that deficiency in C3 in mice provides protection from allergen- [42, 43], pollutant- [44], and RSV-induced [45] airway hyper-responsiveness.

Another possibility for a lack of association is that the -245T>C polymorphism is related to phenotypes not measured (e.g., intermediate phenotypes related to specifically to complement, or bronchial hyper-reactivity (BHR)). In murine studies, Karp et al. [8] identified C5 as a susceptibility locus for allergen-induced airway hyper-responsiveness, a surrogate for BHR. They also demonstrated that blockade of the C5a receptor with a C5R1 antagonist resulted in marked, dose-dependent inhibition of IL-12 in primary human monocytes [8]. However, the same group showed that a 5' deletion in the C5 gene itself correlated with allergen-induced AHR across inbred strains. These data suggest that perhaps genetic variation in other molecules in the pathway (i.e., C5) are important, rather than C5R1. Finally, there is the possibility that C5 and its receptor have no role in the pathogenesis of human asthma. Thus further studies of the contribution of genes regulating the complement system to asthma susceptibility are clearly warranted.

Although the C5R1 (T-245C) polymorphism may provide a valuable tool to assess the risk for C5a-associated disorders, it does not appear to be associated with asthma and/or atopy in these three independent populations.

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References

- Thompson CB. New insights into V(D)J recombination and its role in the evolution of the immune system. *Immunity* 1995; 3:531–9.
- Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science* 1996; 272:50–4.
- Medzhitov R, Janeway CAJ. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 1997; 91:295–8.
- Kobayashi K, Inohara N, Hernandez LD et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002; 416:194–9.
- Medzhitov R, Janeway CAJ. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002; 296:298–300.
- Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 1996; 271:348–50.
- Gerard NP. In: Barnes PJ et al. eds. *Asthma*. Philadelphia: Lippincott-Raven, 1997; 639–51.
- Karp CL, Grupe A, Schadt E et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000; 1:221–6.
- Krug N, Tschernig T, Erpenbeck VJ et al. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med* 2001; 164 (10 Part 1):1841–3.
- Kikuchi Y, Kaplan AP. A role for C5a in augmenting IgG-dependent histamine release from basophils in chronic urticaria. *J Allergy Clin Immunol* 2002; 109:114–8.
- Gerard NP, Gerard C. The chemotactic receptor for human C5a anaphylatoxin. *Nature* 1991; 349:614–7.
- Gerard NP, Hodges MK, Drazen JM et al. Characterization of a receptor for C5a anaphylatoxin on human eosinophils. *J Biol Chem* 1989; 264:1760–6.
- Fureder W, Agis H, Willheim M et al. Differential expression of complement receptors on human basophils and mast cells. Evidence for mast cell heterogeneity and CD88/C5R1 expression on skin mast cells. *J Immunol* 1995; 155:3152–60.
- Gasque P, Chan P, Fontaine M et al. Identification and characterization of the complement C5a anaphylatoxin receptor on human astrocytes. *J Immunol* 1995; 155:4882–9.
- Haviland DL, McCoy RL, Whitehead WT et al. Cellular expression of the C5a anaphylatoxin receptor (C5R1): demonstration of C5R1 on nonmyeloid cells of the liver and lung. *J Immunol* 1995; 154:1861–9.
- Lacy M, Jones J, Whittemore SR et al. Expression of the receptors for the C5a anaphylatoxin, interleukin-8 and FMLP by human astrocytes and microglia. *J Neuroimmunol* 1995; 61:71–8.
- Braun M, Davis AER. Cultured human glomerular mesangial cells express the C5a receptor. *Kidney Int* 1998; 54:1542–9.
- Drouin SM, Kildsgaard J, Haviland J et al. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol* 2001; 166:2025–32.
- Laudes JJ, Chu JC, Huber-Lang M et al. Expression and function of C5a receptor in mouse microvascular endothelial cells. *J Immunol* 2002; 169:5962–70.
- Bao L, Gerard NP, Eddy RLJ et al. Mapping of genes for the human C5a receptor (C5R1), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics* 1992; 13:437–40.
- Gerard NP, Bao L, Xiao-Ping H et al. Human chemotaxis receptor genes cluster at 19q13.3–13.4. Characterization of the human C5a receptor gene. *Biochemistry* 1993; 32:1243–50.
- Ober C, Cox NJ, Abney M et al. Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. *Hum Mol Genet* 1998; 7:1393–8.
- Ober C, Tsalenko A, Parry R et al. A second-generation genome-wide screen for asthma-susceptibility alleles in a founder population. *Am J Hum Genet* 2000; 67:1154–62.
- CSGA. The Collaborative Study on the Genetics of Asthma: a genome-wide search for asthma susceptibility loci in ethnically diverse populations. *Nat Genet* 1997; 15:389–92.
- Dizier MH, Besse-Schmittler C, Guilloud-Bataille M et al. Genome screen for asthma and related phenotypes in the french EGEA study. *Am J Respir Crit Care Med* 2000; 162:1812–8.
- Smith LM, Sanders JZ, Kaiser RJ et al. Fluorescence detection in automated DNA sequence analysis. *Nature* 1986; 321:674–9.
- McCombie WR, Heiner C, Kelley JM et al. Rapid and reliable fluorescent cycle sequencing of double-stranded templates. *DNA Sequence* 1992; 2:289–96.
- Illi S, von Mutius E, Lau S et al. Early childhood infectious diseases and the development of asthma at school age: a birth cohort study. *Br Med J* 2001; 322:390–5.
- Kulig M, Klettke U, Wahn V et al. Development of seasonal allergic rhinitis during the first 7 years of life. *J Allergy Clin Immunol* 2000; 106:832–9.
- Lau S, Illi S, Sommerfeld C et al. Early exposure to house-dust mite and cat allergens and development of childhood asthma: a cohort study. Multicentre Allergy Study Group. *Lancet* 2000; 356:1392–7.
- Bergmann RL, Berhmann KE, Lau-Schadendorf S et al. Atopic disease in infancy. The German Multicenter Atopy Study (MAS-90). *Ped Allergy Immunol* 1994; 5 (Suppl. 1):19–25.
- Nickel R, Wahn U, Hizawa N et al. Evidence for linkage of chromosome 12q15–q24.1 markers to high total serum IgE concentrations in children of the German Multicenter Allergy Study (MAS '90). *Genomics* 1997; 46:159–62.
- Mathias RA, Bickel CA, Beaty TH et al. A genealogical analysis of the Tangier Island, Virginia, population. *J Phys Anthropol* 2000; 112:29–38.
- Barnes KC, Freidhoff LR, Horowitz EM et al. Physician-derived asthma diagnoses made on the basis of questionnaire data are in good agreement with interview-based diagnoses and are not affected by objective tests. *J Allergy Clin Immunol* 1999; 104 (4 Part 1):791–6.
- Barnes KC, Neely JD, Duffy DL et al. Linkage of asthma and total serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. *Genomics* 1996; 37:41–50.
- Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; 52:506–16.
- Marceau F, Bachvarova M, Bergeron J et al. Characterization of a polymorphism in the coding region of the human C5a anaphylatoxin receptor. *Immunogenetics* 1999; 49:618–9.
- Quandt K, Frech K, Karas H et al. MatInd and MatInspector – new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acid Res* 1995; 23:4878–84.

- 39 Lazarus R, Vercelli D, Palmer LJ et al. Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. *Immunol Rev* 2002; 190:109–25.
- 40 Liu X, Beatty TH, Deindl P et al. Associations between total serum IgE levels and the six potentially functional variants within genes IL4, IL13 and IL4R α in German children: Multicenter Atopy Study (MAS). *J Allergy Clin Immunol* 2003; 112:382–8.
- 41 Okinaga S, Slattery D, Humbles A et al. C5L2, a nonsignaling C5A binding protein. *Biochemistry* 2003; 42:9406–15.
- 42 Humbles AA, Lu B, Nilsson CA et al. A role for C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 2000; 406:998–1001.
- 43 Bautsch W, Hoymann HG, Zhang Q et al. Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *J Immunol* 2000; 165:5401–5.
- 44 Walters DM, Breyse PN, Schofield B, Wills-Karp M. Complement factor 3 mediates particulate matter-induced airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2002; 27:413–8.
- 45 Polack FP, Teng MN, Collins PL et al. A role for immune complexes in enhanced respiratory syncytial virus disease. *J Exp Med* 2002; 196:859–65.