

ORIGINAL ARTICLE

Variants in the gene encoding C3 are associated with asthma and related phenotypes among African Caribbean families

KC Barnes¹, AV Grant², D Baltadzhieva¹, S Zhang¹, T Berg¹, L Shao¹, A Zambelli-Weiner¹, W Anderson³, A Nelsen³, S Pillai³, DP Yarnall³, K Dienger⁴, RG Ingersoll⁵, AF Scott⁵, MD Fallin², RA Mathias⁶, TH Beaty², JGN Garcia⁷ and M Wills-Karp⁴

¹Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, MD, USA;

²Department of Epidemiology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA; ³Glaxo-SmithKline, Research Triangle Park, NC, USA; ⁴Division of Immunobiology, Children's Hospital Research Foundation, Children's Hospital Medical Center, Cincinnati, OH, USA; ⁵McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA; ⁶Inherited Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Baltimore, MD, USA and ⁷Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, MD, USA

Proinflammatory and immunoregulatory products from C3 play a major role in phagocytosis, respiratory burst, and airways inflammation. C3 is critical in adaptive immunity; studies in mice deficient in C3 demonstrate that features of asthma are significantly attenuated in the absence of C3. To test the hypothesis that the C3 gene on chromosome 19p13.3–p13.2 contains variants associated with asthma and related phenotypes, we genotyped 25 single nucleotide polymorphism (SNP) markers distributed at intervals of ~1.9 kb within the C3 gene in 852 African Caribbean subjects from 125 nuclear and extended pedigrees. We used the multiallelic test in the family-based association test program to examine sliding windows comprised of 2–6 SNPs. A five-SNP window between markers rs10402876 and rs366510 provided strongest evidence for linkage in the presence of linkage disequilibrium for asthma, high log[total IgE], and high log[IL-13]/[log[IFN- γ]] in terms of global P-values ($P=0.00027$, 0.00013 , and 0.003 , respectively). A three-SNP haplotype GGC for the first three of these markers showed best overall significance for the three phenotypes ($P=0.003$, 0.007 , 0.018 , respectively) considering haplotype-specific tests. Taken together, these results implicate the C3 gene as a priority candidate controlling risk for asthma and allergic disease in this population of African descent.

Genes and Immunity (2006) 7, 27–35. doi:10.1038/sj.gene.6364267; published online 15 December 2005

Keywords: asthma; C3; total IgE; IL-13; INF- γ ; haplotype

Introduction

The acquired (adaptive) immune response, which relies upon antigen-specific responses of lymphocytes, depends upon signals provided by the more ancient innate immune system regarding the origin of the antigen (e.g., self vs non-self) and the type of response needed.^{1–4} Complement is a major effector of innate immunity, playing an essential role in differentiating between innocuous and harmful antigens.⁵ Specifically, activation products derived from C3 (C3a, C3b, and iC3b) play a major role in phagocytosis, respiratory burst, and inflammation (for a review, see Sunyer⁶). Paradoxically, C3 also plays a role in adaptive immunity

as foreign particles are taken up by complement receptors present on antigen-presenting cells (APCs) such as B lymphocytes and presented to T cells via major histocompatibility complex (MHC) class II molecules.^{7–9}

Allergic asthma is an excellent model for understanding the interface between the two multifaceted, immunological networks of innate and adaptive immunity. Asthma is a complex disease of the lungs characterized by airflow obstruction, airway hyper-responsiveness, and airway inflammation. Although asthma is multifactorial in origin, development of an IgE-mediated response to common aeroallergens is the single strongest identifiable predisposing factor for its development.^{10,11} IgE-mediated inflammation is characterized by the generation of IL-4 and IL-13 cytokines in response to allergen, which in turn promotes Th2-type lymphocytes that ultimately lead to inflammatory airway disease. Recent genetic linkage studies in mice have implicated the complement pathway in susceptibility to the development of allergen-driven airway hyper-responsiveness.¹² Specifically, a two base pair deletion

Correspondence: Dr KC Barnes, Johns Hopkins Asthma & Allergy Center – 3A.62A, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, USA.

E-mail: kbarnes@jhmi.edu

Received 14 June 2005; revised 16 August 2005; accepted 16 August 2005; published online 15 December 2005

in the C5 gene located on chromosome 2 is strongly negatively correlated with susceptibility to airway hyper-responsiveness, whereas the wild-type allele confers protection from allergen-driven airway hyper-responsiveness. On the other hand, the presence of C3 has been shown to confer susceptibility to allergic phenotypes when induced by several triggers of asthma including allergen exposure,¹³ RSV,¹⁴ and pollutant exposure.¹⁵ Interestingly, C5a has been shown to drive protective Th1 immune responses through the regulation of the Th1-cytokine, IL-12. On the other hand, C3-deficient mice have been shown to have impaired ability to produce Th2 cytokines in response to allergen challenge.¹³ Collectively, these murine studies suggest that members of the complement system provide an important link between the innate and adaptive immune responses, and that genes encoding the complement family members may be important candidate genes for human asthma.

Indeed, linkage studies on asthma and allergic disease have implicated a region on chromosome 19p, where the gene encoding C3 is located,^{16–18} and a recent study in a Japanese population supported an association between a single C3 variant and a four-single nucleotide polymorphism (SNP) haplotype and bronchial asthma.¹⁹ The C3 gene covers 42.78 kb, from position 6 628 878 to 6 671 660 (NCBI build 35, February 10, 2005) on the reverse strand, and consists of 41 exons.²⁰ Thus, to explore the potential role of SNPs in C3 and risk to human disease, we genotyped a panel of SNPs in the C3 gene to test for association with asthma and allergic disease in a well-characterized set of asthmatic families from a population of African descent. We performed extensive haplotype-based analyses on these markers to further test for linkage in the presence of disequilibrium, a gene controlling risk of asthma and two related quantitative phenotypes, log(IgE) and the ratio of log[IL-13]/[log[IFN- γ]] (Table 1).

Results

The summary statistics for study participants are shown in Table 2. As demonstrated elsewhere,²¹ the mean total IgE concentration (geometric mean of 639 ± 4.6 ng/ml) in the Barbados population is considerably higher than the generally accepted, clinical cutpoint of 242 ng/ml. Age and gender adjusted concentrations among asthmatics (1023.3 ± 3.8 ng/ml) were significantly higher than among nonasthmatics (457.1 ± 4.6 ng/ml; $P < 0.001$). Asthmatics were younger than nonasthmatics (23.1 ± 13.3 and 41.1 ± 15.1 , respectively; $P < 0.001$) as expected, since probands were typically offspring, but there was no significant difference according to gender in the two groups. Thus, even after adjusting for age as a confounding factor, log(tIgE) levels showed statistically significant differences between asthmatics and nonasthmatics.

Unlike with log[total IgE], there were no effects of age or gender on log[serum IL-13] nor log[serum IFN- γ] levels. There was no significant difference in IL-13 levels between asthmatics (731 ± 1033 pg/ml) and nonasthmatics (596 ± 1390 pg/ml; $P = 0.128$), nor were there significant differences in IFN- γ levels when comparing asthmatics (414.3 pg/ml) and nonasthmatics (344 pg/ml;

$P = 0.614$). Neither log[serum IL-13] nor log[serum IFN- γ] levels were correlated with log[total IgE] levels. Serum IL-13 and IFN- γ concentrations were highly correlated with each other ($r = 0.6037$, $P < 0.0001$). When IL-13 and IFN- γ levels were analyzed as the log[IL-13]/log[IFN- γ] ratio, we did not find significant correlation with log[IgE] ($r = -0.0436$, $P = 0.28$).

Single-marker analyses

Figure 1 illustrates the pairwise linkage disequilibrium (LD) between each of the 25 C3 markers using the program HaploView. Markers spanned 46 193 bp and revealed five blocks (clusters) of eight, two, three, two, and two SNPs, respectively, as defined by the algorithm of Gabriel *et al.*²² This block structure resulted in eight orphan SNPs with the longest stretch of five individual SNPs all with little or no LD between the last two blocks (rs163913, rs2230205, rs421147, rs237554, rs2241392, rs344548, rs2241393, rs344550).

In two-point tests for linkage and LD between each of the 25 SNPs individually and traits associated with allergic disease, family-based association test (FBAT) analyses showed only modest evidence for linkage and LD between several markers and the different phenotypes (vertical lines in Figure 2). Tests of individual SNPs with these three phenotypes are illustrated in Figure 2 by vertical lines, but no SNP showed a P -value < 0.01 , and these vertical lines do not form any distinct region of strong evidence. The only individual marker to demonstrate significant evidence of linkage and LD with asthma was rs393770 ($Z = 2.03$, $P = 0.043$). This same marker, in addition to rs2287848 and rs2230205, showed modest evidence for linkage and LD with higher log[total IgE] levels ($Z = 2.03$, $P = 0.042$; $Z = 2.51$, $P = 0.012$; and $Z = 2.24$, $P = 0.025$, respectively). Marker rs2287848 also showed evidence for the log[IL-13]/log[IFN- γ] ratio ($Z = 2.21$, $P = 0.027$) phenotype, as did marker rs344550 ($Z = 2.89$, $P = 0.004$).

Haplotype analyses

Sliding window haplotype analysis for 2–6 SNPs simultaneously was performed across these 25 markers in the C3 gene. In the interest of brevity, only a subset of the results is presented in Figure 2 and Table 3. Figure 2 illustrates the overall tests of linkage and LD across all markers using four-, five-, and six-marker haplotypes, indicating a peak between markers rs436041 and rs2642207, spanning 9094 bp and overlapping contiguous blocks 1, 2, and 3 revealed in the patterns of LD shown in Figure 1. Over all haplotype windows for asthma and the quantitative traits log[total IgE] and log[IL-13]/[log[IFN- γ]], the lowest P -values for global tests of linkage and LD were 0.00027, 0.00013, 0.003, respectively. These corresponded to the five-SNP window between markers rs10402876 and rs366510. In terms of LD, rs10402876, the first SNP in the five-SNP window, is contained in block 2 and the other SNPs are included in block 1. Marker rs10402876, however, shows high LD with its neighboring SNP ($D' = 0.95$) within block 1. Importantly, this region could not have been identified without considering haplotypes in a windowing approach, and would have been missed completely if single-SNP tests had been used to identify markers for further analysis.

At the 3' end of C3, our windowing approach gave rise to another weaker signal of linkage and LD for the four-

Table 1 C3 single nucleotide polymorphisms (SNPs)

Marker	Public location (Build 35)	Inter-SNP distance (bp)	Allele (frequency) ^a	Type (amino-acid position)
rs379527 ^b	6 627 442	N/A	T (0.859) G (0.141)	3' UTR
rs4807893 ^b	6 628 989	1547	A (0.509) G (0.491)	Exon 41 (P1632P)
rs344550 ^b	6 633 953	4964	C (0.659) G (0.341)	Intron 33
rs2241393 ^c	6 636 304	2351	C (0.718) G (0.282)	Intron 29
rs344548 ^c	6 636 817	513	G (0.667) C (0.333)	Intron 29
rs2241392 ^c	6 636 983	166	C (0.580) G (0.420)	Intron 29
rs237554 ^d	6 637 659	676	G (0.896) A (0.104)	Intron 28
rs344543 ^b	6 638 414	755	C (0.566) G (0.434)	Intron 27
rs8108377 ^b	6 639 860	1446	C (0.634) A (0.366)	Intron 27
rs421147 ^b	6 642 424	2564	A (0.706) C (0.294)	Intron 26
rs438703 ^b	6 643 790	1336	A (0.702) T (0.298)	Intron 26
rs436041 ^b	6 643 796	6	C (0.683) G (0.317)	Intron 26
rs432001 ^c	6 644 683	887	A (0.691) G (0.309)	Intron 24
rs393770 ^c	6 644 885	202	A (0.741) G (0.259)	Intron 24
rs10402876 ^b	6 646 001	1116	G (0.687) C (0.313)	Intron 23
rs10410674 ^b	6 647 178	1177	G (0.772) A (0.228)	Intron 23
rs2287848 ^b	6 647 342	164	C (0.853) T (0.147)	Intron 23
rs423490 ^d	6 648 406	1064	G (0.758) A (0.242)	Exon 21 (A915A)
rs366510 ^b	6 648 829	423	T (0.700) G (0.300)	Intron 19, within splicing Lariat.
rs2642207 ^b	6 652 890	4061	G (0.693) T (0.307)	Intron 19
rs408290 ^b	6 653 022	132	C (0.687) G (0.313)	Intron 19
rs428453 ^b	6 653 157	135	C (0.693) G (0.307)	Exon 19 (V807V)
rs432823 ^b	6 653 246	89	G (0.694) A (0.306)	Intron 18, 2 bases from putative Splicing branch point
rs2230205 ^b	6 660 704	7458	G (0.802) A (0.108)	Exon 14 (T612T)
rs163913 ^d	6 673 635	12 931	C (0.844) T (0.156)	5'UTR

^aMinor allele frequency (MAF) based on African Caribbean founders in the Barbados sample ($N = 270$).

Genotyping platform:

^bLuminex™.

^cABI TaqMan™.

^dIllumina™.

SNP window encompassing markers rs379527, rs4807893, rs344550, and rs2241393 for both log[total IgE] and log[IL-13]/[log[IFN- γ]], with global $P = 0.0067$ and $P = 0.0085$, respectively, but not for asthma. The spacing between these markers is greater than in the first peak region, with a mean distance between SNPs of 3.0 kb. For log[IL-13]/[log[IFN- γ]], a single-SNP test of

linkage and LD for rs344550 was actually more significant than the haplotype results at $P = 0.0039$.

Results from examining these tests for linkage and LD to determine specific 'high-risk' haplotypes are presented in Table 3 for both haplotype-specific and global P -values, focusing on the largest peak illustrated in Figure 2. Haplotype analyses implicated the 'GGCAG'

Table 2 Descriptive characteristics of the Barbados study sample

	All subjects (N = 852)	Founders (N = 268)	Asthmatics (N = 316)	Nonasthmatics (N = 446)
Mean age (years)	34 (8–92)	49 (31–86)	22 (8–90)	40 (10–92)
Male gender (%)	49.53	52.61	52.53	46.64
Asthmatics (%)	37.09	7.84	100	0
log[total IgE] ± s.d. ^a	2.79 ± 0.66	2.78 ± 0.66	3.00 ± 0.58	2.66 ± 0.67
sIL-13 ± s.d. ^b	615.56 ± 1181.02	606.79 ± 1405.25	719.02 ± 1030.01	577.54 ± 1332.61
sIFN-γ ± s.d. ^b	356.86 ± 1049.52	328.71 ± 1158.63	419.24 ± 1011.82	349.92 ± 1160.13
IL-13:IFN-γ ratio ± s.d. ^b	3.72 ± 5.81	3.89 ± 6.08	3.86 ± 5.47	3.59 ± 5.79

^aLog-transformed total IgE concentrations measured in ng/ml, age–sex-adjusted.

^bSerum IL13 and serum IFN-γ concentrations measured in pg/ml. Concentrations of both cytokines were not normally distributed and values were log-transformed before analyses; concentrations were not confounded by age or gender.

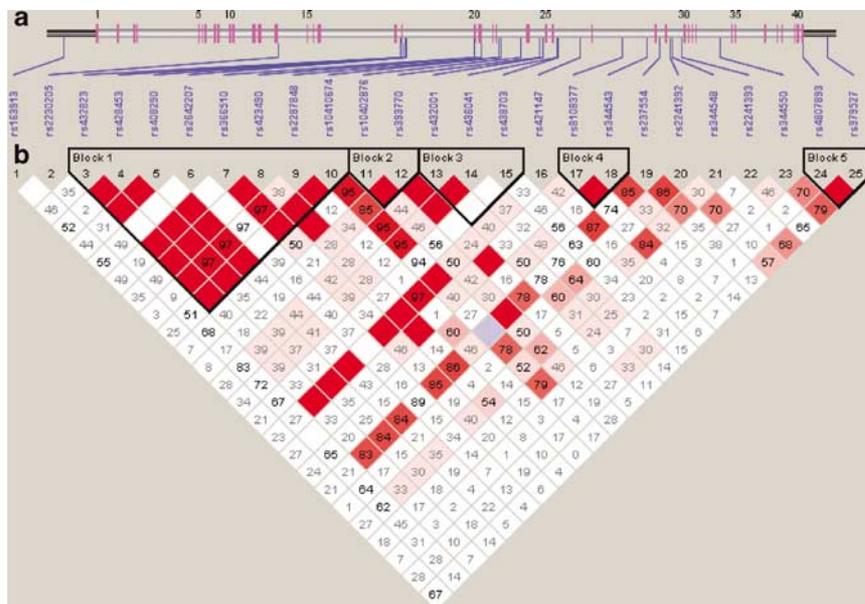


Figure 1 Genomic structure and pairwise D' for 25 SNPs in the gene C3. (a) Illustration of the C3 gene structure indicating exons, introns, and flanking region spanning 3.5 kb. Positioning of the 25 SNPs, referred to by reference sequence numbers, are indicated by vertical lines. Solid regions denote exons, open boxes denote untranslated regions, and introns are shown as connecting lines. The gene is transcribed from left to right. All features are shown to scale. (b) Pairwise LD in 270 or 25 variants in C3. The 25 SNPs are aligned to the pairwise LD plot. Pairwise LD was estimated between each of the markers using the program HaploView (Julian Maller, Developer/MIT). Red squares illustrate strong LD; blue squares illustrate nonsignificant LD; white squares illustrate little/no LD. Four distinct blocks were identified.

haplotype in the five-SNP window (which also had the strongest global P -values; see Figure 2 and Table 3), between markers rs10402876 and rs366510, as being a high-risk haplotype for asthma, log[total IgE] and log[IL-13]/[log[IFN-γ]], with haplotype-specific P -values of 0.028, 0.017, and 0.019, respectively. This haplotype had a frequency of 13.7% among founders.

Smaller subsets of this five-SNP haplotype also showed strong evidence of linkage and LD. The strongest evidence of linkage and LD occurred with a three-SNP haplotype, and building towards the five-SNP haplotype by serially adding a single SNP up- or downstream. A three-SNP haplotype 'GGC' consisting of the first three SNPs in the five-SNP haplotype that had a frequency of 63.6% yielded strongest evidence of linkage and LD for asthma and log[total IgE] ($P = 0.003$ and 0.007 , respectively) considering all specific at-risk haplotypes over all windows within the peak region. Extending these three SNPs to a four- and five-SNP haplotype by going up- or downstream reduced evidence for linkage and LD. When

SNPs were considered in a six-SNP haplotype up- or downstream from 'GGCAG,' evidence for linkage and LD also diminished.

Contrary to this clear pattern for a 'high-risk' haplotype, a similar process applied to search for a 'low-risk' haplotype revealed no single consensus haplotype as protective for these phenotypes (data not shown). While some haplotypes may have a protective effect on asthma and its associated traits, adding an additional SNP up- or downstream typically resulted nonsignificant findings. Moreover, these haplotypes were all present at low frequencies (ranging from 1.3 to 6.2%) for the five-SNP region (rs10402876–rs366510) discussed above. Taken together, these results implicate this 19p region represented by the 'GGCAG' haplotype as a priority region for future searches for causal variants in this population of African descent, and this region can possibly be narrowed down to the section where the first three alleles are 'GGC' considering the combined results of our haplotype analysis.

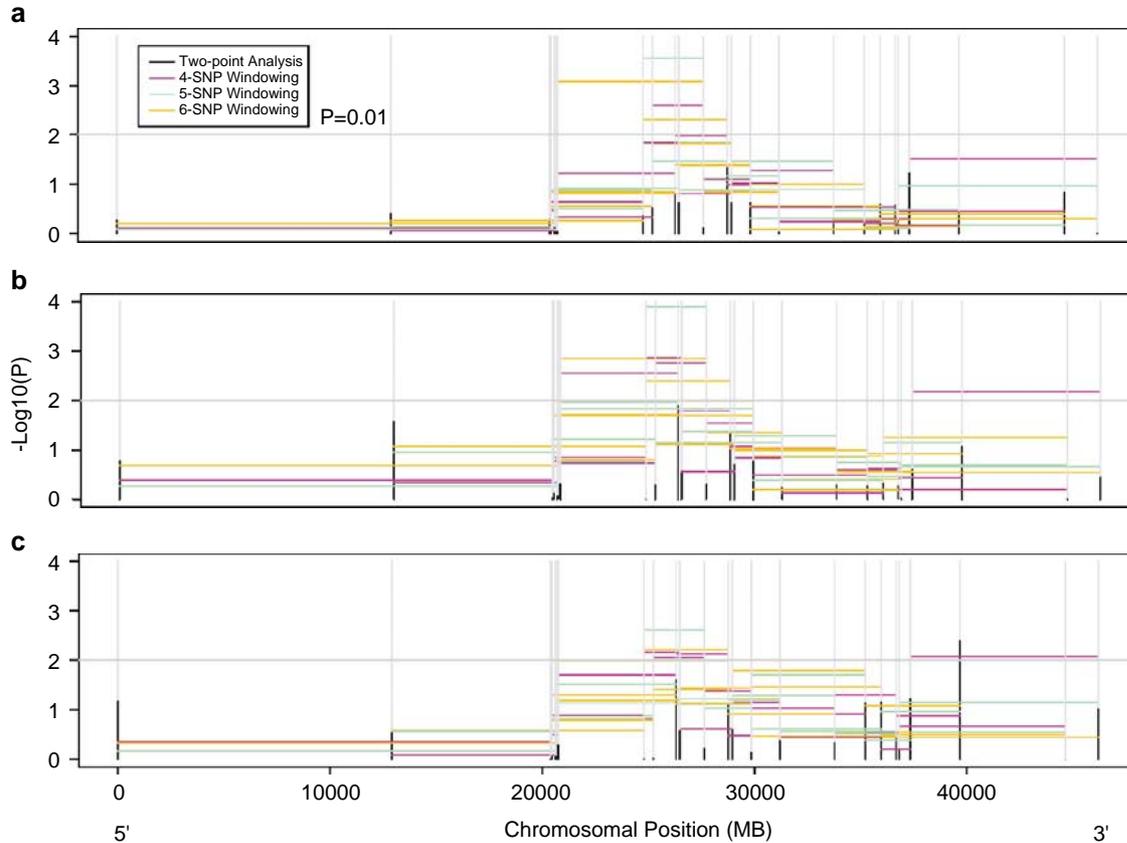


Figure 2 FBAT results showing global $-\log(P)$ against chromosomal location for (a) asthma; (b) $\log[\text{total IgE}]$ concentrations; and (c) $\log[\text{IL-13}]/\log[\text{IFN-}\gamma]$. Two-point and haplotype results for four-, five-, and six-marker windows across the C3 gene are illustrated. Gray vertical lines represent all individual SNPs. Bold vertical lines represent level of significance by two-point analyses. Purple horizontal lines represent all possible 4-marker haplotypes; green horizontal lines represent all possible 5-marker haplotypes; and orange horizontal lines represent all possible 6-marker haplotypes.

Discussion

Based on the important role that the innate immune mediator C3 has been shown to play in murine models of asthma, we explored the potential role of the C3 gene in human asthma. In this study, we identified 25 SNPs from the public database that provided good coverage across the C3 gene (~46 kb; average inter-SNP distance 1925 bp) and which were polymorphic in a population of African descent. Single-marker analyses in 125 African Caribbean families (852 subjects) selected for asthma demonstrated only modest associations between several of the markers and three phenotypes: asthma, serum total IgE levels, and IL-13:IFN- γ concentrations ($P=0.04-0.004$). Two of those individual markers, rs393770 and rs2287848, flanked a 9094 bp region identified from sliding-window haplotype approach by testing 2–6-SNP haplotypes across all 25 markers. A five-SNP haplotype between markers rs10402876 and rs366510 (GGCAG) provided the strongest evidence for linkage and LD to asthma, high $\log[\text{total IgE}]$ and high $\log[\text{IL-13}]/\log[\text{IFN-g}]$ ($P=0.028, 0.017, \text{ and } 0.019$, respectively) for the global test of significance, and was present in 13.7% of founders. A smaller haplotype comprising GGC at the first three markers appeared to constitute a ‘high-risk’ haplotype for asthma ($P=0.003$) ‘high’ tIgE, ($P=0.007$), and ‘high’ IL-13:IFN- γ ratio ($P=0.018$), considering results across all phenotypes.

Since the addition of alleles up- or downstream from this GGCAG haplotype reduced statistical evidence for association, we speculate that this 2.8 kb region can be narrowed down further to the 1.3 kb spanning the first three SNPs and might contain a causal variant(s) relevant to allergic phenotypes. Notably, this C3 gene would not have been identified if the sliding window haplotype approach had not been utilized.

A review of the public database (build 124, dbSNP; <http://www.ncbi.nlm.nih.gov/SNP>) identified two synonymous and one nonsynonymous SNPs within this genomic region; the nonsynonymous (R863K) SNP (rs11569472) was submitted to the public database after our initial set of SNPs was selected, where it was reported at a frequency of only 2.1% (genotype frequency, GG = 95.8% and GA = 4.2%) in 24 African American individuals (12 males/12 females) selected from the human variation panel of 50 African Americans (HD50AA) from the Coriell Cell Repository (http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?pop=595). As a result of the very low frequency of this variant, we only genotyped an initial group of 64 Barbados founders and found that this marker was not polymorphic. No further analyses of this marker were conducted. Thus, this R863K variant cannot be the source of association with asthma and associated phenotypes in this African Caribbean population. All other SNPs genotyped in this study falling within the

Table 3 Haplotypes in the C3 gene associated with asthma, higher log[tIgE], and log[IL-13]/log[IFN-g]

rs436041	rs432001	rs393770	rs10402876	rs10410674	rs2287848	rs423490	rs366510	rs2642207	f	Asthma		log[tIgE]		log[IL-13]/log[IFN-g]	
										P	p _{global}	P	p _{global}	P	p _{global}
			G	G					0.702	0.112		0.106		0.097	
				G	C				0.655	0.077		0.045 *		0.026 *	
					C	A			0.156	0.378		0.184 *		0.118 *	
						A	G		0.200	0.641		0.643 *		0.307 *	
G	G	A							0.335	0.067 *		0.046 *		0.086 *	
	G	A	G						0.262	0.110		0.030		0.045	
			G	G	C				0.636	0.003 ***		0.007 ***		0.018 **	
				G	C	A			0.152	0.153		0.097 *		0.046 *	
					C	A	G		0.141	0.027 **		0.004 ***		0.008 **	
	A	G	G	C					0.428	0.035 *		0.085 *		0.081 **	
			G	G	C	G			0.470	0.063 **		0.085 **		0.231 **	
			G	G	C	A			0.150	0.287 **		0.228 **		0.150 **	
				G	C	A	G		0.141	0.012 *		0.005 **		0.004 **	
G	A	G	G	C					0.258	0.015 *		0.014 *		0.024	
	A	G	G	C	A				0.119	0.202 *		0.357		0.020 *	
		G	G	C	A	G			0.137	0.028 ***		0.017 ***		0.019 **	
G	G	A	G	G	C				0.272	0.025 *		0.013 *		0.017	
	G	A	G	G	C	G			0.133	0.022 *		0.077 **		0.292 *	
	G	A	G	G	C	A			0.118	0.253 *		0.338 *		0.026 *	
		A	G	G	C	A	G		0.109	0.076 **		0.073 **		0.010 **	
			G	G	C	A	G	T	0.127	0.146 ***		0.084 **		0.101 *	

*0.01 < P < 0.05.

** 0.001 < P < 0.01

***P < 0.001.

haplotypes of interest were intronic except for one which was a synonymous variant (rs423490 leading to A915A). The variant rs366510 is near the splicing branch point, but does not appear to be highly conserved, so it is not likely to contribute to the function of any spliceosome. Another possible region of interest is the C3 cleavage site into C3b (exons 1–16) and C3a (exon 16 to the end). Our coverage of SNPs in this region was sparse, and we did not observe evidence of linkage in the presence of disequilibrium for SNPs flanking the cleavage site. Of interest, a previous study demonstrated significant association between asthma and markers in exon 14.¹⁹ Further studies are warranted.

Our findings are consistent with previous studies implicating C3 in asthma pathogenesis. Specifically, data from mice deficient in C3 reveal diminished airway hyper-responsiveness, lung eosinophil levels, and IL-4 production in antigen-challenged lungs as well as reduced antigen-specific IgE and IgG1 responses, and clearly demonstrate that these features of asthma are significantly attenuated in the absence of C3.¹³ Furthermore, C3 has also been implicated in both pollutant-¹⁵ and RSV-induced airway hyper-responsiveness¹⁴ in mouse models. Interestingly, C3 has been shown to be cleaved into its active state by exposure to many of the important triggers of allergic airway disease such as cigarette smoke,²³ house dust mite,²⁴ and by certain airborne pollutants.¹⁵ Our finding that a C3 haplotype in this study was strongly associated with asthma and two related Th2-phenotypes (serum IgE levels and IL-13:IFN- γ ratio) is consistent with some role for the innate mediator C3 in driving the adaptive immune responses. Indeed, C3 levels are elevated in the lungs of asthmatics following segmental allergen challenge.²⁵ Chromosomal regions containing the human C3 gene have also been previously linked with asthma and allergic disease in several different human populations.^{16–18} Moreover, recent findings in a Japanese population implicated both a high-risk and a low-risk SNP haplotype in C3 for bronchial asthma.¹⁹ Taken together, these data provide strong support for C3 as an asthma candidate gene.

In the only other study on C3 variants and asthma, Hasegawa *et al.*¹⁹ demonstrated significant associations between markers in C3 using both a pediatric and adult asthma case–control population from Japan. Specifically, they observed their strongest evidence for association in both groups between asthma and marker rs4807893 ($P=0.010–0.0078$). In the current study, we found evidence for linkage and LD between a four-SNP window including rs4807893 and flanking markers and log[total IgE] (global $P=0.0067$) and log[IL-13]/[log[IFN- γ]] (global $P=0.0085$), but not for asthma. Differences between our findings and the Japanese study may be due to differences in the degree to which these African Caribbean and the Japanese subjects were atopic. After SNPs were selected and genotyped for this study, there were several additional SNPs with high minor allele frequencies in populations of African descent in this region of C3 reported in the public database; our findings indicate that future studies should include these newly deposited SNPs, despite their lower than optimal MAFs since they may be highly relevant to these traits analyzed in this population.

Although we focused exclusively on variants in C3 itself here, it is possible that variants in other genes

important in downstream signaling of the active C3 fragments may also be important. Previous animal studies have demonstrated that mice deficient in both C3 and C3aR have lower airway hyper-reactivity^{13,25,26} and Hasegawa *et al.*¹⁹ also observed an association between a variant in the C3a receptor gene (C3AR1) and severity of childhood asthma. It is also possible that epistatic effects of variants in other genes within the complement pathway (i.e., C5) may contribute further to susceptibility.

In summary, we have demonstrated evidence for linkage and disequilibrium between SNPs in the C3 gene and asthma plus two related quantitative phenotypes in an African Caribbean family study. Using a systematic approach to build haplotypes across 25 SNPs, we have demonstrated the presence of a putative ‘high-risk’ haplotype within a narrow three- to five-SNP window in this region of C3. Findings from this study support a key functional role for C3 in asthma and the Th-2-mediated inflammatory response as measured by total serum IgE and cytokines IL-13 and IFN- γ . Furthermore, these studies highlight the potentially important contribution of polymorphisms in mediators of the innate immune response to dysregulated adaptive immune response such as those observed in human asthma. Future studies to identify the causal variant(s) that focus on the possible effects on gene function will further elucidate the role of the complement system on the pathogenesis of asthma and allergic disease.

Subjects and methods

Subjects

Families were recruited in Barbados, the West Indies, as a part of an ongoing asthma genetics study described elsewhere.^{27,28} Briefly, asthmatic probands were recruited from six polyclinics, two private clinics, and from the Accident and Emergency Department (A&ED) of Queen Elizabeth Hospital (QEH). Patients with a positive family history of asthma or one or more asthmatic siblings were referred by physicians cooperating with on-site study investigators at the University of the West Indies. Nuclear families were recruited and extended when possible. Asthma was defined as (1) a reported history of asthma using a standardized questionnaire,²⁹ (2) a history of physician-diagnosed asthma (past/current), and (3) a reported history of wheezing without an upper respiratory infection (URI) or two out of the following four symptoms: wheezing with a URI, cough without a URI, shortness of breath, and tightness of chest.

Peripheral blood samples were collected from all subjects. Sera were processed and total serum IgE was measured as previously described.²⁷ Briefly, total serum IgE levels were measured in duplicate using the immunochemiluminometric Magic Lite assay (Magic Lite Total IgE Extended Range; CIBA-Corning, Medfield, MA, USA). IgE values were adjusted for both age and gender, then log-transformed to minimize skewness. Serum levels of IL-13 and IFN- γ were measured on all samples by ELISA using specific anti-IL-13 and anti-IFN- γ mAbs obtained from BD Biosciences (San Diego, CA, USA) according to the manufacturer’s instructions. To approximate a normal distribution, serum IL-13 and IFN- γ levels were log-transformed. All participants

except for one parent described their ethnicity as African Caribbean. Informed consent was obtained from all subjects following a protocol approved by the Johns Hopkins Medical Institutions' Joint Committee on Clinical Investigations (JCCI) and the Ministry of Health in Barbados. FBATs on the phenotypes 'asthma', $\log[\text{total IgE}]$, and $\log[\text{IL-13}]/\log[\text{IFN-}\gamma]$ were performed on 852 persons from 125 pedigrees (for both nuclear and extended).

Genotyping

DNA was extracted using standard protocols. Whole genome amplification (WGA) of DNA was prepared by OmniPlex Technology (Rubicon Genomics Inc., Ann Arbor, MI, USA). We used a combined approach to genotype variants in the C3 gene. Initially three SNPs in C3 (rs163913, rs237554, and rs423490) were genotyped using the BeadArray™ technology at Illumina™ (Illumina, San Diego, CA, USA) as part of a high-throughput genotyping study on this Barbados population. In addition to these three SNPs genotyped on the Illumina™ platform, we selected five C3 variants to be genotyped by TaqMan probe-based 5' nuclease assay using Assays on Demand™ (Applied Biosystems, Foster City, CA, USA) because of their ready availability. An additional 92 SNPs were identified, of which 63 were excluded from further analysis because of (1) low minor allele frequency (<10%) in populations of African descent (according to <http://pga.gs.washington.edu/data/c3/database>); (2) redundancy (proximity to another candidate SNP); and/or (3) failure to develop a successful assay. Four SNPs were dropped because they were not in Hardy-Weinberg equilibrium (HWE) (see *Statistical analyses* below). These additional SNPs were genotyped by flow cytometric analysis performed on a Luminex™ 100 flow cytometer as described elsewhere.³⁰ After all SNPs were genotyped and analyzed, a nonsynonymous SNP (rs11569472) that fell within the most significant (five-SNP) haplotype (see Results and Discussion) was genotyped by the TaqMan probe-based 5' nuclease assay using Assays by Design™ (Applied Biosystems, Foster City, CA, USA). However, this marker was not polymorphic in 64 founders and was therefore dropped from further studies.

Figure 1a illustrates the distribution of 25 selected SNPs relative to exons, introns and the 5' and 3' flanking regions for the C3 gene. We identified only four SNPs from the public database in a coding sequence (rs2230205 [Ex14], rs428453 [Ex19], rs423490 [Ex21], rs4807893 [Ex41]); none of which led to an amino-acid substitution. In addition, one SNP in the 3' UTR (rs379527) and one SNP in the 5' UTR (rs163913) were genotyped. The average distance between these SNPs was 1925 bp (ranging 6–12 931 bp). Locations of the 25 SNPs according to the National Center for Biotechnology Information human genome assembly Build 35 are listed in Table 1.

Statistical analyses

Mean age, $\log[\text{total IgE}]$, sIL-13, sIFN- γ levels and mean values for the IL-13:IFN- γ ratio were obtained among all study subjects, founders, and asthmatics and nonasthmatics using STATA 8.2 (Stata Corporation, College Station, TX, USA). Multiple linear regression models were used to test for the effect of age and gender

on $\log[\text{serum IL-13}]$ and $\log[\text{serum IFN-}\gamma]$ levels, and Pearson's correlation coefficients were calculated for the two log-transformed cytokine levels independently and as a ratio and with $\log[\text{IgE}]$. These analyses were conducted using STATA 8.2 (Stata Corporation, College Station, TX, USA).

The SIB-PAIR (v0.99.9) program (<http://www2.qimr.edu.au/davidD/>) was used to detect Mendelian inconsistencies, and genotypes where transmission was found to be inconsistent were set as missing. Departures from HWE proportions defined by χ^2 tests with P -values < 0.01 at each locus were tested among the 270 founders using SIB-PAIR to compare observed vs expected genotype frequencies. Pairwise LD was measured using HaploView (<http://www-genome.wi.mit.edu/personal/jcbarret/haplo>). The composite null hypothesis of no linkage or no LD between each marker and an unobserved gene controlling risk to the discrete phenotype asthma, and two quantitative phenotypes $\log[\text{total IgE}]$ and the ratio of $\log[\text{IL-13}]/\log[\text{IFN-}\gamma]$ was tested using the FBAT.^{31,32} All FBAT analyses were conducted under an additive model, since there was no prior information about a specific dominant or recessive model of inheritance for these phenotypes.

Haplotype analyses were conducted on 25 C3 markers. In FBAT, parental haplotypes are inferred from all genotype information within the nuclear family, and an iterative EM algorithm was used to resolve ambiguous phase. Possible phased genotypes were weighted for all individuals and used to calculate haplotype frequencies among founders. Sliding windows comprising 2–6 SNPs were examined using the multiallelic test in FBAT where all possible haplotypes for sets of contiguous SNPs are considered together as a multiallelic marker, yielding a χ^2 test with degrees of freedom corresponding to the number of distinct haplotypes. Empiric P -values were calculated through permutation analysis using Monte Carlo methods under the null hypothesis of complete independence between haplotypes and the observed phenotypes. FBAT's sampling procedure stops when at least 100 Monte Carlo-based test statistic values are generated and these 100 values are less than the observed test statistic or after 100 000 replicate samples are generated, whichever comes first. This empiric, permutation approach was chosen given the large number of tests performed.

Acknowledgements

We thank all the families and volunteers who graciously participated in this study. We are especially grateful to Dr Kathryn Held, Ms Sharon Patterson, Caitlin Dorer, and Kimberly Donnelly for fieldwork, and to Drs Paul Levett, Raana Naidu, and Harold Watson for facilitating the Barbados study. We thank Robert Munford for helpful discussions and Bo Zhang, Melba Muñoz, and Pat Oldewurtel for technical assistance. This work was supported by National Institutes of Health (NIH) Grants U01 HL66583 and HL67736, and an Allergy and Asthma Foundation of America Young Investigator Award. KCB was supported in part by the Mary Beryl Patch Turnbull Scholar Program.

References

- 1 Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science* 1996; **272**: 50–54.
- 2 Medzhitov R, Janeway CAJ. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 1997; **9**: 295–298.
- 3 Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA *et al*. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002; **416**: 194–199.
- 4 Medzhitov R, Janeway CAJ. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002; **296**: 298–300.
- 5 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**: 348–350.
- 6 Sunyer JO, Boshra H, Lorenzo G, Parra D, Freedman B, Bosch N. Evolution of complement as an effector system in innate and adaptive immunity. *Immunol Res* 2003; **27**: 549–564.
- 7 Jacquier-Sarlin MR, Gabert FM, Villiers MB, Colomb MG. Modulation of antigen processing and presentation by covalently linked complement C3b fragment. *Immunology* 1995; **84**: 164–170.
- 8 Villiers MB, Villiers CL, Jacquier-Sarlin MR, Gabert FM, Journet AM, Colomb MG. Covalent binding of C3b to tetanus toxin: influence on uptake/internalization of antigen by antigen-specific and non-specific B cells. *Immunology* 1996; **89**: 348–355.
- 9 Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. *Ann Rev Immunol* 1998; **16**: 545–568.
- 10 Burrows B, Martinez FD, Haonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989; **320**: 271–277.
- 11 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Ann Rev Immunol* 1999; **17**: 255–281.
- 12 Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ *et al*. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000; **1**: 221–226.
- 13 Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol* 2001; **167**: 4141–4145.
- 14 Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H *et al*. A role for immune complexes in enhanced respiratory syncytial virus disease. *J Exp Med* 2002; **196**: 859–865.
- 15 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**: 413–418.
- 16 Blumenthal MN, Ober C, Beaty TH, Bleecker ER, Langefeld CD, King RA *et al*. Genome scan for loci linked to mite sensitivity: the Collaborative Study on the Genetics of Asthma (CSGA). *Genes Immunity* 2004; **5**: 226–231.
- 17 Lee YA, Wahn U, Kehrt R, Tarani L, Businco L, Gustafsson D *et al*. A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. *Nat Genet* 2000; **26**: 470–473.
- 18 Venanzi S, Malerba G, Galavotti R, Lauciello MC, Trabetti E, Zanoni G *et al*. Linkage to atopy on chromosome 19 in north-eastern Italian families with allergic asthma. *Clin Exp Allergy* 2001; **31**: 1220–1224.
- 19 Hasegawa K, Tamari M, Shao C, Shimizu M, Takahashi N, Mao XQ *et al*. Variations in the C3, C3a receptor, and C5 genes affect susceptibility to bronchial asthma. *Hum Genet* 2004; **115**: 295–301.
- 20 Fong KY, Botto M, Walport MJ, So AK. Genomic organization of human complement component C3. *Genomics* 1990; **7**: 579–586.
- 21 Barnes K, Hizawa N, Neely J, Duffy D, Freidhoff L, Ehrlich E *et al*. Linkage analysis of atopy to chromosome 12q markers in an Afro-Caribbean population. *J Allergy Clin Immunol* 1997; **99**: S476.
- 22 Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B *et al*. The structure of haplotype blocks in the human genome. *Science* 2002; **296**: 2225–2229.
- 23 Kew RR, Ghebrehiwet B, Janoff A. Characterization of the third component of complement (C3) after activation by cigarette smoke. *Clin Immunol Immunopathol* 1987; **44**: 248–258.
- 24 Maruo K, Akaike T, Ono T, Okamoto T, Maeda H. Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease. *J Allergy Clin Immunol* 1997; **100**: 253–260.
- 25 Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y *et al*. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 2000; **406**: 998–1001.
- 26 Bautsch W, Hoymann HG, Zhang Q, Meier-Wiedenbach I, Raschke U, Ames RS *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**: 5401–5405.
- 27 Barnes KC, Neely JD, Duffy DL, Freidhoff LR, Breazeale DR, Schou C *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.
- 28 Barnes KC, Freidhoff LR, Nickel R, Chiu Y-F, Joo S-H, Hizawa N *et al*. Dense mapping of chromosome 12q13.12–q23.3 and linkage to asthma and atopy. *J Allergy Clin Immunol* 1999; **104**: 485–491.
- 29 Barnes KC, Freidhoff LR, Horowitz EM, Mathias RA, Mulkern DM, Bonacum J *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**: 791–796.
- 30 Taylor JD, Briley D, Nguyen Q, Long K, Iannone MA, Li MS *et al*. Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. *Biotechniques* 2001; **30**: 661–666; 668–669.
- 31 Horvath S, Wei E, Xu X, Palmer LJ, Bauer M. Family-based association test method: age of onset traits and covariates. *Genet Epidemiol* 2001; **21**: S403–S408.
- 32 Rabinowitz D, Laird N. A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered* 2000; **50**: 211–223.