TNF polymorphism and bronchoalveolar lavage cell TNF-α levels in chronic beryllium disease and beryllium sensitization

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Background: Beryllium stimulates TNF-α from chronic beryllium disease (CBD) bronchoalveolar lavage (BAL) cells.
Objective: We sought to relate TNF polymorphisms to beryllium-stimulated TNF-α production, to the development of CBD, and to the risk of more severe CBD over time.
Methods: We recruited 147 patients with CBD, 112 beryllium-sensitized subjects, and 323 control subjects; genotyped 5 TNF promoter polymorphisms; and measured beryllium-stimulated and unstimulated BAL cell TNF-α production from a subset of subjects.
Results: Beryllium-stimulated, but not beryllium-unstimulated, BAL cell TNF-α production was significantly increased in patients with CBD compared with that seen in those only sensitized ($P = .0002$). Those subjects with the TNF −857T allele and the only haplotype (haplotype 4) containing this allele demonstrated significantly lower unstimulated BAL cell TNF-α production compared with that seen in noncarriers ($P = .009$). Patients with CBD alone and combined with sensitized subjects carrying the TNF haplotype 1 compared with those without this haplotype had significantly increased beryllium-stimulated BAL cell TNF-α levels ($P = .02$). We found no significant association between patients with CBD, sensitized subjects, and control subjects with any of the TNF promoter polymorphisms or haplotypes. A greater decrease in Pao_2 at maximum exercise was noted in patients with CBD with the −1031C allele ($P = .03$) and with haplotypes other than the TNF haplotype 1 ($P = .01$), 3 (from 5) of which contain the −1031C allele.
Conclusions: The −857T allele and haplotype 1 are associated with BAL cell TNF-α production, indicating a potential role of TNF promoter variants in regulation of TNF production in sensitized subjects and patients with CBD.
Clinical implications: TNF promoter variants are not risk factors for CBD or sensitization. (J Allergy Clin Immunol 2007;119:687-96.)

Key words: *TNF-α, chronic beryllium disease, beryllium sensitization, berylliosis, genetics, polymorphism, bronchoalveolar lavage*

Beryllium exposure in the workplace triggers a beryllium-specific immune response in a minority of those exposed. Specifically, 2% to 10% of exposed workers experience sensitization to beryllium, as demonstrated by a positive response on the beryllium lymphocyte proliferation test (BeLPT) in which their lymphocytes proliferate in response to beryllium *in vitro*.[1,2] Previous studies have shown that beryllium antigen–stimulated T-cell proliferation is MHC class II restricted.[3] Of those with a proliferative response on the BeLPT, some are only beryllium sensitized (BeS), demonstrating an abnormal BeLPT result with no evidence of pulmonary pathology on lung biopsy or physiologic abnormalities. After development of sensitization, some have the granulomatous lung disease chronic beryllium disease (CBD). Granulomatous inflammation is found on lung biopsy with a range of disease manifestations. There are limited physiologic or radiologic abnormalities in a subset, whereas others have evidence of an overwhelming inflammatory response to beryllium, with significant physiologic impairment and evidence of fibrosis on chest radiography. An indicator of the overwhelming inflammatory response in CBD is markedly increased and sustained TNF-α production by CBD bronchoalveolar lavage (BAL) cells.[4,5]

Recent studies have suggested that genetic factors influence an individual's ability to mount an immune response to beryllium. An increased prevalence of HLA-DPB1 alleles containing a glutamic acid at amino acid position 69 (Glu69) has been found in BeS subjects and patients with CBD.[6-9] However, this same polymorphism

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Abbreviations used
BAL: Bronchoalveolar lavage
Be: Beryllium
BeLPT: Beryllium lymphocyte proliferation test
BeS: Beryllium sensitized
CBD: Chronic beryllium disease
NF: Nuclear factor
Pao_2m: Pao_2 at maximal exercise
SNP: Single nucleotide polymorphism

can be found in up to 40% of workers exposed to beryllium without evidence of sensitization or disease,[6] suggesting that other susceptibility factors might be involved in the development of sensitization and disease.

Beryllium antigen stimulates TNF-α production by BAL cells from patients with CBD,[5,10] and in a previous study we have shown that the TNF promoter A allele at the −307 position was associated with greater TNF-α production.[4] Recently, Dotti et al[11] reported that the prevalence of the TNF –307A and TNF –857T alleles was significantly increased in subjects with BeS with and without CBD compared with that seen in beryllium-exposed control subjects without disease, whereas Gaede et al[12] reported that the TNF –307A allele was increased in patients with CBD from the United States.

In the context of these studies, we hypothesized that other TNF promoter polymorphisms would contribute to the magnitude of the beryllium-stimulated BAL cell TNF-α response and subsequent risk of development of CBD and its severity. The aims of this study were therefore (1) to assess the contribution of TNF promoter polymorphisms as risk factors for CBD compared with BeS subjects and beryllium-exposed nondiseased control subjects and their relationship to CBD disease severity and (2) to investigate the association between BAL cell TNF-α production and the same TNF polymorphisms.

METHODS

Study design

Three cohorts[13,14] of beryllium-exposed individuals were studied, and all provided informed consent: patients with CBD (n = 147), BeS subjects (n = 112), and control subjects with beryllium exposure without evidence of sensitization or disease (beryllium nondiseased cohort, n = 323). Full details are included in the Online Repository at www.jacionline.org.

BeS subjects were enrolled if they had evidence of sensitization demonstrated by 2 abnormal BeLPT results or a positive beryllium skin patch test result and no evidence of granulomatous inflammation on transbronchial lung biopsy. Patients with CBD were enrolled if they demonstrated sensitization as above or an abnormal BAL lymphocyte proliferation test and evidence of granulomatous inflammation on lung biopsy.[15] Beryllium nondiseased control subjects were defined as individuals employed in a beryllium industry who had at least one normal blood BeLPT result and no abnormal BeLPT results.

Patients with CBD and BeS subjects underwent clinical evaluation at the time of diagnosis and on follow-up evaluations conducted every 1 to 2 years thereafter, as previously described.[15,16] Control subjects underwent venipuncture and BeLPT. Full details are included in the Online Repository at www.jacionline.org.

Sequence-specific primers and PCR

Genomic DNA from all subjects was extracted from peripheral blood cells by using the Wizard Genomic Purification kit (Promega, Madison, Wis). A total of 5 TNF promoter single nucleotide polymorphisms (SNPs) were identified: −1031(T/C, rs1799964), −863(C/A, rs1800630), −857(C/T, rs1799724), −307(G/A, rs1800629), and −237(G/A, rs361525). The details of primer sequences and PCR conditions were previously described.[17] HLA genotyping was carried out using the method of Bunce and Welsh.[18,19] Haplotype assignment was undertaken with the sophisticated computer program PHASE.[20]

TNF-α protein levels

Freshly isolated BAL cells from patients with CBD and BeS subjects were suspended at 1.0×10^6 cells per milliliter and cultured alone or in the presence of 100 μmol/L $BeSO_4$.[14] Cell supernatants were collected at 24, 48, and 72 hours after stimulation. TNF-α protein concentrations were evaluated by using an ELISA (R&D System, Minneapolis, Minn).[4] The peak production of TNF-α was chosen as the highest level of TNF-α concentration at or after 24 hours.[5,14]

Data analysis

The genotype, phenotype, and allele frequencies were determined by means of direct counting for patients and control subjects. All genotype frequencies were tested for Hardy-Weinberg equilibrium stratified by race.

Continuous variables were compared by using the Student *t* test or the Wilcoxon rank sum test, as appropriate. For 3-way comparisons, the Kruskal-Wallis test was used. Categoric variables were analyzed by using χ^2 and Fisher exact methods. Either a 3-way comparison of patients with CBD, BeS subjects, and control subjects or a 2-way comparison combining patients with CBD and BeS subjects versus control subjects was used to analyze the phenotypic frequencies. To assess the contribution of different genotypes and demographic variables on TNF-α concentration, we used multiple linear regression. Those variables significantly associated with TNF-α production log normalized in univariate analysis were entered into the model by using a stepwise method. Mixed-effects models were used to assess the relationship between TNF polymorphisms and severity of disease in white subjects measured on the basis of longitudinal lung function variables. The time variable used in the longitudinal analysis was time from first exposure to beryllium. Age at test, sex, smoking history, and height were included in the model, along with the allele of interest. Statistical analysis was performed by using JMP-SAS or SAS software (SAS Institute, Cary, NC). All tests were 2-sided.

RESULTS

Demographics of patients and control subjects

The demographics of the patients with CBD, BeS subjects, and beryllium nondiseased control subjects are shown in Table I. On average, the patients were younger than the control subjects ($P < .05$). Current smoking status was different between patients with CBD and BeS subjects ($P < .05$), with the BeS cohort having a greater frequency of current smokers than patients with CBD and control subjects.

TNF polymorphisms and haplotypes and risk of sensitization and disease

Table II summarizes the genotype frequencies of the 5 TNF promoter polymorphisms in the patients with CBD,

TABLE I. Comparison of demographic characteristics among patients with CBD, BeS subjects, and beryllium nondiseased subjects

	Patients with CBD	BeS subjects	Beryllium control subjects
Cases	147	112	323
Sex			
Male	130 (88%)	90 (80%)	286 (88.5%)
Female	17 (12%)	22 (20%)	37 (11.5%)
Race			
White	142 (96.6%)	102 (91.1%)	301 (93.2%)
African American	5 (3.4%)	5 (4.5%)	10 (3.1%)
Asian	0 (0.0%)	3 (2.7%)	2 (0.70%)
American Indian	0 (0.0%)	2 (1.8%)	0 (0.0%)
Hispanic	14 (10.5%)	12 (10.7%)	36 (11.1%)
Age at diagnosis or blood draw (y)	50.3 (27-75)	51 (32-73)	60.5 (38-86)*
Industry			
Nuclear weapons	129 (91.5%)	105 (98.1%)	307 (95.0%)
Beryllium machining facility	12 (7.8%)	2 (1.9%)	16 (5.0%)
Smoking status			
Current	17 (12.1%)	31 (28.4%)†	27 (12.6%)
Former	67 (47.5%)	45 (41.3%)	144 (67.3%)
Never	33 (30.3%)	57 (30.4%)	43 (20.1%)
Treatment			
Ever	37 (25.0%)	0 (0.0%)	NA

NA, Not applicable.
*Age differed between patients with CBD and control subjects and BeS subjects and control subjects ($P < .05$).
†Current smoking status differed significantly between BeS subjects and patients with CBD and BeS subjects and control subjects ($P < .05$) but not between patients with CBD and control subjects.

TABLE II. Genotype frequencies of TNF-α promoter polymorphisms in patients with CBD, BeS subjects, and beryllium nondiseased subjects

		All subjects			White subjects			Hispanic subjects		
		Patients with CBD (n = 147)	BeS subjects (n = 112)	Beryllium control subjects (n = 323)	Patients with CBD (n = 128)	BeS subjects (n = 90)	Beryllium control subjects (n = 275)	Patients with CBD (n = 14)	BeS subjects (n = 12)	Beryllium control subjects (n = 36)
Genotype										
−1031	T/T	61.2	56.3	64.7	64.8	54.4	64	28.6	50	69.4
	T/C	36.1	37.5	31.9	32.8	40	32.8	64.3	33.3	25
	C/C	2.7	6.2	3.4	2.4	5.6	3.2	7.1	16.7	5.6
−863	C/C	73.4	67.9	72.7	75.8	67.8	75.8	50	58.3	77.8
	C/A	25.9	28.6	25.4	23.4	28.9	23.4	50	33.3	22.2
	A/A	0.7	3.5	1.9	0.8	3.3	0.8	0	8.4	0
−857	C/C	82.3	85.7	78.6	81.2	85.6	80.0	85.7	83.3	63.9
	C/T	17	14.3	19.5	18	14.4	18.2	14.3	16.7	33.3
	T/T	0.7	0	1.9	0.8	0	1.8	0	0	2.8
−307	G/G	70.1	64.3	70.3	71.1	64.4	68.7	64.3	66.7	83.3
	G/A	27.9	35.7	27.2	26.6	35.6	29.1	35.7	33.3	11.1
	A/A	2	0	2.5	2.3	0	2.2	0	0	5.6
−237	G/G	87.1	86.6	91.3	91.6	89.1	91.6	71.4	83.3	86.1
	G/A	12.2	13.4	8.4	8	14.4	8	28.6	16.7	13.9
	A/A	0.7	0	0.3	0.4	0	0.4	0	0	0

The values are presented as percentages.

BeS subjects, and beryllium nondiseased control population. All populations were in Hardy-Weinberg equilibrium for all genotype frequencies. No significant differences were found among these 3 groups when the analysis was applied to all subjects or to the white population alone.

We next constructed haplotypes from the 5 TNF polymorphisms and were able to define 6 TNF promoter haplotypes, as shown in Table III. The frequencies of the 6 TNF haplotypes in patients with CBD, BeS subjects, and beryllium nondiseased control subjects were not significantly different.

TABLE III. TNF haplotype frequencies in patients with CBD, BeS subjects, and beryllium nondiseased subjects

						Patients with CBD (n = 147)	BeS subjects (n = 112)	Beryllium control subjects (n = 323)
TNF haplotype	−1031	−863	−857	−307	−237			
1	T	C	C	G	G	120 (81.6%)	85 (75.9%)	252 (77.4%)
2	T	C	C	A	G	44 (29.9%)	40 (35.7%)	96 (29.7%)
3	C	A	C	G	G	39 (26.5%)	36 (32.1%)	88 (27.2%)
4	T	C	T	G	G	26 (17.7%)	16 (14.3%)	69 (21.4%)
5	C	C	C	G	A	19 (12.9%)	15 (13.4%)	28 (8.7%)
6	C	C	C	G	G	1 (0.7%)	1 (0.9%)	2 (0.6%)

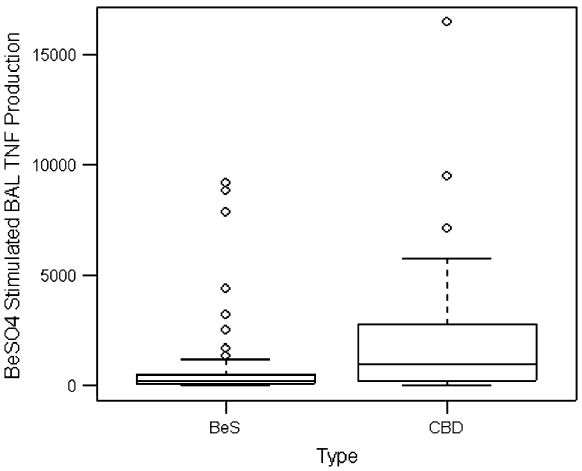

FIG 1. TNF-α production by BAL cells from patients with CBD and BeS subjects after simulation with $BeSO_4$. There was a significant difference between patients with CBD (n = 68; 968 pg/mL [range, 0-16444 pg/mL]) and BeS subjects (n = 69; 204 pg/mL [range, 0-914 pg/mL], $P = .0002$). The values are presented as medians (ranges).

BAL cell TNF-α production and TNF promoter polymorphisms

As has been previously published, there was a significant difference between median $BeSO_4$-stimulated BAL cell TNF-α production in patients with CBD (n = 68; 968 pg/mL [range, 0-16444 pg/mL]) compared with that seen in BeS subjects (n = 69; 204 pg/mL [range, 0-9141 pg/mL], $P = .0002$, Fig 1, Table IV), but no difference was found with unstimulated BAL cell TNF-α production (0 pg/mL [range, 0-5173 pg/mL] for patients with CBD and 9 pg/mL [range, 0-7349 pg/mL] for BeS subjects, Table IV, $P = .2$) and with LPS-stimulated TNF-α production (30,538 pg/mL [range, 361-1,120,412 pg/mL] for patients with CBD and 34,657 pg/mL [range, 554-156,664 pg/mL] for BeS subjects, $P = .3$; data not shown). Limiting the analysis to white subjects only, the $BeSO_4$-stimulated BAL cell TNF-α production was significantly increased in patients with CBD (968 pg/mL [range, 0-16,444 pg/mL]) compared with that seen in BeS subjects (214 pg/mL [range, 0-9141 pg/mL], $P = .001$). Although the trends were similar, there was no significant difference between patients with CBD and BeS subjects in the African American ($P = .5$) and Hispanic ($P = .5$) populations (Table IV).

Table V (and Table E1 in the Online Repository at www.jacionline.org) summarizes the association between TNF promoter polymorphisms, haplotypes, and unstimulated BAL cell TNF-α production. Strikingly, the unstimulated BAL cell TNF-α production was lower in carriers of the TNF –857T allele and TNF haplotype 4, which is the only haplotype to include the −857T allele (n = 19; 0 pg/mL [range, 0-82 pg/mL]) compared with noncarriers of this allele and haplotype (n = 116; 116 pg/mL [range, 0-7349 pg/mL], $P = .009$, Fig 2). Fifteen (78.9%) of 19 −857T allele carriers showed no unstimulated BAL cell TNF-α production.

Table VI (and Table E2 in the Online Repository at www.jacionline.org) summarizes the association between TNF polymorphisms, haplotypes, and $BeSO_4$-stimulated cell TNF-α production. $BeSO_4$-stimulated BAL cell TNF-α production was increased in individuals who carried TNF haplotype 1 (n = 105; 451 pg/mL [range, 0-16444 pg/mL]) compared with haplotype 1 noncarriers (n = 32; 193 pg/mL [range, 0-5784 pg/mL], $P = .02$, Fig 3). We analyzed the patients with CBD and BeS subjects separately and observed the same association in the CBD population ($P = .04$). No other alleles or haplotypes showed significant association with TNF-α production. If we stratify by race, this association is no longer statistically significant ($P = .11$ in white subjects, Table E2 in this article's Online Repository at www.jacionline.org).

HLA-DPB1 Glu69 is not associated with TNF-α production

The frequencies of HLA DPB1-Glu69 carriages were 94.1% for CBD and 92.6% for BeS in patients for whom TNF-α production data were available. There was no difference between $BeSO_4$-stimulated BAL cell TNF-α production in Glu69 carriers (340 pg/mL [range, 0-16,444 pg/mL]) compared with that seen in non-Glu69 carriers (821 pg/mL [range, 0-3233 pg/mL]).

TNF polymorphisms and disease severity

Markers of disease severity for CBD were evaluated for associations with TNF-α alleles and haplotypes in white subjects (Table VII). Comparisons were made when the counts of the alleles or haplotypes were sufficient to make the comparison meaningful and if clinical data were available for the subjects in question to ensure the model converged. Of the white subjects with CBD, 110 of the 128 had at least 1 clinical visit to include in the

TABLE IV. $BeSO_4$-stimulated BAL cell TNF-α production in patients with CBD and BeS subjects

	Patients with CBD		BeS subjects		
	N	TNF-α level (pg/mL)*	N	TNF-α level (pg/mL)*	*P* value†
Unstimulated					
All subjects	68	0 (0-5173)	67	9 (0-7349)	.2
White subjects	57	0 (0-5173)	50	9 (0-5815)	.2
Hispanic subjects	7	118 (0-771)	9	0 (0-7349)	.4
Black subjects	3	0 (0-28.6)	5	35 (0-617)	.09
	CBD		**BeS**		
	N	**TNF-α level (pg/mL)***	**N**	**TNF-α level (pg/mL)***	***P* value**
$BeSO_4$ stimulated					
All subjects	68	968 (0-16444)	69	204 (0-9141)	.0002
White subjects	58	968 (0-16444)	51	214 (0-9141)	.001
Hispanic subjects	7	1480 (0-3749)	9	204 (0-7842)	.5
Black subjects	3	563 (223-4104)	5	279 (97-3233)	.5

*Median (range).
†Wilcoxon rank sum tests were used to compare the continuous variables.

TABLE V. Unstimulated BAL cell TNF-α production (in picograms per milliliter), TNF promoter polymorphisms, and haplotypes in patients with CBD and BeS subjects combined

	Carrier		Noncarrier		
	N	TNF-α level (pg/mL)*	N	TNF-α level (pg/mL)*	*P* value†
Allele					
−1031T	129	0 (0-7349)	6	9.7 (0-204)	.9
−1031C	58	0 (0-5815)	77	0 (0-7349)	.6
−863C	132	0 (0-7349)	3	19.3 (0-204)	NA
−863A	42	0 (0-4193)	93	4260 (0-7349)	.8
−857C	135	0 (0-7349)	0	NA	NA
−857T	19	0 (0-82)	116	0 (0-7349)	.009
−307G	132	0 (0-7349)	3	0 (0-21.6)	NA
−307A	50	16.6 (0-7349)	85	0 (0-5815)	.2
−237G	134	0 (0-7349)	1	0	NA
−237A	17	0 (0-5815)	118	0 (0-7349)	.6
Haplotype					
1	103	0 (0-7349)	32	0 (0-872)	.4
2	50	16.6 (0-7349)	85	0 (0-5815)	.2
3	42	0 (0-4193)	93	0 (0-7349)	.8
4	19	0 (0-82)	116	0 (0-7349)	.009
5	17	0 (0-5815)	118	0 (0-7349)	.6
6	1	0	134	0 (0-7349)	NA

NA, Not applicable.
*Median (range).
†Wilcoxon rank sum tests were used to compare the continuous variables.

analysis. On average, these CBD participants had 4.4 (SD, 3.8) clinical visits. Only clinical data visits up until the time that the patients with CBD were started on corticosteroids are included in this analysis because the effects of corticosteroids on the relevant indices of CBD severity are not known. Comparisons were made between the carriage of the rarer allele in a genotype versus noncarriage at all 5 loci of the TNF promoter polymorphisms and TNF haplotype 1 (−1031 CC/CT [n = 40] vs TT [n = 70], −863 AA/AC [n = 29] vs CC [n = 81], −857 TT/CT [n = 23] vs CC [n = 87], −307 AA/AG [n = 31] vs GG [n = 79], and −237 AA/AG [n = 12] vs GG [n = 98]) and having at least 1 haplotype 1 (n = 20) versus not having a haplotype 1 (n = 90). The data are shown in Table VII and reflect the difference in the changes of lung function index over time (10-40 years since first exposure to beryllium) between the 2 genotypes of interest. Patients with CBD with a TNF −1031C allele on average had a greater loss of FEV_1 (average, 0.01 L/y lower than the −1031TT genotype over the 10- to 40-year period from first exposure, $P = .06$) and Pao_2 at maximal exercise (Pao_2m; 0.39 mm Hg/y lower than the −1031TT genotypes over the 10 to 40 years from first exposure, $P = .03$) over time than patients without a TNF –1031C allele at this marker (Fig 4). Also, patients with CBD without a haplotype 1 had on average a greater reduction in Pao_2m

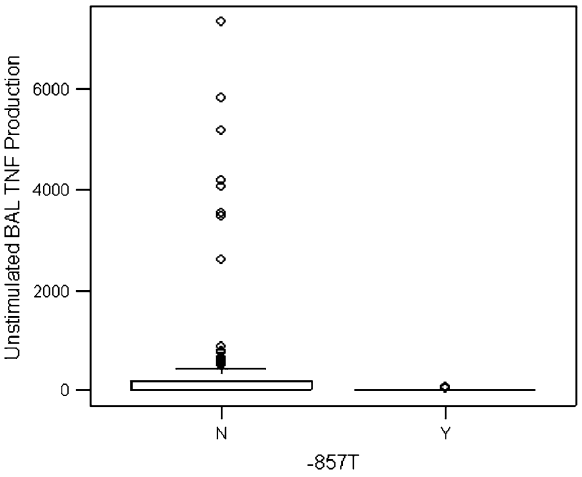

FIG 2. Unstimulated BAL cell TNF-α production in TNF −857T allele carriers and noncarriers. There was a significant difference between the −857T allele carriers (n = 19; 0 pg/mL [range, 0-82 pg/mL]) and noncarriers (n = 116; 116 pg/mL [range, 0-3543 pg/mL]; P = .009). The values are presented as medians (ranges). *Y*, TNF −857T allele carrier; *N*, TNF −857T allele noncarrier.

over time (0.57 mm Hg/y lower than the haplotype 1 subjects over 10 to 40 years from first exposure, P = .01). No associations were noted with the other measures of lung function over time.

DISCUSSION

CBD is a T_H1-mediated granulomatous lung disease restricted by HLA-DP and characterized by an overwhelming production of IL-2 and IFN-γ,[21] along with TNF-α by BAL cells.[4,22] Our previous work indicated that higher beryllium-stimulated BAL cell TNF-α production was associated with more severe symptoms and with a TNF promoter polymorphism, −307A.[4] This was a pilot study with a small number of patients with CBD and insufficient power to address the role of other TNF promoter polymorphisms in TNF-α production and disease severity. In the current study we found that in patients with CBD and BeS subjects, unstimulated BAL cell TNF is associated with carriage of the TNF −857T allele and haplotype 4, which contains the −857T allele. In contrast, TNF haplotype 1 (−1031T, −863C, −857C, −307G, and −237G, a combination of wild-type alleles) carriage was associated with beryllium-stimulated TNF-α production in patients with CBD alone (P = .04) and with BeS subjects and patients with CBD analyzed together (P = .02). Despite these associations, we found no TNF promoter polymorphisms or haplotypes associated with CBD or BeS compared with control subjects. We found associations between the −1031C allele and noncarriage of TNF haplotype 1, which includes the −1031T allele, and more progressive lung function decreases over time.

TNF is an important modulator in granulomatous inflammation. Our current study and previous results support the significant role of TNF in patients with CBD with increased production from CBD beryllium-stimulated BAL cells.[5,14] A number of studies suggest that SNPs in the TNF promoter region are functional, mediating TNF-α transcription and protein production. In our current study the −857T SNP and its haplotype 4 are associated with *de novo* or unstimulated BAL cell TNF-α production. Although the total number of individuals who carried the −857T SNP was small, unstimulated BAL cell production of TNF-α was absent in 78.9%. This indicates that the TNF −857T allele might be protective for TNF-α production. The functional effect of the TNF −857 locus has not been well studied, although a recent study demonstrated increased LPS-stimulated peripheral blood cell TNF from −857CC homozygotes. It also showed that the transcription factor OCT1, which interacts *in vivo* with nuclear factor (NF) κB, only binds the −857T allele, but not the −857C allele, which might inhibit nuclear translocation of NF-κB in the case of the −857T allele.[23] It is not known whether this mechanism explains the spontaneously reduced TNF production from BAL cells from BeS subjects and patients with CBD carrying the −857T allele in this study. Previous work suggests that unstimulated BAL cell TNF is a product of macrophages in contrast to beryllium-stimulated TNF, which is primarily produced by $CD4^+$ T cells.[24] Interestingly, neither the −857T allele nor haplotype 4 were associated with beryllium-stimulated TNF in our study.

TNF haplotype 1 was associated with higher beryllium-stimulated TNF-α, although this would not be significant if corrected for multiple comparisons. Interestingly, the association with haplotype 1 and TNF-α protein levels was not limited to the patients with CBD but was also noted in BeS subjects. These results are contrasted to our previous observation, in which the −307A allele was associated with higher TNF-α production.[4] However, our current results are based on a larger samples size and are not surprising in light of our current understanding of TNF regulation. Other studies to date have provided conflicting results regarding the effect of individual TNF promoter SNPs on TNF production,[25-27] even with the most studied TNF promoter polymorphic site, −307.[28,29] A recent study by Knight et al[30] evaluated TNF mRNA expression and did not find differential expression between the −307 G or A SNPs. They did find differential expression between extended TNF and lymphotoxin-α (LTα) haplotypes but only assessed a limited number of TNF SNPs.

It might be an oversimplification to expect that beryllium-stimulated TNF production would be explained solely by TNF promoter variants when TNF production is controlled at many different levels: it is regulated by factors that affect transcription, signal transduction pathways that converge on NF-κB, translation, and posttranslational regulation. Our previous work established that the $CD4^+$ BAL T cells and not BAL macrophages are the principal source of beryllium-stimulated CBD BAL TNF-α protein production.[24] We know that IL-10 can downregulate beryllium-stimulated TNF-α,[31] and it is likely other beryllium-specific and nonspecific factors might affect

TABLE VI. Median of $BeSO_4$-stimulated BAL cell TNF-α production (in picograms per milliliter), TNF promoter polymorphisms, and haplotypes

	All subjects					Patients with CBD					BeS				
	Carrier		Noncarrier			Carrier		Noncarrier			Carrier		Noncarrier		
	N	Median	N	Median	*P* value	N	Median	N	Median	*P* value*	N	Median	N	Median	*P* value
Allele															
−1031T	131	365	6	159	.1	66	989	2	512	NA	65	210	4	77	.2
−1031C	79	408	58	335	.9	31	997	37	956	.6	27	157	42	303	.3
−863C	134	363	3	153	NA	68	968	0	NA	NA	66	207	3	153	.6
−863A	42	310	95	408	.6	21	997	47	956	.7	21	157	48	303	.3
−857C	137	361	0	NA	NA	68	968	0	NA	NA	69	204	0	NA	NA
−857T	19	30	118	390	.4	9	1298	59	956	NA	10	170	59	210	.3
−307G	134	363	3	215	NA	65	980	3	215	NA	69	204	0	NA	NA
−307A	51	372	80	340	.8	28	692	40	1157	.4	24	320	45	201	.3
−237G	136	350	1	858	NA	67	980	1	858	NA	69	204	0	NA	NA
−237A	17	365	120	350	.8	11	858	57	980	.9	6	216	63	204	.6
Haplotype															
1	105	451	32	193	.02	50	1286	18	301	.04	55	214	14	143	.2
2	52	372	85	340	.7	28	692	49	1158	.4	24	320	45	201	.3
3	42	310	95	408	.6	21	997	47	956	.7	21	158	48	303	.3
4	19	300	118	390	.4	9	1298	59	956	1	10	170	59	210	.3
5	17	365	120	350	.8	11	858	57	980	.9	6	216	63	204	.6
6	1	134	136	363	NA	0	NA	68	968	NA	1	134	68	207	NA

*Wilcoxon rank sum tests were used to compare the continuous variables.

beryllium-stimulated TNF production. Finally, we found no association between BAL cell TNF-α protein levels and HLA-DPB1Glu69, suggesting that unstimulated and beryllium-stimulated TNF production is independent of this shared epitope.

Recently, Dotti et al[11] reported that the prevalence of the TNF –307A and TNF –857T alleles was increased significantly in both patients with CBD (n = 30) and BeS subjects (n = 43) combined compared with beryllium-exposed control subjects (n = 43), whereas no associations were noted with −238, −863, or −1031 SNPs. Using some of the same US patients with CBD, Gaede et al[12] confirmed the association between the TNF –307A allele in US patients with CBD (n = 39) but not in patients with CBD from Europe and Israel. Using the largest population of patients with CBD and BeS subjects to date, we did not find any TNF polymorphisms or haplotypes associated with patients with CBD, BeS subjects, or the 2 combined, similar to results from another US group (personal communication, Erin McCanlies, National Institute of Occupational Health and Safety). The lack of association could be due to misclassification bias; that is, it is likely that some control subjects who are currently without beryllium sensitization and CBD might go on to experience these health effects, whereas those with beryllium sensitization might progress to CBD in the future. These differences in subject classification would affect the genotype frequencies and potentially obscure any real association should it exist. Difference in HLA-DRB1 associations with CBD and beryllium sensitization might account for the varying results because some DRB1 alleles are in linkage disequilibrium with the TNF gene and a high percentage of the US patients with CBD in both the Dotti et al[11] and Gaede et al[12] studies were associated with HLA-DR genotypes not found in other studies to date.[6,9,32,33]

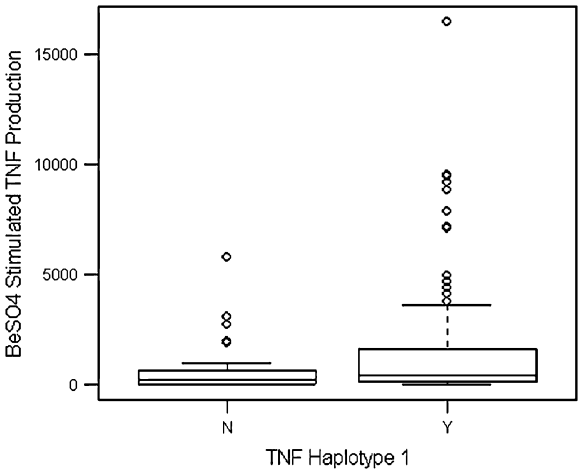

FIG 3. $BeSO_4$-stimulated BAL cell TNF-α concentrations in TNF haplotype 1 carriers and noncarriers. There was a significant difference between the TNF haplotype 1 carriers (n = 105; 451 pg/mL [range, 0-16,444 pg/mL]) and noncarriers (n = 32; 193 pg/mL [range, 0-5784 pg/mL]; *P* = .02). The values are given as medians (ranges). *Y*, TNF haplotype 1 carrier; *N*, TNF haplotype 1 noncarrier.

TNF promoter polymorphisms have been associated with severity of disease, including the granulomatous lung diseases hypersensitivity pneumonitis[34] and sarcoidosis.[17,35] The −307A allele has been observed at increased frequency in patients with sarcoidosis with Lofgren syndrome, a less severe form of the disease with spontaneous resolution,[17,29] whereas other studies have found

TABLE VII. Estimates of influence of TNF variants on pulmonary function severity indicators over time in white patients with CBD

Variant	DLCOU*	FEV_1*	FVC*	FEV_1/FVC ratio*	TLC*	Pao_2b*	Pao_2m*
TNF –1031	mL/min/mm Hg/y	L/y	L/y	Per year	L/y	mm Hg/y	mm Hg/y
CC/CT vs TT	−0.15 (0.09)	−0.01 (0.007)	−0.004 (0.009)	−0.15 (0.10)	0.01 (0.01)	−0.03 (0.13)	−0.39 (0.18)
P value	.12	.06	.61	.12	.24	.83	.03
TNF –863							
CC vs AA/AC	0.15 (0.12)	0.01 (0.009)	0.003 (0.01)	0.17 (0.11)	−0.02 (0.01)	−0.04 (0.17)	−0.07 (0.22)
P value	.19	.16	.77	.14	.13	.80	.75
TNF –857							
CC vs TT/TC	−0.11 (0.12)	−0.01 (0.008)	−0.01 (0.01)	0.007 (0.11)	0.0009 (0.01)	0.12 (0.14)	−0.23 (0.22)
P value	.38	.23	.26	.95	.94	.40	.31
TNF –307							
GG vs AA/AG	0.01 (0.11)	0.0001 (0.008)	−0.0004 (0.01)	0.02 (0.11)	−0.01 (0.01)	0.02 (0.13)	0.03 (0.19)
P value	.91	.99	.96	.85	.41	.90	.86
TNF haplotype 1							
Noncarrier vs carrier	−0.07 (0.13)	0.01 (0.009)	0.01 (0.01)	0.10 (0.12)	0.02 (0.02)	−0.17 (0.29)	−0.57 (0.23)
P value	.58	.22	.30	.42	.16	.29	.01

The values were presented as estimates (SE; value per year).
DLCOU, Diffusion capacity for carbon monoxide uncorrected for hemoglobin; *FVC*, forced vital capacity; *TLC*, total lung capacity; *Pao_2b*, Pao_2 at baseline; *Pao_2m*, Pao_2 at maximal exertion.
*Adjusted by sex, height, smoking history (current, former, never), and age at test.

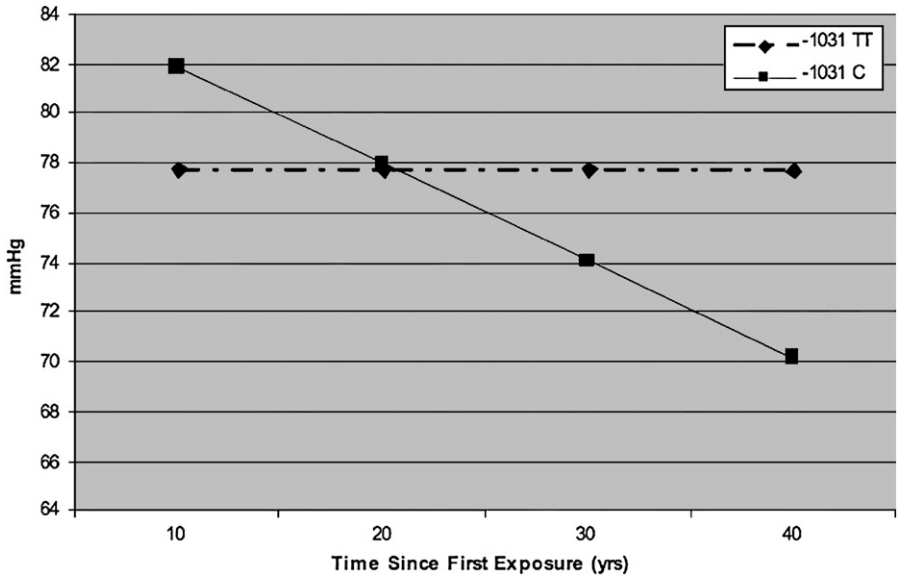

FIG 4. Change in Pao_2m values (in millimeters of mercury per year) by −1031 genotype CC/CT *(solid line)* versus TT *(dashed line)* over time modeled between 10 and 40 years since first beryllium exposure (P = .03) adjusted by sex, height, smoking history (current, former, never), and age at test.

associations between the −307A allele and more severe phenotypes, such as cardiac disease.[36] We were able to assess all of the SNPs and their associations with lung function over time; because of the limited size of the population, changes in lung function could only be analyzed by haplotype 1 over time. We found limited associations with the TNF −1031C allele and those who did not carry the TNF haplotype 1, with lower Pao_2m values over time on average (P = .03 and P = .01, respectively). The −1031C allele was associated with a trend toward lower FEV_1 values over time. Because pulmonary function variables tend to be intercorrelated, we were surprised that we did not find more associations with other lung function variables. This might suggest that these findings represent a false-positive association with multiple comparisons being performed; we did not use Bonferroni correction for multiple comparisons for the assessment of disease severity because it is too conservative when the outcome measures are intercorrelated. We limited this analysis to white subject because of small numbers and inability to analyze the other racial and ethnic groups separately. Ideally, we would have adjusted the results using

population stratification and genomic control because race and ethnicity might affect these variables. Regardless, these results do demonstrate our ability to assess disease progression over time with polymorphisms and haplotypes, which will be important for future genetic studies of disease severity.

In conclusion, our study suggests that constitutive and beryllium-stimulated BAL production is in part determined by TNF polymorphisms and haplotypes. Specifically, the TNF −857T allele is associated with downregulated constitutive production of BAL cell TNF-α, which is probably from macrophages, whereas the TNF haplotype 1 is associated with upregulated beryllium-stimulated BAL cell TNF-α production, which is likely from T cells. The different TNF polymorphisms associated with unstimulated and beryllium-stimulated TNF-α might in part be due to the different cells producing the TNF-α and likely different regulatory pathways. Our study indicates that despite their role in TNF-α production, TNF promoter polymorphisms are not risk factors for CBD or beryllium sensitization. The −1031C TNF promoter polymorphism and noncarriage of the TNF 1 promoter haplotype might be risk factors for disease progression.

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