CC chemokine receptor 5 gene polymorphisms in beryllium disease

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ABSTRACT: CC chemokine receptor 5 (CCR5) is expressed on type-1 T-helper cells, which are involved in the pathogenesis of the granulomatous lung disease chronic beryllium disease (CBD). CCR5 gene (CCR5) polymorphisms are associated with sarcoidosis severity. The present study explores associations between CCR5 polymorphisms and CBD and its disease progression.

Eight CCR5 polymorphisms were genotyped in CBD (n=88), beryllium sensitisation (BeS; n=86) and beryllium-exposed nondiseased controls (n=173) using PCR with sequence-specific primers. Pulmonary function and bronchoalveolar lavage data were examined for associations with genotypes.

There were no significant differences in genotype and allele frequency between CBD, BeS individuals and controls. In CBD, associations were found with decline in forced expiratory volume in 1 s and forced vital capacity and the CCR5 -3458 thymidine (T)T genotype (p<0.0001), and an increase in alveolar–arterial oxygen tension difference at rest (p=0.003) and at maximum exercise (p=0.01) and the -5663 adenine allele. Increased bronchoalveolar lavage lymphocyte numbers were associated with CCR5 -2459 guanine/-2135T (p=0.01) only in the combined CBD and BeS group.

This is the first study showing that CCR5 polymorphisms are associated with worsening pulmonary function over time in CBD, suggesting that CCR5 is important in the progression of pulmonary function in CBD. Further studies would be useful to clarify the mechanism whereby CCR5 polymorphisms affect progression of CBD.

KEYWORDS: Broncholaveolar lavage, CC chemokine receptor 5, chronic beryllium disease, lung function tests, polymorphism

Beryllium exposure in the workplace triggers a beryllium-specific immune response in a minority of those exposed. Specifically, 2–10% of exposed workers develop beryllium sensitisation (BeS), with lymphocytes that proliferate in response to beryllium in the beryllium lymphocyte proliferation test (LPT) (BeLPT) [1, 2]. Previous studies have shown that beryllium-stimulated T-cell proliferation is restricted to major histocompatibility complex class II [3]. Subsequently, trafficking of immune and inflammatory cells is initiated along with cytokine production, resulting in granuloma formation, the hallmark of chronic beryllium disease (CBD).

The chemokines involved in granuloma formation in CBD are unknown. The CC chemokine receptor 5 (CCR5) gene (CCR5), located on chromosome 3p21 and adjacent to CCR2, is implicated in the chemotaxis and activation of leukocyte subsets, including CD4+ type-1 T-helper lymphocytes [4]. CCR5 is polymorphic and some of the variants appear to be functional, although this is not well understood [5, 6].

Of relevance to CBD, an association between CCR5 polymorphisms and sarcoidosis was previously reported [7]. Although an association between CCR5 polymorphisms and presence of disease in sarcoidosis compared to controls was not found, an association between a specific CCR5 haplotype (human haplogroup C (HHC)) and chest radiographic stage of disease was found. Others have reported an increased frequency of a CCR5 32-bp deletion mutation (Δ32) in sarcoidosis [8]. This may suggest that CCR5 polymorphisms are a risk factor for disease or disease severity in granulomatous lung diseases.

This article has supplementary material accessible from www.erj.ersjournals.com

An increase in CCR5 expression has been reported on bronchoalveolar lavage (BAL) cells in sarcoidosis [9–11]. There are no reports of an association between CCR5 polymorphisms and CBD, which is histopathologically identical to sarcoidosis, sharing many of the clinical and radiographic features of sarcoidosis. Against this background, it was hypothesised that CCR5 polymorphisms and/or haplotypes would be associated with CBD and more severe forms of disease, but not with BeS, in which pulmonary granulomatous disease is absent. The aims of the present study were to: 1) assess the contribution of CCR5 polymorphisms and/or haplotypes as risk factors for CBD compared to BeS and beryllium-exposed nondiseased controls; 2) evaluate the association between these polymorphisms and severity markers in CBD; and 3) investigate the association between BAL cellularity and CCR5 polymorphisms and/or haplotypes, in order to determine whether or not there is a functional association between CCR5 polymorphisms and CBD.

METHODS

Study design

A case–control study was conducted that compared Caucasian subjects with CBD (n=88) and BeS (n=86) and subjects with beryllium exposure without evidence of sensitisation or disease (controls; n=173) in order to evaluate CCR5 polymorphisms and their association with CBD. Only Caucasian cases and controls were utilised in order to limit population stratification, as the number of non-Caucasians (e.g. black Americans, Asians, etc.) was too small to permit meaningful analysis. A case comparison study was used to evaluate CCR5 polymorphisms and disease severity in CBD. Cases were enrolled from the clinic at National Jewish Health (Denver, CO, USA). Cases of CBD and BeS had evidence of two abnormal blood BeLPTs. If the cases showed evidence of either noncaseating granulomas or a mononuclear cell infiltrate on lung biopsy, or BAL cell lymphocytosis of $\geqslant 15\%$ and an abnormal BAL cell LPT on bronchoscopy, they were diagnosed as having CBD. If they showed no evidence of these abnormalities, they were diagnosed as having BeS. Controls were undergoing medical surveillance in the same beryllium industries as cases [12–14], and had undergone at least one normal BeLPT. Study subjects provided informed consent according to a protocol approved by the Institutional Review Board at National Jewish Health.

Cases of CBD and BeS underwent venepuncture, a BeLPT, pre- and post-bronchodilator pulmonary function testing and maximum exercise testing with an indwelling arterial line during initial (baseline) and subsequent clinical evaluations [15]. These data were obtained in the Pulmonary Physiology Unit at National Jewish Health using the same clinical protocols over time on the same equipment. Post-bronchodilator pulmonary function variables were used for analysis. Bronchoscopy with BAL, transbronchial biopsy and BAL LPTs were performed for clinical purposes [15]. Control subjects underwent venepuncture and BeLPTs. All subjects completed a modified version of the American Thoracic Society questionnaire [15].

CCR5 genotypes

Genomic DNA was extracted from whole blood using the PAXgene kit (Qiagen, Inc., Valencia, CA, USA). PCR with sequence-specific primers [16, 17] was used to identify eight CCR5 single nucleotide polymorphisms (SNPs): -5663 adenine (A)/guanine (G) (SNP identifier rs2040388), -3900 cytosine (C)/A (rs2856757), -3458 thymidine (T)/G (rs2734225), -2459G/A (rs1799987), -2135T/C (rs1799988), -2086A/G (rs1800023), -1835C/T (rs1800024), and Δ32, which had been investigated in a previous study on sarcoidosis [7].

Data analysis

All genotype frequencies were tested for Hardy–Weinberg equilibrium using Chi-squared goodness-of-fit tests. Normalising transformations were performed on continuous outcome variables when necessary to better approximate model assumptions; these data are expressed as geometric mean $\pm$ geometric SD (GSD). Measures of linkage disequilibrium, the normalised disequilibrium constant (D') and r^2, were calculated using Haploview (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Haplotype blocks were determined with Haploview using the confidence interval (CI) method [18]. Haplotype frequencies were estimated using Haplo.Score [19]. In order to adjust for uncertainty in haplotype assignments, weighted logistic regression models were used to assess associations with disease and disease severity. Comparisons were made when sufficient numbers of observed haplotypes were available. Linear regression was used to determine the influence of genotype/haplotype on cross-sectional continuous outcomes. Mixed-effects models were used to assess longitudinal outcomes and to estimate effects based on different numbers of follow-up visits and different timings of the visits. Age at testing, sex, smoking history, height and time from first beryllium exposure were included in the model, along with the genotype/haplotype and forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), total lung capacity (TLC), diffusing capacity of the lung for carbon monoxide uncorrected for haemoglobin ($D_{L,CO,uncor}$), arterial oxygen tension at rest ($P_{a,O_2,r}$) and during maximal exercise ($P_{a,O_2,max}$), alveolar–arterial oxygen tension difference at rest ($P_{A–a,O_2,r}$) and during maximal exercise ($P_{A–a,O_2,max}$), and workload at maximal exercise (W_{max}). The variable time from first exposure to visit date was restricted to visits ranging 10–40 yrs from first exposure, since there were few visits outside this range from which to make estimates. In order to avoid effects of corticosteroids on the severity of CBD, all subjects' data were included until commencement of corticosteroid use. No adjustment was made for multiple comparisons since this adjustment is too conservative, with the high correlation of outcomes. Statistical analysis was performed using Knowledge STUDIO (Angoss Software Corporation, Toronto, Canada) or SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA). All tests were two sided and a p-value of <0.05 was used to determine significance.

RESULTS

Demographic information

There were no significant sex differences between the three study groups. The control subjects (60.5 ± 11.0 yrs) were older than both the CBD (54.1 ± 10.8 yrs) and BeS subjects (53.5 ± 12.0 yrs) ($p<0.0001$). Ever-smoking was not significantly different across the groups. In the CBD group, 18 (20.5%) patients had received corticosteroids. The BeS subjects and controls were not treated with corticosteroids.

CCR5 polymorphisms

CCR5 promoter polymorphisms were compared across groups, as summarised in table 1. All populations were in Hardy–Weinberg equilibrium for all genotypes. *CCR5* -2459A/G and -2135C/T were in 100% linkage disequilibrium (D'=1). *CCR5* -3900A (odds ratio (OR) 0.35; 95% CI 0.14–0.84; p=0.03), -3458G (OR 0.29; 95% CI 0.11–0.78; p=0.02) and -2459A/-2135C (OR 0.39; 95% CI 0.18–0.86; p=0.03) were decreased in CBD

TABLE 1	Genotype and allele carriage frequencies of CC chemokine receptor 5 gene (*CCR5*) polymorphisms				
Position	Genotype/allele	Controls	CBD	BeS	p-value[#]
Subjects n		173	88	86	
Genotype					
-5663	AA	33.5	36.4	22.1	NS
	AG	45.1	37.5	52.3	
	GG	21.4	26.1	25.6	
-3900	AA	36.4	44.3	44.2	**0.03**[¶]
	AC	48.0	33.0	46.5	
	CC	15.6	22.7	9.3	
-3458	GG	39.5	45.5	45.3	**0.02**[¶]
	GT	48.9	34.1	47.7	
	TT	11.6	20.5	7.0	
-2459/-2135	AA/CC	29.5	36.4	36	**0.03**[¶]
	AG/CT	52.0	36.4	51.2	
	GG/TT	18.5	27.2	12.8	
-2086	AA	39.9	45.5	45.3	NS
	AG	52.6	44.3	50	
	GG	7.5	10.2	4.7	
-1835	CC	83.2	84.1	81.4	NS
	CT	15.6	14.8	18.6	
	TT	1.2	1.1	0.0	
Δ32	GG	79.8	84.1	75.6	NS
	G/del	19.1	15.9	22.1	
	del/del	1.2	0.0	2.3	
Allele carriage					
-5663	A	78.6	73.9	74.4	NS
	G	66.5	63.6	77.9	NS
-3900	A	84.4	77.3	90.7	**0.03**
	C	63.6	55.7	55.8	NS
-3458	G	87.9	79.5	93.0	**0.02**
	T	60.5	54.6	54.7	NS
-2459/-2135	A/C	81.5	72.7	87.2	**0.03**
	G/T	70.5	63.6	64.0	NS
-2086	A	92.5	89.8	95.3	NS
	G	60.1	54.3	54.7	NS
-1835	C	98.8	98.9	100.0	NS
	T	16.8	15.9	18.6	NS
Δ32	G	98.8	100.0	97.7	NS
	del	20.2	15.9	24.4	NS

Data are presented as percentages. CBD: chronic beryllium disease; BeS: beryllium sensitisation; Δ32: *CCR5* 32-bp deletion mutation; A: adenine; G: guanine; C: cytosine; T: thymidine; del: deletion; NS: nonsignificant. [#]: CBD *versus* BeS; [¶]: obtained using 2 × 3 tables and comparing genotype numbers in CBD and BeS.

compared to BeS. However, no significant differences were found between CBD and controls, nor between BeS and controls. Consistent with the allele frequencies, similar differences were found between CBD and BeS when comparing the genotypes at the same three positions (table 1). No other associations were found between any of the other *CCR5* polymorphisms and disease.

The haplotype comprising the -5663/-3900/-3458/-2459/-2135/-2086 SNPs was identified using the CI method in Haploview (fig. 1). Table 2 shows the frequency of common (>1%) *CCR5* haplotypes. There were no significant differences in *CCR5* haplotype between the three groups.

Longitudinal analysis of disease severity in CBD

Mixed-effects models were used to fit lung function measurements from clinical visits, including FEV_1, FVC, TLC, $D_{L,CO,uncor}$, $P_{a,O_2,r}$, $P_{a,O_2,max}$, $P_{A-a,O_2,r}$, $P_{A-a,O_2,max}$ and W_{max}, and covariates, as noted above, along with *CCR5* variants. The mean number of visits was 3.96, with a maximum of 15. The first visit was, on average, 18.1±8.2 yrs from first exposure and the last visit, on average, 27.2±8.8 yrs. Tables 3 and 4 show the mean rates of change in these variables over time in relation to the *CCR5* genotypes of interest, adjusting for age at testing, sex, smoking history, height and time from first beryllium exposure.

On average, CBD subjects homozygous for -5663A showed a significantly greater decrease in FEV_1 over time (p=0.01) than
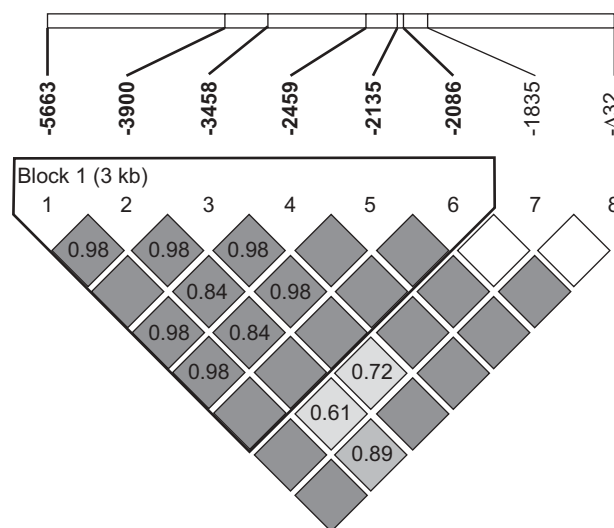
FIGURE 1. CC chemokine receptor 5 gene linkage disequilibrium and haplotype block structure in cases and controls derived using the confidence interval method. Two measures of pairwise linkage disequilibrium used in the Haploview program are normalised disequilibrium constant (*D'*) and logarithm of odds (LOD) scores. Pairwise linkage disequilibrium plots demonstrate the *D'* for the eight polymorphisms in Caucasian cases and controls. The numbers shown represent the *D'* for adjacent pairs; empty squares indicate a *D'* of 1.0 (■: greatest linkage disequilibrium, with an upper confidence bound (UCI) of *D'* of 1.0 and an LOD score of >2; ▨: intermediate linkage disequilibrium, with a UCI of *D'* of 1.0 and an LOD score of ~2; ▨: less information about linkage disequilibrium, with a UCI of *D'* of 1.0 but an LOD score of <2, □: UCI of <1.0 for *D'* and an LOD score of <2). Using the confidence interval method, a haplotype block was identified between the -5663, -3900, -3458, -2459, -2135 and -2086 polymorphisms.

TABLE 2	Common CC chemokine receptor 5 gene haplotype frequencies in chronic beryllium disease (CBD), beryllium sensitisation (BeS) and controls								
Haplotype	-5663	-3900	-3458	-2459	-2135	-2086	Controls	CBD	BeS
Subjects n							346	164	172
1	G	A	G	A	C	A	43.2	44.3	50.6
2	A	C	T	G	T	G	34.0	32.4	29.0
3	A	A	G	A	C	A	8.5	8.6	9.4
4	A	A	G	G	T	A	7.8	7.9	7.4
5	A	C	G	A	C	A	3.1	1.1	1.1
6	A	C	T	G	T	A	1.7	4.5	1.2

Data are presented as percentages; only the common haplotypes (>1%) are shown. A: adenine; G: guanine; C: cytosine; T: thymidine.

did nonhomozygous subjects (fig. 2a). Carriers of the A allele also showed a greater decline in FEV1 than noncarriers (p=0.001) (table 3). A similar trend was seen with these same alleles in relation to FVC. Carriers of the -5663A allele showed a greater decline in $P_{a,O_2,r}$ (p=0.01), as well as an increase in log $P_{A-a,O_2,r}$ (p=0.003) and log $P_{A-a,O_2,max}$ (p=0.01), than did the noncarriers (table 4). Subjects homozygous for the -3900C allele exhibited greater FEV1 (p=0.0002) and FVC decline (p<0.0001) and a greater increase in log $P_{A-a,O_2,r}$ (p=0.04) than noncarriers (tables 3 and 4). Subjects homozygous for -3458T showed greater FEV1 (p<0.0001) and FVC declines (p<0.0001) than nonhomozygous subjects. Those homozygous for -2459G/-2135T (the two loci in complete linkage disequilibrium) exhibited lower FEV1 (p=0.04) and FVCs (p=0.02) than nonhomozygous subjects. Subjects with at least one $\Delta32$ allele showed lower FEV1 (p=0.002) and FVCs (p=0.008) than noncarriers.

The impact of CCR5 haplotype on change in lung function over time was evaluated and similar results were noted (tables 3 and 4). Carriers of haplotype 1 (-5663G/-3900A/-3458G/-2459A/-2135C/-2086A) showed a lesser decline in FEV1 (p<0.0001), FVC (p=0.0004) (fig. 2b), TLC (p=0.01) and $P_{a,O_2,r}$ (p=0.01) (fig. 2c) than did noncarriers. Carriers of haplotype 2 (-5663A/-3900C/-3458T/-2459G/-2135T/-2086G) demonstrated increasing log $P_{A-a,O_2,r}$ (p=0.03) and log $P_{A-a,O_2,max}$ (p=0.01) and a greater decline in FEV1 (p=0.005) over time than noncarriers.

CCR5 polymorphisms and BAL lymphocytes
Comparisons were made between BAL differential cell counts at the first and last visits to clinic of 88 CBD and 78 BeS subjects. There were significant differences between geometric mean$\pm$GSD lymphocyte percentages in BAL from CBD (24.3$\pm$2.35%; n=88) compared with BeS (9.3$\pm$2.24%; n=78;

TABLE 3	Estimates of change in lung function indices by CC chemokine receptor 5 gene (CCR5) polymorphism and haplotype in chronic beryllium disease[#]							
Carrier	FEV1 L·yr^{-1}	p-value	FVC L·yr^{-1}	p-value	TLC L·yr^{-1}	p-value	$D_{L,CO,uncor}$ mL·min^{-1}·mmHg^{-1}·yr^{-1}	p-value
-5663AA	-0.02$\pm$0.007	**0.01**	-0.02$\pm$0.008	**0.01**	-0.02$\pm$0.01	0.08	0.10$\pm$0.09	0.30
-5663A	-0.03$\pm$0.009)	**0.001**	-0.02$\pm$0.01	0.12	-0.006$\pm$0.01	0.61	0.08$\pm$0.11	0.48
-3900CC	-0.03$\pm$0.008	**0.0002**	-0.04$\pm$0.01	**<0.0001**	-0.02$\pm$0.01	0.22	-0.05$\pm$0.11	0.64
-3900C	-0.02$\pm$0.007	**0.007**	-0.02$\pm$0.01	0.06	-0.004$\pm$0.01	0.7	-0.01$\pm$0.09	0.93
-3458TT	-0.03$\pm$0.008	**<0.0001**	-0.05$\pm$0.01	**<0.0001**	-0.02$\pm$0.01	0.2	0.05$\pm$0.11	0.63
-3458T	-0.01$\pm$0.01	0.12	-0.009$\pm$0.01	0.28	-0.009$\pm$0.01	0.4	0.0001$\pm$0.09	0.99
-2459GG/-2135TT	-0.02$\pm$0.008	**0.04**	-0.03$\pm$0.009	**0.02**	-0.01$\pm$0.01	0.36	-0.09$\pm$0.10	0.36
-2459G/-2135T	-0.01$\pm$0.01	0.06	-0.006$\pm$0.01	0.56	-0.02$\pm$0.01	0.07	0.03$\pm$0.10	0.74
-2086AA	0.01$\pm$0.01	0.12	0.009$\pm$0.01	0.28	0.008$\pm$0.01	0.4	0.0001$\pm$0.09	0.99
-1835CC	0.008$\pm$0.02	0.53	0.007$\pm$0.02	0.68	0.01$\pm$0.02	0.44	-0.17$\pm$0.16	0.29
$\Delta32$	-0.03$\pm$0.008	**0.002**	-0.03$\pm$0.12	**0.008**	-0.02$\pm$0.01	0.12	-0.06$\pm$0.12	0.58
Haplotype 1	0.03$\pm$0.007	**<0.0001**	0.03$\pm$0.009	**0.0004**	0.01$\pm$0.01	**0.01**	-0.06$\pm$0.09	0.51
Haplotype 2	-0.02$\pm$0.008	**0.005**	-0.009$\pm$0.01	0.35	-0.004$\pm$0.01	0.71	0.03$\pm$0.10	0.76

Data are presented as mean$\pm$SD, and represent the mean increased rate of decline comparing carriage versus non-carriage of genotypes/alleles/haplotypes. Haplotype 1 is -5663G/-3900A/-3458G/-2459A/-2135C/-2086A; haplotype 2 is -5663A/-3900C/-3458T/-2459G/-2135T/-2086G. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; $D_{L,CO,uncor}$: diffusing capacity of the lung for carbon monoxide uncorrected for haemoglobin; A: adenine; C: cytosine; T: thymidine; G: guanine; $\Delta32$: CCR5 32-bp deletion mutation. [#]: 10–40 yrs from first beryllium exposure.

TABLE 4 Estimates of change in further lung function indices by CC chemokine receptor 5 gene (CCR5) polymorphism and haplotype in chronic beryllium disease[#]

Carrier	$P_{a,O_2,max}$ mmHg·yr⁻¹	p-value	$P_{a,O_2,r}$ mmHg·yr⁻¹	p-value	$P_{A-a,O_2,max}$ mmHg·yr⁻¹	p-value	$P_{A-a,O_2,r}$ mmHg·yr⁻¹	p-value	W_{max} W·yr⁻¹	p-value
-5663AA	-0.12±0.24	0.62	-0.21±0.21	0.33	1.01±1.02	0.49	1.01±1.02	0.49	0.36±0.71	0.61
-5663A	-0.13±0.20	0.50	-0.40±0.16	**0.01**	1.04±1.01	**0.01**	1.06±1.02	**0.003**	-0.73±0.56	0.19
-3900CC	0.07±0.23	0.77	-0.26±0.17	0.12	1.01±1.02	0.62	0.99±1.01	0.73	-1.7±0.64	**0.01**
-3900C	0.15±0.20	0.45	-0.08±0.17	0.65	1.02±1.01	0.07	1.04±1.02	**0.04**	-0.13±0.57	0.82
-3458TT	0.11±0.23	0.62	-0.23±0.17	0.18	0.99±1.02	0.45	1.00±1.02	0.98	-1.8±0.65	**0.01**
-3458T	-0.07±0.19	0.71	-0.06±0.17	0.71	1.22±1.17	0.21	1.17±1.12	0.14	-0.31±0.56	0.58
-2459GG/-2135TT	-0.005±0.21	0.98	0.25±0.16	0.11	1.00±1.02	0.98	1.01±1.02	0.79	-1.2±0.58	**0.05**
-2459G/-2135T	-0.28±0.21	0.17	-0.19±0.18	0.30	1.02±1.01	**0.05**	1.03±1.02	0.06	0.14±0.60	0.81
-2086AA	0.07±0.19	0.71	0.06±0.17	0.70	1.02±1.02	0.15	1.03±1.02	0.09	0.31±0.56	0.58
-1835CC	0.25±0.31	0.42	0.22±0.27	0.40	0.99±1.02	0.63	0.97±1.03	0.34	-0.43±0.84	0.61
Δ32	0.41	0.07	-0.08±0.20	0.69	1.00±1.01	0.96	1.00±1.02	0.95	-1.7±0.67	**0.01**
Haplotype 1	-0.18±0.18	0.32	0.42±0.16	**0.01**	1.00±1.01	0.70	0.99±1.02	0.70	0.73±0.53	0.17
Haplotype 2	-0.12±0.19	0.55	-0.08±0.18	0.67	1.03±1.01	**0.01**	1.04±1.02	**0.03**	0.18±0.57	0.75

Data are presented as mean±SD, or as geometric mean±geometric SD for alveolar–arterial oxygen tension differences (P_{A-a,O_2}). Data represent the mean increased rate of decline comparing carriage versus non-carriage of genotypes/alleles/haplotypes. Haplotype 1 is -5663G/-3900A/-3458G/-2459A/-2135C/-2086A; haplotype 2 is -5663A/-3900C/-3458T/-2459G/-2135T/-2086G. $P_{a,O_2,max}$: arterial oxygen tension during maximal exercise; $P_{a,O_2,r}$: arterial oxygen tension at rest; $P_{A-a,O_2,max}$: P_{A-a,O_2} during maximal exercise; $P_{A-a,O_2,r}$: P_{A-a,O_2} at rest; W_{max}: maximal workload; A: adenine; C: cytosine; T: thymidine; G: guanine; Δ32: CCR5 32-bp deletion mutation. [#]: 10–40 yrs from first beryllium exposure.

p<0.0001) at the first visit to clinic, and for CBD (28.0±1.87%; n=51) compared with BeS (7.0±0.22%; n=47; p<0.0001) at the last visit.

The relationship between BAL lymphocyte percentage and CCR5 polymorphisms was evaluated, comparing the CCR5 polymorphisms, haplotypes and BAL lymphocyte percentages in CBD and BeS combined at the first visit to clinic (table 5). The percentage of BAL lymphocytes was significantly increased in individuals carrying the CCR5 polymorphisms -2459G/-2135T (17.99±2.53%; n=104) compared with noncarriers (12.30±2.64%; n=62; p=0.01). Other CCR5 polymorphism associations were found with both increased and decreased numbers of lymphocytes for CBD and BeS subjects combined. When subjects with CBD and BeS were analysed separately, far fewer associations were demonstrated, although similar trends were apparent (table 1 of online supplementary material).

There was a significant difference in mean BAL lymphocyte percentage between carriers and noncarriers of haplotype 1 in the combined CBD and BeS group (p=0.009) (table 5), with noncarriers of this haplotype showing higher lymphocyte percentages. This haplotype includes all alleles that were associated with a lower BAL lymphocyte percentage (-5663G/-3900A/-3458G/-2459A/-2135C). Carriers of haplotype 2, which includes -2459A/-2135C, showed, on average, higher lymphocyte percentages than noncarriers of this haplotype in the combined CBD and BeS group. Similar, although nonsignificant, trends were seen in the BeS and CBD groups when analysed separately (table 1 of online supplementary material).

DISCUSSION
In the present study, the association between CCR5 polymorphisms in Caucasians with CBD and markers of disease severity is described. There were few significant differences in genotype, allele and haplotype frequency between either CBD or BeS and controls. Interestingly, a greater decline in lung function and worsening gas exchange was found in CBD cases carrying specific CCR5 polymorphisms and haplotypes. Associations were also shown between BAL lymphocyte percentages and CCR5 polymorphisms and haplotypes. Strikingly, these data support the fact that the same CCR5 polymorphisms associated with greater decline in CBD lung function are also associated with greater lymphocyte percentage in the lung. This suggests that CCR5 variants are functional, impacting lymphocyte recruitment into the CBD lung and, in turn, greater decline in lung function and worsening gas exchange.

This is the first study of CCR5 genotypes and haplotypes in CBD. Although the CCR5 -3900A, -3458G and -2459A/-2135C alleles were slightly decreased in CBD compared to BeS, no CCR5 polymorphisms or haplotype was significantly different between CBD and controls or BeS and controls. These findings suggest that CCR5 polymorphisms are not a risk factor for CBD. This is consistent with studies of sarcoidosis, a similar granulomatous lung disease of unknown aetiology. Although one study of Czech sarcoidosis cases found an increased rate of the Δ32 variant in cases compared to controls [8], two studies in UK/Dutch and German populations found no association with any CCR5 genotype or haplotype, including Δ32, and disease risk [7, 20]. It should be noted that the frequencies of case and control genotypes reported in the present study are similar to those noted in these prior studies [7, 20].

In the current study, strong associations were found between changes in static lung function and measures of gas exchange and CCR5 polymorphisms in CBD. Specifically, the -5663AA,
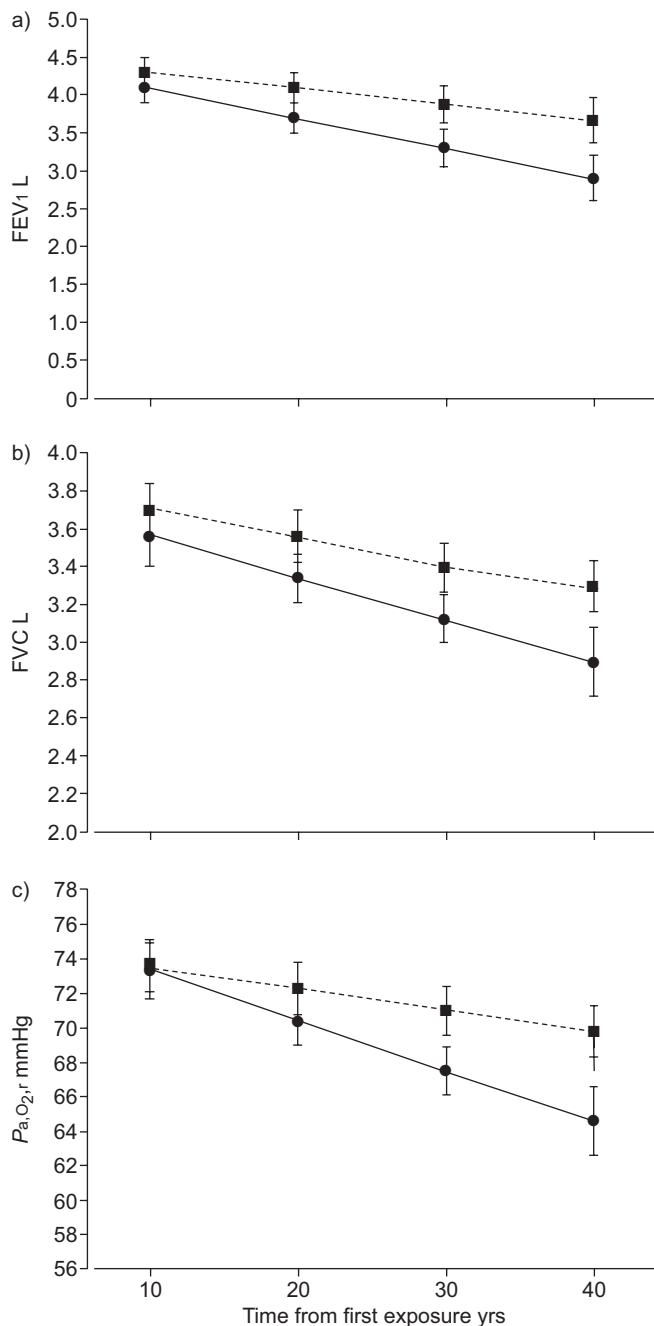
FIGURE 2. Mean change over time in: a) forced expiratory volume in 1 s (FEV1) in -5663 AA homozygotes (——) *versus* individuals with the -5663AG/GG genotype (-------); and b) forced vital capacity (FVC); and c) arterial oxygen tension at rest ($P_{a,O_2,r}$) in those with at least one (------) *versus* no (———) haplotype 1 (-5663G/-3900A/-3458G/-2459A/-2135C/-2086A), modelled ranging 10–40 yrs since first beryllium exposure and adjusted for sex, height (a and b only), smoking history (current, former or never) and age at testing. Data are presented as mean±2SEM, and estimates are based on mixed-effects models. Individuals with the -5663AA genotype showed a greater decline in FEV1 than did the other genotypes (p=0.01). There was a lesser decline in both FVC and $P_{a,O_2,r}$ in haplotype 1 heterozygotes and homozygotes than in those with no haplotype 1 (p=0.01 for both parameters).

-3900CC, -3458TT and -2459GG/-2135TT homozygous genotypes and carriage of the Δ32 allele were associated with a greater decline in FEV1 and FVC, whereas the -5663A and -3900C alleles were associated with worsening measures of gas exchange at rest and during exercise. No associations were noted between any genotype and $D_{L,CO,uncor}$, which may be due to the variability in the measurement of this parameter. Interestingly, some of the genotypes associated with worse lung function over time were the same as those noted to be slightly increased in CBD compared to BeS (-3900CC, -3458TT and -2459GG/-2135TT); this overrepresentation of genotypes in CBD may be due to the association with a more severe disease phenotype in the present CBD population rather than disease susceptibility. Most of the genotypes associated with greater decline in lung function were included in haplotype 2 (-5663A/-3900C/-3458T/-2459T/-2135T/-2086G). Carriers of this haplotype showed a greater decline in FEV1 and increase in $P_{a,O_2,r}$ and $P_{a,O_2,max}$.

The polymorphisms composing haplotype 2 in the current study are also represented in the HHC haplotype that was found to be overrepresented in higher sarcoidosis chest radiograph stages (stage II, III and IV) compared to lower stages (stage 0 and I) [7]. Similarly, the sarcoidosis HHC haplotype carriers also showed significantly lower FEV1 and FVCs on presentation compared with non-HHC carriers [7]. Conversely, the present CBD cases carrying -5663G/-3900A/-3458G/-2459A/-2135C/-2086A (haplotype 1) exhibited better lung function, with higher FEV1, FVC, TLC and $P_{a,O_2,r}$ than noncarriers. These results suggest that *CCR5* variants may be important in granulomatous lung disease progression, as indicated by chest radiography in sarcoidosis, and lung function changes in both sarcoidosis and CBD. Although a second CCR5 sarcoidosis study by FISCHER *et al.* [20] showed the *CCR5* -5663A and -3900C alleles were increased in female patients with Löfgren's syndrome, they did not assess lung function or radiographic stage. As a result, it is difficult to compare our studies with theirs.

In addition to associations between lung function and *CCR5* genotypes/haplotypes, associations were noted between BAL lymphocyte percentages and these same *CCR5* genotypes/haplotypes. Specifically, the BAL lymphocyte percentage was significantly increased in carriers of *CCR5* -2459G/-2135T and haplotype 2 compared to noncarriers of these alleles. Conversely, carriers of *CCR5* -5663G, -3900A, -3458G and -2459A/-2135C alleles and haplotype 1, containing all of these alleles, showed lower BAL lymphocyte percentages than noncarriers. It is possible that these data reflect functional alterations in CCR5 expression important in cell trafficking and cell-to-cell interactions. In this regard, CCR5 is expressed at high levels on CD4+ type-1 T-helper cells and may play a role in enhanced recruitment and activation of lymphocytes and monocytes to the lung and these cells' subsequent immune response [21]. It is possible that enhanced recruitment and activation of BAL lymphocytes may explain the association of the same *CCR5* genotypes and haplotypes with more rapid disease progression.

Previously, KATCHAR *et al.* [10] reported a positive correlation between BAL lymphocyte percentages and the number of CCR5-expressing CD4+ BAL T-cells in sarcoidosis. CAPELLI

TABLE 5	Association between carriage of CC chemokine receptor 5 gene polymorphisms and the percentage of bronchoalveolar lavage lymphocytes[#]					
Position	**Allele**	**Carriers**		**Noncarriers**		**Mean difference (95% CI)**	**p-value[¶]**
		Subjects n	**Lymphocytes %**	**Subjects n**	**Lymphocytes %**		
-5663	A	121	16.78±2.56	45	12.94±2.69	1.29 (0.56–1.73)	0.13
	G	120	14.15±2.64	46	20.29±2.39	0.70 (0.51–0.97)	**0.03**
-3900	A	140	14.44±2.61	26	22.87±2.32	0.63 (0.43–0.94)	**0.03**
	C	90	17.64±2.51	76	13.60±2.69	0.77 (0.58–1.03)	0.08
-3458	G	143	14.73±2.61	23	22.65±2.44	0.65 (0.43–0.99)	**0.04**
	T	88	17.81±2.51	78	13.46±2.66	1.32 (0.99–1.77)	0.06
-2459/-2135	A/C	135	14.59±2.64	31	21.12±2.27	0.69 (0.47–1.00)	**0.05**
	G/T	104	17.99±2.53	62	12.30±2.64	0.69 (0.51–0.93)	**0.01**
-2086	A	154	15.33±2.64	12	20.29±2.10	0.75 (0.43–1.32)	0.32
	G	88	17.81±2.51	78	13.46±2.66	0.76 (0.56–1.01)	0.06
-1835	C	165	NA	1	NA	NA	NA
	T	29	14.15±2.64	137	15.96±2.61	1.13 (0.77–1.66)	0.5
Δ32	G	164	NA	2	NA	NA	NA
	Deletion	35	13.07±2.72	131	16.44±2.56	1.25 (0.88–1.80)	0.21
Haplotype 1		114	13.46±2.66	52	20.09±1.77	0.69 (0.53–0.91)	**0.009**
Haplotype 2		76	18.17±2.51	90	13.46±2.51	1.32 (1.03–1.70)	**0.03**

Data are presented as geometric mean±geometric SD unless otherwise indicated. Haplotype 1 is -5663G/-3900A/-3458G/-2459A/-2135C/-2086A; haplotype 2 is -5663A/-3900C/-3458T/-2459G/-2135T/-2086G. CI: confidence interval; A: adenine; G: guanine; C: cytosine; T: thymidine; Δ32: CCR5 32-bp deletion mutation; NA: not available. [#]: in chronic beryllium disease and beryllium sensitisation combined; [¶]: unpaired t-test.

et al. [22] reported that increased CCR5 expression was observed in both lymphocytes and macrophages from all sarcoidosis patients, along with a trend towards decreased positivity from stage I to III of disease. They also showed that CCR5 expression was significantly reduced in lymphocytes from idiopathic pulmonary fibrosis compared to controls [9]. However, there have been no studies to date evaluating the association between CCR5 expression on BAL cells and CCR5 genotypes or haplotypes in sarcoidosis or other lung disease, including CBD.

The functional correlates of most of the CCR5 polymorphisms are unknown, except for the Δ32 deletion. Homozygosity for this 32-bp deletion results in truncation of the protein, meaning it lacks the transmembrane domain and thus cannot be expressed on the cell surface [23]. The CCR5 promoter polymorphism -2459G/A has been investigated primarily in HIV infection [6, 24–26]. Interestingly, the CCR5 -2459AA genotype has been associated with overexpression of CCR5 and HIV progression [6, 25]. This is discordant with the present findings. In order to clarify the discrepancy of associations with this CCR5 -2459 G/A polymorphism between CBD and HIV infection, functional assay of CCR5 expression in CBD patients is needed, but such experiments are beyond the scope of the current study.

Although use of Bonferroni adjustment of the p-values for multiple comparisons made in the longitudinal analyses was considered, we chose not to do this as it would have been too conservative. That is to say, with the consistency of the correlations in the outcome variables, and the correlation between these variables, the Bonferroni adjustment would have overadjusted the present p-values. If only one or two significant associations had been found, these associations could have been discounted as possibly being due to chance alone because of the multiple comparisons undertaken in the present analysis. However, there were many associations found between disease severity indicators and CCR5 polymorphisms that all showed consistent direction, which supports our not using Bonferroni correction.

In summary, this is the first study to report associations between CCR5 polymorphisms and CBD. Although no overall association between CCR5 polymorphisms and either CBD or BeS was found, associations between worsening change in lung function and CCR5 polymorphisms and CCR5 haplotype 2 were found in CBD. Furthermore, the same variants were shown to be associated with increased BAL lymphocyte percentage, supporting the concept of a functional consequence of this genotype in CBD. Further studies are needed in order to show how these alterations in BAL effected worsening functional changes.

STATEMENT OF INTEREST
None declared.

helpful discussion; and C. Rodriguez for administrative support (all National Jewish Health, Denver, CO, USA); and, most importantly, the workers and patients who participate in these studies and make beryllium-related research possible.

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