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CXCR1 and *CXCR2* haplotypes synergistically modulate cystic fibrosis lung disease

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ABSTRACT: **Cystic fibrosis (CF) lung disease severity is largely independent on the CF transmembrane conductance regulator (*CFTR*) genotype, indicating the contribution of genetic modifiers. The chemokine receptors CXCR1 and CXCR2 have been found to play essential roles in the pathogenesis of CF lung disease. Here, we determine whether genetic variation of *CXCR1* and *CXCR2* influences CF lung disease severity.**

Genomic DNA of CF patients in Germany (n=442) was analysed for common variations in *CXCR1* and *CXCR2* using a single-nucleotide polymorphism (SNP) tagging approach. Associations of *CXCR1* and *CXCR2* SNPs and haplotypes with CF lung disease severity, CXCR1 and CXCR2 expression, and neutrophil effector functions were assessed.

Four SNPs in *CXCR1* and three in *CXCR2* strongly correlated with age-adjusted lung function in CF patients. SNPs comprising haplotypes *CXCR1*_Ha and *CXCR2*_Ha were in high linkage disequilibrium and patients heterozygous for the *CXCR1-2* haplotype cluster (*CXCR1-2*_Ha) had lower lung function compared with patients with homozygous wild-type alleles (forced expiratory volume in 1 s $\leqslant$70% predicted, OR 7.24; $p=2.30\times10^{-5}$). CF patients carrying *CXCR1-2*_Ha showed decreased CXCR1 combined with increased CXCR2 mRNA and protein expression, and displayed disturbed antibacterial effector functions.

***CXCR1* and *CXCR2* genotypes modulate lung function and antibacterial host defence in CF lung disease.**

KEYWORDS: **Chemokines, cystic fibrosis, G-protein coupled receptor, lung function, polymorphism**

Chronic lung disease determines the morbidity and mortality of cystic fibrosis (CF) patients [1]. CF lung disease is characterised by a detrimental feedback loop of bacterial infection and perpetuated inflammation. Although the underlying mechanisms are still poorly understood, previous studies provided evidence that neutrophils represent the key effector cells in this disease condition. CF airway fluids contain millions of activated neutrophils, but these professional phagocytes are inefficient in their antibacterial functionality [2]. Neutrophils are recruited and activated by the chemokine (C-X-C motif) ligand (CXCL)8 through its two cognate seven transmembrane loop G-protein coupled receptors (GPCR) CXCR1 (interleukin-8 receptor α (IL-8Rα)) and CXCR2 (IL-8Rβ), which are both highly expressed on the neutrophil surface. CXCR1 has been identified as a critical component in the pathogenesis of CF lung disease, as CXCR1 mediates antibacterial host defence in CF airways [2]. High CXCR1 surface expression levels were associated with preserved lung function of CF patients and *vice versa*.

Disease severity in CF patients is largely independent of the CF transmembrane conductance regulator (*CFTR*) genotype, indicating the contribution of genetic modifiers [3]. Several genetic modifiers have already been reported in CF patients, including *transforming growth factor β1 (TGFB1)*, *IFRD1*, *MBL2* and recently reported loci on chromosomes

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11p13 and 20q13.2 [4–8]. Based on the functional importance of CXCR1 and CXCR2 in neutrophilic inflammation, and the documented contribution of genetic modifiers to the severity of CF lung disease, we hypothesised that genetic variants regulate CXCR1 and CXCR2 expression levels in CF patients and may have critical impact on CF lung disease severity.

METHODS

Patients

Informed written consent was obtained from all subjects included in the study, their parents or their legal guardians, and all study methods were approved by the local ethics and by the institutional review board (Ludwig Maximilian University of Munich, Munich, Germany). Only subjects who regularly visited our CF care unit at least once every 6 months over the course of the last 5 yrs were included in our studies. In total, 442 CF patients were included in this study. Details of the CF patient population are given in online supplementary table S1. The CF group included 224 male and 218 female patients with a mean±SD age of 21.4±12.6 yrs. Inclusion criteria were the diagnosis of CF by clinical symptoms and positive sweat tests or disease-inducing mutations, forced expiratory volume in 1 s (FEV_1) >25% predicted and being on stable concomitant therapy for at least 2 weeks prior to the study. For 28 patients, no FEV_1 data at the time of blood drawing were available and therefore those patients were not included in the FEV_1 association analyses. Longitudinal FEV_1 values, calculated from a minimum of five consecutive years of CF patient data, were available for 318 CF patients and were used to calculate the FEV_1 predicted at age of 20 yrs, as previously reported by SCHLUCHTER *et al.* [9]. In total, we included 13,256 FEV_1 values in our longitudinal analyses. To compare *CXCR1/2* single-nucleotide polymorphisms (SNPs) between CF and healthy control populations, we included 395 healthy subjects from the KORA population [10]. KORA (Cooperative Health Research in the Region Augsburg) is a regional research platform for population-based surveys and subsequent follow-up studies in the fields of epidemiology, health economics and healthcare research [10]. The KORA F4 study is a follow-up of the KORA S4 study, a population-based health survey conducted in the city of Augsburg, Germany, and two surrounding counties between 1999 and 2001.

Quantitative RT-PCR

Expression levels were quantified in duplicate by real-time quantitative RT-PCR with the use of SYBR green and the iCycler iQ detection system (BioRad, Hercules, CA, USA). Cycle threshold values for genes of interest were normalised to β-actin and used to calculate the relative quantity of mRNA expression. For primer sequences, see online supplementary table S2.

CXCR1/CXCR2 genotyping

Polymorphisms with a minor allele frequency >1% were selected based on the mutation screening performed by VASILESCU *et al.* [11] and genotyped in the aforementioned CF population to investigate the influence of SNPs on CF lung disease. Genomic DNA was extracted from whole blood by a standard salting-out method and DNA samples were genotyped using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Sequenom, San Diego, CA, USA), as described in detail previously [12]. PCR assays and associated extension reactions were designed using the SpectroDESIGNER software (Sequenom). Specific primer sequences are given in online supplementary table S2.

Neutrophil isolation

Neutrophils from peripheral blood were isolated by Ficoll gradient centrifugation. After isolation, neutrophils were washed in PBS, counted and resuspended in RPMI 1640 or Hanks' balanced salt solution (HBSS). The purity of the neutrophil suspensions was >93%, as determined by May–Grünwald–Giemsa staining and staining Ficoll-isolated neutrophil fractions with CD11b and CD16 antibodies for flow cytometry as recently described [13, 14]. Note that peripheral blood was processed immediately after drawing from a single CF centre, thereby excluding transportation or centre-to-centre variation.

Fluoresence-activated cell sorting analysis

CXCR1/2 surface staining was performed as previously described [2]. Briefly, freshly obtained neutrophils from peripheral blood underwent Fc blocking and were then incubated with the respective monoclonal antibodies for 40 min, washed three times and analysed by flow cytometry (FACSCalibur; Becton-Dickinson, Heidelberg, Germany). Calibrator beads were used to adjust the fluoresence-activated cell sorting instrument settings and normalise the data. 10,000 neutrophils were analysed per sample. CXCR1 and CXCR2 were stained on neutrophils using antibodies from BD Biosciences (San Diego, CA, USA). CXCR1 and CXCR2 antibodies from the same clone were used for all stainings performed, and antibody concentrations were normalised to neutrophil numbers, correcting for differences in neutrophil counts among different CF patients. Isotype controls set to a fixed threshold were subtracted from the respective specific antibody expression and the results were reported as mean fluorescence intensity. Calculations were performed with Cell Quest analysis software (Becton-Dickinson, Heidelberg, Germany).

Bacterial killing

Bacterial killing was assessed as described previously [2]. A clinical isolate of a mucoid *Pseudomonas aeruginosa* from a CF patient's sputum was subcultured overnight, grown to stationary phase, washed and pre-opsonised by incubation for 60 min at 37°C in 20% pooled fresh C5a-depleted human serum. After washing twice in PBS, the opsonised *P. aeruginosa* bacteria were resuspended in 1 mL of a mixture of HBSS supplemented with 0.1% gelatine and tryptic soy broth (Difco Laboratories, Detroit, MI, USA). Neutrophils were then incubated at 37°C with bacteria (2×10^7 bacteria·mL^{-1}) at a ratio of five bacteria per neutrophil. Where indicated, CXCL8 (100 nM) was added to the assay to stimulate CXCR1 function. At the times indicated, aliquots of each mixture were removed and *P. aeruginosa* colonies were counted by serial dilution in distilled water and quantitative spread plating. Data are expressed as colony-forming units per millilitre.

Respiratory burst

Neutrophils were incubated, at equal density (2×10^6·mL^{-1}), with dihydrorhodamine-123 stain for 20 min at 37°C. *N*-formyl-methionine-leucine-phenylalanine (1 M) was then added to the cells for 30 min at 37°C. Where indicated, CXCL8 (100 nM) was added to the assay to stimulate CXCR1

function. The respiratory burst of the neutrophils was analysed by measuring the rhodamine-123 fluorescence intensity using flow cytometry.

Statistical analysis

Derived genotype frequencies were compared with the expected allelic population equilibrium based on the Hardy–Weinberg equilibrium test (Pearson Chi-squared) to control for technical genotyping errors. Associations between SNPs and qualitative outcomes were first tested by using Pearson Chi-squared [15] and Fisher's exact test, using a dominant model. Comparisons between quantitative outcomes in two patient groups were performed with the two-sided unpaired t-test, while comparisons between more than two groups for quantitative outcomes were performed with ANOVA. To test associations between SNPs and outcomes in complex models, logistic regression was used for qualitative outcomes and linear regression for quantitative outcomes. Odds ratios and 95% confidence intervals are reported for dichotomous outcomes while the nonstandardised regression coefficient B and the β coefficients are given for quantitative outcomes. Multivariate analysis was used to adjust for potentially confounding factors (age, sex, CFTR genotype and *P. aeruginosa*). Haplotype frequencies were estimated using the expectation-maximisation algorithm [16]. To specify the effects of individual haplotyes, we performed haplotype trend regressions in which the estimated probabilities of the haplotypes are modelled in a logistic regression as independent variables [17]. To account for multiple comparisons, a Bonferroni adjustment was performed. A p-value of <0.002 (0.05 out of 24 tests) was considered to be statistically significant. Where indicated, data are shown as mean ± SEM. Comparisons among all groups were performed with ANOVA and comparisons between two patient groups were performed with the two-sided t-test. Graphs were plotted with Prism 4.0 (Graph Pad Software, San Diego, CA, USA). Statistical analyses were performed with STATA version 8.2 for

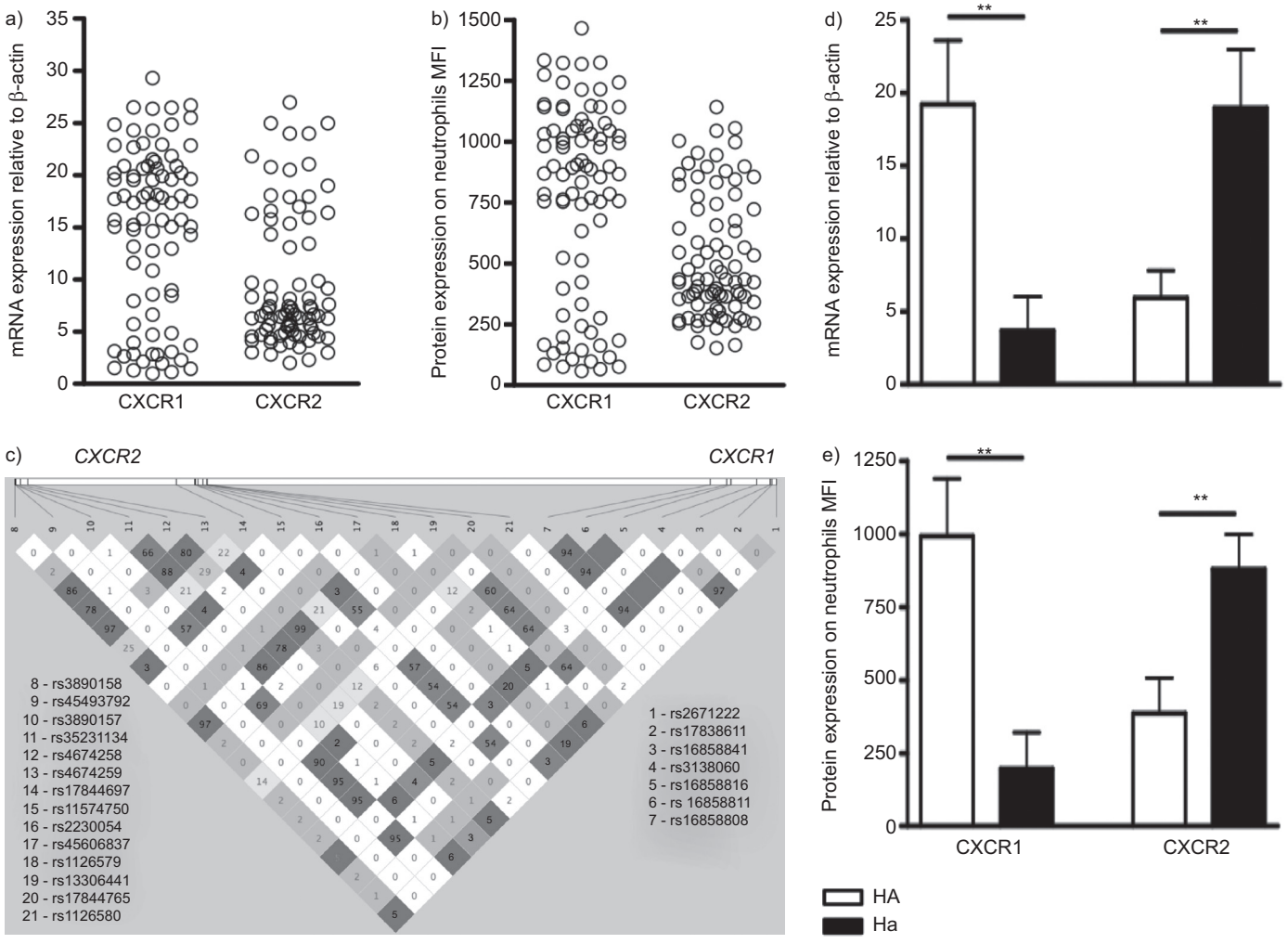

FIGURE 1. CXCR1/CXCR2 expression profiles and *CXCR1/CXCR2* haplotypes in cystic fibrosis (CF) patients. a) CXCR1 and CXCR2 mRNA expression levels were quantified in peripheral blood neutrophils from CF patients by real-time RT-PCR. b) CXCR1 and CXCR2 protein surface expression levels on CF neutrophils were quantified by fluoresence-activated cell sorting. MFI: mean fluorescence intensity. c) Location and linkage disequilibrium (R^2 and D') of *CXCR1* and *CXCR2* polymorphisms genotyped in the CF population. In this plot, each square represents a pairwise comparison between two single-nucleotide polymorphisms (SNPs) and the respective R^2 is given within each square. Darker square colours indicate higher values of D', up to a maximum of 1. SNPs are numbered sequentially, 5′ to 3′, and their relative location is indicated along the top. d) CXCR1 and CXCR2 mRNA and e) protein surface expression levels in CF patients stratified for the HA or Ha haplotypes. **: $p<0.01$.

TABLE 1 *CXCR1* and *CXCR2* tagging single-nucleotide polymorphisms (SNPs) and their association with lung function

SNP	Position	Location/function	Alleles	MAF FEV_1# ⩽70% pred	MAF FEV_1# >70% pred	OR	p-value
CXCR1							
rs2671222	-2668	Promoter	G/A	0.046	0.065	0.66	0.411
rs17838611	-2423	Promoter	G/A	0.027	0.007	3.92	0.028
rs16858841	-2329	Promoter	C/T	0.077	0.015	**6.02**	1.58×10^{-5}
rs3138060	-1566	Intron	C/G	0.044	0.066	0.63	0.167
rs16858816	-143	Intron	C/T	0.066	0.016	**4.61**	2.31×10^{-4}
rs16858811	92	Met/Arg	G/T	0.066	0.014	**5.29**	1.41×10^{-4}
rs16858808	1003	Arg/Cys	C/T	0.052	0.016	3.55	0.003
CXCR2							
rs3890158	-9203	Promoter	A/G	0.468	0.466	1.01	0.948
rs45493792	-9191	Promoter	-/T	0.364	0.370	0.94	0.791
rs3890157	-9185	Promoter	T/G	0.077	0.025	**3.47**	4.73×10^{-4}
rs35231134	-9179	Promoter	-/T	0.510	0.498	1.05	0.822
rs4674258	-8909	Promoter	C/T	0.343	0.370	0.77	0.247
rs4674259	-8490	5′-UTR	A/G	0.382	0.354	1.34	0.741
rs17844697	-270	Intron	G/A	0.423	0.445	0.99	0.953
rs11574750	768	Val/Val	C/T	0.077	0.023	**3.75**	2.77×10^{-4}
rs2230054	786	Leu/Leu	C/T	0.323	0.338	0.88	0.572
rs45606837	936	Leu/Leu	C/T	0.023	0.009	2.67	0.098
rs1126579	1209	3′-UTR	C/T	0.383	0.354	1.34	0.212
rs13306441	1420	3′-UTR	A/G	0.070	0.017	**4.48**	1.64×10^{-4}
Rs17844765	1437	3′-UTR	C/T	0.007	0.006	1.20	0.844
Rs1126580	1441	3′-UTR	C/T	0.433	0.447	0.99	0.955

MAF: minor allele frequency; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; UTR: untranslated region. Bold indicates significance after Bonferroni correction. #: longitudinal FEV_1, age-adjusted (20 yrs) [9].

Windows (STATA Corporation, College Station, TX, USA) and PASW version 18.0 for Mac (SPSS Inc., Chicago, IL, USA).

RESULTS

Expression levels of CXCR1 and CXCR2 observed in peripheral blood neutrophils isolated from CF patients demonstrated two distinct expression populations at both the mRNA and protein levels (fig. 1a and b). Based on a high variability in CXCR1 and CXCR2 mRNA and protein expression (fig. 1a and b and data not shown), we set out to assess whether genetic hot-spots within the *CXCR1* and *CXCR2* genes [11] associate with CXCR1/2 expression levels and CF lung disease severity in a well-characterised CF patient cohort (table 1). 191 CF patients were homozygous for ΔF508, 129 were heterozygous carriers of the ΔF508 allele of CFTR, and 122 had *CFTR* mutations other than ΔF508. 246 patients were positive for *P. aeruginosa* microbiology (bacteria isolated in at least two consecutive sputum samples with a minimum of a 6-month interval). 21 SNPs tagging the *CXCR1* and *CXCR2* loci were genotyped (table 2). All polymorphisms had genotype distributions consistent with the Hardy–Weinberg equilibrium ($p>0.1$) and call rates ranged from 89.1 to 99.1%. The minor allele frequencies of *CXCR1/CXCR2* SNPs showed no significant difference in the CF population compared to an age-matched

TABLE 2 *CXCR1* and *CXCR2* risk allele haplotype combinations and their association with lung function

	Polymorphism position							MAF FEV_1# % pred		OR	p-value
	CXCR1				*CXCR2*			⩽70	>70		
	-2329	-143	92	1003	-9185	768	1420				
***CXCR1*_Ha**	T	T	G	T				0.063	0.014	4.90	2.17×10^{-4}
***CXCR2*_Ha**					G	T	G	0.059	0.017	3.80	0.001
***CXCR1-2*_Ha**	T	T	G	T	G	T	G	0.063	0.010	7.24	2.30×10^{-5}

MAF: minor allele frequency; FEV_1: forced expiratory volume in 1 s; % pred: % predicted. *CXCR1*_Ha and *CXCR2*_Ha combine all risk alleles of *CXCR1* or *CXCR2*, respectively; *CXCR1-2*_Ha combines risk alleles of both *CXCR1* and *CXCR2*. #: longitudinal FEV_1, age-adjusted (20 yrs) [9].

healthy control population (online supplementary table S3). We found that four polymorphisms in *CXCR1* and three polymorphisms in *CXCR2* strongly correlated with age-adjusted lung function [9] in CF patients (table 1). Patients with either haplotype *CXCR1*_Ha or haplotype *CXCR2*_Ha (heterozygous for the SNP cluster) displayed significantly lower age-adjusted longitudinal lung function (FEV_1) than CF patients homozygous for *CXCR1*_HA (FEV_1 $\leqslant$70% pred, OR=4.90) or *CXCR2*_HA (FEV_1 $\leqslant$70% pred, OR=3.80) (table 2). Intriguingly, SNPs comprising both haplotypes *CXCR1*_Ha and *CXCR2*_Ha were in a remarkably high extent of linkage disequilibrium (fig. 1c). Consequently, an even higher risk for the development of reduced lung function was found for the combined haplotype *CXCR1-2*_Ha when compared with homozygous carriers of *CXCR1-2*_HA (FEV_1 $\leqslant$70% pred, OR 7.24) (table 2). CF patients carrying the *CXCR1-2*_Ha haplotype yielded significantly lower CXCR1 mRNA and protein levels combined with higher CXCR2 mRNA and protein levels in peripheral blood neutrophils when compared with *CXCR1-2*_HA CF individuals (fig. 1d and e). The genetic effect of the *CXCR1-2*_Ha haplotype on CXCR1 and CXCR2 mRNA or protein expression was not dependent on circulating serum levels of CXCR1/2 ligands, neutrophil apoptosis or activation status of neutrophils (data not shown).

To determine whether these genetic variants affected neutrophil effector functions, we analysed CXCR1- and CXCR2-mediated antibacterial neutrophil functions in indexed CF patients carrying the *CXCR1-2*_Ha haplotype, in particular, CXCR1-mediated respiratory burst and intracellular killing of *P. aeruginosa* [2]. Indeed, neutrophils from patients carrying *CXCR1-2*_Ha displayed decreased CXCR1-mediated antibacterial functionality (fig. 2).

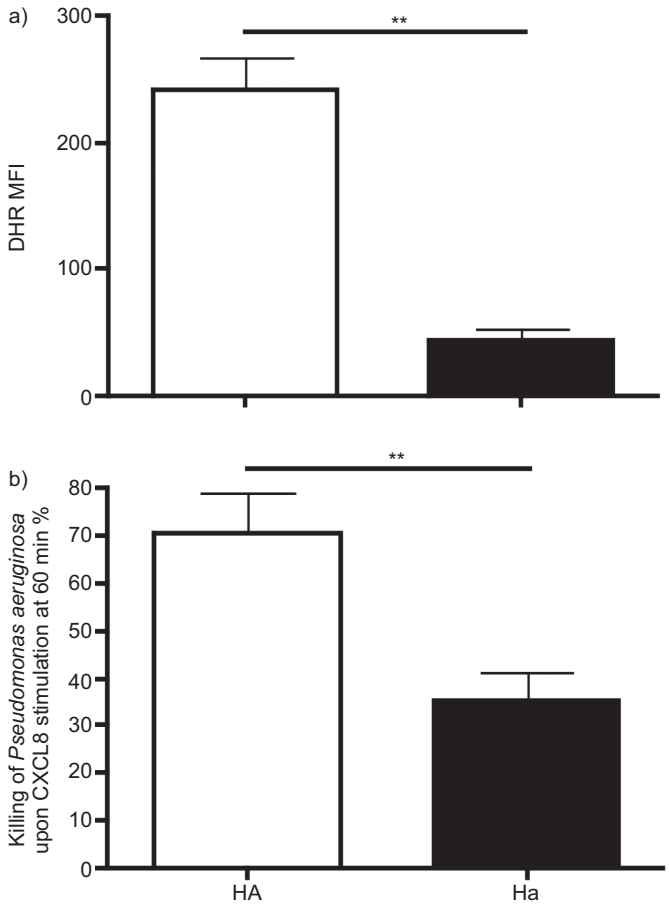

FIGURE 2. *CXCR1/CXCR2* haplotypes modulate antibacterial neutrophil functions in cystic fibrosis (CF) patients. a,b) Neutrophils were isolated from CF patients with the HA (n=3) or Ha (n=3) haplotype and CXCR1- and CXCR2-mediated neutrophil effector functions were analysed as described previously [2]. a) CXCR1-mediated respiratory burst measured by fluorescence-activated cell sorting. After staining with dihydrorhodamine-1,2,3 (DHR) for 20 min at 37°C, cells were stimulated with *N*-formyl-methionine-leucine-phenylalanine (1 M) for an additional 30 min in the presence of CXCL8 (100 nM) and the respiratory burst was analysed by flow cytometry. Results are presented as the mean fluorescence intensity (MFI) of the total neutrophil population. b) CXCR1-mediated killing of *Pseudomonas aeruginosa*. Isolated neutrophils were incubated with pre-opsonised *P. aeruginosa* bacteria at a ratio of five bacteria per neutrophil for 150 min in the presence of CXCL8 (100 nM). **: $p<0.01$.

DISCUSSION

Our results provide strong genetic and functional evidence for a clinically relevant role of *CXCR1* and *CXCR2* haplotypes in modifying CF lung disease. Previous studies identified CXCR1 as a key component in the maintenance and perpetuation of inflammation in CF lung disease [2]: CXCR1 on neutrophils mediates bacterial killing, but is damaged in CF airways proteolytically, thereby favouring infections and sustaining auto-inflammation. These studies demonstrate that high CXCR1 protein expression levels had a protective effect on lung function in CF patients. Inspired by these findings, we systematically analysed associations of *CXCR1/CXCR2* SNPs and haplotypes with CF lung disease severity by means of a candidate gene association study. These genetic investigations identified a *CXCR1/CXCR2* haplotype cluster that had a significant impact on lung function and neutrophil functionality in CF patients.

Additional genetic modifiers for CF lung disease, including *TGFB1*, *IFRD1* and *MBL2* [4–7] have been previously described. Initially, the role of TGF-β1 as a CF modulator was suggested from studies with a case–control setting [6]. However, further studies have shown that designation of the risk allele for *TGFB1* varies between studies, most probably due to transmission ratio distortion and maternal confounder effects, and thus needs to be interpreted with caution [18]. A subsequent genome-wide scan was only able to identify IFRD1 as a CF modifier. Interestingly, *IFRD1* polymorphisms were also significantly associated with variation in neutrophil effector function [7]. Initially, studies of *MBL2* as CF disease modifier gave inconsistent results [6, 19, 20]. However, a recent meta-analysis, taking the majority of available data sets into account, supports *MBL2* as major modifier of CF lung disease [21]. In a recent whole genome-wide approach, two significant loci on chromosomes 11p13 (*EHF-APIP* region) and 20q13.2 were identified that harbour genes of biological relevance for CF [8]. Due to the distinct functionalities of CXCR1, CXCR2, IFRD1 and TGF-β1 [22, 23] and, as the candidate genes on chromosomes 11p13 and 20q13.2 remain as yet unidentified, the comparison of these potential CF lung disease modifiers at genetic, expression and functional levels was out of scope of this study. However, a mutual genetic influence on association results is unlikely given their different chromosomal locations

(*CXCR1/2* Chr 2, *IFRD1* Chr 7 and *TGFB1* Chr 19). Future studies are required to define their inter-relationship and contribution to CF lung disease.

Beyond the statistical association of the *CXCR1/CXCR2* haplotype cluster on longitudinal pulmonary function in our study, we found that CF individuals carrying the haplotype cluster showed disturbed antibacterial effector functionalities, in particular, CXCL8-induced respiratory burst-mediated generation of reactive oxygen species as well as CXCL8-induced killing of *P. aeruginosa*. These studies suggest that the described *CXCR1/CXCR2* haplotype cluster may modulate pulmonary outcome in CF patients through a dysregulation of neutrophilic innate effector functions.

The main limitation of this study is the number of CF patients included. Accordingly, as this study is the first report of a genetic association, these results have to be confirmed by independent investigators in other CF populations. Comparing the distribution of FEV_1 values across allelic groups confirmed the strong association of four polymorphisms in *CXCR1* and three polymorphisms in *CXCR2* with age-adjusted lung function (table 1). Interestingly, we observed that minor allele frequencies of the majority of *CXCR2* SNPs were higher compared with *CXCR1* SNPs (table 1). Similar MAF distributions of *CXCR1* and *CXCR2* SNPs have been demonstrated previously in a control cohort by VASILESCU *et al.* [11]. The frequency of ΔF508 homozygous, ΔF508 heterozygous and non-ΔF508 CF patients did not differ significantly between HA and Ha carriers. Linear regression analysis showed that *CFTR* genotypes had no confounding effects on the *CXCR1*/2 haplotype observed.

Taken together, we have identified a *CXCR1/2* haplotype cluster that is associated with lung function in CF patients, and synergistically affects mRNA and protein expression, thereby modulating neutrophil effector functions. As both CXCR1 and CXCR2 are GPCRs, our results may provide new pharmacological approaches for the treatment of CF lung disease.

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STATEMENT OF INTEREST

A statement of interest for D. Hart can be found at www.erj.ersjournals.com./site/misc/statements.

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