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CC chemokine receptor 5 (CCR5) mRNA expression in pulmonary sarcoidosis☆

Martin Petrek [a,1]*, Agata Gibejova [a,1], Jiri Drabek [a], Frantisek Mrazek [a], Vitezslav Kolek [b], Evzen Weigl [a], Roland M. du Bois [c]

[a] *Department of Immunology, Palacky University, I. P. Pavlova Street 6, Olomouc CZ-775 20, Czech Republic*
[b] *Department of Respiratory Medicine, Palacky University, Olomouc, Czech Republic*
[c] *Interstitial Lung Disease Unit, Royal Brompton Hospital, London, UK*

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Abstract

The CC chemokine receptor 5 (CCR5) binds the chemokine ligands RANTES (CCL5) and MIP-1α (CCL3), which have been implicated in the development of alveolitis in sarcoidosis. We have, therefore, investigated CCR5 mRNA expression in bronchoalveolar lavage fluid (BALF) cells from patients with sarcoidosis. Further, we explored whether there was any association between CCR5 mRNA expression and the presence of the CCR5Δ32 DNA polymorphism. Semiquantitative RT-PCR was used to determine CCR5 mRNA expression from BALF cells from 16 control subjects (C) and 39 patients with sarcoidosis (S). The data on the CCR5Δ32 polymorphism, determined by PCR-SSP, were available for 37 patients. CCR5 mRNA expression was significantly upregulated in sarcoidosis (median ± SEM, C, 0.00 ± 0.07; S, 0.12 ± 0.07; $P < 0.05$). When patients were evaluated according to their CCR5Δ32 genotype, an interesting trend emerged with Δ32 positive patients (wt, mt) expressing less mRNA than the patients with both wild-type alleles (wt, wt): 0.00 ± 0.09, and 0.26 ± 0.09, respectively; $P > 0.05$). In conclusion, upregulation of CCR5 mRNA in BALF of patients with sarcoidosis is consistent with its chemokine ligands RANTES and MIP-1α playing a pivotal role in inflammatory cell recruitment to disease sites. Though the data from this pilot study had no clinical correlations we suggest that further studies are warranted on the role of this Th1 subset marker in the pathogenesis of sarcoidosis.

Keywords: CCR5; Lung inflammation; Th1 lymphocyte recruitment; CCR5Δ32 polymorphism

1. Introduction

Sarcoidosis is a multisystem disorder most frequently affecting the lung that results from the accumulation of CD4+ Th1 cells and macrophages with granuloma formation at disease sites [1]. Recruitment of T-lymphocytes and monocytes from the circulation to the sarcoid lung is enabled by promigratory effect of chemokines [2,3]. The CC chemokines RANTES (CCL5) and MIP-1α (CCL3) have been previously implicated in this process [4,5]. The biological effects of chemokines are mediated through seven transmembrane receptors coupled to G_i-proteins [6]. The CC chemokine receptor 5 (CCR5) is shared by the chemokines RANTES and MIP-1α.

If the CCR5 chemokine ligands (RANTES, MIP-1α) are pivotal to the development of alveolitis in sarcoidosis, we hypothesized that the receptor for these ligands, CCR5, which is characteristic of the Th1 phe-

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* Corresponding author. Tel.: +420-68-585-2285; fax: +420-68-541-5116.
E-mail address: petrekm@fnol.cz (M. Petrek).

[1] M.P. and A.G. contributed equally to this work.

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notype [7–9], may play an important role in the selective recruitment of proinflammatory CD4+ Th1 cells to the sarcoid lung. We have, therefore, investigated CCR5 mRNA expression in bronchoalveolar lavage fluid (BALF) cells from patients with sarcoidosis and compared it with the expression in BALF cells from control subjects.

We have recently shown a positive association between a CCR5 gene polymorphism (a 32 bp deletion in the CCR5 gene, CCR5Δ32) with pulmonary sarcoidosis in the Czech population [10]. We were, therefore, interested to explore whether CCR5 mRNA expression was correlated with this polymorphism.

2. Materials and methods

2.1. Study population

Bronchoalveolar lavage (BAL) was performed according to our standard procedure [11] in 39 patients with sarcoidosis and 16 control subjects. The control subjects showed no clinical signs of lung inflammation and had no lung disease in their medical history at the time of BAL. No individual with abnormal BALF cytology, immunology or microbiology was included in the control group.

The diagnosis of pulmonary sarcoidosis was based on typical clinical features together with granulomas on lung biopsies and supported by the BALF cellular profile (CD4+ lymphocytosis), and was compatible with the criteria contained in the International Statement on Sarcoidosis [12]. With regard to chest radiography, patients were divided into stages (stage 1, $n = 27$; stage 2, $n = 10$; stage 3, $n = 2$). An additional subdivision of patients was established to provide an index of the disease course: patients requiring corticosteroid treatment ($n = 18$) and patients with spontaneous disease resolution ($n = 21$). No patient received corticosteroid treatment before BAL. Detailed clinical characterization of the study groups is shown in Table 1.

The study was performed with the approval of the Ethics Committee of the Medical Faculty of Palacky University Olomouc.

2.2. Semiquantification of CCR5 mRNA expression in BAL cells by RT-PCR

Total cellular RNA was isolated from cells recovered from the BALF using the acid guanidinium thiocyanate–phenol–chloroform method [13] and was reverse transcribed to complementary DNA (cDNA) [14]. Each cDNA sample was amplified in PCR reactions with oligonucleotide primers specific to the CCR5 sequence and also to the sequence of β-actin as a standard. CCR5-specific primers were modified from the previously published primers [15]. For their sequences and details of PCR amplification protocol see Table 2. PCR was carried out in duplicate for each sample and the mean value was calculated. An optimum number of amplification cycles ($n = 41$) was determined to be in exponential phase of amplification (Fig. 1). Authenticity of CCR5 amplification was confirmed by direct sequencing (data not shown). β-Actin specific PCR has been described elsewhere [14].

Table 1
Clinical and laboratory data of investigated subjects

		Sarcoidosis patients		Control subjects	
		$N = 39$	n[a]	$N = 16$	n[a]
Age		47 (25.0–78.0)	39	51 (26.0–79.0)	13
Sex	Male	13	39	9	13
	Female	26	39	4	13
Smoking	Smokers	2	39	4	13
	Non-smokers	31	39	8	13
	Ex-smokers	6	39	1	13
BALF cell concentration	(10^5/ml)	2.5 (0.1–5.4)	37	2.6 (0.5–4.5)	11
BALF differential count	% Macrophages	74.3 (40.0–97.0)	38	91.3 (72.0–96.0)	12
	% Lymphocytes	23.5 (3.0–42.0)	38	8.0 (3.0–28.0)	12
	% Neutrophils	1.5 (0.0–44.0)	38	0.6 (0.0–2.0)	12
	% Eosinophils	0.3 (0.0–4.0)	38	0.03 (0.0–0.30)	12
	% Basophils	0.0 (0.0–0.0)	38	0.0 (0.0–0.0)	12
BALF CD4+/CD8+ T cell	Ratio	6.6 (0.4–33.9)	36	1.5 (0.5–3.6)	12
Corticosteroid treatment	Yes/No[b]	18/21	39	n.e.	n.e.
Chest-radiographic staging	I/II/III/IV	27/10/2/0	39	n.e.	n.e.

BALF, bronchoalveolar lavage fluid; n.e., not evaluated; data are shown as mean values with range (minimum to maximum) in parentheses.
[a] Number of individuals in whom particular data were available.
[b] Treatment initiated only after BAL.

Table 2
PCR protocol of CCR5 amplification and nucleotide sequences of the primers

	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	1 min	1
Denaturation	94	30 s	
Annealing of primers	60	30 s	41
Extension	72	30 s	
Final elongation	72	7 min	1
Primers	Sense	5′-gATggATTATCAAgTgTCAAgT-3′	
	Antisense	5′-gggCTgCgATTTgCTTCAC-3′	

Optical densities (OD) of CCR5 and β-actin amplicons were determined using the software QUANTISCAN (Biosoft, Ferguson, USA). CCR5 mRNA expression was normalized to the expression of the β-actin gene. The optical density ratio (ODR) of CCR5/β-actin was calculated for each sample.

2.3. CCR5Δ32 genotyping

DNA for CCR5Δ32 genotyping was available in 37/39 (94.9%) patients with sarcoidosis. Genotyping for CCR5Δ32 polymorphism was performed as previously described [16]. Briefly, CCR5 wild-type and mutant alleles were typed by PCR using sequence-specific primers (SSP) flanking the region containing the 32 bp deletion: the wild-type allele was detected as a 182 bp fragment; the CCR5Δ32 allele was detected as a 150 bp fragment. In a heterozygous individual, both fragments were detected.

2.4. Statistical analysis

The data are reported as median ± standard error of the mean (SEM). Comparisons of mRNA expression between study groups were carried out using the non-parametric Mann–Whitney *U*-test. Differences with a *P*-value of less than 0.05 were considered statistically significant.

3. Results

3.1. Detection of CCR5 mRNA in BALF cells

In order to investigate expression of mRNA for the chemokine receptor CCR5 in unseparated bronchoalveolar cells of patients with sarcoidosis and control subjects, RNA from their BALF cells was reverse transcribed to cDNA and PCR-specific for CCR5 gene was performed. CCR5 mRNA was detected in 51.3% (20/39) of patients with sarcoidosis but only in 12.5% (2/16) of control subjects, $P = 0.022$ (Fig. 2).

3.2. Semiquantification of CCR5 mRNA expression

The level of CCR5 mRNA expression (optical density ratio, ODR) was determined for each individual by normalizing CCR5 expression to the expression of β-actin gene. CCR5 mRNA expression (ODR, median ± SEM) was significantly higher ($P = 0.045$) in patients with sarcoidosis (S; 0.12 ± 0.07) in comparison with control subjects (C; 0.00 ± 0.07) (Fig. 3). However, CCR5 mRNA expression was not associated with the chest radiographic staging nor with disease course defined by the (non)requirement for corticosteroid therapy (data not shown).

3.3. The relationship between CCR5 mRNA expression and the presence of the CCR5Δ32 polymorphism

We also sought to assess whether CCR5 mRNA expression correlated with the presence of the CCR5Δ32 polymorphism. Twenty-eight of 37 patients (75.7%) were wild-type (wt) homozygotes (CCR5 wt, wt), nine of 37 patients (24.3%) were heterozygotes with the Δ32 mutant allele (mt) (CCR5 wt, mt) and none was mutant-type homozygote (CCR5 mt, mt). In the group of patients without the mutant allele (wt, wt) the ODR of CCR5 mRNA expression reached 1.58. In marked contrast, in patients carrying the mutant allele (wt, mt) it did not exceed 0.70. The median value in 'wt, wt' patients was higher than in 'wt, mt' patients (wt, mt: 0.00 ± 0.09; wt, wt: 0.26 ± 0.09; $P > 0.05$); the difference, however, did not attain significance.

4. Discussion

Chemokines and their receptors play an important role in the selective recruitment of various subsets of leukocytes to disease sites. Recent studies have indicated that some chemokine receptors are differentially expressed on Th1 and Th2 cells: CXCR3 and CCR5 are found predominantly on CD4+ Th1 cells, whereas CCR3, CCR4, CCR8 are restricted to CD4+ Th2 cells [8,9,17]. In this study, we have investigated mRNA expression of the chemokine receptor CCR5 in bron-

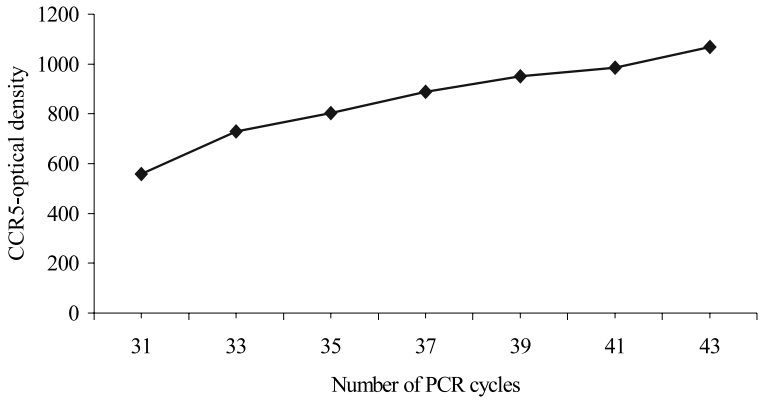

Fig. 1. Kinetic analysis of amplification of CC chemokine receptor (CCR)-5 cDNA. Duplicate samples of reverse transcribed RNA from BALF cells from patient with sarcoidosis were amplified using (CCR)-5 primers for increasing numbers of cycles. The mean CCR5 optical density values were then plotted against the number of cycles ($n = 41$).

choalveolar cells of patients with sarcoidosis, which is a typical Th1 disease [18]. In sarcoidosis, an oligoclonal T helper cell, type 1 (Th1) T-cell-mediated immune response elicited by unknown antigen leads to accumulation of activated Th1 cells at the sites of inflammation and contributes to the granuloma formation [1,2,17,19]. We have shown that CCR5 mRNA is upregulated in patients with sarcoidosis by comparison with control subjects. In our study, CCR5 mRNA expression was determined by semiquantitative RT-PCR. This approach has been standardized and previously validated in the determination of mRNA expression of other chemokines [4,20]. All experiments and measurements have been performed in duplicate. The final PCR protocol was designed from preexperiments to define the exponential phase of amplification from kinetic studies and inaccuracies due to technical problems are unlikely.

Our findings are concordant with previous studies of the CCR5 ligands, RANTES and MIP-1α, and are consistent with the concept that these upregulated chemokines, signaling via upregulated CCR5, are involved in the mechanism of accumulation of inflammatory cells in the sarcoid lung [4,5,21–23].

The CCR5 receptor is expressed on activated/memory $CD26^{high}$ $CD45RA^{low}$ CD45R0 + Th1 lymphocytes as well as monocytes [8,9,24,25]. The significant upregulation of CCR5 mRNA expression in sarcoidosis patients suggests that this receptor may be critical for recruitment of Th1 cells in sarcoidosis. In agreement with this speculation, several reports have demonstrated an important role for CCR5 in attracting Th1 cells to the sites of inflammation in rheumatoid arthritis, multiple sclerosis and crescentic glomerulonephritis diseases associated with a Th1-type immune response [26–29]. To our knowledge, there has been no report to date on the expression of the chemokine receptor CCR5 in inflammatory lung diseases.

The CCR5 mRNA expression was higher in patients with both CCR5Δ32 wild-type allele (wt, wt) in comparison with patients carrying the mutation (wt, mt), and also the maximal value of CCR5 mRNA expression in 'wt, wt' patients exceeded twice that observed in 'wt, mt' patients. However, this difference between the two groups did not attain significance, probably due to small number of subjects. The presence of a 32 bp deletion in the CCR5 loci on both chromosome has been shown to result in a non-functional surface receptor molecule unable to bind chemokine ligands [30]. In our study nine patients carrying the mutation allele were heterozygotes and it is known that heterozygosity

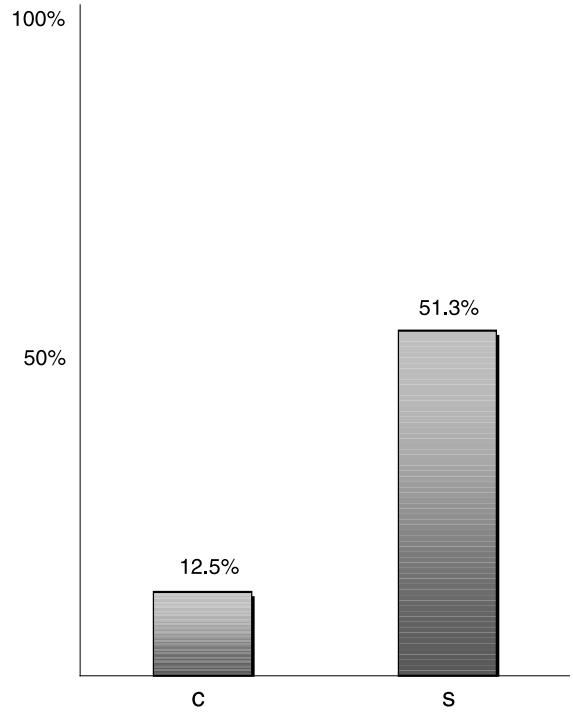

Fig. 2. CC chemokine receptor (CCR)-5 mRNA expression in unseparated BALF cells: proportion (%) of individuals in investigated groups in whom CCR5 mRNA was detected (control subjects, C; sarcoidosis patients, S; $P = 0.022$).

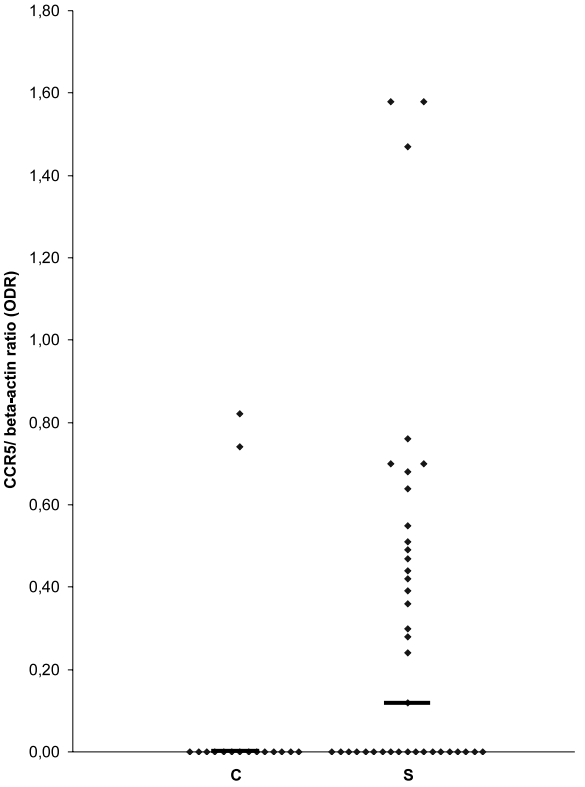

Fig. 3. CC chemokine receptor (CCR)-5 mRNA expression in unseparated BALF cells obtained from control subjects (C; $n = 16$) and patients with sarcoidosis (S; $n = 39$). CCR5 mRNA expression is expressed as ODR (group medians are indicated by horizontal bars). *CCR5 mRNA expression in sarcoidosis patients vs. control group: $P = 0.045$.

in CCR5Δ32 results in a 50% decrease of CCR5 molecule expression on the cell surface [31]. Although the results from our pilot study are limited by the small number of subjects included, they allow the speculation that the presence of CCR5Δ32 mutant allele downregulates not only CCR5 protein but also CCR5 mRNA expression. In this context, it would be interesting to quantify CCR5 protein surface expression and to explore if there is any relationship between CCR5 mRNA and protein levels.

In summary, the detection of upregulated CCR5 mRNA expression in bronchoalveolar cells of patients with sarcoidosis is concordant with its participation in binding upregulated CCR5 ligands in the disease process. Though the results of this pilot study had no clinical correlations we suggest that further studies are warranted to define further the role of this Th1 subset marker in the pathogenesis of sarcoidosis.

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