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Reduced Th1 response in the lungs of HLA-DRB1*0301 patients with pulmonary sarcoidosis

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ABSTRACT: **To investigate why human leukocyte-associated antigen-DRB1*0301 (HLA-DRB1*0301) positive Scandinavian patients have a better prognosis than HLA-DRB1*0301 negative patients, the present authors examined patterns of cytokine expression in bronchoalveolar lavage (BAL) cells and BAL fluid (BALF) from patients with pulmonary sarcoidosis and controls.**

Using real-time PCR, the mRNA expression of selected cytokines in BAL cells from newly diagnosed, untreated nonsmoking patients (n=25) and controls (n=11) was quantified. Cytokine protein levels in BALF from patients (n=34) and controls (n=11) were assessed using cytometric bead array. The patients were evaluated and stratified into two subgroups: HLA-DRB1*0301 positive (all with an acute onset) and HLA-DRB1*0301 negative (all with an insidious onset).

When comparing patients and controls, BAL cells of the patients expressed significantly higher levels of interferon (IFN)-γ and interleukin (IL)-10 mRNA. There were significantly decreased IFN-γ and tumour necrosis factor (TNF)-α mRNA levels, and a tendency toward higher levels of transforming growth factor-β1 mRNA in HLA-DRB1*0301 positive compared with HLA-DRB1*0301 negative patients. Protein levels of IL-1β, IL-2, IL-6, IL-12p70 and TNF-α in BALF were significantly higher in patients. HLA-DRB1*0301 positive patients exhibited tendencies to lower levels of most cytokines in BALF.

In conclusion, the present data show a reduced expression of T-helper cell type-1 cytokines in human leukocyte-associated antigen-DRB1*0301 positive patients, which may relate to their good prognosis.

KEYWORDS: **Bronchoalveolar lavage, cytokine, interferon-γ, tumour necrosis factor-α, sarcoidosis**

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Sarcoidosis, a systemic inflammatory disease of unknown origin, is characterised by an accumulation of activated CD4+ T-cells, which is accompanied by granuloma formation in the involved organ [1]. Pulmonary sarcoidosis resolves either spontaneously or with treatment, or develops into a chronic disease with a risk for fibrosis. An imbalance in the expression of T-helper cell type (Th)1/Th2 cytokines by alveolar cells has been suggested to be of importance for the outcome of a pulmonary immune response in sarcoidosis [2, 3]. A number of studies on mRNA and protein levels of interleukin (IL)-2 and interferon (IFN)-γ in the lungs [4–6] support the concept of sarcoidosis as a Th1-mediated disease. IL-12 acts as an important mediator of the Th1 polarisation in sarcoidosis [7]. Recently, BARBARIN *et al*. [8] reported elevated levels of IL-12p40, but not IL-12p70 protein in the bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis. This may indicate elevated levels of IL-23, since IL-12p40 is also part of the IL-23 heterodimer. In addition to this, the macrophage-derived cytokines IL-1β, IL-6 and tumour necrosis factor (TNF)-α, which have been shown to be important for initiation of inflammatory response and granuloma formation, are expressed at higher levels in patients with active sarcoidosis [9, 10]. Increased levels of TNF-α have, moreover, been reported to correlate with a prolonged disease course in sarcoidosis [2, 11]. Anti-inflammatory cytokines have not been as well characterised in sarcoidosis, although increased levels of IL-10 and transforming growth factor (TGF)-β have been reported in patients with active sarcoidosis [6, 12]. ZISSEL *et al*. [12] reported that patients with active disease and increased TGF-β release in bronchoalveolar

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lavage (BAL) cell culture had a better prognosis with a spontaneous remission within 6 months.

Previous studies by the present authors and other groups demonstrated a strong correlation between human leukocyte-associated antigen-DRB1*0301 (HLA-DRB1*0301) and an acute onset of disease, radiographic pulmonary stage I and a better prognosis with a short disease duration [13–15]. It has also been shown that Scandinavian sarcoidosis patients often have lung-restricted accumulations of CD4+ T-cells using the T-cell receptor (TCR) AV2S3 gene segment [16], which correlates strongly with HLA-DRB1*0301 and in some cases with HLA-DR13 [16, 17]. It is likely that the latter correlation occurs because HLA-DR13 positive patients sometimes carry the HLA-DRB3*0101 allele. The DRB3*0101 molecule is known to be structurally similar to, and to be able to present similar antigenic peptides as, the DRB1*0301 molecule [18].

This study differentiates between sarcoidosis patients with such lung-accumulated AV2S3-expressing T-cells, and those without. In the former group, all were HLA-DRB1*0301 positive except one who was HLA-DR13 positive (called DRB1*0301 positive group), while in the latter group all were DRB1*0301 negative (called DRB1*0301 negative group).

The aim of the study was to characterise the Th1/Th2 cytokine patterns as well as other pro- and anti-inflammatory cytokines in the lungs of sarcoidosis patients, compared with healthy controls. In order to achieve this aim, two newly developed techniques, real-time PCR and cytometric bead array (CBA), were applied to more adequately quantify cytokine mRNA and protein levels in BAL cells and BALF, respectively, from patients and controls.

METHODS

Study subjects

All patients included in this study were consecutive patients referred to the Division of Respiratory Medicine (Karolinska University Hospital, Stockholm, Sweden) for investigation, and all were newly diagnosed with sarcoidosis. All patients had a clinical picture in accordance with pulmonary sarcoidosis, as determined by symptoms, chest radiography and pulmonary function tests, and the diagnosis was established using the criteria by the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) [1]. No patient had received corticosteroid therapy at the time of the study. All patients and controls were nonsmokers. Written informed consent was obtained from all subjects, and the study was approved by the local ethics committee.

A total of 40 patients were included in this study, of whom 25 were analysed for the detection of cytokine mRNA in BAL cells and 34 for cytokine protein levels in BALF. Thus, in 19 male and six female patients, real-time PCR was used for cytokine mRNA detection in BAL cells. These patients were divided into two groups according to human leukocyte antigen (HLA) type: 12 patients (median (range) age 38.5 yrs (30–50); nine males, three females), of whom 11 were HLA-DRB1*0301 positive and one was HLA-DR13 positive, were included in the DRB1*0301 positive group, as previously discussed; 13 patients (median age 39 yrs (27–65); 10 males, three females), who were all HLA-DRB1*0301 negative, were included in the DRB1*0301 negative group. All 12 patients in the DRB1*0301 positive group had an acute onset of the disease, in 10 cases defined as Löfgren's syndrome. In the DRB1*0301 negative group, all patients had an insidious disease onset. Eleven healthy adults (median age 29 yrs (21–46); seven males, four females) were included as controls.

In 34 patients (24 males, 10 females), cytokine levels in BALF were investigated using CBA analysis. These patients were also divided into two groups: 14 HLA-DRB1*0301 positive patients (median age 39.5 yrs (28–50); 10 males, four females) and 20 HLA-DRB1*0301 negative patients (median 43.5 yrs (27–65); 14 males, six females). The DRB1*0301 positive group, all of which had an acute onset of the disease, were defined as Löfgren's syndrome in 13 cases, and all had lung-accumulated T-cells expressing the AV2S3 TCR gene segment. In contrast, none in the DRB1*0301 negative group had such lung T-cell expansions. The patient history did not reveal any difference in environmental exposure between any of the patient groups. The same control group as above has been used for the detection of cytokine levels in BALF. In 19 of the patients, both cytokine mRNA and BALF protein levels were analysed.

BAL and preparation of BALF cells

BAL was performed as previously described [19]. Briefly, fibreoptic bronchoscopy was performed under local anaesthetic. A flexible fibreoptic bronchoscope (OBF Type 1 TR; Olympus Optical Co., Tokyo, Japan) was passed transorally and wedged into the middle-lobe bronchus. Sterile PBS solution at 37°C was instilled in five aliquots of 50 mL and immediately re-aspirated and collected in a siliconised plastic bottle that was kept on ice.

The BALFs recovered were centrifuged at $400 \times g$ for 10 min at 4°C, to separate BAL cells from the supernatant. The supernatant was stored at -70°C until use. The cell pellet was resuspended in RPMI-1640 medium (Sigma-Aldrich, Irvin, UK) and the viability was determined by trypan blue exclusion. Cell differential counts were determined by May-Grünwald-Giemsa staining of cytopin slides. BALF CD4/CD8 T-lymphocyte ratio and TCR AV2S3 expression in BALF cells were determined by flow cytometric analysis (FACScan; Becton Dickinson, Montain View, CA, USA) using monoclonal antibodies (Mabs) against CD3+, CD4+ and CD8+ (Dako Cytomation Norden AB, Solna, Sweden) and anti-human TCR AV2S3-specific Mab clone F1 (Serotec, Oxford, UK), as previously described [20].

RNA extraction and cDNA synthesis

Total RNA was extracted *via* the guanidium thiocyanate-phenol-chloroform technique [21], using RNA Bee (Nordic Biosite, Stockholm, Sweden). Briefly, 300–600 μL RNA Bee was added to 1–2 $\times 10^6$ BAL cells. The samples were incubated for 10 min at room temperature and then stored at -70°C until use. After thawing, about 30–60 μL of chloroform was added to each sample, which was vortexed and kept on ice for 5 min. The samples were centrifuged at 4°C ($12{,}000 \times g$) for 20 min. The upper phase, containing RNA, was transferred to another tube and then an equal volume of ice-cold isopropanol was added. After incubation at -20°C overnight, the samples were centrifuged. The RNA pellets were washed using 75% ice-cold ethanol and dissolved in 20–40 μL diethyl pyrocarbonate-treated water.

To synthesise cDNA, 1 μg of total RNA was incubated in the presence of 20 mM random hexamer primer (Pharmacia Biotech, Uppsala, Sweden) and 200 units Superscript^{TM}II RNase H^{-} Reverse transcriptase (Invitrogen, Lidingo, Sweden) for 10 min at room temperature and then 45 min at 40°C, followed by 5 min at 95°C to inactivate the enzyme. It was then stored at -20°C.

Analysis of cytokine gene expression by real-time PCR

Cytokine gene expression was quantified by real-time PCR using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

A PCR reaction was set up in a 25-μL reaction volume, with 1 × Taqman buffer A, 0.5 U Ampli-Taq gold, $MgCl_2$ concentration optimised for each cytokine (Applied Biosystems), 0.5 mM deoxyribonucleoside triphosphate (Amersham Bioscience, Uppsala, Sweden), 5.0 pmol of each forward and reverse primer (Cybergene AB, Stockholm, Sweden) and 2.5 pmol probe (Cybergene). A measure of 2 μl of the cDNA (diluted 1:3) was used as a template. PCR conditions were optimised for primers, probes and $MgCl_2$. β-actin was used as a housekeeping gene to normalise the values of other genes.

Primers and probes were designed to span either related exon junctions (IL-4, IL-10, IFN-γ, TNF-α and β-actin) or multiple exon junctions separated with long introns (IL-12p40 and TGF-β1; table 1). With such primers, the amplification of contaminating genomic DNA was avoided. The PCR condition was as follows: an initial period of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. All samples were run in duplicates and the mean values calculated.

For relative quantification of expression of cytokine genes in BALF cells, the following arithmetic formula was used: $2^{-\Delta\Delta CT}$ [22], where the amount of target gene was normalised to β-actin (housekeeping gene) and the relative increase of a cytokine in BALF cells was calculated in relation to the mean value of cytokine gene expression in a healthy control group.

Analysis of secreted cytokines by cytometric bead array

Commercially available CBA (BD Biosciences, PharMingen, San Diego, CA, USA) was used for detection of secreted cytokines in BAL supernatant. BAL supernatant was concentrated 650 × using Amicon Ultra-15 Filters (Millipore Corporation, Bedford, MA, USA), the day before cytokine testing was to be performed. The Human Inflammation Kit (BD Biosciences) and Th1/Th2 kit (BD Biosciences) were used, which contain microparticles with six discrete fluorescence intensities, each coated with antibodies to a particular cytokine. This allowed simultaneous detection of multiple soluble analytes in a particle-based immunoassay, measuring IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ protein levels in BAL supernatant. The assays were performed according to the manufacturer's instructions and the data were analysed using the BD Cytometric Bead Array (BD Biosciences) software.

Human leukocyte antigen typing

HLA class II (HLA-DR) typing was carried out on DNA through use of the PCR and amplification with sequence-specific primers [23].

TABLE 1 Sequence of primers and probes for cytokines and β-actin used in real-time PCR

Target sequence	Primer	Sequence 5'–3'	Gene bank accession number	Position
β-Actin	Sense	CCACCATGTACCCTGGCA	NM-001101	981–998
	Probe	AAGATCAAGATCATTGCTCCTCCTGAG		1049–1075
	Antisense	CACATCTGCTGGAAGGTGGA		1121–1140
IFN-γ	Sense	ATATTTTAATGCAGGTCATTCAGATGTA	X01992	234–262
	Probe	TTGAAGAATTGGAAAGAGGAGAGTGACAGAAAAATA		291–327
	Antisense	TGAAGTAAAAGGAGACAATTTGGCT		334–358
TNF-α	Sense	TCTTCTCGAACCCC GAGTGA	AF043342	10–29
	Probe	TAGCCCATGTTGTAGCAAACCCTCAAGCT		38–66
	Antisense	CCTCTGATGGCACCACCAG		142–160
IL-10	Sense	GCTGGAGGACTTTAAGGGTTAC	NM-000572	269–290
	Probe	AGAACCAAGACCCAGACATCAAGGC		355–379
	Antisense	ACAGGGAAGAAATCGATGACAG		434–455
IL-12p40	Sense	TGGAGTGCCAGGAGGACAGT	AF180563	578–597
	Probe	ATGGTGGATGCCGTTCACAAGCTCAA		637–662
	Antisense	TCTTGGGTGGGTCAGGTTTG		705–724
IL-4	Sense	GCCTCACAGAGCAGAAGACTCT	M13982	182–203
	Probe	TGCTGCCTCCAAGAACACAACTGA		234–260
	Antisense	TCTCATGGTGGCTGTAGAACTG		295–316
TGF-β1	Sense	CAGCAACAATTCCTGGCGATAC	NM-000660	890–912
	Probe	CTGCTGGCACCCAGCGACTCG		925–945
	Antisense	AAGGCGAAAGCCCTCAATTT		1007–1026

IFN: interferon; TNF: tumour necrosis factor; IL: interleukin; TGF: transforming growth factor.

Statistical analysis

The Mann-Whitney U-test was used for the comparison of the mRNA and protein level between groups. Correlations between different parameters were determined with Spearman's rank correlation test. Values of $p<0.05$ were regarded as significant.

RESULTS

The BAL cell analyses and pulmonary function parameters are presented in table 2. As expected, the results show higher BAL cell concentrations and higher percentages of lymphocytes in BALF from each patient subgroup compared to controls, while the percentages of macrophages were lower in patients. The vital capacity and diffusing capacity of the lung for carbon monoxide were lower in the HLA-DRB1*0301 negative sarcoidosis patients *versus* HLA-DRB1*0301 positive patients. No other significant differences were found either between patients and controls or between patient subgroups.

Cytokine mRNA expression in BAL fluid cells

The results of real-time PCR for the mRNA expression of IFN-γ, TNF-α, TGF-β1 and IL-10 are summarised in figure 1. A marked increase of IFN-γ m*RNA* in BALF cells from sarcoidosis patients compared with controls was observed ($p=0.0013$; fig. 1a). IL-10 mRNA levels were also increased in sarcoidosis patients ($p=0.016$) compared with controls (fig. 1d). However, no significant differences were found for TGF-β1 mRNA, which was expressed in BALF cells of all individuals (fig. 1c), or IL-12p40 mRNA, which was expressed in BALF cells of 14 patients and six controls (data not shown). IL-4 mRNA expression could only be detected in BALF cells of two patients and one control (data not shown). TNF-α mRNA transcripts did not differ between patients and controls, while significantly reduced levels in HLA-DRB1*0301 positive *versus* negative patients were found ($p=0.05$; fig. 1f). In addition, IFN-γ mRNA was significantly less expressed in HLA-DRB1*0301 positive compared with DRB1*0301 negative patients ($p<0.05$; fig. 1e). Finally, HLA-DRB1*0301 positive patients tended to express higher mRNA levels of TGF-β1 (fig. 1g). A strong correlation was also found between the expression of IFN-γ mRNA and TNF-α mRNA ($r=0.826$; $p<0.0001$) in BALF cells of the entire patient group (fig. 2).

When investigating any association between cytokine mRNA expression and BALF cellular profiles, it was found that IFN-γ mRNA levels correlated positively with the percentages of BALF lymphocytes ($r=0.576$; $p<0.01$; fig. 3a) and negatively with macrophages ($r=-0.556$; $p<0.01$; fig. 3b).

Cytometric bead array analysis of BALF

Concentrated BALF from 34 patients and 11 healthy controls was analysed using CBA. Markedly increased TNF-α ($p<0.05$), IL-1β ($p<0.01$), IL-2 ($p<0.001$), IL-6 ($p<0.01$) and IL-12p70 ($p<0.05$) protein levels were found in sarcoidosis compared with control BALF (fig. 4a–f). A tendency towards elevated levels of IL-10 protein was found in sarcoidosis BALF ($p=0.10$). No differences were detected in the IL-5 protein level in the lavage fluid from patients and controls (data not shown). IL-4 and IFN-γ protein levels were too low for detection with the CBA kit.

TABLE 2 Bronchoalveolar lavage (BAL) analysis and lung function parameters

	Samples used for RT-PCR analyses		Samples used for CBA analyses		Controls
	HLA-DRB1*0301+	DRB1*0301-	DRB1*0301+	DRB1*0301-	For RT-PCR and CBA
Subjects n	12	13	14	20	11
Radiograph stage I/II/III n	6/6/0	2/9/2	6/8/0	4/13/3	ND
BAL analysis					
Recovery %	70 (63–89)	68 (51–83)	73 (54–89)	68 (16–85)	74 (63–88)
Viability %	93 (80–99)	94 (85–100)	95 (80–100)	96 (85–100)	92 (87–98)
Cell concentration $\times 10^6 \cdot L^{-1}$	198 (104–481)	270 (89–746)	161 (93–319)	245 (133–746)	72 (47–165)***
Differential cell counts					
Macrophages %	67 (45–94)	55 (38–87)	65 (45–95)	56 (38–87)	89 (80–95)**
Lymphocytes %	32 (5–52)	38 (11–60)	34 (4–50)	39 (11–60)	9 (6–20)**
Neutrophils %	1 (0–4)	1 (0–8)	1 (0.2–4.2)	0.9 (0–34)	1 (0–2)
Eosinophils %	0 (0–3)	0 (0–3)	0.4 (0.2–3.2)	0.3 (0–1.2)	0 (0–1)
CD4/CD8 ratio	5 (2–19)	6 (1–21)	7 (2–31)	5 (1–21)	ND
Pulmonary function tests % of reference value					
VC	89.5 (80–110)	85.5 (64–108)	91 (80–110)*	87 (65–108)	ND
FEV_1	88.5 (80–105)	83.5 (68–109)	89 (80–105)	80.5 (47–109)	ND
$D_{L,CO}$	91 (85–119)*	73.5 (56–114)	87 (74–119)	78 (56–114)	ND

Data are presented as median (minimum–maximum), unless otherwise stated. RT: real time; CBA: cytometric bead array; ND: not done; VC: vital capacity; FEV_1: forced expiratory volume in one second; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide. *: $p<0.05$ between patient subgroups; **: $p<0.01$ *versus* each patient subgroup; ***: $p<0.001$ *versus* each patient subgroup.

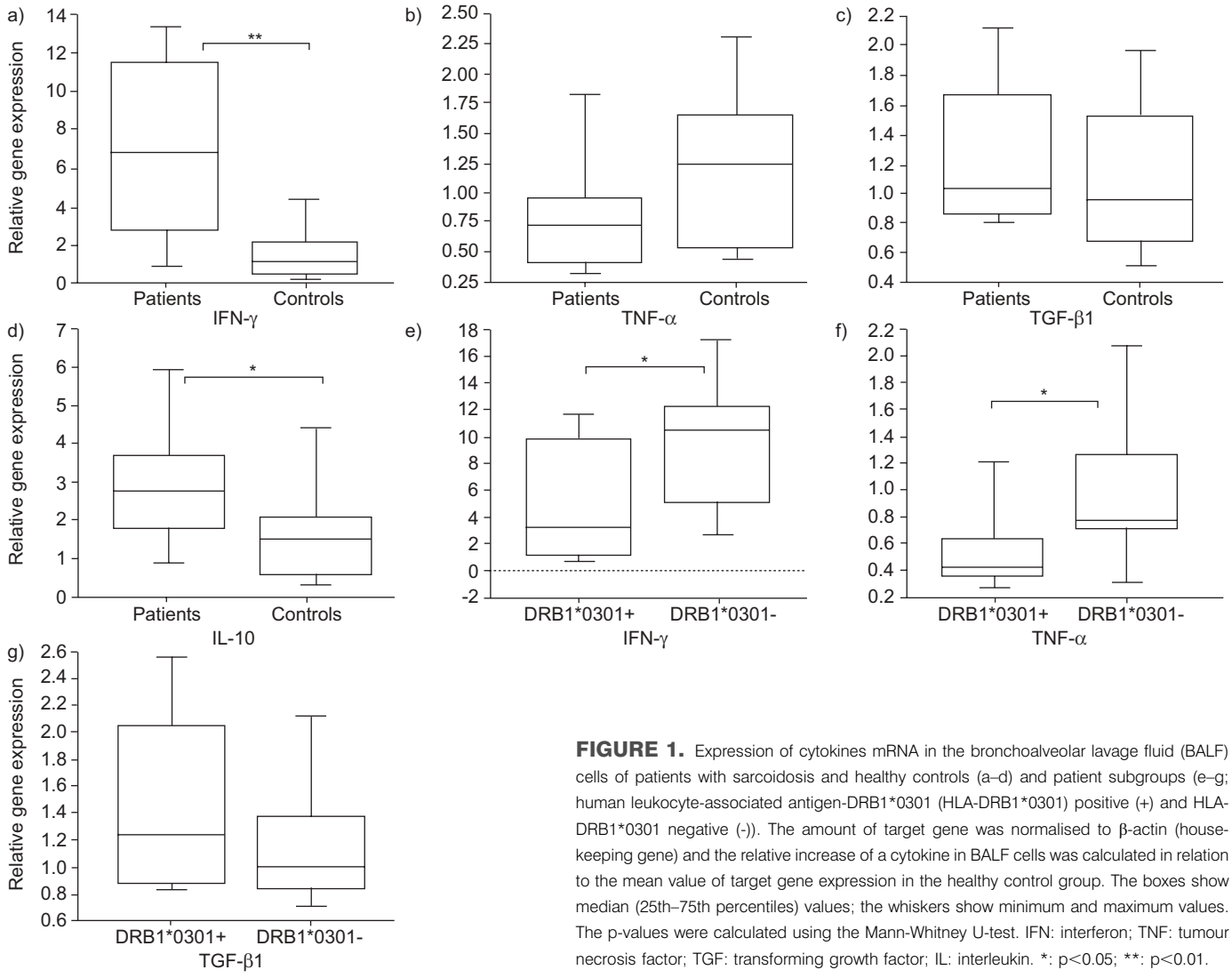

FIGURE 1. Expression of cytokines mRNA in the bronchoalveolar lavage fluid (BALF) cells of patients with sarcoidosis and healthy controls (a–d) and patient subgroups (e–g; human leukocyte-associated antigen-DRB1*0301 (HLA-DRB1*0301) positive (+) and HLA-DRB1*0301 negative (-)). The amount of target gene was normalised to β-actin (housekeeping gene) and the relative increase of a cytokine in BALF cells was calculated in relation to the mean value of target gene expression in the healthy control group. The boxes show median (25th–75th percentiles) values; the whiskers show minimum and maximum values. The p-values were calculated using the Mann-Whitney U-test. IFN: interferon; TNF: tumour necrosis factor; TGF: transforming growth factor; IL: interleukin. *: $p<0.05$; **: $p<0.01$.

Although no statistically significant differences in BALF cytokine protein levels between HLA-DRB1*0301 positive and HLA-DRB1*0301 negative patients were detected, a tendency towards elevated IL-2 levels ($p=0.07$) was observed in HLA-DRB1*0301 negative patients (fig. 4h). Moreover, compared with controls, there were significantly higher protein levels of TNF-α ($p<0.01$), IL-2 ($p<0.001$) and IL-12p70 ($p<0.05$) in HLA-DRB1*0301 negative but not DRB1*0301 positive patients (fig. 4g–i). Finally, it was also noted that IL-10 mRNA expression in sarcoidosis BALF cells associated with IL-10 protein levels in BALF ($r=0.748$; $p<0.01$; fig. 5).

DISCUSSION

This study aimed to investigate Th1/Th2 as well as pro- and anti-inflammatory cytokine profiles in the lungs of patients with sarcoidosis, and to evaluate any differences of such profiles between two distinct subgroups of patients. To do this, cytokine data between HLA-DRB1*0301 positive and DRB1*0301 negative patients were compared, giving consideration to the distinct clinical manifestations in these two categories of patients. DRB1*0301 positive patients were characterised by acute onset and good prognosis. In addition, all patients in the DRB1*0301 positive group, but none in the DRB1*0301 negative group, had AV2S3 positive lung T-cell expansions. The present authors studied the expression of cytokine mRNA and cytokine protein levels in BAL cells and BALF, respectively, in nonsmoking sarcoidosis patients and in controls, using real-time PCR and CBA techniques. To the best of the authors' knowledge, this is the first time that these experimental approaches have been used to investigate cytokine profiles in sarcoidosis.

The well-established concept of sarcoidosis as a Th1-mediated disease is supported by the current data. However, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12), as well as IL-10 as an anti-inflammatory cytokine, were also shown to be increased in the current patients.

Strongly elevated IFN-γ mRNA levels were seen in BAL cells of sarcoidosis compared with normal samples, but IL-4 mRNA levels were undetectable. As with previous studies on pulmonary sarcoidosis that demonstrated elevated expression of Th1 cytokines IL-2 and IFN-γ, and showed little or no detectable expression of Th2 cytokines in BAL cells [3, 24],

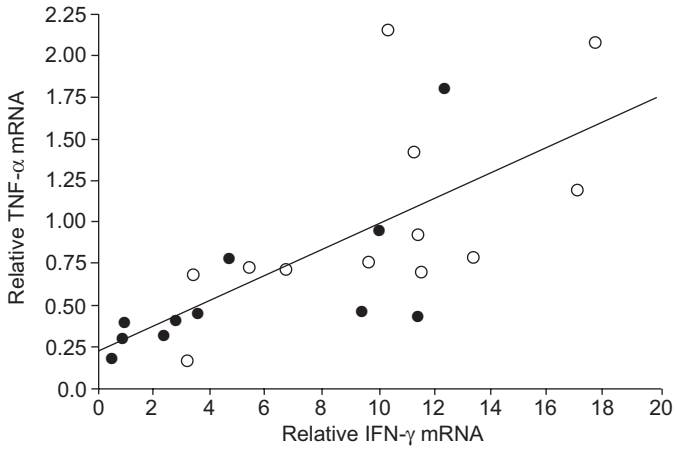

FIGURE 2. Correlation of interferon (IFN)-γ and tumour necrosis factor (TNF)-α mRNA in the bronchoalveolar cells of patients with sarcoidosis. Relative expression of cytokine mRNAs was measured using real-time PCR. Expression of IFN-γ was positively correlated with mRNA levels of TNF-α. The correlations were analysed using Spearman's rank test. ●: DRB1*0301 positive patients; ○: DRB1*0301 negative patients. r=0.836; p<0.0001.

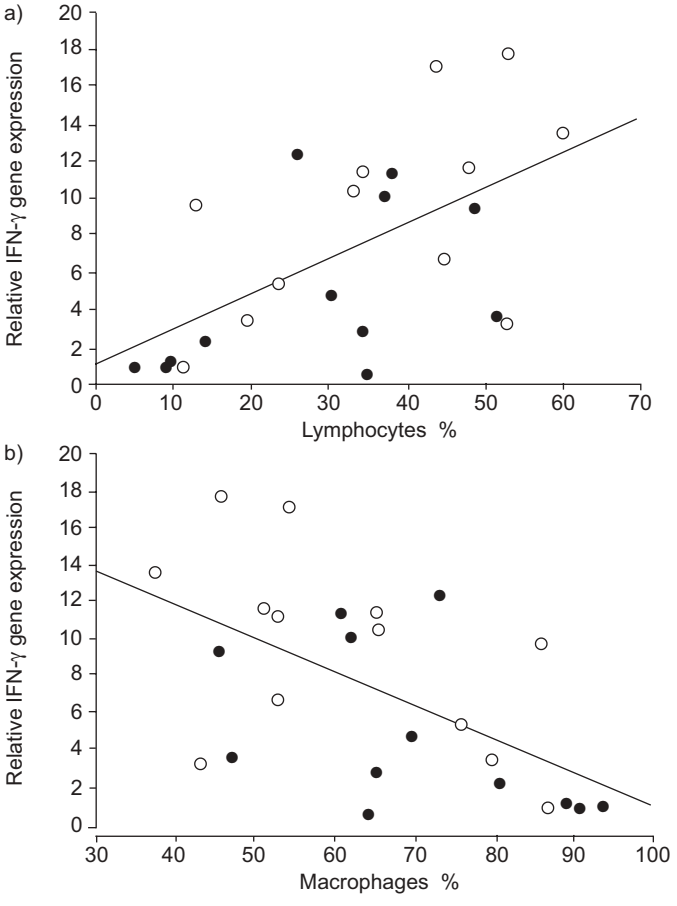

FIGURE 3. Correlation between interferon (IFN)-γ mRNA expression and bronchoalveolar percentages of a) lymphocytes (r=0.576; p<0.01) and b) macrophages (r=0.556; p<0.01) in patients with sarcoidosis. The correlations were analysed using Spearman's rank test. ●: DRB1*0301 positive patients; ○: DRB1*0301 negative patients.

these results are in line with a preferential Th1 cytokine expression at sites of disease in sarcoidosis. No significant changes in the transcript levels for TNF-α or IL-12p40 were found in BAL cells from sarcoidosis patients, which contrasted with some previous studies [24, 25] but was in line with others [4, 7, 26, 27]. Such discrepancies can depend on the choice of patient group, including smoking habits or previous/ongoing treatment, and the sensitivity of the methods. However, in protein level, an increase in IL-12p70 in BAL fluid was noted, which contrasts to the report by BARBARIN *et al*. [8].

As anti-inflammatory mediators, such as IL-10, and T-regulatory cytokines, such as TGF-β, could downregulate inflammation, these were also of interest in the study. Using freshly isolated BAL cells, this study found no significant differences in TGF-β1 mRNA levels between patients and normal samples. However, increased transcripts levels of the anti-inflammatory cytokine IL-10 were noted, which is in line with other studies [4, 6]. The data on TNF-α and IL-12p40 indicate that pulmonary cells other than BAL cells can produce and secrete these cytokines, as they differed significantly in patient and control BALF but not BAL cells. In addition to this, because IL-10 is a potent downregulator of IL-12 and TNF-α [28, 29], increased levels of IL-10 may have a suppressive effect on the BAL cell transcription of TNF-α and IL-12p40. The discrepancy between cytokine mRNA levels in BAL cells and protein levels in BALF could also be due to nontranscriptional regulation and intracellular storage [6], or perhaps differences in cytokine consumption. In addition, other pulmonary cells may contribute to the secretion of these cytokines in BALF. Several studies have suggested pulmonary epithelial cells as potential sources of TNF-α production, a theory that is supported by the fact that TNF-α protein has been localised to alveolar epithelial cells by immunohistochemical staining [30–32]. In contrast, according to the present data, the levels of IL-10 mRNA and protein in the BALF of sarcoidosis patients correlated well, indicating that BALF IL-10 was primarily produced by BALF cells in sarcoidosis.

One important aim of this study was to analyse the Th1/Th2 balance in HLA-DRB1*0301 positive and HLA-DRB1*0301 negative sarcoidosis patients. HLA-DRB1*0301 positive patients, with a very good prognosis [15], showed a tendency towards higher TGF-β1 transcript levels compared with HLA-DRB1*0301 negative patients. This is consistent with results from ZISSEL *et al*. [12], who reported that elevated protein levels of TGF-β1 are associated with good prognosis. The significantly decreased IFN-γ and TNF-α mRNA levels in DRB1*0301 positive patients, indicating a less pronounced Th1 response in BALF cells from this patient group, is consistent with a previous study from the current author group, using intracellular cytokine staining [3]. The finding supports the hypothesis that the Th1/Th2 balance in the lung may play an important role in disease development. The significantly elevated protein levels of TNF-α, IL-2 and IL-12p70 in DRB1*0301 negative but not DRB1*0301 positive patients *versus* controls further supports the concept of a more pronounced Th1 cytokine response in patients with a less favourable prognosis. Clinically speaking, the DRB1*0301 negative patient group had a more marked pulmonary disease, as reflected by more severe chest radiographic staging and more pronounced deterioration in the pulmonary function

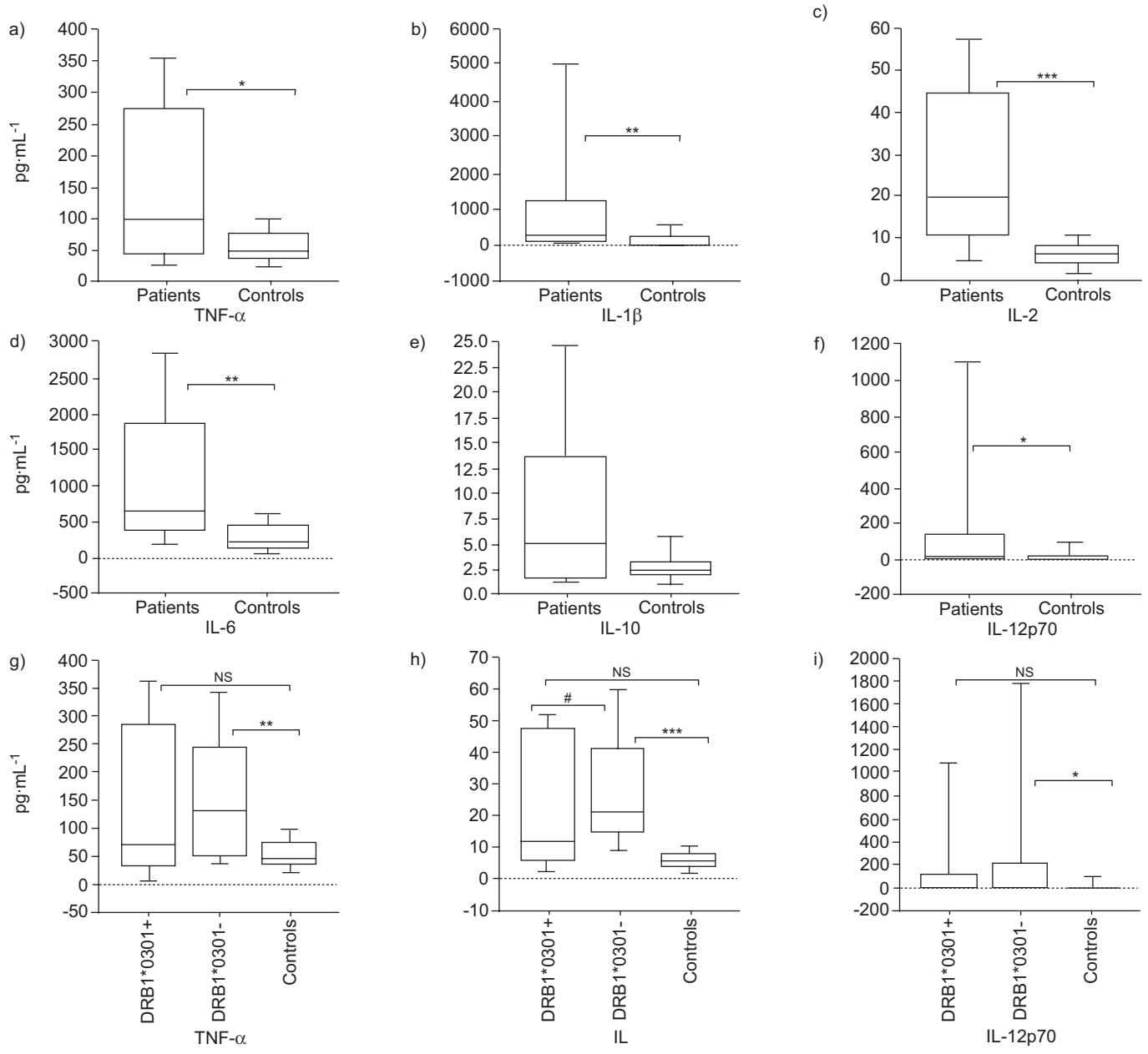

FIGURE 4. Cytokine secretion in bronchoalveolar lavage fluid (BALF). No correction for BALF dilution was applied. Cytokine concentrations in BALF were determined *via* human T-helper cell type (Th)1/Th2 (BD Biosciences, PharMingen, San Diego, CA, USA) and Inflammation CBA (BD Biosciences) kits using flow cytometry in sarcoidosis patients and healthy controls (a–f) and patient subgroups (g–i; human leukocyte-associated antigen-DRB1*0301 (HLA-DRB1*0301) positive (+) compared to HLA-DRB1*0301 negative(-)). Boxes show median (25th–75th percentiles) values; whiskers show minimum and maximum values. The p-values were calculated using the Mann-Whitney U-test. TNF: tumour necrosis factor; IL: interleukin; NS: nonsignificant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; #: $p=0.07$.

parameters. The more severe pulmonary disease in the DRB1*0301 negative patient group could thus be the result of a more pronounced Th1 response with elevated levels of Th1 cytokines. Whether the lower Th1 cytokine levels in DRB1*0301 positive patients are linked to the dramatic T-cell expansions expressing the AV2S3 gene segment and accumulated in the lungs of these patients is not known, and will be investigated in future studies.

The increased IFN-γ and TNF-α mRNA in BAL cells may in part explain the worse prognosis in HLA-DRB1*0301 negative patients, since these two cytokines are believed to be most important for granuloma formation with subsequent fibrosis development. In addition, the strong positive correlation between IFN-γ and TNF-α mRNA levels suggests a positive feedback loop between lymphocytes and macrophages, which may synergise in the formation of granuloma.

In conclusion, by using real-time PCR and cytometric bead array techniques, this study demonstrated significantly higher T-helper cell type-1 cytokine expression in sarcoidosis patients, as well as elevated levels of the anti-inflammatory cytokine

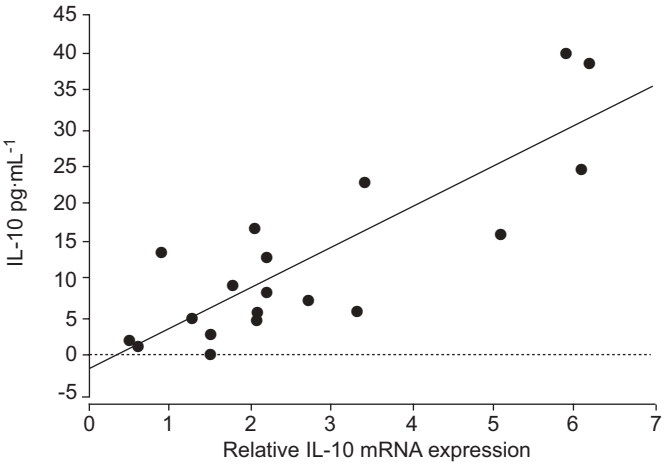

FIGURE 5. Correlation between secreted interleukin (IL)-10 and IL-10 mRNA concentrations in the bronchoalveolar lavage of patients with sarcoidosis. Levels of released IL-10 correlated positively with mRNA levels of IL-10. The correlations were analysed using Spearman's rank test. r=0.748; p=0.0015.

interleukin-10. It is possible that alterations over time in the interferon-γ to interleukin-10 ratio may be related to a spontaneous regression of inflammation in sarcoidosis, but this needs to be investigated. A less pronounced T-helper cell type-1 response and a tendency towards higher levels of transforming growth factor-β1 were associated with human leukocyte-associated antigen-DRB1*0301 positive patients and may explain the spontaneous disease resolution in these patients. Further studies aimed at elucidating the cytokine profile of specific T-cell subsets, in particular the AV2S3-positive T-cells, could help to further explain the pathogenesis of this disease.

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