Single-cell analysis: a novel approach to tumour necrosis factor- α synthesis and secretion in sarcoidosis

P. Pantelidis, D.S. McGrath, A.M. Southcott, R.M. du Bois

Single-cell analysis: a novel approach to tumour necrosis factor- α synthesis and secretion in sarcoidosis. P. Pantelidis, D.S. McGrath, A.M. Southcott, R.M. du Bois. ©ERS Journals Ltd 2002.

ABSTRACT: Tumour necrosis factor (TNF)- α is thought to be a key early cytokine in the pathogenesis of sarcoidosis, despite conflicting data. Largely the product of mononuclear phagocyte activation, it is unclear whether TNF- α production at disease sites is a feature of all mononuclear phagocytes that accumulate there or whether it is secreted by a subset of these cells. Using the reverse haemolytic plaque assay, the aims of this study were to determine if the upregulation of TNF- α could be confirmed and to investigate whether this was monocyte or macrophage specific. The reverse haemolytic plaque assay allows the measurement of cytokine production at a single cell level.

A greater number of alveolar macrophages produced TNF- α compared to autologous monocytes in sarcoidosis but not in controls and, based on cell size, it was confirmed that this was the product of more mature macrophages and that the secretion of TNF- α by monocytes and macrophages was heterogeneous: not all monocytes and macrophages secrete TNF- α . No differences in the average levels of TNF- α secretion by peripheral blood monocytes or alveolar macrophages were observed.

This study has demonstrated that a subset of mononuclear phagocytes, mature macrophages, are responsible for tumour necrosis factor secretion and this could have implications for targeted management in sarcoidosis in the future.

Eur Respir J 2002; 20: 1179–1184.

Interstitial Lung Disease Unit, Dept of Occupational and Environmental Medicine, Royal Brompton Hospital and National Heart and Lung Institute, London, UK.

Correspondence: R.M. du Bois, Interstitial Lung Disease Unit, Dept of Occupational and Environmental Medicine, Royal Brompton Hospital, London, UK, SW3 6NP.
Fax: 44 2073518336
E-mail: r.dubois@rbh.nthames.nhs.uk

Keywords: Haemolytic plaque, macrophages, monocytes, sarcoidosis, tumour necrosis factor

Received: January 25 2002

Accepted after revision: May 12 2002

This study was supported by the Sir Jules Thorn Trust.

Sarcoidosis is a granulomatous disease of unknown aetiology characterised by the presence of multiple epithelioid cell granulomas at disease sites. Pathologically, at an early stage, there is an influx of mononuclear cells followed by lymphocytes. These chronic inflammatory cells then organise spatially into the classic granulomatous response [1–5].

Tumour necrosis factor (TNF)- α is a key early cytokine in a variety of chronic inflammatory responses, including fibrosing alveolitis and granulomatous disease [6–8]. A number of previous publications have demonstrated the upregulation of TNF- α in a variety of contexts including broncho-alveolar lavage (BAL) fluid protein, macrophage protein secretion and macrophage TNF messenger ribonucleic acid content [9–16]. TNF secretion has largely been associated with active disease. However, it is possible that more stable disease may involve continuing TNF secretion at low level to explain the frequent disease flares that are observed.

It is hypothesised that mononuclear phagocyte TNF- α production early in the pathophysiology of granuloma formation is a pivotal factor in the initiation of disease. However, it is not clear whether TNF production in disease sites is a feature of all mononuclear cells that traffic there or whether it is a subset phenomenon.

The current authors have previously demonstrated,

using a reverse haemolytic plaque assay (RHPA), that single-cell production of cytokine can be assayed [16]. This technique has an advantage over routine culture conditions, as the development of cytokine product by individual cells can be localised and measured, which therefore allows for the correlation of cytokine production with cell type.

Taking this background into consideration, there were two aims in this study: 1) to confirm the upregulation of TNF- α and to question whether the upregulation of TNF- α in mononuclear phagocytes at disease sites in sarcoidosis was due to an all-or-nothing phenomenon or whether this was subset-specific; and 2) to evaluate whether there was a low grade continuous secretion of TNF in more chronic, stable disease that may predispose to disease relapse. The more sensitive RHPA technique was used.

Materials and methods

Study population

The study group comprised 10 patients with sarcoidosis (five patients with stage I chest radiography, three patients with stage II, and two patients with grade III) and 10 normal subjects. In the sarcoidosis population, two patients were taking prednisolone

and the remaining eight were on no drugs. All patients had stable disease, *i.e.* no change in symptoms, chest radiographical appearances or pulmonary function tests, in the 6 weeks prior to bronchoscopy.

Patients with sarcoidosis had clinical features consistent with pulmonary sarcoidosis and the diagnosis was supported by a positive lung biopsy in nine patients and a positive Kveim test in three patients. BAL was undertaken as part of clinical diagnosis and staging in the sarcoidosis group.

The normal subjects studied included one current smoker and an exsmoker, who had stopped smoking 7 yrs before lavage. All normal subjects had no evidence of pulmonary disease. Approval for the BAL of the normal volunteers was given by the Royal Brompton Ethics Committee, London, UK. Characteristics of all subjects are summarised in table 1.

Bronchoalveolar lavage

Fibreoptic bronchoscopy and BAL were performed using a standardised technique. Briefly, 5×20 mL aliquots of prewarmed normal saline were instilled into three subsegmental bronchi of the right middle lobe, right lower lobe and lingula, and aspirated immediately by gentle suction. The cells were separated from the lavage fluid by centrifugation at 200×g, 4°C for 7 min and washed twice in Roswell Park Memorial Institute (RPMI) 1640 media containing antibiotics (penicillin 100 U·mL⁻¹ and streptomycin 100 µg·mL⁻¹). Cellular concentrations and recoveries were estimated using crystal violet staining and cellular viability measured using trypan blue (0.4% solution) exclusion. Following cytocentrifuge preparations (Cytospin-2; Shandon Southern Instruments, Sewickly, PA, USA), the remainder of the cells were

Table 1.—Clinical and bronchoalveolar lavage details of the sarcoidosis population and normal healthy controls

	Sarcoid	Normal
Subjects n	10	10
Sex M:F	7:3	7:3
Age yrs	46.5 (27–60)	30.0 (28–34)
Smoking S:EX:NS	0:4:6	1:1:8
Treatment	2	0
Fluid recovery %	52.5 (33.3–61.5)	58.8 (46.6–81.0)
Total BAL cells ×10 ⁵ ·mL ⁻¹ #	2.56 (0.3–5.7)	0.9 (0.1–3.4)
Macr. %	61.5 (28–88)	92.5 (73–98)
Lymph. %	35.0 (9–72)	7.0 (2–26)
Neutr. %	1.0 (0–4)	0.5 (0–3)
Eosin. %	0.0 (0–1)	0.5 (0–1)
Total macr. ×10 ⁵ ·mL ⁻¹ #	1.78 (0.2–2.56)	0.86 (0.12–3.33)
Total lymph. ×10 ⁵ ·mL ⁻¹ #	0.44 (0.11–2.96)	0.05 (0.01–0.15)
Total neutr. ×10 ⁵ ·mL ⁻¹ #	0.05 (0.00–0.22)	0.00 (0.00–0.02)
Total eosin. ×10 ⁵ ·mL ⁻¹ #	0.00 (0.00–0.06)	0.00 (0.00–0.01)

Data are presented as median (range) unless otherwise stated. M: male; F: female; S: current smoker; EX: exsmoker, stopped at least 12 months; NS: nonsmoker; BAL: bronchoalveolar lavage; macr.: macrophages; lymph.: lymphocytes; neutr.: neutrophils; eosin.: eosinophils. #: results expressed per mL BAL fluid.

pelleted and resuspended in complete media, at a count of 1×10⁶ cells·mL⁻¹ for use in the RHPA. Differential cell counts were performed on 200 May-Grünwald-Giemsa-stained cells. The BAL cell details are summarised in table 1.

Isolation of human peripheral blood mononuclear cells

A total of 20 mL of peripheral venous blood were collected into heparinised containers and human peripheral blood mononuclear cells (PBMC) were isolated on an endotoxin-free Histopaque 1077 gradient (Sigma-Aldrich, Poole, Dorset, UK) using the method of BOYUM [17]. The PBMC were washed three times and resuspended at a final concentration of 2×10⁶ cells·mL⁻¹ in complete media, for use in the RHPA.

Reverse haemolytic plaque assay

The assay was performed as previously described [16, 18]. In brief, confluent monolayers of protein-A (Sigma Chemical Co. Ltd, UK)-coated sheep red blood cells (sRBC) (Serotec Ltd, Oxford, UK) and BAL or autologous PBMC cells from each individual were cultured in 30 µL chambers for 12 h, at 37°C, 5% carbon dioxide, with complete media containing 1/50 dilution of rabbit antihuman TNF-α polyclonal antibody (Genzyme Diagnostics, Cambridge, UK) alone or with 10 µg·mL lipopolysaccharide (LPS) (from *Escherichia coli* 055:B5). TNF-α secretion by individual mononuclear phagocytes was visualised as clear plaques of lysed TNF-α/anti-TNF-α-associated sRBC around the TNF-α secreting cells using 1/30 dilution of guinea-pig serum as the source of complement (Gibco, Uxbridge, UK). The viability of the BAL and PBMC cells was evaluated using trypan blue exclusion and TNF-α secreting cells were identified by immunocytochemistry.

To confirm that plaque formation was specific for TNF-α secretion by individual cells: 1) BAL and PBMC cells were omitted from the assay; 2) the anti-TNF-α antibody was replaced with 1:50 normal sheep serum (Dako Ltd, High Wycombe, UK); 3) the complement was omitted; 4) uncoated sRBC were used; and 5) the antibody was pre-absorbed at 4°C overnight with 1 µg·mL⁻¹ recombinant TNF-α. Endotoxin contamination was excluded by assaying TNF-α secretion in the presence of 5 µg·mL⁻¹ endotoxin inhibitor polymyxin B sulphate.

Preliminary time-course cultures of PBMC were performed for 3, 6, 8, 12 and 24 h on two different occasions in order to optimise the culture conditions with respect to easily identifiable haemolytic plaques and cellular viability.

Alveolar macrophages (AM) and peripheral blood monocytes (Mo) were identified following the RHPA by immunolabelling with the monocyte/macrophage-specific KP1 mouse monoclonal antibody (CD68; Dako Ltd) [19]. Briefly, the monolayers were fixed with 1% glutaraldehyde (Grade 1; 25% aqueous solution) for 30 s and permeabilised using Tris-buffered

saline pH 7.4. Visualisation of the KP1-identifiable mononuclear phagocytes was achieved by immunostaining with alkaline phosphatase/anti-alkaline phosphatase (Dako Ltd) [20] together with new fuchsin substrate system (Dako Ltd), containing 5 mM levamisole, and haematoxylin counterstaining.

Single-cell tumour necrosis factor- α quantification

Plaque areas of sRBC lysis around individual TNF- α -secreting cells were measured using Leitz microscopy with Apple Macintosh computer and image analysis package (Improvision UK, Coventry, UK). For each assay condition, 25 lysed plaque areas in each of three replicate chambers were measured. The area occupied by the TNF- α -secreting cells was subtracted from each plaque area. Only BAL cells and PBMC samples with a viability of $\geq 87\%$ were used in the RHPA. The average TNF- α plaque area per cell and percentage of TNF- α secreting cells were calculated for each chamber and the average of the triplicate samples obtained.

A total plaque area per 100 Mo/AM was calculated as follow:

$$\text{Total plaque area} = \text{average plaque area } (\mu\text{m}^2) \times \frac{1}{\text{percentage of TNF-}\alpha \text{ secreting cells}} \quad (1)$$

for each of the triplicate chambers per condition and the total plaque area for each condition was expressed as the average of the triplicate measurements.

The following criteria were applied when measuring plaque area and percentage of plaque-forming cells. 1) Lysis of sRBC around the plaque-forming cell had to be visible and well defined. 2) Plaque measurement was performed on randomly selected nonoverlapping microscope fields. 3) Only plaques formed by single cells were counted: clusters of Mo/AM were not included in the counting. 4) Plaque counting was performed "blind", without knowledge of the cell source in the chambers.

Validation of the assay conditions for the identification of tumour necrosis factor- α secretion

Preliminary time-course experiments using PBMC showed that haemolytic plaque formation was first evident at 6 h. More easily identifiable plaques of sRBC lysis, formed by TNF- α -secreting Mo, were seen after 12 h incubation and the viability of the PBMC cells at the end of the culture period was $>96\%$. At 24 h, larger haemolytic plaques were seen but cellular viability was reduced to $<65\%$. Therefore, for subsequent experiments, cultures were continued for 12 h and cellular viability was monitored.

Plaque formation was specific for TNF- α secretion, and since no plaques were seen when the antibody was replaced with normal rabbit serum, the complement was omitted, uncoated sRBC were used or the antibody was pre-absorbed with recombinant TNF- α . KP1 immunostaining for mononuclear phagocytes

confirmed that all plaque-forming cells were either Mo or AM. No plaque formation was observed after LPS stimulation by any cells other than those stained by the KP1 monoclonal antibody.

Statistical analysis

Group data were distributed nonparametrically and comparisons between groups were made using the Kruskal Wallis test for multiple group comparisons followed by the Mann-Whitney U-test for comparisons between two groups. Correlations were calculated using Spearman's rank test. Differences at the $p < 0.05$ level were considered significant.

Results

Average levels of tumour necrosis factor- α secretion by alveolar macrophages and monocytes

In order to assess whether there is increased TNF- α production and secretion by individual mononuclear phagocytes in subjects with sarcoidosis, the average levels of TNF- α secretion by Mo and AM from subjects with sarcoidosis were assessed and compared with cells obtained from normal individuals.

It was observed that there was no difference between the groups in the average levels of TNF- α secretion by Mo or AM either spontaneously or after LPS stimulation, although LPS stimulation resulted in a small increase in the average levels of TNF- α secretion per cell by Mo and AM from each group of subjects examined. However, lavage cells consistently produced more TNF- α on average than autologous Mo (data not shown).

Heterogeneity of tumour necrosis factor- α secretion

In order to assess whether TNF- α was a constitutive product of all or only a subset of mononuclear phagocytes, the RHPA was used to enumerate the numbers of TNF- α -secreting cells and the levels of secretion by individual cells. Interestingly, it was observed that the secretion of TNF- α by Mo and AM is heterogeneous and that not all Mo and AM secrete TNF- α . Furthermore, of the Mo and AM that did secrete TNF- α , the levels of TNF- α secretion varied considerably between cells.

Figure 1 illustrates the variation in the levels of TNF- α secretion by mononuclear phagocytes from a patient with sarcoidosis in a single RHPA. Plaques of sRBC lysis formed by Mo and AM spontaneously secreting TNF- α over 12-h culture in the RHPA were grouped according to plaque size, and figure 1 illustrates the frequency distribution of these plaque sizes. It can be seen that a greater number of macrophages produce larger plaques than autologous monocytes, although the average plaque sizes of the mononuclear cell populations as a whole were no different.

Fig. 1. –Heterogeneity of tumour necrosis factor (TNF)- α secretion by individual mononuclear phagocytes from a patient with sarcoidosis. Haemolytic plaques obtained after a 12-h culture were assigned to size groupings. Frequency distributions of the percentages of cells with plaques of different areas are shown. ●: monocytes; ○: alveolar macrophages.

Percentages of lung and blood cells secreting tumour necrosis factor- α

In order to quantify the functional heterogeneity of mononuclear phagocytic TNF- α secretion, the percentages of Mo and AM secreting TNF- α either spontaneously or after LPS stimulation were evaluated for all study groups.

A difference was observed between the percentages of AM from subjects with sarcoidosis that secreted TNF- α and the percentages seen with AM from controls (median 17%, range 10–21% for sarcoidosis; 12%, 8–18% for controls; $p < 0.05$) (fig. 2a).

The median percentages of cells synthesising and secreting TNF- α were generally higher for AM compared with autologous Mo for sarcoidosis (AM 17%, Mo 9%) but not for controls (AM 12%, Mo 10%).

LPS stimulation resulted in an increase in the percentage of AM and Mo secreting TNF- α in both groups, with Mo production doubling and more closely approximating AM median levels (fig. 2b).

Total secretion of tumour necrosis factor- α by mononuclear phagocytes

Total amounts of TNF- α secretion by populations of mononuclear phagocytes is dependent on the product of the levels of secretion per cell and the number of Mo and AM secreting TNF- α . In order to evaluate the total levels of TNF- α secretion by these two cell populations in different disease states, the product of the average levels of TNF- α secretion per cell and the percentage of TNF- α secreting mononuclear phagocytes were calculated.

Although sarcoid AM samples produced more total TNF- α than controls, this was not statistically significant. However, after LPS stimulation, spontaneous total TNF- α secretion by AM of sarcoidosis patients was significantly higher than in normal individuals, largely due to an increase in the number

Fig. 2. –Percentage of tumour necrosis factor (TNF)- α secreting monocytes (●) and alveolar macrophages (○) from individual sarcoidosis patients and normal healthy controls. The percentages of monocytes and alveolar macrophages that a) spontaneously secreted TNF- α and b) secreted TNF- α after lipopolysaccharide stimulation during a 12-h culture period are shown. Lines represent the median values in each group. #: $p < 0.044$; *: $p = 0.0063$.

of TNF-secreting cells ($p = 0.001$). No differences were observed for the Mo from the two populations and AM consistently produced more TNF- α than Mo in both groups (data not shown).

Discussion

In this study it has been confirmed: that TNF- α is synthesised and secreted almost exclusively by AM from patients with sarcoidosis; that only a subset of cells were TNF producers; and, importantly, that the difference in secretion between sarcoidosis and controls is due to an increase in the percentage of TNF-secreting cells rather than an increase in production per cell. The goal of the study was not to demonstrate any differences between sarcoid stage. Furthermore, the TNF values for those receiving steroid treatment or with a smoking history were no different than the rest of the population (data not shown). In this regard, in contrast with many previous studies, the continuing secretion of TNF in more stable disease has been demonstrated in this study. In disease that may relapse, it seems logical that there would be low-grade continuing secretion and this was confirmed in this study. By using the more sensitive RHPA assay, it

has been demonstrated that clinically inactive disease may have persistent early cytokine release, consistent with a continuing inflammatory response.

The RHPA is the only methodology that can identify individual cell synthesis and secretion, in contrast with fluorescence-activated cell sorter analysis for example, that can show intracellular product [21], differentiate mononuclear phagocytes from other lower respiratory tract cells, and relate TNF secretion with cell type and size.

The present data are in agreement with other publications on sarcoidosis and TNF [9–15], although an increase in production in stable disease has been shown here in contrast with previous studies that included patients with active or more progressive disease [22]. It has been shown that AM from patients with sarcoidosis and normal individuals secrete more TNF- α than autologous peripheral blood Mo, but that in sarcoidosis the increased production is due to an increase in the percentage of macrophages secreting TNF- α rather than the average product per cell. Although prior studies have shown an increase in TNF- α in sarcoidosis, the present study supplements this information by demonstrating, irrespective of the small and heterogeneous study population, that increased TNF- α arises as a result of increased numbers of TNF- α -secreting cells and not by increased levels of TNF- α production in each cell.

In this study, the cells responsible for the production of TNF- α in the lung were generally greater than $13\ \mu\text{m}^2$, which was the upper limit of the normal of cell diameter observed in the peripheral blood mononuclear group. However, in published data on interleukin (IL)-8 [15] and unpublished observations characterising TNF- α -secreting cells from patients with fibrosing alveolitis (P. Pantelidis), it was confirmed that the majority of these IL-8 and TNF-producing cells bear the RFD 7 phenotype of mature tissue macrophages. This has important implications for targeted management. It is well recognised that a therapeutic goal for the future would be specific treatment. TNF- α production, occurring at the earliest stages of pathogenesis, would be an attractive target. One way of ensuring that the drug was delivered to the microscopic site of origin would be to target specific cell types.

In conclusion, this study has demonstrated that a subset of mature mononuclear phagocytes is responsible for the bulk of tumour necrosis factor production in sarcoidosis. This has implications for targeted approaches to therapy in the future and also for studies of phenotype/function relationships.

References

- Hart LA, Conron M, du Bois RM. Sarcoidosis. *Int J Tuberc Lung Dis* 2001; 5: 791–806.
- Costabel U, Hunninghake GW. ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. *Eur Respir J* 1999; 14: 735–737.
- Muller-Quernheim J. Sarcoidosis: immunopathogenetic concepts and their clinical application. *Eur Respir J* 1998; 12: 716–738.
- Mornex JF, Leroux C, Greenland T, Ecochard D. From granuloma to fibrosis in interstitial lung diseases: molecular and cellular interactions. *Eur Respir J* 1994; 7: 779–785.
- Agostini C, Chilosi M, Zambello R, Trentin L, Semenzato G. Pulmonary immune cells in health and disease: lymphocytes. *Eur Respir J* 1993; 6: 1378–1340.
- Rosenblum GR, Donato NJ. Tumour necrosis factor: a multifaceted hormone. *Crit Rev Immunol* 1989; 9: 21–44.
- Kunkel SL, Chensue SW, Strieter RM, Lynch JP, Remick DG. Cellular and molecular aspects of granulomatous inflammation. *Am J Respir Cell Mol Biol* 1989; 1: 439–447.
- Muller-Quernheim J. The cytokine network in sarcoidosis. *Eur Cytokine Netw* 1996; 7: 13–26.
- Muller-Quernheim J, Pfeifer S, Mannel D, Strausz J, Ferlinz R. Lung-restricted activation of the alveolar macrophage/monocyte system in pulmonary sarcoidosis. *Am Rev Respir Dis* 1992; 145: 187–192.
- Prior C, Knight RA, Herold M, Ott G, Spiteri MA. Pulmonary sarcoidosis: patterns of cytokine release *in vitro*. *Eur Respir J* 1996; 9: 47–53.
- Baughman RP, Strohofer SA, Buchsbaum J, Lower EE. Release of tumour necrosis factor by alveolar macrophages of patients with sarcoidosis. *J Lab Clin Med* 1990; 115: 36–42.
- Steffen M, Petersen J, Oldings M, *et al.* Increased secretion of tumour necrosis factor- α , interleukin-1 β , and interleukin-6 by alveolar macrophages from patients with sarcoidosis. *J Allergy Clin Immunol* 1993; 91: 939–949.
- Bost TW, Riches DWH, Schumacher B, *et al.* Alveolar macrophages from patients with beryllium disease and sarcoidosis express increased levels of mRNA for tumour necrosis factor- α and interleukin-6 but not for interleukin-1 β . *Am J Respir Cell Mol Biol* 1994; 10: 506–513.
- Somoskovi A, Zissel G, Seitzer U, Gerdes J, Schlaak M, Muller-Quernheim J. Polymorphism at position -308 in the promoter region of the TNF-alpha and in the first intron of the TNF-beta genes and spontaneous and lipopolysaccharide-induced TNF-alpha release in sarcoidosis. *Cytokine* 1999; 11: 882–887.
- Martel S, Carre PC, Carrera G, Pipy B, Leophonte PJ. Tumour necrosis factor-alpha gene expression by alveolar macrophages in human lung allograft recipient with recurrence of sarcoidosis. Toulouse Lung Transplantation Group. *Eur Respir J* 1996; 9: 1087–1089.
- Pantelidis P, Southcott AM, Black CM, du Bois RM. Up-regulation of IL-8 secretion by alveolar macrophages from patients with fibrosing alveolitis: a subpopulation analysis. *Clin Exp Immunol* 1997; 108: 95–104.
- Boyum A. Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol* 1976; 5: 9–15.
- Pantelidis P, Southcott AM, Cambrey AD, Laurent GL, du Bois RM. Activation of peripheral blood mononuclear cells in bronchoalveolar lavage fluid from patients with sarcoidosis: visualisation of single cell activation products. *Thorax* 1994; 49: 1146–1151.
- Pulford KA, Rigney AM, Micklem KJ, *et al.* KPI: a new monoclonal antibody that detects a monocyte/

- macrophage associated antigen in routinely processed tissue sections. *J Clin Pathol* 1989; 42: 414–421.
20. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP) complexes. *J Histochem Cytochem* 1984; 32: 219–229.
 21. Nakamura H, Fujishima S, Soejima K, *et al.* Flow cytometric detection of cell-associated cytokines in alveolar macrophages. *Eur Respir J* 1996; 9: 1181–1171.
 22. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M, Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Investig Med* 1998; 46: 223–231.