quots (to obtain more BAL fluid cells for further examination) of 0.9% sterile saline were introduced into the bronchus of the right middle lobe through a fiberoptic bronchoscope. BAL fluids were recovered by gentle suction immediately after the infusion of each aliquot. BAL fluids were filtered through a single layer of sterile gauze. After centrifugation (400 x g for 10 min), the cell pellets were washed twice in PBS. Cells were > 94% viable as determined by the trypan blue exclusion test. Cell differential counts were determined by Wright-Giemsa staining. Evaluation of BAL fluid cells for the proportions of CD3^{+}, CD4^{+}, and CD8^{+} T cells was performed using monoclonal antibodies (MoAbs: Sigma) and flow cytometry. The BAL fluid supernatants of the aliquots obtained from all BD patients and healthy controls were centrifuged again at 500 x g for 30 min. The supernatants were collected and cryopreserved at -30°C for the cytokine measurements. The results of BAL analysis of the aliquots were shown in Table II a, b. Significant differences in the total cell count, percentage of macrophages, and percentage of lymphocytes and neutrophils were observed between active BD patients, silicotic patients and healthy controls (Table II a). Calculations were made in duplicate and expressed as the mean ± SEM.

We did not find any significant difference in the cellular distribution (macrophages, lymphocytes, neutrophils, and CD4/CD8 ratio) in relation to the vascular and pulmonary involvement in active BD (Table IIb).

BAL fluid cell culture
Cells were cultured at 1 x 10^{6} cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) FCS, 2 mM L-glutamine, and 0.1% gentainicin (complete medium) in 24-well plates (Nunc, Denmark) for 48 h at 37°C with 5% CO, in the presence or the absence of LPS (1 µg/ml; *Escherichia coli*, Sigma Aldrich, Germany). To investigate the effects of IL-18 on IFN- production in BAL fluid cells, stimulation with IL-18 was performed. Recombinant IL-18 (R&D sys-

tems, UK) was added at different concentrations. In this analysis 2 x 10^{4} BAL fluid cells were cultured in 0.2 ml of complete medium in 96-well microliter plates for 48 h at 37°C under 5% CO_2, and then culture supernatants were collected.

Analysis of cytokine gene expression
For cytokine gene expression analysis in BAL fluid cells, 5 BD cases and 3 healthy controls were selected. Total RNA of 4x10^{5} cultured BAL fluid cells was isolated using Isogen (Life Technology) and quantified by measurement of absorbance at 260 nm. To synthesize cDNA, 1 µg of total RNA was incubated in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and 5 ng/ml oligo(dT)$_{12-18}$ primers (Pharmacia Biotech, Uppsala, Sweden) for 30 min at 42°C using the reaction conditions described by the manufacturer (Life Technologies), and samples were stored at -80°C. Aliquots of 2 µl of cDNA were amplified by PCR using oligonucleotide primers specific for IFN- , IL-18, and GAPDH. In a DNA thermocycler 480 (Perkin-Elmer/Cetus, Norwalk, CT) each cycle of denaturation was run at 94°C for 1 min, annealing was performed for 1 min at 60°C (IFN- and GAPDH) and at 55°C (IL-18), and extension was performed at 72°C for 1 min. The PCR product was subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. The primers of examined cytokines were as follows: IFN- : 5'-atgaaatatacaagttatatcttggcttt and 3'-gatgctcttcgacctc gaaacagcat; IL-18: 5'-gcttgaatctaaa ttatcagtc and 3'-gaagattcaaattgcatcttat; and GAPDH, 5'-gaaggtgaaggtcgga.

By means of the preliminary PCR amplification we confirmed the optimal number of PCR cycles in the exponential amplification phase for each cytokine and GAPDH. Cycle numbers corresponding to the exponential phase were individually determined for each primer set. The cycles numbers were 45 for 1FN- , and 30 for IL-18 and GAPDH. PCR products were stained by ethidium bromide after electrophoresis, and the intensities of the bands on

photographs of the agarose gels were quantified using a numeric image and optical software.

Immunoassay of BAL fluids and cultured supernatants of BAL fluid cells
BAL fluids were concentrated 10-fold by membrane dialysis using Vivapore (Vivascience, Denmark) for cytokine immunoassays. We measured IFN- in 10-fold concentrated BAL fluids and cultured supernatants using the appropriate commercial ELISA kits (Cytoscreen, BioSource International; Camarillo, CA) (sensitivity, 4 pg/ml) and following the manufacturers' recommendations. The specific ELISA system for human IL-18 was established by Shigehara *et al.* (19). A Maxisorp plate was coated with MoAb 125-2H (20 µg/ml in PBS) at room temperature for 3 h and blocked with PBS containing 1% BSA (Sigma) at 4°C overnight. After washing with PBS containing 0.05% Tween 20 (Sigma), 50 µl of the assay buffer (PBS containing 1% BSA, 5% FCS, and 1 M NaCl) was dispensed, 50 µl of samples and standard human IL-18 were added to the assay buffer, and the plate was incubated at room temperature for 2h.

After washing, peroxidase-conjugated 159-12B (rat IgG2a , 0.5 µg/ml PBS containing 1% BSA, 5% FCS, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-l-propanesulfonate (Sigma Chemicals), and 0.3 M NaCl) was added, and the plate was incubated at room temperature for 2 h. After washing, the substrate solution (100 µl of 0.1 M sodium phosphate citrate buffer containing 0.5 mg/ml *o*-phenylenediamine and 0.003%, H_2O_2, pH 5.0) was added. The reaction was stopped with 100 µl of 1 M H_2SO_4, and the absorbance at 490 nm was measured. In general, the assay was perforned in duplicate. The detectable range of this ELISA was between 10 and 1000 pg/ml. The ELLSA system can detect total IL-18 (proIL-18 and mature IL-18).

Statistical analysis
Descriptive statistics were performed to investigate differences between the study groups using the Mann-Whitney
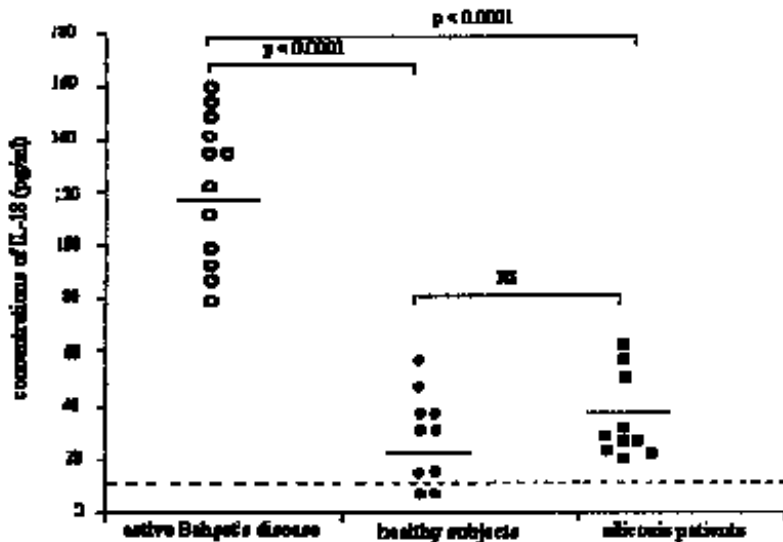
Fig. 1. Concentration of IL-18 in BAL fluid of patients with Behçet's disease (BD),in healthy controls and in silicosis patients. IL-18-BAL fluid was measured by specific ELISA. The dashed line indicates the level of sensitivity of the IL-18 assay (10 pg/ml). Each circle represents a single individual. BD patients had significantly elevated levels of IL-18 in their BAL fluid compared with healthy controls and silicosis patients. All comparisons were made using the Mann U test.

non-parametric test. The correlations between cytokines were assessed using the non-parametric Spearman rank test. All statistical analyses were carried out using the SPSS PC+ statistical package.
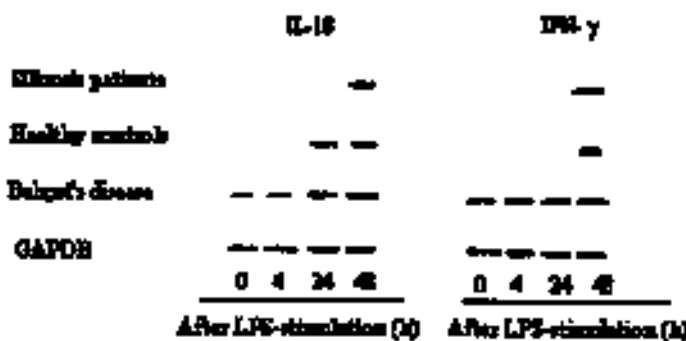
Results

IL-18 protein levels in BAL fluids
We measured protein levels of IL-18 in BAL fluids using a specific ELISA system (Fig. 1). There was a significant difference in BAL fluids IL-18 levels between BD patients (80 - 160 pg/ml, mean 117.16) and healthy controls (12 - 60pg/ml, mean 20.9; p<0.001). There was no statistically significant difference in the concentration of IL-18 (19.8 - 60pg/ml, mean 37.9) between silicosis patients and healthy controls (HC).
In active BD group we found a positive correlation between IL-18 concentration and the percentage of lymphocytes (r = 0.639; p < 0.05). No correlation was observed in silicosis patients (r = 0.137, p = 0.218).

IFN-γ and IL-18 mRNA expression and cytokines production in cultured BAL fluid cells
Freshly isolated BD-BAL cells expressed IL-18 mRNA and IFN- mRNA. IL-18 mRNA and IFN- mRNA expression were also observed at 4h, 24h and 48 h after initial culture with LPS stimulation (Fig. 2). Neither IL-18 mRNA nor IFN- mRNA expression were observed in freshly isolated BAL fluid cells from silicosis patients and healthy subjects. HC cells expressed IL-18 mRNA at 24h and 48h after LPS stimulation, whereas IFN- mRNA expression was observed at 48h. In silicosis patients, both IL-18 and IFN- were only observed at 48h after LPS stimulation.
BD cells released spontaneously IFN- and IL-18 (Fig, 3). Spontaneous release of IL-18 was observed in 2 out of 10 healthy controls. HC cells produced spontaneously lower levels of IFN- than did BD-BAL-fluid cells (p < 0.001). Spontaneous release of IL-18 was observed in one silicosis patient. IFN- levels in silicosis patients were similar to those observed in BD, significantly higher (p < 0.05) than in healthy controls.
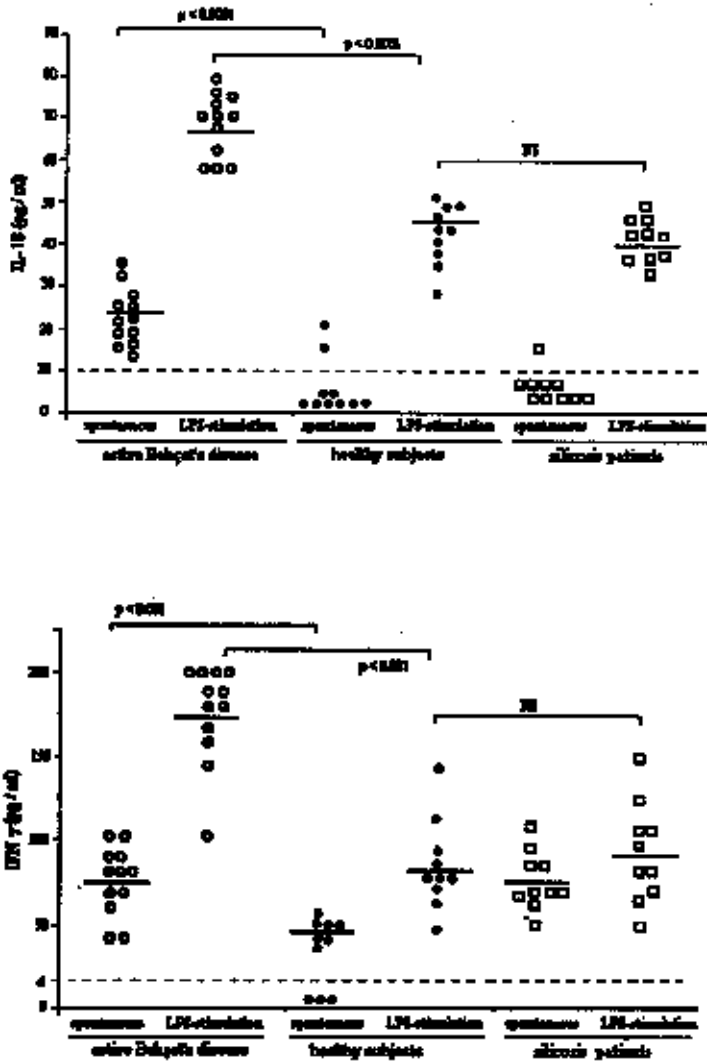LPS stimulation induced a significant increase in IL-18 and IFN- production by BD-BAL fluid cells (Fig. 3). In healthy subjects and disease controls, IL-18 and IFN- -LPS inductions were similar and significantly lower than BD levels (p < 0.001).
There was no correlation (r = 0.0087) between IL-18 and IFN- from unstimulated BAL-fluid cells. After LPS stimulation, a significant positive correlation (r = 0.6755; p = 0.0062) was observed between IL-18 and IFN- production by BD cells. At the opposite, no correlation was found in healthy controls and silicosis patients.
A significant positive correlation was found between the CD4/CD8 ratio and the IFN- level in LPS stimulated BD BAL-fluid cells (r = 0.587, p < 0.001).

Effects of rIL-18 on IFN-γ production
We investigated IFN- production after stimulation of BAL fluid cells with rIL-18 in 5 BD and 2 healthy subjects (Fig. 4). Silicosis patients were not investi-

Fig. 2. Expression of IL-18 mRNA and IFN- mRNA in BAL fluid cells. The PCR product derived from material pooled from 5 BD cases, 3 healthy controls and 7 silicosis patients. IL-18 mRNA and IFN- mRNA in BAL fluid cells were collected and cultured with LPS (1µg/ml). Stimulated BAL fluid cells were recovered at 4, 24 and 48 h. Total RNA of the BAL fluid cells was isolated and cDNA was synthesized using 5 ng/ml oligo(dT)12-18 and reverse transcriptase. The specific primer for IFN- and IL-18 for PCR reaction underwent 45 and 30 cycles, respectively. IL-18 mRNA and IFN- mRNA were observed in freshly isolated BD BAL fluid cells, and after LPS stimulation. The GAPDH gene was used as an internal control.

Fig. 3. Production of IL-18 and IFN- in BAL fluid cells. IFN- and IL-18 production in LPS-stimulated and unstimulated BAL fluid cells of 12 BD patients, 10 healthy controls and 10 silicosis patients. BAL fluid cells were incubated in medium containing 10% FCS with or without LPS for 48 h. The dashed line indicate the level of sensitivity assay (IL-18: 10pg/ml; IFN- : 4 pg/ml). The concentrations of IL-18 and IFN- in culture supernatants were measured using specific ELISA. LPS significantly induced IL-18 and IFN- production in BD patients.

gated due to the absence of sufficient BAL-fluid cells. Under stimulation with rIL-18, an increase in IFN- production was observed in all 5 BD patients, and reached 402 pg/ml (± 23.7) at 100 pg/ml rIL-18 induction. In healthy subjects administration of rIL-18 induced lower levels of IFN- , with a maximum level of 42.6 pg/ml (± 14.3) at 100 pg/ml rIL-18 induction. At all concentrations of rIL-18, IFN- production by BD cells was significantly increased (p < 0.001) compared to healthy controls.

Discussion

Our data demonstrated *in vivo* IL-18 production by lung cells during active pulmonary BD; high levels of IL 18 were demonstrated in the BAL fluids of patients with BD, contrasting with weak production from HC and silicosis subjects. IL-18 and IFN- mRNA expression was observed in BAL fluid cells freshly isolated from BD but not from healthy controls or silicosis patients. IL-18 and IFN- mRNA were also observed in BD-BAL fluid cells after LPS stimulation. In healthy controls and in silicosis patients, IL-18 and
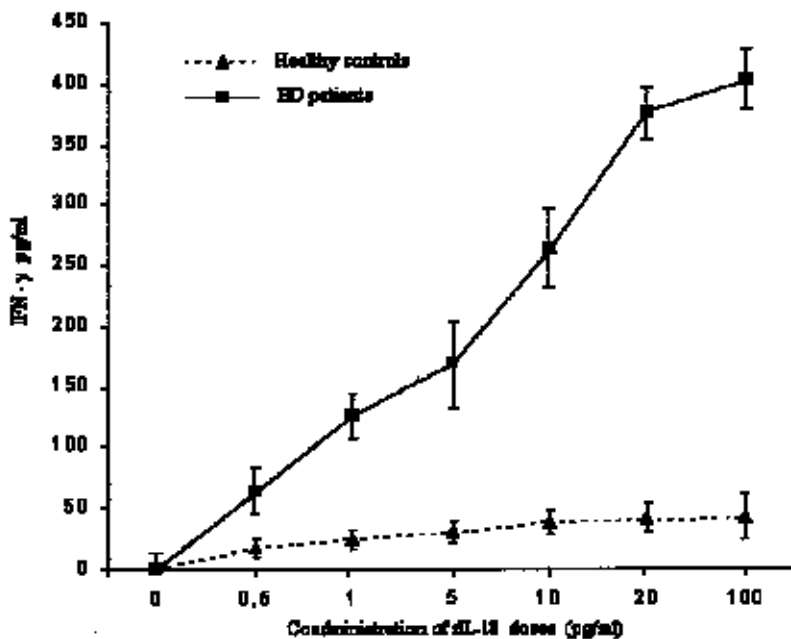
Fig. 4. Effect of human rIL-18 on IFN- production by bronchoalveolar (BAL) fluid cells. BAL fluid cells were incubated with rIL-18 for 48 h. Culture supernatants were collected, and IFN- concentrations were measured by specific ELISA. Data are expressed as the means ± SEM. rIL-18 in a dose dependent manner triggered IFN- secretion in BAL fluid cells from active BD at higher levels than in healthy controls (p< 0.001). Enhancement of IFN- production was dose-dependent. At high concentrations of rIL-18, the production of IFN- reached a plateau. BD patients exhibited greater IFN- induction with the co-administration of rIL-18 at different rIL-18 concentrations (10 and 20 pg/ml). In healthy controls IFN- induction was not significantly different at different doses of rIL-18 (1 and 100 pg/ml).

IFN- mRNA expression were observed 48 hrs after LPS-stimulation. Constitutive IL-18 mRNA expression has been observed in various organs, macrophage lineage cells and lymphocytes (15, 20-22). LPS stimulation induced a significant increase in IL-18 production from BAL fluid cells. LPS-induced IL18 levels were drastically higher in BD than healthy control and silicosis.

High levels of IL-18 production have been reported in sarcoid lungs (19). A correlation between IL-18 and disease-activity in systemic lupus erythematosus has been reported (23). Mononuclear cells of BD are thought be pre-activated by an unknown causative agent or stimuli (4, 5). IL-18 production was markedly enhanced with LPS stimulation in a murine *Propionibacterium acnes*-conditioned liver injury model (24). Activation of alveolar macrophages may be essential to IL-18 production from BD cells; however, silicosis cells did not reach such high IL-18 levels even after LPS stimulation, although macrophages are notably activated due to silica ingestion. The activation pathway of the immune system in BD seemed to be distinct from that observed in silicosis patients, involving more probably lymphocytes. In fact, we demonstrated a positive correlation between the IL-18 concentration and lymphocyte percentages in the active BD group.

Our previous studies demonstrated that Th1 cytokines predominate in active BD (7, 8, 25, 26). Silicosis (27) and sarcoidosis (19) are characterised by a similar shift towards Th1 pathways, associated with IL-18 and IFN- hyperproduction in inflammatory sites. However, increased IL-18 production was also reported in allergic asthma, a Th2 cytokine disease (28). IL-18 should be considered proinflammatory, independent from Th1/Th2. In the Th1 immune response, IL-12 and IL-18 synergistically stimulate IFN- production (11). High levels of IL-12 have been previously described in active BD (8, 26), suggesting early involvement of IL-12 and IL-18 during BD inflammation, resulting in IFN- production. In experimental silicosis, Garn *et al.* (29) re-

ported enhanced IL-12 mRNA levels *in vivo*, whereas IL-18 gene expression was decreased. In our silicosis group we did not find increased IL-18 production and IL-18 mRNA was only expressed at 48h after LPS-stimulation. BAL fluid cells from BD exhibited greater inducibility of IFN- than did healthy controls. The induction of IFN- is highly dependent on CD40/CD40L interaction (30), and significantly enhanced expression of CD40 and CD40L was observed in BD (8). CD40L and IFN- are known to activate monocytes and macrophages to produce reactive nitrogen intermediates and various monokines (30), stimulating antigen-presenting cells to up-regulate class II and costimulatory molecules expression, resulting in antigen-specific T cells activation (31). Mühl *et al.* and Paulukat *et al.* (32, 33) reported induction of IL-18 binding protein (IL-18BP) by IFN- in nonleukocytic cells, suggesting a negative feedback regulation of IL-18. IFN- suppresses constitutive production of IL-18 (34).

Cultured BAL cells from BD and silicosis patients released spontaneously more IFN- than HC, suggesting they were first stimulated *in vivo*. Since IL-18 was originally identified as an IFN-inducing factor (35), we stimulated cultured BAL cells with rIL-18. In response, BD-BAL fluid cells produced markedly greater IFN- levels than did normal BAL fluid cells, reaching a plateau at the highest IL-18 concentrations. This plateau phenomenon in IFN- production may be comprehensive, as IL-18 receptors saturation occurs in high dosages. Similar results were reported in sarcoid BAL fluid cells (19).

In the same way, LPS-stimulated BD fluid cells showed greater induction of IFN- production than HC and silicosis cells. The production of IFN- in silicosis is merely induced by activated macrophages full of silica dust (27). On the other hand, in BD the greater expression of IFN- could be explained by the high CD4/CD8 ratio in BAL fluid cells, and by the increased percentage of CD4+CD45RO+ (25). In BD the BAL CD4/CD8 ratio was increased 2.3-fold compared to healthy controls

and 2.8-fold over silicosis patients. This high CD4/CD8 ratio was significantly correlated with LPS stimulated IFN- production. The proportion of CD45RO+ cells in CD4+ T cells is increased in active BD, indicating a greater proportion of CD4+ T cells with a memory cell phenotype (36, 37).

There is increasing evidence that IL-18 acts as a proinflammatory cytokine and mediates Th1 polarized immune responses (34). Data obtained in sarcoidosis and rheumatoid arthritis indicate that IL-18 is essential during inflammation (13, 19, 38). In the same way, according to our results, IL-18 could play a key role in active BD. Lung inflammatory cells produce an array of immunoregulatory cytokines. The ability to produce IL-18, a potent Th1 proinflammatory cytokine, is of particular relevance. IL-18 is capable of stimulating IL-1 release from activated macrophages (39), inducing in turn increased IFN- and possibly TNF-production by CD4+ T cells, thus affecting the final common pathway of BD immunopathogenesis. Therefore, it is conceivable that IL-18 may fulfill the requisite as a primary initiating cytokine in Th1-mediated diseases, such as BD. Recent animal studies using monoclonal antibody neutralization against IL-18 in organ-specific autoimmune diseases have supported this concept (40).

In summary, our study provides some evidence that IL-18 may play an important role in Th1-mediated disorders, such as BD. Animal studies will test the validity of this hypothesis. An animal model to explain the pathogenesis of BD is under investigation in our laboratory, and further studies are required to understand whether manipulating IL-18 expression may have relevance to the treatment of BD.

In conclusion, our data indicate that IL-18 in BD plays an important role in favouring IFN- synthesis and inducing Th1 cell proliferation. IL-18 may therefore play a role in promoting the local immunoinflammatory response.

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