

## Expression and immune recognition of stress proteins in sarcoidosis and other chronic interstitial lung diseases

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**Summary** Stress proteins (SP) are major immunogens in a number of microbial infections and have been implicated in some autoimmune diseases. The aetiology of sarcoidosis, a non-caseating granulomatous disease, remains unknown, but mycobacteria as well as autoimmunity have been considered. In the present study, patients diagnosed with sarcoidosis and other interstitial lung diseases (ILD), as well as healthy volunteers were studied to determine: (i) the level of expression of SP in alveolar macrophages and blood monocytes; (ii) the serum levels of antibodies specific for mycobacterial SP65 and SP70; and (iii) the reactivity of peripheral blood and alveolar lymphocytes to mycobacterial SP65. Our results suggest that SP are expressed constitutively at high levels in alveolar macrophages, retrieved by bronchoalveolar lavage, from all individuals regardless of health status. In contrast, freshly isolated blood monocytes express low levels of SP, which are, however, readily upregulated following exposure to IFN- $\gamma$  and TNF- $\alpha$ . Lymphocyte reactivity and presence of antibodies against mycobacterial SP may reflect the current state of *in vivo* inflammation rather than the cause of inflammation.

**Key words:** alveolar macrophages, blood monocytes, lung diseases, sarcoidosis, stress proteins.

### Introduction

The stress response is a rapid, complex and transient reprogramming of cellular activities, consisting of induction of gene transcription and synthesis of stress proteins (SP) and post-transcriptional inhibition of normal protein synthesis.<sup>1</sup> The stress response is induced by exposure to supra-optimal temperatures ('heat shock', hence also 'heat shock protein'), as well as many forms of cellular injury including oxidative injury, viral and bacterial infections, exposure to heavy metals, ethanol, or amino acid analogues, nutrient deprivation and changes in pH.<sup>2,3</sup>

Many SP are constitutively expressed in both eukaryotic and prokaryotic cells and are involved in normal cellular functions; for example, SP have been shown to interact with newly made proteins, assisting in their folding and translocation across membranes.<sup>3</sup> In addition, stress induces a modest increase in the cell surface expression of MHC class II antigens,<sup>4</sup> and SP expression may be transiently upregulated during lymphocyte activation and proliferation.<sup>5</sup> These and other findings suggest a possible role for SP in antigen processing, presentation and cellular activation during an immune response.<sup>4,6</sup>

Proteins from a diverse range of pathogenic and commensal micro-organisms have been identified which share sequence homologies with members of the five known

families of vertebrate SP.<sup>3</sup> A frequent finding in studies of the immune responses to bacteria have been that the microbial SP often contain the immunodominant epitopes presented to the host immune system during infection.<sup>7</sup> This has led to the hypothesis that cross-recognition of SP by T cells could be the link between the immune response to infectious agents and autoimmunity.<sup>3,7</sup> Evidence to support this hypothesis includes the finding that human T cell clones specific for a mycobacterial 65 kDa SP sequence also respond to a peptide corresponding to a similar, but not identical, sequence in the human SP65,<sup>8</sup> while other studies have suggested a role for anti-SP immunoreactivity in juvenile chronic rheumatoid arthritis.<sup>9,10</sup> In murine models of mycobacterial immunity it has also been shown that antigen presenting cells (macrophages) stressed by viral infection or heat can become targets for cytotoxic CD8<sup>+</sup> T cells, specific for the 65 kDa mycobacterial SP.<sup>11</sup> Autoimmune diseases in animals with anti-SP reactivity as an important component can be induced with preparations containing mycobacteria, for example, adjuvant arthritis and diabetes.<sup>10,12</sup>

The above hypothesis, however, begs a number of questions before the involvement of SP in autoimmunity can be fully contemplated and explored: (i) what is the level of expression of the various SP under normal steady-state conditions in organ systems most often involved in autoimmune reactions; (ii) how is the expression of these SP regulated during acute and chronic inflammatory processes; and (iii) are the SP, at constitutive and upregulated (inflammation) levels, recognized by the immune system.

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at the T and/or B cell level, in normal or affected individuals? Despite a recent surge in investigations of the potential link between SP and immunopathology, particularly in the area of autoimmune diseases,<sup>12-15</sup> little attention has apparently been given to chronic interstitial lung diseases (ILD) although these include several idiopathic diseases often associated with autoimmune characteristics.<sup>16</sup>

In the present study, patients diagnosed with ILD, including sarcoidosis, an idiopathic granulomatous disease thought to be of autoimmune<sup>16</sup> or mycobacterial origin,<sup>17</sup> as well as healthy volunteers were studied to determine: (i) the level of expression of SP in alveolar macrophages and blood monocytes; (ii) the reactivity of alveolar and peripheral blood lymphocytes to mycobacterial SP65; and (iii) the serum levels of antibodies specific for mycobacterial SP65 and SP70. Our results suggest that SP are expressed constitutively at high levels in alveolar macrophages from all individuals regardless of health status. Lymphocyte reactivity and presence of antibodies against mycobacterial SP may reflect the *in vivo* inflammation rather than being the cause of inflammation.

## Materials and methods

### Human donors

Patient groups under study included individuals with diagnosed sarcoidosis, asbestosis, cryptogenic fibrosing alveolitis (CFAI), various pulmonary malignancies, extrinsic allergic alveolitis (EAA) and silicosis, all defined according to established criteria.<sup>18</sup> Control groups included healthy individuals (non-smokers as well as smokers), individuals with mycobacterial infections, extra-pulmonary lymphoma, and chronic nephrosis/nephritis.

Blood sampling and bronchoalveolar lavage (BAL) were performed, after informed consent was obtained or as part of routine diagnostic or follow-up procedures, at the Sir Charles Gairdner Hospital Department of Respiratory Medicine, Nedlands, WA. Blood samples were also collected from individuals with verified infections, during hospitalization or visits to the outpatient clinic at Royal Darwin Hospital, following informed consent. All procedures were approved by the University of Western Australia Institutional Human Rights Committee.

### Cell samples

A single-segment 300 mL BAL was performed as previously described.<sup>19</sup> The cells were treated with 0.1% lignocaine (Astra, Sydney, NSW, Australia) to detach cells from collection tubes, and separated into density subpopulations on a discontinuous Percoll gradient as described previously.<sup>19</sup> Each interphase was collected separately, and the purity of macrophages determined after three washing steps at 4°C. The fractions with less than approximately 5% contaminating lymphocytes or polymorphonuclear neutrophils (PMN) were pooled and used as 'alveolar macrophages'.

Blood monocytes were similarly enriched for by Percoll density centrifugation following initial isolation of PBMC by Ficoll-Hypaque density centrifugation. Contaminating lymphocytes usually constituted less than 25% of the monocyte preparations.

The differential counts performed on wet samples were confirmed by staining cytospin preparations for non-specific esterase (NSE).<sup>9</sup> T lymphocytes were purified from BAL or PBMC samples by resetting with neuraminidase-treated sheep red blood cells as previously described.<sup>20</sup>

### Stress proteins and monoclonal antibodies

Purified SP65 and SP70, from *Mycobacterium tuberculosis* and *M. bovis*, respectively, were generously supplied by Dr W. Britton (Sydney, NSW, Australia), and by Dr J. van Embden (Bilthoven, The Netherlands).<sup>21</sup> Murine mAb specific for human SP72/73 (clones C92F3A-3 and N27F3-4) were initially generously supplied by Dr W. J. Welch (San Francisco, CA, USA) and subsequently purchased from StressGen (cat no. SPA-810 and SPA-820; Victoria, BC, Canada). Other mAb obtained from StressGen included anti-SP27 (no. SPA-800), anti-SP90 (no. SPA-840) and anti-SP100 (no. SPA-830). The *M. leprae* SP65 specific mAb ML30, which cross-reacts with the human homologue,<sup>22</sup> was kindly supplied by Professor J. Ivanyi (London, UK), and Dr W. van Eden (Utrecht, The Netherlands) generously provided samples of the human SP60-specific mAb LK1 and LK2,<sup>9</sup> both of which were subsequently purchased from StressGen. LK1 is specific for the human SP60, whereas LK2 also recognizes the bacterial homologues.

### Other reagents

Recombinant human cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IFN- $\gamma$ ) and purified fraction V BSA were from Boehringer-Mannheim (Castle Hill, NSW, Australia), prostaglandins and Zymosan A from Sigma (St Louis, MO, USA), [<sup>35</sup>S]-methionine, ECL-reagent and -blot from Amersham (North Ryde, NSW, Australia), Ficoll-Hypaque and Percoll from Pharmacia (Uppsala, Sweden), and RPMI and fetal bovine serum from Gibco (Glen Waverley, Vic., Australia). Human AB serum was obtained from the Red Cross Blood Transfusion Centre, Perth, WA. All disposable tissue culture plastic ware were purchased from Nunc (Roskilde, Denmark), microfuge tubes from Robbins (Baxter Diagnostic, Acacia Ridge, Qld, Australia), and reagents for electroblotting from Bio-Rad Labs (Richmond, CA, USA).

### Stress protein induction and detection

Cell samples ( $2 \times 10^5$  cells/300  $\mu$ L) were resuspended in serum- and methionine-free RPMI and allowed to equilibrate at 37°C for 30 min prior to either heat shock (45°C for 20 min) or exposure to *Chlamydia trachomatis* elementary bodies (0.4–80  $\mu$ g, serovar B or F<sup>22</sup>), opsonized zymosan, 100  $\mu$ g/mL of either IL-1 $\beta$ , IL-6, or IFN- $\gamma$ , 10 ng/mL of TNF- $\alpha$ , or 500 pg/mL of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), PGF<sub>2 $\alpha$</sub> , or PGD<sub>2</sub> for up to 6 h at 37°C. The cells were labelled for 4 h with 20  $\mu$ Ci/mL of [<sup>35</sup>S]-methionine, either during the exposure to stimuli, or, in case of heat shock, following exposure. Labelling was interrupted by 3  $\times$  washing in ice-cold PBS, and the cells were lysed in 50  $\mu$ L Laemmli sample buffer, the DNA sheared and boiled for 6 min. The cell proteins from  $2 \times 10^5$  cells/lane were separated by SDS-PAGE on a 10% resolving gel, and transferred to nitrocellulose by electroblotting. The membranes were probed for SP using mAb specific for the 27, 65, 70, 72/73 and 90 kDa SP (see below), either individually or in combinations, followed by biotinylated goat anti-mouse IgG (Dakopatts, Glostrup, Denmark) and then

streptavidin-biotinylated alkaline phosphatase complex (SABAP; Bio-Rad). Bound complex was visualized using *p*-nitro-blue tetrazolium chloride and 5-bromo-4-indolyl phosphatase (Bio-Rad). Alternatively, following the biotin-conjugate, the membranes were incubated with streptavidin-horseradish peroxidase conjugate and detection was by luminol-based enhanced chemiluminescence (ECL) according to the manufacturers instructions (Amersham).

Metabolically labelled proteins were detected by autoradiography, either preceding or proceeding the immunolabelling process. The nitrocellulose membranes were air-dried, covered in plastic and overlaid with X-ray film (Hyperfilm-MP; Amersham) followed by exposure for 1–4 weeks at  $-80^{\circ}\text{C}$ .

#### Serum analysis

Nitrocellulose membranes with bound mycobacterial SP65 and SP70 were cut into 5 mm strips and incubated for 48 h at  $4^{\circ}\text{C}$  with agitation in blocking buffer (5% skim milk, 1.0 mol/L glycine, 5% goat serum, 1% BSA in double distilled water). The strips were incubated at  $22^{\circ}\text{C}$  for 1 h with serum samples diluted 1:50–1:10<sup>5</sup> in sample buffer (0.1% skim milk, 1% goat serum, 0.1% BSA in Tris-buffered saline with 0.1% Tween-20), followed by biotinylated rabbit anti-human Ig antibody (Dakopatts), and streptavidin-peroxidase conjugate (Boehringer-Mannheim), with washing steps ( $3 \times$  Tris-buffered saline with 0.05% Tween-20) between each antibody incubation. Binding of SP-specific antibody was visualized by ECL, and the bands quantitated by densitometry using an Image Quant laser densitometer and associated software (Molecular Dynamics, Sunnyvale, CA, USA), with standardization against a reference serum from a patient with melioid sepsis. Densitometry readings of 2 or less (equal to non-specific background noise) were considered negative and given a value of 0.

#### Immunocytochemistry

Tissue biopsies were obtained as part of routine diagnostic procedures and preserved by formaldehyde-fixation and paraffin-embedding or by cryo-preservation. Cryo-sections were fixed in 20% acetone in PBS with 0.5% BSA and air-dried.<sup>24</sup> Paraffin was removed from embedded tissues by routine procedures. All sections were immunolabelled for SP using the following murine mAb: ML30,<sup>22</sup> LK1 and LK2,<sup>9</sup> in a triple-layer APAAP-protocol<sup>24</sup> with rabbit-anti-mouse IgG and mouse-anti alkaline phosphatase-AP complexes (Dakopatts) as secondary and tertiary reagents, respectively. Control-mAb included isotype-matched irrelevant Ab and, as 'positive labelling controls', mAb specific for HLA-DR $\alpha$  and CD68.<sup>24</sup> In addition, cytospin preparations of untreated or heat shocked (as for macrophages) HeLa cells were immunolabelled in parallel. All biopsies were labelled and assessed under code. Microphotos were taken using a Leitz photomicroscope and Betachrome-64 film.

#### Flowcytometry

Cells were immunolabelled using murine mAb specific for CD3, CD4, CD19, CD25, CD45RO, CD65 and HLA-DR $\alpha$  (all from Dakopatts) and FITC-conjugated sheep anti-mouse-Ig (Silenus, Sydney, NSW, Australia) in a previously described protocol.<sup>25</sup> Flowcytometry was performed on a FACScan (Becton-Dickenson).

#### T cell blotting

Nitrocellulose membranes with bound mycobacterial SP65 were prepared for T cell assay as described by Abou-Zeid *et al.*,<sup>25</sup> following transfer of a range of SP65-concentrations onto the nitrocellulose using a vacuum mini-blotting apparatus (Bartlett Instruments Melbourne, Vic., Australia). Cell samples were assayed in triplicate for reactivity to nitrocellulose-bound SP65 (NSP65) at concentrations ranging from 0 to 25  $\mu\text{g}/\text{mL}$  in 100  $\mu\text{L}$  microcultures containing  $10^5$  cells/well in supplemented RPMI-1640<sup>26,27</sup> and 5% human AB serum (RPMI-Sup). Cultures were incubated for 7 days at  $37^{\circ}\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ . Control cultures consisted of unstimulated cells and cultures stimulated with Con A (Pharmacia) at 3  $\mu\text{g}/\text{mL}$ , with incubation for 3 days. Fifty microlitres of culture supernatant was removed from each well after 48, 96 and 144 h of incubation, and each time replaced with fresh RPMI-Sup. Cell proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation.<sup>27</sup>

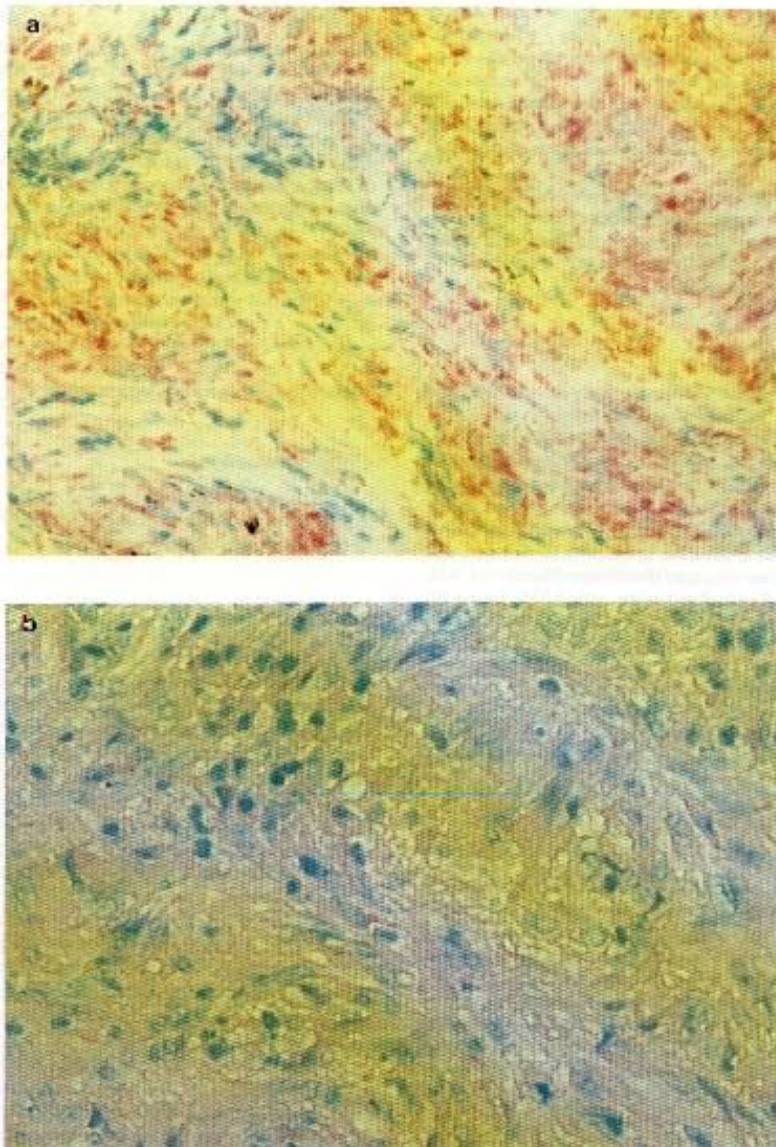
#### Cytokine bioassays

Cell culture supernatants were assayed for the presence of IL-2 and IFN- $\gamma$  as previously described<sup>26,27</sup> using HT-2 and cloned HeLa cells, respectively, as reporter cells.

#### Results

Using mAb with cross-reactivity to mycobacterial and mammalian SP65, as well as a mAb specific for human 60 kDa SP, LK1,<sup>9,22</sup> expression of SP was detected in most biopsies from sarcoidosis-affected tissues (Fig. 1), whereas SP expression was limited to the occasionally retained alveolar macrophage in biopsies from other chronic ILD (Table 1). In sarcoidosis granulomas, positively labelled cells included monocytes-macrophages and epithelioid cells, as judged by intense positive immunolabelling for the CD68 (not shown) and HLA-DR $\alpha$  antigens (Table 1) and morphology, with the former cell type showing the more intense labelling (Fig. 1). Lymphocytes and lung parenchymal cells were negative for SP65 expression. No SP70 expression was detected, except in the occasionally retained alveolar macrophage, in a limited number of biopsies examined from various disease categories (data not shown).

Alveolar macrophages, isolated from both healthy individuals and patients with various chronic ILD constitutively expressed high levels of the 60, 72/73 and 90 kDa SP, which could not be further upregulated under short-term, adherence-free culture conditions, either by heat-shock or exposure to non-opsonized bacteria, or a range of cytokines or prostaglandins, irrespective of health status of the donor (representative examples are shown in Fig. 2a). In the occasional macrophage sample phagocytosis of complement-activated zymosan would cause slight upregulation of the 72/73 kDa SP, but not of the 65 kDa SP (data not shown), and no correlation between this response and disease status was evident. In contrast, freshly isolated blood monocytes expressed only low levels of SP of apparent molecular weight 110, 90, 72/73 and 60 kDa, and were readily induced to upregulate expression of these SP following heat shock or a 4–6 h exposure to



**Fig. 1.** Expression of the SP65 homologue in lung-granulomas in a patient with 'active' sarcoidosis. (a) Biopsy labelled with the mAb ML30. (b) consecutive section labelled with an irrelevant mAb. Original magnification 1150  $\times$ .

IFN- $\gamma$  alone or in combination with TNF- $\alpha$  or prostaglandin (Fig. 2b). In addition, the 72/73 kDa SP were upregulated by IL-1, IL-6, TNF- $\alpha$  and PGF $_{2\alpha}$  in approximately one-third of individuals studied. The monocyte response appeared to be independent of health or smoking status of the donor.

No significant difference between patient groups was apparent when serum samples from healthy individuals

and patients with various ILD or infections were analysed for SP65 reactivity (Fig. 3a). SP70 reactivity was prominent in patients with leprosy and asbestosis, but otherwise absent in all other disease groups (Fig. 3b). Patients with sarcoidosis and verified tuberculosis appeared indistinguishable with respect to their degree of SP65 and SP70 reactivity (Fig. 3). At the time of serum sampling nine of the 12 sarcoidosis patients, six of the 12 leprosy patients,

Table 1. SP- and HLA-DR expression in tissue from patients with interstitial lung diseases.

| Patient | Diagnosis*     | Tissue†      | SP-expression‡ |     | HLA-DR expression§ |
|---------|----------------|--------------|----------------|-----|--------------------|
|         |                |              | ML30/LK1       | LK2 |                    |
| 985     | Necr. gran.    | Tr. b. biop. | -              | ND  | ++                 |
| 334     | CFA/TAA        | Lung         | -              | ND  | +                  |
| 108     | EAA            | Lung         | -              | ND  | -                  |
| 241     | EAA            | Tr. b. biop. | -              | ND  | -                  |
| 037     | CFA            | Lung         | -              | ND  | +                  |
| 083     | CFA/Pneum.     | Lung         | -              | ND  | +                  |
| 655     | CFA/Pneum.     | Lung         | -              | ND  | +                  |
| 449     | CFA            | Lung         | -              | ND  | +                  |
| 550     | CFA            | Lung         | -              | ND  | +                  |
| 947     | CFA            | Lung         | -              | ND  | +                  |
| 458     | Fibrosis       | Tr. b. biop. | -              | ND  | +                  |
| 198     | Fibrosis       | Lung         | -              | ND  | +                  |
| 112     | Bronch. oblit. | Tr. b. biop. | +/-            | ND  | +                  |
| HD      | Sarcoidosis    | Ln. med.     | +++            | +++ | ++++               |
| PT      | Sarcoidosis    | Ln. med.     | +/-            | +   | ++++               |
| CL      | Sarcoidosis    | Ln. med.     | -              | -   | +++                |
| IM      | Sarcoidosis    | Ln. med.     | +++            | +++ | ++++               |
| BN      | Sarcoidosis    | Ln. med.     | ++             | +++ | +++                |
| CN      | Sarcoidosis    | Ln. med.     | ++             | ++  | ++++               |
| DN      | Sarcoidosis    | Ln. med.     | +++            | +++ | +++                |
| TN      | Sarcoidosis    | Ln. med.     | +++ +/+        | -   | +++ +              |
| ES      | Sarcoidosis    | Ln. med.     | +/- +/+        | - + | +++ +              |
| LE      | Sarcoidosis    | Ln. med.     | +/- +          | -   | +++ +              |
| JT      | Sarcoidosis    | Ln. med.     | +++            | ND  | +++ +              |
| AD      | Sarcoidosis    | Ln. med.     | +++ +/+        | +   | +++ +              |
| HK      | Sarcoidosis    | Ln. med.     | + +/-          | +   | +++ +              |
| VY      | Sarcoidosis    | Ln. med.     | +/-            | +/- | +++ +              |
| DE      | Sarcoidosis    | Ln. med.     | + +/+ +        | + + | +++ +              |
| 259     | Sarcoidosis    | Tr. b. biop. | -              | ND  | -                  |
| 638     | Sarcoidosis    | Tr. b. biop. | -              | ND  | ++                 |
| 204     | Sarcoidosis    | Ln. med.     | +++ +          | ND  | +++ +              |
| 577     | Sarcoidosis    | Ln. med.     | +++            | ND  | +++ +              |
| 072     | Sarcoidosis    | Tr. b. biop. | -              | ND  | ++                 |
| 697     | Sarcoidosis    | Tr. b. biop. | -              | ND  | ++                 |
| 496     | Sarcoidosis    | Tr. b. biop. | +              | ND  | +++                |
| 080     | Sarcoidosis    | Tr. b. biop. | +              | ND  | ++                 |
| 052     | Sarcoidosis    | Tr. b. biop. | -              | ND  | +                  |
| 454     | Sarcoidosis    | Tr. b. biop. | -              | ND  | +                  |
| 746     | Sarcoidosis    | Tr. b. biop. | - +            | ND  | +++                |

\*Necr. gran. = necrotizing granuloma of unknown aetiology; CFA = cryptogenic fibrosing alveolitis; EAA = extrinsic allergic alveolitis; pneum. = pneumonia (unspecified infectious aetiology); bronch. oblit. = bronchiolitis obliterans.

†Tr. b. biop. = transbronchial biopsy; Ln. med. = mediastinal lymph node.

‡The proportion of cells positively labelled and the intensity of immunolabelling were judged on a scale from zero (-) to five (+). Generally, the two parameters showed a positive correlation. Labelling with mAb ML30 and LK1 in most cases gave the same result. Where a consistent discrepancy occurred in two or more sections from the same tissue-sample, two values are given (ML30/LK1). ML30 is specific for *M. leprae* SP65, and cross-reacts with the human homologue.<sup>2,3,6</sup> LK1 and LK2 are specific for human SP60, but whereas LK2 cross-react with the prokaryotic homologue, LK1 only recognize the human SP.<sup>6</sup>

§The labelling of the occasionally retained alveolar macrophage is not taken into account in the evaluation, since this was regarded as a 'normal' phenomenon (see Fig. 2).

and seven of the 12 tuberculosis (Tb) patients received no treatment.

When the proliferative responses of T lymphocytes, isolated from healthy individuals and patients suffering from various ILD or mycobacterial infections, to nitrocellulose-bound mycobacterial SP65 were compared, a wide range of responses was observed. However, as for the serum reactivity, no correlation between patient group and responsiveness was apparent (Table 2). Notably, only

in one case of mycobacterial infection (tuberculosis) did the cells respond with a stimulation index (SI) > 2. This patient was the only subject with mycobacterial infection who did not receive antibiotic therapy at the time of PBMC sampling. When a smaller number of BAL T cell samples were tested a range of responses was again observed which showed no correlation with the patient diagnosis (Table 3). No significant deviations from normal phenotypic profiles of PBMC were noted, when the sam-



Fig. 2. Expression of SP in (a) alveolar macrophages and (b) peripheral blood monocytes, following no or short-term (max 6 h) exposure to various stimuli under adherence-free conditions. (a) Electrobloods of SDS-PAGE separated macrophage proteins were probed with a cocktail of SP65, SP70 and SP72/73 specific mAbs, and the binding visualized as described in materials and methods. Lane 1, prestained MW markers; lane 2, 37°C control; lane 3, 45°C heat shock (20 min); lane 4, IL-1; lane 5, TNF- $\alpha$ ; lane 6, IL-6; lane 7, IFN- $\gamma$ ; lane 8, serum-activated zymosan; lane 9, IFN- $\gamma$  + zymosan; lane 10, *Chlamydia trachomatis* serovar B; lane 11, IFN- $\gamma$  + *Chl. trachomatis* B; lane 12, *Chl. trachomatis* serovar F; lane 13, IFN- $\gamma$  + *Chl. trachomatis* F. Arrowheads indicate MW of 73, 72, 70 and 60 kDa, respectively. (b) Autoradiograph of [<sup>35</sup>S]-methionine labelled monocyte proteins separated by SDS-PAGE. Lane 1, 37°C control; lane 2, 45°C heat shock (20 min); lane 3, IL-1; lane 4, TNF- $\alpha$ ; lane 5, IL-6; lane 6, IFN- $\gamma$ ; lane 7, PGF<sub>2</sub>; lane 8, PGD<sub>2</sub>; lane 9, PGF<sub>2</sub> $\alpha$ ; lane 10, IL-1 + TNF- $\alpha$ ; lane 11, IFN- $\gamma$  + TNF- $\alpha$ ; lane 12, IL-6 + TNF- $\alpha$ ; lane 13, IL-1 + IL-6 + TNF- $\alpha$ ; lane 14, IL-1 + PGE<sub>2</sub>; lane 15, IFN- $\gamma$  + PGF<sub>2</sub> $\alpha$ . Arrowheads indicate MW of 90, 70–73 and 60 kDa, respectively.

ples were analysed by flowcytometry (data not shown). Flowcytometric analysis could not be performed on the BAL samples due to insufficient cell numbers; however, previous studies have shown that the CD4:CD8 ratio may be higher in sarcoidosis patients compared to healthy subjects and other ILD groups.<sup>26</sup> No IL-2 or IFN- $\gamma$  were detected in culture supernatants, whether from high or low responders, probably due to genuine lack of production,<sup>27</sup> consumption of low concentrations or non-specific binding of the cytokines to the nitrocellulose particles.

### Discussion

For a human disease to be defined as 'autoimmune' it must fulfil a number of criteria:<sup>28</sup> (i) the autoimmune response must be recognized in the form of an auto-antibody or cell-mediated immune response; (ii) the corresponding antigen must be identified; and (iii) an analogous autoimmune response must be induced in an experimental animal, which subsequently develops a sim-

ilar disease. In recent years, SP have been implicated in a number of human diseases with suspected autoimmune aetiology. However, despite the apparent predominance of responses to bacterial SP at a site of disease, it has proved difficult to implicate such responses in autoimmune disease.<sup>9,10,13</sup> This study demonstrates that the SP65 cognate antigen was indeed expressed at high levels in sarcoidosis granulomas, but not in tissues from other disease groups examined. The upregulation of SP expression in monocytes by, in particular, IFN- $\gamma$  might explain the intense immunolabelling of monocyte-like macrophages in sarcoid granulomas. Other studies have shown that alveolar T lymphocytes from sarcoidosis patients 'spontaneously' release copious amounts of IFN- $\gamma$ ,<sup>29</sup> and IFN- $\gamma$  can be detected *in situ* in the granulomas by immunocytochemistry.<sup>26</sup> Thus, monocytes entering the lung parenchyma from the peripheral blood will be exposed to locally produced IFN- $\gamma$ , contributing to their activation, as typified by high-level expression of MHC class II antigen (Table 1) and monokine production,<sup>30</sup> but also to their protection in a site of active inflammation.<sup>31</sup> As previously described by Jaattela and Wissing,<sup>31</sup> freshly

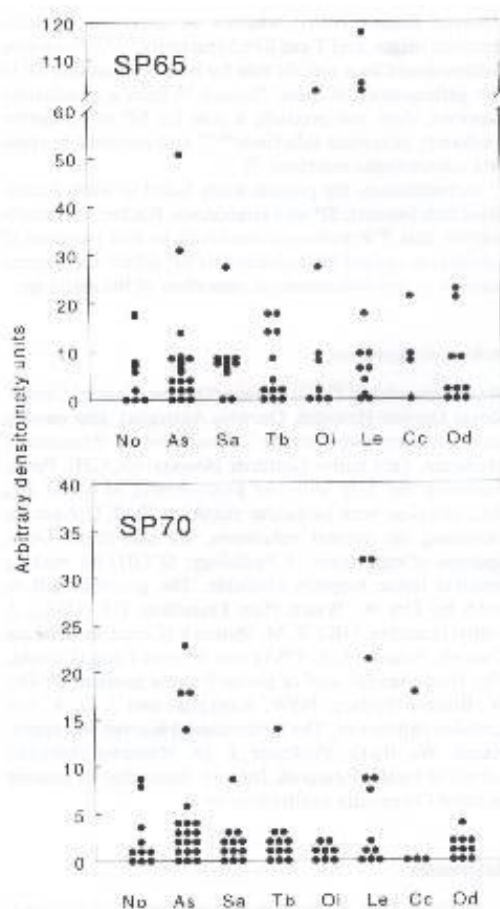


Fig. 3. Serum antibody reactivity to mycobacterial SP65 (a) and SP70 (b) in healthy individuals and patients with ILD or infections, measured by immunoblotting, ECL and densitometry. Data shown are based on measurements of sera at 1:500 dilution. Disease categories: No = normal control subjects, As = asbestosis, Sa = sarcoidosis, Tb = pulmonary tuberculosis, Oi = other pulmonary infections, Le = leprosy, Cc = 'chronic cough', Od = other diseases (including malignancies, pulmonary fibrosis, liver and renal failure).

isolated blood monocytes constitutively express SP, which can be further upregulated upon stimulation. The present study suggests that such upregulation may be selective, depending on the stimulus. In particular the 70 kDa SP complex seemed responsive to modulation by cytokines although with some inter-individual variations, a feature which may provide activated monocytes with a protective mechanism against their own potentially toxic products.<sup>31</sup>

This may also be the explanation for the high constitutive expression of SP by alveolar macrophages, cells which are continuously exposed to a barrage of environmental stress and stimulating factors, including their own pro-

Table 2. *In vitro* proliferative response of peripheral blood T lymphocyte from healthy donors and patients with interstitial lung disease or infections to SP65.

| Diagnosis      | n | SI*               |                     |
|----------------|---|-------------------|---------------------|
|                |   | NSP65             | Con A               |
| Normal         | 6 | 1.2<br>(0.8-2.1)  | 25.3<br>(2.7-109.0) |
| Asbestosis     | 6 | 9.6<br>(1.3-29.2) | 65.8<br>(2.7-208.1) |
| ILD            | 3 | 6.6<br>(1.2-17.1) | 39.5<br>(8.5-66.5)  |
| Chronic cough  | 2 | 2.3<br>(1.1-2.1)  | 60.6<br>(29.7-91.6) |
| Mesothelioma   | 2 | 1.8<br>(1.5-2.1)  | 72.2<br>(6.9-137.6) |
| CFA            | 2 | 1.7<br>(1.2-2.2)  | 4.4<br>(4.4-4.5)    |
| Silicosis      | 1 | 3.1               | 25.5                |
| Infect. pneum. | 2 | 4.3<br>(2.5-6.2)  | 8.1<br>(5.3-10.9)   |
| Tuberculosis   | 5 | 3.0<br>(0.9-10.3) | 7.5<br>(4.8-10.1)   |
| Sarcoidosis    | 7 | 4.3<br>(1.0-21.5) | 11.5<br>(3.0-37.2)  |

\*The stimulation indices (SI) shown are calculated from the cultures (triplicate) with the highest response, with all cell samples tested over a dose-range of 0 to 25 µg/mL of nitrocellulose-bound SP65. Con A was used as a 'positive control' at 3 µg/mL.

Table 3. Proliferative response of BAL T lymphocytes to SP65 and Con A.\*

| Patient no. | Diagnosis      | SI    |        |
|-------------|----------------|-------|--------|
|             |                | NSP65 | Con A† |
| 1           | Sarcoidosis    | 1.9   | 1.7    |
| 2           | Sarcoidosis    | 3.2   | 2.1    |
| 3           | Sarcoidosis    | 2.7   | 2.0    |
| 4           | Sarcoidosis    | 1.9   | 4.9    |
| 5           | Sarcoidosis    | 6.1   | NA‡    |
| 6           | Sarcoidosis    | 1.2   | 1.8    |
| 7           | Chronic cough  | 1.3   | 6.3    |
| 8           | Chronic cough  | 2.6   | 2.9    |
| 9           | Asbestosis/EAA | 1.9   | 2.0    |

\*The stimulation indices (SI) shown are calculated from the cultures (triplicate) with the highest response, with all cell samples tested over a dose-range of 0 to 25 µg/mL of nitrocellulose-bound SP65. Con A was used at 3 µg/mL.

†It should be noted that BAL T lymphocytes characteristically show relatively low responsiveness to mitogens compared to PBMC,<sup>16,20</sup> probably due to *in vivo* microenvironmental influences.<sup>20</sup>

‡Not enough cells available for assaying.

inflammatory products.<sup>32</sup> Alveolar macrophages from all donors, irrespective of the individual's disease status, exhibited high constitutive expression of SP, and no further upregulation followed exposure of the cells to inflammatory mediators, bacteria or opsonized yeast. This is in contrast to findings reported by Clerget and Polla,<sup>33</sup> Kantengwa and Polla,<sup>34</sup> and Cohen *et al.*<sup>35</sup> who all reported

upregulation of several SP in alveolar macrophages following phagocytosis or exposure to environmental stress. The latter study was performed with guinea-pig cells, which might account for at least some of the differences. However, of greater importance is probably the differences in *in vitro* culture condition between this study and the three studies mentioned. Thus, in the present study great care was taken to limit the *ex vivo* manipulations and time in culture prior to 'stimulation', whereas in the earlier studies the cells were cultured for up to 48 h without or with a differentiation-inducing agent and, in one study, on a collagen-coated surface.<sup>23</sup> Other studies have demonstrated that SP gene expression and protein synthesis by macrophages vary with differentiation<sup>36</sup>, indeed that SP may act as inducers of cell differentiation.<sup>37</sup> Since a subset of alveolar macrophages obtained by Percoll density centrifugation was used in the present study, this may have introduced yet another variable precluding direct comparison to earlier studies. However, since a similar procedure was used to obtain peripheral blood monocytes, the response of these cells may be compared to that of the alveolar macrophages.

Although the presence of SP-specific antibodies in various autoimmune conditions have been described,<sup>10,13,14,38,39</sup> the role, if any, of these antibodies in the pathogenesis of the diseases remains unclear. Recently, Kindis-Mügge *et al.*<sup>40</sup> demonstrated similar frequencies of SP70-specific autoantibodies in healthy subjects and SLE patients, and concluded that they are unlikely to play a major role in SLE. Similarly, Kiessling *et al.*<sup>41</sup> concluded that the similarity in SP65-reactivity between healthy subjects and tuberculosis patients might be due to protracted exposure to certain commensal organisms rather than reflect on the mycobacterial infection. In the present study, the SP-antibody reactivities in sarcoidosis and Tb patients were similar and did not differ significantly from the healthy control subjects (Fig. 3). Although based on a relatively small number of individuals there did, however, appear to be agreement between clinical 'activity'/acuteness of the disease process and the SP70 serum reactivity. Whether this response is part of a general 'first-line' immune surveillance response or whether it is playing a specific role in diseases such as asbestosis and leprosy remains to be elucidated.

The T cell response to SP65 also did not show any significant relationship to any one of the disease entities examined here. Rather, as for the antibody responses, a marked proliferative response was only seen in a few patients with particular 'active' disease, whether diagnosed with pulmonary Tb, asbestosis, sarcoidosis or other ILLD (Table 2). This result seems to corroborate the findings of Anderson *et al.*,<sup>42</sup> who demonstrated activation of SP60-specific T cells during inflammatory responses induced in the absence of exogenous bacterial SP65. Likewise, in a model of insulin-dependent diabetes mellitus, a SP65-specific T cell response appears late relative to a response to glutamic acid decarboxylase and detectable insulinitis and, together with responses to a range of other auto-antigens, appears to reflect an ongoing active inflammation rather than play a primary aetiological role.<sup>43,44</sup> Other studies have also failed to show a direct correlation

between disease entity, whether of infectious or autoimmune origin, and T cell SP65 reactivity,<sup>10,45-47</sup> casting further doubt on a specific role for host or microbial SP in the pathogenesis of these diseases.<sup>13</sup> Such a conclusion, however, does not preclude a role for SP in protective immunity to certain infections<sup>48,49</sup> and potential to regulate autoimmune reactions.<sup>10</sup>

In conclusion, the present study failed to show a causative link between SP and sarcoidosis. Rather, the results suggest that T lymphocyte reactivity to and presence of antibodies against (mycobacterial) SP reflect the current state of *in vivo* inflammation regardless of the aetiology.

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