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Antigen-specific multifunctional T cells in sarcoidosis patients with Löfgren´s syndrome

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Running head: T cells responses to mKatG in sarcoidosis

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ABSTRACT

Sarcoidosis is a granulomatous disease of unknown aetiology, mainly affecting the lungs. Recently, T cell responses towards a specific mycobacterial protein, catalase-peroxidase (mKatG), were observed in sarcoidosis patients.

Bronchoalveolar lavage (BAL) fluid and peripheral blood were obtained from a total of 23 sarcoidosis patients, of which 13 had Löfgren´s syndrome and lung-accumulations of TCR AV2S3^{pos} T cells. Using 6-color flow cytometry in combination with intracellular cytokine staining, T cell subsets were studied with regard to IFNγ, TNF and IL-2 production, after stimulation with mKatG or *M. tuberculosis* purified protein derivate (PPD).

Stimulation with mKatG resulted in higher simultaneous IFNγ and TNF production, but less single IFNγ production, from total BAL CD4^{pos} T cells of Löfgren´s patients, as compared to non-Löfgren patients. In contrast, PPD stimulation gave rise to largely similar cytokine responses in both patient subgroups. Furthermore, mKatG stimulated to higher IFNγ production in BAL and blood AV2S3^{pos} T cells than AV2S3^{neg} T cells, whereas the opposite was seen in BAL with PPD stimulation.

Our finding that patients with Löfgren´s syndrome exhibited a more pronounced multifunctional cytokine profile (simultaneous IFNγ and TNF) towards the mycobacterial protein mKatG, may help to explain the distinct disease presentation in this patient subgroup.

Key words: Löfgren´s syndrome, Multifunctional T cells, Mycobacterium tuberculosis, Sarcoidosis, T cells.

INTRODUCTION

Sarcoidosis is a multisystem granulomatous disorder, commonly affecting the lungs. Active disease is characterized by increased numbers of lymphocytes, in particular activated $CD4^{pos}$ T cells, in the lower airways. Increased mRNA and protein levels of interleukin (IL)-2 and interferon (IFN)-γ in the lung support sarcoidosis being a Th1-mediated disease (1-3). IL-2 promotes the Th1 immune response, and contributes to T cell proliferation, whereas IFNγ, together with the T cell and macrophage-derived cytokine tumor necrosis factor (TNF), play important roles in orchestrating maintenance of the characteristic granuloma formation (4, 5).

T cells owe their capacity to recognize virtually any pathogen to their enormously variable T cell receptor (TCR) for antigen. Collectively the T cells in the body encompass millions of different TCR specificities, but each T cell only expresses TCRs with a single specificity. T cells recognize antigens in the form of peptides presented by HLA molecules on antigen-presenting cells, such as dendritic cells and macrophages.

We previously demonstrated that Swedish sarcoidosis patients with the HLA-type DRB1*0301 ($DR3^{pos}$) virtually always have large accumulations, so-called T cell expansions, in their lungs of $CD4^{pos}$ (T-helper) cells with a particular TCR, using the AV2S3 gene segment (6). This suggests that a specific antigen presented by HLA-DR3 molecules has caused the activation and proliferation of the TCR $AV2S3^{pos}$ cells. The lung $AV2S3^{pos}$ $CD4^{pos}$ T cells were found to be significantly more differentiated and activated compared with lung $CD4^{pos}$ T cells expressing other TCR variable gene

segments (7, 8), and they associated with active disease (9). We have also shown that Swedish HLA-DRB1*0301^{pos} (DR3^{pos}) patients often present with an acute disease onset and commonly with Löfgren´s syndrome and normally recover spontaneously within two years.

The aetiology of sarcoidosis is still unknown. However, involvement of mycobacterial species has been proposed, since mycobacterial DNA (10) has been found in sarcoidosis tissues and lymph nodes. Recently, Moller and colleagues identified a specific protein, mycobacterial catalase-peroxidase (mKatG) in sarcoidosis tissue but not in healthy subjects (11). T cell responses against mKatG have been observed in sarcoidosis lung (12, 13) and peripheral blood mononuclear cells (14), but the specificity of T cells responding to mKatG has not been characterized in detail. Although we have data to support that AV2S3^{pos} T cells can recognize mycobacterial antigens (15), we have not been able to firmly establish the specificity of the AV2S3^{pos} lung T cells.

We have previously shown that T cells of the majority of Swedish sarcoidosis patients and healthy PPD^{pos} individuals (i.e. subjects with proven anti-mycobacterial T-cell responses), but not PPD^{neg} individuals, respond to mKatG with IFNγ production in an ELISPOT assay (12). The aim of this study was to elucidate the ability of total lung and blood CD4^{pos} or CD8^{pos} T cells, as well as CD4^{pos} TCR AV2S3^{pos} T cells, of patients with or without Löfgren´s syndrome, to respond to the specific mycobacterial antigen mKatG and to PPD, by measuring the production of IFNγ, TNF and IL-2 with flow cytometry. We also investigated whether these T cells displayed a single- or a multifunctional

cytokine profile in response to antigen stimulation, since T cells of the latter kind have been shown to be particularly potent effector cells.

MATERIALS AND METHODS

Study subjects

The sarcoidosis patients included in this study were referred to the Respiratory Medicine Unit (Karolinska University Hospital, Stockholm, Sweden) for primary diagnostic investigation (except for four already established sarcoidosis patients, who were recruited from their routine check ups at the out-patient clinic). In all patients, the diagnosis was based on chest radiographic findings, pulmonary function tests (vital capacity, forced vital capacity and diffusion capacity for CO), results of bronchoscopy and bronchoalveolar lavage (BAL), and clinical symptoms compatible with sarcoidosis, according to the criteria established by the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) (16). Written informed consent was obtained from all subjects, and the Regional Ethical Review Board approved the study.

A total of 23 sarcoidosis patients participated in the study (clinical and BAL fluid characteristics are given in Table 1). Thirteen patients with an acute disease onset were characterized as having recently diagnosed Löfgren´s syndrome (i.e. erythema nodosum and/or ankle arthritis, fever and bilateral hilar lymphadenopathy with or without lung parenchymal infiltration (17)), (median age 37 yrs (min-max: 29-56); nine males and four females). They all had an increased frequency (a so-called T cell expansion) of TCR AV2S3^{pos} CD4^{pos} T cells in their BAL fluid. The increase (>10.5 % of all CD4^{pos} T cells) was defined as 3 times median AV2S3% of CD4^{pos} T cells in peripheral blood of healthy subjects, i.e. 3×3.5% (18)). Eleven of the patients with Löfgren´s syndrome had haplotype HLA-DRB1*0301 (HLA-DR3), and two had haplotype HLA-DRB3*0101 (the

corresponding HLA molecules are structurally similar, and Scandinavian patients with either haplotypes show an increase above 10.5% of AV2S3^{pos} cells (19, 20)). 10 patients did not have Löfgren´s syndrome, whereof four patients had been followed for 3-20 years (median age 48 yrs (min-max: 33-67); eight males and two females). All had <10.5 % AV2S3^{pos} T cells in their BAL fluid and all were HLA-DRB1*0301^{neg}. Thus, from a phenotypic and immunogenetic point of view the patient groups were rather homogenous, since all Löfgren´s syndrome patients for practical purposes were DR3^{pos}, and all non-Löfgren patients were DR3^{neg}. Out of the four patients with chronic disease, three were treated with oral corticosteroids and one with methotrexate to control the disease activity. No Löfgren´s syndrome patient received oral corticosteroids. No patient in either subgroup showed any signs of a lower respiratory tract infection at least four weeks prior to the bronchoscopy, but one Löfgren´s syndrome patients had an upper respiratory tract infection one week before BAL.

BAL procedure and handling of BAL fluid and whole blood cells

Bronchoalveolar lavage (BAL) was performed as previously described (21). Sample handling is described in the online supplementary material.

Intracellular cytokine staining and flow cytometry

Full details are in the online supplementary material. BAL cells and whole blood were stimulated with recombinant mKatG, purified protein derivative (PPD, from *Mycobacterium tuberculosis*), or a combination of *Staphylococcus enterotoxin* A (SEA)

and *Staphylococcus enterotoxin B* (SEB) as positive control. Unstimulated BAL cells in medium alone, and unstimulated whole blood were used as negative controls.

Statistical analysis

In order to analyze if there were any significant above background responses to the antigens, raw data (without background deduction) was used in the comparisons with medium alone/unstimulated whole blood. In all other analyses background cytokine expression was deducted to focus on the antigen-triggered cytokine production. The Wilcoxon signed rank test was used for comparisons between BAL and whole blood, or comparisons between various cell types in the same individual. Comparisons between different subgroups of sarcoidosis patients were done using the Mann-Whitney U-test. All statistical analyses were performed with GraphPad PRISM 4.03 (GraphPad Software Inc., San Diego, CA, USA). P values <0.05 were regarded as significant.

RESULTS

Cytokine responses towards the mycobacterial protein mKatG in BAL and blood T cells of patients with and without Löfgren´s syndrome

Both in BAL and blood of 13 patients with Löfgren´s syndrome, $CD4^{pos}$ T cells responded with IFNγ production after mKatG and after PPD stimulation, whereas BAL and blood $CD8^{pos}$ T cells responded with IFNγ production only after mKatG stimulation (fig. 1A and B and Supplementary table). The patterns of TNF production in BAL and blood were broadly similar to those for IFNγ , while antigen-specific cells making IL-2 were confined to the $CD4^{pos}$ subset and mainly directed to PPD (Online supplement fig. E1).

Comparing BAL and blood T cell responses, we found that BAL $CD4^{pos}$ T cells responded with IFNγ and TNF production to a significantly higher extent than blood $CD4^{pos}$ T cells after mKatG stimulation, as well as after PPD stimulation. The cytokine response in $CD8^{pos}$ T cells, dominated by IFNγ, did not differ between BAL and blood after mKatG or PPD stimulation (fig. 1C and data not shown).

We also performed the same analysis in ten patients without Löfgren´s syndrome. Overall, patients with Löfgren´s syndrome and non-Löfgren´s syndrome patients exhibited similar cytokine responses towards *in vitro* antigen stimulation (for non-Löfgren´s syndrome data, see fig. E2 in the online supplement). However, some differences were noted, e.g. stimulation with PPD resulted in more BAL $CD4^{pos}$ cells expressing TNF among patients with Löfgren´s syndrome, while mKatG stimulated BAL

$CD4^{pos}$ cells to a similar frequency of IFNγ and TNF production in both patient subgroups (Online supplement fig. E2D). We could not observe any differences between the four treated patients with chronic disease and other non-Löfgrens´s patients regarding any of the analyses in the results section. Likewise, the smokers and the patient with prior upper respiratory infection did not stand out in any particular way.

Cytokine responses towards the mycobacterial protein mKatG in TCR $AV2S3^{pos}$ $CD4^{pos}$ T cells

All Löfgren´s syndrome patients participating in our study had a lung accumulation (so-called expansion) of TCR $AV2S3^{pos}$ $CD4^{pos}$ T cells (median 28% of $CD4^{pos}$), and median blood TCR $AV2S3^{pos}$ $CD4^{pos}$ T cells was 4.4%. We analyzed the percentage of cytokine producing $CD4^{pos}$ $AV2S3^{pos}$ or $AV2S3^{neg}$ T cells that responded to stimuli. mKatG (compared to medium alone) stimulated both BAL $AV2S3^{pos}$ and $AV2S3^{neg}$ T cells to IFNγ production (fig. 2B). However, comparing these two subsets, there was a significantly higher frequency of mKatG-reactive IFNγ-producing cells within the $AV2S3^{pos}$ subset (fig. 2C and Supplementary table). The $AV2S3^{pos}$ and $AV2S3^{neg}$ cells also responded with TNF production, yet to the same extent, whereas the production of IL-2 was very low in both subsets (data not shown). In contrast, after stimulation of BAL cells with PPD the IFNγ, TNF and IL-2 responses were dominant in $AV2S3^{neg}$ cells (fig. 2C and data not shown). That $AV2S3^{pos}$ cells are preferentially stimulated by mKatG is also supported by the finding that the fraction of antigen-triggered IFNγ-producing $CD4^{pos}$ cells that are $AV2S3^{pos}$ is almost doubled after mKatG compared to PPD

stimulation ($p<0.001$, supplemental fig. E3D) and a significant difference ($p<0.01$) is found also for TNF.

Also in peripheral blood mKatG stimulated the AV2S3^{pos} T cells to significantly more IFNγ and TNF production than the AV2S3^{neg} T cells, whereas the IL-2 production did not differ between the subsets (fig. 2C and data not shown).

Spontaneous cytokine production was also assessed and compared between AV2S3pos and AV2S3^{neg} cells (detailed results are in the online supplement). In BAL as well as in peripheral blood, the AV2S3^{pos} cells were to a higher extent expressing TNF and IL-2.

Comparisons between BAL and blood regarding the fractions of AV2S3^{pos} and AV2S3^{neg} cells that responded to antigenic stimulation are in the online supplement.

Higher proportion of multifunctional T cells reactive with mKatG in patients with Löfgren´s syndrome

The most striking difference between patients with and without Löfgren´s syndrome was discovered when comparing the proportions of single- and multifunctional cytokine-producing cells following mKatG stimulation. Multifunctional T cells, i.e. T cells that simultaneously produce two or more cytokines, are particularly potent effector cells. We analyzed the fractions out of total mKatG or PPD reactive BAL CD4^{pos} T cells, that displayed any of the cytokine patterns single IFNγ, single TNF, or simultaneous IFNγ and TNF production. In other words, the sum of cells that responded to antigen with any of

these three patterns was set to 100%. We found that mKatG stimulated BAL CD4^{pos} T cells of Löfgren´s syndrome patients to significantly less production of IFNγ alone compared to non-Löfgren patients (fig. 3B). In sharp contrast, Löfgren´s patients had a higher mKatG-induced simultaneous IFNγ and TNF production, as well as higher production of TNF alone. PPD stimulation, however, gave rise to similar cytokine profiles in both patient subgroups (fig. 3C).

The cytokine patterns in the BAL AV2S3^{pos} and AV2S3^{neg} T cells mirrored that of total CD4^{pos} T cells (data not shown). Thus the above mentioned more multifunctional profile of mKatG-reactive cells in Löfgren´s patients was a property of CD4^{pos} cells in general, and not related to the particular TCR AV2S3. The numbers of antigen-reactive BAL CD8^{pos} T cells was too low to reliably subdivide them further into single/multifunctional subsets, but generally there was a marked dominance of single IFNγ production (data not shown).

Mycobacterial mKatG and PPD trigger T cells with different cytokine profiles

A comparison between the two antigenic stimuli showed that mKatG and PPD stimulation resulted in different cytokine profiles of BAL CD4^{pos} T cells. mKatG stimulated to significantly more single IFNγ production, and significantly less simultaneous IFNγ and TNF production compared to PPD, as fractions of all antigen-responsive cells (fig. 4). These differences between mKatG and PPD responses were statistically significant also when analyzed in each patient group separately, and most

pronounced in the non-Löfgren patients (data not shown). The production of TNF alone was similar after mKatG or PPD stimulation.

Higher cytokine content in multifunctional T cells

The median fluorescent intensity (MFI) of antibody-labeled cytokines is directly related to the cytokine content on a per-cell basis (22). In our study, the highest MFI values were seen for BAL $CD4^{pos}$ T cells producing two cytokines simultaneously, as compared to BAL $CD4^{pos}$ T cells producing only one of the measured cytokines, both after mKatG or PPD stimulation (fig. 5). The MFI analyses were only performed in patients with Löfgren´s syndrome. A statistical comparison of MFI values showed that the multifunctional T cells produced approximately 3-fold more IFNγ and 3-fold more TNF after mKatG stimulation, and approximately 8-fold more IFNγ and 7-fold more TNF after PPD stimulation, as compared to cells producing either of the cytokines alone. These differences in relative cytokine content increases are statistically significant ($p=0.0002$ for both IFNγ and TNF), i.e. PPD induces multifunctional cells with relatively higher per cell cytokine content than mKatG.

In blood, the MFI profiles mirrored those in BAL (online supplement fig. E4).

Cytokine response in $CD27^{pos}$ or $CD27^{neg}$ T cells of sarcoidosis patients

CD27 is a co-stimulatory T cell surface marker expressed on naïve T cells and lost after prolonged activation. In BAL, the majority of $CD4^{pos}$ T cells were $CD27^{neg}$, whereas the

dominating subset in blood was CD27^{pos} T cells. The result from an analysis of 13 patients (of which five had Löfgren´s syndrome), showed that BAL CD4^{pos} CD27^{neg} T cells produced more IFNγ in response to mKatG or PPD as compared to CD4^{pos} CD27^{pos} T cells (fig. 6). In contrast, the CD4^{pos} CD27^{pos} T cells produced more IFNγ in whole blood. There were no differences between patient subgroups. BAL CD8^{pos} T cells, either expressing or lacking CD27 expression, responded to mKatG to the same extent (data not shown).

DISCUSSION

In the present study we investigated the T cell responses in BAL and blood of patients with and without Löfgren´s syndrome, after *in vitro* stimulation with mycobacterial proteins. Interestingly, we found that the BAL $CD4^{pos}$ T cells of Löfgren´s syndrome patients responded to mKatG with a significantly more pronounced multifunctional cytokine profile, as well as TNF alone, in contrast to non-Löfgren´s syndrome patients that exhibited a more distinct single-functional cytokine profile, with production of IFNγ alone. Furthermore, we show for the first time that a specific mycobacterial antigen, mKatG, triggers both BAL and blood $CD4^{pos}$ TCR $AV2S3^{pos}$ T cells to cytokine production. In addition, lung and blood $CD4^{pos}$ as well as $CD8^{pos}$ T cells responded to mKatG stimulation.

We demonstrated a positive IFNγ as well as TNF $CD4^{pos}$ T cell response to mKatG and PPD in patients with Löfgren´s syndrome, as well as without Löfgren´s syndrome, in (with a few exceptions) both BAL and blood. $CD4^{pos}$ T cells have long been known to be important effector cells involved in controlling mycobacterial infections by producing IFNγ (23). The strong $CD8^{pos}$ response (predominantly IFNγ production) to mKatG, but not to PPD, is noteworthy. Studies in mice models have demonstrated that $CD8^{pos}$ T cells are more important in anti-mycobacterial immune responses than previously appreciated (24-26). One may hypothesize that $CD8^{pos}$ T cells contribute to protective immunity against *M. tuberculosis* infection by a combination of cytotoxic activity and cytokine production. In contrast to our mKatG results, stimulation with PPD gave rise to an IFNγ response only in the $CD4^{pos}$ T cells in BAL and blood, but not in the $CD8^{pos}$ T cell subset.

This indicates a selective recognition of the mycobacterial epitopes among T cells. It has previously been shown that both $CD4^{pos}$ and $CD8^{pos}$ T cells of BAL and peripheral blood respond to mKatG with IFNγ production (12). When comparing the reactivity in BAL and blood, we found a higher cytokine response towards mKatG among BAL $CD4^{pos}$ T cells, indicating that these mKatG-specific T cells are accumulating in the affected organ. However, the $CD8^{pos}$ T cells responded to mKatG to the same extent in BAL and blood. These findings are in agreement with those in U.S. patients (12). Some mKatG-derived peptides may be constituents of PPD, but it is technically challenging to identify all components of this largely degraded protein mixture, although one recent study detected peptide fragments of previously known pathogenic mycobacterial antigens (27). Since the magnitude of BAL anti-mKatG and anti-PPD responses varied both within the Löfgren and non-Löfgren groups, the question arises in what proportion of sarcoidosis patients these antigens are truly important. A previous study from our laboratory and that of Dr. D. Moller involving both Swedish and U.S. patients, as well as PPD^{pos} and PPD^{neg} healthy controls, showed that approximately 50% of patients at either location had significant T cell responses to mKatG (12). Further studies are warranted to define the clinical importance of these responses.

Although we did not include any irrelevant antigen in this study, this has previously been done by other investigators. For example, it was shown that BAL cells from sarcoidosis patients reacted against mycobacterial antigens but not to keyhole limpet hemocyanin (KLH), demonstrating antigen specificity (13, 28). Likewise, peripheral blood cells from a majority of sarcoidosis patients responded to stimulation with mycobacterial antigens

but not lysate from *T. brucei* (29). It would, however, also be of interest to compare blood and BAL responses to a common non-specific antigen such as tetanus or candida, to see if there is a general propensity of memory T cells to accumulate in BAL fluid, or if this is the case particularly with mycobacteria-specific cells in sarcoidosis.

Until now we have not been able to directly demonstrate any antigen specificity among AV2S3^{pos} T cells, accumulating in the lungs of DR3^{pos} Scandinavian sarcoidosis patients (20). However, we previously obtained indirect evidence from *Mycobacterium bovis* BCG-vaccinated DR3^{pos} healthy subjects for anti-mycobacterial reactivity of AV2S3^{pos} cells (15), and in sarcoidosis patients for reactivity against mKatG among these particular T helper cells (12). Our intriguing data in the present study, of mKatG initiating a cytokine response in the lung-accumulated AV2S3^{pos} CD4^{pos} T cells, is the first to show that we have a specific disease-related antigen triggering a subset of these cells. TCR AV2S3^{neg} cells also responded to mKatG, but not to the same extent as AV2S3^{pos} cells with regard to IFNγ production. Similar to our result in BAL, we found that the AV2S3^{pos} T cells in blood responded to mKatG stimulation. As in BAL, the AV2S3^{pos} T cells in blood produced significantly more IFNγ in response to mKatG compared to AV2S3^{neg} T cells. How can we explain that mKatG triggers not only different subpopulations of CD4^{pos} T cells, but also CD8^{pos} T cells? To be recognized by T cells a protein must undergo so-called antigen processing, during which it is cleaved into short peptide fragments. CD4^{pos} T cells typically recognize peptides of 15-25 amino acids length, presented by HLA class II molecules (such as HLA-DR), while CD8^{pos} T cells recognize even shorter peptides. Since mKatG is a protein of over 700 amino acids in length, it is

likely to contain several T cell epitopes, both for different CD4^{pos} cells as well as CD8^{pos} cells. To definitely establish the antigen-specificity of AV2S3 cells will require a confirmatory study, which should also include *in vitro* stimulation with individual mKatG peptides to identify clones of T cells responding to the respective peptide epitopes.

The reason for the significantly higher spontaneous TNF and IL-2 production in BAL and blood AV2S3^{pos} cells compared to AV2S3^{neg} cells is not known, but it is tempting to speculate that the AV2S3^{pos} T cells have encountered their antigen *in vivo*, and thus are more activated. That would also be consistent with our recent phenotypic analysis of these cells regarding expression of differentiation and activation markers (8). If *in vivo* antigen triggering of AV2S3^{pos} is the case, it may also explain why they have a limited capacity to be further triggered by antigen *in vitro* to produce these two cytokines, in contrast to IFNγ which is produced to a higher extent by AV2S3^{pos} than AV2S3^{neg} cells after mKatG stimulation.

The fact that AV2S3^{pos} T cells are associated with good prognosis and spontaneously resolving disease (9) in combination with our finding that they secrete effector cytokines upon mKatG and PPD stimulation, suggest that their function is to eliminate an offending antigen. This is also supported by other studies from our group, showing that the TCR AV2S3^{pos} CD4^{pos} T cells are effector cells rather than FoxP3^{pos} regulatory T cells (8, 30). In addition, we have found that these cells are more activated and more differentiated than the AV2S3^{neg} T cell subset (7, 8).

Why do not all AV2S3^{pos} cells respond with cytokine production? It is essential to remember that the AV2S3^{pos} cell population is not a T cell clone. The variable alpha chain can associate with different variable beta chains (31). Mallone *et al* showed that different T cell clones can exhibit different avidity towards the same antigen, with the outcome that some clones only proliferate, whereas others both proliferate and produce cytokines in response to a given antigen concentration (32). This could explain why not all AV2S3^{pos} cells respond to *in vitro* mKatG stimulation with cytokine production.

It has become increasingly apparent that T cell responses are functionally heterogeneous, and that the extent to which Th1 cells are multifunctional, i.e. secrete two or more of the cytokines IFNγ, TNF and IL-2, correlates with disease outcome in several infections (33). Multifunctional Th1 cells have in mice models been associated with enhanced protection against various pathogens, including *M. tuberculosis* (22, 34-36). The induction of multifunctional T cells is now a goal in clinical vaccine development (37, 38).

A striking difference was found between patients with and without Löfgren´s syndrome, when examining the single- or multifunctional cytokine profile of the BAL CD4^{pos} T cells reactive to mKatG. Our data revealed that mKatG stimulated to a smaller fraction of antigen-reactive cells with single IFNγ production, but relatively more cells with simultaneous production of IFNγ and TNF, or single TNF production, in patients with Löfgren´s syndrome. In contrast, PPD stimulation gave rise to similar cytokine patterns in both patient subgroups. However, in the Löfgren group, the degree of multifunctional profile did not differ between AV2S3^{pos} and AV2S3^{neg} cells but was rather a property of

$CD4^{pos}$ cells in general in this patient subset. The fact that all Löfgren´s patients were $DRB1*0301^{pos}$ (or expressed the structurally very similar DRB3*0101) served to increase the possibility to detect differences between patient subgroups with distinct clinical phenotypes, since $DR3^{pos}$ Löfgren´s patients have been shown to have a particularly good prognosis (39). Thus, although the overall magnitude of anti-mKatG responses is similar in patients with or without Löfgren´s syndrome, these findings suggest that the quality of T cell responses against a limited number of mycobacterial antigens, such as mKatG, may be of critical importance for disease outcome. It may be that the difference between these two forms of sarcoidosis is not due to different antigens, but due to qualitative differences (in particular degree of multifunctionality) in the cytokine response to key antigens. In addition to mKatG, the response profile to other mycobacterial antigens probably also is of importance, as indicated by the higher PPD-induced TNF production from BAL $CD4^{pos}$ T cells of patients with Löfgren´s syndrome. A follow-up study of these patients will yield information as to whether the relative proportions of single/multifunctional cells have clinical implications and might be used as a biomarker to predict prognosis.

In the present study we also compared the T cell cytokine profiles induced by the two antigenic stimuli. The results showed that, in both patient groups, mKatG compared to PPD stimulated to a different cytokine pattern. Stimulation with mKatG favored significantly more single IFNγ secretion, whereas PPD stimulation resulted in significantly more of simultaneous IFNγ and TNF production, but very little secretion of IFNγ alone. Furthermore, the average per cell cytokine content of PPD-reactive

multifunctional T cells was particularly high. In addition to chemical properties, this capacity to preferentially induce cells producing IFNγ alone over multifunctional cells (supposedly more efficient effector cells also in sarcoidosis) may in part explain the persistence of mKatG, especially in non-Löfgren patients. In contrast, mycobacterial antigens with PPD-like properties may be better at triggering T cells with a high capacity to support antigen elimination.

By studying the median fluorescent intensity (MFI) of intracellular cytokines we found that the multifunctional $CD4^{pos}$ T cells had the highest MFI values, i.e. more of each cytokine produced from each cell, compared to single cytokine-producing $CD4^{pos}$ T cells. Although this particular analysis was only performed in lung and blood cells of Löfgren´s patients, the results are in line with a previous study in humans (37). It remains to be elucidated which is of greater functional importance – that multifunctional T cells have a bigger repertoire of cytokines, or that the amount of each cytokine per cell is higher. However, as suggested by animal studies, both factors are probably of importance (22).

T cell differentiation is associated with loss of the co-stimulatory molecule CD27, and BAL T cells were predominantly $CD27^{neg}$, in contrast to the situation in blood, indicating a more differentiated T cell population in the affected organ. We found that BAL $CD27^{neg}$ $CD4^{pos}$ T cells contained a higher frequency of cells producing IFNγ in response to mKatG or PPD, as compared to BAL $CD27^{pos}$ $CD4^{pos}$ T cells, while in blood the highest frequency of antigen-specific IFNγ-producing cells were found in the $CD27^{pos}$

CD4^{pos} T cell subset. The findings in BAL, but not in blood, are similar to what has been reported regarding beryllium-specific T cell responses in patients with chronic beryllium disease (CBD), a granulomatous disorder that shows great similarities with sarcoidosis (40). The differences between sarcoidosis and CBD may be related to differences in antigen localization, and duration of antigen exposure.

For the first time we show that a specific mycobacterial antigen, mKatG, stimulates a subset of the CD4^{pos} TCR AV2S3^{pos} T cells, previously found accumulating in the lungs of HLA-DRB1*0301^{pos} sarcoidosis patients, typically with Löfgren´s syndrome. In addition, a more pronounced multifunctional cytokine pattern of the mKatG-specific BAL CD4^{pos} T cells in Löfgren´s syndrome patients compared to corresponding cells in non-Löfgren´s patients, might suggest that patients with Löfgren´s syndrome eliminate antigen more efficiently and thereby obtain a better prognosis. It is noteworthy that strong responses to mKatG were detected not only in CD4^{pos}, but also in CD8^{pos} T cells. The function of mKatG-reactive CD8^{pos} cells should be further investigated.

A deeper understanding of specific T cell responses associated with recovery versus chronic disease should ultimately enable the design of novel, antigen-specific therapies.

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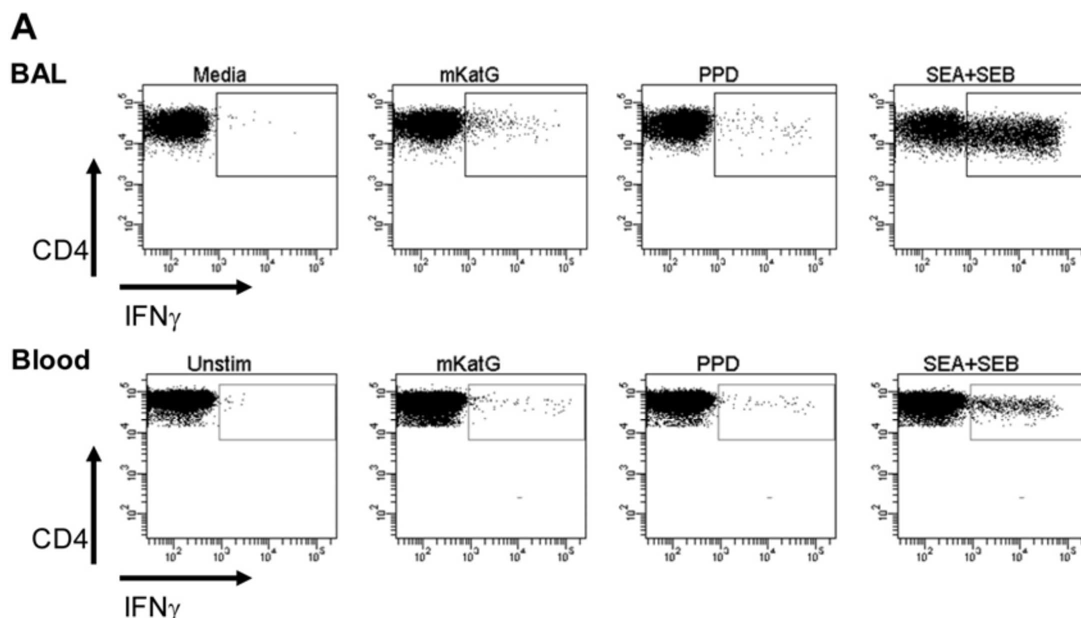
FIGURE LEGENDS

Figure 1. T cells in BAL and blood of patients with Löfgren´s syndrome respond with IFNγ production towards mycobacterial proteins. Representative flow cytometry plots of IFNγ production in BAL or blood CD4^{pos} T cells, in response to mKatG or PPD (media alone or unstimulated whole blood were used as negative controls; superantigens *Staphylococcus enterotoxin* A (SEA) and SEB were used as positive control) (A). Percentages of IFNγ-expressing BAL and blood CD4^{pos} or CD8^{pos} T cells, in response to mKatG or PPD (B). The statistical analyses in (B) were done using raw data, i.e. background (cytokine production in BAL after incubation in media alone, or in unstimulated whole blood) was not deducted. Comparison between BAL and blood in the percentages of IFNγ-expressing CD4^{pos} or CD8^{pos} T cells after stimulation with mKatG or PPD (C). The statistical analyses in (C) were done after deduction of background. Horizontal lines depict median percentage of cytokine-expressing cells. Wilcoxon signed rank test, * p<0.05, ** p<0.01, *** p<0.001.



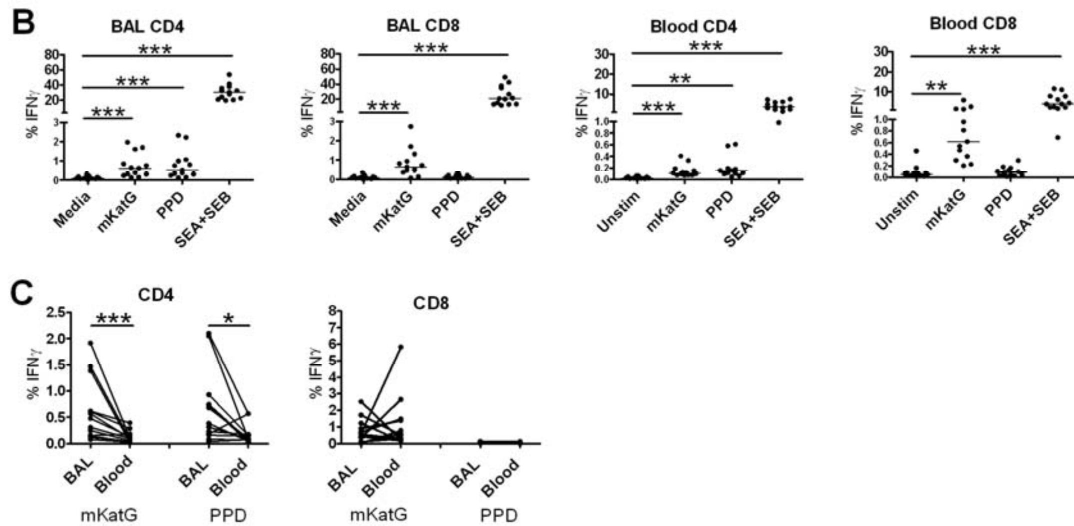


Figure 2. Greater BAL and blood reactivity towards mKatG in TCR AV2S3^{pos} compared to AV2S3^{neg} CD4^{pos} T cells of patients with Löfgren´s syndrome. Representative flow cytometry plots of IFNγ production in BAL and blood TCR AV2S3^{pos} or AV2S3^{neg} T cells, in response to mKatG or PPD (media alone or unstimulated whole blood were used as negative controls; superantigens *Staphylococcus enterotoxin* A (SEA) and SEB were used as positive controls) (A). Comparisons of the percentages of IFNγ-expressing BAL TCR AV2S3^{neg} and AV2S3^{pos} CD4^{pos} T cells in media alone and after stimulation with mKatG (B). The statistical analyses in (B) were done using raw data, i.e. background (cytokine production in BAL after incubation in media alone) was not deducted. Percentages of IFNγ-expressing BAL and blood TCR AV2S3^{neg} and AV2S3^{pos} CD4^{pos} T cells, in response to mKatG or PPD (C). The statistical analyses in (C) were done after deduction of background. Horizontal lines depict median percentage of cytokine-expressing cells. Wilcoxon signed rank test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s.=not significant.

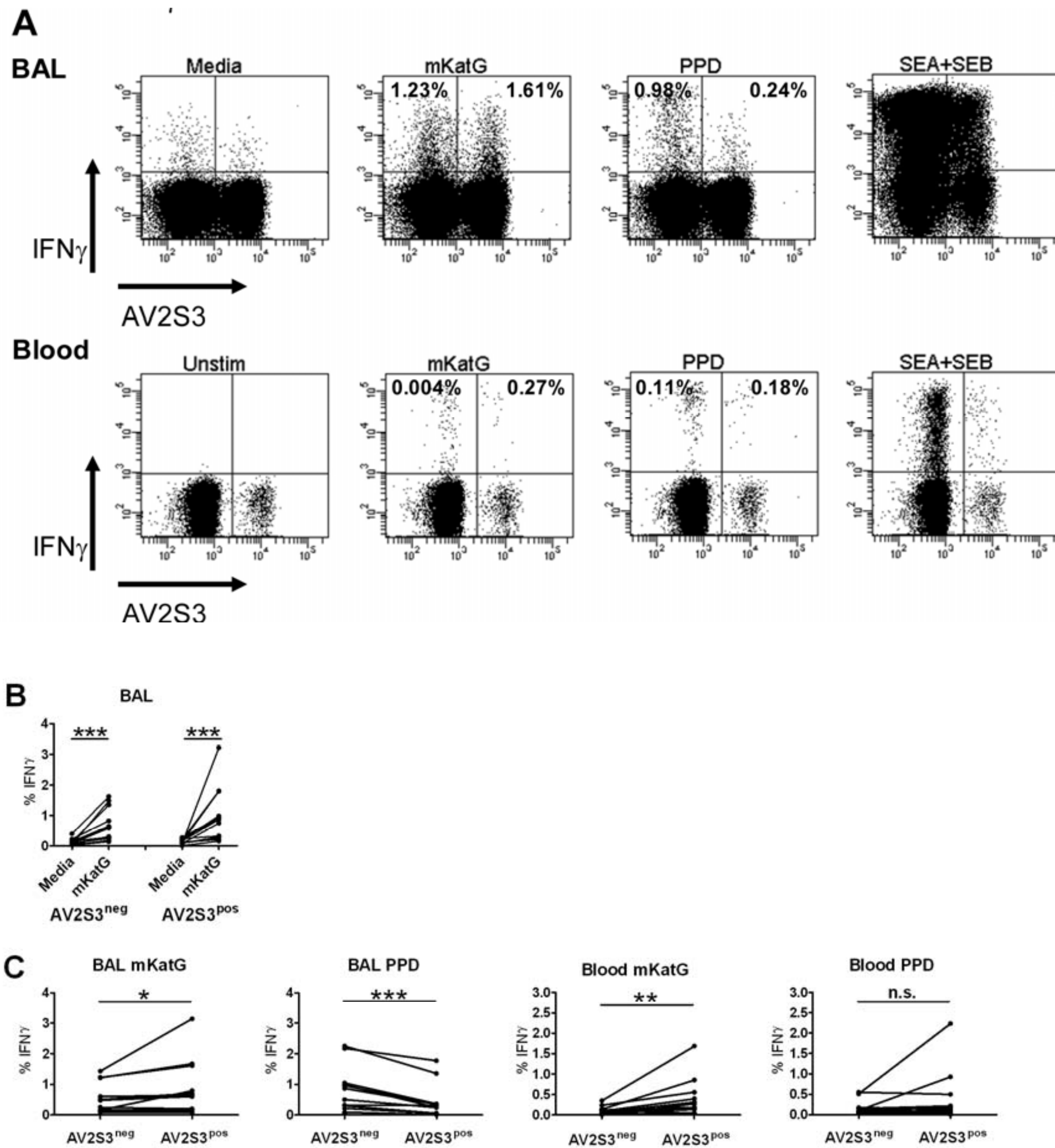


Figure 3. Higher proportion of multifunctional BAL CD4^{pos} T cells reactive with mKatG in Löfgren´s patients compared to non-Löfgren´s syndrome patients. Representative flow cytometry plots , gated on CD4^{pos} BAL T cells, of IFNγ versus TNF production in response to mKatG and PPD (A). Percentage of single IFNγ, single TNF, or simultaneous IFNγ and TNF (i.e. multifunctional) production as a fraction of the BAL

CD4^{pos} T cells that responded to mKatG (B) or PPD (C) stimulation. The sum of all cells responding with any of these cytokine patterns was set to 100%. The statistical analyses were done after deduction of background. Horizontal lines depict median percentage of cytokine production. Mann-Whitney U-test, * p<0.05, ** p<0.01.

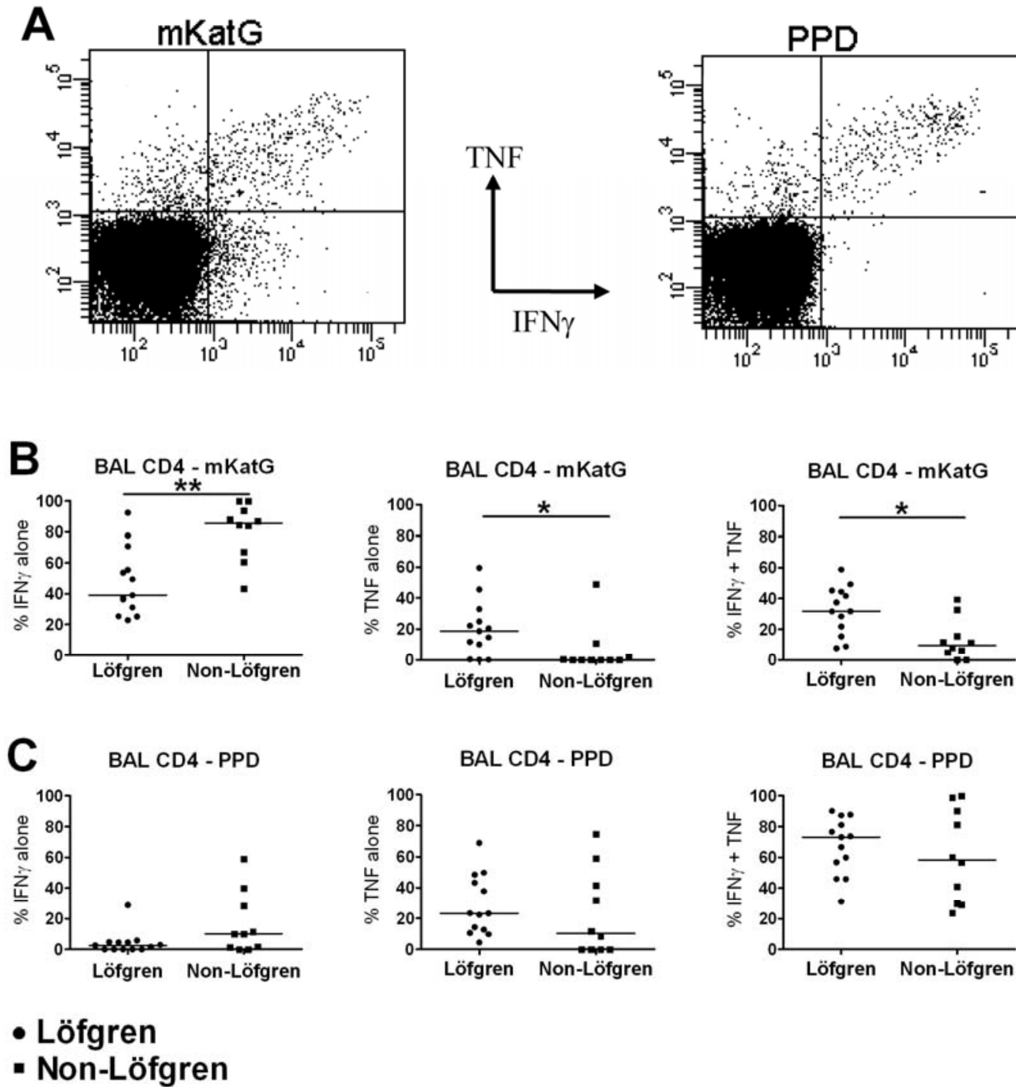


Figure 4. Mycobacterial mKatG stimulates to more single IFNγ production compared with PPD in BAL CD4^{pos} T cells of patients with sarcoidosis. A statistical comparison of the fractions of total mKatG or PPD reactive CD4^{pos} T cells, displaying the cytokine patterns single IFNγ, single TNF, or simultaneous IFNγ and TNF (i.e. multifunctional) production. The sum of all cells responding with any of these cytokine patterns was set to 100%. The statistical analyses were done after deduction of background. Horizontal lines depict median percentage of cytokine-expressing cells. Wilcoxon signed rank test, ** $p<0.01$, *** $p<0.001$.

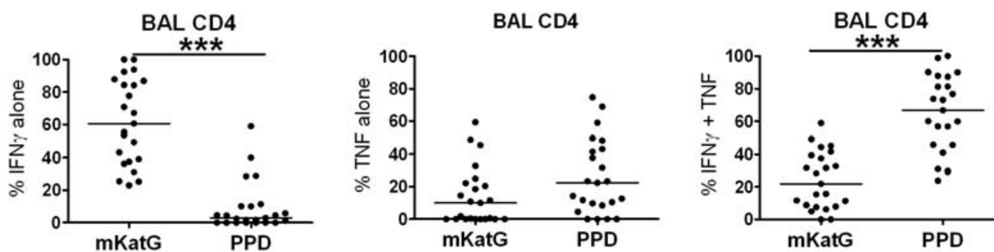


Figure 5. Greater median fluorescent intensity (MFI) for cytokine staining of multifunctional CD4^{pos} T cells of BAL. MFI for IFNγ and TNF respectively, of single IFNγ, single TNF, or simultaneous IFNγ and TNF (i.e. multifunctional) production in BAL CD4^{pos} T cells after stimulation with mKatG or PPD. Only Löfgren´s syndrome patients were analyzed. The statistical analyses were done after deduction of background. Horizontal lines depict median percentage of cytokine production. Wilcoxon signed rank test, * $p<0.01$, *** $p<0.001$.

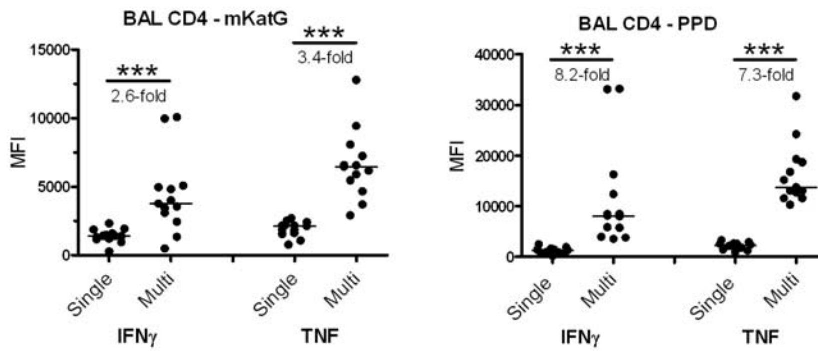
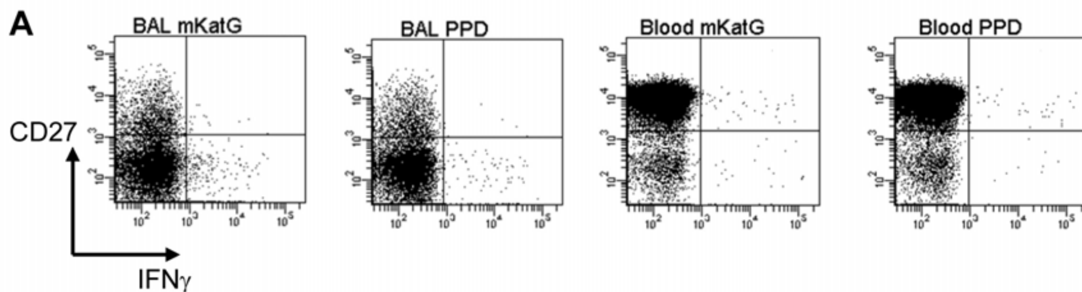


Figure 6. Higher BAL CD27^{neg}, but blood CD27^{pos}, T cell reactivity towards mycobacterial proteins. Representative flow cytometry plots of IFNγ production in BAL and blood CD27^{neg} or CD27^{pos} T cells in response to mKatG or PPD (media alone or unstimulated whole blood were used as negative controls; superantigens *Staphylococcus enterotoxin* A (SEA) and SEB were used as positive controls; data not shown) (A). Percentages of IFNγ-expressing CD27^{neg} or CD27^{pos} BAL or blood CD4^{pos} T cells after mKatG or PPD stimulation (B). The statistical analyses were done after deduction of background. Wilcoxon signed rank test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.



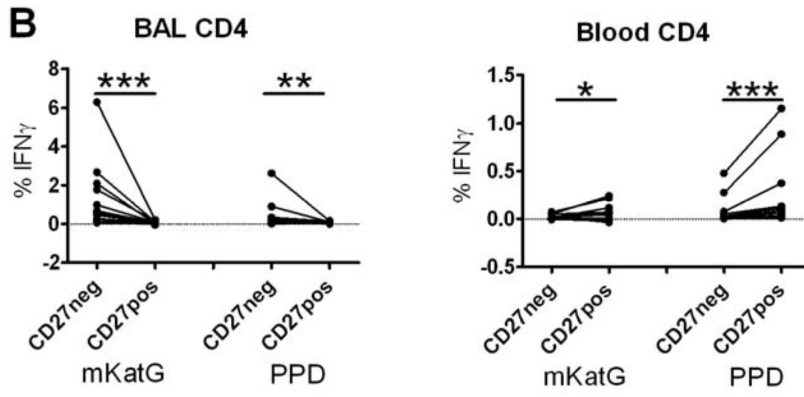

B
BAL CD4
% IFNγ
***
**
CD27neg
CD27pos
mKatG
PPD
Blood CD4
*
***

Table 1. CHARACTERIZATION OF SARCOIDOSIS PATIENTS

	Löfgren (n=13)	**Non-Löfgren (n=10)**
Sex, male/female	9/4	8/2
Age, yr	37 (29-56)	48 (33-67) *
Smoker (yes/ex/never)	3/2/8	1/4/5
X-ray stage (0/I/II/III/IV)	0/10/3/0/0	0/2/3/2/3
BAL analyses		
Recovery (% of instilled volume)	67 (60-72)	59 (55-68)
Viability (%)	94 (93-96)	95 (93-98)
Total cell conc. ($*10^6$/L)	172 (99-248)	160 (125-191)
Total cell number ($*10^6$)	26 (13-38)	23 (16-32)
BAL differential cell counts		
Macrophages (%)	77 (68-85)	76 (64-86)
Macrophages ($*10^6$/L)	128 (81-174)	124 (90-154)
Lymphocytes (%)	23 (13-30)	22 (13-34)
Lymphocytes ($*10^6$/L)	33 (13-66)	27 (14-67)
Neutrophils (%)	1.0 (0.4-1.7)	1.6 (0.4-2.3)
Neutrophils ($*10^6$/L)	1.3 (0.9-2.9)	2.5 (0.4-3.3)
Eosinophils (%)	0.2 (0-0.4)	0.4 (0.2-0.8)
Eosinophils ($*10^6$/L)	0.3 (0-0.5)	0.6 (0.2-1.3)
BAL CD4/CD8 ratio	5.0 (2.5-11)	4.5 (3.5-9.0)
BAL AV2S3 (% of CD4 in BAL)	28 (22-37)	3.5 (2.5-6.7) ***
Blood AV2S3 (% of CD4 in blood)	4.4 (3.9-5.2)	n.d.
Pulmonary function tests		
VC (%)	96 (86-104)	90 (78-99)
FEV_1 (%)	93 (76-102)	90 (75-101)
DL_{CO} (%)	94 (91-107) (n=12)	82 (24-83) (n=3)

Pulmonary function tests (values show % of predicted): VC Vital capacity, % of reference value; FEV_1 Forced expiratory volume in one second**;** DL_{CO} Diffusing capacity

of the lung for carbon monoxide**.** Data are shown as median (p25-p75), except for age that is shown as median (min-max), n.d. = not determined. Mann-Whitney U-test * $p<0.05$, *** $p<0.001$.