EUROPEAN RESPIRATORY *journal*

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Original article

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Please cite this article as: Lepzien R, Liu S, Czarnewski P, *et al*. Monocytes in sarcoidosis are potent TNF producers and predict disease outcome. *Eur Respir J* 2021; in press (https://doi.org/10.1183/13993003.03468-2020).

This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

Monocytes in sarcoidosis are potent TNF producers and predict disease outcome

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ABBREVIATIONS: mononuclear phagocyte (MNP), dendritic cell (DC), bronchoalveolar lavage (BAL), tumor necrosis factor (TNF)

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

FUNDING

This work was supported by grants to AS-S from the Swedish Heart-Lung Foundation, the Swedish Research Council, and Karolinska Institutet. JG was supported by the Swedish Heart-Lung Foundation, the Swedish Research Council, through the regional agreement on medical training and clinical research (ALF) between the Stockholm City Council and Karolinska Institutet, The King Gustaf V's and Queen Victoria's Freemason's Foundation, and Karolinska Institutet. PC is financially supported by the Knut and Alice Wallenberg Foundation as part of the National Bioinformatics Infrastructure Sweden at SciLifeLab.

AUTHOR CONTRIBUTIONS

RL, AE, AB, JG, and AS-S planned the study. SK, AE, AB, and JG included patients, performed bronchoscopies and BÖ retrieved clinical information. RL, SL, MN, FB, JP, and GR performed experiments. RL, and AS-S analyzed experimental data. PC performed bioinformatics analysis. MB performed statistical analysis. RL and AS-S co-wrote, and all co-authors edited the manuscript.

KEYWORDS: dendritic cell, monocyte, sarcoidosis, TNF, Löfgren's syndrome.

For Twitter (256 characters):

Phenotypic, transcriptomic and functional mapping of blood and pulmonary mononuclear phagocytes in sarcoidosis patients. We found that frequency and function of pulmonary monocytes at time of diagnosis predict 2-year disease outcome in sarcoidosis.

ABSTRACT (259 Words)

BACKGROUND: Pulmonary sarcoidosis is an inflammatory disease characterized by granuloma formation and heterogeneous clinical outcome. TNF is a proinflammatory cytokine contributing to granuloma formation and high levels of TNF have been shown to associate with progressive disease. Mononuclear phagocytes (MNPs) are potent producers of TNF and highly responsive to inflammation. In sarcoidosis, alveolar macrophages (AMs) have been well studied. However, MNPs also include monocytes/monocyte-derived cells and dendritic cells (DCs) that despite their central role in inflammation are poorly studied in sarcoidosis.

OBJECTIVE: To determine the role of pulmonary monocyte-derived cells and DCs during sarcoidosis.

METHODS: We performed in-depth phenotypic, functional and transcriptomic analysis of MNPs subsets from blood and bronchoalveolar lavage (BAL) fluid from 108 sarcoidosis patients and 30 healthy controls. We followed the clinical development of patients and assessed how the repertoire and function of MNP subsets at diagnosis correlated with 2-year disease outcome.

RESULTS: Monocytes/monocyte-derived cells were increased in blood and BAL of sarcoidosis patients compared to healthy controls. Interestingly, high frequencies of blood intermediate monocytes at time of diagnosis associated with chronic disease development. RNAseq analysis showed highly inflammatory MNPs in BAL of sarcoidosis patients. Furthermore, frequencies of BAL monocytes/monocyte-derived cells producing TNF without exogenous stimulation at time of diagnosis increased in patients that were followed longitudinally. In contrast to AMs, the frequency of TNF producing BAL monocytes/monocyte-derived cells at time of diagnosis was highest in sarcoidosis patients that developed progressive disease.

CONCLUSION: Our data show that pulmonary monocytes/monocyte-derived cells are highly inflammatory and can be used as a predictor of disease outcome in sarcoidosis patients.

INTRODUCTION

Sarcoidosis is a multisystemic T cell driven inflammatory disorder of unknown etiology with broad clinical heterogeneity. The hallmark of sarcoidosis is formation of granulomas that most commonly affect the lungs. Sarcoidosis resolves in 30-40% of the cases within 2 years [1-4]. In Sweden, one third of sarcoidosis patients present with an acute disease onset, referred to as Löfgren's syndrome (LS) that associates with good prognosis. However, two thirds of patients present with gradual onset (non-LS) sarcoidosis and these patients are more likely to develop chronic disease [5]. What dictates disease severity is still unclear, but local immunological events early during disease development likely set the stage for disease progression, in addition to genetic and environmental factors [6].

T cells have been best studied and T cell driven mechanisms contributing to disease pathophysiology have been identified, while innate immune mechanisms in sarcoidosis are less well understood [6]. Several biological markers that predict disease severity, such as serum interleukin 2 receptor, serum amyloid A and tumor necrosis factor (TNF) have been identified, while candidates predicting disease outcome are still lacking [7, 8]. TNF is a multifunctional cytokine produced by a variety of cells and is important for the induction and maintenance of granulomas [9, 10]. *In vitro* cultured alveolar macrophages (AMs) from sarcoidosis patients are major producers of TNF [11, 12]. In sarcoidosis, AMs produce TNF without exogenous stimulation and highest levels of TNF are measured in patients with progressive disease [8]. Thus, drugs targeting TNF are used as a third line treatment in non-LS patients, resulting in noticeable clinical improvement in a subgroup of the treated patients [13].

In addition to AMs, monocytes/monocyte-derived cells and dendritic cells (DCs), collectively called mononuclear phagocytes (MNPs), are found lining the respiratory mucosa [14-16]. Pulmonary monocytes/monocyte-derived cells from healthy controls are potent producers of TNF upon stimulation *in vitro* [14]. However, functional data of pulmonary monocytes/monocyte-derived cells from sarcoidosis patients is currently not available. We previously reported no difference in the distribution of pulmonary monocytes/monocyte-derived cells from non-LS and LS patients but comparison to pulmonary monocytes/monocyte-derived cells from healthy controls is still missing [17]. Blood monocytes, however, were shown to be affected in sarcoidosis. $CD14^{+}CD16^{+}$ intermediate monocytes (IMs), a subset of monocytes, are expanded in circulation of sarcoidosis patients likely due to the systemic inflammation as also observed in other inflammatory diseases [18-21]. Furthermore, higher frequencies of circulating IMs were associated in patients with response to anti-TNF treatment [22]. In contrast to monocytes, distribution of DCs was not altered in blood or BAL of sarcoidosis patients [23, 24].

In the present study, we aimed to determine to what extent pulmonary monocytes/monocyte-derived cells contribute to inflammation in sarcoidosis. In a detailed, longitudinal study, we show that monocytes/monocyte-derived cells actively contribute to inflammation by TNF production and associate with disease progression in non-LS sarcoidosis

MATERIAL AND METHODS

Study design and patient characteristics

The study was approved by the regional ethical review boards in Stockholm and Umeå, Sweden and performed according to the declaration of Helsinki. 108 sarcoidosis patients and 30 healthy volunteers were included in the study and gave written informed consent (Table I). Bronchoscopies were performed at the Karolinska University Hospital, Stockholm or at the University Hospital, Umeå as previously described [17, 25]. All patients were newly diagnosed with pulmonary sarcoidosis as defined by WASOG guidelines [26] based on clinical signs, chest radiography findings, an elevated CD4/CD8 T cell ratio in BAL, or non-caseating granulomas in tissue biopsies (Table I, Figure S1A-C). 20 patients were diagnosed with Löfgren's syndrome (LS) based on clinical signs (acute disease onset, enlarged bilateral hilar lymph nodes, erythema nodosum and/or periarticular tendovaginitis).

In a cohort of 9 non-LS patients, as part of a study with controlled exercise [27], a second bronchoscopy was performed six months after the first one to follow patients longitudinally (Table II).

Disease outcome was assessed 2 years after establishing diagnosis. In this study, 69 patients had passed the two-year mark and were characterized with one of the following disease outcomes: 1) Remission: no symptoms and no chest radiological signs; 2) Chronic stable: Stable pulmonary manifestations without deterioration with no signs of inflammatory activity in laboratory parameters. No or minor chest radiological changes compared to previous assessment, no systemic treatment required; 3) Chronic Progressive: Deterioration of symptoms and impairment in chest radiological signs compared to previous assessment, systemic treatment required.

Single cell preparations from blood and BAL and flow cytometry

Blood and BAL was processed within one hour after retrieval for further applications. Peripheral blood mononuclear cells (PBMCs) were isolated from blood using Ficoll density gradient centrifugation. BAL samples were kept on ice, filtered through a 100 µm nylon filter (Syntab) and centrifuged at 400 X g for 15 min before downstream application. Cell suspensions were incubated with LIVE/DEAD™ Fixable Aqua/Blue Dead Cell Stain Kit (Life Technologies) and Fc receptors blocked using FcR block (Miltenyi) followed by staining with antibodies against surface molecules (Supplementary table I). For intracellular staining, cells were fixed using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen). Briefly, cells were fixed for 20 min at RT followed by staining with antibodies against intracellular molecules (Supplementary table I). Cells were analyzed using an LSRII or LSR Fortessa flow cytometer (both BD) and data were analyzed using FlowJo X software (BD).

Fluorescence activated cell sorting (FACS)

For cell sorting, blood cells were enriched using the RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies). Blood and BAL cells were used within one hour after retrieval from the study subjects and stained with a validated panel of antibodies (Supplementary table I). For cell sorting, a FACS Aria Fusion or AriaIII (both BD) were used. Sorted cells were subsequently resuspended in Qiazol Lysis reagent (Qiagen) and stored at -80°C until further use.

RNA isolation and sequencing

For detailed information on RNA isolation and RNA sequencing, see supplemental material.

Stimulation of PBMCs and BAL cells

PBMCs and BAL cells were cultured at 1 x 10^6 cells/ml in RPMI 1640 (Sigma-Aldrich) containing 10% fetal calf serum (Invitrogen). Cells were cultured for 3h either unstimulated or 1 ug/ml LPS (Sigma-Aldrich, Escherichia coli O111:B4, L4391) was added. 10 ug/ml brefeldin A (Sigma-Aldrich) was added.

ELISA

For detailed information on cytokine analysis using ELISA, see supplemental material.

Statistical analysis

The numeric data are summarized with the median, unless otherwise stated. Statistical analyses were performed using the Mann Whitney U test, the non-parametric Kruskal-Wallis test with Dunn´s test for correction of multiple comparisons, and the paired Wilcoxon signed-rank test. The Spearman's rank correlation coefficient was used for correlation analysis. Predictive models were estimated with logistic regression. The outcome variable was defined as remission vs. no remission (chronic stable and chronic progressive) as well as chronic stable vs. chronic progressive. The predictors entered the models one at a time. The categorical predictors were introduced by means of dummy variables. Data were analyzed using GraphPad Prism version 8.0 (GraphPad Software) and Stata (StataCorp, College Station, TX, USA). Results were considered statistically significant at the level $p < 0.05$.

RESULTS

$CD14^+CD16^+$ monocytes/monocyte-derived cells are increased in blood and BAL of sarcoidosis patients

To investigate the frequencies of mononuclear phagocyte (MNP) subsets in LS and non-LS sarcoidosis compared to healthy controls (HC) we performed multicolor flow cytometry on matched BAL and blood samples. In BAL, frequencies of alveolar macrophages (AMs), identified based on high side scatter and autofluorescence, were reduced in non-LS and LS patients compared to HC (Figure 1B and Figure S1D). In blood and BAL, MNPs were identified as HLA-DR^+ and lineage negative (Figure 1A-B and Figure S1E-F). Further, three monocyte subsets as well as plasmacytoid DCs (PDC) and conventional DCs (cDCs) 1 and cDC2 were identified (Figure 1C). Overall, distribution of MNP subsets differed most distinctly based on their origin from blood or BAL (Figure 1D-J and Figure S2A-B). Still, significant differences were observed in $CD14^+CD16^+$ intermediate monocytes, that were increased in blood of non-LS and LS patients and in BAL of non-LS patients compared to HC (Figure 1E). cDC2 and cDC1 were significantly decreased in BAL and blood, respectively, of non-LS patients compared to HC (Figure 1G-H). With respect to maturation status of MNPs, the most apparent difference was that BAL MNPs were more mature than blood MNPs by upregulation of HLA-DR and CD86. However, MNPs from non-LS patients in blood and BAL expressed higher levels of HLA-DR and CD86 compared to those from HC within each anatomical compartment (Figure S2C-D). In summary, based on flow cytometric analysis, we documented limited but potentially important alterations in the frequency and maturation of DCs and monocytes/monocyte-derived cells in BAL and blood in sarcoidosis patients.

MNPs from sarcoidosis patients present inflammatory gene signature

To further examine MNPs in sarcoidosis, we sorted blood and BAL DCs and monocytes/monocyte-derived cells as well as AMs from BAL of non-LS patients and HC and performed RNA sequencing on in total nine different populations (RNAseq) (Figure 2A). Principal component analysis showed that samples distributed based on tissue source and cell subset rather than their origin from non-LS patients or HC (Figure 2B). We found that genes were upregulated in BAL MNPs related to cell maturation (*CD40, CD80, CD83*), inflammatory response (*TLR3, TLR7*), cytokine signaling (*TNF*, *IL1B*, *CSF1*, *TGFB*) and chemotaxis (*CCR6, CCR7* as well as *CCL2, 19* and *20*) compared to blood MNPs in both non-LS patients and HC (Figure 2C). The difference in mRNA expression of *CCR6*, *CCR7*, *CD207* and *PD-L1*, all critical for the function of MNPs, was confirmed on protein level (Figure 2D and Figure S2C-E). Importantly, we next analyzed gene signatures across cell subsets comparing non-LS patients and HC. We observed upregulation of genes related to TNF, IL-17 and TLR signaling (Figure 2E) in non-LS patients compared to HC. Gene set enrichment analysis of each sequenced MNP subset verified high expression of genes related to the TNF pathway (such as *TNF, IL1B, IL6, NR4A1* and *REL*) as well as other immune related pathways in samples from non-LS patients compared to HC (Figure 2F). Taken together, utilizing RNA sequencing revealed distinct differences in cytokine signaling across MNP subsets upregulated in non-LS patients compared to healthy controls, in particular related to TNF signaling.

Monocytes/monocyte-derived cells from non-LS patients show high TNF gene and protein expression at time of diagnosis and during disease development

Next, we analyzed whether the enriched TNF signaling pathway in individual MNP subsets from non-LS patients had functional implications. The relative expression of *TNF* gene transcripts in MNPs was overall higher in BAL MNPs compared to blood (Figure 3A). Furthermore, *TNF* in each subset from non-LS patients was higher compared to HC MNPs (Figure 3A). To confirm this, we measured TNF protein in individual MNPs using intracellular cytokine staining and flow cytometry, either *ex vivo* or after 3h of culture with and without stimulation (Figure 3B). In blood of all subjects, TNF could only be detected after LPS stimulation in monocytes (Figure 3B and Figure S4A). In contrast, BAL monocytes/monocyte-derived cells showed an accumulation of TNF intracellularly without stimulation (Figure 3B-C). In some patients, adding LPS did not result in higher frequency of TNF expressing cells than the unstimulated condition (Figure S4A). The frequency of unstimulated TNF expressing MNPs was significantly higher in non-LS patients compared to HC and LS patients (Figure 3C). The frequency of unstimulated TNF expressing monocytes/monocyte-derived cells was higher compared to DCs (Figure S4B). Higher frequencies of TNF-producing monocytes/monocyte-derived cells correlated positively with higher frequencies of TNF-producing macrophages or DCs of the same patient (Figure S4C). TNF was detectable in plasma and BAL fluid, however, no correlations were observed between secreted TNF and the frequency of unstimulated TNF-expressing BAL monocytes/monocyte-derived cells (Figure S4D-E).

The IL-6 signaling pathway was also enriched in the RNAseq analysis of MNPs from non-LS patients and we confirmed similar transcriptional and protein expression pattern for IL-6 as seen for TNF (Figure S5A-F). LS patients showed a trend towards higher frequencies of IL-6 expression in unstimulated BAL MNPs compared to non-

LS patients and HC. Frequencies of TNF and IL-6 expressing, unstimulated BAL monocytes/monocyte-derived cells correlated positively in HC and non-LS patients but showed a trend towards negative correlation in LS patients (Figure S5G). In addition, IL-1β was significantly increased in plasma but not BAL fluid in non-LS patients compared to HC or LS patients (Figure S6).

To assess whether the elevated TNF-producing MNPs in BAL was maintained during disease development, we analyzed samples from a study where newly diagnosed sarcoidosis patients followed a controlled physical exercise program but did not receive any treatment [27]. We observed that frequencies of TNF-expressing unstimulated MNPs from non-LS patients increased over time (Figure 3D). Frequency of IL-6 expressing MNPs as well as MNP distribution were not altered during this time (Figure 3D and Figure S7A-C).

Our data suggest that elevated frequencies of TNF-producing monocytes/monocyte-derived cells in BAL, without additional stimulation, mark an important immunological difference between non-LS patients at time of diagnosis from both LS patients and HC.

Monocytes/monocyte-derived cells and DCs are predictors of the disease outcome in sarcoidosis

To determine whether MNP phenotype, cytokine levels or mRNA expression at time of diagnosis associated with two-year disease outcome we used predictive modelling. Out of the 108 patients included in the study, 69 passed the two-year mark at the time of analysis. 18 patients showed remission while 51 had developed chronic disease: 32 patients being chronic stable and 19 patients with chronic progressive disease (Figure 4A). In line with previous reports [28], the probability to

remit disease two years after diagnosis was higher (odds ratio (OR) = 1.32) when the frequency of Vα2.3^{+} T cells was high in BAL as well as when patients were carrying the HLA-DRB1*03 allele (OR = 39.4), which was mostly attributed to LS patients (Figure 4B). Interestingly, patients with higher frequencies of CD14^{+}CD16^{+} intermediate monocytes in blood at time of diagnosis were less likely to show remission after 2 years (odds ratio (OR) = 0.89) (Figure 4C). In contrast, higher frequencies of cDC2 in BAL at time of diagnosis indicate a higher chance to remit disease after two years (OR = 1.15) (Figure 4D). Additionally, patients with higher frequencies of cDC1 (OR = 0.54) and PDCs (OR = 1.34) in BAL were more likely to not develop progressive disease or clear disease after two years, respectively (Figure S8A).

In our dataset, TNF expression by AMs and CD14^{+}CD16^{-} monocytes/monocyte-derived cells did not predict disease outcome, however, TNF expressing unstimulated CD14^{+}CD16^{+} monocytes/monocyte-derived cells at time of diagnosis predicted a progressive disease development (Figure 4E). Neither soluble TNF and IL-6 concentrations in plasma and BAL fluid nor IL-6 producing MNPs from BAL of sarcoidosis patients predicted disease outcome (Figure 4F and Figure S8B-C).

Collectively, our data show that monocytes/monocyte-derived cells are affected by and contribute actively to inflammation in non-LS patients. Furthermore, assessing monocytes/monocyte-derived cells at time of diagnosis can predict the disease course in non-LS sarcoidosis.

DISCUSSION

MNPs are likely central in sarcoidosis pathogenesis as they activate T cells and produce pro-inflammatory cytokines that drive inflammation in sarcoidosis [29]. In the current study, we revealed that the frequency and distribution of monocytes and DCs in blood and BAL at time of diagnosis may predict disease outcome. Furthermore, monocytes/monocyte-derived cells were potent TNF producers contributing to local and systemic inflammation and high frequencies of TNF producing monocytes/monocyte-derived cells associated with progressive disease development in sarcoidosis.

$CD14^+CD16^+$ intermediate monocytes are indicative of systemic inflammation and are elevated in several inflammatory diseases [20, 21]. We and others have also seen elevated frequencies of IMs in non-LS sarcoidosis patients [18, 19, 30-32]. IMs differentiate from $CD14^+CD16^-$ classical monocytes in blood and the process is accelerated during inflammatory conditions [33]. Cytokines in blood likely drive that differentiation. Reports on elevated plasma concentrations of TNF or IL-6 could not be confirmed in our cohort (Figure S4D and Figure S5F) [34, 35]. However, other cytokines may contribute to inflammation. As our RNAseq data indicated, *IL-1β* or *CSF-1* genes were upregulated in BAL MNPs that could be released into circulation and contribute to inflammation resulting in the increase of $CD14^+CD16^+$ intermediate monocytes. Increased expression of *IL-1β* by BAL MNPs indicates an upregulation of the inflammatory NLRP3 pathway as previously shown to be activated in sarcoidosis [36]. In BAL, increased levels of TNF and IL-6 were observed on both gene expression and protein levels compared to HC indicating local inflammation and may explain the expansion of $CD14^+CD16^+$ monocytes/monocyte-derived cells

in the lung of non-LS patients. Pro-inflammatory cytokines in the lung of sarcoidosis patients were also observed before [37]. Another explanation for the expansion of $CD14^+CD16^+$ monocytes/monocyte-derived cells in BAL could be increased migration of $CD14^+CD16^-$ classical monocytes to the lung that subsequently differentiate to $CD14^+CD16^+$ monocytes/monocyte-derived cells. RNAseq data showed increased *CCL2* gene expression by BAL MNPs. CCL2 is the ligand for CCR2, which is highly expressed on $CD14^+CD16^-$ classical monocytes. Upon migration to the lung, the differentiation of $CD14^+CD16^-$ into $CD14^+CD16^+$ monocytes/monocyte-derived cells is known to be influenced by the tissue environment [33, 38]. Due to the younger HC cohort, we cannot rule out that age-related effects influence the presence of $CD14^+CD16^+$ monocytes/monocyte-derived cells in non-LS patients compared with HC.

In the current study, we also show that pulmonary monocytes/monocyte-derived cells themselves contribute significantly to inflammation by production of proinflammatory TNF. We have previously shown that pulmonary monocytes/monocyte-derived cells could respond to TLR stimulation by producing TNF [14]. However, here we found high frequencies of TNF producing pulmonary monocytes/monocyte-derived cells even without stimulation. Our data complements the finding that in addition to AMs as the principal producer of TNF in sarcoidosis patients [11] also other TNF-producing immune cells contribute to inflammation in sarcoidosis. Most reports studying monocytes in sarcoidosis have been limited to blood. A study on blood monocytes showed a decrease in IL-10 producing regulatory monocytes in sarcoidosis patients compared with HC further strengthening the pro-inflammatory role of monocytes [39]. Less IL-10 produced by monocytes resulted in

impaired suppression of T cell proliferation possibly contributing to the exaggerated T cell alveolitis observed in sarcoidosis [39]. Additionally, blood monocytes from sarcoidosis patients responded with higher TNF and IL-6 production after stimulation compared with controls [18]. This supports our RNAseq data that blood monocytes are highly inflammatory although to a different extent as BAL monocytes/monocyte-derived cells that released TNF spontaneously. While AMs are numerous in BAL fluid and undoubtedly play an important role in sarcoidosis, analysis of bulk BAL cells may mask the contribution of less frequent monocytes/monocyte-derived cells and DCs [40]. By studying the transcriptome of individual MNP subsets, we could also identify differences in gene expression between AMs, monocytes/monocyte-derived cells and DCs (Figure 2F). AMs showed upregulated genes related to the reactive oxygen species pathway, TGFβ or the fatty acid metabolism. Interestingly, CSF1 was upregulated by MNPs in BAL of non-LS patients that favor the differentiation of alternatively activated macrophages [41]. In support of this, MNPs in BAL of non-LS patients also express high levels of TGFβ compared to controls, also favoring alternatively activated macrophage polarization [42]. Potentially, single-cell RNAseq could better reveal the heterogeneity of classically and alternatively activated AMs in sarcoidosis. It is likely that the balance between pro- and anti-inflammatory macrophages contribute to disease progression and resolution. Collectively, these findings mark significant differences between AMs and monocytes/monocyte-derived cells in the lungs of sarcoidosis patients.

Serum amyloid A (SAA) and soluble IL-2 receptor were markers found in patients with progressive disease [7, 8]. Our RNA data confirmed that SAA was increased non-LS patients compared to HC. In addition to disease severity markers, prognostic

factors are needed to determine disease outcome of the patients that a deterioration of symptoms can be intervened early on. We found that high frequencies of blood monocytes and lower frequencies of BAL DCs at time of diagnosis are predictors of disease outcome as patients were less likely to resolve disease after two years. To assess if frequencies of monocytes can be used as a predictive tool in the clinic, a prospective multi-center study would be desirable to obtain larger patient numbers to account for differences in location, age, gender and ethnicity. Functionally, unstimulated TNF-producing BAL monocytes/monocyte-derived cells but not AMs, predicted chronic progressive disease development. This is in complementing previous observations where TNF production by AMs were observed in patients with progressive disease [8]. A strength of our study is the use of flow cytometry and intracellular cytokine staining that allows identification of TNF expression on a single-cell level compared to ELISA that measures total secreted TNF without revealing the source of the cytokine production. Additionally, we used a short 3-hour culture for TNF to accumulate with the advantage to primarily induce cytokine production in MNPs rather than T cells. Since the frequency of $CD14^{+}CD16^{+}$ intermediate monocytes at time of diagnosis correlates with a chronic disease course after two years, it is of interest whether inhibition or modulation of monocyte migration by targeting the CCR2-CCL2 signaling axis could be explored as new treatment target [8, 43].

Collectively, our data reveal an important role for monocytes/monocyte-derived cells in sarcoidosis. While monocytes are pro-inflammatory, AMs are important for regeneration as well as tissue repair and DCs most likely are essential in the T cells response that has to be elucidated in further studies. Continued in-depth analysis of

MNPs may help to better understand the clinical heterogeneity and may pave the way to identify biomarkers and treatment options to help sarcoidosis patients clear the disease.

ACKNOWLEDGEMENTS

We thank all patients and volunteers who have contributed clinical material to this study. We also thank Frida Holmström and the staff at the Department of Public Health and Clinical Medicine, Division of Medicine/Respiratory Medicine, Umeå University Hospital, as well as Margitha Dahl, Heléne Blomquist, Sussi Schedin, and Benita Dahlberg at the Lung Research Unit at Karolinska University Hospital, Stockholm for excellent technical assistance in sample acquisition and preparation. We also thank Diana Ekman from the National Bioinformatics Infrastructure Sweden (NBIS) for bioinformatics advice, and Sara Falck-Jones and Roosa Vaitiniemi from the Smed-Sörensen lab for assistance in collecting clinical data. PC is financially supported by the Knut and Alice Wallenberg Foundation as part of the National Bioinformatics Infrastructure Sweden at SciLifeLab.

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FIGURE LEGENDS

Figure 1. Elevated frequencies of monocytes/monocyte-derived cells in blood and BAL of sarcoidosis patients compared to healthy controls.

Pseudocolor plots from **(A)** blood and **(B)** BAL of one representative non-LS patient are shown to illustrate the gating strategy used to identify MNPs. In BAL, alveolar macrophages were identified based on high autofluorescence and high side scatter characteristics. Among single, live, $CD45^+$ leukocytes, MNPs were identified expressing HLA-DR but are negative for lineage markers. **(C)** Plasmacytoid dendritic cells (PDC) (purple) were identified based on expression of CD123, while CD11c identified myeloid cells. From the $CD11c^+$ cells, monocytes/monocyte-derived cells ($CD14^+CD16^-$ classical monocytes (CM), $CD14^+$ $CD16^+$ intermediate monocytes (IM), and $CD14^-$ $CD16^+$ non-classical monocytes (NCM)) were identified. Subsequently, cDC1 and cDC2 were identified by their expression of CD141 or CD1c, respectively. **(D-J)** Violin plots show frequencies of **(D-F)** monocyte and **(G-J)** DC subsets out of live, $HLA\text{-}DR^+$ lineage negative cells in PBMCs and BAL in non-LS and LS sarcoidosis patients and healthy controls (HC). HC blood n = 25, BAL n= 28; non-LS blood n = 64, BAL n= 76; LS blood n = 14, BAL n = 16. Dotted lines indicate median, 25th and 75th percentile. Statistical analysis was performed using the non-parametric Kruskal-Wallis with Dunn´s test for correction of multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 2. Proinflammatory gene sets enriched in MNPs from sarcoidosis patients compared to healthy controls.

(A) Overview shows the workflow for samples used for RNA sequencing. Table shows number of samples of MNP subsets from HC and non-LS patients that passed quality control for further analysis. **(B)** Principal component analysis (PCA) of normalized and batch-corrected read counts based on MNP subset, gender and tissue. The percentage of variance explained by the respective principal component is indicated in parenthesis. **(C)**

Heatmap shows differentially expressed genes in MNPs comparing blood and BAL. Module 1 contains genes with higher and module 2 with lower gene expression in BAL compared to blood. Selected genes are highlighted and annotated to GO terms. **(D)** Validation of DGE genes for CCR6 (HC n=5, non-LS n=17), CCR7 (HC n=6, non-LS n=19) shown as percentage of CM, and PD-L1 (HC n=2, non-LS n=6) shown as median fluorescence intensity (MFI) in $CD14^{+}CD16^{-}$ classical monocytes/monocyte-derived cells from blood and BAL in HC and non-LS patients. Statistical analysis was performed using the non-parametric Mann-Whitney U unpaired t-test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. **(E)** Heatmap shows differentially expressed genes in MNPs comparing HC and non-LS patients. Module 1 contains genes with higher expression and module 2 genes with lower expression in non-LS patients compared to HC. Selected genes are highlighted and annotated to GO terms and KEGG pathways. **(F)** Heatmap shows gene set enrichment analysis (GSEA) of differentially expressed genes with annotated hallmark genes comparing MNP subsets from blood and BAL of non-LS patients with HC.

Figure 3. Monocytes/monocyte-derived cells from BAL of sarcoidosis patients with excessive TNF production without stimulation

(A) Graph shows the normalized relative *TNF* gene count from blood and BAL of MNPs in HC (open bars) and non-LS patients (filled bars). **(B)** Contour plots show intracellular TNF staining in $CD14^{+}CD16^{-}$ classical monocytes/monocyte-derived cells from blood and BAL of two non-LS patients stained *ex vivo* and after 3h culture without stimulation and after adding LPS (1ug/mL) in the presence of Brefeldin A (10ug/mL). **(C)** Bar graphs show the frequency of TNF-expressing $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes/monocyte-derived cells and AM from blood and BAL of HC (blood n=17, BAL n=23), non-LS (blood + BAL n=39) and LS patients (blood n=12, BAL n=14) after 3h culture without stimulation. Bars indicate the median. **(D)** Graphs show percentage of TNF and IL-6 expressing $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells in BAL from non-LS patients after 3h culture without

stimulation at time of diagnosis and after 6 months. Color coding identifies individual patients and lines connect each patient at time of diagnosis and after 6 months. Statistical analyses were performed using the non-parametric Kruskal-Wallis with Dunn's test for correction of multiple comparisons and the non-parametric paired Wilcoxon signed-rank test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Figure 4. High frequencies of monocytes and unstimulated TNF expression indicate disease progression in non-LS sarcoidosis.

(A) Overview of included patients at time of diagnosis and follow up after 2 years with having either remitted disease or developed chronic stable or chronic progressive disease. **(B)** Plots show predictive modelling of the general experimental parameters $Va2.3^+$ T cells in sarcoidosis patients and HLA-DRB1*03 status. Plots show the probability to clear the disease within two years after establishing diagnosis. Dotted lines indicate the 95% confidence interval. **(C + D)** Plot shows predictive modelling of **(C)** cDC2 in BAL and **(D)** $CD14^+CD16^+$ intermediate monocytes in blood of sarcoidosis patients. Dotted lines indicate the 95% confidence interval. **(E + F)** Violin plots show **(E)** TNF expression after 3h culture without stimulation by $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes/monocyte-derived cells, and AMs at time of diagnosis and **(F)** TNF concentrations in plasma and BAL fluid based on the outcome. Statistical analyses were performed using the non-parametric Kruskal-Wallis with Dunn's test for correction of multiple comparisons and predictive modelling using linear regression. *$p < 0.05$. OR: odds ratio.

Figure 1

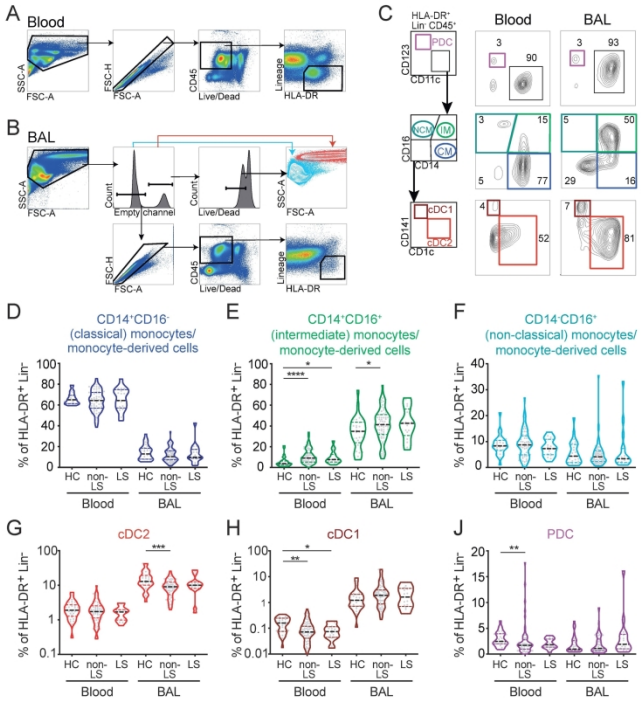

A
Blood
SSC-A
FSC-A
FSC-H
FSC-A
CD45
Live/Dead
Lineage
HLA-DR
B
BAL
SSC-A
FSC-A
Count
Empty channel
Count
Live/Dead
SSC-A
FSC-A
FSC-H
FSC-A
CD45
Live/Dead
Lineage
HLA-DR
C
HLA-DR+ Lin- CD45+
PDC
CD123
CD11c
Blood
BAL
3
90
3
93
NCM
IM
CM
CD16
CD14
3
15
5
77
5
50
29
16
cDC1
cDC2
CD141
CD1c
4
52
7
81
D
CD14+CD16- (classical) monocytes/ monocyte-derived cells
% of HLA-DR+ Lin-
HC
non-LS
LS
Blood
BAL
E
CD14+CD16+ (intermediate) monocytes/ monocyte-derived cells
F
CD14-CD16+ (non-classical) monocytes/ monocyte-derived cells
G
cDC2
H
cDC1
J
PDC

Figure 2

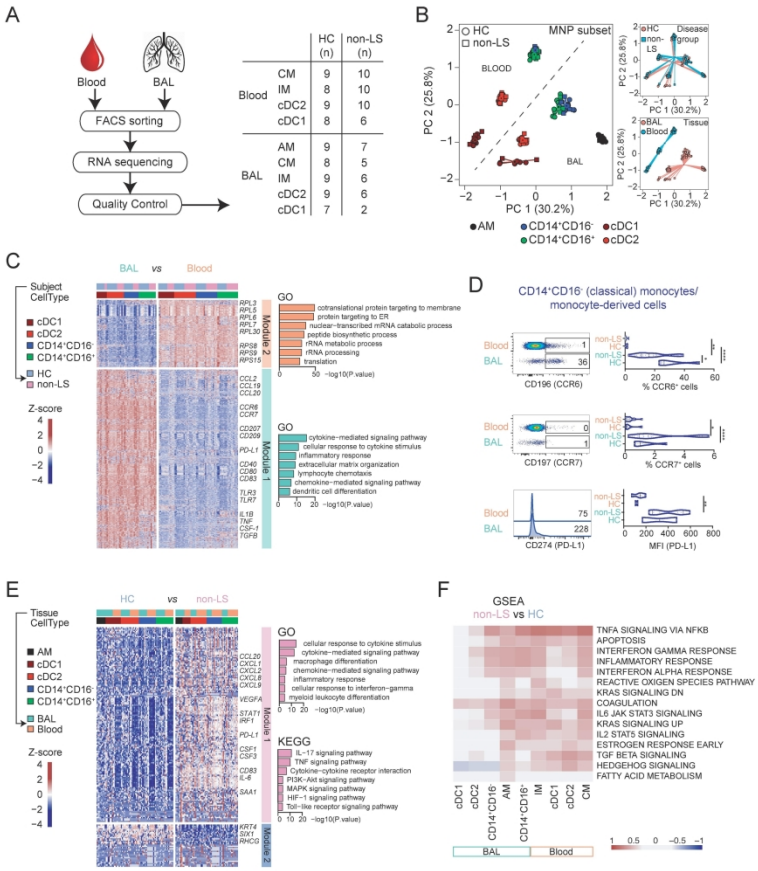

A
Blood
BAL
FACS sorting
RNA sequencing
Quality Control
HC (n)
non-LS (n)
Blood CM 9 10
IM 8 10
cDC2 9 10
cDC1 8 6
BAL AM 9 7
CM 8 5
IM 9 6
cDC2 9 6
cDC1 7 2
B
HC
non-LS
MNP subset
BLOOD
BAL
PC 2 (25.8%)
PC 1 (30.2%)
Disease group
Tissue
AM
CD14+CD16-
CD14+CD16+
cDC1
cDC2
C
BAL vs Blood
Subject
CellType
cDC1
cDC2
CD14+CD16-
CD14+CD16+
HC
non-LS
Z-score
Module 2
Module 1
RPL3 RPL5 RPL6 RPLT RPL30 RPS8 RPS9 RPS15
CCL2 CCL19 CCL20 CCR6 CCR7 CD207 CD209 PD-L1 CD40 CD80 CD83 TLR3 TLR7 IL1B TNF CSF-1 TGFB
GO
cotranslational protein targeting to membrane
protein targeting to ER
nuclear-transcribed mRNA catabolic process
peptide biosynthetic process
rRNA metabolic process
rRNA processing
translation
−log10(P.value)
GO
cytokine-mediated signaling pathway
cellular response to cytokine stimulus
inflammatory response
extracellular matrix organization
lymphocyte chemotaxis
chemokine-mediated signaling pathway
dendritic cell differentiation
−log10(P.value)
D
CD14+CD16- (classical) monocytes/ monocyte-derived cells
Blood
BAL
non-LS
HC
CD196 (CCR6)
% CCR6+ cells
CD197 (CCR7)
% CCR7+ cells
CD274 (PD-L1)
MFI (PD-L1)
E
HC vs non-LS
Tissue
CellType
AM
cDC1
cDC2
CD14+CD16-
CD14+CD16+
BAL
Blood
Z-score
Module 1
Module 2
CCL20 CXCL1 CXCL2 CXCL8 CXCL9 VEGFA STAT1 IRF1 PD-L1 CSF1 CSF3 CD83 IL-6 SAA1 KRT4 SIX1 RNASE3
GO
cellular response to cytokine stimulus
cytokine-mediated signaling pathway
macrophage differentiation
chemokine-mediated signaling pathway
inflammatory response
cellular response to interferon-gamma
myeloid leukocyte differentiation
−log10(P.value)
KEGG
IL-17 signaling pathway
TNF signaling pathway
Cytokine-cytokine receptor interaction
PI3K-Akt signaling pathway
MAPK signaling pathway
HIF-1 signaling pathway
Toll-like receptor signaling pathway
−log10(P.value)
F
GSEA
non-LS vs HC
TNFA SIGNALING VIA NFKB
APOPTOSIS
INTERFERON GAMMA RESPONSE
INFLAMMATORY RESPONSE
INTERFERON ALPHA RESPONSE
REACTIVE OXIGEN SPECIES PATHWAY
KRAS SIGNALING DN
COAGULATION
IL6 JAK STAT3 SIGNALING
KRAS SIGNALING UP
IL2 STAT5 SIGNALING
ESTROGEN RESPONSE EARLY
TGF BETA SIGNALING
HEDGEHOG SIGNALING
FATTY ACID METABOLISM
cDC1
cDC2
CD14+CD16-
AM
CD14+CD16+
IM
cDC1
cDC2
CM
BAL
Blood

Figure 3

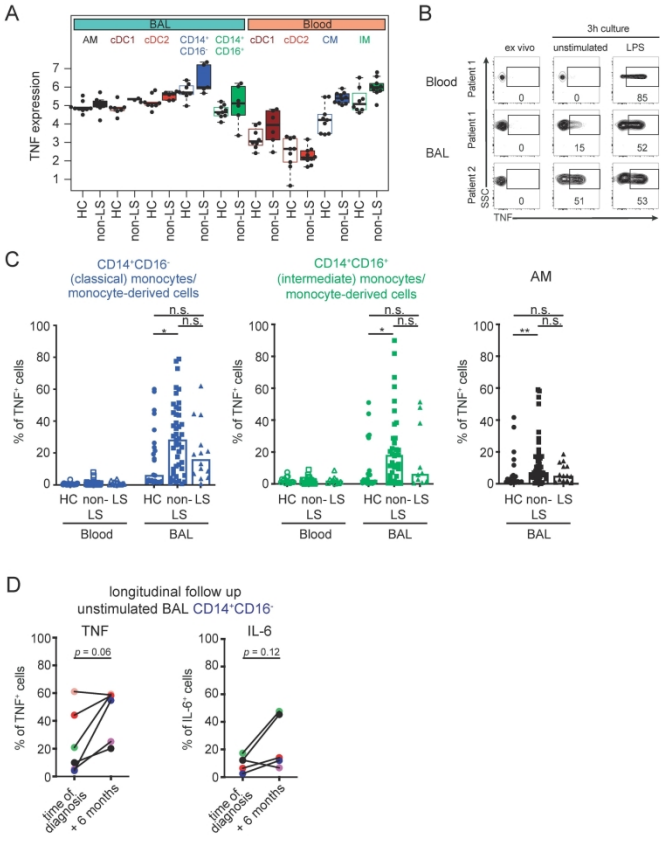
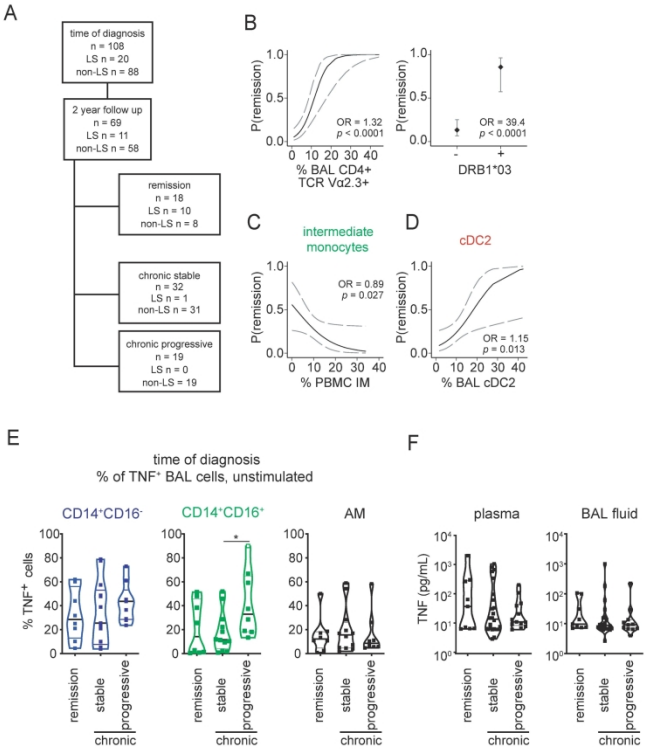

A
BAL
Blood
AM
cDC1
cDC2
CD14+ CD16-
CD14+ CD16+
cDC1
cDC2
CM
IM
TNF expression
HC
non-LS
B
3h culture
ex vivo
unstimulated
LPS
Blood
BAL
Patient 1
Patient 2
SSC
TNF
0
85
15
52
51
53
C
CD14+CD16- (classical) monocytes/ monocyte-derived cells
CD14+CD16+ (intermediate) monocytes/ monocyte-derived cells
AM
% of TNF+ cells
n.s.
*
**
HC
non-LS
Blood
BAL
D
longitudinal follow up
unstimulated BAL CD14+CD16-
TNF
IL-6
p = 0.06
p = 0.12
% of TNF+ cells
% of IL-6+ cells
time of diagnosis
+ 6 months

Figure 4


A
time of diagnosis
n = 108
LS n = 20
non-LS n = 88
2 year follow up
n = 69
LS n = 11
non-LS n = 58
remission
n = 18
LS n = 10
non-LS n = 8
chronic stable
n = 32
LS n = 1
non-LS n = 31
chronic progressive
n = 19
LS n = 0
non-LS = 19
B
P(remission)
OR = 1.32
p < 0.0001
% BAL CD4+ TCR Vα2.3+
OR = 39.4
p < 0.0001
DRB1*03
C
intermediate monocytes
OR = 0.89
p = 0.027
% PBMC IM
D
cDC2
OR = 1.15
p = 0.013
% BAL cDC2

E
time of diagnosis
% of TNF+ BAL cells, unstimulated
CD14+CD16-
CD14+CD16+
AM
% TNF+ cells
remission
stable
progressive
chronic
F
plasma
BAL fluid
TNF (pg/mL)

Table I. Clinical characteristics of healthy controls and sarcoidosis patients at time of diagnosis

		non-Löfgren's syndrome (n=88)	Löfgren's syndrome (n=20)	Healthy controls (n=30)
Sex (male/female)		65/23	16/4	20/10
Age		47 (38-56)	39 (36-44.5)	25 (24-30.5)
Chest radiographic stage (0/I/II/III/IV)		1/18/41/13/2	0/9/10/0/0	N/A
Smoking status (Never/Ex/Smoker)		60/22/5	7/10/3	25/0/5
Extrapulmonary involvement (eye/spleen/kidney/skin)		3/1/1/1	0	N/A
Lung function (% of predicted)	VC	94 (81.5-102) (n = 54)	94 (86-102) (n = 13)	N/A
	DL_{CO}	90 (81-103.5) (n = 49)	93 (87.5-105) (n = 10)	N/A
	FEV1	86 (75.5-99) (n = 53)	87 (81-98.5) (n = 13)	102.6 (93.6-108.5)
BAL fluid characteristics	cell concentration ($x10^6$ cells/L)	155 (114-212) (n = 81)	284 (122-405) (n = 16)	29 (13-81)
	% macrophages	75 (61-83)	80 (60-87)	90 (85-93)
	% T cells	23 (14.5-36.5)	16 (11-36)	9 (5-13)
	% neutrophils	1.45 (0.8-3)	1.8 (1-3)	1.5 (0.6-2.4)
	% eosinophils	0.2 (0-1)	0.7 (0.4-1.6)	0.2 (0-0.6)
	CD4/CD8 ratio	6.1 (3.5-8.7)	6.3 (3.65-7.8)	2.6 (1.7-4.8) (n = 27)
	$V\alpha2.3^+$ $CD4^+$ T cells	4 (3.2-6.3) (n = 81)	16.2 (2.9-29) (n = 16)	N/D
serum ACE		42 (25-60) (n = 80)	45 (33-67) (n = 19)	N/D
serum Albumin		38 (37-41) (n = 86)	40 (37-42) (n = 19)	40 (37-42) (n = 15)
2-year disease outcome (remission/stable/progressive)		8/31/19	10/1/0	N/A

Table I. Clinical characteristics of sarcoidosis patients at time of diagnosis and healthy controls. Data is shown as median ($25\text{-}75^{th}$ percentile). Chest radiography staging defined as follows; Stage I: hilar/mediastinal lymph node enlargement; Stage II: lymph node enlargement and lung parenchyma infiltrates; Stage III: lung parenchyma infiltrates; Stage IV: pulmonary fibrosis. Ex: ex-smoker (considered smoke free for > 1 year); VC: vital capacity; DL_{CO}: diffusing capacity of the lung for carbon monoxide; FEV_1: forced expiratory volume in one second; BAL: bronchoalveolar lavage; ACE: angiotensin-converting enzyme; N/D: not determined; N/A: not applicable.

Table II. Clinical characteristics of a cohort of non-LS patients included in a controlled physical exercise study.

		Exercise cohort* (n=9)	
		TD	TD + 6
Sex (male/female)		7/2	
Age		47.5	
Chest radiographic stage (I/II/III/IV)		1/4/4/0	1/4/4/0
Smoking status (Never/Ex/Smoker)		8/1/0	
Lung function (% of predicted)	TLC	88	89
	DL_{CO}	98	97
	FEV1	90	87
BAL fluid characteristics	cell concentration ($x10^6$ cells/L)	152	215
	% macrophages	63	77
	% T cells	33	20
	% neutrophils	3.1	1.8
	% eosinophils	0.7	0.6
	CD4/CD8 ratio	6.8	7.6

Table II. Clinical characteristics of patients included in the *exercise study [27]. TD: time of diagnosis, TD+6: 6 months after time of diagnosis. Data is shown as median at the two sampling time points. For chest radiography staging and abbreviations see Table I.

Monocytes in sarcoidosis are potent TNF producers and predict disease outcome

Rico Lepzien, Sang Liu, Paulo Czarnewski, Mu Nie, Björn Österberg, Faezzah Baharom, Jamshid Pourazar, Gregory Rankin, Anders Eklund, Matteo Bottai, Susanna Kullberg, Anders Blomberg, Johan Grunewald, and Anna Smed-Sörensen

ONLINE DATA SUPPLEMENT

Methods Supplement

RNA isolation and sequencing

RNA was isolated using the miRNeasy micro kit (Qiagen) according to the manufacturer's protocol. Briefly, chloroform was added to cell homogenates and centrifuged. The aqueous phase was mixed with 1.5 volumes of 100% ethanol. The samples were transferred to a RNeasy MinElute spin column and centrifuged. After, the column was washed with RPE buffer and spun down followed by addition of 80% ethanol and centrifugation. RNA was eluted in a collection tube using RNAse-free water. RNA quantity and quality were assessed using a bioanalyzer and samples with an RNA integrity number > 6 used for further application. Library preparation was performed by Novogene, using TruSeq Stranded mRNA Library Prep Kit (poly-A selection*)* when the total RNA amount was more than 100ng, if the sample total RNA amount was lower than 100ng, the library preparation was done with SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech). Sequencing was performed using a HiSeq-4000 platform (Illumina) with a 2x150-bp pair-end sequencing setup and the sequencing depth was 20 M reads/sample.

RNA-seq data analysis

Pair-ended reads were evaluated for sequencing quality using FASTQC, were trimmed for adaptor sequences and aligned using TopHat2 [1] with HGC38 human genome. Further analysis was then performed in R. Counts were summed up to the gene level and focusing the analysis of protein-coding genes using Biomart's [2] transcript annotation from Ensembl. Genes detected in fewer than 2 samples with counts per million (CPM) values lower than 1 were considered lowly expressed and

removed from the analysis. Unsupervised analysis and visualization of results were done using principal component analysis (PCA) on scaled and centered gene expression and agglomerative hierarchical clustering (HC) using Pearson correlation as distance matrix and “ward.D2” as linkage method. We observed that the library preparation method used for RNAseq influenced sample-wise relative log gene expression (Figure S3A), resulting in a general shift in gene expression as samples separated in PC1 by the library preparation method (Figure S3B). This was corrected using ComBat from sva package [3] preserving sorted cell grouping. Differential expression using EdgeR package [4] was done in several occasions depending on the comparison in question. A model depicting library preparation method, gender, disease group, tissue source and cell type was done to evaluate overall trends in gene expression (y) across those conditions:

$$y \sim LibraryPrep + Gender + DiseaseGroup + TissueSource + CellType \quad \text{(EQ.1)}$$

At a second instance, pair-wise comparisons in EdgeR were done to individually test the effect of disease status (non-LS versus HC) on each cell type per tissue (EQ.2):

$$y \sim DiseaseGroup \quad \text{(EQ.2)}$$

Similarly, pair-wise comparisons were done to test the effect of the tissue (blood vs. lung) on each cell type per disease status (EQ.3):

$$y \sim TissueSource \quad \text{(EQ.3)}$$

In all those comparisons above (EQ.1, 2 and 3), genes with fold change (FC) above 2 and false discovery rate (FDR) below 1e-4 were considered significant.

Functional analysis of differentially expressed genes (sorted by fold change) was done with EnrichR package [5] for gene list hypergeometric test (results from EQ.1) or with gene set enrichment analysis (GSEA) [6] using the fGSEA package [7] for each of the pair-wise comparisons described above (results from EQ.2 and EQ.3). The Gene

Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the list of hallmark gene set from the Molecular Signatures Database (MSigDB) were used for enrichment testing.

ELISA

For detection of TNF, IL-6, and IL-1β in plasma and BAL fluid, the human TNF, IL-6, and IL-1β DuoSet ELISA (all R&D Systems) were used according to the manufacturer's instructions. Briefly, plates were coated with capture antibodies and incubated overnight. Plates were washed 4 times with wash buffer (PBS + 0.05% Tween 20) followed by blocking with reagent diluent for one hour at room temperature (RT). Subsequently, undiluted samples and serial dilution of standard were added and incubated for 2h at RT. Prior to the incubation, BAL fluid was concentrated 20x using Amicon Ultra-4 Centrifugal Filter Units (Merck). Next, the detection antibody was added and incubated for 2h at RT followed by incubation with Streptavidin-HRP for 20 min at RT and color reagent A + B (mixed 1:1) for 20 min at RT before adding stop solution and analysis on an ELISA reader. OD values were measured at 450nm and concentrations interpolated from the standard curve.

References

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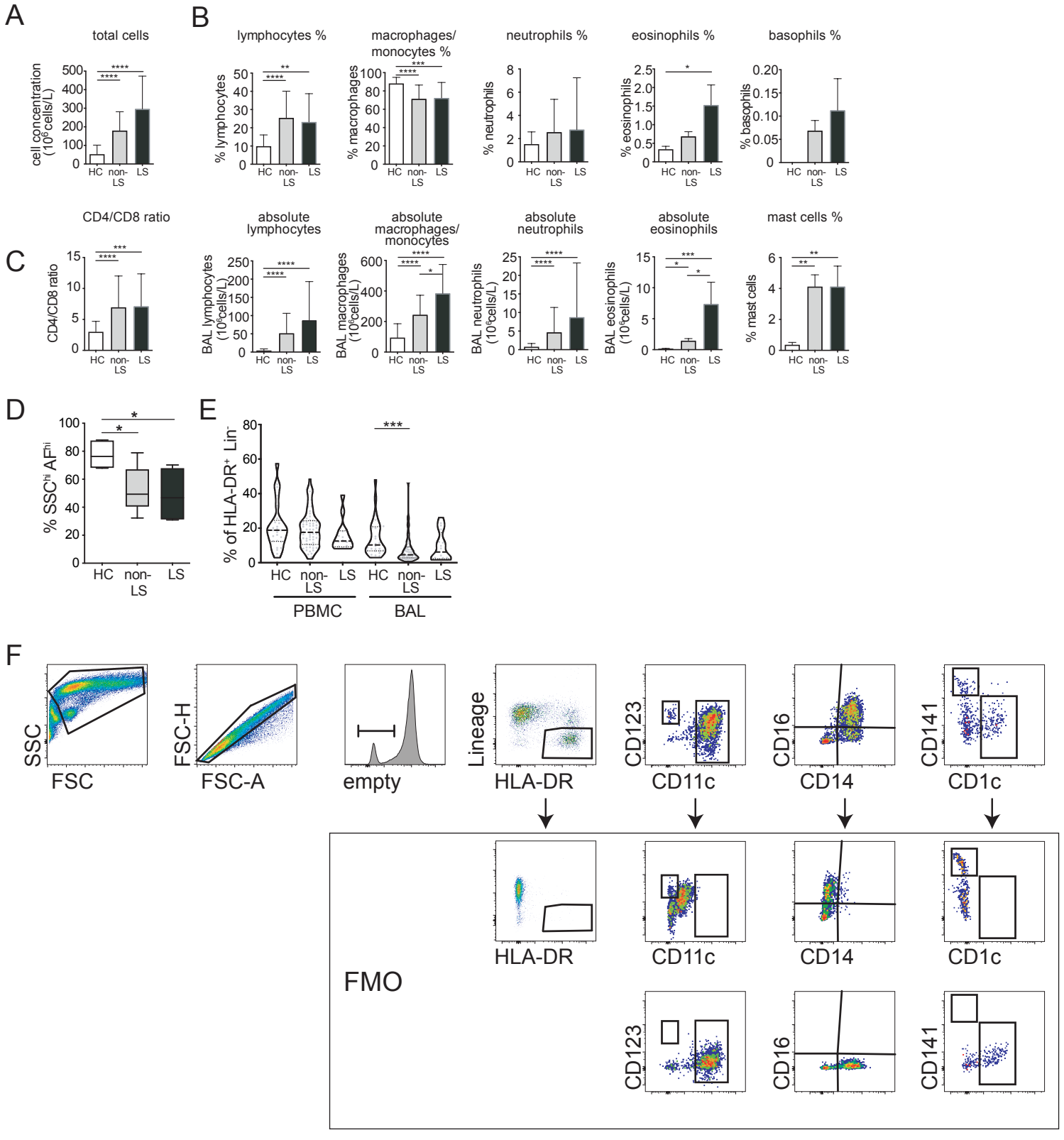
3. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics (Oxford, England)* 2012: 28(6): 882-883.
4. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* 2010: 26(1): 139-140.
5. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, Koplev S, Jenkins SL, Jagodnik KM, Lachmann A, McDermott MG, Monteiro CD, Gundersen GW, Ma'ayan A. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic acids research* 2016: 44(W1): W90-97.
6. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 2005: 102(43): 15545-15550.
7. Korotkevich G, Sukhov V, Sergushichev A. Fast gene set enrichment analysis. *bioRxiv* 2019: 060012.

Supplementary table I. Antibodies used for surface and intracellular staining.

Antibodies	**Source**	**Identifier**
Surface antibodies		
anti-CD1c FITC human (clone AD5-8E7)	Miltenyi	130-090-507
anti-CD1c PE-Vio770 human (clone AD5-8E7)	Miltenyi	130-110-538
anti-CD3 APC-Cy7 human (clone SK7)	BD	341090
anti-CD3 FITC human (clone UCHT1)	BD	555332
anti-CD11c APC human (clone S-HCL-3)	BD	333144
anti-CD11c V450 human (clone B-Ly6)	BD	560369
anti-CD14 BV510 human (clone M5E2)	BioLegend	301842
anti-CD14 PE human (clone MoP9)	BD	359412
anti-CD16 AF700 human (clone 3G8)	BioLegend	302026
anti-CD19 APC-Cy7 human (clone HIB19)	BioLegend	302218
anti-CD19 FITC human (clone HIB19)	BioLegend	302205
anti-CD20 APC-Cy7 human (clone L27)	BD	335829
anti-CD20 FITC human (clone 2H7)	BD	555622
anti-CD45 APC human (clone HI30)	BD	555485
anti-CD56 APC-Cy7 human (clone HCD56)	BioLegend	318332
anti-CD56 FITC human (clone HCD56)	BioLegend	318304
anti-CD66abce APC-Vio770 human (clone TET2)	Miltenyi	130-119-847
anti-CD66abce FITC human (clone TET2)	Miltenyi	130-116-522
anti-CD86 BV650 human (clone FUN-1)	BD	563412
anti-CD123 PerCP-Cy5.5 human (clone 7G3)	BD	558714
anti-CD141 PE human (clone AD5-14H12)	Miltenyi	130-113-318
anti-CD141 VioBlue human (clone AD5-14H12)	Miltenyi	130-113-320
anti-HLA-DR APC-Cy7 human (clone L243)	BD	335831
anti-HLA-DR PE-Cy5 human (clone G46-6)	BD	555813
anti-HLA-DR PE-TR human (clone TU36)	Life Technologies	MHLDR17
Intracellular antibodies		
anti-IL-6 FITC human (clone MQ2-13A5)	BioLegend	551104
anti-TNF APC human (clone MAb11)	BD	554514

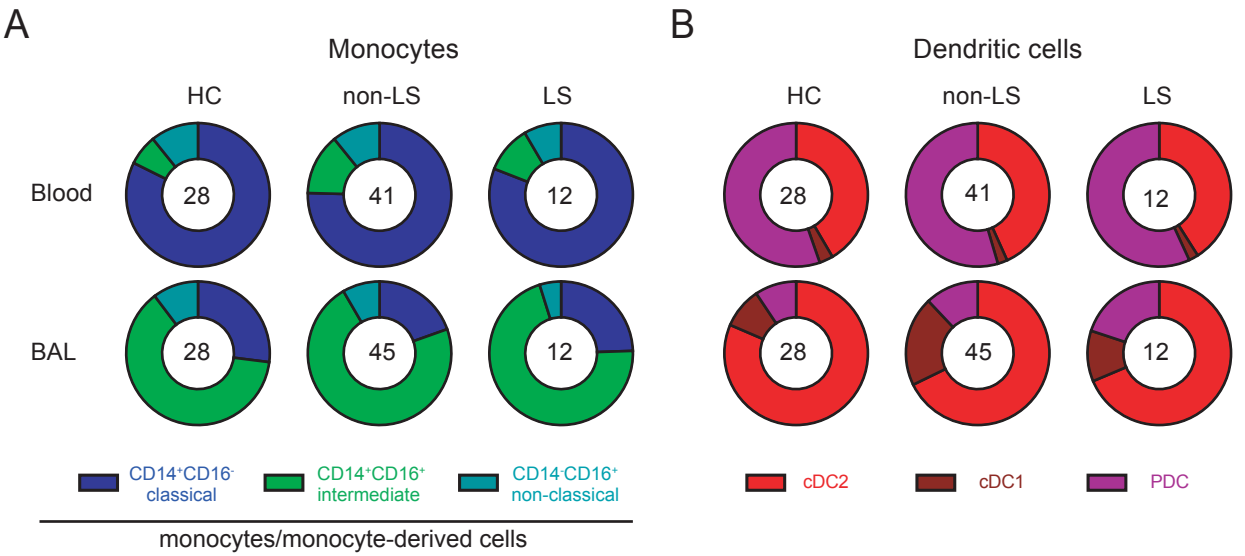
Figure S1

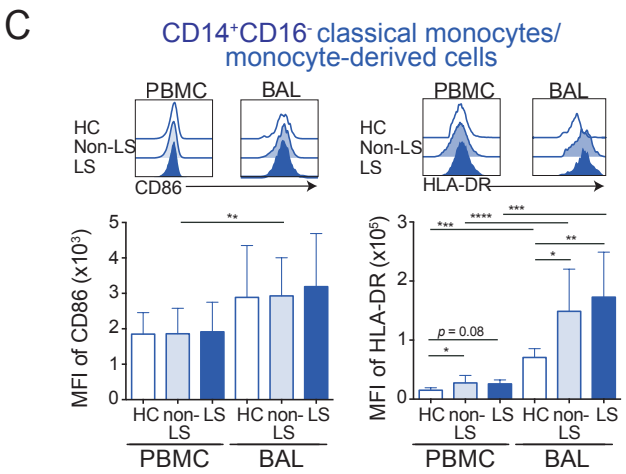

A
total cells
cell concentration (10^6cells/L)
B
lymphocytes %
% lymphocytes
macrophages/ monocytes %
% macrophages
neutrophils %
% neutrophils
eosinophils %
% eosinophils
basophils %
% basophils
CD4/CD8 ratio
absolute lymphocytes
BAL lymphocytes (10^6cells/L)
absolute macrophages/ monocytes
BAL macrophages (10^6cells/L)
absolute neutrophils
BAL neutrophils (10^6cells/L)
absolute eosinophils
BAL eosinophils (10^6cells/L)
mast cells %
% mast cells
C
CD4/CD8 ratio
D
% SSC^hi AF^hi
E
% of HLA-DR+ Lin-
PBMC
BAL
HC
non-LS
LS
F
SSC
FSC
FSC-H
FSC-A
empty
Lineage
HLA-DR
CD123
CD11c
CD16
CD14
CD141
CD1c
FMO

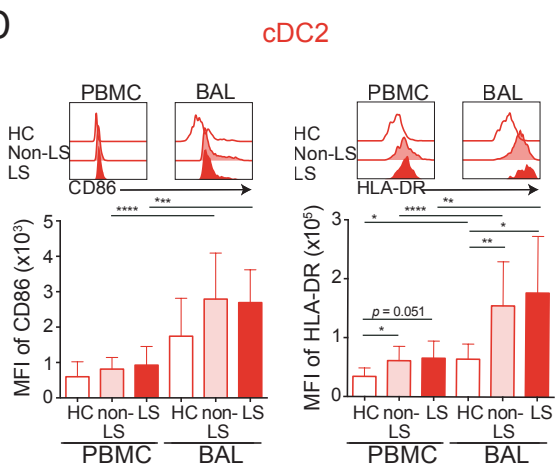
Supplementary figure 1. Cellular composition of BAL

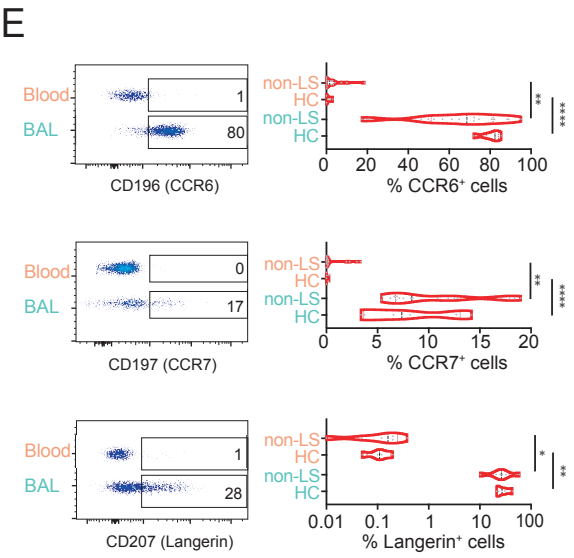
(A) Bar graph shows total BAL cells as concentration per litre. Graphs show mean ± SD. **(B)** Differential count of BAL cells from HC (n=30), non-LS (n=88) and LS patients (n=20). Graphs show the percentage (upper row) as well as the absolute numbers (lower row) of lymphocytes, macrophages/monocyte-derived cells, neutrophils, eosinophils, and basophils. Graphs show mean ± SD. **(C)** CD4/CD8 ratio in BAL as assessed by flow cytometry and staining against surface antigens to identify CD4 and CD8 positive. Graph shows the CD4/CD8 ratio in BAL as mean ± SD. HC n=30, non-LS n=81, and LS patients n=16. **(D)** Bar graphs show the percentage of alveolar macrophages as assessed by flow cytometry. SSC^{hi} and autofluorescence qualified for identification (see gating strategy figure 1B). Box plots show median ± IQR. HC n=6, non-LS n=17, and LS patients n=5. **(E)** Violin plot shows the frequency of HLA-DR+ lineage negative MNPs in blood and BAL (see gating strategy figure 1B). Dotted lines indicate median and 25th and 75th percentile. HC n=28, non-LS n=76, and LS patients n=16. Statistical analysis was performed using the non-parametric Kruskal-Wallis with Dunn´s test for correction of multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(F)** Fluorescence minus one (FMO) controls for HLA-DR, CD123, CD11c, CD14, CD16, CD1c, and CD141.

Figure S2


A
Monocytes
HC
non-LS
LS
Blood
BAL
28
41
12
28
45
12
CD14+CD16- classical
CD14+CD16+ intermediate
CD14-CD16+ non-classical
monocytes/monocyte-derived cells
B
Dendritic cells
HC
non-LS
LS
28
41
12
28
45
12
cDC2
cDC1
PDC


C
CD14+CD16- classical monocytes/ monocyte-derived cells
PBMC
BAL
HC
Non-LS
LS
CD86
HLA-DR
MFI of CD86 (x10³)
MFI of HLA-DR (x10⁵)
p = 0.08
HC non- LS
LS
PBMC
BAL


D
cDC2
PBMC
BAL
HC
Non-LS
LS
CD86
HLA-DR
MFI of CD86 (x10³)
MFI of HLA-DR (x10⁵)
p = 0.051
HC non- LS
LS
PBMC
BAL

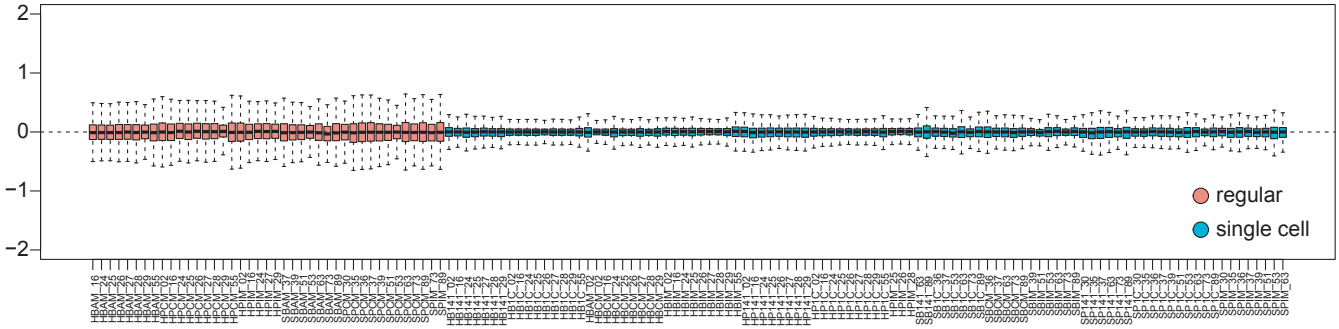

E
Blood
BAL
1
80
CD196 (CCR6)
non-LS
HC
% CCR6⁺ cells
0
17
CD197 (CCR7)
% CCR7⁺ cells
1
28
CD207 (Langerin)
% Langerin⁺ cells

Supplementary figure 2. MNP frequencies and maturation differ between blood and BAL.

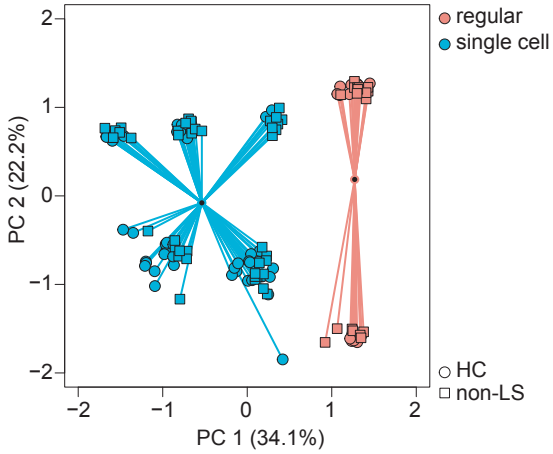
(A + B) Pie charts show the summary in distribution of different **(A)** monocytes/monocyte-derived cells and **(B)** DC subsets in blood and BAL of HC, non-LS and LS patients. Numbers inside the pie chart indicate the number of subjects. **(C + D)** Maturation of **(C)** $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells and **(D)** cDC2 were assessed by measuring the geometric mean fluorescence intensity of HLA-DR and CD86 in cells from blood and BAL of HC, non-LS and LS patients. Histogram show a representative example of HLA-DR and CD86 expression from a HC, non-LS patient and LS patient. Bar graphs show the summary as mean ± SD. Statistical analysis was performed using the non-parametric Kruskal-Wallis with Dunn´s test for correction of multiple comparisons. **(E)** Validation of differentially expressed genes for CCR6 (HC n=5, non-LS n=17), CCR7 (HC n=6, non-LS n=19), and CD207 (Langerin) (HC n=2, non-LS n=6) shown as an example in cDC2 from blood and BAL in HC and non-LS patients. Dotted lines indicate median, 25th and 75th percentile. Statistical analysis was performed using the non-parametric Mann-Whitney U unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$.

Figure S3

A


2
1
0
−1
−2
regular
single cell

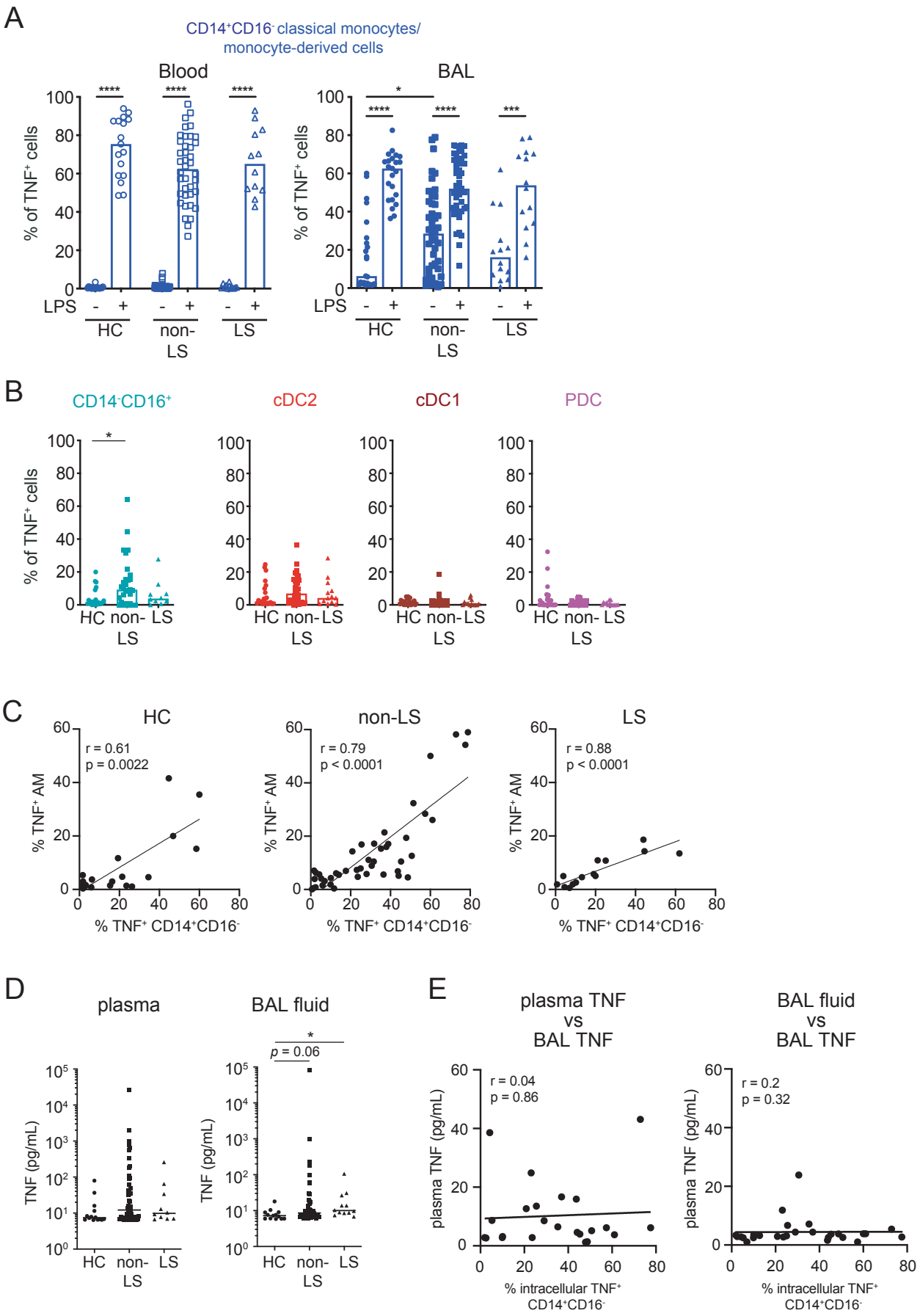
B


2
1
0
−1
−2
PC 2 (22.2%)
−2
−1
0
1
2
PC 1 (34.1%)
regular
single cell
HC
non-LS

Supplementary figure 3. Impact of library preparation on gene expression.

(A) Relative log expression (RLE) plot for individual samples, grouped by library preparation method. **(B)** Principal component analysis of uncorrected normalised RNA-seq data, coloured and grouped by library preparation method.

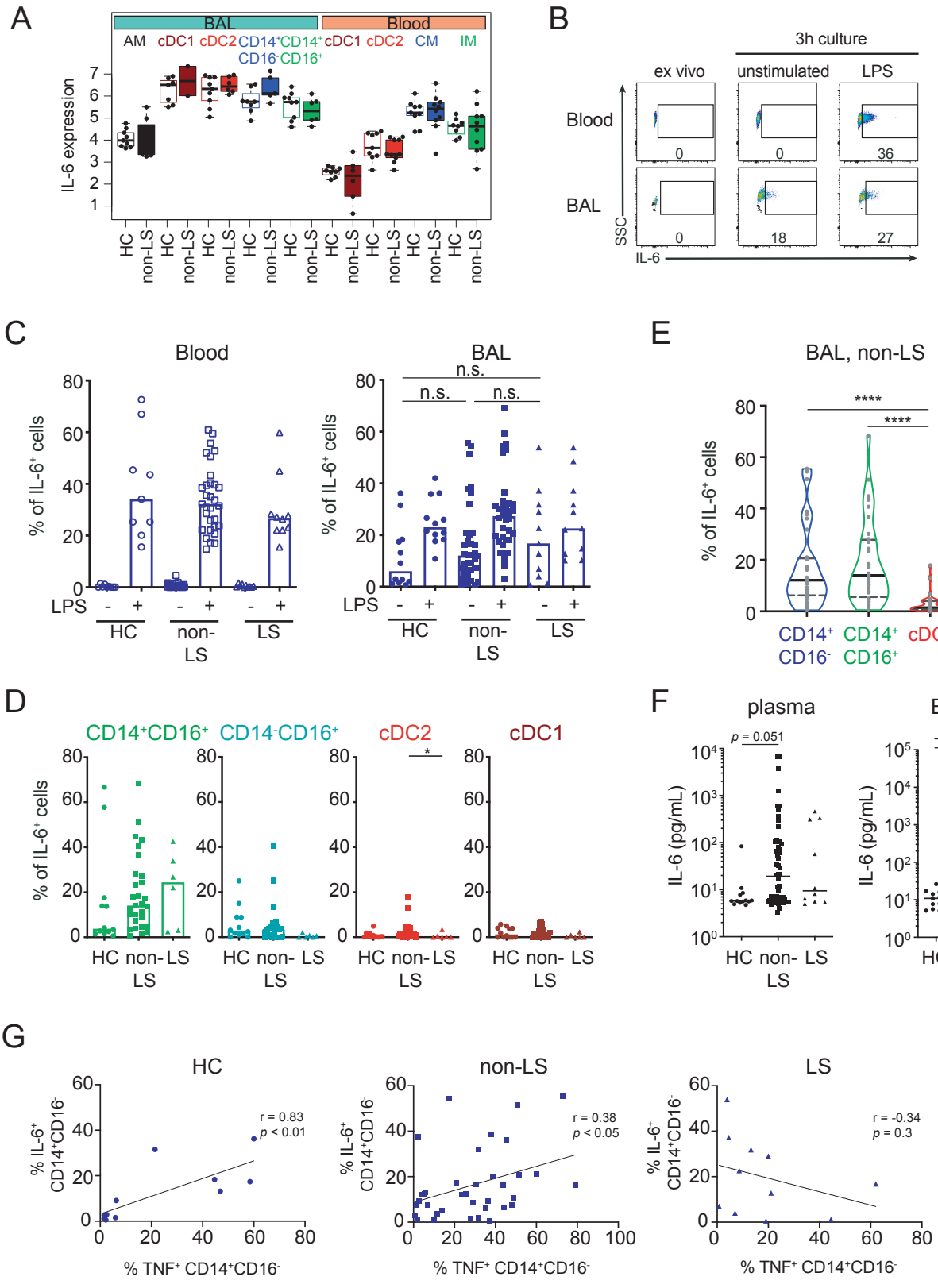
Figure S4


A
CD14+CD16- classical monocytes/
monocyte-derived cells
Blood
BAL
% of TNF+ cells
LPS
HC
non-LS
LS
B
CD14-CD16+
cDC2
cDC1
PDC
C
HC
non-LS
LS
r = 0.61
p = 0.0022
r = 0.79
p < 0.0001
r = 0.88
p < 0.0001
% TNF+ AM
% TNF+ CD14+CD16-
D
plasma
BAL fluid
p = 0.06
TNF (pg/mL)
E
plasma TNF
vs
BAL TNF
BAL fluid
vs
BAL TNF
r = 0.04
p = 0.86
r = 0.2
p = 0.32
plasma TNF (pg/mL)
% intracellular TNF+
CD14+CD16-

Supplementary figure 4. Intracellular TNF expression in BAL MNPs do not correlate with soluble TNF in plasma or BAL fluid.

(A) Bar graphs show the frequency of TNF-expressing $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells from blood (open symbols) and BAL (filled symbols) of HC (circles), non-LS (squares) and LS patients (triangles) after 3h culture with and without LPS stimulation. Bars indicate the median. **(B)** BAL MNPs from HC, non-LS and LS patients were cultured for 3 hours in the presence of BFA and TNF expression determined by intracellular staining and analysis by flow cytometry. Bar graphs show the median. **(C)** Correlation of intracellular TNF expression in AM and $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells after 3h culture without stimulation in HC, non-LS and LS patients. r shows Spearman's correlation coefficient. **(D)** TNF concentration in plasma and BAL fluid of HC, non-LS and LS patients was measured using ELISA. BAL fluid was concentrated 20x prior to the ELISA. Graphs show individual values and line indicated the median. **(E)** Correlation of intracellular TNF expression by BAL $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells after 3h culture without stimulation and concentrations of soluble TNF in plasma or BAL fluid. r shows Spearman's correlation coefficient. Statistical analysis was performed using the non-parametric Kruskal-Wallis with Dunn´s test for correction of multiple comparisons. For correlation analysis the Spearman's rank test was used. $*p < 0.05$, $**p < 0.01$.

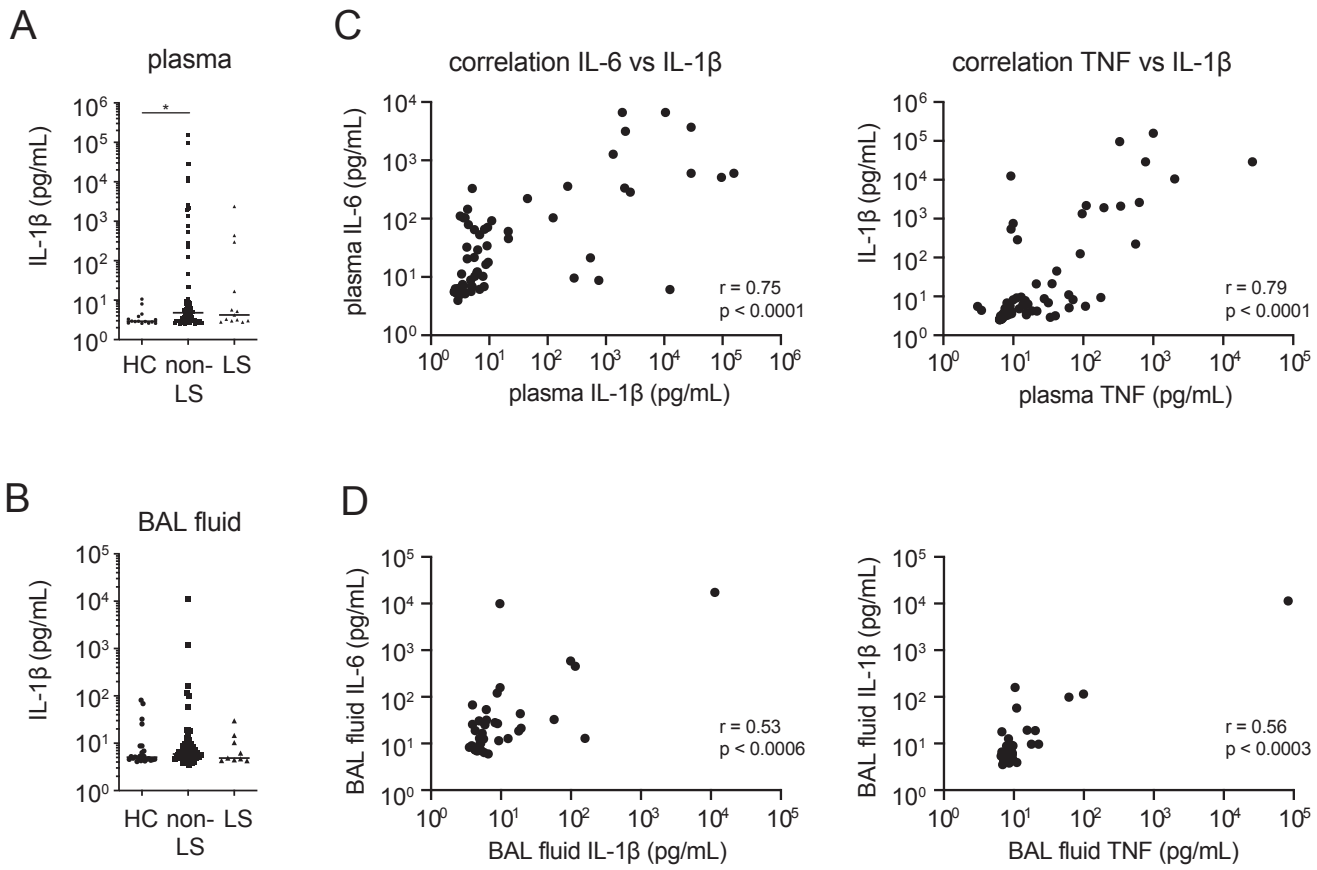
Figure S5


A
BAL
Blood
AM
cDC1
cDC2
CD14+CD14+ CD16- CD16+
cDC1
cDC2
CM
IM
IL-6 expression
HC
non-LS
B
3h culture
ex vivo
unstimulated
LPS
Blood
BAL
SSC
IL-6
0
0
36
0
18
27
C
Blood
BAL
% of IL-6+ cells
LPS
HC
non-LS
LS
n.s.
n.s.
n.s.
E
BAL, non-LS
****
****
% of IL-6+ cells
CD14+ CD16-
CD14+ CD16+
cDC2
D
CD14+CD16+
CD14-CD16+
cDC2
cDC1
% of IL-6+ cells
*
HC
non-LS
LS
F
plasma
BAL fluid
p = 0.051
*
**
IL-6 (pg/mL)
HC
non-LS
LS
G
HC
non-LS
LS
% IL-6+ CD14+CD16-
r = 0.83
p < 0.01
r = 0.38
p < 0.05
r = -0.34
p = 0.3
% TNF+ CD14+CD16-

Supplementary figure 5. IL-6 expression by unstimulated pulmonary monocytes in sarcoidosis patients.

(A) Graph shows the relative IL-6 gene expression count from blood and BAL of the individual subsets in HC (open bars) and non-LS patients (filled bars). **(B)** Pseudocolour plots show IL-6 expression in $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells from blood and BAL of one representative non-LS patients stained *ex vivo* and after 3h culture without stimulation and after adding LPS (1ug/mL) in the presence of Brefeldin A (10ug/mL). **(C)** Bar graphs show the summary as frequency of IL-6 expression in $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells from blood and BAL of HC (circles, n=12), non-LS (squares, n=36) and LS patients (triangles, n=11) after 3h culture with and without LPS stimulation. Bars indicate the median. **(D)** BAL MNPs from HC, non-LS and LS patients were cultured for 3 hours in the presence of BFA and IL-6 expression determined by intracellular staining and analysis by flow cytometry. Bar graphs show the median. **(E)** Violin plots show a summary of IL-6 expression by BAL $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes/monocyte-derived cells and cDC2 from non-LS patients cultured for 3h without stimulation. Black lines indicate the median, 25^{th} and 75^{th} percentile. **(F)** IL-6 concentrations in plasma and BAL fluid of LS patients (plasma n=10, BAL fluid n=12), non-LS patients (plasma n=56, BAL fluid n=52) and HC (plasma and BAL fluid n=13) was assessed using ELISA. BAL fluid was 20x concentrated prior to the ELISA. **(G)** Graphs show the correlation of unstimulated TNF and IL-6 expression in $CD14^{+}CD16^{-}$ monocytes/monocyte-derived cells from BAL of HC (n=12), non-LS (n=11), and LS patients (n=36). r indicates Spearman's correlation coefficient. Statistical analyses were performed using the non-parametric Kruskal-Wallis test with Dunn's test for correction of multiple comparisons and Spearman's correlation analysis. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

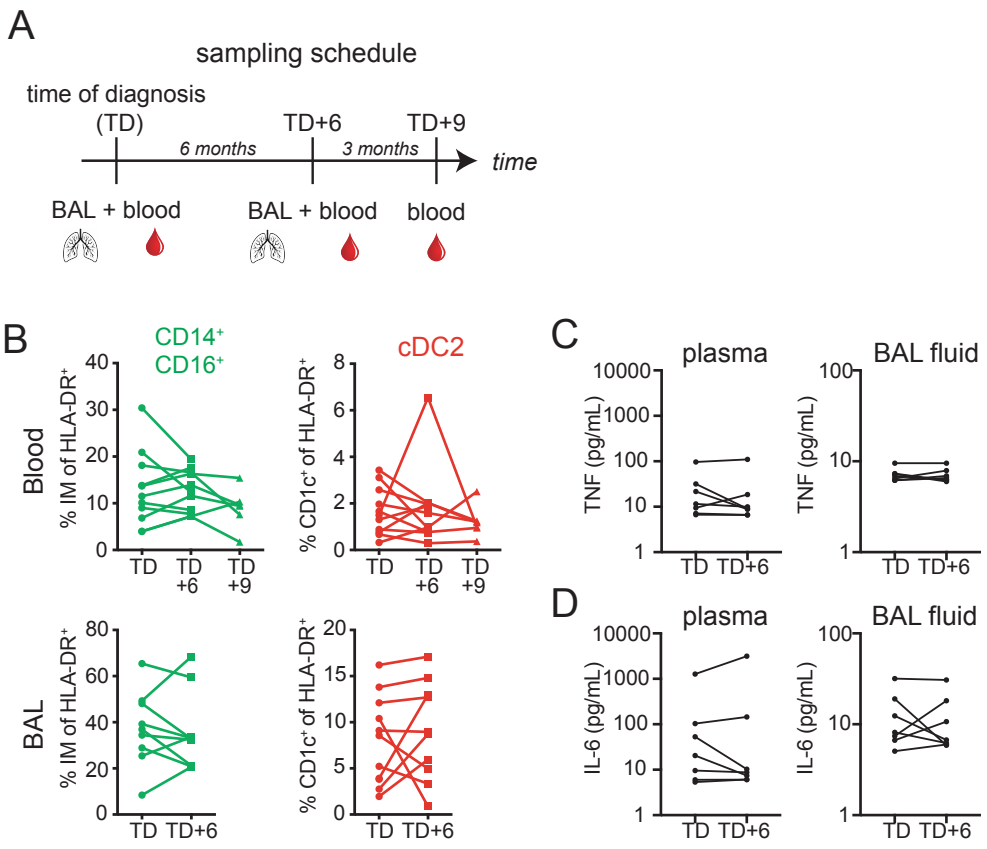
Figure S6


A
plasma
IL-1β (pg/mL)
HC
non-LS
LS
*
C
correlation IL-6 vs IL-1β
plasma IL-6 (pg/mL)
plasma IL-1β (pg/mL)
r = 0.75
p < 0.0001
correlation TNF vs IL-1β
IL-1β (pg/mL)
plasma TNF (pg/mL)
r = 0.79
p < 0.0001
B
BAL fluid
IL-1β (pg/mL)
HC
non-LS
LS
D
BAL fluid IL-6 (pg/mL)
BAL fluid IL-1β (pg/mL)
r = 0.53
p < 0.0006
BAL fluid IL-1β (pg/mL)
BAL fluid TNF (pg/mL)
r = 0.56
p < 0.0003

Supplementary figure 6. IL-1β expression in plasma and BAL fluid.

(A + B) IL-1β concentrations in serum and BAL fluid of healthy controls (HC), non-LS and LS patients. **(C + D)** Corelation of IL-1β and IL-6 or TNF concentration in plasma or BAL fluid of non-LS patients. HC: plasma n=15, BAL fluid n=24; non-LS: plasma n= 81, BAL fluid n=56; LS: plasma n=13, BAL fluid n=9. Statistical analysis was performed using the non-parametric Kruskal-Wallis test with Dunn's test for correction of multiple comparisons. *$p < 0.05$.

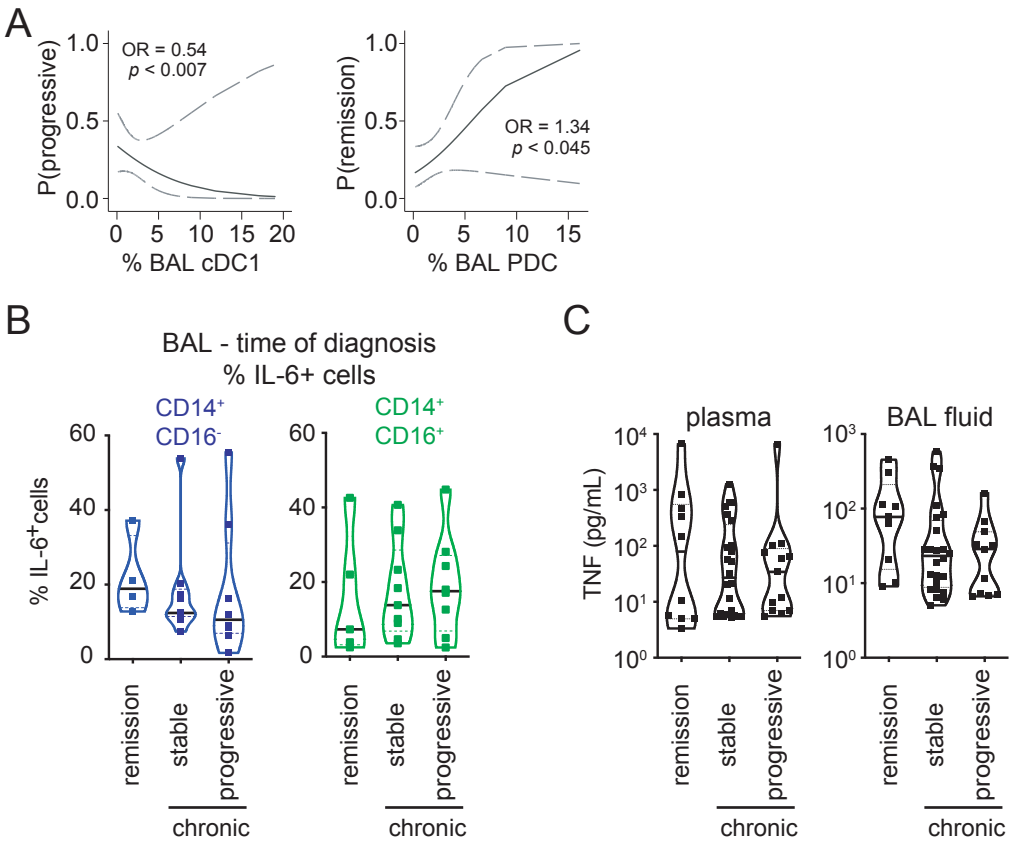
Figure S7


A
sampling schedule
time of diagnosis
(TD)
TD+6
TD+9
6 months
3 months
time
BAL + blood
BAL + blood
blood
B
CD14+
CD16+
cDC2
Blood
BAL
% IM of HLA-DR+
% CD1c+ of HLA-DR+
TD
TD+6
TD+9
C
plasma
BAL fluid
TNF (pg/mL)
D
IL-6 (pg/mL)

Supplementary figure 7. Frequencies of MNPs over time.

(A) A cohort of non-LS patients was included in a separate study that required the patients to undergo a first bronchoscopy at time of diagnosis (TD) to collect blood and BAL. A second bronchoscopy was performed 6 months later (TD + 6) to collect blood and BAL and a third time point another 3 months later (TD + 9) to collect blood only. **(B)** Frequencies of $CD14^{+}CD16^{+}$ monocytes/monocyte-derived cells and cDC2 in non-LS patients over the time course of 9 months in blood (upper row) and BAL (lower row). Lines connect individual patients. **(C + D)** Graphs show the concentrations of **(C)** TNF and **(D)** IL-6 measured in plasma and BAL fluid of the patients at time of diagnosis and after 6 months. Lines connect each individual. Statistical analyses were performed using the non-parametric paired Wilcoxon signed-rank test.

Figure S8


A
P(progressive)
OR = 0.54
p < 0.007
% BAL cDC1
P(remission)
OR = 1.34
p < 0.045
% BAL PDC
B
BAL - time of diagnosis
% IL-6+ cells
CD14+ CD16-
CD14+ CD16+
% IL-6+cells
remission
stable
progressive
chronic
C
plasma
BAL fluid
TNF (pg/mL)
remission
stable
progressive
chronic

Supplementary figure 8. DCs are predictors of disease severity in sarcoidosis.

(A) Plots show predictive modelling of frequencies of cDC1 and PDCs in BAL of sarcoidosis patients at time of diagnosis and probability for disease outcome. **(B + C)** Violin plots show **(B)** TNF expression after 3h culture without stimulation by $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes/monocyte-derived cells and AM at time of diagnosis and **(C)** TNF concentrations in plasma and BAL fluid and were grouped based on the outcome. Statistical analyses were performed using the non-parametric Kruskal-Wallis with Dunn's test for correction of multiple comparisons and predictive modelling using linear regression. *$p < 0.05$. OR: odds ratio.