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ORIGINAL PAPER

The inflammatory fingerprint reveals immune cell populations associated with disease activity in cardiac sarcoidosis

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Abstract

Objective Sarcoidosis is a systemic granulomatous inflammatory disease and patients with cardiac involvement are at increased risk of adverse events. Pathophysiologic processes leading to myocardial inflammation and fibrosis are yet to be determined. Therefore, characterization of the immune response leading to enhanced disease activity and portending poor prognosis of patients with cardiac sarcoidosis (CS) is crucial.

Methods Twenty-six patients with biopsy-proven sarcoidosis were prospectively enrolled for evaluation of suspected CS and disease activity was determined by hybrid cardiac PET/MR imaging. We then analyzed the peripheral blood of individuals with active CS (aCS), chronic CS (cCS), extracardiac sarcoidosis (noCS), and healthy controls using a 36-color spectral flow cytometry and immunoassay panel.

Results Analysis of the inflammatory fingerprint in patients with CS uncovered 56 characteristic immune cell populations. Immunophenotyping of the inflammatory cells revealed distinctive differences between healthy individuals and patients with sarcoidosis. Further, the abundance of the cell populations was associated with cardiac manifestation and disease activity. A critical shift of lymphocytes, innate immune cells, and monocyte subsets occurred in patients with CS compared to extracardiac sarcoidosis and healthy individuals. In addition, cytokine/chemokine expression was aberrant in patients with CS and may contribute to the cardiac pathophysiology of sarcoidosis.

Conclusions Comprehensive characterization of the inflammatory fingerprint reveals changes in frequency and phenotype of several immune cell populations associated with cardiac sarcoidosis. Our results may add further knowledge to the pathophysiology of cardiac sarcoidosis, allowing a better stratification of patients with high disease activity who seem to benefit most from immunosuppressive therapy.

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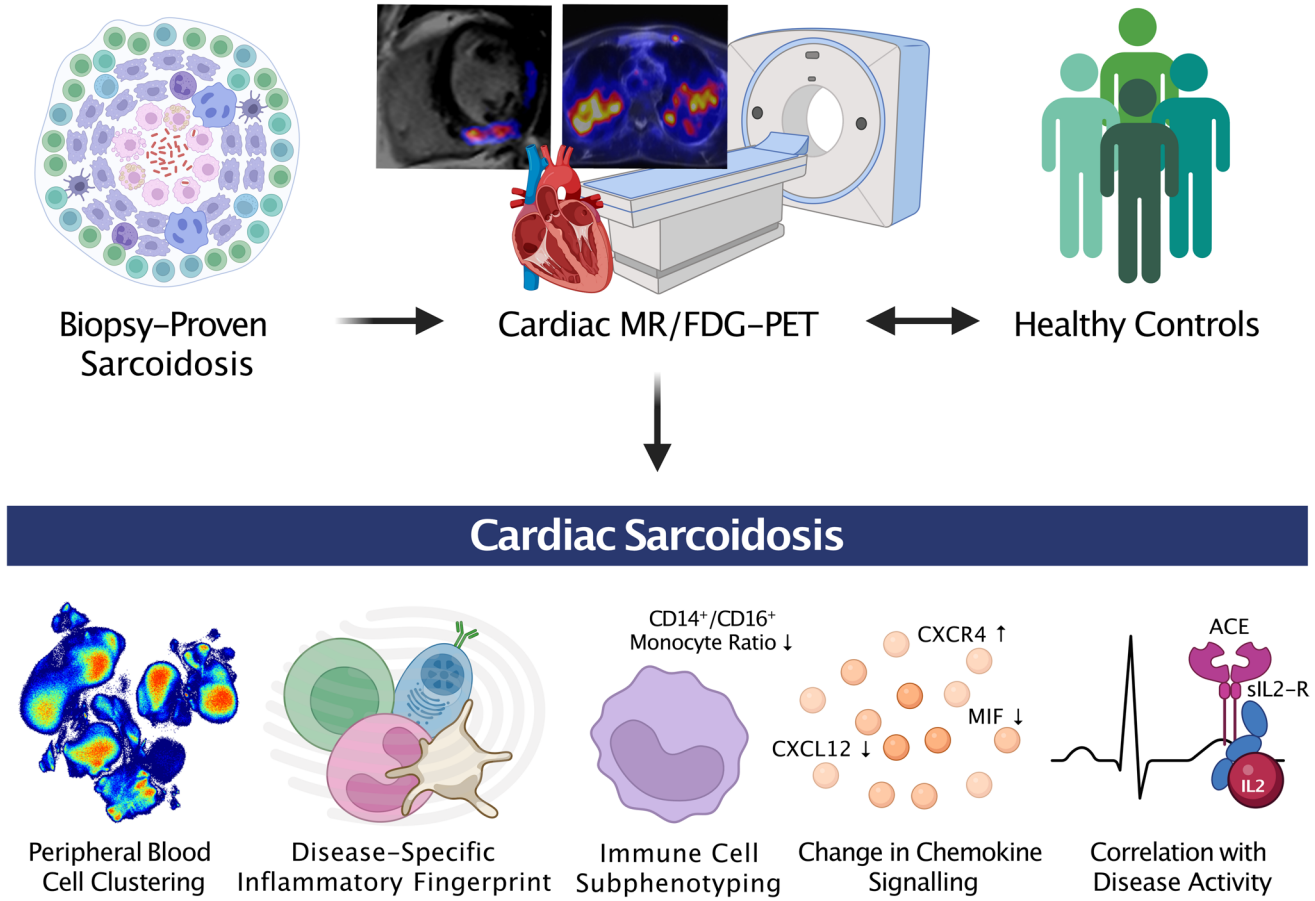
Graphical Abstract



Keywords Cardiac sarcoidosis · Immunophenotyping · Spectral flow cytometry · Inflammation · Immune cells

Introduction

Sarcoidosis is a systemic granulomatous disorder of idiopathic origin with a heterogenous variety of clinical symptoms [1]. The inflammatory disease commonly affects thoracic organs including the heart [2, 3]. In case of cardiac involvement, granuloma formation may lead to reversible inflammation with potential progression to irreversible fibrosis resulting in adverse events including heart failure or malignant arrhythmia [4–6] The prevalence of cardiac sarcoidosis (CS) has been widely described and cardiac involvement may lead to potentially life-threatening events [7, 8]. Especially in patients with a transition from acute (and potential reversible) towards persistent inflammation and fibrosis, anti-inflammatory treatment is a pivotal cornerstone for a successful therapy [3, 6, 9]. Therefore, early and accurate stratification of disease activity in patients with CS is an unmet need to attenuate disease progression and thus, to prevent potentially fatal outcome [8, 10]. Only recently, we were able to show that comprehensive simultaneous hybrid CMR/FDG-PET imaging leads to improved diagnosis of CS and classification of disease activity [11, 12]. Thus, detection of coexisting cardiac inflammation and fibrosis facilitates differentiation of active (aCS) from chronic (cCS) CS [11]. Inflammatory biomarkers including interleukin-2 receptor or angiotensin-converting enzyme are key players in the diagnosis of inflammatory diseases such as CS but reflect underlying pathophysiological pathways only partially [13–15]. Therefore, the assessment of distinct inflammatory signaling cascades and cell signatures in patients with CS might enlighten the expected course of the disease with either favorable or unfavorable outcome. Besides, $CD4^+$ and $CD8^+$ lymphocytes, innate immune cells and especially monocytes are pivotal in the formation of granulomatous diseases such as sarcoidosis [16, 17]. Nonetheless, the inflammatory fingerprint of patients with CS has yet to be determined and might provide striking diagnostic, therapeutic, and even prognostic perspectives.

Methods

Study population

Twenty-six ($n = 26$) patients with biopsy-proven extracardiac sarcoidosis were enrolled in this prospective, consecutive study. All patients underwent cardiovascular magnetic resonance (CMR) coupled with ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging for suspected CS as described previously [11].

Concisely, aCS was defined as abnormalities of cardiac tissue in CMR with concomitant focal or focal-on-diffuse myocardial FDG uptake in the presence of otherwise suppressed myocardial glucose uptake on PET imaging.

Contrarily, cCS was defined as pathological CMR tissue and no myocardial FDG uptake (successful suppression). Ultimately, noCS was defined as the absence of myocardial tissue abnormalities on CMR imaging. Exclusion criteria of hybrid CMR/FDG-PET imaging were defined as homogeneous myocardial FDG uptake affecting the entire left ventricular tissue, as well as quantitative excess of cardiac FDG uptake compared to liver uptake. Peripheral blood samples were collected via venipuncture in an ambulatory setting during the morning hours, prior to cardiac MR/FDG-PET imaging, in order to minimize circadian variation. To reduce glucose levels, which could otherwise confound FDG uptake, all patients followed a strict low-carbohydrate diet the day before CMR/FDG-PET and blood sampling and fasted overnight for at least 12 h. Further, medication on admission was assessed prior to blood sampling and CMR/FDG-PET, and all patients completed a standardized questionnaire on medication history, comorbidities, and cardiovascular risk factors. Immunosuppressive therapy was initiated either following histological confirmation of extracardiac sarcoidosis, based on disease severity, or after the diagnosis of aCS determined by CMR/FDG-PET findings. In accordance with international guidelines, corticosteroids were administered as first-line therapy, while azathioprine was used as a second-line agent in patients experiencing toxicity from prolonged corticosteroid use. No other immunosuppressive agents besides corticosteroids and azathioprine were used in this study. Twenty-nine ($n = 29$) healthy individuals served as controls and were free from any comorbidities (e.g., cardiovascular disease, diabetes mellitus, chronic infectious disease) and were not receiving any form of chronic pharmacological treatment. Baseline characteristics of healthy controls (HC) are provided in Supplementary Table S1.

The study was approved by the Ethics Committee at the Medical Faculty of the Eberhard Karls University and at the University Hospital of Tübingen and all patients gave written informed consent. The experiments were performed in accordance with the highest ethical standards as laid down within the Declaration of Helsinki.

Isolation of peripheral blood mononuclear cells and platelets

Citrated whole blood samples of patients with sarcoidosis and healthy controls (HC) were processed immediately after peripheral venipuncture. Therefore, peripheral blood mononuclear cells (PBMCs) and platelets were analyzed in diluted whole blood as described previously [18, 19]. A detailed description of cellular isolation is outlined in the Supplementary methods section.

Flow cytometry staining

Isolated PBMCs were diluted with DNaseI solution and thereafter, cells were centrifuged, washed, and pre-stained to exclude dead cells. Blocked cells were incubated with human IgG to prevent unspecific binding of antibodies to Fc receptors. Subsequently, extracellular staining of PBMCs was performed with specific antibodies listed in supplementary Table S2. Finally, cells were washed and prepared again to block non-specific binding of antibodies to cellular constituents. Stained cells were acquired by spectral flow cytometry with the SpectroFlo software (Aurora flow cytometer; Cytek Bioscience, CA, USA). Further details of the processing methods and chemicals are given in the Supplementary material.

Flow cytometry data analysis

To account for autofluorescence and overlapping spectra, unmixing was performed with unstained cells and single-stained controls. A classical gating strategy was applied to the 36-color panel using the OMIQ software (Dotmatics, MA, USA). Scaling and compensation of the raw data were adjusted prior to generation of subsamples with 1.5×10^6 events/sample. Thereafter, FlowAI algorithm was carried out for quality control with flow rate 0.1, alpha 0.01, and dynamic range check integrating both limits merging time, fluorescent channels, autofluorescence, gating and subsampling on 1×10^6 cells/group. Normalization of data was applied for all fluorescent channels using fdaNorm prior to scaling and subsample gating of living 5×10^5 CD45$^+$ cells/group. Uniform Manifold Approximation and Projection (UMAP) analysis and visualization was performed to depict cellular sub-populations of distinct disease groups. In the dimensionality reduction analysis, all files and fluorescent parameters were used but live/dead, CD45 and autofluorescence were excluded from clustering. Finally, FlowSOM clustering algorithm was carried out integrating features of CD1c, CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD20, CD25, CD27, CD28, CD38, CD56, CD57, CD123, CD127, CD141, CCR7, HLA-DR, IgD, IgM, IgG, and TCR γδ with 10 training iterations, k = 100 consensus metaclustering, and Euclidian distance metric.

Cytometric immunoassay including cytokines/chemokines

Cytokines and chemokines were determined by LEGENDPlex Inflammation Panel 1 and LEGENDPlex Proinflammatory Chemokine Panel (BioLegend, CA, USA) according to the manufacturer's guidelines as described recently [19]. Resuspended frozen plasma samples of all individuals were analyzed using a FACS Lyric (BD Biosciences, NJ, USA). Data acquisition and interpretation were performed with the LEGENDPlex Data Analysis Software (BioLegend, CA, USA).

Statistical analysis

Pre-processed data were analyzed using JMP® Pro Version 17.1 (SAS Institute, NC, USA) and different software packages in R (R foundation for Statistical Computing, Vienna, Austria). Non-normally distributed data were analyzed using the Mann–Whitney U test; normally distributed data were compared with Student's t-test. Mean values are presented as mean ± standard deviation (SD) in case of Gaussian distribution or as median and interquartile range (IQR) as suitable. Categorical data are presented as total numbers and proportions and were analyzed by chi-squared test. Expression of inflammatory cells were analyzed using a false discovery rate (FDR, Benjamini-Hochberg) controlling procedure to correct significance levels ($p < 0.05$) for FDR ≤ 5% of cells varying between patients with sarcoidosis and healthy individuals. For statistical comparisons, fold change was calculated as ratio between groups for each cell population. Correlation data of flow cytometry were analyzed by pattern hunter in R package "MetaboAnalystR" and is based on Pearson's product-moment correlation coefficient. For visualization of correlation matrix. Spearman's rank correlation coefficient was implemented in R package "corrplot". For high-dimension reduction of immune cell clusters, we applied orthogonal partial least square discriminant analysis (OPLS-DA) with 5-fold internal cross-validation and permutation testing. OPLS-DA and volcano plots were generated with "MetaboAnalystR".

Results

Determination of immunological sub-phenotypes in patients with sarcoidosis

In the present study we prospectively analyzed the inflammatory fingerprint including immune cell signatures in a consecutive cohort of patients with biopsy-proven sarcoidosis utilizing a multicolor flow cytometry approach (Fig. 1A). Baseline characteristics of patients with sarcoidosis ($n = 26$) including clinical and laboratory parameters are summarized in Table 1. Recently, we described a differentiation of active (aCS) from chronic cardiac (cCS) sarcoidosis by resonance/fluorodeoxyglucose positron emission tomography [11]. Therefore, all patients underwent hybrid CMR/FDG-PET to determine cardiac involvement and disease activity of sarcoidosis (Fig. 1B). Thus, fourteen (53.9%) patients presented with aCS, eight (30.8%) patients were classified as cCS whereas MRI of four (15.4%) patients did not exhibit cardiac involvement of sarcoidosis (no CS) (Fig. 1A).

The population's median age was 46.5 (IQR 35.8–54.8) years, and nine (34.6%) patients were female. LVEF did not vary between patient subgroups, but end-systolic volume was significantly ($p = 0.033$) lower in patients without cardiac involvement of sarcoidosis (44ml, IQR 35.3–3.5) in contrast to patients with CS (67ml, IQR 58–78.5). Co-medication including anti-inflammatory drugs as well as important laboratory parameters including soluble IL-2 receptor (sIL-2R) and angiotensin-converting enzyme (ACE) activity did not differ significantly among disease groups (Table 1).

To screen for novel markers eligible to distinguish between sarcoidosis and healthy individuals, we performed clustering of inflammatory sub-phenotypes in patients with CS. Therefore, peripheral blood mononuclear cells (PBMCs) were stained with a 36-color antibody panel (Fig. 1C) and analyzed by spectral flow cytometry.

UMAP for dimensionality reduction unveiled 56 characteristic immune cell populations and disclosed a divergence in abundance of distinct immune cell clusters between patients with sarcoidosis and healthy controls (Figs. 1D and 2A).

To elucidate specific changes in the inflammatory fingerprint associated with biopsy-proven sarcoidosis, we investigated the relative abundance of immune cell populations compared to healthy controls (Supplementary Fig. S1). We found that eighteen cell populations were significantly ($p < 0.05$) up-regulated in patients with sarcoidosis compared to healthy controls (Fig. 2B). Significantly up-regulated clusters mostly comprised of immune cells belonging to the category of $CD14^+$ and $CD16^+$ monocytes, B lymphocytes, $CD4^+$ and $CD8^+$ T cells as well as the $CD24^{high}$ NK cell subpopulation. Among the eight clusters that were down-regulated in patients with sarcoidosis compared to healthy individuals, we exclusively found cells belonging to the class of $CD4^+$ and $CD8^+$ T cells (Fig. 2B).

To evaluate whether critical shifts in the entirety of the immune cell cluster occur between patients with sarcoidosis and healthy individuals, we performed orthogonal partial least square discriminant analysis (OPLS-DA) including all detected cell populations of this study. Clustering of sarcoidosis patients showed a perceptible separation compared to the healthy controls indicating a crucial alteration of the inflammatory phenotype (Fig. 2C).

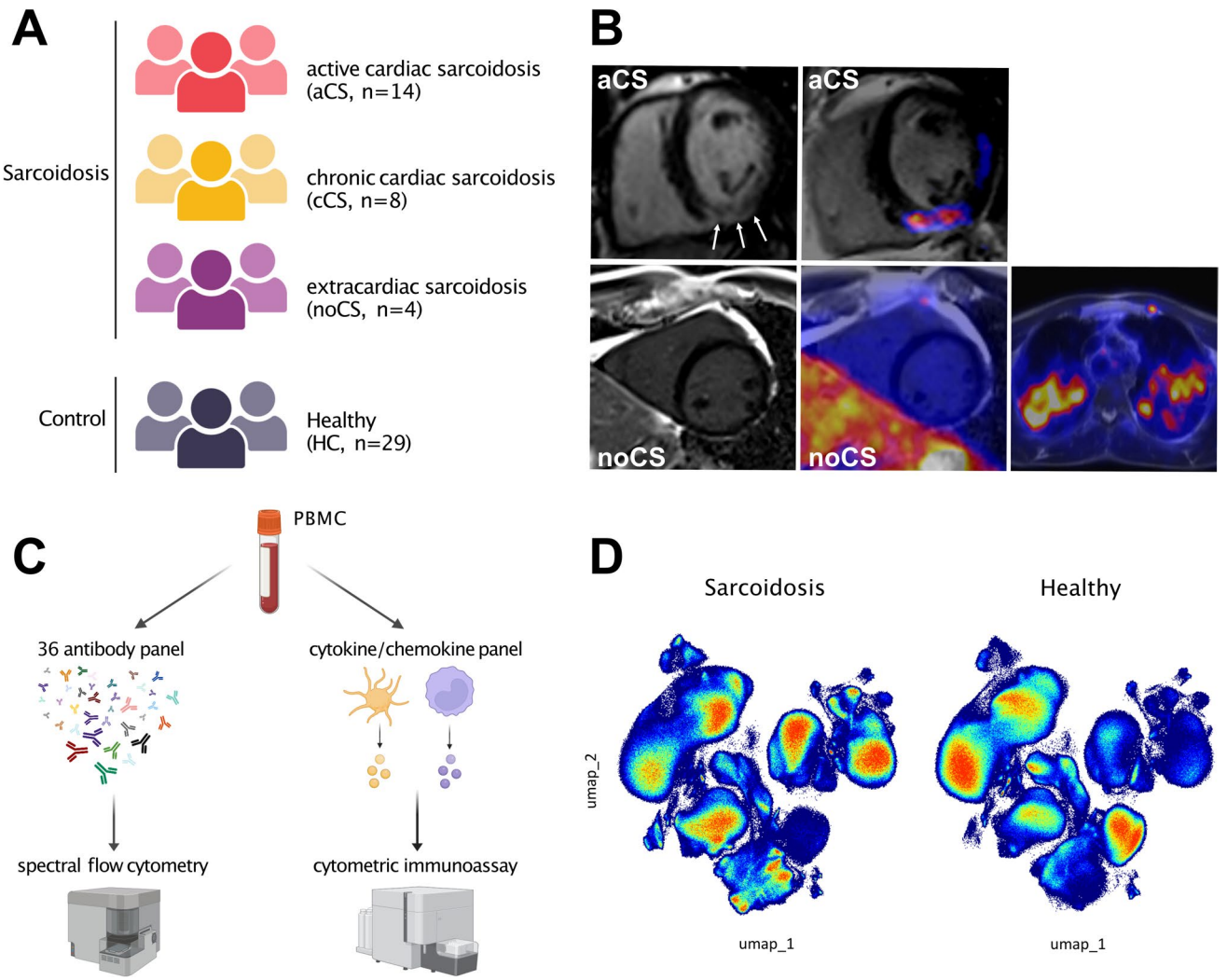


Fig. 1 Multicolor flow cytometry enables characterization of subphenotypes in patients with sarcoidosis. **A** Enrollment of patients with active (aCS) or chronic cardiac sarcoidosis (cCS), extracardiac sarcoidosis (noCS), and healthy individuals included in this study (**B**) Representative images of hybrid cardiac magnetic resonance/fluorodeoxyglucose positron emission tomography. In the top left image non-ischemic late gadolinium enhancement (LGE) is observed in the inferior left-ventricular wall (indicated by arrows). In the top right image PET overlay reveals active inflammation with correspondingly increased FDG uptake in a patient with aCS. The bottom image section represents absence of LGE and complete suppression of myocardial FDG uptake in patients with noCS. High inflammatory activity is observed in pulmonary sarcoidosis granulomas and thoracic lymph nodes. **C** High-dimensional data analysis workflow of peripheral mononuclear blood cells (PBMCs). A multicolor flow cytometry and cytometric immunoassay were employed to detect immune cell populations associated with disease activity of sarcoidosis patients. **D** Transformation of multicolor flow cytometry data by uniform manifold approximation and projection (UMAP) dimensions. The density plots depict merged analyses of all samples according to the patient subgroup

The inflammatory fingerprint of patients with sarcoidosis varies with cardiac involvement and disease activity

To further elucidate changes in the inflammatory signature related to CS, we assessed differences in the abundance of the cellular clusters from sarcoidosis patients with and without cardiac involvement (Supplementary Fig. S2). Comparison of patients with aCS and noCS unveiled significant ($p < 0.05$) up-regulation of activated, double-negative memory B cells, plasmablasts, and neutrophils. Contrarily, MAIT cells and $CD8^+$ intermediate/terminal effector T cells were significantly ($p < 0.05$) down-regulated in patients with aCS when compared to patients with noCS (Fig. 3A). Plasmablasts were significantly ($p < 0.05$) increased, whereas exclusively $CD4^+$ early effector T cells and $CD8^+CD24^+CCR7^+CD45RA^+$ T cells were decreased in patients with cCS when compared to patients with noCS (Fig. 3A). Interestingly, exclusively T cells (double-negative T cells, $CD4^+$

Table 1 Baseline characteristics of the sarcoidosis patient population. Significant ($p < 0.05$) values are highlighted

	All ($n = 26$)	aCS ($n = 14$; 53.9%)	cCS ($n = 8$; 30.8%)	noCS ($n = 4$; 15.4%)	p-value
Female, *n* (%)	9 (34.6)	4 (28.6)	4 (50)	1 (25)	0.542
Age (years), median (IQR)	46.5 (35.8–54.8)	46.5 (39.8–59.8)	41.5 (31–53.8)	51 (36.5–56.5)	0.571
Body mass index (kg/m^2), median (IQR)	26.6 (24.5–29.1)	27.9 (24.8–29.4)	24.7 (18.5–27.2)	25 (22.3–28.4)	0.332
Cardiovascular risk factors					
Arterial hypertension, n (%)	9 (34.6)	3 (21.4)	4 (50)	2 (50)	0.312
Hyperlipidemia, *n* (%)	4 (15.4)	2 (14.3)	2 (25)	0 (0)	0.520
Diabetes mellitus, *n* (%)	2 (7.7)	1 (7.1)	1 (12.5)	0 (0)	0.741
Current smoking, *n* (%)	6 (23.1)	5 (35.7)	0 (0)	1 (25)	0.160
Ex Smoking > 6 mo, *n* (%)	4 (15.4)	0 (0)	2 (25)	2 (50)	**0.033**
Previous syncope, *n* (%)	1 (3.9)	1 (12.5)	0 (0)	0 (0)	0.640
Medication on admission					
Steroids, *n* (%)	12 (46.2)	8 (57.1)	2 (25)	2 (50)	0.342
Steroid dose (mg), median (IQR)	0 (0–10)	3.75 (0–10)	0 (0–7.5)	5 (0–10)	0.389
Azathioprine, *n* (%)	2 (7.7)	2 (14.3)	0 (0)	0 (0)	0.395
NSAID, *n* (%)	2 (7.7)	0 (0)	1 (12.5)	1 (25)	0.211
ACEI/ARB, *n* (%)	7 (26.9)	4 (28.6)	1 (12.5)	2 (50)	0.378
Ca channel antagonists, *n* (%)	1 (3.9)	0 (0)	0 (0)	1 (2)	0.057
β-blockers, *n* (%)	6 (23.1)	3 (21.4)	2 (25)	1 (25)	0.978
Diuretics, *n* (%)	3 (11.5)	2 (14.3)	0 (0)	1 (25)	0.395
Statins, *n* (%)	1 (3.9)	1 (12.5)	0 (0)	0 (0)	0.640
Laboratory parameters					
Renal function (GFR), median (IQR)	95.4 (73.5–112.2)	91.2 (72.1–119.9)	98.3 (79.2–109.9)	87.3 (61.9–106)	0.709
Leukocytes (10^9/l), median (IQR)	6. (5.4–8.3)	6.1 (4.7–8)	6.4 (4.9–7.7)	9.8 (6.9–36.1)	0.118
C-reactive protein (mg/dl), median (IQR)	0.2 (0.1–0.8)	0.2 (0.1–0.4)	0.1 (0.1–0.8)	0.8 (0.1–1.9)	0.807
sIL-2R (U/ml), median (IQR)	549 (426–920)	549 (394–641)	542 (456.8–1427.8)	731 (118.3–1447.5)	0.807
ACE-Activity, (U/l), median (IQR)	39 (29–77)	35 (29–56)	77 (26–88)	71 (71)	0.498
CK (U/l), median (IQR)	109 (68.3–127.5)	112 (74.8–146.3)	99 (69–188.5)	10 (59–130)	0.763
Total cholesterol (mg/dL), median (IQR)	208 (176–239)	208 (176–239)	227 (153–263)	180 (18)	0.785
Triglycerides (mg/dL), median (IQR)	138 (125–333)	17 (123–354)	133 (54–187)	138 (138)	0.707
HbA1c (%), median (IQR)	5.3 (5.3–5.6)	5.4 (5.2–5.8)	5.3 (5.2–5.6)	5.4 (5.4)	0.803
hs-Troponin I (ng/ml), median (IQR)	3 (3–8)	3 (3–5)	10 (3–17)	3 (3–3)	0.643
NT-proBNP (ng/ml), median (IQR)	73 (53.8–834.3)	73 (53.8–834.3)	120 (68–172)	NA	1.000
Cardiac MR imaging					
LVEF (%), median (IQR)	58 (55.5–64.5)	57 (50.5–63.5)	60.8 (55.5–65.5)	62 (56.8–72.5)	0.279
LVEDV (mm), median (IQR)	154 (131.5–168.8)	157 (148–178)	161.2 (118.3–170.4)	123.5 (106.3–141.5)	0.070
ESV (ml), median (IQR)	63 (52–75)	67 (58–91)	65.2 (50.3–73)	44 (35.3–53.5)	**0.033**
SV (ml), median (IQR)	89 (72.5–98)	92 (74–99)	86 (73.5–98.5)	73.5 (69.3–101.8)	0.721
NYHA class					
I, *n* (%)	16 (61.5)	6 (42.9)	6 (75)	4 (100)	0.075
II, *n* (%)	8 (30.8)	7 (50)	1 (12.5)	0 (0)	0.065
III, *n* (%)	1 (3.9)	1 (7.1)	0 (0)	0 (0)	0.640
IV, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	
New electrocardiographic changes at follow-up					
Bundle branch block, *n* (%)	3 (11.5)	3 (21.4)	0 (0)	0 (0)	0.234
Atrial fibrillation, *n* (%)	2 (7.7)	1 (7.1)	1 (12.5)	0 (0)	0.741

ACEI/ARB angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, *aCS* active cardiac sarcoidosis, *cCS* chronic cardiac sarcoidosis, *CK* creatine kinase, *ESV* end-systolic volume, *hs-Troponin I* high sensitive troponin I, *IQR* interquartile range, *LVEDV* left ventricular end-diastolic volume, *LVEF* left ventricular ejection fraction, *NA* not available, *noCS* extracardiac sarcoidosis, *NSAID* non-steroidal anti-inflammatory drugs, *NT-proBNP* N-terminal prohormone of brain natriuretic peptide, *NYHA* New York Heart Association, *sIL-2R* soluble IL-2 receptor, *SV* stroke volume

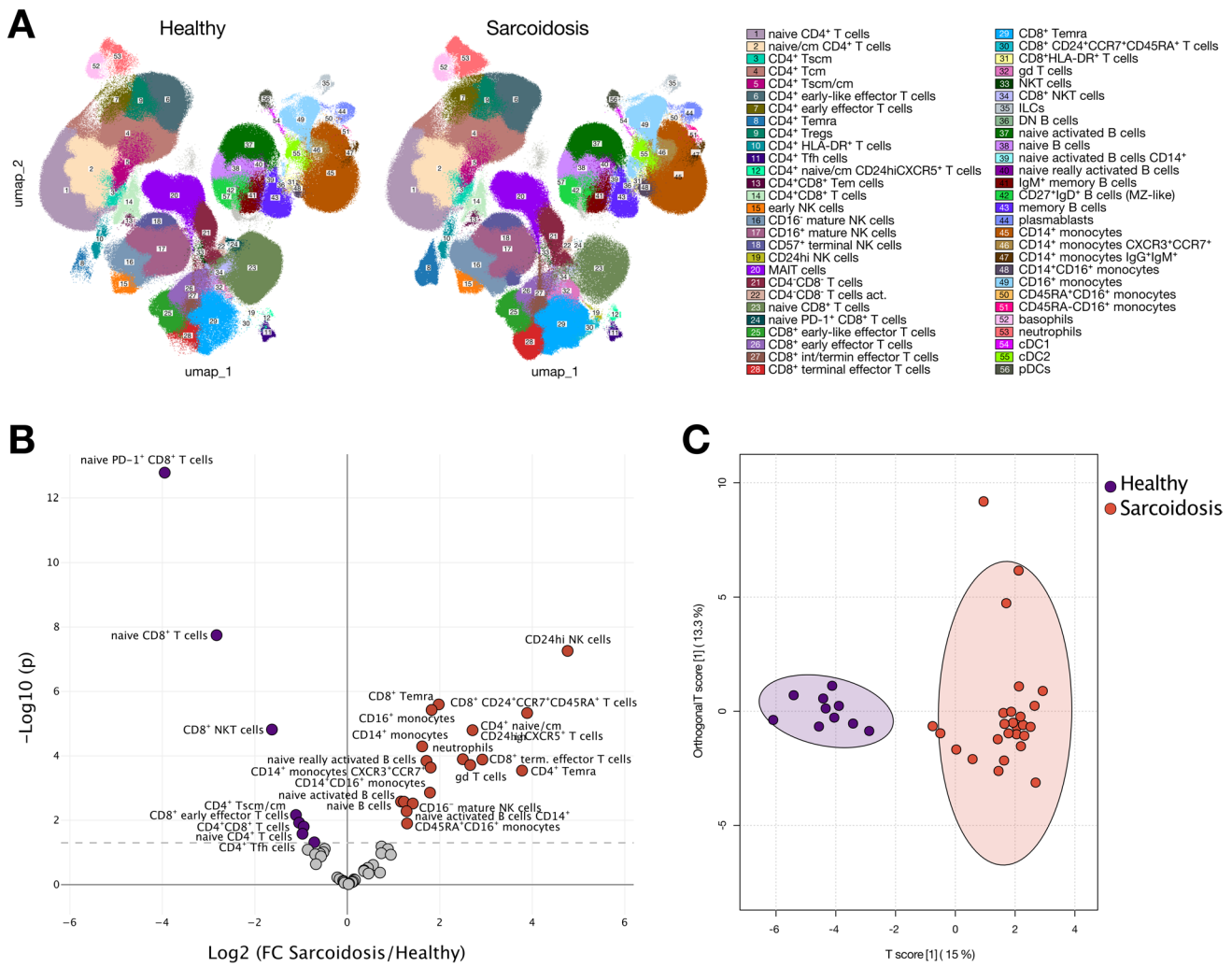


Fig. 2 The immune cell signature is critically altered in sarcoidosis. **A** UMAP analyses including merged events of patients with sarcoidosis and healthy individuals highlighting abundance of 56 individual immune cell clusters detected by 36-color spectral flow cytometry. **B** Results of spectral flow cytometry data were Log2-transformed, and fold change of means was depicted in volcano plot displaying significant alterations between patients with sarcoidosis and healthy individuals ($p < 0.05$, FDR < 0.05, 18 up-regulated, 8 down-regulated cell clusters in sarcoidosis patients). **C** Orthogonal partial least squares (OPLS) discriminant analysis of significantly altered immune cell clusters reveals a critical separation of patients with sarcoidosis and healthy individuals

Temra, naïve $CD8^+$ T cells, and $CD8^+$ NKT cells) were down-regulated in patients with aCS when compared to cCS. On the other hand, innate immune cells including $CD14^+CD16^+$ monocytes, $CD45RA^-CD16^+$, and neutrophils, as well as naïve activated $CD14^+$ B cells and $CD8^+CD24^+CCR7^+CD45RA^+$ T cells were up-regulated in patients with aCS compared to cCS (Fig. 3A). OPLS-DA analyses of all detected inflammatory cells depicted a major separation of patients with CS when compared to patients with noCS. Thus, this indicates a substantial shift of the inflammatory cells in patients with cardiac involvement compared to patients with noCS. However, a less prominent segregation was observed between patients with aCS and cCS (Fig. 3B). In addition to the observed alteration of immune cells, a phenotypical change of the inflammatory landscape occurred in patients with aCS and cCS when compared to patients with noCS or healthy individuals (Fig. 3C). Here, immune cell clusters comprised B and T cells, followed by $CD14^+$ and $CD16^+$ monocytes.

Innate immune cells including monocytes determine the inflammatory landscape and are significantly related to cardiac sarcoidosis

It was striking that relative abundance of mostly $CD16^+$ monocytes was highest in patients with CS when compared to those with noCS or healthy individuals (Fig. 4A). Subgroup comparison unveiled that fraction of $CD16^+$ cells

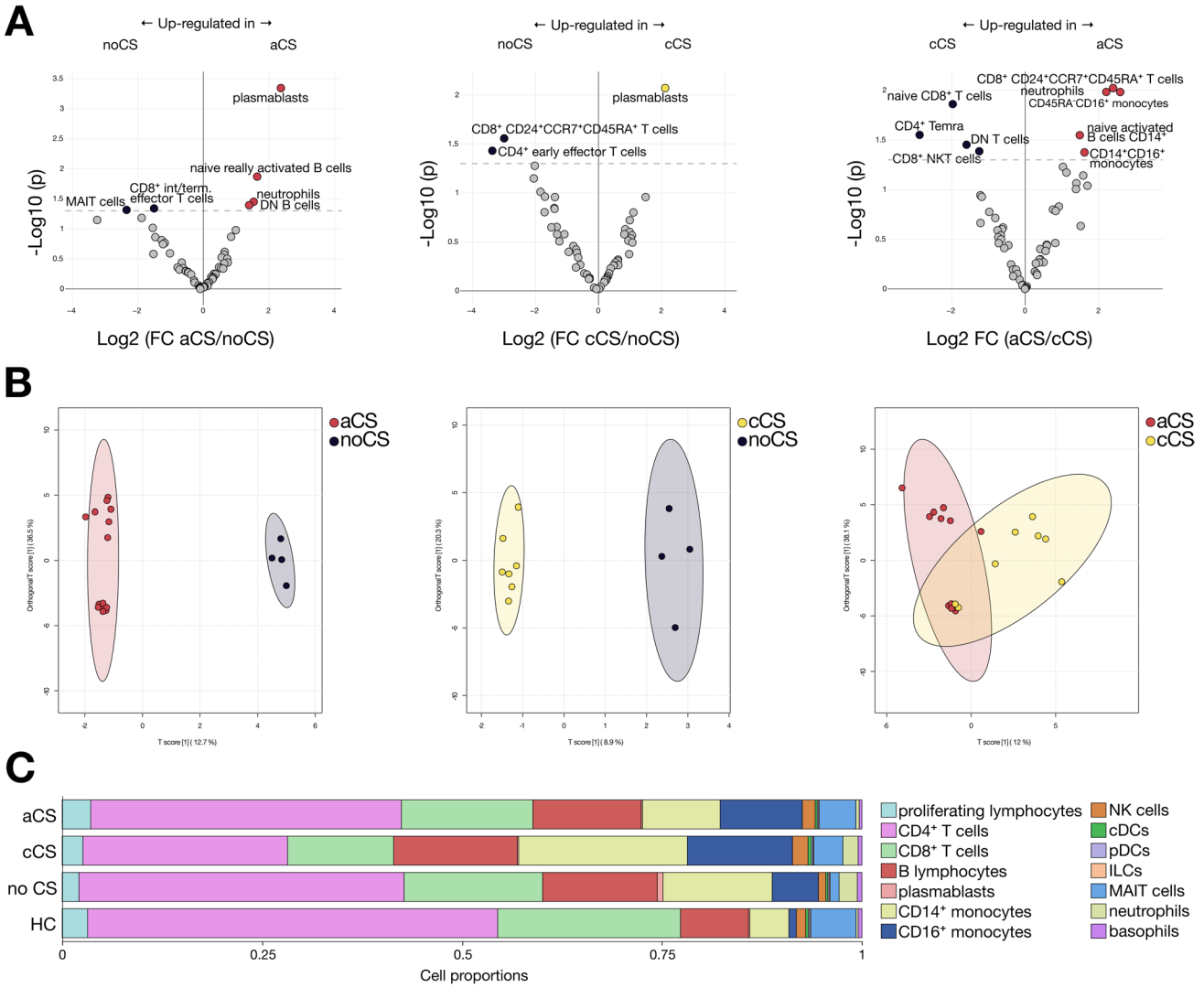


Fig. 3 Characteristic changes of immune cell populations occur in patients with cardiac sarcoidosis and essentially vary with disease activity. **A** Results of spectral flow cytometry data (56 immune cell clusters) comparing patients with aCS to noCS, cCS to noCS, and aCS to cCS as displayed by individual volcano plots ($p < 0.05$). **B** Orthogonal partial least squares (OPLS) discriminant analysis of significantly altered immune cell clusters comparing patients with aCS to noCS, cCS to noCS, and aCS to cCS. A distinct segregation of patients with aCS and cCS implements a crucial impact of changes in immune cell clusters on disease activity. **C** Bar plot comparing the proportion of cell types detected in patients with sarcoidosis and healthy individuals

was significantly ($p < 0.001$) increased in patients with aCS (median 0.08, IQR 0.05–0.11) and cCS (median 0.1, IQR 0.08–0.17) when compared to patients with noCS (median 0.06, IQR 0.03–0.08) or healthy individuals (median 0.01, IQR 0.01–0.01) (Supplementary Fig. S3). Similarly, the fraction of $CD14^+$ cells was significantly ($p = 0.003$) increased in patients with cCS (median 0.14, IQR 0.08–0.33) and aCS (median 0.08, IQR 0.04–0.11) compared to noCS (median 0.11, IQR 0.08–0.22), or healthy controls (median 0.04, IQR 0.03–0.06) (Supplementary Fig. S4). In depth juxtaposition of $CD14^+$ and $CD16^+$ cells uncovered mostly monocyte subpopulations, followed by $CD16^+$ mature NK cells and activated $CD14^+$ B cells (Fig. 4A). Most strikingly, the proportion of $CD14^+$ and $CD16^+$ cells was critically associated with disease activity and cardiac involvement of sarcoidosis. Thus, the $CD14^+/CD16^+$ ratio was lowest in patients with aCS (median 1.26, IQR 0.46–2.68; $p < 0.001$), cCS (median 1.35, IQR 0.7–4.31; $p = 0.034$), followed by patients without CS (median 2.54, IQR 1.72–3.4; $p = 0.020$) and significantly reduced when compared to healthy individuals (median 5.89, IQR 3.24–6.79) (Fig. 4B). Interestingly, $CD4^+/CD8^+$ ratio implemented in the diagnostic algorithm of extracardiac sarcoidosis did not differentiate significantly between

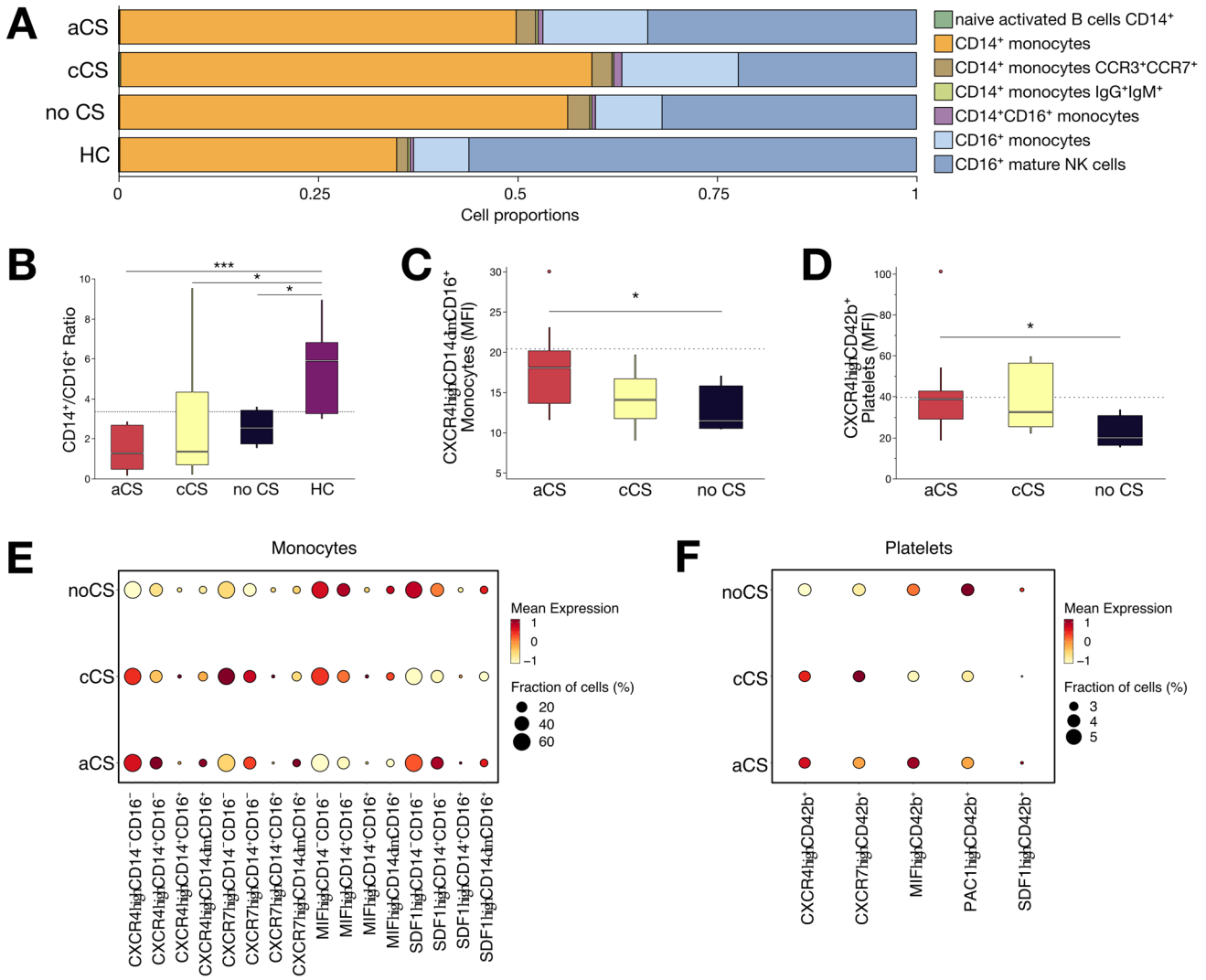


Fig. 4 Distinctive monocyte populations dictate the inflammatory fingerprint, which aligns with the disease activity observed in sarcoidosis patients. **A** Bar plot depicting the proportions of essential $CD14^+$ and $CD16^+$ immune cells mainly belonging to the class of monocytes. **B** Box plots comparing the significantly ($p < 0.05$) altered expression of $CD14^+/CD16^+$ antigens between patients with aCS, cCS, no CS and healthy individuals. Thus, the $CD14^+/CD16^+$ ratio is highly dependent on disease activity in patients with CS. **C** Box plots comparing the expression of CXCR4 in non-classical monocytes significantly ($p < 0.05$) enhanced in patients with aCS compared to noCS. **D** Comparison of $CXCR4^{high}$ circulating platelets illustrates significant up-regulation in patients with aCS in contrast to noCS. **E** Dot plot illustrating the cytokine/chemokine expression of various monocyte populations in patients with sarcoidosis. **F** Cytokine/chemokine panel of circulating platelets in patients with sarcoidosis. (* $p < 0.05$; *** $p < 0.001$)

patients with CS, noCS, or healthy individuals. However, the relative abundance of T cell subpopulations was heterogenous among patients with sarcoidosis and healthy controls (Supplementary Figs. S5 and S6).

Chemokine and cytokine signaling distinguishes between patients with cardiac and extracardiac sarcoidosis

To spotlight pathophysiological cascades contributing to inflammation and granuloma formation in patients with sarcoidosis, we analyzed cytokine/chemokine expression of monocytes and platelets in patients with aCS, cCS, and noCS (Fig. 4C–F). Here, we found highest abundance of chemokine receptors CXCR4, CXCR7, chemokine-like macrophage inhibitory factor (MIF), and stromal cell-derived factor 1 (SDF1/CXCL12) in monocyte subsets (Fig. 4E). Further, the pro-inflammatory chemokine receptor CXR4 was significantly increased in $CD14^{dim}CD16^+$ monocytes of patients with aCS in contrast to those with noCS (Fig. 4C). Interestingly, circulating platelets also served as source of pro-inflammatory chemokines and cytokines indulging

CXCR4, CXCR7, MIF, glycoprotein IIb (PAC1), and SDF-1 (Fig. 4F). Likewise, CXCR4 was significantly ($p < 0.05$) increased in platelets of patients with aCS when compared to those of patients with no CS (Fig. 4D). Interestingly, pro-inflammatory CXCR4 ligands SDF1 and MIF were down-regulated in platelets of patients with aCS compared to healthy individuals, hinting at a potential compensating mechanism of platelets in the inflammatory signaling of patients with CS (Supplementary Fig. S7).

Immune cells and inflammatory signatures are significantly related and predict clinical characteristics and outcome in patients with cardiac sarcoidosis

Assessment of sIL-2 receptor (sIL-2R) and ACE activity is fundamental in diagnostics of sarcoidosis. Thus, we hypothesize that a shift in the inflammatory landscape is associated with differential expression of sarcoidosis biomarkers. Therefore, we performed comprehensive correlation analysis of immune cell clusters with important clinical biomarkers sIL-2R and ACE activity (Fig. 5A, B). We found that sIL-2R significantly ($p < 0.05$) correlated with $CD24^{high}$ NK cells, T cell subpopulations ($CD4^+$ naive/cm $CD24^{high}CCR5^+$ T cells, γδ T cells) as well as $CD14^+CCR3^+CCR7^+$ monocytes (Fig. 5A). Further, sIL-2R concentration was inversely associated with abundance of mainly $CD4^+$ and $CD8^+$ T cell subpopulations as well as memory B cells, early NK cells, and innate lymphatic cells (ILCs) (Fig. 5A). ACE activity significantly ($p < 0.05$) correlated with abundance of $CD14^+$ and $CD14^+CD16^+$ monocytes as well as $CD14^+$ naïve activated B cells (Fig. 5B). ACE activity was inversely associated with abundance of predominantly $CD4^+$ and $CD8^+$ T cells alongside conventional and plasmacytoid dendritic cells (cDCs, pDCs), ILCs, basophils, NK cells, and memory B cells (Fig. 5B). In addition, we found that abundance of immune cells associated with cardiac sarcoidosis as well as clinical inflammation-related cytokine/chemokine levels significantly ($p < 0.05$) correlated with important clinical baseline parameters (Fig. 5C).

Next, we performed comparison of the immunophenotype in individuals with new-onset electrocardiographic changes during the clinical follow-up. We found that eight immune cell clusters were significantly ($p < 0.05$, FDR < 5%) up-regulated in patients with adverse electrophysiological changes compared to patients without new-onset arrhythmia or ECG abnormalities (Fig. 5D). Here, mostly inflammatory cells belonging to the class of T cells, NK cells, $CD14^+$ and $CD16^+$ monocytes, ILCs as well as intensity of $CXCR7^+$ platelets was significantly higher in patients with electrophysiological events.

Contrarily, eleven cell populations were down-regulated in patients with new changes in ECG. Among inflammatory clusters that were down-regulated, we found mainly MIF^{high} monocytes, T cell subpopulations, naïve and activated B cells, and cDCs (Fig. 5D).

Discussion

In patients with cardiac sarcoidosis, granuloma formation and myocardial inflammation portend an increased risk of developing adverse cardiovascular events. In this novel study we investigated the inflammatory landscape of patients with cardiac sarcoidosis to elucidate that: (i) abundance of immune cell populations critically varies between patients with sarcoidosis and healthy individuals, (ii) a distinct cellular immune signature is associated with cardiac involvement and disease activity in patients with sarcoidosis, and (iii) pro-inflammatory cells and mediators are associated with the clinical course of cardiac sarcoidosis. Thus, the novel results of this study may further elucidate the complex, and yet not well understood, pathophysiologic pathways in cardiac sarcoidosis.

Previously, simultaneous hybrid CMR/FDG-PET imaging was shown to precisely separate active from chronic disease in CS [11]. Myocardial inflammation detected by cardiac imaging might be potentially reversible whereas chronic fibrosis is irreversible and yields adverse prognosis [8, 12].

Over the past decade, novel cellular and molecular markers have emerged as prominent targets for the diagnosis and treatment of extracardiac sarcoidosis. However, clinical studies investigating the inflammatory landscape in CS and its association to disease activity and clinical outcome are missing so far.

In this prospective study we characterized the inflammatory fingerprint in a consecutive cohort of patients with CS and elucidated changes of distinct immune cell populations and cytokine/chemokine concentrations. We found that mainly monocytes, neutrophils as well as B and T cells and NK cells were up-regulated in patients with sarcoidosis compared to healthy individuals. In line with previous studies, up-regulation of monocytes/macrophages reflects migration of those innate immune cells to cardiac tissue, where they maintain the inflammatory process and promote granuloma formation [20, 21]. Likewise, over-expression of CD24 and chemokine receptors CXRC3, CXCR5, and CCR7 in patients with sarcoidosis implicates enhanced trafficking of cellular clusters belonging to the humoral and cell-mediated adaptive immune system [22–24]. On the other hand, predominantly immature T cells were down-regulated in patients with sarcoidosis. Interestingly, CD4 and CD8 lymphopenia and down-regulation of NKT cells were shown to correlate with early cardiac involvement and a severe course of the disease [25, 26].

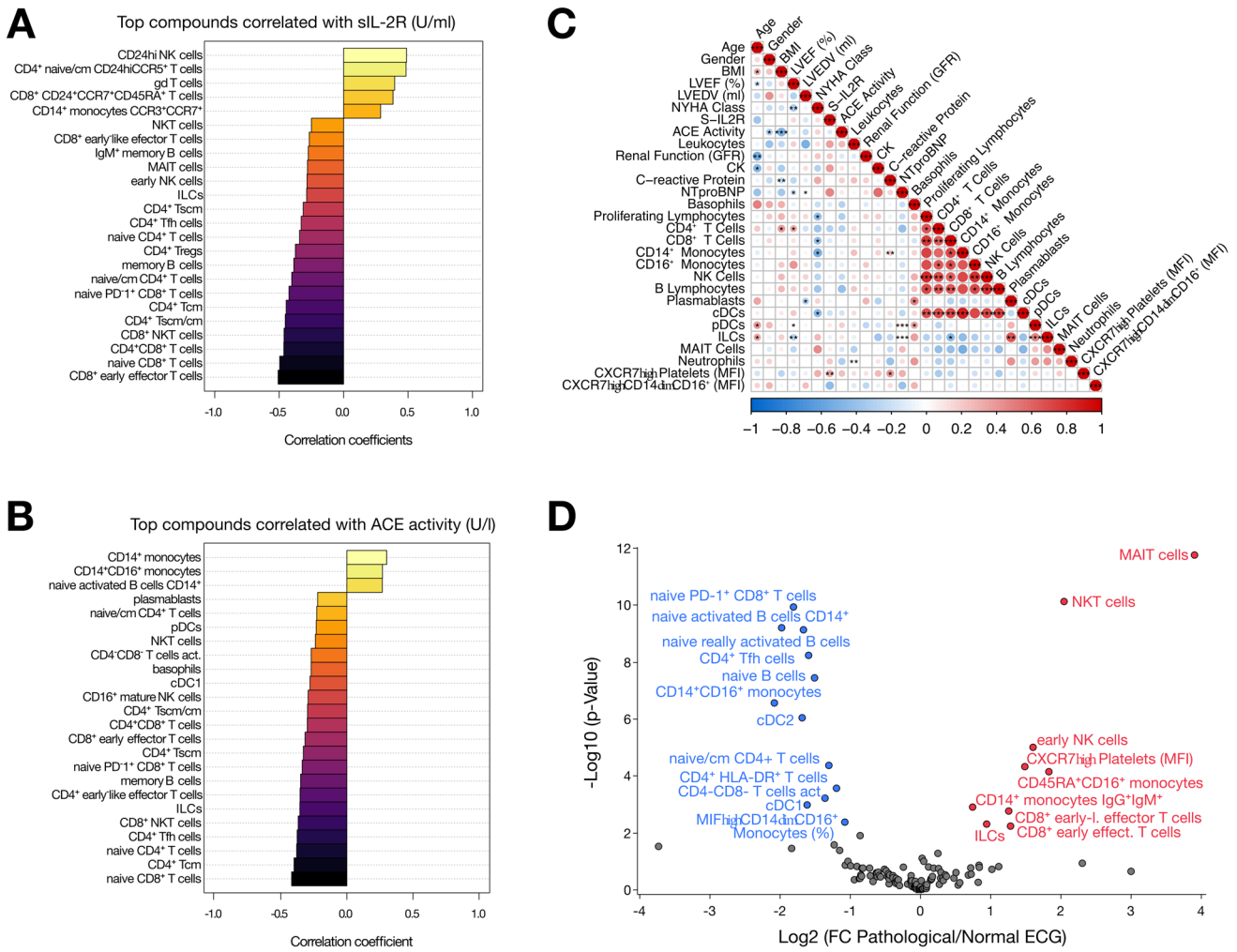


Fig. 5 The Immune cell signature and cytokine/chemokine profile correlate with clinically relevant parameters in patients sarcoidosis. **A** Significant correlations ($p < 0.05$) of immune cells populations with soluble IL-2 receptor concentrations with the referring Spearman´s ρ. **B** Significant correlations ($p < 0.05$) of immune cells populations with soluble angiotensin-converting enzyme concentrations with the referring Spearman´s ρ. **C** Matrix correlogram derived from individual correlation analyses of clinical parameters alongside ex vivo assays. Spearman´s ρ is colored and color intensity and size are plotted proportional to correlation coefficients. **D** Volcano plot highlighting significantly ($p < 0.05$) immune cell populations and chemokine expression in patients with electrocardiographic changes (e.g. bundle branch block, new-onset arrhythmia) during the clinical follow-up

Furthermore, early detection of cardiac sarcoidosis appears to be of utmost clinical interest as the diagnosis of cardiac involvement is often delayed and highly associated with poor prognosis [27, 28]. In this study, we found that the abundance of various immune cell phenotypes is associated with cardiac manifestation of sarcoidosis. In patients with CS mostly B cells and plasmablasts were upregulated in contrast to patients with no CS, hinting at an antibody-dependent autoimmunity in CS, as the humoral immune system is critical for cardiac granuloma formation [24, 29]. Likewise, upon manifestation of CS, neutrophils were increased whereas most T cell populations were downregulated in contrast to patients with no CS. In line with previous findings, an increased neutrophil lymphocyte ratio (NLR) might hint at active sarcoidosis [30]. As described recently, hybrid cardiac imaging seems to be a powerful non-invasive imaging tool for the detection of active cardiac inflammation, potential monitoring of the disease progression, with the technique of LGE predicting clinical outcome in patients with sarcoidosis [8, 11, 12]. In this study, we provide evidence that a critical shift of predominantly monocytes occurs in patients with a CS when compared to c CS hinting at a transition towards persistent inflammation. Strikingly, a suppressed $CD14^+/CD16^+$ ratio was highly associated with disease activity of CS. Thus, determination of monocyte populations to predict disease activity of CS might outperform conventional T cell markers CD4/CD8 which were shown to lack specificity and sensitivity in

patients with sarcoidosis [31, 32]. In line with previous studies suggesting that $CD14^{-}CD16^{+}$ monocytes are key players in granulomatous disease, it is tempting to speculate that a low $CD14^{+}/CD16^{+}$ ratio may hint at cardiac sarcoidosis [33]. Interestingly, this observation contrasts with recent findings indicating that $CD14^{+}$ monocytes are expanded in individuals with elevated corticosteroid levels. Therefore, particularly patients with cardiac sarcoidosis and a low $CD14^{+}/CD16^{+}$ ratio may benefit from early and intensified immunosuppressive treatment.

Monocytes are key players of the innate immunity, promote granuloma formation and are associated with disease activity in patients with sarcoidosis [20, 34]. Only recently, inflammatory cells including monocytes and platelets were shown to be a major source of circulating cytokines/chemokines promoting inflammation and autoimmunity [30, 35, 36]. Recently, $CD14^{dim}CD16^{+}$ monocytes were shown to play an important role in inflammatory homeostasis and predict outcome in patients with cardiovascular disease [18, 37]. In this study, levels of CXCR4 in platelets and non-classical monocytes were significantly associated with disease acuity thus, may indicate an underlying pathophysiological mechanism contributing to cardiac inflammation in sarcoidosis as described previously [38]. It is well-known that myocardial fibrosis is associated with poor prognosis in various cardiomyopathies [39, 40]. Thus, these findings of an altered immune cell landscape in patients with sarcoidosis might contribute to the understanding of cardiac remodeling and pathophysiological cascades promoting adverse cardiovascular events.

Further, determination of the immune signature in patients with CS might enable novel diagnostic perspectives and potential new pharmacological targets to modify disease progression.

Limitations and perspectives

Due to the intention to derive novel hypotheses promoting disease activity in patients with CS, the significance of this study is confined by the *in-vitro* assays and the observational study design with a limited number of patients. Thus, the generalizability of this study may be limited by the small cohort size and disparities in group sizes may limit statistical power and affect the robustness of multivariate analyses. To support the robustness of novel findings and to address the risk of type I errors, we applied false discovery rate (FDR) correction throughout the analysis. For multivariate analysis, we employed unsupervised UMAP clustering, which reduces overfitting and effectively captures biologically and clinically relevant patterns in heterogeneous datasets [41]. Using supervised PLS-DA analyses in our study cohort, we demonstrate that the impact of immunosuppressive comedication (e.g., corticosteroids) on the observed inflammatory fingerprint within subgroups is minor compared to the influence of disease severity and cardiac involvement in sarcoidosis patients (i.e., aCS, cCS, and noCS) (Supplementary Fig. S8). However, a larger prognostic study is warranted to contextualize these findings and to further elucidate the impact of the inflammatory landscape on granuloma formation, myocardial inflammation, and disease progression in patients with cardiac sarcoidosis. Nonetheless, the findings of our manuscript are strengthened by the prospective and multilevel experimental approach eligible to determine the association of an altered immune landscape and CS. Although we do not yield direct evidence, it is tempting to speculate that distinct immune cells and cytokines/chemokines contribute to disease progression of patients with CS and amplify the cardiovascular risk for adverse events. However, given the exploratory nature of the study, a potential impact of comorbidities and comedication including immunosuppressive therapy on the immunological fingerprint cannot be fully precluded. Especially in the setting of infiltrative disease necessitating anti-inflammatory treatment, expression of immune cell clusters in this study might similarly reflect, disease-, tissue- and treatment-related changes of the inflammatory fingerprint [16, 42, 43].

Ultimately, a better profiling of immune signatures in sarcoidosis patients may be a powerful tool to individually tailor disease-modifying, anti-inflammatory therapies and thereby minimize disease burden and improve clinical outcome.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Declarations

Competing interests The authors declare that they have no no competing interests.

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