The NLRP3 inflammasome pathway is activated in sarcoidosis and involved in granuloma formation

Christine Huppertz^{1,9}, Benedikt Jäger^{2,3,4,9}, Grazyna Wieczorek¹, Peggy Engelhard^{3,4}, Stephen J. Oliver¹, Franz-Georg Bauernfeind⁵, Amanda Littlewood-Evans¹, Tobias Welte^{6,7}, Veit Hornung⁸ and Antje Prasse^{2,4,6,7}

Affiliations: ¹Dept of Autoimmunity, Transplantation and Inflammation, Novartis Institutes for BioMedical Research, Basel, Switzerland. ²Fraunhofer ITEM, Hannover, Germany. ³Faculty of Biology, Albert Ludwig University Freiburg, Freiburg, Germany. ⁴Dept of Pneumology, University Medical Center Freiburg, Freiburg, Germany. ⁵Dept of Internal Medicine III, University Hospital Bonn, Bonn, Germany. ⁶Dept of Pulmonology, Medical School Hannover, Hannover, Germany. ⁷DZL, BREATH, Hannover, Germany. ⁸Gene Center and Dept of Biochemistry, Ludwig-Maximilians-Universität München, Munich, Germany. ⁹Both authors contributed equally.

Correspondence: Antje Prasse, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. E-mail: prasse.antje@mh-hannover.de

@ERSpublications

This pilot study underscores a pivotal role for the NLRP3 inflammasome and MIR-223 in the pathogenesis of sarcoidosis. A beneficial effect of a NLRP3 inflammasome pathway inhibitor and an anti-IL-1 β antibody on granuloma formation is demonstrated. <http://bit.ly/36StvLe>

Cite this article as: Huppertz C, Jäger B, Wieczorek G, *et al.* The NLRP3 inflammasome pathway is activated in sarcoidosis and involved in granuloma formation. *Eur Respir J* 2020; 55: 1900119 [<https://doi.org/10.1183/13993003.00119-2019>].

ABSTRACT Sarcoidosis is a disease characterised by granuloma formation. There is an unmet need for new treatment strategies beyond corticosteroids. The NLRP3 inflammasome pathway is expressed in innate immune cells and senses danger signals to elicit inflammatory interleukin (IL)-1 β ; it has recently become a druggable target. This prompted us to test the role of the NLRP3 inflammasome and IL-1 β pathway in granuloma formation and sarcoidosis.

19 sarcoid patients and 19 healthy volunteers were recruited into this pilot study. NLRP3 inflammasome activity was measured in bronchoalveolar lavage (BAL) cells and lung and skin biopsies using immunohistochemistry, Western blot, reverse-transcriptase PCR and ELISA. For *in vivo* experiments we used the trehalose 6,6'-dimycolate-granuloma mouse model and evaluated lung granuloma burden in miR-223 knockout and NLRP3 knockout mice, as well as the treatment effects of MCC950 and anti-IL-1 β antibody therapy.

We found strong upregulation of the NLRP3 inflammasome pathway, evidenced by expression of activated NLRP3 inflammasome components, including cleaved caspase-1 and IL-1 β in lung granuloma, and increased IL-1 β release of BAL cells from sarcoid patients compared to healthy volunteers ($p=0.006$). mRNA levels of miR-223, a micro-RNA downregulating NLRP3, were decreased and NLRP3 mRNA correspondingly increased in alveolar macrophages from sarcoid patients ($p<0.005$). NLRP3 knockout mice showed decreased and miR-223 knockout mice increased granuloma formation compared to wild-type mice. Pharmacological interference using NLRP3 pathway inhibitor MCC950 or an anti-IL-1 β antibody resulted in reduced granuloma formation ($p<0.02$).

In conclusion, our data provide evidence of upregulated inflammasome and IL-1 β pathway activation in sarcoidosis and suggest both as valid therapeutic targets.

This article has an editorial commentary: <https://doi.org/10.1183/13993003.00149-2020>

This article has supplementary material available from erj.ersjournals.com

Received: 17 Jan 2019 | Accepted after revision: 18 Dec 2019

Copyright ©ERS 2020

Introduction

Sarcoidosis is a systemic disease characterised by non-necrotising granuloma formation in genetically susceptible individuals [1–4]. The exact causative trigger factor is still unknown and there are likely to be various triggers [1, 5]. Findings suggest that pathogen-associated molecular patterns (PAMPs) derived from mycobacteria or propionibacteria may trigger an exaggerated immune response in sarcoid patients [5, 6]. It is thought that a misled host response to otherwise harmless PAMPs amplifies the immune response in sarcoidosis [3]. Moller's group showed that serum amyloid A (SAA) was highly expressed by sarcoid granuloma [7]. If needed, patients are treated with immunosuppressants, often for years or even decades. Long-term use of steroid treatment by patients with sarcoidosis often results in increased burden of comorbid side-effects, and new treatment concepts are urgently warranted [1, 2, 8, 9].

Over the past three decades there have been multiple studies to suggest that macrophages from sarcoidosis patients produce a broad spectrum of M1 cytokines that include tumour necrosis factor- α , CXCL10 and interleukin (IL)-1 β [10–17]. Several reports indicated a pathogenic role for IL-1 β in sarcoidosis [11–16, 18–20], almost 30 years ago. However, the IL-1 β family has recently regained tremendous interest due to the molecular delineation of the so-called inflammasome pathway, an intracellular multiprotein complex that plays a crucial role in the production and release of biologically active IL-1 β by macrophages [17, 21]. The most studied inflammasome is the NLRP3 inflammasome, which was found to be critically involved in the pathogenesis of several diseases including silicosis, tuberculosis and Alzheimer's disease [22–24]. Among others, silicate crystals and amyloid fibrils can activate the NLRP3 inflammasome [25–28]. NLRP3 inflammasome assembly requires two signals, a priming signal which results in the transcription of pro-IL-1 β and NLRP3, and a second signal that promotes indirect activation of the inflammasome by factors such as ATP or nigericin [17, 21]. NLRP3 inflammasome oligomerisation leads to the activation of caspase-1 and expression of the functionally active caspase-1 p10 and p20 subunits [17]. Activated caspase-1 subsequently cleaves pro-IL-1 β into the biologically active form of IL-1 β . Based on these new insights in inflammasome and IL-1 β immunobiology, several new treatment strategies blocking the NLRP3 inflammasome and IL-1 β signalling have been developed and even approved for certain diseases [29].

On the background of these findings we became interested in the role of the NLRP3 inflammasome in sarcoidosis. We observed that this inflammasome pathway is indeed active in human samples of sarcoidosis patients compared to healthy volunteers. Furthermore, using a mouse model of pulmonary granuloma formation, we could determine the significance of IL-1 β and inflammasome components contributing to the pathology of sarcoidosis.

Methods

Study population

This study enrolled 19 patients with sarcoidosis, six patients with interstitial lung disease in the context of scleroderma and 19 healthy volunteers (table 1). The study was conducted at the University Medical Center Freiburg, Germany and was approved by the local ethics committee. All subjects provided written informed consent before participating in the study. In 18 of the 19 sarcoidosis patients, bronchoscopy was performed within the routine diagnostic work-up at initial diagnosis. Sarcoid disease activity of the lung was defined as “mild” for inactive lung disease with normal pulmonary function and no respiratory symptoms, “moderate” for active lung disease with no requirement for immunosuppressive treatment according to the guidelines proposed by the American Thoracic Society/European Respiratory Society [30], or “severe” for active lung disease with the requirement for immunosuppressive treatment. These patients categorised with severe disease activity with need for immunosuppressive treatment had severe lung parenchymal (>20%) involvement and an inflammatory phenotype (e.g. elevated levels of soluble IL-2R and bronchoalveolar lavage (BAL) lymphocytosis). We did not recruit patients with advanced, non-inflammatory, fibrotic disease into this study. All included patients classified as suffering from severe disease underwent bronchoscopy and BAL during the routine diagnostic work-up at initial diagnosis.

BAL, transbronchial biopsies

BAL, transbronchial and skin biopsies were performed in sarcoidosis patients as part of their routine diagnostic work-up in accordance to a standardised protocol [31–33]. Further details are given in the supplementary material.

Histopathology and immunohistochemistry of human biopsies

Paraffin tissue sections from human transbronchial biopsies or skin biopsies were stained with haematoxylin and eosin and immunohistochemically, using primary antibodies specific for various markers. Further details are given in the supplementary material.

TABLE 1 Clinical characteristics of sarcoidosis patients and control subjects

	Healthy volunteers	Scleroderma patients	Sarcoidosis patients	Sarcoidosis lung disease activity		
				Mild	Moderate	Severe
Total subjects	19	6	19	5	3	11
Female	13	5	7	3	0	4
Male	6	1	12	2	3	7
Age years	29±10	49±14	48±14*	47±19	39±7	51±13
Chest radiograph type I/II/III/IV	N/A	N/A	2/14/1/2	2/3/0/0	0/3/0/0	0/8/1/2
FVC %	115±5	71±22	93±23*	118±9	93±20	82±18 [‡]
D_{LCO} %	N/A	43±21	74±20	85±23	86±21	65±13
Need for immunosuppressive treatment N/Y	N/A	6 Y	5 N/12 Y	4N/1Y [#]	3 N	11 Y
BAL differential						
Macrophages %	87±6	80±13	55±20*	64±27	61±23	49±16
Lymphocytes %	10±6	8±8	43±20*	34±27	38±23	48±16
Neutrophils %	2±1	10±7	2±1	2±2	1±0	2±1
BAL cell recovery ×10⁶ cells·100 mL⁻¹	6.2±2.4	17.0±5.0	14.4±8.1*	7.1±4.8	15.5±8.2	17.4±7.7 [‡]
Neopterin nmol·L⁻¹	N/A	N/A	15.7±8.5	12.2±6.5	9.4±2.5	18.9±9.1
sIL-2R U·mL⁻¹	N/A	N/A	1646±1702	963±805	744±219	2203±2032

Data are presented as n or mean±SD. Statistical comparisons between groups were performed using the Mann-Whitney U-test. FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; N: no treatment; Y: treatment; BAL: bronchoalveolar lavage; sIL-2R: soluble interleukin-2 receptor; N/A: not applicable. #: due to eye involvement, not due to pulmonary activity. *: p<0.05 in the comparison healthy volunteers *versus* sarcoid patients; [‡]: p<0.05 in the comparison sarcoid patients with mild disease *versus* severe disease.

Immunoblot analysis of caspase-1 p20 activity

BAL cells were stimulated with or without the NLRP3 activation protocol described in detail in the supplementary material and supernatants were precipitated with methanol/chloroform. Cleaved caspase-1 (p20) was detected in the supernatants using primary antibody rabbit mAb cleaved caspase-1 (Cell Signalling Technology, Danvers, MA, USA).

Micro-RNA analysis and reverse-transcriptase PCR

Alveolar macrophages were sorted from BAL cells from sarcoid patients and healthy volunteers on a MoFlo Astrios/Beckman Coulter (Indianapolis, IN, USA) cell sorter based on cell characteristics in the forward/sideward scatter and typical autofluorescence of alveolar macrophages. Total cellular micro-RNA from 10⁶ sorted alveolar macrophages was isolated using mirVana™ isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). Further details are given in the supplementary material.

Animal studies

NLRP3^{-/-} mice (B6 strain background) were kindly provided by Vishva M. Dixit (Genentech, South San Francisco, CA, USA). MiR-223^{-/-} mice (B6.Cg-Ptprca Mir223tm1Fcaml/J), their respective wild-type (B6.SJL-Ptprca^a Pepc^b/BoyJ) control mice and C57BL/6J wild-type mice were purchased from The Jackson Laboratory (Sacramento, CA, USA). For further details, see supplementary material.

Murine pulmonary granuloma model induced by trehalose 6,6'-dimycolate

Trehalose 6,6'-dimycolate (TDM, ALX-581-210-M001; Enzo Life Sciences, Loerrach, Germany) from *Mycobacterium tuberculosis* was prepared in a water-in-oil emulsion using incomplete Freund's adjuvant as described previously [34, 35] and injected into the tail vein. To assess pulmonary granuloma formation, lungs were harvested on day 7 after TDM injection for all studies. In addition, wild-type mice were administered vehicle (PBS) or MCC950, a NLRP3 inflammasome pathway inhibitor, at 10 mg·kg⁻¹ intraperitoneally on days 0, 1, 2, 4 and 6 after TDM injection. In another experiment, wild-type mice were administered subcutaneously with either vehicle (PBS), isotype control antibody anti-cyclosporine (Novartis, Basel, Switzerland) (200 µg per mouse) or anti-IL-1β antibody (Novartis) (200 µg per mouse) on day -1 and again 3 days after TDM injection. Further details are given in the supplementary material.

Results

Study cohort

Clinical characteristics and BAL differentials are given in table 1. Of the 19 patients with sarcoidosis, 11 had severe lung disease activity with significantly decreased lung function and requirement for immunosuppressive treatment because of lung involvement, three had moderate and five had mild lung

disease activity (table 1). None of the patients classified as moderate or mild were in need of immunosuppressive treatment because of lung involvement. All six scleroderma patients had significant lung disease and underwent bronchoscopy because of respiratory deterioration and need for immunosuppressive treatment. All of the study participants were nonsmokers. All subjects were of Caucasian ethnicity except for one African-American. All sarcoid patients were treatment-naïve except one patient with severe disease who received low-dose prednisone (5 mg·day⁻¹). As expected, BAL of sarcoid patients contained increased total cell counts and a significantly higher proportion of lymphocytes compared to BAL from healthy volunteers, while percentage of alveolar macrophages was reduced (table 1). The mean age of all sarcoid patients was significantly increased compared to the mean age of healthy volunteers ($p<0.0001$), while the age across the patients grouped by pulmonary disease activity did not differ significantly. Importantly, there was no statistically significant effect of age on all outcomes described below.

Evidence of inflammasome activation and IL-1 β expression in sarcoid granulomas

Sarcoid granulomas were composed of CD68⁺ macrophages (figure 1a and b). Expression of active caspase-1 depicted with an antibody against cleaved/active caspase-1 (for characterisation see supplementary figure S1) was shown in areas that corresponded to CD68⁺ granulomas frequently containing multinucleated giant cells (figure 1d and e). This pattern was observed in almost all cases of transbronchial and skin biopsies (figure 1a–f, supplementary figures S2 and S3). Immunoreactivity for IL-1 β was observed within the granulomas in all biopsies (figure 1g and h, supplementary figures S2 and S3). Sarcoid granulomas were thus composed primarily of CD68⁺ macrophages which stained positively for active caspase-1 and IL-1 β . For IL-1 β staining, a blinded, semi-quantitative scoring by an expert pathologist was performed and revealed a trend for higher IL-1 β expression in lung granulomas from patients with severe lung disease compared with patients with milder disease ($n=13$, $p=0.1111$; figure 1l).

Evidence for an activated NLRP3 inflammasome pathway in BAL cells and monocytes derived from sarcoid patients

The constitutive release of IL-1 β from sarcoid BAL cells was significantly increased compared to levels determined for BAL cells from healthy volunteers ($p=0.0065$; figure 2a) or scleroderma patients ($p=0.038$) (figure 2a). Furthermore, BAL cells from the sarcoid patients with severe disease showed significantly increased constitutive IL-1 β production compared with healthy volunteers ($p=0.0161$; figure 2b). Compared to constitutive IL-1 β release, upon *ex vivo* stimulation of the NLRP3 inflammasome with lipopolysaccharide (LPS)/nigericin, there was at least a 10-fold increase in IL-1 β production from BAL cells derived from healthy volunteers and from sarcoid patients (figure 2a–c). BAL cells obtained from sarcoidosis patients elicited significantly more IL-1 β compared to those from healthy volunteers upon NLRP3 inflammasome stimulation ($p<0.0001$; figure 2c), and this ability increased with the activity of lung disease (mild *versus* severe disease activity, $p=0.0020$; figure 2d). Similar findings were obtained upon NLRP3 inflammasome stimulation with LPS/ATP (figure 2d). Western blot analysis confirmed active IL-1 β production by BAL cells (supplementary figure S4). Expression and activation state of the caspase-1 enzyme was studied in BAL cells from healthy volunteers and sarcoid patients by Western blotting. We found increased constitutive expression of the activated caspase-1 p20 in the conditioned medium of unstimulated BAL cells from sarcoid patients compared to BAL cells from healthy volunteers (figure 2e). Levels of activated caspase-1 p20 were enhanced in conditioned medium of LPS/nigericin- or LPS/ATP-stimulated BAL cells, again with BAL cells derived from sarcoid patients containing higher levels than healthy-volunteer samples (figure 2e).

In addition, in the same cohort we studied inflammasome activation of monocytes isolated from peripheral blood. Of note, the NLRP3 inflammasome and IL-1 β release is differently activated and regulated in monocytes compared to macrophages. Monocytes from sarcoid patients showed an increased release in IL-1 β following LPS stimulation in contrast to monocytes from healthy volunteers ($p=0.0069$; supplementary figure S5).

Since miR-223 has been shown previously to downregulate NLRP3 transcription in mouse bone-marrow derived cells by our group [36], we studied the RNA expression of miR-223 and NLRP3 in fluorescence-activated cell-sorted alveolar macrophages by reverse-transcriptase PCR. We found a significant decrease in relative miR-223 expression ($p=0.0002$; figure 2f) and a significant increase in relative NLRP3 expression ($p=0.0023$; figure 2g) in sorted alveolar macrophages from sarcoid patients compared to healthy volunteers. Thus, reduced miR-223 levels probably contribute to increased NLRP3 expression and NLRP3 inflammasome pathway activation in sarcoidosis.

Downstream effects of IL-1 β signalling are upregulated in sarcoid patient samples

Because of the increased IL-1 β levels observed in sarcoid patients, we became interested in downstream effects of IL-1 β signalling and investigated the expression of IL-1 receptor antagonist (IL-1ra) and IL-8,

FIGURE 1 Evidence for inflammasome and interleukin (IL)-1 β pathway activation in sarcoid lung granulomas. a–k) Representative photomicrographs of consecutive sections of tissues derived by transbronchial biopsy (TBB) from a sarcoid patient with severe lung disease activity. a–i) TBB tissue was immunohistochemically stained using antibodies against the macrophage lineage marker CD68 (a, b), activated caspase-1 (d, e) and IL-1 β (g, h). c, f, i) Negative control stainings were obtained by applying respective isotype control antibodies. j, k) TBB tissue was stained with haematoxylin and eosin (H&E) for histopathological assessment, indicating non-necrotising sarcoid granuloma. Scale bars=100 μ m (a, d, g, j), 200 μ m (b, c, e, f, h, i, k). l) Quantification of IL-1 β immunoreactivity in lung granuloma is depicted in relation to pulmonary disease activity. IL-1 β staining was blindly scored semi-quantitatively for TBBs derived from 13 patients.

both of which are inducible by IL-1 β . Constitutive levels of IL-1 α released from BAL cells cultured *ex vivo* for 24 h were highly elevated for sarcoid patients compared to healthy volunteers ($p=0.0038$; figure 3a) and increased with disease activity ($p=0.0001$; figure 3b). In addition, the levels of the neutrophil chemoattractant IL-8 released by BAL cells were increased in sarcoidosis ($p=0.0074$ and $p=0.0004$; figure 3c

FIGURE 2 Evidence for an activated NLRP3 inflammasome pathway in bronchoalveolar lavage (BAL) cells from sarcoid patients. **a–e**) BAL cells from healthy volunteers (HV), patients with scleroderma interstitial lung disease (SSc) or patients with sarcoidosis (Sarcoid) were left unstimulated for 6 h (**a**, **b**) or stimulated for 6 h with lipopolysaccharide (LPS)+nigericin (Nig) or LPS+ATP to activate the NLRP3 inflammasome (**c–e**) and levels of IL-1 β and the cleaved subunit caspase-1 p20 were measured in the cell culture supernatants by ELISA and Western blotting. **a**, **c**) IL-1 β levels from healthy volunteers ($n=18$), sarcoid patients ($n=17$) and patients with SSc-ILD ($n=6$); **b**, **d**) IL-1 β levels from sarcoid patients across pulmonary disease activity. **e**) Representative immunoblot analysis for the cleaved caspase-1 subunit p20, which results from caspase-1 activation by the inflammasome. **f**, **g**) In an additional study, alveolar macrophages were sorted from BAL cells from 20 severe sarcoid patients with progressive disease and need for combined immunosuppressive treatment and seven healthy volunteers; **f**) miR-223 expression relative to MammU6 and **g**) NLRP3 relative to GAPDH expression was determined by reverse-transcriptase PCR. Bars depict the median. *: $p<0.05$, **: $p<0.01$, Mann-Whitney U-test.

and **d**). We found a statistically significant correlation between IL-1 α and IL-8 levels ($r^2=0.860$, $p<0.0001$; supplementary figure S6) and between constitutive IL-1 β and IL-8 production ($r^2=0.559$, $p=0.0006$; supplementary figure S6). Neutrophils comprised only a low fraction of the overall BAL cell populations recovered from both sarcoid patients and healthy volunteers, as expected. However, neutrophil counts when normalised per total cell counts/100 mL BAL fluid were increased in sarcoid patients compared to healthy volunteers ($p=0.0256$), and were especially significantly increased in patients with severe compared to mild disease ($p=0.0018$; table 1). Furthermore, blood neutrophil counts in sarcoid patients with severe lung disease activity were significantly elevated compared to healthy volunteers ($p=0.0458$; figure 3e).

FIGURE 3 Constitutive interleukin-1 receptor antagonist (IL-1ra) and IL-8 release from bronchoalveolar lavage (BAL) cells and increased neutrophil counts in sarcoidosis are indicative of IL-1 β pathway activation. a–d) BAL cells from healthy volunteers (HV) or sarcoid patients were cultured without further stimulation for 24 h and levels of IL-1ra [a, b] and IL-8 [c, d] were measured in the conditioned medium. a) IL-1ra production of BAL cells from healthy volunteers (n=17) and sarcoid patients (n=18); b) IL-1ra production of BAL cells from sarcoid patients across pulmonary disease activity. c, d) IL-8 production from BAL cells accordingly (HV n=13, sarcoid n=18). e, f) Blood neutrophil counts determined by fluorescence-activated cell-sorting for HV (n=17) and sarcoid patients (n=16) and in accordance to pulmonary disease activity. Bars depict the median. *: p<0.05, **: p<0.01, Mann-Whitney U-test.

Upregulated SAA levels in sarcoid patients perpetuate NLRP3 inflammasome activity

As SAA has been proposed as a main driver in the pathogenesis of sarcoidosis and as an activator of the NLRP3 inflammasome [7], we analysed the expression and effects of SAA in the context of the NLRP3 inflammasome pathway; the details of these analyses are provided in supplementary figure S7.

The NLRP3 inflammasome pathway is active in a mouse model of granuloma formation

The glycolipid TDM when injected intravenously induces pulmonary granuloma in mice. Representative photomicrographs of pulmonary granuloma induced by TDM in wild-type mice are shown in figure 4a–c and supplementary figures S8 and S9. Total granuloma burden of the left lobe of the lung was counted and exemplified in figure 4b. We investigated the functional responsiveness of the murine BAL cells to NLRP3 inflammasome stimulation by LPS/nigericin (and LPS/ATP). We found increased IL-1 β release from the TDM-treated *versus* untreated mice (p=0.0006; figure 4d), reflecting an increased inflammasome activation in this model. Consecutive immunohistochemically stained sections revealed an accumulation of Iba-1⁺ macrophages (figure 4e and f, supplementary figures S8 and S9) and expression of IL-1 β in the granuloma corresponding to the same area (figure 4g, supplementary figure S9). Furthermore, we could

FIGURE 4 Trehalose 6,6'-dimycolate (TDM)-induced pulmonary granuloma in mice resemble human sarcoid granuloma. Involvement of the NLRP3 inflammasome pathway and reduced pulmonary granuloma burden in mice treated with compounds targeting the NLRP3 inflammasome. a) Haematoxylin and eosin (H&E) stain of lung harvested on day 7 from C57/BL6 wild-type (WT) mice challenged with TDM. b) Counting strategy for granuloma burden of the left lobe of the lung. c) High magnification of typical TDM-induced granulomas in mouse lung. d) Interleukin (IL)-1 β release from BAL cells stimulated *ex vivo* with lipopolysaccharide/nigericin (6 h) was determined in the supernatants. e) H&E-stained section of lung granulomas; red and green circles indicate granuloma formation. Higher magnification images are shown in f and g. f–h) Immunohistochemistry of the TDM-induced granuloma using antibodies against f) the mouse macrophage lineage marker Iba-1, g) IL-1 β or h) serum amyloid A (SAA). i–k) H&E stains of lung tissues from i) WT mice, j) miR-223 $^{-/-}$ mice and k) NLRP3 $^{-/-}$ mice 7 days after TDM injection (each $n=6$). l) Granuloma counts of lung tissues from WT mice, miR-223 $^{-/-}$ mice and NLRP3 $^{-/-}$ mice. m) H&E stains of lungs from TDM-treated C57/BL6 WT mice treated with vehicle or the NLRP3 inflammasome pathway inhibitor MCC950 (each $n=2$). n) Reduced granuloma counts in lung tissues from MCC950-treated mice compared to vehicle-treated mice. o) H&E stains of lung from TDM-treated C57/BL6 WT mice treated with isotype control antibody or anti-IL-1 β antibody. p) Significantly reduced granuloma counts in lung tissues from anti-IL-1 β -treated mice compared to mice treated with isotype control antibody or vehicle (each $n=8$). Data are presented as individual values and mean $\pm$ SD. Scale bars a, b) 1000 μ m, c) 10 μ m, e–g, i–k) 500 μ m. *: $p<0.05$, **: $p<0.01$; d, n) unpaired t-test, l, p) unpaired ANOVA.

show SAA staining in the TDM-induced granulomas (figure 4h, supplementary figure S9). To address the impact of the NLRP3 inflammasome and miR-223 on granuloma formation, we analysed the effects of genetic ablation of these molecules in the TDM model. We found significantly increased granuloma counts in lungs from miR-223 $^{-/-}$ mice ($p=0.0016$) and reduced granuloma counts in lungs from NLRP3 $^{-/-}$ mice ($p<0.0001$) compared to wild-type mice (figure 4i–l). Moreover, in miR-223 knockout mice we observed larger and more confluent granuloma than in wild-type or NLRP3 $^{-/-}$ mice.

To assess the effects of pharmacological intervention of the NLRP3 inflammasome pathway, the NLRP3 inflammasome pathway inhibitor MCC950 was administered for 7 days to the TDM-treated wild-type

mice, resulting in a significant reduction of pulmonary granuloma formation ($p=0.002$; figure 4m and n). Similarly, treatment with anti-IL-1 β antibodies significantly reduced granuloma counts compared to vehicle control ($p=0.0184$; figure 4o and p) and isotype control antibody ($p=0.021$; figure 4p).

Discussion

Sarcoidosis is a granulomatous disease with a high unmet medical need which urgently requires new treatment strategies. In this article we demonstrate the significance of the inflammasome as a key factor in sarcoidosis development and disease severity and identify this pathway as a potential treatment target for sarcoidosis. We show that inflammasome components and active IL-1 β are expressed by macrophages and multinucleated giant cells within the sarcoid granuloma. Furthermore, in contrast to healthy volunteers, the NLRP3 inflammasome is constitutively activated in alveolar macrophages from patients with sarcoidosis and levels of NLRP3 inflammasome activation correlate with disease activity. These results demonstrate clearly that NLRP3 inflammasome activation takes place within the granuloma and appears to be initiated by signals present in the lesion. Furthermore, we provide supportive preclinical data in an experimental model of sarcoidosis in which granuloma formation is induced by the mycobacterial derived glycolipid TDM, to demonstrate the amplifying role of NLRP3 inflammasome activation in pulmonary granuloma formation.

Immunohistochemistry revealed that both active caspase-1 and IL-1 β protein are highly expressed by multinucleated giant cells within the sarcoid granuloma as well as by sarcoid tissue macrophages, but not in normal lung and skin. BAL cells from patients with sarcoidosis spontaneously released both IL-1 β and the active subunit of caspase-1 in contrast to cells derived from healthy volunteers or scleroderma patients. Moreover, a stimulation protocol resulting in NLRP3 inflammasome activation demonstrated NLRP3 inflammasome hyperactivation in sarcoid BAL cells compared to healthy cells. Recent findings highlight the crucial role of inflammasomes as sensors of pathogens and danger signals [14–16]; the body needs to differentiate between harmless and deleterious PAMPs and damage-associated molecular patterns, and inflammasomes help direct the innate immune responses and maintain lung tissue homeostasis. Thus, under normal conditions, inflammasome activation and IL-1 β production are tightly regulated because of their potentially deleterious effects [17, 21]. However, in our sarcoid patient cohort we found dysregulated NLRP3 inflammasome activation and consecutive IL-1 β release to be highly increased in patients with severe disease, and a need for immunosuppressive treatment. Almost 30 years ago researchers noted a correlation of BAL fluid IL-1 β levels with disease activity [13, 14], increased IL-1 β gene expression of sarcoid alveolar macrophages and increased IL-1 α levels [20, 37, 38], which we now attribute to enhanced inflammasome activity in this study. Of note, in familial sarcoidosis, the Blau syndrome, a mutation in the Nod2 gene causing activation of NF- κ B via an alternative NLR pathway was described [39, 40]. Moreover, in our sarcoid cohort, increased IL-1 β release was associated with downstream IL-1R1 signalling events such as elevated IL-1 α and IL-8 levels and ensuing neutrophil influx. Higher BAL neutrophil counts were reported in more severely affected patients with need for immunosuppressive treatment. [41, 42]. In addition, increased IL-1 β production by antigen-presenting cells such as macrophages and increased IL-1R signalling drives generation of TH17.1 cells, which were recently found to be increased in sarcoidosis and may act as a key pathogenetic element [43–45]. Thus, our findings demonstrate that constitutive NLRP3 inflammasome activation in sarcoidosis appears to be of both pathophysiological and clinical relevance.

Recent immunohistopathological analysis shows accumulation of the acute phase protein SAA in the centre of the sarcoid granuloma in close proximity to multinucleated giant cells [7, 46]. Indeed, by its distribution and proximity to macrophages and correlation with CD3⁺ cell influx, the authors claim that SAA coordinates innate and adaptive immune responses to aid development of granulomatous inflammation in sarcoidosis and potentially other granulomatous diseases. Furthermore, the authors use a murine experimental model of sarcoidosis to substantiate their theory that SAA contributes to development of granulomas. Others suggest NLRP3 inflammasome activation by SAA in human and mouse macrophages *in vitro*, in human psoriatic keratinocytes *ex vivo* and preclinically in murine asthma models [25–27]. We observed that in line with previously published data, serum SAA levels were increased in our cohort of sarcoid patients [47, 48]. Following stimulation with SAA, BAL cells from patients with sarcoidosis released IL-1 β in contrast to cells derived from healthy volunteers. Furthermore, SAA-induced IL-1 β production by peripheral blood monocytes was efficiently blocked by the caspase-1 inhibitor VX-740 and the NLRP3 inflammasome inhibitor MCC950. In the light of a previous publication [7], our data suggest that SAA may be a potential endogenous damage-associated molecular pattern capable of triggering NLRP3 inflammasome activation in sarcoidosis. To substantiate this, we also found accumulation of SAA in the granulomatous lesions in our murine model, where mycobacteria-derived TDM evokes pulmonary granuloma. Further, TDM-induced granuloma formation was markedly attenuated in NLRP3^{−/−} mice compared to wild-type animals.

One additional mechanism by which NLRP3 inflammasome activation is regulated is through miR-223, which directly targets NLRP3 expression levels [36, 49, 50]. In comparison to alveolar macrophages from healthy volunteers, miR-223 expression levels of sarcoid alveolar macrophages were significantly reduced, while NLRP3 expression was significantly upregulated. Since NLRP3 expression levels are closely correlated with NLRP3 inflammasome activity, our findings suggest that reduced miR-223 expression of sarcoid macrophages may contribute to the constitutive activation of the NLRP3 inflammasome. Since miR-223-deficient mice showed an increase in NLRP3 inflammasome activity and granuloma formation compared to the respective wild-type animals in the sarcoidosis model *in vivo*, this assumption is reinforced. Thus, our data suggest a pivotal role of the NLRP3 inflammasome and miR-223 depletion in amplifying granuloma formation.

We used the same TDM granuloma model to test therapeutic strategies which interfere with NLRP3 inflammasome activation and IL-1 β signalling. MCC950 is a recently developed small molecule inhibiting NLRP3 inflammasome assembly and activation [29]. Mice treated with MCC950 showed significantly reduced lung granuloma burden following TDM stimulation compared to vehicle-treated mice, suggesting that this new treatment strategy has a direct effect on granuloma formation. Moreover, mice treated with anti-IL-1 β antibody showed significantly reduced granuloma burden following TDM stimulation compared to mice treated with vehicle or isotype control antibody. Taken together, these data suggest that the NLRP3 inflammasome pathway is active in this mouse model of sarcoidosis.

Our findings are limited by the fact that TDM-induced granuloma formation in mice is acute and transient with spontaneous resolution that occurs after 10 days. Furthermore, ultrastructural analysis showed accumulation of macrophages but not epithelioid cells in the centre of the evolving granuloma [51]. However, we lack currently any accepted mouse model for sarcoidosis. In our hands the TDM model is superior to all other models that we tested. Similar to granuloma in human sarcoidosis, TDM-induced granulomas are composed of multinucleated giant which express IL-1 β and SAA. Moreover, as with sarcoidosis, TDM-induced granuloma is considered to involve mycobacterial PAMPs as a possible trigger in the initial formation of granuloma. Mycobacterial-derived PAMPs have been identified in sarcoid granuloma and Kveim reagent [6, 52]. Based on these considerations, we think that the TDM-induced granuloma model is one of the best currently available models for granuloma formation in sarcoidosis. However, future *in vivo* studies are needed to analyse the effect of NLRP3 inflammasome blockade on already established lung granuloma. Our human studies are limited by the fact that we were not able to detect NLRP3 expression in sarcoid granuloma by immunohistochemistry due to a lack of a specific antibody giving reliable stainings in formalin-fixed tissues. Although our reverse-transcriptase PCR experiments and functional experiments suggest NLRP3 inflammasome activation upregulated in macrophages of patients with severe sarcoidosis and need for immunosuppressive treatment, we cannot exclude that other inflammasome subtypes may additionally be activated. Moreover, since we did not perform positron emission tomography/computed tomography scanning in our two sarcoid cohorts we cannot provide the information whether NLRP3 inflammasome activation and NLRP3 expression is increased in BAL cells of patients with advanced fibrotic disease and less inflammation.

In conclusion, our studies underscore a role for the NLRP3 inflammasome in sarcoidosis. Our data suggest a pivotal role of NLRP3 inflammasome activation in granuloma formation and demonstrate a beneficial effect of a NLRP3 inflammasome pathway inhibitor on granuloma formation. These data warrant future studies addressing NLRP3 inflammasome inhibition in patients with sarcoidosis. The clinical relevance of inflammasome activation in sarcoidosis pathobiology is currently being explored in an ongoing trial of therapeutic IL-1 β blockade in patients with active pulmonary sarcoidosis (www.clinicaltrials.gov NCT02888080).

Acknowledgements: We would like to thank Victoria Wirtz (Fraunhofer ITEM, Hannover, Germany) and Marija Curcic, Antje Marcantonio and Gaelle Elain (all Novartis, Basel, Switzerland) for expert technical assistance.

Author contributions: Conception and design: C. Huppertz, B. Jäger, G. Wieczorek S.J. Oliver, F-G. Bauernfeind, V. Hornung and A. Prasse. Experiments with human materials and analyses: C. Huppertz, B. Jäger, G. Wieczorek, S.J. Oliver, F-G. Bauernfeind and A. Prasse. Experiments with mice and murine materials and analyses: C. Huppertz, P. Engelhard, B. Jäger, G. Wieczorek, F-G. Bauernfeind and A. Prasse. Manuscript preparation: C. Huppertz, B. Jäger, G. Wieczorek, S.J. Oliver, P. Engelhard, A. Littlewood-Evans, F-G. Bauernfeind, V. Hornung and A. Prasse.

Conflict of interest: C. Huppertz is an employee of and owns shares in Novartis Pharma AG. B. Jäger has nothing to disclose. G. Wieczorek is an employee of and owns shares in Novartis Pharma AG. P. Engelhard has nothing to disclose. S.J. Oliver is an employee of and owns shares in Novartis Pharma AG. F-G. Bauernfeind has nothing to disclose. A. Littlewood-Evans is an employee of and owns shares in Novartis Pharma AG. T. Welte reports grants from Novartis, during the conduct of the study; personal fees for advisory board work from Novartis, outside the submitted work. V. Hornung has nothing to disclose. A. Prasse reports grants from Novartis, during the conduct of the study; grants and personal fees for lectures from Boehringer Ingelheim and AstraZeneca, personal fees for lectures from Roche, Novartis and Sanofi Aventis, outside the submitted work.

Support statement: Part of this work was funded by a research grant from Novartis to the University Medical Center Freiburg (PI: A. Prasse). Further funding was provided by DZL BREATH. Funding information for this article has been deposited with the Crossref Funder Registry.

References

- Valeyre D, Prasse A, Nunes H, *et al.* Sarcoidosis. *Lancet* 2014; 383: 1155–1167.
- Baughman RP, Culver DA, Judson MA. A concise review of pulmonary sarcoidosis. *Am J Respir Crit Care Med* 2011; 183: 573–581.
- Chen ES, Moller DR. Sarcoidosis – scientific progress and clinical challenges. *Nat Rev Rheumatol* 2011; 7: 457–467.
- Spagnolo P, Rossi G, Trisolini R, *et al.* Pulmonary sarcoidosis. *Lancet Respir Med* 2018; 6: 389–402.
- Moller DR. Potential etiologic agents in sarcoidosis. *Proc Am Thorac Soc* 2007; 4: 465–468.
- Eishi Y. Etiologic link between sarcoidosis and *Propionibacterium acnes*. *Respir Investig* 2013; 51: 56–68.
- Chen ES, Song Z, Willett MH, *et al.* Serum amyloid A regulates granulomatous inflammation in sarcoidosis through Toll-like receptor-2. *Am J Respir Crit Care Med* 2010; 181: 360–373.
- Vorselaars AD, van Moersel CH, Deneer VH, *et al.* Current therapy in sarcoidosis, the role of existing drugs and future medicine. *Inflamm Allergy Drug Targets* 2013; 12: 369–377.
- Broos CE, Wapenaar M, Looman CWN, *et al.* Daily home spirometry to detect early steroid treatment effects in newly treated pulmonary sarcoidosis. *Eur Respir J* 2018; 51: 1702089.
- Müller-Quernheim J, Pfeifer S, Männel D, *et al.* Lung-restricted activation of the alveolar macrophage/monocyte system in pulmonary sarcoidosis. *Am Rev Respir Dis* 1992; 145: 187–192.
- Kline JN, Schwartz DA, Monick MM, *et al.* Relative release of interleukin-1 β and interleukin-1 receptor antagonist by alveolar macrophages. A study in asbestos-induced lung disease, sarcoidosis, and idiopathic pulmonary fibrosis. *Chest* 1993; 104: 47–53.
- Steffen M, Petersen J, Oldigs M, *et al.* Increased secretion of tumor necrosis factor- α , interleukin-1- β , and interleukin-6 by alveolar macrophages from patients with sarcoidosis. *J Allergy Clin Immunol* 1993; 91: 939–949.
- Mikuniya T, Nagai S, Takeuchi M, *et al.* Significance of the interleukin-1 receptor antagonist/interleukin-1 β ratio as a prognostic factor in patients with pulmonary sarcoidosis. *Respiration* 2000; 67: 389–396.
- Pueringer RJ, Schwartz DA, Dayton CS, *et al.* The relationship between alveolar macrophage TNF, IL-1, and PGE2 release, alveolitis, and disease severity in sarcoidosis. *Chest* 1993; 103: 832–838.
- Prior C, Knight RA, Herold M, *et al.* Pulmonary sarcoidosis: patterns of cytokine release *in vitro*. *Eur Respir J* 1996; 9: 47–53.
- Marshall BG, Wangoo A, Cook HT, *et al.* Increased inflammatory cytokines and new collagen formation in cutaneous tuberculosis and sarcoidosis. *Thorax* 1996; 51: 1253–1261.
- Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013; 13: 397–411.
- Talreja J, Talwar H, Ahmad N, *et al.* Dual inhibition of Rip2 and IRAK1/4 regulates IL-1 β and IL-6 in sarcoidosis alveolar macrophages and peripheral blood mononuclear cells. *J Immunol* 2016; 197: 1368–1378.
- Rastogi R, Du W, Ju D, *et al.* Dysregulation of p38 and MKP-1 in response to NOD1/TLR4 stimulation in sarcoid bronchoalveolar cells. *Am J Respir Crit Care Med* 2011; 183: 500–510.
- Kern JA, Lamb RJ, Reed JC, *et al.* Interleukin-1- β gene expression in human monocytes and alveolar macrophages from normal subjects and patients with sarcoidosis. *Am Rev Respir Dis* 1988; 137: 1180–1184.
- Bauernfeind F, Hornung V. Of inflammasomes and pathogens – sensing of microbes by the inflammasome. *EMBO Mol Med* 2013; 5: 814–826.
- Hornung V, Bauernfeind F, Halle A, *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 2008; 9: 847–856.
- Dostert C, Pétrilli V, Van Bruggen R, *et al.* Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008; 320: 674–677.
- Cassel SL, Eisenbarth SC, Iyer SS, *et al.* The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci USA* 2008; 105: 9035–9040.
- Ather JL, Ckless K, Martin R, *et al.* Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J Immunol* 2011; 187: 64–73.
- Niemi K, Teirilä L, Lappalainen J, *et al.* Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. *J Immunol* 2011; 186: 6119–6128.
- Yu N, Liu S, Yi X, *et al.* Serum amyloid A induces interleukin-1 β secretion from keratinocytes via the NACHT, LRR and PYD domains-containing protein 3 inflammasome. *Clin Exp Immunol* 2015; 179: 344–353.
- Halle A, Hornung V, Petzold GC, *et al.* The NALP3 inflammasome is involved in the innate immune response to amyloid- β . *Nat Immunol* 2008; 9: 857–865.
- Coll RC, Robertson AA, Chae JJ, *et al.* A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med* 2015; 21: 248–255.
- Hunninghake GW, Costabel U, Ando M, *et al.* ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 1999; 16: 149–173.
- Prasse A, Katic C, Germann M, *et al.* Phenotyping sarcoidosis from a pulmonary perspective. *Am J Respir Crit Care Med* 2008; 177: 330–336.
- Prasse A, Zissel G, Lützen N, *et al.* Inhaled vasoactive intestinal peptide exerts immunoregulatory effects in sarcoidosis. *Am J Respir Crit Care Med* 2010; 182: 540–548.
- Prasse A, Pechkovsky DV, Toews GB, *et al.* A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *Am J Respir Crit Care Med* 2006; 173: 781–792.
- Guidry TV, Hunter RL, Actor JK. Mycobacterial glycolipid trehalose 6,6'-dimycolate-induced hypersensitive granulomas: contribution of CD4⁺ lymphocytes. *Microbiology* 2007; 153: 3360–3369.
- Perez RL, Roman J, Roser S, *et al.* Cytokine message and protein expression during lung granuloma formation and resolution induced by the mycobacterial cord factor trehalose-6,6'-dimycolate. *J Interferon Cytokine Res* 2000; 20: 795–804.

- 36 Bauernfeind F, Rieger A, Schildberg FA, *et al.* NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 2012; 189: 4175–4181.
- 37 Wewers MD, Saltini C, Sellers S, *et al.* Evaluation of alveolar macrophages in normals and individuals with active pulmonary sarcoidosis for the spontaneous expression of the interleukin-1 β gene. *Cell Immunol* 1987; 107: 479–488.
- 38 Rolfe MW, Standiford TJ, Kunkel SL, *et al.* Interleukin-1 receptor antagonist expression in sarcoidosis. *Am Rev Respir Dis* 1993; 148: 1378–1384.
- 39 Besnard V, Calender A, Bouvry D, *et al.* G908R NOD2 variant in a family with sarcoidosis. *Respir Res* 2018; 19: 44.
- 40 Bello GA, Adrianto I, Dumancas GG, *et al.* Role of NOD2 pathway genes in sarcoidosis cases with clinical characteristics of Blau syndrome. *Am J Respir Crit Care Med* 2015; 192: 1133–1135.
- 41 Ziegenhagen MW, Rothe ME, Schlaak M, *et al.* Bronchoalveolar and serological parameters reflecting the severity of sarcoidosis. *Eur Respir J* 2003; 21: 407–413.
- 42 Drent M, Jacobs JA, de Vries J, *et al.* Does the cellular bronchoalveolar lavage fluid profile reflect the severity of sarcoidosis? *Eur Respir J* 1999; 13: 1338–1344.
- 43 Broos CE, Koth LL, van Nimwegen M, *et al.* Increased T-helper 17.1 cells in sarcoidosis mediastinal lymph nodes. *Eur Respir J* 2018; 51: 1701124.
- 44 Zielinski CE, Mele F, Aschenbrenner D, *et al.* Pathogen-induced human TH17 cells produce IFN- γ or IL-10 and are regulated by IL-1 β . *Nature* 2012; 484: 514–518.
- 45 Deng J, Yu XQ, Wang PH. Inflammasome activation and Th17 responses. *Mol Immunol* 2019; 107: 142–164.
- 46 Huho A, Foulke L, Jennings T, *et al.* The role of serum amyloid A staining of granulomatous tissues for the diagnosis of sarcoidosis. *Respir Med* 2017; 126: 1–8.
- 47 Bargagli E, Magi B, Olivieri C, *et al.* Analysis of serum amyloid A in sarcoidosis patients. *Respir Med* 2011; 105: 775–780.
- 48 Rothkrantz-Kos S, van Dieijen-Visser MP, Mulder PG, *et al.* Potential usefulness of inflammatory markers to monitor respiratory functional impairment in sarcoidosis. *Clin Chem* 2003; 49: 1510–1517.
- 49 Haneklaus M, Gerlic M, Kurowska-Stolarska M, *et al.* Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1 β production. *J Immunol* 2012; 189: 3795–3799.
- 50 Neudecker V, Haneklaus M, Jensen O, *et al.* Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. *J Exp Med* 2017; 214: 1737–1752.
- 51 Soler P, Bernaudin JF, Basset F. Ultrastructure of pulmonary granulomatosis induced in rats by intravenous complete Freund's adjuvant. *Virchows Arch A Pathol Anat Histol* 1975; 368: 35–50.
- 52 Schupp JC, Tchaptchet S, Lützen N, *et al.* Immune response to *Propionibacterium acnes* in patients with sarcoidosis – *in vivo* and *in vitro*. *BMC Pulm Med* 2015; 15: 75.