

## **Supplement to**

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

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## Supplementary Methods

### *Bronchoalveolar lavage, transbronchial biopsies and laboratory chemistry*

Bronchoalveolar lavage (BAL) was performed in healthy volunteers and sarcoidosis patients as part of their routine diagnostic work-up in accordance to a standardized protocol.<sup>1-3</sup> The BAL was pooled, filtered through two layers of gauze, and centrifuged at 500 g for 10 minutes at 4°C. The cells were counted and cell smears were stained with May-Grunwald-Giemsa stain (Merck, Germany) for the cell differentials. Following this, BAL cells were further processed for functional assays, sorting of alveolar macrophages and RNA extraction as described below. In addition, transbronchial biopsies (TBB) were obtained from sarcoid patients during the routine diagnostic work-up and further processed for histopathology and immunohistochemistry. Skin biopsies from sarcoid patients were also obtained within the routine diagnostic work-up at the Department of Dermatology, University Medical Center Freiburg. The standard laboratory chemistry parameters, serum sIL-2R and serum neopterin, were measured by routine laboratory chemistry at the University Medical Center Freiburg using a standardized protocol.<sup>1</sup> Serum levels of SAA were determined by ELISA (Invitrogen/Thermo Fisher Scientific, USA).

### *Histopathology and immunohistochemistry of human biopsies*

Tissues from human transbronchial biopsies or skin biopsies were fixed for 24 hours in 10% normal buffered formalin, dehydrated through graded alcohol and xylene and embedded in paraffin. Three-µm-thick sections were cut and stained with hematoxylin and eosin (H&E) and immunohistochemically, using primary antibodies specific for human macrophage lineage marker CD68 (clone KP-1, Dako, Denmark, 1:500), for cleaved caspase-1, D210 (rabbit polyclonal, YC0002, ImmunoWay Biotechnology, USA, 1:50) and IL-1β (goat polyclonal, raised against *E. coli*-derived recombinant human IL-1β /IL-1F2 Ala117-Ser269, AF-201-NA, R&D Systems, UK, 1:10) and secondary reagents, including for CD68, a goat-anti mouse-biotinylated antibody (Vector Laboratories, USA, 1:200); for caspase-1, the Envision+ system anti-rabbit (Dako, Ready to use kit); and for IL-1β, a horse anti-goat-biotinylated antibody (Vector Laboratories, 1:200). Details of the methods are provided in table below. Negative control staining was performed using matched concentrations of isotype control antibodies. Sections from human healthy skin and tonsils were stained in parallel. To further assess specificity of the cleaved/active caspase-1 antibody, staining was performed on paraffin-embedded THP-1 cells with and without prior stimulation with 100



ng/ml LPS for 4h. Human skin biopsies and TBB samples were examined blindly and semiquantitatively scored for IL-1 $\beta$  staining intensity: 0 – no cells, 1 - very few cells, 2 - few cells, 3 - moderate number of cells, 4 - many cells, 5 - extensive number of cells.

All biopsy samples were digitalized using ScanScope XT slide scanner (Aperio, Leica Biosystems, Switzerland).

#### **Primary antibodies and methods used for IHC**

Marker	Clone	Vendor	Procedure	Pretreatment	Dilution/ Conc.	Incubation	Detection
CD68	KP-1	Dako	Ventana	sCC1	1:500	1 hour, 37°C	DAB Map
Caspase-1, cleaved	Rabbit pAb	Immuno-way YC0002	Manual	borate buffer MW 45 min	1:50 20ug/ml	ON, +4°C	Envision HRP
IL-1 beta	Goat pAb	R&D Systems AF-201-NA	Manual	Envision Flex low, PT-Module 20 min	1:10 10ug/ml	1 hour, RT	ABC-HRP

#### ***NLRP3 inflammasome activation of BAL cells***

IL-1 $\beta$  production by BAL cells was assessed following NLRP3 inflammasome stimulation. Human BAL cells were cultured in macrophage SFM/Gibco medium (serum free medium; Life technologies) supplemented with 1% penicillin/streptomycin (Biochrom). Human BAL cells ( $10^5$  cells/100 $\mu$ l media; 96-well plates) were stimulated with 1 $\mu$ g/ml LPS (Fluka Biochemika) for 4h followed by 10 $\mu$ M nigericin (Sigma Aldrich) or 1mM ATP (Sigma Aldrich) for a further 2h. Cell supernatants were collected 6h after stimulation to determine levels of IL-1 $\beta$  release by ELISA and Western Blot. The concentration of IL-1 $\beta$  was determined using an ELISA (Human IL-1 $\beta$ / IL-1F2 DuoSet, R&D) according to the manufacturer's instructions.

#### ***Determination of cytokine production by BAL cells following SAA stimulation.***

Human BAL cells were cultured at  $10^6$  cells/ml in 24-well plates in 1 ml RPMI 1640 medium supplemented with 2% human serum and 1% penicillin/streptomycin and stimulated with or without 1 $\mu$ g/ml SAA (recombinant human apo-SAA, PeproTech, Germany). Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient and cultured under the same conditions. Conditioned medium was harvested after 24h and stored at -80°C. The concentrations of IL-1 $\beta$ , IL-1ra, IL-8 and TNF- $\alpha$  in the conditioned medium were determined by ELISA (all R&D Duosets) or by homogenous time resolved fluorescence (HTRF; Cisbio Bioassays, France) as specified in the manufacturers' instructions.



### ***Immunoblot analysis of caspase-1p20 activity and active IL-1 $\beta$ release***

BAL cells were stimulated with the NLRP3 activation protocol described above and supernatants were precipitated with methanol/chloroform. Upon centrifugation, the upper phase was removed and methanol added to the lower phase prior to another centrifugation step. The supernatant was discarded and the pellet was incubated at 55 °C for 10 minutes and resuspended in Laemmli-buffer. Samples were separated using 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Cleaved caspase-1 (p20) was detected using primary antibody rabbit mAb cleaved caspase-1 (Asp297) (D57A2) (1:200) (Cell Signalling Technology) and IL-1 $\beta$  was detected using primary rabbit polyclonal anti IL-1 $\beta$  antibody (1:1000) (Abcam, ab2105) with the secondary antibody goat anti-rabbit (H+L)-HRP conjugate (1:3000) (BioRad Laboratories). Enhanced chemiluminescence (Clarity™ Western ECL Substrate/BioRad) was used for detection with ChemiDoc™ MD Imaging System (BioRad).

### ***MicroRNA analysis and RT-PCR***

Alveolar macrophages were sorted from BAL cells from an additional cohort of sarcoid patients and healthy volunteers on a MoFlo Astrios/Beckman Coulter cell sorter based on cell characteristics in the forward/sideward scatter and typical autofluorescence of AMs. In all experiments purity of sorted macrophages exceeded 99%. Total cellular miRNA from 106 sorted AMs was isolated using mirVana™ isolation kit (Thermo Fisher Scientific) and tailed using poly(A) polymerase (Fermentas). Poly(A) RNA was treated with DNase I (Fermentas) and reverse transcribed with M-MuLV reverse transcriptase (Fermentas) using a PolyT adapter (5'-GCGAGCACAGAATTAATACGACTCACTATAGG(T)18VN-3'). The obtained cDNA was analyzed by Real-Time PCR (Light Cycler/Roche) with the following primers: huNLRP3 (5'-AGAATGCCTTGGGAGACTCA-3' and 5'-CAGAATTCACCAACCCCAGT-3'), resulting in a 93 bp product, exon 6/7 overlapping; miR-223 (5'-TGTCAGTTTGTCAAATACCCCA-3' and 5'-GCGAGCACAGAATTAATACGAC-3'), resulting in a 72 bp product (consisting of miR-223 mature sequence and the PolyT adapter); GAPDH (5'-ACAGTCAGCCGCATCTTCTT-3' and 5'-GTTAAAAGCAGCCCTGGTGA-3') and MammU6 (hsaU6F 5'-AAATTCGTGAAGCGTTCCAT-3' and uni-R 5'-GCGAGCACAGAATTAATACGAC-3') as reference. The expression of NLRP3 and of



miR-223 was normalized to GAPDH expression or MammU6 expression, respectively, and plotted as arbitrary units on a linear scale.<sup>4</sup>

### ***Isolation of monocytes from peripheral blood mononuclear cells (PBMC)***

Human PBMCs were isolated from human blood of healthy volunteers and patients with sarcoidosis by density gradient centrifugation in Ficoll-Paque™ PLUS / GE Healthcare-Life Sciences medium. Human monocytes were isolated from PBMCs of healthy volunteers and patients with sarcoidosis using a commercially available EasySep™ Human Monocyte enrichment kit (StemCell Technologies).

### ***Testing NLRP3 inflammasome activation in monocytes***

Monocytes from 19 sarcoid patients and 18 healthy volunteers (same cohort as BAL cells) were isolated as described above and immediately plated at a concentration of  $1 \times 10^6$ /well in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin and stimulated for 4 hours with either LPS (Fluka Biochemika) ( $1 \mu\text{g/ml}$ ), SAA (recombinant human apo-SAA, PeproTech, Germany) ( $1 \mu\text{g/ml}$ ). After 4h of culture supernatants were harvested and stored at  $-80^\circ\text{C}$  for IL-1 $\beta$  cytokine measurement by ELISA (R&D Duoset).

### **Testing the effect of NLRP3 inflammasome inhibition in SAA stimulated monocytes**

Human CD14 monocytes from healthy volunteer-derived PBMCs were isolated as described above and stimulated at  $10^5$  cells/ 200  $\mu\text{l}$  in 96 well plates with or without  $1 \mu\text{g/ml}$  SAA (recombinant human apo-SAA, PeproTech, Germany) for 24 h. Pralnacasan (VX-740) ( $0.01 \mu\text{M}$ ,  $0.1 \mu\text{M}$ ,  $1 \mu\text{M}$  and  $10 \mu\text{M}$ ), MCC950 (Tocris) ( $0.01 \mu\text{M}$ ,  $0.1 \mu\text{M}$ ,  $1 \mu\text{M}$  and  $10 \mu\text{M}$ ) or dimethylsulfoxide control were added 30 min prior to SAA.<sup>5</sup> IL-1 $\beta$  production following SAA stimulation was measured in the conditioned medium by homogenous time resolved fluorescence (HTRF; Cisbio Bioassays, France) as specified in the manufacturers' instructions.

### ***Animal studies***

NLRP3  $-/-$  mice (B6 strain background) were kindly provided by Dr. Dixit from Genentech.<sup>6</sup> MiR-223  $-/-$  mice (B6.Cg-Ptprca Mir223tm1Fcml/J) (JAX stock #013198)<sup>7</sup> their



respective wildtype (B6.SJL-Ptprca Pepcb/BoyJ) (JAX stock #002014)<sup>8-10</sup> control mice and wildtype (WT) control mice (C57BL/6J) (JAX stock #000664) were purchased from the Jackson Laboratory/USA. All mouse procedures were carried out in accordance to Home Office German regulations and the Animals (Scientific Procedures) Act 1986 and were approved by the respective local Ethical Review Body (Regierungspräsidium Freiburg, Germany (AZ: 35/9185.81/G-07/05; AZ: 35-9185.81/G-10/38) and LAVES, Oldenburg, Germany (AZ: 33.19-42502-04-15/1861; AZ: 33.19-42502-04-15/2018).

### ***Murine pulmonary granuloma model induced by trehalose 6,6'-dimycolate (TDM)***

TDM (Enzo Life Sciences GmbH, Germany) from mycobacterium tuberculosis was prepared in a water in oil emulsion using incomplete Freund's Adjuvant (IFA: vehicle fluid: phosphate buffered saline with 3.2% incomplete Freund's-Adjuvant (Sigma Aldrich) and 0.2% Tween80 (Sigma Aldrich) as described.<sup>11,12</sup> Six to twelve weeks old NLRP3<sup>-/-</sup>, miR-223<sup>-/-</sup>, B6.SJL-Ptprca Pepcb/BoyJ (WT) and C57BL/6J (WT) mice were intravenously injected via the tail vein with 1 µg TDM in 10 µl water in oil emulsion (IFA/PBS and 0.2% Tween80)/ g body weight with the only exception of miR-223<sup>-/-</sup> mice which received 0.5 µg TDM in 10 µl water in oil emulsion (IFA/PBS and 0.2% Tween80)/g body weight. To assess pulmonary granuloma formation, lungs were harvested on day 7 after TDM injection for all studies (n=6 per group). For IL-1β production by BAL cells after NLRP3 inflammasome activation, mice were sacrificed on day 3 and BAL was performed as described.<sup>13</sup> Six to twelve weeks old C57BL/6J WT mice (n=10 per group) were administered vehicle (PBS) or MCC950, a NLRP3 inflammasome pathway inhibitor, at 10 mg/kg intraperitoneally on day 0, 1, 2, 4, and 6 after TDM injection. In an additional experiment, six to twelve weeks old C57BL/6J WT mice (n=8 per group) were administered subcutaneously with either vehicle (PBS), isotype control antibody anti-cyclosporine (Novartis) [200µg/mouse] or anti-IL1β antibody (Novartis) [200µg/mouse] on day -1 and again 3 days after TDM injection.

### ***Histopathology and immunohistochemistry of mouse lung tissue***

Murine lungs were fixed in 10% normal buffered formalin for 24 hours, cut to tissue sections, dehydrated overnight and embedded in paraffin. Paraffin tissue sections from murine lung lobes (3µm) were stained with H&E and immunohistochemically using antibodies specific for mouse macrophage lineage marker Iba-1 (Wako Chemicals USA Inc., USA, 1:500), mouse IL-1β (Abcam, UK, 1:800), and SAA (Novus Biologicals, UK, 1:150).



Granuloma load per lung section was counted semi-quantitatively as exemplified in the results section. All biopsy samples were digitalized using ScanScope XT slide scanner (Aperio, Leica Biosystems, Switzerland) or Mirax Scan 150 BF/FL (Zeiss, Germany).

#### **Primary antibodies and methods used for IHC**

Marker	Clone	Vendor	Procedure	Pretreatment	Dilution/ Conc.	Incubation	Detection
Iba-1	Rabbit pAB	Wako	Ventana	sCC1	1:500	1 hour, 37°C	DAB Map
IL-1b	Rabbit pAb	Abcam	Ventana	sCC1	1:800	3 hours, 37°C	OmniMap +TSA HQ
SAA	Reu86.1	Novus	Ventana	sCC1	1:150	1 hour, RT	OmniMap

#### ***Statistics***

Statistical analyses were performed with Graph Pad Prism Software v6.01. Comparisons between groups from clinical data was done by Mann-Whitney U test, assuming non-parametric, non-Gaussian distribution, and by unpaired t-test or ANOVA for preclinical data as specified in the figure legends. To assess relationships and potential correlation between 2 parameters,  $r^2$  values were determined using the linear regression function in Graph Pad Prism Software. For clarity, in the figures, 2 stars denote anything with a significance of  $p < 0.01$ , while the precise  $p$  values are mentioned in the results section (unless they reach levels of  $p < 0.0001$ .)



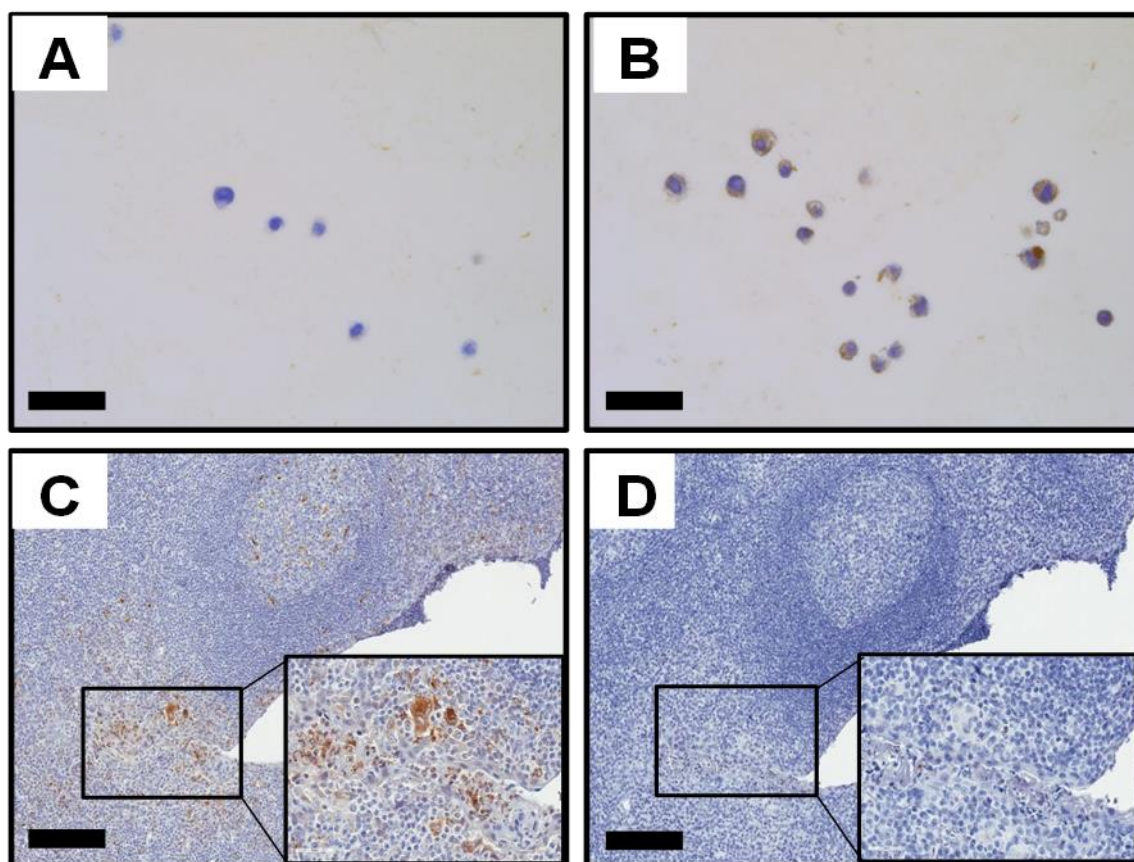
## Supplementary Results

### **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 also as an activator of the NLRP3 inflammasome, we analyzed the expression and effects of SAA in the context of the NLRP3 inflammasome pathway. We found that serum levels of SAA were significantly increased in sarcoid patients in comparison to HV ( $p = 0.0497$ , Fig. S6A). Upon ex vivo stimulation with SAA, sarcoid BAL cells, especially those derived from the patients with severe lung disease, elicited significantly higher IL-1 $\beta$  levels than HV BAL cells ( $p = 0.0184$ , Fig. S6B and data not shown). Furthermore, SAA-induced IL-1 $\beta$  release from PBMCs derived from sarcoid patients versus HV was also significantly higher ( $p = 0.0182$ , Fig. S6C). In order to confirm SAA as an activator of the NLRP3 inflammasome as was recently postulated, we tested the effects of the published NLRP3 inflammasome pathway inhibitor MCC950 and an inhibitor of caspase-1, VX-740 on SAA-mediated IL-1 $\beta$  release of CD14 $^{+}$  monocytes isolated from peripheral blood from healthy donors. Both inhibitors were able to reduce IL-1 $\beta$  release from the SAA-stimulated monocytes in a dose-dependent manner (Fig. S6D).



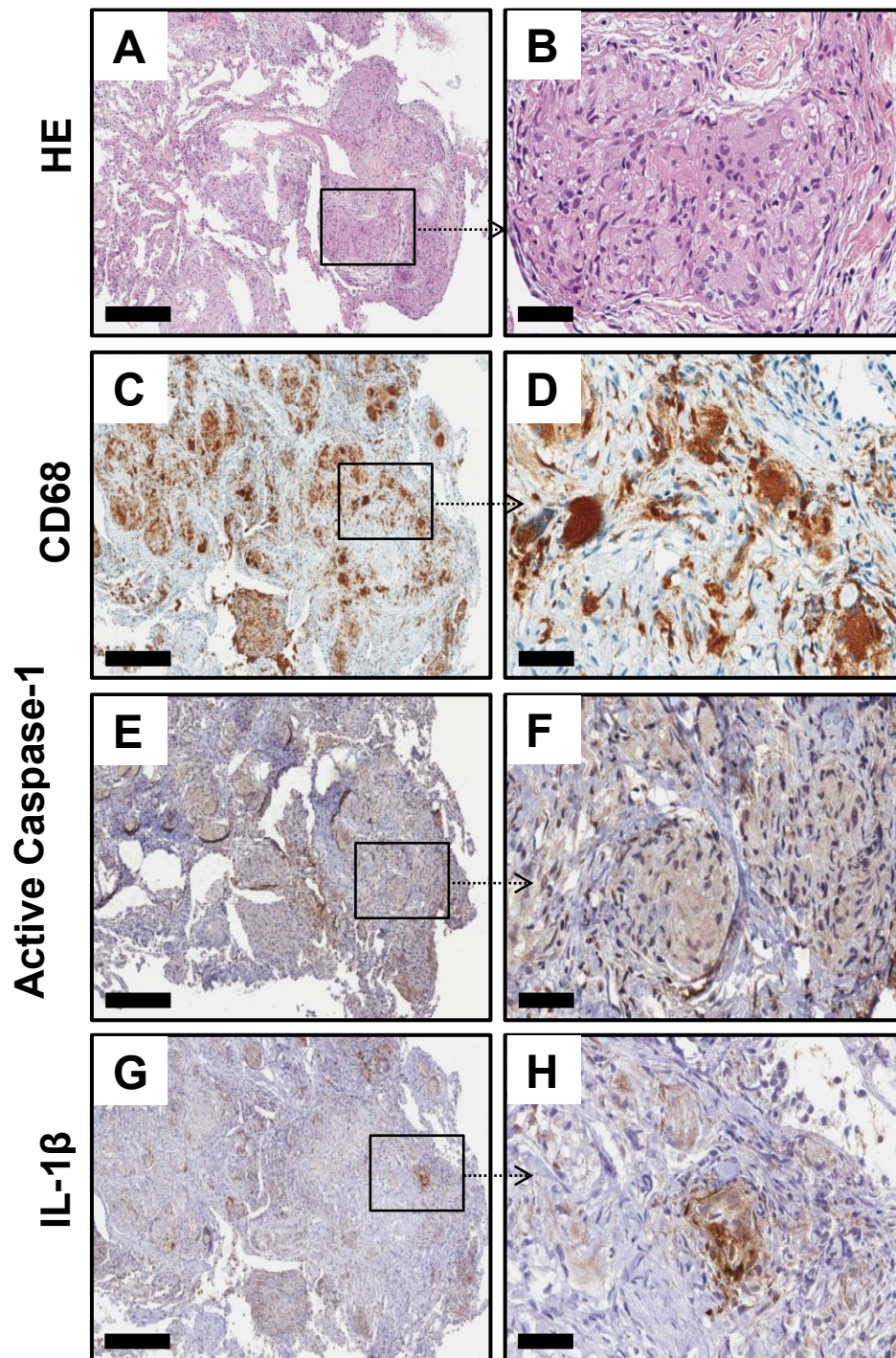
## Supplementary Figure S1



**Figure S1: Additional characterization of active caspase-1 antibody.** (A,B) THP-1 cells were left unstimulated (A) or stimulated with LPS (100ng/ml) for 4 h (B), paraffin-embedded, and stained immunohistochemically using an antibody against activated caspase-1. The antibody staining is only positive for LPS stimulated THP-1 cells which express active (cleaved) caspase-1 in the cytoplasm. The scale bar represents 50  $\mu$ m. (C,D) Representative photomicrographs of consecutive sections of human tonsils stained immunohistochemically using an antibody against CD68 (C) or activate caspase-1 (D). Macrophages from human tonsil tissue are negative for active caspase-1. The scale bar represents 200  $\mu$ m.



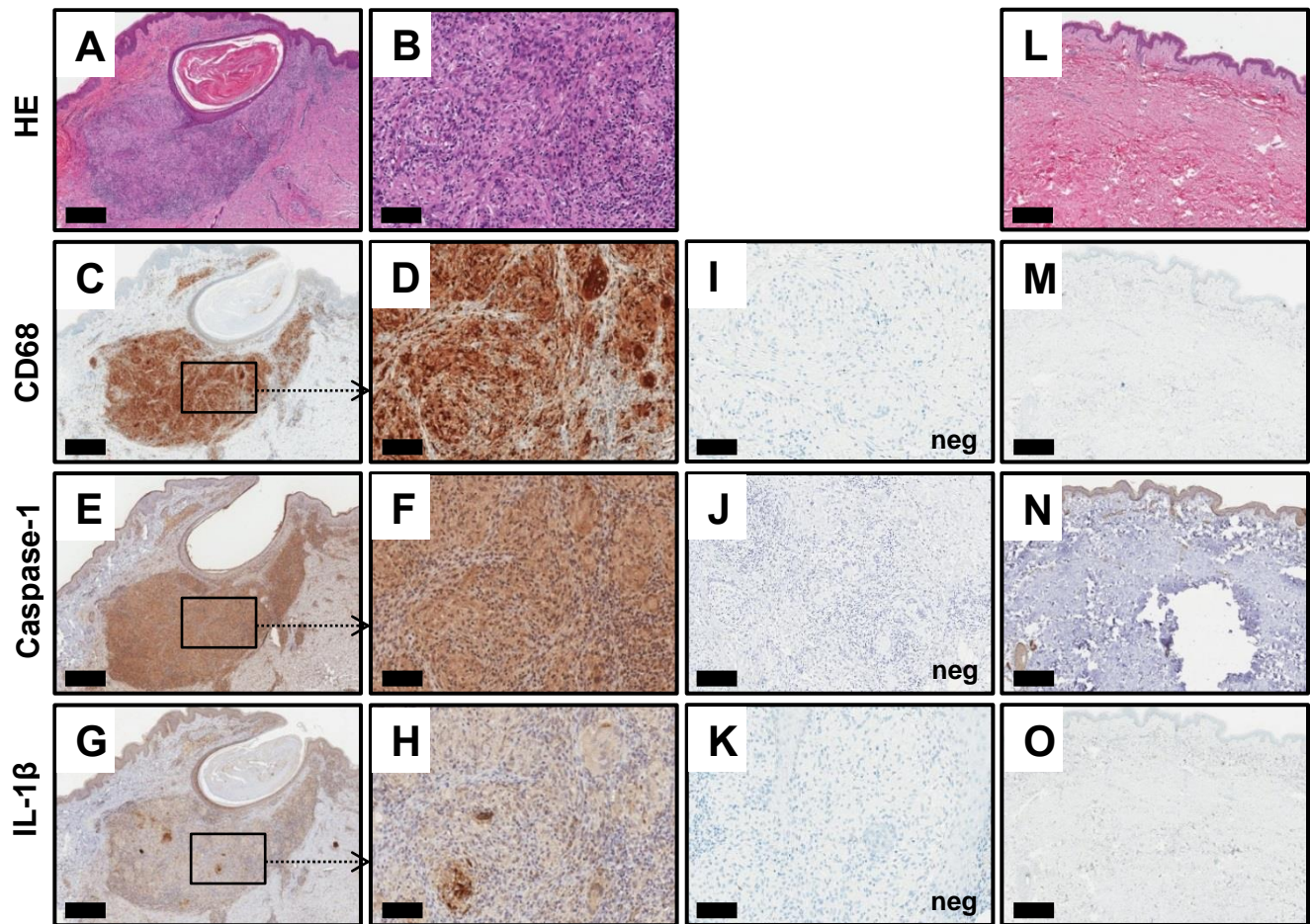
## Supplementary Figure S2



**Figure S2: Evidence for inflammasome and IL-1 $\beta$  pathway activation in sarcoid lung granulomas.** (A-H) Representative original photomicrographs of consecutive sections of tissues derived by transbronchial biopsy (TBB) from an additional sarcoid patient (severe disease and different from the patient in Fig. 1). (A, B) TBB tissue was stained with hematoxylin eosin (H&E) for histopathological assessment. (C-H) TBB tissue was stained immunohistochemically using antibodies against the macrophage lineage marker CD68 (C,D), activated caspase-1 (E,F), or IL-1 $\beta$  (G,H). The scale bar represents 300  $\mu$ m (panel A,C,E,G) and 50  $\mu$ m (panel B,D,F,H).



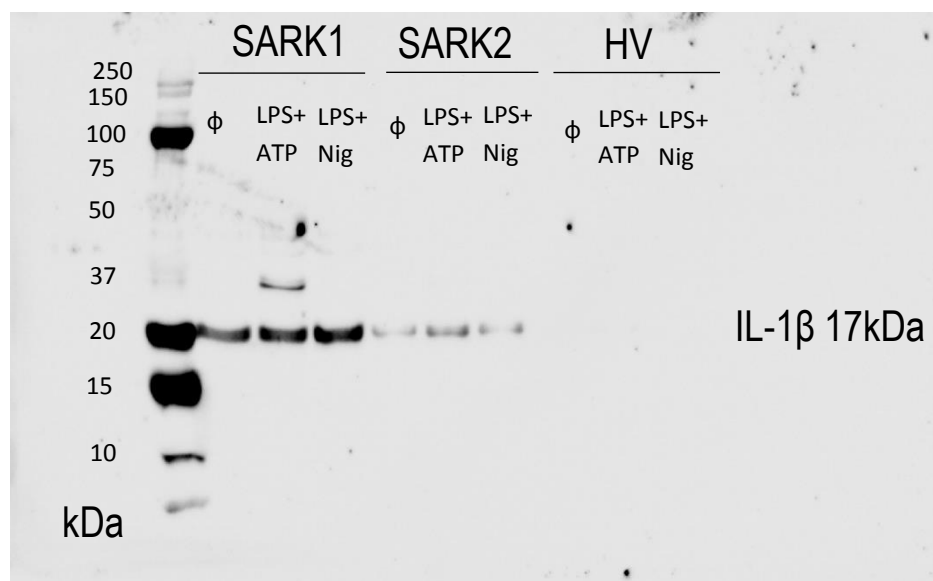
## Supplementary Figure S3



**Figure S3: Evidence for inflammasome and IL-1 $\beta$  pathway activation in sarcoid skin granulomas.** (A-K) Representative original photomicrographs of skin biopsy tissue from a sarcoid patient. (A,B) Skin tissue was stained with hematoxylin eosin (H&E) for histological assessment, indicating non-necrotizing sarcoid granuloma. (C-K) Skin tissue was stained immunohistochemically using antibodies against the macrophage lineage marker CD68 (C,D), activated caspase-1 (E,F), or IL-1 $\beta$  (G,H). (I-K) Negative control stainings were obtained by applying respective isotype control antibodies. (L-O) Negative stainings obtained with the antigen-specific antibodies for skin from a healthy donor. The scale bar represents 500  $\mu$ m in panels A,C,E,G and L,M,N,O, and 100  $\mu$ m in panels B,D,F,H and I,J,K.



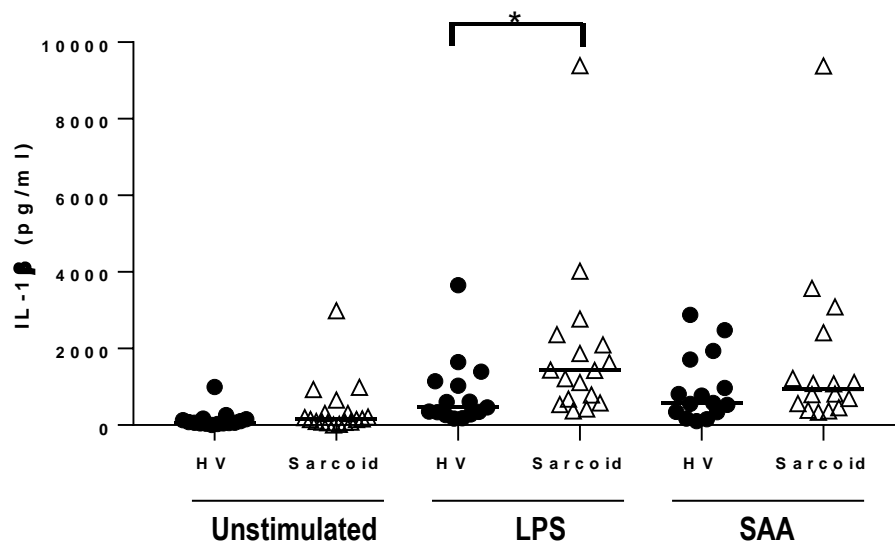
## Supplementary Figure S4



**Figure S4: Western blot confirming active IL-1 released by sarcoid BAL cells.** Representative Western blot of an experiment in which supernatant of BAL cells derived from either sarcoid patients (SARK1 and SARK2) or from healthy volunteer (HV) were used. BAL cells were either unstimulated ( $\phi$ ) or stimulated with LPS for 4 hours and then stimulated w/wo ATP or nigericin (Nig) for further 2 hours. Western blot shows the production of the active, 17 kDalton form of IL-1 $\beta$ . The inactive 31 kDalton pro-IL-1 $\beta$  form was much less released. Abbreviations: kDa: kilo Dalton, SARC: sarcoidosis, HV: healthy volunteer, Nig: nigericin.



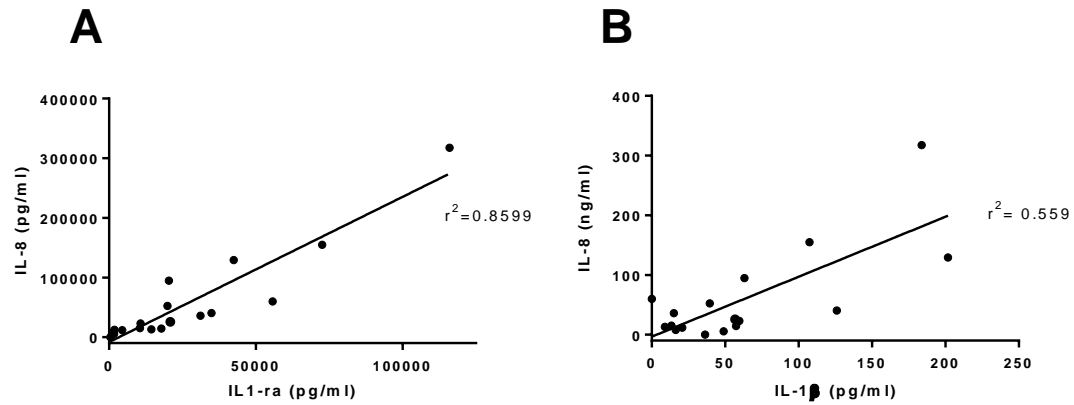
## Supplementary Figure S5



**Figure S5: Significantly increased IL-1 $\beta$  production by sarcoid monocytes following LPS stimulation.** Monocytes were isolated from PBMCs of 19 sarcoid patients and 18 healthy volunteers (same study cohort as BAL cell experiments) and cultured for 4h w/wo stimulation with either LPS (1 $\mu$ g/ml) or SAA (1 $\mu$ g/ml). Following LPS but not SAA stimulation, sarcoid monocytes showed a significantly increased production of IL-1 $\beta$  ( $p=0.007$ ) while in unstimulated monocytes there was a trend towards higher IL-1 $\beta$  production by sarcoid monocytes but comparison did not reach statistical significance ( $p=0.058$ ). \* $p<0.05$



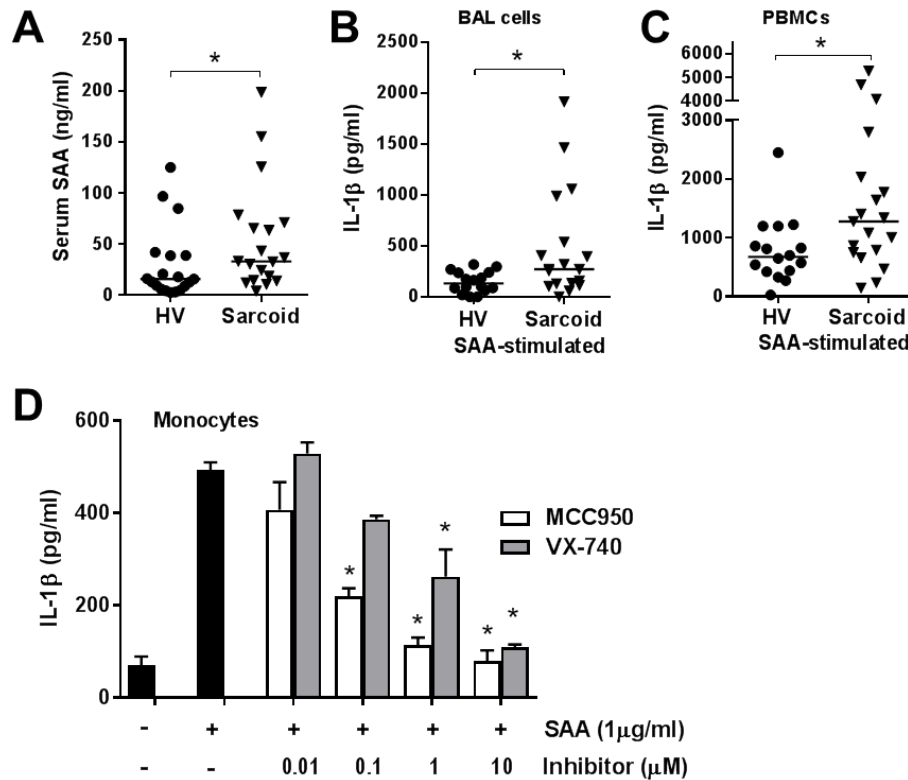
## Supplementary Figure S6



**Figure S6: Close correlation of constitutive IL-1ra, IL-8 and IL-1 $\beta$  production levels of BAL cells from sarcoidosis patients.** (A) Significant correlation between IL-1ra and IL-8 production levels ( $r^2 = 0.860$ ,  $p < 0.0001$ ). (B) Significant correlation between IL-1 $\beta$  and IL-8 production levels ( $r^2 = 0.559$ ,  $p = 0.0006$ ).



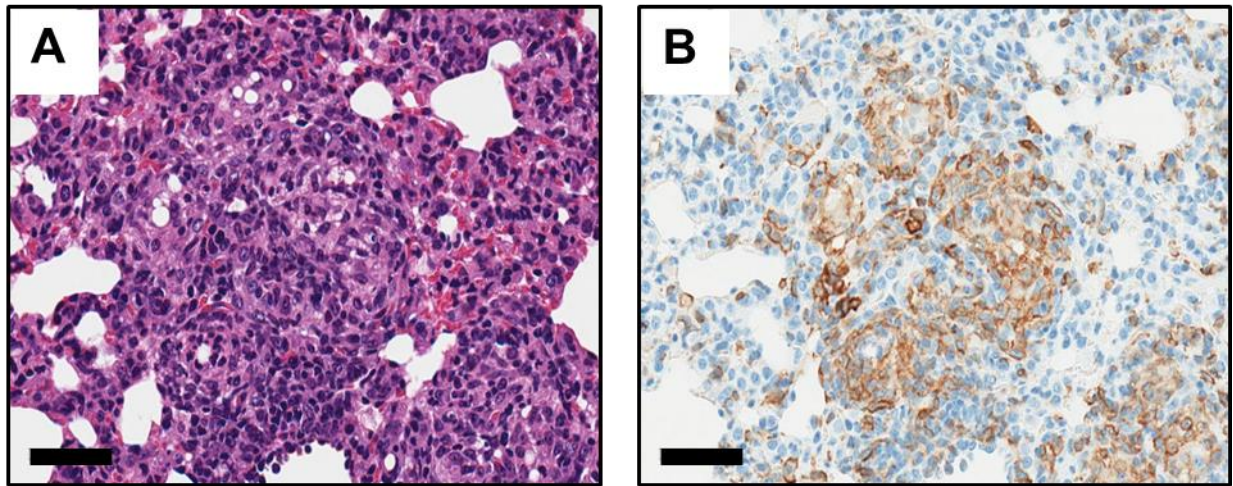
## Supplementary Figure S7



**Figure S7: Higher SAA serum levels and responsiveness of BAL cells and PBMCs to ex vivo SAA stimulation in sarcoidosis.** (A) SAA levels measured in serum samples of HV ( $n=19$ ) or patients with sarcoidosis ( $n=19$ ). (B) BAL cells (HV  $n=16$ , SARC  $n=18$ ) or (C) PBMCs (HV  $n=16$ , SARC  $n=19$ ) from HV or sarcoid patients were stimulated with SAA for 24h and levels of IL-1 $\beta$  measured in the supernatants by homogenous time resolved fluorescence. The bars in panels A-C depict the median.  $*p<0.05$ , Mann-Whitney U test. (D) CD14 monocytes were isolated from the peripheral blood from healthy volunteers and stimulated with SAA for 24h with or without a caspase-1 inhibitor VX-740 or the NLRP3 inflammasome pathway inhibitor MCC950. IL-1 $\beta$  production following SAA stimulation was measured in the conditioned medium. The data indicate mean  $\pm$  SEM from triplicate measurements of one out of 2 experiments with similar outcome.  $*p<0.05$  for SAA-stimulated group w/wo compound treatment, ANOVA followed by Dunnett's multiple comparisons test.



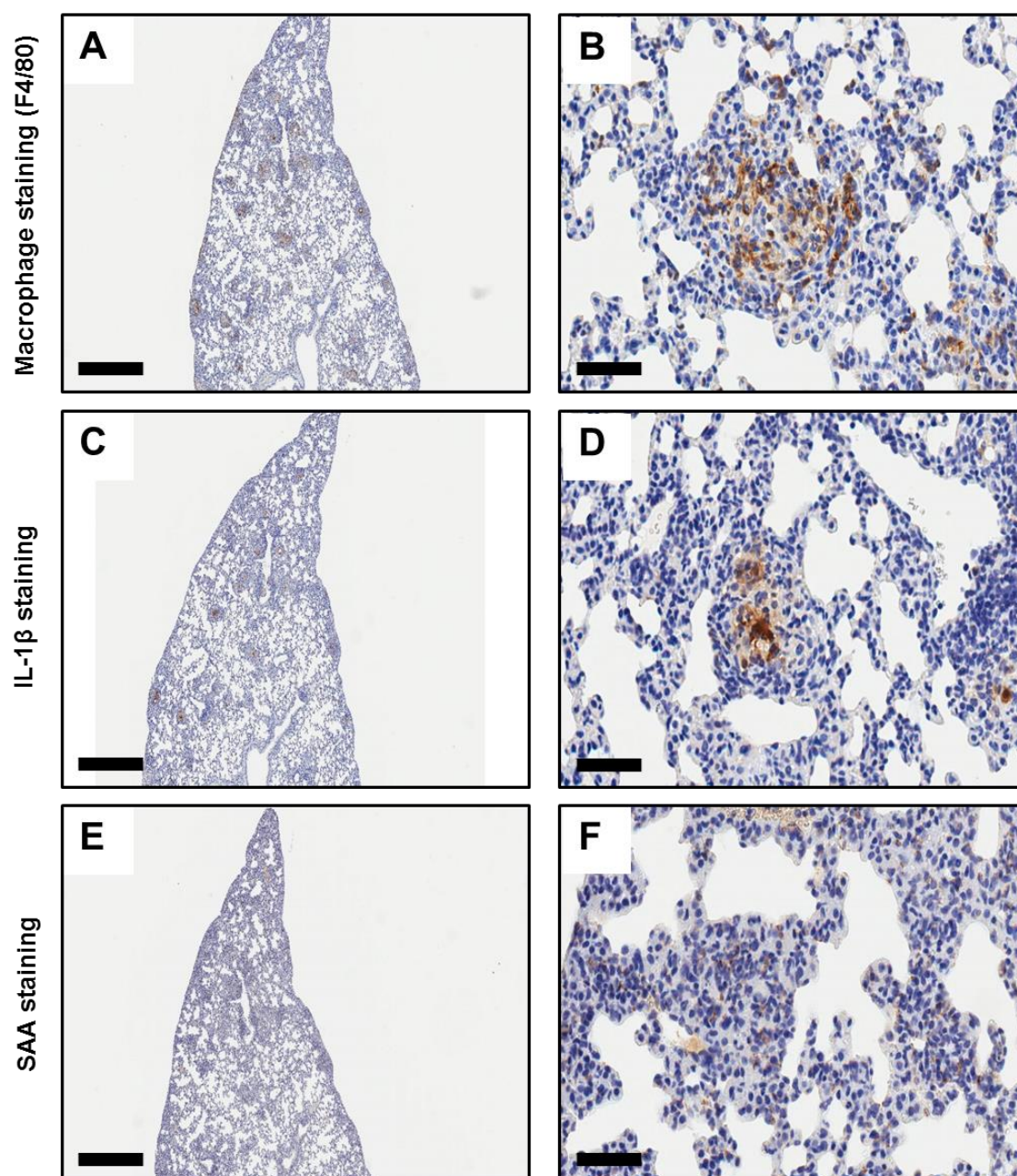
## Supplementary Figure S8



**Figure S8. TDM induced pulmonary granuloma in mice resemble human sarcoid granuloma.** (A) High magnification of nonnecrotizing TDM-induced granuloma in lung of a WT mouse (hematoxylin and eosin stain). (B) Sections were stained by immunohistochemistry using antibodies against the macrophage lineage marker Iba-1. The scale bar represents 50  $\mu\text{m}$ .



## Supplementary Figure S9



**Figure S9. TDM induced pulmonary granuloma in mice consists of macrophages and express IL-1 $\beta$  and SAA.** (A,B) F4/80 staining demonstrates accumulation of macrophages in TDM-induced nonnecrotizing lung granuloma of WT mice. (C,D) IL-1 $\beta$  expression of presumably macrophages in the center of TDM-induced lung granuloma of WT mice. (E,F) SAA expression in the center of TDM-induced lung granuloma of WT mice. The scale bar represents 50  $\mu$ m.



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