Adenosine deaminase 2 as a biomarker of macrophage activation syndrome in systemic juvenile idiopathic arthritis

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Supplementary materials

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Supplementary Methods

Diagnostic criteria: The following diagnostic criteria were utilized: 2017 American Heart Association Guidelines for KD [1]; 1997 American College of Rheumatology (ACR) revised criteria for SLE [2], and 2004 International League of Associations for Rheumatology (ILAR) Classification Criteria for JIA [3]. The diagnosis of JDM was made clinically based on presence of a typical skin rash, symmetric muscle weakness, muscle enzyme elevation, and magnetic resonance imaging results compatible with diffuse muscle inflammation.

The medical record of each case was reviewed by a pediatric rheumatologist on the investigational team. Patients with sJIA were further stratified into inactive sJIA, active sJIA without MAS, or active sJIA with MAS. Disease activity and MAS status were determined based on medical record and also analyzed using the 2016 ACR/EULAR MAS Classification Criteria for MAS complicating sJIA [4]. While all patients with a clinical diagnosis of MAS displayed ferritin levels > 684 ng/mL, 4 patients did not meet the minor criteria and 4 patients met one minor criterion (either elevated AST or low platelets), but did not fulfil the full criteria due to the lack of fibrinogen and/or triglycerides measurements at the time of sample collection.

Quantification of ADA2 activity: ADA2 activity was measured in human plasma, serum, or cell culture supernatant by a spectrophotometric assay which quantifies the adenosine-dependent generation of ammonia in the presence of EHNA, a selective inhibitor of ADA1 [5-7]. The final concentrations of adenosine and EHNA were adjusted to 12 mM (saturating for ADA2) and 0.1 mM, respectively. The modified spectrophotometric ADA2 assay was then fully validated (i.e., for linearity, range, precision, accuracy, specificity, robustness, and ruggedness), and results were shown to agree closely with ADA2 activity measured with an independent HPLC-based assay that directly quantifies the conversion of adenosine to inosine (MS Hershfield, NJ Ganson, SJ Kelly, unpublished). This assay has been used in the Clinical Laboratory

Improvement Amendments (CLIA)-certified laboratory of one of the coauthors (MSH) to test more than 1000 individuals for DADA2.

***In vitro* stimulation:** PBMC from 4 healthy donors were isolated using Ficoll centrifugation. Cells were cultured in complete RPMI with 10% fetal bovine serum and penicillin-streptomycin (Thermo Fisher, Waltham, MA). PBMC (1×10^5 / well in 96 well plate) were stimulated with selected inflammatory cytokines (Peprotech, Rocky Hill, NJ) or TLR ligands (Invivogen, San Diego, CA). The supernatant was collected after 5 days to determine ADA2 activity. Cytokines used in this study included: IL-1β (20 ng/ml), IL-4 (20 ng/ml), IL-6 (50 ng/ml), IL-10 (20 ng/ml), IL-12 (20 ng/ml), IL-17 (20 ng/ml), IL-18 (20 ng/ml), IFN-α (10^3 U/mL), IFN-γ (10^3 U/mL), TGF-β (20 ng/ml), PAMC3SK4 (10 µg/mL), LPS (100 ng/mL), and R848 (100 ng/mL). Monocyte depletion was performed using the anti-CD14 magnetic beads and monocyte enrichment was performed using a Human Monocyte Isolation Kit (all from Miltenyi Biotec, Cambridge, MA) according to manufacturer's instructions.

Cytokine measurement: Ferritin, IL-18, and CXCL9 were quantified using enzyme-linked immunosorbent assays (ELISA) following manufacturer's instructions. Human ferritin ELISA was obtained from Crystal Chem (Elk Grove Village, IL) while human IL-18 and CXCL9 ELISA kits were purchased from R&D Systems (Minneapolis, MN). IL-10 and total TGF-β were measured in a subset of patients with active sJIA with or without MAS using a custom Legendplex bead-based immunoassay (Biolegend, San Diego, CA). All assays were performed following the instructions provided by the manufacturer.

Flow cytometry: PBMC isolated by Ficoll centrifugation were fixed in 4% paraformaldehyde for 10 minutes, washed in PBS, and resuspended in permeabilization buffer (Ebioscience, San Diego, CA). Cells were washed with PBS with 2% FBS and stained with an optimized amount of rat anti-human/mouse CD11b, and monoclonal mouse anti-ADA2 (clone 3D11, Sigma) or isotype control. After washing, cells were resuspended and acquired using a Becton-Dickinson

FACS Canto II flow cytometer and analyzed with FCS Express 5 software (De Novo Software, Glendale, CA). Live cells were identified by size and singlets were gated for analysis, monocytes and lymphocytes were separated by forward scatter and CD14 staining.

Confocal microscopy: Bone marrow cells from a patient with MAS were fixed using 4% paraformaldehyde for 15 minutes followed by permeabilization using PBS with 0.2% saponin and 1% bovine serum albumin (BSA) for 30 minutes. After washing with PBS with 1% BSA and blocking in PBS with 5% rabbit or mouse serum, cells were incubated with polyclonal rabbit anti-ADA2 (clone N2C3, Genetex, Irvine, CA), monoclonal mouse anti-ADA2 (3D1), or corresponding isotype antibodies overnight. Cells were washed and incubated with fluorophore-conjugated secondary antibodies to rabbit or mouse for 1 hour. After washing with PBS with BSA, cells were stained with mouse anti-human CD68 and Hoechst 33342 before final washes and cytospun onto slides for mounting with FluorMount G (Thermo Fisher). Images were acquired using a Zeiss Axio Observer Z1 Inverted Microscope with Zeiss LSM 800 with Airyscan confocal system (Zeiss, Oberkochen, Germany).

References

1. McCrindle BW, Rowley AH, Newburger JW, Burns JC, Bolger AF, Gewitz M, et al. Diagnosis, Treatment, and Long-Term Management of Kawasaki Disease: A Scientific Statement for Health Professionals From the American Heart Association. Circulation. 2017 Apr 25; 135(17):e927-e999.

2. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis and rheumatism. 1997 Sep; 40(9):1725.

3. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. The Journal of rheumatology. 2004 Feb; 31(2):390-392.

4. Ravelli A, Minoia F, Davi S, Horne A, Bovis F, Pistorio A, et al. 2016 Classification Criteria for Macrophage Activation Syndrome Complicating Systemic Juvenile Idiopathic Arthritis: A European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. Arthritis Rheumatol. 2016 Mar; 68(3):566-576.

5. Lee PY, Huang Y, Zhou Q, Schnappauf O, Hershfield MS, Li Y, et al. Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2. The Journal of allergy and clinical immunology. 2018 Oct; 142(4):1363-1365 e1368.

6. Muraoka T, Katsuramaki T, Shiraishi H, Yokoyama MM. Automated enzymatic measurement of adenosine deaminase isoenzyme activities in serum. Analytical biochemistry. 1990 Jun; 187(2):268-272.

7. Slaats EH, Asberg EG, van Keimpema AR, Kruijswijk H. A continuous method for the estimation of adenosine deaminase catalytic concentration in pleural effusions with a Hitachi 705 discrete analyser. Journal of clinical chemistry and clinical biochemistry Zeitschrift fur klinische Chemie und klinische Biochemie. 1985 Oct; 23(10):677-682.

Supplementary Table S1. Demographics of healthy controls and patients.

	n	Age (yr)	% Female	Active disease
Healthy Controls (Children)	174	8.3 ± 5.0	48%	n/a
Healthy Controls (Adults)	150	32.1 ± 8.1	50%	n/a
Kawasaki disease	25	2.9 ± 1.9	40%	100%
Systemic lupus erythematosus	14	13.1 ± 4.2	79%	79%
Juvenile dermatomyositis	13	10.7 ± 6.0	62%	77%
Juvenile idiopathic arthritis	121	11.4 ± 6.2	53%	72%
oligoarticular JIA or polyarticular JIA	19	13.9 ± 5.1	65%	88%
psoriatic arthritis or enthesitis-related arthritis	14	11.9 ± 3.8	50%	100%
systemic juvenile idiopathic arthritis	88	10.6 ± 6.7	57%	65%
sJIA - inactive disease	31	11.1 ± 7.5	56%	0%
sJIA - active disease, without MAS	34	10.9 ± 6.0	53%	100%
sJIA - active disease, with MAS	23	8.6 ± 7.2	65%	100%

Supplementary Table S2. Utility of MAS biomarkers based on the optimal cut-off value

	Optimal Cut-off	Youden's index	Sensitivity	95%CI	Specificity	95%CI	Likelihood ratio
ADA2	25.7 U/L	0.8589	91.3	72.0 - 98.9	94.6	81.8 - 99.3	16.9
Ferritin	731 ng/mL	0.8824	100.0	88.2 - 100.0	88.2	72.6 - 96.7	8.5
IL-18	68,363 ng/ml	0.8532	95.0	75.1 - 99.9	90.3	72.4 - 98.0	9.8
CXCL9	203 ng/mL	0.6742	90.0	68.3 - 98.7	77.4	58.9 - 90.4	4.0

Supplementary Table S3. Utility of MAS biomarkers using the upper limit of normal as cut-off

	Upper limit of normal	Sensitivity	95%CI	Specificity	95%CI	Likelihood ratio
Ferritin	120 ng/mL	100.0	85.2 - 100.0	50.0	32.4 - 67.7	2.0
IL-18	580 ng/ml	100.0	83.2 - 100.0	19.4	7.5 - 37.5	1.2
ADA2	27.8 U/L	87.1	66.4 - 97.2	94.6	81.8 - 99.3	16.1
CXCL9	121 ng/mL	95.0	75.1 - 99.9	64.5	45.4 - 80.8	2.7

Supplementary Figure S1

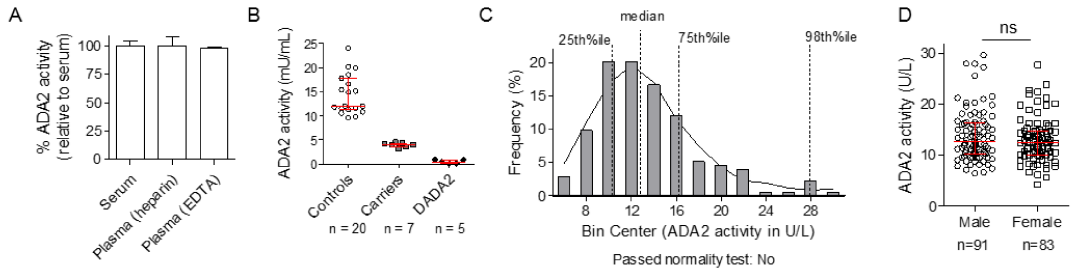

Figure S1. Establishing the normal range of peripheral blood ADA2 activity in healthy pediatric controls. A) Comparison of ADA2 levels in serum and plasma with different anticoagulants from healthy donors (n = 2 per condition; normalized to serum activity). B) Comparison of ADA2 levels in healthy controls (n = 20), patients with DADA2 (n = 5) and heterozygous carriers (n = 7). C) Distribution of plasma ADA2 activity in healthy children (n = 174). The upper limit of normal is defined as the 98th percentile. D) Comparison of ADA2 activity in healthy male (n = 91) vs. female children (n = 83). Median and interquartile range are displayed in scatter dot plots.

Supplementary Figure S2

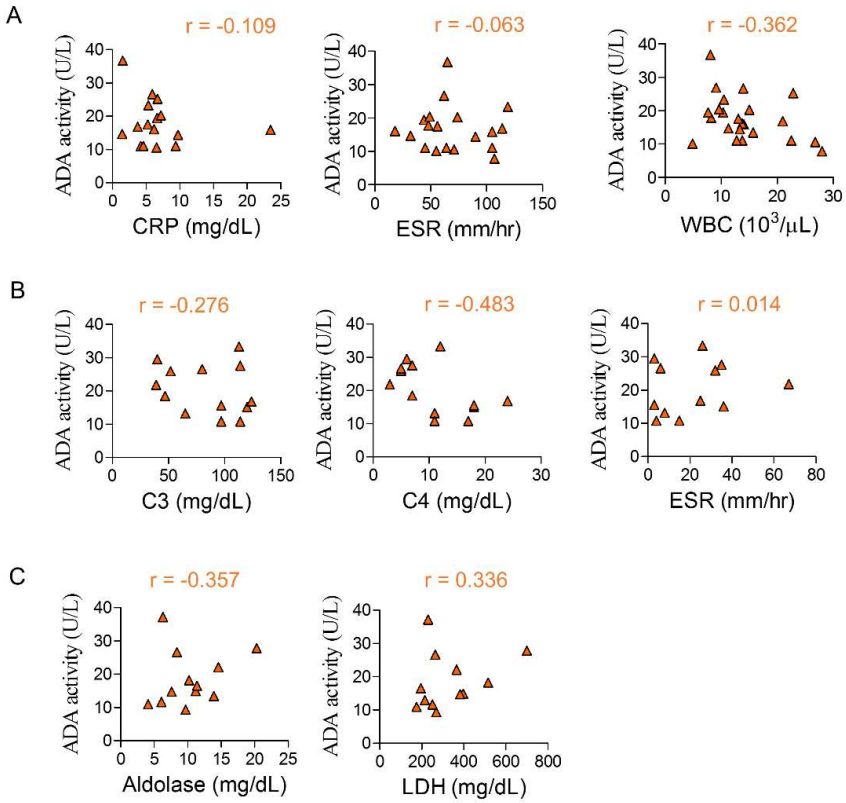

Figure S2. ADA isoenzyme levels do not correlate with markers of inflammation or disease activity. A) Comparison of ADA2 with WBC, ESR and C-reactive levels in patients with Kawasaki disease (n = 25). B) Comparison of ADA isoenzyme levels with ESR, complement C3 and C4 in patients with pSLE (n = 13). C) Comparison of ADA isoenzymes levels with aldolase and LDH in children with dermatomyositis (n = 12). $p > 0.05$ for all comparisons in panels A, B and C.

Supplementary Figure S3

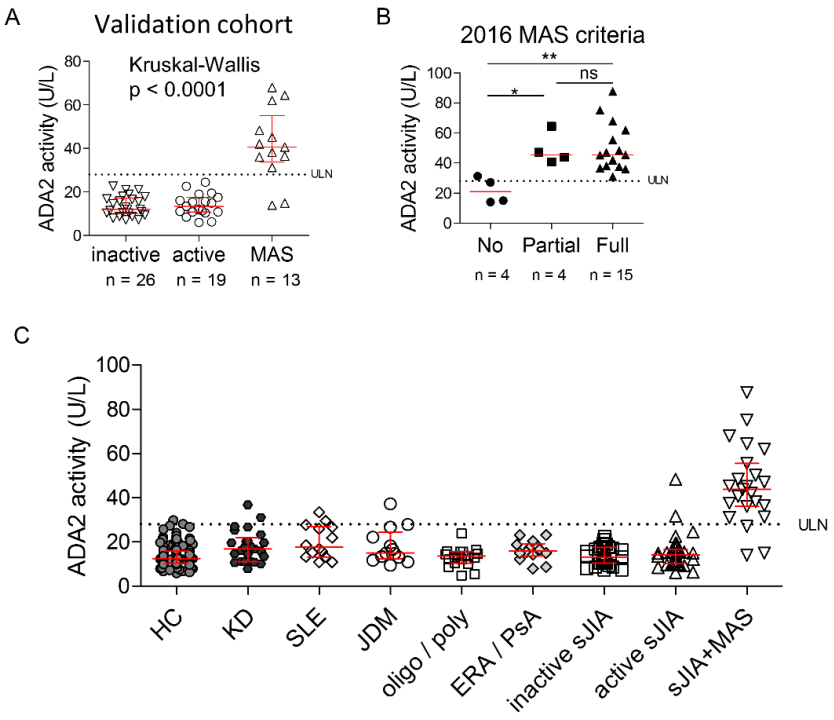

Figure S3. Validation of ADA2 elevation in MAS associated with sJIA. A) Serum ADA2 activity measurement from an independent validation cohort of sJIA patients (n = 58). B) Evaluation of the 2016 MAS Classification Criteria for all patients with clinical diagnosis of MAS in this study (n = 23). C) Display of plasma / serum ADA2 activity in healthy controls and all patient. Median and interquartile range are displayed in scatter dot plots. * p< 0.05, ** p < 0.01

Supplementary Figure S4

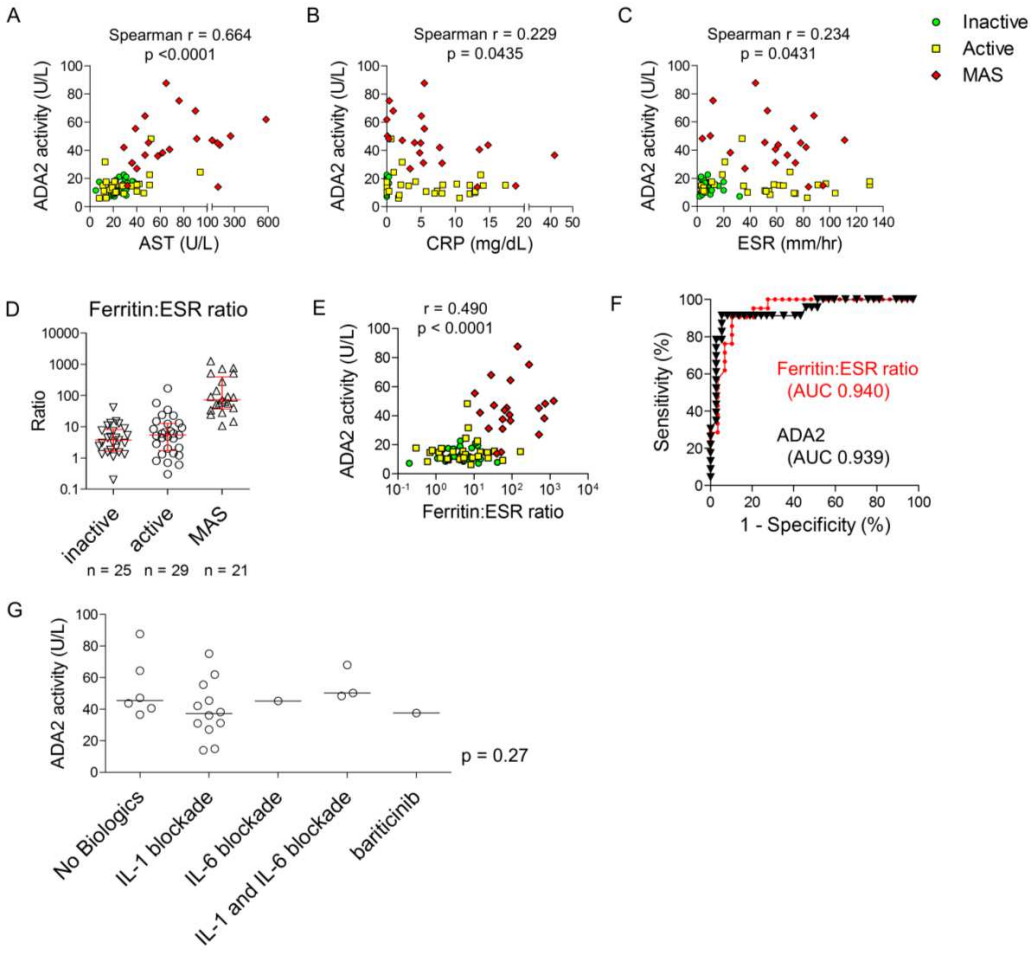

Figure S4. Comparison of ADA2 and other biomarkers. Correlations of ADA2 with A) AST, B) ESR and C) CRP. D) Comparison of ferritin : ESR ratio in sJIA patients. E) Correlation between ADA2 and ferritin:ESR ratio in sJIA patients. F) ROC curve of ADA2 and ferritin:ESR ratio in distinguishing MAS from active sJIA. G) Comparison of ADA2 activity levels in MAS patients grouped by the usage of biologic therapy. IL-1 blockade: anakinra or canakinumab; IL-6 blockade: tocilizumab. For panels A, B, C and E, all sJIA patients (inactive, active and MAS groups) were included for calculation of Spearman's rank correlation coefficient.

Supplementary Figure S5

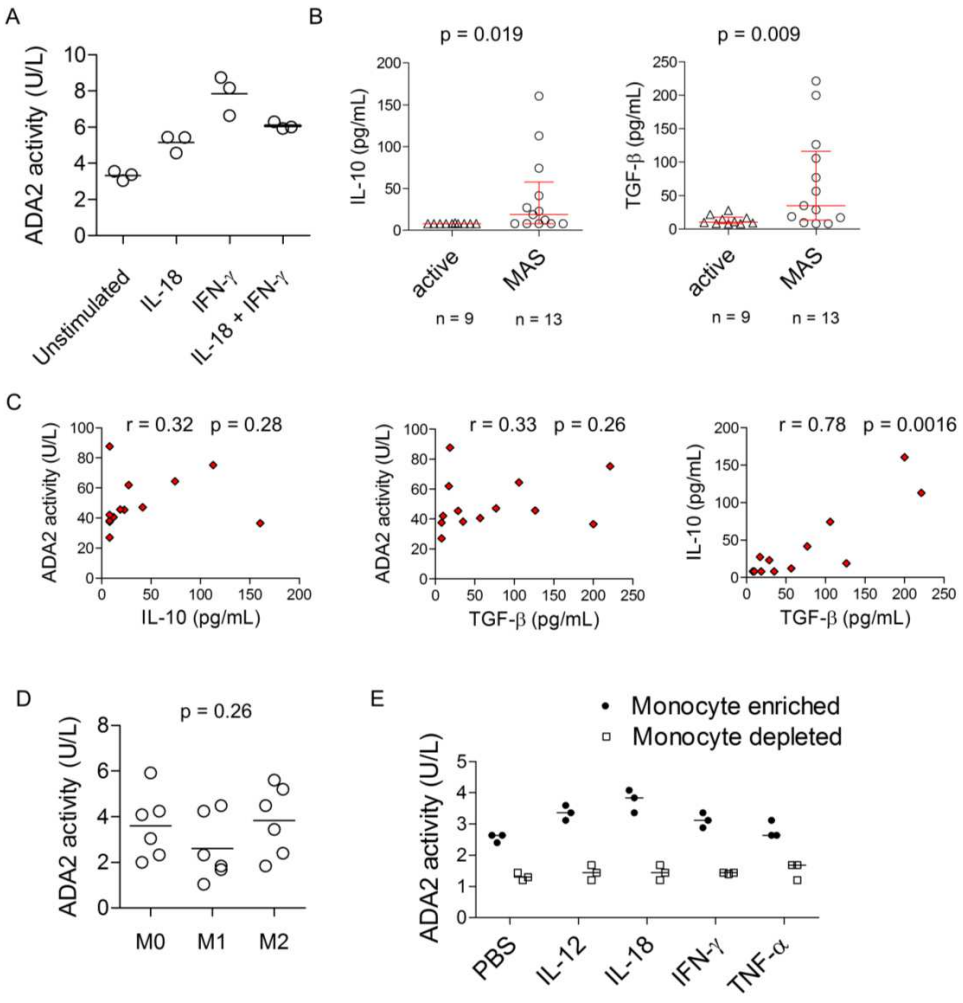

Figure S5. Mechanism and source of ADA2 production in MAS. A) ADA2 activity in the supernatant of healthy donor PBMC stimulated with IL-18, IFN-γ, or IL-18 + IFN-γ combined. B) Plasma IL-10 and TGF-β levels in active sJIA patients with (n =13) or without MAS (n= 9) determined using a multiplex assay. C) Correlations between IL-10, TGF-β and ADA2 activity levels in patients with MAS (n = 13). D) ADA2 activity in the supernatant of MCSF-induced monocyte-derived macrophages primed with M1 (LPS + IFN-γ) or M2 stimulus (IL-4) for 3 days. Data represent duplicates from three healthy donors. D) ADA2 activity in the supernatant of monocyte-enriched PBMC and monocyte-depleted PBMC following cytokine stimulation for 5 days. Dots represent results from 3 healthy donors per condition in panels B and C.

Supplementary Figure S6

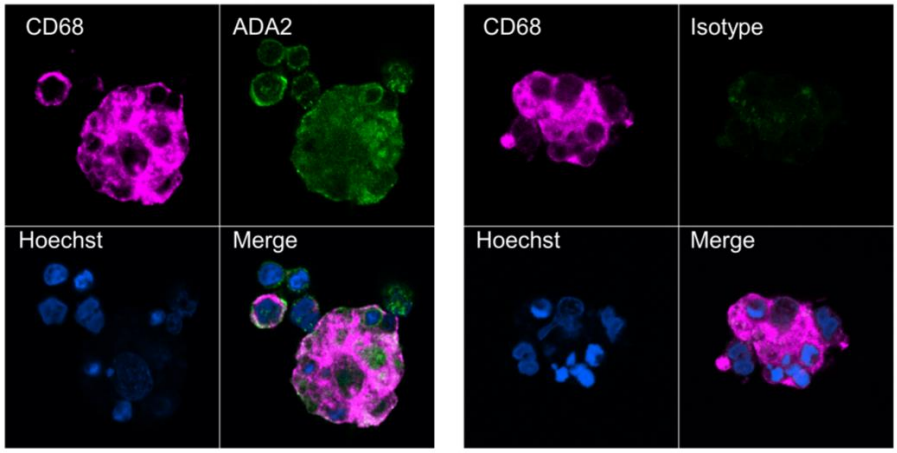

Figure S6. Confocal microscopy of ADA2 expression in bone marrow CD68+ macrophages from a patient with overt MAS using a monoclonal antibody to ADA2 (left) or an isotype control antibody (right).