

Hepcidin as a predictive factor and therapeutic target in erythropoiesis-stimulating agent treatment for anemia of chronic disease in rats

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Supplemental Methods

Animals

Female Lewis rats (Charles River Laboratories, Sulzfeld, Germany) were kept on a standard rodent diet (180 mg Fe/kg, C1000 from Altromin, Lage, Germany) until they reached an age of 6 to 8 weeks and a body weight of 140 to 160 g. The animals had free access to food and water and were kept according to institutional and governmental guidelines in the animal quarters of the Innsbruck Medical University with a 12-hour light-dark cycle and an average temperature of 20°C plus or minus 1°C. Design of the animal experiments was approved by the Austrian Federal Ministry of Science and Research (BMWF-66.011/0155-II/3b/2011).

ESA doses were freshly adjusted to 500 µL with 0.85% saline per rat. LDN-193189 was freshly dissolved in 500 µL 0.85% saline per rat.

Small weekly blood samples (300 µL) from tail vein punctures were used for complete blood counts (CBC) analysis on a Vet-ABC animal blood counter (Scil Animal Care Company, Viernheim, Germany) and serum hepcidin measurements.

At the end of the experiments, rats were anesthetized and blood was taken by retrobulbar puncture. CBC analysis was performed and serum was prepared for serum hepcidin measurement.

Serum iron was measured using the QuantiChrom iron assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions.

Serum hepcidin determination

Determination of hepcidin in rat serum was performed by mass spectrometry as detailed elsewhere ¹.

RNA preparation from tissue, reverse transcription, and TaqMan real-time PCR

Total RNA preparation from nitrogen-frozen rat tissue, reverse transcription of 4 µg RNA, and TaqMan or EvaGreen real-time polymerase chain reaction (PCR) were performed as previously described ².

The following primers and TaqMan probes were used for probe based real-time PCR:

rat *Hamp*: fw 5'-TGAGCAGCGGTGCCTATCT-3', rv 5'-CCATGCCAAGGCTGCAG-3', probe FAM-CGGCAACAGACGAGACAGACTACGGC-BHQ1; rat glucuronidase beta (*Gusb*): fw 5'-ATTACTCGAACAATCGGTTGCA-3', rv 5'-ACCGGCATGTCCAAGGTT-3', probe FAM-CGTAGCGGCTGCCGGTACCACT-BHQ1.

The following primers were used for EvaGreen green based real-time PCR:

Rat IL-6 fw 5'-CTGTCTCGAGCCCACCAGGAA-3', rv 5'-GGCTGGAAGTCTCTTGCGGA-3', *TNF-alpha* fw 5'-AACTTCGGGGTGATCGGTCC-3', rv 5'-GCTTGGTGGTTTGCTACGACG-3' and *IFN-gamma* fw 5'-AGGAACTGGCAAAAGGACGGTA-3', rv 5'-CAGGTGCGATTTCGATGACACT-3'. Melting curve analysis was performed with each run to test specificity in EvaGreen assays and agarose gel electrophoresis of PCR products was performed with first primer tests to ensure a single product. Real-time PCR was performed at least in duplicates on a Bio-Rad CFX96 light cycler. Ssofast Probes Supermix and Ssofast EvaGreen Supermix (Bio-Rad, Vienna, Austria) were used according to the manufacturer's instructions. Relative quantities were calculated using Bio-Rad's CFX Manager software based on a standard curve calculated from four serial tenfold dilutions of the standard. A standard curve was pipetted on each individual plate.

Western blotting

Cytoplasmic protein was extracted from freshly isolated tissue and Western blotting was performed as previously described³. Anti-ferritin antibody (2 µg/mL, Dako, Austria), anti-rat-ferroportin-antibody¹ or anti-actin (2 µg/mL, Sigma, Germany) were used as described previously³.

Nuclear extracts were prepared from freshly isolated tissue using a commercially available kit (NE-PER, Thermo scientific, Rockford, USA). Western blotting of nuclear extracts was performed as described for cytoplasmic extracts. Phospho-Smad1/Smad5/Smad8-antibody (0.1 µg/mL, Cell Signaling Technology, Inc., Danvers, USA) and TATA binding protein (1TBP18, final concentration 0.1µg/mL from Abcam, Cambridge, UK) were used as described previously³.

Immunofluorescence (IF)

Formalin fixed and paraffin-wax embedded rat spleen and duodenum samples were cut at 3 µm thickness.

After deparaffinization in xylenes and rehydration in graded ethanol, a proteinase based antigen retrieval was performed. Slides were incubated in 60 µg/mL proteinase K (Roche, Vienna, Austria) with 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, Sigma) pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma) and 0.5% Triton X-100 (Sigma) for 20' at room temperature (RT).

After blocking unspecific binding with 1% BSA (fraction V, Sigma) in 1x phosphate buffered saline (PBS) (pH 7.4) for 5' at RT, sections were incubated with 1:200 rabbit anti-Fp1 antibody (NBP1-21502, Novus Biologicals, Germany) in PBS/1% BSA for 1h at RT.

After three washes in PBS, donkey anti rabbit Alexa 555 (Invitrogen, Germany) 1:800 in PBS/1% BSA was applied as secondary antibody for 30' at RT.

After three more washes, slides were coverslipped with Roti-Mount FluorCare DAPI (Carl Roth, Germany).

Micrographs were taken with constant exposure conditions for each organ on a Zeiss Axioskop 2 microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a ProgRes C14plus camera (Jenoptik, Jena, Germany) using the ProgRes CapturePro 2.8.8 software for capturing.

Quantification of macrophage iron transport

For macrophage iron uptake and release studies, resident peritoneal macrophages were harvested from control and anemic rats by peritoneal lavage immediately after the anesthetized animals had been killed by cervical dislocation. A total of 0.5×10^6 peritoneal macrophages were seeded in 12-well plates in 750 μ L RPMI medium containing 5% fetal calf serum (endotoxin free FCS gold, PAA, Pasching, Austria), 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma). Macrophages were allowed to adhere for 20 minutes and were then washed extensively to remove non adherent cells. After a resting period of 2 to 4 hours, cells were washed 3 times with serum-free RPMI containing 25 mM HEPES and then incubated therein. Cellular iron efflux from isolated rat macrophages was determined as described ⁷ by a ⁵⁹Fe based method. Briefly, cells were loaded with 5 μ M ⁵⁹Fe-citrate for 4 hours. For iron uptake measurements, cells were washed, harvested, and cellular radioactivity was measured in a gamma counter. For iron release, after the 4 hour iron loading cells were washed and incubated in ⁵⁹Fe free medium for 2 hours. The supernatants were used for gamma counting.

Bone marrow smears

For preparation of bone marrow smears both ends of the harvested rat femur were cut off. The bone was then placed in a specially prepared ⁴ 1.5 mL tube and centrifuged (2 min., 100 x g). Harvested bone marrow cells were immediately diluted in 100 μ L 1xPBS with 1 mM EDTA,

three smears were prepared from the total volume and stained using May-Grünwald-Giemsa (panoptic) technique.

Semiquantitative myelograms and morphologic description were done in a blinded fashion by a hematologist experienced in the analysis of rodent bone marrow smears. Representative fields from three smears per animal were evaluated for cellularity and expressed as averaged percentage of the visual fields.

In each representative visual field cellularity was estimated and 100 cells were counted with respect to G:E ratio and maturation profiles (myelograms) in all hematopoietic lines. For evaluation a Zeiss Axioscope 40 microscope with 5x, 10x, 40x and 100x lenses was used. Representative fields were photo documented on a pixel link system.

Flow cytometry

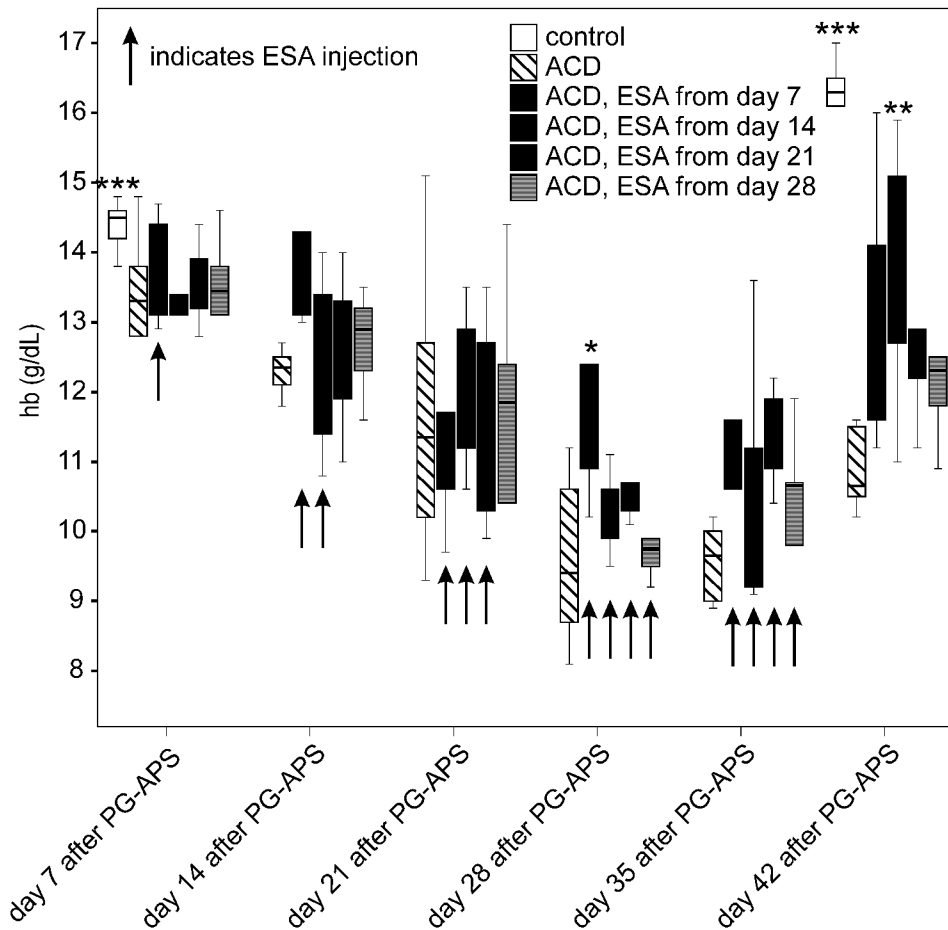
Data were collected on a FACSVerser instrument (BD) and analyzed using FlowJo software (TreeStar Inc). Using FACSVerser allows for cell counting while performing FACS analysis. Fluorochrome-conjugated antibodies were purchased from BD: APC-anti-rat erythroid cells (clone HIS49), PE-anti-CD71 (clone OX-26), V450-anti- CD11b (clone WT.5), and from AbD Serotec: FITC-anti-CD44. Bone marrow from rat femurs was extruded into PBS supplemented with 5 mM EDTA and dissociated. Cell suspensions were dissolved in PBS supplemented with sterile 2% FBS and 0.5% BSA and cells were then costained with the antibodies listed above.

Statistics

Statistical analysis was carried out using SPSS software package version 17.1 (SPSS Inc., Chicago, IL).

Supplemental Methods References

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4. Dobson KR, Reading L, Haberey M, Marine X, Scutt A. Centrifugal Isolation of Bone Marrow from Bone: An Improved Method for the Recovery and Quantitation of Bone Marrow Osteoprogenitor Cells from Rat Tibiae and Femurae. *Calcif Tissue Int*. 1999;65(5):411–3.



Supplemental Figure S1. Blood hemoglobin and serum hepcidin changes in ACD rats treated with darbepoetin alfa.

This figure shows the time course of blood hemoglobin as supplement to the endpoint data depicted in Fig. 2.

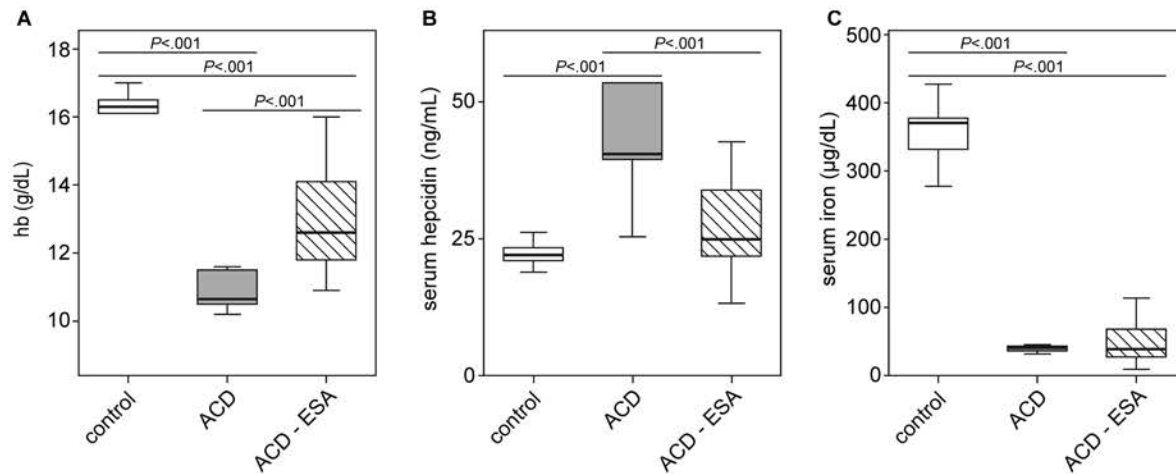
ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Controls received no PG-APS. Groups of ACD rats were treated with darbepoetin alfa (ESA) for 14, 21, 28 or 35 days before euthanasia.

An arrow in the graph indicates when the particular group received ESA treatment.

Data are depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). Groups are non-ACD controls (blank), ACD (diagonal lines) and darbepoetin alfa (ESA) treated ACD groups (shades of grey decreasing with treatment duration). The group treated for 35 days is darkest, followed by treatment for 28 days, 21 days and finally 14 days with the lightest grey. n=10 for controls, n=6 for ACD and n=5 to 6 for each ACD-ESA group.

Statistical significance of differences was calculated using ANOVA and Bonferroni-Holm correction for multiple comparisons. Significant differences (control versus all other groups

and ACD-ESA versus ACD) are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Other changes were not significant.



Supplemental Figure S2. Blood hemoglobin, serum hepcidin and iron levels in ACD rats treated with darbepoetin alfa.

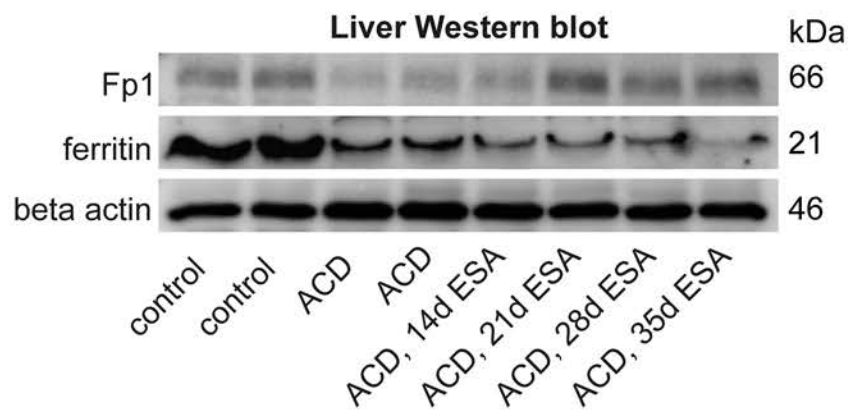
ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Controls received no PG-APS. Groups of ACD rats were treated with darbepoetin alfa (ESA) for either 35, 28, 21 or 14 days before euthanasia. For this supplemental figure, all ESA data were pooled into one group. See Figure 2 for separate data points.

Endpoint data are depicted, as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers).

Statistical significances of differences were calculated using ANOVA and Bonferroni-Holm correction for multiple comparisons. All groups were compared to each other. Only significant differences are depicted.

(A) Blood hemoglobin, (B) serum hepcidin and (C) serum iron are shown.

n=10 for controls, n=6 for ACD and n=22 for ACD-ESA.

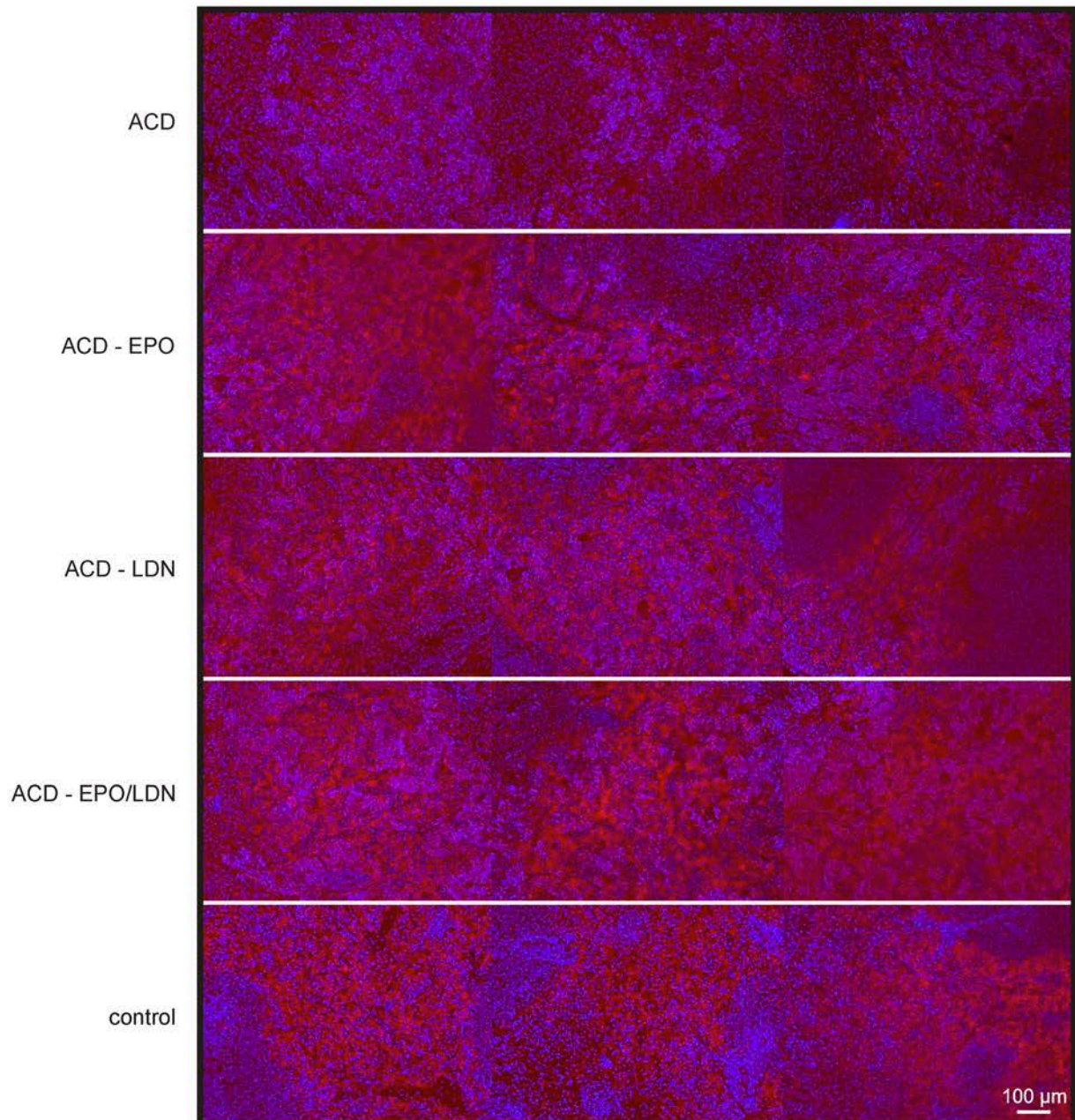


Supplemental Figure S3. Liver iron metabolism in ACD after ESA treatment.

ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Controls received no PG-APS. Groups of ACD rats were treated with darbepoetin alfa (ESA) for 14, 21, 28 or 35 days before euthanasia.

n=10 for controls, n=6 for ACD and n=5 to 6 for each single ACD-ESA group, see Fig.2.

One representative out of at least three western blots of liver Fp1 and ferritin are shown. Beta actin was used as a loading control.

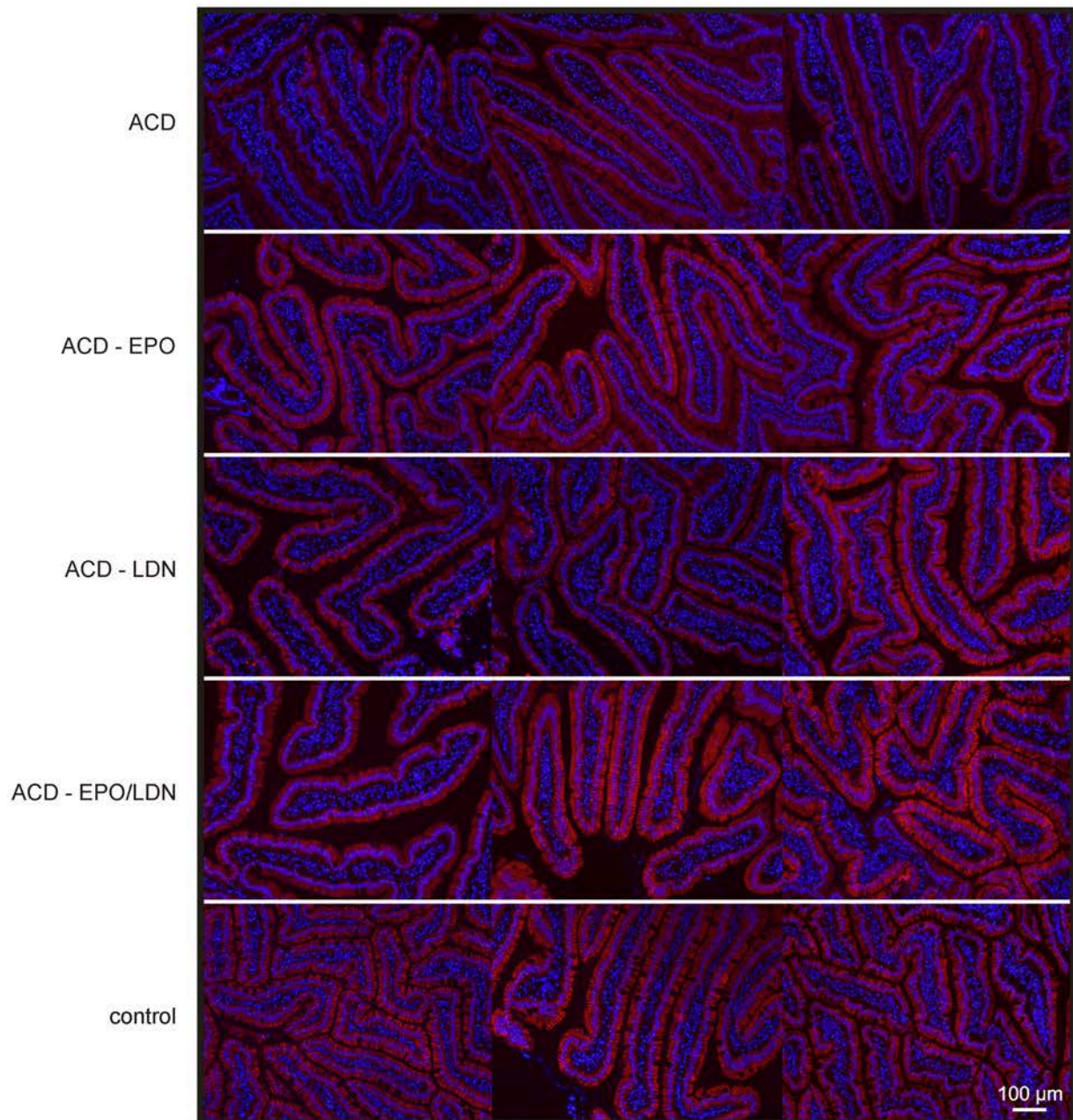


Supplemental Figure S4. Splenic Fp1 immunofluorescence in ACD rats treated with ESA and/or LDN-193189.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN or a combination of both was started on day 21. Controls received no PG-APS. On day 42 all animals were euthanized. n=7/group.

Fp1 immunofluorescence was performed on sections of formalin fixed, paraffin embedded spleens. Fp1 is shown in red (Alexa 555). Nuclei are shown in blue (DAPI counterstain).

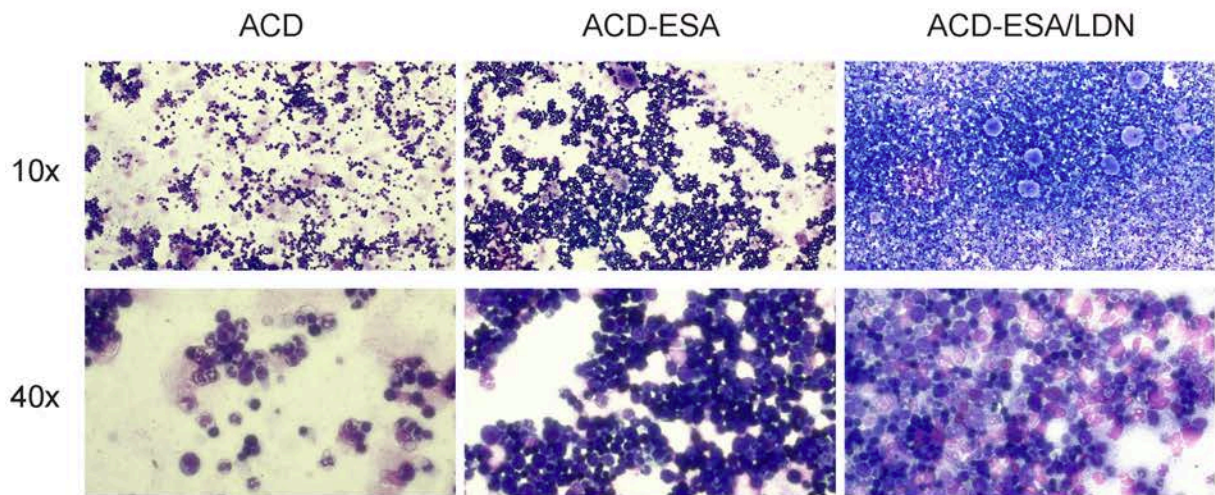
Micrographs of three different individuals per group are depicted.



Supplemental Figure S5. Duodenal Fp1 immunofluorescence in ACD rats treated with ESA and/or LDN-193189.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN or a combination of both was started on day 21. Controls received no PG-APS. On day 42 all animals were euthanized. n=7/group.

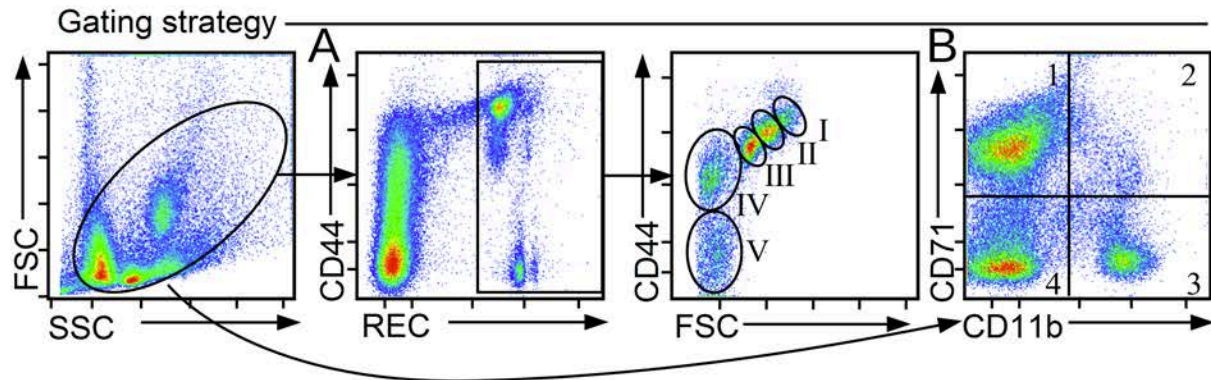
Fp1 immunofluorescence was performed on sections of formalin fixed, paraffin embedded duodena. Fp1 is shown in red (Alexa 555). Nuclei are shown in blue (DAPI counterstain). Micrographs of three different individuals per group are depicted.



Supplemental Figure S6. Bone marrow smears of ACD rats with and without treatment.

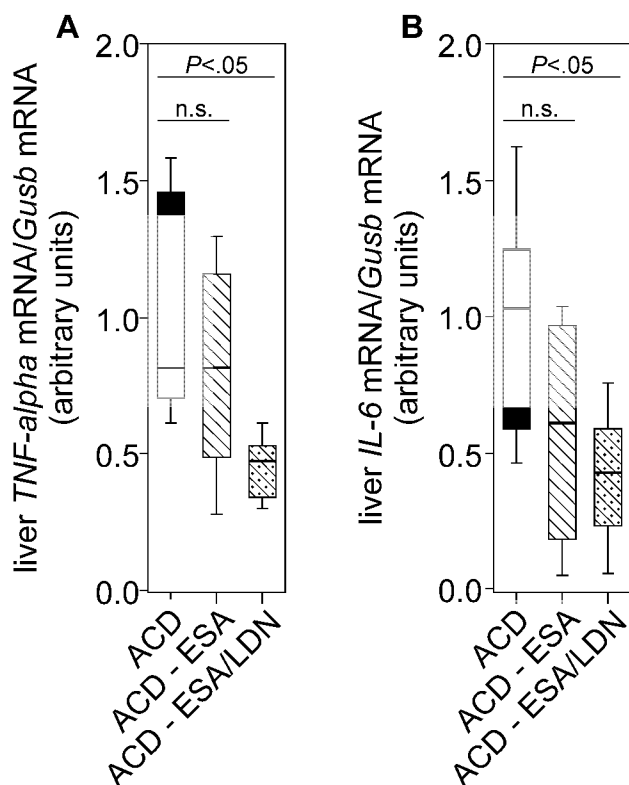
ACD was induced by injection of PG-APS in rats on day 0. Groups of ACD rats were treated from day 21 with darbepoetin alfa (ESA) or with ESA and LDN combined. On day 42 all animals were euthanized. n=7 per group.

Three bone marrow smears per animal were prepared and stained using May-Grünwald-Giemsa as detailed in Supplemental Methods. Representative micrographs for each group are shown. Pictures were taken with a Zeiss Axioscope 40 microscope with a pixel link system at an original magnification of 10x or 40x.



Supplemental Figure S7. Gating strategy for FACS analysis of bone marrow cells.

The two different gating strategies are shown. **(A)** Bone marrow cells were stained with antibodies against rat-erythroid-cells (REC) and CD44. The REC positive population was subsequently plotted according to forward scatter on the x-axis and CD44 expression on the y-axis, so as to identify 5 different stages of erythroide differentiation, corresponding to proerythroblasts (I), basophilic cells (II), polychromatic cells (III), orthochromatic cells and reticulocytes (IV), and mature RBCs (V). **(B)** Bone marrow cells were stained with antibodies against CD11b and CD71. All bone marrow cells were plotted according to CD11b expression on the x-axis and CD71 expression on the y-axis, to identify 4 different groups. CD71⁺/CD11b⁻ marrow erythroid cells (1), CD71⁺/CD11b⁺ and CD71⁻/CD11b⁺ marrow mononuclear cells (2,3) and others (4).



Supplemental Figure S8. ESA effect and ESA/LDN-193189 combined effect in ACD on liver pro-inflammatory cytokine expression.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA) or a combination of ESA and LDN was started on day 21. On day 42 all animals were euthanized. n=7/group.

Groups are ACD (grey), ACD-ESA (diagonal lines) and ACD-ESA/LDN (dots and lines). Data are depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). n=7 for each group.

Liver *TNF-alpha* mRNA (A) and liver *IL-6* mRNA (B), as measured by real-time RT PCR, were normalized to the expression of the housekeeping gene *Gusb* and expressed as abundance relative to the mean of ACD (arbitrary units).

Statistical significance of differences between groups was calculated using ANOVA with Dunnett's test for multiple comparisons versus ACD.

Supplemental Table S1. Red blood cell counts in ACD rats treated with LDN-193189 and/or ESA.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN or a combination of both was started on day 21. Control animals received no PG-APS. On day 42 all animals were euthanized.

Day 42 hemoglobin (hgb), hematocrit (hct), rbc (red blood cell count), mean cellular volume (mcv), mean cellular hemoglobin (mch) and mean cellular hemoglobin concentration (mchc) are shown as means and standard error of the mean (SEM).

	hgb (g/dL)		hct (%)		rbc ($\times 10^6/\mu\text{L}$)		mcv (fL)		mch (pg)		mchc (g/dL)	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
control n=16	16.14	0.12	47.09	0.56	8.47	0.08	55.59	0.35	19.07	0.08	34.33	0.32
ACD n=21	10.92	0.23	34.94	0.74	7.51	0.14	46.55	0.31	14.54	0.12	31.28	0.21
ACD-ESA n=14	13.17	0.29	42.92	0.94	8.97	0.17	47.71	0.32	14.67	0.12	30.67	0.12
ACD-LDN n=13	13.38	0.40	42.96	1.43	8.45	0.27	50.69	0.71	15.85	0.19	31.19	0.19
ACD-ESA/LDN n=14	15.77	0.45	49.75	1.56	9.28	0.28	53.85	0.87	17.03	0.29	31.74	0.23
control - ESA n=5	23.14	0.58	73.93	2.23	12.04	0.41	61.40	1.08	19.26	0.37	31.33	0.21

Supplemental Table S2. White blood cell count in ACD rats treated with LDN-193189 and/or ESA.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN or a combination of both was started on day 21. Control animals received no PG-APS. On day 42 all animals were euthanized.

Day 42 white blood cell count (wbc) and the percentage of lymphocytes, monocytes, granulocytes and eosinophils are shown as means and standard error of the mean (SEM).

	wbc ($\times 10^3/\mu\text{L}$)		lymphoc. (%)		monoc. (%)		granuloc. (%)		eosinoph. (%)	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
control n=16	4.66	0.35	49.91	1.67	11.89	0.48	36.98	1.81	1.22	0.12
ACD n=21	22.80	0.86	15.22	1.06	6.14	0.25	71.31	1.18	7.32	0.36
ACD-ESA n=14	23.19	0.81	12.97	1.65	5.75	0.37	75.62	2.03	5.67	0.53
ACD-LDN n=13	22.61	1.11	9.75	0.89	5.05	0.40	79.20	1.79	6.00	0.97
ACD-ESA/LDN n=14	21.15	1.15	11.10	1.59	4.68	0.57	76.97	2.33	7.25	0.63
control - ESA n=5	7.25	0.85	48.25	2.54	12.40	0.50	37.12	2.54	2.23	0.27