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

Short title: Role of hepcidin in ESA treatment for ACD

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Abstract

Anemia of chronic disease is a multifactorial disorder, resulting mainly from inflammation-driven reticuloendothelial iron retention, impaired erythropoiesis, and reduced biological activity of erythropoietin. Erythropoiesis-stimulating agents have been used for anemia of chronic disease treatment, however, with varying response rates and potential adverse effects. Serum concentrations of hepcidin, a key regulator of iron homeostasis, are increased in anemia of chronic disease patients and linked to its pathogenesis, because hepcidin blocks cellular iron egress, thus limiting iron availability for erythropoiesis. We tested whether serum hepcidin levels can predict and affect the therapeutic efficacy of erythropoiesis-stimulating agent treatment using a well-established rat model of anemia of chronic disease. We found that high pre-treatment hepcidin levels correlated with an impaired hematological response to an erythropoiesis-stimulating agent in anemia of chronic disease rats. Combined treatment with an erythropoiesis-stimulating agent and an inhibitor of hepcidin expression, LDN-193189, significantly reduced serum hepcidin levels, mobilized iron from tissue stores, increased serum iron levels and more effectively improved hemoglobin levels than erythropoiesis-stimulating agent or LDN-193189 monotherapy. In parallel, both the erythropoiesis-stimulating agent and erythropoiesis-stimulating agent/LDN-193189 combined reduced the expression of cytokines known to inhibit erythropoiesis.

We conclude that serum hepcidin levels can predict the hematological responsiveness to erythropoiesis-stimulating agent therapy in anemia of chronic disease. Pharmacological inhibition of hepcidin formation improves the erythropoiesis-stimulating agent’s therapeutic efficacy, which may favor a reduction of erythropoiesis-stimulating agent dosages, costs and side effects.
Introduction

Anemia of chronic disease (ACD), also termed anemia of chronic inflammation (ACI), is the most prevalent anemia among hospitalized patients $^{1,2}$ and is primarily found in subjects suffering from diseases with associated chronic immune activation, such as cancer, autoimmune diseases, chronic infection or dialysis dependent renal failure $^{1-5}$.

A central mechanism, by which chronic immune activation causes anemia, is the retention of iron in the reticuloendothelial system $^{6-9}$, causing a “functional iron deficiency” and consequently an insufficient iron supply for erythropoiesis $^{10}$. In addition, cytokine mediated effects on erythropoietin functionality and erythrocyte half-life, along with antiproliferative effects of cytokines and radicals on the proliferation and differentiation of erythroid progenitors, further contribute to ACD/ACI pathogenesis $^{2,11-13}$.

Hepcidin is a primarily liver derived peptide that orchestrates body iron homeostasis upon binding to cellular iron exporter ferroportin (Fp1) resulting in its internalization and degradation with subsequent reduction of cellular iron egress $^{14}$. As hepcidin is an acute phase protein, its levels are increased in inflammation $^{7,15,16}$, which is of pivotal importance for the reticuloendothelial iron retention underlying ACD $^{3,6,7,9,10,17}$.

Accordingly, neutralization of hepcidin by specific anti-hepcidin antibodies or spiegelmers $^{18,19}$ as well as pharmacological inhibition of hepcidin formation could either reverse iron retention in the reticuloendothelial system and/or improve anemia in different animal models of inflammatory anemia $^{20,21}$. In line with this, serum hepcidin has been suggested as a useful marker for patients with ACD $^{22}$.

Overexpression of hepcidin in mice or injection of recombinant hepcidin causes a hypochromic anemia and impaired response to endogenous erythropoietin $^{10,23}$. Of interest, erythropoietin has been shown to decrease hepcidin expression $^{24,25}$ via induction of erythropoiesis $^{25,26}$. Although erythropoietin treatment decreased liver Hamp (the gene encoding for hepcidin) expression in a mouse model of generalized inflammation $^{27}$, erythropoietin treatment could only partially correct anemia in this model $^{28}$.

As the development of anemia negatively affects the patients’ quality of life and impairs basic physiological functions such as cardiovascular performance $^{29,30}$ treatment of anemia with erythropoiesis-stimulating agents (ESA; epoetin or darbepoetin alfa) was introduced into clinical practice. This therapy can significantly improve the patients’ quality of life $^{29,30}$ but can also result in undesirable side effects, like thrombophilia, stroke and death, as observed in cancer patients or subjects with end stage renal disease $^{30-32}$. Due to ESA effects on plasma volume, the risk of thrombosis could depend on the required ESA dose $^{30}$. Interestingly, at
least in dialysis patients, the risk of cardiovascular events is associated with an impaired hematological responsiveness to ESA. Furthermore, ESA treatment is ineffective in specific patients suffering from ACD/ACI, even if true iron deficiency has been ruled out. Clinically useful biomarkers which predict the efficacy of ESA treatment are desirable, but have not been identified thus far. Due to its pivotal role in iron metabolism and the fundamental role of iron in erythropoiesis, hepcidin is a good candidate for such a predictor. Therefore, we used a well-established arthritis model (PG-APS) in rats, which resembles characteristics of human ACD and studied the biological interaction between hepcidin and ESA treatment in a prospective fashion.

**Methods**

**Animals**

Female Lewis rats were kept on a standard rodent diet until they reached an age of 6 to 8 weeks and a body weight of 140 to 160 g. All treatments were performed by intraperitoneal (i.p.) injection. Chronic inflammation (arthritis) causing ACD was induced as described, using group A streptococcal peptidoglycan-polysaccharide (PG-APS) (Lee Laboratories, Grayson, GA) at a total dose of 15 µg rhamnose/g body weight. For a short term ESA experiment, untreated ACD rats were compared to ACD rats treated with 500 U of recombinant human erythropoietin (rhEPO, epoetin alfa, EPREX, Janssen Cilag). One group received the rhEPO treatment once (on day 16 after PG-APS injection), the other group thrice on three consecutive days, starting from day 14 after PG-APS injection. Rats were sacrificed on day 17 after PG-APS injections. In all following experiments, rats were euthanized 42 days after the induction of arthritis. In these long term experiments, darbepoetin alfa (Aranesp, Amgen, at 10 µg/kg body weight) was used because of its longer half life. Darbepoetin alfa treatments were performed weekly for the indicated periods. Another treatment modality was the administration of LDN-193189 (LDN, Axon Medchem, Groningen, Netherlands), which was used at a dose of 3 mg/kg body weight and given every second day. First, rats were treated with darbepoetin alfa for 14, 21, 28 or 35 days before euthanasia. One group of ACD rats received no darbepoetin and control rats were not injected with PG-APS.
In another experimental setup, ACD rats either received no drug or were treated with
darbepoetin alfa alone or in combination with LDN for 21 days before euthanasia. In a further
series of independent experiment, ACD animals were injected with LDN alone.
For flow cytometry analysis of bone marrows, we compared healthy controls, untreated ACD
rats and ACD rats treated with darbepoetin alfa, LDN or both for 21 days before euthanasia.
For determination of hemoglobin and hepcidin levels over time, small blood samples (300
µL) were taken weekly by tail vein puncture from every animal.

**Analysis of rat specimens**
For details on complete blood counts (CBC), serum hepcidin \(^7\) and serum iron measurements,
Western blotting \(^7,36\), RT real-time PCR \(^37\), macrophage iron export measurements \(^7\),
immunofluorescence, bone marrow smears and bone marrow flow cytometry please see the
supplemental information.

**Statistics**
Comparisons between multiple groups in this study were performed by ANOVA with
Bonferroni-Holm correction or Dunnett’s test for multiple comparisons. A corrected p value
below 0.05 was regarded as significant.

Further details on the methods used for this study can be found in the supplement.

**Results**
*ESA therapy inhibits hepatic Hamp mRNA expression in vivo in a rodent model of anemia of
chronic disease.*
Following our working hypothesis, we first tested if ESAs affect hepcidin expression in a rat
model of ACD. Therefore, we injected rhEPO (500 U i.p.) into rats once or on three
consecutive days starting two weeks after induction of ACD upon PG-APS injection. As
shown in Fig. 1A, both a single as well as three consecutive doses of rhEPO, significantly
decreased liver *Hamp* mRNA expression as compared to untreated animals. Importantly, this
was paralleled by reduced serum hepcidin levels in the respective groups (Fig. 1B).

*Long term effect of ESA treatment in ACD*
As the short term experiments proved the effectiveness of rhEPO treatment to reduce the
hepatic expression of hepcidin in this specific model, we conducted long term experiments to
study the effects of such interventions on ACD correction. Fig. 2A shows hemoglobin concentrations of ACD animals at day 42 after injection of PG-APS. Depending on the assigned group, animals received ESA treatment for 14, 21, 28 or 35 days, respectively. Darbepoetin alfa (10 µg/kg) was used for this experiment because of its longer half life.

Fig. 2A shows the trend towards higher hemoglobin in ESA treated groups, though this change became significant only for the 28 days ESA treatment group (p<0.01). All ACD groups, either ESA treated or untreated, presented significantly lower hemoglobin levels at the end of the observation period than healthy controls (p<0.001, Fig. 2A).

We found serum hepcidin to be reduced by ESA treatment (Fig. 2B), though statistical significance was reached not for all ESA groups. However, serum hepcidin levels in the ESA treated animals were not significantly different from controls, while untreated ACD animals had increased serum hepcidin concentrations (p<0.01, Fig. 2B). Interestingly, the ESA mediated reduction of hepcidin levels did not translate into significant changes of serum iron concentrations (Fig. 2C), and serum iron remained below control levels in all ACD groups (p<0.001, Fig. 2C).

Differences between the single ESA treatment groups (n=5 to 6 per group) where small and not significant for hemoglobin, serum hepcidin and serum iron. However, the overall effect of ESA for pooled data of ACD rats from all treatment periods showed a significant increase of hemoglobin and serum iron levels (p<0.001, Suppl. Fig. S2)

When performing Western blot analysis in the spleen after the termination of the experiment (day 42), we found that Fp1 protein levels (Fig. 3A) were lower in ACD as compared to control rats, and this reduction was partially antagonized by ESA treatment in a time dependent fashion (Fig. 3A). Accordingly, spleen ferritin levels were higher in ACD (Fig. 3A) as compared to control animals, whereas ferritin expression was declining with prolongation of ESA therapy (Fig. 3A).

These observations in the spleen are paralleled by corresponding changes in the liver (Suppl. Fig. S3).

To specifically investigate the functional effects of ESA therapy on iron homeostasis on a cellular basis, we studied peritoneal macrophages from healthy controls and from ACD rats, with or without 35 days of darbepoetin alfa treatment, in vitro. We found that $^{59}$Fe release from macrophages derived from ACD rats was significantly lower than from macrophages of control animals (p<0.001, Fig. 3B), while ESA treatment of ACD rats significantly increased macrophage iron release (p<0.05, Fig. 3B), which, however, remained lower than in control animals (p<0.001, Fig. 3B). Importantly, these alterations of macrophage iron export nicely
correspond to the changes in spleen Fp1 expression observed in ACD animals with and without ESA treatment (Fig. 3 A-B). The effects seen on $^{59}$Fe release (Fig. 3B) are not caused by changes in $^{59}$Fe uptake, because uptake is increased in ACD (p<0.001, Fig. 3C) and not significantly changed by ESA treatment, with a trend towards decrease (Fig. 3C).

**Hepcidin predicts response to ESA treatment**

Based on these observations and being aware of the regulatory effects of hepcidin on Fp1 expression 14, which largely determines circulating iron levels and iron availability for erythropoiesis 21, and because we observed variations in the responsiveness of rats to ESA treatment (Fig. 2A, Supplemental Fig. S1), we questioned, whether pretreatment serum hepcidin levels may determine the responsiveness of ACD rats to ESA therapy. Mean serum levels of hepcidin in ACD rats at 7, 14 and 21 days after induction of inflammation did not differ significantly (48.4+/−12.5 ng/mL, 62.7+/−18.8 ng/mL, 50.2+/−22.2 ng/mL, mean+/−standard deviation, respectively), indicating that there was no general change over time in pretreatment serum hepcidin levels before initiation of ESA and/or LDN therapy.

We thus performed a correlation analysis between serum hepcidin levels in ACD rats immediately before the start of ESA therapy in the different treatment groups and the consecutive change of hemoglobin concentrations over two weeks, because it took at least two weeks until a significant change in hemoglobin levels upon ESA treatment could be observed (Supplemental Fig. S1).

By performing Spearman rank correlation analysis we observed a highly significant negative correlation between pre-treatment serum hepcidin levels and the relative increase of hemoglobin after two weeks of ESA therapy (Fig. 4A, rho=-0.557, p<0.01). In individuals with pre-treatment serum hepcidin levels above 40 ng/ml, blood hemoglobin levels on average declined even with ESA treatment over two weeks of follow up (Fig. 4B), while lower pre-treatment hepcidin levels (below 40 ng/mg) predicted a better hematological response to ESA therapy (p<0.01, Fig. 4B). This indicates that high pre-treatment hepcidin levels in rats with ACD are associated with a poor hematological response to ESA therapy.

**Hepcidin inhibition improves the therapeutic efficacy of ESA treatment**

Based on the observations made thus far, we questioned whether inhibition of hepcidin formation may increase the therapeutic efficacy of ESA. We thus performed a new series of experiments, and investigated the combined effects of LDN-193189, a small-molecule inhibitor of BMP type I receptors, which reduces hepcidin expression in models of ACD 21,
and ESA therapy. Therefore, anemic rats received either darbepoetin alone on a weekly basis or a combination of darbepoetin plus LDN. Importantly, the combined treatment with ESA/LDN resulted in a faster and more sustained increase of hemoglobin levels as compared to treatment with ESA alone (Fig. 5A).

To see whether the combined effects of ESA and LDN treatments differed from that observed after sole LDN treatment, we analyzed the changes of hemoglobin levels following sole LDN treatment of ACD rats. Interestingly, hemoglobin levels of ACD rats were similar after three weeks of either ESA or LDN treatment (Fig. 5E), but neither regimen could normalize hemoglobin levels to the values observed in untreated, healthy controls (Fig. 5E, p<0.01). Of note, combined ESA/LDN treatment resulted in significantly higher hemoglobin levels than treatment with LDN alone (Fig. 5E, p<0.05) providing further evidence for synergistic effects of LDN and ESA to reverse inflammatory anemia.

At the end of the treatment period, both liver 

Hamp mRNA (Fig. 5B) and serum hepcidin levels (Fig. 5C), were significantly reduced in the ESA/LDN treated ACD animals as compared to untreated ACD rats (p<0.05 and p<0.01). In line with the predicted effect of LDN toward BMP type I receptor mediated SMAD phosphorylation we found a strong reduction of SMAD1,5,8 phosphorylation in the livers of ACD rats (Fig. 5J) after combined treatment with ESA/LDN, whereas sole ESA treatment had little effect, which resembles the differences in the reduction of hepcidin expression between the two treatment regimens (Fig. 5 B-C, J).

The functional relevance of serum hepcidin changes is supported by immunofluorescence analysis in spleen (Supplemental Fig. S4) and duodenum (Supplemental Fig. S5). The cell surface expression of Fp1 in spleen macrophages and duodenal enterocytes is strongly reduced in ACD as compared to control animals, whereas treatment with ESA or LDN and most prominently combined ESA/LDN enhanced Fp1 protein cell surface expression in these two organs (Supplemental Fig. S4 and S5).

Serum iron levels were not altered by sole ESA treatment, whereas the combination of ESA with LDN resulted in a significant increase of serum iron concentrations (p<0.05, Fig. 5D).

When analyzing hematological parameters in the different treatment groups we found that MCV and MCH were not affected by ESA treatment alone as compared to untreated ACD rats, while the administration of LDN (p<0.001, Fig. 5F-G) and the combination of ESA/LDN (p<0.001, Fig. 5 F-G) increased these indices.

*ESA/LDN treatment ameliorates erythropoiesis in ACD rats*
Upon investigation of bone marrow composition (for sample bone marrow micrographs see Supplemental Fig. S6) we found an increased cellularity after treatment with ESA alone (p<0.01, Fig. 5H) which became more prominent by ESA/LDN combined treatment (p<0.001, Fig. 5H). This was paralleled by a pronounced and highly significant (p<0.001, Fig. 5I) shift in the ratio of granulopoiesis to erythropoiesis upon both ESA and ESA/LDN treatment. These data demonstrate an expansion of the erythropoietic lineage in the bone marrow which was more pronounced with ESA/LDN than sole ESA treatment.

To further analyze the effects of combined treatment on erythropoiesis, we performed FACS analysis on bone marrows. We studied the percentage of erythroid cells (CD71^+CD11b^-) (Suppl. Fig. S7B) and further analyzed maturation stages of erythroid differentiation using anti-rat-erythroid-cells (REC) and anti-CD44 antibodies (Suppl. Fig. S7A).

In agreement with Richardson et al. 38 we found a dramatic decrease in CD71^+CD11b^- erythroid cells in the bone marrow of ACD rats compared to control rats (p<0.001, Fig. 6A,C). When studying erythroid differentiation, we found a significantly reduced number of orthochromatic cells/reticulocytes in the ACD rats when compared to controls (p<0.001, Fig. 6B,D), while other precursors were not reduced (Fig. 6B).

Both ESA and LDN treatment resulted in a trend towards increased numbers of CD71^+CD11b^- erythroid cells (Fig. 6E) and orthochromatic cells/reticulocytes (Fig. 6F) in the bone marrow, but the combination of ESA and LDN resulted in a more sustained and significant increase in the number of CD71^+CD11b^- erythroid cells and orthochromatic cells/reticulocytes (p<0.05, Fig. 6E-F). The FACS results are in accordance with the results obtained from the bone marrow smears (Fig. 5H-I).

The complete blood count data from ESA/LDN experiments can be found in Supplemental Tables S1 and S2. Of note, neither treatment resulted in a change in total white blood cell counts (Suppl. Table S2) as compared to ACD animals.

**Anti-inflammatory effect of ESA/LDN treatment**

As erythropoietin has recently been shown to inhibit pro-inflammatory immune effector pathways 39 and because hepcidin exerts immune modulatory effects 40,41 we questioned, whether part of the erythropoiesis-stimulating activity of ESA and/or LDN can be traced back to alterations of immune activation states. Indeed, we found that ESA significantly reduced TNF-alpha mRNA expression in the spleen (p<0.05, Fig. 7A), and this effect was even more pronounced in combination with LDN (p<0.001, Fig. 7A). Similarly, ESA/LDN treatment significantly inhibited spleen IFN-gamma (p<0.01, Fig. 7B) and IL-6 (p<0.01, Fig. 7C).
mRNA expression. To rule out that this effect was due to substitution of macrophages by hematopoietic cells in the spleen we also studied TNF-alpha (Suppl. Fig. S8A) and IL-6 (Suppl. Fig. S8B) mRNA expression in the livers and found comparable alterations of their expression following treatment with ESA and LDN.

Discussion
Herein, we provide evidence for two important, clinically relevant functions of the master regulator of iron homeostasis, hepcidin. First, in predicting the response to ESA therapy in inflammatory anemia and second in showing that inhibition of hepcidin formation can accelerate and potentiate the hematological response of ESA resulting in normalization of hemoglobin levels even in the presence of severe inflammation.

Erythropoiesis is the main consumer of iron in the human body, and the majority of iron needed for this process originates from macrophages which take up and degrade senescent erythrocytes resulting in re-utilization of iron. The transfer of iron from macrophages to the circulation is largely controlled by the interaction of hepcidin with the iron export protein Fp1. Thus, the high hepcidin levels found in ACD block macrophage iron egress and contribute to an iron restricted erythropoiesis.

Here we demonstrate in a rat model of ACD that higher serum hepcidin levels predict a poorer hematological response to ESA treatment. However, the benefit/risk-ratio of ESA therapy of anemia is still a matter of discussion, which makes the availability of predictive diagnostic markers desirable.

The negative correlation between high circulating hepcidin levels and the response to ESA therapy most likely relates to the fact that higher hepcidin activity is associated with a reduced availability of iron for erythropoiesis. Our observation is in agreement with the observation by Prentice and co-workers who found that hepcidin is the major predictor of iron incorporation into red cells in anemic children in Africa. As hepcidin is regulated by various stimuli such as hypoxia, anemia, iron or inflammation it is of importance to consider which regulatory mechanism underlies the poor predictive effect of high hepcidin levels for the response to ESA therapy.

Importantly, hepcidin is differently regulated by iron and inflammation and recent evidence suggests that iron deficiency dominates over inflammation mediated regulation of hepcidin expression in animal models of inflammatory anemia and in patients with inflammation or undergoing phlebotomy.
Thus, the negative association between ESA responsiveness and hepcidin levels may be due to enhanced inflammation and cytokine activities which, first, promote macrophage iron retention and erythrophagocytosis, second, block ESA activity via inhibition of erythropoietin receptor expression and/or functionality on erythroid progenitor cells and presumably reduce the expression/activity of erythroid regulators of hematopoiesis such as GDF15 along with apoptosis inducing effects of several cytokines toward erythroid progenitor cells.

However, serum hepcidin might prove as a good marker to identify those ACD patients who may respond to ESA therapy, thereby avoiding unnecessary treatment cycles. Nonetheless, the predictive diagnostic value of hepcidin may be different in other disorders such as chronic renal failure, where higher hepcidin levels are also a consequence of reduced renal clearance.

We could demonstrate that ESA treatment reduced serum hepcidin and liver Hamp mRNA expression in rats with ACD. In accordance with the changes in hepcidin levels, ESA treatment of ACD rats reduced iron storage in the spleen as evidenced by reduced ferritin levels and increased iron mobilization from macrophages of ACD animals.

However, this ESA driven iron mobilization was insufficient, as demonstrated by a non-significant increase of serum iron levels and unaffected MCV and MCH which also translated to minimum improvement of anemia. Importantly, high hepcidin levels were associated with a reduced therapeutic efficacy of ESA, which prompted us to study the combined effects of ESA and the pharmacological inhibitor of hepcidin expression, LDN.

Compared to both LDN and ESA monotherapy, the combination of LDN and ESA resulted in an improved and faster hematological response, and hemoglobin levels reached the threshold observed in non-inflamed control animals. The LDN/ESA combination therapy was also superior to the monotherapies in increasing MCV and MCH and expanding the number of erythroid cells and orthochromatic cells/reticulocytes in the bone marrow.

This goes along with the observation that hepcidin inhibition by shRNA mediated knock down prior to induction of acute anemia upon injection of heat inactivated Brucella melitensis into mice or application of anti-hepcidin antibodies could partly prevent anemia development upon concomitant injection of ESA. However, in this model neither an effect of hepcidin inhibition alone nor an effect of the combined treatments toward improvement of an existing anemia could be demonstrated. Our results thus provide novel evidence on the potential therapeutic efficacy of this combined treatment in a rodent model which resembles the pathophysiology of human ACD.
This leads to the questions toward the mechanism underlying the improved therapeutic efficacy of combination treatment.

Combining ESA treatment with LDN improved hemoglobin and significantly affected serum iron, MCV and MCH, indicating that ESA therapy benefits from supportive iron mobilization through hepcidin antagonization in ACD.

Furthermore, ESA/LDN treatment reduced the expression of pro-inflammatory cytokines, such as IL-6, TNF-alpha or IFN-gamma. These proteins contribute to inflammatory anemia by promoting macrophage iron retention, blocking duodenal iron absorption, by reducing erythrocyte half-life, by impairing erythroid progenitor proliferation and differentiation and/or blocking the biological activity of erythropoietin. IFN-gamma, a T-helper cytokine, causes anemia by reducing erythrocyte half-life, by blocking the proliferation of erythroid progenitor cells, or by reducing macrophage iron egress via inhibition of Fpl transcription. Mechanistically, part of these anti-inflammatory effects can be traced back to erythropoietin mediated inhibition of pro-inflammatory NF-κB driven immune effector pathways in macrophages. Accordingly, erythropoietin has been demonstrated to improve the clinical course of inflammation driven auto-immune disorders by this pathway.

Interestingly, we found a more sustained reduction of pro-inflammatory cytokines with combined ESA/LDN treatment than with ESA alone. The blockage of hepcidin expression and/or the BMP pathway by LDN may thus exert anti-inflammatory effects. This is supported by the finding that hepcidin by itself causes immune modulatory effects. A BMP-4 dependent stress erythropoiesis was shown to be partly responsible for erythropoietin induced improvement of hemoglobin levels in zymosan induced anemia. However, the fact that the BMP receptor inhibitor LDN strongly improves the erythropoietin response rules out that this mechanism is responsible for the anti-inflammatory effects observed in our model.

Apart from reducing hepcidin expression by LDN, other therapeutic strategies to block the biological activity of hepcidin, such as soluble hemojuvelin, anti-hepcidin antibodies or spiegelmers may also prove efficient to ameliorate the therapeutic response of ESAs.

In summary, we provide evidence that predicting the response to ESA therapy by measuring pre-treatment hepcidin levels and upon applying a combined treatment of ESA and hepcidin antagonizing agents can improve the therapeutic efficacy of ESA therapy. In patient treatment secondary benefits could be a reduction of dosages, costs and most importantly undesired or serious adverse effects of ESA treatment. Once anti-hepcidin strategies become clinically available this strategy of combination therapy has to be studied in a randomized prospective
fashion also keeping in mind to evaluate the effect of anemia correction on the course of the diseases underlying ACD.

Authorship and Disclosures

Contribution: M.T., I.T. and G.W. designed the research, controlled and analyzed the data, and wrote the paper; M.T., I.T., M.N., A.S., T.S., M.A., D.H., M.S., D.W., W.P., W.W., A.T.M. and D.R.W. performed the experiments; all authors checked the final version. Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


**Figure 1.** ESA therapy inhibits hepatic *Hamp* mRNA induction *in vivo* in a rodent model of anemia of chronic disease.

ACD was induced in female Lewis rats by i.p. injection of PG-APS. Animals were then either treated i.p. with 500 U erythropoietin alfa (ESA) on day 16 after PG-APS (ACD, 24h ESA group) or on day 14, 15 and 16 after PG-APS injection (ACD, 72h ESA group). All animals were sacrificed on day 17. n=9 per group.

*Hamp* mRNA levels in the livers were measured by real-time RT-PCR (A). Data are normalized to the expression of the housekeeping gene *Gusb* and expressed as abundance relative to the mean of control (arbitrary units) and depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). Hepcidin peptide was measured in serum (B).

ESA groups were compared against ACD using ANOVA and Bonferroni-Holm correction for multiple comparisons.
Figure 2. 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.

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. * indicates a significant difference versus each single other group with p<0.001.

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

n=10 for controls, n=6 for ACD and n=5 to 6 for each ACD-ESA group.
Figure 3. Spleen and macrophage iron metabolism following ESA treatment.

(A) 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. One representative out of at least three Western blots of spleen Fp1 and ferritin (A) are shown. Beta actin was used as a loading control. n=10 for controls, n=6 for ACD and n=5 to 6 for each single ACD-ESA group, see Fig.2.

In another experiment (B-C), peritoneal macrophages were taken from control rats, ACD rats (42 days after PG-APS injection), and ESA treated ACD rats (35 days of weekly ESA treatment). The cells were loaded with Fe\(^{59}\)-citrate for 4 hours. Afterwards, the radioactivity was measured in the washed, harvested cells (B). Other cells were washed and incubated for 2 hours of culture in Fe\(^{59}\) free medium. At the end, gamma counting was performed on supernatants to determine iron release (C). Data are depicted, as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). Data were normalized to control (arbitrary units). Statistical significances were calculated using ANOVA and Bonferroni-Holm correction for multiple comparisons. n=12 for controls and n=8 for ACD and ACD-ESA for macrophage experiments.
Figure 4. Correlation of pre-treatment serum hepcidin levels with changes of hemoglobin concentrations following ESA treatment in ACD rats.

ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Groups of ACD rats were treated with darbepoetin alfa (ESA) for either 35, 28, 21 or 14 days before euthanasia. Data is from the same ACD animals as in Fig. 2 and Fig. 3A. Serum hepcidin concentrations prior to onset of ESA treatment of ACD animals are plotted against the relative change of hemoglobin over the first two weeks of ESA treatment (hemoglobin after two weeks of ESA treatment minus hemoglobin concentrations before ESA treatment).

(A) The linear regression is shown. Spearman’s rank correlation reveals a highly significant negative correlation between these two parameters (\(\rho=-0.557, \ p<0.01\)).

(B) Animals are divided into two groups according to low (<40 ng/ml) and high (>40 ng/ml) pre-treatment serum hepcidin levels. Data are depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). The statistical significance of the difference in hemoglobin change between the two groups was calculated by Student’s t-test (\(p<0.01\)).
Figure 5. Effects of ESA and/or LDN-193189 on hemoglobin levels and erythropoiesis in rats with inflammatory anemia

ACD was induced by injection of PG-APS in rats on day 0. Groups of ACD rats were treated from day 21 onwards with darbepoetin alfa (ESA) or with ESA and LDN in combination. On day 42 all animals were euthanized. Accordingly, the treatment duration was always 21 days. Data are depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). n=7 for each group.

The trend of blood hemoglobin over the complete treatment period is depicted in (A). Groups are ACD (grey), ACD-ESA (diagonal lines), ACD-ESA/LDN (dots and lines). All other figures (B-J) show the endpoint of treatment. Liver Hamp mRNA (B), as measured by real-time RT PCR, was normalized to the expression of the housekeeping gene Gusb and expressed as abundance relative to the mean of ACD (arbitrary units). Serum hepcidin (C) was measured by mass spectrometry and serum iron (D) by colorimetry. In (E-F) age matched, untreated, non ACD controls (n=10), and LDN only treated ACD animals (n=6, also treated from day 21) are included for comparison.

Bone marrow cellularity and the ratio of granulopoiesis to erythropoiesis are shown in (H, I). The SMAD1/5/8 complex was detected by Western blotting in liver nuclear extracts (J). TATA binding protein (1TBP18 antibody) was detected in the respective samples as a nuclear loading control. One representative out of three blots is shown.

Statistical significance of differences between groups was calculated using ANOVA with Bonferroni-Holm correction for multiple testing.
Figure 6: ESA and LDN-193189 treatment ameliorate defective marrow erythropoiesis in ACD rats.

ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN-193189 or a combination of both was started on day 21. Controls received no PG-APS. On day 42 all animals were euthanized for marrow analysis by flow cytometry. n=7/group.

All details on the gating strategy are shown in Suppl. Fig. S7. Representative blots for control and ACD rats are shown in (A) and (B). The numbers in (A) and (B) are the mean percentages of all animals for each gate ± standard error of the mean (SEM). The total number of marrow erythroid cells (CD71+CD11b−) per femur (C-D) and the total number of orthochromatic cells/reticulocytes (CD44dim,FCSlow) per femur (E-F) are shown as mean ± SEM. Student’s t-test was applied for pairwise comparison (C,E) and ANOVA with Dunnett’s test for multiple comparisons versus ACD (D,F). *p < 0.05, **p < 0.01.
Figure 7. Effects of ESA or ESA/LDN-193189 treatment on spleen cytokine expression in ACD rats.

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.

Spleen TNF-alpha mRNA (A), spleen IFN-gamma mRNA (B) and spleen IL-6 mRNA (C), 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 Bonferroni-Holm correction for multiple testing. Data were logarithmized to reach homogeneity of variance.
(A) Spleen Western blot

- Fp1: control, control, ACD, ACD, ACD, 14d ESA, ACD, 21d ESA, ACD, 28d ESA, ACD, 35d ESA
- Ferritin: control, control, ACD, ACD, ACD, 14d ESA, ACD, 21d ESA, ACD, 28d ESA, ACD, 35d ESA
- Beta actin: control, control, ACD, ACD, ACD, 14d ESA, ACD, 21d ESA, ACD, 28d ESA, ACD, 35d ESA

(B) Peritoneal macrophage Fe$^{59}$ release (arbitrary units)

- Control: P < .001, ACD: P < .001

(C) Peritoneal macrophage Fe$^{59}$ uptake (arbitrary units)

- Control: P < .001, ACD-ESA: n.s.
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.

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.

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

The following primers were used for EvaGreen green based real-time PCR:
Rat IL-6 fw 5'-CTGTCTCGAGCCCACCAGGA-3', rv 5'-GGCTGGAAGTCTCTTGCGGA-3', TNF-alpha fw 5'-AACTTCGGGGTGATCGGTCC-3', rv 5'-GCTTGGTGGTTTGCTACGACG-3' and IFN-gamma fw 5'-AGGAACTGGGCAAAGGACGGTA-3', rv 5'-CAGGTGCAGATTGCATGACACT-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 described. Anti-ferritin antibody (2 μg/mL, Dako, Austria), anti-rat-ferroportin-antibody or anti-actin (2 μg/mL, Sigma, Germany) were used as described previously.

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 previously.

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 x 10^6 peritoneal macrophages were seeded in 12-well plates in 750 µL 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 µM 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 µL 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 FACSVerse instrument (BD) and analyzed using FlowJo software (TreeStar Inc). Using FACSVerse 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


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).

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<th>hct (%)</th>
<th>rbc (x10^6/µL)</th>
<th>mcv (fL)</th>
<th>mch (pg)</th>
<th>mchc (g/dL)</th>
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<tr>
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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).

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<tr>
<th></th>
<th>wbc (x10³/µL)</th>
<th>lymphoc. (%)</th>
<th>monoc. (%)</th>
<th>granuloc. (%)</th>
<th>eosinoph. (%)</th>
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<tr>
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Mean and standard error of the mean (SEM).