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

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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 the treatment of anemia of chronic disease, although with varying response rates and potential adverse effects. Serum concentrations of hepcidin, a key regulator of iron homeostasis, are increased in patients with anemia of chronic disease and linked to the pathogenesis of this disease, because hepcidin blocks cellular iron egress, thus limiting availability of iron 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 hematologic response to an erythropoiesis-stimulating agent in rats with anemia of chronic disease. 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 improved hemoglobin levels more effectively than did the 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 hematologic 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, 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, auto-immune 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 the pathogenesis of ACD/anemia of chronic inflammation.[2,11–13]

Hepcidin is a primarily liver-derived peptide that orchestrates body iron homeostasis upon binding to the 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 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 a 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 patients' quality of life[29,30] but can also result in undesirable side effects, such as thrombophilia, stroke and death, as observed in cancer patients or subjects

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with end-stage renal disease.[30–32] Due to the effects of ESA on plasma volume, the risk of thrombosis could depend on the dose of ESA required.[30] Interestingly, at least in dialysis patients, the risk of cardiovascular events is associated with impaired hematologic responsiveness to ESA.[33] Furthermore, ESA treatment is ineffective in some patients suffering from ACD/anemia of chronic inflammation,[2,4] even if true iron deficiency has been ruled out.[34]

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.

We, therefore, used a well-established arthritis model in rats, which has features resembling human ACD,[7,35] 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 elsewhere,[21] using group A streptococcal peptidoglycan-polysaccharide (PG-APS) (Lee Laboratories, Grayson, GA, USA) 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 the following experiments, rats were euthanized 42 days after the induction of arthritis. In these long-term experiments, darbepoetin alfa (Aranesp, Amgen) was used, at a dose of 10 μg/kg body weight, because of its longer half life. Darbepoetin alfa treatment was given weekly for the indicated periods.

Another treatment modality was the administration of LDN-193189 (LDN, Axon Medchem, Groningen, the 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 experiments, 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

Details on complete blood counts, serum hepcidin[7] and serum iron measurements, western blotting,[7,36] real-time reverse transcriptase polymerase chain reaction analysis,[37] macrophage iron export measurements,[7] immunofluorescence, bone marrow smears and bone marrow flow cytometry are provided in the *Online Supplement.*

Statistics

Comparisons between multiple groups in this study were performed by ANOVA with the Bonferroni-Holm correction or the Dunnett test for multiple comparisons. A corrected P value less than 0.05 was regarded as statistically significant.

Further details on the methods used for this study can be found in the *Online Supplement*.

Results

Erythropoiesis-stimulating agent therapy inhibits hepatic Hamp mRNA expression in vivo in a rodent model of anemia of chronic disease

Following our working hypothesis, we first tested whether ESA treatment affects hepcidin expression in a rat model of ACD. We, therefore, injected rhEPO (500 U i.p.) into rats once or on three consecutive days starting 2 weeks after induction of ACD by PG-APS injection. As shown in Figure 1A, both a single as well as three consecutive doses of rhEPO, significantly decreased liver *Hamp* mRNA expression as compared to that in untreated animals. Importantly, this was paralleled by reduced serum hepcidin levels in the respective groups (Figure 1B).

Long-term effect of erythropoiesis-stimulating agent treatment in anemia of chronic disease

As the short-term experiments proved the effectiveness of rhEPO treatment at reducing the hepatic expression of hepcidin in this specific model, we conducted long-term experiments to study the effects of such interventions on the correction of ACD. Figure 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. Darbepoetin alfa (10 μg/kg) was used for this experiment because of its longer half-life.

Figure 2A shows the trend towards higher hemoglobin concentrations in ESA-treated groups, although this change became significant only for the group treated with the ESA for 28 days (P<0.01). All ACD groups, whether ESA-treated or untreated, had significantly lower hemoglobin levels at the end of the observation period than had healthy controls (P<0.001, Figure 2A).

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

Differences between the single ESA treatment groups (n=5 to 6 per group) were small and not significant for hemoglobin, serum hepcidin and serum iron. However, on the basis of pooled data from the ACD rats from all treatment periods, the overall effect of ESA was a significant increase of hemoglobin and serum iron levels (P<0.001, *Online Supplementary Figure S2*)

When performing western blot analysis of the spleen after the termination of the experiment (day 42), we found

that Fp1 protein levels (Figure 3A) were lower in ACD rats than in control rats, and that this reduction was partially antagonized by ESA treatment in a time-dependent fashion (Figure 3A). Accordingly, splenic ferritin levels were higher in ACD rats than in control animals, whereas ferritin expression declined with prolongation of ESA therapy (Figure 3A). These observations in the spleen were paralleled by corresponding changes in the liver (*Online Supplementary Figure 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 that from macrophages of control animals (P<0.001, Figure 3B), while ESA treatment of ACD rats significantly increased macrophage iron release (P<0.05, Figure 3B), which, however, remained lower than in control animals (P<0.001, Figure 3B). Importantly, these alterations of macrophage iron export corresponded nicely with the changes in spleen Fp1 expression observed in ACD animals with and without ESA treatment (Figure 3A,B). The effects seen on ^{59}Fe release (Figure 3B) were not caused by changes in ^{59}Fe uptake, because uptake was increased in ACD (P<0.001, Figure 3C) and not significantly changed by ESA treatment, with a trend towards a decrease (Figure 3C).

Hepcidin levels predict response to erythropoiesis-stimulating agent 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 (Figure 2A, *Online Supplementary Figure S1*), we questioned whether pre-treatment serum hepcidin levels may determine the responsiveness of ACD rats to ESA therapy.

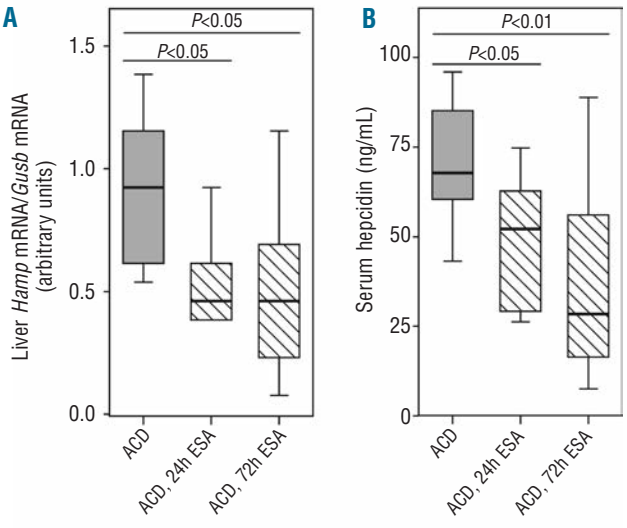

Figure 1. ESA therapy inhibits hepatic *Hamp* mRNA induction *in vivo* in a rodent model of ACD. ACD was induced in female Lewis rats by i.p. injection of PG-APS. Animals were then treated i.p. with 500 U erythropoietin alfa (ESA) on day 16 after PG-APS (ACD, 24h ESA group) or on days 14, 15 and 16 after PG-APS injection (ACD, 72h ESA group). All animals (n=9 per group) were sacrificed on day 17. *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 the Bonferroni-Holm correction for multiple comparisons.

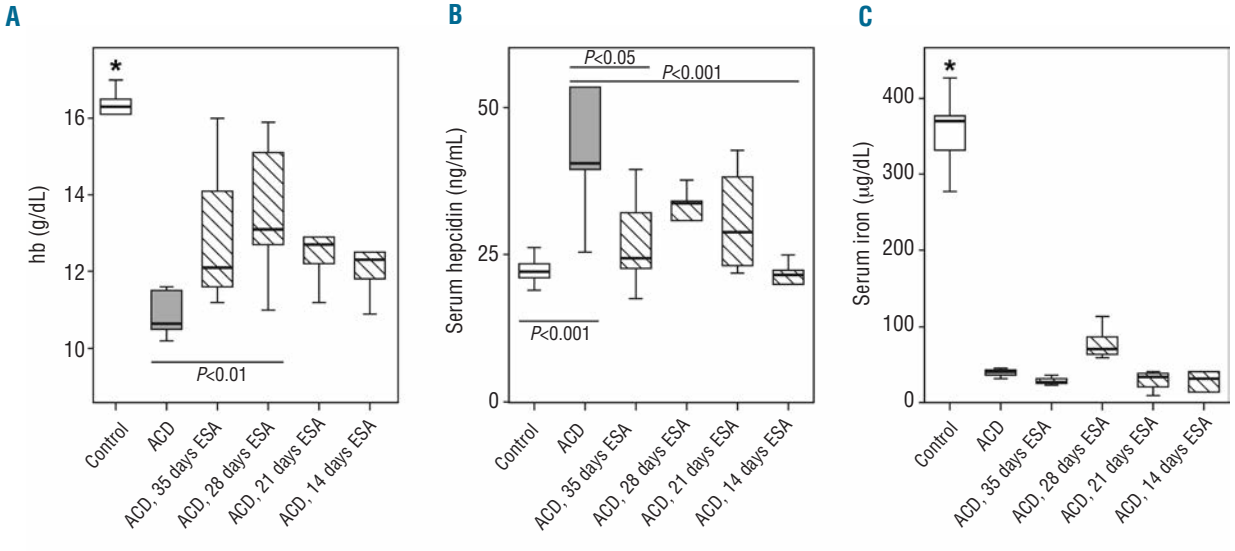

Figure 2. Blood hemoglobin, serum hepcidin and iron levels in ACD rats treated with darbepoetin alfa. ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Controls received no PG-APS. Groups of ACD rats were treated with darbepoetin alfa (ESA) for either 35, 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 the 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 (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.

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, and 50.2±22.2 ng/mL, mean ± standard deviation, respectively), indicating that there was no general change over time in pre-treatment 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 2 weeks, because it took at least 2 weeks until a significant change in hemoglobin levels upon ESA treatment could be observed (*Online Supplementary Figure S1*).

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 2 weeks of ESA therapy (Figure 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 2 weeks of follow up (Figure 4B), while lower pre-treatment hepcidin levels (below 40 ng/mg) predicted a better hematologic response to ESA therapy ($P<0.01$, Figure 4B). This indicates that high pre-treatment hepcidin levels in rats with ACD are associated with a poor hematologic response to ESA therapy.

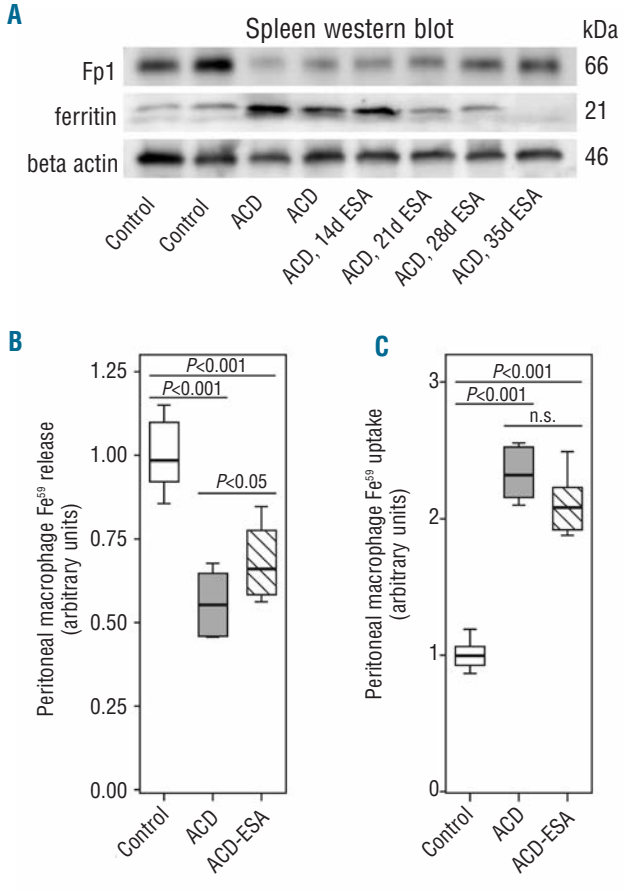

Figure 3. Spleen and macrophage iron metabolism following ESA treatment. (A) ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Controls received no PG-APS. Groups of ACD rats were treated with darbepoetin alfa (ESA) for either 35, 28, 21 or 14 days before euthanasia. One representative blot out of at least three western blots of spleen Fp1 and ferritin 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 Figure 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 h. Afterwards, the radioactivity was measured in the washed, harvested cells (B). Other cells were washed and incubated for 2 h 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 the Bonferroni-Holm correction for multiple comparisons (n=12 for controls and n=8 for ACD and ACD-ESA for macrophage experiments).

Hepcidin inhibition improves the therapeutic efficacy of erythropoiesis-stimulating agent 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, a small-molecule inhibitor of bone morphogenetic protein (BMP) type I receptors, which reduces hepcidin expression in models of ACD,[21] and ESA therapy. Therefore, anemic rats were given 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 (Figure 5A).

To see whether the combined effects of ESA and LDN treatments differed from those 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 3 weeks of either ESA or LDN treatment (Figure 5E), but neither regimen could normalize hemoglobin levels to the values observed in untreated, healthy controls (Figure 5E, $P<0.01$). Of note, combined ESA/LDN treatment resulted in significantly higher hemoglobin levels than treatment with LDN alone (Figure 5E, $P<0.05$), providing further evidence for synergistic effects of LDN and ESA in the reversal of inflammatory anemia.

At the end of the treatment period, both liver *Hamp* mRNA (Figure 5B) and serum hepcidin levels (Figure 5C), were significantly lower in the ESA/LDN-treated ACD animals than in the 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 (Figure 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 (Figure 5B,C,J).

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

these two organs (*Online Supplementary Figures 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 ($P<0.05$, Figure 5D).

When analyzing hematologic parameters in the different treatment groups we found that mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) were not affected by ESA treatment alone, while the administration of LDN ($P<0.001$, Figure 5F,G) and the combination of ESA/LDN ($P<0.001$, Figure 5F,G) increased these indices.

Erythropoiesis-stimulating agent/LDN treatment ameliorates erythropoiesis in rats with anemia of chronic disease

Upon investigation of bone marrow composition (for sample bone marrow micrographs see *Online Supplementary Figure S6*) we found an increased cellularity after treatment with ESA alone ($P<0.01$, Figure 5H); this increase in cellularity was even more prominent after combined ESA/LDN treatment ($P<0.001$, Figure 5H). This was paralleled by a pronounced and highly significant ($P<0.001$, Figure 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 treatment than with ESA treatment alone.

To further investigate the effects of combined treatment on erythropoiesis, we performed FACS analysis on bone marrow samples. We studied the percentage of erythroid cells (CD71+CD11b-) (*Online Supplementary Figure S7B*) and further analyzed maturation stages of erythroid differentiation using anti-rat-erythroid-cells and anti-CD44 antibodies (*Online Supplementary Figure S7A*).

In agreement with Richardson *et al.*[38] we found dramatically fewer in $CD71^{+}CD11b^{-}$ erythroid cells in the bone marrow of ACD rats than in control rats ($P<0.001$, Figure 6A,C). When studying erythroid differentiation, we found a significantly lower number of orthochromatic cells/reticulocytes in the ACD rats than in the control animals ($P<0.001$, Figure 6B,D), while other precursors were not reduced (Figure 6B).

Both ESA and LDN treatment resulted in a trend towards increased numbers of $CD71^{+}CD11b^{-}$ erythroid cells (Figure 6E) and orthochromatic cells/reticulocytes (Figure 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 ($P<0.05$, Figure 6E,F). The FACS results are in accordance with the results obtained from the bone marrow smears (Figure 5H-I)

The complete blood count data from ESA/LDN experiments are presented in *Online Supplementary Tables S1* and *S2*. Of note, neither treatment resulted in a change in total white blood cell counts (*Online Supplementary Table S2*) as compared to those in ACD animals.

Anti-inflammatory effect of erythropoiesis-stimulating agent/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 could be traced back to alterations of immune activation states. Indeed, we found that ESA significantly reduced *TNF-alpha* mRNA expression in the spleen ($P<0.05$, Figure 7A), and this effect was even more pronounced when combined ESA/LDN was administered ($P<0.001$, Figure 7A). Similarly, ESA/LDN treatment significantly inhibited spleen *IFN-gamma* (P<0.01, Figure 7B) and *IL-6* ($P<0.01$, Figure 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* (*Online Supplementary Figure S8A*) and *IL-6* (*Online Supplementary Figure S8B*) mRNA expression in the liver and found comparable alterations of their expression following treatment with ESA and LDN.

Discussion

Here we provide evidence of two important, clinically relevant functions of the master regulator of iron home-

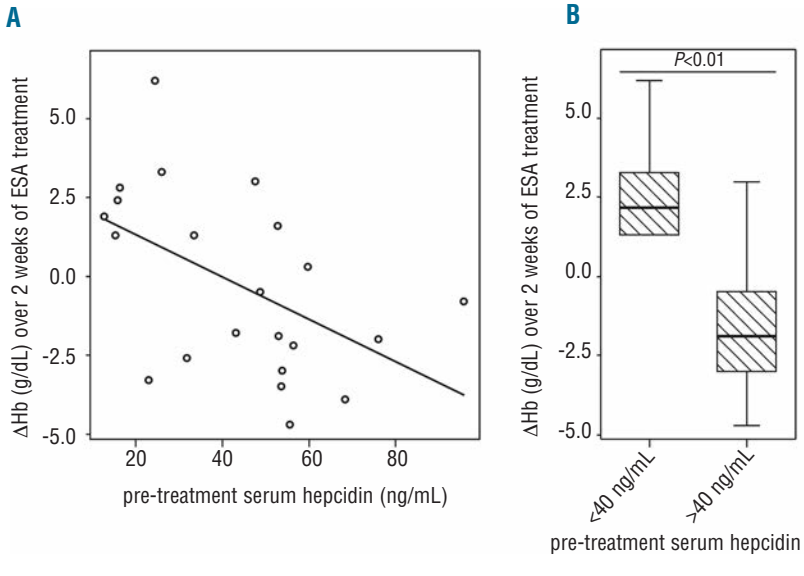

Figure 4. Correlation of pre-treatment serum hepcidin levels with changes of hemoglobin concentrations following ESA treatment in ACD rats. ACD was induced by injection of PG-APS in rats on day 0. On day 42 all animals were euthanized. Groups of ACD rats were treated with darbepoetin alfa (ESA) for either 35, 28, 21 or 14 days before euthanasia. Data are from the same ACD animals as in Figure 2 and Figure 3A. Serum hepcidin concentrations prior to onset of ESA treatment of ACD animals are plotted against the relative change of hemoglobin (ΔHb) over the first 2 weeks of ESA treatment (hemoglobin after 2 weeks of ESA treatment minus hemoglobin concentrations before ESA treatment). (A) The linear regression is shown. Spearman 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 the Student t-test ($P<0.01$).

ostasis, 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 hematologic response to 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.[6,42–44] 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 iron-restricted erythropoiesis.[3,7,9,10,22]

Here we demonstrate in a rat model of ACD that higher serum hepcidin levels predict a poorer hematologic response to ESA treatment. However, the benefit/risk-ratio of ESA therapy of anemia is still a matter of discussion,[30–32] which makes the availability of predictive diagnostic markers desirable.

The negative correlation between high circulating hepcidin levels and the response to ESA therapy is most likely related 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 *et al.*, who found that hepcidin is the major predictor of iron incorporation into red cells in anemic chil-

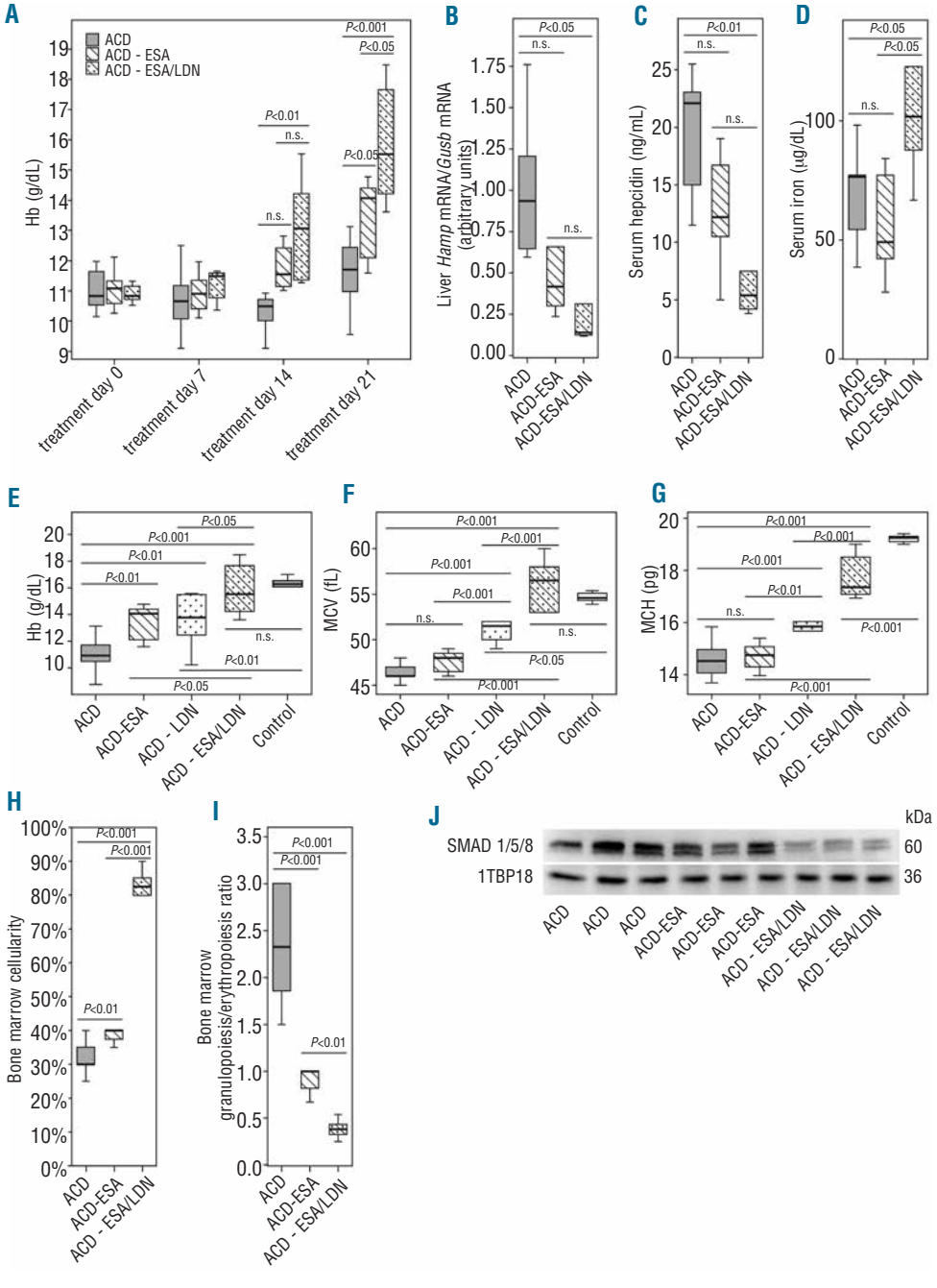

Figure 5. Effects of ESA and/or LDN-193189 on hemoglobin (Hb) levels and erythropoiesis in rats with inflammatory anemia. ACD was induced by injection of PG-APS in rats on day 0. Groups of ACD rats were treated from day 21 onwards with darbepoetin alfa (ESA) or with ESA and LDN in combination On day 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 shown in (A). Groups are ACD (gray), 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 reverse transcriptase polymerase chain reaction, 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 treated only with LDN (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 blot out of three is shown. Statistical significance of differences between groups was calculated using ANOVA with the Bonferroni-Holm correction for multiple testing.

dren in Africa.[45] As hepcidin is regulated by various stimuli such as hypoxia, anemia, iron and inflammation,[3,15,17] it is important 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.[21,27,46,47]

Thus, the negative association between ESA responsiveness and hepcidin levels may be due to enhanced inflammation and cytokine activities which promote macrophage iron retention and erythrophagocytosis, but also 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[48] along with apoptosis-inducing effects of several cytokines toward erythroid progenitor cells.[2,11]

However, serum hepcidin might prove to be a good marker for identifying 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

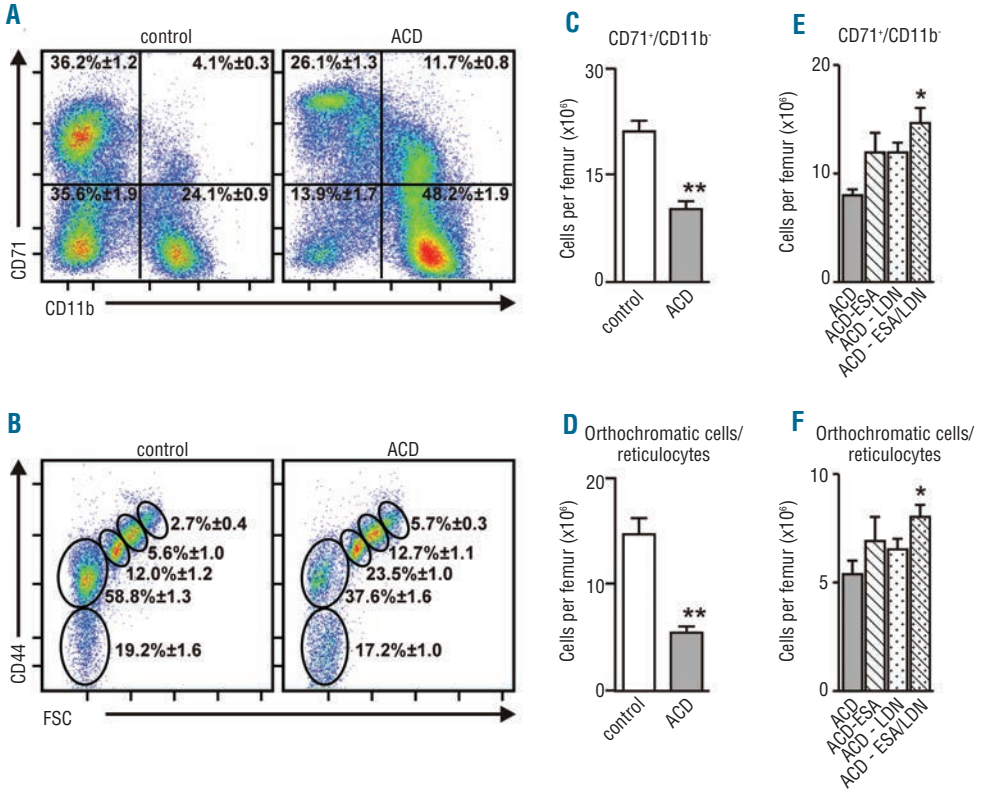

Figure 6. ESA and LDN treatment ameliorates defective marrow erythropoiesis in ACD rats. ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA), LDN or a combination of both was started on day 21. Controls received no PG-APS. On day 42 all animals were euthanized for marrow analysis by flow cytometry (n=7/group). All details on the gating strategy are shown in *Online Supplementary Figure 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 ($CD44^{dim}$,FCS^{low}) per femur (E-F) are shown as mean ± SEM. The Student t-test was applied for pairwise comparison (C,E) and ANOVA with the Dunnett test for multiple comparisons *versus* ACD (D,F). *P<0.05, **P<0.01.

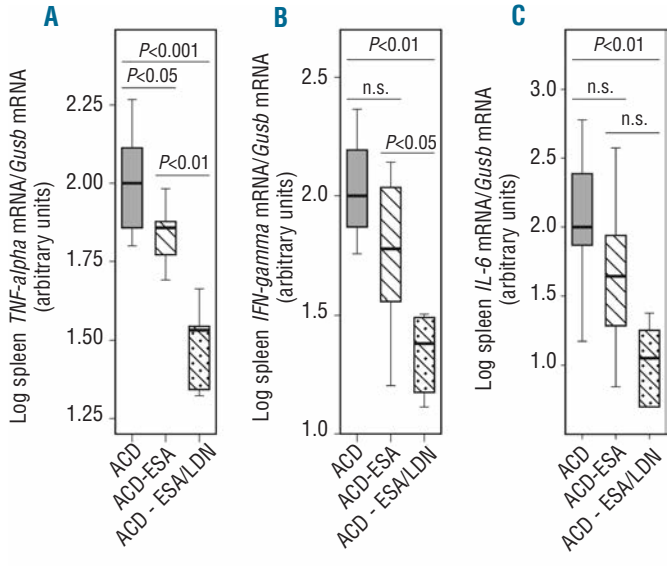

Figure 7. Effects of ESA or ESA/LDN treatment on spleen cytokine expression in ACD rats. ACD was induced by injection of PG-APS in rats on day 0 and treatment with darbepoetin alfa (ESA) or a combination of ESA and LDN was started on day 21. On day 42 all animals were euthanized (n=7/group). Groups are ACD (gray), ACD-ESA (diagonal lines) and ACD-ESA/LDN (dots and lines). Data are presented 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 reverse transcriptase polymerase chain reaction, 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 the Bonferroni-Holm correction for multiple testing. Data were logarithmized to reach homogeneity of variance.

as chronic renal failure, in which higher hepcidin levels are also a consequence of reduced renal clearance.[22]

We demonstrated 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 into 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.[20,21]

Compared to both LDN and ESA monotherapy, the combination of LDN and ESA resulted in an improved and faster hematologic 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 is compatible 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.[19] 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 of the potential therapeutic efficacy of this combined treatment in a rodent model which resembles the pathophysiology of human ACD.

This leads to questions regarding the mechanism underlying the improved therapeutic efficacy of combination treatment.

Combining ESA/LDN treatment improved the hemoglobin concentration and significantly affected serum iron, MCV and MCH, indicating that ESA therapy benefits from supportive iron mobilization through hepcidin antagonism in ACD. Furthermore, ESA/LDN treatment reduced the expression of pro-inflammatory cytokines, such as IL-6, TNF-α and IFN-γ. These proteins contribute to inflammatory anemia by promoting macrophage iron retention,[8] blocking duodenal iron absorption,[49] reducing erythrocyte half-life,[50] impairing erythroid progenitor proliferation and differentiation[2,11] and/or blocking the biological activity of erythropoietin.[12,13] IFN-γ, a T-helper cytokine, causes anemia by reducing erythrocyte half-life,[50] by blocking the proliferation of erythroid progenitor cells,[2,11] or by reducing macrophage iron egress via inhibition of Fp1 transcription.[8] Mechanistically, part of these anti-inflammatory effects can be traced back to erythropoietin-mediated inhibition of pro-inflammatory nuclear factor-κB-driven immune effector pathways in macrophages.[39] Accordingly, erythropoietin has been demonstrated to improve the clinical course of inflammation-driven auto-immune disorders by this pathway.[39]

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.[41] A BMP-4-dependent stress erythropoiesis was shown to be partly responsible for erythropoietin-induced improvement of hemoglobin levels in zymosan-induced anemia.[28] 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 at improving the therapeutic response to ESA therapy.[18–21]

In summary, we provide evidence that predicting the response to ESA therapy by measuring pre-treatment hepcidin levels and applying a combined treatment of ESA and hepcidin-antagonizing agents can improve the therapeutic efficacy of ESA therapy. 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 would have to be studied in a randomized prospective fashion also keeping in mind that it would be important to evaluate the effect of correcting the anemia on the course of the diseases underlying ACD.

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Authorship and Disclosures

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References

1. Matzner Y, Levy S, Grossowicz N, Izak G, Hershko C. Prevalence and causes of anemia in elderly hospitalized patients. Gerontology. 1979;25(2):113-9.
2. Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med. 2005;352(10):1011-23.
3. Ganz T, Nemeth E. Iron sequestration and anemia of inflammation. Semin Hematol. 2009;46(4):387-93.
4. Weiss G, Schett G. Anaemia in inflammatory rheumatic diseases. Nat Rev Rheumatol. 2013;9(4):205-15.
5. Babitt JL, Lin HY. Mechanisms of Anemia in CKD. J Am Soc Nephrol. 2012;23(10):1631-4.
6. Pantopoulos K, Porwal SK, Tartakoff A, Devireddy L. Mechanisms of mammalian iron homeostasis. Biochemistry (Mosc). 2012;51(29):5705-24.
7. Theurl I, Aigner E, Theurl M, Nairz M, Seifert M, Schroll A, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. Blood. 2009;113(21):5277-86.
8. Ludwiczek S, Aigner E, Theurl I, Weiss G.

Cytokine-mediated regulation of iron transport in human monocytic cells. Blood. 2003;101(10):4148-54.
9. Theurl I, Mattle V, Seifert M, Mariani M, Marth C, Weiss G. Dysregulated monocyte iron homeostasis and erythropoietin formation in patients with anemia of chronic disease. Blood. 2006;107(10):4142-8.
10. Roy CN, Mak HH, Akpan I, Losyev G, Zurakowski D, Andrews NC. Hepcidin antimicrobial peptide transgenic mice exhibit features of the anemia of inflammation. Blood. 2007;109(9):4038-44.
11. Means RT Jr. Recent developments in the anemia of chronic disease. Curr Hematol Rep. 2003;2(2):116-21.
12. Cazzola M, Ponchio L, Benedetti F de, Ravelli A, Rosti V, Beguin Y, et al. Defective iron supply for erythropoiesis and adequate endogenous erythropoietin production in the anemia associated with systemic-onset juvenile chronic arthritis. Blood. 1996;87 (11):4824-30.
13. Jelkmann W. Regulation of erythropoietin production. J Physiol. 2011;589(6):1251–8.
14. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. science. 2004;306(5704):2090-3.
15. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002;110(7):1037-44.
16. Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood. 2005;106(5):1864-6.
17. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. Cell. 2010;142 (1):24-38.
18. Schwoebel F, van Eijk LT, Zboralski D, Sell S, Buchner K, Maasch C, et al. The effects of the anti-hepcidin Spiegelmer NOX-H94 on inflammation-induced anemia in cynomolgus monkeys. Blood. 2013;121(12):2311-5.
19. Sasu BJ, Cooke KS, Arvedson TL, Plewa C, Ellison AR, Sheng J, et al. Antihepcidin antibody treatment modulates iron metabolism and is effective in a mouse model of inflammation-induced anemia. Blood. 2010;115 (17):3616-24.
20. Steinbicker AU, Sachidanandan C, Vonner AJ, Yusuf RZ, Deng DY, Lai CS, et al. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. Blood. 2011;117(18): 4915-23.
21. Theurl I, Schroll A, Sonnweber T, Nairz M, Theurl M, Willenbacher W, et al. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. Blood. 2011;118(18): 4977-84.
22. Kroot JJC, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. Clin Chem. 2011;57 (12):1650-69.
23. Rivera S, Nemeth E, Gabayan V, Lopez MA, Farshidi D, Ganz T. Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs. Blood. 2005;106(6):2196-9.
24. Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A, Vaulont S. Hepcidin, a new iron regulatory peptide. Blood Cells Mol Dis. 2002;29(3):327-35.
25. Liu Q, Davidoff O, Niss K, Haase VH. Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. J Clin Invest. 2012;122(12):4635-44.
26. Sasaki Y, Noguchi-Sasaki M, Yasuno H, Yorozu K, Shimonaka Y. Erythropoietin stimulation decreases hepcidin expression through hematopoietic activity on bone marrow cells in mice. Int J Hematol. 2012;96(6):692-700.
27. Lasocki S, Millot S, Andrieu V, Lettéron P, Pilard N, Muzeau F, et al. Phlebotomies or erythropoietin injections allow mobilization of iron stores in a mouse model mimicking intensive care anemia. Crit Care Med. 2008;36(8):2388-94.
28. Millot S, Andrieu V, Letteron P, Lyoumi S, Hurtado-Nedelec M, Karim Z, et al. Erythropoietin stimulates spleen BMP4-dependent stress erythropoiesis and partially corrects anemia in a mouse model of generalized inflammation. Blood. 2010;116(26): 6072-81.
29. Littlewood TJ, Bajetta E, Nortier JWR, Vercammen E, Rapoport B. Effects of epoetin alfa on hematologic parameters and quality of life in cancer patients receiving nonplatinum chemotherapy: results of a randomized, double-blind, placebo-controlled trial. J Clin Oncol. 2001;19(11):2865-74.
30. Spivak JL, Gascón P, Ludwig H. Anemia management in oncology and hematology. Oncologist. 2009;14(Suppl 1):43-56.
31. Pfeffer MA, Burdmann EA, Chen C-Y, Cooper ME, de Zeeuw D, Eckardt K-U, et al. A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. N Engl J Med. 2009;361(21):2019-32.
32. Bohlius J, Schmidlin K, Brillant C, Schwarzer G, Trelle S, Seidenfeld J, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials. Lancet. 2009;373(9674):1532-42.
33. Solomon SD, Uno H, Lewis EF, Eckardt K-U, Lin J, Burdmann EA, et al. Erythropoietic response and outcomes in kidney disease and type 2 diabetes. N Engl J Med. 2010;363(12):1146-55.
34. Goodnough LT, Nemeth E, Ganz T. Detection, evaluation, and management of iron-restricted erythropoiesis. Blood. 2010;116(23):4754-61.
35. Coccia MA, Cooke K, Stoney G, Pistillo J, Del Castillo J, Duryea D, et al. Novel erythropoiesis stimulating protein (darbepoetin alfa) alleviates anemia associated with chronic inflammatory disease in a rodent model. Exp Hematol. 2001;29(10):1201-9.
36. Ludwiczek S, Theurl I, Muckenthaler MU, Jakab M, Mair SM, Theurl M, et al. Ca2+ channel blockers reverse iron overload by a new mechanism via divalent metal transporter-1. Nat Med. 2007;13(4):448-54.
37. Theurl M, Theurl I, Hochegger K, Obrist P, Subramaniam N, van Rooijen N, et al. Kupffer cells modulate iron homeostasis in mice via regulation of hepcidin expression. J Mol Med. 2008;86(7):825-35.
38. Richardson CL, Delehanty LL, Bullock GC, Rival CM, Tung KS, Kimpel DL, et al. Isocitrate ameliorates anemia by suppressing the erythroid iron restriction response. J Clin Invest. 2013;123(8):3614-23.
39. Nairz M, Schroll A, Moschen AR, Sonnweber T, Theurl M, Theurl I, et al. Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-κB-inducible immune pathways. Immunity. 2011;34(1): 61-74.
40. De Domenico I, Zhang TY, Koening CL, Branch RW, London N, Lo E, et al. Hepcidin mediates transcriptional changes that modulate acute cytokine-induced inflammatory responses in mice. J Clin Invest. 2010;120(7): 2395-405.
41. Pagani A, Nai A, Corna G, Bosurgi L, Rovere-Querini P, Camaschella C, et al. Low hepcidin accounts for the proinflammatory status associated with iron deficiency. Blood. 2011;118(3):736-46.
42. Delaby C, Pilard N, Puy H, Canonne-Hergaux F. Sequential regulation of ferroportin expression after erythrophagocytosis in murine macrophages: early mRNA induction by haem, followed by iron-dependent protein expression. Biochem J. 2008;411(1): 123-31.
43. Knutson MD, Oukka M, Koss LM, Aydemir F, Wessling-Resnick M. Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin. Proc Natl Acad Sci USA. 2005;102(5):1324-8.
44. Soe-Lin S, Apte SS, Andriopoulos B, Andrews MC, Schranzhofer M, Kahawita T, et al. Nramp1 promotes efficient macrophage recycling of iron following erythrophagocytosis in vivo. Proc Natl Acad Sci USA. 2009;106(14):5960-5.
45. Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H, et al. Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. Blood. 2012;119(8):1922-8.
46. Theurl I, Schroll A, Nairz M, Seifert M, Theurl M, Sonnweber T, et al. Pathways for the regulation of hepcidin expression in anemia of chronic disease and iron deficiency anemia in vivo. Haematologica. 2011;96(12): 1761-9.
47. Mast AE, Schlumpf KS, Wright DJ, Johnson B, Glynn SA, Busch MP, et al. Hepcidin level predicts hemoglobin concentration in individuals undergoing repeated phlebotomy. Haematologica. 2013;98(8):1324-30.
48. Tanno T, Bhanu NV, Oneal PA, Goh S-H, Staker P, Lee YT, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med. 2007;13(9):1096-101.
49. Yamaji S, Sharp P, Ramesh B, Srai SK. Inhibition of iron transport across human intestinal epithelial cells by hepcidin. Blood. 2004;104(7):2178-80.
50. Libregts SF, Gutiérrez L, Bruin AM de, Wensveen FM, Papadopoulos P, Ijcken W van, et al. Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. Blood. 2011;118(9): 2578-88.