Mini Review

Up-regulation of hepcidin by interleukin-6 contributes to anemia of inflammation in multicentric Castleman's disease (MCD)

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   Multicentric Castleman's disease (MCD) is a rare lymphoproliferative disorder characterized by overproduction of interleukin-6 (IL-6). Anemia is a common symptom of MCD but its mechanism has been poorly understood. Hepcidin is an IL-6-induced key regulator of iron metabolism and has a major role in anemia of inflammation (AI). Our study showed that treatment with tocilizumab (an anti-IL-6 receptor antibody) resulted in long-term reductions of serum hepcidin-25 in MCD patients, which was accompanied by progressive normalization of iron-related parameters. In *in vitro* experiments, IL-6-induced hepcidin mRNA expression in hepatocytes was completely inhibited with tocilizumab and partially with erythropoietin (EPO), but enhanced by bone morphogenetic protein (BMP) and patient's serum. Our findings and evidence published elsewhere, leads us to suggest that, although multiple factors affect hepcidin levels, IL-6 plays an essential role in the induction of hepcidin which leads to anemia in MCD. Therefore, IL-6 blockage may constitute a promising molecular targeting therapy for AI.

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Introduction

   Dysregulated overproduction of IL-6 from affected lymph nodes has been identified as responsible for systemic manifestations of multicentric Castleman's disease (MCD), such as general fatigue, fever, anemia, polyclonal hypergammaglobulinemia, hypoalbuminemia, and an increase in acute phase proteins[1]. Previous studies showed that tocilizumab treatment dramatically alleviated symptoms and biochemical abnormalities of MCD[2,3]. MCD-associated anemia is characterized by hypochromic microcytic anemia with anemia of inflammation (AI) features such as low levels of serum iron, hemoglobin and transferrin, but its mechanism has not been elucidated until the discovery of hepcidin.

   Hepcidin is a key regulator of body iron homeostasis and increased hepcidin synthesis is thought to contribute to the AI[4], which is commonly observed in patients with chronic

inflammatory disorders. Inflammatory cytokines, mainly IL-6, can induce hepcidin expression through the Janus kinase signal transducer and activator of transcription 3 signal (JAK/STAT3) pathway and this induction reportedly depends on an intact BMP/SMAD pathway as well[5-7]. We used tocilizumab-treated MCD patients as models to investigate the role of IL-6-induced hepcidin in AI. This report summarizes our findings and discusses the mechanism of IL-6 regulation of hepcidin in the clinical AI setting.

Castleman's disease and roles of Interleukin-6

Castleman's disease (CD) is a relatively rare lympho-proliferative disorder with hyperplastic lymph nodes characterized histologically by follicular hyperplasia and capillary proliferation associated with endothelial hyperplasia. CD has been classified histopathologically as either hyaline-vascular or plasma cell type. The hyaline-vascular type is generally localized, while the plasma-cell type, as well as being localized, can also be diffuse (multicentric CD, or MCD) with multiple organ involvement. Enlarged lymph nodes with massive plasma cell infiltration in the interfollicular regions of hyperplastic lymph follicles, accompanied by systemic chronic inflammatory findings, are typical manifestations of MCD[8]. Although the etiology of CD is not yet fully understood, dysregulated production of IL-6 from affected lymph nodes has been identified as responsible for systemic manifestations of CD[1], such as general fatigue, fever, anemia, polyclonal hypergammaglobulinemia and an increase in acute phase proteins. The clinical abnormalities of localized form may resolve after excision of the affected lymph nodes; MCD, on the other hand, is often refractory to treatment and has a severe prognosis. MCD can also be associated with HIV infection, POEMS syndrome, amyloidosis, renal insufficiency and increased risk of malignancies, especially Kaposi⁻s sarcoma and lymphoma[9-12].

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis. IL-6 exerts its action via a cell surface receptor complex consisting of two functional membrane proteins: a ligand-binding chain (IL-6R, α-chain) and a non-ligand-binding but signal-transducing chain (β-chain, gp130)[13]. When bound to IL-6R on the cell surface, IL-6/IL-6R complex becomes associated with gp130, resulting in signal transduction. Tocilizumab, a humanized
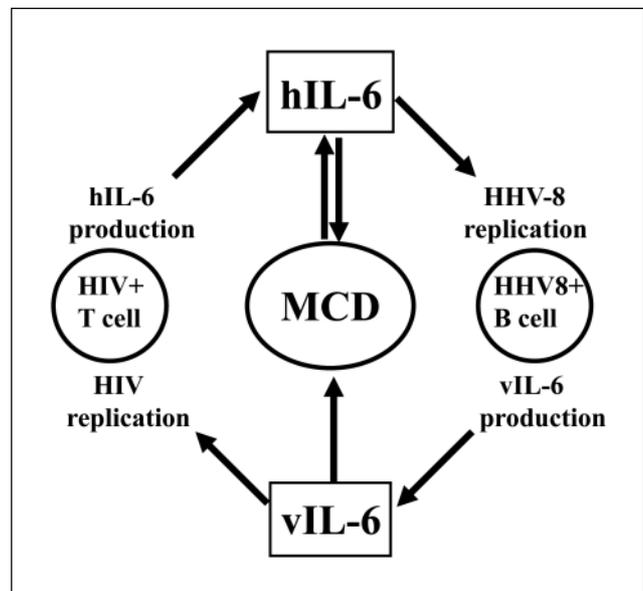
Fig.1 Schematic diagram of our hypothesis showing cross-talk between HIV and HHV-8 via IL-6 in MCD patients dually infected with HIV/HHV-8

Stimulation of HHV-8-infected B cells by hIL-6 leads to HHV-8 replication, and replication of HHV-8 can lead to vIL-6 expression, which can activate HIV replication in HIV infected T-cells. The mutual regulation of HIV and HHV-8 replication by huIL-6 and vIL-6 may constitute a vicious circle and lead to deterioration of the clinical manifestations of MCD.

anti-IL-6 receptor antibody, can bind both cell surface IL-6R and soluble IL-6R so as to inhibit formation of the IL-6/IL-6R complex and has been successfully used to alleviate MCD symptom[2,3].

The human herpes virus-8 (HHV-8) has been frequently reported as playing a role in the pathogenesis of MCD. The HHV-8 sequence has been detected in more than 60% of MCD patients infected with HIV and 20-40% of those who were not. HHV-8 has been shown to produce a viral IL-6 analogue (vIL-6) that displays many biologic functions of human IL-6 (hIL-6). vIL-6 also can activate STAT3 and stimulate the growth of certain factor-dependent cells, promote hematopoiesis, and induce vascular endothelial growth factor (VEGF)[14]. In fact, vIL-6 mRNA has been detected in MCD lesions[15] and induces huIL-6 production from MCD-derived cell lines[16]. Based on these results and our previous finding[17] that hIL-6 induces HHV-8 reactivation and, conversely, that vIL-6 activates HIV replication, we suggest that an interaction may occur via IL-6 between HIV and HHV-8 in dually infected MCD patients (Fig.1).
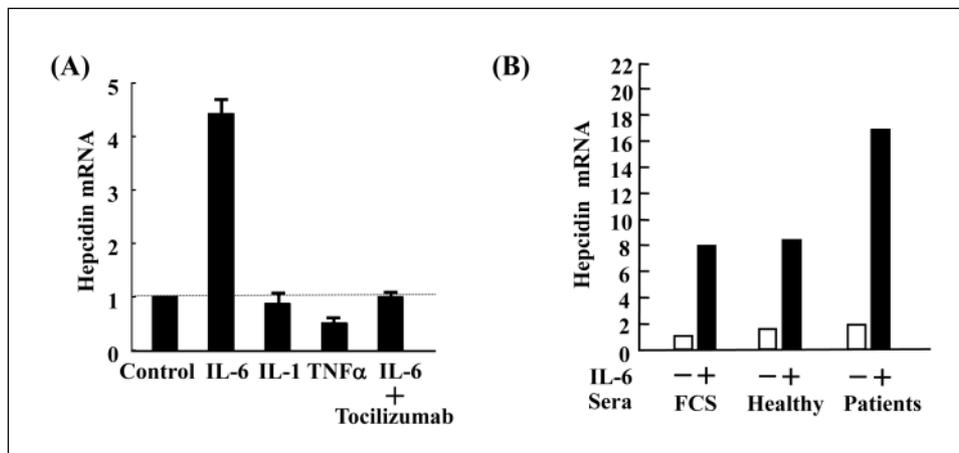
Fig.2

(A): Stimulatory effects of IL-6, IL-1 and TNF-α on hepcidin mRNA and Inhibitory effects of tocilizumab on IL-6-induced hepcidin mRNA expression in vitro. Tocilizumab (25 ng/mL) was incubated with the PLC/PRF/5 hepatocyte cell line for 30 minutes before IL-6 (10 ng/mL), IL-1 (1 ng/ml) and TNF-α (10 ng/ml) stimulation. Hepcidin mRNA was assayed with quantitative real-time PCR 8 hours after IL-6 stimulation. (B): Effect of MCD patient sera on IL-6-induced hepcidin expression in PLC/PRF/5 cells. The graph shows hepcidin mRNA expression in PLC/PRF/5 cells treated with a culture medium containing 10% of either fetal calf serum (FCS), serum from healthy volunteers (n=5) or serum from MCD patients (n=7). Cells stimulated with IL-6 (10 ng/mL) showed an 8-fold increase in hepcidin mRNA expression compared with nonstimulated controls. Serum from MCD patients enhanced IL-6-induced hepcidin expression by a mean factor of 16.5, whereas serum from the healthy volunteers had no such effect.

This cross-talk may constitute a vicious circle and thus lead to deterioration of the clinical manifestations, so that blockade of the IL-6 action with tocilizumab may cut through this vicious circle and constitute a new therapeutic strategy for MCD patient with HIV/HHV-8 infection.

Hepcidin expression induced by IL-6, BMP or other serum factor(s) in MCD

Hepcidin is not only an iron regulatory hormone but also an acute-phase reactant[18]. Under inflammatory conditions, inflammatory cytokines, and IL-6 in particular, play a central role in hepcidin induction, while STAT3 is the major transcription factor responsible for the up-regulation of hepcidin via the IL-6 transduction pathway during inflammation[5,6]. IL-6 stimulates hepcidin expression in isolated hepatocytes and in hepatoma cell lines. Administration of IL-6 to human subjects stimulates an increase in hepcidin production and results in low serum iron[19]. Mice lacking IL-6 fail to induce hepcidin and do not become hypoferremic after treatment with endotoxin[19,20]. All the findings of these *in vitro* and *in vivo* studies suggest that IL-6-induced hepcidin is a potent mediator of AI.

Consistent with these findings, our quantitative real-time polymerase chain reaction (PCR) examination of hepatoma cell lines showed that IL-6, but not IL-1 or TNF-α induced hepcidin, moreover, mRNA expression was completely inhibited with tocilizumab, indicating that IL-6 acts directly on hepcidin expression in hepatocytes and that tocilizumab is an effective inhibitor of hepcidin induction by IL-6 (Fig.2A).

The BMP-SMAD pathway is another major signaling pathway for activation of hepcidin expression[21]. BMP2, 4, 6, and 9 belong to a bone morphogenetic protein (BMP) subfamily. *In vitro* studies have demonstrated that each of these BMPs markedly increases hepcidin expression in hepatocytes through the receptor-activated Smad1/5/8[22-24]. It has further been shown that mutation of the BMP-response element in the hepcidin promoter severely impairs hepcidin expression in response to IL-6[7], and that inhibition of the BMP-SMAD pathway blocked the IL-6-induced hepcidin[25]. The findings suggest that the BMP/SMAD pathway is required for a normal hepcidin response to inflammation. We also observed elevated serum levels of BMP2 and BMP4 in the MCD patients and found that IL-6-induced hepcidin mRNA in hepatoma cell lines was augmented in the presence of BMP2 (data not shown), indicating a synergistic effect by IL-6 and BMP2 on hepcidin induction.
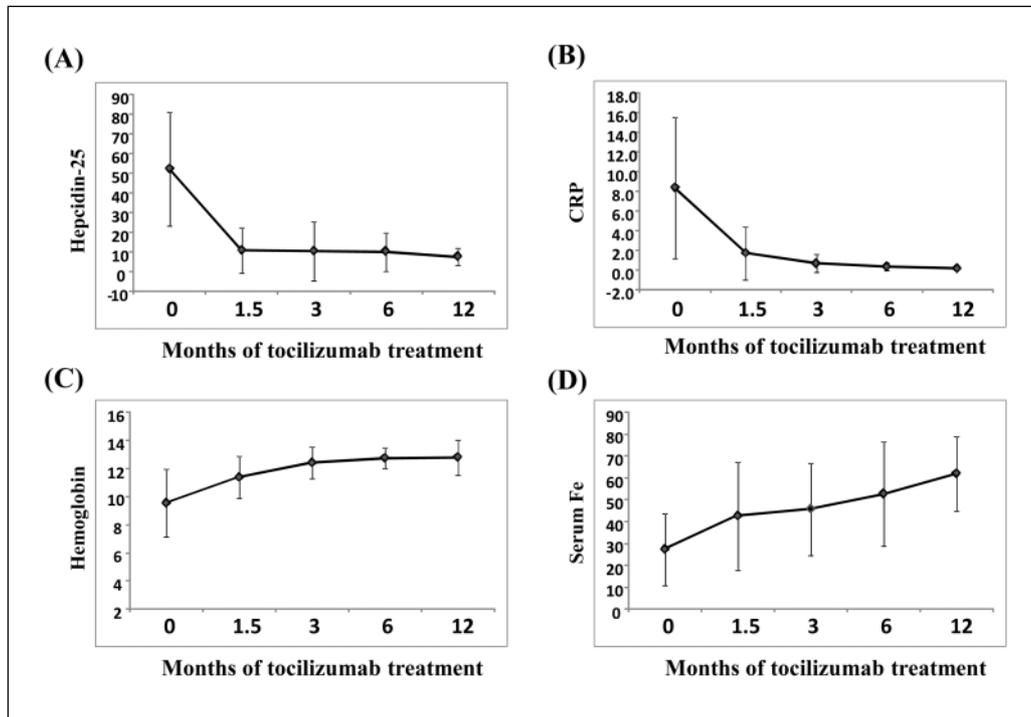
Fig.3 Tocilizumab treatment resulted in hepcidin suppression and improvement of anemia in MCD patients. Long-term effects were assessed at 1.5, 3, 6, and 12 months after the initiation of tocilizumab therapy. Serum hepcidin-25 was quantified with a liquid chromatography-tandem mass spectrometry based assay system. Other AI-related parameters were measured with standard laboratory techniques.

(A) Serum level of hepcidin-25, (B) CRP, (C) Hb, and (D) Serum iron.

To clarify whether MCD patient serum contains humoral factors other than IL-6 which influence hepcidin expression, we used real-time PCR to investigate whether such serum affected IL-6-induced hepcidin mRNA expression in PLC/PRF/5 cells. The addition of patient serum to the culture to a concentration of 10% enhanced IL-6-induced hepcidin expression more than did IL-6 alone (Fig.2B), suggesting that enhancing factor(s) were present in the MCD patients' sera, which were involved in IL-6-induced hepcidin expression by acting on the IL-6 signal pathway. We hypothesize that BMP2/4 is a promising candidate enhancing factor, but this needs further investigation.

IL-6 blockage inhibits hepcidin production and improves anemia in MCD patients

We previously reported that the blockage of IL-6 with tocilizumab significantly improved the systemic manifestations of MCD, including Hb level[2,3], and suggested that overproduction of IL-6 may be associated with anemia in MCD patients. Kawabata et al.[26] reported that serum hepcidin levels were reduced within 24 hours in two MCD patients treated with tocilizumab, indicating that IL-6 may induce hepcidin production in MCD. To determine whether IL-6-induced hepcidin directly causes AI and whether IL-6 blockage remains effective during long-term treatment, we investigated the long-term effects of tocilizumab treatment on serum levels of hepcidin-25 (a major form of the active hepcidin peptide, which is composed of 25 residues and promotes internalization and degradation of ferroportin, a cell surface iron transporter) and iron-related parameters in 10 MCD patients who also were diagnosed with AI. Tocilizumab (8 mg/kg body weight) was administered intravenously at 2-week intervals for 12 months. The initial serum level of hepcidin-25 tended to be elevated with an average of 52 ng/mL, while mean CRP value was 4.9 mg/dL, Hb was 11.6 g/dL, serum iron was 39 μg/dL and ferritin was 77 ng/mL. Long-term administration of tocilizumab resulted in a continuing decrease and eventual normaliza-

tion of serum hepcidin-25 levels after 12 months for all patients (Fig.3A). This long-term reduction in serum hepcidin levels was followed by a progressive decline in CRP and gradual improvement of the anemia status of all patients because mean Hb, Fe, RBC, and MCV values increased, and ferritin decreased after initiation of treatment, and this effect continued until the end of the study period ($p < 0.05$ paired t test vs baseline). Part of our results is shown in Figure 3B-D. The suppression of hepcidin production as a result of tocilizumab treatment, accompanied by improvement of iron metabolism parameters, indicates that excessive hepcidin production induced by elevated IL-6 contributed to AI in the MCD patients.

We noted in particular that the serum hepcidin-25 level correlated significantly and positively with ferritin (Pearson correlation test, $r=0.67$, $p=0.021$), but not with the other parameters before tocilizumab administration. Furthermore, tocilizumab administration resulted in down-regulation of serum ferritin accompanied by a parallel decrease in serum hepcidin levels. This is explained by the fact that ferritin is a cellular storage protein for iron, and that an elevated ferritin level generally reflects excessive iron storage as commonly observed in AI. We speculate that excessive hepcidin leads to an increase in iron storage and diminishes the amount of serum iron available for Hb synthesis and erythrocyte production, which leads in turn to inflammatory anemia in MCD. Our results suggest that iron absorption in intestine cells and iron release from macrophages were enhanced following the suppression of hepcidin up-regulation by tocilizumab.

Hepcidin production is regulated by other factors in MCD

Although inflammatory cytokines, mainly IL-6, have been shown to increase hepcidin expression and this induction is responsible for AI as noted above, antagonistic signals for hepcidin regulation can occur simultaneously under pathogenic conditions. MCD is a good example to demonstrate the interaction between positive and negative signals in hepcidin regulation.

In our MCD patients, the initial serum levels of hepcidin-25 before tocilizumab administration were generally elevated but varied widely (8-256 ng/mL), whereas serum IL-6 levels were markedly elevated in most of our MCD cases (mean value: 128 pg/ml). Moreover, serum hepcidin-25 correlated well with ferritin (Pearson correlation test:

$r=0.67$, $p=0.021$), but not with IL-6, so that serum hepcidin-25 levels did not seem to be controlled by that of serum IL-6 alone. We therefore hypothesized that other antagonistic factors may counteract hepcidin induction by IL-6. Anemia has been proven to be a negative regulator for hepcidin production[27]. It was noteworthy that three cases in our study had severe anemia with respective Hb levels of 5.7, 6.3, and 7.2 g/dL in conjunction with low levels of serum hepcidin-25 (44, 14, 8 ng/ml) before tocilizumab administration. This finding suggests that very severe anemia probably counteracts the effect of IL-6 on hepcidin production.

In anemia, an increase in erythropoiesis requires an increase in iron for maintaining homeostasis, while hepcidin production is down-regulated to allow for an increase in iron adsorption and release from macrophages. In our study, we investigated the effect of erythropoietin (EPO) on hepcidin in MCD. First, we noticed that there is an inverse relationship between baseline levels of EPO and Hb in the MCD patients we examined (Pearson correlation test: $r=-0.68$, $p=0.043$), (Fig.4A) and tocilizumab treatment resulted in a progressive decrease in serum EPO (Fig.4B), indicating that anemia may have caused erythropoiesis through feedback mechanism. To test whether hepcidin production is affected by erythropoietic activity, the effect of recombinant EPO (rEPO) on IL-6-induced hepcidin expression was analyzed in human hepatoma cell lines. We found that IL-6-induced hepcidin mRNA expression was down-regulated by rEPO in a dose-dependent manner in these cells (Fig.4C), suggesting that anemia could mediate hepcidin suppression through Epo or erythropoietic activity in order to maintain iron homeostasis. Our results are consistent with those of previously published studies[28-30] and confirm that EPO negatively regulates hepcidin either *in vivo* or *in vitro*.

Recent evidence has shown that in addition to EPO, growth differentiation factor-15 and twisted gastrulation-1 also mediate hepcidin suppression in response to augmented hematopoietic activity[31-33]. In addition, SMAD7 and TMPRSS6 (a membrane-bound serine protease matriptase-2) have been identified as inhibitors of hepcidin expression in response to iron-controlled regulation of hepcidin[34,35]. Hypoxia resulting from amemia is also thought to inhibit hepcidin expression. It is therefore quite possible that other negative regulators down-regulated the serum hepcidin level in MCD.
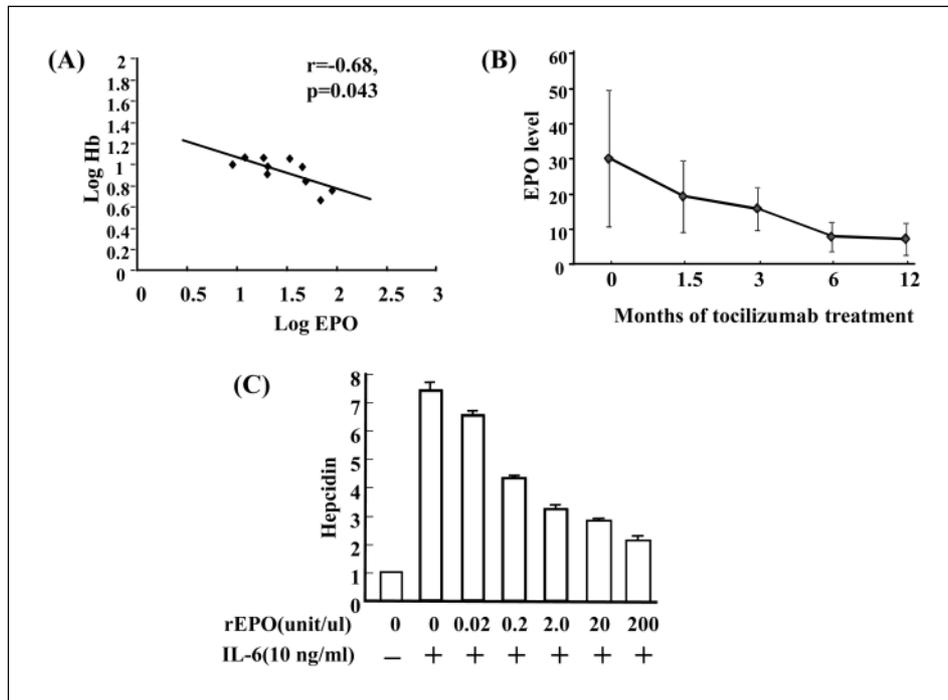
Fig.4 Suppression of IL-6-induced hepcidin by EPO

(A): an inverse association between EPO and Hb in serum of MCD patients.

(B): long-term treatment with tocilizuma resulted in reduction of serum EPO in MCD patients.

(C): Effect of recombinant human EPO on IL-6-induced hepcidin expression in Hep3B cells. rEPO suppressed IL-6-induced hepcidin expression in a dose-dependent manner.
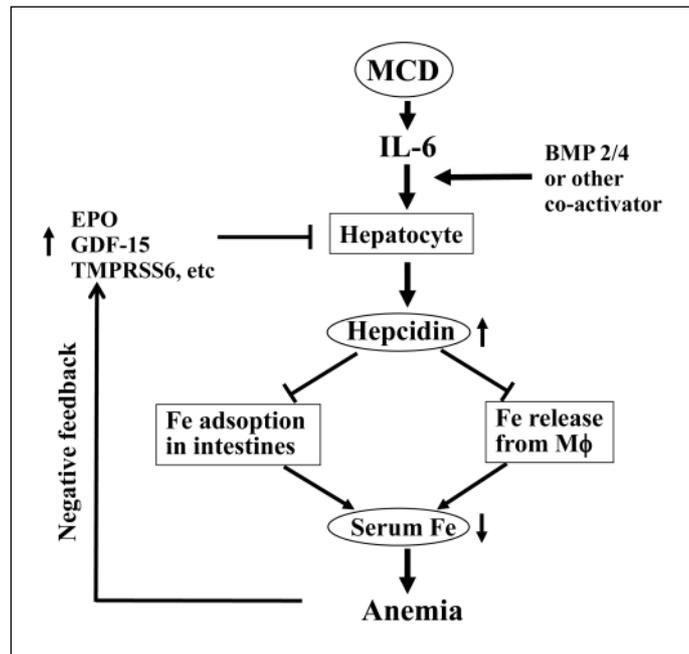
Fig.5 Schematic representation of a proposed model of mechanism
of hepcidin-mediated anemia development in MCD patients

→ toward; ⊣ opposite

Conclusions

We conclude on the basis of our findings and those of others that overproduction of IL-6 associated with MCD induces hepcidin upregulation, which may be enhanced by BMPs and as yet unknown serum factors. Further, excessive hepcidin inhibits the release of recycled iron from macrophages (Mϕ) and absorbed iron from intestine cells, which restrict the iron supply for hemoglobin synthesis and causes anemia. In turn and functioning as feedback mechanism, anemia suppresses hepcidin production through upregulation of EPO and other hepcidin-suppressing factors (Fig.5). This explains why tocilizumab therapy suppresses hepcidin production and improves anemia of inflammation (AI) through blocking the IL-6 pathway which plays an essential role in AI.

Conflicts-of-Interest

The authors have no financial conflicts of interest.

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