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Intestinal Inflammation Modulates Expression of the Iron-Regulating Hormone Hepcidin Depending on Erythropoietic Activity and the Commensal Microbiota

Nanda Kumar N. Shanmugam,* Estela Trebicka,* Ling-lin Fu,*† Hai Ning Shi,* and Bobby J. Cherayil*

States of chronic inflammation such as inflammatory bowel disease are often associated with dysregulated iron metabolism and the consequent development of an anemia that is caused by maldistribution of iron. Abnormally elevated expression of the hormone hepcidin, the central regulator of systemic iron homeostasis, has been implicated in these abnormalities. However, the mechanisms that regulate hepcidin expression in conditions such as inflammatory bowel disease are not completely understood. To clarify this issue, we studied hepcidin expression in mouse models of colitis. We found that dextran sulfate sodium–induced colitis inhibited hepcidin expression in wild-type mice but upregulated it in IL-10–deficient animals. We identified two mechanisms contributing to this difference. Firstly, erythropoietic activity, as indicated by serum erythropoietin concentrations and splenic erythropoiesis, was higher in the wild-type mice, and pharmacologic inhibition of erythropoiesis prevented colitis-associated hepcidin downregulation in these animals. Secondly, the IL-10 knockout mice had higher expression of multiple inflammatory genes in the liver, including several controlled by STAT3, a key regulator of hepcidin. The results of cohousing and fecal transplantation experiments indicated that the microbiota was involved in modulating the expression of hepcidin and other STAT3-dependent hepatic genes in the context of intestinal inflammation. Our observations thus demonstrate the importance of erythropoietic activity and the microbiota in influencing hepcidin expression during colitis and provide insight into the dysregulated iron homeostasis seen in inflammatory diseases. *The Journal of Immunology, 2014, 193: 1398–1407.

Inflammatory bowel disease (IBD) and other chronic inflammatory conditions can lead to dysregulation of iron homeostasis and the consequent development of an iron-refractory anemia known as the anemia of inflammation (AI) (1). The pathogenesis of AI is related to abnormally elevated expression of the peptide hormone hepcidin, which is secreted by the liver and functions as the central regulator of systemic iron metabolism (2). Hepcidin acts by binding to the macrophage and enterocyte plasma membrane protein ferroportin, causing it be internalized and degraded (3). Because ferroportin is the sole means by which iron that is absorbed from the diet or recycled from aged erythrocytes is exported into the circulation, hepcidin-dependent alterations in ferroportin levels play a major role in controlling serum iron concentrations. The expression of hepcidin itself, which is regulated exclusively at the level of transcription (4), is induced by increased tissue and serum iron and inhibited by conditions such as anemia and hypoxia that raise iron requirements. Thus, the hepcidin–ferroportin axis is a key component of a negative-feedback loop that maintains systemic iron homeostasis.

Hepcidin expression is also increased by inflammatory signals. In relatively simple models of inflammation, such as the injection of turpentine or LPS, IL-6 has been shown to be an important mediator of hepcidin upregulation by virtue of its ability to activate the transcription factor STAT3 (5–8). Whether IL-6 plays the central role in increasing hepcidin expression in clinical inflammatory diseases remains to be determined. The inflammation-induced increase in hepcidin expression leads to ferroportin downregulation, with consequent impairment of iron absorption from the gut and decreased release of iron from phagocytes involved in erythrocyte turnover. As a result, serum iron concentrations fall, compromising erythropoiesis and leading to the development of AI. AI is particularly difficult to treat because the associated downregulation of ferroportin results in poor absorption of oral iron supplements (1).

With recent advances in understanding of the role of hepcidin in iron metabolism and with the availability of methods to measure the hormone in biological samples (9, 10), there has been increasing interest in examining hepcidin levels in patients with IBD. Somewhat unexpectedly, whereas several studies have documented increases in serum or urinary hepcidin in the patients, correlating with elevated IL-6 in some cases, others have found no...
difference from controls or even decreased hepcidin levels (11–16). Although differences in methodologies and coexisting iron deficiency could account for some of the discrepant findings, the results of these studies suggest that intestinal inflammation does not consistently lead to hepcidin upregulation. It is not always clear why hepcidin levels go up in some patients with IBD and not in others, a reflection of our incomplete understanding of the various factors that influence hepcidin expression in the context of inflammation. It is important to elucidate the role of such factors to identify patients at risk for developing AI, to clarify AI pathogenesis, and to devise strategies to prevent and treat this condition.

We have been working to shed light on this issue by studying hepcidin expression in mouse models of IBD. Our earlier experiments have shown that hepcidin expression is elevated in some of the models, such as piroxicam-induced colitis in IL-10 knockout (KO) mice and T cell transfer colitis in lymphocyte-deficient mice, whereas it is downregulated in dextran sulfate sodium (DSS)–induced colitis in wild-type (WT) animals (17, 18). We extend this line of investigation in the present work to elucidate the mechanisms involved in these differences. By comparing the effects of DSS colitis on hepcidin expression in WT versus IL-10 KO mice, we show that variations in erythropoietic activity play an important role in determining whether hepcidin goes up or down in response to intestinal inflammation. We also show that the composition of the intestinal microbiota influences colitis-associated alterations in the expression of several STAT3-regulated genes in the liver, including hepcidin. Our results provide new insights into the mechanisms involved in the abnormalities of iron metabolism associated with conditions such as IBD.

Materials and Methods

Animal studies
WT C57BL/6 mice, 5–6 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility at Massachusetts General Hospital. IL-10 KO mice (C57BL/6 background) were also obtained from The Jackson Laboratory, bred at Massachusetts General Hospital, and maintained under conditions identical to the WT animals. Mice of both sexes were used, and experimental groups were matched by age and sex. Colitis was induced as described previously (19) by administering 3% DSS (36–50 kDa; MP Biomedicals, Solon, OH) in the drinking water for 7 d. In some experiments, the WT and KO mice were cohoused in the same cage for 2 wk, starting at the age of 3 wk, before initiating DSS administration. Tissues were harvested at necropsy following carbon dioxide euthanasia and processed as described below. All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (protocol number 2008N000061, animal welfare assurance number A3596-01).

Fecal transplantation
Groups of 3-wk-old WT and IL-10 KO mice were administered a mixture of antibiotics in their drinking water (ampicillin 1 g/liter, neomycin 1 g/l, metronidazole 1 g/l, and vancomycin 0.5 g/l) that has been shown previously to deplete the gut microbiota (20). The antibiotics were discontinued 2 wk after stopping the antibiotic treatment, the mice were gavaged with a fecal slurry made by homogenizing in PBS pooled stool pellets and cecal contents of either WT or IL-10 KO donor mice (three donor mice for each pool). Two weeks after the fecal transplantation, the recipient mice were treated with DSS for 7 d as described above.

Analysis of gene expression in the liver
At necropsy, pieces of liver were excised and homogenized in TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA was prepared according to the manufacturer’s guidelines and used for quantitative RT-PCR as described in detail earlier (21). In some experiments, RNA was prepared using the RNeasy Mini-Kit (Qiagen, Valencia, CA) and following the manufacturer’s instructions. Results were similar regardless of the method of RNA preparation. Relative expression was calculated using the 2−ΔΔCt method after normalizing to the housekeeping transcript GAPDH. Primers used to amplify hepcidin and GAPDH transcripts have been described previously (21). Primers for amplification of the STAT3-regulated genes haptoglobin, hemopexin, IL-1 receptor antagonist (IL-1RN), serum amyloid A1 (SAA1), and SAA3 are listed in Supplemental Table I.

Microarray analysis
Microarray-based liver gene expression analysis was carried out at the Partners Center for Personalized Genomic Medicine using total RNA samples prepared from 4 WT and 4 IL-10 KO mice treated with DSS for 7 d. After passing quality control, total liver RNA (0.1 μg) was converted to biotinylated cDNA using the Ambion WT Expression amplification kit (Life Technologies, Grand Island, NY) and the Affymetrix WT Terminal Labeling kit (Affymetrix, Santa Clara, CA). Labeled cDNA (5.5 μg) was fragmented and hybridized for 16 h at 45°C to Affymetrix GeneChip Mouse 2.0 ST arrays in an Affymetrix 645 hybridization oven (Affymetrix). The arrays were washed and stained in the Affymetrix Fluidics Station 450 (Affymetrix) and then scanned using the Affymetrix GeneChip Scanner 3000 7G running Affymetrix Gene Command Console v. 3.2 (Affymetrix). The microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE2168 (http://www.ncbi.nlm.nih.gov/geo/).

Bioinformatics analysis was carried out at the Massachusetts General Hospital Biomedical Informatics Core. Robust multiarray average (22) was used to analyze the Affymetrix microarray probeset expression data (23) in R/BioConductor (R v. 3.0.1, BioConductor v. 2.12). The limma package in R was used to evaluate differential gene expression (24). The p values were corrected for multiple tests using the Benjamini and Hochberg correction method. Genes that were expressed at significantly higher levels in the IL-10 KO mice were analyzed for overrepresented pathways using DAVID EASE (25) with the Gene Ontology Biological Processes set. They were also evaluated for enriched pathways using the Gene Set Enrichment Analysis (GSEA) tool (26). The GSEA approach is summarized in http://www.gene-effects.co.uk/2012/pathway-analysis-using-the-gene-set-enrichment-analysis-gsea-tool/. In brief, as described in the GSEA manual, “all genes are first ranked by their signal-to-noise ratio, then the enrichment score (ES) is calculated by ‘walking’ down the ranked list of genes, increasing a running-sum statistic when a gene is in the set and decreasing it when it is not. The magnitude of the increment depends on the correlation of the gene with a phenotype. The ES is the maximum deviation from zero encountered in walking the list. A positive ES indicates gene set enrichment at the top of the ranked list; a negative ES indicates gene set enrichment at the bottom of the ranked list.”

For the analysis of STAT3-regulated genes, we reviewed the literature and identified a set of 26 such genes expressed in the liver (27–33). An enrichment test of the STAT3-regulated genes was carried out using the Fisher exact and Pearson χ2 tests to determine if there was a significant overlap with the set of genes expressed at higher levels in the IL-10 KO mice. The results indicated significant overlap (p = 7.476 × 10−5 by Fisher exact test; p = 6.191 × 10−12 by Pearson χ2 test with Yates’s continuity correction). A heat map of the STAT3-regulated genes was generated.

Colon IL-6 production and histological analysis
At necropsy, the entire colon was excised and gently flushed with PBS. Half-centimeter segments of the distal end were excised and incubated overnight in complete medium. The culture supernatants were then used to estimate the levels of IL-6 by ELISA, as previously described (21). The cytokine concentration was normalized to the total protein concentration of the homogenate of the corresponding colon segment and expressed as nanograms per milligram. The remainder of the colon was opened lengthwise, rolled into a “Swiss roll” preparation, and fixed in 3.7% formaldehyde in PBS. The roll was embedded in paraffin and 5-μm sections processed for H&E staining at the Department of Pathology, Massachusetts General Hospital. The stained sections were scored in a blinded fashion using a previously described scoring system that evaluates inflammatory infiltration and tissue damage (34).

Flow cytometry analysis
The spleen was excised at necropsy after dissecting it free of other tissue. After recording the weight, a single-cell suspension was prepared by homogenizing the spleen through a sterile 70-μm mesh in 1 ml PBS. Without lysing erythrocytes, aliquots of the cells were washed, treated with Fc block (BD Biosciences, San Jose, CA), stained with FITC-conjugated anti-Ter119 and PE-conjugated anti-CD71 (eBioscience, San Diego, CA), and fixed in 4% formaldehyde. The cells were then analyzed on an Accuri C6 flow cytometer (BD Biosciences) to determine the proportion of CD71+ Ter119+ erythropoietic precursors. Analysis gates were set using forward and side scatter and the FITC and PE fluorescence of an unstained sample.
Serum erythropoietin

Serum erythropoietin concentrations were determined using an ELISA kit from R&D Systems (Minneapolis, MN) and following the manufacturer's guidelines.

Serum iron

Serum iron was determined as described previously (18) using a colorimetric assay kit from Thermo Scientific (Waltham, MA) as recommended by the manufacturer.

Stool bacterial PCR

Stool DNA was prepared using the QIAamp mini-kit (Qiagen, Germantown, MD), and subjected to quantitative PCR analysis with 16S ribosomal DNA primers specific for Bacteriodetes and Firmicutes (35), Bacteroides fragilis, and segmented filamentous bacteria (36), as well as with universal bacterial 16S ribosomal DNA primers. The sequences of the primers are indicated in Supplemental Table I. The \( \Delta \Delta^{\text{Ct}} \) method was used to obtain a measure of the relative proportions of individual bacterial groups by normalizing the cycle threshold (Ct) obtained with group-specific primers to the Ct obtained with the universal 16S primers.

Statistical analysis

Means and SEs, calculated from the results of multiple experiments in most cases, are displayed in the figures. The Student's \( t \) test was used to compare results between groups (Prism v6.0c; GraphPad, San Diego, CA). A \( p \) value <0.05 was considered to be statistically significant. Statistically significant differences are indicated with asterisks, with the \( p \) values and sample numbers being specified in the figure legends. The \( p \) values that are close to significance are indicated in the figures themselves.

Results

Based on our earlier observation that piroxicam-induced colitis in IL-10-deficient mice was associated with hepcidin upregulation (18), we examined the effect of another epithelium-disrupting colitogenic agent, DSS. In the IL-10 KO mice, induction of colitis by oral administration of 3% DSS was associated with a significant increase in hepcidin expression that occurred over the course of 7 d (Fig. 1A, Supplemental Fig. 1). This increase was in marked contrast to the effects of DSS colitis in WT mice, in which, as we have shown previously (19), hepcidin expression was downregulated (Fig. 1A). Because the difference in colitis-associated hepcidin expression between the WT and IL-10 KO mice was maximal at 7 d of DSS treatment, we used this time point for all subsequent experiments. It should also be mentioned that despite some interexperimental variation in the expression levels of hepcidin (possibly related to minor differences in age of the mice, sex composition of the groups, or DSS consumption), the contrasting behavior of the colitis-associated change in hepcidin expression in the WT and IL-10 KO mice after 7 d of DSS, downregulation in WT and upregulation in IL-10 KO, was absolutely consistent. A decrease in serum iron concentration, corresponding to the elevation of hepcidin, was observed in the IL-10–deficient mice treated with DSS, whereas there was little or no change in the case of the WT animals (Fig. 1B). Examination of colon histology indicated that the severity of the DSS-induced colitis was greater in the IL-10 KO mice than in the WT animals based on inflammatory infiltration, disruption of epithelial architecture, and subepithelial edema (Supplemental Fig. 2). However, when we compared colonic expression of IL-6, the major inflammatory cytokine that has been shown to induce hepcidin upregulation (5–8), we found that the levels were similar in the WT and KO mice (Fig. 1C). Thus, differential IL-6 expression by the colon is unlikely to account completely for the difference in hepcidin expression between the WT and IL-10 KO mice. Colonic production of TNF-\( \alpha \), a cytokine that we have shown previously to inhibit hepcidin expression (19), also did not differ between the two types of animals (data not shown). IL-6 and TNF-\( \alpha \) were not detectable in serum samples of any of the mice.

**FIGURE 1.** Contrasting effects of DSS colitis on hepcidin expression in WT and IL-10 KO mice are not explained by differences in IL-6. (A) Liver hepcidin mRNA expression in WT and IL-10 KO mice treated with DSS for 7 d. \( *p = 0.01, **p = 0.0002 \) \( (n = 10–13 \) mice/group). (B) Serum iron concentrations in WT and IL-10 KO mice treated with DSS for 7 d. \( *p < 0.0001 \) \( (n = 8–10 \) mice/group). (C) IL-6 concentrations in the supernatants of colon explants from WT and IL-10 KO mice treated with DSS for 7 d. \( *p = 0.0002, **p = 0.01 \) \( (n = 6–12 \) mice/group).

During necropsy at the end of the DSS treatment period, we noticed a striking difference in spleen size between the WT and IL-10-deficient animals. In the WT mice, DSS colitis was associated with a clear and statistically significant increase in spleen weight, whereas DSS colitis in the IL-10 KO animals did not significantly alter this parameter (Fig. 2A). DSS colitis was also...
accompanied by an increase in the proportion of immature, CD71+ Ter119+ erythropoietic precursors in the spleen of both WT and IL-10 KO mice, but this increase was significantly greater in the WT animals (Fig. 2B, 2C). These results indicate that DSS colitis leads to extramedullary erythropoiesis and that this process is significantly reduced in the IL-10 KO mice, thus accounting for the attenuation of splenomegaly in these animals. Consistent with these findings, the serum concentration of erythropoietin, the major erythropoiesis-inducing hormone (37), was markedly elevated by DSS colitis in the WT mice and significantly higher than in the IL-10-deficient animals (Fig. 2D).

Increased erythropoietin levels, resulting from hypoxia or from exogenous administration of the hormone, have been shown previously to inhibit hepcidin expression (38). This erythropoietin-induced hepcidin downregulation is mediated by increased circulating levels of certain members of the TGF-β superfamily, such as growth differentiation factor 15, that are produced by erythrocyte precursors during increased erythropoietic activity and inhibit hepcidin expression (2, 38, 39). To determine whether such a mechanism might contribute to the DSS colitis-associated hepcidin downregulation in the WT mice, we adopted a previously used strategy and treated the animals with carboplatin, an inhibitor of erythropoiesis (38). Carboplatin treatment significantly inhibited the colitis-induced splenomegaly and the associated increase in splenic erythropoietic precursors (Fig. 3A and data not shown). Importantly, the carboplatin treatment completely prevented the downregulation of hepcidin associated with DSS colitis (Fig. 3B). Carboplatin inhibited DSS-induced IL-6 production by the colon (Supplemental Fig. 3A), and so its effects on hepcidin expression cannot be explained by changes in the level of colonic IL-6. The results thus indicate that increased erythropoietic activity inhibits hepcidin expression in the context of intestinal inflammation and suggest that the variation in erythropoiesis between the WT and IL-10-deficient mice is one of the factors responsible for the difference in colitis-associated hepcidin expression between these groups of animals.

To identify other factors that might be involved in the differential hepcidin expression in the colitic WT and IL-10 KO mice, we compared their hepatic transcriptional profiles at the end of the period of DSS administration. The results of the microarray analysis revealed a striking enrichment of transcripts involved in the inflammatory response among those that were significantly higher in the IL-10-deficient mice compared with the WT animals (Fig. 4A). Several of these enriched transcripts were derived from genes that are known targets of STAT3 (27–33), including hepcidin (indicated as Hamp, for hepcidin antimicrobial peptide in Fig. 4B). Confirming the microarray findings, we showed by quantitative RT-PCR that several of the STAT3-regulated genes were expressed at significantly higher levels in the IL-10 KO mice than in the WT animals following DSS treatment. These results indicate that DSS colitis is associated with greater upregulation of hepatic proinflammatory transcripts in the IL-10-deficient mice than in WT animals and that many of these transcripts are controlled by STAT3.

We were interested in identifying the mechanism responsible for the greater STAT3-dependent gene expression in the livers of the colitic IL-10 KO mice. Our results indicate that the differences in colitis-associated expression of hepcidin between the WT and IL-10 KO mice cannot be explained by differences in colon production of IL-6 (Fig. 1C), suggesting that this cytokine is unlikely to be the sole driver of the enhanced hepatic STAT3 activation in these mice. The only other STAT3-activating cytokine that has been implicated in regulating hepcidin expression is IL-22. Exogenously administered IL-22 has been shown to increase hepcidin

![FIGURE 2.](https://example.com/figure2)
FIGURE 2. DSS colitis induces greater erythropoietic activity in WT than in IL-10 KO mice. (A) Spleen weights in WT and IL-10 KO mice treated with DSS for 7 d. *p = 0.0004, **p = 0.007 (n = 9–18 mice/group). (B) Representative FACS plots showing CD71 and Ter119 staining of splenocytes from control and 7 d DSS-treated WT and IL-10 KO mice. (C) Proportions of CD71+Ter119+ erythropoietic precursors in the spleens of WT and IL-10 KO mice, control or DSS treated for 7 d. *p = 0.0003, **p = 0.013, ***p = 0.003 (n = 5 mice/group). (D) Serum erythropoietin concentrations in WT and IL-10 KO mice treated with DSS for 7 d. *p = 0.001, **p = 0.005, ***p = 0.004 (n = 8–12 mice/group).
expression in vitro and in vivo (40, 41), but the physiologic role of this cytokine in regulating hepcidin expression in inflammatory states is not clear. Nevertheless, we compared colon IL-22 expression in the WT and IL-10 KO mice and could find no difference (data not shown). We next considered the possibility that differences in the intestinal microbiota between the two types of mice might influence hepcidin expression, either by directly affecting STAT3 activity in the liver or indirectly by altering the colonic or systemic inflammatory milieu. To test this idea, we cohoused IL-10-deficient mice with WT animals for 2 wk before

**FIGURE 3.** Carboplatin (Cp)-mediated inhibition of erythropoiesis in WT mice prevents DSS colitis-induced hepcidin downregulation. (A) Spleen weights in WT mice treated with Cp during DSS colitis. *p = 0.04, **p = 0.002 (n = 7 mice/group). (B) Liver hepcidin mRNA expression in WT and IL-10 KO mice treated with Cp during DSS colitis. *p = 0.026, **p = 0.036 (n = 7 or 8 mice/group).

**FIGURE 4.** Liver transcriptional profiling reveals enrichment of proinflammatory and STAT3-dependent genes among those that are expressed at higher levels in the IL-10 KO mice with DSS colitis. (A) GSEA demonstrating the enrichment of inflammatory response transcripts among those expressed at higher levels in the IL-10 KO mice. (B) Heat map showing Z-scores for 26 STAT3-regulated genes (listed on the right) in livers of four WT and four IL-10 KO mice, with each column corresponding to an individual sample. These genes overlapped significantly with those expressed at higher levels in the IL-10 KO mice (*p = 7.476 \times 10^{-5} by Fisher exact test; p = 6.191 \times 10^{-12} by Pearson’s χ² test with Yates’s continuity correction).
initiating DSS administration to allow sharing of their commensal flora. We confirmed that the cohousing had an effect on the microbiota by using PCR analysis of 16S rRNA genes in stool DNA to assess the relative proportions of Bacteroidetes and Firmicutes, the major gut commensal bacterial phyla in mice and humans (42). The IL-10-deficient animals cohoused with the WT mice demonstrated a significant increase in the relative proportion of Firmicutes compared with their non-cohoused counterparts (Fig. 5A). We found that cohousing had similar effects on two other groups of commensals, segmented filamentous bacteria and Bacteroides fragilis (Fig. 5B, 5C). The effect was particularly marked in the case of B. fragilis: without cohousing, the IL-10 KO mice had a significantly higher proportion of B. fragilis relative to the WT animals, whereas the IL-10 KO mice cohoused with the WT animals had B. fragilis levels comparable to the WT (Fig. 5C). The cohousing had a striking inhibitory effect on the colitis-induced increase in hepcidin expression in the KO mice (Fig. 5D) and also inhibited the colitis-associated upregulation of four out of five other STAT3-regulated genes in these animals, including hemopexin, haptoglobin, SAA3, and IL-1RN (Fig. 6A–E). Although the expression of the fifth STAT3-regulated gene, SAA1, was also significantly higher in the colitic IL-10 KO mice, it was not affected by cohousing (Fig. 6C). The cohousing did not significantly alter spleen size or colon IL-6 in the IL-10-deficient mice (Supplemental Fig. 3B, 3C). Interestingly, cohousing did not affect the colitis-induced downregulation of hepcidin in the WT mice (data not shown).

To confirm and substantiate the effects of cohousing on colitis-associated changes in hepcidin expression, we carried out fecal transplantation experiments. As described in detail in the Materials and Methods section, groups of WT and IL-10 KO mice were treated with a mixture of oral antibiotics to deplete their gut microbiota and then administered (by oral gavage) homogenates of stool and cecal contents from either WT or IL-10 KO mice. Two weeks after the fecal transplantation, stool DNA from the transplant recipients was analyzed by PCR for the presence of B fragilis as an indicator of the efficacy of the transplantation. Both WT and IL-10 KO recipients of WT fecal material had low proportions of B. fragilis, similar to the WT donor, whereas the WT and IL-10 KO recipients of IL-10 KO fecal material had high proportions of B. fragilis, similar to the IL-10 KO donor (Fig. 7A). These findings indicate that the transplantation was at least partially effective. The recipient mice were then treated with DSS according to our usual protocol and liver hepcidin expression assessed on day 7. Liver RNA samples from DSS-treated nontransplanted WT and IL-10 KO mice were included in the analysis for the purposes of comparison. As shown in Fig. 7B, transplantation of WT fecal material into IL-10 KO mice completely prevented colitis-induced upregulation of hepcidin in the recipients, similar to the results of the cohousing experiment. Interestingly, transplantation of IL-10 KO fecal material into IL-10 KO mice did not restore colitis-induced hepcidin upregulation in the recipients. Transplantation of IL-10 KO or WT fecal material into WT recipients resulted in slightly higher colitis-associated hepcidin expression than in nontransplanted WT mice, but these differences were not statistically significant. Similar results were obtained when we examined the expression of the STAT3-regulated gene.
SAA3 (Fig. 7C). We also characterized the effects of the fecal transplantation on spleen weight and colon IL-6 production (Supplemental Fig. 3D, 3E). Unlike in the cohousing experiments, fecal transplantation did have appreciable effects on colitis-induced splenomegaly. The IL-10 KO recipients of either WT or IL-10 KO fecal material had larger spleens than the WT recipients, with the difference reaching statistical significance in the recipients of the WT flora. The degree of splenomegaly in the IL-10 KO recipients was similar to that in WT mice treated with DSS (compare Supplemental Fig. 3D with Fig. 2A), raising the possibility that increased erythropoietic activity in the IL-10 KO recipients of WT flora could be a factor in the suppressed colitis-associated hepcidin expression in these mice (Fig. 7B). Fecal transplantation also had effects on DSS-induced colon IL-6 production (Supplemental Fig. 3E), with the WT and IL-10 KO recipients of IL-10 KO fecal material having higher IL-6 levels than the corresponding recipients of WT fecal material. However, the effects on colon IL-6 did not correlate with the effects on hepcidin expression.

The results of both the cohousing and fecal transplantation experiments indicate that alterations in the microbiota of the IL-10–deficient mice caused by exposure to the WT microflora had an inhibitory effect on colitis-induced hepcidin expression. Taken together, these findings strongly suggest that the commensal flora has an important influence on hepcidin expression in the context of intestinal inflammation. Interestingly, neither cohousing with WT mice nor transplantation of WT fecal material had consistent effects on serum iron concentrations in the IL-10 KO mice with colitis (data not shown) despite the clear effects on hepcidin expression. Although we do not have a definitive explanation for this outcome, it could reflect the consequences of variations in blood loss from the inflamed colon, differences in food intake, or the influence of iron-regulating mechanisms that do not involve hepcidin.

**Discussion**

Our studies reveal important roles for two factors, erythropoietic activity and the commensal microbiota, in the regulation of hepcidin expression in colitis.
hepcidin expression during intestinal inflammation. Earlier work has shown that systemic *Salmonella* infection or the injection of bacterial products such as LPS can induce extramedullary erythropoiesis in the spleen (43-45). The findings reported in this study demonstrate that DSS colitis also induces splenic erythropoiesis, possibly reflecting the systemic translocation of LPS that has been documented in both murine and human colitis (46, 47). The colitis-associated erythropoietic activity had an inhibitory effect on hepcidin expression as indicated by the results of carboplatin treatment in the WT mice. This inhibition is probably mediated by factors, such as growth differentiation factor 15 and twisted gastrulation 1, that are produced by erythropoietic precursors and are known to suppress expression of the hormone (39, 48). Interestingly, there was a clear difference in splenic erythropoiesis between the colitic WT and IL-10 KO mice, with the activity being significantly greater in the WT animals. This difference provides a plausible explanation for our observation that DSS colitis causes hepcidin to be downregulated in the WT mice but upregulated in the IL-10 KO animals. Although extramedullary erythropoiesis is usually not a feature of human inflammatory disorders, anemia caused by associated abnormalities such as iron sequestration, deficient iron intake, or bleeding can increase erythropoietic activity and could thus lead to hepcidin downregulation. This idea is supported by clinical observations in patients with IBD showing that individuals with concurrent anemia usually have lower levels of hepcidin (15, 16).

The explanation for the decreased erythropoietin levels and lower erythropoietic activity in the IL-10-deficient mice is not clear. It appears to be at least partially independent of the microbiota because spleen size was not significantly affected by cohousing the IL-10 KO mice with WT animals (Supplemental Fig. 3B). It could reflect a consequence of the IL-10 deficiency per se, but whether it represents a direct influence of IL-10 on erythropoietin expression and erythropoiesis or an indirect outcome of IL-10-dependent alterations in intestinal inflammation remains to be seen. The available data on the effects of IL-10 on erythropoiesis are inconsistent and do not allow firm conclusions to be drawn. For example, a study on the effects of IL-10 in a murine in vitro culture model demonstrated that the cytokine promoted erythropoietin-induced burst-forming unit-erythroid (BFU-E) and CFU-erythroid growth (49), whereas studies in humans showed that IL-10 inhibited erythropoietin-independent growth of peripheral blood-derived BFU-E from patients with polycythemia vera as well as erythropoietin-induced growth of BFU-E from normal individuals (50, 51). An alternative explanation for the lower erythropoietin expression in the IL-10 KO mice is that it is secondary to the higher hepcidin expression in these animals. Elevated hepcidin leads to decreased serum iron concentrations and a consequent lowering of intracellular iron in most tissues including, presumably, the renal peritubular fibroblasts that produce erythropoietin. When intracellular iron concentrations fall, erythropoietin expression is inhibited, a mechanism that is believed to allow coordination of erythropoiesis with iron availability (52, 53). Clearly, further investigation will be required to determine how exactly IL-10 deficiency affects erythropoietin expression and erythropoiesis in the context of intestinal inflammation.

In addition to the effects of erythropoietic activity, the composition of the commensal microbiota also has a significant influence on colitis-associated changes in hepcidin expression, as indicated by the results of our cohousing and fecal transplantation experiments. Although both the cohousing and the fecal transplantation strategies have their limitations, and neither one can be considered to be conclusive on its own, the results of the two experiments taken together provide persuasive evidence in support of an important role for the microbiota in influencing hepcidin expression during intestinal inflammation. In each of these experiments, when the IL-10 KO mice were exposed to the WT flora, there was a clear inhibitory effect on their colitis-associated hepcidin expression, whereas WT mice exposed to the IL-10 KO flora did not alter their expression of hepcidin.
Because every batch of WT and IL-10 KO mice that we used consistently demonstrated colitis-associated hepcidin down- or upregulation, respectively, the simplest interpretation of these results is that the WT mice have an intestinal microflora that inhibits hepcidin expression, whereas the IL-10-deficient mice do not, and that the WT flora actively inhibits colitis-associated hepcidin upregulation in the KOs when transmitted by cohousing or fecal transplantation. Note that we cannot exclude that the commensal population of the IL-10 KO mice includes organisms that promote hepcidin expression and that the survival or growth of such organisms is suppressed following exposure to the WT microbiota. It is not clear why the WT mice are resistant to the influence of the IL-10 KO flora (at least with respect to hepcidin expression) in both the cohousing and fecal transplantation experiments. It is possible that the relevant commensals of the IL-10 KO mice are not able to colonize the WT animals. It is also possible that the relevant commensals of the IL-10 KO mice are simply not transmissible under the conditions of our experiments, an idea that is consistent with our observation that transplantation of IL-10 KO fecal material into IL-10 KO recipients did not recapitulate the colitis-induced hepcidin upregulation observed in the unmanipulated KO animals. These possibilities are quite plausible given the complexity of the microbiota and the diverse growth and niche requirements of individual bacterial groups. Further investigation will be required to identify the commensal organisms that have the putative positive and negative effects on hepcidin expression. Although there is a striking difference between the WT and IL-10 KO mice in the relative proportion of B. fragilis present in their stool (Figs. 5C, 7A), it is unlikely that this organism is solely responsible for the differences in colitis-associated hepcidin expression observed in these animals. IL-10 KO recipients of WT fecal material display the low proportion of B. fragilis characteristic of WT mice, correlating with low colitis-associated hepcidin expression; however, although WT and IL-10 KO recipients of IL-10 KO fecal material acquire the high proportion of B. fragilis characteristic of IL-10 KO mice, their colitis-associated hepcidin expression is low or only slightly elevated (Fig. 7B). The issue of identifying the relevant hepcidin-regulating commensal organisms is of more than academic interest because clarification of the mechanism responsible for the effects of the gut flora on hepcidin expression may make it possible to develop therapeutic strategies to inhibit abnormally elevated hepcidin levels by manipulating the microbiota. It will also be important to determine how IL-10 deficiency affects gut microbiota composition. IL-10 plays important roles in regulating multiple aspects of innate and adaptive immune defenses in the gastrointestinal tract (54, 55), and further studies will be required to determine which of these IL-10–dependent functions is involved in the microbiota alterations that influence colitis-associated hepcidin expression.

The effect of the microbiota on hepcidin expression in the IL-10 KO mice appears to be mediated by changes in STAT3 activation, which could reflect an indirect influence that acts via microbiota-dependent changes in intestinal or systemic inflammatory milieu. The increased severity of colitis in the IL-10–deficient mice is consistent with this idea. However, differences in colitis-induced hepcidin expression do not correlate with differences in colonic production of IL-6 in our cohousing and transplantation experiments, suggesting that this cytokine is not the only driver of STAT3 activation in this context. Our findings raise the possibility that IL-6 may act redundantly with another cytokine, perhaps IL-22, to activate STAT3 and increase hepcidin expression. An alternative possibility is that the effect of the microbiota on STAT3 activation and hepcidin expression is a direct influence of the gut flora on liver inflammation. Whenever there is a breach of the intestinal lining, as occurs during colitis, gut commensals or their products are translocated across the damaged epithelium and transported via the portal circulation to the liver (47, 56). Activation of TLRs expressed on various cell types in the liver can occur by this mechanism and has been shown to contribute to acute and chronic hepatic inflammation (56). Based on these observations, it seems plausible that differences in microbiota composition could directly affect STAT3-dependent expression of hepcidin by influencing the activation of hepatic pattern recognition receptors during DSS colitis. The results of our fecal transplantation experiments, which indicate clear alterations in splenic size in the IL-10 KO recipients (Supplemental Fig. 3D), raise the possibility that another mechanism by which the microbiota might influence hepcidin expression is by modulating colitis-associated erythropoietic activity. However, because alterations in splenic size are not observed in the cohousing studies (Supplemental Fig. 3B), it is possible that microbiota composition may affect hepcidin expression by mechanisms that may be dependent on or independent of changes in erythropoietic activity. Regardless of the exact mechanism by which the microbiota influences STAT3 activation in the liver, our experiments suggest that inhibition of this transcription factor may represent a viable strategy for lowering abnormally elevated hepcidin levels in individuals with IBD.

Our findings support the general idea that the level of hepcidin expression in inflammatory conditions such as IBD is determined by the net effect of a combination of positively acting and inhibitory factors. We have shown previously that TNF-α is one of the inhibitory influences (18), and we now add erythropoietic activity to this category. Some components of the intestinal microbiota may also act negatively, although others may promote hepcidin expression. Differences in the relative contributions of the positive and negative factors could help to explain the variations in hepcidin levels that have been observed in clinical studies of IBD. Our findings also suggest that both STAT3 and the microbiota represent viable targets for therapeutic interventions that are directed at controlling hepcidin expression.

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