Letter to *Blood*

To the editor:

Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B

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Activin B, which is strongly induced by inflammatory stimuli in the mouse liver, has recently appeared as a potent inductor of hepcidin in vitro, via the crossactivation of noncanonical SMAD1/5/8 signaling.[1,2] To confirm the cause and effect relationships among activin B, Smad1/5/8 phosphorylation, and hepcidin in vivo, we challenged $Inhbb^{-/-}$ mice[3] (deficient in activin B) with lipopolysaccharide (LPS) or infected them with an *Escherichia coli* septicemic strain, as indicated in supplemental Methods (available on the *Blood* Web site).

To examine whether, as observed in human hepatoma cell lines and in mouse primary hepatocytes, activin B also stimulates canonical SMAD2/3 and noncanonical SMAD1/5/8 signaling in vivo, we compared by western blot analysis Smad2 and Smad5 phosphorylation levels in the liver of wild-type and $Inhbb^{-/-}$ mice 4 hours after LPS stimulation (Figure 1A) or *E coli* infection (Figure 1B). Whereas these inflammatory stimuli strongly induce Smad2 and Smad5 phosphorylation in wild-type mice, none of these Smad effectors are activated in mice deficient in activin B. These data demonstrate that activin B, whose messenger RNA (mRNA) expression is strongly induced after LPS administration (Figure 1C) or *E coli* infection (Figure 1D), is required for activation of Smad1/5/8 signaling in these mice.

In parallel to activating SMAD1/5/8 phosphorylation in vitro, activin B was also shown to induce hepcidin expression.[1,2] Remarkably, pretreatment of hepatocytes with the BMP type I receptor inhibitor LDN-193189 prevented both the induction of SMAD/5/8 phosphorylation and the upregulation of the hepcidin (*HAMP*) gene expression by activin B,[1] suggesting that, at least in vitro, the effect of activin B on hepcidin expression was entirely attributable to the activation of noncanonical SMAD signaling. Since activin B also activates noncanonical Smad1/5/8 signaling in vivo, we expected that induction of hepcidin expression in response to LPS administration or *E coli* infection would be impaired in $Inhbb^{-/-}$ mice. However, the magnitude of hepcidin mRNA induction and its evolution over time was unexpectedly similar in wild-type and in $Inhbb^{-/-}$ mice challenged with LPS (Figure 1E) or infected with *E coli* (Figure 1F), pointing out the limitations of in vitro studies. To confirm the data at the protein level, serum hepcidin was quantified by competitive enzyme-linked immunosorbent assay (ELISA) in wild-type and in $Inhbb^{-/-}$ mice before and 4 hours after a LPS challenge. As suggested by the quantitative polymerase chain reaction (PCR) data, and similarly to wild-type mice, $Inhbb^{-/-}$ mice produce on average 3 times more hepcidin after endotoxin administration (Figure 1G). These results show that neither activation of Smad1/5/8 signaling nor activin B induction is necessary for upregulation of hepcidin production by inflammatory stimuli such as LPS administration or *E coli* infection. Hepcidin induction during inflammation was previously shown to be due at least in part to direct transcriptional regulation by the interleukin-6/STAT3 pathway.[4-6] Four hours after challenge with LPS or infection

with *E coli*, Stat3 phosphorylation was similarly induced in wild-type and $Inhbb^{-/-}$ mice (Figure 1A-B), suggesting a preponderant role of Stat3 activation in hepcidin induction by these inflammatory stimuli.

Mice lacking the iron-inducible Smad1/5/8-activating ligand Bmp6 have very low basal hepcidin levels.[7] However, they respond to LPS by inducing liver expression of *Inhbb* as much as do wild-type mice[1] and, as shown in Figure 2A, this leads to a similar induction of Smad1/5/8 phosphorylation in the 2 categories of mice. Importantly, despite a marginally significant increase in the amount of circulating hepcidin 4 hours after a LPS challenge in $Bmp6^{-/-}$ mice, the level reached after stimulation remains similar to that in unchallenged wild-type animals and ~3 times lower than that in LPS-challenged wild-type mice (Figure 2B). These data highlight a lack of proportionality between Smad1/5/8 signaling and hepcidin production in the inflammatory context.

Circulating iron and tissue iron both activate the Smad1/5/8 signaling cascade in the hepatocyte, which leads to the induction of not only hepcidin mRNA but also the mRNA of other targets, such as *Id1* and *Smad7*.[8,9] We therefore examined whether activation of Smad1/5/8 phosphorylation by inflammation was also accompanied by an induction of *Id1* and *Smad7* gene expression. As shown in Figure 2C-F, neither LPS nor *E coli* infection led to the induction of *Id1* or *Smad7* mRNA in the liver of either wild-type or $Inhbb^{-/-}$ mice. *Id1* mRNA expression was strongly repressed at the earliest time point (2 hours) but returned to baseline at 4 hours (Figure 2C-D). *Smad7* mRNA expression was also strongly repressed at 2 hours post-LPS challenge or post–*E coli* infection, and it remained below baseline for the whole time period (Figure 2E-F). Notably, the kinetics of expression of these 2 genes were similar in wild-type and in $Inhbb^{-/-}$ mice. These data point out major differences between Smad1/5/8 activation by iron and by inflammation. Indeed, whereas there is a good correlation between Smad1/5/8 activation by iron and induction of *Hamp*, *Id1*, and *Smad7* gene expression,[8,9] such a concordance is totally lacking in the inflammatory context. It would be interesting to know whether activation of Smad1/5/8 signaling by inflammatory stimuli takes place in liver cells other than the hepcidin-producing hepatocytes, as this would explain the discrepancies observed between iron and inflammation.

To determine whether activin B gene expression is induced in any type of inflammation, we infected mice with different pathogens (bacteria and parasite) and injected them with turpentine to cause sterile tissue abscess. As shown in supplemental Figure 1, liver *Inhbb* mRNA expression was induced not only in a mouse model of infection with the gram-negative extracellular pathogenic bacteria *E coli* but also in mice infected with the gram-negative intracellular pathogenic bacteria *Salmonella enterica* serovar Typhimurium or the
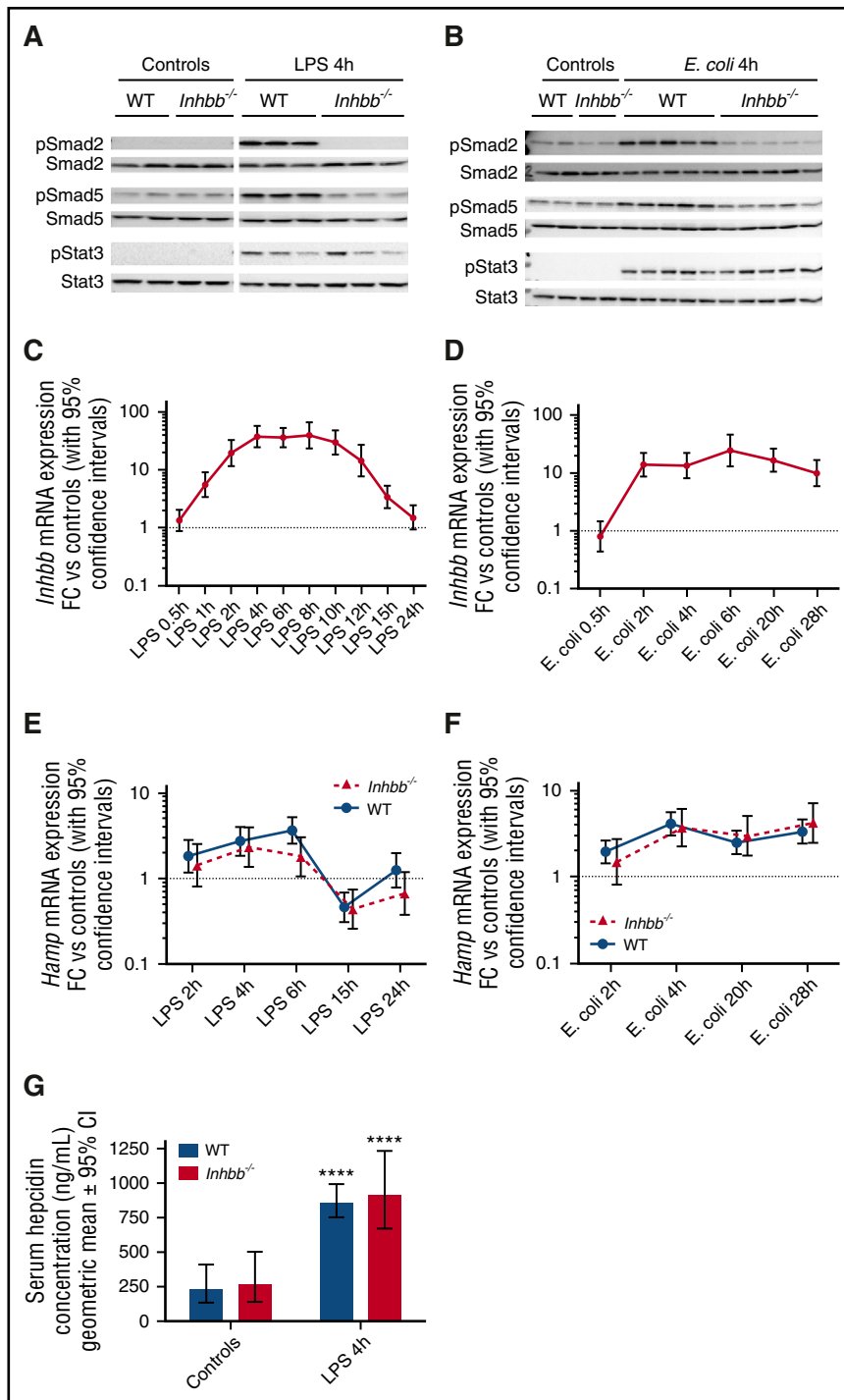
Figure 1. Although Smad1/5/8 signaling is not activated by inflammatory stimuli in the absence of activin B, this has no impact on the induction of hepcidin expression. (A-B) Fresh protein extracts were prepared from livers of wild-type (WT) and activin B (*Inhbb*)–deficient mice 4 hours after a LPS challenge or infection with *E. coli*. Phospho-Smad2, phospho-Smad5, phospho-Stat3, total Smad2, total Smad5, and total Stat3 were detected by immunoblot techniques on a Chemidoc MP Imaging System (Bio-Rad). (C-F) Evolution over time of activin B (*Inhbb*) (C-D) and hepcidin (*Hamp*) (E-F) mRNA expression was examined in wild-type mice and (for hepcidin) *Inhbb*$^{-/-}$ mice challenged with LPS or infected with *E. coli*. Point estimates of the fold change (FC) in gene expression relative to baseline ($2^{-\Delta\Delta Ct}$) are shown on the graphs, together with their 95% confidence intervals (CIs). When the lower limit of the fold-change CI exceeds 1, gene expression is significantly induced relative to baseline. (G) Serum hepcidin levels were measured by competitive ELISA in wild-type and *Inhbb*$^{-/-}$ mice at baseline and 4 hours after LPS challenge. Values shown are geometric means ± 95% CIs. Comparisons of log-transformed serum hepcidin levels were made by 2-way analysis of variance followed by Sidak's multiple comparison tests of planned contrasts. Results of comparisons with baseline levels in mice of the same genotype are shown above the bars. ****P < .0001.

gram-positive *Staphylococcus aureus*. However, despite notable induction of hepcidin (*Hamp*) mRNA, liver *Inhbb* mRNA expression was not significantly increased in the malaria model induced by *Plasmodium berghei* K173–infected red blood cells or in the sterile inflammation induced by turpentine. These data suggest that production of activin B by the liver is a biomarker of bacterial infection rather than a key player in anemia of inflammation.

Bmp6$^{-/-}$ mice have strong impairment of Bmp signaling, likely similar to the one achieved when treating wild-type animals with the BMP type I receptor inhibitor LDN-193189 or the BMP ligand antagonist ALK3-Fc. When challenged with LPS, they hardly increase

their hepcidin production to the level seen in unchallenged wild-type mice. Much higher circulating hepcidin levels would be expected if the inflammation and iron (BMP) signals had additive transcriptional effects on hepcidin promoter. Rather, our in vivo observations suggest transcriptional synergy between these 2 signals and explain why lowering one of them using LDN-193189 or ALK3-Fc is sufficient to attenuate the induction of hepcidin gene expression by various inflammatory stimuli.[10-12] A similar attenuation is observed when BMP signaling is genetically impaired, as here in *Bmp6*$^{-/-}$ mice or in mice with liver-specific deletion of Alk3.[13] The present data are compatible with the previously proposed synergy between interleukin-6/STAT3
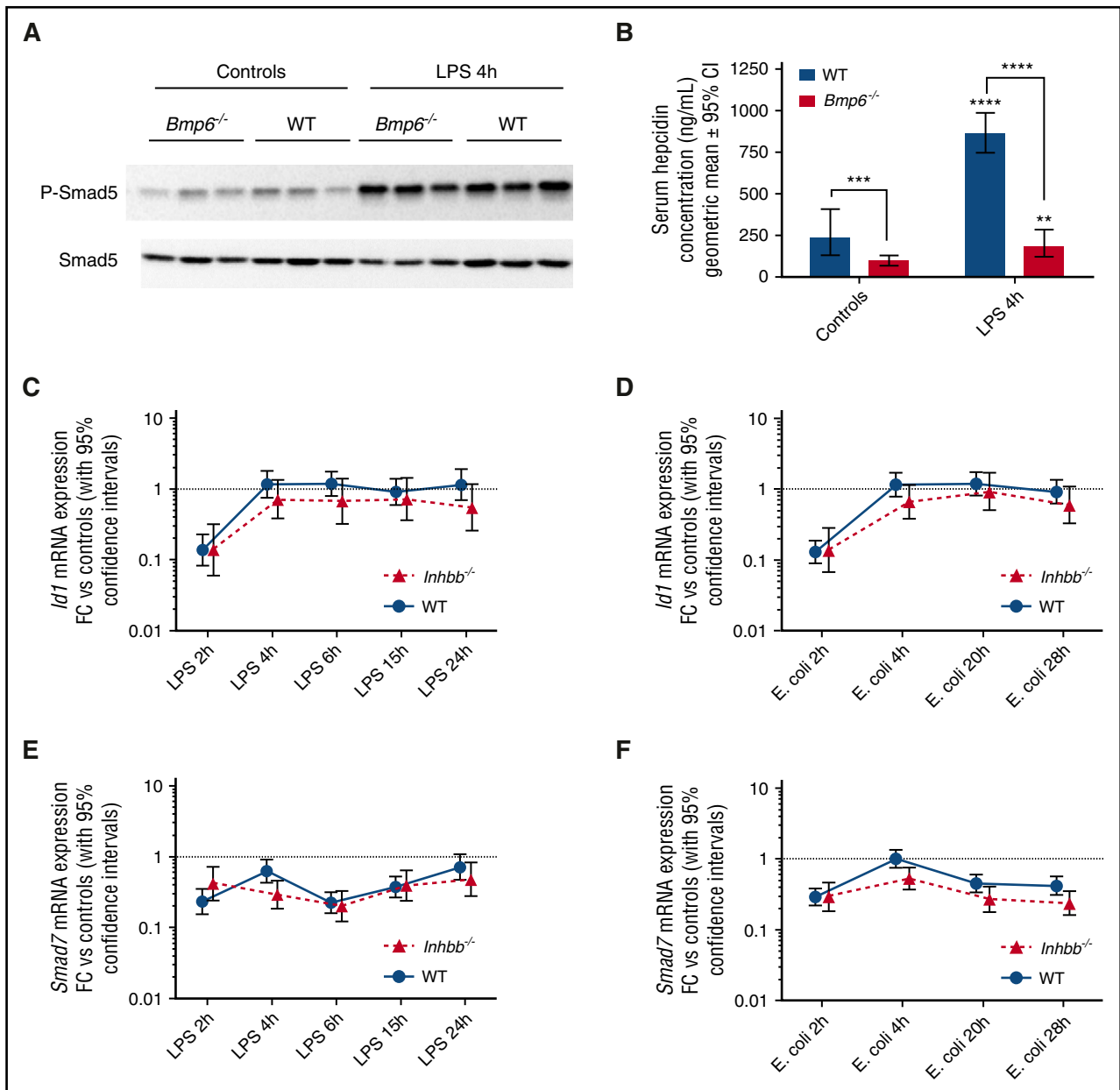
Figure 2. In contrast to Smad1/5/8 activation by iron, activation by inflammatory stimuli does not lead to the expected induction in hepcidin and has no impact on the expression of its hepatocyte targets, Id1 and Smad7. (A) Fresh protein extracts were prepared from livers of wild-type (WT) and *Bmp6*$^{-/-}$ mice. Phospho-Smad5 and total Smad5 were detected by immunoblot techniques on a Chemidoc MP Imaging System (Bio-Rad). (B) Serum hepcidin levels were measured by competitive ELISA in wild-type and *Bmp6*-deficient mice at baseline and 4 hours after LPS challenge. Values shown are geometric means ± 95% confidence intervals (CIs). Comparisons of log-transformed serum hepcidin levels were made by 2-way analysis of variance followed by Sidak's multiple comparison tests of planned contrasts. Results of comparisons with baseline levels in mice of the same genotype are shown above the bars. Results of comparison between wild-type and *Bmp6*$^{-/-}$ mice are indicated by connecting lines. **$P < .01$; ***$P < .001$; ****$P < .0001$. (C-F) Evolution over time of *Id1* (C-D) and *Smad7* (E-F) mRNA expression was examined in wild-type and *Inhbb*$^{-/-}$ mice challenged with LPS or infected with *E coli*. Point estimates of the fold change (FC) in gene expression relative to baseline ($2^{-\Delta\Delta Ct}$) are shown on the graphs, together with their 95% CIs. When the upper limit of the fold-change CI is below 1, gene expression is significantly repressed relative to baseline.

and BMP/SMAD signaling in regulating hepcidin.[14] They show that full induction of hepcidin expression by inflammatory stimuli requires a functional BMP6-activated signaling pathway in the hepatocyte but is independent of activin B and its activation of Smad1/5/8 signaling that likely occurs in other cells of the liver.

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The online version of this article contains a data supplement.

Contribution: C.B.-F. challenged the mice with LPS and performed reverse transcription PCR and western blotting experiments. A.G. infected the mice with the different pathogens and performed reverse transcription PCR experiments.

C.L. was in charge of mouse genotyping, challenged the mice with plasmodium and turpentine, and performed hepcidin ELISAs. O.G. helped with mouse experiments. D.M. helped with data analysis and interpretation. P.M. and E.O. were involved in the design of infection experiments. H.C. and M.-P.R. led and supervised the project through all stages, helped with data analyses, and wrote the manuscript with suggestions and comments from all authors.

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