

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

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Material and Methods

Murine models. B6.129S4-*Inhbb*^{tm1Jae/J} (*Inhbb*^{-/-}; deficient in activin B) mice present with a failure of eyelid fusion at birth and females show increased gestation time and decreased nursing ability¹. We therefore mated *Inhbb*^{+/-} mothers to *Inhbb*^{-/-} fathers to obtain the *Inhbb*^{-/-} males used in this study. To examine the importance of activin B on hepcidin upregulation by inflammation, 7 to 8-week-old *Inhbb*^{-/-} mice and wild-type controls on the same genetic background (C57BL/6) were challenged with an intraperitoneal injection of LPS (serotype 055:B5; Sigma; 1µg/g body weight) and livers were harvested at different time points (baseline, 2h, 4h, 6h, 15h and 24h) following injection. B6.129S4-*Inhbb*^{-/-} mice and wild-type controls were also infected with *E. coli* SP15 (10⁸ CFU; footpad injection) and livers were harvested at baseline, 2, 4, 20 and 28 hours post-infection. To examine the correlation between Smad1/5/8 signaling and hepcidin levels, 7 to 8-week-old *Bmp6* null mice (*Bmp6*^{m1Rob})² and wild-type mice on a CD1 background were challenged with an intraperitoneal injection of LPS (1µg/g body weight) and livers were harvested 4 hours later. Induction of activin B was also examined in the liver of C57BL/6 mice infected intraperitoneally with *Salmonella enterica* serovar typhimurium SL1344 (10⁴ CFU), *Staphylococcus aureus* Newman (2x10⁸ CFU), or of mice that received *Plasmodium berghei* K173-infected red blood cells (10⁶) or a subcutaneous intracapsular injection of oil of turpentine (5mL/kg; Sigma). Mice were sacrificed at the indicated times 2 hours to 11 days after injection. All experiments were performed on males and mice received a diet with standard iron content (250 mg iron/kg; SAFE, Augy, France). The number of mice challenged at each time point is indicated on Supplementary Table 1. Experimental protocols were approved by the Midi-Pyrénées Animal Ethics Committee.

Quantitation of mRNA levels. Total RNA from mouse liver was extracted using Isol-RNA lysis reagent (5 PRIME). cDNA was synthesized using MMLV-RT (Promega). The sequences of the primers for the *Inhbb*, *Hamp*, *Id1*, and *Smad7* target genes and the reference gene *Hprt* are listed in Supplementary Table 2. Quantitative PCR (Q-PCR) reactions were prepared with LightCycler® 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics) and run on a LightCycler® 480 System (Roche Diagnostics). ΔCt values were obtained by subtracting the reference gene Ct to the target gene Ct.

Serum hepcidin. Serum hepcidin levels were quantified using the Intrinsic LifeSciences (La Jolla, CA) Hepcidin-Murine Compete™ competitive ELISA.

Protein extraction. Livers were homogenized in a FastPrep®-24 Instrument for 15 sec at 4 m/s. The lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5mM EDTA, pH 8, 0,1% NP-40) included inhibitors of proteases (complete protease inhibitor cocktail, Roche Applied Science) and of phosphatases (phosphatase inhibitor cocktail 2, Sigma-Aldrich). Liver proteins were quantified using the Bio-Rad Protein Assay.

Western blot analysis. Fresh protein extracts were diluted in Laemmli buffer (Sigma-Aldrich), incubated for 5 minutes at 95° C, and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Amersham). Membranes were blocked with 5% of dry milk in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.15% Tween 20). They were incubated with a rabbit antibody to phospho-Smad5 (Epitomics), phospho-Smad2 (Cell Signaling), or phospho-Stat3 (Cell Signaling) at 4° C overnight, and washed with TBS-T buffer. Following incubation with a goat anti-rabbit IgG antibody (Cell Signaling) conjugated to horseradish peroxidase (HRP), enzyme activity was visualized by an ECL-based detection system (Millipore). Blots were then stripped and reprobed with a rabbit antibody to Smad5 (Epitomics), Smad2 (Cell Signaling), or Stat3 (Cell Signaling) 2 hours at RT before incubation with the goat anti-rabbit HRP-linked antibody. Blot imaging and analysis was performed on a Chemidoc MP Imaging System (Bio-Rad) with the Image Lab software.

Statistical analyses. Means of quantitative PCR $\Delta\Delta C_t$ values and serum hepcidin (log-transformed) in mice of the different genotypes were compared with ANOVA (one- or two-way, as applicable) followed by Sidak's multiple comparison tests of planned contrasts between pairs of means and calculation of 95% confidence intervals (CI) for the $\Delta\Delta C_t$ s. Point estimates of the fold changes in gene expression between 2 groups ($2^{-\Delta\Delta C_t}$) and their 95% CI were obtained by antilog2 transformation of the means and confidence limits of $-\Delta\Delta C_t$ s³.

References.

1. Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev.* 1994;8(4):414-427.
2. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ. Mice lacking Bmp6 function. *Dev Genet.* 1998;22(4):321-339.
3. Yuan JS, Reed A, Chen F, Stewart CN, Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics.* 2006;7:85.

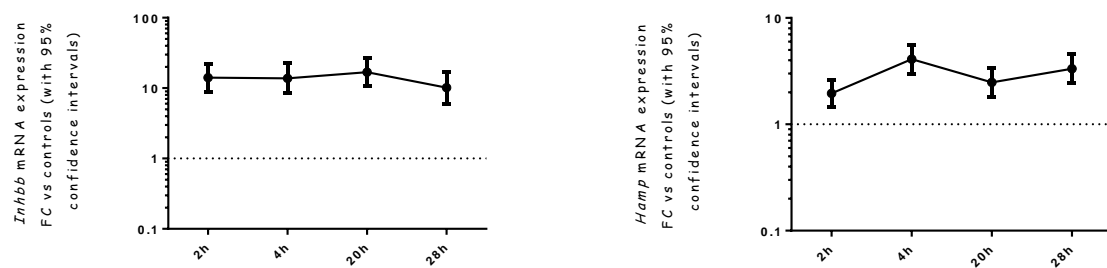
Supplementary Table 1. Number of mice (all males) used in the different inflammation models

genotype	stimulus/strain	time-point					
	LPS	baseline	2h	4h	6h	15h	24h
WT	C57BL/6	12	6	9	13	8	5
<i>Inhbb</i> ^{-/-}	C57BL/6	28	4	5	5	5	4
WT	CD1	4		4			
<i>Bmp6</i> ^{-/-}	CD1	4		4			
	<i>E. coli</i>	baseline	2h	4h	20h	28h	
WT	C57BL/6	11	22	18	17	15	
<i>Inhbb</i> ^{-/-}	C57BL/6	9	4	8	6	6	
	<i>Salmonella</i>	baseline	6h	D1	D2	D3	D4
WT	C57BL/6	11	6	5	6	3	2
	<i>S. aureus</i>	baseline	2h	4h	14h	48h	
WT	C57BL/6	8	3	6	5	5	
	<i>P. berghei</i>	baseline	D2	D3	D5	D7	D11
WT	C57BL/6	8	5	5	5	5	5
	turpentine	baseline	6h	24h	48h	96h	
WT	C57BL/6	9	5	6	9	3	

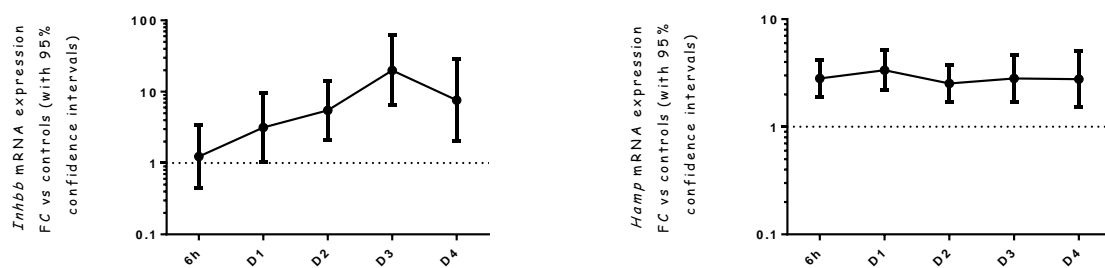
Supplementary Table 2. Sequences of the primers used in quantitative PCR reactions

	Forward (5' -> 3')	Reverse (5' -> 3')
Hprt	CTG-GTT-AAG-CAG-TAC-AGC-CCC-AA	CAG-GAG-GTC-CTT-TTC-ACC-AGC
Inhbb	TCA-GCT-TTG-CAG-AGA-CAG	GAA-GAA-GTA-CAG-GCG-GAC
Hamp	AAG-CAG-GGC-AGA-CAT-TGC-GAT	CAG-GAT-GTG-GCT-CTA-GGC-TAT-GT
Id1	ACC-CTG-AAC-GGC-GAG-ATC-A	TCG-TCG-GCT-GGA-ACA-CAT-G
Smad7	GCA-GGC-TGT-CCA-GAT-GCT-GT	GAT-CCC-CAG-GCT-CCA-GAA-GA

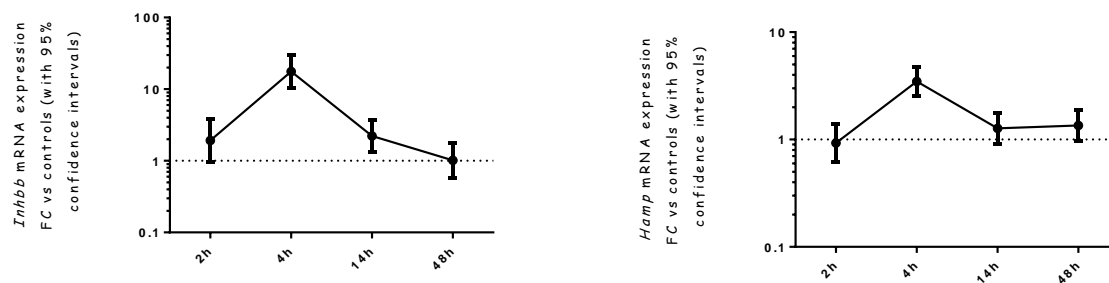
Supplementary Fig. 1. Activin B is upregulated in mouse models of bacterial infection but not in models of malaria or sterile inflammation. Evolution over time of the mRNA expression of the activin B (*Inhbb*) and hepcidin (*Hamp*) in wild-type mice infected with *E. coli*, *Salmonella enterica* serovar typhimurium, *Staphylococcus aureus*, *Plasmodium berghei* K173-infected red blood cells, or in mice injected with turpentine. Point estimates of the fold changes 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.



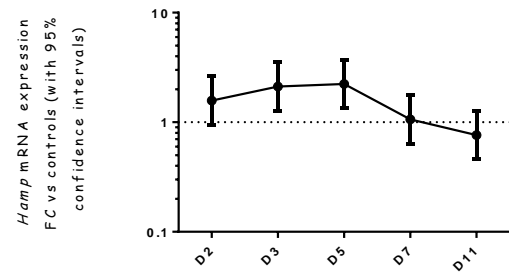
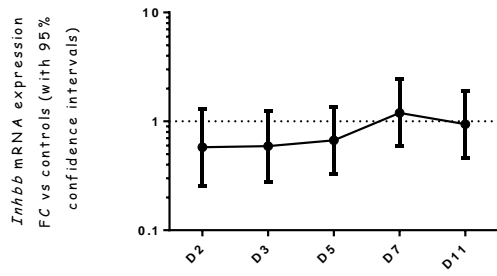
E. coli sepsis



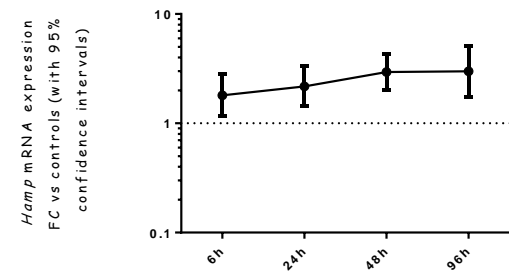
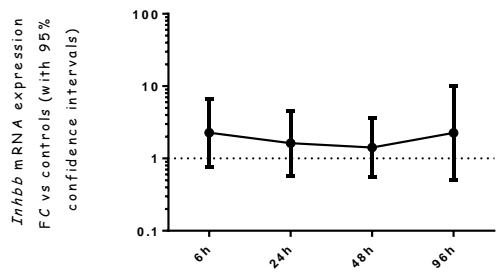
Salmonella infection



S. aureus infection



Plasmodium berghei infection



Sterile abscess induced by turpentine