## Title

Hepcidin-Induced Hypoferremia Is a Critical Host Defense Mechanism against the Siderophilic Bacterium Vibrio vulnificus



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# Hepcidin-Induced Hypoferremia Is a Critical Host Defense Mechanism Against the Siderophilic Bacterium Vibrio vulnificus

--Manuscript Draft--

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| **Manuscript Number:** | CELL-HOST-MICROBE-D-14-00525R2 |
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| **Abstract:** | Hypoferremia, mediated by increased production of hepcidin, develops early during infections. It has been proposed but never demonstrated that this response is a host defense mechanism to restrict iron availability for extracellular pathogens. Hereditary hemochromatosis, an iron overload disease caused by hepcidin deficiency, is associated with lethal infections by siderophilic bacteria. To elucidate the mechanisms of this susceptibility, wild-type and hepcidin-deficient mice with varying iron load were infected with Vibrio vulnificus, and their inflammatory responses, iron regulation and survival analyzed. Although systemic iron overload facilitated the infection, hepcidin-induced reactive hypoferremia protected the host from acute mortality by restricting bacterial growth. Timely administration of hepcidin agonists rescued the susceptible hepcidin-deficient mice from death, regardless of iron overload. Our study demonstrates the host defense role of hepcidin in infections with siderophilic pathogens, and indicates that hepcidin agonists may improve the outcome of these lethal infections in patients with hereditary hemochromatosis or thalassemia. |
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Cover Letter

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October 27, 2014

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Dear Editor,

We are submitting a revised version of our Research Article titled "**Hepcidin-induced hypoferremia is a critical host-defense mechanism against the siderophilic bacterium *Vibrio vulnificus***". We have followed the instructions in the Authors Checklist to prepare the final submission.

As we stated in our original cover letter, the manuscript documents the effect of a new mechanism of innate immunity on microbial growth and shows how it can be manipulated to treat infections. Hypoferremia is a very early response to infections. The decrease in extracellular iron is caused by cytokine-mediated induction of the iron-regulatory hormone hepcidin. In turn, increased hepcidin triggers iron sequestration in macrophages. Although long suspected to function as an innate immune response against extracellular pathogens, the host defense role of hepcidin-induced hypoferremia has never been experimentally verified.

In the current manuscript, we demonstrate for the first time that hepcidin has an important role in host defense and that hepcidin agonists can be used to treat infections. Specifically, we show that hepcidin is essential for survival after infection with *Vibrio vulnificus, a* deadly siderophilic bacterial pathogen. The protective effect of hepcidin was mediated by its ability to rapidly decrease serum iron which restricted bacterial growth.

Patients with hereditary hemochromatosis, a common genetic disease caused by hepcidin deficiency, are predisposed to infections with *Vibrio vulnificus* and other siderophilic bacteria. In particular, *V. vulnificus* causes a fulminant infection with more than 50% mortality. In a murine model of hereditary hemochromatosis, treatment with hepcidin agonists mitigated the high mortality caused by *V. vulnificus*. The dramatic effectiveness of the new treatment even after the infection was established was unexpected. We believe that the new host defense mechanism and the corresponding therapeutic strategy will be of great interest to your readers. The subject is also timely because of increasing reports of lethal *V. vulnificus* infections in the popular media, e.g.:
http://www.huffingtonpost.com/2013/10/11/vibrio-vulnificus-florida-bacteria-salt-water-31-infected_n_4086268.html

Thank you for your consideration,

Tomas Ganz

Tomas Ganz, Ph.D., M.D.
Professor of Medicine and Pathology

**Response to the Reviewers' Comments**

**Reviewer #1**

*Hepcidin is a small peptide that regulates iron levels in the host. Production of hepcidin is upregulated during infection, causing a reduction in circulating iron levels. This process has been proposed as a host defense mechanism to sequester nutrient iron away from invading pathogens, though this has not been demonstrated experimentally. In this manuscript, the authors demonstrate that hepcidin is crucial for the survival of the host during infection with Vibrio vulnificus, a gram-negative bacterium that causes disease characterized by significant mortality in the presence of high iron. Altering iron levels through the diet significantly impacted infection with V. vulnificus, with mice on a high iron diet having higher bacterial burdens than those on a low iron diet. Moreover, infection of mice lacking hepcidin resulted in higher bacterial burdens and increased mortality. The lack of hepcidin corresponded to higher iron levels in the serum and livers of infected mice fed a high iron diet. The importance of hepcidin in regulating circulating iron levels during infection was also supported by treatment of infected mice with a hepcidin agonist, which significantly reduced serum iron levels as well as bacterial burden in hepcidin knockout mice. These data demonstrate that hepcidin plays a critical role in reducing available iron as a defense against invading pathogens.*

*Comments:*

*1. The survival and bacterial burdens of both wild type and Hamp-/- mice on a regular diet should be included.*

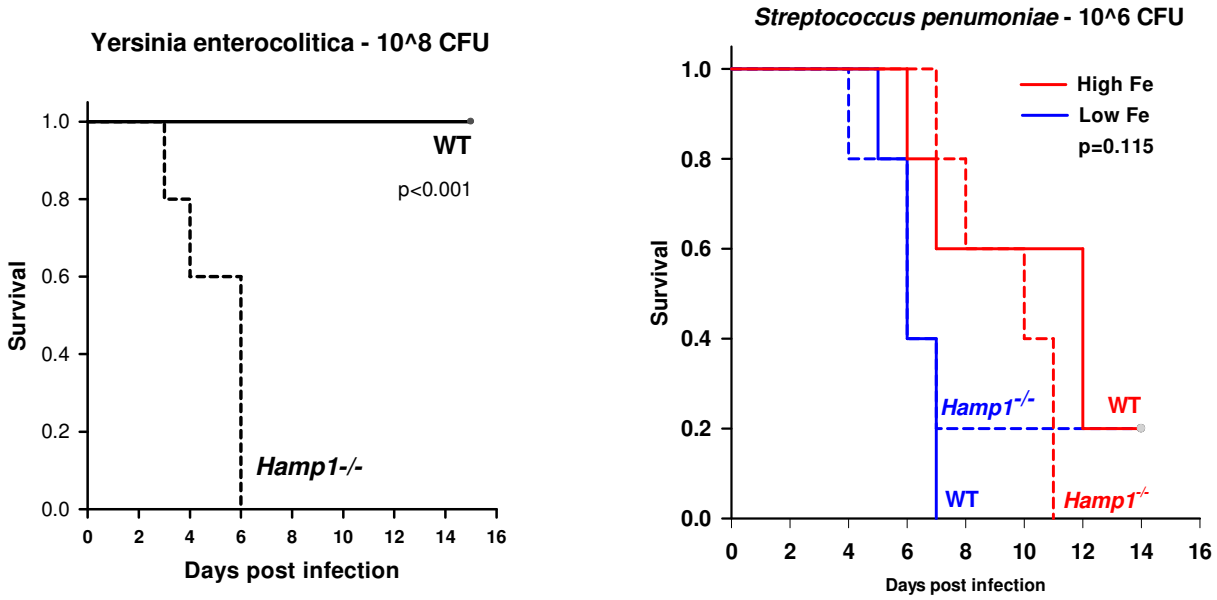
In all our experiments, Hamp1$^{-/-}$ mice were fed a regular diet, not a high-iron diet. We have now clarified this in several figure legends. We did not use high-iron diet in Hamp-/- mice because the concentration of iron in regular diet is sufficient to promote iron loading of Hamp-/- mice as demonstrated by the iron measurements in the liver (Figure 3D). We also corrected Table S2, to indicate that Hamp1$^{-/-}$ mice were on a standard diet rather than high-iron diet.

High-iron diet (10,000 ppm Fe) was used only for the WT mice infected with 300, 10^3 and 10^5 CFUs since the regular diet was not sufficient to load these mice to iron levels that could be compared with the hepcidin-deficient mice. However, we did use WT mice on regular diet in Figure S6: infection with 10^7 CFU resulted in 100% mortality and infection with 10^6 CFU resulted in only 30% mortality. Based on this, we expect that WT mice on a regular diet would not be susceptible to infection with 10^3 or 10^5 CFU, and the proposed experiments would lead to an unnecessary sacrifice of animals.

*2. The authors should consider including an infection with a "non-siderophilic" pathogen that still requires iron for infection. This would demonstrate that the phenotypes are not specific to V. vulnificus infection.*

We agree that the evaluation of the proposed mechanism should be extended to other pathogens and we are currently working on that issue using both non-siderophilic and siderophilic bacteria. However, we are planning to include these data in a different manuscript since they require a complex analysis in which disease progression, severity and iron dependence varies by pathogen. Nevertheless, we provide evidence to the reviewer showing that iron-loaded Hamp1$^{-/-}$ mice are more susceptible than WT to infection with the siderophilic bacterium *Yersinia enterocolitica.* Interestingly, we could not detect either an early increase in hepcidin or development of hypoferremia in this infection model (data not shown), suggesting a somewhat different mechanism of hepcidin-and iron-related susceptibility of this pathogen compared to V. vulnificus. For the non-siderophilic pathogen *Streptococcus pneumoniae,* both WT and Hamp1-/- mice show the same susceptibility to infection, with no obvious iron dependence. Iron-loaded animals even displayed a trend toward improved survival.

We changed the title of the manuscript to clarify that we are only presenting results related to *V. vulnificus.*




*3. The authors should consider including a strain of V. vulnificus that cannot acquire iron (such as the VuuA knockout) to confirm that growth in serum from iron-loaded or hepcidin deficient mice is dependent on the specific uptake of iron by the bacterium and not some other factor in the serum that is enhancing growth.*

The use of a *V. vulnificus* mutant that cannot acquire iron would provide a useful tool to confirm that the growth of the bacteria in serum is specifically dependent on the iron uptake. The reviewer suggested the use of the *VuuA* mutant, a bacterium that lacks the ferric vulnibactin receptor and therefore is unable to acquire iron from transferrin and the siderophore vulnibactin. This mutant was previously shown to be less virulent than the wild-type strain (Webster AC and Litwin CM, Infect Immun, 2000), however it was still able to cause infection. This happens because *V. vulnificus* has other means to acquire iron, namely through hemin receptors (*Hupa/Hvta*) and hydroxamate siderophores. In addition, Kim *et al* demonstrated that prior to the use of transferrin and vulnibactin as iron sources, *V. vulnificus* requires non-transferrin bound iron (NTBI) to initiate their growth, through an uncharacterized transporter (Kim CM *et al*, J Infect Dis, 2007). Thus, it would be difficult to address the role of iron uptake by *V. vulnificus* in our model, as we would have to use multiple mutations alone and in combination, with no mutation available for the important but as yet unknown NTBI transporter. We believe that the various iron conditions included in the manuscript (dietary iron manipulation and treatment with minihepcidins *in* vivo, as well as bacterial growth analysis *in vitro* using iron-depleted and iron-loaded serum iron, and supplementation with ferric ammonium citrate and deferiprone) are sufficient to conclude that iron is the critical factor driving bacterial growth.

**Reviewer #2**

*The hypoferremia associated with hepcidin induction that occurs during many acute infections is widely assumed to represent a host anti-infective effect, acting through depriving iron accessibility to invading microorganisms. Arezes et al correctly identify that the critical role of hepcidin in protective hypoferremia has never been formally shown. They attempt to test this hypothesis by investigating the responses of wild-type C57BL/6 mice and Hamp1-/- mice (maintained on diets with different iron contents, administered for different lengths of time) to infection with Vibrio vulnificus. They show that Hamp1-/- mice are more susceptible than wild-type to infection, and link this primarily to the ability to mount a rapid hypoferremic response. The work presented is novel, and the experiment in which hepcidin knockout animals are rescued from lethal infection by addition of minihepcidin agonists post-infection is convincing and exciting. The main limitation relates to the fact that Hamp1-/- mice, even after dietary iron manipulation, do not have equivalent iron status to wild-types throughout their development or at the time of experiment; therefore the wild-types are not ideal controls; some conclusions in the paper from experiments in which WT and KO are directly compared should therefore be*

*moderated. Also the interpretation of some aspects of figure 3 needs clarifying.*

*Specific comments:*

*1. Title: 'siderophilic infections' is too general as the authors only consider one infection - this should be revised to e.g. '…against the sideropohilic bacterium, Vibrio vulnificus' or similar.*

We changed the title according to the reviewer's suggestion.

*2. Introduction: is there evidence that patients with HH fail to mount hypoferremic responses to inflammation / infection as suggested? A reference should be provided, or else it should be made clear that this is hypothetical (or this line of argument could be removed). Hfe-/- mice appear to be able to mount a good hypoferremic response to LPS (Wallace et al, Blood, 2011). As the main interpretation of manuscript relates to ability to mount a hypoferremic response, the parallel between Vibrio infections here and susceptibility of HH patients (which is presumably linked to excess NTBI rather than due to a failure to make a hypoferremic response) may fall down. Does transferrin iron represent an iron source for Vibrio vulnificus? Is the hypoferremic response more important for removing NTBI or for decreasing Tf saturation?*

As noted in Figure 1 of the Wallace paper, the ability to generate hypoferremia in response to LPS declines as the hemochromatosis phenotype becomes more severe (WT>HFE>TfR2>TFR2/HFE). To our knowledge, there are no clinical studies that evaluated hypoferremic response to infection in HH patients. Similarly to Hfe-/- mice, it is likely that many HH patients, particularly those with HFE mutations, are able to increase hepcidin in response to infection. This may explain why relatively few HH patients develop infections with siderophilic bacteria. However, unlike the mouse models, HH patients have highly variable baseline hepcidin levels and variable severity of iron overload (e.g. Ryan et al. Acta Haematol. 2014 Sep 26;133(2):155-161), due to genetic and environmental modifiers. The presence of comorbid factors such as alcohol consumption or liver failure may contribute to the HH patients' susceptibility to infection as these factors decrease baseline hepcidin production and may blunt its response to inflammation. We have added these considerations in the Discussion.

The reviewer also points out that excess NTBI, rather than a deficient hypoferremic response, may be the reason why HH patients and *Hamp1-/-* mice are more susceptible to infection. To address this hypothesis we included new data in which we compare the NTBI values for WT mice and Hamp1-/- mice (although we already had Hamp1-/- data in the original submission, we repeated the measurements for Hamp1-/- in the same experiment to allow for an accurate comparison between all the groups). Only iron-loaded Hamp1-/- mice had increased NTBI at baseline, whereas iron-depleted Hamp1-/- and both groups of WT mice had similarly low NTBI. Because even iron-depleted Hamp1-/- mice are more susceptible to *V. vulnificus* infection than WT mice, NTBI levels cannot be the only explanation. We now clarify in the text (page 7-8) that both reactive hypoferremia and the baseline levels of transferrin-bound and non-transferrin bound iron represent determinants in the susceptibility to V. vulnificus infection. The article by Kim CM *et al*, J Infect Dis, 2007 similarly proposed that the removal of both TBI and NTBI is important at different stages: NTBI in the beginning of growth and TBI during exponential growth. Our hypothesis that the hypoferremic response is at least as important as baseline NTBI levels is also supported by our post-infection treatment with minihepcidin in Hamp1-/- mice, which significantly improved mouse survival. This outcome would not be possible if baseline NTBI was the main factor driving the infection.

*3. Methods: The authors attempt to address the inherent differences in baseline iron status between Hamp1-/- with wild-type through dietary manipulation. According to the methods, the iron-loading diets were non-equivalent and also the times of the WT and KO mice on altered iron diets were different: "Starting at 6 weeks of age, WT mice were fed an iron--poor (4 ppm Fe) or iron--rich (10000 ppm Fe) diet (Harlan) for 2 weeks. Starting at 4--5 weeks of age, Hamp1--/-- mice were fed an iron--poor (4 ppm Fe) or regular diet (270 ppm Fe) for 4--6 weeks before the infection". However the figures*

*(e.g. Figure 3) give the impression that the iron diets were the same. The figure or legend should be clarified to point this out, as it is important.*

We appreciate this suggestion and have added that information to the legends of Figure 1 and 3.

*4. The interpretation of Figure 3 needs revisiting. The authors reduce the infecting dose to 300 CFU in order to enable survival to beyond 16h. Do Hamp1-/- mice succumb to infection with this dose? The evidence that Hamp1-/- mice on low iron diets were more susceptible to this inoculum than WT does not seem to be presented; Like WT mice, most knockouts on low iron diet did not have detectable CFU in blood or liver according to figure 3A - would these therefore survive the infection? In the results section describing figure 3 the authors state "After infection, iron-loaded Hamp1-/- mice had CFU in both blood and liver, as opposed to their WT counterparts, in which no CFU were detectable for most of the mice (Figure 3A). This result confirms that the lack of hepcidin favors bacterial replication and dissemination." The second sentence in the quotation is not supported by the finding of no CFU in most Hamp1-/- mice on iron low diets. Similarly, the authors state "The different susceptibility of WT and Hamp1-/- to V. vulnificus infection is not attributable to their baseline iron differences because iron-depleted Hamp1-/- mice were much more susceptible to infection even though they had lower liver iron stores than iron-overloaded WT mice" - but this susceptibility difference is not apparent from the similarly low CFU counts shown in Figure 3A. The claim that the difference in baseline iron status (figure 3D) between WT and KO does not account for 'the effect' is not directly supported unless the authors can show that the Hamp1-/- mice actually are more susceptible to this specific dose. The difference in susceptibility between 'iron-loaded' WT and KO in this experiment may still relate in part to the differing baseline iron status shown in figure 3D - have the authors measured LPI/NTBI in their iron-loaded WT mice?*

We thank the reviewer for these suggestions. While iron-loaded Hamp1-/- mice were indeed terminally ill 16h after infection with 300 CFU, iron-depleted Hamp1-/- mice were not yet moribund at that time point and we do not know whether they would have succumbed at a later time-point because all groups were euthanized at the same time. Therefore, we removed the conclusion that Hamp1-/- were more susceptible at this specific dose. We also removed the sentence: “the different susceptibility of WT and Hamp1-/- to V. vulnificus infection is not attributable to their baseline iron differences because iron-depleted Hamp1-/- mice were much more susceptible to infection even though they had lower liver iron stores than iron-overloaded WT mice". We agree that we cannot draw this conclusion using only the CFU data. However, this conclusion is fully supported by the survival data in Figure 1. We have now included liver iron measurements in Figure 1 to make our point clear.

We also added NTBI data for WT mice in Figure 3, as explained in the previous comment.

We have extensively rewritten the section referring to Figure 3 and made it clearer that multiple factors may determine the replication and dissemination of V. vulnificus in vivo: baseline concentrations of transferrin-bound and non-transferrin bound iron, as well as reactive hypoferremia (all of which are affected by hepcidin concentrations).

*5. In figure 3C, despite the absence of hepcidin, serum iron levels still appear lower in infected than non-infected mice (significant in the case of the iron-loaded mice), albeit to a lesser extent than WT. The first sentence of the following section titled 'Hepcidin levels increase early after….', specifically "Only WT but not Hamp1-/- animals decreased serum iron after infection" should therefore be revised. Could transcriptional regulation of ferroportin during inflammation (e.g. Ludwiczek et al, Blood, 2003) account for the decrease in serum iron in the iron -loaded Hamp1 -/- mice, so that hepcidin might not be the sole mediator of hypoferremia in this context? Have the authors measured e.g. splenic or liver ferroportin levels in these groups?*

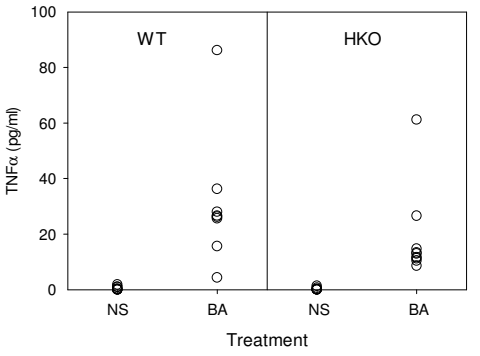
We agree and have corrected the statement. We now state: “The slight decrease in serum iron may be a consequence of decreased food intake, a common trait in sick mice, or due to direct effects of inflammatory cytokines on ferroportin mRNA (Ludwiczek et al)”.

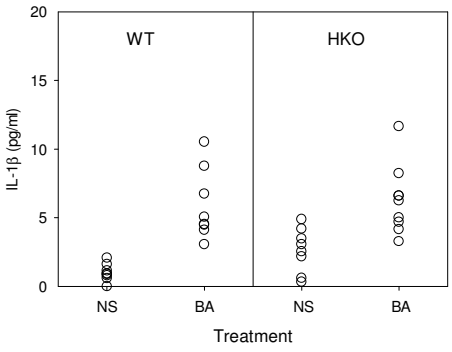
*6. Can the authors speculate why the hypoferremia in WT iron-loaded mice infected with a low dose (300 CFU) appears considerably more marked than with the high dose in Figure 4C? Is it due to the differences in the timing of sampling?*

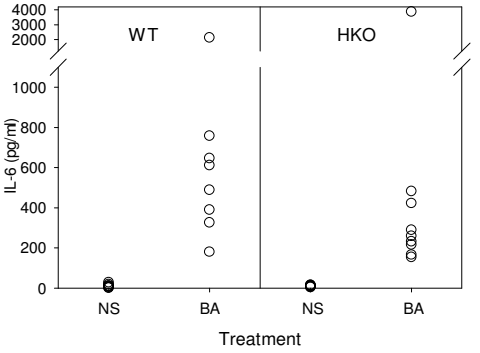
The serum iron measurements of WT mice in both Figures 3 and 4 are from infections with 10^5 CFU (now clarified in the legend), thus the dose was not different. However, these infections were performed at different times and using different sets of mice so we would expect some inter-experimental variation. Furthermore, in Figure 3 the serum iron was measured at 16h, while in Figure 4 the last time point was at 10h. Interestingly, the difference between the two experiments was not in the level of hypoferremia after infection (30 µM in Figure 3 and 35 µM in Figure 4), but rather in the baseline serum iron (65 µM in Figure 3 and 53 µM in Figure 4).

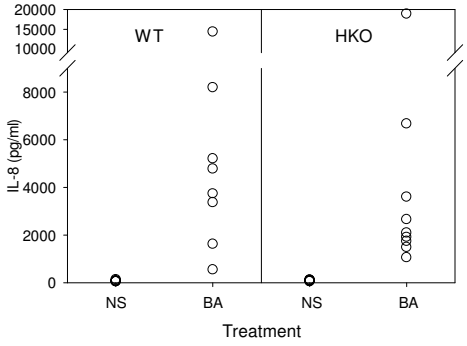
*7. All together, the data suggest that a *combination* of infecting bacterial dose, baseline iron status (possibly including NTBI), and the ability to mount a hypoferremic response are determinants of ability to survive V. vulnificus infection. However immunological differences owing to the differing iron status during early development could potentially play a role as iron status can effect innate immune signaling (eg Wang et al JCI 2009 on iron and TLR4, and work from the Weiss group on iron and IFN-gamma signaling). Related to this it could be mentioned in the discussion out that the Hamp1-/- mice, even if kept on a low iron diet, have an altered tissue distribution of iron compared to low iron WT mice (even if serum iron levels are similar), so that the baseline iron statuses are not the same.*

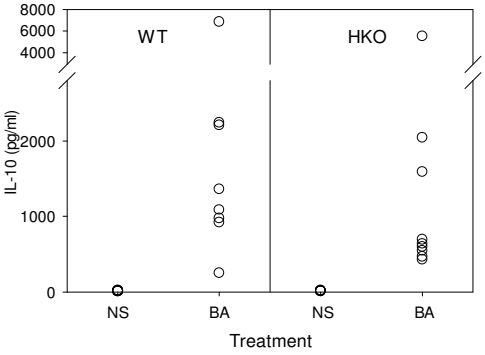
We now include in the discussion the possibility that altered tissue iron distribution in Hamp1-/- mice may affect the immunological response to the bacterial challenge. We attempted to test this hypothesis experimentally by comparing the cytokine induction in WT and Hamp1-/- after a sterile inflammatory stimulus (heat-killed Brucella abortus). We did not use V. vulnificus in this experiment because cytokine response would be affected by the difference in the severity of infection among these groups. After this inflammatory stimulus, the cytokine profile was quite similar between WT and Hamp1-/- mice (data provided below). As this test did not provide any support for immunological differences between Hamp1-/- and WT mice, we did not include it in the paper.

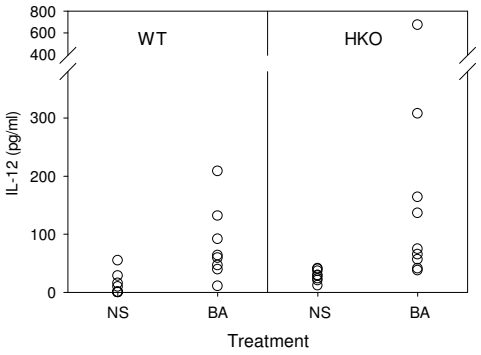


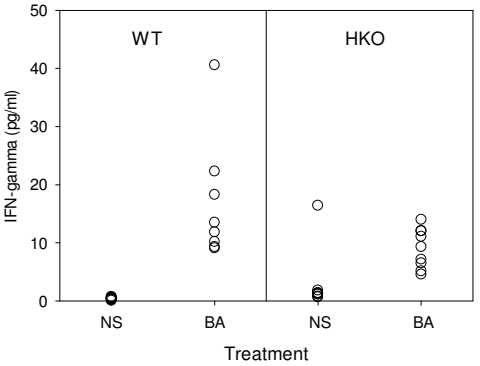



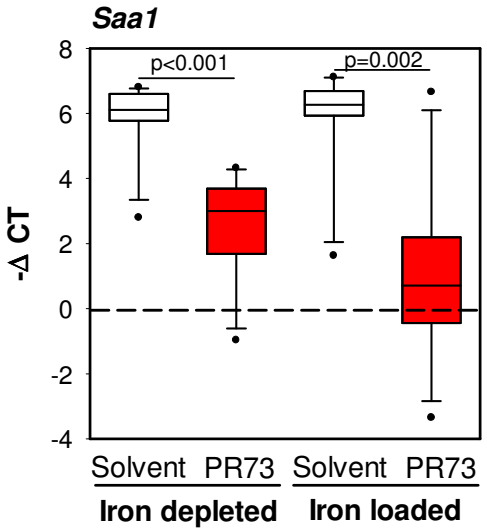

Plasma cytokine concentrations 1 day after intraperitoneal injection of heat-killed *Brucella abortus* (BA) or saline. BA significantly induces each of the cytokines in both WT mice and hepcidin KO (HKO) mice (n=8-9 per group). Accounting for multiple testing, there is not a significant effect of genotype (WT vs HKO) on any of the cytokines.

*8. The authors do not refer to the paper by Pan et al Dev Comp Immunol. 2012 "Insights into the antibacterial and immunomodulatory functions of tilapia hepcidin (TH)2-3 against Vibrio vulnificus infection in mice". A fish hepcidin is used successfully to inhibit Vibrio vulnificus infection in mice. The TH2-3 hepcidin alters immune function and in the mice. Although they do not measure iron status in the mice, the TH2-3 hepcidin is reportedly iron-regulated in tilapia, although it is not apparent whether it could interact with murine ferroportin. Nevertheless this paper is relevant and should be discussed and cited.*

We thank the reviewer for mentioning this article and we added it to Discussion.

*9. Did (pre- or concurrent) treatment of mice with minihepcidin have immunomodulatory activities and influence the inflammatory response to infection?*

Because minihepcidin treatment prevented the growth of V. vulnificus (Figure 5), the severity of infection was drastically different between solvent-treated and minihepcidin-treated mice. Accordingly, increase in the hepatic Saa-1 mRNA expression was much greater in solvent-treated than minihepcidin-treated mice (data provided below). This milder inflammatory response in minihepcidin-treated mice is much more likely to be a consequence of milder infection than to be a consequence of the immunomodulation related to iron metabolism changes. For this reason, we did not include the Saa-1 data in the manuscript.




*Saa1* mRNA expression in the liver of *Hamp1*-/- mice (either iron-depleted or iron-loaded), treated with 100 nmol of PR73 (red bars) or solvent (white bars) 24h and 3h before infection with 300 CFU V. vulnificus. Mice were euthanized 16h after infection. The dashed line represents the average baseline expression of *Saa1* mRNA in non-infected mice. Data were normalized to the expression of β-actin. n=10-11 per group.

Minor points:

*1. Minor point: The role of activin B in hepcidin induction was shown in response to LPS stimulus but not in the context of infection in the cited study.*

The reviewer is correct and we changed that sentence accordingly.

*2. I think it would be worth adding the data from Figure S1A to the main manuscript.*

We would prefer to leave the data in Supplemental Information since they are mostly a confirmation of previous studies that used iron injections instead of dietary iron loading to increase susceptibility of WT mice to *V. vulnificus* infection.

*3. The labeling in the text and supplementary data of Figures S3 and S4 needs to be checked and corrected. It would help to also quote the infecting dose in the legend.*

The text in the manuscript was wrong and we corrected this oversight. We also included the infecting dose in the legends.

*4. A schematic diagram depicting the method used in Figure 7 might enhance clarity.*

We thank the reviewer for this suggestion and have included a new supplementary figure (Figure S7) to help clarify the experimental setup.

**Reviewer #3**

*This is a clearly written, carefully conducted study showing the role of hepcidin in the hypoferremia of infection. The use of the hepcidin deficient mice and dietary manipulation of iron levels, rather than injecting iron to create overload, are strengths of the approach. I have only minor comments on the data.*

*1. The text (past paragraph on page 7) indicates that wild type but not Hamp- mice decreased serum iron after infection. While the level of hypoferremia in the Hamp- mice does not drop to the level of the wild type, there is a significant drop in serum iron levels in the iron-loaded Hamp- mice following infection. Some discussion of the drop in iron levels is warranted.*

We agree that the statement was inaccurate and have corrected it. We now state: “The slight decrease in serum iron may be a consequence of decreased food intake, a common trait in sick mice, or due to direct effects of inflammatory cytokines on ferroportin mRNA”.

*2. Minihepcidin resulted in marked hypoferremia but liver iron levels did not change. Is it known where the serum iron is transferred prior to storage in the liver?*

After minihepcidin treatment, iron stops being supplied to the serum because it is sequestered in the macrophages of the spleen and liver and in duodenal enterocytes (Ramos et al Blood 2012). Although iron is sequestered in the stores under the effect of minihepcidin, the baseline amount of iron present in the stores is much bigger than the serum iron compartment, so even if all the serum iron was added to stores, the net iron increase would be difficult to detect within only a day or two.

Graphical Abstract

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# Hepcidin-Induced Hypoferremia Is a Critical Host Defense Mechanism Against the Siderophilic Bacterium *Vibrio vulnificus*

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**Running title:** Hepcidin protects against siderophilic infections

**SUMMARY**

Hypoferremia, mediated by increased production of hepcidin, develops early during infections. It has been proposed but never demonstrated that this response is a host defense mechanism to restrict iron availability for extracellular pathogens. Hereditary hemochromatosis, an iron overload disease caused by hepcidin deficiency, is associated with lethal infections by siderophilic bacteria. To elucidate the mechanisms of this susceptibility, wild-type and hepcidin-deficient mice with varying iron load were infected with *Vibrio vulnificus,* and their inflammatory responses, iron regulation and survival analyzed. Although systemic iron overload facilitated the infection, hepcidin-induced reactive hypoferremia protected the host from acute mortality by restricting bacterial growth. Timely administration of hepcidin agonists rescued the susceptible hepcidin-deficient mice from death, regardless of iron overload. Our study demonstrates the host defense role of hepcidin in infections with siderophilic pathogens, and indicates that hepcidin agonists may improve the outcome of these lethal infections in patients with hereditary hemochromatosis or thalassemia.

**INTRODUCTION**

Iron, required as a co-factor for many important biological processes, is an essential nutrient for nearly all living organisms. The requirement for this metal places it in a critical role at the host-pathogen interface: microbes evolved complex means to acquire iron from the host (Marx, 2002; Schaible and Kaufmann, 2004) and the host evolved the ability to resist infection by sequestering iron so it is less available to microbes.

Hereditary hemochromatosis, a common genetic iron overload disease (Ganz and Nemeth, 2011), increases susceptibility to infections with *Vibrio vulnificus* and *Yersinia enterocolitica* (Khan et al., 2007), gram-negative bacteria classified as "siderophilic" because their pathogenicity is enhanced by excess iron (Weinberg, 2008, 2009). *Vibrio vulnificus* causes fulminant sepsis with mortality higher than 50% in susceptible patients including those with hereditary hemochromatosis and other iron overload conditions (Horseman and Surani, 2011) but it does not cause severe illness in healthy individuals. It is not known which specific manifestations of hereditary hemochromatosis predispose to infection with siderophilic microbes: liver injury, tissue iron loading, high baseline plasma iron concentrations or the inability to lower iron concentrations in plasma in response to infections.

Hereditary hemochromatosis is caused by deficiency of the iron-regulatory hormone hepcidin (Ganz and Nemeth, 2011). Hepcidin is a 25 amino acid peptide secreted by hepatocytes. It controls iron concentrations in extracellular fluid and blood plasma by regulating the amount of ferroportin, the sole known cellular iron exporter. Ferroportin transports absorbed, recycled or stored iron from tissues into plasma (Donovan et al., 2005). Hepcidin binding to ferroportin triggers its degradation, resulting in decreased transfer of iron to plasma and consequently hypoferremia (Nemeth et al., 2004b). During infections or in response to injection of microbial molecules, hepcidin production is greatly enhanced (Armitage et al., 2011; Rodriguez et al., 2014), stimulated by inflammatory cytokines including IL-6

(Nemeth et al., 2004a; Rodriguez et al., 2014) and possibly activin B (Besson-Fournier et al., 2012). It has been proposed that hepcidin-mediated hypoferremia functions as a host defense mechanism that evolved to restrict iron availability for pathogen growth (Drakesmith and Prentice, 2012; Ganz, 2009) but this has never been demonstrated. Hepcidin was also reported to have direct bactericidal activity *in vitro* (Krause et al., 2000; Park et al., 2001), but the effect is seen only at unphysiologically high concentrations.

Here we demonstrate that hepcidin has a critical role in host defense against *V. vulnificus* by inducing reactive hypoferremia during early phases of infection. In addition, we show that acute pre- or post-exposure treatment of susceptible mice with hepcidin agonists mitigates the high mortality caused by this pathogen.

## RESULTS

### Hepcidin is required for resistance to *V. vulnificus* infection

Iron is thought to be required for rapid growth of *V. vulnificus* and lethality during infections, as was previously demonstrated in mice injected with iron-dextran (Starks et al., 2000; Wright et al., 1981). To examine whether the iron-regulatory hormone hepcidin affects the response to infection, we compared the severity of the *V. vulnificus* infection in wild-type (WT) and hepcidin knock-out (*Hamp1*$^{-/-}$) mice. Mice were iron-depleted or iron-loaded by dietary manipulations, and infected subcutaneously with a low ($10^3$ CFU) or moderate ($10^5$ CFU) inoculum of *V. vulnificus*. In both iron-depleted (Figure 1A) and iron-loaded (Figure 1B) conditions *Hamp1*$^{-/-}$ mice were significantly more susceptible than WT mice: iron-loaded *Hamp1*$^{-/-}$ died within one day after infection, iron-depleted *Hamp1*$^{-/-}$ within next several days, whereas WT mice survived the infection. WT mice were susceptible to *V. vulnificus* infection only when iron-loaded (Table S1) and infected with a large inoculum of *V. vulnificus* ($10^6$ CFU). Under those conditions, iron-loaded mice died within 2 days after infection while most of the iron-depleted mice still survived (Figure S1A), confirming that iron has a striking effect on *V. vulnificus* lethality. The differential susceptibility of WT and *Hamp1*$^{-/-}$ to *V. vulnificus* infection was not attributable to their baseline liver iron differences because iron-depleted *Hamp1*$^{-/-}$ mice were much more susceptible to infection even though they had lower liver iron stores than iron-overloaded WT mice, as measured in a parallel set of mice maintained under the same conditions as the mice used for the survival experiments (Figure 1C). As *V. vulnificus* is an extracellular pathogen (Gulig et al., 2005), intracellular hepatocyte iron is not likely to play a direct role in the growth of these bacteria.

To examine whether extracellular iron concentrations alter the growth rate of *V. vulnificus*, we assessed bacterial growth *ex vivo* in sera collected from uninfected iron-loaded or iron-depleted WT animals. *V. vulnificus* growth was greatly enhanced in serum derived from iron-loaded compared to iron-depleted

mice (Figure S1B). Supplementing iron-depleted serum with ferric ammonium citrate (FAC) also enhanced bacterial growth. Conversely, addition of the synthetic iron chelator deferiprone to iron-rich serum prevented bacterial growth. Interestingly, growth in low-iron serum was similar to the growth under standard conditions in LB-N broth. This indicates that *V. vulnificus* can grow adequately even with limited iron availability but that excess iron greatly increases the growth rate. In iron-rich serum, bacteria underwent ~18 divisions within 3 hours, or one division every 10 min, an astoundingly rapid growth rate.

Human infections with *V. vulnificus* and their laboratory models often terminate in septic shock (Shan et al., 2012). To verify that this mechanism contributed to rapid death of iron-loaded mice, we inspected H&E stained skin (site of injection) and liver sections of *Hamp1*$^{-/-}$ mice injected with $1x10^3$ CFU of *V. vulnificus* or saline. The venules in the skin and hepatic circulation of infected mice were greatly dilated compared with saline-treated mice (Figure 2A-D and E-H). The vasodilatation was accompanied by erythrocyte sludging characteristic of circulatory shock. In the skin, massive aggregates of bacteria were visible in the perivascular space (Figure 2E-H). In the liver, no bacteria were seen but there was neutrophilic infiltration in perivenular spaces (Figure S2). These findings are consistent with endotoxemic shock.

To determine whether the susceptibility of *Hamp1*$^{-/-}$ mice to severe *V. vulnificus* infection was due to greater bacteremia and bacterial dissemination, we assayed bacterial CFU counts in blood and liver of iron-depleted and iron-loaded WT and *Hamp1*$^{-/-}$ mice 16 h after infection. Liver CFU counts likely represent extracellular bacteria as *V. vulnificus* does not appear to invade cells (Gulig et al., 2005). To allow *Hamp1*$^{-/-}$ mice to survive long enough to undergo analysis at the 16 h time point, we injected all groups with a small inoculum of bacteria (300 CFU). After infection with this low dose, only iron-loaded *Hamp1*$^{-/-}$ mice, but not iron-depleted *Hamp1*$^{-/-}$ or either of the WT groups, had CFU in blood and liver

(Figure 3A). For the WT groups, the iron-dependent difference in CFUs was observed at a higher inoculum ($10^5$ CFU, Figure 3B). These data indicate that in both *Hamp1*$^{-/-}$ and WT mice, bacterial replication and dissemination is highly dependent on the host's iron status. As shown in Figure 1, mortality from *V. vulnificus* infection did not correlate with baseline liver iron concentrations, therefore we hypothesized that the difference in baseline serum iron concentrations, baseline non-transferrin bound iron (NTBI) and/or the ability to generate hypoferremia may explain the disparate susceptibility of *Hamp1*$^{-/-}$ and WT mice and the iron dependence of bacterial growth *in vivo*. Indeed, WT mice lowered their serum iron concentrations in response to infection (Figure 3C, mean of 26 µM in iron-depleted and 30 µM in iron-loaded infected mice), whereas *Hamp1*$^{-/-}$ mice had a much smaller decrease in serum iron (mean of 63 µM in iron-depleted and 52 µM in iron-loaded infected mice), insufficient to affect the replication of *V. vulnificus*. The slight decrease in *Hamp1*$^{-/-}$ serum iron may be a consequence of decreased food intake, a common trait in sick mice, or due to direct effects of inflammatory cytokines on ferroportin mRNA (Ludwiczek et al., 2003).

Among *Hamp1*$^{-/-}$ mice, despite similar serum iron concentrations in iron-loaded and iron-depleted groups, the iron-loaded group had higher CFU counts in the blood and liver (Figure 3A). This difference in bacterial burden may be related to the difference in the serum concentrations of NTBI, a known source of iron for *V. vulnificus* (Kim et al., 2007). NTBI was only detected in iron-loaded mice (1.28± 0.60 LPI units), but not in the iron-depleted group (Figure 3D), correlating with the bacterial burden. WT mice had low NTBI levels, similar to those in iron-depleted *Hamp1*$^{-/-}$ mice, suggesting that NTBI did not contribute to bacterial growth in WT mice. Among WT mice infected with the high inoculum ($10^5$ CFU), the iron-loaded group had higher CFU counts in the blood and liver than the iron-depleted group (Figure 3B), despite the similar degree of hypoferremia 16 h after infection (Figure 3C). As NTBI levels were not significantly different between these two groups, it is likely that serum iron concentrations during the early stages of infection (prior to the hepcidin increase and hypoferremia) also contribute to

susceptibility to *V. vulnificus* infection. Thus, our data suggest that baseline concentrations of transferrin-bound and non-transferrin bound iron as well as reactive hypoferremia, all of which are controlled by hepcidin, determine the replication and dissemination of *V. vulnificus* in vivo.

**Hepcidin levels increase early after *V. vulnificus* infection**

Only WT but not *Hamp1*$^{-/-}$ animals were able to generate significant hypoferremia, suggesting that hepcidin mediates inflammatory hypoferremia during infection. To confirm this hypothesis we analyzed hepatic hepcidin mRNA and serum hepcidin protein at 3, 6 and 10 hours after infection with $10^5$ *V. vulnificus* in iron-loaded and iron-depleted WT mice. As expected, iron-loaded mice had higher levels of hepcidin mRNA and protein compared to iron-depleted mice at all time points (Figure 4A,B). By 6 h after infection, both iron-depleted and iron-loaded mice acutely increased their hepatic hepcidin mRNA (Figure 4A) as well as serum hepcidin concentration (Figure 4B) compared to uninfected mice. At 10 h after infection, serum hepcidin increased further in iron-depleted mice and stabilized at very high levels in iron-loaded mice (Figure 4B). These increases in hepcidin concentrations were accompanied by a significant decrease in serum iron at 6 h and 10 h in iron-depleted mice (Figure 4C), and a trend toward decreased serum iron in iron-loaded mice. The latter may not have decreased serum iron as efficiently because iron–loaded hepatocytes, macrophages and enterocytes express more ferroportin (Chua et al., 2006; Delaby et al., 2005; Zoller et al., 2002).

IL-6 is thought to be the predominant stimulus for hepcidin synthesis during inflammation (Nemeth et al., 2004a; Rodriguez et al., 2014). We found dramatically increased serum concentrations of this pro-inflammatory cytokine in infected mice starting at 3 h after infection (Figure 4D), with no differences between iron-depleted and iron-loaded mice. A similar increase was also observed for the inflammatory cytokines KC-GRO, IL-12p70, IL-1β, TNF-α, IL-10 and IL-2, while IFN-γ, IL-4 and IL-5 remained unchanged (Figure S4C). We also observed early increases in hepatic *Saa1* and *Inhbb* mRNA (Figure S4A and B).

Interestingly, at 16h after infection, no statistically significant differences in hepcidin were observed between infected and saline-treated WT mice, either at the hepatic mRNA or serum protein levels (Figure S3A,B), despite increased inflammatory markers, including hepatic *Saa1* mRNA (Figure S3C) and proinflammatory cytokines in the serum (Figure S3E). *Inhbb* mRNA, coding for the $\beta_B$-subunit of activin B, a protein involved in an alternative pathway for hepcidin regulation by inflammation, was also increased in infected iron-loaded groups (Figure S3D). These data imply that hepcidin production in the liver was rapidly increased early in the course of infection by inflammatory mediators induced by bacterial infection. Elevated hepcidin in turn caused acute hypoferremia. The hypoferremia appears to counterregulate hepcidin so that by 16 h hepcidin levels in infected WT mice were no longer different from uninfected controls.

**Minihepcidin PR73 protects against mortality from infections with *Vibrio vulnificus***

To further examine the importance of early hepcidin activity for resistance to *V. vulnificus* infection, we tested the therapeutic effect of minihepcidin PR73 (a synthetic hepcidin agonist, Figure S5) in *Hamp1*$^{-/-}$ mice. We used minihepcidin because it has greater potency and longer lasting effect than full-length hepcidin (Ramos et al., 2012). Mice were pretreated with PR73 24h and 3h before infection and were euthanized 16h after s.c. infection with 300 CFU of *V. vulnificus*. Administration of minihepcidin caused marked hypoferremia (3 µM in iron-depleted and 9 µM in iron-loaded mice) (Figure 5A). Liver iron stores did not change likely because of the short duration of the experiment (40 h) (Figure 5B). Importantly, minihepcidin treatment resulted in decreased numbers of bacteria in blood and liver of both iron-depleted and iron-loaded mice (Figure 5C,D). Pretreatment with PR73 also markedly improved survival: while most of the non-treated *Hamp1*$^{-/-}$ mice died within 2 days after infection with $10^3$ or $10^5$ CFU of *V. vulnificus*, mice treated with minihepcidin survived, regardless of their iron load and regardless of the number of bacteria administered (Figure 6A,B).

To test whether minihepcidins are effective not only for the prevention but also post-exposure treatment of *V. vulnificus* infection, we injected PR73 in iron-loaded *Hamp1*$^{-/-}$ mice 3 h after infection with $10^3$ CFU of bacteria. Even delayed injection (post exposure treatment) of PR73 significantly increased the survival of these highly susceptible mice (Figure 6C).

To determine if the anti-Vibrio effect of minihepcidin was due to iron restriction of bacterial growth or a direct bactericidal effect of the PR73 peptide, we measured the growth and killing of a *V. vulnificus* strain marked by a chloramphenicol-resistance plasmid, similarly to a published approach (Figure S7) (Gulig et al., 1997). The marker plasmid only replicates in the presence of arabinose, which is not present in significant amounts in mouse serum. Bacteria were grown *ex vivo* in sera that were obtained from *Hamp1*$^{-/-}$ mice treated with PR73 minihepcidin or with a solvent control. Iron concentrations of these sera are provided in Table S2. After incubating bacteria in the mouse sera for 2 h, CFU counts were determined on plates that allow only plasmid-containing bacteria to grow (supplemented with chloramphenicol and arabinose) or plates that allow all Vibrio bacteria to grow (no chloramphenicol/arabinose). Because the marker plasmid does not replicate in bacteria growing in serum, the reduction in CFU of plasmid-containing Vibrio reflects the killing of the inoculum. The number of total Vibrio CFU reflects both growth and killing of bacteria whereas the ratio of total to plasmid-containing bacteria (T/P) reflects growth only (Gulig et al., 1997) (Figure S7).

After incubation in *ex vivo* sera, total CFU were 15-20-fold lower in sera of minihepcidin-treated *Hamp1*$^{-/-}$ mice compared to the sera of solvent-treated *Hamp1*$^{-/-}$ mice (Figure 7A), regardless whether the donor mice were on low or high iron diet, mirroring the *in vivo* CFU results (Figure 5C,D). In contrast, CFU of the plasmid-containing bacteria were merely 1.7-1.9-fold lower in minihepcidin-treated sera relative to the solvent sera (Figure 7B), demonstrating that the peptide had only a slight bactericidal effect in serum. T/P ratios (i.e. bacterial growth) were about ten-fold lower for sera from the minihepcidin-

treated mice compared with the solvent controls (Table S2), indicating that minihepcidin-triggered hypoferremia significantly decreased bacterial replication. This effect of hypoferremia on Vibrio growth was also replicated using sera from noninfected WT mice that were fed low- or high-iron diet (Figure 7C,D). Moderately hypoferremic sera (because of low-iron diet) slowed down Vibrio growth compared to iron-rich sera (because of high-iron diet), as shown by a more than 10-fold decreased T/P ratio (Table S2), without affecting the number of plasmid-containing bacteria (Figure 7D). Thus, minihepcidin-induced hypoferremia rather than a direct antimicrobial effect of minihepcidin was responsible for slower Vibrio replication.

Like healthy humans, WT mice, which appropriately respond to infection by inducing endogenous hepcidin, are highly resistant to *V. vulnificus* infection. When very large inocula of *V. vulnificus* ($10^6$ and $10^7$ CFU) were administered to WT mice, minihepcidins did not further protect them from lethal infection (Figure S6), arguing against a direct microbicidal effect of these peptides *in vivo*.

**DISCUSSION**

The growth of pathogens in their hosts is critically dependent on the pathogens' ability to capture and utilize iron. The hosts have evolved several countermeasures to restrict the bioavailability of iron and effectively starve the microbes (Ganz, 2009). Hepcidin-induced hypoferremia has been proposed as an important host defense mechanism (Drakesmith and Prentice, 2012; Ganz, 2009), but specific support for its role has been lacking. In this study, we show that acute hypoferremia is an important host defense mechanism against the siderophilic bacterial pathogen *V. vulnificus*.

After infection with *V. vulnificus*, iron-loaded mice had greater bacterial CFU burden in blood and the liver. Moreover, *ex vivo* sera that had excess iron promoted the rapid growth of *V. vulnificus*. This is not

a result of the inability of these bacteria to utilize iron at its physiologic concentration because bacteria grew well even in sera with relatively low iron levels. Rather, high iron levels trigger extraordinarily rapid *V. vulnificus* growth, with bacteria dividing every 10 min. We speculate that high iron concentrations, or possibly the related presence of one or more non-transferrin bound iron (NTBI) forms in the environment of *V. vulnificus,* activate a genetic program for very rapid growth, leading to septicemia, high cytokine concentrations, evidence of septic shock at necropsy and early mortality. The early mortality of susceptible humans after infection with these bacteria suggests that a similar program may be activated during human infections with *V. vulnificus*.

In our mouse models, the order of susceptibility to rapid mortality from *V. vulnificus* was *Hamp1*$^{-/-}$ iron-overloaded > *Hamp1*$^{-/-}$ iron-depleted > WT iron-overloaded > WT iron-depleted. This together with studies of the effect of serum iron on *V. vulnificus* growth ex vivo indicated that plasma iron concentrations during the course of infection determined the growth of *V. vulnificus* and host mortality. *Hamp1*$^{-/-}$ mice were more susceptible to lethal infection, even when they had the same baseline serum iron and lower liver iron stores than iron loaded WT mice. This suggests that acute hypoferremia after infection is a major iron-related determinant of resistance to *V. vulnificus*.

WT mice developed hypoferremia within hours after infection with *V. vulnificus*, and this response was dependent on early induction of hepcidin, preceded by a rise in inflammatory cytokines such as IL-6 and activin B. IL-6 acts as an inflammatory signal that triggers hepcidin expression in the liver through the JAK/STAT3 pathway (Nemeth et al., 2004a; Verga Falzacappa et al., 2007; Wrighting and Andrews, 2006). Activin B, through SMAD1/5/8 signaling (Besson-Fournier et al., 2012), is also a stimulus for hepcidin production in response to inflammation. We found that expression of *Inhbb,* encoding the activin $\beta_B$-subunit, is increased prior to the hepcidin response, suggesting that the two pathways may cooperate in the induction of hepcidin during infection.

To further examine if hepcidin-induced hypoferremia is an effective component in the innate immune response against *V. vulnificus*, we analyzed the therapeutic effects of minihepcidins in *Hamp1*$^{-/-}$ mice. Minihepcidins are synthetic hepcidin agonists previously shown to bind ferroportin and induce its endocytosis and proteolysis, thus preventing iron overload in hepcidin-deficient mice (Preza et al., 2011; Ramos et al., 2012). In the current study, the short-term minihepcidin treatment robustly decreased serum iron without altering liver iron stores. Both pretreatment with minihepcidin and administration 3h after the infection protected the highly susceptible *Hamp1*$^{-/-}$ mice from dying from infection, even with a large inoculum of *V. vulnificus*. Minihepcidin-treated mice had much lower concentrations of bacterial CFU in the blood and liver, presumably because of a lower bacterial growth rate due to decreased plasma iron. WT mice with their intact endogenous hepcidin response to infection were not further protected by minihepcidins injections. This limitation is not clinically important because healthy humans do not develop severe *V. vulnificus* infections. Another study (Pan et al., 2012) showed that treatment with tilapia hepcidin TH(2-3) did increase survival rate in Balb/C mice infected with *V. vulnificus*, and that TH(2-3) had both antimicrobial and immunomodulatory effects. However, effects on iron metabolism were not investigated and it is not known if TH(2-3) interacts with murine ferroportin.

Since hepcidin and its fragments have microbicidal effects *in vitro* (Park et al., 2001), we tested if the minihepcidin had a bactericidal effect on *V. vulnificus*. By using a *V. vulnificus* strain containing a marker plasmid, pGTR905, that functions as a replicon only in the presence of arabinose (a sugar not found in relevant concentrations in animal tissues), we demonstrated that the minihepcidin-containing serum had only a slight bactericidal effect that was not sufficient to explain the dramatically lower number of total bacterial CFU. Rather, the slower growth of bacteria in sera from minihepcidin-treated mice was caused by the low iron concentrations in those sera. The dependence of *V. vulnificus* growth in *ex vivo* sera on the serum iron concentrations was confirmed in WT mice where serum iron was manipulated not by the administration of minihepcidin but by changing the dietary iron content. The composition of

sera is also not favorable for the direct microbicidal activity of hepcidin which is dependent on acidic pH that is not physiologic in serum (Maisetta et al., 2010).

We cannot exclude the possibility that immunological differences exist between *Hamp1*$^{-/-}$ and WT mice, even when *Hamp1*$^{-/-}$ mice are kept on a low iron diet. Hepcidin deficiency results in altered tissue iron distribution and iron-depleted macrophages, and this could potentially affect innate immune signaling (Oexle et al., 2003; Wang et al., 2009). However, the ability of minihepcidins to rapidly alleviate the mortality of *Hamp1*$^{-/-}$ mice from *V. vulnificus* infection would argue that any immunological differences between *Hamp1*$^{-/-}$ and WT mice are minor or acutely reversible by hepcidin or its analogs. A recent study showed that genes encoding proteins involved in *V. vulnificus* iron acquisition (siderophore receptor *vuuA* and components of siderophore synthase) were induced in bacteria collected from infected patients (Bisharat et al., 2013). Deletion of the *vuuA* gene, which impaired iron uptake from both transferrin and siderophore vulnibactin, resulted in reduced virulence in mice (Webster and Litwin, 2000). However, despite the evident link between the iron uptake and virulence of *V. vulnificus*, the molecular mechanism by which excess iron reprograms *V. vulnificus* for very rapid growth is still elusive.

Human hereditary hemochromatosis is a well-established risk factor for lethal infection with *V. vulnificus.* However, the severity of hepcidin deficiency and iron overload in humans is highly variable, particularly in the most common form caused by the C282Y HFE mutations (Waalen et al., 2002). The variability is attributable to both genetic modifiers (e.g. gender) and environmental factors (e.g. alcohol intake). Therefore, patients with mild or normal phenotypes, will likely not be at increased risk of siderophilic infections. Indeed, studies in HFE knockout mice showed appropriate decrease in serum iron in response to LPS (Wallace et al., 2011). Only when a second hemochromatosis gene TfR2 is ablated is the hypoferremic response to LPS lost (Wallace et al., 2011). Like the HFE mouse model, in the absence of additional genetic or comorbid factors, most patients with HFE mutations may appropriately

respond to infections by increasing hepcidin and developing hypoferremia. In contrast, the loss of hepcidin in *Hamp1*$^{-/-}$ mice generates a particularly severe form of the disease and it nearly completely ablates the hypoferremia of infection. We hypothesize that those hemochromatosis patients with very low baseline hepcidin and impaired hepcidin response to infection, either because of the pattern of causative mutations and/or the presence of comorbid factors, will have increased susceptibility to infections with siderophilic bacteria.

Hepcidin deficiency in humans, such as in fully penetrant forms of hereditary hemochromatosis, is associated with the appearance of NTBI in plasma (Brissot et al., 2012). NTBI was shown to promote the growth of *V. vulnificus* in cirrhotic ascites (Kim et al., 2007) even better than holotransferrin. Our results also demonstrate that the growth of *V. vulnificus in vivo* was highly enhanced in sera containing NTBI. One effect of therapeutically-induced hypoferremia in iron overload would be the loss of NTBI from blood plasma. Further studies are required to elucidate the requirement of NTBI for Vibrio growth in serum and *in vivo*, and its potential mechanisms.

In summary, hepcidin plays an important role in host defense against siderophilic bacteria such as *V. vulnificus* by controlling baseline plasma iron concentrations and mediating hypoferremia in response to infection. These effects prevent the rapid growth of *V. vulnificus* so that other innate immune mechanisms are sufficient to control the infection. Pointing to potential therapeutic applications, pre- or post-infection administration of hepcidin agonists to hepcidin-deficient mice increased their resistance to *V. vulnificus* infection and protected them from consequent mortality.

## EXPERIMENTAL PROCEDURES

### Preparation of bacteria

*Vibrio vulnificus* CMCP6 strain was grown in Luria-Bertani broth (Becton Dickinson) containing 0.85% NaCl (LB-N), and bacterial stocks were stored at -80°C in LB-N with 35% glycerol. The day before the experiment, 50 µl of *V. vulnificus* was diluted 1:1000 in LB-N and grown at room temperature overnight. On the day of infection, the culture was diluted 1:30 in LB-N and shaken at 37 °C until the optical density at 600nm reached 0.3. Bacteria were then harvested by centrifugation (13800g for 10 min at 4 °C) and suspended in 0.9% sodium chloride injection solution (Hospira) at the required concentrations.

Bacterial CFU were counted by plating 6 µl of serial dilutions of blood, homogenized liver or bacterial suspension on LB-N plates containing 1.5% agar and enumerating the bacterial colonies after incubation at 37°C.

### Animal studies

All experiments involving animals were approved by the University of California, Los Angeles (UCLA) Office of Animal Research Oversight. C57BL/6 wild-type mice were obtained from Charles River Laboratories. Hepcidin-1 knockout (*Hamp1*$^{-/-}$) mice (originally provided by Dr. Sophie Vaulont (Lesbordes-Brion et al., 2006) and backcrossed by us onto the C57BL/6 background using marker—assisted accelerated backcrossing (Ramos et al., 2012)) were bred in our vivaria. Only male mice were used. WT and *Hamp1*$^{-/-}$ mice were iron-depleted or iron-loaded using dietary manipulations. Starting at 6 weeks of age, WT mice were fed an iron-poor (4 ppm Fe) or iron-rich (10000 ppm Fe) diet (Harlan) for 2 weeks. Starting at 4-5 weeks of age, *Hamp1*$^{-/-}$ mice were fed an iron-poor (4 ppm Fe) or standard diet (270 ppm Fe) for 4-6 weeks before the infection. The earlier start and longer regime of low-iron diet in *Hamp1*$^{-/-}$ was necessary to achieve substantial iron depletion because these mice are already iron-overloaded at a young age.

For survival experiments, animals were infected by subcutaneous (s.c.) injection (100 µl) in the interscapular area with $1x10^3$-$1x10^7$ CFU of *V. vulnificus* suspended in 0.9% sodium chloride (Hospira). Control mice received equivalent injections of saline only. Mice were observed for 4 days or were euthanized earlier by isofluorane (Clipper) inhalation if they became moribund.

For tissue and blood collection, WT and *Hamp1*$^{-/-}$ were infected with 300 CFU (and $1x10^5$ only in WT mice) and control animals received saline-only injections. Mice were euthanized 16-18h after infection, were exsanguinated, and their livers were harvested. For histhopathology analysis, *Hamp1*$^{-/-}$ mice were injected with $1x10^3$ CFU of *V. vulnificus* or saline, euthanized 10-12h after infection and a piece of the liver and the injection site (skin) were collected into tubes containing 4% formalin.

To analyze early changes in iron, hepcidin and pro-inflammatory cytokines after infection, WT mice (fed an iron-poor or iron-rich diet for 2 weeks) were injected with $1x10^5$ CFU of *V. vulnificus* or saline and animals were euthanized at 3, 6 or 10 h after infection.

To test the therapeutic effects of minihepcidin, mice were injected intraperitoneally with 100 nmol of minihepcidin PR73 dissolved in 100 µl of SL220 (Preza et al., 2011; Ramos et al., 2012) (a PEG-phospholipid based solubilizer, NOF Corp.), whereas control groups were injected with the same amount of solvent. For minihepcidin pretreatment studies, animals were injected 24h and 3h before infection. For treatment studies, animals received minihepcidin or solvent injection 3h after the infection. In both experiments, surviving animals also received minihepcidin or solvent injection, 24 and 48 h after infection.

**Peptide synthesis**

Minihepcidin PR73 was synthesized as a C-terminal amide using standard solid-phase Fmoc chemistry and was purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC). Its

purity was evaluated by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC. From N- to C-terminus the primary sequence of PR73 was: iminodiacetic acid, L-threonine, L-histidine, L-3,3-diphenylalanine, L-β-homoproline, L-arginine, L-cysteine, L-arginine, L-β-homophenylalanine, 6-aminohexanoic acid, iminodiacetic acid palmitylamide (Ida-Thr-His-Dpa-bhPro-Arg-Cys-Arg-bhPhe-Ahx-Ida(NHPal)-CONH2).

**Serum and liver iron measurements**

Blood was collected in serum separator tubes (BD) and allowed to clot at room temperature for 30 min followed by centrifugation for 5 min, 4500 rpm at 4 C. The serum was transferred to new tubes for serum iron quantification using the colorimetric Iron-SL assay (Sekisui Diagnostics) following the manufacturer's instructions. For liver iron measurements, the tissue was homogenized and a 75 µl sample was added to 1125 µl of protein precipitation solution (0.53M HCl and 5.3% trichloroacetic acid, Fisher Scientific). The samples were heated at 100 °C for 1 hour, centrifuged for 10 minutes (13000 rpm at 4°C), and the supernatant was used for the Iron-SL assay, measuring the absorbance at 595 nm relative to Iron AA Standard (Ricca Chemical Company). Results are expressed as µg Fe per gram of wet liver weight.

**Histopathology analysis**

Liver and skin sections were fixed in 4% formalin for 12h, imbedded in paraffin, cut in 5-micron sections and stained with hematoxylin and eosin at the UCLA Translational Pathology Core Laboratory. Skin sections were also stained using the Gram Stain for Tissue kit (Sigma) following the manufacturer's instructions. Microscopic images were acquired on a Nikon Eclipse E600 microscope using Nikon Plan Fluor 4x/0.13, Plan Apo 10×/0.45, Plan Apo 40×/0.95 and Plan Fluor 100x/1.30 objectives with a Spot RT3 2MP Slider camera and Spot 5.0 software. Pictures were assembled using Adobe Photoshop CS5.

**Labile plasma iron quantification**

Labile plasma iron (LPI), a redox-active form of non-transferrin bound iron(Esposito et al., 2003), was measured using the FeROS eLPI kit (aFerrix) according to the manufacturer's instruction. Briefly, 20 µl of mouse serum was plated in duplicate in 96-well plates. Serum was treated with 100 µl of LPI reagent containing buffered 2,3-dihydrorhodamine and ascorbate, in the presence or absence of an iron chelator. Fluorescence resulting from oxidation of 2,3-dihydrorhodamine after exposure to reactive oxygen species was measured in a Gemini XS microplate reader (Molecular Devices) using 495 nm for excitation and 525 for emission. A kinetic measurement was performed every 2 minutes for 40 minutes. The ΔFU/min for each sample, with or without the iron chelator, was converted to LPI units by comparing it with the standard values obtained in the same test. Values higher than 0.2 were considered indicative for the presence of NTBI.

**Gene expression analysis**

RNA from mouse liver was extracted by TRIzol Reagent (life technologies), and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer's instructions. Real-time quantification of transcripts was performed in duplicates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using Sso Advance SYBR Green Supermix (Bio-Rad) and specific primers for *Hamp1* (fwd: TTGCGATACCAATGCAGAAGA; rev: GATGTGGCTCTAGGCTATGTT), *Inhbb* (fwd: CTCCGAGATCAT CAGCTTTGC, rev: GGAGCAGTTTCAGGTACAGCC), *Saa1* (fwd: AGTCTGGGCTGCTGAGAAAA; rev: ATGTCTGTTGGCTTCCTGTG) and *Actb* (fwd: ACCCACACTGTGCCCATCTA; rev: CACGCTCGGTCAGGATCTTC). Data were normalized to the expression of β-actin.

**Serum hepcidin assay**

Mouse hepcidin ELISA was performed as previously described (Kim et al., 2014). Mouse hepcidin-1 monoclonal antibodies, Ab2B10 (capture) and AB2H4-HRP (detection), as well as synthetic mouse hepcidin-25, were a generous gift from Amgen. High binding 96-well EIA plates (Corning Costar) were coated overnight at room temperature with 50 µl/well of 3.6 µg/ml Ab2B10 in 0.2 M carbonate-bicarbonate buffer pH 9.4 (Pierce - Thermo Scientific). Plates were washed two times with wash buffer (PBS, 0.5% Tween-20) and then blocked for 45 minutes with 200 µl/well blocking buffer (PBS, 1% BSA, 1% goat serum, 0.5% Tween-20). Serum samples (previously diluted in blocking buffer at 1:10000 for iron-loaded mice or 1:1000 for iron-depleted mice) and standards were incubated for one hour at room temperature (50 µl/well), washed four times with wash buffer, incubated for one hour with 50 µl/well of 130 ng/ml Ab2H4-HRP, washed 4 times and then developed with 100 µl/well Ultra-TMB substrate (Thermo Scientific) for 15 min in the dark at room temperature. The reaction was stopped by adding 50 µl 2M sulfuric acid, and the absorbance was measured at 450 nm using a 96-well plate reader (Molecular Devices).

**Analysis of the bactericidal and bacteriostatic effects of minihepcidin on *V. vulnificus***

To analyze the mode of action of minihepcidin PR73, we used *V. vulnificus* CMCP6 containing pGTR905, a marker plasmid that confers resistance to chloramphenicol, as described in Starks *et al* (Starks et al., 2006). The plasmid replicates only in the presence of arabinose, which is not found in mouse tissues. Thus, the loss of plasmid from the bacterial population during growth in mouse serum is a measure of bactericidal activity. The principle is graphically illustrated in Gulig *et al* (Gulig et al., 1997).

The plasmid-containing bacterial inoculum was first grown in LB-NAC, LB-N broth supplemented with 1% L-arabinose (Sigma) and 5 µg/ml chloramphenicol (Sigma), to enable and select for plasmid replication. The bacteria were then washed, suspended in LB-N at $1x10^5$ CFU/ml and 10 µl added to 40 µl of mouse

serum in 96-well plates. Serial 5-fold dilutions were performed in serum. Serum samples were obtained from *Hamp1*$^{-/-}$ mice kept on an iron-poor or standard diet for 4-6 weeks and injected 24 h and 3 h before blood collection with 100 nmoles PR73 in SL220 or with SL220 only (5 mice per group). Sera were also collected from WT mice kept on iron-poor or iron-rich diet for 2 weeks (5 mice per group). For each mouse group, sera was pooled and used for *in vitro* experiments. 96-well plates with bacteria were incubated for 2 h at 37 C with shaking (300 rpm), using triplicates for each condition. Five µl from each well was plated on LB-N and LB-NAC plates (containing 1.5% agar) and CFU were counted for each plate.

**Quantification of inflammatory cytokines in serum**

Inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70 and TNF-α) were measured in mouse serum using the Proinflammatory Panel 1 (mouse) kit (Meso Scale Discovery) following manufacturer's instructions. Briefly, 50 µl of standards and 2-fold diluted samples were added to a 10-spot multiplex plate pre-coated with capture antibodies, and the plate was incubated at room temperature with shaking for 2 hours. After washing 3 times with wash buffer (PBS supplemented with 0.05% Tween-20), 25 µl of detection antibody was added to each well and incubated for 2 hours, washed 3 times and incubated with 150 µl of 2X read buffer (provided with the kit). The plate was read in a chemiluminescence reader (SECTOR Imager 2400, Meso Scale Discovery), and data were analyzed using the MSD Discovery workbench software (Meso Scale Discovery).

**Statistical analysis**

The statistical significance of differences between groups was evaluated using Student *t* test if data were normally distributed or Mann-Whitney *U* test if this condition was not met. Survival differences were analyzed using Kaplan-Meier survival curves and Log-Rank test. All statistics were done using Sigmaplot 12.5 (Systat Software).

**AUTHORS CONTRIBUTION**

JA and YB designed and performed experiments, analyzed data, and wrote the paper; GJ, VG, and EV performed experiments and analyzed data, PR designed and prepared minihepcidins; PAG developed V. vulnificus strains, contributed to experimental design and wrote the paper; and TG and EN designed experiments, analyzed data and wrote the paper.

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**FIGURE LEGENDS**

**Figure 1. *Vibrio vulnificus* infection is highly lethal in hepcidin-deficient mice**. Kaplan-Meier survival curves for iron depleted **(A)** and iron loaded **(B)** mice, after infection with $1x10^3$ (dashed lines) or $1x10^5$ (solid lines) CFU of *V. vulnificus* (n=4-11 in each group). WT and *Hamp1*$^{-/-}$ mice were iron-depleted or iron-loaded by dietary modification (WT: 4 ppm or 10,000 ppm Fe diet for 2 weeks; *Hamp1*$^{-/-}$: 4 ppm or standard diet for 4-6 weeks, see Methods). By multifactorial Kaplan-Meier Log-Rank analysis, differences in survival between WT and *Hamp1* $^{-/-}$ mice were significant in both iron depleted (combined CFU, $p<0.05$), and iron-loaded conditions (combined CFU, $p<0.001$). **(C)** Liver iron stores were measured in a parallel set of mice and confirmed the effective modulation of iron stores by dietary iron manipulation (n=5-10 per group). For liver iron measurements, statistical significance was assessed using student's *t* test if data were normally distributed (**$p<0.01$) or Mann-Whitney *U* test if they were not normally distributed ($^{++}p<0.01$). See also Figure S1.

**Figure 2: Local *V. vulnificus* proliferation induces vasodilation, leukostasis and erythrocyte sludging.** Skin sections at the site of injection with saline (A, C, E and G) or 1 x $10^3$ CFU of *V. vulnificus* (B, D, F, H) in *Hamp1*$^{-/-}$ mice. Sections were stained with hematoxylin and eosin (A-D) or Gram stain and tartrazine (E-H). **Panels A-B** and magnified in **C-D:** Dilated venules (v) and arterioles (a), leukostasis and erythrocyte sludging are seen in the skin of *V. vulnificus*-infected mice compared with saline-injected controls**.** **Panels E-F** and magnified in **G-H:** Numerous bacteria (purple) are seen in perivascular spaces of infected mice (arrow). See also figure S2.

**Figure 3. Iron-dependent dissemination of *V. vulnificus* in blood and liver.** Wild-type and *Hamp1*$^{-/-}$ mice were iron-depleted or iron-loaded by dietary modification (WT: 4 ppm or 10,000 ppm Fe diet for 2 weeks; *Hamp1*$^{-/-}$: 4 ppm or standard diet for 4-6 weeks, see Methods), and infected with *V. vulnificus* (n=5-10 per group). **(A)** and **(B)** Bacterial counts in blood and liver 16 h after infection with 300 CFU (A) and $1x10^5$CFU (B). Each symbol represents one mouse (iron depleted in blue and iron loaded in red); black solid lines represent the mean of CFU counts; black dotted line represents the lower limit of detection of CFU counts (calculated as half of the minimum detectable CFU counts). **(C)** Serum iron levels of WT ($1x10^5$CFU) and *Hamp1*$^{-/-}$(300 CFU) mice (white fill = saline groups, grey fill = *V. vulnificus*

groups). Unlike WT mice which decreased their serum iron to the mean of ~30 µM, *Hamp1*$^{-/-}$ mice did not develop marked hypoferremia after infection. **(D)** Measurement of non-transferrin bound iron in serum of iron-depleted and iron-loaded WT and *Hamp1*$^{-/-}$ mice prior to infection (n=4-6 per group). Statistical significance was assessed using student's *t* test if data were normally distributed (*p<0.05; **p<0.01; ***p<0.001) or Mann-Whitney *U* test if they were not ($^{+}$p<0.05; $^{++}$p<0.01). See also Figure S3.

**Figure 4. WT mice respond to *V. vulnificus* infection by rapidly increasing plasma hepcidin concentration.** WT mice, either iron-depleted (blue) or iron-loaded (red), were injected with $10^5$ CFU *V. vulnificus* (solid lines) or saline (dashed lines) and euthanized after 3, 6 or 10 hours. **(A)** Hepatic *Hamp1* mRNA expression. **(B)** Serum hepcidin concentration. **(C)** Serum iron concentration. **(D)** The inflammatory response to infection was confirmed by serum IL-6 assay. Each point represents the mean ± standard deviation (n=5). Statistical significance was assessed using student's *t* test if data were normally distributed (*p<0.05; **p<0.01; ***p<0.001) or Mann-Whitney *U* test if they were not ($^{+}$p<0.05; $^{++}$p<0.01). See also Figure S4.

**Figure 5. Minihepcidin PR73 decreases infection by *V. vulnificus* in *Hamp1*$^{-/-}$ mice.** *Hamp1*$^{-/-}$ mice (either iron-depleted or iron-loaded) were treated with 100 nmol of PR73 (red symbols) or solvent (white symbols) 24h and 3h before infection with 300 CFU *V. vulnificus*. Mice were euthanized 16h after infection. N=10-11 per group. **(A)** Serum iron was markedly decreased in PR73-injected mice, with no difference in serum iron between solvent-injected iron-depleted and iron-loaded mice. **(B)** Liver iron was higher in iron-loaded than iron-depleted mice as expected. PR73 administration did not result in significant changes in liver iron stores. **(C)** and **(D)** *V. vulnificus* was undetectable in blood and liver in PR73-treated mice, in contrast to mice treated with saline. Each dot represents one mouse; black solid lines represent the mean of CFU counts. The black dotted line represents the lower limit of detection of CFU counts (calculated as half of the minimum detectable CFU counts). Statistical significance was assessed using student's *t* test if data were normally distributed (**p<0.01) or Mann-Whitney *U* test if they were not ($^{+}$p<0.05; $^{++}$p<0.01). See also Figure S5.

**Figure 6. Minihepcidin PR73 prevents death due to *V. vulnificus* infection in *Hamp1*$^{-/-}$ mice.** Kaplan-Meier survival curves, n=4-5 per group. Both iron-depleted **(A)** and iron-loaded **(B)** *Hamp1*$^{-/-}$ mice survived the infection with $10^3$ and $10^5$ CFU *V. vulnificus* when treated with 100 nmol PR73 (red) before the infection. **(C)** *Hamp1*$^{-/-}$ mice were resistant to infection even if PR73 was administered 3h after infection. Statistically significant differences in survival between solvent- and PR73-treated mice were assessed using the Log-Rank survival analyzes: $p<0.05$ for iron-depleted, $10^3$ CFU; $p<0.01$ for the other groups. See also Figure S6.

**Figure 7. Minihepcidin PR73 acts in serum by slowing bacterial growth.** Serum was collected from iron-depleted or iron-loaded *Hamp1*$^{-/-}$ mice that were injected with PR73 or solvent, and from iron-depleted or iron-loaded WT mice (not treated with PR73). Serum iron concentrations are shown in Table S2. *V. vulnificus* carrying the non-replicating marker plasmid pGTR905 was incubated in these sera *in vitro* for 2 h, and CFU were determined either on plates without chloramphenicol (**total** bacteria) or plates with chloramphenicol and arabinose (allows growth of only **plasmid-containing** bacteria). **(A)** PR73 greatly reduced total bacterial yield, which reflects the sum of bacteriostatic and bactericidal effects. **(B)** PR73 only slightly reduced the yield of plasmid-containing bacteria indicating only a small bactericidal effect. **(C)** The number of total bacteria was much higher in serum from iron-loaded WT mice than in serum from iron-depleted mice, as expected. **(D)** Different serum iron concentrations did not affect the yield of plasmid-containing bacteria indicating that hypoferremia by itself does not have a bactericidal effect. Each vertical bar represents the mean, and error bars represent standard deviation for 3 independent experiments (with 3 replicates in each experiment). The black dotted line represents the number of plasmid-containing bacteria after growth that diluted plasmid copy number to 1 plasmid per bacterium (thus bacteria in the original inoculum carried 5 plasmids per bacterium). Statistical significance was assessed using student's *t* test (*$p<0.05$; **$p<0.01$; ***$p<0.001$). See also Figure S7.

Figure 1
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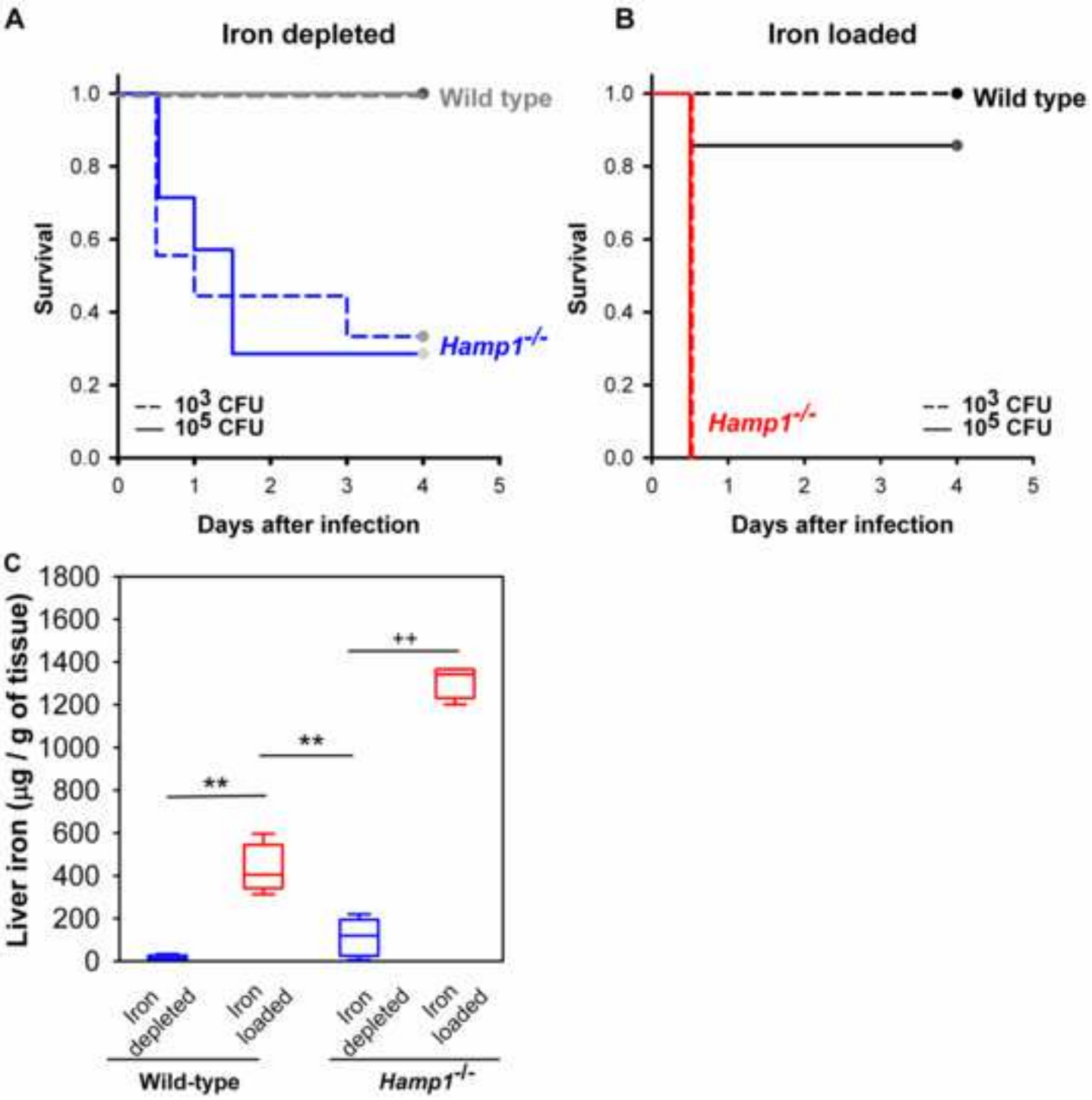

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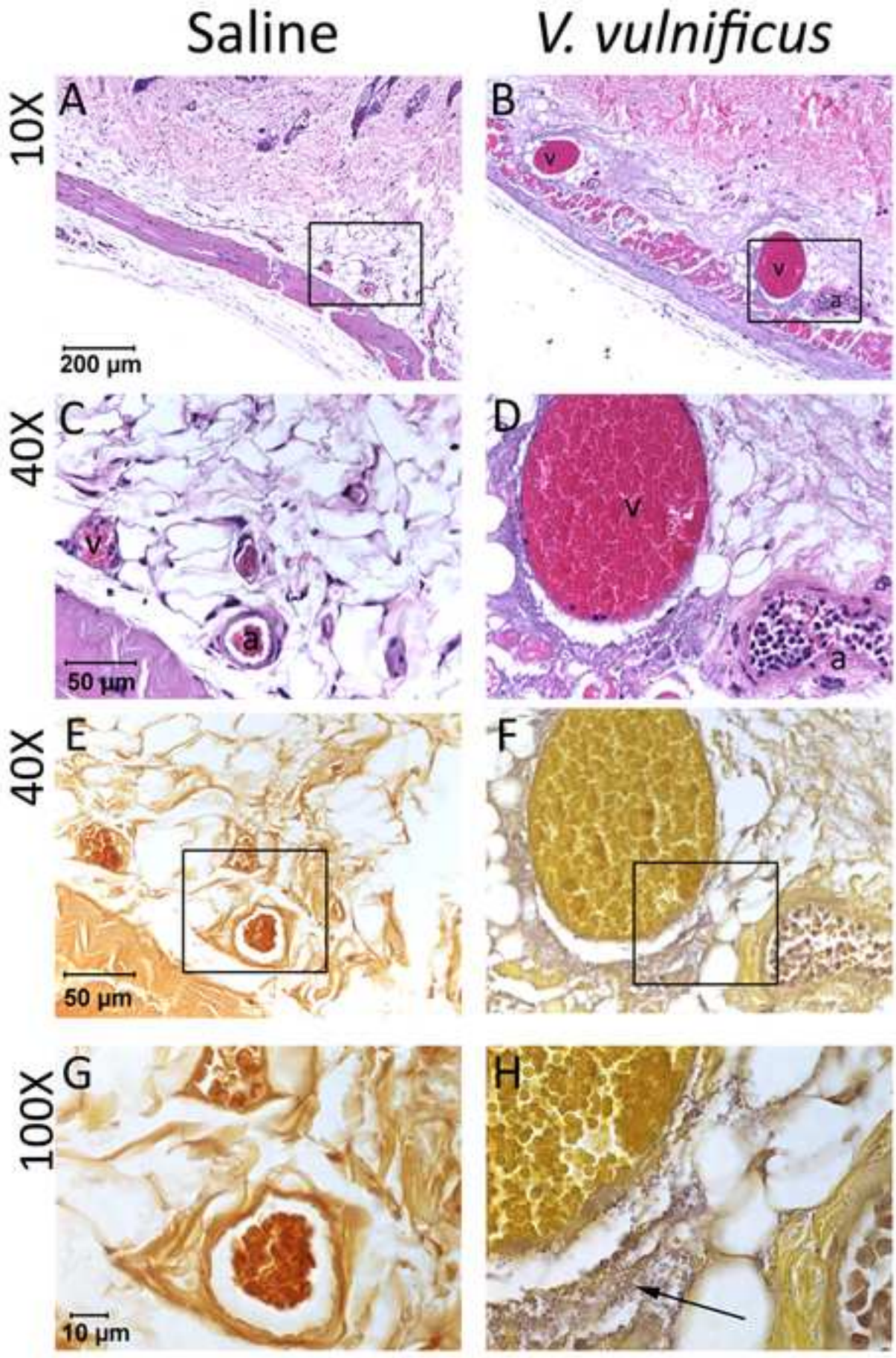

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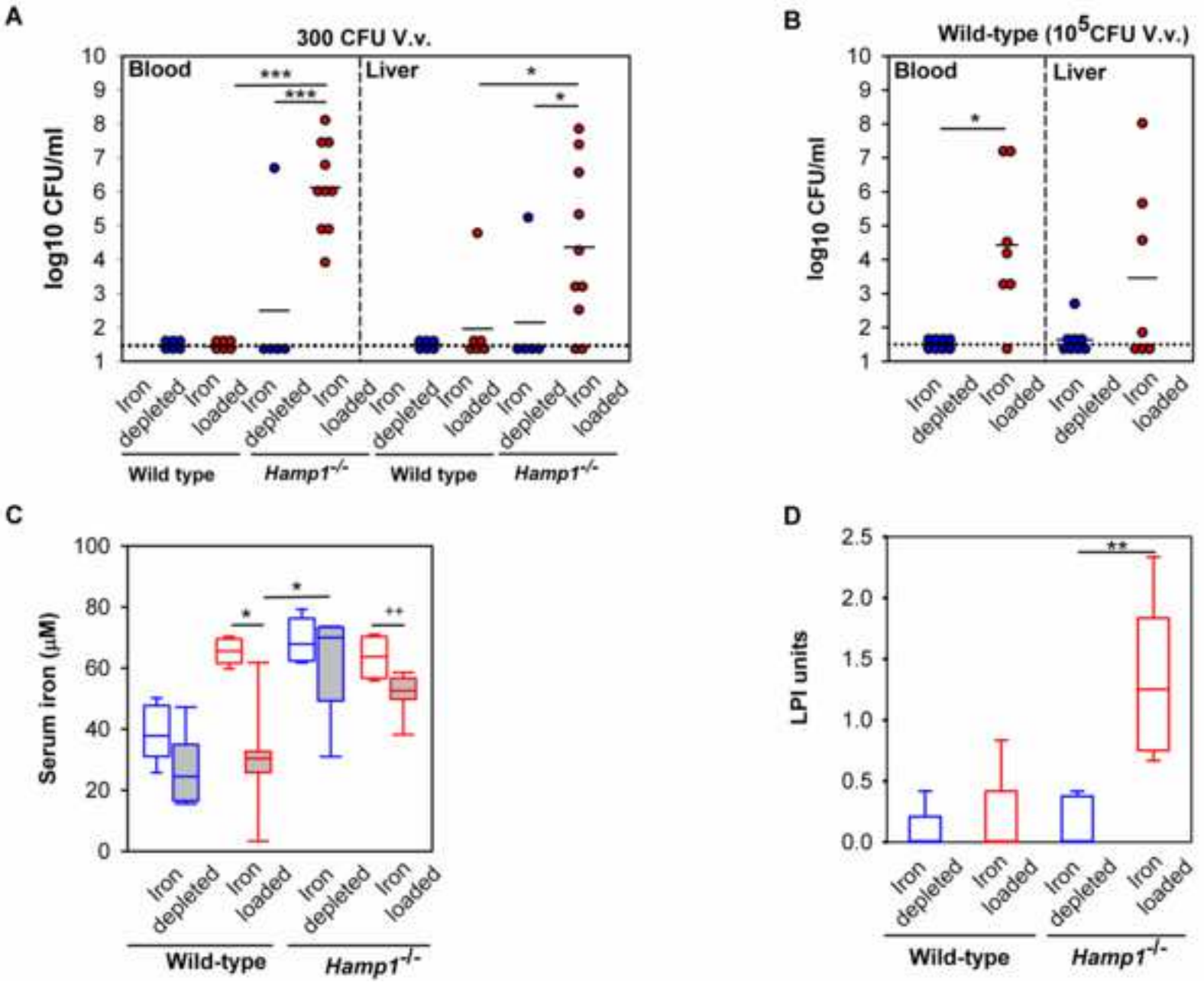

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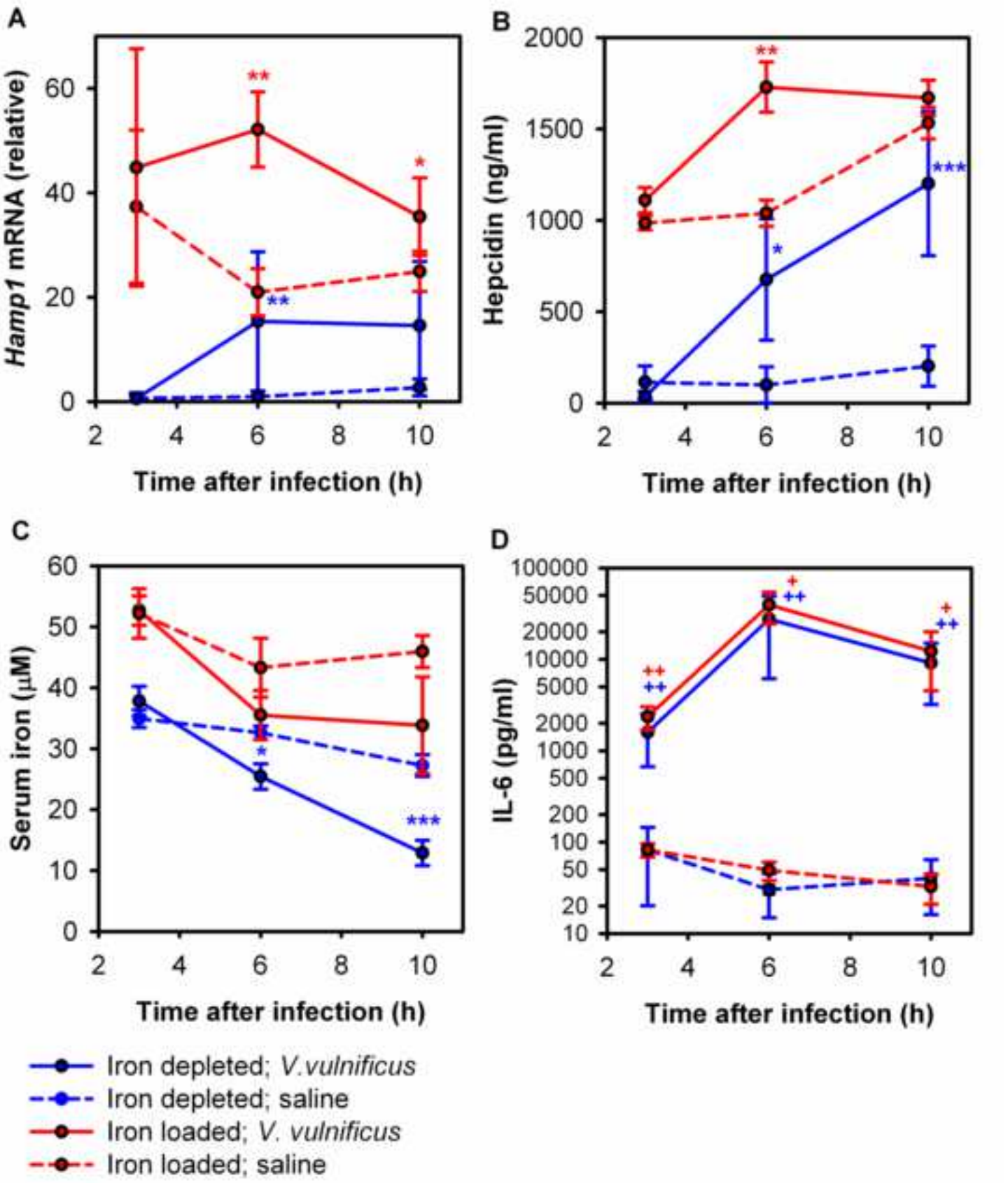

Figure 5
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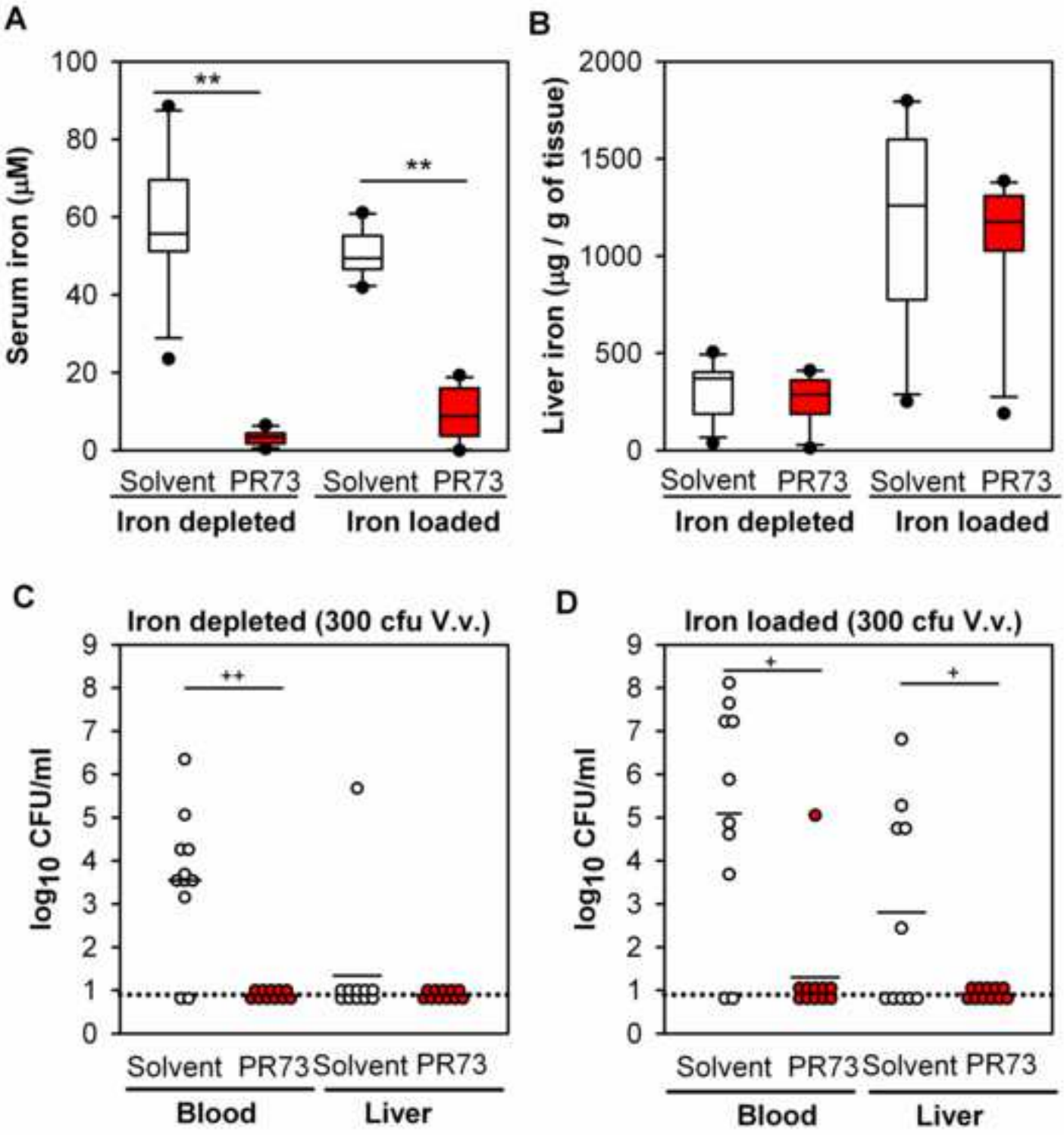

Figure 6
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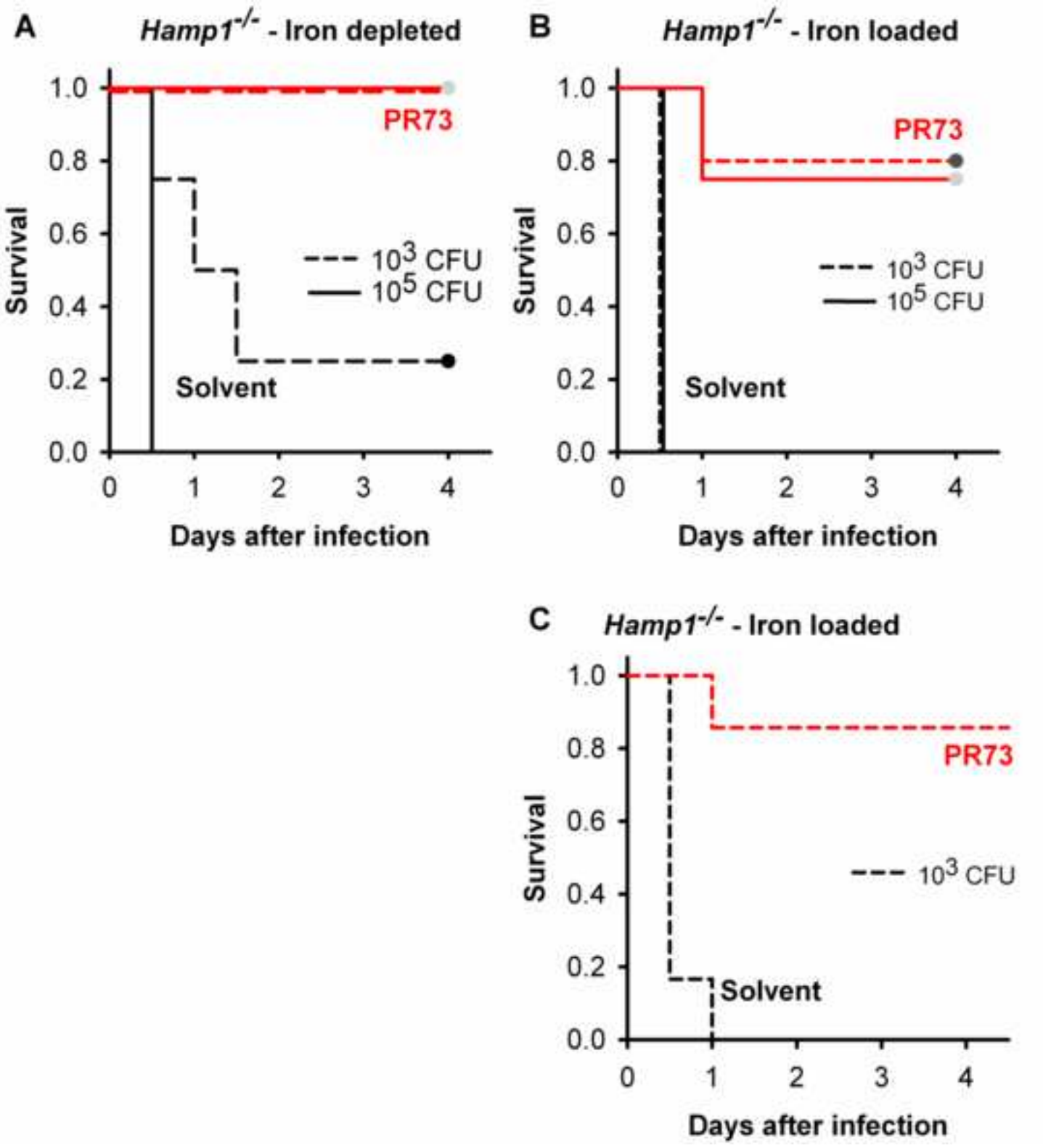

Figure 7
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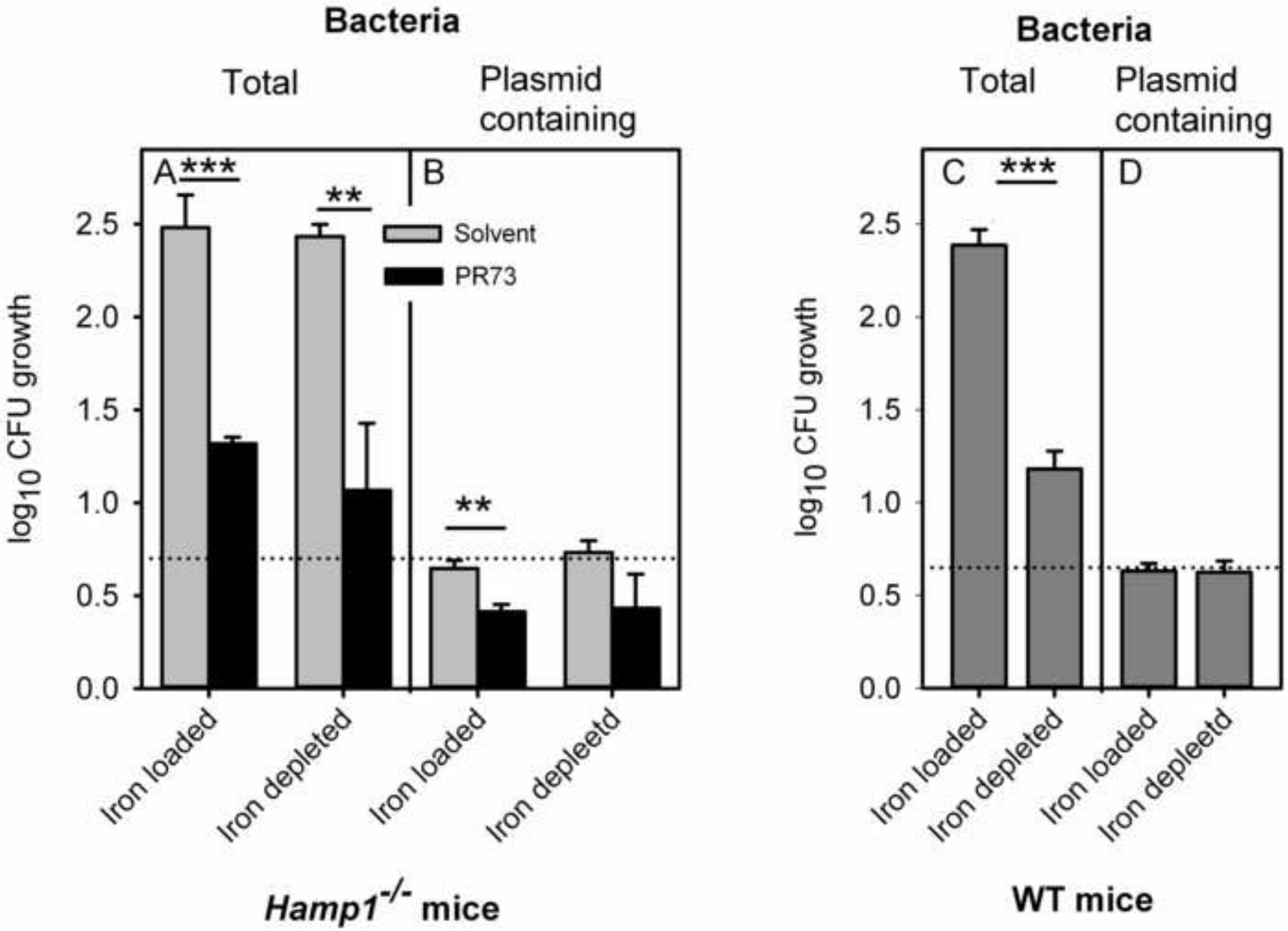

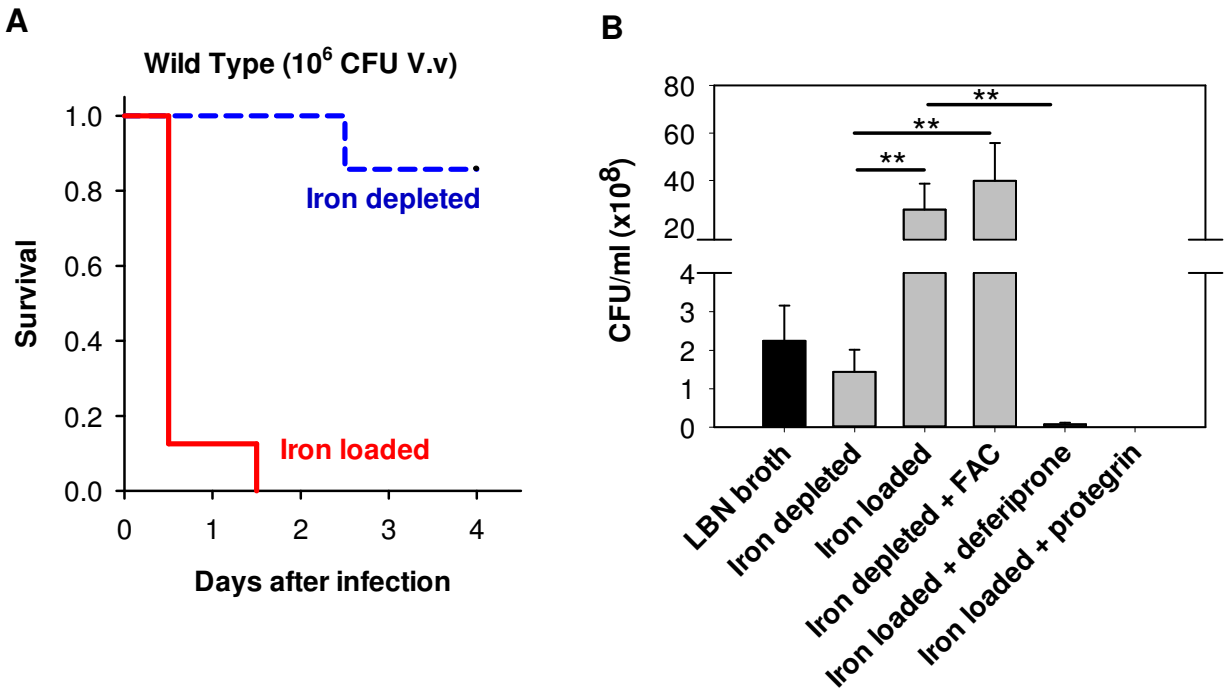


**Figure S1, Related to Figure 1. *V. vulnificus* pathogenicity and growth are greatly enhanced by high iron concentrations. (A)** Kaplan-Meier survival curves for 8-10 week-old WT mice, after s.c. infection with $1x10^6$ CFU of *V. vulnificus.* Iron-loaded mice (red line) died very rapidly, whereas iron-depleted mice (blue line) survived the infection, with the difference significant at $p<0.001$ by log-rank survival analysis. **(B)** Serum was collected from iron-depleted and iron-loaded WT mice. *V. vulnificus* ($1x10^4$ CFU/ml) was incubated for 3 h at 37 $^oC$ with shaking (300 rpm) in LB-N broth (black fill), or in different sera (gray fill) as indicated. Bacterial growth in LB-N broth and low-iron serum (26 µM Fe) was similar, but it was greatly increased in high-iron serum (60 µM Fe) and in iron-depleted serum supplemented with FAC (added to a final concentration of 100 µM iron). Bacterial growth was impaired in high iron serum treated with the iron chelator deferiprone (150 µM). Protegrin (0.4mg/ml) addition to high-iron serum killed all the bacteria. Bars represent mean + standard deviation (n=7). Statistical significance was assessed using student's *t* test (**$p<0.01$).

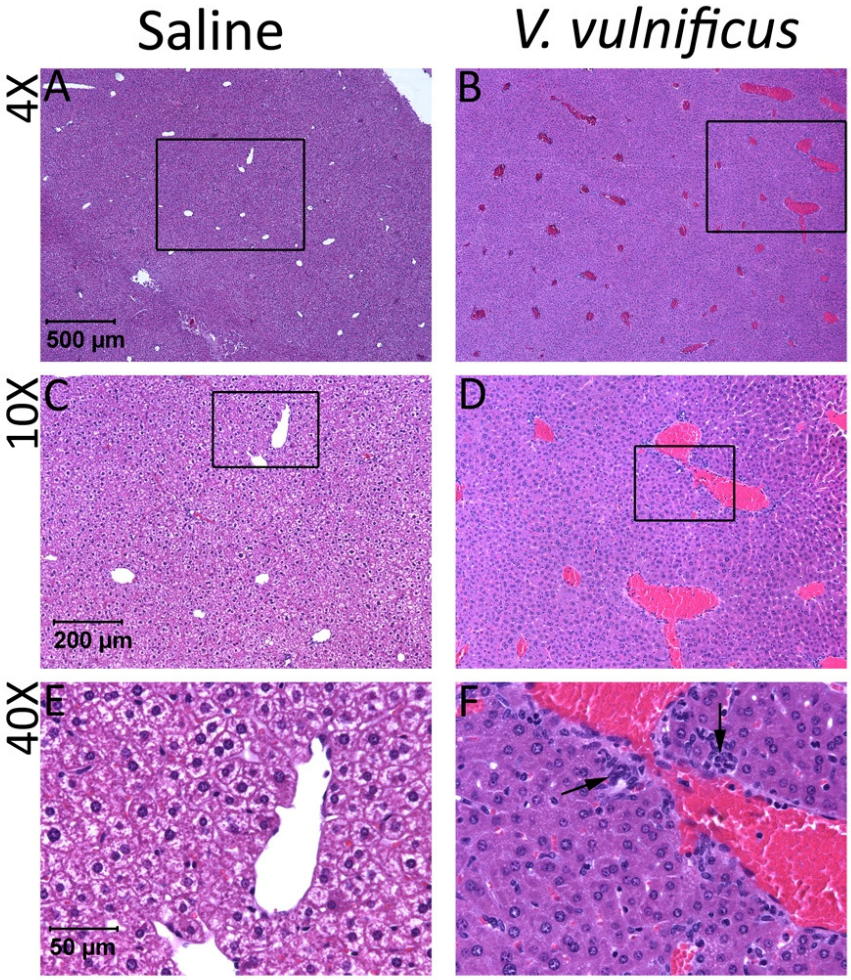


**Figure S2, Related to Figure 2. *V. vulnificus* infection causes inflammation and erythrocyte sludging in the liver.** Liver sections (stained with hematoxylin and eosin) of *Hamp1*$^{-/-}$ mice injected with saline (A, C, E) or 1 x $10^3$ CFU of *V. vulnificus* (B, D, F): rectangular regions are magnified. **(A-D)** Veins and venules in infected mice are dilated and congested with aggregated erythrocytes (B,D), unlike the saline controls (A,C). **(E-F)** Perivascular neutrophil infiltration (arrows) in infected mice (F) but not in saline controls (E).

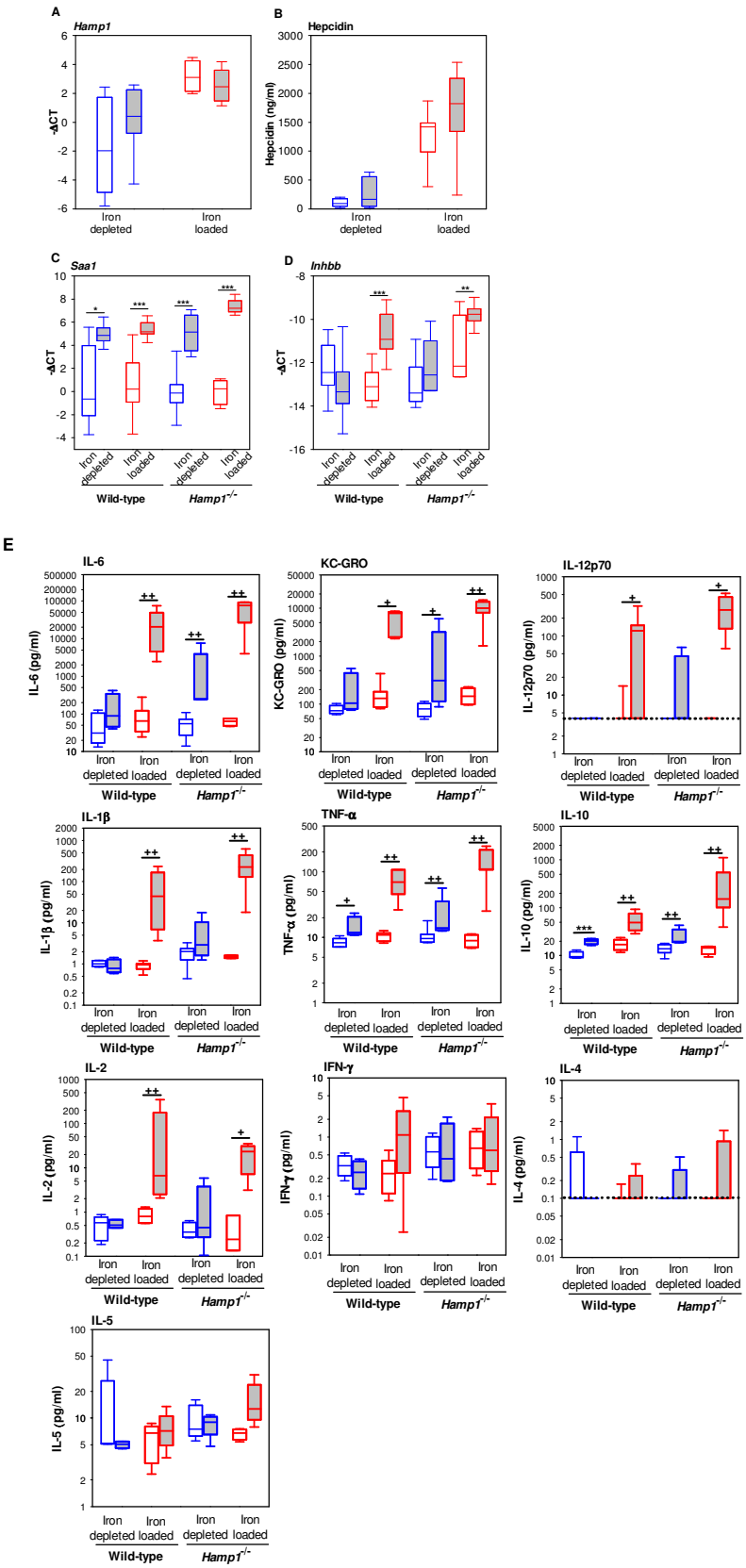
A
Hamp1
-ΔCT
Iron depleted
Iron loaded
B
Hepcidin
Hepcidin (ng/ml)
C
Saa1
D
Inhbb
Wild-type
Hamp1-/-
E
IL-6
IL-6 (pg/ml)
KC-GRO
KC-GRO (pg/ml)
IL-12p70
IL-12p70 (pg/ml)
IL-1β
IL-1β (pg/ml)
TNF-α
TNF-α (pg/ml)
IL-10
IL-10 (pg/ml)
IL-2
IL-2 (pg/ml)
IFN-γ
IFN-γ (pg/ml)
IL-4
IL-4 (pg/ml)
IL-5
IL-5 (pg/ml)

**Figure S3, Related to Figure 3. Liver hepcidin mRNA is no longer significantly increased by 16 h after infection, despite evidence of inflammation.** Iron-depleted (blue) and iron-loaded (red) WT and *Hamp1*$^{-/-}$ mice were injected with saline (white fill) or with *V. vulnificus* (grey fill, $10^5$ CFU for WT and 300 CFU for *Hamp1*$^{-/-}$), n=6-8 per group. **(A)** Hepatic *Hamp1* mRNA. **(B)** Serum hepcidin. **(C)** Hepatic *Saa1* mRNA. **(D)** Hepatic *Inhbb* mRNA. Measurements of mRNA concentration are shown as -ΔCt relative to actin mRNA. **(E)** A panel of 10 inflammatory cytokines was analyzed in serum from iron-depleted and iron-loaded WT and *Hamp1*$^{-/-}$ mice 16 hours after *V. vulnificus* infection (grey bars) or saline injection (white bars). Infected mice presented higher levels of IL-6, KC-GRO, IL-12p70, IL-1β, TNF-α, IL-10 and IL-2. No changes were observed for IFN-γ, IL-4 and IL-5. The black dotted line represents the lower limit of detection of the assay. Statistical significance was assessed using student's *t* test if data were normally distributed (*p<0.05; **p<0.01; ***p<0.001) or Mann-Whitney *U* test if they were not ($^{+}$p<0.05; $^{++}$p<0.01), n=6-8 per group.

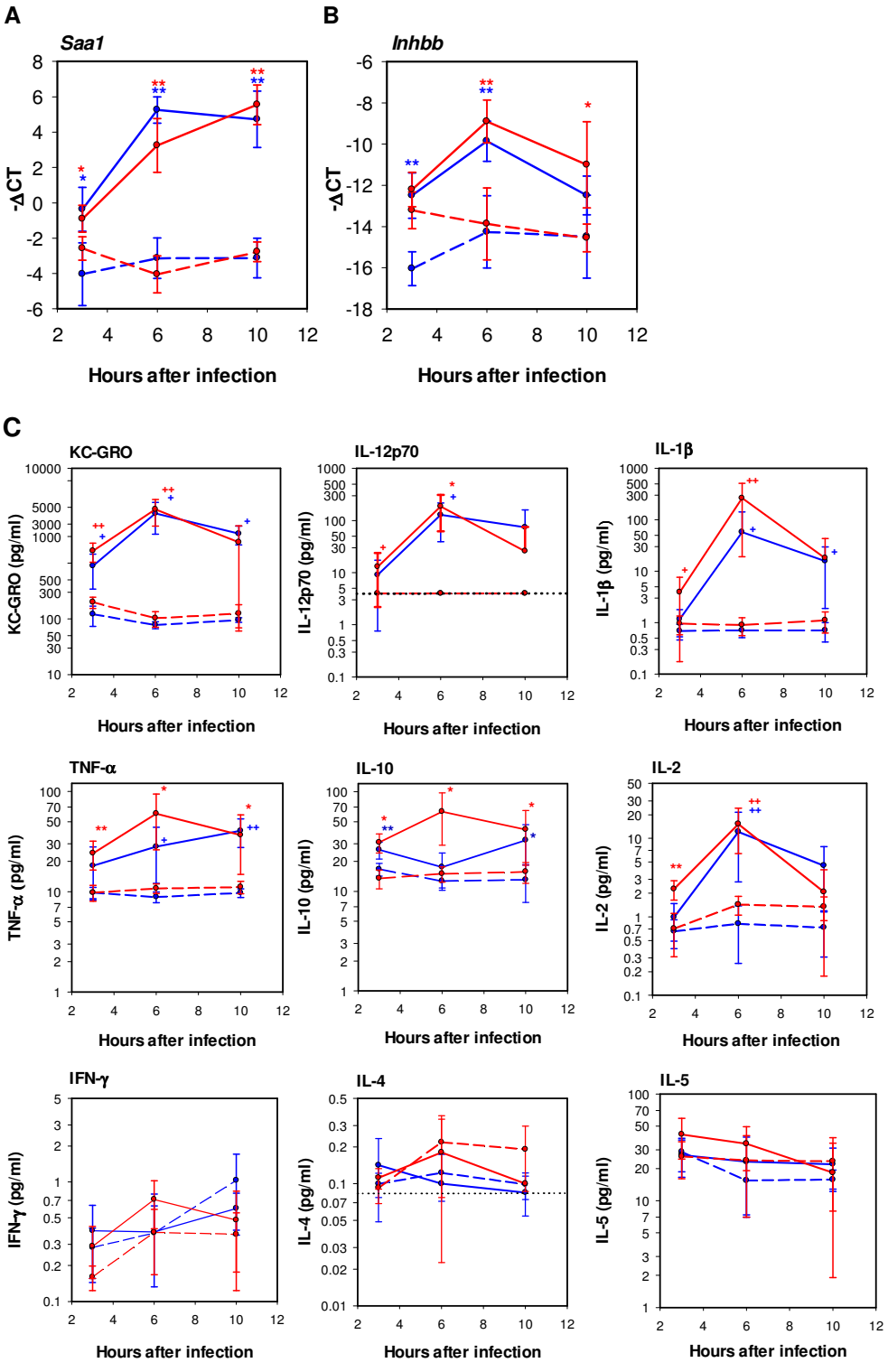
A
Saa1
-ΔCT
Hours after infection
B
Inhbb
-ΔCT
Hours after infection
C
KC-GRO
KC-GRO (pg/ml)
Hours after infection
IL-12p70
IL-12p70 (pg/ml)
Hours after infection
IL-1β
IL-1β (pg/ml)
Hours after infection
TNF-α
TNF-α (pg/ml)
Hours after infection
IL-10
IL-10 (pg/ml)
Hours after infection
IL-2
IL-2 (pg/ml)
Hours after infection
IFN-γ
IFN-γ (pg/ml)
Hours after infection
IL-4
IL-4 (pg/ml)
Hours after infection
IL-5
IL-5 (pg/ml)
Hours after infection
Iron-depleted; saline
Iron-depleted; V. vulnificus
Iron-loaded; saline
Iron-loaded; V. vulnificus

**Figure S4, related to Figure 4. Inflammation is induced early during infection.** qRT-PCR from liver samples was performed to analyze the mRNA expression of *Saa1* **(A)** *and Inhbb* **(B)** in iron-depleted (blue) and iron-loaded (red) WT mice, 3, 6 and 10 hours after infection with $10^5$ CFU *V. vulnificus* (solid lines) or saline injections (dashed lines). All the infected groups increased *Saa1* and *Inhbb* mRNA starting at 3 hours after infection. No differences were observed between iron-loaded and iron-depleted mice. Gene expression results are represented as -ΔCt relative to actin mRNA. Each dot represents the mean, and error bars represent standard deviation (n=5). **(C)** A panel of 9 pro-inflammatory cytokines was analyzed in serum from iron-depleted (blue) and iron-loaded (red) WT mice, 3, 6 and 10 hours after *V. vulnificus* infection (solid lines) or saline injection (dashed), n=5 per group. Infected mice presented higher levels of KC-GRO, IL-12p70, IL-1β, TNF-α, IL-10 and IL-2, starting at 3 hours after infection. No changes were observed for IFN-γ, IL-4 and IL-5. The black dotted line represents the lower limit of detection of the assay. Statistical significance was assessed using student's *t* test if data were normally distributed (*$p<0.05$; **$p<0.01$***$p<0.001$) or Mann-Whitney *U* test if they were not ($^{+}p<0.05$; $^{++}p<0.01$).

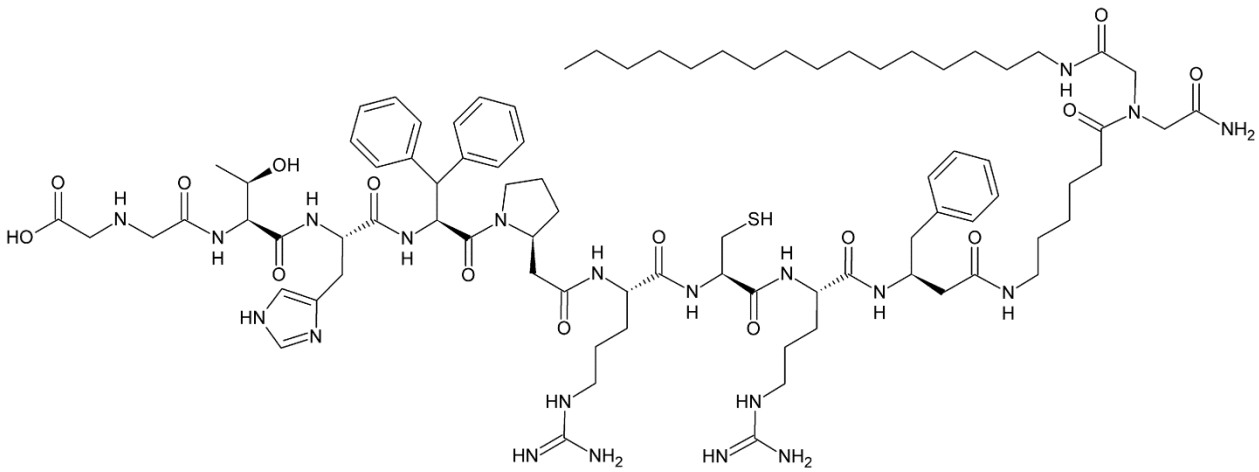

**Figure S5, Related to Figure 5. Molecular structure of minihepcidin PR73.** From N- to C-terminus the primary sequence of PR73 was: iminodiacetic acid, L-threonine, L-histidine, L-3,3-diphenylalanine, L-β-homoproline, L-arginine, L-cysteine, L-arginine, L-β-homophenylalanine, 6-aminohexanoic acid, iminodiacetic acid palmitylamide (Ida-Thr-His-Dpa-bhPro-Arg-Cys-Arg-bhPhe-Ahx-Ida(NHPal)-CONH2).

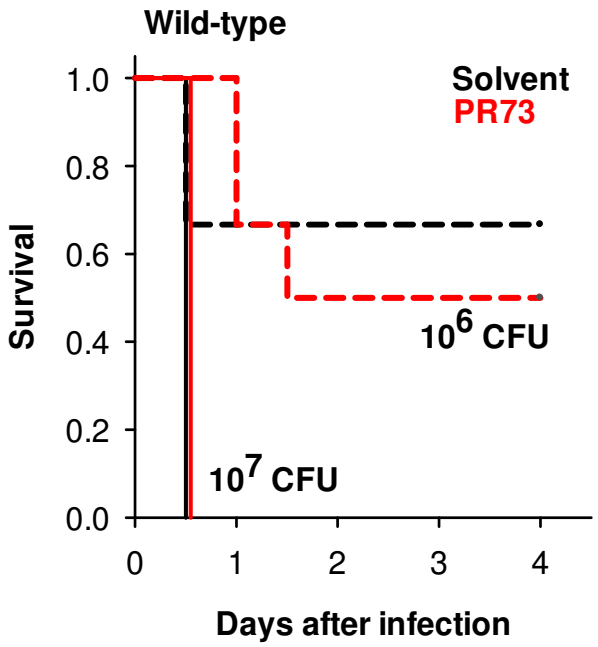


**Figure S6, Related to Figure 6. Minihepcidin does not further protect WT mice from death due to *V. vulnificus* infection.** Kaplan-Meier survival curves for 8-10 week-old WT mice (kept on a standard diet, 270 ppm Fe), treated with minihepcidin PR73 (red lines) or solvent (black lines), 24h and 3h before infection with $1x10^6$ CFU (dashed lines) or $1x10^7$ CFU (solid lines) *V. vulnificus.* No statistically significant differences were found between solvent and PR73 treated mice for each bacterial inoculum. N=5-6 per group.

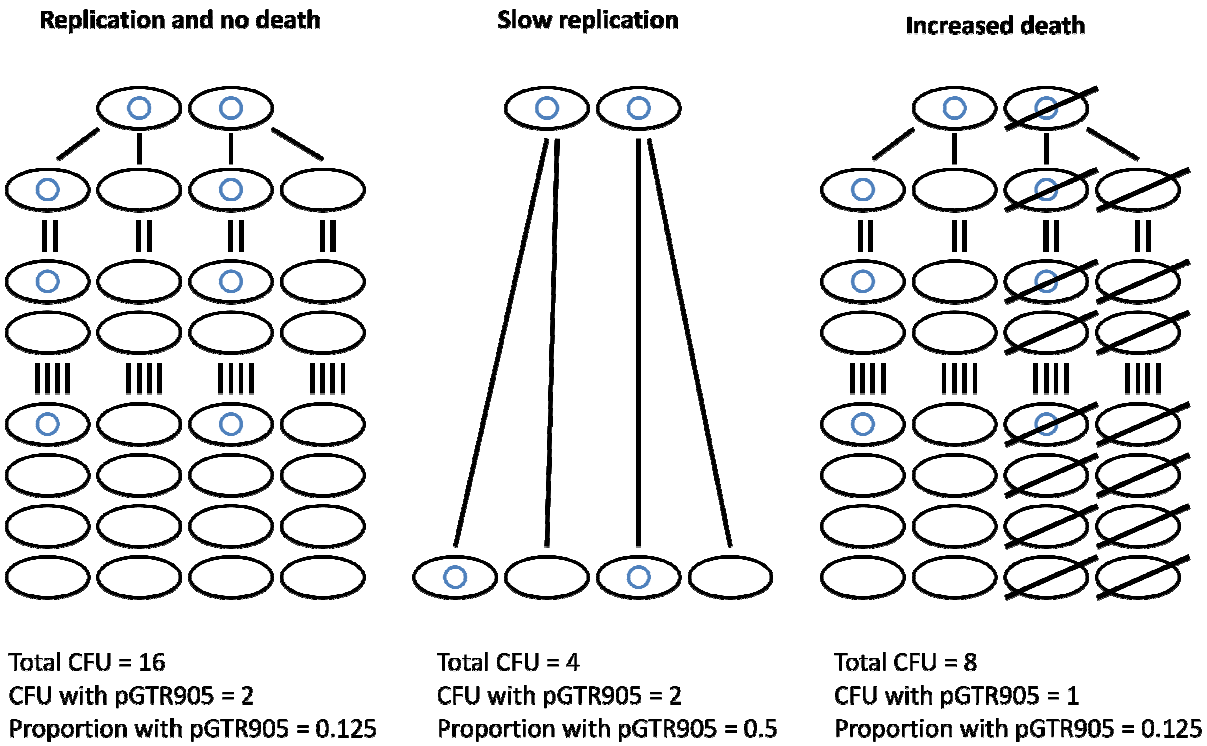


**Figure S7, Related to Figure 7. Use of plasmid pGTR905 to differentiate the replication rate from the death rate of V. vulnificus in mouse serum (adapted from Gulig et al, 1997).** The marker plasmid (blue circle) only replicates in the presence of arabinose, which is not present in significant amounts in mouse serum. **Left panel:** under standard conditions (normal replication and no death), a representative population of 2 plasmid-containing bacteria after three divisions yields 16 bacteria, 2 of which contain the plasmid (proportion of plasmid-containing bacteria = 0.125). **Central panel:** if PR73 decreases the replication rate (e.g. only one division occurs within the same time period), the total number of bacteria will be lower (4 CFU) and the proportion of plasmid-containing bacteria will be higher (0.5). **Right panel:** if PR73 increases killing without affecting the replication rate (e.g. half of the bacteria are killed), the total number of bacteria will be lower (8 CFU), but the proportion of plasmid-containing bacteria will be the same as in standard conditions (0.125). Therefore, the total number of bacteria reflects both growth and killing of bacteria whereas the ratio of total to plasmid-containing bacteria reflects growth only.

**Table S1, Related to Figure 1. Serum and liver iron concentrations in WT mice fed an iron-poor (4 ppm Fe) or iron-rich (10000 ppm Fe) diet for 2 weeks (n=8 per group)**

| | Serum Fe (μM) | Liver iron (μg/g of wet tissue) |
|---|---|---|
| **4 ppm diet** | 38 ± 9 | 18 ± 9 |
| **10,000 ppm diet** | 65 ± 4 | 425 ± 109 |
| **p-value** | <0.001 | <0.001 |

**Table S2, Related to Figure 7. Serum iron concentrations and bacterial growth in sera *ex vivo***

| | Solvent sera | | Minihepcidin sera | |
|---|---|---|---|---|
| | **Serum iron** | **$Log_{10}$(T/P)** | **Serum iron** | **$Log_{10}$(T/P)** |
| ***Hamp1*$^{-/-}$ mice, standard diet** | 66 µM | 1.84±0.21 | 13 µM | 0.90±0.02** |
| ***Hamp1*$^{-/-}$ mice, low-iron diet** | 64 µM | 1.70±0.13 | 4 µM | 0.63±0.53* |

Means ± SD are shown. *p=0.028, **p=0.0017 compared to solvent sera

| | High-iron diet | | Low-iron diet | |
|---|---|---|---|---|
| | **Serum iron** | **$Log_{10}$(T/P)** | **Serum iron** | **$Log_{10}$(T/P)** |
| **WT mice** | 64 µM | 1.75±0.06 | 37 µM | 0.56±0.15* |

Means ± SD are shown. *p=0.0002 compared to high-iron sera