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Epidermal hepcidin is required for neutrophil response to bacterial infection

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Introduction
Necrotizing fasciitis (NF) is an infection characterized by widespread necrosis of the skin, subcutaneous tissues, and fascia that was first described by Hippocrates in the 5th century (1). The standard treatment of NF consists of broad-spectrum antibiotics, extensive surgical debridement, and supportive care. However, even with current state-of-the-art treatment, NF frequently takes a fulminating course and is still associated with high mortality rates up to 35% (1). Group A Streptococcus (GAS) is considered the most common cause of NF associated with bacteremia and shock. Upon detection of these Gram-positive pyogenic bacteria, neutrophil recruitment is critical to the resolution of infection (2). However, GAS is equipped with a magnitude of virulence factors, allowing the pathogen to uniquely counteract each antibacterial strategy of neutrophils (3).

Hepcidin was originally identified as a cationic antimicrobial peptide (AMP) by its close structural similarity to the beta defensins but is now also recognized as a key iron regulatory hormone (4). Hepcidin is mainly produced by the liver in conditions of high iron, infection, or inflammation. Hepcidin controls plasma iron levels by binding to ferroportin (FPN), the only known iron exporter, and inducing its degradation (5). Patients with iron overload are well known to be associated with a predisposition to a variety of infections. Hepcidin contributes to innate immunity by decreasing plasma iron levels, providing an iron-restricted internal milieu inhospitable to microbes (6).

Besides the liver, an increasing number of studies showed that hepcidin is also expressed in other tissues (7–10). We previously demonstrated that hepatic hepcidin is sufficient to ensure systemic iron homeostasis in physiological conditions (11), suggesting that production of hepcidin by other tissues may have local roles. It may have a role at the site of infections and/or in poorly perfused tissues, inaccessible by systemic hepcidin from the circulation. The putative expression and local role of hepcidin in the skin, a major site of AMP production, are not known. We have employed our recently generated mouse model, in which the hepcidin gene can be spatiotemporally inactivated, to explore the putative expression and role of hepcidin in the skin in the context of GAS infection.

Results and Discussion
We examined hepcidin expression on skin biopsies derived from patients suffering from GAS NF (detailed in Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI126645S1). Hepcidin staining of...
Hepcidin expression was induced in the skin of infected mice (Figure 1C) and clearly detected in the keratinocytes, as visualized by keratin 14 (K14) staining (Figure 1D).

To probe the functional significance of keratinocyte-derived hepcidin in vivo, we developed a mouse model of keratinocyte-specific hepcidin deficiency (Hamp1Δker) by crossing Hamp1lox/lox mice with K14cre+ mice (Figure 1E). We observed an efficient truncation of the floxed Hamp1 allele in the epidermis of the Hamp1Δker mice, but not in the Hamp1lox/lox mice or K14cre+ mice (Supplemental Figure 3; see complete unedited blots in the supplemental material). Systemic iron parameters were unchanged between Hamp1lox/lox and Hamp1Δker mice (Figure 1F), in agreement with our previous study using human liver tissue sections was used as a positive control (Supplemental Figure 1). Hepcidin expression was higher and more widespread in the skin of NF patients than in the skin of a healthy subject, especially in keratinocytes, the predominant cell type in the epidermis (Figure 1A). Hepcidin mRNA expression was induced (Figure 1B) in a human 3D organotypic skin model (Supplemental Figure 2) as a direct consequence of GAS infection.

To investigate the role of hepcidin in the development of NF, we used an established model of necrotizing soft tissue infection (12, 13) where a strain of GAS, isolated from a patient with NF (14), is introduced subcutaneously into a shaved area on the flank of a mouse. Compared with skin biopsies of healthy mice, hepcidin expression was induced in the skin of infected mice (Figure 1C) and clearly detected in the keratinocytes, as visualized by keratin 14 (K14) staining (Figure 1D).
demonstrated bacteriostatic activities against GAS (Figure 2A), hepcidin had neither bactericidal (Figure 2A) nor bacteriostatic activities (Figure 2B). Moreover, primary keratinocytes derived from Hamp1lox/lox and Hamp1Δker mice displayed the same bactericidal activity against this pathogen (Figure 2C). We therefore ruled out a direct antimicrobial effect of hepcidin on these bacteria.

AMPs have been reported to have pleiotropic effects and influence a host’s inflammatory responses during infection (15). We therefore asked whether hepcidin could have an immunomodulatory role in keratinocytes. For this purpose, we performed a cytoplex on the supernatant of murine primary keratinocytes incubated with 0.36 μM and 3.6 μM synthetic hepcidin. Interestingly, hepcidin induced a dose-dependent increase of the key neutrophil chemokine CXCL1 but not of the other inflammatory cytokines we tested (Figure 2D). The capacity of mouse hepcidin to induce CXCL1 in primary keratinocytes was confirmed by ELISA (Supplemental Figure 5), as was the capacity of human hepcidin to induce the production of IL-8, the human functional homolog of CXCL1, in the human HaCat keratinocyte cell line and in a human 3D organotypic skin model (Figure 2E).

To investigate the mechanisms by which hepcidin protected against the spread of GAS infection, we first determined the putative bacteriostatic and bactericidal effects of hepcidin against GAS in vitro. While the well-known antimicrobial peptide LL-37 demonstrated bacteriostatic activities against GAS (Figure 2A), hepcidin had neither bactericidal (Figure 2A) nor bacteriostatic activities (Figure 2B). Moreover, primary keratinocytes derived from Hamp1lox/lox and Hamp1Δker mice displayed the same bactericidal activity against this pathogen (Figure 2C). We therefore ruled out a direct antimicrobial effect of hepcidin on these bacteria.

AMPs have been reported to have pleiotropic effects and influence a host’s inflammatory responses during infection (15). We therefore asked whether hepcidin could have an immunomodulatory role in keratinocytes. For this purpose, we performed a cytoplex on the supernatant of murine primary keratinocytes incubated with 0.36 μM and 3.6 μM synthetic hepcidin. Interestingly, hepcidin induced a dose-dependent increase of the key neutrophil chemokine CXCL1 but not of the other inflammatory cytokines we tested (Figure 2D). The capacity of mouse hepcidin to induce CXCL1 in primary keratinocytes was confirmed by ELISA (Supplemental Figure 5), as was the capacity of human hepcidin to induce the production of IL-8, the human functional homolog of CXCL1, in the human HaCat keratinocyte cell line and in a human 3D organotypic skin model (Figure 2E).
The cognate receptor of hepcidin is the iron exporter FPN, questioning the role of FPN/iron in the induction of CXCL1 by hepcidin. The stimulatory effect of hepcidin on CXCL1 was reduced by the addition of a drug preventing the interaction of hepcidin with the iron exporter FPN (16) (Figure 2F). These data suggest that hepcidin, in primary keratinocytes, induces CXCL1 through a FPN-dependent pathway. Binding of hepcidin to FPN is well known to induce its internalization and degradation, resulting in an increase of intracellular iron (5). In corroboration with the action of hepcidin on FPN, incubation of primary keratinocytes with iron stimulated CXCL1 production (Figure 2G).

In agreement with the in vitro results showing that hepcidin stimulates CXCL1 production in primary keratinocytes, the in vivo keratinocyte CXCL1 production in response to GAS infection was lower in Hamp1Δker mice than in Hamp1lox/lox littermates, as shown by IHC (Figure 3C) and ELISA (Figure 3B) on skin biopsies. As a consequence of the lower CXCL1 production, less neutrophil recruitment was observed in the skin of Hamp1Δker mice compared with that of control littermates, as shown by IHC (Figure 3C) and by cytometry analysis (Figure 3D). This defect in the ability of keratinocyte-derived hepcidin to recruit neutrophils at the site of infection translated into a decrease in the necrotic skin lesion size of the Hamp1Δker mice as compared with controls (Figure 3E). Subcutaneous injection of CXCL1 into GAS-infected Hamp1Δker mice (Figure 3F) significantly decreased the number of bacteria to even below that found in the lesions of Hamp1lox/lox mice (Figure 3G). These results strongly suggest that the lack of CXCL1 production in Hamp1Δker mice was responsible for their susceptibility to GAS infection. Altogether, these results suggest that hepcidin is critical for regulating CXCL1 production in keratinocytes and that it may tune the magnitude of the neutrophil recruitment in the immune response.

We next investigated the possible advantages of indirect production of CXCL1 through hepcidin during GAS infection. GAS is equipped with a quantity of neutrophil resistance factors, allowing the pathogen to uniquely counteract each antibacteri-
al strategy of neutrophils (3). One of the principal mechanisms of GAS-immune escape is the production of surface-associated serine proteases by GAS, such as SpyCEP (also designated ScpC), which cleaves human IL-8/mouse CXCL1 to suppress chemokine-mediated neutrophil recruitment (17), or SpeB, which allows GAS to translocate across the epithelial barrier by degrading several host plasma and matrix proteins (18). To examine whether hepcidin was a target of SpyCEP or SpeB, synthetic hepcidin and CXCL1 (as a control) were incubated with purified SpyCEP, SpeB, or PBS overnight. The digestion products were examined by mass spectrometry analyses. As shown by the MALDI spectrum (Figure 4A), CXCL1 was cleaved, as expected, into 2 fragments of 5.9 kDa and 1.3 kDa. In contrast, hepcidin was not cleaved in the presence of SpyCEP (Figure 4A, bottom panel) or SpeB (Supplemental Figure 6). This suggests that hepcidin could serve as a reservoir to maintain a steady-state level of CXCL1 in the context of infection. CXCL1 is phylogenetically ancient and is expressed in Dictostelium discoideum, whereas hepcidin-like peptides appeared more recently during evolution, for example in teleost fishes (Supplemental Figure 7). Considering the theory that host-pathogen interactions coevolve, we could speculate that whereas GAS has already evolved to counteract the activity of CXCL1, it has not yet developed a virulence factor able to neutralize the activity of hepcidin. Because of hepcidin resistance to bacterial protease activities such as SpyCEP or SpeB, and in view of its unanticipated immunomodulatory role, we also asked whether local hepcidin injection could have a therapeutic effect on the systemic spread of bacteria in a NF model. Twenty-four hours after GAS infection, 1 μg synthetic hepcidin or PBS was subcutaneously injected at the bacterial inoculation site, followed by 2 injections of 500 ng hepcidin or PBS for 2 consecutive days (Figure 4B). As expected, hepcidin-treated mice showed an increase in neutrophil recruitment (Figure 4C). In contrast to the PBS-treated mice, which exhibited systemic signs of infection including weight loss (Figure 4D), rough hair coat, and hunched posture (data not shown), hepcidin-treated mice did not present any signs of systemic disease and accordingly recovered their initial weight. Remarkably, whereas all the control mice presented with systemic bacterial dissemination (as shown by the number of bacteria in the spleen), 7 of the 9 hepcidin-treated mice showed absolutely no bacterial dissemination.
Author contributions
MM, SL, SC, SMS, and JRRM designed the experiments, carried out experiments, and performed data analysis. AF, ASZ, CH, CG, NO, and JWD provided essential reagents and scientific advice. JRRM and CP designed the experiments and supervised the project. CP wrote the manuscript.

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