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Graphical abstract

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Epidermal hepcidin is required for neutrophil response to bacterial infection

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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) NF is considered the most common cause of NF, with a mortality rate of up to 35% (1). Group A Streptococcus (GAS) is considered the most common cause of NF. GAS is a pyogenic bacteria and neutrophil recruitment is critical to the resolution of infection (2). However, GAS is equipped with a magnitude of virulence factors, allowing the pathogen to evade the immune system and spread 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) NF is considered the most common cause of NF, with a mortality rate of up to 35% (1). Group A Streptococcus (GAS) is considered the most common cause of NF. GAS is a pyogenic bacteria and neutrophil recruitment is critical to the resolution of infection (2). However, GAS is equipped with a magnitude of virulence factors, allowing the pathogen to evade the immune system and spread infection from an initial tissue focus. Unexpectedly, this effect was due to its ability to promote production of the CXCL1 chemokine by keratinocytes, resulting in neutrophil recruitment. Unlike CXCL1, hepcidin is resistant to degradation by major GAS proteases and could therefore serve as a reservoir to maintain steady-state levels of CXCL1 in infected tissue. Finally, injection of synthetic hepcidin at the site of infection can limit or completely prevent systemic spread of GAS infection, suggesting that hepcidin agonists could have a therapeutic role in NF.

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) NF is considered the most common cause of NF, with a mortality rate of up to 35% (1). Group A Streptococcus (GAS) is considered the most common cause of NF. GAS is a pyogenic bacteria and neutrophil recruitment is critical to the resolution of infection (2). However, GAS is equipped with a magnitude of virulence factors, allowing the pathogen to evade the immune system and spread infection from an initial tissue focus. Unexpectedly, this effect was due to its ability to promote production of the CXCL1 chemokine by keratinocytes, resulting in neutrophil recruitment. Unlike CXCL1, hepcidin is resistant to degradation by major GAS proteases and could therefore serve as a reservoir to maintain steady-state levels of CXCL1 in infected tissue. Finally, injection of synthetic hepcidin at the site of infection can limit or completely prevent systemic spread of GAS infection, suggesting that hepcidin agonists could have a therapeutic role in NF.
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The expression of hepcidin 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 ($\text{Hamp}^1\Delta_{\text{ker}}$) by crossing $\text{Hamp}^1\text{lox}/\text{lox}$ mice with K14cre+ mice (Figure 1E). We observed an efficient truncation of the floxed $\text{Hamp}^1$ allele in the epidermis of the $\text{Hamp}^1\Delta_{\text{ker}}$ mice, but not in the $\text{Hamp}^1\text{lox}/\text{lox}$ mice or K14cre+ mice (Supplemental Figure 3; see complete unedited blots in the supplemental material). Systemic iron parameters were unchanged between $\text{Hamp}^1\text{lox}/\text{lox}$ and $\text{Hamp}^1\Delta_{\text{ker}}$ mice (Figure 1F), in agreement with our previous study.

**Figure 1. Keratinocyte hepcidin prevents bacterial systemic spread.** IHC with or without primary antibody detecting (A) hepcidin (in brown) on sections of cutaneous human biopsies of GAS NF patients and healthy control using PerkinElmer’s Luminara multilabel slide scanner Panoramic Viewer software. (B) Real-time reverse transcription PCR (qPCR) for hepcidin from GAS-infected human 3D organotypic skin equivalent model; n=4 per group. (C) qPCR for hepcidin in murine GAS-infected skin; n $\geq$ 3 per group. (D) IHC for hepcidin (in blue) and K14 (in brown) on cutaneous biopsies of WT mice challenged or not with GAS. Scale bars: 100 μm. Leica DMI3000B microscope, Leica DFC310FX camera, S/0.4; Leica LAS Core Software. (E) Generation of $\text{Hamp}^1\Delta_{\text{ker}}$ mice. (F) Plasma iron, ferritin, transferrin, and skin iron levels in $\text{Hamp}^1\text{lox}/\text{lox}$ and $\text{Hamp}^1\Delta_{\text{ker}}$ mice; n $\geq$ 4 per group. (G) Bacterial count in skin, blood, and spleen of $\text{Hamp}^1\text{lox}/\text{lox}$ and $\text{Hamp}^1\Delta_{\text{ker}}$ mice 4 days after injection with GAS; n $\geq$ 10 per group. Statistical analysis was performed using a Mann Whitney test (B, C, F, and G) or a 2-way ANOVA followed by Tukey’s test for weight kinetics (H). *P < 0.05; **P < 0.01; ***P < 0.001.
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demonstrated bacteriostatic activities against GAS (Figure 2A), hepcidin had neither bactericidal 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).

These mice were infected with GAS in the NF model (14). Keratinocyte hepcidin staining was not detectable in the Hamp1Δker mice (Supplemental Figure 4), confirming that the stained hepcidin peptide is of skin but not of liver origin. Four days after infection, Hamp1Δker mice had a significantly higher number of bacteria than the Hamp1lox/lox littermates at the lesion site (10⁶ vs 10⁵ CFU/mg) but also in the blood (10⁴ vs 10² CFU/mL) and in the spleen (5 × 10⁴ vs 38 CFU/g) (Figure 1G). Hamp1Δker mice also lost more weight than the Hamp1lox/lox mice, further underlining the higher morbidity in these mice (Figure 1H). These data indicate that keratinocyte production of hepcidin is important in limiting the ability of GAS to replicate within the necrotic skin tissues and to disseminate from the initial focus of infection into the bloodstream and systemic organs.

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.

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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 3A) 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-
gen 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.

Figure 4. Hepcidin is resistant to SpyCEP cleavage and has a therapeutic role in NF. (A) Mass spectroscopy analysis of CXCL1 or hepcidin incubated overnight with SpyCEP or PBS. Electrospray ionization generated a series of multiply charged ions (indicated as m/z; mass-to-charge ratio) from which the average molecular mass (m) of each was deduced. The blue arrows indicate uncleaved peptide peaks at 7.8 kDa (CXCL1) and 2.7 kDa (hepcidin). Red arrows show the cleavage products of CXCL1 with a small (1.3 kDa) and a big (5.9 kDa) fragment. (B) Therapeutic protocol. (C) Neutrophil count (3 measurements per individual mouse were averaged); n = 6 per group. (D) Weight variation and (E) bacterial count in spleen of WT infected mice treated with PBS or hepcidin (n = 9, red square) or PBS (n = 9, black square) during 4 days. Statistical analysis was performed using a Student’s t test (C), a 2-way ANOVA followed by Tukey’s test (D), or a Mann Whitney test (E). *P < 0.05.
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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Methods

See Supplemental Methods.

Study approval. For human studies, written informed consent to the protocol was obtained for all subjects and was approved by the Institutional Review Board and the regional ethics committee Paris IV (IRB 2016/40NICB and IRB 00003835). The collection of personal data was approved by the “Commission Nationale de l’Informatique et des Libertés.”

The animal studies described here were reviewed and approved (agreement no. CEEA34.CP.003.13) by the “Président du Comité d’Éthique pour l’Expérimentation Animale Paris Descartes” and are in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals (Council of Europe, ETS 123, 1991).