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IL-22 Controls Iron-Dependent Nutritional Immunity Against Systemic Bacterial Infections

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Abstract

Host immunity limits iron availability to pathogenic bacteria, but whether immunity limits pathogenic bacteria from accessing host heme, the major source of iron in the body, remains unclear. Using *Citrobacter rodentium*, a mouse enteric pathogen and *Escherichia coli*, a major cause of sepsis in humans as models, we find that interleukin-22, a cytokine best known for its ability to promote epithelial barrier function, also suppresses the systemic growth of bacteria by limiting iron availability to the pathogen. Using an unbiased proteomic approach to understand the mechanistic basis of IL-22 dependent iron retention in the host, we have identified that IL-22 induces the production of the plasma hemoglobin scavenger haptoglobin and heme scavenger hemopexin. Moreover, the anti-microbial effect of IL-22 depends on the induction of hemopexin expression, while haptogloblin is dispensable. Impaired pathogen clearance in infected *Il22*$^{-/-}$ mice was restored by hemopexin administration and hemopexin-deficient mice had increased pathogen loads after infection. These studies reveal a previously unrecognized host defense mechanism regulated by IL-22 that relies on the induction of hemopexin to limit heme availability to bacteria leading to suppression of bacterial growth during systemic infections.

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Introduction

Iron is an essential nutrient for nearly all microorganisms, including pathogenic bacteria (1). In mammals, the majority of iron is found inside erythrocytes, contained in the prosthetic heme groups of hemoglobin (2). Given their strict requirement for iron, pathogenic bacteria evolved strategies to trigger hemolysis as the means to extract iron from hemoglobin (1). Bacterial pathogens possess several mechanisms to overcome the limited availability of iron in the circulation or at local niches. These include production of siderophores that bind iron at extremely high-affinity, heme acquisition systems, and mechanism for the uptake of transferrin- and lactoferrin-bound iron (1). Host immunity encompasses several mechanisms that limit host iron availability to pathogenic bacteria (3-6), a defense strategy known as nutritional immunity (7). This host defense strategy involves a reduction of circulating or local iron by inhibition of cellular iron export and the induction of cellular iron import by host cells via hepcidin-dependent and independent mechanisms (3-6). Whether nutritional immunity limits pathogenic bacteria from accessing host heme, the major source of iron, remains unclear.

Interleukin-22 (IL-22) is an important cytokine that promotes early host defense, epithelial barrier function and tissue repair at mucosal surfaces (8, 9). In response to bacterial infection, IL-22 is produced by several immune cells, leading to the induction of innate antimicrobial molecules that are thought to promote host defense and intestinal barrier protection against pathogens (10). The protective function of IL-22 was revealed by the observation that *Il22*$^{-/-}$ mice are highly susceptible to the attaching and effacing mouse pathogen *Citrobacter rodentium.* In the absence of IL-22, there was marked intestinal damage and bacterial translocation in orally infected mice (8, 9). IL-22 signals through a heterodimeric receptor complex comprised of an ubiquitously expressed IL-10R2 subunit and an epithelial-specific IL-22RA1 subunit (11, 12). Because of the highly restricted expression of the IL-22RA1 subunit, IL-22 stimulates epithelial cells on certain organs such as the liver and kidney as well as epithelial barriers including the skin and the intestine (13). Using *C. rodentium* and *E. coli* infection models, we report that IL-22 induces a systemic protective response that is mediated by hemopexin, a plasma heme scavenger produced in the liver, that limits the availability of heme-iron to the microbes and suppresses bacterial systemic growth.

Results

IL-22 promotes pathogen clearance and host survival after systemic infection with *C. rodentium*

Consistent with earlier studies (8, 9), *Il22*$^{-/-}$ mice succumbed early to oral infection with *C. rodentium*, which was associated with increased pathogen load in blood, liver, and spleen (Fig. S1). Systemic *C. rodentium* infection induced rapid production of IL-22 in plasma (Fig. 1A). To determine whether IL-22 controls pathogen growth systemically, wild-type (WT) and *Il22*$^{-/-}$ mice were infected with *C. rodentium* intravenously and monitored for survival and pathogen load in the blood. Surprisingly, >90% of the *Il22*$^{-/-}$ mice succumbed to intravenous infection compared to ~20% of the WT mice (Fig. 1B). Mortality of infected *Il22*$^{-/-}$ mice was associated with impaired pathogen clearance, resulting in increased

pathogen load in blood (Fig. 1C). Pathogen-induced lethality in *Il22*$^{-/-}$ mice required live bacteria because the animals did not succumb when injected intravenously with heat-killed *C. rodentium* (Fig. S2). While antibody production is critical to control *C. rodentium* oral infection (14, 15), the production of pathogen-specific IgM and IgG was not impaired in *Il22*$^{-/-}$ mice, as compared with WT mice (Fig. S3). To ascertain the role of IL-22 in controlling pathogen growth, we pre-treated *Il22*$^{-/-}$ mice with recombinant IL-22 prior to intravenous *C. rodentium* infection and assessed the pathogen loads in the blood. Administration of IL-22 reduced pathogen load (Fig. 1D). In our mouse colony, ~ 90% of the bacteria present in the liver of orally infected *Il22*$^{-/-}$ mice were *C. rodentium* although a few commensals, particularly *Lactobacillus spp.*, were also identified (Fig. S1G). To determine whether *C. rodentium* infection could induce mortality in the absence of commensals, we pre-treated WT germ-free (GF) mice with an IL-22 neutralizing antibody and infected the mice with the pathogen via the oral route**.** Neutralization of IL-22 reduced the survival of GF mice after oral *C. rodentium* infection (Fig. 1E), which was associated with increased pathogen loads in blood, liver and spleen (Fig. 1F-H). Collectively, these results indicate that IL-22 limits the systemic expansion of *C. rodentium* and promotes host survival.

IL-22 regulates plasma hemopexin and haptoglobin after pathogen infection

To understand further the mechanism by which IL-22 promotes systemic pathogen clearance, we set to identify, by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS**)** analysis, plasma proteins regulated by IL-22 before and after infection of WT and *Il22*$^{-/-}$ mice with *C. rodentium* intravenously. Consistent with previous studies (16, 17), several proteins including serum amyloid-A1, serum amyloid-A2, inter-alpha-trypsin inhibitor heavy chain, adiponectin, C-reactive protein, alpha-2-macroglobulin and complement factors were reduced in the plasma of infected *Il22*$^{-/-}$ mice compared with infected WT mice (Fig. 2). In addition, the induction of the plasma extracellular heme scavenger hemopexin (HPX) (18) and the hemoglobin scavenger haptoglobin (HP) (19) in response to *C. rodentium* infection was severely impaired in *Il22*$^{-/-}$ mice compared to WT mice (Fig. 2). This was associated with the accumulation of extracellular hemoglobin α- and β-globin chains in the plasma of infected *Il22*$^{-/-}$ mice compared to WT mice (Fig. 2). Importantly, intraperitoneal administration of IL-22 restored the induction of HP and HPX expression in the plasma of *Il22*$^{-/-}$ mice compared to *Il22*$^{-/-}$ mice treated with control protein (Fig. 2).

Hemolysis driven by bacterial virulence factors promotes pathogen growth in *IL22*$^{-/-}$ mice

Accumulation of extracellular hemoglobin α- and β-globin chains in the plasma of *Il22*$^{-/-}$ mice suggested that IL-22 deficiency is associated with augmented hemolysis after systemic *C. rodentium* infection and/or with impaired disposal of extracellular hemoglobin from plasma**.** To verify that hemoglobin proteins are regulated by IL-22, we assessed the amounts of α-globin in the plasma of uninfected and infected WT and *Il22*$^{-/-}$ mice by immunoblotting. The analysis confirmed that the amounts of α-globin were increased in the plasma of *Il22*$^{-/-}$ mice in response to intravenous or oral infection with *C. rodentium* (Fig. 3A,B). Because scavenging of extracellular hemoglobin dimers by HP prevents the release of the prosthetic heme groups of hemoglobin (20, 21), we asked whether induction of HP by

IL-22 was associated with inhibition of heme accumulation in plasma. *Il22*$^{-/-}$ mice infected via the intravenous or oral route accumulated heme in plasma whereas infected WT mice did not (Fig. 3C). Consistent with a role of hemoglobin release from lysed erythrocytes in promoting pathogen growth, intravenous administration of lysed erythrocytes to *C. rodentium* infected WT mice increased their pathogen loads in the blood (Fig. 3D). The filament protein EspA as well as EspB and EspD are required for the assembly of the translocon of the enterocyte effacement (LEE)-encoded T3SS and promote the formation of EspB/EspD pores that mediate erythrocyte lysis in vitro (22). To assess whether *C. rodentium* translocators induce erythrocyte lysis, mouse erythrocytes were incubated *in vitro* with WT and *C. rodentium* mutants deficient in EspA, EspB and Ler, the global regulator of the LEE that is critical for pathogen virulence (23). The WT bacterium, but not the EspA, EspB and Ler mutants, induced erythrocyte lysis (Fig. 3E). To determine whether EspB is important for hemolysis associated with systemic growth and lethality of *C. rodentium* in vivo, we infected *Il22*$^{-/-}$ mice with WT or the EspB mutant and monitored the pathogen loads in blood as well as mouse survival. Notably, deficiency of EspB was associated with reduced pathogen loads and absence of lethality when compared to the WT bacterium that caused 100% mortality in *Il22*$^{-/-}$ mice (Fig. 3F,G). *Ler* expression was detected in the blood of mice after intravenous infection with *C. rodentium* (Fig. S4A). Furthermore, the reduced ability of the EspB mutant to grow systemically was not associated with reduced expression of *ler* in infected WT or *Il22*$^{-/-}$ mice (Fig. S4B). In contrast to the EspB mutant, the pathogen load of a *C. rodentium* mutant deficient in EspH, a T3SS effector that regulates phagocytosis (24), was comparable to that of the WT bacterium in infected *Il22*$^{-/-}$ mice (Fig. S5). The Ler mutant that regulates the expression of LEE-encoded proteins including EspB, also exhibited reduced pathogen loads and failed to cause mortality in *Il22*$^{-/-}$ mice when compared to the WT bacterium (Fig. S6). Importantly, growth of the EspB mutant was enhanced *in vivo* by intravenous administration of lysed erythrocytes (Fig. 3H). Furthermore, EspB and Ler promoted heme accumulation in plasma after intravenous infection of *Il22*$^{-/-}$ mice with *C. rodentium* (Fig. 3I and Fig. S6). In contrast, the amounts of iron and the unsaturated iron-binding capacity in plasma before and after infection with *C. rodentium* were comparable in WT and *Il22*$^{-/-}$ mice (Fig. S7). Thus, IL-22 regulates heme, but not free iron, accumulation in the plasma following systemic *C. rodentium* infection. These results indicate that hemolysis driven by LEE-encoded EspB translocator and ensuing release of heme in plasma promotes *C. rodentium* growth *in vivo.*

IL-22-dependent induction of hemopexin inhibits heme-mediated bacterial growth

Analysis of plasma proteins by LC-MS/MS revealed that HPX and HP are positively regulated by IL-22 in response to systemic *C. rodentium* infection (Fig. 2). Immunobloting analysis of plasma proteins confirmed that IL-22 is required for induction of HPX and HP in response to intravenous and oral *C. rodentium* infection (Fig. 4A,B,C). The IL-22 receptor is expressed on epithelial cells including hepatocytes, the major site of HPX and HP production (25, 26). Consistently, intraperitoneal administration of IL-22 induced HPX and HP mRNA in the liver (Fig. S8). To determine whether *C. rodentium* infection induces anti-bacterial activity in plasma, *C. rodentium* colonies were monitored *in vitro* after incubation with plasma from uninfected and infected mice. Plasma from infected WT mice inhibited *C. rodentium* colony formation compared to plasma of uninfected WT mice (Fig. 4D). Notably,

plasma from infected *Il22*$^{-/-}$ mice failed to limit colony formation compared to the plasma of infected WT mice (Fig. 4D). Consistently, intravenous administration of recombinant IL-22 to *Il22*$^{-/-}$ mice restored the plasma anti-bacterial activity (Fig. 4E). To identify the mechanism underlying this anti-bacterial activity in plasma, we fractionated plasma proteins from IL-22-treated mice by column chromatography and assessed the pathogen inhibitory activity in the fractions by agar plating assay. Immunoblotting analysis showed that the presence of the heme scavenger HPX, but not the hemoglobin scavenger HP, correlated with the ability of the plasma fractions to suppress *C. rodentium* colony formation (Fig. 4F). To determine whether heme promotes *C. rodentium* growth, the pathogen was incubated *in vitro* with and without synthetic heme (i.e. hemin) in the presence of increasing concentrations of HPX. In the absence of HPX, hemin promoted robust *C. rodentium* colony formation in vitro (Fig. 4G), while addition of HPX, at a concentrations range found in mouse plasma, i.e. ~500-1000 μg/ml (27), inhibited hemin-driven pathogen growth in a dose-dependent manner (Fig. 4G). Hemoglobin also promoted robust *C. rodentium* colony formation in vitro, which was inhibited by HPX in a dose-dependent manner (Fig. 4H) whereas HP did not (Fig. S9). Furthermore, exogenous HPX largely restored the anti-bacterial activity of the plasma isolated from infected *Il22*$^{-/-}$ mice in vitro (Fig. 4I). Collectively, these results suggest that once released from extracellular hemoglobin, heme promotes *C. rodentium* growth, a pathogenic effect countered via a mechanism mediated by the IL-22-dependent induction of the plasma heme scavenger HPX.

IL-22 limits heme-mediated *E. coli* growth via hemopexin

E. coli, a Gram-negative bacterium that normally inhabits the intestine, is a major cause of bacteremia and sepsis in humans (28, 29)**.** As it was observed with *C. rodentium*, hemin promoted robust colony formation of an *E. coli* strain isolated from the mouse intestine which was inhibited by HPX in vitro (Fig. 5A). Furthermore, administration of recombinant IL-22 to *Il22*$^{-/-}$ mice restored the ability of plasma to suppress *E. coli* colony formation in vitro (Fig. 5B), which was strictly dependent on the expression of endogenous HPX (Fig. 5C). Furthermore, *Il22*$^{-/-}$ and *Hpx*$^{-/-}$ mice were more susceptible and had more bacterial loads than WT mice after intravenous infection with *E. coli* (Fig. 5D,E,F,G).These results indicate that *E. coli* colony formation is promoted by heme which is inhibited by IL-22-mediated induction of HPX.

IL-22 requires hemopexin, but not haptoglobin, to suppress pathogen growth *in vitro* and *in vivo*

We next assessed the requirement for HPX and HP in IL-22-mediated suppression of bacterial growth. Importantly, the ability of plasma from IL-22-treated WT mice to suppress *C. rodentium* colony formation was comparable to that of plasma from *Hp*$^{-/-}$ mice treated with IL-22 (Fig. 6A). In contrast, the plasma of *Hpx*$^{-/-}$ and *Hpx*$^{-/-}$*Hp*$^{-/-}$ mice treated with IL-22 was impaired in suppressing *C. rodentium* growth which was comparable to the plasma of untreated WT mice (Fig. 6B,C). To confirm that IL-22 suppresses pathogen growth *in vivo* via a mechanism involving heme neutralization by HPX, WT, *Hpx*$^{-/-}$, *Hp*$^{-/-}$, and *Hpx*$^{-/-}$*Hp*$^{-/-}$ mice received IL-22 intraperitoneally and then the mice were infected with *C. rodentium* intravenously. While administration of IL-22 induced a comparable reduction of pathogen loads in the blood of WT and *Hp*$^{-/-}$ mice (Fig. 6D), this anti-bacterial effect

was lost in *Hpx*$^{-/-}$ and *Hpx*$^{-/-}$*Hp*$^{-/-}$ mice (Fig. 6E,F). Thus, IL-22 requires HPX, but not HP, to suppress pathogen growth *in vitro* and *in vivo.* To assess whether HPX is sufficient to promote pathogen clearance in the absence of IL-22 administration, WT, *Hpx*$^{-/-}$, *Hp*$^{-/-}$, and *Hpx*$^{-/-}$*Hp*$^{-/-}$ mice were infected with *C. rodentium* intravenously and pathogen loads and mouse survival were monitored in infected mice. There was impaired pathogen clearance in *Hpx*$^{-/-}$ and *Hpx*$^{-/-}$*Hp*$^{-/-}$ mice, but not *Hp*$^{-/-}$ mice, compared to WT mice (Fig. 6G). Furthermore, administration of HPX reduced the pathogen load in *Il22*$^{-/-}$ mice compared to mock-treated mice after intravenous (Fig. 6H) and oral infection (Fig. 6I,J). Together, these results indicate that IL-22 limits the growth of *C. rodentium* via HPX *in vivo*.

Discussion

IL-22 has been primarily linked to the regulation of host defense, cellular proliferation and tissue repair at intestinal barriers (10). In the current studies, we provide evidence for a role of IL-22 in protecting the host against the enteric pathogen *C. rodentium* and *E. coli* by mediating the production of HPX in plasma. Although produced by immune cells in response to microbial stimuli, IL-22 stimulates its heterodimeric IL-22RA-1/IL-10R receptor on epithelial cells including hepatocytes that are known to produce large amounts of HPX (18). Induction of HPX in response to systemic infections was so far thought to protect the infected host from heme-induced cell toxicity, inflammation and multi-organ dysfunction (30), without interfering with the host pathogen load and as such conferring disease tolerance to systemic bacterial infections (30, 31). This protective effect of HPX and its removal by macrophages and parenchymal cells has also been extended to several other pathologies associated with varying degrees of hemolysis (25). Our work demonstrates that IL-22-induced HPX also confers resistance to systemic bacterial infections by limiting the heme availability to bacteria. Pathogens possess several mechanisms to overcome iron-limiting defenses induced by the host including siderophores, hemophores and heme/hemoprotein receptors (1). Thus, pathogens capable of colonizing iron-poor niches are likely to be less susceptible to IL-22-mediated HPX induction. *C. rodentium* and *E. coli* appear to rely on strategies to acquire iron from heme when they colonize the plasma and systemic organs. *C. rodentium* and *E. coli* produce enterobactin, a siderophore, and its receptor FepA to acquire iron and may express functional heme uptake systems (32, 33). Our results suggest that *C. rodentium* promotes hemolysis and heme acquisition through pore formation via T3SS-mediated factors and specifically EspB/EspD. EspH, a T3SS effector that regulates phagocytosis, was dispensable for pathogen growth in *IL22*$^{-/-}$ mice. These findings suggest that the T3SS promotes bacterial growth by inducing hemolysis. However, there maybe additional factors including compromised ability to block phagocytosis that might contribute to the impaired ability of T3SS mutants to infect the mice. Further work is needed to understand how *C. rodentium* and *E. coli* acquire iron from heme in vivo.

Our studies indicate that pathogenic bacteria such as A/E pathogens take advantage of virulence strategies such as expression of Ler-dependent factors normally used to colonize the intestinal epithelium to survive in alternative niches such as the blood in the event of causing bacteremia. Administration of IL-22-binding protein (IL-22-BP), a natural antagonist of IL-22 signaling, has been reported to enhance the systemic clearance of commensal bacteria in a model of polymicrobial sepsis (34). Further work is needed to

determine whether the protective function of IL-22-BP is mediated through the induction of HPX and/or another mechanism. Our results suggest that strategies to scavenge heme in plasma, such as that afforded by the administration of IL-22, HPX or other approaches may be beneficial in the treatment of systemic bacterial infections.

Material and Methods

Study design

The aim of this study was to elucidate and characterize the mechanism by which IL-22 mediates systemic protection against bacterial infection using the *C. rodentium* and *E. coli* models in mice. The experimental design involved in vivo and in vitro experiments, including protein identification by LC-tandem MS, chromatographic fractionation of plasma proteins, immunoblotting, histological analysis, reverse transcription polymerase chain reaction (RT-PCR) analysis and bacterial colony enumeration. The animal experiments were not randomized. The investigators were not blinded to the allocation during experiments and analyses unless otherwise indicated. Experimental replication is indicated in the figure legends.

Animals

Six to eight weeks old WT C57BL6 mice were bred in our animal facility. *Il22*$^{-/-}$, *Hp*$^{-/-}$, *Hpx*$^{-/-}$, *and Hp*$^{-/-}$*Hpx*$^{-/-}$, mice on C57BL6 background have been described (35, 36) and bred under specific pathogen-free (SPF) conditions at the University of Michigan Cancer Center. *Il22*$^{-/-}$ mice were obtained from Genentech. GF mice were bred and maintained at the GF Animal Core Facility of the University of Michigan. The animal studies were conducted under protocols approved by the University of Michigan Committee on Use and Care of Animals.

Citrobacter rodentium infection

Kanamycin (Km)-resistant WT *Citrobacter rodentium* strain DBS120 (pCRP1::Tn5) was a gift of Dr. David Schauer, Massachusetts Institute of Technology. The isogenic *C. rodentium Δler* mutant strain has been described (37). *C. rodentium* strains with nonpolar deletion mutants of *espA, espB and espH* have been described (23). For inoculations, bacteria were grown overnight in Luria-Bertani (LB) broth supplemented with Km (50 μg/ml) with shaking at 37°C. Mice were infected by oral gavage with 0.2 mL of PBS containing approximately 1×10^9 colony-forming units (CFU) of *C. rodentium*, or by intravenous injection with 0.2 mL of PBS containing approximately 5×10^7 CFU of *C. rodentium*. The investigators were not blinded to allocation during experiments. To mimic the continuous source of bacteremia observed in *Il22*$^{-/-}$ mice orally infected with the pathogen, mice were infected with 5×10^7 CFU of *C. rodentium* intravenously daily for 9-10 days. To assess the role of HPX in *C. rodentium* clearance in vivo, *Il22*$^{-/-}$ mice were orally infected with the pathogen (1×10^9 CFU) and the mice were treated intravenously with purified HPX (Athens Research and Technology; 2 mg/mouse) or saline on day 3, 4, 5 and 6 post-infection. For systemic infections, HPX (2 mg/mouse) or saline (sham) was injected intravenously on three consecutive days before infection or control (sham) into *Il22*$^{-/-}$ mice and then the mice were infected with 5×10^7 CFU of *C. rodentium* intravenously. To determine pathogen loads in the

feces, fecal pellets were collected from individual mice, homogenized in cold PBS and plated at serial dilutions onto MacConkey agar plates containing 50 μg/ml Km, and the number of CFU was determined after overnight incubation at 37°C. To determine bacterial number in the blood, liver and spleen, mice were euthanized at various time points post-infection and blood or tissue samples were plated onto MacConkey agar containing 50 μg/ml Km, and the number of CFU was determined after overnight incubation at 37°C.

IL-22 neutralization and administration

To deplete endogenous IL-22 in mice, anti-IL-22 (150 μg/mouse per dose; 8E11; Genentech) or the equivalent amount of mouse isotype-matched control antibody (to ragweed; 10D9.1E11.1F12; Genentech) were injected to the mice 3 times a week. To treat mice with IL-22, mice were injected intravenously with Fc-IL-22 (50 μg per dose; PRO312045; Genentech) or the equivalent amount of mouse isotype-matched control antibody (to ragweed; 10D9.1E11.1F12; Genentech) daily for 3 days, and 6 hrs after from final injection.

Escherichia coli infection

E. coli strain NI1076 was isolated from the mouse intestine mice as described (17). For infections, bacteria were grown overnight in Luria-Bertani (LB) broth with shaking at 37°C. Mice were infected by intravenous injection with 0.2 mL of PBS containing approximately 2×10^8 CFU of *E. coli.*

Bacterial growth assays

1×10^5 CFU/mL of *C. rodentium* or *E. coli* were incubated in DMEM in the presence of 500 μg/mL of Apo-transferrin (Athens Research and Technology) to chelate free iron. To assess bacterial growth, hemin (2.5 μg/ml; Sigma) or hemoglobin (25 μg/ml; Sigma) was added to the culture medium in the presence and absence of different concentrations of HPX (Athens Research and Technology) or HP (Athens Research and Technology).

Statistical analysis

Statistical significance was calculated as indicated in the figure legends using GraphPad Prism 6 software. Log-rank test was used to assess mouse survival. Non-parametric Mann-Whitney test was used for pair comparisons and Dunn's test for multiple comparisons. Differences were considered significant when p values were less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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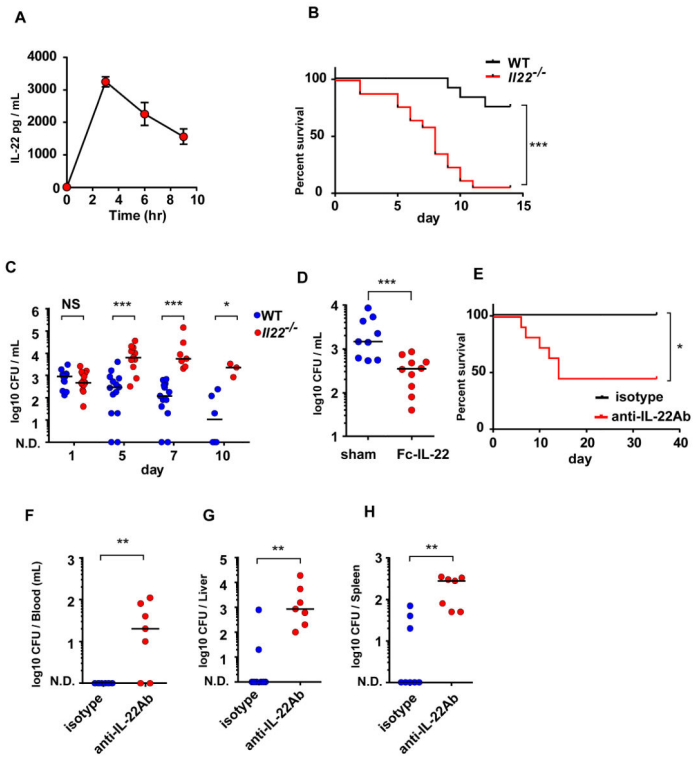

Figure 1. IL-22 promotes pathogen clearance and host survival after systemic infection with *C. rodentium*

A, Production of IL-22 after a single intravenous injection of *C. rodentium* (5×10^7 CFU) into WT mice (n=4). IL-22 amounts in plasma on indicated time points after injection are shown. **B,** Survival of WT (n= 12) and *Il22*$^{-/-}$ (n= 17) mice after intravenous pathogen infection. Mice were injected daily with 5×10^7 CFU of *C. rodentium* from day 0 to 9 to mimic bacteremia resulting from oral infection of *Il22*$^{-/-}$ mice. **C,** Pathogen loads in the blood on the indicated time points after systemic *C. rodentium* infection using bacteremia model employed in panel B. **D,** The effect of IL-22 administration on bacterial clearance. *Il22*$^{-/-}$ mice were treated with Fc-IL-22 or control protein (sham), and pathogen loads in blood were measured 18 hrs after a single intravenous injection with 5×10^7 CFU of *C. rodentium*. **E,** Mouse survival of GF mice pre-treated with anti-IL-22 neutralizing antibody (n= 11) or isotype-matched control antibody (n=9) infected orally with *C. rodentium* (1×10^9 CFU). **F-H,** Pathogen loads in the blood (**F**), liver (**G**), and spleen (**H**) of GF mice pre-treated with anti-IL-22 neutralizing antibody or control antibody. Pathogen loads were measured on day 7 after oral *C. rodentium* infection. The data in panels **A-H** are representative of at least 2 independent experiments. Each circle represents one mouse (**C, D, F, G, H**). Median values are indicated by a horizontal bar. NS, not significant, *p<0.05, **p<0.01, ***p<0.001, log-rank test (**B, E**), Mann-Whitney test (**C, D, F, G, H**).

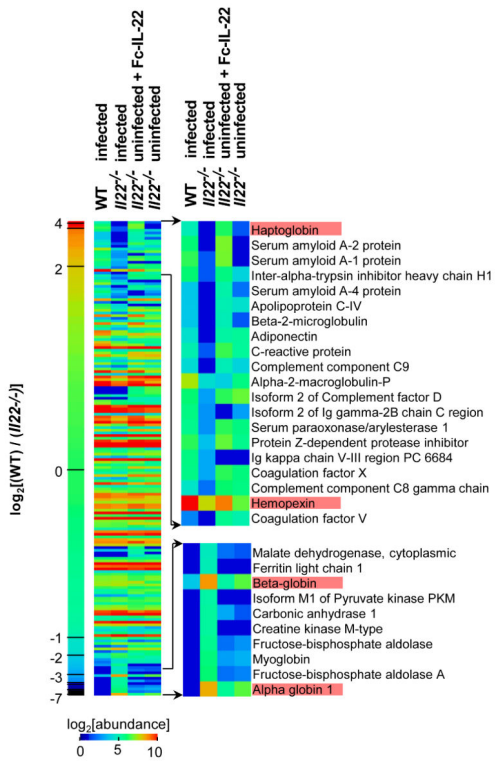

Figure 2. IL-22-mediated regulation of plasma proteins after *C. rodentium* infection

Heat-map analysis of protein abundance in plasma samples from indicated mice. Protein analysis was performed by LC-MS/MS. Left panel shows the abundance of all detected proteins. Right panel shows proteins increased (upper) or decreased (lower) in samples from infected *IL22*$^{-/-}$ mice compared to samples from infected WT mice or *Il22*$^{-/-}$ mice treated with Fc-IL-22 compared to *Il22*$^{-/-}$ treated with control protein. Plasma samples were collected from mice on day 7 after intravenous *C. rodentium* infection (infected WT and *Il22*$^{-/-}$ mice). Data are representative of two different experiments. HPX, HP, α- and β-globin are boxed in light red.

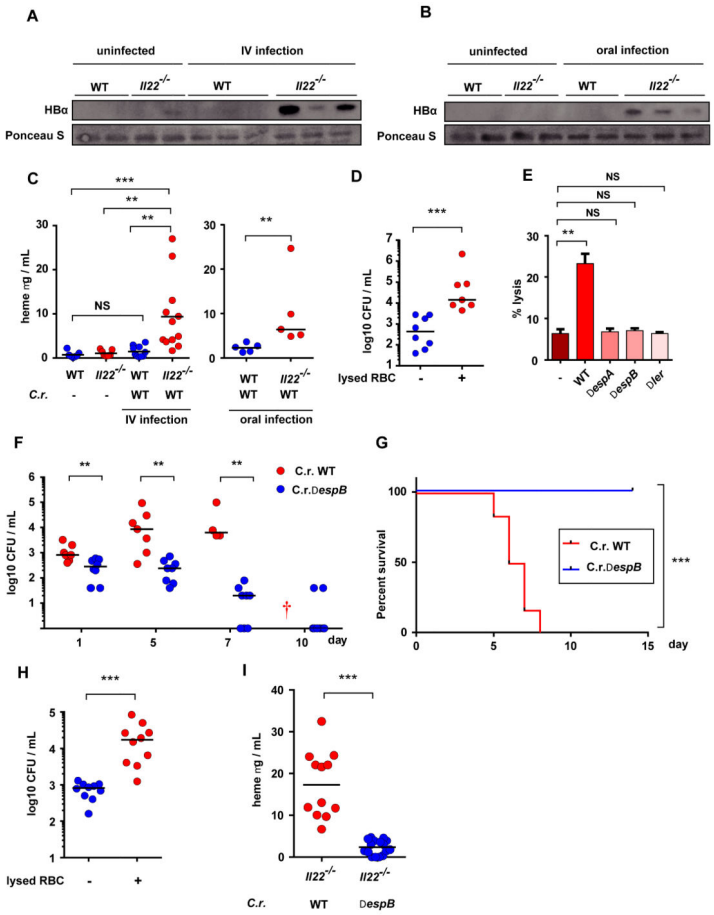

Figure 3. Erythrocyte lysis and heme release via the EspB translocator promote pathogen growth in vivo

A-B, Detection of hemoglobin subunit alpha (HBα) in the plasma of uninfected and infected *Il22*$^{-/-}$ mice. Mice were infected intravenously using bacteremia model (**A**) or orally (**B**) with *C. rodentium*. Plasma samples (n= 3 mice for each group) were imunoblotted with an antibody against HBα. Ponceau S staining of the gel is also shown. **C,** The amounts of heme in plasma were measured in uninfected mice and mice infected intravenously (left panel) or orally (right panel) with *C. rodentium* (C. r.). **D,** Administration of lysed erythrocytes enhances pathogen growth in vivo. *Il22*$^{-/-}$ mice were co-injected intravenously with lysed red blood cells (RBC) or vehicle and 5×10^7 CFU of *C. rodentium* and pathogen loads in blood were measured at 18 hrs post-infection. **E**, Erythrocyte lysis induced by WT and indicated mutant *C. rodentium* strains. Data are shown as mean of quintuplicates ± s. d. **F,** Pathogen loads in blood in bacteremia model used in Fig. 1B. *Il22*$^{-/-}$ mice were infected intravenously with WT or *espB* mutant *C. rodentium* strains. Blood samples were collected at the indicated time points. † indicates all mice succumbed. **G,** Survival of *Il22*$^{-/-}$ mice in the bacteremia model after infection with WT (n= 6) or *espB* mutant (n=6) *C. rodentium* strains. **H,** Loads of *espB* mutant *C. rodentium* in blood of *Il22*$^{-/-}$ mice at 18 hrs post-infection with or without lysed RBC. **I,** The amounts of heme in plasma of indicated mice were measured after intravenous infection with WT or *espB* mutant *C. rodentium (C. r.)* strains. The data in panels **A-I** are representative of at least two independent experiments.

Each circle (**C, D, F, H, I**) represents one mouse. Median values are indicated by a horizontal bar. NS, not significant, *p<0.05, **p<0.01, ***p<0.001, Dunn's test (**C,** left panel, **E**), Mann Whitney test (**C** right panel, **D, F, H, I**), log-rank test (**G**).

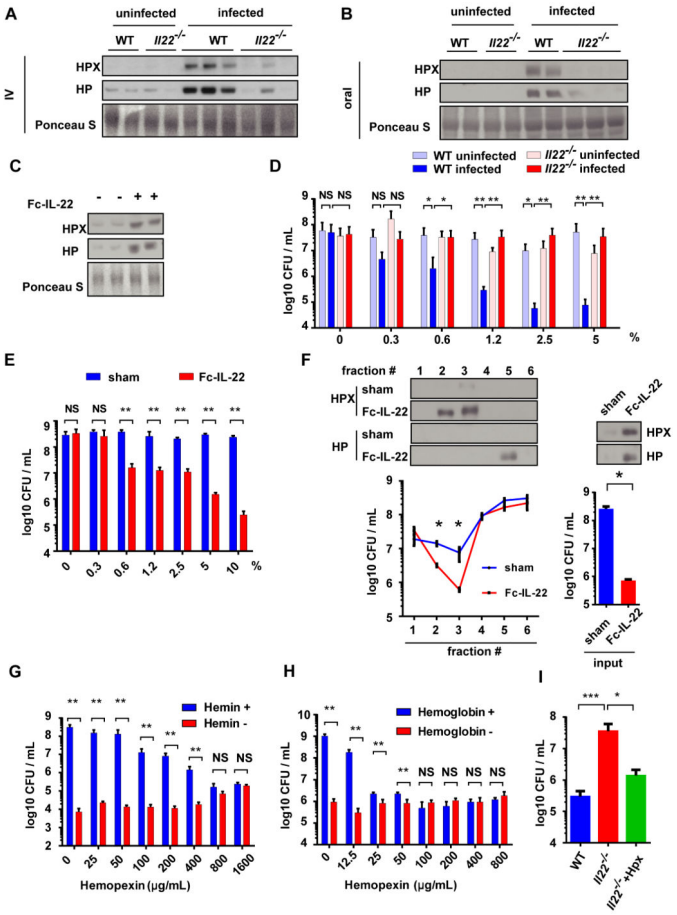

Figure 4. Induction of HPX by IL-22 is essential for inhibition of *C. rodentium* growth in the presence of heme

A, B. Detection of HPX and HP in the plasma of uninfected and infected animals. WT (n= 2-3 per group) or *Il22*$^{-/-}$ (n= 2-3 per group) mice were left uninfected or infected with WT *C. rodentium* i. v. (**A**) or orally (**B**) and the plasma samples were collected and immunoblotted with antibodies against HPX (Hpx) and HP (Hp). Staining of gels with Ponceau S is also shown. **C,** Detection of HPX and HP in the plasma of *Il22*$^{-/-}$ mice left untreated (−) or treated with recombinant Fc-IL-22. **D,** Growth of *C. rodentium* in the presence of plasma from WT and *Il22*$^{-/-}$ mice. Bacterial growth was measured by plating. **E,** Growth of *C. rodentium* in the presence of plasma from *Il22*$^{-/-}$ mice treated with Fc-IL-22 or control protein (sham). Experiments were performed as in panel **D**. **F,** Mice were treated with Fc-IL-22 or control protein, and plasma samples were fractionated by column chromatography. Sequential fractions were pooled into 6 fractions that were analyzed for their ability to promote *C. rodentium* colony formation and for the presence of HPX or HP by immunoblotting. Analysis of unfractionated plasma samples is shown on right panel. **G,** Hemin promotes *C. rodentium* colony formation which is inhibited by HPX. Bacteria were incubated with and without hemin in the presence of indicated concentration of HPX. Bacterial growth was measured by plating. Data are shown as mean of sextuplicate cultures ± s. d. **H,** Hemoglobin promotes *C. rodentium* colony formation which is inhibited by HPX. Bacteria were incubated with and without hemoglobin in the presence of indicated

concentration of HPX. Bacterial growth was measured by plating. **I,** Addition of HPX enhances the ability of plasma from *Il22*$^{-/-}$ mice to inhibit *C. rodentium* colony formation. Data in **D** and **E** represent means of six different mouse samples ± s. d. Data in **G** and **H** represent means of 6 technical replicates. The data in panels **A-I** are representative of at least two independent experiments NS, not significant, *p<0.05, **p<0.01, ***p<0.001, Dunn's test (**D, I**), Mann Whitney test (**C** right panel, **E, F, G, H**).

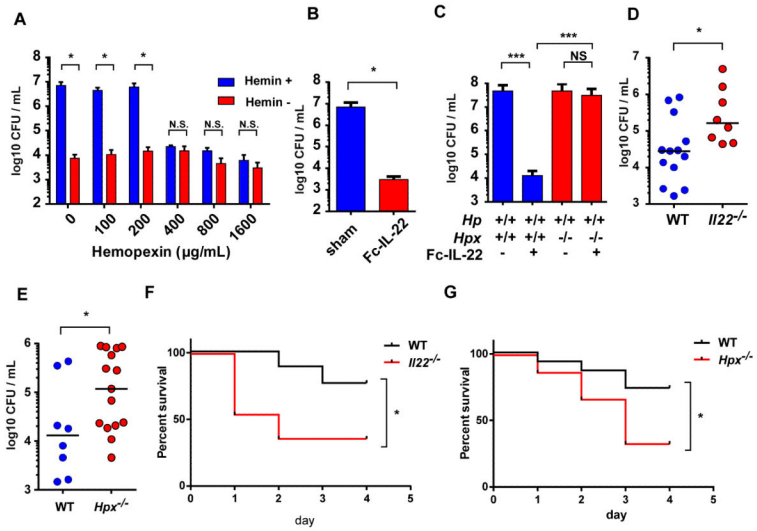

Figure 5. Induction of HPX by IL-22 inhibits *E. coli* growth

A, Hemin promotes *E. coli* growth in vitro which is inhibited by HPX. Bacteria were incubated with and without hemin in the presence of indicated concentration of HPX. Bacterial growth was measured by plating. Data are shown as mean of quadruplicate cultures ± s. d. **B**, Growth of *E. coli.* in the presence of plasma from *Il22*$^{-/-}$ mice treated with Fc-IL-22 or control protein (sham). Data is shown as mean values ± s. d. **C**, Role of HPX in IL-22-mediated inhibition of *E. coli* growth. Growth of *E. coli* in the presence of plasma from WT and *Hpx*$^{-/-}$ mice pre-treated with Fc-IL-22 or control protein (−). Data are shown as the means of 13 technical replicates ± s. d. **D, E,** Bacterial loads in the blood after a single intravenous injection of *E. coli.* Blood samples were collected from WT (**D, E**), *Il22*$^{-/-}$ mice (**D**) and *Hpx*$^{-/-}$ mice (**E**) at 18 hrs post-infection. **F, G,** Survival of WT (n= 9) and *Il22*$^{-/-}$ (n= 11) (**F**) and WT (n=15) and *Hpx*$^{-/-}$ mice (n= 15) (**G**) after intravenous *E. coli* infection. The data in panels **A-E** are representative of at least two independent experiments. Data in panels **F** and **G** are results from two pooled experiments. Each symbol represents one mouse. Median values are indicated by a horizontal bar. NS, not significant, *p<0.05, Mann Whitney test (**A, B, D, E**), ***p<0.001, Dunn's test (**C**), *p<0.05, log-rank test (**F, G**).

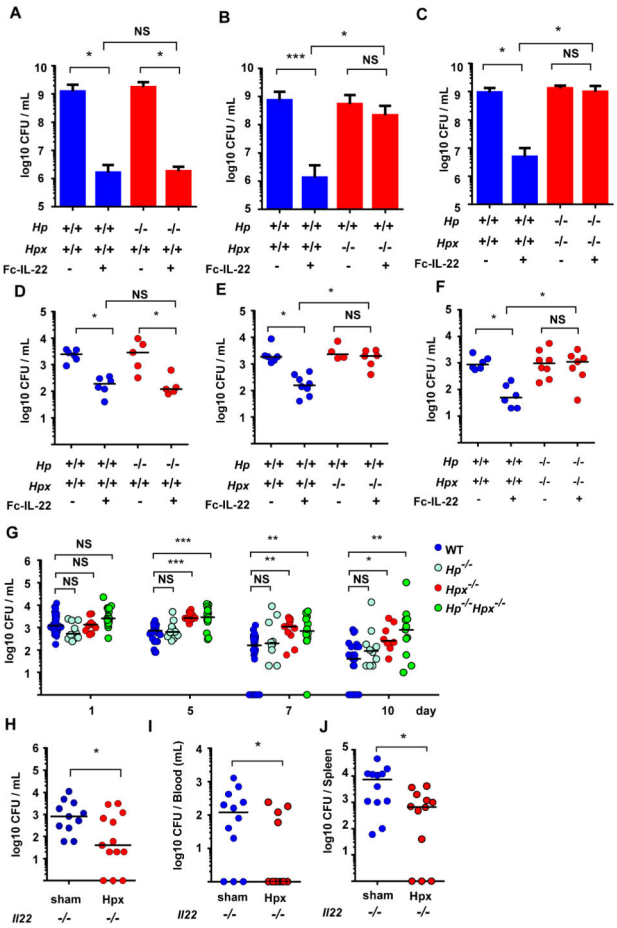

Figure 6. HPX, but not HP, is essential for inhibition of bacterial growth *in vivo*

A-C, Role of HPX and HP in IL-22-mediated inhibition of *C. rodentium* growth. Growth of *C. rodentium* in the presence of plasma samples from WT, $Hp^{-/-}$, $Hpx^{-/-}$, or $Hp^{-/-}Hpx^{-/-}$ mice pre-treated with Fc-IL-22 or control protein (−). Data are shown as mean of at least six different mouse samples ± s. d. **D-F,** WT, $Hp^{-/-}$, $Hpx^{-/-}$, or $Hp^{-/-}Hpx^{-/-}$ mice were treated with Fc-IL-22 or control protein and infected with 5×10^7 CFU of *C. rodentium* i. v. after Fc-IL-22 or control protein administration. Pathogen loads in blood 18 hrs after infection are shown. **G**, Pathogen loads in the blood of indicated mice after *C. rodentium* infection using the bacteremia model shown in Fig. 1B. Pathogen loads in blood at the indicated time points are shown. **H,** HPX or saline (sham) was injected intravenously into $Il22^{-/-}$ mice and then the mice were infected with 5×10^7 CFU of *C. rodentium* intravenously. Pathogen loads in blood were measured 18 hrs after infection. **I-J**, $Il22^{-/-}$ mice were infected orally with 10^9 CFU of *C. rodentium* and purified HPX or saline (sham) was injected intravenously into the mice on day 3, 4, 5 and 6 after infection. Pathogen loads in blood (H, I) and spleen (J) were measured on day 7 after infection. Each symbol represents one mouse (**D-J**). Median values are indicated by horizontal bar (**D-J**). The data in panels **A-J** are representative of at least two independent experiments. NS, not significant *p<0.05, **p<0.01, ***p<0.001, Dunn's test (**A-G)**, Mann Whitney test (**H-J**).