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Hepatocytes and neutrophils cooperatively suppress bacterial infection by differentially regulating lipocalin-2 and NETs

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Abstract

Lipocalin-2 (LCN2)/neutrophil gelatinase-associated lipocalin (NGAL), a key anti-bacterial protein, are highly elevated in patients with end-stage liver disease that is often associated with bacterial infection. LCN2 is expressed at high levels in both hepatocytes and neutrophils; however, how hepatocyte- and neutrophil-derived LCN2 cooperate to combat bacterial infection remains unclear. Here by studying hepatocyte- and myeloid-specific *Lcn2* knockout mice in two models of systemic and local *Klebsiella pneumoniae* infections, we demonstrated that hepatocytes played a critical role in controlling systemic infection by secreting LCN2 protein into the circulation post intraperitoneal injection of bacteria, whereas neutrophils were more important in combating lung local infection by carrying LCN2 in their specific granules to the local infection site post intratracheal intubation of bacteria. Both hepatocyte- and myeloid cell-derived LCN2 were required against bacterial infection in peritoneal cavity and liver necrotic areas post intraperitoneal injection of *Klebsiella pneumoniae*. LCN2/NGAL protein was detected in neutrophil extracellular traps (NETs) in activated neutrophils from mice and humans. Disruption of the *Lcn2* gene in neutrophils abolished LCN2 on NETs, whereas deletion of this gene in hepatocytes did not affect LCN2 protein on NETs. Genetic deletion of the *Lcn2* gene globally or specifically in neutrophils

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did not affect NET formation, but reduced the bactericidal effect of NETs *in vitro*. Finally, NGAL positive NETs were detected in the liver from patients with various types of liver diseases.

Conclusion—Both hepatocytes and neutrophils combat bacterial infection through the production of LCN2. Extracellular LCN2 secreted by hepatocytes limits systemic bacterial infection; whereas neutrophils carry LCN2 protein to the local site and against local bacterial infection through NETs.

Keywords

Klebsiella pneumoniae; myeloid cells; liver; neutrophil gelatinase-associated lipocalin

Introduction

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin [NGAL] (human ortholog of mouse *Lcn2* gene), is one of the most extensively investigated acute phase proteins because its levels are highly elevated (up to ~6μg/ml in the serum[(1)]) under all inflammatory conditions [(2)] including in liver disease.[(3)] It is well documented that LCN2 plays a critical role in host defense against bacterial infection by sequestering iron-containing siderophores.[(4)] Siderophores are amongst the strongest soluble Fe^{3+} binding agents known, and bacteria obtain iron by producing siderophores. LCN2 can bind to and subsequently sequester iron-containing siderophores, thereby preventing bacteria uptake of iron and limiting their growth.[(4)] In contrast to its well-established bacteriostatic function, the other functions of LCN2 are less clear and reports regarding this area are controversial. For example, the experimental data suggest that LCN2 promotes liver injury and inflammation in alcoholic steatohepatitis [(5, 6)] and nonalcoholic steatohepatitis (NASH),[(7)] but other studies suggest that LCN2 protects against acute liver injury induced by hepatotoxins,[(8)] and promotes liver regeneration.[(1)] Clinical studies have revealed that serum NGAL levels are markedly elevated in various types of liver diseases, and NGAL is a predictor for mortality and bacterial infection in patients with liver cirrhosis,[(9–12)] suggesting that LCN2/NGAL is associated with bacterial infection in liver cirrhosis. It is well known that both hepatocytes and neutrophils are two important cell types to produce LCN2/NGAL and combat bacterial infection;[(1, 13)] however, how these two types of cells cooperate to combat bacterial infection via the production of LCN2/NGAL remains unclear.

The liver is a critical innate immunity organ in the body against invading microorganisms by secreting a large number of innate immunity proteins including LCN2/NGAL into the blood. [(14)] LCN2 is highly elevated in the serum in mice post bacterial infection (from ~60 ng/ml at basal levels to ~6μg/ml post infection).[(1)] Such highly elevated circulating LCN2 levels are mainly secreted by hepatocytes [(1)] and play a critical role in inhibiting bacterial infection by sequestrating iron-chelating siderophores.[(4)] Interestingly, neutrophils also express high levels of LCN2, which are usually stored in their specific granules.[(13)] Both the extracellular LCN2 produced by hepatocytes and the intracellular LCN2 stored in the granules of neutrophils likely play important roles in host defense against bacterial infection;[(15)] however, how these two forms of LCN2 interact and combat bacterial infection is not fully understood.

Neutrophils are the most abundant innate immune cells in the human body. To control bacterial infection, neutrophils migrate from peripheral blood to infected tissues and perform their functions against bacterial infection through three strategies: phagocytosis, degranulation and the formation of neutrophil extracellular traps (NETs). Formation of NETs is a recently identified mechanism to kill bacteria by neutrophils via both cell death-dependent and -independent pathways.[16, 17] The death pathway termed NETosis is associated with nuclear delobulation, loss of cellular polarization, chromatin decondensation and plasma membrane rupture.[16, 17] The cell death-independent pathway is mediated by expelling nuclear chromatin and releasing granule proteins through degranulation.[16, 17] Conversion of histone arginines to citrullines by peptidyl arginine deiminase 4 (PAD4) is a critical step of NET formation following specific types of stimulation, which reduces the strong positive charge of histones, weakens histone-DNA binding, and subsequently unwraps the nucleosomes.[18] Proteomic analyses of NET protein composition have detected up to 80 proteins in NETs including several granule proteins: neutrophil elastase, myeloperoxidase (MPO), defensins, NGAL etc.[19–21] The granule protein LCN2/NGAL was also identified in zymography analyses of purified NETs in which a 125-kDa high molecular weight gelatinase, a complex of MMP-9 and NGAL,[22] was detected.[23] However, the role of LCN2 in NET formation and function has not been systematically investigated.

Our previous study has shown that hepatocyte-derived LCN2 is secreted to circulating blood to inhibit bacterial infection.[1] Given that LCN2 is also stored in the specific granules of neutrophils, the role of intracellular LCN2 is not clear. Thus, we generated myeloid-specific *Lcn2* knockout (KO) ($Lcn2^{Mye-/-}$) mice besides with hepatocyte-specific *Lcn2* KO ($Lcn2^{Hep-/-}$) and double KO mice, subjecting them to LPS injection and *K. pneumoniae* infection models. Using these mice, we demonstrate that both hepatocytes and myeloid cells combat bacterial infection through the production of LCN2. Extracellular LCN2 protein secreted by hepatocytes plays a critical role in limiting systemic bacterial infection; whereas intracellular LCN2 protein from neutrophils is a component of NETs, playing a critical role in suppressing local bacterial infection through NETs.

Materials and Methods

Animals

$Lcn2^{flox/flox}$ mice were described previously.[1] Hepatocyte-specific *Lcn2* knockout (KO) mice ($Lcn2^{Hep-/-}$) and myeloid-specific *Lcn2* KO mice ($Lcn2^{Mye-/-}$) were generated by a few steps including crossing $Lcn2^{flox/flox}$ mice with Albumin Cre mice and Lysosome Cre mice (The Jax Laboratory, Bar Harbor, ME), respectively. $Lcn2^{Hep-/-}$ mice were then crossed with $Lcn2^{Mye-/-}$ mice to obtain four lines of mice: $Lcn2^{Hep-/-}$, $Lcn2^{Mye-/-}$, double KO ($Lcn2^{DKO}$), and $Lcn2^{flox/flox}$ mice. The littermate $Lcn2^{flox/flox}$ mice were used as wild-type (WT) control. *Lcn2* global KO mice ($Lcn2^{-/-}$) on a C57BL/6N background were described previously.[1] All mice were cared for in accordance with NIH guidelines. All animal studies were approved by the NIAAA Animal Care and Use Committee.

Animal models

For lipopolysaccharides (LPS) model, mice were injected intraperitoneally with LPS from *Escherichia coli* 026:B6 (Sigma, St. Louis, MO) in a dose of 1mg/kg. For systemic bacterial infection model, mice were injected intraperitoneally with 100μl of a low dose of *K. pneumoniae* strain 43816 (5×10^3 CFU)(ATCC, Manassas, VA) or a high dose of 5×10^4 CFU. For lung bacterial infection model, mice were given *K. pneumoniae* strain 43816 (5×10^6 CFU) through a noninvasive intratracheal intubation as described.[24] All mice were sacrificed under anesthesia by inhalation of isoflurane.

Human liver samples

Cirrhotic liver tissues from different etiologies (eg. nonalcoholic steatohepatitis [NASH], viral hepatitis C [HCV], autoimmune hepatitis [AH]), and liver tissues from acetaminophen-induced liver failure (APAP) were obtained through the Liver Tissue Procurement and Distribution System (NIH Contract #HHSN276201200017C), University of Minnesota. Severe alcoholic hepatitis (SAH) patient and control human livers were obtained from the Clinical Resources for Alcoholic Hepatitis (1R24AA025017), Johns Hopkins University as described previously.[25] All samples were collected by snap-freezing in liquid nitrogen.

NET formation and quantification *in vitro* and *in vivo*

NET quantification was to co-stain with a nuclear marker (eg. DNA, cit-histone) and a neutrophil granule marker (eg. MPO, elastase, LCN2/NAGL). In the current paper, several different combinations were used to quantify NETs. Histone citrullination specifically occurs during NET formation,[18] so we used co-staining of cit-histone and a neutrophil marker for *in vivo* NET quantification. See details in supporting materials.

Statistical Analysis

GraphPad Prism 7 (GraphPad Software, La Jolla, CA) was used to analyze data. To compare values obtained from three or more groups, a one-way ANOVA was used, followed by Tukey post-hoc test. To compare values between two groups, unpaired T test was used. Data were presented as means ± SEM, with a significant difference at the level of $P<0.05$.

Other methods are described in supporting materials.

Results

LPS injection elevates intracellular LCN2 expression in neutrophils and extracellular LCN2 secretion from hepatocytes

LPS injection upregulated LCN2 protein and *Lcn2* mRNA expression in different mouse organs including liver, kidney, intestine and spleen (Supporting Fig. 1A, B). Among them, the liver had the highest fold induction of *Lcn2* mRNA post LPS injection (Supporting Fig. 1B), which was further investigated. As illustrated in Fig. 1A, hepatic *Lcn2* mRNA was markedly upregulated more than 200-fold with peak effect 6 hours after LPS injection. But surprisingly, immunohistochemistry analysis revealed that strong LCN2 protein staining was observed in non-parenchymal/immune cells but not in hepatocytes with strongest staining 6 hours post injection (Fig. 1B, supporting Fig. 2A). To further confirm whether strong LCN2

protein is expressed in neutrophils, we performed double staining of LCN2 and MPO. As illustrated in Fig. 1C and supporting Fig. 2B, more than 80% LCN2 positive staining was overlapped with MPO staining in liver tissues from mice 6 hours post LPS injection, suggesting strong LCN2 protein is detected in intrahepatic neutrophils post LPS injection. Interestingly, the number of $LCN2^+$ cells was markedly decreased with only approximately 40% and 20% MPO^+ cells being LCN2 positive 12 and 24 hours post LPS injection, which may be because LCN2 proteins were secreted through the granule release at later time points after neutrophil activation. Moreover, LCN2 protein was barely detected in the normal livers from mice without LPS treatment (control group) (Fig. 1B–C). Finally, qRT-PCR analyses demonstrated that *Lcn2* mRNA levels were highly upregulated in the isolated intrahepatic neutrophils from LPS-treated mice (Fig. 1D).

To examine whether hepatocytes expressed LCN2, hepatocytes were isolated and subjected to qRT-PCR and western blot analyses. As illustrated in Fig. 1D, *Lcn2* mRNA levels in hepatocytes were upregulated by more than 200-fold in LPS-treated mice compared to control mice. LCN2 protein expression was detected at low levels in these isolated hepatocytes with the peak effect occurring 12 hours post LPS injection (supporting Fig. 2C).

The above data revealed that *Lcn2* mRNA levels were highly elevated but LCN2 protein levels were detected at low levels in hepatocytes, while high LCN2 protein levels were detected in neutrophils post LPS injection, suggesting that LCN2 protein is rapidly secreted from hepatocytes but is stored in neutrophils. To confirm this observation, we generated hepatocyte-specific *Lcn2* ($Lcn2^{Hep-/-}$) knockout mice and myeloid-specific *Lcn2* ($Lcn2^{Mye-/-}$) knockout mice. As illustrated in Fig. 1E, serum LCN2 protein levels were significantly elevated in wild-type (WT) mice post LPS injection (~ 6μg/ml). Compared to WT mice, $Lcn2^{Hep-/-}$ mice had significantly lower serum levels of LCN2 protein (~1μg/ml). There was a trend toward a decrease in serum LCN2 levels in $Lcn2^{Mye-/-}$ mice when compared with WT mice but did not reach statistical difference (Fig. 1E). Serum ALT levels were comparable among these three groups (Fig. 1E).

Deletion of the *Lcn2* gene in hepatocytes or in myeloid cells has minimal effect on LPS-induced inflammation

LCN2 was reported to play a role in inducing neutrophil infiltration and inflammation,[7, 26] but this notion was not confirmed by other studies.[15] To further clarify the role of LCN2 in neutrophil and macrophage recruitment and inflammation, we examined hepatic MPO^+ neutrophils, $F4/80^+$ macrophages, and cytokines, and serum cytokines in WT, $Lcn2^{Hep-/-}$, and $Lcn2^{Mye-/-}$ mice treated with LPS. As illustrated in Fig. 1F, immunostaining analyses revealed that MPO^+ cells and $F4/80^+$ cells were detected equally among all three groups. As expected, $LCN2^+$ cells were barely detected in $Lcn2^{Mye-/-}$ mice, but detected equally between WT and $Lcn2^{Hep-/-}$ mice. Quantitative RT-PCR and flow cytometric analyses also confirmed that hepatic expression of the neutrophil marker *Ly6g* and intrahepatic $CD11b^+Gr1^+$ neutrophil numbers were comparable among all three groups (supporting Fig. 3A). In addition, we also measured expression of CD62L levels, a marker of neutrophil activation (downregulation of CD62L represents neutrophil activation). As shown in supporting Fig. 3A, intrahepatic $CD11b^+Gr1^+CD62L^+$ neutrophil numbers were comparable among WT,

Lcn2$^{Hep-/-}$, and *Lcn2*$^{Mye-/-}$ mice treated with LPS, suggesting that LCN2 does not affect neutrophil activation and that the decreased serum LCN2 level in LPS-treated *Lcn2*$^{Hep-/-}$ mice is not due to the reduction of neutrophil activation. Moreover, the number and percentage of circulating neutrophils, the number of NF-κB p65^{+} cells in the liver, serum levels of several cytokines, and hepatic expression of *F4/80*, *Tnfa* and *Il-6* mRNAs in all three groups were comparable (Supporting Fig. 3B–E). Finally, *in vitro* experiments revealed that LPS treatment induced similar expression levels of mRNAs and proteins of several cytokines in neutrophils from WT and *Lcn2* KO mice (Supporting Fig. 4A–B).

Hepatocytes and myeloid cells cooperatively fight against systemic bacterial infection through LCN2

To test the role of myeloid cell- and hepatocyte-derived LCN2 in the control of bacterial infection, we generated four lines of mice, including *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice to test the mortality post infection of *K. pneumoniae*. As illustrated in Fig. 2A, both *Lcn2*$^{Hep-/-}$ and *Lcn2*^{DKO} (*Lcn2*$^{Hep-/-Mye-/-}$) mice began to die around 12 hours and were all dead within 36 hours after injection of a high dose (5×10^4 CFU) of *K. pneumoniae*. On the other hand, many of WT and *Lcn2*$^{Mye-/-}$ mice survived until 40 hours (Fig. 2A). Next, we injected mice with a lower dose (5×10^3 CFU) of *K. pneumoniae* and sacrificed them 24 hours post-infection. Serum ALT, AST, ALP levels were not different among these four groups (Fig. 2B). Serum LCN2 levels were elevated (~ 2.5 μg/ml) in *Lcn2*$^{f/f}$ mice, such elevation was significantly reduced in *Lcn2*$^{Hep-/-}$ and *Lcn2*^{DKO} mice (Fig. 2C). There was a trend reduction in serum LCN2 protein levels in *Lcn2*$^{Mye-/-}$ mice compared with *Lcn2*$^{f/f}$ mice, but did not reach statistical difference (Fig. 2C). In agreement with levels of LCN2 protein, bacterial burden in peripheral blood and the liver was higher in *Lcn2*$^{Hep-/-}$ and *Lcn2*^{DKO} mice, but was comparable in *Lcn2*$^{Mye-/-}$ mice when compared with WT *Lcn2*$^{f/f}$ mice (Fig. 2C).

In addition, peritoneal lavage fluid LCN2 levels were elevated (~ 2.8μg/ml) in *Lcn2*$^{f/f}$ mice and significantly reduced in all other three groups (Fig. 2D). There was a trend of reduction in peritoneal lavage fluid LCN2 protein levels in *Lcn2*^{DKO} mice compared with *Lcn2*$^{Hep-/-}$ and *Lcn2*$^{Mye-/-}$ mice, but did not reach statistical difference (Fig. 2D). Bacterial burden in peritoneal lavage fluid was significantly higher in all three lines of mutant mice than that in WT *Lcn2*$^{f/f}$ mice, which is in consistent with LCN2 protein levels (Fig. 2D).

LCN2 is expressed in neutrophils intracellularly and secreted by hepatocytes post bacterial infection

As illustrated in Fig. 3A, injection of *K. pneumoniae* intraperitoneally caused similar levels of liver necrosis among *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice. Immunohistochemistry analyses revealed that LCN2 proteins were detected strongly in neutrophils in necrotic areas and weakly in hepatocytes in *Lcn2*$^{f/f}$ mice. Interestingly, LCN2 was mainly detected in neutrophils in necrotic area in *Lcn2*$^{Hep-/-}$ mice while only weak diffused LCN2 staining was detected in hepatocytes from *Lcn2*$^{Mye-/-}$ mice. No LCN2 protein staining was detected in *Lcn2*^{DKO} mice. Furthermore, greater *K. pneumoniae* staining per necrotic area was found in *Lcn2*^{DKO} mice compared to the other three groups (Fig. 3A, B). There was a trend of elevation in the density of *K. pneumoniae* staining in

Lcn2$^{Hep-/-}$ and *Lcn2*$^{Mye-/-}$ mice when compared to *Lcn2*$^{f/f}$ mice, but did not reach statistical difference (Fig. 3A, B).

There were a large number of neutrophils recruited into necrotic area in the liver post *K. pneumoniae* infection, but there were no differences in neutrophil numbers per necrotic area among all four groups (Fig. 3A, B). F4/80 staining was also performed to examine macrophages. As illustrated in Supporting 5A, strong F4/80^{+} macrophage staining was detected in non-necrotic areas in the liver tissues from *K. pneumoniae*-treated mice. Interestingly, F4/80 staining was not detected in necrotic areas. Lack of F4/80 staining in necrotic areas may be due to macrophage death or downregulation of F4/80 markers in macrophages. The number of macrophages in non-necrotic areas was comparable among *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ mice. In addition, hepatic expressions of *F4/80, Il-6, and Tnfa* mRNAs, and TLR4 and p65 NF-κB proteins were similar among these three groups post *K. pneumoniae* infection (Supporting Fig. 5B).

Myeloid cells fight against lung local bacterial infection via the production of LCN2

Next, we tested the role of hepatocyte LCN2 and myeloid LCN2 in a model of lung local bacterial infection induced by intratracheal intubation of *K. pneumoniae* (5×10^6 CFU). As illustrated in Fig. 4A, bronchoalveolar lavage fluid LCN2 levels were significantly elevated (~6μg/ml) in *Lcn2*$^{f/f}$ mice post infection, and such elevation was markedly reduced in *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice (less than 2.5μg/ml) but not in *Lcn2*$^{Hep-/-}$ mice (left panel, Fig. 4A). In addition, serum LCN2 levels were markedly elevated (~6μg/ml) in *Lcn2*$^{f/f}$ mice, and such elevation was markedly reduced in all other three groups (right panel, Fig. 4A). There was a tendency of higher serum LCN levels in *Lcn2*$^{Mye-/-}$ compared to *Lcn2*$^{Hep-/-}$ and *Lcn2*^{DKO} mice, but did not reach statistical difference (right panel, Fig. 4A).

Next, we measured bacterial load in bronchoalveolar lavage fluid and blood. Compared to *Lcn2*$^{f/f}$ mice, *Lcn2*$^{Hep-/-}$ mice had comparable levels of bacterial burden in bronchoalveolar lavage fluid; while *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice had higher levels of bacterial burden (Fig. 4B). In addition, in this intratracheal intubation model, blood bacterial load levels were ten times lower than those in bronchoalveolar lavage fluid (Fig. 4B). These blood bacterial burden levels were comparable among *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$, and *Lcn2*$^{f/f}$ mice; whereas *Lcn2*^{DKO} mice had higher levels when compared to *Lcn2*$^{f/f}$ mice (Fig. 4B).

Immunohistochemistry analyses revealed that LCN2 levels were highly expressed in lung tissue from *Lcn2*$^{f/f}$ and *Lcn2*$^{Hep-/-}$ mice after bacterial infection, such expression levels were markedly reduced in *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice (Fig. 4C), which is in agreement with LCN2 levels in bronchoalveolar lavage fluid as shown in Fig. 4A. Immunohistochemistry analyses demonstrated that a similar number of MPO^{+} neutrophils was recruited into the lungs in all four groups post infection (Fig. 4C). Quantitative RT-PCR analyses revealed that expression of *Ly6g, Il-6,* and *Tnfa* mRNA was highly upregulated in the lungs from *K. pneumoniae* bacteria-infected mice compared to saline-treated control mice (Fig. 4D). Expression of these inflammatory mediators was similar among these four groups except *Tnfa* expression that was lower in *Lcn2*$^{Hep-/-}$ mice than in *Lcn2*$^{f/f}$ mice post infection (Fig. 4E).

F4/80 and IBA-1 staining were also performed to examine macrophages. As illustrated in Supporting Fig. 6, few F4/80$^+$ macrophage staining was detected in the lung tissues from *K. pneumoniae*-treated mice, but a large number of IBA-1$^+$ macrophages was detected. These results suggest that infiltrating macrophages in the lungs express low levels of F4/80 but high levels of IBA-1. The number of IBA-1$^+$ macrophages in the lung was comparable among *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ mice, suggesting that LCN2 does not affect lung macrophage infiltration in this local bacterial infection model.

Intracellular but not extracellular LCN2 is a component of NETs

NETs play an important role in the anti-bacterial infection by neutrophils.[16, 27] Thus, we tested whether LCN2 is also a component of murine NETs and contributes to NET formation. Mouse neutrophils were isolated from bone marrow and stimulated with PMA or calcium ionophore A23187, two well-known stimulators for NET formation in neutrophils, [16] to study NETs *in vitro*. The gold standard for NET quantification is to co-stain with a nuclear marker (e.g.: DNA, cit-histone) and a neutrophil granule marker (e.g.: MPO, elastase, LCN2/NAGL).[28] As illustrated in Fig. 5A, B, LCN2 is co-localized with cit-histone in NETs structure, indicating that LCN2 is a component of mouse NETs. However, LCN2 staining was not detected in all NETs, and only approximately 30% of mouse NETs had LCN2 staining (Fig. 5C).

As expected, LCN2 was not detected in NETs in PMA-stimulated LCN2 KO neutrophils (Fig. 5C). We then asked whether extracellular LCN2 can integrate into NETs. To test this hypothesis, we added recombinant LCN2 protein or culture medium from LPS-treated hepatocytes (containing LCN2) into PMA-stimulated LCN2 KO neutrophils. No positive LCN2 staining was detected in NETs from PMA-stimulated LCN2 KO neutrophils (Fig. 5C, Supporting Fig. 7), suggesting extracellular LCN2 from hepatocytes or other sources does not integrate into NETs and that the source of LCN2 in NETs is from intracellular neutrophil granules.

LCN2 is not required for NET formation *in vitro* and *in vivo*

To test whether LCN2 is required for NET formation, we isolated neutrophils from WT and *Lcn2* KO mice, and stimulated them with PMA or co-cultured with *K. pneumoniae.* The degree of NETosis, as detected by cit-histone immunostaining, was comparable in WT and *Lcn2* KO neutrophils stimulated with PMA (Fig. 6A, B) or *K. pneumoniae* (supporting Fig. 8), suggesting intracellular LCN2 in neutrophils is not required for NET formation in isolated neutrophils. To determine whether extracellular LCN2 regulates NETosis, *Lcn2*KO neutrophils were incubated with recombinant LCN2 protein and subsequently stimulated with PMA or *K. pneumoniae*. As illustrated in Fig. 6A, B and supporting Fig. 8, incubation with recombinant LCN2 did not affect NETosis in *Lcn2* KO neutrophils *in vitro*.

Next, we investigated whether LCN2 is required for NET formation *in vivo* in mice infected with *K. pneumoniae.* Immunofluorescence microscopy revealed co-staining of MPO and cit-histone in the liver samples (Fig. 6C), suggesting that intra-hepatic neutrophils developed NETs post *K. pneumoniae* infection. However, there were no differences in NET formation among *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice (Fig. 6C, D). Furthermore, we

quantified NET formation in serum and peritoneal lavage fluid by an ELISA-based cit-histone-DNA binding test. In *Lcn2*$^{f/f}$ WT mice, *K. pneumoniae* infection caused a 3-fold and 1.8-fold increase in NET products in serum and peritoneal lavage fluid compared to PBS-treated control group, respectively. Similar elevation of NET products in serum and peritoneal lavage fluid was also observed in *Lcn2*$^{Hep-/-}$, *Lcn2*$^{Mye-/-}$ and *Lcn2*^{DKO} mice (Fig. 6E). Overall, these results indicate that, in contrast to other neutrophil granule proteins, LCN2 is not required for NET formation in mice.

LCN2 contributes to the antibacterial functions of NETs in an iron-dependent manner

It is well documented that circulating LCN2 plays an important role in suppressing bacterial growth.[1] We asked whether LCN2 affects neutrophil phagocytosis and degranulation, which both are important for neutrophils to attack bacterial infection. As illustrated in Supporting Fig. 9A–B, the levels of neutrophil phagocytosis were comparable in the neutrophils from *Lcn2*$^{f/f}$, *Lcn2*$^{Hep-/-}$, and *Lcn2*^{DKO} mice, and the levels of degranulation were also comparable in the neutrophils from WT and *Lcn2* KO mice. These data suggest that LCN2 does not affect neutrophil phagocytosis and degranulation. Next we wondered whether the anti-bacterial functions of LCN2 is mediated via the NETs. To answer this question, the ability of NETs to kill bacteria was assessed *in vitro*. As illustrated in Fig. 7A, incubation with NETs markedly inhibited bacterial growth in iron free culture medium, and NETs from WT and *Lcn2* KO mice had the same degree of inhibition against *K. pneumoniae* growth *in vitro*. Because LCN2 inhibits bacterial infection by sequestering iron-containing siderophores,[4] we performed NET-mediated bacterial killing in iron containing medium. As illustrated in Fig. 7B, adding ferric iron markedly reduced the anti-bacterial functions of NETs and increased bacterial growth in a dose-dependent manner. In the absence of iron (0 μM) or at high doses of iron (10 and 100 μM), there were no differences in bacterial growth from the incubation with NETs of WT and *Lcn2* KO neutrophils. However, at low doses of iron (0.1 and 1 μM), bacterial growth from the incubation with *Lcn2* KO NETs was greater compared to that from WT NETs. These results suggest that LCN2 present in NETs has antibacterial properties.

NGAL/LCN2 is a component of human NETs that infiltrate hepatic tissue in liver diseases

Healthy human blood neutrophils were isolated and stimulated with PMA, and were subjected to immunofluorescent staining. As illustrated in Fig. 8A, NGAL (human ortholog of mouse LCN2) co-localized with elastase in the majority of neutrophil NETs, confirming that NGAL is a component of NETs from human neutrophils.[21] Interestingly, almost all elastase^{+} NETs were stained with NAGL (Fig. 8A). Furthermore, immunofluorescent staining was also performed in liver samples from patients with various types of liver diseases. Co-localization of intracellular NGAL and cit-histone was frequently observed in liver samples from patients with severe alcoholic hepatitis (SAH) (Fig. 8B), cirrhotic liver tissues from HCV, NASH, AH, and liver tissues from acetaminophen-induced acute liver failure (APAP) (supporting Fig. 10) with the highest number in SAH (Fig. 8C). These results suggest that NGAL containing NETs frequently infiltrate into the liver in a variety of liver diseases.

Discussion

Several novel and interesting findings are presented in the current study. First, hepatocytes and myeloid cells cooperatively control bacterial infection through the production of LCN2. Second, hepatocytes play a more important role in controlling systemic bacterial infection while myeloid cells are more vital to combat lung local infection by generating LCN2. Third, we demonstrated for the first time that hepatocyte LCN2 does not bind to NETs but that neutrophils are the source of LCN2 present in NETs. Fourth, while LCN2 is not required for NET formation, it contributes to the bacteriostatic functions of NETs. Finally, NGAL/LCN2 containing NETs are detected in various types of liver diseases.

LCN2 is reported to be induced under numerous pathological conditions in many organs such as liver, kidneys, lungs, bone and brain.[2, 3] For example, LCN2 was found to be highly elevated in the serum, various types of fluids and tissues in mouse models and patients with liver disease.[3] However, which cell types are responsible for such high elevation remains unclear. In the current study, we demonstrated that after LPS injection, both hepatocytes and intra-hepatic neutrophils expressed *Lcn2* mRNA, but LCN2 protein was mainly detected in neutrophils by immunohistochemistry analysis. Genetic deletion of the *Lcn2* gene in myeloid cells abolished LCN2 protein expression in intra-hepatic neutrophils from LPS-treated mice, but only slightly (no statistical difference) reduced serum LCN2 protein levels. In contrast, genetic deletion of the *Lcn2* gene in hepatocytes did not affect LCN2 protein expression in intra-hepatic neutrophils but markedly reduced serum levels of LCN2 protein. Collectively, after LPS injection, LCN2 protein is highly upregulated in hepatocytes and subsequently secreted into circulation, which contributes to elevation of serum LCN2 protein. LCN2/NAGL protein contains a signal peptide that promotes its secretion,[29] which contributes to its secretion in hepatocytes. In contrast, LCN2/NAGL in neutrophils is stored in the specific granules, so neutrophils can carry LCN2/NAGL to the local infection area.[27]

Similar to LPS-induction of LCN2, infection with *K. pneumoniae* also elevates LCN2 protein with secreted form in hepatocytes and with intracellular form in neutrophils. Through generation of these different forms of LCN2, hepatocytes and neutrophils differentially combat systemic and local bacterial infection. Genetic deletion of the *Lcn2* gene in hepatocytes ($Lcn2^{Hep-/-}$) reduced circulating LCN2 levels and increased bacterial loads in the blood post systemic *K. pneumoniae* infection (via the intraperitoneal injection) but did not affect LCN2 levels and bacterial loads in bronchoalveolar lavage fluid in a model of lung local infection induced by intratracheal intubation of *K. pneumoniae*. In contrast, genetic deletion of *Lcn2* gene in myeloid cells ($Lcn2^{Mye-/-}$) markedly reduced LCN2 levels and increased bacterial loads in bronchoalveolar lavage fluid in this intratracheal intubation lung infection model but did not affect LCN2 and bacterial loads in the systemic *K. pneumoniae* infection model. Our data suggest that secretion of LCN2 protein by hepatocytes plays an important role in systemic bacterial infection while neutrophils carry LCN2 protein in specific granules to combat local bacterial infection in the lungs. Interestingly, LCN2 protein from both hepatocytes and myeloid cells seems to be important to combat bacterial infection in peritoneal capacity and necrotic area in the liver to limit

bacteria growth after intraperitoneal injection of *K. pneumoniae*, which is probably because LCN2 protein from both cell types can reach to peritoneal capacity and liver necrotic area.

It was reported that LCN2 played a role in recruiting neutrophils.[26] However, in another study, it was suggested that the absence of LCN2 in neutrophils didn't affect their ability to accumulate at site of infection.[15] In our study, we found that global or cell-specific deletion of the *Lcn2* gene neither affected circulating neutrophils nor affected the infiltrating neutrophils in the liver and lungs in both models of intraperitoneal injection and intratracheal intubation of *K. pneumoniae* infection as well in the LPS injection model, suggesting circulating LCN2 or intracellular LCN2 in neutrophils does not affect neutrophil recruitment and activation in these models we tested. In addition, hepatic and serum levels of several pro-inflammatory cytokines were equal among WT and three lines of *Lcn2* conditional KO mice as demonstrated in the current paper. *In vitro* treatment with recombinant LCN2 protein did not upregulate expression of several cytokines (IL-6, IL-10, MCP-1, TNF-α, and IFN-γ) in both neutrophils and hepatocytes (data not shown). Collectively, our data suggest that LCN2 plays a minor role in regulating inflammation in several models we used in the current paper.

Formation of NETs is a recently identified mechanism to immobilize and kill bacteria by neutrophils.[16] NETs contain several neutrophil granule proteins such as neutrophil elastase and MPO.[30] LCN2 was identified in purified NETs from zymography analyses of gelatinase [23] and from proteomic analyses[21]. Through immunohistochemistry analyses, we visualized LCN2 protein in the NETs from activated mouse neutrophils and NGAL protein from activated human neutrophils *in vitro* and *in vivo*, supporting that LCN2/NGAL is a component of NETs in mice and humans. Interestingly, LCN2 protein was detected in only approximately 30% NETs in mouse neutrophils whereas most human NET samples contained NGAL. Thus, NGAL could be used as marker of human NETs. Neutrophil granule proteins such as neutrophil elastase and MPO were reported to contribute the formation of NETs,[30] although some other studies do not support this notion.[31] In the present study, we demonstrated that genetic deletion of the *Lcn2* gene in myeloid cells or hepatocytes did not affect the levels of NETs in the liver and serum from *K. pneumoniae*-infected mice. Neutrophils from $Lcn2^{-/-}$ mice generated the same number of NETs *in vitro* as those from WT mice after incubation with PMA or *K. pneumoniae*. However, while not required for NET formation, the NETs from $Lcn2^{-/-}$ neutrophils had reduced bacteriostatic activity in the presence of iron than those from WT neutrophils. Thus, the data from our studies suggest that LCN2 is not required for NET formation but contributes to the anti-bacterial function of NETs in an iron-dependent manner. NETs without LCN2 have strong ability to inhibit/kill bacteria in an iron-independent manner, thus LCN2 is incorporated into NETs as a by-product following bacterial infection and does not play a role in the control of bacterial infection when local iron is absent. However, when local iron levels are elevated, LCN2 in the NETs likely enhances the bacteriostatic functions of NETs.

Finally, $NAGL^+$ NETs were detected in the liver samples from various types of liver diseases. Given well-documented bacteriostatic functions of LCN2, these $NAGL^+$ NETs, together with circulating LCN2 protein likely plays an important role in host defense against bacterial infection in liver disease, especially in the end-stage liver disease that is often

associated with bacterial infection. However, whether and how LCN2 regulates inflammation during bacterial infection and during chronic sterile inflammation in liver disease remain obscure. As mentioned in the introduction, the role of LCN2 in regulating inflammation has been controversial in various types of diseases, including liver disorders. [3] In the acute bacterial infection model we used in the current paper, genetic deletion of the *Lcn2* gene in hepatocytes or myeloid cells, or both had little impact on inflammatory responses, suggesting that LCN2 may not have an important role in regulating inflammation in acute bacterial infection; however, we cannot rule out that LCN2 may play a role in the control of inflammatory response during chronic bacterial infection. Furthermore, it was reported that LCN2 either promoted liver injury and inflammation [5–7] or protected against acute liver injury [1, 8] in various liver injury models with sterile inflammation. Thus, the role of LCN2 in the control of sterile inflammation requires further studies to clarify.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	aspartate aminotransferase
BALF	bronchoalveolar lavage fluid
CFU	colony-forming unit
Cit-histone	citrulline Histone H3
Ctrl	control
DAPI	4′,6-Diamidino-2-Phenylindole
IFN	interferon
IL	interleukin
KO	knockout
K. pneumoniae	*Klebsiella pneumoniae*
LCN2	lipocalin-2

***Lcn2*$^{f/f}$**	*Lcn2*$^{flox/flox}$
***Lcn2*$^{Hep-/-}$**	hepatocyte-specific *Lcn2* knockout
***Lcn2*$^{Mye-/-}$**	myeloid-specific *Lcn2* knockout
***Lcn2*^{DKO}**	hepatocyte and myeloid-specific *Lcn2* knockout
LPS	lipopolysaccharides
MCP	monocyte chemotactic protein
MPO	myeloperoxidase
NGAL	neutrophil gelatinase-associated lipocalin
NETs	neutrophil extracellular traps
PLF	peritoneal lavage fluid
PMA	phorbol 12-myristate 13-acetate
rLCN2	recombinant LCN2
TNF	tumor necrosis factor
WT	wide-type

References

Author names in bold designate shared co-first authorship

1. Xu MJ, Feng D, Wu H, Wang H, Chan Y, Kolls J, et al. Liver is the major source of elevated serum lipocalin-2 levels after bacterial infection or partial hepatectomy: a critical role for IL-6/STAT3. Hepatology. 2015; 61:692–702. [PubMed: 25234944]
2. Xiao X, Yeoh BS, Vijay-Kumar M. Lipocalin 2: An Emerging Player in Iron Homeostasis and Inflammation. Annu Rev Nutr. 2017; 37:103–130. [PubMed: 28628361]
3. Asimakopoulou A, Weiskirchen S, Weiskirchen R. Lipocalin 2 (LCN2) Expression in Hepatic Malfunction and Therapy. Front Physiol. 2016; 7:430. [PubMed: 27729871]
4. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002; 10:1033–1043. [PubMed: 12453412]
5. Wieser V, Tymoszuk P, Adolph TE, Grander C, Grabherr F, Enrich B, et al. Lipocalin 2 drives neutrophilic inflammation in alcoholic liver disease. J Hepatol. 2016; 64:872–880. [PubMed: 26682726]
6. Cai Y, Jogasuria A, Yin H, Xu MJ, Hu X, Wang J, et al. The Detrimental Role Played by Lipocalin-2 in Alcoholic Fatty Liver in Mice. Am J Pathol. 2016; 186:2417–2428. [PubMed: 27427417]
7. Ye D, Yang K, Zang S, Lin Z, Chau HT, Wang Y, et al. Lipocalin-2 mediates non-alcoholic steatohepatitis by promoting neutrophil-macrophage crosstalk via the induction of CXCR2. J Hepatol. 2016; 65:988–997. [PubMed: 27266617]
8. Borkham-Kamphorst E, van de Leur E, Zimmermann HW, Karlmark KR, Tihaa L, Haas U, et al. Protective effects of lipocalin-2 (LCN2) in acute liver injury suggest a novel function in liver homeostasis. Biochim Biophys Acta. 2013; 1832:660–673. [PubMed: 23376114]

9. Markwardt D, Holdt L, Steib C, Benesic A, Bendtsen F, Bernardi M, et al. Plasma cystatin C is a predictor of renal dysfunction, acute-on-chronic liver failure, and mortality in patients with acutely decompensated liver cirrhosis. Hepatology. 2017; 66:1232–1241. [PubMed: 28545169]
10. Cullaro G, Kim G, Pereira MR, Brown RS Jr, Verna EC. Ascites Neutrophil Gelatinase-Associated Lipocalin Identifies Spontaneous Bacterial Peritonitis and Predicts Mortality in Hospitalized Patients with Cirrhosis. Dig Dis Sci. 2017; 62:3487–3494. [PubMed: 29098551]
11. Barreto R, Elia C, Sola E, Moreira R, Ariza X, Rodriguez E, et al. Urinary neutrophil gelatinase-associated lipocalin predicts kidney outcome and death in patients with cirrhosis and bacterial infections. J Hepatol. 2014; 61:35–42. [PubMed: 24613364]
12. Ariza X, Graupera I, Coll M, Sola E, Barreto R, Garcia E, et al. Neutrophil gelatinase-associated lipocalin is a biomarker of acute-on-chronic liver failure and prognosis in cirrhosis. J Hepatol. 2016; 65:57–65. [PubMed: 26988732]
13. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993; 268:10425–10432. [PubMed: 7683678]
14. Zhou Z, Xu MJ, Gao B. Hepatocytes: a key cell type for innate immunity. Cell Mol Immunol. 2016; 13:301–315. [PubMed: 26685902]
15. Cramer EP, Dahl SL, Rozell B, Knudsen KJ, Thomsen K, Moser C, et al. Lipocalin-2 from both myeloid cells and the epithelium combats Klebsiella pneumoniae lung infection in mice. Blood. 2017; 129:2813–2817. [PubMed: 28396494]
16. Branzk N, Papayannopoulos V. Molecular mechanisms regulating NETosis in infection and disease. Semin Immunopathol. 2013; 35:513–530. [PubMed: 23732507]
17. Sorensen OE, Borregaard N. Neutrophil extracellular traps - the dark side of neutrophils. J Clin Invest. 2016; 126:1612–1620. [PubMed: 27135878]
18. Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol. 2009; 184:205–213. [PubMed: 19153223]
19. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009; 5:e1000639. [PubMed: 19876394]
20. Dwyer M, Shan Q, D'Ortona S, Maurer R, Mitchell R, Olesen H, et al. Cystic fibrosis sputum DNA has NETosis characteristics and neutrophil extracellular trap release is regulated by macrophage migration-inhibitory factor. J Innate Immun. 2014; 6:765–779. [PubMed: 24862346]
21. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013; 5:178ra140.
22. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem. 2001; 276:37258–37265. [PubMed: 11486009]
23. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann Rheum Dis. 2015; 74:1417–1424. [PubMed: 24570026]
24. Cai Y, Kimura S. Noninvasive intratracheal intubation to study the pathology and physiology of mouse lung. J Vis Exp. 2013:e50601. [PubMed: 24300823]
25. Khanova E, Wu R, Wang W, Yan R, Chen Y, French SW, et al. Pyroptosis by Caspase11/4-Gasdermin-D Pathway in Alcoholic Hepatitis. Hepatology. 2017
26. Liu Z, Petersen R, Devireddy L. Impaired neutrophil function in 24p3 null mice contributes to enhanced susceptibility to bacterial infections. J Immunol. 2013; 190:4692–4706. [PubMed: 23543755]
27. Borregaard N, Sorensen OE, Theilgaard-Monch K. Neutrophil granules: a library of innate immunity proteins. Trends Immunol. 2007; 28:340–345. [PubMed: 17627888]

28. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, Ishizu A. NETosis markers: Quest for specific, objective, and quantitative markers. Clin Chim Acta. 2016; 459:89–93. [PubMed: 27259468]

29. Bundgaard JR, Sengelov H, Borregaard N, Kjeldsen L. Molecular cloning and expression of a cDNA encoding NGAL: a lipocalin expressed in human neutrophils. Biochem Biophys Res Commun. 1994; 202:1468–1475. [PubMed: 8060329]

30. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010; 191:677–691. [PubMed: 20974816]

31. Martinod K, Witsch T, Farley K, Gallant M, Remold-O'Donnell E, Wagner DD. Neutrophil elastase-deficient mice form neutrophil extracellular traps in an experimental model of deep vein thrombosis. J Thromb Haemost. 2016; 14:551–558. [PubMed: 26712312]

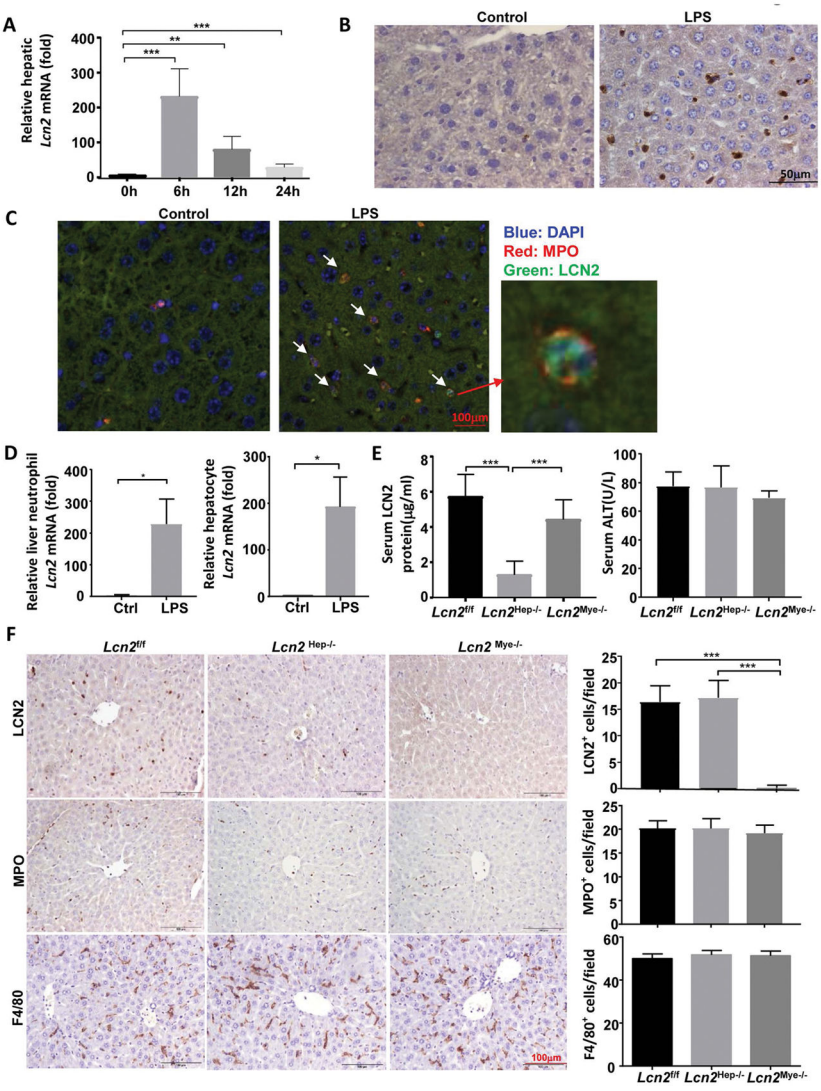

Fig. 1.

LPS injection elevates intracellular LCN2 expression in neutrophils and extracellular LCN2 secretion from hepatocytes. (A) Mice were given LPS (1mg/kg) intraperitoneally and then sacrificed various time periods post injection. Liver tissues were subjected to qPCR for *Lcn2*. (B–D) Mice were given LPS (1mg/kg) intraperitoneally or saline (Control) and sacrificed 6 hours later. Liver tissues were subjected to immunohistochemistry with anti-LCN2 antibody (Panel B), or immunofluorescence with anti-LCN2 (green) and anti-MPO (red) antibody (Panel C). Liver neutrophils and hepatocytes were isolated and subjected to qPCR for *Lcn2* (Panel D). (E–F) $Lcn2^{f/f}$ (WT), $Lcn2^{Hep-/-}$, $Lcn2^{mye-/-}$ mice were injected with LPS (1 mg/kg) for 6 hours, serum LCN2 protein and ALT levels were measured (panel E). Liver tissues were subjected to immunohistochemistry with anti-LCN2, anti-MPO or anti-F4/80 antibody. LCN2, MPO and F4/80 positive cells per field were quantified (Panel F). Representative images are shown in panels B, C, and F. Values represent means ±SEM (n=4–8). **P*<0.05; ***P*<0.01; ****P*<0.001.

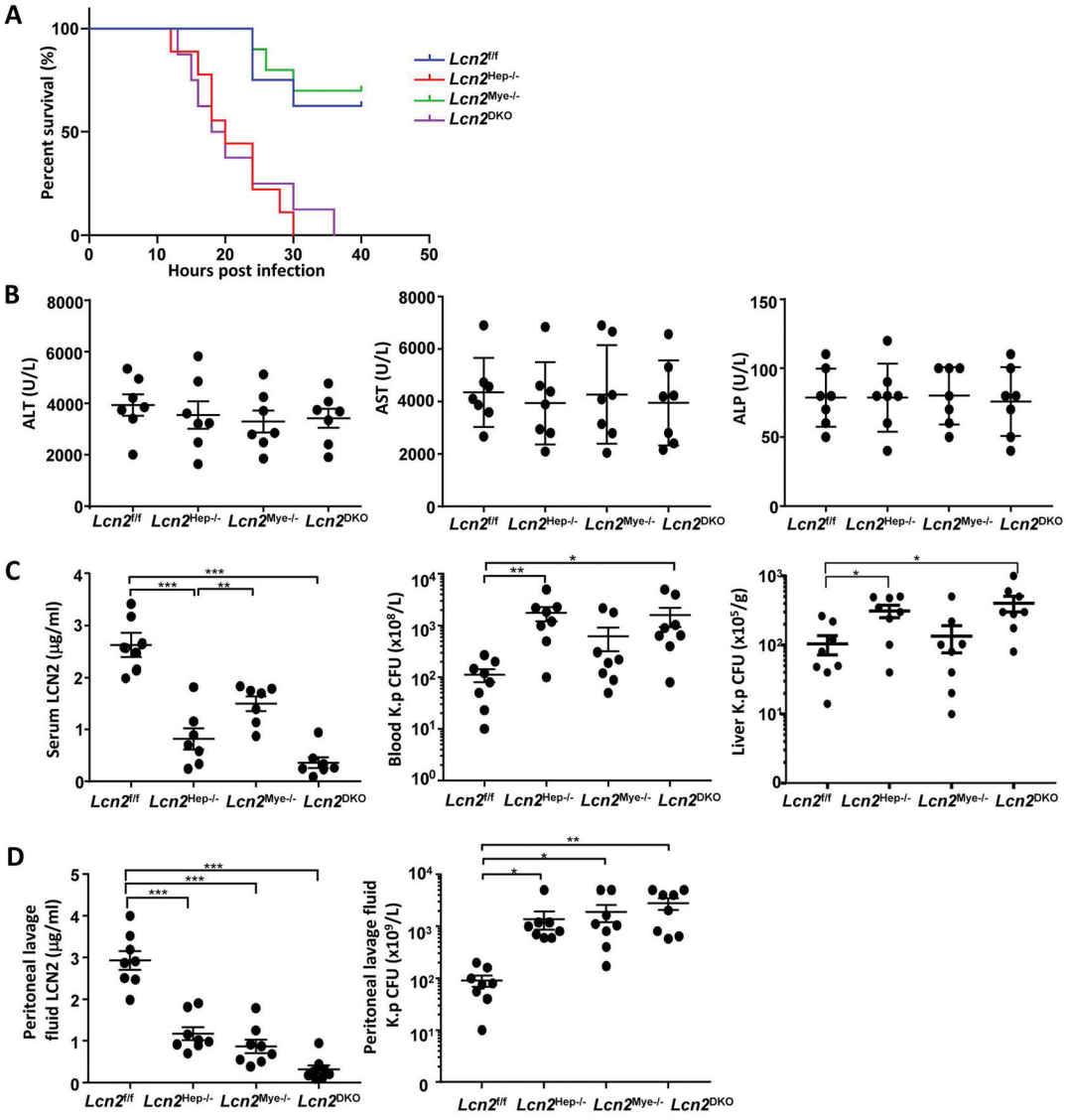

Fig. 2.

Hepatocytes and myeloid cells cooperatively control systemic bacterial infection via the production of LCN2. (A) Mice were injected intraperitoneally with a high dose (5×10^4 CFU) of *K. pneumoniae*, and the survival rate until 40 hours was measured (n=10 in each group). (B–D) Mice were injected intraperitoneally with a low dose (5×10^3 CFU) of *K. pneumoniae* and sacrificed after 24 hours. Serum ALT, AST, ALP, and LCN2 levels were measured. The blood and liver samples were collected for bacterial CFU measurement. Peritoneal lavage fluid samples were collected for LCN2 protein measurement or for CFU measurement. Viable bacteria were determined by colony counts as total CFU per mL of blood/peritoneal lavage fluid. ALP: Alkaline phosphatase; *K.p.: K. pneumoniae.* Values represent means ±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

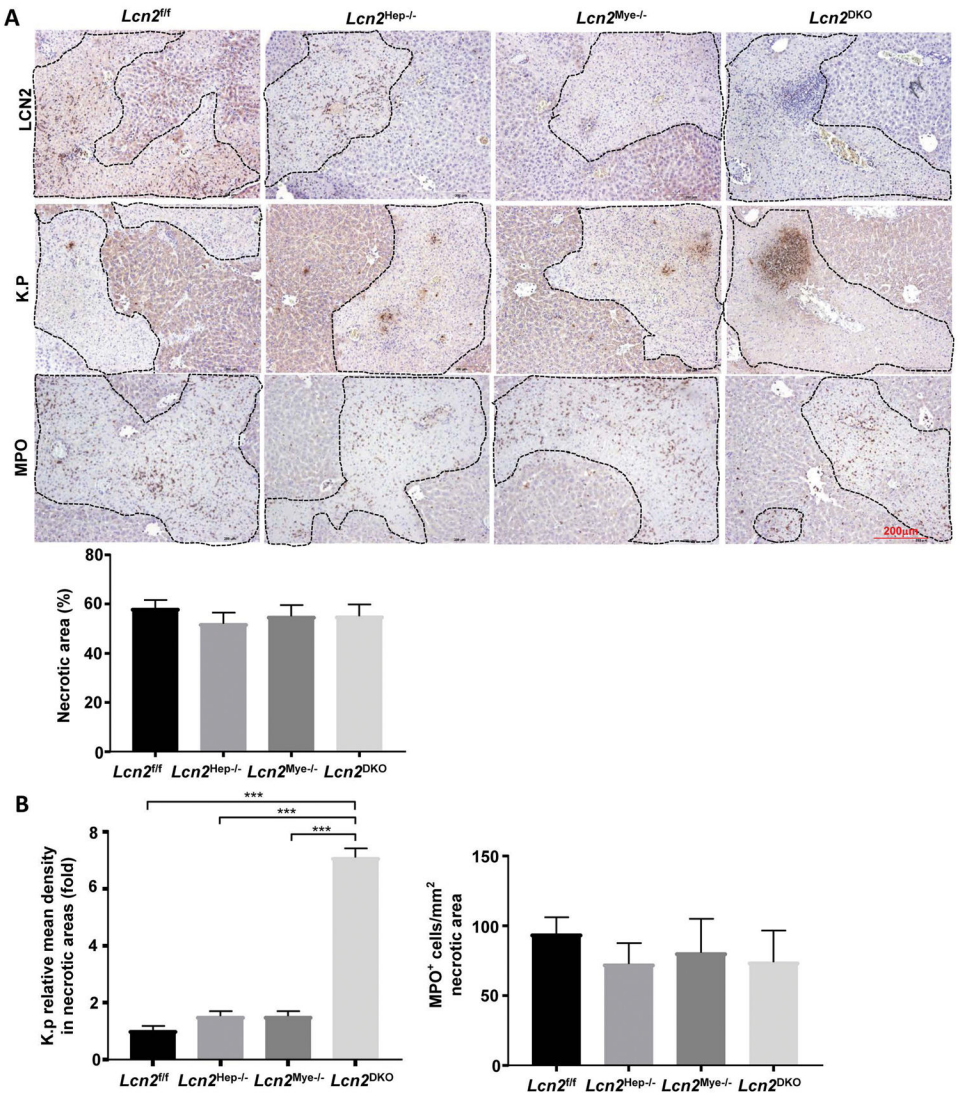

Fig. 3.

Both hepatocyte- and neutrophil-derived LCN2 combat *K. pneumoniae* in necrotic areas in the liver post infection. Mice were injected intraperitoneally with a dose (5×10^3 CFU) of *K. pneumoniae* and sacrificed 24 hours later. (A) Representative immunohistochemistry staining photographs of *K. pneumoniae*, LCN2 and MPO in the liver are shown. Necrotic areas are circled by dot lines and were quantified. Note: MPO^+ neutrophils are accumulated in necrotic areas with high levels of LCN2 expression. Brown staining indicates positive staining. (B) Quantification of *K. pneumoniae* was calculated by relative mean density/necrotic area. Quantification of MPO^+ cells/necrotic area was calculated. Values represent means ±SEM (n=8). *** $P<0.001$.

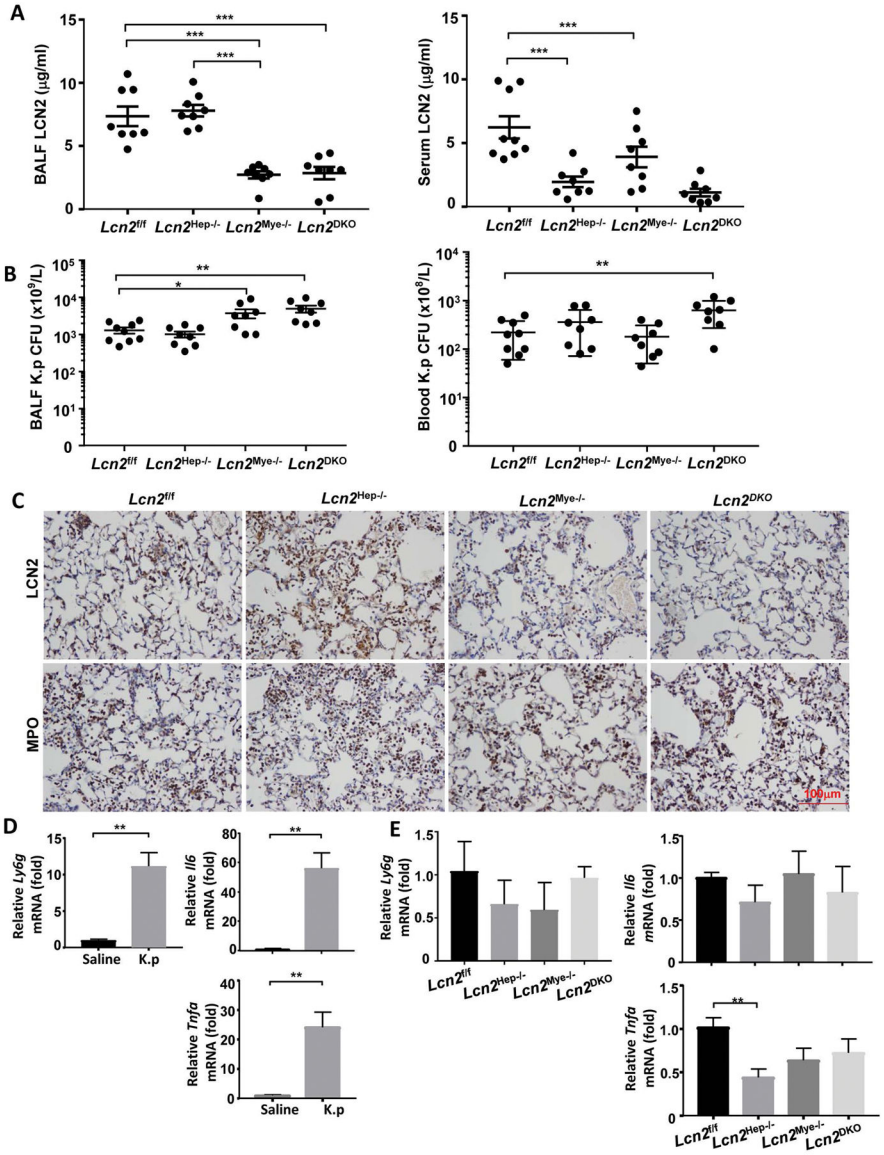

Fig. 4.

Myeloid cell- but not hepatocyte-derived LCN2 combats lung local bacterial infection. Mice were given a dose (5×10^6 CFU) of *K. pneumoniae* by intratracheal incubation and sacrificed 24 hours post-infection. (A) Bronchoalveolar lavage fluid (BALF) and serum were collected and subjected to an ELISA for LCN2 protein. (B) BALF and blood were collected 24 hours post-infection, and viable bacteria were determined by colony counts as total CFU per mL of blood/BALF. (C) Representative immunohistochemistry staining photographs of LCN2 and MPO in the lungs are shown. (D) Lung tissues from saline- or *K. pneumoniae*-treated WT mice were subjected to qRT-PCR analyses. (E) Lung tissues from *K. pneumoniae*-treated mice were subjected to qRT-PCR analyses. Values represent means ±SEM (n=8–9). **P*<0.05; ***P*<0.01; ****P*<0.001.

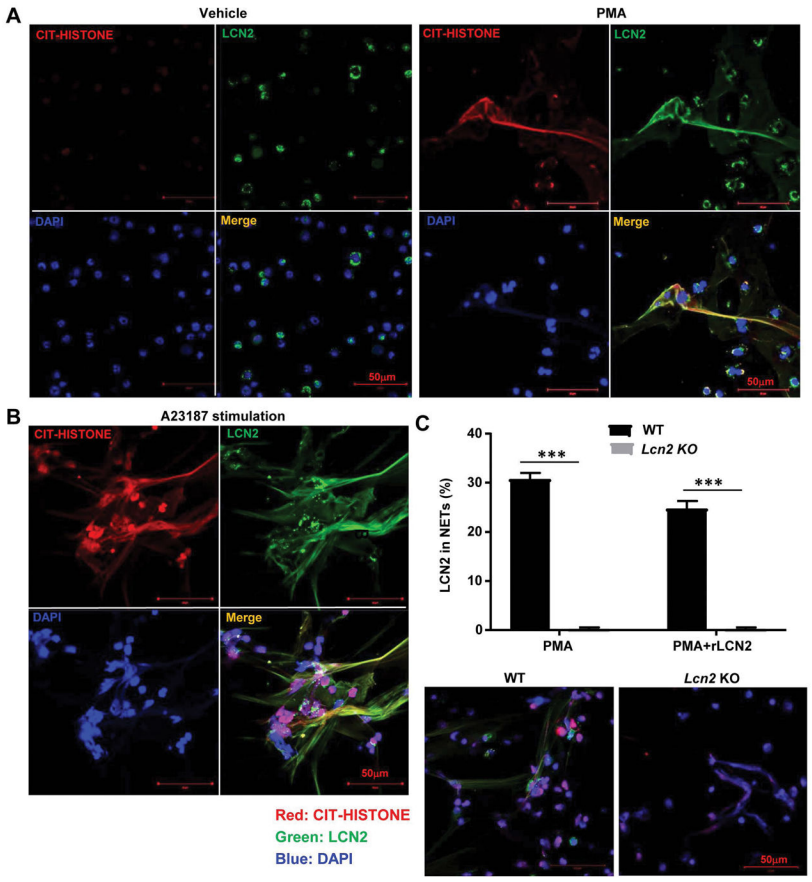

Fig. 5.

LCN2 is a component of NETs. (A, B) Bone marrow neutrophils were isolated from WT mice and stimulated with vehicle or 100nM PMA or 100nM A23187 for 4 hours. Representative immunofluorescent staining photographs of cit-Histone, LCN2, DAPI are shown by confocal microscopy. (C) Bone marrow neutrophils were isolated from WT and *Lcn2* KO mice, and incubated with 2000 ng/ml recombinant LCN2 protein and 100nM PMA for 4 hours. Quantification of LCN2 positive NETs was performed. Representative immunofluorescent staining photographs of cit-Histone, LCN2, DAPI from WT and *Lcn2* KO neutrophils are shown by confocal microscopy. Values represent means ± SEM from three independent experiments. ***P<0.001.

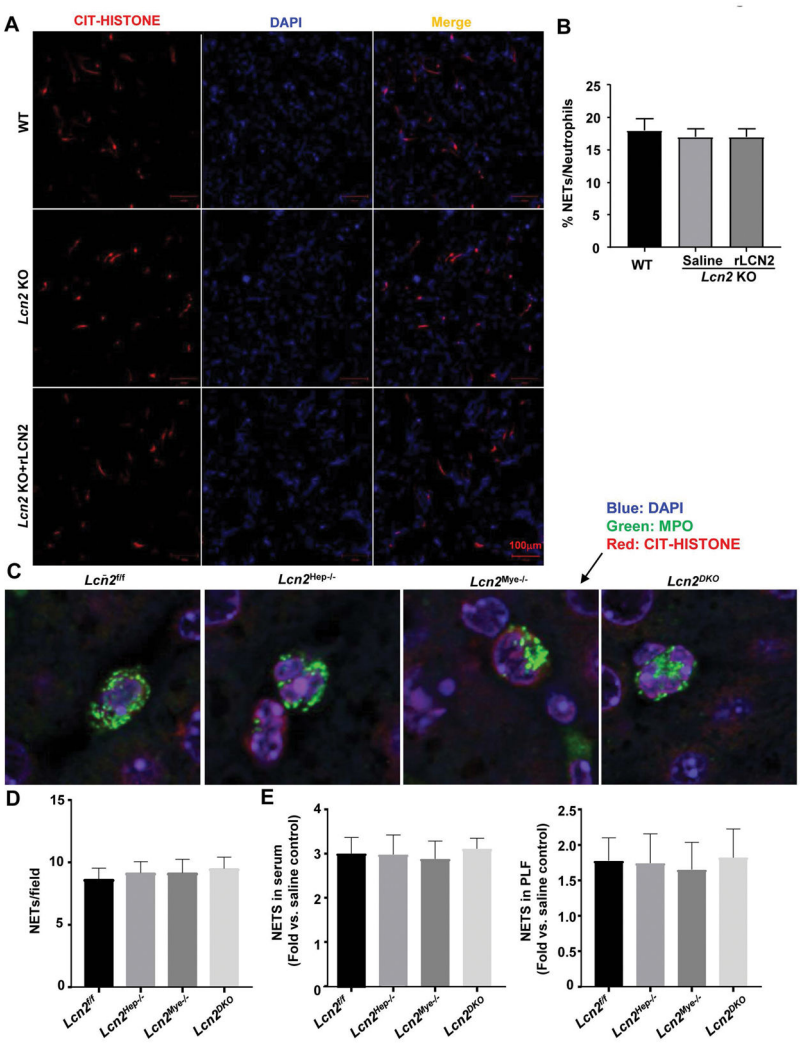

Fig. 6.

LCN2 is not required for NET formation *in vitro* and *in vivo*. (A, B) Bone marrow neutrophils from WT or *Lcn2* KO mice were isolated and stimulated with 100nM PMA for 4 hours. Representative immunofluorescent staining photographs of cit-Histone, LCN2, DAPI are shown by confocal microscopy (panel A). Quantification of NETosis per field was conducted (panel B). (C–E). Mice were given a dose (5×10^3 CFU) of *K. pneumoniae* intraperitoneally and sacrificed 24 hours post-infection. Liver tissues were collected and subjected to immunofluorescent staining with MPO and cit-Histone. Representative photographs are shown (panel C). Quantification of MPO and cit-histone double positive cells per field was conducted (panel D). Serum and peritoneal lavage fluid (PLF) were collected and subjected to an ELISA based cit-histone-DNA binding test (panel E). In panel E, values in PLF and serum from saline-treated mice were set as 1. Values represent means ±SEM (n=8).

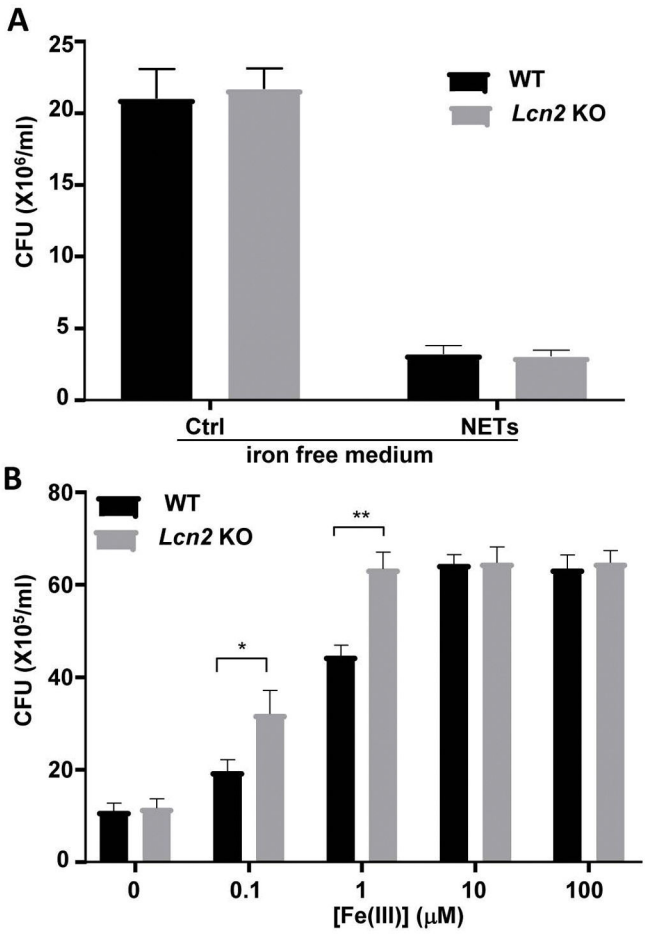

Fig. 7.

LCN2 contributes to NET-associated bacterial killing in an iron-dependent manner. (A) Bone marrow neutrophils from WT and *Lcn2* KO mice were stimulated with A23187 to induce NETs (NET group) or without A23187 (Control). NETs were isolated and then incubated with *K. pneumoniae* in an iron-free medium for 16 hours. Bacterial growth was determined by counting CFU. (B) NETs from WT or *Lcn2* KO mice were incubated with *K. pneumoniae* in the presence of different concentrations of $FeCl_3$ for 16 hours. Bacterial growth was determined by counting CFU. Values represent means ± SEM from three independent experiments. **P*<0.05; ***P*<0.01.

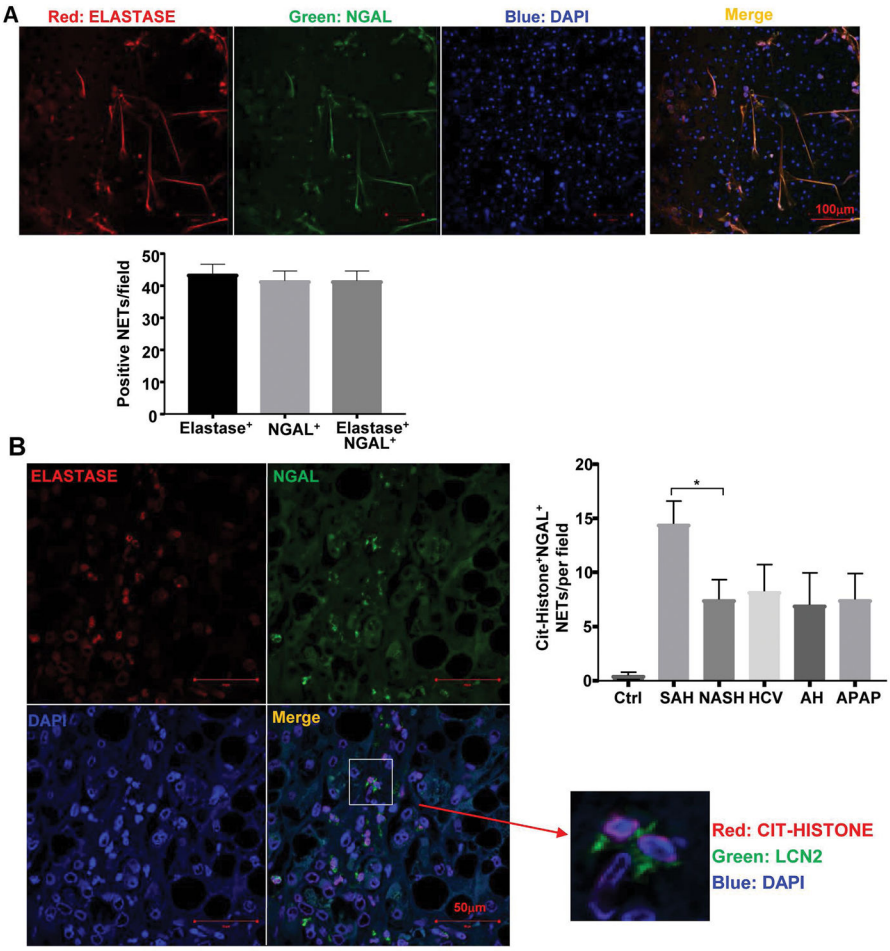

Fig. 8.

NGAL is a component of NETs from human neutrophils *in vivo* and *in vitro*. (A) Human neutrophils were isolated and stimulated with 100nM PMA for 4 hours. Representative immunofluorescent staining photographs of elastase, NGAL, DAPI are shown by confocal microscopy. Elastase^{+}NGAL^{+}, Elastase^{+}, or NGAL^{+} NETs were counted. (B) Representative immunofluorescence photographs of cit-histone and NGAL in the liver from patients with severe alcoholic hepatitis (SAH). (C) Cit-histone+NGAL+ NETs were counted from human cirrhotic liver tissues of SAH, NASH (non-alcoholic steatohepatitis), HCV (hepatitis C virus), AH (autoimmune hepatitis), or from acetaminophen-induced acute liver failure (APAP). Values represent means ± SEM (n=5–7). *P<0.05