Interleukin 22 (IL-22) is an IL-10-related cytokine produced by T helper 17 (Th17) cells and other immune cells that sends signals via IL-22 receptor alpha 1 (IL-22Ra1), which is expressed on epithelial tissues, as well as hepatocytes. IL-22 has been shown to have hepatoprotective effects that are mediated by signal transducer and activator of transcription 3 (STAT3) signaling. However, it is unclear whether IL-22 can directly regulate antimicrobial programs in the liver. To test this hypothesis, hepatocyte-specific IL-22Ra1 knockout (Il22Ra1<sup>Hep<sup>/−</sup>−</sup>) and Stat3 knockout (Stat3<sup>Hep<sup>/−</sup>−</sup>) mice were generated and subjected to intra-abdominal infection with Klebsiella pneumoniae, which results in liver injury and necrosis. We found that overexpression of IL-22 or therapeutic administration of recombinant IL-22 (rIL-22), given 2 h postinfection, significantly reduced the bacterial burden in both the liver and spleen. The antimicrobial activity of rIL-22 required hepatic IL22Ra1 and Stat3. Serum from rIL-22-treated mice showed potent bacteriostatic activity against K. pneumoniae, which was dependent on lipocalin 2 (LCN2). However, in vivo, rIL-22-induced antimicrobial activity was only partially reduced in LCN2-deficient mice. We found that rIL-22 also induced serum amyloid A2 (SAA2) and that SAA2 had anti-K. pneumoniae bactericidal activity in vitro. These results demonstrate that IL-22, through IL-22Ra1 and STAT3 singling, can induce intrinsic antimicrobial activity in the liver, which is due in part to LCN2 and SAA2. Therefore, IL-22 may be a useful adjunct in treating hepatic and intra-abdominal infections.
effects of IL-22. These data support further studies of IL-22 as a potential adjunctive treatment in intra-abdominal infection.

**MATERIALS AND METHODS**

**Animals.** Stat3-floxed (Stat3^{floxed}) mice in the C57BL/6 background were obtained as a kind gift from Mark H. Kaplan. Liver-specific IL-22-transgenic (TG) mice in the C57BL/6 background were generated as described previously (17). Lcn2^{+/−} mice in the C57BL/6 background have been previously described (18). Albumin-Cre mice in the C57BL/6 background and C57BL/6 wild-type mice were obtained from The Jackson Laboratory. IL-22Rα1-floxed (IL22ra1^{floxed}) mice in the C57BL/6 background were designed in our laboratory and made at Ozgene. LoxP sites were engineered to flank exons 3. All mice were bred in specific-pathogen-free rooms within animal facilities at the University of Pittsburgh. To generate liver-specific Stat3- or IL22r1α-deficient mice, we crossed Stat3^{floxed} or IL22r1α^{floxed} mice to albumin-Cre mice, respectively (see Table S1 and Fig. S1 in the supplemental material).

**Animal models.** For survival experiments, mice were intraperitoneally (i.p.) injected with K. pneumoniae (ATCC 43816) at a dose of 1 × 10^{3} to 3 × 10^{3} CFU per mouse. The mice were then monitored every 12 h for 7 days. For the IL-22 treatment model, mice were injected i.p. with 10^{4} CFU of K. pneumoniae. After 2 h, the mice received, by i.p. injection, vehicle or recombinant mouse IL-22 (rIL-22; provided by Xiaojing Yan, Generon Corporation) at 1 μg/g of body weight. After 18 h of treatment, the mice were sacrificed and the blood, liver, and spleen tissues were collected. All animal studies were approved by the Institutional Animal Care and Use Committee of the LSU Health Sciences Center or the University of Pittsburgh. Littermate Cre-negative floxed mice were used as WT controls for hepatocyte-specific knockout mice.

**Sample processing.** For bacterial CFU determinations, liver (100 mg) and spleen tissues were collected, homogenized in phosphate-buffered saline (PBS), and cultured on LB agar plates at 37°C overnight. A portion of liver tissue was also fixed in 10% formalin for hematoxylin-and-eosin (H&E) staining. Liver total mRNA was isolated using TRIzol reagent (Invitrogen). Alanine transaminase (ALT) was measured in serum at specified time points using the Vitros DT60 II chemistry system (Ortho-Clinical Diagnostics, Inc.). Serum LCN2 concentrations were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

**Histology.** Liver tissue was collected after mice were sacrificed and was fixed in 10% neutral buffered formalin. The fixed tissue was then embedded, sectioned, deparaffined, and stained with H&E. The histology scores of liver tissue sections were determined by a blinded examiner. The scoring criteria were as follows: 0, naive; 1, patchy necrosis; 2, patchy necrosis and hemorrhage; 3, patchy necrosis, hemorrhage, and loss of capsule/rupture of capsule; and 4, patchy necrosis, hemorrhage, loss of capsule/rupture of capsule, and bacterial abscess.

**Human hepatocyte assay.** Fresh human hepatocytes were obtained from Triangle Research Labs (Research Triangle Park, NC). All donors had no recent tobacco use, rare alcohol use, and were negative for HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV). The company provided cell culture medium, and the cells were cultured following the company’s protocol. The cells were stimulated with human IL-22:FC for 24 h. (IL-22:FC is a fusion protein with IL-22 complexed with the constant region of the fragment crystallizable [FC] of human IgG2). The supernatants were saved as conditioned medium for bacteriostatic and bactericidal assays. The total cellular RNA was isolated from the cell pellet using TRIzol reagent.

**Antimicrobial assays.** To assay bactericidal activity, serum or conditioned medium was cocultured with K. pneumoniae for 2 h at 37°C and then plated on an LB plate overnight at 37°C to determine the remaining CFU. To assay bacteriostatic activity, diluted serum or conditioned medium was incubated with K. pneumoniae in a 96-well plate and growth kinetics were assessed over 9 h in a heat-controlled shaking microplate reader and read at an optical density of 600 nm (OD_{600}) every hour.

**RNA sequencing.** Total RNA from liver tissue was isolated using TRIzol (Thermo Fisher) and further purified using the Qiagen RNeasy miniprep cleanup kit (Qiagen). Each sample was assessed using a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher) and Agilent Tapestation 2200 (Agilent Technologies). Sequencing libraries were generated using the Illumina TruSeq RNA Access library prep kit (Illumina). Cluster generation and 75-bp single-read single-indexed sequencing was performed on Illumina NextSeq 500 (Illumina). All assays used were completed by following the manufacturer’s protocol. For sequencing analysis, raw reads from Illumina NextSeq 500 in fastq format were trimmed to remove adapter/primer sequences. The trimmed reads were then aligned using BWA against the Mouse genome build 37.2 in GeneSifter edition for next generation sequencing (Geospiza) (19).

**Statistical analysis.** All data are presented as the mean results ± standard errors of the means (SEM). Statistical analysis was performed with a commercially available statistical software program (GraphPad Prism; GraphPad Software, Inc.). Data were tested for differences using analysis of variance (ANOVA) for mixed and random-effect models, followed by the Tukey-Kramer range test. The Student t test was performed to compare values between two groups. The log rank test was performed for survival curves. P values of less than 0.05 were considered statistically significant. All data were analyzed, and significant differences between groups are marked with asterisks in the figures.

**RNA sequencing Short Read Archive accession number.** RNA sequencing (RNA-seq) data were deposited in the Short Read Archive under accession number PRJNA306248.
Furthermore, these mice showed significantly reduced serum ALT levels (Fig. 2C) and significantly less liver necrosis (Fig. 2D and E). We next determined whether IL-22 administration induced antimicrobial activity in the serum. To assay this, we incubated sera from vehicle- or IL-22-treated mice (harvested at 24 h after IL-22 treatment) and assessed the ability of the serum to inhibit/delay the growth of *K. pneumoniae* in medium (serum comprised 10% of the final well volume). *K. pneumoniae* growth in medium in the absence of serum is shown in Fig. 2F (line a). Naïve serum (Fig. 2F, line b) showed no inhibition of growth. In contrast, serum from IL-22-treated mice (Fig. 2F, line c) showed potent inhibition/delay in growth. There was also some inhibition.
seen for serum from mice that were infected with *K. pneumoniae* regardless of whether they were treated with IL-22 or vehicle (Fig. 2F, lines d and e). IL-22 itself did not result in any *in vitro* inhibition or delay of *K. pneumoniae* (data not shown).

The therapeutic benefit of IL-22 requires hepatic IL-22Ra1/STAT3 signaling. IL-22 signals to hepatocytes via its heterodimeric receptor complex of IL-22 receptor alpha 1 (IL-22Ra1) and IL-10R2 and can lead to signal transduction via STAT3 (20, 21).
requirement for gp130, we treated mice with an anti-gp130 antibody, showing that the effect of rIL-22 treatment significantly reduced the burden of K. pneumoniae at 24 h postinoculation in the livers of control mice but not in those of II22ra1Hep−/− mice (Fig. 3A). Again, IL-22 treatment significantly reduced the burden of K. pneumoniae at 24 h postinoculation in the livers of control mice but not in those of II22ra1Hep−/− mice (Fig. 3A). Similarly, rIL-22 treatment was associated with reduced serum ALT levels in control mice (Fig. 3C), whereas II22ra1Hep−/− mice had significantly higher ALT levels than control mice (Fig. 3C). Consistent with IL-22 signaling via STAT3, studies of rIL-22 in liver-specific Stat3 knockout (Stat3Hep−/−) mice showed similar results. Compared to control mice, Stat3Hep−/− mice showed substantially greater K. pneumoniae burdens in the liver at 20 h postinoculation, and there was no reduction with rIL-22 (Fig. 3D). Similar effects were observed with serum ALT levels as well (Fig. 3E). Based on the fact that endogenous STAT3 but not IL-22Ra1 was required for control of K. pneumoniae growth in the liver, we hypothesized that the gp130 signaling pathway may be more critical for STAT3 activation in this model. To determine the requirement for gp130, we treated mice with an anti-gp130 antibody. Compared to the results for control mice, anti-gp130 antibody significantly exacerbated the hepatic K. pneumoniae bacterial burden. Strikingly, the administration of rIL-22 could rescue the anti-gp130 antibody-treated mice, showing that the effect of rIL-22 is independent of gp130 signaling (Fig. 3F).

**IL-22 control of K. pneumoniae is partially dependent on lipocalin 2.** Hepatocytes are a major source of the antimicrobial protein lipocalin 2 (LCN2) after bacterial infection, and its response is dependent on STAT3 signaling and can be induced by IL-22 (22). We first measured the serum LCN2 concentrations in K. pneumoniae-infected IL-22TG mice. Twenty-four hours after K. pneumoniae infection, IL-22TG mice had significant higher serum LCN2 levels than mice not infected with K. pneumoniae, as well as K. pneumoniae-infected WT mice (Fig. 4A). To determine whether LCN2 mediated the serum antimicrobial activity we had observed with rIL-22 treatment, we treated WT or Lcn2−/− mice with rIL-22 and assayed their sera for anti-K. pneumoniae antimicrobial activity. Sera from IL-22-treated WT mice showed significant inhibition/delay of K. pneumoniae growth, equivalent to the effect of piperacillin-tazobactam (Fig. 4B). In contrast, serum from rIL-22-treated Lcn2−/− mice did not result in any significant inhibition/delay of K. pneumoniae growth (Fig. 4B), suggesting that LCN2 is a major mediator of the in vitro serum antimicrobial activity after rIL-22 treatment.

We next determined whether LCN2 was required for hepatic control of K. pneumoniae in vivo by administering rIL-22 treatment to WT or Lcn2−/− mice. Interestingly, the liver K. pneumoniae burden was still reduced in rIL-22-treated Lcn2−/− mice.
(Fig. 4C), suggesting both LCN2-dependent and LCN2-independent mechanisms for IL-22 in vivo.

Role of serum amyloid A2 in IL-22-mediated control of K. pneumoniae. To further understand the antimicrobial mechanisms of IL-22 in the liver, we used RNA sequencing (RNA-seq) to assess gene expression in the livers of rIL-22-treated mice. After 24 h of treatment with rIL-22, liver Saa2 and Saa1 were significantly upregulated (Fig. 5A; also see Table S2 in the supplemental material). In particular, Saa2 was also upregulated in IL-22Ra1 WT control mice treated with rIL-22 (Fig. 5B). We then stimulated fresh human hepatocytes with human rIL-22 for 24 h. These cells showed a significant increase in the expression of SAA2 after rIL-22 stimulation (Fig. 5D). We next analyzed the putative structure of SAA2 using the RaptorX structure prediction algorithm. A ribbon diagram (Fig. 5C) shows that SAA2 has four putative alpha helices. The first helix (28 amino acids) is amphipathic, which has been associated with antimicrobial activity of alpha-helical antimicrobial peptides. Next, we assessed whether recombinant human SAA2 shows antimicrobial activity in vitro. Indeed, incubation of SAA2 with K. pneumoniae resulted in dose-dependent bactericidal activity (Fig. 5E).

DISCUSSION

IL-22 is an IL-10 family member that signals via IL-22Ra1, which is largely localized on epithelial cells, as well as hepatocytes (20, 23). IL-22 has been shown to play a critical role in mucosal immunity at barrier surfaces, including the gastrointestinal tract and in the lung (24). In the gut, IL-22 regulates the expression of antimicrobial proteins, such as Reg3γ (25), and can also increase fucosylation through the upregulation of Fut2, resulting in enhanced resistance to pathogens (26–28). IL-22 can also activate STAT1 and increase antiviral immunity in the gut (23). In the lung, IL-22 can induce the expression of Reg3γ (25) and Lcn2 (10), which can mediate host resistance against certain Gram-positive and Gram-negative bacteria, respectively. Based on some of these activities and its protection against epithelial damage, IL-22 is in a clinical trial for gut-related graft-versus-host disease (ClinicalTrials registration no. NCT02406651).

In the liver, IL-22 has been shown to be hepatoprotective in several models of liver injury (29). In addition, IL-22 has been shown to attenuate liver fibrosis, in part by inducing senescence of hepatic stellate cells (30). This axis also appears to be important in humans, as greater IL-22 responses were associated with less liver fibrosis in a cohort of patients with Schistosoma japonicum infection (31). IL-22 signaling can also be regulated by a decoy receptor encoded by the gene IL22Ra2, which encodes an IL-22 binding protein. Single-nucleotide polymorphisms in the gene appear to correlate with transcript levels, as well as liver fibrosis (31).

Whether IL-22 is effective in intra-abdominal infection is unclear. We chose an intraperitoneal model with a fixed dose of K. pneumoniae as it is less variable than cecal ligation and puncture and causes a reproducible liver injury. Both overexpression and therapeutic administration of IL-22 resulted in substantially reduced liver injury, as well as reduced bacterial burdens in the liver, demonstrating an IL-22-induced antimicrobial effect. This effect was intrinsic to hepatic IL-22Ra1 and STAT3 signaling, as liver-specific IL-22Ra1- or STAT3-deficient mice did not demonstrate any
antimicrobial activity. LCN2, a gene product regulated by IL-22, could mediate bacteriostatic activity but lacked bactericidal activity against *K. pneumoniae*. Moreover, the IL-22-mediated reduction in hepatic bacterial growth was partial in LCN2-deficient mice.

It has been shown that this strain of *K. pneumoniae* encodes lipocalin 2-sensitive (enterochelin) and lipocalin 2-resistant (salmochelin and yersiniabactin) siderophores (32). *K. pneumoniae* bacteria that are enterochelin gene deletion mutants but retain the expression of lipocalin 2-resistant siderophores also show resistance to lipocalin 2 in *in vivo* infection. However, the expression of enterochelin in the presence of lipocalin 2-resistant siderophores, such as yersiniabactin, still confers a degree of lipocalin 2 sensitivity in a lung infection model (32). These data are consistent with our data in this intra-abdominal infection model. Importantly, though, we also observed a lipocalin 2-independent antimicrobial activity of IL-22 in *in vivo*.

RNA-seq analysis showed the induction of serum amyloid proteins that can function as opsonins (33). Analysis of their putative structure also showed several alpha helices similar to antimicrobial peptides. Indeed, recombinant SAA2 showed bactericidal activity against *K. pneumoniae* in *in vitro*. Values represent the mean results ± SD (*n* = 3 to 5 replicates). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

ACKNOWLEDGMENTS

We acknowledge Hillary Cleveland, Chad Harrington, and Matthew Stefanovski for their technical support.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Mingquan Zheng, William T Horne, Jeremy McAleer, Derek Pociask, and Jay Kolls under grant number R37 HL079142. HHS | National Institutes of Health (NIH) | NIAID provided funding to Taylor Eddens under grant number F30AI114146.

REFERENCES


