Article

Interleukin-22 Regulates the Complement System to Promote Resistance against Pathobionts after Pathogen-Induced Intestinal Damage

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SUMMARY

Pathobionts play a critical role in disease development, but the immune mechanisms against pathobionts remain poorly understood. Here, we report a critical role for interleukin-22 (IL-22) in systemic protection against bacterial pathobionts that translocate into the circulation after infection with the pathogen *Clostridium difficile*. Infection with *C. difficile* induced IL-22, and infected $Il22^{-/-}$ mice harbored high numbers of pathobionts in extraintestinal organs despite comparable pathogen load and intestinal damage in mutant and wild-type mice. Pathobionts exhibited increased resistant against complement-mediated phagocytosis, and their intravenous administration resulted in high animal mortality. Selective removal of translocated commensals rescued $Il22^{-/-}$ mice, and IL-22 administration enhanced the elimination of pathobionts. Mechanistically, IL-22 augmented bacterial phagocytosis by increasing the expression and bacterial binding of complement C3. Our study demonstrates an unexpected role for IL-22 in controlling the elimination of pathobionts that enter the systemic circulation through the regulation of the complement system.

INTRODUCTION

The intestine of humans and animals harbors diverse microbial species that provide numerous benefits to their hosts (Kamada et al., 2013; Round and Mazmanian, 2009). However, the gut microbiota also includes potentially virulent species, called pathobionts, that can cause disease when intestinal homeostasis is disrupted, particularly in immunocompromised hosts (Chow and Mazmanian, 2010; Kamada et al., 2013). Indeed, disruption of the healthy microbiota by several mechanisms, including antibiotic treatment, results in dysbiosis, which can lead to the accumulation of pathobionts and disease (Kamada et al., 2013; Pham

and Lawley 2014). However, the mechanisms whereby pathobionts give rise to disease remain poorly understood. *Clostridium difficile* is a Gram-positive anaerobic bacterium that overgrows during dysbiosis in patients who have received broad-spectrum antibiotics and causes infectious diarrhea and pseudomembranous colitis (Carroll and Bartlett 2011). The severity of *C. difficile* infection varies from mild diarrhea to septic shock and multiple organ failure, associated with high mortality (Eaton and Mazuski, 2013; Sunenshine and McDonald, 2006). Moreover, even after treatment with antibiotics such as vancomycin and metronidazole, which eradicate *C. difficile*, 7% of patients will still die from *C. difficile* infection (Carroll and Bartlett, 2011). Although the cause of severe complications and mortality associated with *C. difficile* infection remains poorly understood, animal studies suggest that commensal bacteria contribute to disease severity. For example, after *C. difficile* infection, mice lacking interleukin-1β (IL-1β), inflammasome components, or the innate immune receptor Nod1 exhibit high mortality associated with impaired neutrophil recruitment to areas of intestinal damage and increased translocation of commensal bacteria (Hasegawa et al., 2011; Hasegawa et al., 2012). Thus, translocated commensal bacteria might contribute to *C. difficile*-infection-induced mortality, but the mechanisms by which commensal bacteria from the intestine accumulate in extraintestinal tissues and the immune factors that protect the host against their accumulation remain largely unknown.

IL-22, a cytokine produced by RORγt[+] innate lymphoid cells, T helper 17 (Th17) cells, Th22 cells, and γδ T cells, has been implicated in the control of commensal bacteria in the intestine (Sonnenberg et al., 2011). Secretion of IL-22 from intestinal immune cells is regulated by IL-23 and IL-1β, the latter of which is produced largely via the inflammasome (Sutton et al., 2009). IL-22 enhances the expression of antimicrobial proteins that limit the association between certain intestinal bacteria and the epithelium (Brandl et al., 2007; Zheng et al., 2008). Furthermore, IL-22 regulates the repair of damaged epithelium and maintains homeostasis of the microbiota (Sa et al., 2007, Zheng et al., 2008). Moreover, IL-22 plays an important role in host protection against enteropathogens, such as *Citrobacter rodentium* (Aujla et al., 2008; Basu et al., 2012; Satoh-Takayama et al., 2008; Schulz et al., 2008). Unlike *C. difficile* infection, which induces

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acute intestinal dysfunction through the production of epithelium-damaging toxins A and B (TcdA and TcdB, respectively) (Kuehne et al., 2010), intestinal disease triggered by *C. rodentium* develops more slowly, and pathogen clearance is dependent on acquired immunity (Mundy et al., 2005). Here, we report that IL-22-deficient mice exhibited increased mortality after oral infection with *C. difficile*, and mice were rescued by selective antibiotic treatment that removed commensal bacteria, but not *C. difficile.* The intestinal burden of *C. difficile* and the extent of intestinal damage were not altered in infected *Il22*$^{-/-}$ mice. Instead, IL-22 enhanced the expression of complement C3, which contributed to the clearance of specific pathobionts from peripheral organs. Hence, our study underscores a critical role for IL-22 in protecting the host beyond the intestine by facilitating the systemic clearance of intestinal pathobionts.

RESULTS

IL-22-Deficient Mice Are More Susceptible to *C. difficile* Infection

To assess the role of IL-22 in *C. difficile*-induced colitis, we infected antibiotic-treated mice with the pathogen that recapitulates many of the disease features observed in humans (Chen et al., 2008; Hasegawa et al., 2011). We detected increased production of IL-22 in the intestine, lung, and liver of mice 24 hr after infection with *C. difficile* (Figure 1A). We also detected marginal induction of IL-17 in cecum after *C. difficile* infection (Figure S1A, available online). To assess the source of increased IL-22, we infected *Rag1*$^{-/-}$ mice, which lack T and B cells, and found that increased IL-22 amounts after *C. difficile* infection were independent of RAG1, whereas no significant IL-17 induction was detected in the absence of RAG1 (Figures S1A and S1B). This suggests that the source of increased IL-22 is innate cells, but not mature T and B cells, including Th17, Th22, and γδ T cells. To investigate the role of IL-22 in host protection during *C. difficile* infection, we assessed the survival of *Il22*$^{-/-}$ mice after *C. difficile* infection and found strikingly higher mortality rates in *Il22*$^{-/-}$ mice than in wild-type (WT) mice (Figure 1B). We also found no significant impact of RAG1 deficiency on mouse survival after *C. difficile* infection (Figure S1C), suggesting that T-cell-derived cytokines, including IL-17 in this case, are dispensable for protection against *C. difficile* infection. To understand the cause of increased mortality in *Il22*$^{-/-}$ mice associated with *C. difficile* infection, we analyzed tissue histology and found a similar degree of intestinal damage, including epithelial disruption, submucosal edema, and infiltration of inflammatory cells (including neutrophils), in *Il22*$^{-/-}$ and WT mice 3 days after *C. difficile* infection (Figures 1C–1E). Consistently, flow cytometric analysis showed similar numbers of neutrophils (CD11b$^+$ Ly6G$^+$) and macrophages (CD11b$^+$F4/80$^+$) in the intestines of *Il22*$^{-/-}$ and WT mice 2 days after *C. difficile* infection (Figures 1F–1H). Additionally, *Il22*$^{-/-}$ and WT mice produced comparable amounts of CXCL1, a chemokine that is important in the recruitment of neutrophils (Figure 1I). We also found comparable weight loss in *Il22*$^{-/-}$ and WT mice and similar numbers of TUNEL-positive apoptotic cells in the intestinal epithelium after *C. difficile* infection (Figures S1D–S1F). These results indicate that deficiency of IL-22 does not affect the extent of intestinal damage or recruitment of inflammatory cells after *C. difficile*

infection and suggest that IL-22 contributes to protection against *C. difficile*-infection-induced mortality through other host defense mechanisms.

Reduction of Gammaenterobacteria Protects IL-22-Deficient Mice from Lethality after *C. difficile* Infection

The observation that *Il22*$^{-/-}$ and WT mice exhibit comparable intestinal epithelial damage and inflammatory response after *C. difficile* infection suggests that epithelial damage per se is unlikely to be the main factor that leads to the death of infected *Il22*$^{-/-}$ mice. Furthermore, similar numbers of *C. difficile* were found in the feces of infected *Il22*$^{-/-}$ and WT mice (Figure S1G), indicating that IL-22 does not protect the host by promoting clearance of *C. difficile* in the intestine. In addition, *C. difficile* was not detected in the lung or liver of *Il22*$^{-/-}$ mice, indicating that the increased mortality of *Il22*$^{-/-}$ mice was not caused by systemic translocation of *C. difficile* itself (Figure S1H). Analysis of the gut microbiota by Illumina sequencing of the 16S rRNA gene revealed that IL-22 deficiency did not significantly alter the composition of the fecal microbiota in uninfected and *C. difficile*-infected mice (Figures S1I–SK, Table S1). We also found that IL-22 deficiency did not affect the survival rate of mice infected with a streptomycin-resistant strain of *Salmonella enterica* serovar Typhimurium, whose robust colonization requires elimination of commensals by streptomycin treatment (Figure S1L). We therefore hypothesized that IL-22 might play an important role in the systemic clearance of translocated commensal bacteria after *C. difficile* infection. To test this, we determined the numbers of commensals in extraintestinal organs of *Il22*$^{-/-}$ and WT mice before and after *C. difficile* infection. Notably, increased numbers of commensals were detected in all organs tested, despite similar amounts of fecal bacteria in *Il22*$^{-/-}$ and WT mice (Figure 2A, Figure S1G). Because no significant differences in bacterial numbers were found between the organs of uninfected *Il22*$^{-/-}$ and WT mice (Figure 2A), these results indicate that in the absence of IL-22, *C. difficile* infection results in increased numbers of commensals in extraintestinal tissues. Consistently, the concentrations of serum aspartate transaminase (AST) and alanine transaminase (ALT), which are markers of liver damage, were significantly higher in *Il22*$^{-/-}$ mice than in WT mice 3 days after *C. difficile* infection (Figures 2B and 2C), most likely reflecting tissue damage associated with increased burdens of translocated bacteria in the liver of *Il22*$^{-/-}$ mice after *C. difficile* infection. Collectively, these results indicate that IL-22 regulates the clearance of translocated intestinal bacteria in extraintestinal organs after *C. difficile* infection.

To determine the contribution of translocated commensals to the pathogenesis of *C. difficile* infection, we investigated next the survival rate of *C. difficile*-infected *Il22*$^{-/-}$ mice in the absence or presence of ciprofloxacin, an antibiotic that effectively kills many commensals, but not *C. difficile*, in vitro (Figure S1M). Quantitative PCR analysis using bacterial-group-specific primers showed that ciprofloxacin treatment reduced the numbers of total bacteria and enterobacteria ~8-fold and 30-fold, respectively, but it did not affect the numbers of *C. difficile* in feces (Figure 2D). Histological analysis showed that *C. difficile* infection induced a similar degree of epithelial damage and submucosal edema in ciprofloxacin-treated and control mice (Figure S1N), which is consistent with comparable numbers of *C. difficile* in
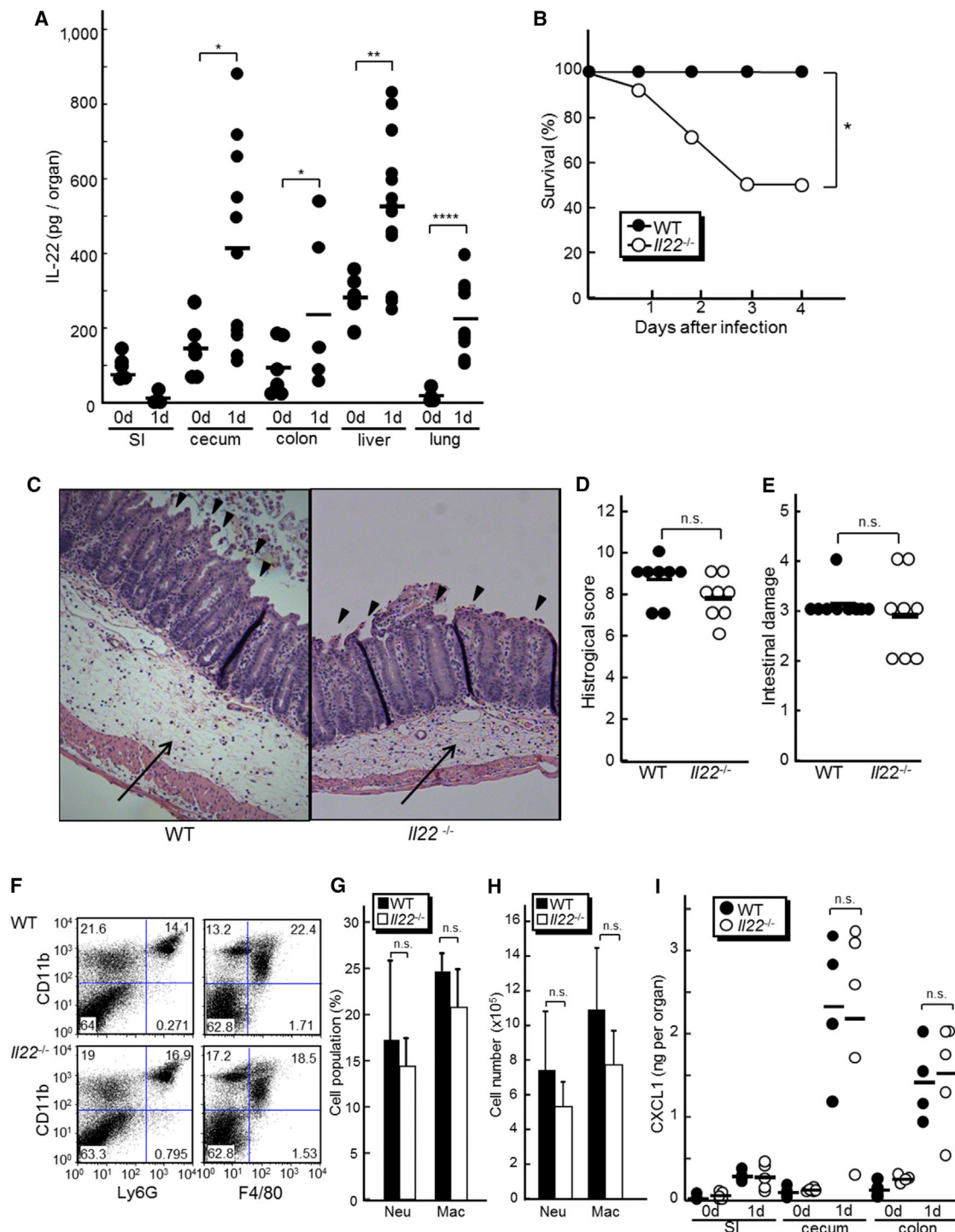
Figure 1. *Il22*^{−/−} Mice Are More Susceptible to *C. difficile* Independently of Epithelial Damage and Neutrophil Recruitment

(A) WT mice were infected with 10^8 cfu of *C. difficile* by gastric gavage after antibiotic treatment as described in the Supplemental Experimental Procedures. The amounts of IL-22 in indicated organs from uninfected (0 d, n = 6) and mice infected for 24 hr (1 d, n = 10) were determined by ELISA. SI, small intestine. Results are representative of three independent experiments.

(B) Survival of *Il22*^{−/−} and WT mice infected with *C. difficile* was monitored for 14 days (n = 16 per group). All mice were infected by gastric gavage with 10^8 cfu of *C. difficile*. No additional deaths were observed beyond 5 days after infection.

(C) Representative histology of ceca from *Il22*^{−/−} and WT mice 3 days after infection. Arrows and arrowheads show submucosal edema and epithelial damage, respectively (200× magnification). The images are representative of ten mice per group.

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antibiotic-treated and untreated mice. Notably, ciprofloxacin treatment completely rescued $Il22^{-/-}$ mice from *C. difficile*-infection-induced lethality (Figure 2E). Importantly, ciprofloxacin treatment dramatically reduced the number of bacteria translocated in extraintestinal organs after *C. difficile* infection (Figure 2F). Furthermore, Illumina sequencing revealed that ciprofloxacin treatment strongly reduced the number of Enterobacteriales species in the liver and feces (Figure 2G). Taken together, these results suggest that translocated commensal bacteria after *C. difficile* infection contribute to the death of $Il22^{-/-}$ mice, which can be rescued by ciprofloxacin treatment to limit the extent of commensal-bacteria translocation.

Translocation of Specific Species of Pathobionts after *C. difficile* Infection Contributes to Lethality in IL-22-Deficient Mice

To investigate the role of individual bacterial species in *C. difficile*-induced disease, we further characterized the composition of the bacteria present in the liver of *C. difficile*-infected $Il22^{-/-}$ mice and isolated dominant bacteria from the organs of *C. difficile*-infected mice to assess their pathogenicity. The most abundant bacteria in the liver after *C. difficile* infection were Enterobacteriaceae species (including several belonging to the *Enterobacter cloacae* complex), *Klebsiella pneumonia*, and *Escherichia coli* (Figure 2G). These bacteria represented ~89% of anaerobically cultivable bacterial species detected as operational taxonomic units (OTUs) in Figure 2G. In addition, Lactobacillales species, including *Enterococcus faecalis* and *Lactobacillus murinus*, were identified as the second dominant (~1% of total bacteria) group in the liver after *C. difficile* infection (Figure 2G). To evaluate the ability of these dominant bacterial species to cause lethality, we infected WT mice with individual bacterial clones by intravenous (i.v.) administration to mimic bacterial translocation after *C. difficile* infection. WT mice infected with enterobacterial species isolated from the tissues of *C. difficile*-infected $Il22^{-/-}$ mice (NI1077, NI1107, NI1078, and NI1188) died by day 3 after bacterial administration (Figure 3A), whereas all mice infected with two Lactobacillales species (NI1079 and NI1118) also isolated from the liver survived (Figure 3A). Importantly, i.v. administration of the same number of 16S rRNA phylotype-matched enterobacterial strains (NI796 and NI1185) isolated from the mouse feces did not result in lethality (Figure 3A). These results suggest that pathogenicity of enterobacterial species is strain dependent. Consistently, administration of pathobiont strains, but not that of control phylotype-matched strains of Enterobacteriaceae, induced liver damage, as assessed by ALT and AST concentrations in serum (Figures 3B and 3C). Collectively, our data suggest that particular strains of enterobacterial pathobionts that accumulate and translocate into extraintestinal organs during *C. difficile* infection display remarkable lethality once in the circulation.

Enterobacterial Pathobionts Isolated from *C. difficile*-Infected $Il22^{-/-}$ Mice Are Resistant to Complement-Mediated Phagocytosis

The differential ability of individual commensals to induce tissue damage and lethality in vivo could be due to differences in cytotoxicity. However, both pathobiont and nonpathobiont commensal strains showed similar cytotoxicity and cytokine induction in vitro (Figures S2A–S2C). Therefore, we hypothesized that pathobiont strains possess a unique ability to escape elimination from host tissues. To test this, we determined the survival of individual commensals after i.v. administration into WT mice. Comparison of bacterial numbers in the blood, liver, and lung of infected mice showed that pathobionts had higher survival rates than control commensal bacterial strains (Figure 4A, Figure S3A). Moreover, the survival rates of individual commensals were proportional to their ability to induce host lethality and organ damage, as revealed by release of AST and ALT into the circulation after inoculation in mice (Figure 3). These results suggest that pathobionts cause detrimental tissue damage by evading host protective immunity.

Previous studies have shown a critical role for neutrophils in the elimination of translocated commensals at intestinal sites in the mouse model of *C. difficile* infection (Hasegawa et al., 2010; Hasegawa et al., 2012). Therefore, we hypothesized that the enterobacterial pathobionts isolated from $Il22^{-/-}$ mice after *C. difficile* infection have the ability to avoid elimination by neutrophils or other phagocytic cells. Indeed, isolated pathobionts were more resistant than control phylotype-matched commensal strains to both killing by neutrophils (Figure 4B) and phagocytosis by macrophages (Figure 4C). To determine more comprehensively the susceptibility to phagocytosis of translocated pathobionts that survived in organs such as the liver after *C. difficile* infection, we examined the sensitivity to phagocytosis of 100 randomly selected bacterial clones isolated from the livers and feces of $Il22^{-/-}$ mice 3 days after *C. difficile* infection. The proportion of phagocytosis-resistant commensal clones was significantly higher in the livers of *C. difficile*-infected $Il22^{-/-}$ mice than in the feces of the same mice (Figure S3B). These results suggest that some translocated commensals are able to evade phagocytosis and thus survive in the mouse organs after *C. difficile* infection.

Phagocytosis of bacteria is facilitated by opsonization with immunoglobulins (Igs) and complement factors (Noris and Remuzzi 2013). Therefore, we tested the ability of the sera from $Rag1^{-/-}$ and $C3^{-/-}$ mice, which are deficient in Ig production and all complement pathways, respectively (Noris and Remuzzi 2013), to facilitate phagocytosis of pathobionts and control commensals. The sera from $C3^{-/-}$ mice poorly promoted the phagocytosis of

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(D and E) The overall pathology (D) and epithelial-damage (E) scores are based on the analysis of ceca on day 3 postinfection. Results are representative of five independent experiments.

(F–H) Immune cells isolated from ceca and colons of $Il22^{-/-}$ and WT mice (n = 5 per group) were analyzed by flow cytometry 2 days postinfection. (F) Percentage of cells labeled with anti-CD11b and anti-Ly-6G or anti-CD11b and anti-F4/80 mAbs are shown. (G and H) Percentage (G) and cell number (H) ± SD of CD11b⁺Ly-6G⁺ (neutrophils) or CD11b⁺F4/80⁺ (macrophages) are shown. Results are representative of two independent experiments.

(I) The amounts of CXCL1 in the indicated tissues of $Il22^{-/-}$ and WT mice (n = 5 per group) were determined by ELISA 24 hr postinfection. SI, small intestine. Results are representative of three independent experiments.

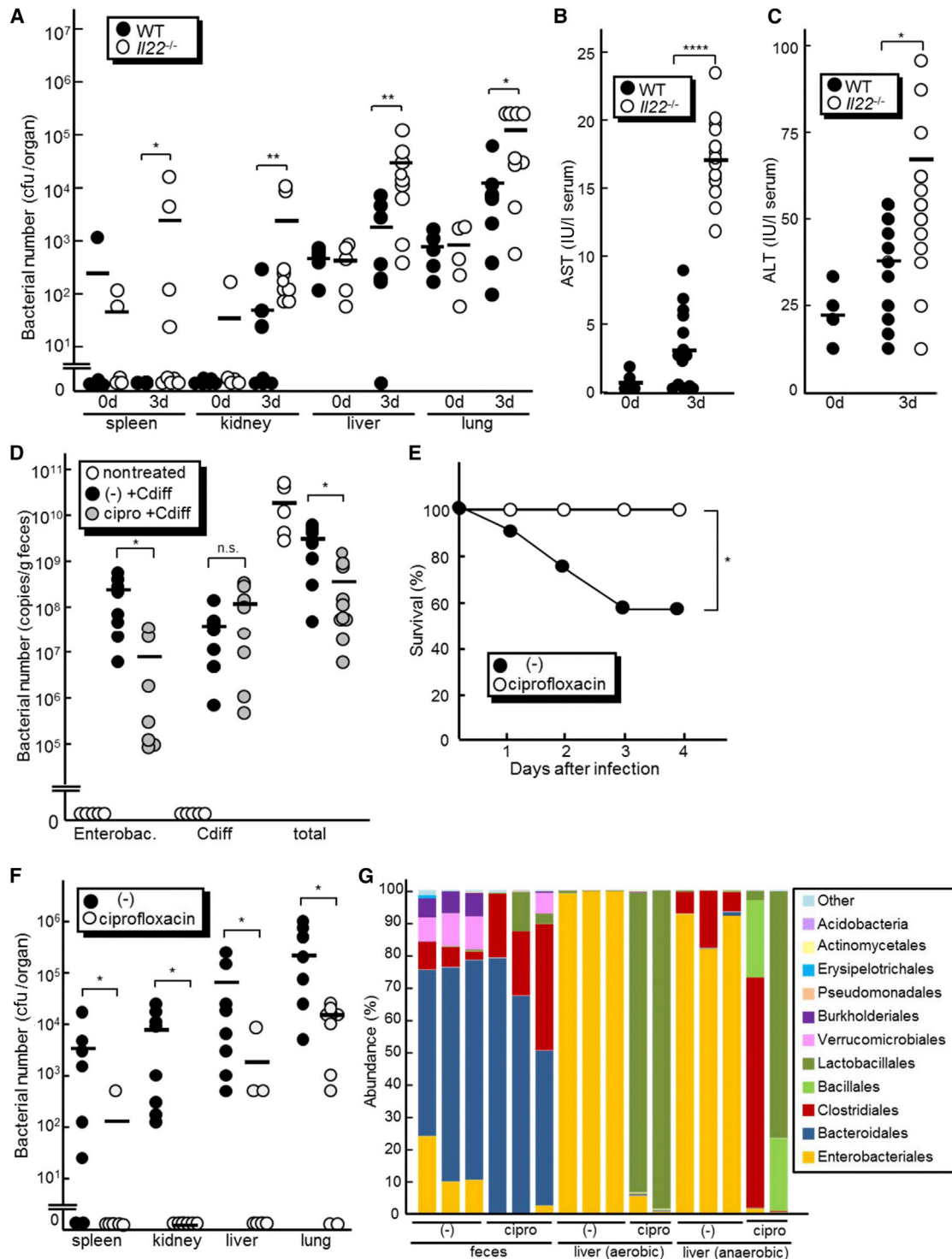Error bars indicate means. *p < 0.05; **p < 0.01; ****p < 0.0001; n.s., not significant (p > 0.05). See also Figure S1.

Figure 2. Increased Load of Commensals in the Peripheral Organs of Infected *Il22*$^{-/-}$ Mice

(A) The number of total cultivable bacteria in spleen, kidney, liver, lung, and feces from *Il22*$^{-/-}$ (n = 9) and WT (n = 8) mice infected with 10^8 cfu of *C. difficile*. Bacterial loads were determined on days 0 and 3 postinfection by plating on nonselective BHI.

(B and C) The concentrations of AST (B) and ALT (C) in sera from *Il22*$^{-/-}$ (n = 12) and WT (n = 10) mice 3 days after *C. difficile* infection. Results are representative of two independent experiments.

(D–G) *Il22*$^{-/-}$ mice were treated with 417 mg/kg ciprofloxacin in drinking water or were left alone (−) after *C. difficile* infection. (D) The numbers of total bacteria, enterobacteria, and *C. difficile* in feces of ciprofloxacin-treated and untreated control mice (n = 7 per group) on day 2 postinfection were determined by quantitative RT-PCR. (E) Survival of ciprofloxacin-treated (n = 8) and untreated (n = 7) mice infected with *C. difficile* was monitored for 14 days. No additional
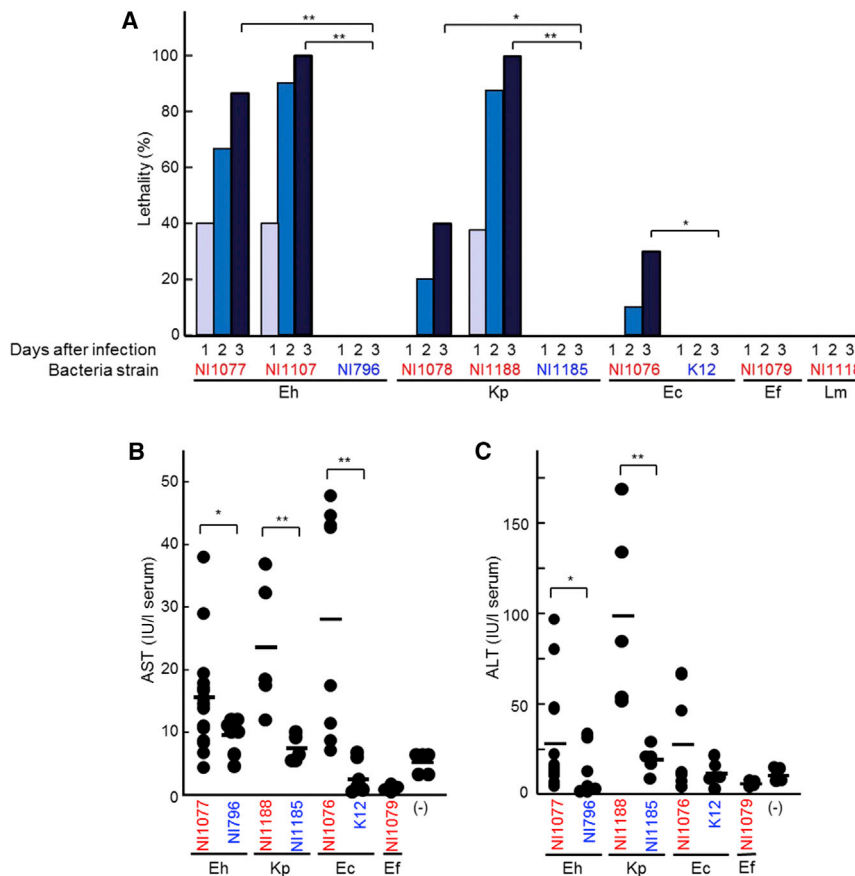
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Figure 3. Enterobacteria Isolated from Liver of Mice Infected with *C. difficile* Exhibit Enhanced Lethality after i.v. Administration

Enterobacteriaceae *E. hormaechei* (Eh) NI1077, NI1109, and NI796; *K. pneumoniae* (Kp) NI1078, NI1188, and NI1185; *E. coli* NI1076 and K12; Lactobacillales *E. faecalis* (Ef) NI1079; and *L. murinus* (Lm) NI1118 were administered (2 × 10^8 cfu) to WT mice intravenously.

(A) Survival of infected mice (n = 10) was monitored for 14 days. No additional deaths were observed beyond 5 days after infection. Results are representative of three independent experiments.

(B and C) The concentrations of AST (B) and ALT (C) in the serum 3 days after infection. Strains isolated from the liver after *C. difficile* infection are indicated in red, whereas control strains (isolates from the murine intestine and K-12) are indicated in blue.

Error bars indicate means. *p < 0.05; **p < 0.01. Results are representative of three independent experiments. See also Table S1.

all tested bacteria by macrophages, although pathobiont strains were more resistant to C3-mediated phagocytosis (Figure 4D). In contrast, the sera from *Rag1*$^{-/-}$ and WT mice showed a similar ability to facilitate phagocytosis of both groups of bacteria (Figure 4D). These results suggest that C3, but not antibodies, is important for phagocytosis of commensal bacteria. To test whether pathobionts are less susceptible to complement-mediated phagocytosis by inhibiting either C3 activation or C3 deposition on their surface, we incubated the bacteria in fresh murine serum as a complement source for 30 min. After incubation, we detected comparable production of C3 subfragments by immunoblotting, suggesting that pathobiont and control bacterial strains elicit similar complement activation (Figure 4E, lower panel). To assess the amounts of C3 subfragments deposited on bacteria, we removed unbound C3 by extensive washing and immunoblotted bacterial extracts with anti-C3 antibody. Notably, C3 deposition was lower in pathobionts than in phylotype-matched commensals (Figure 4E, upper panel). These results indicate that pathobionts exhibit a reduced ability to induce C3 deposition but a similar ability to induce C3 processing. Similar amounts of bacteria-bound C3 were detected when bacteria were incubated with serum from *Rag1*$^{-/-}$ and WT mice (Fig-

ures S3C and S3D), indicating that C3 deposition by commensals is dependent on the lectin or alternative pathway, but not the classical Ig-mediated pathway. To further test which complement pathway is involved in C3 deposition on commensal bacteria, we tested the ability of commensals to bind mannose-binding lectin (MBL) proteins, which are responsible for C3 deposition in the lectin pathway (Noris and Remuzzi 2013). We found that the tested commensals did not bind MBL proteins (Figures S3E and S3F), suggesting that C3 deposition on intestinal bacteria is mediated by the alternative pathway.

Recombinant IL-22 Improves the Survival of *C. difficile*-Infected Mice by Enhancing Systemic Clearance of Intestinal Pathobionts

Il22$^{-/-}$ mice showed defective clearance of translocated pathobionts in peripheral organs after *C. difficile* infection (Figure 4A). We therefore determined whether systemic administration of recombinant IL-22 protects *Il22*$^{-/-}$ mice from *C. difficile*-infection-induced mortality. Intraperitoneal (i.p.) administration of IL-22 rescued mice from mortality after *C. difficile* infection (Figure 5A), which was associated with reduced numbers of translocated bacteria in the liver and lung (Figure 5B). To further investigate the ability of IL-22 to confer systemic protection against pathobionts, we used NI1077, one of the dominant pathobionts that translocate into extraintestinal organs after *C. difficile* infection. We found that i.v. administration of pathobiont NI1077 induced IL-1β production, but no or minimal induction of IL-22 in the liver, lung, or cecum (Figures S4A–S4E). In contrast, high amounts of

deaths were observed beyond 5 days after infection. (F) The number of total cultivable bacteria in spleen, kidney, liver, and lung from ciprofloxacin-treated (n = 9) and untreated (n = 8) mice on day 3 infection with *C. difficile*. Results are representative of two independent experiments. (G) Taxonomic composition of fecal bacteria and cultivable bacterial populations in the liver of ciprofloxacin-treated and untreated mice on day 3 after *C. difficile* infection (n = 2 or 3 per group). Error bars indicate means. *p < 0.05; **p < 0.01; ****p < 0.0001; n.s., not significant (p > 0.05). See also Figure S2.
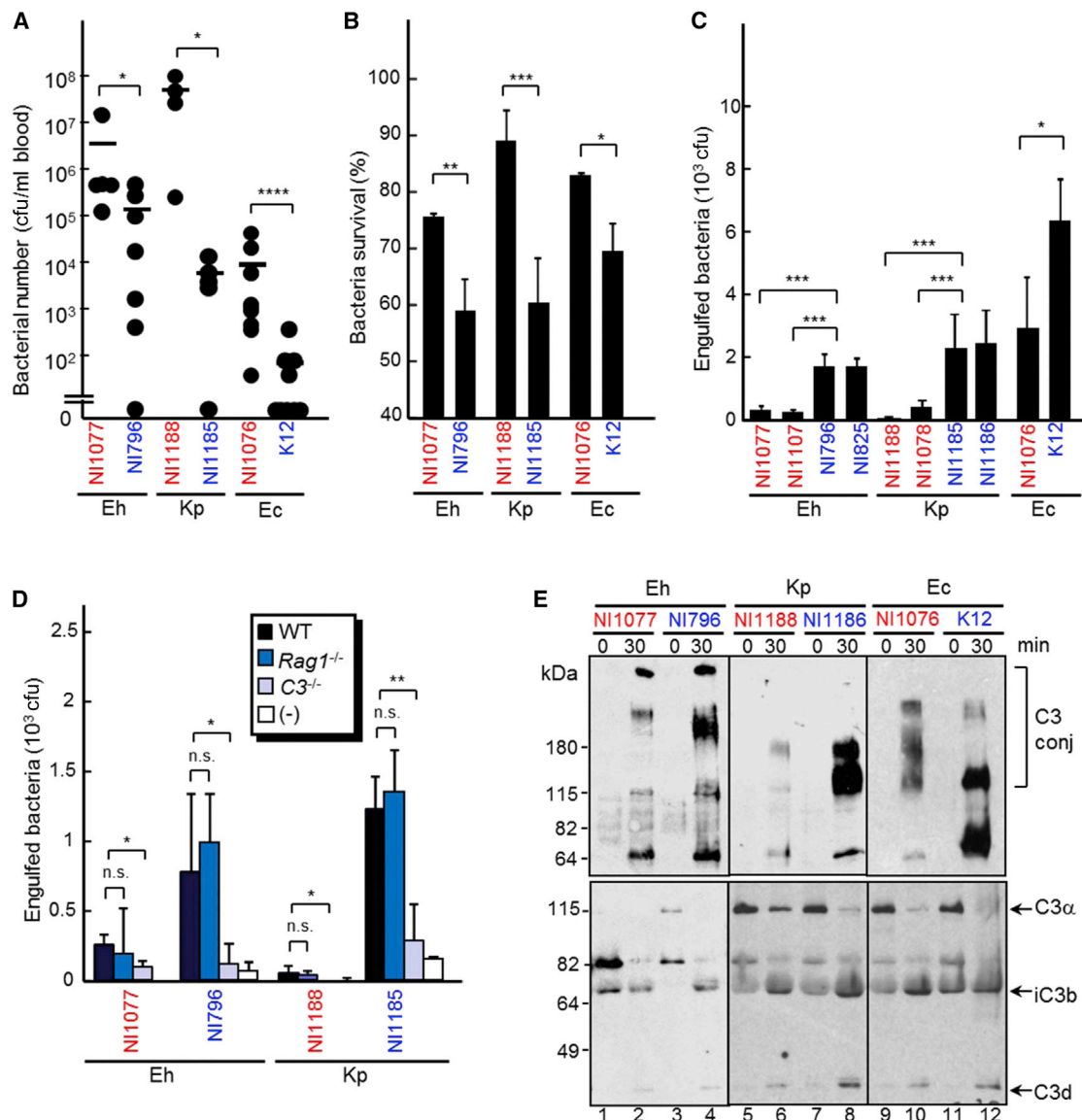
Figure 4. Increased Survival of Enterobacteria in Tissues Is Associated with Impaired Phagocytosis by Neutrophils

(A) WT mice were infected intravenously with 1×10^8 cfu of *E. hormaechei* (Eh) NI1077 and NI796; *K. pneumoniae* (Kp) NI1078 and NI1188; and *E. coli* NI1076 and K12. The number of bacteria in the blood from mice (n = 7 per group) on day 2 after infection was determined by plating on BHI media.

(B) Neutrophils were incubated with 5×10^3 cfu of the indicated strains in 2.5% fresh mouse serum for 2 hr. Surviving bacteria were counted by plating on BHI.

(C) Bone marrow macrophages were cultured with the indicated strains at moi 1:1 in the presence of 5% fresh mouse serum. The cells were treated with gentamycin 20 min after infection. Internalized living bacteria were counted by plating on BHI.

(D) Bone marrow macrophages were cultured with the indicated strains at moi 1:1 in 5% fresh mouse serum from WT, *Rag1*$^{-/-}$, or *C3*$^{-/-}$ mice. The cells were treated with gentamycin 20 min after infection. Internalized bacteria were counted by plating on BHI.

(E) C3 processing and deposition on indicated strains. A total of 5×10^8 cfu of bacteria were incubated in mouse serum for 30 min, and C3 subfragments deposited on bacteria (upper panel) and total reaction mixture (lower panel) were detected by anti-C3 antibody. C3 conj, C3 conjugated with bacteria.

Error bars indicate means ± SD. The strains that were isolated from liver after *C. difficile* infection are indicated in red, whereas control intestinal strains are indicated in blue. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant (p > 0.05). Results are representative of four independent experiments. See also Figure S3.

IL-22 were detected after *C. difficile* infection (Figure 1A). Thus, robust induction of IL-22 requires intestinal infection in the *C. difficile* infection model. To assess the role of IL-22 in protection against exposures to pathobionts, we intravenously injected NI1077 into *Il22*$^{-/-}$ and WT mice and found comparable mortality (Figure S4F). These results suggest that IL-22 protects the

host only when robustly induced after *C. difficile* intestinal infection. To assess the protective ability of IL-22, we pretreated mice with recombinant IL-22 and then intravenously injected NI1077 into *Pycard*$^{-/-}$ mice deficient in *C. difficile*-infection-induced production of IL-22 in the intestine (Figures S4G and S4H). We used *Pycard*$^{-/-}$ mice to avoid the induction of IL-22 because
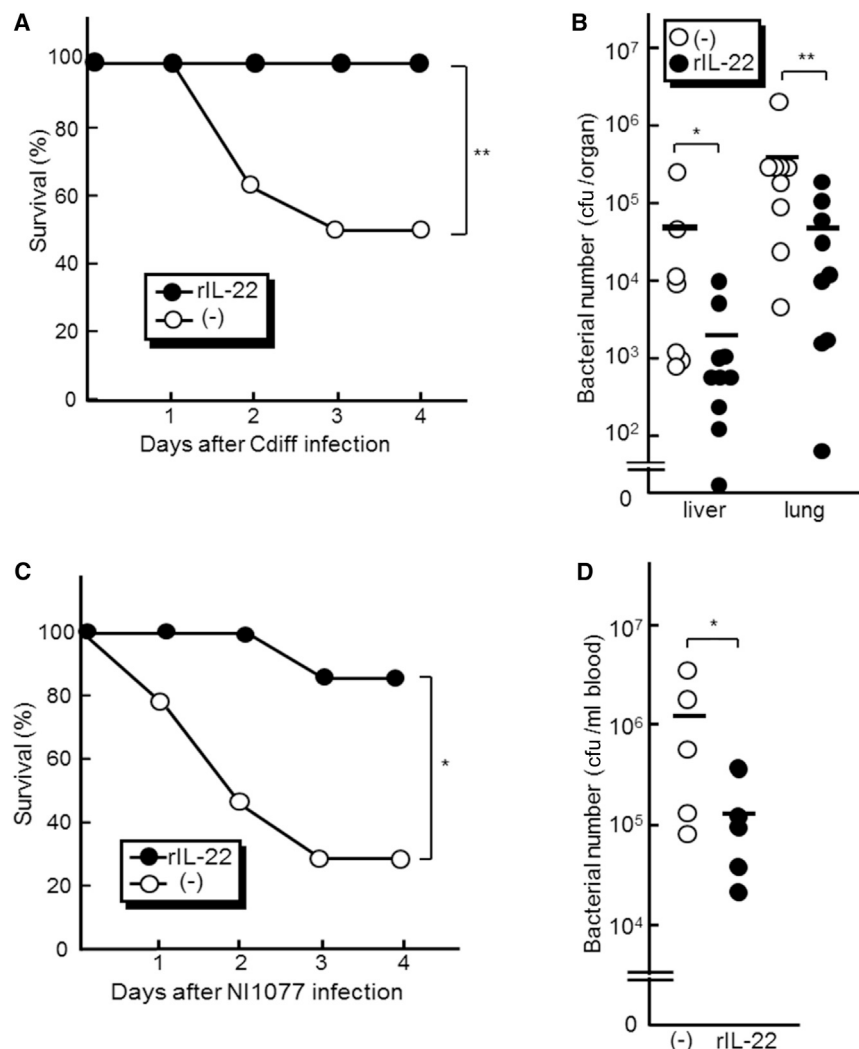
Figure 5. i.p. Administration of Recombinant IL-22 Protects the Host from Systemic Infection with Pathobionts

(A and B) $Il22^{-/-}$ mice were treated with recombinant IL-22 (1 μg per mouse) or mock by i.p. injection 12 hr after infection with 10^8 cfu of *C. difficile*. (A) The survival of infected mice was monitored for 7 days. No additional deaths were observed beyond 4 days after infection. Cdiff, *C. difficile*. (B) The number of total cultivable bacteria in liver and lung from $Il22^{-/-}$ (n = 10 per group). The bacterial number in the tissues on day 3 postinfection was determined by plating on BHI media. Results are representative of three independent experiments. (C and D) $Pycard^{-/-}$ mice were pretreated with recombinant IL-22 (1 μg per mouse) (n = 6) or mock (n = 10) by i.p. injection 1 day prior to i.v. infection with 2×10^8 cfu of the pathobiont NI1077. (C) The survival of NI1077-infected mice was monitored for 7 days. No further deaths were observed beyond 4 days after infection. (D) The bacterial number in blood on day 1 postinfection was determined by plating on BHI media (n = 5 per group).
*p < 0.05; **p < 0.01. Results are representative of three independent experiments. See also Figure S4.

$Pycard^{-/-}$ mice exhibit impaired production of IL-1β, which is required for IL-22 production in the liver and lung after i.v. administration of NI1077 (Figures S4A–S4C). Notably, administration of recombinant IL-22 improved the survival of NI1077-infected $Pycard^{-/-}$ mice, which was associated with reduced numbers of NI1077 in the blood (Figures 5C and 5D). These results suggest that IL-22 protects the host by inducing systemic immune responses that promote the clearance of harmful pathobionts and thus leads to improved host survival after *C. difficile* infection.

IL-22 Enhances Elimination of Commensal Bacteria during *C. difficile* Infection by Inducing C3 Expression and Deposition

To determine the mechanisms by which IL-22 enhances the host protective response against translocated pathobionts after *C. difficile* infection, we first tested the expression of β-defensins DEFB4 and DEFB14, whose human homologs are induced by IL-22 stimulation in keratinocytes (Wolk et al., 2004). We found increased *Defb4* mRNA expression in the cecum after *C. difficile* infection but undetectable *Defb14* mRNA expression

in WT and $Il22^{-/-}$ mice (Figure S5A). Neutrophils isolated from $Il22^{-/-}$ and WT mice showed similar bactericidal activity (Figure S5B), indicating that IL-22 does not affect the bactericidal activity of neutrophils. To further study the immune responses elicited by IL-22, we reviewed the Gene Expression Omnibus data set (accession numbers GDS3226 and GSE44091) of gene expression in IL-22-treated or TcdA- and TcdB-treated murine intestines (Zheng et al., 2008; D'Auria et al., 2013). In addition to showing increased expression of genes known to be induced by IL-22, such as those encoding S100 proteins and acute phase proteins (Liang et al., 2010; Sonnenberg et al., 2012), analysis of the data sets revealed increased expression of *C3* in IL-22-treated and TcdA- and TcdB-treated mice (Figures S5C and S4D). Therefore, we determined whether IL-22 regulates the induction of C3 expression in response to *C. difficile* infection. Quantitative RT-PCR analyses showed that the mRNA expression of *C3*, but not *Cfd* (complement factor D, a component of the alternative complement pathway), was increased in the liver and intestine 2 days after *C. difficile* infection (Figures 6A and 6B). We also confirmed the induction of other known IL-22-regulated genes after *C. difficile* infection, including those encoding S100 proteins and acute phase proteins (Figures S5E–S5H). Notably, the induction of *C3* was significantly lower in $Il22^{-/-}$ mice than in control WT mice (Figures 6A and 6B). We also found that the induction of *C3* was independent of RAG1 (Figure S5I). Furthermore, injection of recombinant IL-22 induced *C3* mRNA expression in WT mice (Figure 6C) and increased the expression of *C3* in the liver of $Il22^{-/-}$ mice after *C. difficile* infection (Figure 6A).
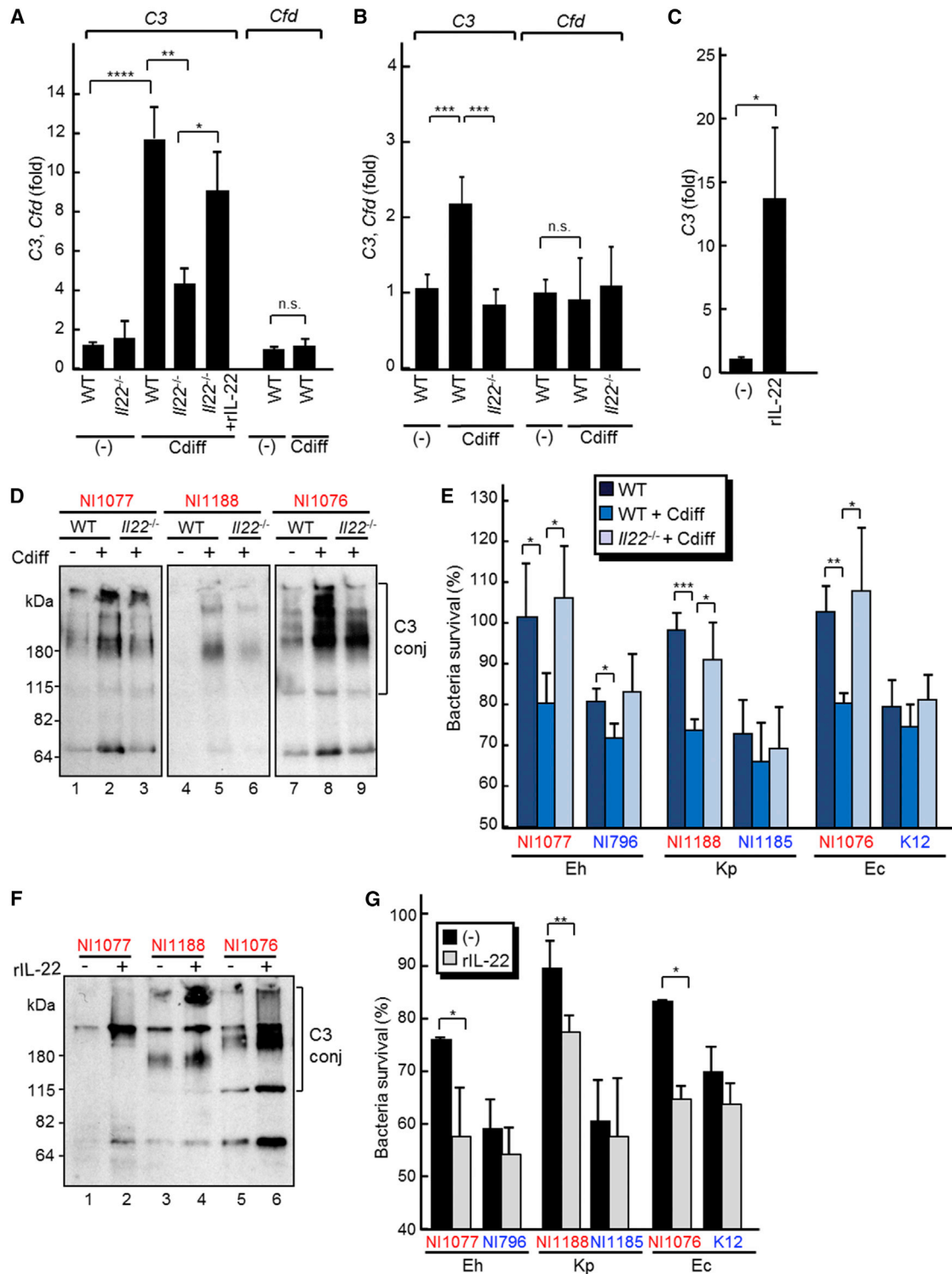
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Figure 6. IL-22 Controls *C. difficile*-Infection-Induced C3 Expression and Bacterial Deposition

(A and B) $Il22^{-/-}$ and WT mice were infected with 10^8 cfu of *C. difficile*. The expression of *C3* and *Cfd* mRNAs in liver (A) and cecum (B) of $Il22^{-/-}$ (n = 12) and WT (n = 19) mice on day 0 (−) and day 2 postinfection was determined by quantitative RT-PCR. Cdiff, *C. difficile*.

(C) WT mice (n = 5 per group) were treated with or without recombinant IL-22 (1 μg per mouse) by i.p. injection. *C3* expression in liver was determined by quantitative RT-PCR.

(D) C3 deposition on the indicated bacterial strains was detected as described in Figure 4 after incubation with sera from $Il22^{-/-}$ and WT mice infected (+) or uninfected (−) with *C. difficile*. Cdiff, *C. difficile*; C3 conj, C3 conjugated with bacteria.

(legend continued on next page)

We additionally performed immunoblotting analysis to verify that C3 amounts in serum were also increased in *C. difficile*-infected WT and *Il22*$^{-/-}$ mice (Figure S5J). Comparison of *C3* expression in organs revealed the liver as the potential major source of increased *C3* after IL-22 treatment (Figure S5K). Indeed, stimulation of the human hepatocyte cell line HepG2 with IL-22 increased *C3* expression, which was inhibited by incubation with the STAT3-specific inhibitor WP1066 (Figure S5L). In addition, the amounts of C3 deposition on pathobionts were higher when the bacteria were incubated with sera from infected WT mice than when they were incubated with sera from uninfected WT mice (Figure 6D, compare lanes 1 and 2; 4 and 5; and 7 and 8). Moreover, C3 deposition by pathobionts was lower when the bacteria were incubated with sera from infected *Il22*$^{-/-}$ mice than when they were incubated with sera from infected WT mice (Figure 6D, compare lanes 2 and 3; 5 and 6; and 8 and 9). Likewise, the bactericidal activity of neutrophils was enhanced by incubation with sera from *C. difficile*-infected WT mice, but not from *Il22*$^{-/-}$ mice (Figure 6E). Furthermore, the sera from mice treated with IL-22 enhanced both C3 deposition on pathobionts (Figure 6F) and the bactericidal activity of neutrophils (Figure 6G). These results indicate that IL-22 promotes C3 expression and deposition on bacteria and thereby enhances the ability of neutrophils to kill pathobionts.

To determine the role of C3 in IL-22-mediated protective responses, we determined the effect of C3 depletion by cobra venom factor (CVF) on the ability of serum to augment pathobiont killing by neutrophils. Immunoblotting analysis showed that C3 amounts in the serum were increased after IL-22 treatment (Figures S5M and S5N). Injection of CVF depleted C3 in naive and infected mice after treatment with IL-22 (Figure S5N). Notably, C3 depletion by CVF was confirmed by the disappearance of the C3 peak in the chromatographic profile and of the C3 α chain in Coomassie blue staining (Figures S5M and S5N, lower panel), indicating that C3 is highly abundant in the serum, consistent with the published serum-protein database (Nanjappa et al., 2014). Because IL-22 can potentially induce multiple serum proteins, we separated serum proteins by UnoQ column chromatography and determined whether the C3-containing fraction would specifically augment pathobiont killing by neutrophils. Immunoblotting analysis showed that C3 was separated from most of other serum proteins and was detected in fraction 3 (Figures S5M and S5N). The serum from IL-22-treated mice showed increased protein amounts in fraction 3 as well as in other fractions, whereas CVF-treated mice with and without IL-22 coinjection showed reduced protein amounts only in fraction 3 (Figure S5M), consistent with the finding by Coomassie blue staining. Using UnoQ column fractionation, we found that the presence of C3 (fraction 3) correlated with increased pathobiont killing activity by neutrophils, which was enhanced by IL-22 and blocked by C3 depletion with CVF (Figure S5O). Therefore, C3,

but not other abundant serum proteins, appears to be particularly important for pathobiont-killing activity by neutrophils. To determine whether C3 is important for host mortality and bacterial elimination after *C. difficile* infection and i.v. injection of the pathobiont NI1077, we treated mice with CVF to deplete C3 before *C. difficile* infection or pathobiont i.v. administration. We found that CVF-treated mice were more susceptible to both *C. difficile* infection (Figure S5P) and i.v. administration of the pathobiont (Figure S5R). Importantly, IL-22 injection failed to rescue CVF-treated mice from mortality induced by i.v. administration of the pathobiont (Figure S5R). Consistently, we found that bacterial load was higher in the liver and lung of *C. difficile*-infected mice that had been pretreated with CVF (Figure S5Q) and in the blood of pathobiont-infected mice than in mice pretreated with CVF or mice without CVF treatment (Figure S5S). Collectively, these results suggest that C3 plays an important role in IL-22-mediated pathobiont elimination during *C. difficile* infection and protects the host from pathobiont-induced lethality.

DISCUSSION

In this study, we have demonstrated a protective role for IL-22 in host defense against *C. difficile* infection. *Il22*$^{-/-}$ mice displayed higher mortality than did WT mice after *C. difficile* infection despite a comparable degree of intestinal damage and pathogen burden. In *Il22*$^{-/-}$ mice after *C. difficile* infection, we found increased translocation of enterobacterial pathobionts to extraintestinal organs, including the liver, which was associated with increased degree of liver injury. These pathobionts exhibited high pathogenicity when present in the blood, which was associated with their resistance to complement-mediated phagocytosis and killing by phagocytes. Importantly, IL-22 was required for induction of C3 after *C. difficile* infection, and IL-22 administration protected the mice from pathobiont accumulation in extraintestinal organs and pathobiont-associated mortality. Therefore, our study highlights an important role for IL-22 in limiting pathobiont-induced systemic disease and identifies a mechanism by which pathobionts can evade host immunity.

IL-22 has been shown to be critical for host defense against other pathogens (Aujla et al., 2008; Basu et al., 2012; Satoh-Takayama et al., 2008; Schulz et al., 2008). In addition, IL-22 has been implicated in the clearance of *Alcaligenes* species in lymphoid tissues (Sonnenberg et al., 2012) and affects the composition of the microbiota in mice infected with *S. enterica* Typhimurium (Behnsen et al., 2014). However, we found no significant differences between *Il22*$^{-/-}$ mice and WT mice in the microbiota composition before or after *C. difficile* infection, suggesting that IL-22 does not regulate the composition of microbiota in all infection models. In other infection models, IL-22 protects the host against pathogens via the induction of

(E) Neutrophils were incubated with the indicated strains in mouse sera from *Il22*$^{-/-}$ and WT mice infected or uninfected with *C. difficile*. The surviving bacteria were counted by plating on BHI. Cdiff, *C. difficile*.
(F and G) WT mice were treated with or without recombinant IL-22 (1 μg per mouse) by i.p. injection. (F) C3 deposition on the indicated bacterial strains was detected after incubation with sera from WT mice treated with recombinant IL-22. C3 conj, C3 conjugated with bacteria. (G) Neutrophils were incubated with the indicated strains with mouse sera from WT mice treated with or without recombinant IL-22. The survival of bacteria was assessed by plating on BHI.
Error bars indicate means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant (p > 0.05). Results are representative of three independent experiments. See also Figure S5.

antimicrobial peptides, such as RegIII proteins, and enhances the repair of the intestinal epithelium, at least in part, by preventing epithelial cell death (Brandl et al., 2007; Zheng et al., 2008). Deficiency in IL-22 also results in increased translocation of intestinal bacteria into the liver after *C. rodentium* infection. Although IL-22 protects the host by regulating the elimination of *C. rodentium* (Basu et al., 2012), IL-22 did not affect the number of *C. difficile* in the intestine. This suggests that IL-22 can also promote host survival through mechanisms distinct from pathogen elimination. Our studies highlight the importance of systemic protection against pathobionts after they are translocated into peripheral organs as a result of intestinal epithelial barrier defects induced by *C. difficile* infection. In the *C. difficile* infection model, IL-22 plays a critical role in complement-mediated elimination of pathobionts that have disseminated systemically, at least in part, by upregulating C3 expression. Therefore, IL-22 protects the host not only by promoting intestinal barrier function but also by promoting protective systemic immune responses. In the *C. difficile* infection model, inflammasome-mediated IL-1β production is also critical for both local (intestinal) and systemic immune responses by facilitating neutrophil recruitment and promoting elimination of commensals after *C. difficile* infection (Hasegawa et al. 2011; Hasegawa et al. 2012). IL-1β is also important for IL-22 production by innate lymphoid cells (Sutton et al., 2009). It is possible that the host has additional protective mechanisms against infection to link local to systemic immune responses against pathogens and pathobionts. Moreover, *C. difficile* infection induces several proteins, including S100 proteins and acute phase proteins, in an IL-22-dependent manner. S100 proteins are known to regulate C3 expression (Schonthaler et al., 2013). Therefore, S100 might also contribute to increased C3 expression during *C. difficile* infection. C3 activation by bacteria results in production of C3a and the downstream factor C5a (Noris and Remuzzi 2013). C3a and C5a are known to be involved in the recruitment of IL-1β-producing inflammatory cells, inflammasome activation, and IL-22 induction (Liu et al., 2011; Laudisi et al., 2013; Noris and Remuzzi 2013). Therefore, innate responses mediated by complement fragments IL-1β and IL-22 might exert a positive-feedback loop as a part of the complex host defense mechanism that confers both intestinal and systemic protection against pathobiont-induced morbidity. Complement fragments and IL-22 are also known to be involved in liver repair (Strey et al., 2003; Radaeva et al., 2004). Therefore, signaling mediated by complements IL-1β and IL-22 might also play an important role during the recovery phase after *C. difficile* infection. Fully understanding the mechanisms by which the host coordinates local and systemic immunity will require additional studies.

After *C. difficile* infection, a subset of patients with pseudomembranous colitis develop sepsis and multiple organ failure, which are associated with high mortality (Eaton and Mazuski, 2013; Sunenshine and McDonald, 2006). The elimination of commensals by antibiotics improves host survival after *C. difficile* infection in the mouse model, suggesting a potential therapeutic application. *C. difficile* possesses toxins that disrupt epithelial barrier function (Kuehne et al., 2010), which subsequently leads to translocation of commensals and immunostimulatory molecules through the damaged intestinal epithelium. Neutrophils that are recruited into the damaged intestine can eliminate many of the translocated commensals after *C. difficile* infection (Hasegawa et al., 2011; Hasegawa et al., 2012). However, we have shown here that certain pathobionts are resistant to host defense responses, including phagocytosis, and eventually translocate into peripheral organs to cause organ damage. Therefore, host mechanisms to boost systemic protection against translocated pathobionts, such as those mediated through IL-22, are important for survival against *C. difficile* infection and could be exploited in the treatment against *C. difficile* infection. Our study has shown that γ-enterobacterial commensals as one of the dominant pathobionts responsible for complications of *C. difficile* infection in the mouse model. Importantly, individual strains within the same γ-enterobacterial species display differential sensitivity to complement-mediated phagocytosis and cause varying degrees of lethality to the host. One of the complement-resistance mechanisms exhibited by bacteria is capsular polysaccharides (CPSs) on the bacterial surface, which can shield bacteria from recognition by complement factors (Rautemaa and Meri, 1999). In addition, the outer structures of lipopoly(oligo)saccharides (LPSs or LOSs) are crucial for protecting bacteria against host immune responses, including complement activity (Roberts, 1996). The complement-resistant structural determinants of CPSs and LPSs are highly diverse among enterobacteria (Rautemaa and Meri, 1999). Therefore, the differential sensitivity of individual strains within the same bacterial species to complement might be ultimately dependent on their surface structures. Our study highlights the importance of complement resistance in the pathogenesis of commensal-mediated intestinal disease. Therefore, understanding the mechanisms by which pathobionts acquire complement resistance will be critical for treating systemic complications caused by gut commensal bacteria. The presence of commensals is important to suppress *C. difficile* expansion in the intestine, and elimination of competitive commensals by antibiotics results in overgrowth of *C. difficile* and *C. difficile*-infection-related disease (Carroll and Bartlett, 2011). Currently, elimination of *C. difficile* with vancomycin and/or metronidazole is the first-line treatment for *C. difficile*-infected patients (Rupnik et al., 2009). However, some patients suffer relapsing disease that is refractory to standard antibiotic treatment. Transplantation of fecal microbiota from healthy donors has been shown to be effective for the prevention of disease recurrence (Koenigsknecht and Young, 2013; Lo Vecchio and Cohen, 2014). Because individual strains within the same species possess differential resistance against host elimination and can give rise to varying degrees of disease severity, it will be important to assess in detail the composition of the donor microbiota in fecal transplants to avoid transplanting potential pathobionts to patients already sickened by infection and dysbiosis.

EXPERIMENTAL PROCEDURES

Bacterial Strains

C. difficile VPI10463 and *E. coli* K-12 were cultured as previously described (Hasegawa et al., 2012). *Enterobacter hormaechei* NI1077, NI1109, and NI796; *Klebsiella pneumoniae* NI1078, NI1188, and NI1185; *E. coli* NI1076; *E. faecalis* NI1079; and *L. murinus* NI1118 were isolated from the intestine of *C. difficile*-infected or uninfected mice and cultured at 37°C in brain heart infusion (BHI) medium (BD) under an aerobic environment, and their 16S rRNA gene phylotypes were determined as described in Hasegawa et al. (2012).

Mice

WT, $C3^{-/-}$, and $Rag1^{-/-}$ C57BL/6 (B6) mice were obtained from the Jackson Laboratory. $Il22^{-/-}$ mice in B6 background were a gift from Dr. Wenjun Ouyang (Genentech). All mice were housed, bred, and maintained under specific pathogen-free conditions as described in Hasegawa et al. (2012). Mice in different cages or derived from different sources were cohoused for 2 weeks for normalization of the microbiota composition before experiments. The mouse studies were approved by the University of Michigan Committee on Use and Care of Animals.

C. difficile Infection and Analyses

Eight-week-old mice were infected with 10^8 colony-forming units (cfu) of C. difficile VPI10463 after antibiotic treatment as described in Hasegawa et al. (2012). Postinfection analyses were performed as described in the Supplemental Experimental Procedures.

Analysis of Microbiota Composition

Bacterial DNAs were extracted from mouse intestines as described in Hasegawa et al. (2012). The V4 region of the 16S rRNA gene (252 bp) was sequenced with an Illumina MiSeq sequencer, and ≈22,000 reads were analyzed by Mothur (Schloss et al., 2009; Kozich et al., 2013). OTUs were classified into taxonomic phylotypes at >97% identity with the use of Mothur (Schloss et al., 2009).

Preparation of Intestinal Immune Cells and Flow Cytometric Analysis

Intestinal lamina propria cells were prepared from mice 2 days postinfection, and immunostaining and flow cytometric analysis were performed as previously described in Hasegawa et al. (2012). In brief, isolated lamina propria cells were stained with fluorescein-isothiocyanate-labeled CD11b monoclonal antibody (mAb; M1/70), allophycocyanin-labeled mAb F4/80 (BM8), and phycoerythrin-labeled Ly-6G mAb (1A8). Isotype-matched antibodies (BD) were used as controls. Dead cells were excluded with 7-AAD staining. Neutrophils were defined as $CD11b^+Ly-6G^+$ cells, and macrophages were defined as $CD11b^+F4/80^+$ cells.

Assay of Systemic Infection

Mice were injected with 2×10^8 cfu of isolated commensals intravenously. Bacterial number in the blood on day 3 was determined by serial plating of diluted blood, and colonies were counted on BHI plates after 24 hr incubation under aerobic conditions.

Assay of Phagocytosis and Bacterial Killing

Bone-marrow-derived macrophages were cultured with isolated bacteria (multiplicity of infection [moi] 1:1) for 20 min with or without 5% fresh mouse serum and then treated with 50 μg/ml gentamycin for 1 hr after five washes with PBS. The cells were lysed with 0.1% Nonidet P-40 in PBS and plated on BHI. Under these conditions, 91% ± 3% of bacteria survived in macrophages for 2 hr, and 97.2% ± 0.7% of bacteria were killed within 24 hr. For the assay of bacterial killing, mouse peritoneal neutrophils were collected from the i.p. cavity 4 hr after i.p. injection of thioglycollate medium. Next, 5×10^5 neutrophils were incubated with 5×10^3 bacteria with 2.5% (v/v) fresh mouse serum for 2 hr. Bacterial survival was assessed by plating on BHI.

Immunoblotting for C3 Deposition

A total of 5×10^8 bacteria were incubated in 50% (v/v) mouse serum for 30 min at 37°C. Unbound C3 was removed from bacterial pellets by washing with ice-cold PBS. Samples and control 1/200 total reaction mixtures were boiled in Laemmli's buffer and separated by 10% SDS-PAGE (1×10^8 bacteria per lane). For C3 deposition, C3 was examined by immunoblotting using anti-mouse C3d antibody (R&D). For the binding assay of MBL proteins, MBLs were examined by immunoblotting using anti-mouse MBL1 and MBL2 antibodies (gifts from Steffen Thiel, Aarhus University in Denmark). For equal loading, aliquots from each sample were plated, and equal amounts of colony-forming units were lysed.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software version 6.0 (GraphPad Software). Differences between two groups were evaluated with an unpaired two-tailed Student's t test. False discovery rate q values were calculated by Mothur (Schloss et al., 2009) for comparison of OTU abundances. For multiple group comparisons, statistical analysis was performed with one-way ANOVA and then the Dunnett's or Sidak's test as a post hoc test. The survival rate of infected mice was analyzed with the log-rank test. Differences at $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.09.010.

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