Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10

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Type I interferon (IFN) α/β is critical for host defense. During endotoxicosis or highly lethal bacterial infections where systemic inflammation predominates, mice deficient in IFN-α/β receptor (IFNAR) display decreased systemic inflammation and improved outcome. However, human sepsis mortality often occurs during a prolonged period of immunosuppression and not from exaggerated inflammation. We used a low lethality cecal ligation and puncture (CLP) model of sepsis to determine the role of type I IFNs in host defense during sepsis. Despite increased endotoxin resistance, IFNAR$^{-/-}$ and chimeric mice lacking IFNAR in hematopoietic cells display increased mortality to CLP. This was not associated with an altered early systemic inflammatory response, except for decreased CXCL10 production. IFNAR$^{-/-}$ mice display persistently elevated peritoneal bacterial counts compared with wild-type mice, reduced peritoneal neutrophil recruitment, and recruitment of neutrophils with poor phagocytic function despite normal to enhanced adaptive immune function during sepsis. Importantly, CXCL10 treatment of IFNAR$^{-/-}$ mice improves survival and decreases peritoneal bacterial loads, and CXCL10 increases mouse and human neutrophil phagocytosis. Using a low lethality sepsis model, we identify a critical role of type I IFN–dependent CXCL10 in host defense during polymicrobial sepsis by increasing neutrophil recruitment and function.

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Abbreviations used: ANOVA, analysis of variance; CASP, colon ascendens stent peritonitis; CLP, cecal ligation and puncture; IFNAR, IFN-α/β receptor; SIRS, systemic inflammatory response syndrome.

Despite advancements in our understanding of innate and adaptive immunity, applying this knowledge to the treatment of sepsis has proven difficult. Sepsis causes a dramatic systemic inflammatory response syndrome (SIRS) but is also capable of causing dysfunction in both the innate and adaptive branches of the immune system (Bone, 1996; Döcke et al., 1997; Hotchkiss and Karl, 2003). In patients receiving appropriate resuscitation, the early systemic SIRS phase is rarely lethal, and patients increasingly succumb to secondary infections and organ failure during a prolonged period of immunosuppression and failure in host defense (Bone, 1996; Hotchkiss and Karl, 2003). Understanding both phases of human sepsis requires the application of appropriate animal models that can produce a SIRS response that can be overcome to allow the study of host defense during the more prolonged phases in sepsis. To this end, we use the cecal ligation and puncture (CLP) model of sepsis that creates a significant but not overwhelming SIRS response, allowing us to dissect factors that control host defense during prolonged sepsis (Scumpia et al., 2006, 2007; Delano et al., 2007).

Many factors modulate innate and adaptive immune responses and link the two branches of immunity during infection. Type I IFNs, including IFN-α, IFN-β, and IFN-ω, are ubiquitious cytokines originally shown to inhibit viral infection (Lindenmann et al., 1957; Isaacs and Burke, 1959). Recently, type I IFNs were shown to act downstream of Toll-like receptor signaling (Uematsu and Akira, 2007) to induce a specific gene activation signature, including induction of chemokines such as CXCL10 and MCP-5 (Toshchakov et al., 2002), and antimicrobial/antiviral response genes (Sadler and

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Williams, 2008). Type I IFNs also serve as a link between the innate and adaptive immune systems (Hoebe and Beutler, 2004), participating in autoimmunity (Blanco et al., 2001) and viral (Müller et al., 1994) and bacterial infection (O'Connell et al., 2004; Mancuso et al., 2007). Their functions in severe bacterial infection remain controversial, as signaling through the type I IFN pathway has detrimental consequences in certain bacterial infections (O'Connell et al., 2004; Martin et al., 2009) but protective effects in others (Mancuso et al., 2007). Importantly, in response to severe endotoxicosis or a highly inflammatory model of bacterial peritonitis, colon ascendens stent peritonitis (CASP; Maier et al., 2004), absence of the receptor for type I IFN (IFN-α/β receptor [IFNAR]; Müller et al., 1994) was shown to be protective by decreasing the systemic hyperinflammation associated with these two models (Mahieu et al., 2006; Weighardt et al., 2006).

In this report, we dissected the effects of type I IFN signaling on host immunity to a model of polymicrobial sepsis that more closely approximates the magnitude and mortality seen in human disease. We find that IFNAR$^{-/-}$ mice are more sensitive to CLP mortality, particularly at later time points. We attribute this increased sensitivity to a failure in hematopoietic cells, particularly neutrophils, to clear the infection, and not to an exaggerated inflammatory response. We demonstrate that when the SIRS response does not predominate, type I IFN is required for host defense in sepsis, and that impaired induction of the IFN-inducible CXCL10 is at least in part responsible for this protection.

RESULTS AND DISCUSSION

Deficiency of type I IFN worsens survival to CLP peritonitis but improves survival to endotoxicosis

While investigating whether type I IFN participates in the adaptive immune suppression that occurs during sepsis (Scumpia et al., 2006; Scumpia et al., 2007), we used our low lethality model of CLP that consistently causes 10–20% mortality. To our surprise, we observed that beginning 2–3 d after sepsis induction, IFNAR$^{-/-}$ mice began to die, whereas control SEV129 mice continued to survive. By 7 d after CLP, only 45% of IFNAR$^{-/-}$ mice were alive compared with 95% of the wild-type SEV129 mice (Fig. 1 A).

Because previous reports found that the absence of type I IFN signaling improves survival to endotoxin- or TNF-induced severe shock (Mahieu et al., 2006; Huys et al., 2009), and improves outcome in the CASP model of severe bacterial peritonitis (Weighardt et al., 2006), we injected wild-type SEV129 and IFNAR$^{-/-}$ mice with a lethal dose of bacterial LPS (500 μg/kg or ∼10 mg/kg of body weight). Unlike in CLP-induced sepsis, in a single pilot experiment, IFNAR$^{-/-}$ mice were highly resistant to endotoxin-induced mortality, confirming previous reports that type I IFN can participate in systemic inflammation (IFNAR$^{-/-}$ mortality of 8% vs. SEV129 mortality of 83%; P = 0.0006; Fig. 1 B).

Together with delayed mortality in the low lethality CLP model, these data suggest that an exaggerated systemic inflammatory response present in highly inflammatory models (Mahieu et al., 2006; Weighardt et al., 2006; Huys et al., 2009), which can be augmented by type I IFN, may not be playing as large a role in our model.

Type I IFN signaling does not play a major role in CLP-induced systemic inflammation

To investigate whether type I IFN had an effect on systemic cytokine responses in our CLP model, Luminex multiplex analysis was performed on plasma samples over the early course of CLP. Type I IFN signaling did not play a major role in the global cytokine or chemokine response after CLP. Preliminary data shows that plasma levels of most cytokines measured, including TNF-α, IL-6, IL-1β, and IL-10 were comparable between SEV129 and IFNAR$^{-/-}$ mice (data not depicted for IL-10; Fig. 2 A). In fact, IFNAR$^{-/-}$ mice produced significantly higher levels of KC, a chemokine highly induced during bacterial infection, 6 h after CLP than their wild-type counterparts (Fig. 2 A). The only cytokine significantly decreased in IFNAR$^{-/-}$ mice was the IFN-inducible chemokine CXCL10, which was decreased by >60% when compared with wild-type levels (IP-10; Fig. 2 A).

It is important to note that the early inflammatory response in lethal endotoxicosis and the CASP model is often several magnitudes higher than seen in human sepsis, as determined by plasma cytokine concentrations. In mouse endotoxicosis, for example, serum TNF and IL-6 concentrations can often reach the nanogram and microgram per milliliter levels (Eskandari et al., 1992), which are concentrations often three logs higher than those seen in critically ill humans (Rogy et al., 1994). During CASP peritonitis, a large bacterial load is

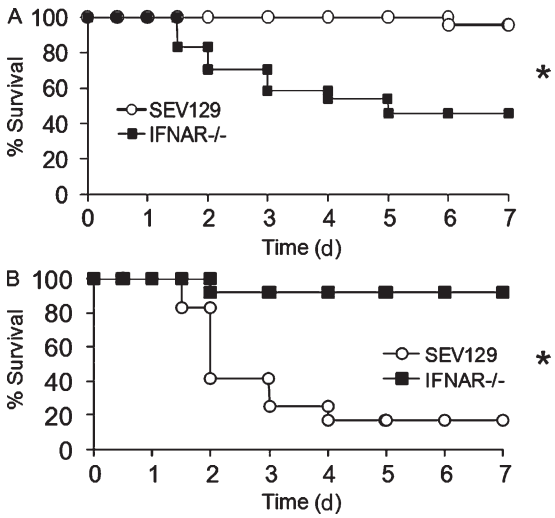

Figure 1. Type I IFN deficiency worsens CLP but not endotoxicosis survival. (A) SEV129 and IFNAR$^{-/-}$ mice underwent CLP surgery with a 27-gauge needle, and survival was monitored. The figure is the combination of two separate experiments with similar results (n = 24 per group; *, P = 0.0002 using Fisher's exact test). (B) SEV129 and IFNAR$^{-/-}$ mice were given an i.p. injection of 500 μg *E. coli* O111:B4 LPS, and survival was monitored for 7 d. The experiment was performed once (n = 12 per group; *, P = 0.0006 using Fisher's exact test).

continuously released into the peritoneum, resulting in a large initial inflammatory response (Maier et al., 2004). Similar to endotoxin shock, the absence of type I IFN decreases the magnitude of the systemic inflammatory response and is protective in this model (Weighardt et al., 2006). When mice are given a large bacterial burden, the presence of type I IFN propagates inflammation and worsens outcome (Martin et al., 2009). Alternatively, when mice are infected with smaller doses of bacteria causing a smaller inflammatory response, the presence of type I IFN appears to be protective by promoting bacterial clearance, although the mechanism has not been fully explored (Mancuso et al., 2007).

IFNAR$^{-/-}$ mice have decreased peritoneal bacterial clearance compared with SEV129 mice

Because the early systemic inflammatory response did not seem to participate in the increased mortality in IFNAR$^{-/-}$ mice, we next examined the effect of type I IFN on bacterial clearance. At various time points after CLP, bacterial content in the peritoneal lavage fluid was quantified. At 12 h after CLP, IFNAR$^{-/-}$ mice displayed decreased bacterial loads in the peritoneum compared with wild-type SEV129 animals, although this difference was not significant (Fig. 2 B). At 48 and 96 h after CLP, IFNAR$^{-/-}$ mice displayed a significant increase in bacterial load compared with the wild-type SEV129 animals ($P < 0.05$ at both time points; Fig. 2 B). IFNAR$^{-/-}$ mice failed to clear persistent infection, suggesting a defect in host defense that may explain the increased mortality in IFNAR$^{-/-}$ mice. It is important to note that the failure of mice to clear bacteria at later time points may reflect the mouse model of sepsis chosen and does not fully recapitulate human sepsis, where high doses of antibiotics are frequently used.

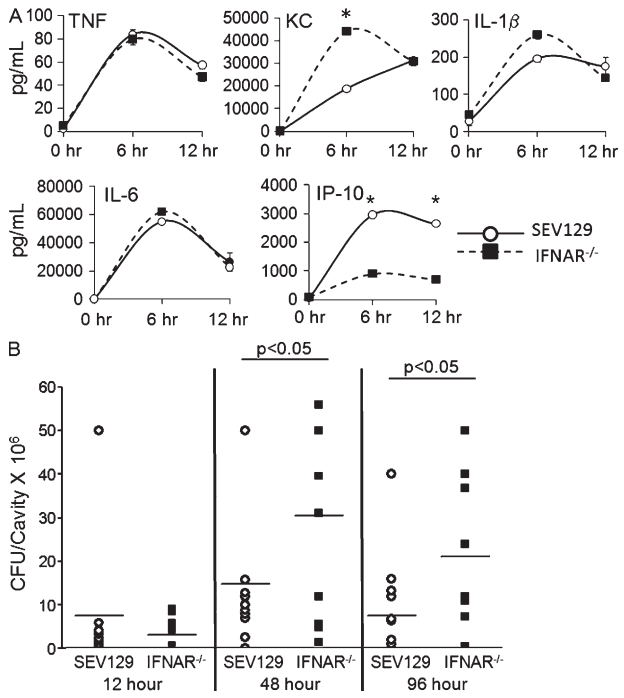

Figure 2. Type I IFN signaling does not play a role in inflammation associated with CLP. (A) SEV129 and IFNAR$^{-/-}$ mice underwent CLP surgery and were sacrificed at 0, 6, and 12 h after surgery. Serum cytokine levels from peripheral blood were determined by MILLIPLEX MAP Mouse Cytokine/Chemokine–Premixed 22 Plex kits. Select cytokines in this figure include TNF, KC, IL-6, IL-1β, and IP-10. Each time point was performed once ($n = 3$ per group per time point; *, $P < 0.05$ using the Student's *t* test). Error bars indicate SD. (B) SEV129 and IFNAR$^{-/-}$ mice underwent CLP surgery and were sacrificed at 12, 48, and 96 h after surgery. Bacteremia was determined from peritoneal lavage fluid plated on sheep blood agar. Each point represents CFUs from one mouse. The experiment was performed three times ($n = 3$ per group; $P < 0.05$ using the Student's *t* test). Horizontal bars indicate means.

Type I IFN signaling in the hematopoietic system is needed for survival to CLP

Because functioning leukocytes are critical to host defense and type I IFNs are pleiotropic cytokines that affect how all cells respond to infection, we next examined whether type I IFN signaling in the hematopoietic system was required for sepsis survival. Chimeras were created to test the radiosensitive hematopoietic and the radioresistant parenchymal systems. In brief, either SEV129 or IFNAR$^{-/-}$ mice were lethally irradiated and reconstituted with BM from either SEV129 or IFNAR$^{-/-}$ mice, creating (a) wild-type mice reconstituted with wild-type BM, (b) wild-type mice with a hematopoietic system that lacked type I IFN signaling, or (c) mice lacking IFN expression in parenchymal cells but possessing intact type I IFN signaling in their hematopoietic cells. Complete chimerism was confirmed using an antibody to the IFNAR1 receptor in circulating peripheral blood leukocytes 14 d after reconstitution (Fig. S1).

Using these chimeras, we find that mice with defective type I IFN signaling in their hematopoietic system demonstrate a 40% increase in mortality when compared with the wild-type mice reconstituted with a wild-type hematopoietic system ($P = 0.006$; Fig. 3). Mice that possessed an IFNAR$^{-/-}$ parenchymal system (IFNAR$^{-/-}$ mice) but were reconstituted with a normal hematopoietic system demonstrate a <15% decrease in survival compared with wild-type reconstituted mice ($n = 26$ mice; $P = 0.55$; not depicted), indicating the importance of type I IFN signaling within the hematopoietic system in host defense during septic peritonitis.

Both neutrophil recruitment and function require intact type I IFN signaling

Because phagocytes are the primary mediators of bacterial clearance during septic peritonitis, we investigated whether type I IFN modulates neutrophil and macrophage responses. At various time points after CLP, we examined neutrophil and macrophage counts as well as phagocytic function within the peritoneal cavity. As expected, CLP induces peritoneal neutrophil influx starting as early as 12 h after CLP in wild-type and IFNAR$^{-/-}$ mice. At this early time point, we find a small and insignificant decrease in the total number of Gr-1^{high}CD11b^{+} neutrophils of IFNAR$^{-/-}$ mice when

compared with wild-type mice (Fig. 4 A). By 48 h, however, neutrophil recruitment is significantly less in IFNAR$^{-/-}$ mice, indicating that type I IFN signaling is required for early neutrophil recruitment after CLP (Fig. 4 A). Consistent with increasing bacterial loads in the peritoneum at 96 h after CLP, IFNAR$^{-/-}$ mice actually have an increased number of peritoneal neutrophils, whereas the peritoneal neutrophil counts in wild-type mice continue to decline with their continued clearance of bacteria (Fig. 4 A). These data suggest that IFNAR$^{-/-}$ neutrophils are failing to clear the bacteria at this late time point.

We next examined whether macrophage recruitment was altered by deficient type I IFN signaling. We found that 12 h after CLP, there is a modest recruitment of macrophages to the peritoneum, but this is ~10-fold lower than the recruitment of neutrophils (Fig. 4 B). Consistent with macrophages participating in later phases of inflammation, 48 h after CLP, macrophage recruitment increases and this continues through 96 h after CLP (Fig. 4 B). Although we found a trend to decreased macrophage recruitment in IFNAR$^{-/-}$ mice at both 48 and 96 h after CLP, these data failed to reach significance (Fig. 4 B).

Because phagocytosis is an important function of both neutrophils and macrophages, we next examined whether type I IFN participates in the phagocytic function of peritoneal neutrophils by measuring their ability to engulf fluorescent beads ex vivo. As expected, neutrophil phagocytic function decreases over time after CLP in both wild-type and IFNAR$^{-/-}$ mice. Interestingly, although the ability to phagocytose stabilized at days 2 and 4 in wild-type mice, phagocytic function of IFNAR$^{-/-}$ mice continued to decline (Fig. 4 C). Although phagocytic function in macrophages also declined, IFNAR deficiency did not alter macrophage phagocytic function (Fig. 4 D). This inability to fully activate neutrophil phagocytic function, coupled with the earlier deficiency in neutrophil recruitment, likely explains the increased bacterial load seen after sepsis in IFNAR$^{-/-}$ mice.

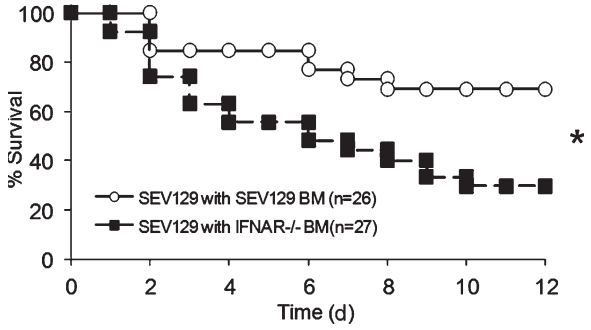

Figure 3. Type I IFN signaling in the hematopoietic system is needed for survival to CLP. SEV129 mice irradiated and reconstituted with BM from SEV129 or IFNAR$^{-/-}$ mice underwent CLP surgery with a 27-gauge needle, and survival was monitored for 12 d. The figure is the combination of two separate experiments with similar results (n = 26 for the SEV129 group and 27 for the IFNAR$^{-/-}$ group; *, P = 0.0006 using Fisher's exact test).

Enhanced adaptive immune responses in septic IFNAR$^{-/-}$ mice

We next determined whether type I IFN participates in adaptive immune suppression during sepsis. As expected, septic wild-type mice displayed impaired antibody production (IgM and IgG_{2a}) after immunization with a T cell–dependent antigen, NP-KLH. To our surprise, sham-treated IFNAR$^{-/-}$ mice displayed severely impaired responses when compared with wild-type sham mice, but septic IFNAR$^{-/-}$ mice display similar IgM production and enhanced NP-specific IgG_{2a} production compared with sham IFNAR$^{-/-}$ mice (Fig. S2). This suggests that the increased mortality in IFNAR$^{-/-}$ mice during sepsis cannot be easily explained by changes in adaptive immunity.

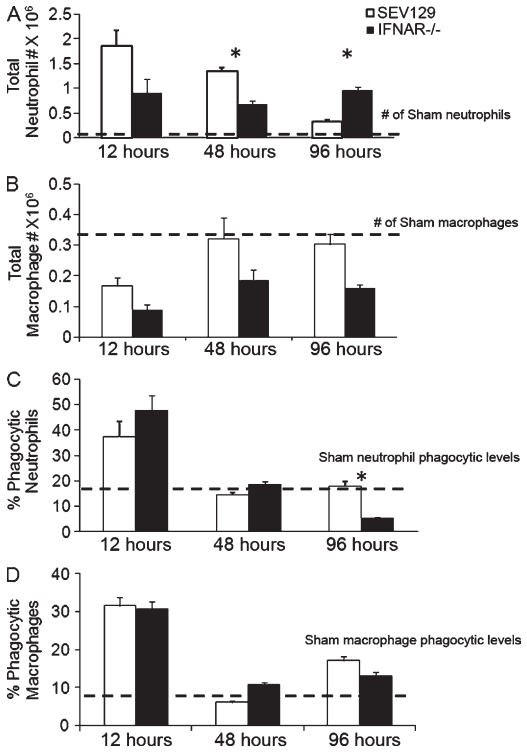

Figure 4. Neutrophil and macrophage recruitment and phagocytic function require intact type I IFN signaling. SEV129 wild-type and IFNAR$^{-/-}$ mice underwent CLP surgery and were sacrificed at 12, 48, and 96 h after surgery. Peritoneal cells were stained extracellularly for flow cytometry. (A) Total neutrophil counts were determined by gating on Gr1$^+$CD11b$^+$ cells. (B) The total number of macrophages was determined by gating on CD11b$^+$F4/80$^+$ cells. The dashed lines indicate the total number of neutrophils (A) or macrophages (B) found in sham animals. (C and D) 100,000 cells were incubated with FITC beads for 30 min at 37°C. Cells were washed two times with PBS and stained extracellularly for flow cytometry. (C) Cells were first gated on neutrophils (Gr1$^+$CD11b$^+$ cells), and then FITC$^+$ cells were considered phagocytic. (D) Cells were first gated on macrophages (CD11b$^+$F4/80$^+$ cells), and then FITC$^+$ cells were considered phagocytic. The dashed lines indicate sham phagocytic levels. *, P < 0.05 using the Student's t test. Error bars indicate SD. The data shown for all panels were obtained with 10 mice per group performed over three independent experiments.

Administration of CXCL10 improves outcome by decreasing bacteremia in IFNAR$^{-/-}$ mice

Type I IFN activates many host defense pathways that participate in host protection during sepsis. We found that plasma CXCL10 concentrations were decreased in IFNAR$^{-/-}$ mice. CXCL10 is a potent neutrophil chemoattractant (Zeng et al., 2005) that has been deemed an important host defense in bacterial peritonitis in mice (Ness et al., 2003), and is known to increase in human neonatal sepsis (Ng et al., 2006) and adults (Olszyna et al., 1999). We examined whether replacement of CXCL10 may improve survival in IFNAR$^{-/-}$ mice.

Indeed, treatment of IFNAR$^{-/-}$ mice with CXCL10 6 and 72 h after CLP improved survival compared with IFNAR$^{-/-}$ mice treated with vehicle control ($P = 0.01$ using Fisher's exact test; Fig. 5 A), and brought survival close to wild-type levels (54% in SEV mice vs. 50% in IFNAR$^{-/-}$ mice with CXCL10). Similarly, a single dose of CXCL10 6 h after CLP caused an increase in bacterial peritoneal clearance, resulting in decreased peritoneal bacterial counts 48 h after CLP ($P = 0.01$ using the Mann-Whitney *U* test; Fig. 5 B).

CXCL10 administration increases peritoneal neutrophil function and recruitment in wild-type and IFNAR$^{-/-}$ mice

We next determined whether CXCL10 treatment improved bacterial clearance in wild-type and IFNAR$^{-/-}$ mice by affecting neutrophil recruitment and phagocytic function. We find that in both wild-type and IFNAR$^{-/-}$ mice, a single dose of CXCL10 increases phagocytic neutrophil recruitment 18 h after treatment ($P = 0.001$ using one-way analysis of variance [ANOVA]; Fig. 5 C). There was a trend toward increased recruitment of neutrophils in IFNAR$^{-/-}$ mice, but this did not achieve significance.

Treatment of human neutrophils with CXCL10 improves phagocytosis

Septic adult peripheral blood neutrophils demonstrate reduced phagocytic activity during sepsis (Kaufmann et al., 2006; Danikas et al., 2008; Taneja et al., 2008). Although no human studies have examined whether type I IFN is increased in sepsis, CXCL10 was shown to be an early marker of sepsis in neonates and adults. Whether CXCL10 mediates neutrophil phagocytosis in humans, or whether levels during sepsis are sufficient or sustained long enough to mediate adequate neutrophil recruitment and phagocytic activity is unknown. In this report, we find that although human blood neutrophils ($CD66b^+CD16^{hi}$ cells) have a low phagocytic activity, treatment for 4 h with CXCL10 doubles their phagocytic ability (Fig. 6 A). Furthermore, treatment with CXCL10 for 2 h improves LPS-mediated neutrophil phagocytosis ($P < 0.001$ using one-way ANOVA; Fig. 6 B). These data suggest that CXCL10 treatment may improve neutrophil phagocytosis in sepsis.

In summary, our study identifies type I IFN responsiveness in the hematopoietic system as a protective factor in bacterial sepsis. We demonstrate a role of type I IFN in neutrophil recruitment and phagocytic function during the later stages of bacterial peritonitis. Without type I IFN–dependent neutrophil recruitment and activation, mice fail to clear bacteria, resulting in a persistently elevated bacterial burden. Previously, CXCL10 blockade was shown to worsen outcome to sepsis (Ness et al., 2003). We further demonstrate that maximal induction of CXCL10 during sepsis requires type I IFN, and administration of CXCL10 alone restores neutrophil recruitment and improves bacterial clearance in IFNAR$^{-/-}$ mice. In addition, treatment of human neutrophils with CXCL10 improves their phagocytic ability. These data provide evidence for a dual role of type I IFN in bacterial host defense,

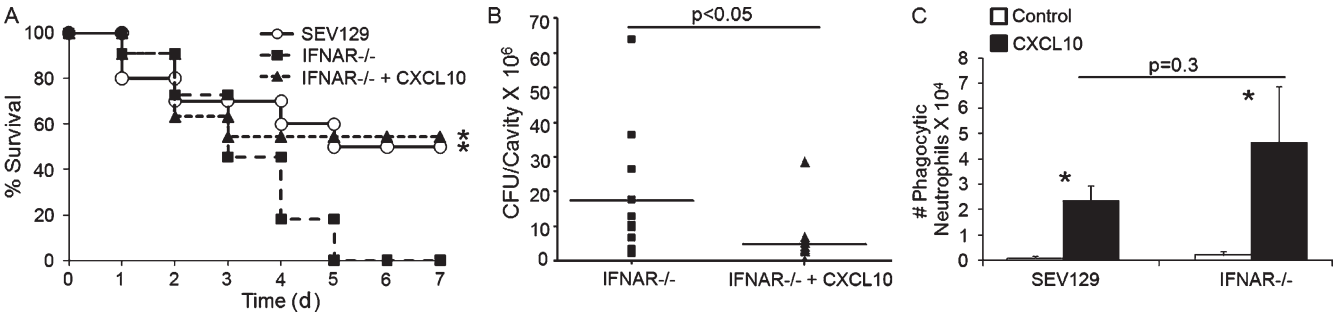

Figure 5. CXCL10 improves outcome by decreasing bacteremia in IFNAR$^{-/-}$ mice. (A) SEV129 wild-type mice ($n = 10$), IFNAR$^{-/-}$ mice ($n = 11$), or IFNAR$^{-/-}$ mice with 100 ng CXCL10 6 h and on day 3 after CLP ($n = 11$). Survival was monitored for 7 d. There was a 50% survival advantage in SEV129 wild-type compared with IFNAR$^{-/-}$ mice, and a 54% survival advantage in IFNAR$^{-/-}$ + CXCL10 compared with IFNAR$^{-/-}$ mice. *, $P = 0.01$ using Fisher's exact test. Data are from a single experiment with 10 mice in the SEV129 group and 11 mice in the IFNAR$^{-/-}$ and IFNAR$^{-/-}$ + CXCL10 groups. Two independent survival experiments were performed with consistent results. (B) IFNAR$^{-/-}$ mice underwent CLP surgery, and 6 h after surgery mice were injected with PBS or 100 ng of recombinant IP-10. Mice were sacrificed 48 h after CLP, and bacteremia was determined from dilutions of peritoneal lavage fluid obtained aseptically. Each point represents CFUs from one mouse. $P < 0.05$ using the Student's *t* test. Horizontal bars indicate means. The figure represents data from two independent experiments using four mice per group with similar results. (C) SEV129 wild-type and IFNAR$^{-/-}$ mice were injected with 100 ng CXCL10. Peritoneal cells were harvested 18 h later and were examined by flow cytometry for phagocytic neutrophils ($Gr1^+CD11b^+$ cells containing $FITC^+$ latex beads). *, $P = 0.001$ using one-way ANOVA; *, $P < 0.05$ using the Tukey post hoc analysis. Error bars indicate SD. The figure represents data from three independent experiments using at least four mice per group with similar results.

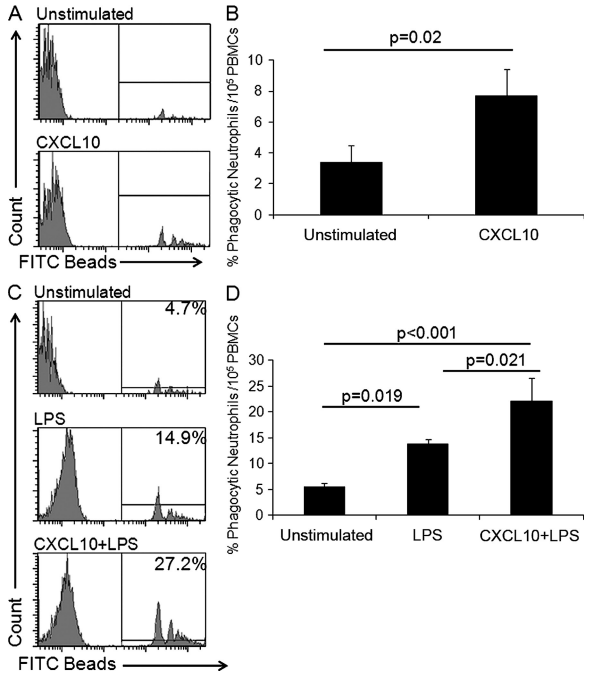

Figure 6. CXCL10 treatment improves human neutrophil phagocytic function in vitro. Whole-blood leukocytes were collected using Histopaque 1119. 10^5 unstimulated cells, cells treated with 100 ng CXCL10 for 4 or 2 h followed by an 18-h stimulation with 1 µg/ml LPS, or cells stimulated with LPS alone for 18 h were plated in round-bottom 96-well plates. FITC beads were added and cells were incubated at 37°C for 30 min. Cells were washed and stained for neutrophil markers ($CD66b^+CD16^{hi}$). (A) Representative histograms of the percentage of phagocytic neutrophils from unstimulated (top) or CXCL10 treatment for 4 h (bottom). (B) Graphical analysis of the percentage of phagocytic neutrophils from one healthy control ($P = 0.019$ using the Student's *t* test). (C) Representative histograms of the percentage of phagocytic neutrophils from unstimulated (top), LPS-stimulated (middle), and CXCL10-treated + LPS-treated leukocytes (bottom). (D) Graph of the percentage of phagocytic neutrophils from one healthy control ($P < 0.001$ using one-way ANOVA). For all panels, treatment was performed in triplicate and the experiment was performed on three healthy controls with similar results. Error bars indicate SD.

contributing to either detrimental inflammation when systemic inflammation predominates or neutrophil-mediated host defense when optimal bacterial clearance is necessary for host survival.

Type I IFN is required for survival in the much less lethal model of polymicrobial sepsis. A similar difference between these models was also found in mice deficient in the Toll-like receptor adaptor protein MyD88. Although MyD88 deficiency was protective against CASP peritonitis (Weighardt et al., 2002), MyD88 deficiency was found to worsen outcome to CLP-induced peritonitis, presumably by decreasing host defense factors, resulting in increased bacterial dissemination (Peck-Palmer et al., 2008). Similarly, in a neonatal group B streptococcus sepsis model, $MyD88^{-/-}$ neonates succumbed to a low-dose inoculum of bacteria, whereas wild-type mice survived, and the opposite was true of a high-dose inoculum of bacteria (Mancuso et al., 2004). Other pleiotropic molecules such as complement C5a receptors and HMGB1 can also show immunomodulatory effects on host defense and inflammation depending on the severity of the septic insult (Rittirsch et al., 2008). These studies, in concordance with our own, highlight the plasticity of immunomodulatory signaling cascades and their ability to participate in either detrimental systemic inflammatory cascades or protective host defense responses depending on the bacterial burden.

MATERIALS AND METHODS

Human cell-culture studies. The collection of peripheral venous blood from unidentified human volunteers was approved by the Institutional Review Board of the University of Florida. No identifying personal information was collected, and all volunteers signed informed consent.

Mice. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine. IFN-α/βR/A129 mice on the 129S6/SvEv background (H-2b; $IFNAR^{-/-}$) and wild-type SEV129 mice were purchased from B&K Universal. All mice were maintained at the University of Florida College of Medicine.

Endotoxicosis and CLP. For induction of endotoxicosis, mice were injected with 500 µg LPS isolated from *Escherichia coli* strain O111:B4 (Sigma-Aldrich) i.p. For induction of polymicrobial sepsis, mice underwent CLP or a sham procedure, as previously described (Delano et al., 2007), to obtain a mortality of 10–20% in control mice by 10 d. In brief, a laparotomy was performed, the cecum was isolated, and ~0.5 cm of cecum was ligated below the ileocecal valve and punctured through and through with a 27-gauge needle. Sham operation was performed by isolating the cecum without ligation or puncture.

Isolation of human leukocytes. Leukocytes were harvested using a Histopaque 1119 density gradient. In brief, whole blood was gently added to a layer of Histopaque 1119 (Sigma-Aldrich) and centrifuged at 700 *g* for 30 min at room temperature. A clear opaque layer of cells was visible within the gradient. This layer containing mixed leukocytes was harvested and washed with PBS. Cells were counted using a hemocytometer and plated in round-bottom 96-well plates in RPMI 1640 containing 10% FBS (Cellgro), and then stimulated ex vivo with the recombinant CXCL10. We recognize that the responses to CXCL10 treatment reflect both the direct effects of the cytokine on the neutrophil and macrophage populations, as well as effects secondary to CXC10 on other contained leukocyte populations.

Administration of CXCL10 (IP-10). For in vivo chemokine treatment, each mouse was injected i.p. with 100 ng of mouse recombinant IP-10 (R&D Systems) or PBS 6 h after surgery, and if experiments lasted longer than 3 d, again on day 3 (Vasquez and Soong, 2006). For ex vivo studies, recombinant human CXCL10 (R&D Systems) was reconstituted, as suggested by the manufacturer, in sterile PBS, and human leukocytes were treated with 100 ng CXCL10 for 2 h followed by stimulation with 1 µg/ml LPS. In some cases, the leukocytes were stimulated with CXCL10 for 4 h followed by a phagocytosis assay.

Phagocytosis assay. 10^5 cells were incubated with 10^8 yellow-green fluorescent polystyrene microspheres (FluoSpheres; Invitrogen) for 30 min at 37°. Cells were then washed and stained for additional markers (see Flow cytometry).

Flow cytometry. Immediately after mice were euthanized, 10 ml of physiological saline was injected into the peritoneal cavity and was lavaged repeatedly. Cells were washed in PBS, pelleted, and subsequently stained for flow cytometry. Mouse peritoneal cells were characterized using anti-Gr1–PE, anti-CD11b–FITC, and F4/80 allophycocyanin–Alexa Fluor 750. Human neutrophils were characterized using anti-CD66b–biotin (R&D Systems),

followed by a streptavidin–PerCP-Cy5.5 conjugation and anti-CD16–PE. Cells were counted and flow cytometry was performed using antibodies purchased from BD unless otherwise stated. Samples were acquired and analyzed on a flow cytometer (LSR II; BD). At least 10^4 live (SYTOX Blue$^-$; Invitrogen) cells were analyzed.

Chimeras. SEV129 or IFNAR$^{-/-}$ mice were treated with 1,000 rads of γ radiation. Mice were reconstituted with BM from either SEV129 or IFNAR$^{-/-}$ mice 24 h later. Mice were placed on antibiotic water 1 wk before irradiation and were maintained on antibiotic water for 6 wk after reconstitution. Chimeras were verified by staining blood collected 14 d after reconstitution with an antibody to the extracellular domain of the IFNAR1 subunit of IFNAR (BioLegend). Survival studies were performed 7–8 wk after reconstitution.

Determination of bacterial load. Peritoneal bacterial counts were determined by culturing 100 µl of serially diluted peritoneal washings on sheep's blood agar plates (Thermo Fisher Scientific) at 37°C in 5% CO_2. Plates were counted after 24 h of culture.

Multiplex cytokine analysis. Assessments of cytokine profiles from the proliferation assay were performed using a commercially available multiplexed kit (MILLIPLEX MAP Mouse Cytokine/Chemokine–Premixed 22 Plex; Millipore). Simultaneous measurement of 22 cytokines/chemokines was performed. All assays were performed according to the manufacturer's protocols. Samples were diluted 1:1 in the assay buffer provided with the kit, and at least two replicate wells were plated per sample. Cytokine concentrations were determined using BeadView software (Millipore).

Statistics. Differences in survival were determined by Fisher's exact test. Continuous variables were first tested for normality and equality of variances. Differences among groups were evaluated by the Student's *t* test or one-way ANOVA with the Tukey post hoc analysis where stated. Significance was determined at the 95% confidence level.

Online supplemental material. Fig. S1 shows verification of IFNAR$^{-/-}$/SEV129 chimeras. Fig. S2 depicts adaptive immune antibody responses to NP-KLH immunization. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091959/DC1.

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