IL-10 administration reduces PGE-2 levels and promotes CR3-mediated clearance of *Escherichia coli* K1 by phagocytes in meningitis

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Ineffectiveness of antibiotics in treating neonatal *Escherichia coli* K1 meningitis and the emergence of antibiotic-resistant strains evidently warrants new prevention strategies. We observed that administration of interleukin (IL)-10 during high-grade bacteremia clears antibiotic-sensitive and -resistant *E. coli* from blood of infected mice. Micro-CT studies of brains from infected animals displayed gross morphological changes similar to those observed in infected human neonates. In mice, IL-10, but not antibiotic or anti-TNF antibody treatment prevented brain damage caused by *E. coli*. IL-10 administration elevated CR3 expression in neutrophils and macrophages of infected mice, whereas infected and untreated mice displayed increased expression of FcγRI and TLR2. Neutrophils or macrophages pretreated with IL-10 ex vivo exhibited a significantly greater microbicidal activity against *E. coli* compared with cells isolated from wild-type or IL-10$^{-/-}$ mice. The protective effect of IL-10 was abrogated when CR3 was knocked-down in vivo by siRNA. The increased expression of CR3 in phagocytes was caused by inhibition of prostaglandin E-2 (PGE-2) levels, which were significantly increased in neutrophils and macrophages upon *E. coli* infection. These findings describe a novel modality of IL-10–mediated *E. coli* clearance by diverting the entry of bacteria via CR3 and preventing PGE-2 formation in neonatal meningitis.

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Abbreviations used: CSF, cerebrospinal fluid; LDH, lactate dehydrogenase; MDA, malondialdehyde; MRI, magnetic resonance image; PGE-2, prostaglandin E-2; PGES-2, prostaglandin synthase E-2; rh, recombinant human; SIRS, systemic inflammatory response syndrome.

Escherichia coli K1 is the most common cause of meningitis in premature infants (46%), whereas it is the second most common agent in full-term neonates (15%; Bonacorsi and Bingen, 2005; Shah et al., 2005). Mortality rates of 5% are recorded in children in the developed world, but these rise to ∼30% in developing countries (Bedford et al., 2001; Houdouin et al., 2008). Despite recent advances in antibiotic therapy and supportive care, bacterial sepsis and meningitis caused by *E. coli* remain a serious disease (Mulder et al., 1984; de Louvois et al., 1991). Although the mortality rates can be reduced by antibiotic treatment, the neurological sequelae in 30–40% of the survivors lead to mental retardation, hearing loss, and other complications (Kim, 2003). Ventriculitis frequently accompanies neonatal meningitis, particularly when caused by *E. coli* K1, *Enterobacter sakazakii,* and other Gram-negative organisms (Jones et al., 2004). A recent surge in antibiotic-resistant strains of *E. coli* K1 may significantly increase the mortality and morbidity rates (Boyer-Mariotte et al., 2008; Dubois et al., 2009). In addition, the prognosis of meningitis is difficult until the bacteria reach the cerebrospinal fluid (CSF), by which time greater amounts of proinflammatory cytokines are circulating in the blood and the progression of brain damage has begun. Treatment with antibiotics during high bacteremia releases significant amounts of endotoxin, which often causes septic shock and, ultimately, organ dysfunction. Therefore, alternative avenues to treat and prevent this deadly disease are needed.

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 6 1307-1319
www.jem.org/cgi/doi/10.1084/jem.20092265

The Journal of Experimental Medicine

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A certain threshold of bacteremia is required for the adherence of *E. coli* to the cerebrovascular endothelium and for subsequent crossing of the blood–brain barrier (Xie et al., 2004), indicating that the bacterium in circulation must evade host defense mechanisms. Complement and phagocytes are responsible for the clearance of bacterial pathogens at early stages of infection (van Lookeren Campagne et al., 2007). Our studies demonstrated that *E. coli* K1 avoids complement attack by binding to C4b-binding protein, a modulator of the classical complement pathway via outer membrane protein A (Prasadarao et al., 2002; Wooster et al., 2006; Maruvada et al., 2008). Neutrophils and macrophages form an important line of defense against invading pathogens and phagocytose pathogens through a variety of surface receptors, especially FcγRI and CR3 (Isberg and Tran Van Nhieu, 1994; Aderem and Underhill, 1999; McCoy and O'Neill, 2008). Neutrophils often increase in number during sepsis, a stage preceding meningitis, and represent an important source of proinflammatory cytokines (Pinheiro da Silva and Soriano, 2009). Neutrophils are programmed to undergo constitutive apoptosis in the absence of prosurvival stimuli in keeping with their short-lifespan (Kennedy and DeLeo, 2009). Critically, apoptotic cells also serve as a source of antigen for antigen-presenting DCs. However, *E. coli* K1 also interacts with DCs to suppress both maturation and antigen presentation (Mittal and Prasadarao, 2008). The fact that macrophage apoptosis might also benefit the host is supported by the observation that many bacteria have evolved mechanisms to facilitate survival within macrophages (Sansonetti, 2001). Our studies demonstrate that *E. coli* enters macrophages by increasing the expression of FcγRI and TLR2 (Mittal et al., 2010) and multiply, indicating that *E. coli* utilizes several strategies for survival during the progression of infection that results in meningitis.

Although the neonatal inflammatory response is considered intrinsically hyporesponsive, the clinical observation is that neonates more often develop a severe systemic inflammatory response syndrome (SIRS) during sepsis than children and adults (Pillay et al., 1994; Schultz, et al., 2002). Pathophysiological events of sepsis suggest that proinflammatory molecules that initiate SIRS trigger the release of antiinflammatory molecules to limit inflammation (Bone et al., 1997). The antiinflammatory response, which is primarily mediated by IL-10 and TGF-β, is referred to as the compensatory antiinflammatory response syndrome (CARS; Powell, 2000). Therefore, an imbalance between SIRS and CARS is responsible for the exaggerated inflammatory response in neonates, and thus, for the high morbidity and mortality of preterm infants during infection (Duggan et al., 2001; Schultz et al., 2004). However, the role of IL-10 in *E. coli* infection is virtually unknown. In this study, a well-established newborn mouse model of meningitis using WT and IL-$10^{-/-}$ mice was used to determine the role of IL-10 in the pathogenesis of meningitis by *E. coli* K1. Furthermore, we examined the effect of IL-10 administration on the progression and resolution of infection.

RESULTS

IL-10 is required for host survival, pathogen control, and prevention of hyperinflammatory immune response in neonatal *E. coli* K1 meningitis

To investigate the role of IL-10, 3-d-old WT and IL-$10^{-/-}$ mice were infected intranasally with 10^3 CFU of *E. coli* K1 and various parameters of the resulting infection and disease were compared. Analysis of serum samples at various time points revealed that IL-10 production peaked around 12 h after infection, whereas peak production of TNF, IFN-γ, IL-1β, IL-12, and IL-6 was observed at 72 h after infection in WT mice (Fig. 1, A and B, and Fig. S1, A–D). In contrast, these proinflammatory cytokines were observed in significantly higher quantities by 24 h after infection in IL-$10^{-/-}$ mice ($P < 0.01$ compared with WT animals; Fig. 1 B and Fig. S1, A–D). A high level of bacteremia (10^6 CFU of bacteria) was observed in WT animals starting from 48 h, whereas IL-$10^{-/-}$ mice showed similar bacteremia levels by 12 h after infection (Fig. 1 C). The WT animals developed meningitis as demonstrated by positive CSF cultures, between 72 and 96 h after infection, whereas IL-$10^{-/-}$ mice showed the pathogens in CSF by 16 h. IL-$10^{-/-}$ animals succumbed to infection within 48 h even at an inoculum size of 10^2 CFU of *E. coli* (unpublished data). Histopathological examination of the brains at 72 h (WT) and 24 h (IL-$10^{-/-}$) after infection indicated that the pathological conditions in this model are very similar to that of humans. Spotty neuronal necrosis and scattered apoptotic bodies were seen both in the cortex and hippocampal dentate gyrus in the brain of *E. coli* K1–infected mice compared with control uninfected animals (Fig. 1 D). Acute inflammation in the brain and spreading along penetrating vessels into the cerebral cortex and white matter was prominent. A high number of neutrophils was observed at the base of the brain involving leptomeninges, cisternal areas, and choroidal fissures. Increase in cellularity was conspicuous in the molecular layer of cerebral cortex. Acute hemorrhage in the choroid plexus was also observed. These pathological changes were much more pronounced in the brains of IL-$10^{-/-}$ mice compared with WT animals. On par with these results, the tissue damage markers lactate dehydrogenase and malondialdehyde (MDA) were increased dramatically in brains of IL-$10^{-/-}$–infected animals in comparison to WT-infected mice, whereas glutathione levels decreased, indicating oxidative stress (Fig. S1, E–G). Quantification of the influx of various cells into brain tissue showed a moderate influx of microglia, granulocytes, B cells, and macrophages in WT mice, which was even more prominent in IL-$10^{-/-}$ mice (Fig. 1, E–H). In contrast, the influx of $CD4^+$ and $CD8^+$ T cells were similar up to 24 h after infection in both these animals (Fig. S2, A and B). IL-10 production in brain also robustly increased by 12 h after infection and declined by 72 h, which correlated with IL-10 mRNA, as RT-PCR showed high amounts of IL-10 transcripts in infected animals compared with control uninfected mice (Fig. S2, C–E). These results suggest that IL-10 plays a vital role in controlling the inflammatory response during the acute phase; however, the production of

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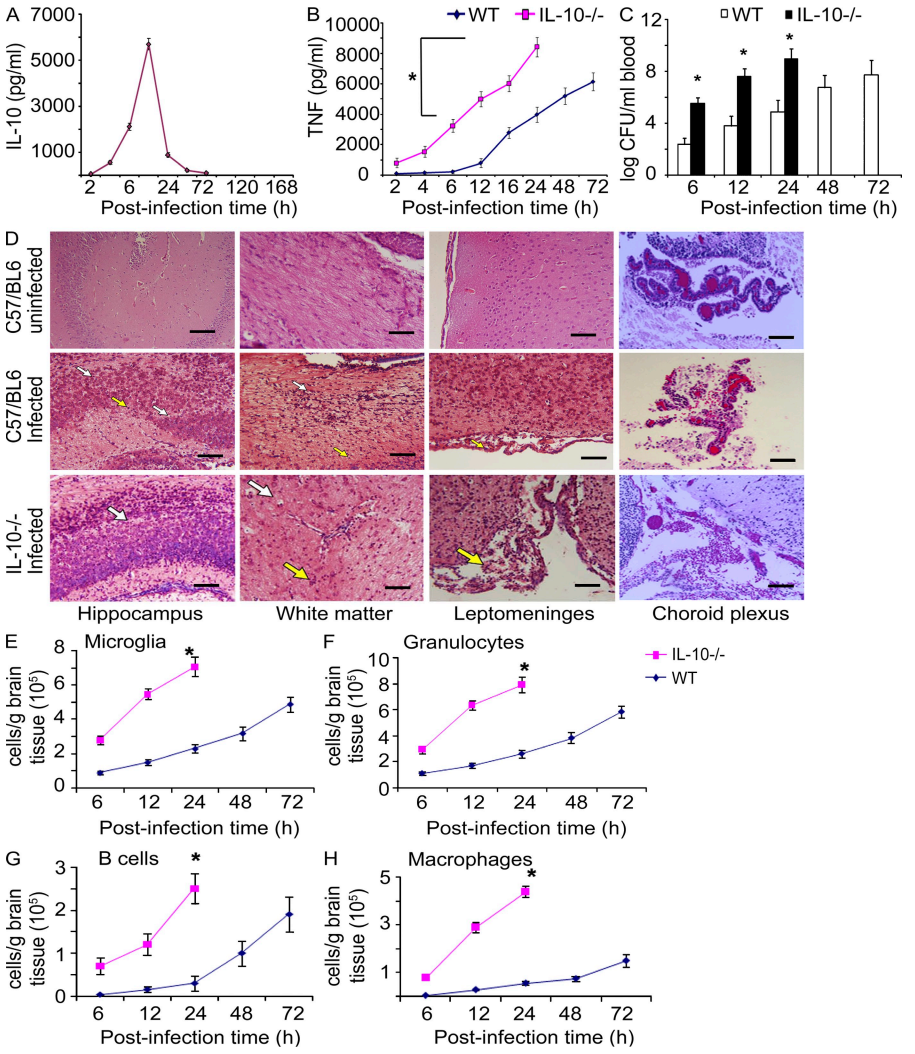

Figure 1. Cytokine production, pathogen load, and brain histopathology in *E. coli* K1–infected mice. WT and IL-10$^{-/-}$ mice at day 3 were infected intranasally with 10^3 CFU of *E. coli* K1, and the production of cytokines was examined by ELISA. The peak production of IL-10 was observed at 12 h after infection in WT mice (A). IL-10$^{-/-}$ animals showed higher production of serum TNF and blood bacterial load compared with WT mice (B and C). H&E staining of the brain sections revealed greater infiltration of neutrophils and apoptosis of neurons in the hippocampus in brains of infected mice compared with control uninfected mice. Significant damage to leptomeninges and hemorrhage in white matter was also observed (D). These pathological changes were much more pronounced and severe in IL-10$^{-/-}$ mice. Yellow arrows indicate neutrophil infiltration and white arrows indicate the apoptosis of neurons. Half of the infected brains from WT and IL-10$^{-/-}$ mice were homogenized and subjected to flow cytometry. IL-10$^{-/-}$ animals infected with *E. coli* succumbed to infection by 24 h, therefore the data are shown up to 24 h. Microglia, B cells, granulocytes, and macrophages were significantly increased in IL-10$^{-/-}$ mice compared with WT mice (E–H). The results were obtained from six independent experiments with 12 animals per group. Data represent mean ± SEM. *, $P < 0.001$ by two-tailed Student's *t* test. Bars, 10 µm.

IL-10 was down-regulated during the chronic phase of *E. coli* K1 infection.

Administration of IL-10 during high-grade bacteremia improves the outcome of *E. coli* K1 meningitis

To examine whether the administration of IL-10 would protect these animals, a single dose of various concentrations of recombinant human IL-10 (rh-IL-10) or PBS was injected intraperitoneally into mice at 48 h after infection. Doses of 0.1 µg and 1.0 µg per animal did not show a significant difference in the bacterial load compared with infected WT animals. However, administration of 2.5 or 5 µg of rh-IL-10 after 48 h after infection, by which time the bacteremia levels were already high, rescued the animals from infection by 75 and 100%, respectively (unpublished data). The bacterial count and inflammatory response in rh-IL-10–treated (5 µg) animals was reduced to basal levels in blood, and the animals appeared normal by 6 d after infection (Fig. 2, A and B). This rescue by rh-IL-10 also prevented, and probably reversed, the macrophage and PMN infiltration into the brain, and thus resolved the inflammation and significantly reduced the immunopathology (Fig. 2 C). Similarly, IL-10$^{-/-}$ animals were also rescued from *E. coli* infection by rh-IL-10 administration at 12 h after infection (unpublished data). Despite the survival of *E. coli* K1–infected infants as a result of antibiotic treatment, the neurological sequelae impose long-lasting effects on the quality of their life (Shah et al., 2005; Karageorgopoulos et al., 2009). In addition, the number of *E. coli* K1 strains that are resistant to third generation antibiotics is on the rise, and therefore the morbidity and mortality rates might increase considerably (Ramdani-Bouguessa et al., 2006; Boyer-Mariotte et al., 2008; Dubois et al., 2009). Therefore, to examine whether rh-IL-10 administration also prevents antibiotic-resistant *E. coli* infection, newborn mice were infected with cefotaxime-resistant or sensitive strains of *E. coli* K1 (*E. coli* CTX^R or *E. coli* CTX^S) and treated with cefotaxime starting at 48 h after infection (10 mg/kg body weight). The antibiotic therapy failed to rescue the *E. coli* CTX^R–infected animals treated with cefotaxime, and all the mice succumbed to infection by 96 h after infection, whereas animals given *E. coli* CTX^S survived. In addition, the animals infected with *E. coli* CTX^R and treated with cefotaxime still showed high levels of bacteremia and CSF cultures were positive for meningitis, whereas animals with *E. coli* CTX^S

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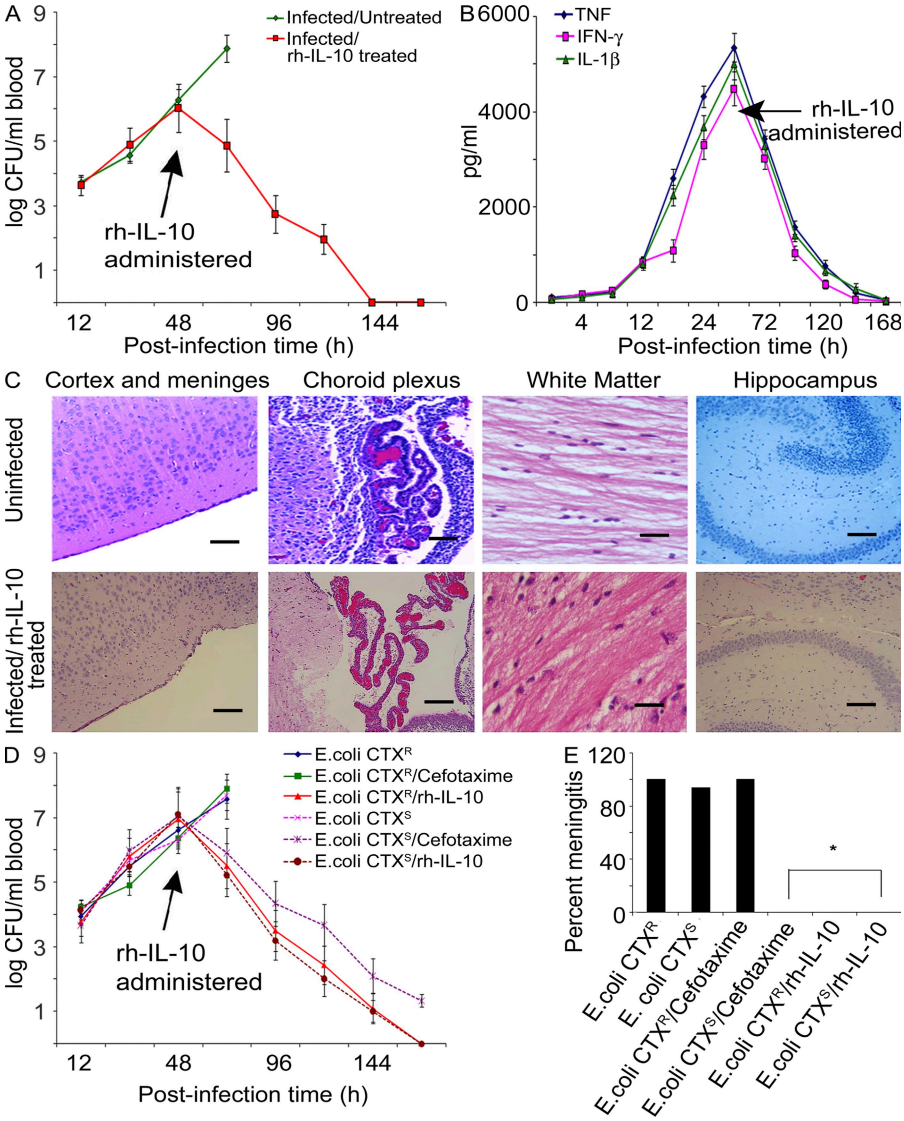

Figure 2. Administration of exogenous rh-IL-10 decreases bacteremia and the occurrence of meningitis in newborn mice induced by *E. coli* K1. (A) Infant mice were infected with 10^3 CFU of *E. coli* and, after 48 h, 5 µg of rh-IL-10 in 10 µl of saline was administered for each animal. Blood was collected at various time points, and the levels of bacteremia were determined by plating the dilutions on blood agar containing antibiotics. Infected and untreated mice were sacrificed at 72 h after infection for ethical reasons. (B) Various cytokines measured in the rh-IL-10–administered animals demonstrated that the levels of proinflammatory cytokines reduced to basal levels. (C) Histological examination of brain sections revealed normal morphology of the brain comparable to control uninfected mice without any infiltration of neutrophils and apoptosis of neurons in the hippocampus in rh-IL-10–treated animals. (D) Some groups of animals were infected with cefotaxime-resistant *E. coli* (*E. coli* CTX^R) or cefotaxime-sensitive *E. coli* (*E. coli* CTX^S). At 48 h after infection, infected mice were treated with cefotaxime or rh-IL-10, blood was collected and enumerated by plating on antibiotic containing agar. (E) CSF samples were collected from all animals either at 72 or 168 h after infection and cultured in broth containing appropriate antibiotics. The data are representative of 6 independent experiments with 12 animals in each group. Data represent mean ± SEM. *, $P < 0.001$ by two-tailed Student's *t* test. Bars, 20 µm.

demonstrated declining bacteria levels over the time (Fig. 2, D and E). In contrast, the animals infected with *E. coli* CTX^R and treated with rh-IL-10 (5 µg/animal) survived well and showed no signs of bacteremia and the occurrence of meningitis found in mice with *E. coli* CTX^S infection (Fig. 2, D and E). To test whether cefotaxime treatment of newborn mice reveals normal brain histology, pathological examination of brain tissues from animals infected with *E. coli* CTX^R and CTX^S was performed. A significant loss of neuronal cells was observed, supporting the notion that antibiotics cannot restore normalcy in the brain (Fig. 3 A). This indicates that the neurological sequelae in infants, despite treatment with antibiotics, could be caused by the residual damage to the brain. In contrast, the histopathology of the brains obtained from mice infected with *E. coli* CTX^R and treated with rh-IL-10 showed no signs of damage (Fig. S2 F). Notably, injection of 1 µg of rh-IL-10 along with cefotaxime restored the morphology of brain and other organs to normal condition (unpublished data). These results demonstrate that administration of exogenous IL-10 protects newborn mice from brain damage and the occurrence of meningitis by controlling proinflammatory cytokines and by enhancing the clearance of *E. coli* K1 during later stages of infection.

E. coli K1 infection causes damage to the brain similar to that of in humans

Although the histology of infected brains of rodents have been compared in some studies (Glode et al., 1977; Ballok et al., 2004), which showed similar neuronal damage to that of human brains, the differences in the morphology of the whole brains have not established. Therefore, to examine whether the gross morphological changes of infected mouse brain are similar to that of human brain in *E. coli* infection, virtual histology (micro-CT) of the brains of infected mice was performed. Magnetic resonance images (MRIs) of a neonate with *E. coli* K1 meningitis admitted to CHLA were also examined and compared (Fig. 3, B and C, and Videos 1 and 2). In both mice and the neonate, we observed hydrocephalus, gray

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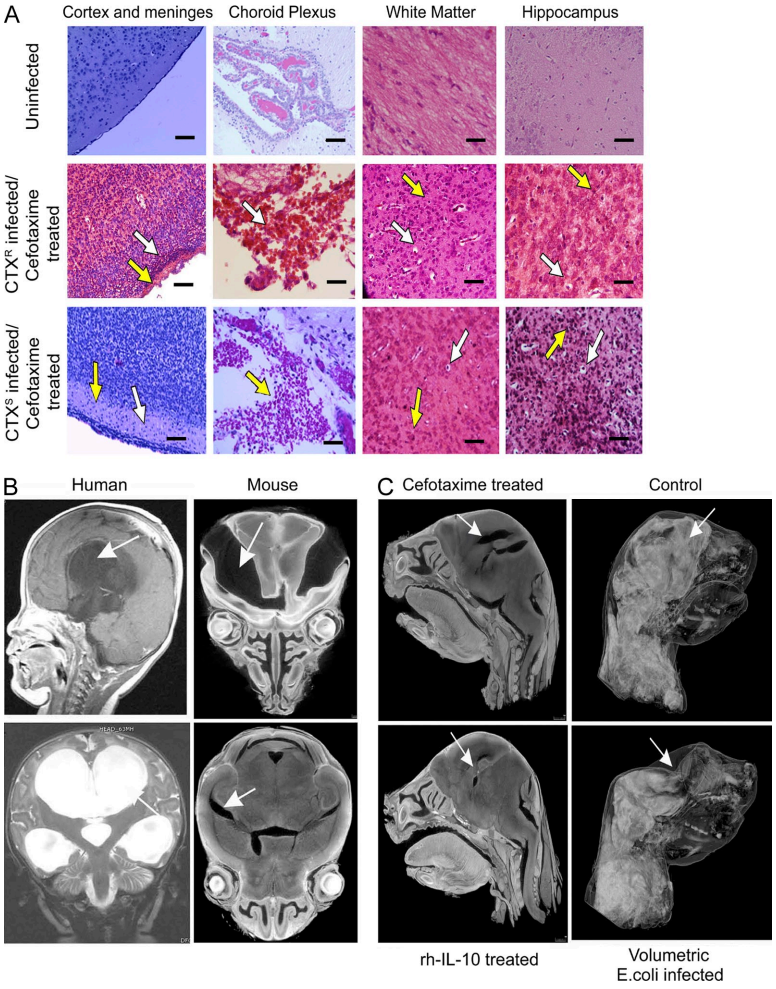

Figure 3. Histopathology and micro-CT of brains from mice, and MRIs of a human neonate infected with *E. coli*. (A) H&E staining of brain sections from animals infected with *E. coli* CTX^S or CTX^R followed by antibiotic treatment demonstrate that *E. coli* CTX^S–induced pathology still remained. Yellow arrows indicate neutrophil infiltration and white arrows indicate the apoptosis of neurons. (B) MRIs of a neonate with *E. coli* K1 meningitis admitted to CHLA and micro-CT images of infant mice infected with *E. coli* K1. (C) Saggital sections of newborn mice treated with rh-IL-10 or cefotaxime and total volume pictures of brains of infant mice uninfected and infected with bacteria. The arrows indicate dilation of ventricles and loss of neurons. Three independent experiments were performed with 10 mice per group for histopathology experiments. Two animals per group were used for micro-CT studies. Bars, 10 µm.

matter, and white matter volume loss and dilatation of the lateral ventricles. These results indicate that in newborn mice and humans, *E. coli* K1 meningitis caused significant brain damage. Brains of infected and antibiotic-treated animals still showed significant edema (Video 3). However, no such brain damage was visible in rh-IL-10–treated animals (Video 4). Furthermore, volumetric analysis of infected brains demonstrated a significant loss of a portion of the forebrain in mice upon infection, which was prevented by rh-IL-10 treatment (Fig. 3 C and Videos 5–7).

Anti-TNF antibody pretreatment did not prevent the occurrence of *E. coli* K1 meningitis in newborn mice

The production of high amounts of proinflammatory cytokines, especially TNF in mice infected with *E. coli* K1, might be responsible for the exacerbated pathophysiology and organ dysfunction observed in these animals. Because the administration of rh-IL-10 reduced proinflammatory cytokines at one end of the treatment regimen and cleared the pathogen on the other end, it is reasonable to speculate that suppressing the overproduction of TNF may also produce protective effects similar to that of rh-IL-10. Neutrophils and macrophages provide protection at the site of infection and are the primary source of inflammatory cytokines. Therefore, we examined the production of TNF production in neutrophils and macrophages infected with *E. coli* K1 in vitro at various time points and also the efficacy of anti-TNF antibody to neutralize the produced cytokine. The untreated or isotype-matched control antibody-treated neutrophils or macrophages infected with *E. coli* produced TNF in detectable levels at 2 h after infection and increased 8-fold at 6 h after infection (Fig. 4 A and Fig. S3 A). Preincubation of the phagocytes with anti-TNF antibody completely neutralized the TNF production. The survival of *E. coli* K1 in anti-TNF antibody pretreated neutrophils or macrophages was not affected although the bacteria were completely taken up by the cells by 6 h after infection (Fig. 4, B and C, and Fig. S3, B and C). In addition, anti-TNF antibody failed to rescue the neutrophils or macrophages from *E. coli* K1–mediated apoptosis (Fig. 4 D and Fig. S3 D). To examine the effect of anti-TNF antibody in vivo, infected animals were treated with anti-TNF antibody at 48 h after infection. Mice infected with *E. coli* with or without administration of control antibody showed a gradual increase in TNF production in both blood and brain, whereas administration of anti-TNF antibody neutralized the production of the cytokine by 72 h after infection, and hence was not detectable by ELISA (Fig. 5, A and B). However, the bacterial load and the occurrence of meningitis in anti-TNF antibody–treated animals were similar to that of untreated or control antibody-treated mice ($\log_{10}$ 8.0 ± 0.5 CFU of *E. coli* in the blood and with 90% positive CSF cultures at 72 h after infection; Fig. 5, C and D). Histological examination of the brain sections from anti-TNF antibody–treated animals exhibited similar damage to the brain compared with control antibody or untreated animals (Fig. 5 E). These findings suggest that neutralizing the production of TNF does not confer protection against *E. coli* K1 meningitis unlike the administration of rh-IL-10.

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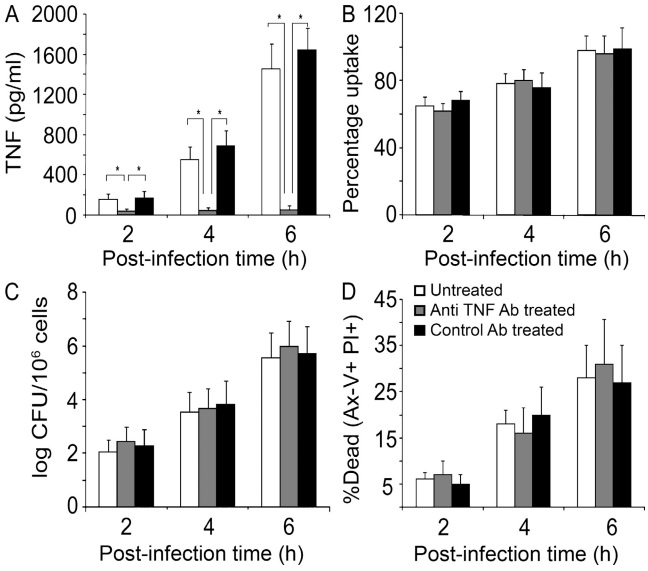

Figure 4. Effect of Anti-TNF antibody treatment on phagocytosis of *E. coli* by neutrophils: Neutrophils were isolated from the blood of mice pretreated with anti-TNF antibody, isotype matched control antibody, or left untreated and infected with *E. coli*. The production of TNF was assessed at different infection times by ELISA (A). The uptake (B) and intracellular bacteria (C) were also determined after infecting the cells at a multiplicity of infection of 10 (cell to bacteria ratio, 1:10). The viability of neutrophils was examined by Annexin V and propidium iodide staining (D). Data represent mean ± SEM and are representative of three independent experiments performed in triplicate. *, $P < 0.001$ by two-tailed Student's *t* test.

IL-10 treatment increases neutrophil and macrophage phagocytosis of *E. coli* by up-regulation of CR3 expression

The ability of rh-IL-10 treatment to clear the pathogen from the circulation of infected mice suggests increased phagocytosis of the bacteria by neutrophils and/or macrophages probably mediated by IL-10R. Therefore, we examined whether IL-10R1 is expressed in uninfected mouse neutrophils by flow cytometry. The data revealed that IL-10R1 expressed in good quantity on the surface of neutrophils (Fig. 6 A). Furthermore, the effect of rh-IL-10 treatment of neutrophils before bacterial infection on the phagocytic activity of neutrophils isolated from WT animals was examined ex vivo. *E. coli* K1 enters and survives in neutrophils isolated from WT mice; however, neutrophils pretreated with rh-IL-10 showed enhanced uptake of *E. coli* K1 and killing of bacteria within 6 h after infection (Fig. 6, B and C). Neutrophils from WT mice infected with *E. coli* K1 underwent rapid apoptosis compared with cells from rh-IL-10–treated animals (Fig. 6 D). Neutrophils from IL-$10^{-/-}$ mice showed no capacity to kill the bacteria and underwent apoptosis very rapidly ex vivo. They could, however, be rescued from *E. coli*–induced apoptosis with rh-IL-10 pretreatment. Similar results were observed with neutrophils isolated from human peripheral blood (Fig. S4, A–C). Collectively, these data indicate that bacteria might prevent bactericidal events in neutrophils either by suppressing the apoptotic mechanisms once inside neutrophils or by modulating their entry into the cells, such that the normal phagocytic arsenal is circumvented. To test the latter hypothesis, we examined the expression of surface pathogen associated recognition receptors on neutrophils isolated from mice infected with *E. coli* K1. Flow cytometry analysis revealed increased expression of TLR2 and FcγRI (CD64), but reduced expression of TLR4 and CR3 (Fig. 6 E). Notably, neutrophils from infected and rh-IL-10–treated mice showed significantly increased expression of CR3 and TLR4 and down-regulation of TLR2 and FcγRI. A similar receptor profile was observed on human neutrophils (Fig. S4 D). Introducing siRNA to CR3 into neutrophils in vitro further substantiated the role of CR3 in the phagocytosis of *E. coli* K1 by neutrophils after rh-IL-10 treatment. *E. coli* entered and survived in neutrophils transfected with CR3-siRNA and pretreatment with rh-IL-10 had no effect on uptake and killing of the bacterium (Fig. 6, F and G). The protective effect of rh-IL-10 on the apoptosis of infected neutrophils was also abrogated by suppressing the expression of CR3 (Fig. 6 H). These data suggest that rh-IL-10 administration up-regulates the expression of CR3 in neutrophils leading to enhanced killing of *E. coli* K1. In addition to neutrophils, macrophages also play an important role in the clearance of invading pathogens. Therefore, we also examined the effect of rh-IL10 on the phagocytosis of *E. coli* and surface expression of various receptors upon infection in macrophages. Peritoneal macrophages from both WT and IL-$10^{-/-}$ exhibited similar uptake and intracellular survival of *E. coli*, whereas rh-IL-10 treatment reduced the survival of bacteria in these cells significantly (Fig. S5, A and B). The viability and the expression of surface receptors were also similar to that of the expression observed in neutrophils (Fig. S5, C and D). In addition, no difference in uptake, survival, apoptosis, and receptor profile of CR3 knockout macrophages even after treatment with rh-IL-10 compared with control siRNA-treated macrophages was observed (Fig. S5, E–H). This indicates that rh-IL-10 enhances CR3 expression so that the bacteria enter through CR3 for efficient destruction by phagocytes.

IL-10 treatment fails to protect mice that lack CR3 expression against *E. coli*–induced meningitis

To confirm that CR3 expression is critical in vivo for rh-IL-10 mediated protection against *E. coli* infection and brain damage, CR3 expression in mice was suppressed using Invivofectamine reagent. Groups of animals were transfected with premixed control siRNA or CR3-siRNA and Invivofectamine reagent by intraperitoneal injection. CR3 expression in neutrophils, peritoneal macrophages, liver, and spleen was then examined by flow cytometry. The expression of CR3 was completely absent in neutrophils, macrophages, and other tissues of transfected mice (Fig. 7 A and not depicted). Determination of bacterial load in the blood of CR3-siRNA–transfected and –infected animals showed that *E. coli* entered the blood within 2 h and multiplied to a $\log_{10}$ CFU of >5 by 24 h similar to that of control siRNA transfected mice (Fig. 7 B). Administration

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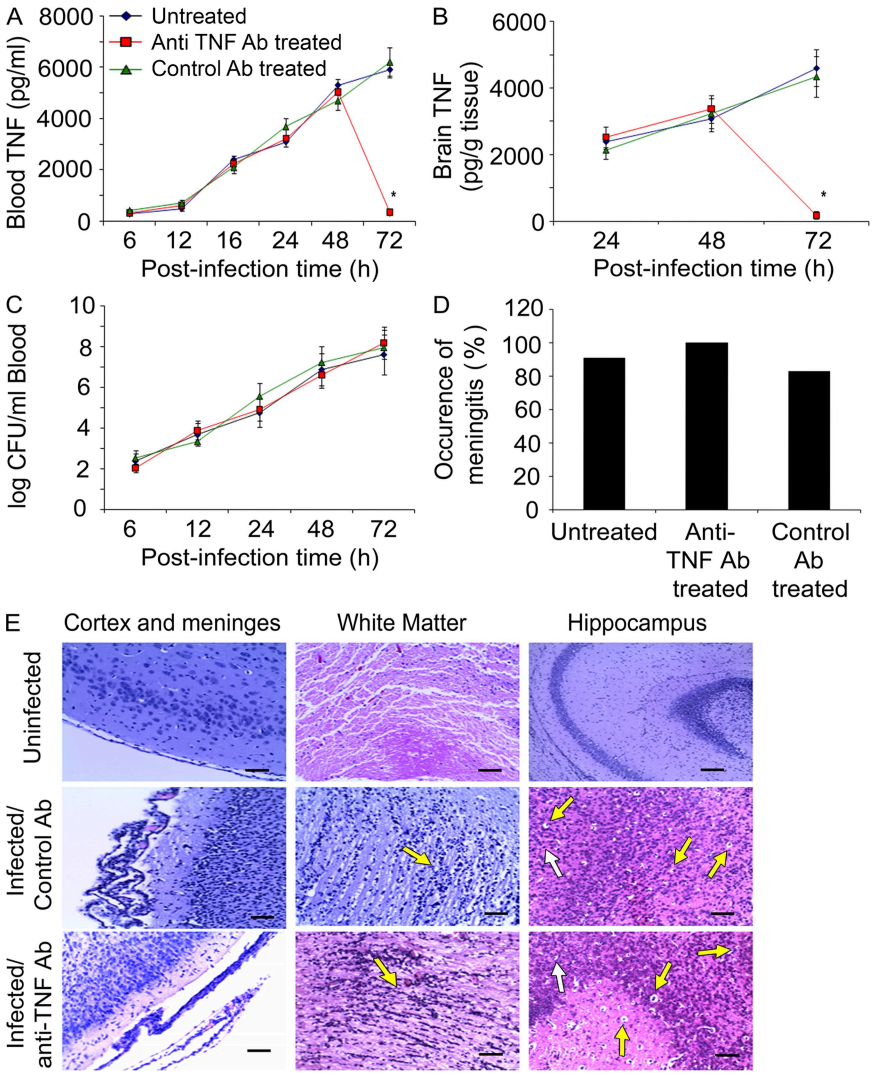

Figure 5. Anti-TNF antibody administration fails to rescue the mice from *E. coli* K1 meningitis. 3-d-old mice were infected with *E. coli* and, after 48 h, 5 µg of anti-TNF antibody in 10 µl of saline was administered for each animal. The production of TNF was assessed in blood (A) and brain homogenates (B) of infected mice at different times after infection by ELISA. TNF levels were reduced to basal levels due to anti-TNF antibody treatment in these animals by 72 h after infection. Blood was collected at various time points, and the levels of bacteremia were determined by plating the dilutions on blood agar containing antibiotics (C). CSF samples were collected from all animals at 72 h after infection and cultured in broth containing appropriate antibiotics (D). Brains were harvested from infected and uninfected mice, and the brain sections were subjected to H&E staining (E). Yellow arrows indicate neutrophil infiltration and white arrows indicate the apoptosis of neurons. The results were obtained from three independent experiments with 12 animals per group. Data represent mean ± SEM. *, $P < 0.001$ by two-tailed Student's *t* test. Bars, 20 µm.

of rh-IL-10 in control siRNA transfected animals reduced the bacterial load, whereas it had no effect on CR3 knocked down animals. The animals showed positive CSF cultures and histopathological analysis revealed brain damage and infiltration of neutrophils similar to that of WT animals infected with *E. coli* (Fig. 7, C and D). The protective effect of rh-IL-10 administration during high-level bacteremia on cytokine production in CR3 knocked down mice was completely abrogated (Fig. 7 E, only TNF data are depicted). Neutrophils from CR3-siRNA–transfected and –infected animals revealed greater expression of TLR2 and FcγRI and did not change in animals with rh-IL-10 treatment (Fig. 7 F). Similar results were also observed with peritoneal macrophages isolated from CR3 knocked down mice (unpublished data). These results collectively demonstrate that CR3 expression was significantly suppressed during the progression of the disease by *E. coli* K1 and the protective effects of rh-IL-10 are caused by restoration of CR3 expression in neutrophils and macrophages.

IL-10–induced suppression of prostaglandin E-2 (PGE-2) is responsible for enhanced expression of CR3 and bactericidal activity of neutrophils and macrophages

Two important mediators of inflammatory response during bacterial meningitis are nitric oxide and PGE-2 (Kim, 2003). Although there is limited information on the role of prostanoids, such as PGE-2 in meningitis, one clinical study showed that 72 of 80 infants and children with meningitis exhibited elevated levels of CSF PGE-2 (Mustafa et al., 1990). Notably, PGE-2 has also been involved in down-regulation of CR3 expression in monocytes and macrophages (Zeidler et al., 2000). Therefore, to examine whether PGE-2 is responsible for rh-IL-10–induced protection against meningitis caused by *E. coli*, the production of PGE-2 in neutrophils and macrophages was examined. Neutrophils infected with the bacteria showed significantly greater production of PGE-2 over the time in comparison to uninfected cells, which levels were reduced by >80% by rh-IL-10 treatment (Fig. 8 A). In contrast, *E. coli*–infected neutrophils in which PGE-2 expression is suppressed by introducing siRNA to PGE synthase 2 (PGES-2) demonstrated no production of PGE-2 in comparison to control siRNA-transfected cells (Fig. 8 B). The PGES-2 siRNA–transfected neutrophils, despite engulfing the bacteria in a manner similar to that of control siRNA/neutrophils, killed very efficiently and no detectable bacteria were observed by 6 h after infection (Fig. 8, C and D). In contrast, intracellular *E. coli* in control siRNA/neutrophils survived and

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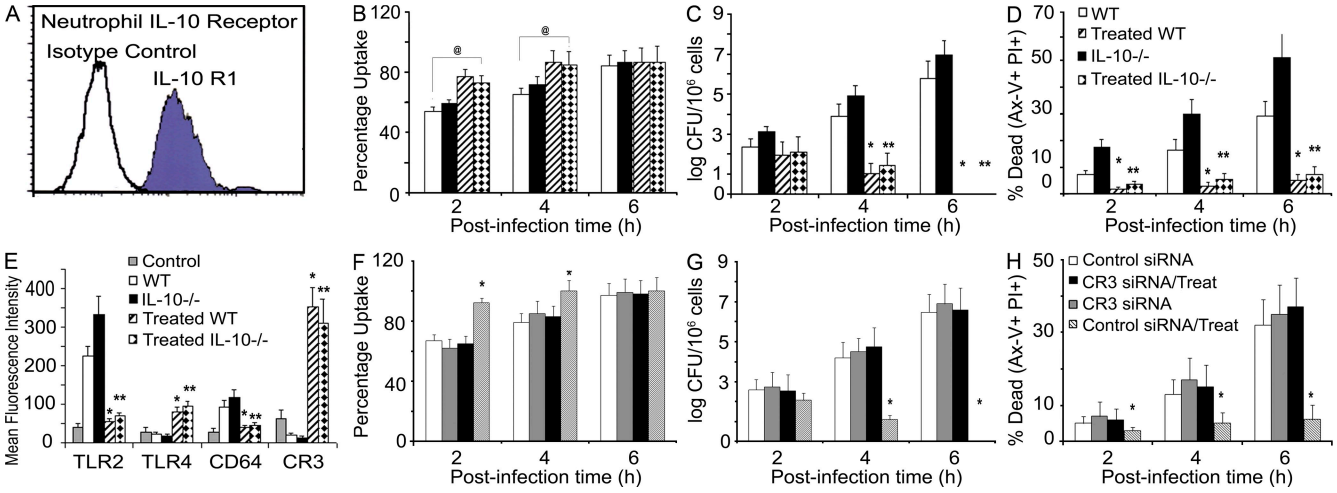

Figure 6. IL-10 treatment increased the phagocytosis of *E. coli* by neutrophils via up-regulation of CR3 expression. Neutrophils were isolated from the blood of WT mice and the expression of IL-10R1 was examined by flow cytometry (A). The uptake of *E. coli* (B) and intracellular bacteria (C) were then assessed after infecting the cells with *E. coli* at an MOI of 10. Further, the viability of the cells was examined by Annexin V and propidium iodide staining and analyzed by flow cytometry (D). The expression of TLR2, TLR4, CR3, and FcγRI (CD64) was examined on the surface of neutrophils by flow cytometry after infection with *E. coli* K1 and graphed after subtracting the values of isotype matched controls (E). In separate experiments, neutrophils were transfected with control siRNA or CR3 siRNA and treated with rh-IL-10. The uptake (F), intracellular survival (G) of bacteria, and viability of neutrophils (H) were determined upon infection with *E. coli*. The experiments were performed five times in triplicate independently, and the error bars represent SEM. The decrease or increase in various parameters was statistically significant with rh-IL-10 treatment compared with untreated conditions. *, $P < 0.01$; **, $P < 0.001$; @, $P < 0.04$ by two-tailed Student's *t* test.

multiplied over the same time period. Similarly, suppression of PGES-2 expression in neutrophils renders the cells resistant to apoptosis despite infection with bacteria (Fig. 8 E). In addition, *E. coli*–infected PGES-2 siRNA/neutrophils showed increased expression of CR3 and TLR4 and decreased expression of TLR2 and FcγRI, similar to expression levels observed in rh-IL-10–pretreated and infected neutrophils (Fig. 8 F). We therefore examined whether mice infected with *E. coli* induce the production of PGE-2. On par with the in vitro data, the production of PGE-2 in blood increased twofold by 72 h in comparison to the levels produced at 24 h after infection and administration of rh-IL-10 at 48 h after infection significantly reduced circulating PGE-2 by 72 h (Fig. 8 G). The levels of PGE-2 in the brains of infected animals exhibited a similar increase. These levels decreased to baseline after rh-IL-10 treatment (Fig. 8 H). Similar data on PGE-2 production was also obtained with peritoneal macrophages (Fig. S6, A–F), substantiating the role of PGE-2 in rh-IL-10–induced protective effects during the development of *E. coli* K1 meningitis.

DISCUSSION

Currently, the primary mode of treatment of *E. coli* meningitis is limited to antibiotics, which reduced the mortality and frequency of complications, but the incidence of serious neurological disability remains alarmingly high (Kim, 2003). Trivedi et al. (2007) showed that focal anisotropy values were significantly greater in patients with *E. coli* infection after antibiotic treatment than in the controls, indicating that the inflammation is still active. Moreover, the emergence of antibiotic-resistant *E. coli* further complicates treatment options (Dubois et al., 2009), warranting the necessity of new treatments that control both bacterial load and inflammatory response. Our findings provide novel insights into the role of IL-10 in the control of pathogen load at times of high-grade bacteremia in a newborn mouse model of *E. coli* meningitis. Although we expected that exogenous administration of IL-10 would decrease disease severity because of suppression of the proinflammatory response, its effect in the control of pathogen load was most surprising. In the majority of situations, IL-10 has a suppressive effect on both macrophages and dendritic cells (Moore et al., 2001; Demangel et al., 2002) and most of the animal models have shown that IL-10 inhibits antimicrobial response while protecting from immunopathology (Hunter et al., 1997; Wilson et al., 2005). In contrast to this paradigm, IL-10 plays the opposite role in *E. coli* infection; it helps control the phagocytic activity of immune cells to clear the pathogens while reducing inflammation. Some studies have reported that persistent bacteremia is the result of loss of pathogen clearance caused by apoptosis of lymphocytes (Hotchkiss et al., 2001; Alileche et al., 2005; Wesche-Soldato et al., 2007). Also, there is evidence that patients dying from sepsis have markedly increased lymphocyte apoptosis in the spleen (Toti et al., 2004; Felmet et al., 2005). Recently, it has been shown that IL-10 generates pathogen-clearing phagocytes (monocytes and macrophages), which are resistant to complement lysis, and thereby enabled to survive longer in a hostile inflammatory environment (Koch et al., 2009). Our findings suggest that IL-10 treatment prevents neutrophil and macrophage apoptosis induced by *E. coli*, and thereby increases

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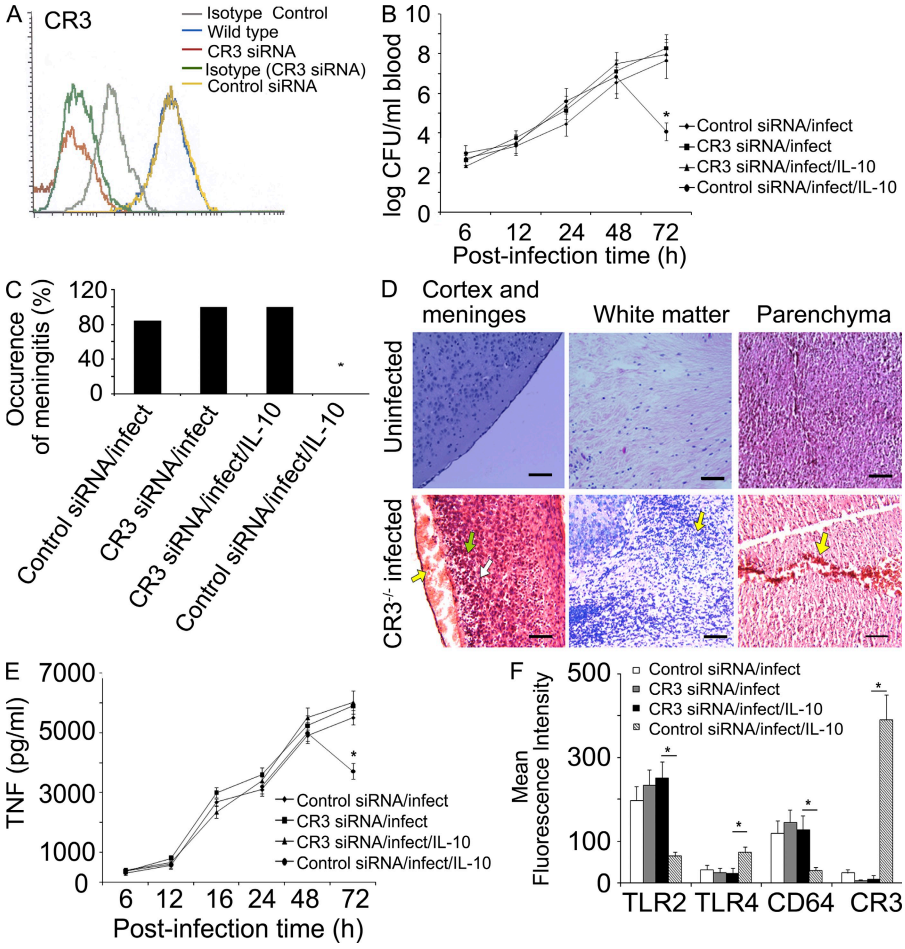

Figure 7. The protective effect of rh-IL-10 is abrogated in CR3 knockdown mice. Newborn mice were injected with CR3 siRNA in Invivofectamine reagent to knockdown the expression of CR3, as described in Materials and methods. The suppression of CR3 in neutrophils was evaluated by flow cytometry using an anti-CR3 antibody. Isotype-matched antibodies were also used as controls for $CR3^+$ and $CR3^-$ cells (A). Mice were infected with *E. coli* K1 and the bacterial load in blood was examined at different times after infection (B). CSF samples were collected from the animals at 72 h after infection and cultured in broth containing appropriate antibiotics (C). Histopathological examination of brain sections revealed significant damage in brains of CR3 knockdown mice, even after treatment with rh-IL-10 compared with control uninfected mice (D). There was no significant difference in the TNF production in control, CR3 knockdown, or CR3 knockdown and rh-IL-10–treated mice compared with control siRNA transfection followed by rh-IL-10 treatment (E). In addition, the receptor profile of neutrophils was unaffected despite treatment with rh-IL-10 in comparison to control treatment (F). The results were obtained from six independent experiments with 12 animals per group. Data represents mean ± SEM. *, $P < 0.001$ by two tailed Student's *t* test. Bars, 10 µM.

the phagocytosis of the bacteria. In animal models using adult mice, it has been shown that both Gram-negative bacteria and mycobacteria induced the recruitment of neutrophils, which secrete abundant amounts of IL-10 (Zhang et al., 2009). In acute mycobacterial infection, neutrophil-derived IL-10 controlled the inflammatory response of dendritic cells, monocytes, and macrophages in the lung (Zhang et al., 2009). However, diminished production of IL-10 mRNA and IL-10 protein in term and preterm neonates compared with adults was observed after stimulating with LPS (Schultz et al., 2004). We also observed decreased IL-10 production and increased levels of proinflammatory cytokines in blood and brain of *E. coli*–infected neonatal mice 24 h after infection. Therefore, IL-10 produced by neutrophils may not be enough, and excessive IL-10 quantities may be required to subdue the inflammatory response and improve phagocytosis of pathogens by neutrophils and macrophages in neonates.

Our studies also demonstrated that the IL-10–induced bactericidal activity of neutrophils and macrophages is caused by increased expression of CR3 and TLR4, which was suppressed during the infection without treatment. The role of CR3-mediated clearance of *E. coli* from the blood was further substantiated in mice by suppressing CR3 expression using siRNA and Invivofectamine. Because CR3 plays a role in transmigration of neutrophils from the blood to the site of inflammation, it is possible that lack of occurrence of meningitis in CR3 knockdown mice could be caused by inefficient migration of neutrophils, which may be acting as reservoirs for *E. coli* multiplication during the early stages of infection. However, mice deficient in CR3 have been developed and found to have normal migration of neutrophils in the peritoneal cavity after chemical and TNF stimulus (Lu et al., 1997). LFA-1 has been shown to overshadow the contribution of CR3 for neutrophil migration and adhesion to endothelial cells, indicating that CR3 is not absolutely necessary (Ding et al., 1999). In agreement, histopathological sections of brains of CR3 knocked down and infected mice also exhibited neutrophil infiltration similar to that of WT-infected mice, indicating neutrophil migration is not defective in CR3-suppressed mice. Furthermore, increased formation of PGE-2 was observed in the blood of *E. coli*–infected mice and also by neutrophils and macrophages ex vivo upon infection. Biogenesis of PGE-2 has been shown to occur through TLR2 signaling during apoptotic neutrophil uptake by macrophages in acute BCG infection (D'Avila et al., 2008). The PGE-2 production observed in *E. coli* K1 meningitis corroborates this mechanistic concept, as we also observed significant increase in TLR2 production in neutrophils and macrophages upon infection with *E. coli* K1.

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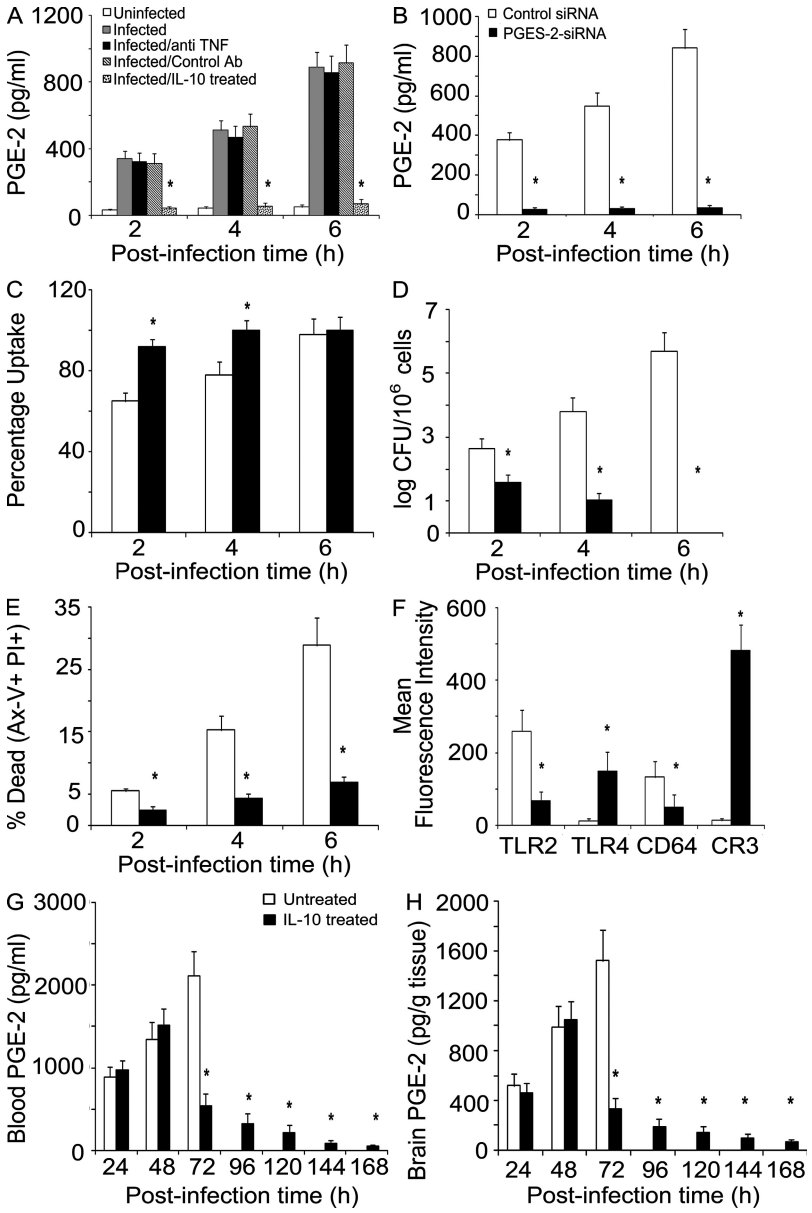

Figure 8. *E. coli* K1 induces the production of PGE-2 in vitro and in vivo. Neutrophils isolated from the blood of WT mice were infected with *E. coli* ex vivo and the production of PGE-2 was determined by EIA (A). Neutrophils, when transfected with PGES-2 siRNA, significantly reduced the production of PGE-2 compared with control siRNA-transfected cells (B). Assessment of the uptake (C), intracellular bacteria (D), and viability of neutrophils (E) demonstrated that lack of PGE-2 production enhanced phagocytosis and prevented apoptosis of neutrophils, although the uptake was not affected. The receptor profile of PGES-2 or control siRNA transfected neutrophils infected with *E. coli* in vitro was examined by flow cytometry (F). In addition, the production of PGE-2 was examined in blood (G) and brain homogenates (H) of *E. coli*–infected mice with or without rh-IL-10. The ex vivo studies were performed three times independently in triplicate, whereas data from in vivo studies were obtained from six independent experiments with 12 animals for each group. Data represent mean ± SEM.
*, $P < 0.001$ by two-tailed Student's *t* test.

IL-10 administration suppressed the formation of PGE-2 by neutrophils and macrophages, in which case the expression of TLR2 in these cells suppressed and induced the expression of CR3. It has been demonstrated that IL-10 inhibits the production of PGE-2 induced by LPS in monocytes (Niiro et al., 1994); however, PGE-2 generated during efferocytosis enhanced the production of IL-10 (Medeiros et al., 2009). Thus, the relationship between IL-10 and PGE-2 may be dependent on the biological phenomena that are taking place or the presence of other pro- and antiinflammatory cytokines in the milieu. Collectively, these results suggest that *E. coli* entry via receptors other than CR3 target PGE-2 to enhance bacterial survival and replication in host cells.

Studies in experimental animals and humans with bacterial meningitis have shown that two forms of neuronal injury are predominant: necrotic cortical injury and apoptotic hippocampal injury, as seen in *E. coli*–infected mice in this study (Nau et al., 1999; Kim, 2003). Neuronal injury associated with bacterial meningitis probably involves many microbial and host factors. Adjunct therapy with dexamethasone has been shown to reduce hearing impairment and other neurological sequelae in children with *H. influenzae* type b meningitis, but dexamethasone was shown to aggravate hippocampal injury in experimental models of pneumococcal meningitis (Wald et al., 1995; Leib et al., 2003). In contrast to these observations, IL-10 treatment completely prevented and/or restored the damaged brain as seen in micro-CT images and histopathology. Prevention of meningitis after reaching a threshold level of bacteremia is a very important therapeutic strategy, as the diagnosis of meningitis is difficult until after the bacteria reaches the central nervous system. The finding of prevention of meningitis caused by antibiotic-resistant strains by exogenous IL-10 is significant to treat these patients. This function indicates that an adjunct therapy containing a regimen of antibiotics and small quantities of IL-10 would be possible to treat neonates. Since IL-10 therapy has already been used in adults for some autoimmune diseases like psoriasis, it is possible that administration of small quantities of exogenous IL-10 would not have adverse effects on neonates (Friedrich et al., 2002). Although, the concept of treating neonates with *E. coli* infections by exogenous IL-10 remains to be tested, these findings provide a strong rationale for exploration of IL-10 therapies beyond antibiotic treatment alone. This IL-10 therapy may also form the basis of future therapeutic strategies to treat meningitis.

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MATERIALS AND METHODS

Animals. Breeding pairs of C57BL/6 IL-10$^{-/-}$ mice and C57BL/6 WT mice were obtained from The Jackson Laboratory.

Infectious organisms. *E. coli* K1 is a rifampicin-resistant mutant of *E. coli* K1 strain RS 218 (serotype O18:K1:H7) and is sensitive to cefotaxime (CTX^S; Prasadarao et al., 1996). Cefotaxime-resistant strain of *E. coli* (CTX^R) was isolated from CSF of a newborn as previously described (Dubois et al., 2009). Bacteria were grown in brain heart infusion broth (Difco Laboratories) with appropriate antibiotics.

Newborn mouse model of meningitis. The animal studies were approved by the institutional animal care and use committee of the Saban Research Institute at CHLA and followed National Institutes of Health guidelines for the performance of animal experiments. 3-d-old mice were randomly divided into various groups and infected intranasally with 10^3 CFU/10 µl of bacteria. Control mice received pyrogen-free saline through the same route. Blood was collected from the tail or facial vein at different times after infection and plated on LB agar containing rifampicin. CSF samples were collected aseptically under anesthesia by cisternal puncture and directly inoculated into broth containing antibiotics in which the growth of *E. coli* was considered positive for meningitis. Mice were perfused intracardially with 0.9% saline to remove blood and contaminating intravascular leukocytes. Various tissues were aseptically harvested and homogenized in sterile PBS, and the bacterial counts were determined by plating 10-fold serial dilutions on rifampicin LB agar plates.

IL-10 and anti-TNF antibody treatment. To assess the effect of rh-IL-10 on meningitis, groups of 3-d-old mice were infected with *E. coli* CTX^S or *E. coli* CTX^R and treated intraperitoneally at 48 h after infection with various doses of rh-IL-10 (0.1–5 µg/animal; (PeproTech), with cefotaxime (10 mg/kg body weight) or with cefotaxime and rh-IL-10 (1 µg/animal) together. For anti-TNF antibody treatment, groups of 3-d-old mice were infected with *E. coli* CTX^S and treated intraperitoneally at 48 h after infection with one dose of 5 µg/animal of murine anti-TNF antibody or isotype-matched control antibody (R&D Systems).

Histopathology. Half of the tissue samples were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. 4–5-µm-thick sections were cut using a Leica microtome and stained with hematoxylin and eosin (H&E; Mittal et al., 2009b).

Determination of cytokine and PGE-2 levels. The levels of TNF, IL-1β, IL-6, IL-10, IL-12p70, and IFN-γ, were measured in blood and brain homogenates of infant mice infected with *E. coli* using ELISA kits from Biosource according to the manufacturer's instructions. PGE-2 levels were determined by EIA kit (Cayman Chemicals).

Quantitative assessment and phenotypic characterization of brain leukocytes. Characterization of leukocytes derived from brains of newborn mice was done using flow cytometry as described previously (Mittal and Prasadarao, 2008) using various antibodies obtained from eBioscience. Murine microglia and macrophages were identified by staining with anti-CD45 (LAC)-biotin and anti-F4/80-FITC, respectively followed by avidin-PE/Cy5. Inflammatory leukocytes recruited to the brain are CD45^{high} F4/80$^-$, macrophages are CD45^{high} F4/80$^+$, and microglia are CD45^{low} F4/80$^+$. CD4$^+$ and CD8$^+$ T lymphocytes were stained with rat anti–mouse-CD4 followed by goat anti–rat-phycoerythrin and anti-CD8-FITC. B lymphocytes were detected by staining with anti-CD45R (B220)-FITC and anti-CD45 (LCA)-biotin followed by avidin-phycoerythrin/Cy5. Granulocytes were stained with anti-Ly6-G (GR-1), followed by goat anti–rat-PE and F4/80-FITC. Granulocytes were defined as Ly6-G$^+$ F4/80$^-$. Control staining included incubation of brain-derived leukocytes with unlabeled or fluorochrome-labeled isotype-matched control antibodies. Flow cytometry was performed on a FACScan instrument (BD) and the data were analyzed with CellQuest Software.

RT-PCR. For detection of IL-10 by RT-PCR, RNA was isolated from the brains of uninfected and infected mice with TRIZOL-LS-reagent (Invitrogen). RNA was quantified using a NanoDrop machine. RT-PCR was performed using the following primer sequences: sense, 5′-ACCTGGTAGAAGTGATGCCCCAGGCA-3′; antisense, 5′-CTATGCAGTTGATGAAGATGTACCA-3′. Mouse GAPDH was used as an internal control, using the following primer sequences: sense, 5′-CATCACCATCTTCCAGGAGCG-3′; antisense, 5′-GAGGGGCCATCCACAGTCTTC-3′. Negative controls without RT were performed in parallel for every reaction, to exclude amplification of contaminating DNA. The amplified products were separated on a 1% agarose gel and were stained with ethidium bromide. The gels were photographed, and optical densities were determined by using a computer imaging analysis system (Visitron Systems GmbH). RT-PCR was performed three times and the mean density of PCR products was determined to calculate the ratio of IL-10 to GAPDH mRNA.

Mouse brain micro-CT imaging (virtual histology). Formalin-fixed specimens were sent to Numira Biosciences for micro-CT imaging. Specimens were stained with a proprietary contrast agent. A high-resolution, volumetric micro-CT scanner (µCT40; ScanCo Medical) was used to scan the tissue with the following parameters: 10 µm isometric voxel resolution at 200 ms exposure time, 2,000 views, and 10 frames per view. The micro-CT generated DICOM files were converted into a file format compatible with the image processing software applications. Using Teem (Version 1.10.0; http://teem.sourceforge.net/), SCIRun (Scientific and Computing Imaging Institute, University of Utah, Salt Lake City, Utah), and Numira's proprietary software tools, images of the samples were generated.

Phagocytosis and analysis of surface receptors. Neutrophils were isolated from the peripheral blood of the human volunteers and mice as described previously (Criss and Seifert, 2008; Luo and Dorf, 2001). IRB of CHLA approved the blood withdrawal protocol from human volunteers. Macrophages were isolated from the peritoneal cavity of mice as previously described (Mittal et al., 2009a). In some experiments, neutrophils and macrophages were transfected with CR3 siRNA (Invitrogen; Oligo ID MSS205544), PGES-2 siRNA (Ambion; Oligo ID 97139), or respective control siRNA using Lipofectamine 2000+ (Invitrogen) transfecting reagent. Phagocytosis of bacteria by neutrophils and macrophages was determined by gentamicin protection assays (Mittal and Prasadarao, 2008). The expression of TLR2, TLR4, CR3, and FcγRI on the surface of neutrophils and macrophages isolated from infected mice was determined by flow cytometry after staining with respective antibodies coupled to various fluorochromes, and at least 10,000 events were collected for analysis. Neutrophils and macrophages were gated using GR-1 and F4/80 staining, respectively, as well as forward and side scatter parameters. Results are expressed as mean fluorescence intensity subtracted from isotype-matched and respective uninfected controls.

Suppression of CR3 expression using Invivofectamine reagent. To suppress CR3 expression, newborn mice at day 1 were injected with CR3 siRNA or control siRNA in Invivofectamine reagent (premixed in 1:1 ratio, 10 nmol in 10 µl; Invitrogen). Invivofectamine is a proprietary lipid-based nontoxic transfection reagent for in vivo RNAi applications. This reagent stabilizes siRNA in vivo and knocks down the expression of gene of interest. Mice were given four doses of CR3 siRNA intraperitoneally; two doses before infection, one dose immediately after administration of bacteria, and final dose 24 h after infection.

Statistical analysis. For statistical analysis of data, analysis of variance, Fischer test, Wilcoxon signed rank test, χ^2 test, and Student's *t* test were applied. P values <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows cytokine production and tissue damage markers in *E. coli*–infected WT and IL-10$^{-/-}$ mice. Fig. S2 shows CD4$^+$ and CD8$^+$ T cell responses, IL-10 production, and brain pathology

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upon *E. coli* infection in WT and IL-10$^{-/-}$ mice. Fig. S3 depicts the effect of TNF neutralization on the phagocytic ability and viability of *E. coli*–infected macrophages in vitro. Fig. S4 shows the phagocytosis, receptor profile, and viability of human neutrophils infected with *E. coli* in vitro. Fig. S5 shows the phagocytic ability, apoptosis, and receptor profile of peritoneal macrophages pretreated with IL-10 before infection with *E. coli*. Fig. S6 shows the effect of silencing PGES-2 on the ability of peritoneal macrophages to engulf *E. coli*, prevent apoptosis, and increase the expression of surface receptors compared with control siRNA-treated cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092265/DC1.

We thank Elaine Tuomanen and Grace Aldrovandi for their suggestions and encouragement during the progress of this work. We also thank Scott Filler, Barbara Driscoll, and Mary Kearns-Jonker for critical reading of the manuscript.

This work was supported by RO1 AI40567 to N.V. Prasadarao.

The authors have no conflicting financial interests.

Submitted: 20 October 2009
Accepted: 14 April 2010

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