**Abstract**

Neonatal meningitis due to *Escherichia coli* K1 is a serious illness with unchanged morbidity and mortality rates for the last few decades. The lack of a comprehensive understanding of the mechanisms involved in the development of meningitis contributes to this poor outcome. Here, we demonstrate that depletion of macrophages in newborn mice renders the animals resistant to *E. coli* K1 induced meningitis. The entry of *E. coli* K1 into macrophages requires the interaction of outer membrane protein A (OmpA) of *E. coli* K1 with the alpha chain of Fcγ receptor I (FcγRI, CD64) for which IgG opsonization is not necessary. Overexpression of full-length but not C-terminal truncated FcγRI in COS-1 cells permits *E. coli* K1 to enter the cells. Moreover, OmpA binding to FcγRIa prevents the recruitment of the γ-chain and induces a different pattern of tyrosine phosphorylation of macrophage proteins compared to IgG2a induced phosphorylation. Of note, FcγRIα−/− mice are resistant to *E. coli* infection due to accelerated clearance of bacteria from circulation, which in turn was the result of increased expression of CR3 on macrophages. Reintroduction of human FcγRI in mouse FcγRIα−/− macrophages in *vivo* increased bacterial survival by suppressing the expression of CR3. Adoptive transfer of wild type macrophages into FcγRIα−/− mice restored susceptibility to *E. coli* infection. Together, these results show that the interaction of FcγRI alpha chain with OmpA plays a key role in the development of neonatal meningitis by *E. coli* K1.

**Introduction**

Professional phagocytes, including neutrophils and macrophages (MO) express a specific set of phagocytic receptors that recognize, bind to and mediate internalization of microbial pathogens [1,2,3]. Although MO receptor-mediated phagocytosis generally leads to the destruction of the pathogen, certain receptor-ligand interactions allow for a permissive environment in which the pathogen can thrive and even proliferate. MO provide a barrier that pathogens must overcome to adhere to and penetrate into tissues. Nonetheless, diverse strategies are used by different bacterial pathogens to subvert phagocytes. *Escherichia coli* K1 causes meningitis in neonates, which remains a significant problem for the last few decades with case fatality rates ranging from 5 to 40% of infected neonates [4,5,6,7]. Despite treatment with advanced antibiotics, up to 30% of survivors exhibit neurological sequelae such as hearing impairment, mental retardation, and hydrocephalus. Furthermore, due to the emergence of antibiotic resistant strains, mortality rates may significantly increase in future [8]. The crossing of the mucosal epithelium and the invasion of small subepithelial blood vessels by *E. coli* K1 represent critical early steps in the pathogenesis of meningitis. During initial colonization, *E. coli* K1 encounters several host defense mechanisms such as complement, neutrophils, and MO on its path to the blood-brain barrier (BBB). However, very little is known about the mechanisms by which *E. coli* K1 finds a niche to avoid these host defenses. Our previous studies demonstrated that *E. coli* K1 evades complement attack by binding to the complement pathway regulator C4bp via outer membrane protein A (OmpA), which subsequently cleaves C3b and C4b complement proteins [9,10]. In addition, lack of significant quantities of C9, a terminal complement component necessary for the formation of the membrane attack complex, in neonatal population gives an additional opportunity for *E. coli* K1 to survive in the blood [10]. However, our studies have shown that an inoculum of >10^3 CFU/ml of *E. coli* K1 is required to resist serum bactericidal activity [11], indicating that the bacteria must take a refuge in certain cells to survive and multiply during the initial stages of infection, when fewer bacteria are present in the blood.

Despite the importance of MO in innate and adaptive immunity, the interaction of *E. coli* K1 with these cells is poorly defined. MO
**Author Summary**

*Escherichia coli* K1 is the most common cause of meningitis in premature infants; the mortality rate of this disease ranges from 5% to 30%. A better understanding of the pathogenesis of *E. coli* K1 meningitis is needed to develop new preventative strategies. We have shown that outer membrane protein A (OmpA) of *E. coli* K1, independent of antibody opsonization, is critical for bacterial entrance and survival within macrophages. Using a newborn mouse model, we found that depletion of macrophages renders the animals resistant to *E. coli* K1 induced meningitis. OmpA binds to α-chain of Fcy-receptor I (FcγR) in macrophages, but does not induce expected gamma chain association and signaling. FcγRα knockout mice are resistant to *E. coli* K1 infection because their macrophages express more CR3 and are thus able to kill bacteria with greater efficiency, preventing the development of high-grade bacteremia, a pre-requisite for the onset of meningitis. These novel observations demonstrate that inhibiting OmpA binding to FcγRα is a promising therapeutic target for treatment or prevention of neonatal meningitis.

MO in the pathogenesis of *E. coli* K1 induced meningitis, MO were depleted in newborn mice by the administration of carrageenan [27,28]. MO readily ingest carrageenan in contrast to lymphocytes, which are not actively phagocytic and lack a well-developed lysosomal complex. Due to its unique secondary and tertiary structure, carrageenan is resistant to biochemical degradation by lysosomal glycosidases. Carrageenan containing phagolysosomes eventually rupture due to osmotic swelling. The consequent release of hydrolytic enzymes into the cytosol causes irreversible damage and eventual lysis of MO [29]. Following three days of carrageenan administration starting at day 1 after birth, the animals showed >95% depletion of MO from livers and spleens, as shown by flow cytometry after staining with F4/80 antibody (5.33% ± 0.4% before and 0.17% ± 0.1% after carrageenan treatment) (Figure 1A). However, treatment with carrageenan did not affect B cells (39.81% ± 0.7% before and 40.19% ± 0.9% after carrageenan treatment), CD4+ T cells (17.56% ± 0.5% before and 18.02% ± 0.6% after carrageenan treatment), CD8+ T cells (21.1% ± 0.4% before and 25.3% ± 0.3% after carrageenan treatment), DCs (5.67% ± 1.2% before and 6.09% ± 0.9% after carrageenan treatment), or PMNs (3.98% ± 1.2% before and 4.13% ± 1.4% after carrageenan treatment) in spleens of MO-depleted mice compared with untreated mice (Figure S1). The MO-depleted mice were then infected with 10^5 CFU of *E. coli* K1 by intranasal instillation and examined for progression of the disease as previously described [28]. Control animals (n = 15 for each group) developed bacteremia at 6 h post-infection, which was increased to 5.5 log_{10} CFU per ml of blood by 48 h (Figure 1B). In contrast, the MO-depleted mice, despite having a similar number of bacteria in the blood at 6 h, cleared these bacteria from the circulation by 48 h post-infection. In agreement with the bacteremia levels, >90% of control mice developed meningitis at 72 h after infection, whereas none of the MO-depleted animals showed signs of meningitis and all survived beyond 7 days (Figure 1C). Determination of serum cytokine levels at various times post-infection revealed that control animals produced an initial burst of IL-10, which peaked at 12 h, and then declined to basal levels by 48 h (Figure 1D). In contrast, the pro-inflammatory cytokines, TNF-α, IFN-γ, IL-1β, IL-6 and IL-12p70 only became detectable in the blood at 12 h post-infection and peaked by 72 h (Figure 1D and Figure S2). Of note, although the MO-depleted mice had early production of pro-inflammatory cytokines, their levels were significantly lower than those in the control mice. In these mice, IL-10 levels progressively rose during the initial stages of infection and peaked at 72 h at which time the bacteria were completely cleared from the circulation (Figure 1D).

Histopathological examination of control mice infected with *E. coli* K1 revealed marked infiltration of PMNs in the leptomeningeal and ventricular spaces (Figure 1E). The hippocampus was also inflamed and there was apoptosis of neurons, as indicated by pyknotic nuclei in Ammon’s horn. Acute hemorrhage and inflammation was observed, most prominently in the white matter of the brain. The cortex and molecular layer had increased cellularity due to inflammatory exudates. The MO-depleted mice, however, did not reveal such pathological changes. Blood brain barrier (BBB) leakage is the hallmark of neonatal meningitis. Therefore we used the Evans blue extravasation method to quantify BBB leakage in both the control and MO-depleted mice [28]. The dye was injected intraperitoneally at 68 h post-infection and after four hours, the brains were removed and Evans blue concentration determined. A marked increase in the permeability of the BBB was observed in infected WT animals, which was significantly reduced in MO-depleted mice, (p<0.001 by student’s t test) (Figure 1F). Furthermore, the number of *E. coli* K1 entering the brain was approximately 6.0 log_{10} CFU in control animals,
Figure 1. Depletion of MØ in newborn mice prevented the occurrence of meningitis by *E. coli* K1. Newborn mice were administered α-carrageenan once a day for three days after birth. Spleens and livers were harvested, homogenized and the cells in the homogenates were subjected to flow cytometry after staining with F4/80 antibodies. Cells from untreated animals and those stained with isotype-matched antibodies were used as controls. The graphs show the CFU/ml of blood and the percent meningitis over time. The histological images illustrate the differences in the cortex and meninges, white matter, and parenchyma between WT and MØ-depleted mice. The box plots compare Evans Blue and CFU/g brain tissue levels between WT and MØ-depleted groups. 
controls (A). The MØ-depleted animals were infected with 10^7 CFU of E. coli K1 by intranasal instillation and blood was collected at different post-infection times. Various dilutions of the blood were plated on agar containing rifampicin (B). Cerebrospinal fluid was collected from the same animals aspartically by cisternal puncture and inoculated into LB broth containing antibiotics (C). Blood collected from these animals was also used to measure the presence of TNF-α or IL-10 by ELISA (D). At 72 h post-infection, animals were sacrificed due to a moribund situation for ethical reasons, and the brains were harvested, fixed, paraffin sections prepared and stained with Hematoxylin and Eosin (E). Neutrophil infiltration (black arrow) was observed in the cortex and meninges in brains of WT mice along with apoptosis of neurons indicated by perinuclear halo (yellow arrows). White matter showed increased cellularity due to inflammatory exudates. Pyknotic nuclei (yellow arrows) and inflammatory cells (black arrow) were observed in the hippocampus, suggesting apoptosis of neurons. In contrast, no such pathological changes were seen in the brains of MØ-depleted mice. In some experiments, the animals were injected intraperitoneally with Evans blue at 68 h post-infection. The animals were sacrificed at 72 h the brains were harvested, then homogenized and the concentration of Evans blue determined (F). Brain homogenates from infected animals were used as controls. Half of the brain from each animal was homogenized and the presence of E. coli K1 determined by plating the homogenates on antibiotic containing agar (G). The data represent mean values ± SE of three separate experiments with a total of 15 animals per group. Histopathology is from one animal that is representative of similar results from the rest of the experimental group. Blood brain barrier leakage and the bacterial burden in MØ-depleted animals were statistically different when compared with the untreated and infected animals, *p<0.001 by Student’s t test. Scale bars, 20 μM.

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whereas the brains of the MØ-depleted animals contained very few bacteria (Figure 1G). These results demonstrate that MØ may be important for E. coli K1 to reach a required level of bacteremia, which is critical for the establishment of neonatal meningitis.

**OmpA interaction with FcγRIa is requisite for E. coli K1 binding to, and entry into, MØ**

Our previous studies have shown that OmpA+ E. coli binds and enters MO in vitro irrespective of opsonization status of the bacteria [14]. OmpA− E. coli, although entered in lower numbers but failed to survive inside MO. This indicates that OmpA mediated entry into MO enables OmpA+ E. coli K1 to resist the normal antimicrobial mechanisms of MO. Therefore, to understand the nature of the macrophage surface structures that interact with E. coli K1, biotin-labeled cell surface proteins of THP-1 cells differentiated into MO (THP-M) and RAW 264.7 cells were incubated with OmpA+ E. coli, OmpA− E. coli or a laboratory E. coli HB101. Bound proteins were then released and analyzed by western blotting with streptavidin peroxidase. A small number of proteins bound to all the bacteria from both the cells. However, OmpA+ E. coli prominently bound to the 110 and 70 kDa proteins from both THP-M and RAW 264.7 cells, whereas OmpA− E. coli bound only to the 110 kDa protein (Figure 2A). Although some proteins bound to HB101 were of similar molecular mass to those bound to OmpA+/OmpA− E. coli, other proteins showed different binding patterns. Based on their molecular masses, we speculated that the proteins binding to E. coli K1 could be Toll-like receptor-4 (110 kDa) and FcγRIa (CD64, 72 kDa). Since OmpA+ E. coli specifically bound to the 70 kDa protein in contrast to OmpA− E. coli, the blots were reprobed with an anti-FcγRI antibody, which reacted with the 70 kDa protein, suggesting that OmpA+ E. coli binds to FcγRIa. Of note, treating the bacteria with 40% pooled human serum did not alter the binding, indicating that opsonization with complement and/or with non-specific antibody did not alter bacterial interaction with macrophage surface proteins.

Next, we used blocking antibodies to determine the contribution of OmpA-FcγRIa interaction in E. coli entry into MO. OmpA+ E. coli was incubated with Fab fragments of anti-OmpA antibody (polyclonal) prior to addition to MO. In other experiments, the RAW 264.7 cells were pre-treated with antibodies to FcγRI, CR3, TLR2, TLR4 or the mannose receptor prior to addition of OmpA+ E. coli. Isotype matched antibodies or anti-S-fimbria antibodies were used as controls. Both anti-OmpA and anti-FcγRI antibodies reduced the number of bound and intracellular E. coli K1 by ~80%, whereas other antibodies showed no significant inhibition (Figure 2B). To verify that the anti-FcγRI antibody actually inhibited FcγRI-mediated phagocytosis, the effect of this antibody on the entry of zymosan coated with fluorescent-labeled IgG2a that occurs via FcγRI was also determined. The internalized zymosan particles were counted per 100 cells after quenching the external fluorescence by Trypan Blue [30]. As predicted, anti-FcγRI antibodies significantly inhibited the entry of opsonized zymosan (Figure 2C).

MO pretreated with the anti-FcγRI antibody were also infected with Group B streptococcus (GBS) pre-treated with C3-deficient serum (for deposition of C3 and to avoid bacterial killing by serum), which is known to enter MO through the CR3 receptor [31,32,33]. The internalization of GBS, however, was not affected by pretreatment with anti-FcγRI antibody, suggesting that it did not interfere with CR3 receptor function in MO (Figure 2D). However, as expected, anti-CR3 antibodies significantly blocked the binding and entry of GBS into RAW 264.7 cells. To further confirm the role of OmpA interaction with MO in E. coli entry into MO, OmpA+ E. coli was purified from OmpA+ E. coli and reconstituted into liposomes as previously described [34], which were used to pre-treat RAW 264.7 cells prior to adding the bacteria (Figure 2E). The liposomes containing OmpA blocked both binding and intracellular survival of E. coli K1 by approximately 50%, whereas liposomes containing outer membrane proteins from OmpA− E. coli did not show such inhibition. Increasing concentrations of OmpA liposomes showed no further increase in the inhibition, indicating that the structure of OmpA in liposomes may not be optimal to that of OmpA on E. coli K1 to bind to FcγRIa.

The fate of OmpA+ E. coli after phagocytosis by RAW 264.7 cells was examined by immunocytochemistry after differential staining. Extracellular bacteria were stained with FITC labeled secondary antibody (green) and the intracellular bacteria were stained with a TRITC labeled secondary antibody (red) after incubation with primary anti-S-fimbria antibody. As shown in Figure 2F, a number of OmpA+ E. coli bound to RAW 264.7 cells, whereas very few OmpA− E. coli bound at 30 min post-infection. Analysis of intracellular bacteria over time revealed that OmpA+ E. coli multiplied, whereas OmpA− E. coli were degraded inside the cells. Collectively, these studies suggest that the OmpA of E. coli K1 interacts with regions of FcγRIa similar to those involved in the binding of Fc and that this interaction enables the organism to enter MO. In addition, the data suggest that other receptors that recognize pathogen-associated molecules may not play a significant role in MO binding and entry of E. coli K1. However, entry through other receptors in the absence of OmpA-FcγRIa interaction renders the bacteria susceptible to macrophage killing.

**FcγRIa gene silencing by RNA interference abolishes E. coli K1 binding to and entry into MØ**

To confirm the role of FcγRIa in OmpA+ E. coli entry of MO, short hairpin RNA (shRNA) sequences for murine FcγRIa and CR3 in pGeneClip Neomycin vectors were used to transfect RAW
Figure 2. OmpA interaction with FcγRIa is necessary for binding to, and entry of, E. coli K1 in RAW 264.7 cells. (A) The surface proteins of RAW 264.7 cells and THP-1 differentiated into macrophages (THP-M) were labeled with NHS-LC-Biotin and the membrane proteins prepared. OmpA+ or OmpA− E. coli, with or without treatment with 40% pooled human serum for 10 min, or HB101 were incubated with 2 μg of biotinylated proteins for 1 h, washed, the bound proteins released, and subjected to SDS-PAGE. The proteins were then transferred to a nitrocellulose and immunoblotted with streptavidin peroxidase. The blots were stripped and reprobed with anti-FcγRI antibody. (B) RAW 264.7 cells were incubated with various antibodies prior to the addition of E. coli K1. Similarly, OmpA+ E. coli were incubated with anti-OmpA antibodies for 1 h on ice prior to
264.7 cells. Suppression of FcγRIa and CR3 gene transcription and expression was verified by RT-PCR and flow cytometry, respectively. The respective shRNA suppressed the transcription of FcγRIa and CR3 considerably, but had no effect on GAPDH, TLR2 or TLR4 mRNA transcript levels (Figure 3A). On par with changes in transcription levels, the surface expression of FcγRIa and CR3 was significantly reduced, while TLR2 and TLR4 expression was unaltered (Figure 3B). There was >90% reduction in the OmpA+ E. coli phagocytosed by FcγRIa-shRNA/RAW cells compared to control or CR3-shRNA/RAW cells (p<0.001 by two-tailed t-test) (Figure 3C). This reduction was due to inefficient binding of E. coli K1 to these cells, as less than 30% of bacteria were bound by the FcγRIa-shRNA/RAW cells compared to non-transfected or control-shRNA transfected cells. In contrast, both binding and intracellular survival of GBS were not affected in FcγRIa-shRNA/RAW cells, whereas CR3-shRNA transfection caused significant reduction in both of these processes (Figure 3D). Immunocytochemistry of E. coli K1 infected FcγRIa-shRNA/RAW cells revealed that very few cells ingested bacteria and were killed within 2 h post-infection (Figure 3E, fragmented bacteria). However, E. coli K1 entered and replicated in CR3-shRNA/RAW cells similar to control RAW cells. Comparable results were also obtained with THP-M cells transfected with shRNA specific to human FcγRI (data not shown). To further confirm that lack of FcγRIa expression rendered bacteria susceptible to macrophage killing, FcγRIa-shRNA/RAW cells infected with E. coli K1 were examined by transmission electron microscopy. Although few numbers of FcγRIa-shRNA/RAW cells engulfed E. coli K1, several of them were either degraded or in the process of degradation by 1 h post-infection and were completely killed by 8 h post-infection (Figure 3F). In contrast, CR3-shRNA/RAW cells showed intact bacteria in endosomes undergoing significant multiplication by 8 h post-infection. Taken together these results demonstrate that OmpA-FcγRIa interaction is critical for E. coli K1 to bind to, enter and survive in MO.

**OmpA of E. coli K1 binds to FcγRIa and induces a distinct signaling pattern**

One important question to address in these studies is how OmpA of E. coli K1 binds to FcγRIa at the same region as the Fc-region of IgG in the context of whole blood. Generally, specific or even non-specific IgG in circulation binds invading bacteria and thereby presents the pathogen to FcγR receptors on MO. Therefore, it is possible that OmpA+ E. coli may be displacing IgG for binding to FcγRI. We tested this hypothesis by performing two different competitive binding experiments. First, OmpA− E. coli were coated with anti-S-fimbria antibody and added to FcγRIa/CO1 cells treated with cytochalasin D to prevent internalization. The cells were washed and then various quantities of OmpA+ E. coli were added and incubated for 10 min. After washing the monolayers, the number of OmpA− E. coli that remained bound to COS-1 cells were determined by plating on agar containing tetracycline (OmpA+ E. coli is sensitive to tetracycline). As shown in Figure 5A, IgG2a opsonized OmpA− E. coli bound COS-1 cells in significantly greater numbers compared to unopsonized bacteria and progressively more bacteria were released from the cells as more OmpA+ E. coli were added to the wells. In contrast, OmpA− E. coli could not displace bound OmpA− E. coli. In separate experiments, peritoneal MO were incubated with FITC-IgG2a (1 µg) for 1 h in the presence of cytochalasin D, washed and then various quantities of OmpA+ E. coli or OmpA− E. coli were added. The cells were incubated for 10 min, washed and the amount of FITC-IgG that remained bound to the MO was determined by flow cytometry. As shown in Figure 5B, the amount of FITC-IgG2a bound to peritoneal MO was decreased when OmpA+ E. coli were added, whereas addition of OmpA− E. coli had no effect. These
Figure 3. Suppression of FcγRIα expression using shRNA prevents *E. coli* K1 entry into RAW 264.7 cells. (A) RAW 264.7 cells were transfected with plasmids containing shRNA to FcγRIIa or CR3, total RNA was isolated and subjected to RT-PCR using specific primers. GAPDH primers were used as internal controls. (B) FcγRIIa−/− and CR3−/RAW cells were further subjected to flow cytometry using antibodies to FcγRI, CR3, TLR2 and TLR4. Mean fluorescence intensities were plotted after subtracting the values of isotype-matched controls. (C and D) Total cell bound and intracellular bacteria (measured by gentamicin protection assay) were determined after infecting FcγRIIa−/− and CR3−/RAW cells with *E. coli* K1 or Group B streptococcus. *E. coli* K1 and GBS that were bound or intracellular in control cells were taken as 100%. (E) Immunocytochemistry of *E. coli* K1 entered into FcγRIIa−/− and CR3−/RAW cells after differential staining as described in Materials and Methods. Scale bars, 10 μM. (F) FcγRIIa−/− and CR3−/RAW cells
were infected with *E. coli* K1 for varying periods, fixed and subjected to transmission electron microscopy as described in Materials and Methods. Photomicrographs at 1 h and 8 h post-infection are shown and arrows indicate vacuoles containing bacteria or empty vacuoles. Invasion experiments were performed in triplicate and were independently done three times. Data represent mean ± SD and the decrease in bound or intracellular bacteria was statistically significant when compared with control shRNA/RAW 264.7 cells, *p*<0.001 by Student’s *t* test. Scale Bars 1.0 μm. doi:10.1371/journal.ppat.1001203.g003

results indicate that the interaction of *E. coli* K1 with FcγRIα via OmpA can displace bound IgG2a.

Binding to the γ-chain of FcγRIα is crucial for inducing the antimicrobial activity of MO [20]. Since OmpA binding to FcγRIα prevented the killing of the bacteria, we hypothesize that *E. coli* K1 interaction with FcγRIα avoids the association of the γ-chain. Consistent with this assumption, OmpA+/ *E. coli* interaction with MO in the presence or absence of IgG2a opsonization induced far less γ-chain association with FcγRIα in comparison to OmpA− *E. coli*, as shown by immunoprecipitation studies (Figure 5C). Similarly, OmpA+/ *E. coli* induced a distinct tyrosine phosphorylation pattern of macrophage cytoplasmic proteins compared to OmpA− *E. coli* oposanized with IgG2a (Figure 5D). Taken together, these studies suggest that the interaction of *E. coli* K1 with FcγRIα can displace the bound IgG2a, which is mediated by OmpA. They also indicate that OmpA/FcγRI interaction induces novel signaling patterns, which may abrogate the normal antimicrobial response of these cells.

FcγRIα−/− mice are resistant to *E. coli* K1 infection and do not develop high degree of bacteremia

To confirm the role of FcγRIα in the pathogenesis of *E. coli* K1 meningitis, FcγRIα−/− mice were used for infection studies. MO isolated from FcγRIα−/− mice did not express FcγRIα but had unchanged expression of other FcγRs, TLRs, mannose receptor and CR3 were unchanged compared to normal littermates (data not shown). The newborn animals were intranasally infected with *E. coli* K1 and examined for disease progression. Of note, the FcγRIα−/− animals did not develop bacteremia even at a 100 fold higher infectious dose, even though *E. coli* K1 entered the circulation within two hours of infection (Figure S3A). In contrast, wild type (WT) animals showed 7.0 log10 CFU of *E. coli* K1 in blood at 72 h post-infection (Figure 6A). The FcγRIα−/− mice did not develop meningitis even when infected with a 100-fold greater inoculum (data not shown). These mice did not show any signs of meningitis even after 7 days of infection, whereas 90% of WT mice showed positive CSF cultures by 72 h post-infection (Figure 6B). Cytokine analysis in the sera of these animals demonstrated that infected WT animals generated significant amounts of TNF-α, IL-1β, IL-6, IFN-γ and IL-12, but FcγRIα−/− mice did not (Figure 6C and Figure S3B–D). On the other hand, IL-10 production peaked at 24 h post-infection and subsequently returned to basal levels in WT mice, whereas FcγRIα+/− mice showed increased IL-10 production at 72 h post infection (Figure 6D). We next examined brain-blood barrier leakage in FcγRIα−/− mice. Infection with *E. coli* K1 caused no leakage in FcγRIα−/− mice, whereas WT animals had significant leakage of Evans blue dye (Figure 6E). Furthermore, no bacterial colonies were detected in the brains of FcγRIα−/− mice, while WT animals had a high bacterial load (Figure 6F). Similarly, the pathology of the brains from FcγRIα−/− mice revealed no infiltration of neutrophils, neuronal damage or gliosis, which are the characteristic pathological features of *E. coli* K1 meningitis observed in WT bacteria infected mice (Figure 6G). In contrast, infection of FcγRIα−/− mice with GBS resulted in significant bacteremia and development of meningitis (Figure S4A–G). Together these results suggest that FcγRIα expression is critical for *E. coli* K1 to achieve high-grade bacteremia and for subsequent development of meningitis in newborn mice.

FcγRIα−/− MØ exhibit greater expression of CR3 and TLR4 and produce lower levels of inducible nitric oxide

Our studies have shown that MO isolated from *E. coli* K1 infected mice exhibited increased expression of FcγRI and TLR2, as well as increased production of nitric oxide (NO) due to iNOS activation [28]. We also observed that upregulation of CR3 expression on MO led to enhanced killing of *E. coli* K1, whereas this effect was completely abrogated in CR3 siRNA transfected MO *in vitro*. Other investigators have also demonstrated that CR3, TLR2 and TLR4 play important roles in the phagocytic ability of MO [35–42]. Therefore, we examined whether the inability of *E. coli* K1 to survive in FcγRIα−/− mice was due to altered expression of surface receptors using flow cytometry. Peritoneal MO isolated from infected FcγRIα−/− mice exhibited increased expression of CR3 and TLR4, but lower expression of TLR2 (Figure 7A). These cells also produced lower or negligible quantities of inducible NO upon challenge with *E. coli* K1, whereas MO from WT mice generated six-fold higher amounts of NO at 6 h post-infection (Figure 7B). Furthermore, *E. coli* K1 binding to, and entry into, bone marrow-derived MO (BMDMs) from FcγRIα−/− mice were significantly lower compared to WT MO (Figure 7C and D). Some bacteria entered FcγRIα−/− BMDMs, but they were killed within a short period of time as determined by immunocytochemistry (data not shown). To substantiate the role of FcγRI in *E. coli* K1 entry, FcγRIα−/− BMDMs were transfected with hFcγRIα, FcγRIα-CT or FcγRII and then used for binding and invasion assays. As shown in Figure 7C, *E. coli* K1 binding to FcγRIα−/− BMDMs/FcγRIα and FcγRIα−/− BMDMs/FcγRIα-CT increased significantly compared to FcγRIα−/− BMDMs and FcγRIα−/− BMDMs/FcγRII. However, entry was limited to binding to FcγRIα−/− BMDMs/FcγRIα cells only. These results suggest that FcγRIα expression is critical for *E. coli* K1 binding to, and entry into, MO and that the C-terminal domain plays a significant role for the entry. FcγRIα−/− BMDM transfected with a FcγRIα construct exhibited decreased expression of TLR4 and CR3 and increased expression of FcγRI and TLR2 in comparison with FcγRIα−/− BMDM after challenge with *E. coli* K1 (p<0.01) (Figure 7E). Transfection with FcγRIα-CT, however, resulted in only a partial increase or decrease of these surface molecules. In contrast, FcγRIα−/− BMDM transfected with FcγRII showed basal level expression of these molecules. Confirming the requirement of FcγRIα interaction with *E. coli* K1 to induce NO production, FcγRIα−/− BMDMs/FcγRIα cells generated greater quantities of NO by 6 h post-infection as compared to FcγRI−/+ BMDMs/FcγRI-CT and FcγRI−/+ BMDMs/FcγRII cells (Figure 7E). Taken together, these data suggest that FcγRIα interaction with OmpA of *E. coli* K1 is necessary for suppression of CR3 and TLR4 expression and to enhance the expression of FcγRI and TLR2, and maximal NO production.

FcγRIα−/− mice reconstituted with FcγRIα+/+ MØ are susceptible to *E. coli* K1 meningitis

To confirm the role of FcγRIα expression on MO in the pathogenesis of *E. coli* K1 meningitis, FcγRIα−/− mice were
Figure 4. FcγRIα expression is sufficient to facilitate E. coli K1 invasion of COS-1 cells. COS-1 cells were transfected with plasmids containing FcγRIα, FcγRI-CT or FcγRII and the expression of the recombinant proteins was determined by Western blotting (A) from total cell lysates or by flow cytometry (B) using anti-Myc antibody. COS-1 cells transfected with pcDNA3 were used as a control (Mock). E. coli K1 binding to, and invasion of, transfected COS-1 cells were performed as described in Materials and Methods (C). Purified Myc-FcγRIα or BSA (control) was incubated with OmpA+ or OmpA− E. coli for 1 h on ice. Bacteria were washed, the bound proteins were released and were subjected to SDS-PAGE. The proteins

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reconstituted with FcγRIα+/+ or FcγRIα−/− MO and then infected with *E. coli* K1. FcγRIα−/− mice that received FcγRIα+/+ MO showed higher blood bacterial numbers compared with animals replenished with FcγRIα+/+ MO (Figure 3A). 94% of CSF cultures were positive for *E. coli* K1 in FcγRIα+/+ MO reconstituted mice, whereas all cultures were sterile in animals that received FcγRIα−/− MO (Figure 3B). BBB disruption was significant in FcγRIα+/+ MO-replenished animals compared to FcγRIα−/− MO reconstituted mice (Figure 3C). Higher numbers of bacteria were also recovered from the brains of mice replenished with FcγRIα+/+ compared to animals those received FcγRIα−/− MO (Figure 3D). These results confirm that FcγRIα expression on MO is critical for the onset of *E. coli* K1 meningitis.

**Discussion**

The host response to infection starts with the identification of invading microorganisms via innate immune surveillance systems [43]. Nonetheless bacterial pathogens utilize very effective mechanisms to avoid host defenses in order to promote successful replication and dissemination [44]. MO provide an important innate and adaptive immune coverage in the host, although their importance in *E. coli* K1 meningitis is unexplored. In the present study, we demonstrate that the expression of FcγRIα-chain in MO is critical for the survival of *E. coli* K1 inside these immune cells by using MO-depleted and FcγRIα−/− mice. It is tempting to speculate that the ability of *E. coli* K1 to survive inside MO might enable these bacteria to infect the central nervous system via a "Trojan horse" mechanism. Pathogens that naturally infect the central nervous system, such as *Brucella*, *Listeria*, and *Mycobacterium*, have been demonstrated to use this mode of entry [43,46]. We observed that the interaction of OmpA with FcγRIα in MO is critical for bacterial binding to, entry into, and subsequent survival in these cells. Generally various FcγRs recognize microbes coated with either specific or non-specific antibodies. However a select number of microbes have developed methods to avoid this recognition. Protein A of *S. aureus* is known to bind to the Fc portion of the antibodies so that it avoids interacting with FcγRI, whereas most other microbes either downregulate phagocytic mechanisms or avoid phagocytosis entirely [47,48]. This study therefore depicts the first evidence that a bacterial protein binds directly to FcγRIα to divert anti-microbial mechanisms.

Our competitive inhibition studies demonstrated that OmpA interacts with FcγRIα and can displace the binding of Fc portion of IgG. Therefore, it is possible that the bacteria in circulation, despite being coated with non-specific IgG, interact with MO via FcγRIα for binding to and entering the cells for subsequent multiplication. OmpA− *E. coli* could not survive in MO, suggesting that the interaction of OmpA with FcγRIα induces survival strategies or suppresses anti-microbial pathways in MO. However, OmpA− *E. coli* has been shown to express reduced levels of type 1 fimbiae and susceptible to chemical stresses [49,50]. Therefore, it is possible that OmpA− *E. coli* could be less capable of dealing with macrophage-induced stresses. *Listeria*, *Shigella*, and *Rickettsia* escape from the phagosome to the cytosol to avoid destruction in phagolysosomes [51]. Other pathogens interfere with the normal biogenesis of phagolysosomes, thus leading to the formation of replicative vacuoles [52,53]. Since *E. coli* K1 continue to multiply inside phagosomes, one can speculate that phagosomes containing OmpA+ *E. coli* avoid lysosomal fusion by blocking phagosome maturation. The receptors expressed on the surface of MO play a decisive role in the course of infection, whether pathogens are killed or the MO machinery is taken over by the microbes [54]. Receptors like TLR2, TLR4 and CR3 have been implicated in the phagocytic ability of MO [55,56,57]. Downregulation of CR3 expression on the surface of MO has been associated with the decrease in the phagocytosis of pathogens and hence survival inside MO [58]. TLR2 expression has been shown to prolong survival of *Staphylococcus aureus* inside phagosomes in MO, which may be a strategy adopted by this pathogen to evade innate immunity. On par with this concept, TLR2 or MyD88 KO mice have been demonstrated to be resistant to sepsis, indicating that TLR2 mediated signaling is playing an important role in the survival of bacterial pathogens [59]. Activation of MO through TLR4 has been shown to direct the induction of Th1 and Th-17 cells, which mediate protective cellular immunity to * Bordetella pertussis* by enhancing the bac tericidal activity of MO [60]. It is still to be determined whether TLR2 expression upon *E. coli* K1 infection has any role in the pathogenesis of meningitis.

We recently demonstrated that iNOS−/− mice and aminoguanidine (iNOS specific inhibitor) treated MO showed enhanced expression of CR3 and TLR4 and very low levels of TLR2 and FcγRI, indicating that iNOS suppression results in decreased expression of FcγRI [28]. In agreement with these studies, we showed here that lack of FcγRI in MO prevented the production of inducible NO and increased the expression of CR3 and TLR4, indicating that OmpA-FcγRIα interaction is critical for manipulating the surface expression of CR3 and TLRs in MO. Our current results indicate that in *E. coli* K1 pathogenesis, FcγRI interaction with OmpA enhances the expression of TLR2, which in turn can be utilized by the bacteria as a receptor to modulate the efficiency of phagosome formation. Alternatively, *E. coli* K1 interaction with FcγRIα activates non-microbial mechanisms for the bacterial survival in MO. Our studies have demonstrated that *E. coli* K1 infected MO also exhibit increased expression of gp96, a known chaperone for TLR2 and TLR4 [28]. These interactions may also induce effector proteins into MO by *E. coli* K1 that eventually are responsible for the control of macrophage environment. Further studies are in progress to examine these possibilities. As cytokines are known to modulate MO microbicidal activity, it is also possible that the surface expression of TLRs and CR3 could be controlled by the circulating cytokines in *E. coli* K1 infection. Of note, we have demonstrated that IL-10 administration suppressed the expression of FcγRI and enhanced the expression of TLR4 and CR3, which in turn prevented the survival of *E. coli* K1 in MO [61]. In contrast, for several other pathogens, circulating IL-10 supports intracellular replication, indicating that *E. coli* K1 pathogenesis is distinct from that induced by other bacterial pathogens [62].

Previous studies have shown that the cytoplasmic (CY) domain of FcγRIα plays an important role in phagocytosis and antigen presentation [63]. However, lack of the CY domain neither alters the association of γ-chain with FcγRIα nor influences the tyrosine phosphorylation of γ-chain in response to receptor specific cross-
In contrast to these findings, we observed that OmpA binding to FcγRIα did not induce the association of c-chain despite the presence of the CY domain. This binding also induced a different tyrosine phosphorylation response in MØ. Therefore, the CY domain of FcγRIα induces signaling events independent of c-chain during the invasion of E. coli K1. Similarly, Qin et al demonstrated that the CY domain induces different gene expression in murine MØ compared to MO stably transfected with CY-deleted FcγRIα. Alteration of signal transduction pathways to impair FcγR-mediated phagocytosis has also been observed in HIV infected MO, which have downregulated the expression of the γ-subunit. Moreover, direct interaction of periplakin with the CY domain of human linking [63]. In contrast to these findings, we observed that OmpA binding to FcγRIα did not induce the association of γ-chain despite the presence of the CY domain. This binding also induced a different tyrosine phosphorylation response in MØ. Therefore, the CY domain of FcγRIα induces signaling events independent of γ-chain during the invasion of E. coli K1. Similarly, Qin et al demonstrated that the CY domain induces different gene expression in murine MØ compared to MO stably transfected with CY-deleted FcγRIα. Alteration of signal transduction pathways to impair FcγR-mediated phagocytosis has also been observed in HIV infected MO, which have downregulated the expression of the γ-subunit. Moreover, direct interaction of periplakin with the CY domain of human

**Figure 5.** *E. coli* K1 binds to FcγRIα via OmpA and induces a distinct signaling. (A) OmpA− *E. coli* were coated with IgG2a for 1 h on ice, washed and then added to COS-1 cells pre-treated with cytochalasin D. After one hour of incubation, the cells were washed and OmpA+ *E. coli* were added at an MOI of 10 and 100. The cells were incubated for 10 min, washed, and the bound OmpA− *E. coli* enumerated as described in Materials and Methods. (B) Peritoneal MØ pre-treated with cytochalasin D were incubated with FITC-IgG2a for 30 min, washed, and further incubated with OmpA− or OmpA− at an MOI of 10 or 100 for 10 min. The cells were washed and subjected to flow cytometry to determine the bound levels of IgG2a Cells without the addition of IgG2a were used as a control. (C) Immunoprecipitation of total cell lysates obtained from RAW 264.7 cells infected with OmpA+ or OmpA+ *E. coli* or Zymosan with anti- FcγRI antibody was followed by Western blotting with antibodies to γ-chain or FcγRI. (D) Total cell lysates of RAW 264.7 cells infected with OmpA+ or OmpA− *E. coli* were subjected to Western blotting with anti-phospho-tyrosine antibodies. Competitive inhibition studies were performed at least four times in triplicate and the data represent mean ± SD. The decrease in the number of bacteria attached to COS-1 cells or MFI was statistically significant compared to IgG2a coated OmpA− *E. coli*, *p<0.001 by Student’s t test. doi:10.1371/journal.ppat.1001203.g005

**Figure 5.** *E. coli* K1 binds to FcγRIα via OmpA and induces a distinct signaling. (A) OmpA− *E. coli* were coated with IgG2a for 1 h on ice, washed and then added to COS-1 cells pre-treated with cytochalasin D. After one hour of incubation, the cells were washed and OmpA+ *E. coli* were added at an MOI of 10 and 100. The cells were incubated for 10 min, washed, and the bound OmpA− *E. coli* enumerated as described in Materials and Methods. (B) Peritoneal MØ pre-treated with cytochalasin D were incubated with FITC-IgG2a for 30 min, washed, and further incubated with OmpA− or OmpA− at an MOI of 10 or 100 for 10 min. The cells were washed and subjected to flow cytometry to determine the bound levels of IgG2a Cells without the addition of IgG2a were used as a control. (C) Immunoprecipitation of total cell lysates obtained from RAW 264.7 cells infected with OmpA+ or OmpA+ *E. coli* or Zymosan with anti- FcγRI antibody was followed by Western blotting with antibodies to γ-chain or FcγRI. (D) Total cell lysates of RAW 264.7 cells infected with OmpA+ or OmpA− *E. coli* were subjected to Western blotting with anti-phospho-tyrosine antibodies. Competitive inhibition studies were performed at least four times in triplicate and the data represent mean ± SD. The decrease in the number of bacteria attached to COS-1 cells or MFI was statistically significant compared to IgG2a coated OmpA− *E. coli*, *p<0.001 by Student’s t test. doi:10.1371/journal.ppat.1001203.g005
Figure 6. FcγRIα−/− mice are resistant to E. coli K1 induced meningitis. (A) Wild type (WT) and FcγRIα−/− mice were infected intranasally at post-natal day 3 with 10⁵ CFU of E. coli K1. At various time points blood was collected, diluted, and bacteria enumerated by plating on agar containing antibiotics. (B) Cerebrospinal fluid samples from experimental and control animals were collected and inoculated into LB broth containing antibiotics and incubated overnight at 37 °C. Positive cultures indicate the occurrence of meningitis. (C and D) TNF-α and IL-10 concentrations in the blood of WT and FcγRIα−/− animals infected with E. coli were measured by ELISA. (E) Blood-brain barrier leakage in infected animals was measured by Evans blue extravasation method as described in Materials and Methods. (F) The bacterial load in the brains of infected animals was determined by plating brain homogenates on agar containing antibiotics. (G) Brain halves from experimental and control animals were fixed, paraffin embedded, sectioned and stained with H & E. Cortex and meninges showed severe inflammation (black arrow) along with apoptosis of neurons (yellow arrow) in the brains of WT infected mice. White matter revealed increased cellularity due to inflammatory exudate (black arrow). Neutrophil infiltration (black arrow) and apoptosis of neurons (yellow arrow) were observed in hippocampus. On the contrary, no such pathological changes were seen in the...
FCγRIIa can confer unique properties on this receptor [66]. It should be noted that there are significant differences in the cytoplasmic regions of human and murine FCγRIIa. However, our data demonstrate that the interaction of OmpA induced a similar response in both human and murine MO. In summary, our studies provide the first evidence that a bacterial protein interacts directly with FCγRIIa in order to bind to and enter MO and manipulates the intracellular signaling for bacterial survival and multiplication. The new repertoire of interaction also suggests that MO function may be manipulated by targeting additional epitopes without activating MO microbicidal function. This strategy will be useful for devising novel methods of therapy for other diseases involving FCγRIIa in addition to neonatal E. coli K1 meningitis.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal care and Use Committee (IACUC) of The Saban Research Institute of Childrens Hospital Los Angeles (Permit number: A3276-01). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Bacteria

E. coli E44, a rifampin-resistant mutant of E. coli K1 strain RS 218 (serotype O18:K1:H7), has been isolated from the cerebrospinal fluid of a neonate with meningitis and invades human brain microvascular endothelial cells (HBMEC) [34]. E91, a derivative of E44 in which ompD gene is disrupted (designated as OmpA−E. coli) and HB101 (a laboratory E. coli strain that expresses K-12 capsular polysaccharide) are noninvasive in HBMEC [34]. Group B streptococcus type III strain COH-1 used in these studies was provided by Dr. Craig Rubens of Seattle Children’s Hospital, Seattle [67]. All bacteria were grown in brain heart infusion broth with appropriate antibiotics as necessary. Bacterial media were purchased from Difco laboratories (Detroit, MI).

Cell culture and reagents

Murine MO cell line RAW 264.7, human macrophage like cells, THP-1 and COS-1 cells were obtained from American Type Culture Collection (Manassas, VA). COS-1 cells were stably transfected with cDNA encoding human FCγRIa, a mutant form of FCγRIa containing a stop codon after first amino acid of the cytoplasmic domain (Lys315→Stop 315) (FCγRI-CT), or with human FCγRII [67]. Anti-FCγRII (blocks the binding of FC-portion of IgG to FCγRI), anti-CD11b, anti-CD32, anti-MR, anti-TLR2, anti-TLR4 and anti-Myc antibodies were obtained from Cell signaling. Purified IgG2a and FITC-IgG2a were obtained from Sigma (St. Louis, MO). Anti- IgG 96 antibody was raised in our lab as previously described [34,68]. Anti-phospho-tyrosine antibody (4G10) was obtained from BD Sciences and all secondary antibodies coupled to various fluorophores were obtained from Bio-Rad Labs (Hercules, CA).

Bacterial invasion assays

Confluent MO monolayers in 24-well plates were incubated with 1×10⁶ E. coli K1 (multiplicity of infection of 10) in experimental medium (1:1 mixture of Ham’s F-12 and M-199 containing 5% heat-inactivated fetal bovine serum) for 60 min at 37°C, whereas COS-1 cell monolayers were infected with E. coli K1 at an MOI of 100 for 1.5 h. The monolayers were washed three times with RPMI 1640 and further incubated in experimental medium containing gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. The monolayers were washed again and lysed with 0.5% Triton X-100. The intracellular bacteria were enumerated by plating on sheep blood agar. In duplicate experiments, the total cell associated bacteria were determined as described for invasion except that the gentamicin step was omitted.

Generation of FCγRIa− and CR3− RAW 264.7 cells

SureSilencing shRNA plasmids to mouse FCγRI and CR3 (CD11b) in the pGeneClip Neomycin Vector were obtained from Super Array Inc., (Frederick, MD). RAW 264.7 cells were transfected with shRNA plasmids using Lipofectamine 2000 and later selected for G418 resistant colonies.

Biotinylation of MO membrane proteins

The cell surface proteins of THP-1 cells differentiated into MO (THP-M) and RAW 264.7 cells were biotinylated by adding to 0.1 M sodium bicarbonate buffer (pH 8.0) containing 0.5 mg/ml NHS-LC-Biotin (Pierce Co, Rockford, IL) at a final protein concentration of 2 mg/ml in tissue culture flasks. The flasks were incubated on ice for 1 h, the cells were extensively washed with ice-cold PBS and solubilized in 5% Triton X-100 in PBS. Total membranes from the cells were isolated following extensive dialysis against PBS and then were concentrated using Centricon tubes (Millipore, Bedford, MA; 10-kDa cut-off). Biotinylated proteins (2–5 μg) were incubated with various bacteria from a 5-nl overnight culture in a volume of 0.5 ml at 37°C on a rotator for 1 h. The bacteria were then centrifuged and the pellets were washed three times with PBS containing 0.1% Triton X-100. After a final wash, the bound proteins were released with Laemmli buffer in the presence of β-mercapto-ethanol and analyzed by SDS-PAGE. The separated proteins were transferred to nitrocellulose and immunoblotted with streptavidin coupled to peroxidase. The protein bands were visualized by ECL reagent (Amer sham Biosciences, Piscataway, NJ).

RNA isolation and RT-PCR

Total RNA was isolated from various transfected RAW 266.4 cells with TRIzol-LS-reagent (Gibco BRL, Gaithersburg, MD) and quantified using a nanodrop machine. RT-PCR was performed using the following primer sequences: FCγRIa (321 bp) FP 5’-TCCATTCTTGAGGAAAATACTGACG-3’ and RP 5’-GTTTGGCTGTGGTTTGAGACC-3’; TLR2 (459 bp) FP 5’-TGAGTGGAGAAATA TGGAG-3’ and RP 5’-CTTGCACTCTCA- TAACTCCTGTC-3’; TLR4 (506 bp) FP 5’-AGC TTTCCTTATAAG-3’ and RP 5’-GAATAGGGAGCGCCA- CTTG-3’. GAPDH (479 bp), FP 5’-CACAGTCCATGCGCAT- CACTG-3’ and RP 5’- TACTCTTGGAG GCCATGTG-3’.

Negative control assays without primers were performed in parallel for every reaction. The amplified products were separated on a 1% agarose gel and were stained with ethidium bromide.
Figure 7. Alteration of surface receptor expression in MØ obtained from WT and FcγRIα−/− mice upon infection with E. coli K1. (A) Peritoneal MØ from infected WT and FcγRIα−/− mice were isolated, stained with antibodies to FcγRI, TLR2, TLR4 and CR3, and then subjected to flow cytometry. Data are presented after subtracting the mean fluorescence intensity (MFI) of isotype-matched control. (B) The production of NO by MØ infected with E. coli K1 isolated from WT and FcγRIα−/− mice was measured as nitrite by the Griess method. (C and D) Bone marrow derived MØ (BMDMs) from FcγRIα−/− mice were transfected with FcγRIα, FcγRIα-CT or FcγRII and used for E. coli K1 binding and invasion assays. (E) Flow cytometry of FcγRIα−/− BMDMs transfected with FcγR constructs were infected with E. coli K1 for 6 h, washed and then subjected to flow cytometry after staining with antibodies to FcγRI, TLR2, TLR4, or CR3. MFI values for control-uninfected cells were subtracted from the values of infected cells.
Flow cytometry

Expression of FcγRI, CR3, TLR2 and TLR4 was detected by staining with appropriate FITC-, phycoerythrin (PE)-, PE-CY5.5-, or allophycocyanin (APC)-coupled mouse monoclonal antibodies (eBiosciences, San Diego, CA). Cells were first pre-incubated for 20 min with IgG blocking buffer to mask non-specific binding sites and then further incubated with the indicated antibodies or an isotype control antibody for 30 min at 4°C. The cells were subsequently washed three times with PBS and fixed with BD Cytofix (BD Biosciences). Cells were then analyzed by four-color flow cytometry using FACS calibur Cell Quest Pro software (BD Biosciences, San Jose, CA). Side and forward scatter parameters for which F4/480 was used as a MO-gating marker, which formed the collection gate and at least 5000 events within this gate were collected for analysis.

Depletion of MØ

Newborn C57BL/6 mice were injected intraperitoneally with (20-mg/Kg body weight) κ-carrageenan (Sigma, St. Louis, MO) on days 1, 2 and 3 before infecting with E. coli. In control groups, mice were treated with equal volumes of saline.

Newborn mouse model of meningitis

Three-day old mice were randomly divided into various groups and infected intranasally with 10^7 CFU of bacteria. Control mice received pyrogen free saline through the same route. Blood was collected from the tail or facial vein at designated times post-infection and plated on LB agar containing rifampicin to assess bacteremia and level of infection. CSF samples were collected aseptically under anesthesia by cisternal puncture and directly inoculated into broth containing antibiotics. Mice were perfused aseptically under anesthesia by cisternal puncture and directly inoculated into broth containing antibiotics. Mice were perfused intracardially with 0.9 % saline to remove blood and contaminants intravascular leukocytes. Brains were aseptically removed and homogenized in sterile PBS. Bacterial counts in all tissues were determined by plating ten-fold serial dilutions on rifampicin LB agar plates. Growth of E. coli in rifampicin containing LB broth from the CSF samples was considered positive for meningitis [28].

Characterization of liver and spleen leukocytes

Determination of leukocytes in livers and spleens of untreated and carrageenan treated mice was done using flow cytometry [61]. PMNs were identified by staining with anti-Ly6-G (GR-1) followed by goat anti-rat- phycocerythrin (PE). CD4^+ and CD8^+ T lymphocytes were stained with rat anti-mouse-CD4 followed by goat anti-rat-PE and anti-CD8-FTTC. DCs were stained with APC conjugated anti-CD11c antibody. B lymphocytes were detected by staining with anti-CD45R (B220)-FTTC. Flow cytometry was performed on a FACSscan instrument (BD Biosciences, CA) and the data were analyzed with Cell Quest Software.

Immunoprecipitation and Western blotting

Total cell lysates of RAW 264.7 cells infected with bacteria for varying time periods were centrifuged at 16,000 X g for 20 min at 4°C. The supernatants were collected and the protein contents determined. For immunoprecipitation studies, 300–500 µg of protein was incubated with the appropriate antibody overnight at 4°C, washed and further incubated for 1 h with protein A-agarose. The immune complexes were washed four times with cell lysate buffer and the proteins bound to agarose were eluted in SDS sample buffer for further analysis by Western blotting. Portions of the cell lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide electrophoresis gel. The proteins were transferred to a nitrocellulose membrane, which was then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.03% Tween 20 (TBST) for 2 h at room temperature. The blot was then incubated with the primary antibody overnight at 4°C in 5% BSA/TBST. The blot was then washed with TBST and further incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the blot was washed four times with TBST for 1 h, developed with SuperSignal chemiluminescence reagent, and exposed to x-ray film to visualize the proteins.

Transmission electron microscopy

RAW 264.7 cells were incubated with E. coli K1 at an MOI of 10 for varying times, washed and then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1. All samples were washed three times in 0.1 M cacodylate buffer for 15 minutes each. The cells were then post-fixed for 20 minutes in 1% osmium tetroxide at 4°C followed by addition of EtOH (60%). Samples were dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each), then into propylene oxide (two time, 15 min each), and into a 1:1 propylene oxide/Eponate, left overnight, capped, at room temperature. The propylene oxide/Eponate mixture was decanted off and replaced with 100% Eponate mixture. The samples were polymerized at 70°C for 48 h. Thin sections (~80 nm) were cut using a diamond knife, mounted on un-coated 300 mesh copper grids and stained with 5% uranyl acetate for 20 min. Photographs were taken with a transmission electron microscope (JEOL, JEM 2100 LaB6) equipped with a Gatan Ultra Scan 1000 CCD camera.

Competitive binding assays

COS-1 cells were grown in 24-well tissue culture plate to confluence and then treated with 0.5 µg/ml of cytochalasin D for 30 min prior to addition of antibiotics. OmpA^− E. coli were incubated with anti-S-fimbria antibody for 1 h, on ice, washed, and then added to the COS-1 monolayers at an MOI of 100 for 1 h. OmpA^− E. coli alone infected monolayers served as controls in these experiments. The monolayers were then washed to remove unbound bacteria and incubated with OmpA^+ E. coli at an MOI of 10 and 100 for 10 min, washed the monolayers, and then dissolved in 150 µl of PBS containing 0.3% Triton X-100. Serial dilutions were made and plated on agar containing tetracycline (12.5 µg/ml) in which only OmpA^+ E. coli grow. The number of CFU was counted and determined the percent displacement by OmpA^+ E. coli. In some experiments, FITC-IgG2a (1 µg) was incubated with cytochalsin-D treated peritoneal MO while rotating the test tube at a low speed for 30 min and washed to remove unbound IgG. Various inocula of OmpA^+ E. coli or OmpA^− E. coli were added to the cells and incubated for 10 min, washed and the bound FITC-IgG was determined by flow cytometry.

Differential staining of E. coli K1

RAW 264.7 cells were grown in eight-well chamber slides and infected with E. coli K1 as described above. The monolayers were
Figure 8. Adoptive transfer of FcγRla<sup>−/−</sup> MØ into FcγRla<sup>−/−</sup> mice restored the susceptibility to E. coli K1 meningitis. FcγRla<sup>−/−</sup> mice were reconstituted with FcγRla<sup>−/−</sup> MØ by intraperitoneal injection as described in Materials and Methods. Blood was withdrawn at various time points and bacteremia levels enumerated by plating the serial dilutions on agar containing antibiotics (A). Cerebrospinal fluid obtained from the same animals as described in A were directly inoculated into LB broth containing antibiotics. Positive broth cultures were considered positive for the occurrence of meningitis (B). In addition, blood brain barrier leakage (C) and brain bacterial load (D) were determined as described in Materials and Methods. Increase in these parameters in FcγRla<sup>−/−</sup> MØ reconstituted mice was statistically significant compared with FcγRla<sup>−/−</sup> MØ reconstituted animals, *p<0.001 by Student’s t test. Results are representative of four independent experiments with 12 animals per group. Data represent mean ± SE.

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then washed with PBS and fixed in 2% paraformaldehyde for 10 min at room temperature. Subsequently, anti-S-fimbria antibody (1:1000 dilution) was added to the cells and incubated for 1 h at room temperature. The cells were then washed with PBS and incubated with secondary antibodies conjugated to FITC for 30 min at room temperature. The monolayers were washed four times with PBS and incubated with excess amounts of secondary antibody coupled to horseradish peroxidase for 1 h at RT to block the external primary antibody sites. After thorough washing of the cells, the monolayers were permeabilized with 5% normal goat serum in phosphate-buffered saline containing 1% Triton X-100 (NGS/PBST) for 30 min. The cells were again incubated with anti-S-fimbria antibody for 1 h in Triton/NGS/PBST buffer, washed and further incubated with secondary antibody coupled to Cy3 for 30 min. The cells were washed again, the chambers removed, and the slides mounted in Vectashield (Vector Laboratories) anti-fade solution containing 4’, 6-diamidino-2-phenylindole. Cells were viewed using a Leica (Wetzlar, Germany) DMRA microscope with Plan-apochromat ×40/1.25 NA and ×63/1.40 NA oil immersion objective lenses. Image acquisition was with a SkyVision-2/VDS digital CCD (12-bit, 1280×1024 pixel) camera in unbinned or 2×2-binned models into EasyFISH software, saved as 16-bit monochrome, and merged as 24-bit RGB TIFF images (Applied Spectral Imaging Inc., Carlsbad, CA). The images were assembled and labeled using Adobe Photoshop 7.0.

**Determination of the BBB leakage**

BBB permeability was quantitatively evaluated by detection of extravasated Evans blue dye [28]. Briefly, 2% Evans blue dye in saline was injected intraperitoneally into infected or uninfected mice and after 4 h, mice were deeply anesthetized with Nembutal and transcardially perfused with PBS until colorless perfusion fluid was obtained from the right atrium. Brains from infected animals were harvested, weighed and homogenized. Tissue supernatant was obtained by centrifugation and protein concentration was determined. Evans blue intensity was determined on a microplate reader at 550 nm. Calculations were based on external standards dissolved in the same solvent. The amount of extravasated Evans blue dye was quantified as micrograms per milligram protein.

**Isolation of peritoneal MØ and adoptive transfer of MØ**

Peritoneal MØ were isolated from mice according to the method of Mittal et al [28,69,70]. Briefly, the mouse peritoneal cavity was exposed carefully without disrupting blood vessels and 2–3 ml of RPMI was slowly injected. The lavage was collected and cultured in tissue culture flasks for 2 h at 37°C under 5% CO₂ to allow adherence of MØ. Non-adherent cells were removed and the flasks washed three times with Hanks’ solution. The adherent cells were harvested from the flasks using a rubber policeman and were resuspended in 10% FCS-RPMI 1640 medium. MO were then positively selected using Miltenyi Biotec kit and purity examined by FACS analysis using F4/80 antibody, which then positively selected using Miltenyi biotech kit and percentage were resuspended in 10% FCS-RPMI 1640 medium. MØ were cultured in tissue culture flasks for 2 h at 37°C. Half of the brain was fixed in 10% buffered formalin, routinely processed and embedded in paraffin. 4–5 μm sections were cut using a Leica microtome and stained with hematoxylin and eosin (H & E). Pictures were taken using a Zeiss Axiosview Microscope connected to a JVC 3-chip color video camera and read by the pathologist in a blinded fashion.

**Histopathology**

Half of the brain was fixed in 10% buffered formalin, routinely processed and embedded in paraffin. 4–5 μm sections were cut using a Leica microtome and stained with hematoxylin and eosin (H & E). Pictures were taken using a Zeiss Axiosview Microscope connected to a JVC 3-chip color video camera and read by the pathologist in a blinded fashion.

**Cytokine assays**

Cytokine (TNF-α, IL-1β, IL-6, IL-12 p70 and IL-10) levels in sera from various animals were determined using Biosource ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Statistical analysis**

For statistical analysis of the data, two tailed Fischer test, Wilcoxon signed rank test and Student’s t-test were applied and p value <0.05 was considered statistically significant.

**Supporting Information**

**Figure S1** Analysis of various cell types in MO-depleted mice. MO were depleted in newborn mice by the administration of carrageenan as described in Materials and Methods. Spleens and livers were harvested, homogenized, and the cells in the homogenates were subjected to flow cytometry for analysis of neutrophils (A), dendritic cells (DCs) (B), CD4+ T cells (C), CD8+ T cells (D) and B cells (E).

**Figure S2** Cytokine production in MO-depleted mice infected with *E. coli* K1. WT and MO-depleted newborn mice were infected with 10⁷ CFU of *E. coli* K1 by intranasal instillation, blood samples were collected at various times, and the concentrations of IL-β (A), IL-6 (B), IFN-γ (C) and IL-12 (D) determined by ELISA as described in Materials and Methods. The data represent means ± SD of three independent experiments with five animals in each group. The decrease in the cytokines in MO-depleted animals was statistically significant compared to WT animals, *p<0.001 by Student’s t test.*

**Figure S3** Bacteremia and cytokines levels in WT and *FcrRIa−/−* mice infected with *E. coli* K1. (A) WT and *FcrRIa−/−* mice at day 3 were infected with *E. coli* K1, blood samples collected at various times, dilutions made and plated on blood agar containing antibiotics. The levels of IL-1β (B), IL-6 (C), IL-12 (D) in the blood were quantified. Bacteremia and cytokines levels were determined using *FcrRIa−/−* mice infected with *E. coli* K1. (A) WT and *FcrRIa−/−* mice at day 3 were infected with *E. coli* K1, blood samples collected at various times, dilutions made and plated on blood agar containing antibiotics. The levels of IL-1β (B), IL-6 (C), IL-12 (D) in the blood were quantified.
samples were determined by ELISA. The data represent means ± SD of three separate experiments performed in triplicate with fifteen animals in each group. 

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Figure S4  Bacterial load and the occurrence of meningitis in newborn mice infected with Group B streptococcus. (A) WT and FcγR1a−/− mice at day 3 after birth were infected with 10^9 CFU of GBS intranasally. Blood was collected at 24, 48 and 72 h post-infection, dilutions were made, and plated on agar. (B) CSF samples were collected aseptically by cisternal puncture and inoculated directly into LB broth, and positive CSF cultures were considered positive for the occurrence of meningitis. (C) At 72 h infection, dilutions were made, and plated on agar. CSF cultures were considered positive for meningitis. The data represent mean ± SD of three separate experiments with four animals each group.

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References

1. Hornf MF, Wick MJ, Rhen M, Normark S (2002) Bacterial strategies for overcoming host innate and adaptive immune responses. Nat Immunol 3: 1033–1040.
2. Sansonetti PJ (2000) Phagocytes, a cell biology view. J Cell Sci 113: 3355–3356.
3. Underhill DM, Ozinsky A (2002) Phagocytosis of microbes: complexity in action. Annu Rev Immunol 20: 325–352.
4. Keese U, Piskounov NV (1999) Models of experimental bacterial meningitis. Role and limitations. Infect Dis Clin North Am 13: 549–577.
5. Leib SL, Tauber MG (1999) Pathogenesis of bacterial meningitis. Infect Dis Clin North Am 13: 527–548.
6. Pong A, Bradley JS (1999) Bacterial meningitis and the newborn infant. Infect Dis Clin North Am 13: 711-733.
7. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, et al. (2002) Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 347: 240-247.
8. Dubois D, Prasadarao NV, Mittal R, Brev, L, Roujou-Gris M, et al. (2009) CTX-M beta-lactamase production and virulence of Escherichia coli K1. Emerg Infect Dis 15: 1880-1890.
9. Prasadarao NV, Blom AM, Villoutreix BO, Linsangan LC (2002) A novel interaction of outer membrane protein A with C6b binding protein mediates serum resistance of Escherichia coli K1. J Infect Med 169: 6352-6360.
10. Wooster DG, Maruvada R, Blom AM, Prasadarao NV (2006) Logarithmic phase Escherichia coli K1 efficiently avoids serum killing by promoting C3b-mediated C9b and C6b degradation. Immunology 117: 482-493.
11. Maruvada R, Blom AM, Prasadarao NV (2002) Effects of complement regulators bound to Escherichia coli K1 and Group B Streptococci on the interaction with host cells. Immunology 124: 265-276.
12. Dale DC, Bower L, Liles WC (2000) The phagocytes: neutrophils and monocytes. Blood 110: 935-945.
13. Mukhopadhyay S, Pluddemann A, Gordon S (2009) Macrophage pattern recognition receptors in immunity, homeostasis and self tolerance. Adv Exp Med Biol 653: 1-14.
14. Sukumar SK, Shinada H, Prasadarao NV (2003) Entry and intracellular replication of Escherichia coli K1 in macrophages require expression of outer membrane protein A. Infect Immun 71: 5951–5961.
15. Barth E, Fischer G, Schreiber EM, Vollmeyer J, Georjoff M, et al. (2001) Differences in the expression of CD94 and nCD14 on polymononuclear cells and on monocytes in patients with septic shock. Cytokine 14: 299-302.
16. Brandtzaeg P, van Deuren M (2002) Current concepts in the role of the host innate immunity. J Immunol 178: 4917–4925.
17. Hoffmann JJ (2009) Neutrophil CD64: a diagnostic marker for infection and inflammation. Clin Chem Lab Med 47: 301-307.
18. Noel GJ, Katz SL, Edelson PJ (1994) The role of C3 in mediating binding and ingestion of group B Streptococcus serotype III by murine macrophages. Pediatr Res 30: 114-119.
19. Prasadarao NV, Wiss CA, Weiser JN, Sats M, Huang SH, et al. (1996) Outer membrane protein A of Escherichia coli contributes to invasion of brain microvascular endothelial cells. Infect Immun 64: 146-153.
20. Drevets DA, Leenen PJ, Campbell PA (1993) Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of Listeria monocytogenes by phagocytic macrophages. J Immunol 151: 5431-5439.
21. Gafa V, Manches O, Pastor A, Drouet E, Ambroise-Thomas P, et al. (2005) Human cytomegalovirus downregulates complement receptors (CR3, CR4) and decreases phagocytosis by macrophages. J Med Virol 76: 361–366.
22. Dreven DS, Leenen PJ, Campbell PA (1996) Complement receptor type 3 mediates phagocytosis and killing of Listeria monocytogenes by TNF-alpha and IFN-gamma-stimulated macrophage precursor hybrid. Cell Immunol 169: 1–6.
23. Mosser D (1994) Receptors on phagocytic cells involved in microbial recognition. Immunol Rev 150: 99–114.
24. Watanabe I, Ishiki M, Shiratsuchi A, Nakanishi Y (2007) TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity. J Immunol 178: 4917–4925.
25. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, et al. (2005) Macrophage receptors and immune recognition. Immunol Rev 199: 901–944.
26. Weiss DJ, Souza CD, Evanon OA, Sanders M, Rutherford M (2008) Bovine monocyte TLR2 receptors differentially regulate the intracellular fate of Mycobacterium avium subsp. paratuberculosis and Mycobacterium avium subsp. avium. J Leukoc Biol 83: 48–55.
27. Higgins SC, Jarnicki AG, Lavelle EC, Mills KH (2006) TLR4 mediates vaccine-induced protective cellular immunity to Bordetella pertussis role of IL-17-producing T cells. J Immunol 177: 7800–7809.
28. Daciev L, Gorvel JP (2010) Bacterial manipulation of innate immunity to promote infection. Nat Rev Microbiol 8: 117–129.
29. Adey D, Underhill DM (1995) Structure/function relationships of Fc gamma receptors in phagocytosis. Semin Immunol 7: 45-54.
30. Beekman JM, Bakken JE, van de Winkel JG, Leusen JH (2004) Direct interaction between FcgammaRI (CD64) and periplakin controls receptor endocytosis and ligand binding capacity. Proc Natl Acad Sci U S A 101: 10392–10397.
31. Drevets DA, Leenen PJ, Campbell PA (1993) Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of Listeria monocytogenes by phagocytic macrophages. J Immunol 151: 5431-5439.
46. Lam GY, Brunell JH (2008) Cell biology: A \textit{Listeria} escape trick. Nature 455: 1186–1187.

47. Sjodahl J (1977) Structural studies on the four repetitive Fc-binding regions in protein A from \textit{Staphylococcus aureus}. Eur J Biochem 78: 471–490.

48. Solca A, Medesan C, Laky M, Onica D, Sjoquist J, et al. (1987) Effect of protein A of \textit{Staphylococcus aureus} on the binding of monomeric and polymeric IgG to Fc receptor-bearing cells. Immunology 58: 173–179.

49. Wang Y (2002) The function of OmpA in \textit{Escherichia coli}. Biochem Biophys Res Commun 292: 396–401.

50. Smith SG, Mahon V, Lambert MA, Fagan RP (2007) A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol Lett 275: 1–11.

51. Celli J, Finlay BB (2002) Bacterial avoidance of phagocytosis. Trends Microbiol 10: 232–237.

52. Ernst JD (2000) Bacterial inhibition of phagocytosis. Cell Microbiol 2: 379–386.

53. Rosenberger CM, Finlay BB (2003) Phagocyte sabotage: disruption of Macrophage receptors and immune recognition. Annu Rev Immunol 21: 901–944.

54. Kedzierska K, Ellery P, Mak J, Lewin SR, Crowe SM, et al. (2002) HIV-1 down-modulates gamma signaling chain of FC gamma R on human macrophages: a possible mechanism for inhibition of phagocytosis. J Immunol 168: 2905–2903.

55. Mittal R, Sharma S, Chhibber S, Harjai K (2008) Contribution of free radicals in macrophage secretory products to urovirulence of \textit{Pseudomonas aeruginosa}. Comp Immunol Microbiol Infect Dis 29: 12–26.

56. Maruvada R, Prasadaro NV, Rubens CE (2009) Acquisition of factor H by a novel surface protein on group B \textit{Streptococcus} promotes complement degradation. Faseb J 23: 3967–3977.

57. Mittal R, Prasadaro NV (2002) Identification of \textit{Escherichia coli} outer membrane protein A receptor on human brain microvascular endothelial cells. Infect Immun 70: 4535–4563.

58. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K (2009) Contribution of macrophage secretory products to urovirulence of \textit{Pseudomonas aeruginosa}. FEMS Immunol Med Microbiol 57: 156–164.

59. Mittal R, Sharma S, Chhibber S, Harjai K (2006) Effect of macrophage secretory products on elaboration of virulence factors by planktonic and biofilm cells of \textit{Pseudomonas aeruginosa}. Comp Immunol Microbiol Infect Dis 29: 12–26.

60. Mittal R, Prasadaro NV (2010) Nitric oxide/cGMP signaling induces \textit{Escherichia coli} K1 receptor expression and modulates the permeability in human brain endothelial cell monolayers during invasion. Cell Microbiol 12: 67–83.