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gp96 expression in neutrophils is critical for the onset of *Escherichia coli* K1 (RS218) meningitis

Rahul Mittal¹ and Nemani V. Prasadarao^{1,2,3,*}

¹Division of Infectious Diseases, Department of Pediatrics, The Saban Research Institute, Children's Hospital Los Angeles, CA 90027.

²Department of Surgery, The Saban Research Institute, Children's Hospital Los Angeles, CA 90027.

³Keck School of Medicine, University of Southern California, Los Angeles, CA 90027.

Abstract

Despite the fundamental function of neutrophils (PMNs) in innate immunity, their role in *Escherichia coli* K1 (EC-K1) induced meningitis is unexplored. Here we show that PMN-depleted mice are resistant to EC-K1 (RS218) meningitis. EC-K1 survives and multiplies in PMNs for which outer membrane protein A (OmpA) expression is essential. EC-K1 infection of PMNs increases the cell surface expression of gp96, which acts as a receptor for bacterial entry. Suppression of gp96 expression in newborn mice prevents the onset of EC-K1 meningitis. Infection of PMNs with EC-K1 suppresses oxidative burst by down regulating *rac1*, *rac2* and *gp91^{phox}* transcription both *in vitro* and *in vivo*. The interaction of loop 2 of OmpA with gp96 is essential for EC-K1-mediated inhibition of oxidative burst. These results reveal that EC-K1 exploits surface expressed gp96 in PMNs to prevent oxidative burst for the onset of neonatal meningitis.

Bacterial meningitis remains a serious disease resulting in high morbidity and mortality, despite the use of potent antibiotics¹⁻³. This unfavorable outcome is due to our incomplete knowledge about the pathogenesis of the disease for developing new modes of prevention. *Escherichia coli* K1 (EC-K1) is one of the most common pathogens causing neonatal meningitis⁴. The disease is fatal in 5-40% of infected neonates and causes neurological sequelae in up to 30% of survivors⁵. Studies in experimental models of hematogenous meningitis have shown that a certain threshold of bacteremia is necessary for the development of meningitis^{6,7}. Thus, EC-K1 must avoid host defense mechanisms for proliferation in blood and/or tissues to reach a high level of bacteremia. Intracellular

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*Corresponding author: Nemani V. Prasadarao, Division of Infectious Diseases, MS #51, Children's Hospital Los Angeles and Keck School of Medicine, University of Southern California, 4650 Sunset Blvd., Los Angeles, CA 90027, Tel: 323-361-5465, Fax: 323-361-2867, pnenani@chla.usc.edu.

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survival of EC-K1, therefore, represents a fundamental pathogenicity mechanism during the onset of meningitis. Several virulence factors have been implicated in the ability of EC-K1 (RS218) to cause meningitis, which include S-fimbria, IbeA, IbeB, cytotoxic necrotizing factor (CNF), TraJ and outer membrane protein A (OmpA)^{8–13}. OmpA is one of the major outer membrane proteins of EC-K1 and plays crucial roles in maintaining the integrity of outer membrane and in bacterial conjugation^{14–16}. However, our studies demonstrate that OmpA is important for binding of EC-K1 to human brain microvascular endothelial cells (HBMEC) and subsequent invasion¹⁰. OmpA expression has also shown to be essential for survival of EC-K1 in immune cells such as macrophages and dendritic cells as well as to resist serum bactericidal activity^{17–19}. We have subsequently shown that mutation of three residues in loops 1 and 2 prevent the interaction of EC-K1 with HBMEC and neutrophils²⁰. Nonetheless, the role of neutrophils and how their interaction with EC-K1 OmpA contributes to the pathogenesis of EC-K1 meningitis is not well explored.

Binding of microorganisms to PMN surface-receptors generates signals that regulate the response of the phagocyte and killing of the microbial intruder^{21–23}. Killing may be achieved through production of reactive oxygen species (ROS) at the expense of cytosolic NADPH^{24,25}. However, pathogenic microbes develop strategies to avoid PMN killing by suppressing ROS generation²⁶. Several studies have indicated that heat shock proteins such as gp96 can chaperone antigenic peptides and promote their delivery to antigen-presenting cells for presentation to T cells²⁷. gp96 is an endoplasmic paralog of heat shock protein 90 and has been shown to play a variety of roles in mammalian organisms^{28–30}. Although gp96 is generally localized to the endoplasmic reticulum, it may gain extracellular access after cell activation, infection or necrotic cell death^{31–33}. Our previous studies have shown that Ecgp96 (a homologue of gp96) acts as a receptor for OmpA of EC-K1 on HBMECs¹⁰. Therefore, we sought to investigate whether the interaction of EC-K1 with PMNs requires gp96. We observed that EC-K1 enters and survives in PMNs using gp96 as a receptor and suppresses the oxidative burst of PMNs for which OmpA expression is required. Interestingly, PMN-depleted or gp96 suppressed mice were resistant to EC-K1 meningitis, indicating that bacteria utilize PMNs as a replication permissive niche during initial stages of infection.

RESULTS

PMN depletion in newborn mice prevents EC-K1 meningitis

To determine whether neutrophils play a crucial role in the pathogenesis of EC-K1 meningitis, newborn mice were depleted of PMNs by injecting mAb RB6-8C5 starting at Day 1 and then infected intranasally with EC-K1 at Day 3. Flow cytometry analysis of spleen cells revealed complete depletion of PMNs in RB6-8C5 injected animals compared to WT or control antibody (C-Ab) injected mice (Figure 1a). Unexpectedly, PMN-depleted mice survived without any visual signs of distress despite infection with EC-K1 beyond 7days whereas WT and C-Ab treated mice succumbed to infection by 96h post-infection (Figure 1b). PMN-depleted mice showed enhanced survival even at higher doses of EC-K1 (Supplementary Figure S1a and b). A small number of bacteria that entered were cleared from circulation to undetectable levels by 48h post-infection in PMN-depleted mice,

whereas WT and C-Ab treated animals showed high degree of bacteremia (Figure 1c). CSF cultures were negative in PMN-depleted animals, indicating the absence of meningitis (Figure 1d). The levels of proinflammatory cytokines, TNF- α , IL-1 β , IL-6 and IL-12 were significantly higher in WT and C-Ab treated infected mice compared to the levels in PMN-depleted mice (Supplementary Figure S1c–f). Neither blood-brain barrier leakage nor bacterial load in the brains was observed in PMN-depleted mice (Figure 1e, f). Histopathological examination of brain sections exhibited severe gliosis and increased cellularity in the molecular layer of the cortex in WT and C-Ab treated mice (Figure 1g). Marked apoptosis indicated by pyknotic nuclei (inset) and infiltration of neutrophils was also observed in dentate gyrus in the hippocampus, while leptomeninges showed severe inflammation. In contrast, the brains from PMN-depleted mice showed no such pathological conditions.

Our observation that EC-K1 manipulates the function of macrophages for survival and cause meningitis³⁴ led us to hypothesize that PMNs provide initial safe havens for EC-K1 and subsequently hijack macrophages for their survival due to short life span of PMNs. To test this, we depleted PMNs in mice infected with EC-K1 24 h post-infection. Interestingly, we observed that depleting PMNs after infection (PI-PMN-depleted) failed to prevent the onset of meningitis by EC-K1 (Figure 2a). No statistical difference in blood bacterial load of PI-PMN-depleted, C-Ab treated or WT mice was observed and 90% of mice in all groups had meningitis with high bacterial titers in the brains (Figure 2b–d). To further confirm these findings, PMNs and macrophages were isolated from WT infected mice at 6 and 24h post-infection and intracellular bacteria enumerated. PMNs isolated at 6h post-infection, not at 24 h post-infection, showed significantly greater number of bacteria inside the cells (Figure 2e). In contrast, no bacteria were recovered from macrophages at 6 h but only at 24 h post-infection. These results suggest that PMNs provide initial niche for EC-K1 and due to short life span of PMNs, the bacteria subsequently take refuge in macrophages to survive and multiply.

EC-K1 suppresses oxidative burst in PMNs for survival

To examine whether EC-K1 survives inside PMNs, OmpA⁺ *E. coli* (E44) and OmpA⁻ *E. coli* (E91) were incubated with PMNs at an MOI of 10 for varying times. Although both bacteria entered PMNs efficiently, only OmpA⁺ *E. coli* survived in PMNs whereas OmpA⁻ *E. coli* were killed within 2 h post-infection (Figure 3a, b). To confirm whether OmpA expression is critical for survival in PMNs, OmpA⁻ *E. coli* (E91) transfected with OmpA plasmid (pOmpA⁺) or null plasmid (pOmpA⁻) were used for phagocytosis assays. pOmpA⁺ *E. coli* survived in PMNs whereas pOmpA⁻ *E. coli* were killed, indicating that OmpA expression is crucial for the survival (Figure 3a, b). Furthermore, EC-K1 mutants that lack S-fimbria (*sfa*), IbeA (*ibeA*), type-1 fimbria (*fimH*) or CNF (*cnf*) were used to examine the role of these virulence factors for survival in PMNs. All the mutants expressing OmpA were able to enter, survive and persist in PMNs similar to OmpA⁺ *E. coli* (Figure 3c d). These findings confirm that other virulence factors, except OmpA, contribute insignificantly for the survival of EC-K1 in PMNs. To examine whether the survival of EC-K1 in PMNs was due to the suppression of ROS production, we monitored the ability of primary human PMNs to generate ROS following infection with EC-K1. Unstimulated

PMNs did not produce detectable ROS, whereas PMNs treated with phorbol myristate acetate (PMA) potently generated ROS within 10 min (Figure 3e). OmpA⁺ *E. coli* failed to induce ROS production while a strong oxidative burst was documented in OmpA⁻ *E. coli* infected PMNs. ROS production in response to OmpA⁻ *E. coli* was first detected after 5min and reached a maximum between 40–45 min. Similar increase in oxidative burst was also observed with pOmpA⁻ *E. coli* but not when infected with pOmpA⁺ *E. coli*, *E. coli* *ibeA*, *E. coli* *cnf*, *E. coli* *sfa*, or *E. coli* *fimH* strains (Figure 3f). These data suggest that OmpA expression in EC-K1 and its interaction with PMNs is critical to prevent the oxidative burst and for survival in the cells.

Role of OmpA and effect of opsonization on ROS production

To confirm the role of OmpA in preventing the production of ROS by PMNs, bacteria were pretreated with antibodies to OmpA before infecting PMNs. Anti-OmpA antibody pretreatment or addition to the medium along with OmpA⁺ *E. coli* (E44) led to a significant enhancement in the oxidative burst in PMNs, indicating that interaction of OmpA with PMNs is required for EC-K1-mediated suppressive effect on the oxidative burst capacity (Figure 4a). Additionally, the effect of opsonization on oxidative burst was examined by pretreating the bacteria with pooled human serum (PHS) or heat inactivated PHS and then adding to PMNs. There was no increase in the ROS generation in PMNs infected with opsonized bacteria, which was similar to the untreated bacteria (Figure 4b). This indicates that serum components may not play a significant role in the interaction of OmpA⁺ *E. coli* with PMNs. Next, to determine whether viable EC-K1 interaction with PMNs is necessary for ROS generation, PMNs were infected with heat-killed (HK) OmpA⁺ *E. coli*. Interestingly, HK-bacteria triggered strong ROS generation in PMNs that persisted for 60min (Figure 4c). However, when a mixture of viable and HK-bacteria in the ratio of 1:10 or 1:100 were added to PMNs, the PMN oxidative burst was completely prevented (Figure 4d). We also observed that direct contact between PMN and bacteria is required because PMNs separated from OmpA⁺ *E. coli* by 0.4 μ m-pore transwells showed strong oxidative burst (Supplementary Figure S2a). The reason for this oxidative burst may be due to the contribution of factors released by OmpA⁺ *E. coli*, which passed through transwells and enhanced PMN ROS production. The bacterial culture supernatant from live OmpA⁺ *E. coli* or bacterial lysates was found to be a potent stimulator of oxidative burst in PMNs (Supplementary Figure S2b). OmpA⁺ *E. coli* could suppress the ROS generation elicited by PMA or LPS prior to or after treatment of PMNs (Figure 4e). OmpA⁺ *E. coli* pre-treated with chloramphenicol (Cm) elicited a strong oxidative burst in PMNs compared with untreated bacteria (Figure 4f). However, a delay in adding Cm by 6 h, which allowed protein synthesis led to suppression of oxidative burst in OmpA⁺ *E. coli* infected PMNs. An increase in the ROS production was seen when tetracycline (tet), a cell-permeable antibiotic, was added to PMNs, thus preventing the multiplication of internal bacteria (Figure 4f). These results suggest that live OmpA⁺ *E. coli* that is capable of de novo protein synthesis actively inhibits the PMN respiratory burst.

OmpA⁺ *E. coli* reduces *rac1*, *rac2* and *gp91^{phox}* transcription

To assess whether EC-K1 had any effect on the expression of *rac1*, *rac2* and *gp91^{phox}* genes, encoding the multi-component NADPH oxidase complex responsible for oxidative

burst in PMNs, RT-PCR was performed. A low level of *rac1* transcript was detected in uninfected PMNs, which level was further dropped in OmpA⁺ *E. coli* infected cells, whereas OmpA⁻ *E. coli* infection or PMA treatment enhanced the transcription levels (Figure 5a). OmpA⁻ *E. coli* and PMA stimulated PMNs exhibited a significant increase in *rac2* and *gp91^{phox}* mRNA levels, whereas only low levels of transcripts was observed in OmpA⁺ *E. coli* infected cells. Quantitative analysis of PCR products normalized to *gapdh* levels revealed 70, 90 and 50% decrease in the expression of *rac1*, *rac2* and *gp91^{phox}*, respectively in OmpA⁺ *E. coli* infected PMNs when compared to untreated cells (Figure 5b). In contrast, PMNs incubated with OmpA⁻ *E. coli* or PMA showed two-fold increase in the transcription of these genes compared to unstimulated PMNs. Downregulation of Rac1, Rac2, and *gp91^{phox}* at the protein levels was also demonstrated upon OmpA⁺ *E. coli* infection, and upregulation of these molecules in OmpA⁻ *E. coli* infected or PMA stimulated PMNs (Figure 5c, d). Similarly, PMNs obtained from the spleens of *E. coli* infected mice showed decreased levels of *rac1*, *rac2* and *gp91^{phox}* as compared to PMNs from uninfected mice (Figure 4e, f and Supplementary Figure S2c, d). Overall these data suggest that EC-K1 inhibits oxidative burst in PMNs both *in vitro* and *in vivo* by downregulating the expression of Rac1, Rac2 and *gp91^{phox}*.

gp96 expression in PMNs is vital for EC-K1 entry

We have demonstrated that Ecgp96 (HSP90 β form) is a receptor for OmpA of EC-K1 in HBMEC¹⁰. Therefore, we theorize that gp96 on the surface of PMNs may be playing a role in the infection. To test this, PMNs infected with EC-K1 were examined for the expression of gp96. OmpA⁺ *E. coli* (E44) increased the expression of gp96 on PMNs upon infection by seven-fold in comparison to uninfected cells (Figure 6a). In contrast, the expression of gp96 in PMNs infected with OmpA⁻ *E. coli* (E91) was similar to that of uninfected PMNs. Next, we determined whether gp96 plays a role in the entry of EC-K1 in PMNs by suppressing the expression using siRNA. Although a majority of gp96 is confined to the cytosol, under certain conditions, some portions of gp96 translocate to plasma membrane³¹. Therefore, both extracellular and intracellular expression of gp96 was examined after the introduction of siRNAs. The expression of gp96 was considerably reduced in all siRNA transfected PMNs while control-siRNA transfected cells showed no decrease in the expression (Figure 6b, c). RT-PCR also revealed a drastic reduction in the transcript levels of gp96 in gp96-siRNA-1 transfected PMNs upon infection with OmpA⁺ *E. coli* (Figure 6d). Of note, the entry of EC-K1 was inhibited by 80% in gp96-siRNA-1 transfected PMNs (Figure 6e). Nonetheless, OmpA⁺ *E. coli* that entered in low numbers were killed completely by 6 h post-infection in gp96-siRNA-1 transfected PMNs (Figure 6f). Since gp96 siRNA-1 significantly reduced the expression of gp96 in PMNs, this siRNA was used for subsequent experiments. No oxidative burst was observed in gp96⁻ PMNs infected with EC-K1 due to the lack of entry of a sufficient number of bacteria to mount ROS generation (Figure 5g). Since knocking down gp96 can have an effect on other receptors, we blocked gp96 interaction with OmpA of EC-K1 by anti-gp96 antibodies. The entry, survival and the production of ROS were significantly reduced when PMNs pretreated with anti-gp96 antibody (Supplementary Figure S3a–c). These results suggest that EC-K1 utilizes gp96 on PMNs to enter and subsequently suppress the generation of oxidative burst.

Suppression of gp96 expression *in vivo* prevents meningitis

If survival of EC-K1 inside PMNs represents a significant contribution to the pathogenesis of meningitis, then limiting access of the organism to this intracellular site should prevent the onset of meningitis. To test this, we suppressed the expression of gp96 in mice using gp96-siRNA-1 and InvivoFectamine reagent^{33,35}. No stimulation of immune response was detected with the administration of siRNA and InvivoFectamine reagent as the cytokine levels (TNF- α , IL-1 β , IL-6 and IL-12) were similar between treated and untreated mice (Supplementary Figure S4a–d). Both extracellular and intracellular gp96 expression in PMNs obtained from spleen, peritoneum and liver was significantly suppressed in transfected mice, whereas control siRNA had no effect (Figure 7a and Supplementary Figure S5a). RT-PCR confirmed knockdown of gp96 in PMNs in mice even after infection with EC-K1 (Figure S4b). gp96 KD mice showed better survival compared to WT or control siRNA treated mice, which succumbed to infection by 96 h post-infection (Figure 7b). No bacteremia or blood-brain barrier leakage was observed in gp96 KD mice, whereas blood bacterial numbers or Evans blue leakage increased with post-infection time in WT mice (Figure 7c, d). CSF cultures were positive in 90 and 100% of WT and control siRNA treated animals, respectively whereas all the CSF cultures were sterile in gp96 KD mice (Figure 7e). No bacteria were recovered from the brains of gp96 KD mice and histopathological examination of brain tissues revealed no brain damage (Figure 6f and Supplementary Figure S5c). Since lack of PMNs prevents the onset of meningitis in newborn mice, these data suggest that gp96 expression in PMNs may be critical for the onset of meningitis by EC-K1.

Loop 2 of OmpA interacts with gp96 in PMNs

OmpA is a 325 amino acid protein with eight transmembrane domains and four extracellular loops¹⁵. Therefore, next to determine which loops of OmpA interact with gp96 in PMNs, three to four amino acids of OmpA extracellular loops were mutated to alanines as shown in Figure 8a²⁰. For convenient presentation of data, the loop mutants were divided into four groups. Mutations in loop 2 could not prevent the oxidative burst as compared to OmpA⁺ *E. coli* (E44) or other loop mutants (Figure 8b). To examine whether the interaction of gp96 with loop 2 domains in PMNs is necessary for EC-K1 entry, the expression of gp96 was suppressed using siRNA and then the cells infected with EC-K1. Loop 2 mutants (L2Ms) unexpectedly entered gp96⁻ PMNs with a four-fold more frequency than other bacteria (Figure 8c). However, L2Ms were killed in gp96⁻ PMNs in a short time; whereas other loop mutants although entered in low numbers were killed by 4h post-infection (Figure 8d). Since our previous studies revealed that CR3 expression in PMNs plays a crucial role in killing of EC-K1, we speculated that L2Ms may have entered PMNs via CR3 and subsequently could not survive due to the oxidative burst³⁵. Therefore, we examined the expression of CR3 on PMNs upon infection with various OmpA loop mutants. L2Ms increased the expression of CR3, while other mutants showed CR3 expression similar to that of OmpA⁺ *E. coli* (Figure 8e). To confirm the role of CR3 in the entry of L2Ms, we suppressed the expression of CR3 in PMNs by siRNA, which reduced CR3 expression significantly (Supplementary Figure S6a). Interestingly, L2Ms were unable to enter and survive in CR3 knockdown PMNs whereas other mutants entered and survived (Figure 8f, g). No oxidative burst was observed in CR3 knocked down PMNs and infected with L2Ms (Figure 8h). Additionally, blocking of

CR3 interaction with L2M by a CR3 blocking peptide XVA143 prevented the entry of L2Ms into PMNs (Supplementary Figure S6b–h), indicating that loop 2 regions dictate the interaction with either gp96 or CR3 depending on the peptide sequence. To confirm the role of CR3 and gp96, PMNs were transfected with gp96 and CR3 siRNA and then used for EC-K1 phagocytosis assays. None of the EC-K1 strains was able to enter and survive inside PMNs (Supplementary Figure S7a, b). There was no oxidative burst observed in PMNs and could be due to the interaction of a low number of bacteria with the cells (Supplementary Figure S7c). These results indicate that loop 2 of OmpA in EC-K1 interacts with gp96 on PMNs to enter, survive and prevent oxidative burst. Lack of this interaction enhances the expression of CR3 by which EC-K1 enters, however could not survive in PMNs.

DISCUSSION

Pathogenic bacteria have evolved efficient strategies to overcome the weaponry of neutrophils to win the combat^{36–38}. An interesting observation in this study is that depletion of PMNs in newborn mice renders them resistant to EC-K1 infection. This indicates that interaction between PMN and EC-K1 interaction is a critical step in the pathogenesis of neonatal meningitis. Since the bacterial numbers did not increase in PMN-depleted mice, PMNs may act as prime safe havens for the multiplication during initial stages of infection necessary to reach high-grade bacteremia. In addition, PMNs containing EC-K1 may disseminate bacteria to distal sites or to present to macrophages for a second round of multiplication. Concurrently, depletion of PMNs 24 h post-infection in mice could not protect them from the onset of meningitis. Another compelling observation is that OmpA⁺ *E. coli* significantly upregulates the expression of gp96 in PMNs, which in turn acts as a receptor for OmpA for the entry of EC-K1 into the cells. Of note, silencing the expression of gp96 by siRNA in PMNs prevented the entry of EC-K1. Confirming the role of gp96 in EC-K1 induced pathogenesis, gp96 knockdown mice were resistant to EC-K1 meningitis, which is due to lack of high-grade bacteremia. The present study provides both *in vitro* and *in vivo* evidence that EC-K1 can exploit the inflammatory response of the host to facilitate its own survival by gaining access to a locale within PMN using gp96 where it is shielded from being killed.

Upon contact with pathogens, PMNs undergo oxidative burst leading to the production of a large array of ROS³⁹. NADPH oxidase system plays a major role in the production of ROS^{40–45}. Since Rac1, Rac2 and gp91^{phox} play an essential role in the generation of oxidative burst in neutrophils, we examined the levels of these molecules in PMNs infected with EC-K1. Interestingly, we observed significant downregulation in the expression of Rac1, Rac2 and gp91^{phox} at both gene and protein levels. Although a number of pathogens has been demonstrated to modulate PMN oxidative metabolism, EC-K1 appears to utilize a unique mechanism to accomplish the downregulation of oxidative burst in PMNs. *Helicobacter pylori* and *Salmonella enterica* serovar typhimurium have been shown to stimulate the ROS generation in PMNs by redirecting NADPH oxidase activity away from the phagosome distinct than EC-K1, which prevents oxidative burst in PMNs by suppressing the expression of NADPH oxidase components^{46,47}. *Streptococcus pyogenes* has been shown to trigger ROS generation in PMNs, however this pathogen up-regulates oxidative stress response genes to avoid intracellular killing within PMN phagosomes⁴⁸. Other

bacteria such as *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* inhibit phagocytosis by PMNs employing a variety of effector proteins like YopT and YopO²⁶. However, we observed that EC-K1-mediated suppression of oxidative burst in PMNs depends on OmpA as EC-K1 mutants that were defective in the expression of other virulence factors including FimH, CNF, S-fimbrial adhesin, and ibeA failed to trigger the production of ROS. However, OmpA⁻ *E. coli* triggered a strong oxidative burst in PMNs, indicating that OmpA but not other virulence factors play an important role in the ability of EC-K1 to down-regulate oxidative burst in PMNs. Interestingly, EC-K1 also blocked PMA or LPS induced oxidative burst in PMNs unlike *Neisseria gonorrhoea* which do not prevent PMA mediated oxidative burst in PMNs⁴⁹. Besides, this EC-K1-mediated suppressive effect on ROS generation requires live proliferating bacteria and a direct contact between bacteria and PMNs. Bacterial protein synthesis is required for EC-K1 to block the PMN oxidative burst since bacteriostatic concentrations of chloramphenicol prevent this effect induced by the bacterium. Nevertheless bacterial lysates, killed bacteria and bacterial supernatants were able to induce strong ROS production in PMNs. This finding implies that PMNs have the ability to recognize and respond to EC-K1 products; however, live bacteria overcome their stimulatory effects. Therefore, it is possible that OmpA interaction with gp96 on PMNs induces virulence factors in EC-K1 that are responsible for the prevention of oxidative burst.

Our recent studies have shown that loops 1, 2 and 4 are important for interaction of OmpA with gp96 in HBMEC for EC-K1 invasion⁵⁰. We also demonstrate that loop 2 interaction with PMNs is critical for survival of EC-K1 *in vitro* and for the establishment of meningitis²⁰. In support of these studies, the data here demonstrate that loop 2 interaction of OmpA with gp96 controls the survival of the bacterium in PMNs. Lack of loop 2 interaction with gp96 enhanced the expression of CR3, which was subsequently used by the bacteria to enter PMNs. However, entry through CR3 resulted in complete elimination of bacteria from PMNs, indicating that loop 2-gp96 interaction activates signaling mechanisms for survival in PMNs. Similarly, EC-K1 interaction with macrophages suppresses the expression of CR3 during the onset of meningitis³⁵. Of note, in macrophages, OmpA of EC-K1 interacts with Fc-gamma receptor I for entering the cells although both Fc-gamma receptor I and gp96 expression increased upon infection with EC-K1³⁴. In addition, we observed that depletion of PMNs at later stages of infection did not affect the outcome of EC-K1 meningitis. This suggests that EC-K1 finds a niche in PMNs for its multiplication during the initial stages and subsequently manipulates the function of macrophages to reach high-grade bacteremia. Therefore, PMNs and macrophages play an important role at different temporal stages of infection in the pathogenesis of EC-K1 meningitis. Taken together, these results suggest that EC-K1 exploits PMNs by interacting with gp96 via loop 2 of OmpA to suppress the anti-bactericidal activity of the cells. Therefore, blocking this initial interaction would be an attractive strategy for developing therapeutic avenues to preventing this deadly disease in neonates.

METHODS

Bacterial strains

All strains used in this study were derived from a cerebrospinal fluid isolate of EC-K1 strain RS218 (serotype O18:K1:H7). E44 is spontaneous rifampin-resistant mutant of RS 218 strain (designated as OmpA⁺ *E. coli*) and E91 is a derivative of E44 that expresses no OmpA¹⁰. E91 (OmpA⁻ *E. coli*) was transformed with pUC19 containing the entire *ompA* gene and the pUC19 plasmid alone to obtain pOmpA⁺ *E. coli* and pOmpA⁻ *E. coli*, respectively. EC-K1 strains that lack S-fimbriae (*sfa*), type 1 fimbriae (*fimH*), *ibeA* gene (*ibeA*), and cytotoxic necrotizing factor 1 (*cnf*) are described elsewhere^{8-13,18,19}. All bacteria were grown in Luria broth with appropriate antibiotics as necessary. Bacterial lysates were prepared by suspending EC-K1 strains in 0.5% Triton X-100 in PBS and sonicated briefly (30-s pulses) over a period of 3 min on ice. Cellular debris was removed by centrifugation followed by filtration through 0.2- μ m pore size filters. The absence of live bacteria was confirmed by plating the lysates on blood agar plates and incubation overnight at 37°C.

Isolation of human PMNs

Heparinized venous blood was obtained from consented healthy volunteers following a protocol approved by the Children's Hospital Los Angeles Institutional Review Board. Dextran-sedimented PMNs were purified on a Ficoll-Hypaque gradient as previously described²⁵. PMNs were re-suspended at 1×10^7 cells per ml in Dulbecco's PBS (without calcium and magnesium; Mediatech) containing 0.1% dextrose and kept on ice until use. PMN preparations were >95% pure as determined by flow cytometry as well as cytospin preparations and morphologically assessment by phase-contrast microscopy. PMNs were >99% viable as monitored by trypan blue exclusion and Annexin-V-PI staining.

Determination of entry and intracellular bacteria in PMNs

PMNs (1×10^6 cells ml⁻¹) were washed three times in culture medium without antibiotics and varying concentrations of bacteria were added to the tubes. PMNs and bacteria were then incubated for 1 h at 37°C. Gentamicin was added to PMN-bacteria co-culture tubes at a final concentration of 100 μ g ml⁻¹ and incubated for an additional 60 min at 37°C. PMN-bacteria cocultures were washed three times in RPMI 1640 containing no antibiotics and reconstituted with antibiotic-free culture medium. Tubes were then assessed immediately for intracellular bacteria (time period 0) or placed again at 37°C in culture medium containing 100 μ g ml⁻¹ gentamicin. To determine number of bacteria entered inside PMNs, PMN-bacteria cocultures (described above) were washed twice and cells were lysed with 100 μ l of 0.5% Triton X-100. The released intracellular bacteria were enumerated by plating 20 μ l each of PMN-bacteria co-culture supernatant on blood agar plates. Counts in supernatant was subtracted from the initial count to calculate the number of bacteria entered inside PMNs^{18,33,35}. Results are expressed as percentage of bacteria taken up by PMNs at respective sampling time intervals.

Luminol-dependent chemiluminescence (LDCL)

A total of 10^6 PMNs were incubated in Morse's defined medium (MDM)⁵¹ in the presence of 20 μ M luminol (Sigma), at a final volume of 1 ml. For stimulation experiments, PMNs were incubated with live or heat killed EC-K1 at a multiplicity of infection of 10 (cell to bacteria ratio, 1:10). LDCL was measured in an LKB-Wallac 1250 luminometer (Perkin Elmer) every 5 min for 1 h at 37°C, and the counts per second (CPS) of LDCL were averaged per condition between triplicate tubes. Untreated and 10 ng ml⁻¹ PMA-treated PMNs served as negative and positive controls, respectively to determine ROS production. In some experiments, PMNs were pre-exposed to 10 mM diphenylidene idonium hydrochloride (DPI) (Sigma) for 10 min or XVA143 (10 μ M) prior to the addition of bacteria for 1h. XVA143, a β 2 integrin allosteric antagonist (CR3 inhibitor), was generously provided by Dr. N. Fotouhi (Roche)⁵². Experiments were repeated at least five times, using PMNs from a different donor for each triplicate.

RT-PCR

Total RNA from uninfected, *E. coli*-infected or PMA treated human PMNs was isolated using RNA extraction kit (Qiagen) and then reverse transcribed using a RT-PCR kit (Qiagen). The primer sequences used were as follows: Rac1 (forward 5'-AGGAAGAGAAAATGCCTG-3'; reverse 5'-AGCAAAGCGTACAAA GGT-3'); Rac2 (forward 5'-TAGATGGGTCTGATCCTC-3'; reverse 5'-AGAAAAGACCATCAACGA-3'); gp91phox (forward 5'-ATGGGGAACTGGG CTGTGAAT-3'; reverse 5'- CCTC ATGGAAGAGACAAGTTAGAA-3').

Flow Cytometry

The levels of gp96, Rac1, Rac2 and gp91^{phox} in PMNs were determined by flow cytometry. The cells were fixed and permeabilized in cytofix and cytoperm buffer (BD Biosciences,) followed by incubation with IgG blocking buffer to mask nonspecific binding sites. After incubation, cells were incubated with gp96, Rac1 (Abcam), Rac2 or gp91^{phox} (Santa Cruz biotechnology) antibody. The cells were then washed and incubated with secondary antibody conjugated to Alexa Fluor 488 antibody (Invitrogen). For extracellular staining of gp96, cells were fixed in cytofix, washed, incubated with IgG blocking buffer followed by incubation with primary and secondary antibodies. gp96 antibody was generated in our laboratory as described earlier¹⁰. The cells were then analyzed by flow cytometry using FACS Calibur Cell Quest Pro software (BD Biosciences) and at least 10,000 events were gated.

siRNA transfection

PMNs were transfected with CR3 siRNA (oligo identification number HSS179976) or gp96 siRNA [oligo identification numbers HSS110955 (gp96-siRNA-1), HSS110953 (gp96-siRNA-2) and HSS186409 (gp96-siRNA-3), Invitrogen] using Lipofectamine 2000 plus (Invitrogen) according to the manufacturer's instructions. Briefly, 20 pmols of CR3 or gp96 siRNA in 100 μ l of Lipofectamine 2000 plus was added to PMNs (5×10^5) suspended in Opti-MEM® I Reduced Serum Medium (Invitrogen) having no antibiotics and incubated at 37°C for 6h. After incubation the medium was replaced with RPMI 1640 containing L-

Glutamine, sodium pyruvate, nonessential amino acids, 1% penicillin/streptomycin, and 10% fetal calf serum. Cells were harvested 24 h after transfection and CR3 or gp96 knockdown was determined by flow cytometry. The viability of the cells was assessed by trypan blue exclusion and Annexin-V-PI staining which revealed >90% viability of the transfected cells.

Newborn mouse model of meningitis

The animal studies were approved by the Institutional Animal Care and Use Committee (IAUAC) of the Saban Research Institute at CHLA and followed National Institutes of Health guidelines for the performance of animal experiments. Breeding pairs of C57BL/6 wild-type (WT) mice were obtained from Jackson Laboratories. Three-day-old mouse pups were randomly divided into various groups and infected intranasally with different doses of bacteria or pyrogen free saline^{33,35,53}. Blood was collected from the tail or facial vein at different time periods of post-infection, dilutions were made, and plated on rifampicin LB agar plates. CSF samples were collected aseptically by cisternal puncture under anesthesia and directly inoculated into broth containing appropriate antibiotics. Bacterial counts in blood and brain tissue homogenates were determined by plating ten-fold serial dilutions on rifampicin LB agar plates. Growth of EC-K1 in rifampicin containing LB broth from CSF samples was considered positive for meningitis. PMNs were depleted in mice by the administration of mAb RB6-8C5 as described in earlier studies^{32,54}. Mice were given a total of four doses of RB6, two doses at 12 h interval (100 µg) on Day 1 and similar doses at Day 2 and infected with EC-K1 on Day 3. Control group received similar doses of isotype-matched antibody. PMNs were also depleted in EC-K1 infected mice 24 h post-infection by injecting two doses of RB6-8C5 antibody 6 h apart. Similar doses of isotype-matched antibody were injected in control mice. The depletion of PMNs was confirmed by flow cytometry using anti-Gr1 antibody.

Suppression of gp96 expression in newborn mice

gp96 expression was suppressed in neonatal mice by siRNA to gp96 and InvivoFectamine as described earlier^{35,53}. Briefly, newborn mice were given four doses of gp96 siRNA (10 nmol; oligo identification number MSS205545, Invitrogen) intraperitoneally; two doses before infection on Day 1 and Day 2, one dose immediately after administration of bacteria, on Day 3 and final dose at 24 h after infection on Day 4. The suppression of gp96 in splenic PMNs on Day 3 and Day 4 was confirmed by flow cytometry and RT-PCR.

Determination of BBB leakage

The quantitative evaluation of BBB permeability was done by detection of extravasated Evans blue dye^{33,55}. Briefly, mice were injected intraperitoneally with 2% Evans blue dye in saline and after 4 h, animals were deeply anesthetized with Nembutal and transcatheterially perfused until colorless perfusion fluid was obtained from the right atrium. After decapitation, brain tissue was removed, weighed, homogenized and centrifuged. The protein concentration was then determined in the tissue supernatants. Evans blue intensity was determined by a microplate reader at 550 nm. Results were expressed in micrograms of Evans blue dye per milligram protein.

Statistical analysis

Statistical significance was determined by a paired, two-tailed Student's *t* test, ANOVA and Fisher's exact test. Values of $p < 0.05$ were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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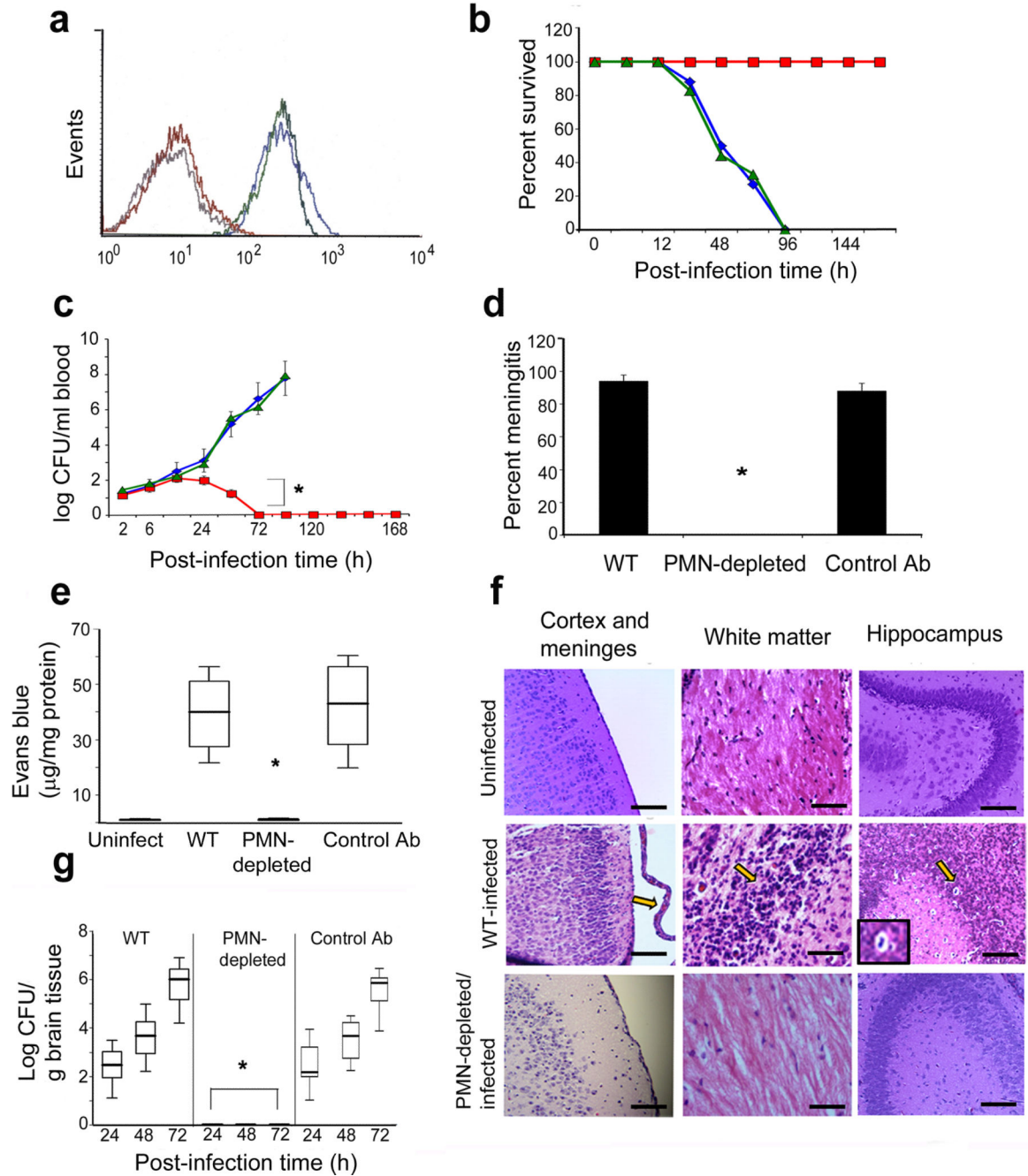


Figure 1. PMN depletion renders newborn mice resistant to *E. coli* K1 meningitis

Newborn mice were injected four doses of mAb RB6-8C5 between Day 1 and Day 3 after birth or control antibody and the depletion of PMNs in spleen was examined by flow cytometry. Isotype-matched antibody (grey line), WT (blue line), PMN-depleted (red line) or control antibody (green line) (a). WT (blue line), PMN-depleted (red line) or control antibody (green line) treated mice at Day 3 were infected with *E. coli* K1 and the percentage of survival was assessed (b). Bacteremia levels in these animals were determined by plating various dilutions of blood samples collected at indicated time points on agar containing

antibiotics (c). Cerebrospinal fluids (CSF) at 72 h post-infection were cultured in antibiotic containing Luria broth to assess the occurrence of meningitis (d). The blood-brain barrier permeability was determined by the Evans blue extravasation as described in Methods section (e). Bacterial load in the brains of WT, PMN-depleted and control antibody treated mice was evaluated after 72 h post-infection (f). Brains from WT and PMN-depleted mice infected with *E. coli* K1 were removed and subjected to histopathological examination after staining with H and E (g). The data represent mean values \pm SD and is representative of five independent experiments with 18 animals in each group. * $p < 0.01$ PMN-depleted versus WT or control Ab treated mice by two tailed *t* test and ANOVA. Arrows indicate neutrophil infiltration in leptomeninges or white matter and apoptosis of neurons in hippocampus. Scale bars 20 μ M.

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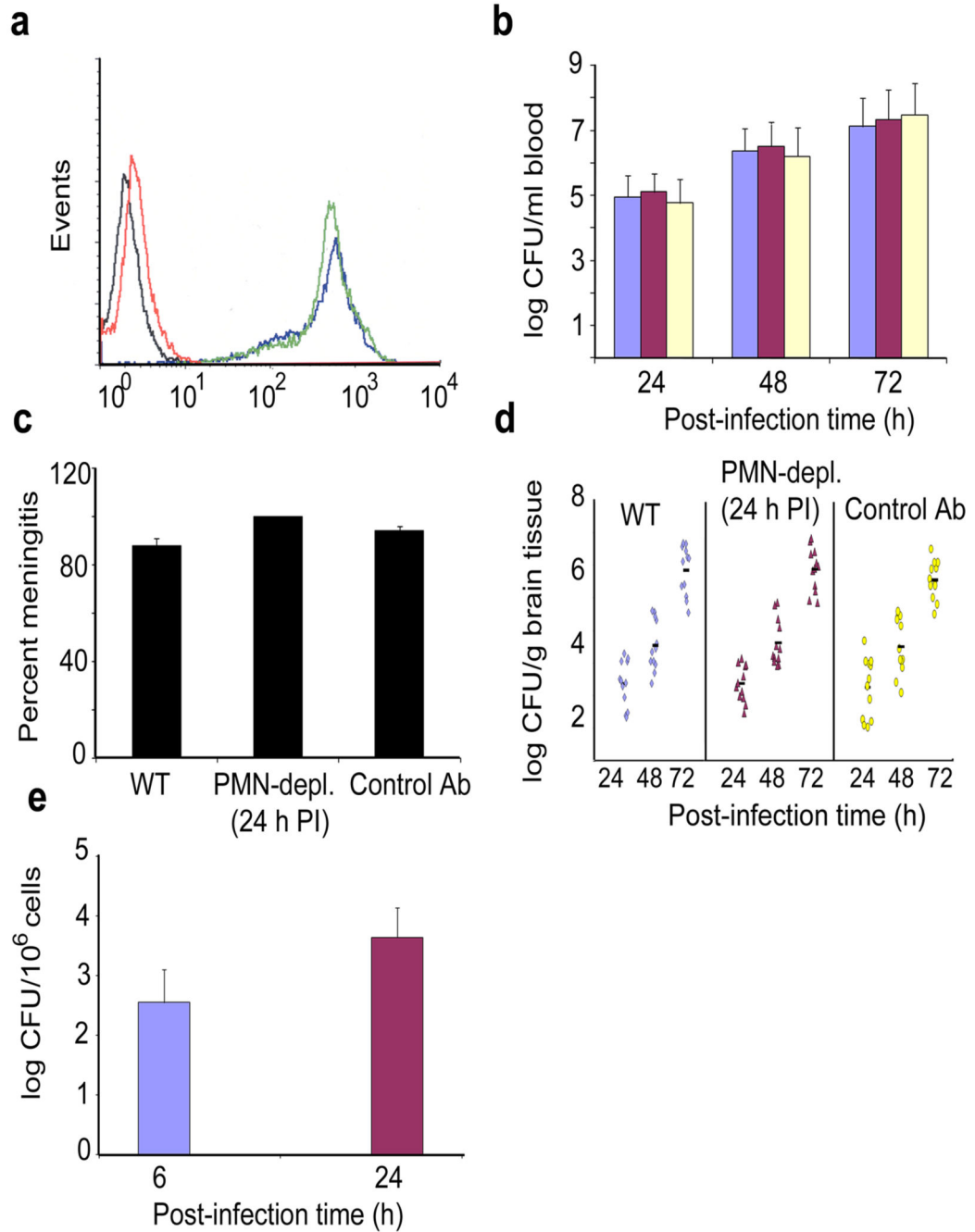


Figure 2. PMN-depletion in mice after infection with *E. coli* K1 fails to prevent the onset of meningitis

Mice were infected with *E. coli* K1 (E44) and PMNs were depleted 24 h post-infection as described in Methods section. The depletion of PMNs was confirmed by flow cytometry. Isotype-matched antibody (grey line), WT (blue line), PMN-depleted (red line) or control antibody (green line) (a). Blood from WT (blue bar), PMN depleted (red bar) or control antibody treated (yellow bar) mice was plated on antibiotic containing agar to determine bacteremia levels (b), CSF (c) and brain samples from WT (blue), PMN depleted (red) or

control antibody treated (yellow) animals (d) were analyzed for the presence of *E. coli* K1 by growing the bacteria in antibiotic containing broth or on agar. PMNs (blue bar) and macrophages (red bar) were isolated from lungs of infected mice at 6 and 24 h post-infection and number of intracellular bacteria enumerated by plating on agar containing antibiotics (e). The data represent mean values \pm SD and is representative of five independent experiments with 12 animals in each group.

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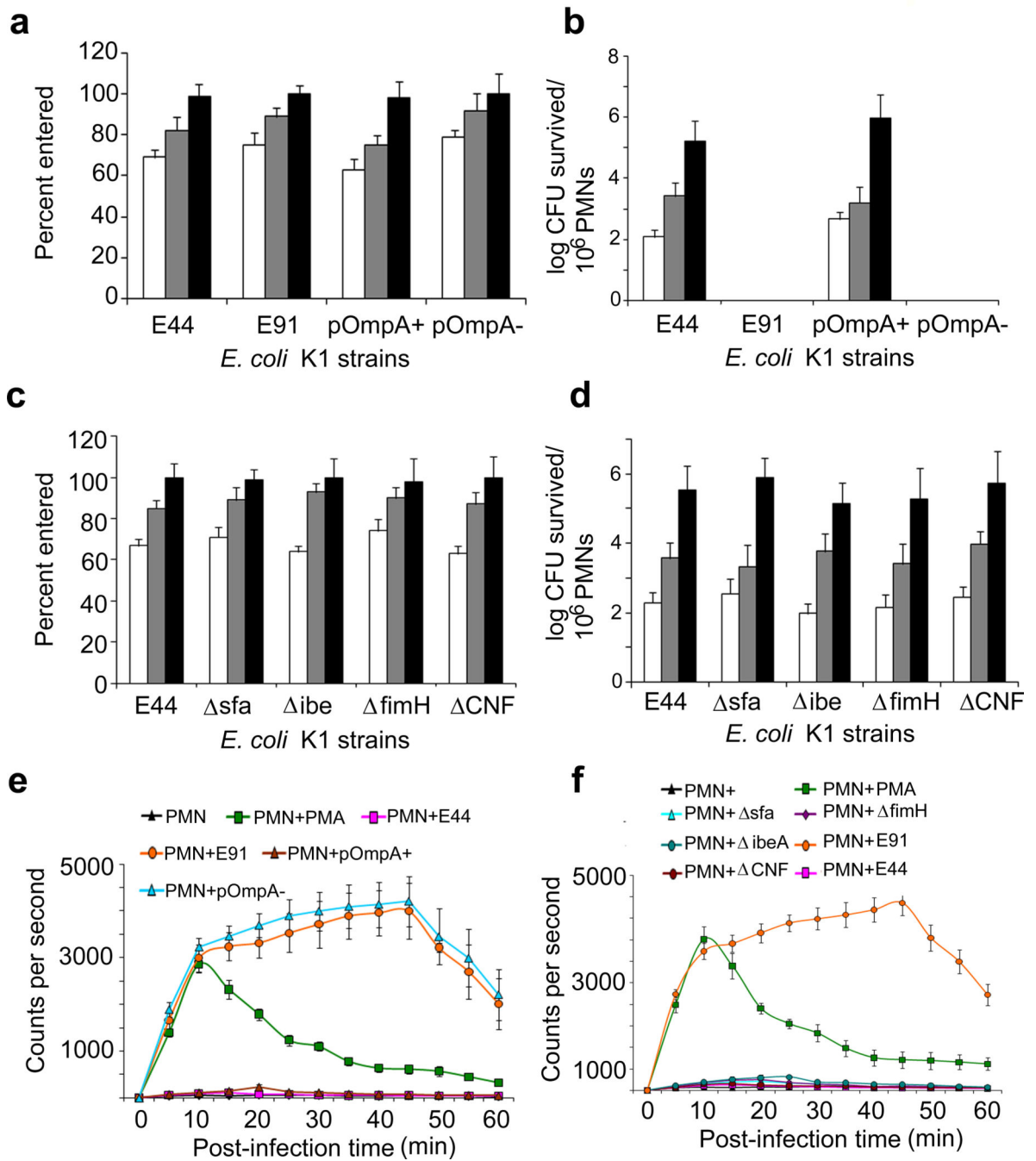


Figure 3. Phagocytosis and oxidative burst in *E. coli* K1 infected PMNs

Various strains of *E. coli* K1 were incubated with PMNs at a multiplicity of infection of 10 (PMN to bacteria ratio, 1:10) for indicated time points. The percentage uptake (a and c) and the number of intracellular bacteria (b and d) were determined by gentamicin protection assays at 2 h (white bar), 4 h (grey bar) and 6 h (black bar). In separate experiments, PMNs were left uninfected (PMN), stimulated with PMA, exposed to OmpA⁺ *E. coli* K1 (E44), OmpA⁻ *E. coli* K1 (E91), *E. coli* *ibeA*, *E. coli* *CNF*, *E. coli* *sfa*, *E. coli* *fimH* or plasmid complemented strains (pOmpA⁺ and pOmpA⁻) of *E. coli* K1 and the oxidative burst was

measured using luminol-dependent chemiluminescence as described in Methods section (e, f). The data represent mean values \pm SD of triplicate samples and is a representative of seven independent experiments.

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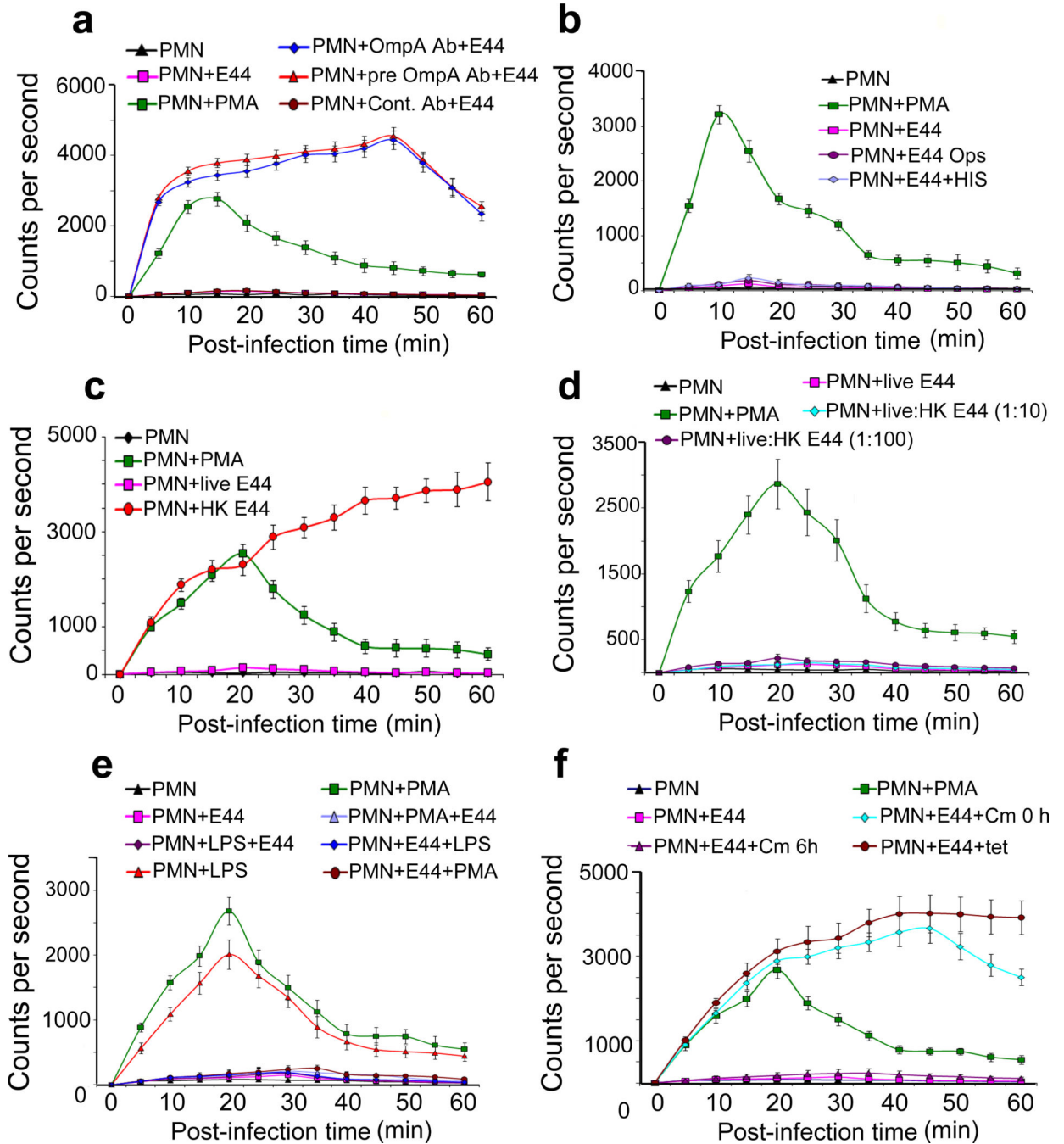


Figure 4. Effect of anti-OmpA antibody and chloramphenicol on oxidative burst in *E. coli* K1 infected PMNs

PMNs were left uninfected, stimulated with PMA or infected with anti-OmpA antibody or control antibody treated *E. coli* K1. In these experiments anti-OmpA antibodies were incubated with *E. coli* K1 prior to infection (PMN+pre OmpA Ab+E44) or added at the same time (PMN+OmpA Ab+E44) (a). PMNs were also infected with *E. coli* K1 unopsonized, opsonized with pooled human serum (Ops) or treated with heat inactivated serum (HIS) (b). In separate experiments, PMNs were stimulated with heat killed (HK)

bacteria alone (c) or a mixture of heat killed and live bacteria (d). In addition, PMNs were first treated with PMA or LPS and then infected with OmpA⁺ *E. coli* K1 (E44) or vice versa (e). Chloroamphenicol (Cm) was added to PMN-bacteria co-culture immediately (PMN +E44+Cm 0 h) or after 6 h (PMN+E44+Cm 6 h) (F). Similarly, tetracycline (5 µg ml⁻¹) was also added to PMN-bacteria co-culture (PMN+E44+tet). Oxidative burst was measured as described in Methods section after all these treatments. Data are expressed as mean values ± SD of triplicate samples and representative of five independent experiments

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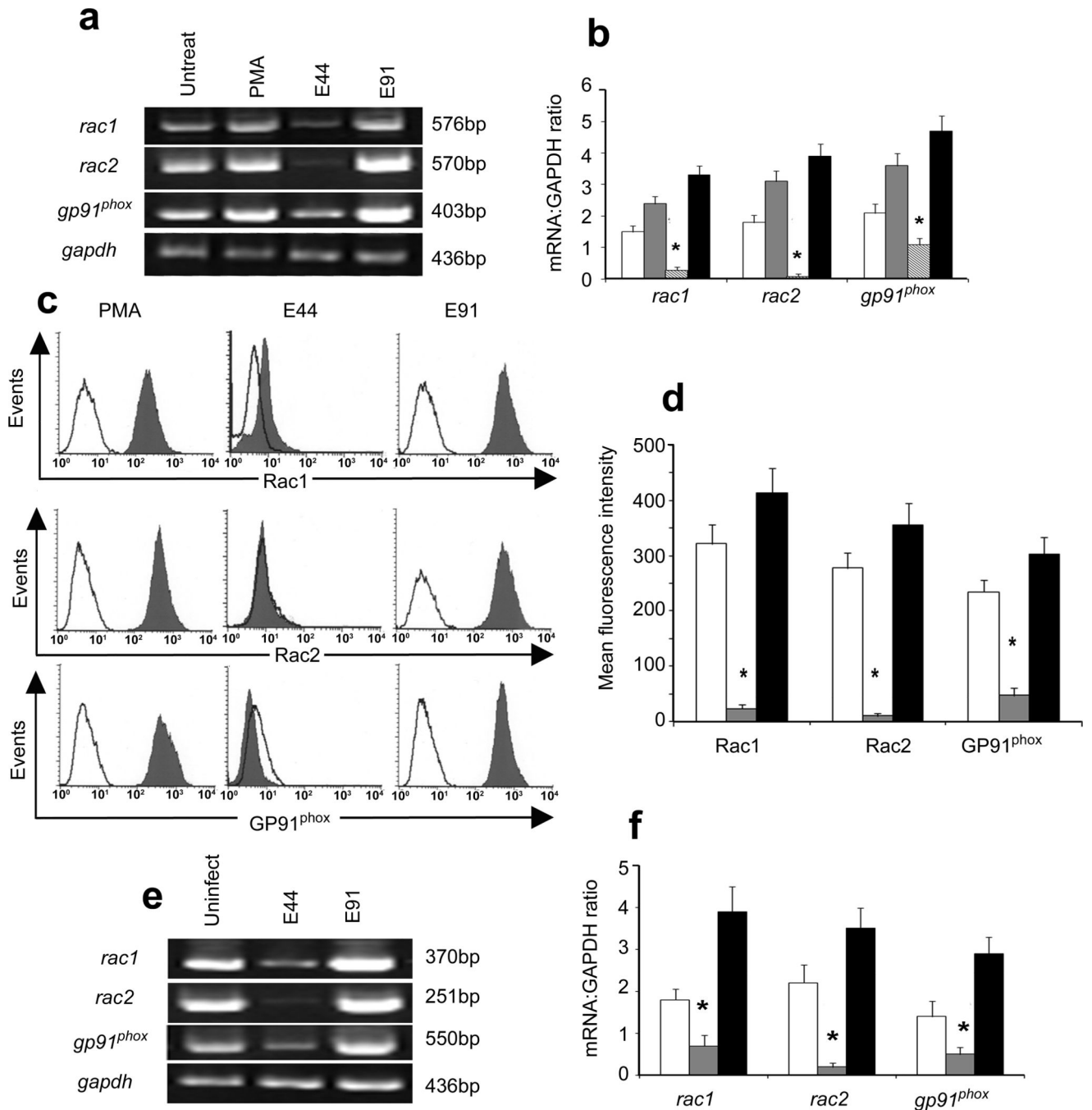


Figure 5. *E. coli* K1 downregulates the transcription of NADPH oxidase components in PMNs RT-PCR using primers for *rac1*, *rac2*, *gp91^{phox}* and *gapdh* and total RNA isolated from PMNs untreated, PMA stimulated, OmpA+ *E. coli* (E44) infected, or OmpA- *E. coli* (E91) infected was performed (a). The densities of the transcripts from three different experiments were determined and mean values \pm SD are expressed as a ratio of *rac1*, *rac2*, *gp91^{phox}* mRNA and *gapdh* mRNA in untreated (white bar), PMA treated (grey bar), E44 infected (shaded bar) or E91 infected (black bar) PMNs (b). In separate experiments, PMNs treated with PMA, OmpA+ *E. coli* (E44) or OmpA- *E. coli* (E91) were washed, fixed and the

protein levels of Rac1, Rac2 and gp91^{phox} were determined by flow cytometry using specific antibodies (c). For quantitative measurements, the geometric mean fluorescence intensities were plotted for Rac1, Rac2 and gp91^{phox} in PMA treated (white bar), E44 infected (grey bar) or E91 infected (black bar) PMNs (d). RT-PCR analysis of expression of *rac1*, *rac2* gp91^{phox} and *gapdh* mRNA in spleen PMNs isolated from uninfected, OmpA+ *E. coli* (E44) infected or OmpA- *E. coli* (E91) infected mice (e). For quantitation, the transcription levels were determined by densitometry of bands from three different experiments and mean values \pm SD are expressed as a ratio of *rac1*, *rac2* gp91^{phox} mRNA and *gapdh* in PMA treated (white bar), E44 infected (grey bar) or E91 infected (black bar) PMNs (f). The results presented in a-d are representative of seven independent experiments. *p<0.01 by two tailed *t* test and ANOVA.

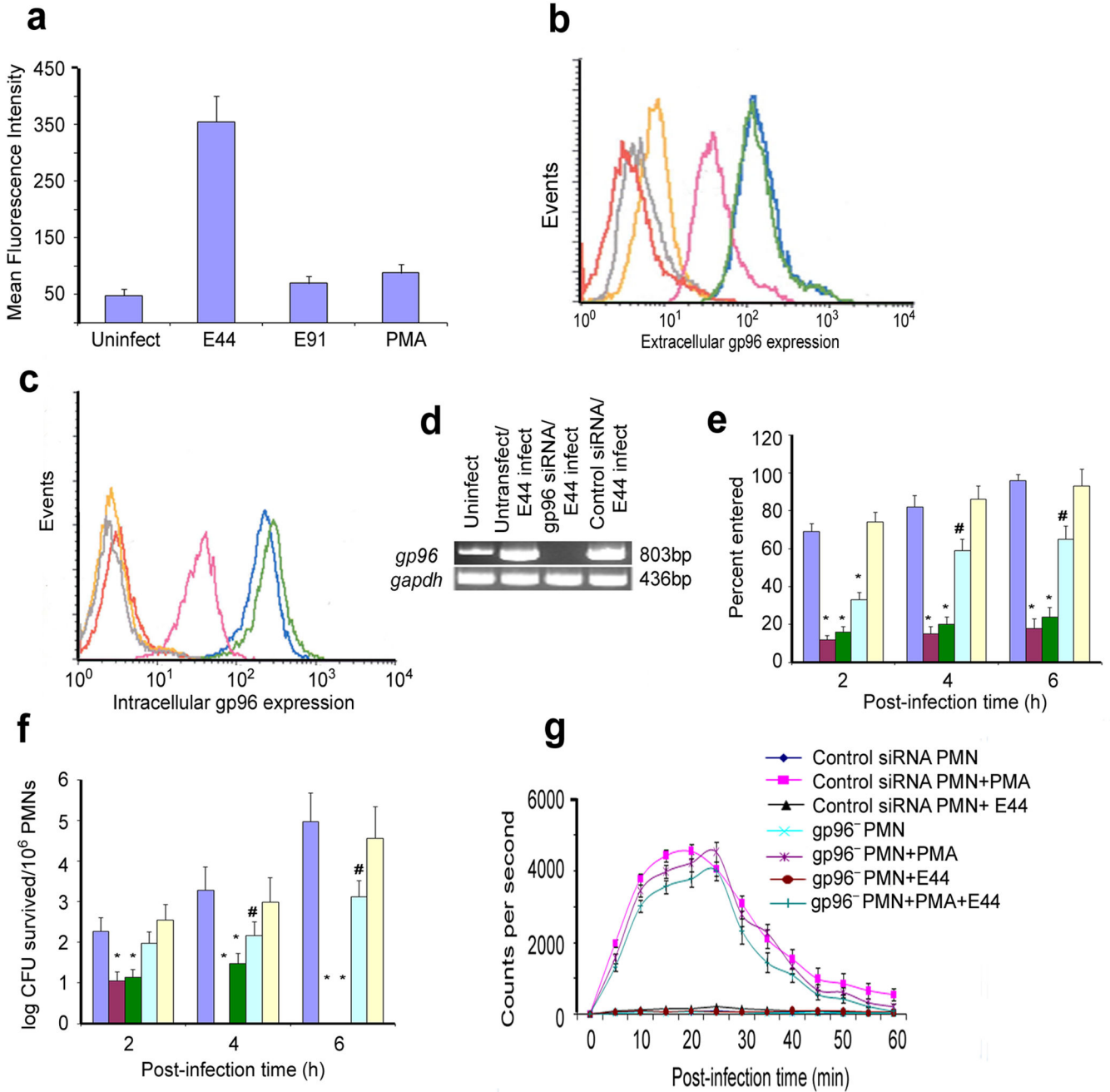


Figure 6. The expression of gp96 in PMNs is critical for *E. coli* K1 entry and suppression of oxidative burst

PMNs were infected with *OmpA*⁺ *E. coli* (E44), *OmpA*⁻ *E. coli* (E91), stimulated with PMA or left without infection. The expression of gp96 on the surface of PMNs was determined by flow cytometry. The data are presented as geometric mean fluorescence intensity (MFI) of logarithmic data subtracted from isotype-matched controls (a). In separate experiments, PMNs were transfected with three gp96 siRNAs (red line, orange line and pink line for gp96-siRNA-1, gp96-siRNA-2, gp96-siRNA-3, respectively) or control siRNA (green line), and the expression of extracellular (b) or intracellular (c) gp96 in PMNs was

measured by flow cytometry. Isotype-matched control antibody (grey line) or untransfected PMNs (blue line) were used as controls. RT-PCR performed with gp96 specific primers and total RNA isolated from gp96-siRNA-1 or control siRNA transfected PMNs or untransfected cells (d). The entry (e) and intracellular survival (f) of bacteria in PMNs transfected with gp96-siRNA-1 (red bar) or gp96-siRNA-2 (green bar) or gp96-siRNA-3 (blue bar) or control siRNA (yellow bar) or untransfected (purple bar) was determined. In addition, oxidative burst was also determined in gp96-siRNA-1 transfected PMNs and then treated with PMA, OmpA+ *E. coli* (E44) or PMA+E44 (g). Data are expressed as the mean values \pm SD of triplicate samples and are representative of five independent experiments. The oligo numbers for gp96-siRNA-1, gp96-siRNA-2, and gp96-siRNA-3 were HSS110955, HSS110953 and HSS186409, respectively. * $p < 0.01$ or # $p < 0.05$ by two tailed *t* test and ANOVA.

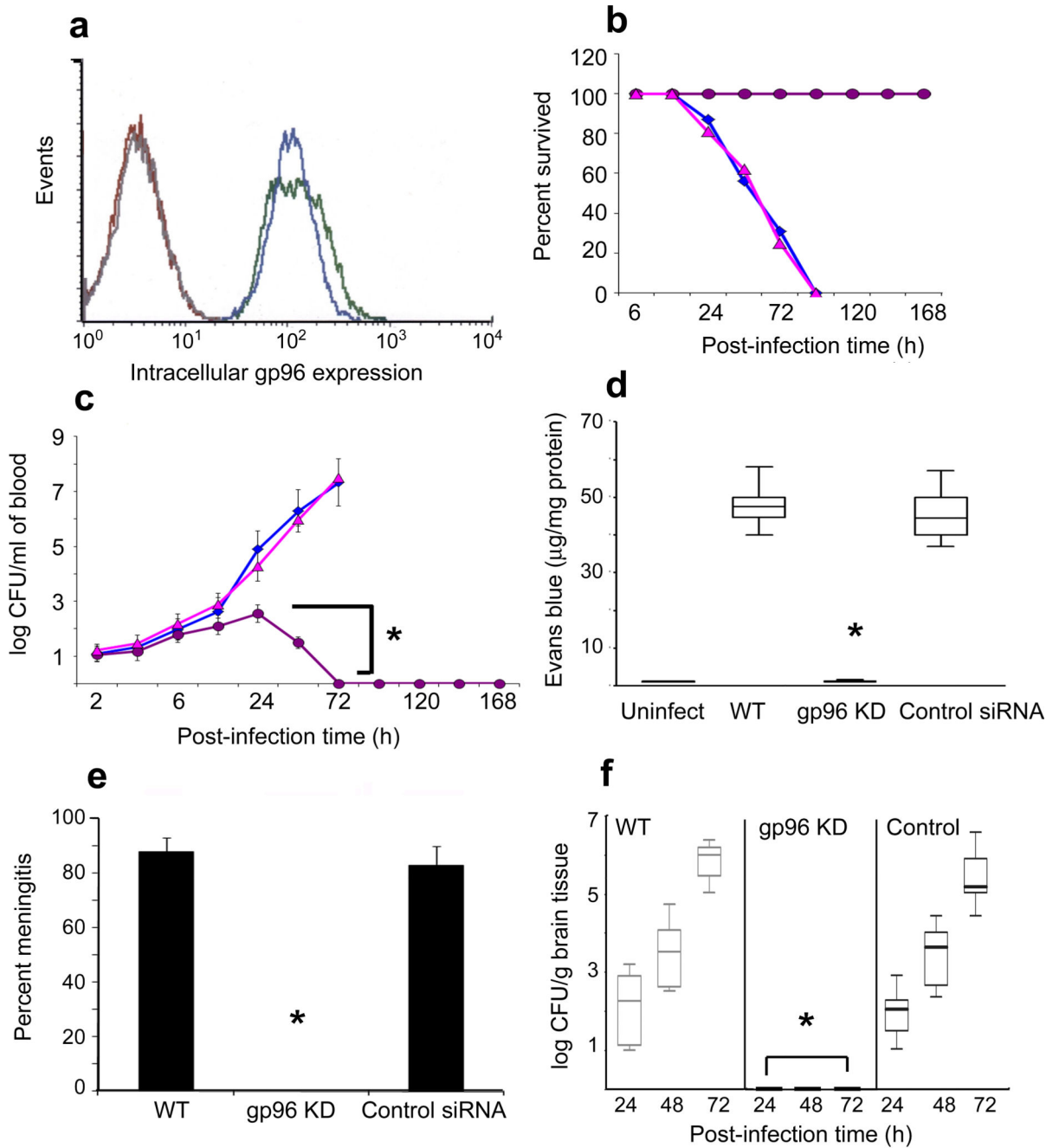


Figure 7. Suppression of gp96 in newborn mice provides protection against *E. coli* K1 meningitis

Newborn mice were injected with gp96-siRNA-1 to knockdown (KD) the expression of gp96 as described in Methods section and extracellular expression of gp96 in PMNs obtained from spleen of WT (blue line), gp96 KD (red line) or control siRNA treated (green line) animals was assessed by flow cytometry (a). The grey line represents isotype-matched antibody control. WT (diamonds), gp96 KD (circles) or control siRNA treated (triangles) mice at Day 3 were infected with *E. coli* K1 and the survival of animals was recorded up to 7 days post-infection (b). Bacteremia levels in WT (diamonds), gp96-KD (circles) or control

siRNA treated (triangles) and infected mice were determined at various times post-infection (c). In separate experiments, gp96 KD or WT mice infected with *E. coli* K1 were injected with Evans blue dye and leakage was determined as described in Methods sections (d). CSF samples (e) and brains (f) were collected and the presence of bacteria determined by culturing in antibiotic containing broth or on agar. The data represent mean values \pm SD and is representative of five independent experiments with 18 animals in each group. * $p < 0.01$ by two tailed *t* test, Fisher's exact test and ANOVA.

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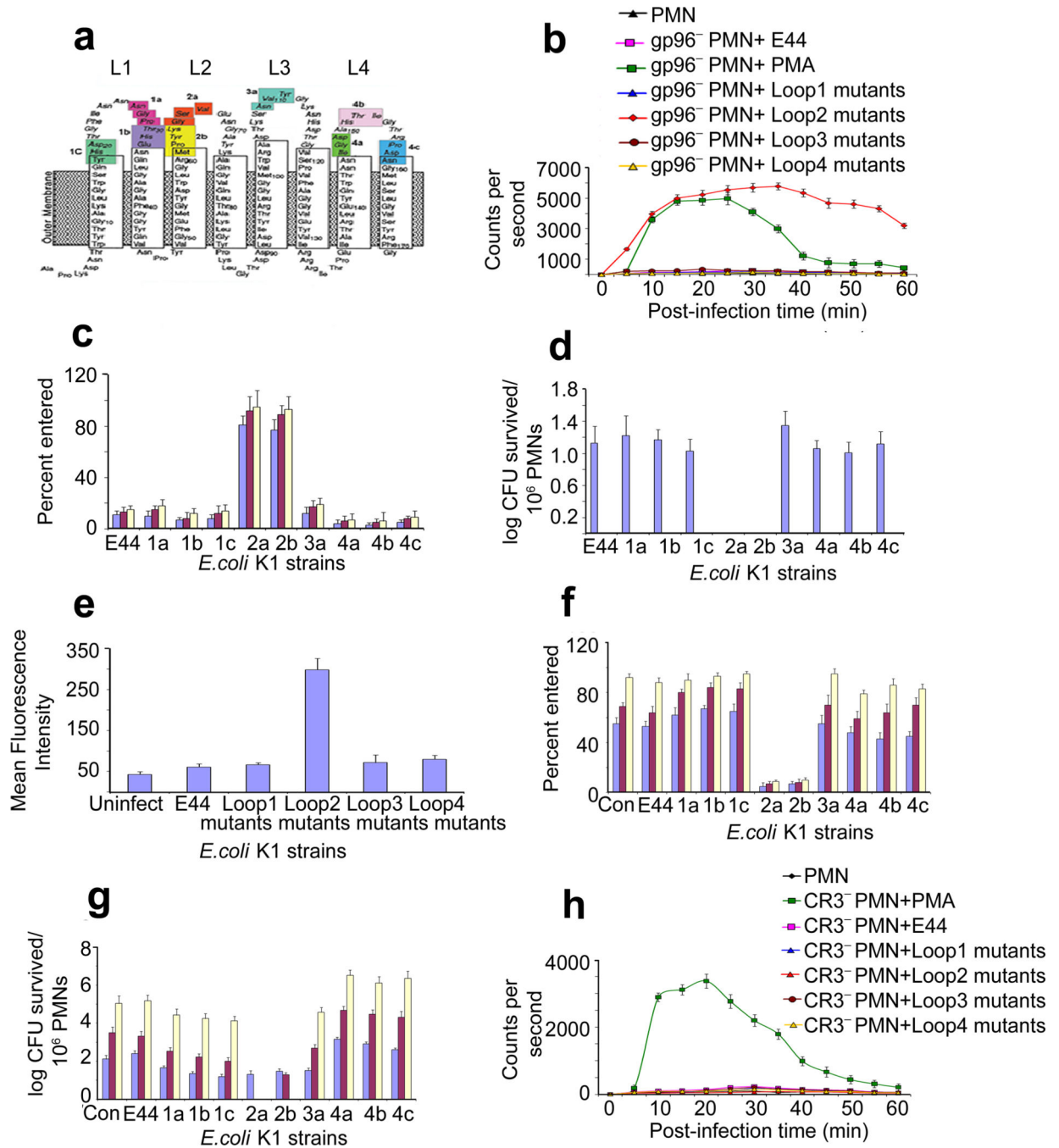


Figure 8. Interaction of loop 2 of OmpA with gp96 in PMNs is necessary for the entry of *E. coli* K1 and suppression of ROS production

Various *E. coli* K1 (E44) mutants were generated by mutating three amino acids to alanines in the extracellular loops of OmpA (highlighted) (a). PMNs were transfected with gp96-siRNA-1 (gp96⁻ PMNs) and oxidative burst capacity of PMNs infected with *E. coli* K1 mutants was examined (b). In addition, entry (c) and survival (d) of *E. coli* K1 mutants was determined in gp96-siRNA-1 transfected PMNs at 2 h (purple bar), 4 h (maroon bar) and 6 h (yellow bar) post-infection. CR3 expression on the surface of PMNs infected with *E. coli* K1

mutants was analyzed by flow cytometry. Mean fluorescence intensities were graphed (e). In separate experiments, PMNs were transfected with CR3 siRNA (CR3⁻ PMNs) and the entry (f), survival (g) and oxidative burst capacity (h) of PMNs infected with *E. coli* K1 mutants was determined. Results are expressed as mean values \pm SD of triplicate samples and are representative of five independent experiments.

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