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***Escherichia coli* K1 induces pterin production for enhanced expression of Fcγ receptor I to invade RAW 264.7 macrophages**

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Abstract

Macrophages serve as permissive niches for *Escherichia coli* (*E. coli*) K1 to attain high grade bacteremia in the pathogenesis of meningitis in neonates. Although pterin levels are a diagnostic marker for immune activation, the role of macrophages in pterin production and in the establishment of meningitis is unknown. Here, we demonstrate that macrophages infected with *E. coli* K1 produce both neopterin and biopterin through increased expression of GTP-cyclohydrolase 1 (GCH1). Of note, increased production of biopterin enhances the expression of Fc-gamma receptor I (CD64), which in turn, aided the entry of *E. coli* K1 in macrophages while increased neopterin suppresses reactive oxygen species (ROS), thereby aiding bacterial survival. Inhibition of GCH1 by 2, 4-Diamino-6-hydroxypyrimidine (DAHP) prevented the *E. coli* K1 induced expression of CD64 in macrophages *in vitro* and the development of bacteremia in a newborn mouse model of meningitis. These studies suggest that targeting GCH1 could be therapeutic strategy for preventing neonatal meningitis by *E. coli* K1.

Keywords

Neopterin; Biopterin; macrophages; *E. coli* K1; meningitis

1. Introduction

Guanosine triphosphate (GTP) is the biosynthetic source for neopterin production due to the action of GTP-cyclohydrolase I (GCH1). 7, 8-dihydroneopterin and neopterin are the products of GTP cleavage in macrophages and dendritic cells [1]. Although the biological relevance of neopterin secretion from macrophages remains unknown, measurement of

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neopterin levels is a clinical marker for the diagnosis of malignant disorders and cell-mediated immune activation. Experiments have suggested that neopterin can inhibit the activity of xanthine oxidase and NADPH oxidase, thus blocking the production of reactive oxygen species [2]. Lipopolysaccharide (LPS), tumor necrosis factor- α and γ -interferon stimulate pterin production in immune cells and endothelial cells [3, 4]. Tetrahydrobiopterin (6*R*-*L*-erythro-1, 2-dihydroxypropyl 2-amino-4-hydroxy-5, 6, 7, 8-tetrahydropteridine; BH₄) is another biosynthetic product of GCH1 activity and functions as a co-factor for nitric oxide synthase in the generation of nitric oxide (NO) from *L*-arginine [5]. Previous studies have demonstrated that activation of macrophages with LPS and IFN- γ and IL-12 induced parallel increases in NO and intracellular BH₄ levels [4].

Our studies have shown that *E. coli* K1, which causes neonatal meningitis, enters and suppresses antimicrobial activities of macrophages to survive inside them, multiply, and finally escape into the bloodstream in large numbers to cross the blood-brain barrier. *E. coli* K1 utilizes Fc-gamma receptor I (CD64) to bind to and enter macrophages. This is evident by the lack of invasion of *E. coli* K1 in both bone-marrow derived and peritoneal macrophages isolated from CD64^{-/-} mice [6]. In agreement, CD64^{-/-} mice are resistant to *E. coli* K1 infection due to their inability to attain threshold levels of bacteremia required for the onset of meningitis, suggesting that CD64 expression in macrophages is critical for the pathogenesis. Of note, the expression of outer membrane protein A (OmpA) is critical for the survival of *E. coli* K1 in macrophages, indicating that the interaction of OmpA with CD64 contributes to alteration of macrophage function [6]. Studies have also shown that patients with sepsis show increased levels of neopterin, CD64 and CR3 in monocytes [7]. Elevated serum neopterin levels were also observed in patients infected with *Shigella* [8]. Since high biopterin and neopterin levels were also reported in patients with bacterial meningitis [9], we sought to examine whether *E. coli* K1 interaction with macrophages produce neopterin and biopterin and they contribute to the entry of the bacterium into the cells.

2. Materials and methods

2.1. Bacterial strains, antibodies and other reagents

Escherichia coli (OmpA+ *E. coli*) is a spontaneous rifampin-resistant mutant of strain RS 218 (serotype O18:K1:H7), which was isolated from the cerebrospinal fluid of a newborn with meningitis [10]. OmpA- *E. coli* is a mutant of RS218 that expresses no OmpA and cannot survive in macrophages [10]. Antibodies to GCH1, iNOS, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies tagged to various fluorophores were purchased from Invitrogen (Carlsbad, CA). FuGENE HD reagent from Roche (Indianapolis, IN) was used for plasmid transfection and Lipofectamine from Sigma (St. Louis, MO) was used for siRNA transfection. DAHP was purchased from Sigma (St. Louis, MO). Griess reagent was purchased from Promega (Madison, WI). 6-methylpterin (internal standard), D-neopterin, L-Biopterin were purchased from Shricks laboratories (Jona, Switzerland). Ascorbic acid was obtained from Calbiochem (La Jolla, USA). Lugol's Iodine was purchased from Electron Microscopy Sciences (Hatfield, PA, USA).

2.2. *E. coli* invasion assays

Peritoneal and RAW 264.7 macrophages were cultured in 24-well plates as described earlier [6] and incubated with 10⁶ CFU of *E. coli* in experimental medium (1:1 mixture of Ham's F-12 and M-199 containing 5% heat-inactivated fetal bovine serum) for 90 min at 37°C in CO₂ incubator. The monolayers were washed three times with RPMI 1640 and incubated further with experimental medium containing gentamicin (100 μ g /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 determined by plating the dilutions on sheep blood agar. To enumerate the total cell associated bacteria, the experiments were performed without gentamicin step.

2.3. Determination of pterin levels

Pterins (biopterin and neopterin) were measured as described previously by HPLC [11]. The entire sample preparation was performed in dark environment using brown eppendorf tubes. Confluent monolayers of RAW 264.7 macrophages in 100 mm dishes were washed three times with cold PBS and suspended in 100 μ l of PBS, sonicated at a relative output of 0-5 for 30 sec and treated with 50 μ l of 10% trichloroacetic acid on ice for 30 min. RAW 264.7 macrophages cell lysates samples were centrifuged at $10,000 \times g$ at 4°C for 10 min and the supernatants were subjected for iodine oxidation. 125 μ l of sample was mixed with 200 nM neopterin, 100 nM biopterin and 10 μ l of internal standard (6-methylpterin). 30 μ l of 1N HCl was added, and the oxidation was started by the addition of 10 μ l acidic iodine solution. The tube was kept at room temperature for 60 min. Subsequently, ascorbic acid (15 μ l) was added to the mixture to reduce excess iodine. The solution was filtered using acro-disc syringe filter. 100 μ l of the solution was injected to HPLC (Dionex, Thermo Fisher Scientific now, USA). Separation was performed on a C18 Spherisorb, 5 μ m pre-column (10 $\times$ 4.6 mm) and ODS-1 Spherisorb analytical column (250 $\times$ 4.6 mM) (both from Waters, Milford, MA, USA), using 1.25 mmol/L potassium hydrogen phosphate buffer, with 6% (v/v) methanol at a flow rate 1.3 mL/min from 0 to 11.5 mins, and 1.7 mL/min from 13 to 26 mins by their native fluorescence at λ_{EX} : 350 nm, λ_{EM} : 450 nm using a fluorescence detector.

2.4. Immunoprecipitation and Western Blotting

RAW 264.7 macrophages were grown to confluence and infected with bacteria in the presence or absence of DAHP treatment for varying times and immunoprecipitation and Western blotting were performed as described earlier [11]. The protein expression levels were quantitated by measuring the intensity of the bands using ImageJ software (<http://imagej.nih.gov/ij/>).

2.5. Flow cytometry and NO estimation as nitrite

RAW 264.7 macrophages were infected with OmpA+ *E. coli* with or without DAHP pre-treatment for various periods and surface expression of CD64 was determined by flow cytometry and supernatants from the respective samples were used to determine nitrite levels using Greiss reagent [11].

2.6. Superoxide anion generation assay

Superoxide anion generation was measured using the superoxide anion assay kit (Sigma, catalog no. CS1000) following the manufacturer's protocol using Phorbol 12-myristate (PMA) as a superoxide anion stimulator and superoxide dismutase (SOD) as a superoxide scavenger. Approximately 1×10^5 cells of RAW 264.7 macrophages were incubated with DMEM (10% Serum and 5.5 mM glucose) in 96 well clear bottom white plate. Confluent macrophages were washed three times with HBSS solution and re-suspended with 100 l of assay medium consists of luminol and enhancer solution. Macrophages were immediately infected with OmpA+ *E. coli* and OmpA- *E. coli* infection (1:10 MOI in assay medium) with or without PMA pre-treatment. The samples were mixed and chemiluminescence was measured every 15 min for 2 h in a luminometer (Perkin Elmer). Cells were maintained at 37°C during the experiment.

2.7. Newborn mouse model of meningitis

The animal studies were approved by the IACUC of Children's Hospital of Los Angeles and followed guidelines for the performance of animal experiments implemented by National Institutes of Health. C57BL/6J timed-pregnant mice were purchased from Jackson Laboratories (Bar Harbor, ME). Three day-old mouse pups were infected intranasally with 10^3 CFU of bacteria in pyrogen-free saline as previously described [11]. To evaluate the effect of DAHP on OmpA+ *E. coli* meningitis, DAHP (100 mg/kg body weight) was injected intraperitoneally at the time of infection and two more doses at 6 h intervals prior to and after the infection. 5 μ l of facial vein blood was collected aseptically from all the pups in the respective groups at 72 h post-infection to determine the bacterial load by plating ten-fold serial dilutions on rifampicin LB agar plates.

2.8. Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 11.0) and values presented as mean $\pm$ standard deviation (SD). Significant differences ($p < 0.05$) between the groups were determined using the unpaired Student's *t*-test.

3. Results

3.1. RAW 264.7 macrophages produce neopterin by activating GCH1 upon *E. coli* K1 infection

Since we observed pterin production in the brains of newborn mice infected with *E. coli* K1 [11], we sought to examine whether macrophages, which are permissive niches for replication of the bacterium, have any role in the pterin generation. GCH1 is a critical enzyme in the production of pterins [4]. Therefore, RAW 264.7 macrophages infected with OmpA+ *E. coli* or OmpA- *E. coli* for varying times and the total cell lysates were initially subjected to Western blotting with anti-GCH1 antibodies. OmpA+ *E. coli* induced the expression of GCH1 between 30 and 60 min post-infection by two fold compared with control uninfected cells, which levels started declining by 90 min (Fig. 1A and B). In contrast, OmpA- *E. coli* showed only a marginal increase in the GCH1 expression. HPLC analysis of total neopterin levels revealed that OmpA+ *E. coli* induced more neopterin than the levels produced by uninfected cells or OmpA- *E. coli* infected cells (Fig. 1C). Neopterin and its reduced forms, namely, dihydro- and tetrahydro-neopterins have been shown to maintain reactive oxygen levels in macrophages [12]. Therefore, we determined the production of superoxide in RAW 264.7 macrophages upon infection. In agreement with neopterin levels, OmpA+ *E. coli* infected macrophages showed significantly lower superoxide production compared with control, OmpA- *E. coli* or PMA treated cells. RAW 264.7 macrophages pre-treated with PMA and then infected with OmpA+ *E. coli* also exhibited lower production of superoxide compared with PMA alone treated cells. OmpA- *E. coli* infected macrophages could not prevent the superoxide production induced by PMA, whereas superoxide dismutase treated cells reduced the levels by 90% (Fig. 1D). These experiments suggest that OmpA+ *E. coli* effectively scavenges superoxide produced by macrophages by inducing GCH1/neopterin expression.

3.2. RAW 264.7 macrophages also produce biopterin to induce NO production

Our previous studies have demonstrated that *E. coli* K1 infected HBMEC generates biopterin, which in turn is responsible for activation iNOS [11]. Additionally, suppression of pterin production by GCH1 inhibitor, DAHP and iNOS inhibitor, aminoguanidine (AG) blocked the replication of *E. coli* K1, which event is required to reach high grade bacteremia in newborn mice. Since our studies also demonstrated that macrophages act as a replicative

niche for *E. coli* K1, we examined whether the bacterial infection of RAW 264.7 macrophages also induces biopterin production. Similar to that of neopterin production, total biopterin levels were also elevated in OmpA+ *E. coli* infected macrophages compared with control or OmpA- *E. coli* infected cells (Fig. 2A). Given the concept that tetrahydro-biopterin (BH4) acts as a cofactor for iNOS, we also determined the BH4 concentration. The levels of BH4 produced by OmpA+ *E. coli* infected macrophages were significantly higher than the levels induced in uninfected or OmpA- *E. coli* infected cells. The ratio of BH4 to BH2 was 3 times (75 nmoles of BH4 versus 25 nmoles of BH2), which may promote iNOS coupling to L-arginine, leading to increase in NO production (Fig. 2A-C). In agreement with the BH4 production, Western blotting analysis of macrophages infected with OmpA+ *E. coli* showed increased expression of iNOS at 30 and 60 min post-infection (Fig. 2D). Further, the dimerization of iNOS increased upon *E. coli* K1 infection. Pre-treating the RAW 264.7 macrophages with DAHP, an inhibitor of GCH1, prior to infection with OmpA+ *E. coli* significantly blocked the iNOS expression. We further measured the NO production in the cell lysates, which data revealed that OmpA+ *E. coli* infection of RAW 264.7 macrophages significantly induced the production of NO compared with control cells, which was inhibited by DAHP treatment. These results indicate that GCH1 activity, which leads to the production of biopterin besides neopterin, is critical for iNOS expression and subsequent production of NO in macrophages infected with *E. coli* K1.

3.3. GCH1 inhibitor, DAHP prevents *E. coli* K1 invasion in RAW 264.7 macrophages

We have previously demonstrated that *E. coli* K1 utilizes Fc-gamma receptor I (CD64) for binding to and entering into macrophages. Lack of CD64 expression in macrophages significantly reduced the invasive capacity of the bacterium into the cells [6]. Since, our studies also demonstrate that *E. coli* K1 could not attain required bacteremia in DAHP pre-treated newborn mice [11], we speculated that DAHP treatment of macrophages render the cells non-permissive for *E. coli* K1 to become safe havens for multiplication. Therefore, the effect of DAHP pretreatment on *E. coli* K1 binding (total cell associated) and intracellular survival was evaluated in RAW 264.7 macrophages. DAHP inhibited both total cell associated and intracellular *E. coli* K1 in a dose and time dependent manner (Fig. 3A and B). At 5 mM concentration of DAHP for 3 h incubation, the total cell associated bacteria was reduced by 50% ($6 \pm 0.7 \times 10^5$ CFU/well versus $3 \pm 0.5 \times 10^5$ CFU/well, $p < 0.05$) and the intracellular bacteria was reduced by 75% ($3 \pm 0.5 \times 10^5$ CFU/well versus $2.5 \pm 0.5 \times 10^4$ CFU/well, $p < 0.05$). However, treating macrophages with DAHP after infection with *E. coli* K1 did not have any effect on total cell associated/invasion (data not shown), implying that DAHP pre-treatment did not cause increased killing of the bacteria inside macrophages. DAHP pre-treatment also did not show any anti-bacterial effects on *E. coli* K1 even at 10 mM concentration and cytotoxic effects on RAW 264.7 macrophages up to 6 h in culture (data not shown), indicating that the observed differences in total cell associated or intracellular are not due to lack of sufficient bacteria in the medium. In addition, peritoneal macrophages were isolated and examined whether DAHP also prevents *E. coli* K1 binding and invasion in primary macrophages. As shown in Fig 3C, both the total cell associated and invaded bacteria were significantly lower in DAHP treated cells compared to the levels of untreated (control) macrophages. These results suggest that DAHP treatment of macrophages reduces the binding of *E. coli* K1 to the cells, thereby the invasion.

3.4. Inhibition of GCH1 activity with DAHP downregulates expression of CD64 in RAW 264.7 macrophages and prevents bacteremia in newborn mice

E. coli K1 infection of macrophages induces the expression of CD64, which in turn serves as a receptor for the bacterium to bind to and invade the cells [6]. Since inhibition of GCH1 by DAHP prevents the entry of *E. coli* K1 into RAW 264.7 macrophages, we examined the expression of CD64 in these cells with or without DAHP treatment followed infection with

E. coli K1. As expected, *E. coli* K1 infected RAW 264.7 macrophages showed increased expression of CD64 from 30 min post-infection, whereas pretreatment with DAHP using IC₅₀ concentration reduced the levels by 50% (Fig. 4A and B). Further decrease in CD64 levels was also observed with higher concentrations of DAHP despite infection with *E. coli* K1 (data not shown). Similar results showing a decrease in CD64 levels were also obtained when the infected RAW 264.7 macrophages subjected to flow cytometry analysis using anti-CD64 antibodies (Fig. 4C). Similarly, bone marrow derived macrophages and peritoneal macrophages cultured *ex vivo* also showed attenuated total cell associated and intracellular bacteria in the presence of DAHP. Flow cytometry analysis of CD64 in these cell types in the presence of DAHP also exhibited reduced surface expression of CD64, similar to RAW 264.7 macrophages (data not shown). Since our previous studies demonstrated that inhibition of iNOS activity reduced the entry of *E. coli* K1 into macrophages *in vivo*, it is possible that DAHP treated prevents inducible NO production as shown previously, thereby preventing the CD64 expression on the surface of the cells to act as receptors for *E. coli* K1 [14]. We demonstrated that oral administration of DAHP to newborn mice prior to inoculation of *E. coli* K1 protected the mice from the onset of meningitis [11]. To understand further how DAHP pretreatment prevents the onset of meningitis in the newborn mice, we determined the bacteremia levels in these mice 72 h post-infection. In agreement with the previous concept that high grade bacteremia is a pre-requisite for the bacterium to enter the brain, we observed high levels of bacteria in the blood of *E. coli* K1 infected mice (Fig. 4D). DAHP treated animals shown no signs of bacteremia, suggesting that *E. coli* K1 could not establish high grade bacteremia probably due its inability to enter macrophages due to suppression of CD64 expression for multiplication in the presence of DAHP.

4. Discussion

Our studies have clearly demonstrated that macrophages play an important in the onset of meningitis by *E. coli* K1 as depletion of macrophages in newborn mice prevents the disease progression. Therefore, it is possible that macrophages provide a replicative niche required for the development of a certain threshold of bacteremia, which is a pre-requisite for the pathogenesis. Of note, *E. coli* K1 utilizes CD64 as a receptor for binding to and entering macrophages in which the bacterium survives for multiplication. However, it is not clear how the interaction of OmpA of *E. coli* K1 with CD64 controls the anti-phagocytic machinery of macrophages. Here, we demonstrate that *E. coli* K1 interaction with macrophages induces the production of neopterin and biopterin by activating GCH1. Lack of OmpA expression could not elicit pterin production as the bacterium could not efficiently associate with macrophages. Neopterin production in rat peritoneal macrophages suppresses the effect of superoxide generated from NADPH oxidase [2]. Our studies also revealed that OmpA+ *E. coli* infected RAW 264.7 macrophages inhibited the production of superoxide even after treatment with PMA. Therefore, *E. coli* K1-induced neopterin production might suppress superoxide mediated bactericidal activity of macrophages. Neopterin produced by RAW 264.7 macrophages upon *E. coli* K1 infection may re-enter the cells and be intracellularly reduced to di-hydro or tetra-hydro neopterin to exhibit antioxidant activities as previously described [3].

Dimerization of iNOS is required for optimal enzymatic activity for which multiple cofactors including calmodulin, heme, FAD, NADPH, FMN and BH₄ are important [13]. *E. coli* K1 interaction with human brain microvascular endothelial cells (HBMEC) induced biopterin levels, which in turn is responsible for increased production of NO [11]. The interdependence of pterins and NO production was further confirmed by the absence of *E. coli* K1 induced meningitis in newborn mice in which iNOS or GCH1 activity was suppressed with inhibitors [11, 14]. Parallel to HBMEC, *E. coli* K1 infection also increased expression of total biopterin in RAW 264.7 macrophages and a concomitant increase in

iNOS dimerization and NO production. We also measured BH4 after sample oxidation, which is widely accepted to represent the amount of intracellular BH4, and it significantly increased in RAW 264.7 macrophages infected with *E. coli* K1. The decrease in iNOS dimerization and NO production (as total nitrite) upon pre-treatment with DAHP further indicate the interdependence of GCH1-mediated production of biopterin and iNOS activity in macrophages. The survival of *E. coli* K1 in RAW 264.7 macrophages despite increased levels of NO is in contrast to the mechanism utilized by *Chlamydomophila pneumoniae*, an obligate intracellular bacterium that causes respiratory infection. Increased chlamydial growth in RAW 264.7 macrophages with an iNOS inhibitor as well as in iNOS-deficient macrophages clearly indicates that growth of the bacteria in macrophage is sensitive to NO [15].

Unexpectedly in this study, inhibition of GCH1 activity with DAHP significantly reduced the total cell association and entry of *E. coli* K1 in macrophages (both RAW 264.7 macrophages and peritoneal). The decreased invasion frequency of the bacterium into RAW 264.7 macrophages is due to the inhibition of CD64 upregulation on the surface induced by *E. coli* during the infection process. Studies from our lab and Hwu et al revealed that GCH1 activity depends on gp96 and Hsp90 (a homologue of gp96), respectively, indicating that GCH1 mediated production of inducible NO induces gp96 expression, which may assist GCH1 and CD64 maturation/translocation [11, 16]. In addition, the increased production of NO also induces the CD64 expression on the cell surface for bacteria to bind and invade macrophages. Therefore, inhibiting pterin production by targeting GCH1 could be a good therapeutic target for preventing neonatal meningitis by *E. coli*.

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Fig. 1. Infection of RAW 264.7 macrophages with *E. coli* K1 induces GCH expression, neopterin production and suppresses super oxide production

(A) Confluent monolayers of RAW 264.7 macrophages were infected with OmpA+ *E. coli* or OmpA- *E. coli* for varying periods, total cell lysates prepared and subjected to Western blotting with anti-GCH1 or anti-β-actin antibodies. (B) Densitometric analysis of the protein bands from panel A. The values represent an average of areas of bands from three blots performed independently and the error bars represent standard deviation. (C) RAW 264.7 macrophages infected with OmpA+ *E. coli* or OmpA- *E. coli* for 1 h, total cell lysates prepared and subjected to HPLC analysis as described in the Methods. (D) Similarly, RAW 264.7 macrophages were infected with or without PMA pre-treatment as described in the Methods. The supernatants were collected and the production of superoxide was determined, and expressed as luminescence units (LU).

Fig. 2. Inhibition of GCH1 activity in RAW 264.7 macrophages with DAHP prevents iNOS dimerization and NO production

RAW 264.7 macrophage monolayers were infected with OmpA+ *E. coli* or OmpA- *E. coli* for varying periods (only 60 min data shown), total cell lysates prepared and total biopterin (A), biopterin + BH2 (B) or BH4 (C) levels were determined by HPLC. The total cell lysates obtained from infected RAW 264.7 macrophages were subjected to Western blotting under non-denatured and denatured conditions with anti-iNOS or β-actin antibodies (D). Furthermore, supernatants were collected from the same experiments and the total amount NO produced was determined after converting it to nitrite using Greiss reagent (E). All the experiments were performed at least three times.

Fig. 3. Pre-treatment of RAW 264.7 macrophages and peritoneal macrophages with DAHP prevents the binding and invasion of *E. coli* K1

RAW 264.7 macrophages (A and B) or peritoneal macrophages (C) in 24 well plates were pre-treated with various concentrations of DAHP (A) or for varying times with 5 mM DAHP (B and C) prior to infection with *E. coli* K1. Total cell associated or intracellular bacteria were determined as described in Methods. The experiments were performed in triplicate at least three times and the error bars represent standard deviation from the mean values. The total cell associated and intracellular bacteria in Panels A and B are presented as percent values taking control cell parameters as 100%, whereas in Panel C, the data are shown as an absolute number of bacteria.

Fig. 4. DAHP blocks the increased expression of CD64 in RAW 264.7 macrophages upon *E. coli* infection

RAW 264.7 macrophages were left untreated or incubated with 5 mM DAHP for 1 h followed by infection with *E. coli* K1 for varying periods, total cell lysates prepared and subjected to Western blotting using anti-CD64 or anti-β-actin antibodies (A). Densitometric analysis of CD64 and β-actin bands were performed from three separate blots using Image J software, and areas of CD64 normalized to β-actin areas were graphed. The error bars represent standard deviation from the mean values (B). In separate experiments, the RAW 264.7 macrophages were treated as in A and subjected to flow cytometry using anti-CD64 antibodies (C). Mean fluorescence intensities were graphed. The experiments were performed three times and the error bars represent mean values ± standard deviation. Newborn mice on day 3 were pre-treated with DAHP 6 h prior to infection with *E. coli* K1. At 72 h post-infection, blood was withdrawn from the animals, and total bacteria present in the blood were determined by plating the dilutions on LB agar containing rifampicin. * No colonies were observed in all the pups pre-treated with DAHP and infected with *E. coli* (D).