Bacterial Lipoprotein Induces Resistance to Gram-Negative Sepsis in TLR4-Deficient Mice via Enhanced Bacterial Clearance[1]

Gavin C. O'Brien, Jiang Huai Wang,[2] and H. Paul Redmond

TLRs are highly conserved pathogen recognition receptors. As a result, TLR4-deficient C3H/HeJ mice are highly susceptible to Gram-negative sepsis. We have previously demonstrated that tolerance induced by bacterial lipoprotein (BLP) protects wild-type mice against polymicrobial sepsis-induced lethality. In this study, we assessed whether pretreatment of C3H/HeJ mice with BLP could induce resistance to a subsequent Gram-negative *Salmonella typhimurium* infection. Pretreatment with BLP resulted in a significant survival benefit in TLR4-deficient C3H/HeJ mice ($p < 0.0002$ vs control C3H/HeJ) after challenge with live *S. typhimurium* (0.25×10^6 CFU/mouse). This survival benefit was associated with enhanced bacterial clearance from the circulation and in the visceral organs ($p < 0.05$ vs control C3H/HeJ). Furthermore, pretreatment with BLP resulted in significant increases in complement receptor type 3 (CR3) and FcγIII/IIR expression on polymorphonuclear neutrophils (PMNs) and macrophages ($p < 0.05$ vs control C3H/HeJ). There was impaired bacterial recognition and phagocytosis in TLR4-deficient mice compared with wild-type mice. However, a significant augmented uptake, ingestion, and intracellular killing of *S. typhimurium* by PMNs and peritoneal macrophages was evident in BLP-pretreated C3H/HeJ mice ($p < 0.05$ vs control C3H/HeJ). An up-regulation of inducible NO synthase and increased production of intracellular NO were observed in peritoneal macrophages from BLP-pretreated C3H/HeJ mice ($p < 0.05$ vs control C3H/HeJ). Depletion of PMNs did not diminish the beneficial effects of BLP with regard to both animal survival and bacterial clearance. These results indicate that BLP, a TLR2 ligand, protects highly susceptible TLR4-deficient mice from Gram-negative sepsis via enhanced bacterial clearance. ***The Journal of Immunology,*** **2005, 174: 1020–1026.**

Toll-like receptors are a highly conserved family of type I transmembrane proteins characterized by an extracellular domain with leucine-rich repeats and a cytoplasmic Toll/IL-1R homology domain. Thus far, only 10 mammalian TLRs have been deciphered, yet these 10 receptors are thought to recognize and orchestrate the innate immune response to a vast morass of invading pathogens (1). It is believed that TLRs overcome this apparent discrepancy in receptor to pathogen ratio by functioning as pattern recognition receptors, specifically via the recognition of pathogen-associated molecular patterns (PAMPs)[3]. PAMPs are conserved structures within pathogens that activate macrophages, monocytes, and neutrophils to produce cytokines and low molecular mass mediators, thus initiating the innate immune response.

PAMPs tend to be invariant molecular structures that are essential for the survival of the pathogen and thus are far less likely to variability because mutations affecting these structures tend to result in death of the pathogen itself (2). The best-known and most exhaustively studied PAMP is endotoxin or LPS, a major structural component of Gram-negative bacteria. A wealth of biochemical and genetic evidence has identified TLR4 as the receptor that mediates cellular activation in response to LPS (3, 4). Conversely, it is known that TLR4-deficient C3H/HeJ mice are hyporesponsive to the biological effects of LPS (5). PAMPs for TLR2 include a variety of agonists derived from Gram-positive organisms such as peptidoglycan (6) and lipoteichoic acid (7–9). TLR2 plays a crucial role in the host defense against Gram-positive bacteria and their cell wall components. Therefore, it seems that TLR2 and TLR4 are activated primarily by different PAMPs to initiate the host response to Gram-positive and Gram-negative bacterial infection, respectively. This is further illustrated by the fact that TLR2 (but not TLR4) knockout mice are highly susceptible to Gram-positive bacteria, especially *Staphylococcus aureus* (10), whereas TLR4 (but not TLR2) knockout mice are highly susceptible to Gram-negative bacteria such as *Salmonella typhimurium* (5, 11).

The hyporesponsiveness of TLR4-deficient C3H/HeJ mice to LPS has been a stimulus to immunological research for >30 years (12). The apparently paradoxically high susceptibility of these C3H/HeJ mice to live Gram-negative *S. typhimurium* infection was somewhat clarified when the genetic defect was found to correlate with a mutation at a single locus, then termed the defective *Lps* allele (*Lps*^{d} allele) (11). In 1997, Medzhitov et al. (13) described the first human homologue of Toll, now referred to as TLR4. Subsequently, the mutation in the *Lps*^{d} allele was gratifyingly found to correlate with the TLR4 mutation in these C3H/HeJ mice (14).

Several strategies have been explored over the years in attempts to modulate the host response to sepsis. In the early 1960s, it was

Department of Academic Surgery, National University of Ireland, Cork University Hospital, Cork, Ireland

Received for publication April 16, 2004. Accepted for publication November 9, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by The Irish Research Council for Science, Engineering and Technology–the Basic Research Grants Scheme SC/2003/27.

[2] Address correspondence and reprint requests to Dr. Jiang Huai Wang, Department of Academic Surgery, National University of Ireland, Cork University Hospital, Cork, Ireland. E-mail address: jh.wang@ucc.ie

[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; BLP, bacterial lipoprotein; CR3, complement receptor type 3; PMN, polymorphonuclear neutrophil; iNOS, inducible NO synthase; DAF-FM, 4-amino-5-methylaimno-2′,7′-difluorescein.

0022-1767/05/$02.00

observed that animals receiving a low-dose pre-exposure to LPS had a markedly reduced mortality when rechallenged with a "lethal" injection of endotoxin (15). This phenomenon is termed "endotoxin tolerance." Endotoxin tolerance is observed in vivo, with a decreased febrile response and an escape from lethality, as well as in vitro, with reduced production of inflammatory cytokines in response to secondary stimulation by LPS (16). Recently, there has been a dramatic resurgence in interest in the mechanisms responsible for the LPS-tolerant phenotype. It was hypothesized that LPS tolerance occurred via alteration of the intracellular signaling pathways of LPS, since LPS tolerance involves mobilization of NF-κB and a predominant accumulation of p50/p50 homodimers of NF-κB transcription factors in LPS-tolerant cells (17). It has subsequently been shown that endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface TLR4 expression (16). This is an adaptive host response which may represent an essential regulatory mechanism during Gram-negative bacterial infection. The clinical applicability of LPS tolerance, however, is limited. We have previously demonstrated that LPS tolerance affords protection only against the lethal effects of a subsequent LPS challenge. LPS tolerance fails to confer protection against either a lethal bacterial (*S. aureus* and *S. typhimurium*) infection or polymicrobial sepsis (18).

Recently, in our laboratory, we have focused our attention on a lesser-studied bacterial agonist, bacterial lipoprotein (BLP). BLP is characterized by a unique NH_3-terminal lipo-amino acid, *N*-acyl-*S*-diacylglycerol cysteine and is the most abundant protein in the outer membrane of Gram-positive bacteria. BLP serves as a PAMP for Gram-positive and Gram-negative bacteria, initiating an innate immune response via TLR2. TLR2-deficient cells are sensitive to LPS stimulation but have no response to Gram-positive bacterial cell wall components (19). When these cells are transfected with TLR2, they respond to BLP stimulation (20). Furthermore, two recent studies have shown that activation of target cells by highly purified LPS is through TLR4 only and not through TLR2 (3, 21). These data indicate that, in contrast to TLR4, TLR2 is the major receptor for BLP. This accounts for the fact that BLP can both activate a variety of host inflammatory cells to produce proinflammatory cytokines and also to induce lethal shock in both LPS-responsive C3H/HeN mice and LPS-hyporesponsive C3H/HeJ mice (22). We have also shown that pre-exposure of mice to a sublethal dose of BLP induces a BLP tolerance that protects against a subsequent BLP challenge (23). This BLP tolerance can also be induced in human THP-1 monocytes where it is associated with a diminished production of TNF-α and IL-6 as well as suppressed MAPK phosphorylation and NF-κB activation. We have demonstrated that induction of BLP tolerance is associated with down-regulation of TLR2 expression (24). Furthermore, we have shown that BLP tolerance, in addition to protection against a BLP challenge, also protects against endotoxin shock through a cross-tolerance to LPS-induced lethality. More importantly, unlike LPS tolerance, induction of BLP tolerance confers protection against both live bacterial (*S. aureus* and *S. typhimurium*) sepsis and polymicrobial sepsis induced by cecal ligation and puncture (18). This represents a significant potential target for study in sepsis immunology and therapeutic development.

With this in mind, the aim of this study was, first, to determine whether BLP, a TLR2 agonist, could protect TLR4-deficient C3H/HeJ mice against live Gram-negative sepsis and, second, if this proved to be true, to determine the underlying mechanisms involved in this process.

Materials and Methods

Bacterial cell wall components and bacteria

BLP, a synthetic bacterial lipopeptide (Pam_3Cys-Ser-Lys_4-OH; Boehringer Mannheim Biochemica), was endotoxin free as confirmed by the *Limulus* amebocyte assay (Charles River Endosafe). *Staphylococcus typhimurium* was obtained from the National University of Ireland culture collection. Bacteria were cultured at 37°C in trypticase soy broth (Merck), harvested at mid-logarithmic growth phase, washed twice, and resuspended in PBS (Invitrogen Life Technologies) for in vivo use. The concentration of resuspended bacteria was determined and adjusted spectrophotometrically at 550 nm.

BLP pretreatment and S. typhimurium *infection*

Pyrogen-free male C3H/HeN and C3H/HeJ mice (8–10 wk old and 18–22 g) were purchased from Harlan. Mice were housed in barrier cages under controlled environmental conditions (12/12 h of light/dark cycle, 55 ± 5% humidity, 23°C) and had free access to standard laboratory chow and water. Animals were fasted 12 h before experiments and allowed water ad libitum. All animal procedures were conducted in the University Biological Services Unit under a license from the Department of Health and Children (Republic of Ireland).

Mice were pretreated with BLP by i.p. injection of 10 mg/kg BLP 24 h before septic challenges. Control mice received an equal volume (200 μl) of PBS 24 h before septic challenges. Gram-negative sepsis was induced via i.p. injection of 200 μl of PBS containing live *S. typhimurium* (0.25×10^6 CFU/mouse). Survival was monitored for at least 14 days.

Enumeration of bacteria in blood and organs

Control and BLP-pretreated C3H/HeN and C3H/HeJ mice were culled at 24 and 48 h after i.p. injection of live *S. typhimurium* (0.25×10^6 CFU/mouse). Samples of blood were taken and the dissected lungs, liver, and spleen were homogenized. Serial 10-fold dilutions of whole blood and organ homogenates in sterile water containing 0.5% Triton X-100 (Sigma-Aldrich) were plated on trypticase soy agar (Merck) and cultured for 24 h at 37°C for determination of bacterial CFU.

Neutrophil depletion

C3H/HeJ mice were injected i.p. with cyclophosphamide (Sigma-Aldrich) at a dose of 250 mg/kg on day 0 and 100 mg/kg on day 3 to produce neutropenia. On day 4, BLP pretreatment was conducted as described above. Twenty-four hours later, control and BLP-pretreated mice received an i.p. injection of 200 μl of PBS containing 0.25×10^6 CFU of viable *S. typhimurium*, and animal survival was monitored for at least 14 days. The number of *S. typhimurium* in the blood and organs was also determined as described above.

Peritoneal macrophage isolation

Peritoneal cells were isolated from C3H/HeN and C3H/HeJ mice by peritoneal lavage using ice-cold PBS. Cells were incubated with RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS (Invitrogen Life Technologies) in 24-well plates (Falcon) at 37°C in 5% CO for 90 min, and the adherent cells were used as peritoneal macrophages. Peritoneal macrophages (2×10^5 cells/well) were further cultured in fresh RPMI 1640 medium supplemented with 10% FCS.

FACScan analysis of CR3 and FcγIII/IIR expression

Heparinized blood and peritoneal lavage were collected from C3H/HeN, control, and BLP-pretreated C3H/HeJ mice and dual-stained with anti-Ly-6G (a marker for murine polymorphonuclear neutrophils (PMNs; BD Pharmingen), anti-F4/80 Ag (a marker for murine macrophages; Serotec), anti-complement receptor type 3 (CR3; BD PharMingen), and anti- FcγIII/IIR (BD Pharmingen) mAbs conjugated with PE or FITC. PE- or FITC-conjugated anti-mouse isotype-matched Abs (BD Pharmingen) were used as negative controls. Erythrocytes were lysed using lysis buffer (BD Biosciences). FACScan analysis was performed from at least 5,000 events for detecting expression of CR3 and FcγIII/IIR on PMNs (Ly-6G-positive cells) and macrophages (F4/80-positive cells) using CellQuest software (BD Biosciences).

Analysis of bacterial uptake, phagocytosis, and intracellular killing

Staphylococcus typhimurium was heat-killed at 95°C for 20 min and labeled with 0.1% FITC (Sigma-Aldrich) in 0.1 M carbonate buffer (pH 9.5) at 37°C for 60 min. Heparinized blood and peritoneal lavage collected from

C3H/HeN, control, and BLP-pretreated mice were incubated with 1×10^6 CFU/ml heat-killed, FITC-labeled *S. typhimurium* at 37°C for 15 min. Bacterial uptake by PMNs and peritoneal macrophages was assessed by FACScan analysis. PMN and peritoneal macrophage populations were identified by their positive staining for anti-Ly-6G and anti-F4/80, respectively. Bacterial phagocytosis was further determined after the external fluorescence of the bound, but noningested bacteria was quenched with 0.025% crystal violet (Sigma-Aldrich). Intracellular bacterial killing was determined as previously described (18). Briefly, peritoneal macrophages were cultured in RPMI 1640 medium containing 10% FCS and incubated with live *S. typhimurium* (macrophage:bacteria = 1:20) at 37°C for 60 min in the presence of cytochalasin B (5 μg/ml; Sigma-Aldrich) to assess extracellular bacterial killing or absence of cytochalasin B to assess total bacterial killing. After macrophages were lysed, total and extracellular bacterial killing were determined by incubation of serial 10-fold dilutions of the lysates on tryptone soy agar (Merck) plates at 37°C for 24 h. Intracellular bacterial killing was calculated according to the total and extracellular bacterial killing.

Detection of inducible NO synthase (iNOS) expression and intracellular NO production

The expression of iNOS in peritoneal macrophages was detected by Western blot analysis. Briefly, peritoneal macrophages pooled from three control or three BLP-pretreated C3H/HeJ mice were lysed in ice with lysis buffer (1% Triton X-100, 20 mM Tris, 137 mM NaCl, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Protein concentrations were determined using a micro-BCA protein assay reagent kit (Pierce). The proteins were denatured at 95°C for 10 min in loading buffer (60 mM Tris, 2.5% SDS, 10% glycerol, 5% 2-ME, and 0.01% bromphenol blue). Aliquots containing equal amounts of total protein from each sample were separated in SDS-PAGE and transblotted onto nitrocellulose membranes (Schleicher & Schuell Microscience). After blocking for 2 h with TBS containing 0.1% Tween 20 and 6% nonfat milk, membranes were probed overnight at 4°C with anti-iNOS pAb (Chemicon). Blots were further incubated for 1 h with secondary HRP-conjugated anti-rabbit IgG (DakoCytomation) and developed using an ECL detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. The intracellular NO formation in peritoneal macrophages was detected by using the fluorescent probe 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM) diacetate (Molecular Probes) as previously described (25, 26). Briefly, peritoneal cells isolated from control and BLP-pretreated C3H/HeJ mice by peritoneal lavage were incubated in RPMI 1640 medium without phenol red (Invitrogen Life Technologies). Cells were stained with anti-F4/80 Ag mAb conjugated with PE (Serotec) for identifying the macrophage population and further loaded with 5 μM DAF-FM diacetate for 30 min. FACScan analysis for detecting intracellular NO formation in peritoneal macrophages (F4/80-positive cells) was performed from at least 10,000 events on a flow cytometer (BD Biosciences) to detect the log of the mean channel fluorescence intensity with an acquisition of FL1 using CellQuest software (BD Biosciences).

Statistical analysis

All data are presented as the mean ± SD. Statistical analysis was performed using the log rank test for survival studies and the Mann-Whitney *U* test for all others. Differences were judged statistically significant when the $p < 0.05$.

Results

BLP induces resistance to Gram-negative sepsis in TLR4-deficient mice

We previously demonstrated that BLP is capable of inducing cross-tolerance to both LPS and live bacterial (*S. aureus* and *S. typhimurium*) challenges in mice (18). We hypothesized that BLP could protect TLR4-deficient mice against Gram-negative sepsis, to which these mice are highly susceptible. In our first experiment, TLR4-deficient C3H/HeJ mice were randomized to either receive 10 mg/kg BLP i.p. 24 h before bacterial challenge or to receive a control i.p injection of PBS. After 24 h, each mouse received 0.25×10^6 CFU of live *S. typhimurium* i.p. Survival was monitored for at least 14 days. Pretreatment with BLP conferred a statistically significant survival benefit against *S. typhimurium*-induced lethality compared with control mice ($p = 0.0002$; Fig. 1). Therefore, we conclude that BLP, a TLR2 ligand, induces resistance to *S. typhimurium* infection and thus protects TLR4-deficient mice against Gram-negative sepsis.

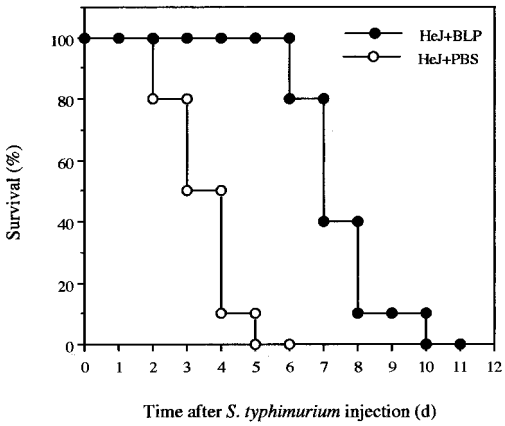

FIGURE 1. Pretreatment with BLP protects C3H/HeJ mice against Gram-negative sepsis. Following pretreatment with BLP, TLR4-deficient C3H/HeJ mice were challenged with live *S. typhimurium* (0.25×10^6 CFU/mouse). Survival was monitored for at least 14 days. The Kaplan-Meier survival graph shows BLP-pretreated animals ($n = 10$) were conferred with a statistically significant survival benefit over the control animals ($n = 10$), with a $p < 0.0002$.

BLP induces resistance to Gram-negative sepsis in TLR4-deficient mice via enhanced bacterial clearance

Having shown a survival benefit, we hypothesized that BLP may confer this benefit via enhanced bacterial clearance. To test this hypothesis, we challenged three groups of mice. The groups consisted of a control group of C3H/HeN mice with normal TLR4 and two groups of TLR4-deficient C3H/HeJ mice, one-half of which were pretreated with BLP (10 mg/kg). Following exposure to 0.25×10^6 CFU of live *S. typhimurium*, mice were culled at 24 and 48 h, respectively. At each time point, samples of blood and homogenates of lungs, liver, and spleen were cultured at 37°C for 24 h whereupon bacterial CFU were enumerated and compared. At the 24-h time point (Fig. 2*a*), we see the significant differences in bacterial dissemination in the bloodstream, lungs, and livers of control C3H/HeJ mice compared with the control C3H/HeN mice, highlighting the grossly dysfunctional Gram-negative bacterial clearance capabilities in animals with a mutated TLR4 ($p < 0.05$). We also saw how BLP pretreatment leads to a dramatic reinstatement of bacterial clearance function to TLR4-deficient mice. Even at this early time point of 24 h, pretreatment with BLP resulted in a dramatic attenuation in bacterial CFU counts in both the bloodstream and the lungs of the C3H/HeJ mice compared with their control counterparts ($p < 0.05$). BLP led to bacterial clearance rates from the bloodstream and lungs which approach those of the wild-type C3H/HeN mice.

At the 48-h time point (Fig. 2*b*), we note a gross difference in bacterial CFU counts between C3H/HeN mice and control C3H/HeJ mice in all samples ($p < 0.05$). Pretreatment with BLP resulted in a significantly enhanced bacterial clearance from the blood and all of the solid viscera of the C3H/HeJ mice compared with the control C3H/HeJ animals ($p < 0.05$). We conclude that BLP protects TLR4-deficient mice against Gram-negative sepsis via enhanced bacterial clearance.

Enhanced PMN and macrophage activation in BLP-pretreated TLR4-deficient mice

We determined CR3 and FcγIII/IIR expression on PMNs and peritoneal macrophages from control and BLP-pretreated TLR4-deficient mice. Pretreatment with BLP resulted in an increase in the

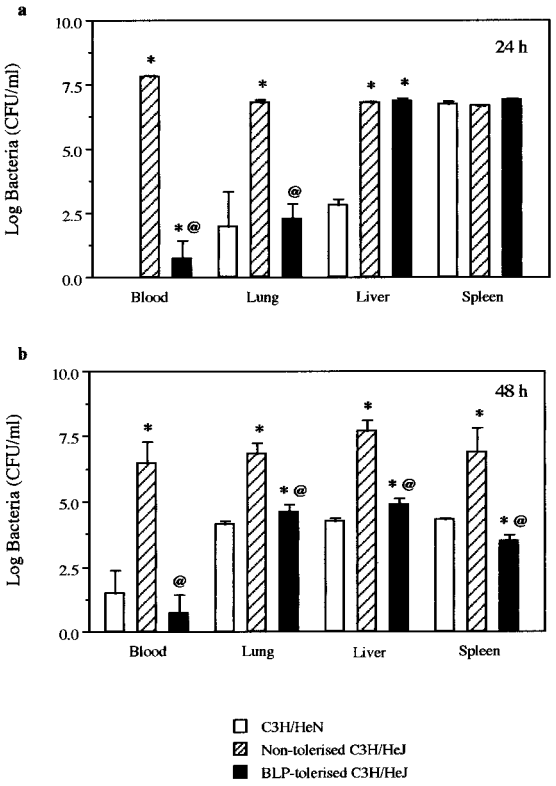

FIGURE 2. Bacterial clearance rates are enhanced in BLP-pretreated C3H/HeJ mice. Wild-type C3H/HeN mice, control, and BLP-pretreated TLR4-deficient C3H/HeJ mice were challenged with 0.25×10^6 CFU of live *S. typhimurium* per mouse. Bacterial CFU were counted in the blood and visceral organs collected at 24 h (*a*) and 48 h (*b*) as described in *Materials and Methods*. Data are expressed as the mean ± SD of five mice per time point. *, $p < 0.05$ vs C3H/HeN mice; @, $p < 0.05$ vs control C3H/HeJ mice.

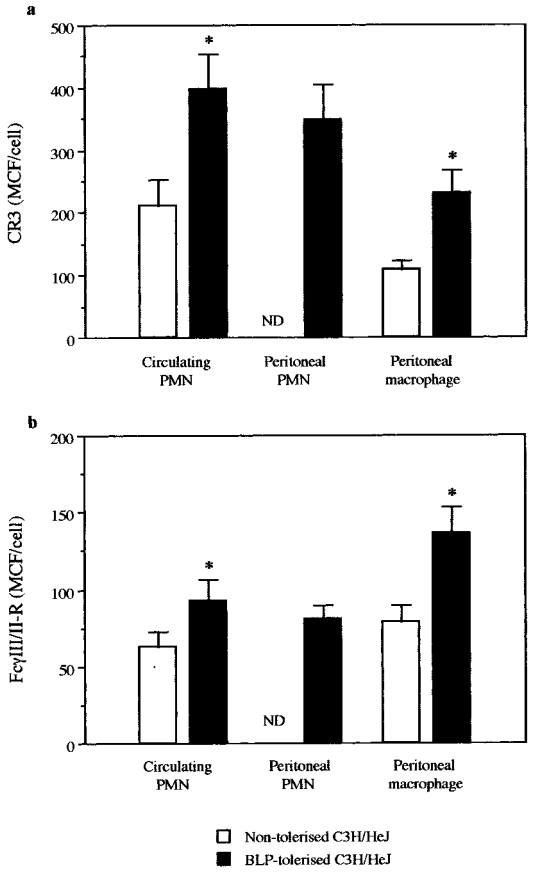

FIGURE 3. BLP pretreatment up-regulates CR3 and FcγIII/IIR expression on C3H/HeJ mouse PMNs and peritoneal macrophages. FACScan analysis of whole blood and peritoneal lavage from control and BLP-pretreated TLR4-deficient C3H/HeJ mice was performed after dual staining with anti-Ly-6G, anti-F4/80, anti-CR3, or anti-FcγIII/IIR mAbs conjugated with PE or FITC. CR3 (*a*) and FcγIII/IIR (*b*) expression on PMNs and peritoneal macrophages was determined and expressed as mean channel fluorescence (MCF) per cell as described in *Materials and Methods*. Data are presented as mean ± SD of 10 mice/group. *, $p < 0.05$ vs control C3H/HeJ mice.

circulating PMN population and a recruitment of PMNs into the peritoneal cavity. There was an associated increase in surface expression of CR3 and FcγIII/IIR expression on these cells ($p < 0.05$; Fig. 3). In addition, BLP pretreatment significantly up-regulated the expression of these two receptors on peritoneal macrophages ($p < 0.05$) (Fig. 3).

Increased bacterial recognition and bactericidal activity in BLP-pretreated TLR4-deficient mice

We next assessed the ability of PMNs and peritoneal macrophages to recognize, phagocytose, and kill bacteria. In this experiment, we compared cells from wild-type C3H/HeN mice with both control and BLP-pretreated C3H/HeJ mice. After incubation with heat-killed or live *S. typhimurium*, PMNs and peritoneal macrophages from control mice had significantly less uptake, phagocytosis, and intracellular killing compared with cells from C3H/HeN mice with normal TLR4 receptors ($p < 0.05$) (Fig. 4). Pretreatment of C3H/HeJ mice with BLP led to significant increases in PMN and macrophage uptake and phagocytosis of *S. typhimurium* ($p < 0.05$; Fig. 4*a*). Furthermore, intracellular killing of *S. typhimurium* by peritoneal macrophages was significantly enhanced in BLP-pretreated mice compared with control C3H/HeJ mice ($p < 0.05$; Fig. 4*b*).

Up-regulation of iNOS expression and increased intracellular NO production in BLP-pretreated TLR4-deficient mice

To further identify possible mechanisms involved in the enhanced bactericidal activity observed in BLP-pretreated TLR4-deficient mice, we assessed the expression of iNOS and the production of intracellular NO in peritoneal macrophages from control and BLP-pretreated C3H/HeJ mice. Western blot analysis revealed that iNOS expression was significantly up-regulated in peritoneal macrophages after pretreatment with BLP in TLR4-deficient mice, whereas this protein was undetectable in peritoneal macrophages from control C3H/HeJ mice (Fig. 5*a*). Furthermore, assessment of intracellular NO formation using the fluorescent probe DAF-FM diacetate-based FACScan analysis showed that the increased production of intracellular NO by the peritoneal macrophages from BLP-pretreated C3H/HeJ mice was statistically significant when compared with NO levels produced by the peritoneal macrophages from control C3H/HeJ mice ($p < 0.05$; Fig. 5, *b* and *c*).

BLP-afforded protection against Gram-negative sepsis is partly independent of neutrophil function

To determine whether PMNs play a crucial role in BLP-afforded protection against Gram-negative bacterial infection in TLR4-deficient C3H/HeJ mice, we repeated both survival study and bacterial clearance experiments, but first depleted the C3H/HeJ mice of their neutrophil population using i.p. cyclophosphamide injection (mean reduction of 96% PMNs, data not shown). Although neutrophil depletion resulted in a faster mortality rate in both groups,

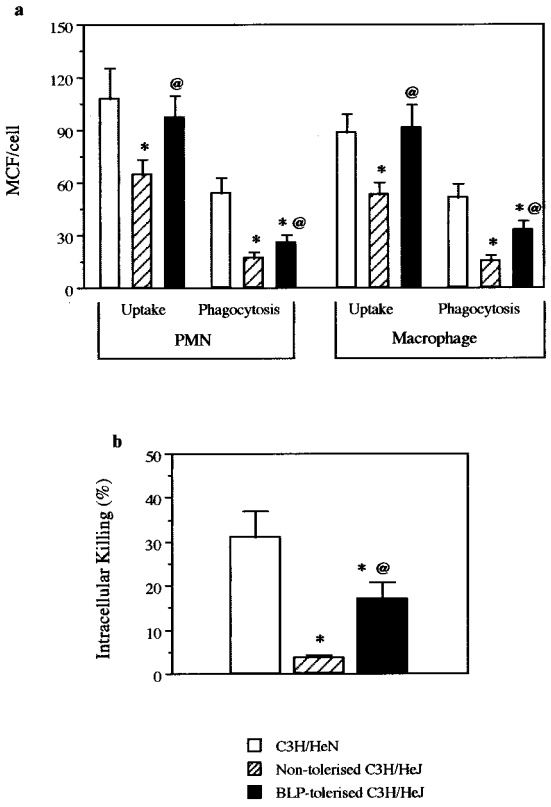

FIGURE 4. Enhanced bacterial recognition, phagocytosis, and intracellular killing in BLP-pretreated C3H/HeJ mice. *a,* Bacterial uptake and phagocytosis by circulating PMNs and peritoneal macrophages from wild-type C3H/HeN, control, and BLP-pretreated TLR4-deficient C3H/HeJ mice were assessed by FACScan analysis as described. Data are expressed as mean ± SD of five separate experiments. *, $p < 0.05$ vs C3H/HeN mice; @, $p < 0.05$ vs control C3H/HeJ mice. *b,* Intracellular bacterial killing by peritoneal macrophages was determined as described. Data are presented as mean ± SD of five independent experiments. Each experiment was conducted in triplicate. *, $p < 0.05$ vs C3H/HeN mice; @, $p < 0.05$ vs control C3H/HeJ mice.

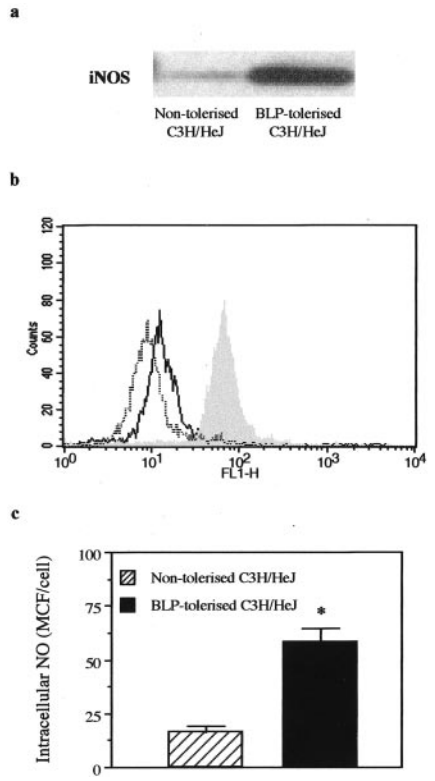

FIGURE 5. BLP pretreatment up-regulates iNOS expression and increases intracellular NO production in C3H/HeJ peritoneal macrophages. *a,* Cellular extracts were prepared from peritoneal macrophages and subjected to Western blot analysis. iNOS expression was detected using anti-mouse iNOS pAb. The results presented are representative of three independent experiments. *b,* Intracellular NO production in peritoneal macrophages was detected by the fluorescent probe, DAF-FM diacetate, on FACScan analysis. The filled histogram is DAF-FM-stained macrophages from BLP-pretreated C3H/HeJ mice, the dark line histogram is DAF-FM-stained macrophages from control C3H/HeJ mice, and the dotted line histogram is nonstained macrophages used as control. A representative of three separate assays is shown. *c,* Summary of intracellular NO production in peritoneal macrophages from control and BLP-pretreated C3H/HeJ mice. Data are expressed as the mean ± SD and are representative of three separate experiments. *, $p < 0.05$ vs control C3H/HeJ mice.

pretreatment of C3H/HeJ mice with BLP resulted in a significant survival benefit over the control mice ($p = 0.0043$; Fig. 6). Furthermore, in neutropenic C3H/HeJ mice, BLP pretreatment resulted in a statistically significant increase in bacterial clearance from the blood, lungs, and spleens compared with the control neutropenic C3H/HeJ mice at the 24- and 48-h time points ($p < 0.05$; Fig. 7).

Discussion

Despite significant advances in our understanding of the molecular and genetic basis of sepsis and its associated immunologic response, sepsis remains a problem worldwide. Sepsis is the primary cause of death in the noncoronary intensive care unit (27) and septic shock is estimated to be responsible for >100,000 deaths annually in the United States (28). In most institutions, Gram-negative bacteria account for ~50% of all bacterimias, with mortality estimated at 10% for normal individuals and 30% in immunocompromised patients (29).

The cloning of TLR4 and its identification as the primary LPS receptor in mammals prompted the discovery that specific mutations in the *TLR4* gene (Asp299Gly and Thr399Ile) are associated with a diminished airway response to inhaled LPS in normal human volunteers (30). Subsequently, it has been shown that within an intensive care unit-based septic shock cohort, patients with a TLR4 polymorphism had more severe disease and an increased frequency of Gram-negative infections. These findings indicate that mutations in the TLR4 may predispose patients to Gram-negative septic shock (31). A similar study in a human septic-shock population suggested an association between a novel TLR2 (Arg753Gln) polymorphism and Gram-positive staphylococcal infection (32).

We have previously shown that BLP, a TLR2 agonist, is capable of inducing cross-tolerance to *S. typhimurium*, Gram-negative bacteria which signals via TLR4. In this article, we have gone on to show that BLP pretreatment can protect TLR4-deficient mice against live *S. typhimurium* challenge, to which these mice are extremely susceptible. The main mechanism underlying this process seems to be associated with enhanced bacterial clearance. Pretreatment of TLR4-deficient mice with BLP leads to enhanced clearance from the bloodstream and greatly reduced end-organ bacterial dissemination of *S. typhimurium* compared with control animals.

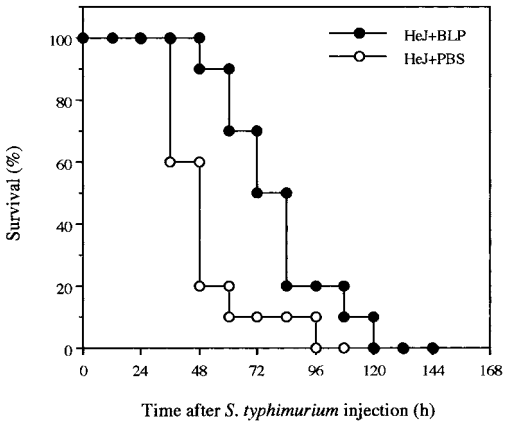

FIGURE 6. Pretreatment with BLP enhances survival in neutrophil-depleted C3H/HeJ mice following live *S. typhimurium* challenge. TLR4-deficient C3H/HeJ mice were depleted of their neutrophil population via i.p. cyclophosphamide administration. Control ($n = 10$) and BLP-pretreated ($n = 10$) C3H/HeJ mice were subsequently challenged with live *S. typhimurium* (0.25×10^6 CFU/mouse). Survival was monitored at 12-hour intervals for at least 14 days. The Kaplan-Meier survival graph shows that neutrophil-depleted BLP-pretreated C3H/HeJ animals were conferred with a statistically significant survival benefit over the neutrophil-depleted control C3H/HeJ mice, with a $p = 0.0043$.

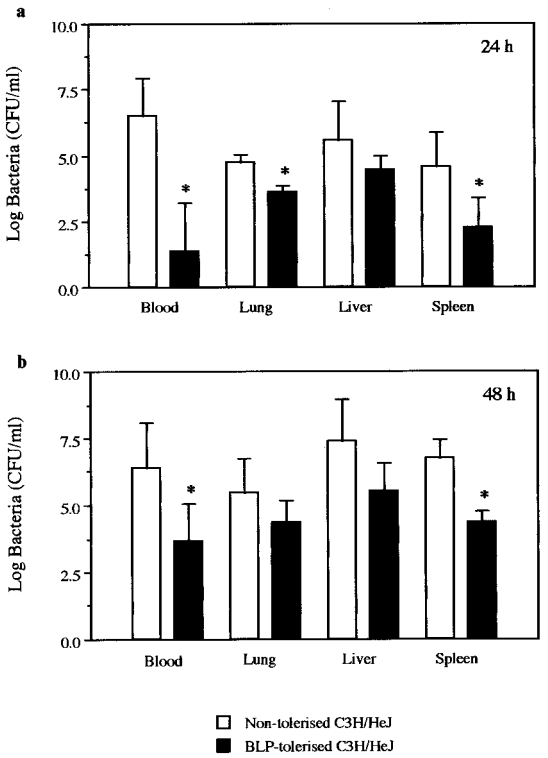

FIGURE 7. BLP-pretreated neutrophil-depleted C3H/HeJ mice show enhanced bacterial clearance. After depleting the neutrophil population, control and BLP-pretreated TLR4-deficient C3H/HeJ mice were challenged with live *S. typhimurium* (0.25×10^6 CFU/mouse). Bacterial CFU were counted in the blood and visceral organs collected at 24 h (*a*) and 48 h (*b*) as described. Data are expressed as the mean ± SD of five mice per time point. *, $p < 0.05$ vs control, neutrophil-depleted, C3H/HeJ mice.

Since C3H/HeJ mice have mutated TLR4 receptors, it is clear that the enhanced bacterial clearance cannot be due to an up-regulation or modification of TLR4 but rather due to enhanced nonspecific antibacterial function. The uptake, phagocytosis, and killing of invading bacteria are mediated via activation of CR3 and FcγIII/IIR (33, 34). Overexpression of these two receptors is associated with enhanced bacterial clearance (35). To investigate possible mechanisms contributing to the enhanced host defense against Gram-negative sepsis observed in BLP-pretreated mice, we assessed CR3 and FcγIII/IIR expression on PMNs and peritoneal macrophages. Pretreatment with BLP resulted in an increased PMN population in the circulation and peritoneal cavity, with overexpression of CR3 and FcγIII/IIR on PMNs as well as on peritoneal macrophages. To determine whether this altered phenotype of PMNs and peritoneal macrophages is associated with BLP-afforded protection against Gram-negative sepsis, we further assessed the ability of these cells to recognize, ingest, and kill *S. typhimurium*. PMNs and peritoneal macrophages from BLP-pretreated TLR4-deficient mice, in keeping with the activation of phagocytic receptors on these cells, exhibited increased bacterial recognition, phagocytosis, and intracellular killing. The enhanced bacterial recognition and microbicidal activity observed in BLP-pretreated TLR4-deficient mice may contribute toward their improved survival over control TLR4-deficient mice following lethal Gram-negative sepsis.

Next, we attempted to identify possible molecular mechanisms that contribute to the enhanced peritoneal macrophage-mediated intracellular killing of *S. typhimurium* observed in BLP-pretreated TLR4-deficient C3H/HeJ mice. Macrophages, the resident professional phagocytes, are an early sensor of bacterial infection via up-regulation of iNOS expression and NO production. NO produced by macrophages is responsible for the initial microbicidal activity against intracellular pathogens including *S. typhimurium* (36, 37). In iNOS knockout mice, failure to generate NO leads to susceptibility to *S. typhimurium* infection with an increased mortality (37, 38). Furthermore, peritoneal macrophages derived from iNOS knockout mice display impaired intracellular killing of *S. typhimurium* (36). In the present study, we showed that pretreatment with BLP in TLR4-deficient C3H/HeJ mice resulted in up-regulation of iNOS expression and increased production of intracellular NO. This may partially explain the enhanced intracellular killing of *S. typhimurium* by peritoneal macrophages in vitro and the accelerated bacterial clearance in vivo observed in BLP-pretreated C3H/HeJ mice.

PMNs are the first line of defense against bacterial infection. To further assess the mechanisms underlying the enhanced survival and bacterial clearance seen in BLP-pretreated TLR4-deficient mice, we repeated both the survival study and the bacterial clearance studies, first having depleted the mice of their neutrophils using i.p. cyclophosphamide. To our surprise, BLP-pretreated, TLR4-deficient, neutropenic mice again displayed a statistically significant survival benefit over their control counterparts. Despite neutropenia, BLP-pretreated mice showed enhanced bacterial clearance from the bloodstream and visceral organs following live *S. typhimurium* challenge. These findings, in combination with our in vitro findings, suggest that macrophage-mediated phagocytosis and microbicidal activity must play a paramount role in BLP-pretreated TLR4-deficient mice.

The emerging wealth of evidence pointing toward a genetic predisposition in individual patients to develop sepsis will have important ramifications in the clinical setting. Already studies have shown a clinical correlation between TLR4 and TLR2 polymorphisms in humans and the development of Gram-negative and Gram-positive sepsis, respectively (31, 32). Individuals with these naturally occurring polymorphisms represent an extremely high-risk population in terms of the development of postoperative sepsis, for whom, to date, no specific prophylactic measures exist.

With future increased availability of genetic profiling, it will become possible to preoperatively identify patients with TLR polymorphisms. This will allow the application of tailored prophylactic measures to prevent postoperative sepsis and morbidity. Our findings may represent one such measure. Pretreatment with BLP mediates a novel adaptive host response associated with unique protective effects during septic shock. Unlike LPS tolerance, BLP can induce cross-tolerance to LPS, live bacterial, and polymicrobial sepsis-induced lethality. In this study, we have demonstrated the unique ability of BLP to induce resistance to *S. typhimurium* infection in TLR4-deficient mice and thus protect these mice against Gram-negative sepsis.

References

1. Ulevitch, R. J. 2001. New therapeutic targets revealed through investigations of innate immunity. *Crit. Care Med. 29:S8*.
2. Medzhitov, R., and C. A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell 91:295*.
3. Tapping, R. I., S. Akashi, K. Miyake, P. J. Godowski, and P. S. Tobias. 2000. Toll-like receptor 4, but not Toll-like receptor 2, is a signaling receptor for *Escherichia* and *Salmonella* lipopolysaccharides. *J. Immunol. 165:5780*.
4. Akashi, S., R. Shimazu, H. Ogata, Y. Nagai, K. Takeda, M. Kimoto, and K. Miyake. 2000. Cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. *J. Immunol. 164:3471*.
5. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. TLR4-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol. 162:3749*.
6. Iwaki, D., H. Mitsuzawa, S. Murakami, H. Sano, M. Konishi, T. Akino, and Y. Kuroki. 2002. The extracellular TLR2 domain directly binds peptidoglycan derived from *Staphylococcus aureus. J. Biol. Chem. 277:24315*.
7. Schwander, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan and lipoteichoic acid-induced activation is mediated via TLR2. *J. Biol. Chem. 274:17406*.
8. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Recognition of Gram-positive cell wall components by the innate immune system occurs via the Toll-like receptor 2. *J. Immunol. 163:1*.
9. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by lipoproteins through Toll-like receptors. *Science 285:732*.
10. Takeuchi, O., K. Hoshino, and S. Akira. 2000. TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol. 165:5392*.
11. O' Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. McDermott, and S. B. Formal. 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the *Lps* gene. *J. Immunol. 124:20*.
12. Sultzer, B. M. 1968. Genetic control of leukocyte responses to endotoxin. *Nature 219:1253*.
13. Medzhitov, R., P. Preston-Hulbert, and C. Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394*.
14. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in the *Tlr4* gene. *Science 282:2085*.
15. Greisman, S. E., R. B. Hornick, F. A. Carozza, and T. E. Woodward. 1964. Endotoxin tolerance. *J. Clin. Invest. 43:986*.
16. Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, and S. Akira. 2000. Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. *J. Immunol. 164:3476*.
17. Ziegler-Heitbrock, H. W., A. Wedel, W. Schraut, M. Strobel, P. Wendelglass, T. Sternsdorf, P. A. Bauerle, J. G. Haas, and G. Riethmuller. 1994. Tolerance to lipopolysaccharide involves mobilization of nuclear factor κB with predominance of p50 homodimers. *J. Biol. Chem. 269:17001*.
18. Wang, J. H., M. Doyle, B. J. Manning, S. Blankson, Q. D. Wu, C. Power, R. A. Cahill, and H. P. Redmond. 2003. Cutting edge: bacterial lipoprotein induces endotoxin-independent tolerance to septic shock. *J. Immunol. 170:14*.
19. Heine, H., C. J. Kirschning, E. Lien, B. G. Monks, M. Rothe, and D. T. Golenbock. 1999. Cells that carry a null allele for Toll-like receptor 2 are capable of responding to endotoxin. *J. Immunol. 162:6971*.
20. Hirschfield, M., C. J. Kirschning, R. Schwander, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J. Immunol. 163:2382*.
21. Hirschfield, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol. 165:618*.
22. Zhang, H., J. W. Peterson, D. W. Nielsen, and G. R. Klimpel. 1997. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. *J. Immunol. 159:4868*.
23. Doyle, M., J. H. Wang, and H. P. Redmond. 2000. Bacterial lipoprotein induces endotoxin independent tolerance to systemic inflammatory response syndrome in vivo and in vitro. *Surg. Forum 51:191*.
24. Wang, J. H., M. Doyle, B. J. Manning, Q. D. Wu, S. Blankson, and H. P. Redmond. 2002. Induction of bacterial lipoprotein tolerance is associated with suppression of Toll-like receptor 2 expression. *J. Biol. Chem. 277:36068*.
25. Kojima, H., N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, and T. Nagano. 1998. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem. 70:2446*.
26. Marriott, H. M., F. Ali, R. C. Read, T. J. Mitchell, M. K. Whyte, and D. H. Dockrell. 2004. Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection. *FASEB J. 18:1126*.
27. Parillo, J. E., M. M. Parker, C. Natanson, A. F. Suffredini, R. L. Danner, R. E. Cunnion, and F. P. Ognibene. 1990. Septic shock: advances in the understanding of the pathogenesis, cardiovascular dysfunction, and therapy. *Ann. Intern. Med. 113:227*.
28. Mendez, C., A. A. Kramer, K. F. Salhab, G. A. Valdes, J. G. Norman, K. J. Tracey, and L. C. Carey. 1999. Tolerance to shock; an exploration of mechanism. *Ann. Surg. 229:843*.
29. Dunn, D. 1994. Gram-negative bacterial sepsis and sepsis syndrome. *Surg. Clin. North Am. 74:621*.
30. Arbour, N. C., E. Lorenz, B. C. Schutte, J. Zabner, J. N. Kline, M. Jones, K. L. Frees, J. L. Watt, and D. A. Schwartz. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet. 25:187*.
31. Lorenz, E., J. P. Mira, K. L. Frees, and D. A. Schwartz. 2002. Relevance of mutations in the TLR4 receptor in patients with Gram-negative septic shock. *Arch. Intern. Med. 162:1028*.
32. Lorenz, E., J. P. Mira, K. L. Cornish, N. C. Arbour, and D. A. Schwartz. 2000. A novel polymorphism in the Toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect. Immun. 68:6398*.
33. Ehlers, M. R. 2000. CR3: a general purpose adhesion recognition receptor essential for innate immunity. *Microbes Infect. 2:289*.
34. Adarem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol. 17:593*.
35. Weighardt, H., C. Feterowski, M. Veit, M. Rump, H. Wagner, and B. Holzmann. 2000. Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to enhanced innate effector response. *J. Immunol. 165:4537*.
36. Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med. 192:227*.
37. Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J. Exp. Med. 192:237*.
38. Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. C. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity 10:29*.