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## Cutting Edge: Bacterial Lipoprotein Induces Endotoxin-Independent Tolerance to Septic Shock **FREE**

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## CUTTING EDGE

## Cutting Edge: Bacterial Lipoprotein Induces Endotoxin-Independent Tolerance to Septic Shock

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*Tolerance to bacterial cell wall components is an adaptive host response. Endotoxin/LPS tolerance is characterized by a survival advantage against subsequent lethal LPS challenge. However, it is uncertain whether LPS tolerance can afford protection against other septic challenges. In this study, we show that tolerance induced by bacterial lipoprotein (BLP) protects mice against not only BLP-induced lethality, but also LPS-, live bacteria-, and polymicrobial sepsis-induced lethality. In contrast, LPS tolerance offers no survival benefit against the latter two challenges. Furthermore, induction of BLP tolerance results in over-expression of complement receptor type 3 and FcγIII/IIIR on neutrophils (polymorphonuclear neutrophils) and peritoneal macrophages, with increased bacterial recognition and bactericidal activity, whereas LPS-tolerized mice exhibit an impaired ability to ingest and to kill bacteria. These results indicate that BLP tolerance is a novel adaptive host response associated with a unique protective effect during septic shock. The Journal of Immunology, 2003, 170: 14–18.*

Pre-exposure to LPS induces a transient state of cellular hyporesponsiveness to a secondary LPS challenge with diminished production of proinflammatory cytokines, and protection against a subsequent lethal LPS challenge resulting in a significant survival advantage (1, 2). This well-established phenomenon, first described in the 1960s (3), is termed LPS tolerance. It is an adaptive host response which may represent an essential regulatory mechanism during Gram-negative bacterial infection.

Bacterial lipoprotein (BLP),<sup>3</sup> characterized by a unique NH<sub>2</sub>-terminal lipo-amino acid, *N*-acyl-*S*-diacylglycerol cysteine, is the most abundant protein in the outer membrane of both Gram-positive and Gram-negative bacteria. Like LPS, BLP can be released from proliferating *Escherichia coli*, and treatment of bacteria with antibiotics significantly enhances BLP release (4). BLP is known to activate monocytes/macrophages to produce inflammatory cytokines and to induce lethal

shock in both LPS-responsive C<sub>3</sub>H/HeN mice and LPS-hyporesponsive C<sub>3</sub>H/HeJ mice (5–7). Pre-exposure of murine macrophages (8) and human THP-1 monocytes (9) to BLP induces tolerance to the stimulatory effects of BLP but also a cross-tolerance to LPS, which leads us to investigate the protective effect of BLP tolerance in septic shock and to compare this with LPS tolerance.

## Materials and Methods

### Bacterial cell wall components and bacteria

LPS from *E. coli* O55:B5 (Sigma-Aldrich, St. Louis, MO) and BLP, a synthetic bacterial lipopeptide (Pam<sub>3</sub>Cys-Ser-Lys<sub>4</sub>-OH; Boehringer Mannheim Biochemical, Mannheim, Germany) that was endotoxin-free as confirmed by limulus amoebocyte lysate assay (Charles River Endosafe, Charleston, SC), were dissolved in PBS (Life Technologies, Paisley, Scotland, U.K.). *Staphylococcus aureus* 14458 and *Salmonella typhimurium* were obtained from American Type Culture Collection (Manassas, VA) and the National University of Ireland culture collection, respectively. Bacteria were cultured at 37°C in trypticase soy broth (Merck, Darmstadt, Germany), harvested at the mid-logarithmic growth phase, washed twice, and resuspended in PBS for in vivo use. The concentration of resuspended bacteria was determined and adjusted spectrophotometrically at 550 nm.

### Septic shock models

Pyrogen-free male MF-1, C57BL/6, and CD-1 mice (8- to 10-wk old and 18–22 g) were purchased from Harlan (Oxon, U.K.). All animal procedures were conducted under a license from the Department of Health and Children (Republic of Ireland). Tolerance in mice was induced by i.p. injection of 10 mg/kg BLP (BLP tolerance), 10 mg/kg LPS (LPS tolerance), or an equal volume (200 μl) of PBS (no tolerance) 24 h before septic challenges. Nontolerized, BLP-tolerized, and LPS-tolerized male MF-1 mice were injected i.p. with BLP (45 mg/kg), LPS (45 mg/kg), or their combination (30 plus 30 mg/kg). Survival was monitored for at least 10 days. Male C57BL/6 mice were tolerized to BLP or LPS and received an i.p. injection of 200 μl PBS containing live *S. aureus* and *S. typhimurium* (2 × 10<sup>7</sup> plus 2 × 10<sup>6</sup> CFU/mouse). Survival was monitored for at least 14 days. Following induction of BLP or LPS tolerance, male CD-1 mice were subjected to polymicrobial sepsis induced by cecal ligation and puncture (CLP) (10). Briefly, the cecum was exposed through a 1.0–1.5 cm abdominal midline incision in anesthetized mice, ligated at its base with 3-0 silk suture, and punctured twice with an 18-gauge needle. The cecum was then returned to the peritoneal cavity and the abdominal incision was closed. Survival was monitored for at least 10 days.

### Cytokine measurements

We performed additional experiments in mice as described above. Blood samples from nontolerized, BLP-tolerized, and LPS-tolerized mice were collected at

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<sup>3</sup> Abbreviations used in this paper: BLP, bacterial lipoprotein; CLP, cecal ligation and puncture; CR3, complement receptor type 3; PMN, polymorphonuclear neutrophil.

different time points after septic challenges. Serum TNF- $\alpha$ , IL-6, IL-10, and IL-12 were determined by ELISA (R&D Systems, Minneapolis, MN).

#### FACSscan analysis of immunofluorescence

Heparinized blood and peritoneal lavage were collected from nontolerized, BLP-tolerized, and LPS-tolerized mice and dual-stained with anti-Ly-6G (BD PharMingen, San Diego, CA), anti-F4/80 Ag (Serotec, Oxford, U.K.), anti-complement receptor type 3 (CR3; BD PharMingen), and anti-Fc $\gamma$ III/IIIR (BD PharMingen) mAbs conjugated with PE or FITC. PE- or FITC-conjugated anti-mouse isotype-matched mAbs (BD PharMingen) were used as negative controls. Erythrocytes were lysed using lysis buffer (BD Biosciences, Mountain View, CA). FACSscan analysis was performed from at least 5000 events for detecting the expression of CR3 and Fc $\gamma$ III/IIIR on polymorphonuclear neutrophils (PMN; Ly-6G-positive cells) and macrophages (F4/80-positive cells) using CellQuest software (BD Biosciences).

#### Bacterial uptake, ingestion, and intracellular killing

*S. aureus* and *S. typhimurium* were heat-killed at 95°C for 20 min and labeled with 0.1% FITC (Sigma-Aldrich). Heparinized blood and peritoneal lavage collected from nontolerized, BLP-tolerized, and LPS-tolerized mice were incubated with  $1 \times 10^6$  CFU/ml of heat-killed, FITC-labeled *S. aureus* or *S. typhimurium* at 37°C for 15 min. Bacterial uptake by PMN and peritoneal macrophages was assessed by FACSscan analysis. Bacterial ingestion 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 (11). Briefly, peritoneal macrophages were incubated with live *S. aureus* or *S. typhimurium* (macrophage:bacteria = 1:20) at 37°C for 60 min, in the presence or absence of cytochalasin B (5  $\mu$ g/ml; Sigma-Aldrich). After macrophages were lysed, total and extracellular bacterial killing were determined by incubation of serial 10-fold dilutions of the lysates on tryptone soya agar (Merck) plates at 37°C for 24 h. Intracellular bacterial killing was calculated according to the total and extracellular bacterial killing.

#### Statistical analysis

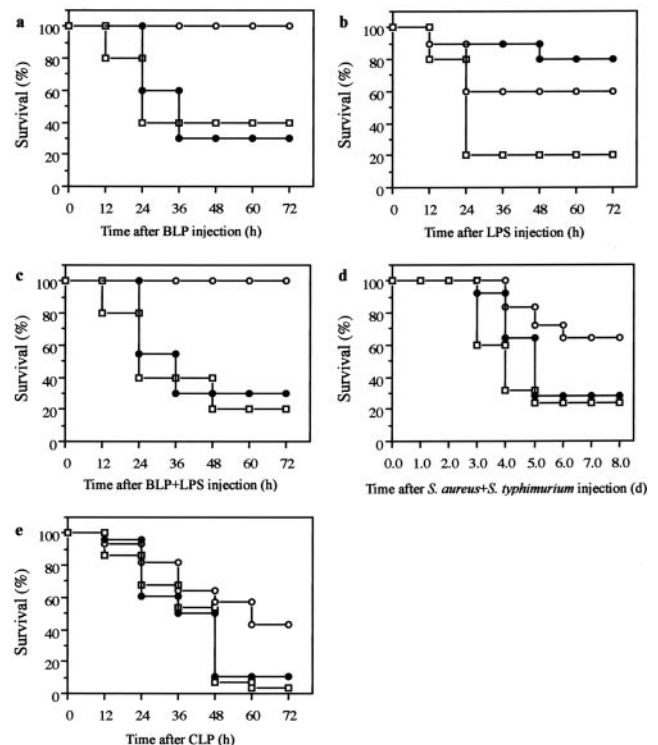
All data are presented as the mean  $\pm$  SD. Statistical analysis was performed using the log rank test for survival studies and the Mann-Whitney *U* test for all others. Differences were judged statistically significant when  $p < 0.05$ .

## Results

### Improved survival after lethal septic challenges in BLP-tolerized mice

Induction of BLP tolerance completely protected mice against BLP-induced lethality with 100% survival as compared with 40% survival in nontolerized mice ( $p = 0.0000$ ; Fig. 1*a*). Furthermore, BLP tolerance significantly improved survival in endotoxic shock, from 20% in nontolerized mice to 60% ( $p = 0.0152$ ; Fig. 1*b*), indicating a cross-tolerance to LPS-induced lethality. Complete protection was also conferred by BLP tolerance in mice challenged with a combination of BLP and LPS ( $p = 0.0000$  vs nontolerized mice; Fig. 1*c*). LPS tolerance protected against its own lethality by reducing the mortality rate from 80% in nontolerized mice to 20% ( $p = 0.0002$ ; Fig. 1*b*). However, it failed to protect mice against lethal BLP challenge and a combined BLP plus LPS challenge (Fig. 1, *a* and *c*).

To determine whether BLP tolerance could afford protection in clinically relevant states, we used two sepsis models, i.e., bacterial infection induced by live *S. aureus* and *S. typhimurium*, and polymicrobial sepsis induced by CLP. Forty percent of nontolerized mice died on day 3 after *S. aureus* and *S. typhimurium* challenge, and only 24% survived up to day 14, whereas BLP-tolerized mice were resistant to *S. aureus* and *S. typhimurium* infection with 100% survival on day 3 and 64% survival up to day 14 ( $p = 0.0008$ ; Fig. 1*d*). BLP tolerance-afforded protection was also observed in mice challenged with CLP-induced polymicrobial sepsis in which survival was significantly improved from 3.6% in nontolerized mice to 43% in BLP-tolerized mice ( $p = 0.0013$ ; Fig. 1*e*). However, induction of LPS tolerance offered no protection against either lethal bac-



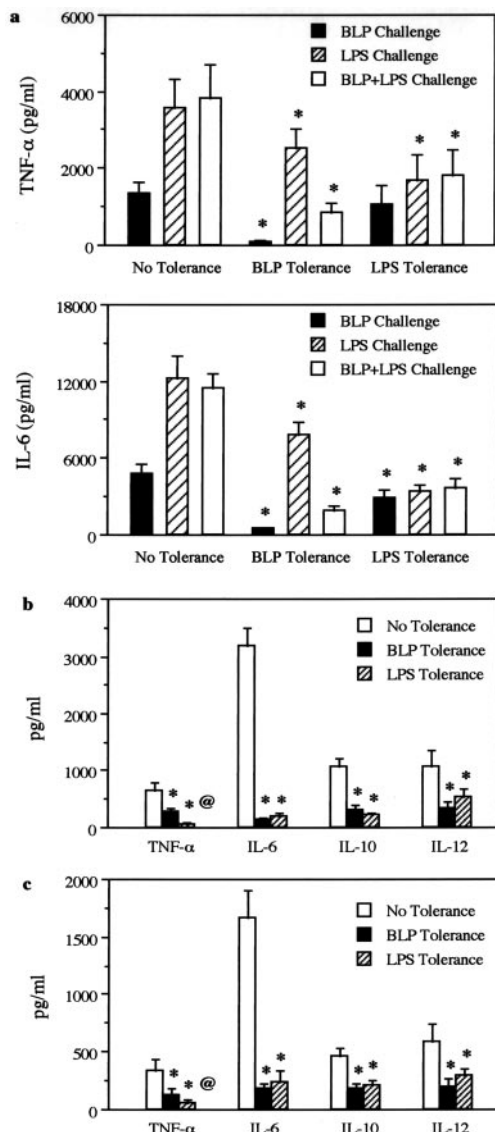
**FIGURE 1.** Induction of BLP tolerance protects mice against different septic challenge-induced lethality. Following induction of BLP tolerance (○), LPS tolerance (●), or no tolerance (□), male MF-1 mice ( $n = 20$  per group) were challenged with BLP (45 mg/kg) (*a*), LPS (45 mg/kg) (*b*), or their combination (30 plus 30 mg/kg) (*c*), male C57BL/6 mice ( $n = 25$  per group) were challenged with a combination of live *S. aureus* and *S. typhimurium* ( $2 \times 10^7$  plus  $2 \times 10^6$  CFU/mouse) (*d*), and male CD-1 mice ( $n = 28$  per group) were challenged with CLP-induced polymicrobial sepsis (*e*). Survival was monitored for at least 14 days in animals challenged with live bacteria or at least 10 days in other groups.

terial infection or polymicrobial sepsis (Fig. 1, *d* and *e*). Furthermore, tolerance induced by other forms of LPS derived from *E. coli* O111:B4 and *S. typhimurium* did not protect mice against lethal BLP challenge, or against microbial sepsis (data not shown).

#### Attenuated inflammatory cytokine production in both BLP- and LPS-tolerized mice

We measured serum levels of TNF- $\alpha$  and IL-6 in BLP-tolerized mice following lethal BLP and LPS challenges and compared them to levels encountered in LPS-tolerized mice. As shown in Fig. 2*a*, induction of BLP tolerance resulted in a near complete attenuation of TNF- $\alpha$  and IL-6 release in mice challenged with lethal BLP and also resulted in a significant reduction in peak serum levels of these two cytokines in mice challenged with lethal LPS. LPS tolerance significantly attenuated the release of TNF- $\alpha$  and IL-6 in LPS-challenged mice as well as IL-6 in BLP-challenged mice, but had no inhibitory effect on TNF- $\alpha$  production induced by lethal BLP challenge (Fig. 2*a*).

Lethal *S. aureus* and *S. typhimurium* challenge in naive mice resulted in an increased release of proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12 as well as anti-inflammatory cytokine IL-10. These increases were significantly attenuated in both BLP-tolerized and LPS-tolerized mice (Fig. 2*b*). Similar results were found in BLP-tolerized and LPS-tolerized mice subjected



**FIGURE 2.** Attenuated pro- and anti-inflammatory cytokine production in BLP- and LPS-tolerized mice. *a*, Serum cytokine levels after MF-1 mice were challenged with lethal BLP, LPS, or their combination. Data shown here are the results of peak serum levels of TNF- $\alpha$  at 90 min and IL-6 at 4 h, and expressed as the mean  $\pm$  SD of five independent experiments. \*,  $p < 0.05$  vs nontolerized mice. *b* and *c*, Serum cytokine levels after C57BL/6 mice were challenged with live *S. aureus* plus *S. typhimurium* (*b*) or after CD-1 mice were subjected to CLP-induced polymicrobial sepsis (*c*). Data shown here are the results of peak serum levels of TNF- $\alpha$  at 2 h, IL-10 and IL-12 at 4 h, and IL-6 at 6 h, and expressed as the mean  $\pm$  SD of five independent experiments. \*,  $p < 0.05$  vs nontolerized mice; @,  $p < 0.05$  vs BLP-tolerized mice.

to CLP-induced polymicrobial sepsis (Fig. 2*c*). Notably, there was a more profound inhibition of TNF- $\alpha$  production observed in LPS-tolerized mice following challenges with lethal bacterial infection or polymicrobial sepsis (Fig. 2, *b* and *c*).

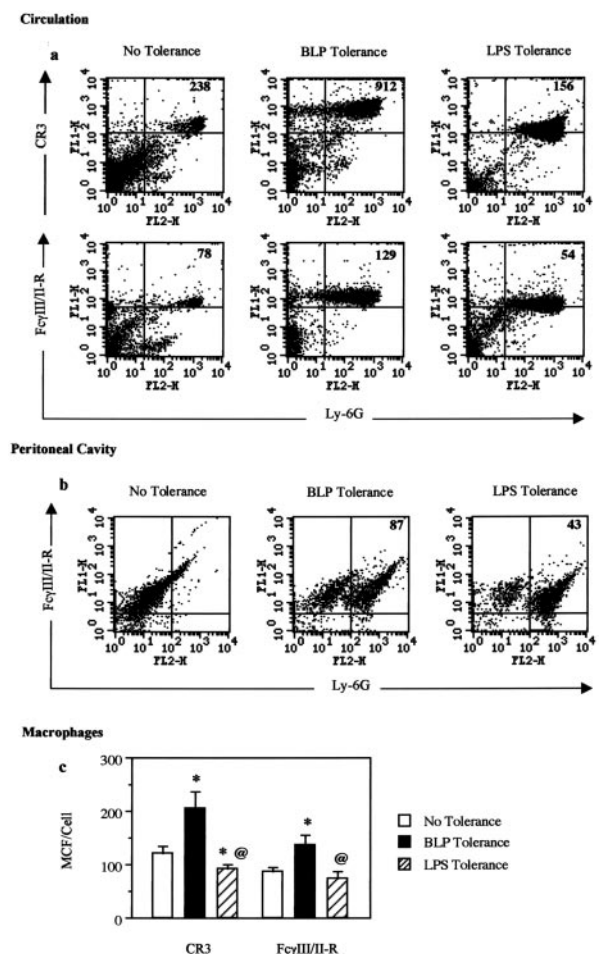
#### Enhanced PMN and macrophage activation in BLP-tolerized mice

We determined CR3 and Fc $\gamma$ III/IIIR expression on PMN and peritoneal macrophages in whole blood and peritoneal lavage collected from nontolerized, BLP-tolerized, and LPS-tolerized mice. Induction of BLP tolerance resulted in an increase in the circulating PMN population and a recruitment of PMN into the peritoneal cavity, with an increased surface expression of

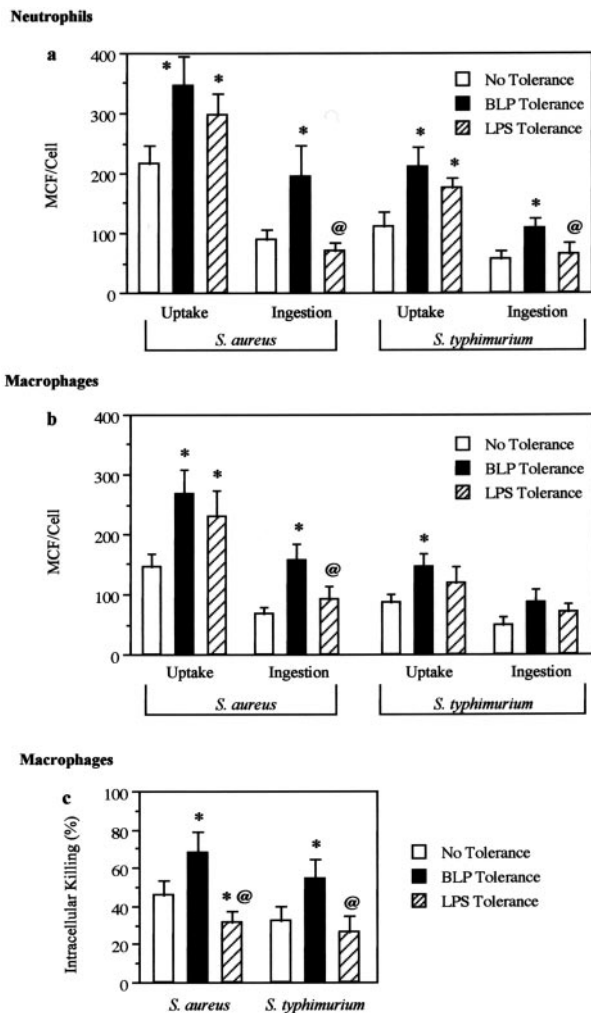
CR3 and Fc $\gamma$ III/IIIR (Fig. 3, *a* and *b*). In addition, BLP tolerance significantly up-regulated the expression of these two receptors on peritoneal macrophages (Fig. 3*c*). In contrast, although LPS tolerance resulted in an increase in PMN numbers in the circulation and peritoneal cavity, there was a down-regulation of CR3 and Fc $\gamma$ III/IIIR expression on PMN and macrophages in LPS-tolerized mice (Fig. 3).

#### Increased bacterial recognition and bactericidal activity in BLP-tolerized mice

We next assessed the ability of PMN and peritoneal macrophages to recognize, phagocytose, and kill bacteria. PMN and macrophages from BLP-tolerized mice had significantly increased uptake and ingestion of both *S. aureus* and *S. typhimurium*, compared with cells from nontolerized mice (Fig. 4, *a* and *b*). Furthermore, intracellular killing of *S. aureus* and *S. typhimurium* by peritoneal macrophages was significantly enhanced in BLP-tolerized mice (Fig. 4*c*). In contrast, although



**FIGURE 3.** Induction of BLP tolerance up-regulates CR3 and Fc $\gamma$ III/IIIR expression on PMN and peritoneal macrophages. *a* and *b*, FACSscan dot plot analysis of CR3 and Fc $\gamma$ III/IIIR expression on PMN in whole blood (*a*) and peritoneal lavage (*b*). Ly-6G<sup>+</sup>/CR3<sup>+</sup> or Ly-6G<sup>+</sup>/Fc $\gamma$ III/IIIR<sup>+</sup> cells indicate PMN population in the circulation and peritoneal cavity. The inserted value (mean channel fluorescence (MCF)/cell) indicates PMN CR3 and Fc $\gamma$ III/IIIR expression. Data are from a single experiment representative of seven performed. *c*, CR3 and Fc $\gamma$ III/IIIR expression on peritoneal macrophages identified by dual-staining with FITC-F4/80 Ag plus either PE-CR3 or PE-Fc $\gamma$ III/IIIR. Data are the mean  $\pm$  SD of seven independent experiments. \*,  $p < 0.05$  vs nontolerized mice; @,  $p < 0.05$  vs BLP-tolerized mice.



**FIGURE 4.** Enhanced bacterial recognition, ingestion, and intracellular killing in BLP-tolerized mice. Bacterial uptake and ingestion by circulating PMN (*a*) and peritoneal macrophages (*b*) were determined by FACScan analysis as described. Data are the mean  $\pm$  SD of seven independent experiments. \*,  $p < 0.05$  vs nontolerized mice; @,  $p < 0.05$  vs BLP-tolerized mice. *c*, Intracellular bacterial killing was determined as described. Data are the mean  $\pm$  SD of five independent experiments. Each experiment was conducted in triplicate. \*,  $p < 0.05$  vs nontolerized mice; @,  $p < 0.05$  vs BLP-tolerized mice.

there was an increased uptake of *S. aureus* and *S. typhimurium* by PMN and macrophages in LPS-tolerized mice, LPS tolerance did not augment bacterial ingestion as seen with BLP tolerance (Fig. 4, *a* and *b*). Indeed, peritoneal macrophages from LPS-tolerized mice showed a significant reduction in intracellular killing of *S. aureus* when compared with nontolerized mice (Fig. 4*c*).

## Discussion

In this study, we have demonstrated that induction of BLP tolerance, in addition to protection against a lethal BLP challenge, also protects against endotoxin shock through a cross-tolerance to LPS-induced lethality. This compares to LPS tolerance which affords protection only against the lethal effects of LPS. More importantly, unlike LPS tolerance, induction of BLP tolerance confers protection against microbial sepsis induced by either live *S. aureus* and *S. typhimurium* infection or CLP. These results indicate that BLP tolerance, in contrast to LPS tolerance,

represents a potential novel adaptive host response that results in a unique protective effect during septic shock. The purity of commercial LPS preparations has recently been questioned because of the potential contamination with microbial protein such as BLP (12, 13). However, no cross-tolerance to BLP was found in LPS-tolerized mice, indicating that the contamination of commercial LPS preparations has no significant effect on in vivo tolerance induction.

In response to bacterial invasion, mononuclear phagocytes produce inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12 (14). Although appropriate amounts of these cytokines are essential for cell-mediated microbicidal activity, excessive production can lead to an uncontrolled inflammatory response, multiple organ failure, and ultimately death (14–16). As the protective effect of LPS tolerance in endotoxin shock is associated with attenuation of proinflammatory cytokine production, in particular TNF- $\alpha$  (1, 2), we compared serum levels of TNF- $\alpha$  and IL-6 following lethal BLP or LPS challenge in BLP-tolerized and LPS-tolerized mice. Induction of BLP tolerance not only blunted the release of TNF- $\alpha$  in mice challenged with lethal BLP, but also reduced peak serum level of TNF- $\alpha$  in mice challenged with lethal LPS. In contrast, LPS tolerance failed to inhibit TNF- $\alpha$  production in BLP-challenged mice, which may partly explain why LPS tolerance conferred no protection against a lethal BLP challenge. In mice challenged with microbial sepsis, both BLP and LPS tolerance significantly reduced the serum levels of TNF- $\alpha$ , IL-6, IL-10, and IL-12. LPS tolerance resulted in a much greater inhibition of TNF- $\alpha$  production, an effect that may be deleterious to host defense against invading bacteria, as certain amounts of endogenous TNF- $\alpha$  are almost certainly required for effective eradication of bacterial infection (17, 18), and blocking TNF- $\alpha$  accelerates bacterial infection (19, 20). These results, in keeping with the survival data, suggest that induction of BLP tolerance inhibits inflammatory responses but, in contrast to LPS tolerance, may simultaneously prevent uncontrolled bacterial infection.

PMN are the first line of host defense against bacterial infection. Invading bacteria are ingested and killed by PMN through activation of CR3 and Fc $\gamma$ III/IIIR which are also involved in macrophage-mediated bacterial phagocytosis (21, 22). Overexpression of these two phagocytic receptors is associated with enhanced bacterial clearance (23). To investigate possible mechanisms contributing to enhanced host defense against microbial sepsis observed in BLP-tolerized mice, we assessed CR3 and Fc $\gamma$ III/IIIR expression on PMN and peritoneal macrophages. Induction of BLP tolerance resulted in an increased PMN population in the circulation and peritoneal cavity, with overexpression of CR3 and Fc $\gamma$ III/IIIR on PMN as well as on peritoneal macrophages. In contrast, LPS tolerance led to a suppressed expression of these two phagocytic receptors. To determine whether this altered phenotype of PMN and peritoneal macrophages is associated with BLP tolerance-afforded protection against lethal bacterial infection, we further assessed the ability of these cells to recognize, ingest, and kill *S. aureus* and *S. typhimurium*. PMN and peritoneal macrophages from BLP-tolerized 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-tolerized mice may contribute to their improved survival over LPS-tolerized mice following lethal microbial sepsis.

Microbial sepsis can lead to an uncontrolled inflammatory response characterized by the excessive release of proinflammatory cytokines, which is host self-destructive. Our findings provide evidence that the development of tolerance to the stimulatory effects of the bacterial cell wall component BLP is host self-controlled and self-protective, and represents a beneficial and not a harmful adaptive host response to bacterial infection. Secondly, we provide evidence that the mechanism by which BLP tolerance protects mice against clinically relevant microbial sepsis directly relates to enhanced bacterial recognition, ingestion, and intracellular killing, demonstrating how BLP-tolerized cells, though hyporesponsive in one sense are also primed for an increased bactericidal activity. Finally, our disparate findings in relation to BLP- and LPS-tolerized mice illustrate the markedly variable effects that different bacterial Ags have on cells of the innate immune system. Though sepsis research has focused for many years on the immune stimulatory actions of Gram-negative endotoxin, our work clearly points toward other bacterial Ags and in particular BLP, a more ubiquitously abundant component of both Gram-negative and Gram-positive bacteria, as a potential target for study in sepsis immunology and therapeutic development.

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