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Bacterial Superantigens Enhance the In Vitro Proinflammatory Response and In Vivo Lethality of the TLR2 Agonist Bacterial Lipopeptide

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Bacterial superantigens are Gram-positive exotoxins that induce proinflammatory cytokine release in vitro, cause lethal shock in vivo, and can be detected in the bloodstream of critically ill patients. They also have a powerful priming effect on the TLR4 agonist LPS. The aim of this study was to investigate the relationship between superantigens and the TLR2 agonist bacterial lipopeptide (BLP). Priming of human monocytes or PBMCs with superantigens significantly enhanced proinflammatory cytokine TNF-α and IL-6 release in response to BLP stimulation. The priming effect of superantigens could be blocked by inhibiting p38 MAPK during the priming phase as opposed to NF-κB or ERK inhibition. This was consistent with higher expression of the phosphorylated p38 after superantigen priming and BLP or LPS stimulation. C57BL/6 mice with superantigen priming (10 µg/mouse) when challenged with BLP (600 µg/mouse) exhibited substantially higher mortality (100%) compared with mice without superantigen priming (zero). Mice given superantigen alone did not demonstrate any signs of illness. Mice challenged with both superantigen and BLP had significantly higher levels of serum TNF-α and IL-6 compared with those of mice challenged with either agent alone. Depletion of the monocyte/macrophage subpopulation significantly reduced the mortality rate from 100 to 20% in superantigen-primed, BLP-challenged C57BL/6 mice, with a 5- to 10-fold decrease in serum TNF-α and IL-6. Our results demonstrate that bacterial superantigens enhance the in vitro proinflammatory cytokine release and in vivo lethality of BLP. This novel finding may help to explain the massive proinflammatory cytokine release seen in superantigen-mediated septic shock. The Journal of Immunology, 2011, 187: 5363–5369.

The incidence of sepsis has increased significantly over the past 30 years (1) and is now the leading cause of death in critically ill patients (2). Sepsis develops when the initial, appropriate response to an infection becomes amplified and then dysregulated (3). Bacterial superantigens are Gram-positive exotoxins that are involved in the pathogenesis of septic shock. They are the most powerful T cell mitogens ever discovered and can stimulate T lymphocytes in an uncontrolled manner resulting in fever, shock, and death (4, 5). Superantigens have also been shown to have a powerful priming effect on Gram-negative endotoxin/LPS. The staphylococcal toxic shock syndrome toxin-1 (TSST-1) enhances the susceptibility of rabbits to a lethal injection of LPS by a factor of ~50,000, and coinjection of LPS and TSST-1 induces TNF-α levels significantly higher than those by injection of similar doses of either toxin alone (3). Recently, studies have shown that superantigens can activate the human immune system independently of T cells by directly binding with the MHC class II receptor and upregulating the surface expression of TLR2 and TLR4 on monocytes (6, 7), which significantly enhances the proinflammatory response of innate immunity to Gram-negative endotoxin/LPS.

Materials and Methods

Bacterial cell wall components and bacterial superantigens

LPS from Escherichia coli O55:B5 (Sigma-Aldrich, St. Louis, MO) and the TLR2 agonist BLP (BMC Microcollections, Tübingen, Germany), a synthetic bacterial lipopeptide (PamCys-Ser-Lys4-OH) that was endotoxin-free as confirmed by the Limulus ameocyte lysate assay (Charles River Endosafe, Charleston, SC), were dissolved in sterile PBS (Lonza, Basel, Switzerland). Staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) from Staphylococcus aureus were purchased from Sigma-Aldrich and reconstituted with sterile PBS.

Isolation and cultures of human PBMCs and monocytes

PBMCs were separated from the whole blood of healthy volunteers by differential gradient centrifugation over endotoxin-free Ficoll-Paque Plus (Amersham Biosciences, Amersham, U.K.) using a method described by Kanof et al. (13). Monocytes were separated from PBMCs through differential gradient centrifugation over iso-osmotic Percoll (GE Healthcare,
Superantigen enhancement of BLP lethality

Aldrich) 24 h prior to superantigen and BLP challenges. Mice without recovery, Gaithersburg, MD).

and IL-6 concentrations in the supernatants or in the serum were assessed collected at different time points after SEA and/or BLP injection. TNF-

injected i.p. with 200 ml BLP or 100 pg/ml LPS for 4 h. Cell-free supernatants from the presence or absence of 25 mg/ml polymyxin B (Sigma-Aldrich) for 6 h and further stimulated with various doses of BLP for 4 h (SEB plus BLP), or pretreated with BLP for 4 h and then incubated with 1 ng/ml SEB for 6 h (BLP plus SEB), or treated with 1 ng/ml SEB and BLP together (SEB/BLP). In addition, isolated human monocytes were treated with various doses of either SEB alone or BLP alone. Cell-free supernatants from SEB- and/or BLP-challenged PBMCs and monocytes were collected and stored at −80°C until analysis. For in vivo experiments, male C57BL/6 mice with or without macrophage depletion were challenged by i.p. injection of either SEA, BLP, or their combination, and blood samples were collected at different time points after SEA and/or BLP injection. TNF-α and IL-6 concentrations in the supernatants or in the serum were assessed by ELISA (eBioscience, Hatfield, U.K.) according to the manufacturer’s instructions. Concentrations of multiple cytokines in the supernatants were also measured by MSD 96-well Cytokine Multiarray (Meso Scale Discovery, Gaithersburg, MD).

Inhibition experiments

Isolated human monocytes plated onto 96-well plates ( Falcon, Lincoln Park, NJ) at 5 × 10⁵ cells/well were primed with 1 ng/ml SEB for 6 h and further stimulated with various doses of BLP for 4 h. Isolated human monocytes plated onto 96-well plates at 5 × 10⁵ cells/well were primed with 1 ng/ml SEB for 6 h in the presence or absence of 25 mg/ml polymyxin B (Sigma-Aldrich) for 6 h and further stimulated with various doses of BLP for 4 h (SEB plus BLP), or pretreated with BLP for 4 h and then incubated with 1 ng/ml SEB for 6 h (BLP plus SEB), or treated with 1 ng/ml SEB and BLP together (SEB/BLP). In addition, isolated human monocytes were treated with various doses of either SEB alone or BLP alone. Cell-free supernatants from SEB- and/or BLP-challenged PBMCs and monocytes were collected and stored at −80°C until analysis. For in vivo experiments, male C57BL/6 mice with or without macrophage depletion were challenged by i.p. injection of either SEA, BLP, or their combination, and blood samples were collected at different time points after SEA and/or BLP injection. TNF-α and IL-6 concentrations in the supernatants or in the serum were assessed by ELISA (eBioscience, Hatfield, U.K.) according to the manufacturer’s instructions. Concentrations of multiple cytokines in the supernatants were also measured by MSD 96-well Cytokine Multiarray (Meso Scale Discovery, Gaithersburg, MD).

Western blot analysis

After BLP or LPS stimulation, isolated human monocytes pretreated with or without superantigens for 6 h were collected at various time periods, washed with ice-cold PBS, and lysed on ice in cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM PMSF and protease inhibitor mixture (Roche, Indianapolis, IN). The resultant lysates were centrifuged, and supernatants containing the cytoplasmic proteins were collected. Protein concentration was determined using a micro BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein extracts were separated on SDS–polyacrylamide gels and trans-blotted onto polyvinylidene difluoride membranes (Schleicher & Schuell, Dassel, Germany). The membrane was blocked for 1 h at room temperature with PBS containing 0.05% Tween 20 and 5% nonfat milk and probed overnight at 4°C with mouse anti-human phosphorylated p38 mitogen-activated protein (MAPK) (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with HRP-conjugated secondary Ab (Dako, Cambridge, U.K.) at room temperature for 1 h, developed with SuperSignal chemiluminescent substrate (Pierce), and captured with LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Sepsis studies

Pyrogen-free, 8- to 10-wk-old, male C57BL/6 mice were purchased from Harlan (Oxon, U.K.). Mice were housed in barrier cages under controlled environmental conditions (12/12 h 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 carried out in a licensed biomedical facility (University Biological Services Unit, University College Cork) under a license from the Department of Health and Children (Republic of Ireland). All animal studies were conducted with ethical approval granted from the University College Cork Ethics Committee.

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Results

Superantigens enhance the proinflammatory cytokine release from human PBMCs and monocytes in response to BLP stimulation

Human PBMCs were stimulated with 1 ng/ml SEB for 6 h and then challenged with BLP at increasing concentrations for 4 h. There was a significantly higher release of TNF-α from superantigen-primed PBMCs in response to BLP at 10 and 100 ng/ml compared with that of naive PBMCs and of PBMCs stimulated with SEB alone (Fig. 1A). When the monocyte subpopulation isolated from PBMCs was treated with either SEB alone or BLP alone at various concentrations, there was only a slight increase in TNF-α release from these monocytes (Fig. 1B); however, there was a significantly higher amount of TNF-α released from monocytes that were primed with superantigen and challenged with BLP at 10 and 100 ng/ml (SEB plus BLP) compared with that of monocytes without superantigen priming or treated with SEB alone (Fig. 1C). To clarify whether superantigens exert a priming or synergistic effect with BLP, we assessed TNF-α release from monocytes that were pretreated with BLP for 4 h and challenged with SEB for 6 h (BLP plus SEB) or stimulated with SEB and BLP together (SEB/BLP). In these circumstances, SEB failed to enhance BLP-stimulated TNF-α release (Fig. 1C), indicating that SEB exerts a priming rather than a synergistic effect on BLP-induced proinflammatory cytokine release. We further used polymyxin B, a specific LPS inhibitor, to confirm that the observed priming effect of SEB on BLP-stimulated TNF-α release was not due to the contamination of LPS. As shown in Fig. 1D, the SEB-associated priming effect could not be blocked by addition of polymyxin B, which clearly rules out any effects from a potential LPS contamination.

Multiarray cytokine analysis was performed next to quantify what effect superantigen priming had on the release of other cytokines from monocytes. Isolated human monocytes were incubated with culture medium or 1 ng/ml SEB for 6 h and further stimulated with 100 ng/ml BLP for 4 h. As shown in Fig. 2, superantigen priming significantly enhanced BLP-stimulated IL-6 release from human monocytes; however, there was no significantly enhanced release of IL-1β, IL-8, IL-10, and IL-12p70 from monocytes primed with superantigens and challenged with BLP.

The superantigen priming effect is dependent upon the p38 MAPK pathway

To further elucidate the intracellular signal transduction pathway involved in the superantigen priming effect, inhibitors to the p38, ERK, and NF-κB pathways were used on human monocytes

macrophage depletion received 200 μl PBS. The depletion of macrophages was verified by FACScan analysis of the F4/80-positive cells in the peritoneal lavage and spleen.

Mice with or without macrophage depletion were randomly divided into three groups, and each group received two i.p. injections, one at 0 h and a second after 6 h. Mice in the superantigen-alone group received i.p. injection of 200 μl PBS containing 10 μg SE, followed by a second injection of 200 μl PBS 6 h later. Mice in the BLP-alone group received i.p. injection of 200 μl PBS, followed by a second injection of 200 μl PBS containing 600 μg BLP 6 h later. Mice in the superantigen plus BLP group received i.p. injection of 200 μl PBS containing 10 μg SE, followed by a second injection of 200 μl PBS containing 600 μg BLP 6 h later. Blood samples were collected at 90 min and 4 h after second i.p. injection. Survival was monitored for at least 7 d.

Statistical analysis

All data are presented as the mean ± SD. Statistical analysis was performed using SPSS version 18 (SPSS, Chicago, IL). The log-rank test was used for survival analysis, and the Mann–Whitney U test was used for all others. Differences were judged statistically significant when p < 0.05.
during the superantigen priming phase. We found that the priming effect of superantigens (1 ng/ml SEB for 6 h) on monocytes was almost completely abrogated by inhibiting the p38 MAPK during the priming phase, as opposed to ERK or NF-kB inhibition (Fig. 3A). When monocytes were primed with SEB (1 ng/ml) for 6 h and further stimulated with various doses of BLP for 4 h (BLP+SEB), or pretreated with BLP for 4 h and further incubated with SEB (1 ng/ml) for 6 h (BLP+SEB), or treated with SEB (1 ng/ml) and BLP together (SEB+BLP), D. Isolated human monocytes were primed with 1 ng/ml SEB in the absence or presence of polymyxin B (25 μg/ml) for 6 h and stimulated with BLP for 4 h. TNF-α concentrations in the culture supernatants were assessed by ELISA. Data are presented as mean ± SD of four to six independent experiments, and each experiment was conducted in triplicate. *p < 0.05 compared with naive or SEB alone.

Superantigens augment BLP- or LPS-stimulated phosphorylation of p38 MAPK in human monocytes

We next analyzed the activation of p38 MAPK in either BLP- or LPS-stimulated human monocytes with or without superantigen priming. Activation of p38 MAPK was determined by assessing the level of phosphorylated p38 expression as this has been shown accurately to measure p38 MAPK activity (18). Superantigen priming of monocytes with 1 ng/ml SEB for 6 h led to an increased expression of phosphorylated p38 at 30 min after BLP stimulation compared with that of cells without superantigen priming (Fig. 4A, 4C). Superantigen alone had no stimulatory effect on phosphorylated p38 expression. When LPS was used as a second stimulant, an early and substantially increased activation of p38 MAPK was observed at 15 min after LPS stimulation in superantigen-primed monocytes compared with nonprimed monocytes (Fig. 4B, 4D).

Superantigens amplify in vivo lethality of BLP via increased proinflammatory cytokine release

To assess whether superantigens enhance the lethality of BLP in vivo, a survival study was carried out in a murine sepsis model.
Mice were divided into three groups: superantigen alone, BLP alone, and superantigen plus BLP. Survival was monitored for at least 7 d. Mice that received superantigen alone (10 μg SEA/mouse) did not demonstrate any signs of illness, and there was a 100% survival after 72 h (Fig. 5A). Similarly, mice that received BLP alone (600 μg/mouse) exhibited a 100% survival after 72 h (Fig. 5B). When SEA (10 μg/mouse) was used in combination with BLP (600 μg/mouse), however, all mice demonstrated severe signs of septic shock, with 100% mortality within 24 h (p = 0.0001 versus either SEA or BLP alone) (Fig. 5C).

Further experiments were then carried out to analyze the circulating proinflammatory cytokine levels in these groups. There were significantly higher levels of serum TNF-α in mice that received superantigen plus BLP compared with those in mice that received superantigen alone or BLP alone (Fig. 5D). This difference was highest at 90 min but still present at 4 h after BLP challenge. There were also significantly higher levels of serum IL-6 at both 90 min and 4 h in the superantigen plus BLP group compared with those in the superantigen-alone and BLP-alone groups (Fig. 5D).

### Discussion

Sepsis describes a complex clinical syndrome that results from a harmful or damaging host response to infection (3). The innate immune system, which is now known to play an important role in the pathogenesis of sepsis, responds rapidly to infection by means of pattern recognition receptors (e.g., TLRs) that interact with highly conserved molecular motifs present on pathogens, such as LPS or BLP (19, 20). Binding of TLRs to these epitopes stimulates intracellular signaling and increases the transcription of proinflammatory cytokines such as TNF-α.

Bacterial superantigens are exotoxins involved in the pathogenesis of Gram-positive septic shock (5). There are 41 known superantigen, the vast majority secreted by *Staphylococcus aureus* and *Streptococcus pyogenes* (4). They are the causative agents in staphylococcal and streptococcal toxic shock syndrome (TSS) and have been implicated in the pathogenesis of atopic...
dermatitis (21), psoriasis (22), Kawasaki syndrome (23), and acute rheumatic fever (24). A unique feature of superantigens is that they possess particular structural and sequence characteristics that result in the shared ability to bypass the mechanisms of conventional, MHC-restricted Ag processing (25). This makes them capable of activating up to 20% of the T cell repertoire in an uncontrolled manner (4). Recently, however, superantigens have been shown to activate the innate immune system independently of T cells (6, 7, 26, 27) through direct binding of superantigens to MHC class II receptors on monocytes.

Although the incidence of TSS has been decreasing (28), Gram-positive infections are becoming more prevalent and are responsible for ≈50% of sepsis cases in the United States (1). Azuma and colleagues (29) analyzed the prevalence of five different classes of superantigens in 78 consecutive patients in the intensive care unit. The detection of superantigens was present in 31% of septic patients without shock and in 41% of patients with septic shock. The role of superantigens in sepsis is not fully understood, but they are known to have a potentiating effect on the TLR4 ligand endotoxin/LPS (6). Because the vast majority of superantigens are secreted by Gram-positive bacteria, the relevance of this synergy is restricted to cases of polymicrobial sepsis or translocation of secreting bacteria (28). Superantigens have been shown to upregulate the expression of both TLR2 and TLR4 on isolated human monocytes (6, 7, 26), which was also observed in the current study (data not shown). Although this upregulation by superantigen of TLRs may play a role in the increased responsiveness of the innate immune system in superantigen-mediated septic shock, other research has shown that in patients with severe sepsis, TLR expression does not correlate well with ligand sensitivity (31, 32). We therefore analyzed the effect of superantigens on other aspects of the TLR-mediated intracellular signal transduction pathway. During TLR signaling, ligand binding to the TLR activates an intracellular cascade of adapter proteins, namely MyD88, IL-1 receptor–associated kinase, and TNFR-activated factor-6.

To confirm further these inhibitory findings, we examined whether superantigens augment BLP- or LPS-stimulated phosphorylation of p38 MAPK in human monocytes. Superantigen

![FIGURE 5. Superantigens amplify BLP-associated proinflammatory cytokine release and lethality in vivo.](http://www.jimmunol.org/)

Mice received SEA alone (10 μg/mouse), BLP alone (600 μg/mouse), or their combination (SEA+BLP) as described in Materials and Methods. A–C, Kaplan–Meier survival curve demonstrating that mice challenged with both SEA and BLP (n = 10) had significantly higher mortality (100%) than mice that received SEA alone (n = 10) or BLP alone (n = 10) (p = 0.0001). D, Serum TNF-α and IL-6 were assessed by ELISA, and data shown are mean ± SD of three mice per time point. *p < 0.05 compared with mice that received SEA alone or BLP alone.

![FIGURE 6. Depletion of macrophages improves survival with attenuated proinflammatory cytokine release in superantigen-primed, BLP-challenged mice.](http://www.jimmunol.org/)

A–C, Kaplan–Meier survival curve demonstrating that after depletion of macrophages, mice that received SEA alone or BLP alone exhibited a 100% survival rate, and mice challenged with both SEA and BLP had an 80% survival rate (n = 10 per group). D, Serum TNF-α and IL-6 were assayed by ELISA, and data shown are mean ± SD of four mice per time point. *p < 0.05 compared with mice that received SEA alone or BLP alone.
alone had little effect on p38 MAPK activation, however when used in combination with BLP or LPS, superantigen priming substantially increased the expression of phosphorylated p38 in both BLP- and LPS-stimulated human monocytes. Importantly, the intensity and duration of phosphorylated p38 expression was stronger and longer in superantigen-primed monocytes after BLP or LPS stimulation, which corresponds with the increased and sustained proinflammatory cytokine release seen in both in vitro and in vivo settings. These novel findings may help to identify potential therapeutic targets for future treatments of superantigen-mediated septic shock.

Previous work has shown that bacterial superantigens enhance the lethality of Gram-negative endotoxin in vivo (35, 36). Schlievert (35) demonstrated that the coadministration of superantigens and endotoxin resulted in a reduction of the LD₅₀ of either toxin given alone by up to 50,000 times. Henne et al. (36) examined the ability of TSST-1 to enhance the production of endotoxin-induced TNF-α in C3H/HeN mice and demonstrated that when mice were injected with 20 μg TSST-1 12 h before exposure to 1 μg endotoxin, serum TNF-α was 20 times higher than that found in mice challenged with endotoxin alone. Although the ability of superantigens to enhance the lethality of endotoxin has been known for a long time, the ability of superantigens to enhance the lethality of other TLR agonists has not yet been examined. In the current study, we investigated whether superantigens could enhance the lethality of the TLR2 agonist BLP in vivo using wild-type C57BL/6 mice. We found that SEA alone did not induce any illness in mice; this corresponds with a previous report on the administration of superantigens to C3H/HeN mice (36), where mice, in contrast to humans, are known to be much more resistant to the toxic effect of superantigens. When given alone, a sublethal dose of BLP at 600 μg/mouse caused mild signs of sepsis, but all mice recovered quickly, and there was no mortality observed in this group. When given in combination, however, SEA significantly enhanced the lethality of BLP, and all mice died within 24 h of BLP challenge. Consistent with the survival result, there were significantly higher levels of serum TNF-α and IL-6 in mice that received both SEA and BLP compared with those in mice that received either agent alone. These increases were most marked at 90 min for serum TNF-α and 4 h for serum IL-6 after BLP challenge. This mirrors the pattern of proinflammatory cytokine release observed in lethal BLP-induced septic shock in MF-1 mice (37) compared with that observed in superantigen-induced toxic shock in HLA-DR1 transgenic mice where a biphasic proinflammatory cytokine release was seen (38). By contrast, in our model, wild-type C57BL/6 mice that received SEA alone had levels of serum TNF-α and IL-6 that were barely detectable, confirming the resistance of this strain of mice to superantigen challenge.

In our in vivo findings that superantigens substantially enhance the lethality of BLP are somewhat discordant with recent work by Chau et al. (39) who demonstrated that the TLR2 agonist peptidoglycan (PGN) protects HLA-DR4 transgenic mice from staphylococcal TSS by attenuation of the superantigen-induced IL-2 response. There are several reasons that may account for this discordance. Attenuation of the superantigen-stimulated IL-2 response by PGN seemingly requires the signal from TLR2 in cordance. Attenuation of the superantigen-stimulated IL-2 response. There are several reasons that may account for this discordance. Attenuation of the superantigen-stimulated IL-2 response by PGN seemingly requires the signal from TLR2 in combination with TLR6, as zymosan that binds to TLR2 and TLR6, but not BLP (Pan3C3k4) that binds to TLR2 and TLR1, inhibited the IL-2 response of PBMCs to superantigens (39). Furthermore, PGN-afforded protection against staphylococcal TSS seen in HLA-DR4 transgenic mice could not be translated into wild-type C57BL/6 mice as these mice did not mount an IL-2 response to superantigens (39). By contrast, our in vivo data have revealed that superantigen priming of C57BL/6 mice renders these mice to be more susceptible to a subsequent BLP challenge.

To test our hypothesis that the superantigen-enhanced in vivo lethality of BLP is monocyte/macrophage dependent, as opposed to T cell dependent, we repeated the survival experiments in macrophage-depleted wild-type C57BL/6 mice. The survival rate in superantigen-primed, BLP-challenged mice was significantly increased from zero in mice without depletion of macrophages to 80% in mice with macrophage depletion. Consistent with an improved survival rate, there was also a significant 5- to 10-fold attenuation of serum TNF-α and IL-6 in these mice. These findings clearly indicate that superantigen-amplified in vivo BLP lethality is mediated through the monocyte/macrophage subpopulation.

In conclusion, we have shown that bacterial superantigens significantly increase the proinflammatory cytokine release from human monocytes in response to BLP stimulation and that this enhanced responsiveness is achieved via an increased activation of the p38 pathway. We have also demonstrated that superantigens amplify the lethality of BLP in vivo through an enhanced proinflammatory cytokine release and that this lethality is dependent on the monocyte/macrophage subpopulation. Taken together, these novel findings may help to explain the massive proinflammatory cytokine release observed in superantigen-mediated septic shock.

Disclosures

The authors have no financial conflicts of interest.

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