Protection against Endotoxic Shock by Bactericidal/Permeability-increasing Protein in Rats

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Abstract

Bactericidal/permeability-increasing protein (BPI) is a neutrophil primary granule protein that inhibits effects of LPS in vitro. The current study examined the effects of BPI on hemodynamics, mortality, and circulating endotoxin and cytokines in conscious rats with endotoxic shock. Catheters were implanted into the right femoral artery and vein. 1 d later, human recombinant BPI (10 mg/kg) or vehicle was intravenously injected immediately, 30 min, or 2 h after intravenous injection of LPS (7.5 mg/kg). Mean arterial pressure (MAP) and heart rate were monitored and blood was collected before and after injection. BPI given immediately or 30 min after LPS prevented the LPS-induced reduction in MAP at 4–8 h and markedly reduced mortality. BPI given 2 h after LPS injection had no protective effect. BPI treated immediately after LPS reduced the circulating levels of endotoxin and IL-6 but increased the circulating levels of TNF. We propose that BPI exerts its protective effect through a TNF-independent mechanism, by inhibiting endotoxin-stimulated production of IL-6. (*J. Clin. Invest.* 1995. 95:1947–1952.) LPS • septic shock • tumor necrosis factor (TNF) • IL-6 • cytokine

Introduction

Circulatory shock resulting from sepsis has become the most common cause of death in intensive care units (1, 2). Despite the complexity of septic shock, endotoxin is generally accepted as a principle bacterial product that is recognized by all mammalian hosts as a major pathogenic entity (2). Endotoxin, a complex of LPS associated with proteins (3), is a major component of the outer membrane of Gram-negative bacteria. When released, endotoxin can cause shock, disseminated intravascular coagulation, multisystem organ failure, and death (4–7). The LPS portion of the endotoxin molecule has profound effects on soluble and cellular host inflammatory systems (6). Plasma concentrations of endotoxin correlate positively with severity of disease and mortality in septic patients (8–10).

The bactericidal/permeability-increasing protein (BPI),[1] one of the neutrophil primary granule proteins, is cytotoxic specifically for Gram-negative bacteria in vitro because of its strong attraction to the outer membrane of the Gram-negative bacterial envelope (11–13). Several lines of evidence indicate that BPI can inhibit effects of LPS in vitro when added extracellularly (14–16). BPI has been shown to bind LPS near the lipid A domain, leading to abrogation of detrimental host responses to LPS (14). BPI also inhibits LPS-stimulated TNF release from monocytes in vitro, and in vivo in the murine lung, and blocks LPS-mediated pyrogenicity in rabbits (16). Further, BPI causes killing of serum-resistant Gram-negative bacteria in vitro and inhibits the bacteria-induced release of TNF in human whole blood (17). Recently, BPI has been found to decrease mortality in mice challenged with endotoxin (18). Taken together, these studies suggest that BPI may have therapeutic potential in severe endotoxemia or endotoxic shock.

The current study was designed to examine the effects of BPI on hemodynamics, mortality, and circulating levels of endotoxin and cytokines in endotoxic shock. We developed a rat model of endotoxic shock in which BP and heart rate (HR) could be observed for 6 d in the conscious, unrestrained state. We show that BPI inhibits the LPS-induced increase in circulating levels of endotoxin and IL-6, prevents the LPS-induced hypotension, and reduces mortality in endotoxic shock, despite causing elevation in circulating levels of TNF.

Methods

Male Sprague-Dawley rats aged 9 wk were obtained from Charles River Laboratories (Wilmington, MA). All rats were maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (0600–1800 h) and fed with a standard rat diet. 2 d after arrival, the rats were anesthetized with Metofane (Pitman-Moore, Inc., Mundelein, IL), and one polyethylene catheter (PE-10 fused with PE-50) filled with heparin-saline solution (50 U/ml) was implanted into the abdominal aorta through the right femoral artery for measurements of BP and HR. A second was implanted into the right femoral vein for drug administration. The catheters were exteriorized at the back of the neck with the aid of

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1. *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; HR, heart rate; MAP, mean arterial pressure.

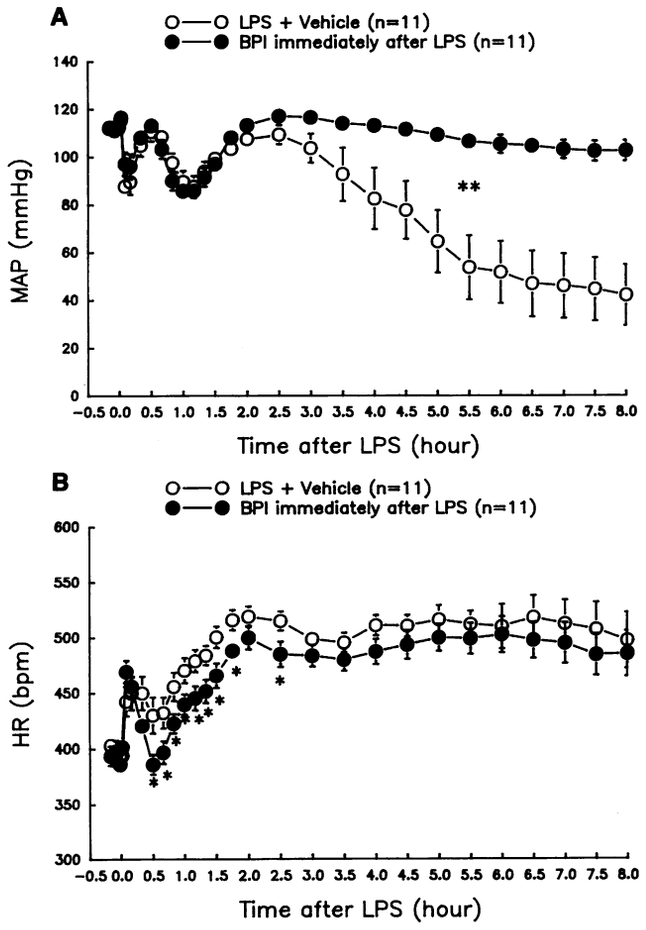

Figure 1. Effects of BPI treatment immediately after LPS injection on MAP and HR. $**P < 0.01$, comparison of MAP at 4–8 h between the vehicle- and BPI-treated group; $*P < 0.05$, compared to the vehicle-treated group at the same time point.

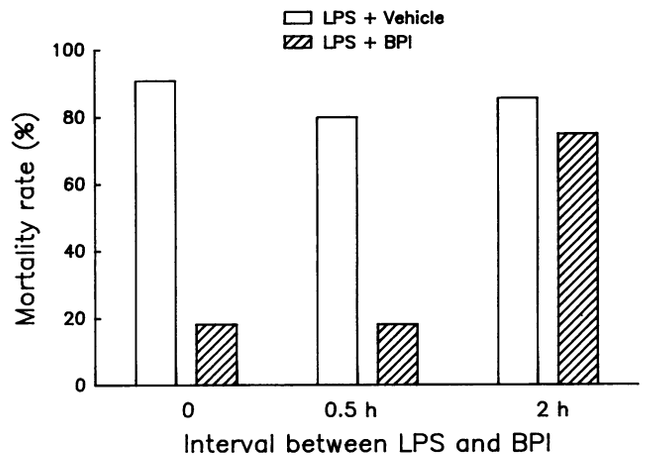

Figure 2. Effect of BPI treatment on mortality rate immediately, 30 min, or 2 h after LPS injection.

a stainless steel wire tunneled subcutaneously and then fixed with fast-polymerizing dental cement. After catheter implantation, all rats were housed individually.

24 h after surgery, the arterial catheter was connected to a pressure transducer (CP-01; Century Technology Co. Inc., Inglewood, CA) coupled to a polygraph (Model 7; Grass Instruments Co., Quincy, MA). Mean arterial pressure (MAP) and HR were monitored simultaneously in conscious, unrestrained rats. After a 45-min stabilization period, each rat received a bolus injection of LPS (7.5 mg/kg) (from *Escherichia coli* serotype 0127:B8; Sigma Chemical Co., St. Louis, MO). A pilot study had shown that the LPS at this dose caused 85–90% death in 24 h in rats. Human recombinant BPI (10 mg/kg) (Incyte Pharmaceutical, Inc., Palo Alto, CA) or vehicle was intravenously administered as bolus injection immediately, 30 min, or 2 h after LPS injection. Previous studies demonstrated that BPI administered intravenously at doses up to 10 mg/kg produced no acute hematologic, biochemical, or pathologic abnormalities in mice or Sprague-Dawley rats (18, 19). MAP and HR were monitored after injection of LPS and BPI or vehicle. If the rat survived, MAP and HR were measured daily for 6 d.

To avoid influences of blood loss on hemodynamics, blood collections were performed in separate groups of rats. The rats were catheterized as above. 24 h after surgery, BPI (10 mg/kg) or vehicle was intravenously injected immediately after the LPS (7.5 mg/kg) injection. Blood (0.5 ml) was collected through the arterial catheter 5 min before and after injection. An equal volume of saline was intravenously injected immediately after blood collection. Serum was separated and stored at −70°C for the measurement of endotoxin, TNF, and IL-6.

Serum endotoxin was measured by a limulus amebocyte lysate assay, which was sensitive down to 0.5 endotoxin units/ml. Serum TNF was measured using a cell-based cytotoxicity assay, sensitive to 0.05 ng of TNF. Serum IL-6 was measured using an IL-6–dependent B-9 hybridoma proliferation assay (20), sensitive to IL-6 at 0.05 ng/ml.

Statistical analysis. Results were expressed as mean±SEM. The parameters at the same time point between the BPI and vehicle group were compared by an unpaired Student's *t* test. The parameters in the same group among the different time points were compared by one way analysis of variance. $P < 0.05$ was considered to be statistical significance.

Results

Effects of BPI given immediately after LPS. LPS administration resulted in a rapid decrease in MAP, which recovered briefly and then underwent a second transient decrease in the vehicle-treated group (Fig. 1 *A*). This early phase was followed by a larger, more sustained decrease in MAP over the next 8 h. Administration of BPI immediately after LPS did not alter the initial transient falls in MAP but prevented the later sustained decrease. There was a significant difference in MAP at 4–8 h after LPS injection between the vehicle- and BPI-treated group ($P < 0.01$, Fig. 1 *A*).

LPS administration rapidly increased HR, which reached a peak level at 2 h, and thereafter remained at this plateau over the subsequent 6 h (Fig. 1 *B*). BPI treatment attenuated the LPS-induced tachycardia significantly at 0.5–2.5 h but not at the later time points.

LPS caused death in 91% (10/11) of the vehicle-treated rats (Fig. 2), but only in 18% (2/11) of the BPI-treated rats.

MAP remained normal in all nine surviving rats in the BPI-treated group and in the one surviving animal in the vehicle-treated group for 6 d after the LPS injection (data not shown). The tachycardiac response to LPS lasted 2–3 d and then returned to normal in surviving rats in both groups (not shown).

Effects of BPI given 30 min after LPS. In this study set, LPS administration caused similar effects on MAP and HR in

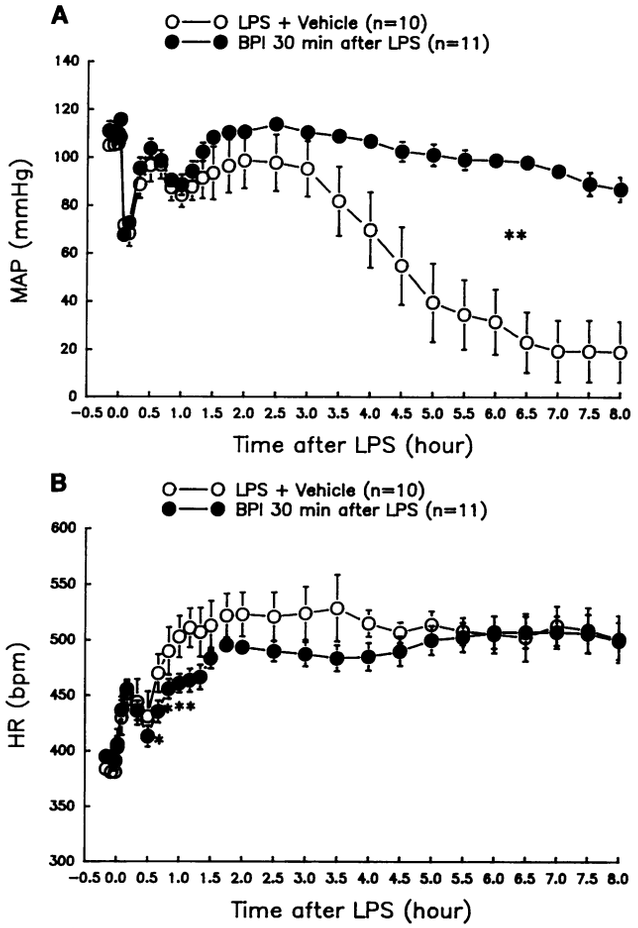

Figure 3. Effects of BPI treatment 30 min after LPS injection on MAP and HR. $**P < 0.01$, comparison of MAP at 4–8 h between the vehicle- and BPI-treated group; $*P < 0.05$, compared to the vehicle-treated group at the same time point.

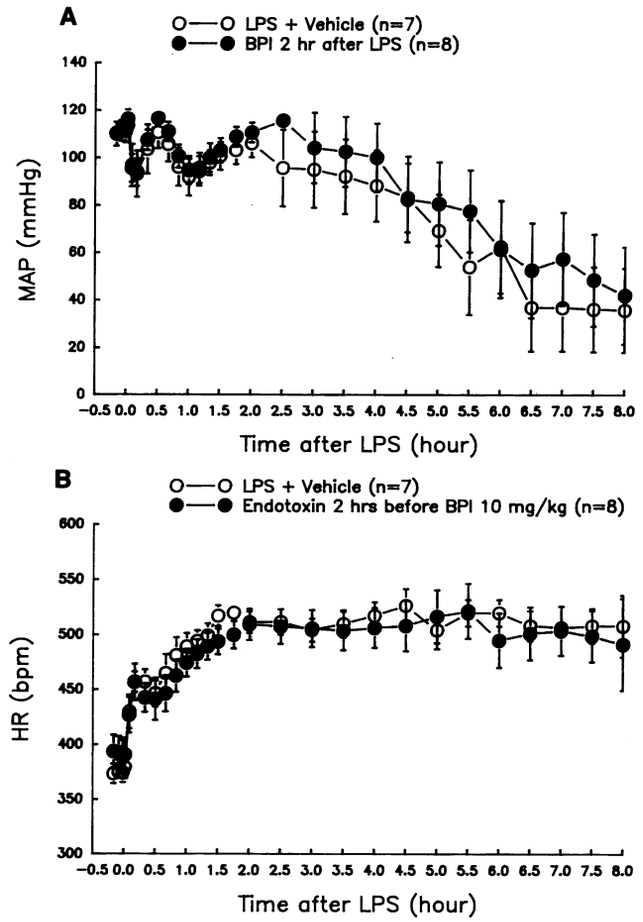

Figure 4. Effects of BPI treatment 2 h after LPS injection on MAP and HR.

the vehicle-treated animals as those described above (Fig. 3). BPI treatment 30 min after LPS injection significantly protected rats from the LPS-induced reduction in MAP measured 4–8 h after LPS injection. BPI also attenuated the LPS-induced tachycardiac response at 40–90 min after LPS, but not at other time points.

The mortality rate was 80% (8/10) and 18% (2/11) in the vehicle- and BPI-treated group, respectively (Fig. 2). MAP was stable for 6 d and tachycardia lasted 3 d in the surviving rats in both groups (data not shown).

Effects of BPI given 2 h after LPS. LPS induced alterations in MAP and HR (Fig. 4), essentially as shown above. BPI treatment 2 h after LPS injection did not significantly affect the LPS-induced hypotensive and tachycardiac responses. Furthermore, when given 2 h after LPS, BPI did not significantly reduce mortality. LPS caused 86% (6/7) mortality in the vehicle-treated rats and 75% (6/8) in the BPI-treated rats (Fig. 2).

Serum endotoxin. Intravenous injection of LPS rapidly and markedly elevated the serum level of endotoxin in the vehicle-treated rats (Fig. 5). Circulating endotoxin reached a peak level at 15 min and then declined by 50% at 45 min, 60% at 75–120 min, and 85% at 240 min. BPI administration immediately after LPS injection significantly inhibited the LPS-induced increase in serum endotoxin (Fig. 5 *A*). BPI lowered the endotoxin levels by 60% at 15 min and by more than 80% at 2–4 h.

Serum TNF. After injection of LPS, TNF in serum rose from undetectable levels to 11 ng/ml at 30 min, to a peak level at 36 ng/ml at 90 min, and then declined back to undetectable levels by 4 h (Fig. 5 *B*). BPI administered immediately after LPS did not significantly affect the levels of TNF over the first 30 min. However, thereafter, TNF rose to significantly higher levels in the BPI-treated animals than in control animals. It should be noted that the TNF detected was active, because it was measured by bioassay.

Serum IL-6. Serum levels of IL-6 rose significantly between 60 and 90 min after LPS injection and reached a peak level at 4 h (Fig. 5 *C*). In BPI-treated animals, the LPS-induced rise in IL-6 levels began as in the vehicle-treated animals, reaching comparable levels at 90 min. However, thereafter, BPI attenuated the rise in IL-6 levels such that at 4 h the IL-6 levels were < 25% of those in the vehicle-treated animals (Fig. 5 *C*).

Discussion

BPI injected soon after LPS challenge markedly reduced mortality in this model of endotoxic shock. Early signs of efficacy

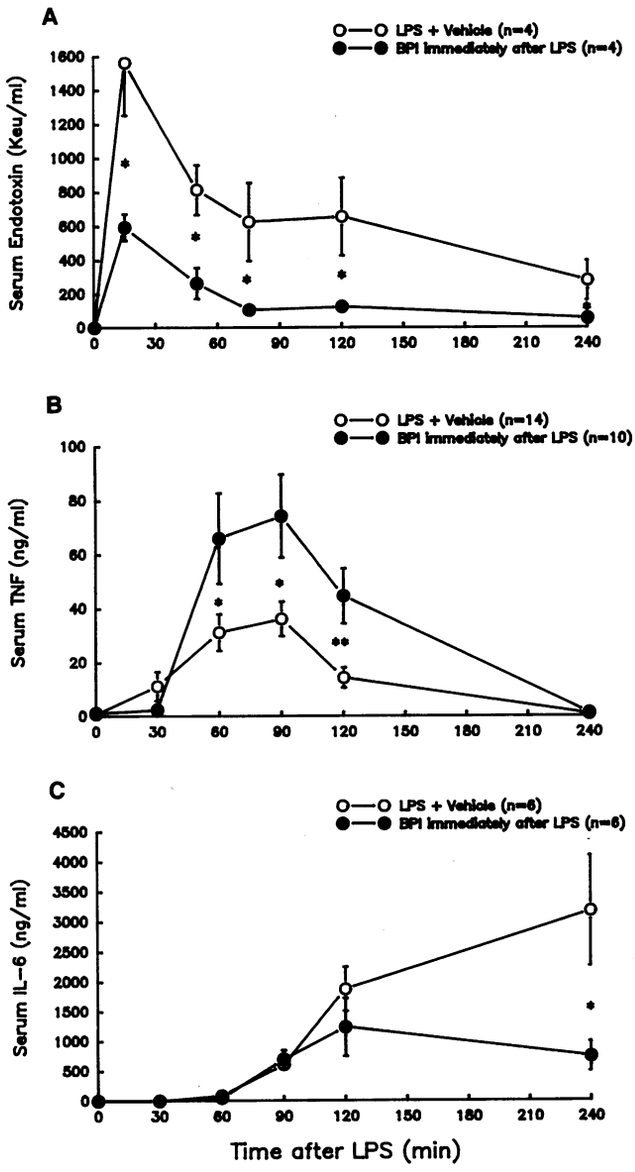

Figure 5. Effect of BPI treatment on serum levels of endotoxin, TNF, and IL-6 immediately after LPS injection. $*P < 0.05$, $**P < 0.01$, comparison between the vehicle- and BPI-treated groups; *Keu,* 1,000 endotoxin units.

were prevention of the LPS-induced hypotension, reduction in circulating endotoxin levels, and inhibition of elevated circulating IL-6 levels. These results indicate that BPI may have potential value for treatment of Gram-negative septic shock.

The MAP pattern observed in the rat model described here shows two phases of shock: (*a*) shock at the early phase, beginning at 5 min and recovering at 1.5–2 h after LPS injection; (*b*) shock at the late phase, beginning at 3.5–4 h after LPS and often leading to death. Heart rate was increased 5 min after LPS and thereafter remained at a substantially elevated level.

It has been shown that the LPS-induced reduction in blood pressure is associated with a decrease in cardiac output and stroke volume in conscious rats (21, 22), and tachycardia occurs as a compensatory response to maintain cardiac output and blood pressure. The current study demonstrated that, in the control animals, LPS caused a marked drop in blood pressure, despite a compensatory tachycardic response. In the BPI-treated animals, a similar tachycardic response was observed; however, in contrast to the controls, blood pressure remained stable. These data suggest that BPI may improve cardiac function by blocking detrimental effects of LPS on the heart.

In the current study, BPI treatment 2 h after LPS injection did not significantly affect LPS-induced hypotension, tachycardia, and lethality. This observation is consistent with the previous finding that BPI administered up to 1 h after LPS provides protection against lethal endotoxin challenge in mice (18). The narrow time window for protection may be attributed to the fact that, in models involving bolus injection of LPS, the highest circulating levels of endotoxin are achieved over the first 2 h. Endotoxin at these levels may be irreversibly fatal. In our study, the circulating endotoxin in the vehicle-treated rats rose to a peak level 15 min after LPS injection and then decreased. At 2 h, the circulating endotoxin level had declined to 40% of this peak level, which was close to the peak level of endotoxin observed in animals that were treated with BPI immediately after LPS injection.

Although BPI substantially reduced circulating endotoxin levels, its actions cannot be simply attributed to endotoxin neutralization. If that were the case, BPI would have been expected to suppress the endotoxin-induced rise in TNF levels. However, our data show that BPI actually increases circulating levels of bioactive TNF in the animals challenged with LPS. The increase in TNF levels may reflect a decrease in the TNF-stimulated production of soluble TNF receptors (TNF inhibitors) rather than an increase in TNF release. Indeed, Rogy et al. (23) have recently found that BPI significantly reduces circulating soluble TNF receptors in baboons with septic shock, supporting this explanation. Our observation that BPI protects against LPS lethality despite a rise in TNF levels suggests that BPI may exert its protective effect by neutralizing or counteracting a subset of, rather than all of the events triggered by, endotoxin.

TNF is thought to be one of the important mediators in septic shock. It has been reported that neutralizing antibodies against TNF or other agents that neutralize TNF protect animals against lethal endotoxin or *E. coli* challenge (24–28). In humans, TNF is released during the early stages of septic shock, and circulating TNF levels might correlate with outcome in severe sepsis (29–31). Nevertheless, some investigators have argued that TNF release is not the central mediator that is most directly responsible for sepsis (32–35). It has been reported that administration of monoclonal antibody to endotoxin before lethal challenge with live *E. coli* protected animals from death but did not inhibit the rise in circulating TNF levels (33). Elevated TNF levels may be necessary but not sufficient for the lethal toxicity of endotoxin.

On the other hand, our data indicate that the protective effects of BPI may be due to its marked inhibition of IL-6 production, which was evident 4 h after LPS injection. A variety of published evidence links this cytokine to septic or endotoxic shock. Elevated serum IL-6 levels are found in the majority of patients with sepsis and show a strong positive correlation with mortality (36–39). In infants with sepsis, IL-6 levels are significantly increased, suggesting that plasma IL-6 is a specific indicator of sepsis (40). In addition, plasma IL-6 levels are significantly elevated in patients and chimpanzees with dissem-

inated intravascular coagulation (41, 42), and anti–IL-6 antibodies inhibit endotoxin-induced activation of the coagulation system (42), suggesting that IL-6 may be a pivotal factor for coagulation activation. Further, several studies suggest that IL-6 may be a terminal mediator of pathological responses to septic shock (43–46). In *E. coli*–infected baboons, treatment with tissue factor pathway inhibitor prevents lethality and lowers IL-6 levels but does not reduce elevated TNF levels (45). In mice with endotoxic shock, IL-6 continues to circulate up to the time of death and correlates with outcomes, suggesting that IL-6 may serve as a prognostic marker for endotoxic shock (47). Anti–IL-6 monoclonal antibodies protect mice against lethal *E. coli* infection or lethal TNF challenge, despite high circulating levels of TNF (46). Passive immunization against leukemia inhibitory factor (D factor) protects mice against the lethal effects of endotoxin and blocks the endotoxin-induced increase in serum IL-6 without altering the endotoxin-induced rise in serum levels of TNF (44). Thus, it seems a reasonable hypothesis that BPI exerts its protective effects in endotoxic shock by attenuating the production of IL-6 through a TNF-independent mechanism. Further studies should be directed to understanding how BPI inhibits endotoxin from stimulating IL-6 accumulation without preventing the rise in TNF levels.

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