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Synthetic Endotoxin-Binding Peptides Block Endotoxin-Triggered TNF-α Production by Macrophages In Vitro and In Vivo and Prevent Endotoxin-Mediated Toxic Shock

Silke Dankesreiter, *Adolf Hoess,[†] Jens Schneider-Mergener,[‡] Hermann Wagner,* and Thomas Miethke[1]*

Lipid A, the conserved portion of endotoxin, is the major mediator of septic shock; therefore, endotoxin-neutralizing molecules could have important clinical applications. Here we show that peptides derived from *Limulus* anti-LPS factor (LALF), bactericidal/permeability increasing protein (BPI) and endotoxin-binding protein, bind to lipid A and block the recombinant LALF/lipid A interaction in vitro. Because their neutralizing capacity in vitro as well as in vivo has been limited, we created hybrid peptides comprising two endotoxin-binding domains. The hybrid molecule LL-10-H-14, containing endotoxin-binding domains from LALF and endotoxin-binding protein, turned out to be the most active peptide within the series of peptides tested here to inhibit the CD14/lipid A interaction and is able in vitro to block the endotoxin-induced TNF-α release of murine macrophages up to 90%. Furthermore, LL-10-H-14 not only reduced peak serum levels of TNF-α of mice when preinjected but also reduced TNF-α levels when given 15 min after the endotoxin challenge. As compared with other peptides, only LL-10-H-14 is able to strongly decrease endotoxin-stimulated TNF-α release by human macrophage cell lines as well as by PBMC. Furthermore, the hybrid peptide is protective against endotoxin-provoked lethal shock. As such, LL-10-H-14 could have prophylactic and/or therapeutic properties in humans for the management of septic shock. ***The Journal of Immunology,*** **2000, 164: 4804–4811.**

Endotoxin is a major constituent of the outer membrane of Gram-negative bacteria and is recognized as a key molecule in the pathophysiology of Gram-negative shock. On release endotoxin interacts with the endotoxin-binding protein (LBP),[2] an acute phase protein synthesized by liver cells (1, 2). LBP catalyzes the binding of endotoxin to CD14, which is the primary receptor for endotoxin and is expressed mainly on macrophages (3). The recently defined membrane proteins Toll-like receptors 2 and 4, which are structurally related to the *Drosophila* protein Toll, appear to be involved in CD14-mediated signal transduction (4, 5). Endotoxin-stimulated macrophages secrete a number of cytokines like TNF-α, IL-1, IL-6, and others, which in toxic concentrations are mediators for the development of septic shock (6, 7). In certain animal models, TNF-α is prime for the lethality induced on injection of endotoxin (8).

To prevent the cascade of endotoxin-induced events in humans, several approaches have been explored. First, Abs directed against endotoxin or the structurally conserved lipid A moiety were developed with the aim to neutralize endotoxins generated by different Gram-negative bacteria. However, these Abs appeared not to recognize natural endotoxins, i.e., lipid A carrying the polysaccharide component (9). mAbs recognizing core structures of endotoxin of different Enterobacteriaceae may be promising in that they neutralize endotoxin-mediated toxicity in vivo (10). However, again their use may be limited because of their lack of cross-reactivity among different kinds of endotoxin. Second, anti-TNF-α Abs or TNF receptor antagonists have also been evaluated as very effective in their potential to protect against lethal endotoxinemia in several animal models (8, 11, 12). However, none of these molecules appeared to be effective in humans. Furthermore, complete neutralization of TNF-α during an infection is probably disadvantageous, because in physiological concentrations TNF-α orchestrates the inflammatory reaction of the infected host (13). Third, application of endotoxin-binding molecules has been investigated which may prevent the endotoxin-LBP-CD14 complex formation. Several candidate molecules with endotoxin-binding and neutralizing capability are known and bactericidal/permeability increasing protein (BPI) may be until now the most promising candidate (14–16). *Limulus* anti-LPS factor (LALF) is a protein from the horseshoe crab with high potential to block endotoxin-mediated activities in animals (17–20). Both BPI and LALF crystal structures are known, and the LALF analysis revealed a detailed view of a potential endotoxin-binding site (21). The site consists of the aa 31–52 and is characterized by an alternating series of positively charged and hydrophobic residues forming a positively charged amphipathic loop (21). Two other endotoxin-binding proteins from mammals, namely bactericidal/permeability increasing protein (BPI) and LPS binding protein (LBP), were proposed to have a similar endotoxin binding site (21). Although unrelated to LALF, endotoxin-binding domains were identified in both proteins using synthetic peptides (2, 22, 23). The endotoxin-binding domains of all three proteins are functionally competent within LBP in domain exchange mutant proteins (24).

Recent approaches to develop molecules that neutralize endotoxin have concentrated on characterizing lipid A-binding regions

*Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany; [†]MorphoSys, Martinsried/Munich, Germany; and [‡]Institute of Medical Immunology, Humboldt University of Berlin, Berlin, Germany

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[1] Address correspondence and reprint requests to Dr. Thomas Miethke, Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Trogerstrasse 9, 81675 Munich, Germany. E-mail address: Thomas.Miethke@lrz.tu-muenchen.de

[2] Abbreviations used in this paper: LBP, endotoxin-binding protein; BPI, bactericidal/permeability increasing protein; LALF, *Limulus* anti-LPS factor; D-GalN, D-galactosamine; PMB, PMB sulfate; rLALF, recombinant LALF; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VLE, very low endotoxin.

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from endotoxin-binding peptides and proteins. This includes synthetic peptides derived from sequences of polymyxin B sulfate (PMB) (25), *Tachypleus* anti-LPS factor (26), recombinant LALF (rLALF) (27), BPI (23), CAP-18 (28, 29), and LBP (22).

Here, we further examined the endotoxin-neutralizing potential of a variety of peptides in in vitro and in vivo studies with the aim to optimize their endotoxin-neutralizing capacity. We developed a series of peptides derived from LALF, BPI, and LBP as well as hybrid peptides, comprising two endotoxin-binding domains. The peptide LL-10-H-14, a hybrid between LALF and LBP, showed exceptional activity. It is able to block endotoxin-induced TNF-α release of murine and human macrophages as well as peripheral blood leukocytes. Most surprisingly, LL-10-H-14 is effective in suppressing TNF-α levels in mice pre- or postinjected with endotoxin and to prevent lethality in the endotoxin/D-galactosamine (D-GalN) mouse model.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were bought from Harlan-Winkelmann (Borchen, Germany). All mice were ordered with an age of 8–10 wk and housed in our own animal facility.

Cell culture

Murine cell lines were cultured in medium containing <0.01 endotoxin units (EU)/ml endotoxin (very low endotoxon (VLE) RPMI 1640, Seromed, Biochrom, Berlin, Germany). The medium was supplemented with 10% (v/v) FCS (Seromed, Biochrom), 5×10^{-5} M 2-ME (Life Technologies, Karlsruhe, Germany), and antibiotics (penicillin G (100 IU/ml medium) and streptomycin sulfate (100 IU/ml medium)).

For culture of the human cell line Mono Mac 6, the VLE RPMI 1640 medium was supplemented with 5% (v/v) human serum (human serum (male), Sigma, Deisenhofen, Germany) and 5×10^{-5} M 2-ME. Human PBMC were also cultured in VLE RPMI 1640 medium but supplemented with 5% (v/v) of the donor serum and 5×10^{-5} M 2-ME.

Reagents

Peptide sequences are given in Table I. All experiments were performed with endotoxin from *Salmonella enteritidis* (Sigma). lipid A, and PMB were purchased from Sigma. D-GalN came from Carl Roth (Karlsruhe, Germany). CD14 was kindly donated by Dr. C. Schütt (Institute of Immunology, Greifswald, Germany). LALF was generously provided by Dr. R. C. Liddington (Dana Farber Cancer Institute, Boston, MA). For the generation of stock solutions, all reagents were dissolved in endotoxin-free water (water for embryo transfer, Sigma).

Peptide synthesis

Peptides were prepared on an automated peptide synthesizer (Abimed Analyes-Technik, Langenfeld, Germany) using a standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) machine protocol. For detection purposes, all peptides were biotinylated using 2 eq of biotin activated with 2 eq of benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate and 4 eq of *N*-methylmorpholine. The cleaved peptides were worked up according to the manufacturer's protocols and purified with reversed phase HPLC to a purity of >95%. Cyclic peptides were obtained by incubating cysteine-containing peptides in 10% DMSO for 24 h at room temperature (30). The peptides were then purified by reversed phase HPLC to a purity of >95%. The characterization of the peptides was done by laser desorption-time of flight mass spectroscopy.

Competitive inhibition of rLALF/lipid A binding

Lipid A (100 μl, 0.5 μg/ml), dissolved in PBS, was coated on a microtiter plate (Polysorp U96, Nunc, Wiesbaden, Germany) for 120 min at 37°C. After blocking with PBST for 20 min (room temperature), the solid phase was incubated for 60 min with 100 μl containing increasing amounts of peptides (0.01–100 μg/ml) mixed with rLALF (0.2 μg/ml, dissolved in PBS plus Tween (PBST)). After three washings with PBST, the ELISA was developed with a rabbit antiserum against rLALF (incubation time, 60 min) and a second anti-rabbit Ab conjugated (Sigma) to alkaline phosphatase (incubation time, 60 min). *p*-Nitrophenyl phosphate (1 mg/ml) was used as a substrate, and the absorbance was quantitated at 405 nm with a microplate reader. Each measurement was performed in duplicate.

Competitive inhibition of CD14/lipid A binding

CD14 A (100 μl, 5 μg/ml), dissolved in PBS, was coated on a microtiter plate (Polysorp U96) for 120 min at room temperature. After blocking with PBST for 30 min (room temperature), the solid phase was incubated for 120 min with 100 μl peptides (10 μg/ml) mixed with FITC-labeled endotoxin (2.5 μg/ml, dissolved in PBST). After three washings with PBST, an alkaline phosphatase-coupled anti-FITC mAb (dissolved 1:2500 in PBST, Boehringer Mannheim, Mannheim, Germany) was added. After another wash cycle, the ELISA was developed with *p*-nitrophenyl phosphate (1 mg/ml) as substrate, and the absorbance was quantitated at 405 nm using a microplate reader. Each measurement was performed in duplicate.

Stimulation protocol

To analyze the inhibitory effect of the peptides on TNF-α release in vitro, the murine macrophage cell line ANA-1 (1×10^5 cells/well), the human monocyte cell line Mono Mac 6 (1×10^5 cells/well), as well as PBMC (5×10^5 cells/well) were incubated with different peptides (5 μg/ml), and 5 min later the cells were stimulated with endotoxin (40 ng/ml for ANA-1 and Mono Mac 6 cells, 5 ng/ml for PBMC) for 30 min (37°C, 5% CO_2). To remove unbound endotoxin and peptide, the cells were washed twice and incubated for another 4 h (37°C, 5% CO_2). At this time point, supernatants were harvested and stored at −20°C until the TNF-α content was measured by ELISA. To analyze cell viability a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described below. Cell cultures were performed in 96-well, flat-bottom microtiter plates (Nunclon surface, Nunc).

Injection protocol

To induce endotoxin shock, mice were preinjected with D-GalN (i.p., 4 mg/mouse in 100 μl) and after 1 h challenged with endotoxin (i.p., 1 μg/mouse in 50 μl). For prevention of shock or systemic TNF-α release mice were pretreated 15 min before the endotoxin injection with peptides (i.p., 100 μg/mouse in 50 μl), PMB (i.p., 100 μg/mouse in 50 μl), or solvent (endotoxin-free water, i.p., 50 μl).

Serum preparation

Treated and control animals were killed and blood was drawn from the heart. Subsequently, the samples were centrifuged at 10,000 rpm (7 min), and the supernatant was collected and stored at −20°C until used in the TNF-α ELISA.

Determination of TNF-α

Murine TNF-α serum levels were determined using commercially available TNF-α ELISA kits (DuoSeT, Genzyme, Cambridge, MA). The assay was performed as described by the manufacturer except that the capture Ab was used in a concentration of 3 μg/ml, experimental samples and standards were incubated overnight (4°C), and the detecting Ab was added in a concentration of 2.1 μg/ml. Serum samples and culture supernatants were used at a dilution of 1:2 and measured twice. Human TNF-α was monitored using the TNF-α ELISA (OptEIA) from PharMingen (San Diego, CA). The assay was performed as described by the manufacturer.

MTT assay

To measure cell viability, the MTT assay was performed. Briefly, the cultured cells (1×10^5 ANA-1 cells/well, 1×10^5 Mono Mac 6 cells/well, or 5×10^5 PBMC/well) were incubated with MTT (420 μg/ml, Sigma), which is metabolized by living cells in 3 h. After solubilization of MTT crystals with HCl-isopropanol (150 μl/well), the OD was determined at 570/690 nm.

Results

Endotoxin-neutralizing potential of LBP

To analyze the potential of peptides derived from LBP, BPI, and LALF to neutralize endotoxin, we compared their ability to block the binding of lipid A to rLALF. The cyclic LALF-derived peptide LALF-14 was chosen in that it has been described as the smallest unit still possessing full endotoxin-binding capacity (Ref. 31 and Table I). Analogues peptides derived from BPI (B-14) and LBP (H-14) were prepared based on the alignment as proposed by Hoess et al. (21). Both, H-14 and B-14 were derived from amino

Table I. *LALF-derived peptides*

Peptide Designation	Sequence	Comment
LALF protein	D-H-E-C-H-Y-R-I-K-P-T-F-R-R-L-K-W-K-Y-K-G-K-F-W-C-P-S	aa 28–54 ≙ binding domain
LL-10	K-P-T-F-R-R-L-K-W-K	LALF aa 36–45, linear
LALF-14	G-C-K-P-T-F-R-R-L-K-W-K-Y-K-C-G	LALF aa 36–47, cyclic
LALF-22	G-C-H-Y-R-I-K-P-T-F-R-R-L-K-W-K-Y-K-G-K-F-W-C-G	LALF aa 31–52, cyclic
LBP-derived peptide		
H-14	C-R-W-K-V-R-K-S-F-F-K-L-Q-C-G	hLBP[a] aa 90–101 ≙ binding domain, cyclic
BPI-derived peptide		
B-14	C-K-W-K-A-Q-K-R-F-L-K-M-S-C-G	hBPI aa 90–101 ≙ binding domain, cyclic
Dimeric peptides		
LL-10-L-11	K-P-T-F-R-R-L-K-W-K-C-K-C-T-F-R-R-L-K-W-K-C-G	Linear + cyclic LALF
LL-10-L-14	K-P-T-F-R-R-L-K-W-K-C-K-P-T-F-R-R-L-K-W-K-Y-K-C-G	Linear + cyclic LALF
LL-10-H-14	K-P-T-F-R-R-L-K-W-K-C-R-W-K-V-R-K-S-F-F-K-L-Q-C	Linear LALF + cyclic hLBP
B-10-H-14	K-K-I-E-S-A-L-R-N-K-C-R-W-K-V-R-K-S-F-F-K-L-Q-C-G	Linear hBPI + cyclic hLBP
Control peptides		
NEU-10	G-A-T-P-Q-D-L-N-T-M-K	Neutral net charge
PA-10	P-R-R-W-T-F-K-L-K-K	LALF aa 36–45, linear, randomized sequence

[a] h, human.

acid regions 90–101 of LBP or BPI, respectively, and comprise 12 aa from the endotoxin-binding domain as well as 2 cysteines at both ends to form a cyclic peptide after oxidation (Table I). The cyclic peptide LALF-22 consists of the complete endotoxin-binding domain of LALF (Table I). As controls, we used a linear peptide comprising the minimal LALF/endotoxin-binding domain (LL-10) and NEU-10 with a neutral net charge. In agreement with Ried et al. (31), we observed that the cyclic peptides LALF-14 (Fig. 1, ○) and H-14 (Fig. 1, ■) efficiently blocked the rLALF/lipid A interaction in contrast to the linear peptide LL-10 (Fig. 1, ●) and the cyclic peptide B-14 (Fig. 1, □). The control peptide NEU-10 was inactive as expected (Fig. 1, ◆). The cyclic peptide LALF-22 (Fig. 1, ▲) was as active as the positive control PMB (Fig. 1, △). At least 10 μg/ml of an active peptide were required to display full activity. Similar results were obtained when the peptides LALF-14, H-14, and B-14 were analyzed for their ability to bind to lipid A or to block the LBP/lipid A interaction (data not shown). Based on these results, the peptides derived from LALF and LBP were further investigated as candidates to block release of TNF-α by endotoxin-stimulated macrophages.

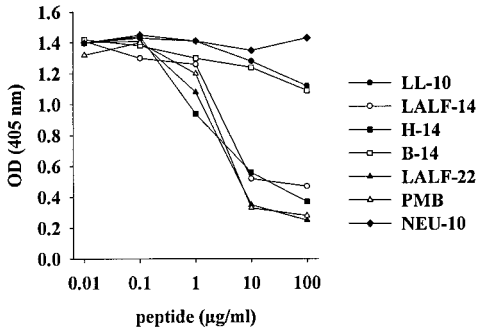

FIGURE 1. Inhibition of the rLALF/lipid A interaction by endotoxin-binding peptides. Microtiter plates were coated with lipid A (0.5 μg/ml) and subsequently incubated with a mixture of rLALF (0.2 μg/ml) and graded doses of the peptides LL-10, LALF-14, H-14, B-14, LALF-22, PMB, and NEU-10. Bound rLALF was detected using an anti-rLALF serum and an alkaline phosphatase-conjugated second Ab. The peptide NEU-10 (neutral net charge) and PMB served as negative and positive control, respectively.

Analysis of endotoxin-binding peptides based on their ability to reduce TNF-α secretion in vitro

TNF-α is a key molecule in the pathophysiology of septic shock (8, 32). It has been shown that Abs against TNF-α protect experimental animals in endotoxin- as well as superantigen-induced lethal shock models, which are considered as model systems for Gram-negative and Gram-positive sepsis, respectively (8, 33, 34). To explore the potential of the endotoxin-binding peptides to inhibit TNF-α release by endotoxin-stimulated macrophages we first evaluated cells of the murine macrophage cell line ANA-1. Accordingly, the peptides LALF-14, H-14, B-14, LALF-22, NEU-10, and PA-10 (another control peptide with a randomized LL-10 peptide sequence) were compared with Polymyxin B (PMB) in their ability to neutralize endotoxin. Most of the cyclic peptides tested failed to block endotoxin-induced TNF-α secretion by the cell line (Fig. 2*A*). However, the cyclic peptide LALF-22 (which consists of the complete endotoxin-binding domain of LALF) was effective in that it blocked up to 65% of the endotoxin-induced TNF-α secretion (Fig. 2*A*). In fact, the inhibitory activity of LALF-22 was comparable with that of PMB (Fig. 2*A*). As expected, the control peptides NEU-10 and PA-10 did not influence TNF-α production in this in vitro system (Fig. 2*A*). To verify that the peptides were not toxic for ANA-1 cells, the metabolic activity of the cells was analyzed using the MTT assay. As demonstrated in Fig. 2*B*, the peptides did not diminish the metabolism of MTT. The statistical significance of the data are presented in Table II. Whereas the extent of LALF-22 mediated inhibition of TNF-α release by ANA-1 cells differed significantly from inhibition by the negative control peptide PA-10, this was not the case for the peptides H-14, B-14, and LALF-14. Because peptides LALF-14 and H-14 bind lipid A and block the LBP/lipid A interaction but failed to neutralize natural endotoxin-induced cytokine production, we conclude that effective peptides, such as LALF-22, need to consist of the complete endotoxin-binding domain.

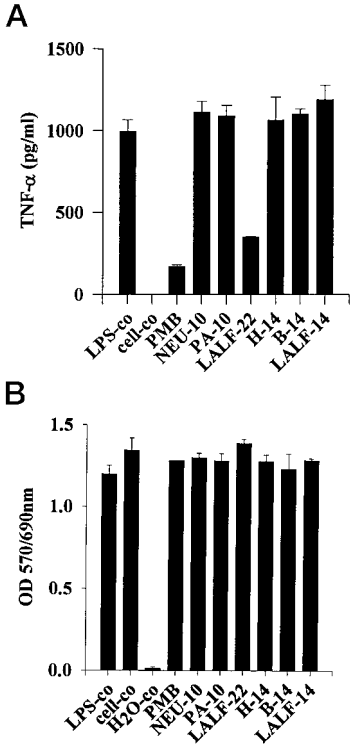

FIGURE 2. *A,* Peptide-mediated inhibition of TNF-α release from endotoxin-stimulated ANA-1 cells. The murine macrophage cell line ANA-1 (1×10^5 cells/well) was stimulated with endotoxin (40 ng/ml) for 30 min. Subsequently, the cells were washed and cultured for another 4 h. At this time point, the supernatant was removed and analyzed for TNF-α by ELISA. Five minutes before the endotoxin stimulus, the indicated peptides (5 μg/ml) were added. The peptides NEU-10 (neutral net charge) and PA-10 (randomized LL-10 sequence) were used as negative controls. PMB (5 μg/ml) served as positive control. LPS-co depicts cells stimulated with endotoxin in the absence of peptides; cell-co shows unstimulated cells. Error bars represent the SD of duplicates. The experiment represents one of three identical experiments. *B,* Viability of endotoxin-stimulated and peptide-treated ANA-1 cells. The viability of ANA-1 cells described in *A* was analyzed by the MTT assay. After removal of the supernatant, MTT (420 μg/ml) was added, and the cells were incubated for 3 h. After solubilization of the MTT crystals with HCl-isopropanol (150 μl/well) the optical density was determined at 570/690 nm. H_2O-co demonstrates a hypotonic negative control where unstimulated cells were incubated with H_2O. Error bars represent the SD of duplicates.

Optimization of endotoxin-binding peptides

Endotoxin has been described to form multimeric complexes (35). In an attempt to increase the biological potential of endotoxin-binding peptides, we reasoned that two endotoxin-binding domains encoded by one peptide might increase the binding activity to endotoxin because of an avidity effect. Therefore, we developed peptides consisting of two endotoxin-binding domains ("dimeric" peptides). It was important for the design of the dimeric peptides to select these peptides with high endotoxin-binding capacity and with as few amino acids as possible because the peptide synthesis efficiency decreases with the growing length of the peptide chain. The peptides LL-10-H-14 and LL-10-L-14 are based on the linear LALF-derived peptide LL-10 fused with the cyclic LBP-derived peptide H-14 or the cyclic LALF-derived peptide LALF-14, respectively (Table I). Peptide B-10-H-14 consists of the linear BPI-derived peptide B-10 plus the cyclic LBP-derived peptide H-14 (Table I). We used the combination of a linear with a cyclic peptide, as the combination of two cyclic peptides with the need of four cysteines would have been to complicated to be prepared. We then compared the ability to block the CD14/endotoxin interaction of the dimeric peptides with the cyclic peptides LALF-14 and H-14. As detailed in Fig. 3, LL-10-H-14, LL-10-L-14, and B-10-H-14 inhibited binding of endotoxin to CD14 to a higher degree than LALF-14 or H-14. Importantly, the potency of LL-10-H-14 to neutralize endotoxin and thus to suppress the release of TNF-α by stimulated ANA-1 cells was higher than that of LALF-22 and was at least as effective as PMB (Fig. 4*A*). The magnitude of suppression of the TNF-α release could not be enhanced by the addition of the single peptides LL-10 and H-14 to the cell culture, indicating that the two endotoxin-binding domains of LL-10-H-14 must be in close proximity for efficient neutralization of endotoxin (Fig. 4*A*). The dimeric peptide LL-10-L-14, in which the cyclic LBP-derived domain was substituted with a LALF-derived cyclic domain, was incapable of blocking TNF-α release (Table I, Fig. 4*B*). Substitution of the linear part of LL-10-H-14 with a linear peptide derived from BPI resulting in the peptide B-10-H-14 (Table I) also diminished its capacity to inhibit TNF-α release (Fig. 4*B*). In addition, the two dimeric proteins LL-10-L-11 (consisting of a linear LALF-derived part plus a shorter cyclic LALF-derived part) and B-10-L-14 (consisting of a linear BPI-derived part plus a cyclic LALF-derived part) displayed no capacity to inhibit TNF-α release

Table II. *Statistical analysis of the data of several independent experiments concerning the peptide-mediated inhibition of endotoxin-induced TNF-α release*[a]

Inhibition of TNF-α Release by ANA-1 Cells: Comparison Between				Inhibition of TNF-α Release by Mono Mac 6 Cells: Comparison Between				Inhibition of TNF-α Release In Vivo: Comparison Between			
Peptide 1	Peptide 2	Result	α[b]	Peptide 1	Peptide 2	Result	α	Peptide 1	Peptide 2	Result	α
NEU-10	PA-10	H0[c]	0.520	NEU-10	PA-10	H0	0.591	NEU-10	PA-10	H0	0.725
PMB	PA-10	H1[d]	>0.001	PMB	PA-10	H1	0.004	PMB	PA-10	H1	>0.001
LALF-22	PA-10	H1	0.001	LALF-22	PA-10	H0	0.709	LALF-22	PA-10	H1	0.008
H-14	PA-10	H0	0.310	H-14	PA-10	H0	0.624	H-14	PA-10	H0	0.276
B-14	PA-10	H0	0.274	B-14	PA-10	H0	0.762	B-14	PA-10	H0	0.604
LALF-14	PA-10	H0	0.426	LALF-14	PA-10	H0	0.528	LL-10	PA-10	H0	0.371
LL-10-H-14	PA-10	H1	>0.001	LL-10-H-14	PA-10	H1	0.007	LALF-14	PA-10	H0	0.126
B-10-H-14	PA-10	H0	0.947	B-10-H-14	PA-10	H0	0.805	LL-10-H-14	PA-10	H1	>0.001
LL-10 + H-14	PA-10	H0	0.612	LL-10 + H-14	PA-10	H0	0.614	B-10-H-14	PA-10	H0	0.734
LL-10-H-14	PMB	H0	0.432	LL-10-H-14	PMB	H0	0.784	LL-10 + H-14	PA-10	H0	0.915
LL-10-H-14	LALF-22	H0	0.096	LL-10-H-14	LALF-22	H1	0.006	LL-10-H-14	PMB	H0	0.905
								LL-10-H-14	LALF-22	H0	0.225

[a] Univariate analysis of variance.
[b] Probability of error.
[c] H0, no significant difference between two peptides, $\alpha > 0.05$.
[d] H1, significant difference between two peptides, $\alpha < 0.05$.

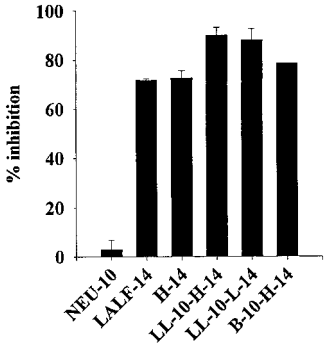

FIGURE 3. Inhibition of the CD14/lipid A interaction by dimeric endotoxin-binding peptides. Microtiter plates were coated with CD14 (5 μg/ml). Subsequently, a mixture of the indicated peptides (10 μg/ml) and FITC-labeled endotoxin (2.5 μg/ml) was added. Bound endotoxin was detected by adding an anti-FITC Ab conjugated with alkaline phosphatase. The peptide NEU-10 (neutral net charge) served as negative control. Error bars depict SD of the results of at least two independent experiments. B-10-H-14 was analyzed only once.

(data not shown). Statistical analysis of the data revealed that LL-10-H-14 differed significantly in its potential to block the release of endotoxin-mediated TNF-α release in comparison to the negative control peptide PA-10 (Table II). There was no difference when PMB and LL-10-H-14, or LL-10-H-14 and LALF-22 were compared (Table II). These data suggested that LL-10-H-14 and LALF-22 display the highest potency for the neutralization of endotoxin among the peptides examined.

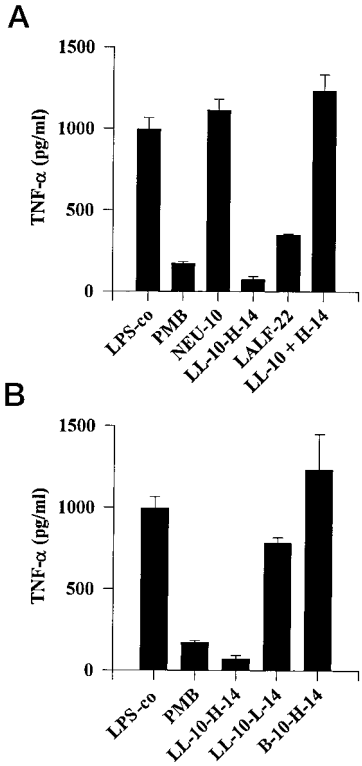

FIGURE 4. Analysis of dimeric peptides. The experiments were performed with the indicated peptides as described in Fig. 2. *A*, Comparison of the activity of LL-10-H-14 with LALF-22, or with the combination LL-10 plus H-14. Error bars depict the SD of duplicates. The comparison between LL-10-H-14 and LALF-22 was performed three times and the comparison between LL-10-H-14 and the combination LL-10 plus H-14 twice, always with identical results. LPS-co represents cells stimulated only with endotoxin. *B*, Comparison of different dimeric peptides. Error bars depict the SD of duplicates. The experiment represents one of two identical experiments.

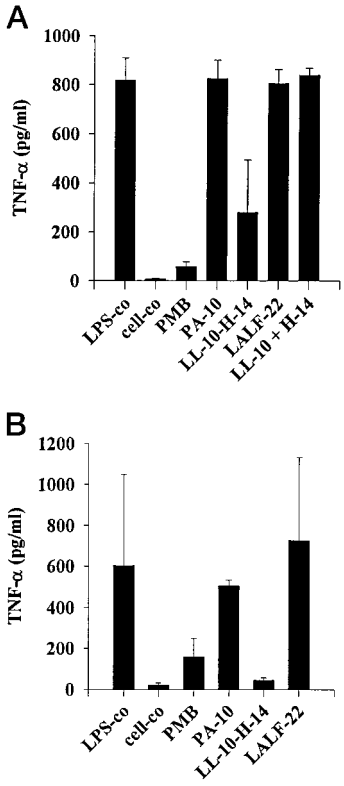

FIGURE 5. Inhibition of TNF-α release from endotoxin-stimulated Mono Mac 6 cells and human PBMC by endotoxin-binding peptides. *A*, The human macrophage cell line Mono Mac 6 (1×10^5 cells/well) was stimulated with endotoxin (40 ng/ml). Five minutes before the endotoxin stimulation, the indicated peptides (5 μg/ml) were added. After 30 min, the cells were washed and subsequently cultured for another 4 h. The supernatant was removed and the TNF-α content was analyzed. LPS-co represents cells stimulated only with endotoxin, cell-co depicts unstimulated cells, and PMB shows cells treated with PMB (5 μg/ml) instead with peptides. Error bars represent the SD of duplicates. The experiments was repeated twice with identical results. *B*, The experiment was performed as above except that 5×10^5 PBMC were stimulated with endotoxin at a concentration of 5 ng/ml. Error bars represent the SD of duplicates.

Endotoxin-induced TNF-α secretion of the human macrophage cell line Mono Mac 6 as well as peripheral blood leukocytes is reduced by endotoxin-binding peptides

To address the question of whether endotoxin-binding peptides are also able to reduce TNF-α release of human cells on endotoxin stimulation, we used the human macrophage cell line Mono Mac 6 and tested the peptides in the same system as described for the murine cell line ANA-1. As demonstrated in Fig. 5*A*, LL-10-H-14 reduced TNF-α levels by ~66%. The observation that LALF-22 was ineffective in blocking TNF-α release from the human Mono Mac 6 cell line contrasted to the results obtained with the murine ANA-1 cell line. As described for murine cells, the combination of peptides LL-10 plus H-14 was also incompetent to block the release of TNF-α by endotoxin-stimulated Mono Mac 6 cells (Fig. 5*A*). A statistical analysis of the data showed that LL-10-H-14 differed significantly from the control peptide PA-10 in its potential to inhibit TNF-α release by Mono Mac 6 cells (Table II). This was not the case for LALF-22. Comparison of LL-10-H-14 with

PMB showed no difference; however, LL-10-H-14 differed significantly from LALF-22 (Table II). Finally, we analyzed human PBL. Fig. 5*B* shows that LL-10-H-14 was also able to diminish the endotoxin-stimulated TNF-α release. Again LALF-22 was ineffective. These data show that LL-10-H-14 is the most active peptide on human cells within the series of peptides analyzed here.

Suppression of TNF-α release in vivo

To address the issue whether endotoxin-binding peptides are functional in vivo we analyzed whether LL-10-H-14 blocks systemic TNF-α release in mice challenged with endotoxin. To this C57BL/6 and BALB/c mice were pretreated with LL-10-H-14 and 15 min later challenged with endotoxin. TNF-α serum levels were determined at the peak response, i.e., 75 min after the endotoxin challenge. Fig. 6*A* demonstrates that LL-10-H-14 inhibited the endotoxin-induced TNF-α serum level by ~75% in both strains of mice and was as effective as PMB. When compared with the linear peptide LL-10 and the cyclic peptides LALF-14 and LALF-22, LL-10-H-14 appeared to be more effective, although there were no differences to LALF-22 (Fig. 6*B*). Statistical evaluation of the data revealed that only LL-10-H-14 and LALF-22 were significantly different to the control peptide PA-10 (Table II). As described for the experiments with murine ANA-1 cells, there was no difference between LL-10-H-14 and LALF-22 (Table II). To evaluate how long the peptide LL-10-H-14 is effective in vivo the time interval between peptide injection and endotoxin challenge was varied from 1 h pre- to 1 h post-endotoxin challenge. TNF-α serum levels were suppressed maximally if peptide was injected in an interval 15 min before or after endotoxin challenge (Fig. 6*C*). Longer intervals of pretreatment or a further delayed injection were less or not effective (Fig. 6*C*). Obviously, LL-10-H-14 is functional in vivo for ~15 to 30 min.

The endotoxin-binding peptide LL-10-H-14 protects mice in the endotoxin/D-GalN model

It is most critical to determine whether a synthetic peptide is able to prevent endotoxin-induced lethality and therefore might be a candidate for treatment of septic shock. We therefore used the murine endotoxin/D-GalN shock model (36). D-GalN sensitizes mice to the toxic effects of TNF-α by several orders of magnitude by an ill-defined, UTP-depleting, hepatotoxic mechanism (37). Pretreatment of D-GalN-sensitized C57BL/6 mice with the peptide 15 min before the endotoxin challenge increased the survival rate to 100% (Fig. 7). In this experiment, mice were sensitized with 4 mg D-GalN/mouse. Higher concentrations of D-GalN (which renders mice more susceptible to TNF-α (36)) reduced the ability of LL-10-H-14 to increase survival of endotoxin challenged mice. Accordingly, sensitization with 8 mg D-GalN/mouse resulted in a survival rate of only 40% of LL-10-H-14 pretreated mice (data not shown).

Discussion

Septic shock is considered as a serious problem in intensive care units. Despite effective antibiotics the lethality of septic shock remains high (38). This problem is likely to increase as more aggressive operations with immunosuppressed patients will be performed. Although our knowledge of the pathophysiology of septic shock increases its complexity is far from being understood. It is, however, accepted that once invading bacteria have triggered the complex cascade leading to septic shock, therapeutic interventions my often be too late in time. Strategies thus would need to be developed which prevent pathophysiological reactions of the infected host. Gram-negative bacteria are the most frequent cause of septic shock and endotoxin, an integral part of the outer membrane

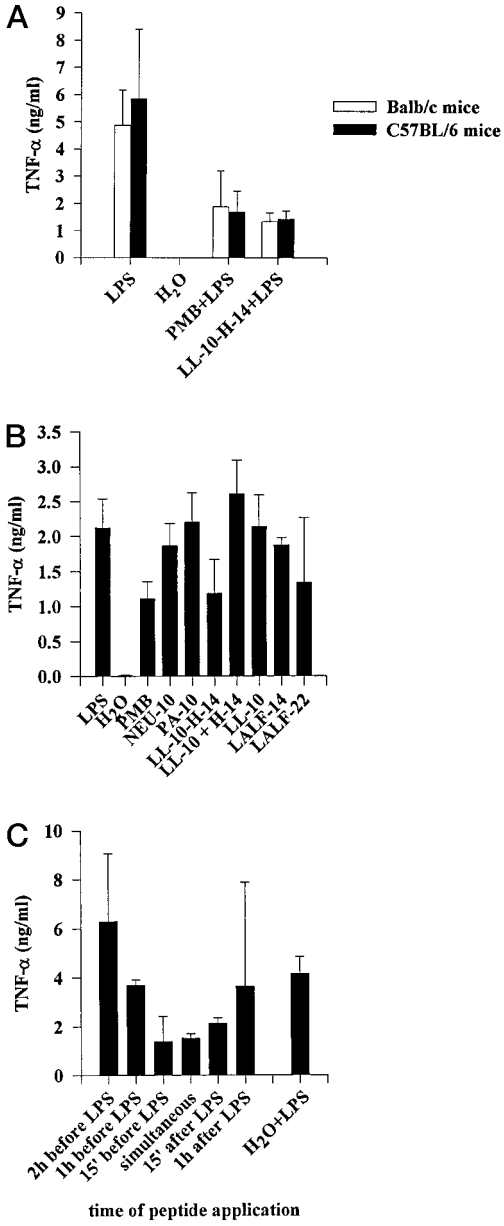

FIGURE 6. *A*, Inhibition of endotoxin-induced TNF-α release in vivo. Groups of BALB/c ($n = 2$) and C57BL/6 ($n = 2$) mice were treated with endotoxin (1 μg/mouse) or PMB (100 μg/mouse) plus endotoxin (1 μg/mouse) or LL-10-H-14 (100 μg/mouse) plus endotoxin (1 μg/mouse). After 75 min, the mice were sacrificed and TNF-α serum levels were analyzed. Control mice were injected only with solvent (H_2O). Error bars depict the SD of two equally treated mice. The experiments represents one of two identical experiments. *B*, Comparison of the in vivo efficiency of the dimeric peptide LL-10-H-14 with cyclic and linear peptides. Groups of BALB/c mice ($n = 3$, PA-10 injected mice $n = 2$) were pretreated with the peptides (100 μg/mouse) indicated. After 15 min, the mice were injected with endotoxin (1 μg/mouse). The mice were sacrificed 75 min after the endotoxin injection, and serum was prepared to quantify the TNF-α content by ELISA. PMB-pretreated mice ($n = 3$, 100 μg/mouse) served as positive control for TNF-α inhibition. LPS represents mice ($n = 3$) treated only with endotoxin; H_2O depicts mice ($n = 3$) injected only with solvent (H_2O, 100 μl/mouse). The error bars represent the SD within groups of mice. *C*, Kinetics of LL-10-H-14-mediated inhibition of TNF-α release in vivo. With respect to the endotoxin injection (1 μg/mouse), groups of BALB/c mice ($n = 2$) were pretreated, coinjected, or subsequently treated with LL-10-H-14 (100 μg/mouse) as indicated. H_2O + LPS represents mice ($n = 2$) injected with endotoxin (1 μg/mouse) and solvent (H_2O, 100 μl/mouse). The error bars represent the SD within groups of mice.

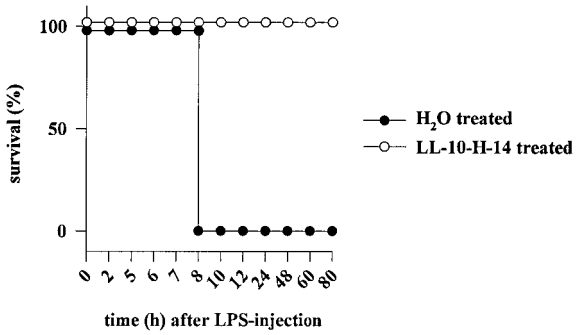

FIGURE 7. LL-10-H-14 prevents lethality in the endotoxin/D-GalN model. Two groups of BALB/c mice ($n = 5$) were pretreated with D-GalN (4 mg/mouse) for 1 h. Subsequently, the mice were challenged with endotoxin (1 μg/mouse). ○, Mice treated with LL-10-H-14 (100 μg/mouse) 15 min before the endotoxin injection; ●, mice that received solvent (H_2O).

of Gram-negative bacteria, is key to initiate the complex reaction pattern of the host (39–41). Here we show that the cyclic peptides H-14, B-14, LALF-14, and LALF-22 derived from the endotoxin-binding molecules LBP, BPI, and LALF, respectively, are able to bind to lipid A and block the rLALF/lipid A (Fig. 1 and Ref. 31) and the LBP/lipid A interaction (data not shown). LBP catalyzes the transfer of endotoxin from the solubilized to the membrane-bound form; i.e., it promotes the binding of endotoxin to the endotoxin receptor CD14 on macrophages, and this process is thought to represent the starting point of the shock cascade (1, 3, 42). In view of the ability of the peptides to block this interaction, we anticipated that they inhibit TNF-α secretion by endotoxin-stimulated macrophages. However, it turned out that the cyclic peptides H-14, B-14, and LALF-14 failed to be effective (Fig. 2*A*). In contrast, the cyclic peptide LALF-22 which consists of the complete endotoxin-binding domain of LALF was more effective (Fig. 2*A*). Thus, inhibition of endotoxin-induced TNF-α release may require larger structures than the minimal endotoxin-binding domains, which could be explained by the decreased affinity of the peptides as compared with the wild-type proteins. Alternatively, the different requirements are related to the structural differences between lipid A used for the binding to rLALF or LBP and natural endotoxin which was taken for the stimulation of macrophages. However, this seems unlikely because H-14 and LALF-14 bind to natural endotoxins of different Enterobacteriaceae (Ref. 31 and data not shown). The reason why LALF-22 was inactive on human cells is at present unclear (Fig. 5). In both in vitro settings, the identical endotoxin was used. Furthermore, LALF-22 was also ineffective when the human cells were stimulated by endotoxin in the presence of FCS, which was also used in the murine system, ruling out the possibility that different LBPs might be responsible for the differences observed (data not shown). The sequences of mouse and human CD14 show 66% amino acid identity (43). Therefore, it is possible that endotoxin is recognized by mouse and human CD14 in such a different way that LALF-22 is able or unable to interfere with this process, respectively.

To further increase the efficiency of endotoxin-binding peptides, we created a series of dimeric peptides, i.e., peptides containing two endotoxin-binding domains. The idea for the development of dimeric peptides was based on the rationale to increase the avidity of the peptide for endotoxin and that natural endotoxin-binding proteins like LBP may bind more than one endotoxin molecule per molecule of LBP (44). The dimeric peptide LL-10-H-14 consisting of the linear LALF-derived peptide LL-10 and the cyclic LBP-derived peptide H-14 displayed the highest activity of the series of peptides tested here to block the release of TNF-α by human and murine cells in repeated experiments (Figs. 4*A* and 5). The combination of the single peptides LL-10 plus H-14 did not result into the same effectiveness (Figs. 4*A*, 5, and 6*B*), emphasizing the importance of two endotoxin-binding domains being in close proximity. The exchange of the cyclic part H-14 with different LALF-derived cyclic peptides as well as substitution of LL-10 with B-10 destroyed the functional capacity of the dimeric peptide (Fig. 4*B*). Thus, endotoxin-binding domains from different endotoxin proteins cannot be exchanged on the peptide level, in contrast to mutant LBP proteins where the endotoxin-binding domain of LBP can be substituted with the domain from LALF as well as BPI without destroying the function of the protein (24).

There is evidence that peptides derived from LBP, BPI and LALF containing the endotoxin-binding domains may inhibit the endotoxin-mediated release of TNF-α in vivo (45). However, the interpretation of these data is clouded because in these experiments the peptides were mixed with endotoxin in vitro and the mixture used for challenge in vivo. Hence, we separated the injection of the peptide and endotoxin in time to mimic more closely the clinical situation. Using this injection protocol, we show that the peptide LL-10-H-14 was able to inhibit endotoxin-triggered TNF-α release in vivo (Fig. 6). Although LL-10-H-14 did not completely impair TNF-α production, mice were protected from endotoxin-mediated lethal shock (Fig. 7). A complete blockade of TNF-α production is probably not desirable because physiological concentrations of TNF-α are required to orchestrate inflammatory reactions aimed to localize bacterial invaders. For example, using the sublethal model of cecal ligation and puncture, Echtenacher et al. (13) showed that mice treated with a neutralizing anti TNF-α Ab succumbed to peritonitis, whereas untreated mice were able to control the infection. The ability of LL-10-H-14 to protect mice from lethal endotoxin shock was confined to a narrow window of D-GalN concentrations. Thus, mice sensitized with higher concentrations of D-GalN were only partially protected, and although the TNF-α levels were reduced they were presumably still sufficient to induce lethal shock.

The peptide LL-10-H-14 was as effective as PMB. Although in some experiments, LL-10-H-14 seemed to be more active, the statistical analysis revealed no differences between the two compounds. The biggest disadvantage of PMB is its well known toxicity which impairs severely its clinical use. Adverse reactions include nephrotoxicity, which is the most serious toxic effect, neurotoxicity, and hypersensitive reactions. We observed no toxic side effects in mice bolus-injected with LL-10-H-14. On the other, we did not analyze the potential toxicity of the compound after prolonged or repeated administration. Clearly, additional experiments need to be done to show that the peptide LL-10-H-14 is less toxic than PMB.

A limitation of the peptide LL-10-H-14 is the fact that the peptide is only active in vivo for ~30 min (Fig. 6*C*). Because of its low m.w., the agent is presumably rapidly cleared via the kidneys. Because endotoxin is given as a bolus injection in the model analyzed here, TNF-α serum levels are increased for only a short period of time (~4 h). In this case, the short period where the peptide LL-10-H-14 is effective in vivo is sufficient to inhibit systemic TNF-α secretion. However, in experimental models of bacterial infection as well as in an infectious clinical situation, a continuous supply of endotoxin is generated. In this situation, peptides like LL-10-H-14 must be either administered continuously or coupled to, e.g., protein carriers to increase their half-life in vivo. Experiments addressing this issue will be performed.

In comparison with murine macrophages, human macrophages are much more sensitive toward endotoxin; i.e., picogram amounts of endotoxin are sufficient to trigger TNF-α secretion in these

cells. Therefore, the effectiveness of the peptide LL-10-H-14 to block endotoxin-mediated TNF-α secretion by human macrophages could be much lower. However, as compared with all other peptides, only LL-10-H-14 was able to block release of TNF-α by endotoxin-stimulated human macrophages and PBMC to an extent similar to that observed with murine macrophages (Fig. 5). We think that the peptide LL-10-H-14 could be the basis for the development of endotoxin-neutralizing drugs for clinical use.

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References

1. Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science 249:1429.*
2. Lamping, N., A. Hoess, B. Yu, T. C. Park, C. J. Kirschning, D. Pfeil, D. Reuter, S. D. Wright, F. Herrmann, and R. R. Schumann. 1996. Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. *J. Immunol. 157:4648.*
3. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249:1431.*
4. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature 395:284.*
5. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. 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 Tlr4 gene. *Science 282:2085.*
6. Waage, A., and T. Espevik. 1988. Interleukin 1 potentiates the lethal effect of tumor necrosis factor α/cachectin in mice. *J. Exp. Med. 167:1987.*
7. Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock: association between interleukin 6, interleukin 1, and fatal outcome. *J. Exp. Med. 169:333.*
8. Beutler, B., I. W. Milsark, and A. C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:869.*
9. Galanos, C., M. A. Freudenberg, F. Jay, D. Nerkar, K. Veleva, H. Brade, and W. Strittmatter. 1984. Immunogenic properties of lipid A. *Rev. Infect. Dis. 6:546.*
10. Di Padova, F. E., H. Brade, G. R. Barclay, I. R. Poxton, E. Liehl, E. Schuetze, H. P. Kocher, G. Ramsay, M. H. Schreier, and D. B. McClelland. 1993. A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. *Infect. Immun. 61:3863.*
11. Nassif, X., J. C. Mathison, E. Wolfson, J. A. Koziol, R. J. Ulevitch, and M. So. 1992. Tumour necrosis factor α antibody protects against lethal meningococcaemia. *Mol. Microbiol. 6:591.*
12. Mathison, J. C., E. Wolfson, and R. J. Ulevitch. 1988. Participation of tumor necrosis factor in the mediation of Gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest. 81:1925.*
13. Echtenacher, B., W. Falk, D. N. Mannel, and P. H. Krammer. 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *J. Immunol. 145:3762.*
14. Elsbach, P. 1998. The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense. *J. Leukocyte Biol. 64:14.*
15. Ooi, C. E., J. Weiss, M. E. Doerfler, and P. Elsbach. 1991. Endotoxin-neutralizing properties of the 25 kD N-terminal fragment and a newly isolated 30 kD C-terminal fragment of the 55–60 kD bactericidal/permeability-increasing protein of human neutrophils. *J. Exp. Med. 174:649.*
16. Marra, M. N., C. G. Wilde, J. E. Griffith, J. L. Snable, and R. W. Scott. 1990. Bactericidal/permeability-increasing protein has endotoxin-neutralizing activity. *J. Immunol. 144:662.*
17. Aketagawa, J., T. Miyata, S. Ohtsubo, T. Nakamura, T. Morita, H. Hayashida, S. Iwanaga, T. Takao, and Y. Shimonishi. 1986. Primary structure of *Limulus* anticoagulant anti-lipopolysaccharide factor. *J. Biol. Chem. 261:7357.*
18. Morita, T., S. Ohtsubo, T. Nakamura, S. Tanaka, S. Iwanaga, K. Ohashi, and M. Niwa. 1985. Isolation and biological activities of *Limulus* anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). *J. Biochem. 97:1611.*
19. Roth, R. I., D. Su, A. H. Child, N. R. Wainwright, and J. Levin. 1998. *Limulus* antilipopolysaccharide factor prevents mortality late in the course of endotoxemia. *J. Infect. Dis. 177:388.*
20. Alpert, G., G. Baldwin, C. Thompson, N. Wainwright, T. J. Novitsky, Z. Gillis, J. Parsonnet, G. R. Fleisher, and G. R. Siber. 1992. Limulus antilipopolysaccharide factor protects rabbits from meningococcal endotoxin shock. *J. Infect. Dis. 165:494.*
21. Hoess, A., S. Watson, G. R. Siber, and R. Liddington. 1993. Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1.5 A resolution. *EMBO J. 12:3351.*
22. Taylor, A. H., G. Heavner, M. Nedelman, D. Sherris, E. Brunt, D. Knight, and J. Ghrayeb. 1995. Lipopolysaccharide (LPS) neutralizing peptides reveal a lipid A binding site of LPS binding protein. *J. Biol. Chem. 270:17934.*
23. Little, R. G., D. N. Kelner, E. Lim, D. J. Burke, and P. J. Conlon. 1994. Functional domains of recombinant bactericidal/permeability increasing protein (rBPI23). *J. Biol. Chem. 269:1865.*
24. Schumann, R. R., N. Lamping, and A. Hoess. 1997. Interchangeable endotoxin-binding domains in proteins with opposite lipopolysaccharide-dependent activities. *J. Immunol. 159:5599.*
25. Rustici, A., M. Velucchi, R. Faggioni, M. Sironi, P. Ghezzi, S. Quataert, B. Green, and M. Porro. 1993. Molecular mapping and detoxification of the lipid A binding site by synthetic peptides. *Science 259:361.*
26. Kloczewiak, M., K. M. Black, P. Loiselle, J. M. Cavaillon, N. Wainwright, and H. S. Warren. 1994. Synthetic peptides that mimic the binding site of horseshoe crab antilipopolysaccharide factor. *J. Infect. Dis. 170:1490.*
27. Hoess, A., J. Schneider-Mergener, and R. C. Liddington. 1995. Identification of the LPS-binding domain of an endotoxin neutralising protein, *Limulus* anti-LPS factor. *Prog. Clin. Biol. Res. 392:327.*
28. Hirata, M., Y. Shimomura, M. Yoshida, S. C. Wright, and J. W. Larrick. 1994. Endotoxin-binding synthetic peptides with endotoxin-neutralizing, antibacterial and anticoagulant activities. *Prog. Clin. Biol. Res. 388:147.*
29. Larrick, J. W., M. Hirata, H. Zheng, J. Zhong, D. Bolin, J. M. Cavaillon, H. S. Warren, and S. C. Wright. 1994. A novel granulocyte-derived peptide with lipopolysaccharide-neutralizing activity. *J. Immunol. 152:231.*
30. Tam, J. P., C. R. Wu, W. Liu, and J. W. Zhang. 1991. Disulfide bond formation in peptides by dimethyl sulfoxide. *J. Am. Chem. Soc. 113:6657.*
31. Ried, C., C. Wahl, T. Miethke, G. Wellnhofer, C. Landgraf, J. Schneider-Mergener, and A. Hoess. 1996. High affinity endotoxin-binding and neutralizing peptides based on the crystal structure of recombinant *Limulus* anti-lipopolysaccharide factor. *J. Biol. Chem. 271:28120.*
32. Beutler, B., and A. Cerami. 1989. The biology of cachectin/TNF: a primary mediator of the host response. *Annu. Rev. Immunol. 7:625.*
33. Lehmann, V., M. A. Freudenberg, and C. Galanos. 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J. Exp. Med. 165:657.*
34. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med. 175: 91.*
35. Brandenburg, K., W. Richter, M. H. Koch, H. W. Meyer, and U. Seydel. 1998. Characterization of the nonlamellar cubic and HII structures of lipid A from *Salmonella enterica* serovar Minnesota by X-ray diffraction and freeze-fracture electron microscopy. *Chem. Phys. Lipids 91:53.*
36. Galanos, C., M. A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization of the lethal effects of endotoxin. *Proc. Natl. Acad. Sci. USA 76: 5939.*
37. Decker, K., and D. Kappler. 1974. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev. Physiol. Biochem. Pharmacol. 71:77.*
38. Glauser, M. P., G. Zanetti, J. D. Baumgartner, and J. Cohen. 1991. Septic shock: pathogenesis. *Lancet 338:732.*
39. Raetz, C. R. 1990. Biochemistry of endotoxins. *Annu. Rev. Biochem. 59:129.*
40. Rietschel, E. T., and H. Brade. 1992. Bacterial endotoxins. *Sci. Am. 267:54.*
41. Ulevitch, R. J., and P. S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol. 13:437.*
42. Wright, S. D., P. S. Tobias, R. J. Ulevitch, and R. A. Ramos. 1989. Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *J. Exp. Med. 170:1231.*
43. Ferrero, E., C. L. Hsieh, U. Francke, and S. M. Goyert. 1990. CD14 is a member of the family of leucine-rich proteins and is encoded by a gene syntenic with multiple receptor genes. *J. Immunol. 145:331.*
44. Tobias, P. S., K. Soldau, J. A. Gegner, D. Mintz, and R. J. Ulevitch. 1995. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J. Biol. Chem. 270:10482.*
45. Battafaraono, R. J., P. S. Dahlberg, C. A. Ratz, J. W. Johnston, B. H. Gray, J. R. Haseman, K. H. Mayo, and D. L. Dunn. 1995. Peptide derivatives of three distinct lipopolysaccharide binding proteins inhibit lipopolysaccharide-induced tumor necrosis factor-α secretion in vitro. *Surgery 118:318.*