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Murine Bactericidal/Permeability-Increasing Protein Inhibits the Endotoxic Activity of Lipopolysaccharide and Gram-Negative Bacteria

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Recognition of LPS by TLR4 initiates inflammatory responses inducing potent antimicrobial immunity. However, uncontrolled inflammatory responses can be detrimental. To prevent the development of septic shock during an infection with Gram-negative bacteria, the immune system has developed mechanisms to neutralize LPS by specialized proteins. In this study, we report the recombinant expression and functional characterization of the mouse homolog of human bactericidal/permeability-increasing protein (BPI). Purified recombinant mouse BPI was able to neutralize LPS-mediated activation of macrophages and to block LPS-dependent maturation of dendritic cells. Recombinant mouse BPI neutralized the capacity of Gram-negative bacteria to activate immune cells, but did not influence the stimulatory properties of Gram-positive bacteria. Unlike human BPI, mouse BPI failed to kill or inhibit the growth of *Pseudomonas aeruginosa*. Together, these data demonstrate that murine BPI is a potent LPS-neutralizing protein that may limit innate immune responses during Gram-negative infections. The Journal of Immunology, 2008, 180: 7546–7552.

During an infection, the innate immune system must rapidly and specifically detect the presence of foreign pathogens to trigger appropriate protective responses. Nonetheless, the duration and intensity of the host inflammatory reaction must be tightly regulated to avoid the fatal consequences of septic shock. Host recognition of bacterial pathogens is mediated mainly by the TLRs that are activated by unique and invariant microbial structures (1). Microbial recognition by the innate arm of the immune system rapidly induces a series of proinflammatory cascades and stimulates a definitive adaptive immune response (2). Numerous studies have demonstrated that the activation of TLR4 by LPS, a complex and abundant glycolipid of the outer membrane of Gram-negative bacteria, is essential to robust host defense (3).

LPS is one of the most potent stimulators of the innate immune system (4). Mammals possess an array of LPS-binding proteins that are referred to as the bactericidal/permeability-increasing (BPI) protein family and are characterized by the expression of a homologous protein domain that is shared by the family members (BPI protein domain). In addition to BPI itself, members of this protein family include the LPS-binding protein (LBP), phospholipid transfer protein, cholesterol ester transfer protein, and the palate lung and nasal epithelium clone (PLUNC) proteins (5, 6). These proteins have been shown to initiate LPS recognition by immune cells or to sequester and neutralize LPS. Specifically, LBP is an acute phase protein produced mainly in the liver and present in the serum that transfers LPS monomers from micelles to the primary LPS-binding receptor CD14 at basal expression levels. At high concentrations, LBP is able to inhibit LPS by delivering LPS to plasma lipoproteins (7). LPS-CD14 interactions lead to the recruitment of transmembrane TLR4 in conjunction with an extraacellular soluble protein termed MD-2, thereby leading to a conformational change in MD-2 which is sensed by TLR4 (8). This sequence of the binding modus of MD-2 after interaction with its ligand to TLR4 could be recently confirmed by a structure model of TLR4 and MD-2 along with the LPS antagonist Eritoran (9). Minute amounts of LPS trigger the formation of this protein complex and activate a powerful cellular response in the host (10). In contrast, the high-affinity binding property of human BPI to the lipid A moiety of LPS at the nanomolar range prevents the transfer of LPS to cellular receptors and facilitates its uptake by macrophages through a phagocytic pathway. This mechanism leads to the elimination of LPS and to the down-regulation of inflammatory cell activation (11). BPI is highly conserved in evolution with orthologs of this protein being expressed in teleost fish (12), rabbit (13), rat (14), mice (15, 16), humans (17), and invertebrates (18).

The initial incorporation of the PLUNC proteins into the BPI family was based solely on structural similarities, although an interaction of PLUNC proteins with LPS has been demonstrated (19). The exact function of the PLUNC proteins is difficult to predict because the BPI family consists of proteins that bind LPS yet mediate opposing biological effects, such as LBP and BPI (20). Short PLUNC1 protein has been shown to limit the growth of *Mycoplasma hominis* inside lung epithelial cells but the precise mechanism for this action remains unsolved (21).

Recently, a mouse homolog of human BPI was identified and the expression and regulation of the expression of this protein was
analyzed (15, 16). In this report, we have analyzed the potential of purified recombinant mouse (rm) BPI protein to interfere with cell activation in response to LPS as well as intact bacteria. We demonstrate that rmBPI specifically neutralizes LPS-mediated activation of APCs.

Materials and Methods

**Mice and bacterial strains**

C57BL/6 mice were purchased from Charles River.

For in vitro stimulation, *Salmonella enterica* serovar Typhimurium (S. typhimurium) NCTC 12023, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Neisseria meningitidis (clinical isolate), Staphylococcus aureus ATCC25923, group B Streptococcus (GBS, clinical isolate) as well as *Listeria monocytogenes* (clinical isolate) were used.

**Cells**

293T HEK cells, RAW264.7 macrophages (American Type Culture Collection), and RAW macrophages stably transfected with a NK-κB luciferase reporter construct (RAW-κB) (22) were cultured with RPMI 1640 supplemented with 5% FCS, 10 mM HEPES, 10 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium).

**Cloning of murine BPI into an expression plasmid, transient and stable transfection, and immunoblotting**

The mouse BPI coding sequence was cloned into the pCMV-FLAG 1 (Sigma-Aldrich) expression vector. A full-length PCR product was obtained using primer pairs to amplify the open reading frame of mouse BPI without the putative leader sequence. A preparative PCR was conducted with the Triple Master Mix (Eppendorf) and separated on an agarose gel. The 1.4-kb DNA fragment of the PCR was purified with a Gel Extraction Kit (Promega) and directly ligated into the plasmid using compatible restriction sites. The ligated plasmids were transformed into *E. coli* DH5α and sequenced using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). To identify recombinant protein expression, the plasmid was transiently transfected into 293T HEK cells using polyethyleneimine (Sigma-Aldrich) (23) and the cell lysates as well as supernatants were collected 48 h later. In case of stable expression, the BPI plasmid construct was transfected along with pcDNA3.1 hygromycin (Invitrogen) to provide selection marker. After selection with hygromycin B, single clones were isolated and the supernatant and cell lysates were collected. Recombinant mouse BPI-FLAG protein was purified from the supernatant by M2 Agarose beads (anti-FLAG M2 Affinity Gel Freezer-safe; Sigma-Aldrich) and dissolved in SDS-PAGE sample buffer. Supernatant as well as cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Thereafter, the blots were incubated with an anti-FLAG M2 Ab (mouse-anti-FLAG mAb; Sigma-Aldrich) and detected with a donkey anti-mouse IgG-HRP (Dianova) using the ECL Plus reagent (GE).

**Generation of an anti-mouse BPI antiseraum**

An antiseraum was generated from two peptides derived from the coding sequence of mouse BPI (peptide 1, CRGHRRDPCPHPPVMRFVPN (257–276 aa) and peptide 2, CQLLSNNGFQLNTDFLR (309–326 aa)). The synthesis of the peptides, coupling to keyhole limpet hemocyanin, immunization of the rabbit, and collection of the serum was performed by Serotec. The antiseraum was purified by affinity chromatography with the peptides used for immunization to obtain a highly purified anti-mouse-specific polyclonal antiseraum.

**Purification of rmBPI**

Supernatants were collected from three independently generated stable clones expressing rmBPI and purified by affinity chromatography using a fast protein liquid chromatographer (ÄKTA; GE). An Anti-FLAG M2 Affinity Gel was prepacked into an empty chromatography column and the supernatant was applied to the column. The bound protein was eluted with 0.1 M glycine (pH 3.5) and the eluted fractions were immediately neutralized with 0.5 M Tris (pH 7.4). Fractions that contained the recombinant protein were subjected to dialysis in PBS (pH 7.4) and transferred to a siliconized tube to avoid the loss of the protein during short-term storage. The protein content were determined by a protein assay based on bichromatic acid (Optima). The purity of the protein fraction was analyzed by SDS-PAGE followed by Coomassie blue staining and by immunoblot using an Ab that detects either the FLAG epitope tag or the murine BPI protein, respectively.

**Generation of dendritic cells (DCs)**

DCs were derived from the bone marrow of isolated mouse femur and tibia. Marrow cells were flushed from the bones, depleted of erythrocytes, and cultured in complete medium with GM-CSF. The culture medium was replaced every third day. On day 7, suspension cells were harvested as immature DCs and plated into 12-well plates.

**Stimulation of cells**

RAW-κB cells as well as DCs were stimulated either in the presence or absence of different amounts of LPS. To determine whether rmBPI was able to neutralize LPS, LPS (E. coli 0111:B4; Sigma-Alrich) was preincubated with 10, 1, 0.1, 0.01, and 0.001 μg/ml rmBPI, respectively, for 30 min before adding it to the cells. In comparison, human purified BPI (Athens Research) was used in the same concentration as rmBPI for the inhibition of LPS-mediated activation of RAW-κB cells. Sixteen hours after stimulation, the supernatants were collected, RAW-κB cell lysates were prepared for determination of luciferase activity, and stimulated DCs were processed for flow cytometry analysis. RAW-κB macrophages were also stimulated in the presence of different amounts of UV-treated bacteria. The efficacy of UV treatment to block bacterial replication was verified in each case by plating the treated and untreated samples on agar plates. The optimal bacterial number to elicit a robust activation of the NF-κB luciferase reporter construct was individually determined for each isolate. Interference of rmBPI with cellular responses was measured by bacterial stimulation in the presence of rmBPI (10, 1, and 0.1 μg/ml). Finally, RAW macrophages were stimulated with an optimal multiplicity of infection (MOI) of live *E. coli*, as determined by the measurement of TNF production into the supernatant. Live *E. coli* stimulation of macrophages was either performed in the presence or absence of rmBPI (10 μg/ml) and supernatants were collected for analysis of its inhibitory capacity.

**Cytokine production**

Mouse TNF, IL-12, and human IL-8 were analyzed from the supernatant of stimulated cells by a standard sandwich ELISA technique using matched Ab pairs (BD Biosciences) according to the manufacturer’s recommendations.

**Measurement of nitrite**

NO$_2^-$ in supernatants was determined by the Griess assay (24).

**Flow cytometry**

Immature bone marrow-derived DCs (BMDCs) were cultured in the presence of 100 ng/ml LPS or 2 μM CpG, respectively. To analyze the inhibitory capacity of rmBPI, the cells were cocultured with different amounts of the recombinant protein. Twenty-four hours after stimulation, the cultures were harvested and stained for the surface expression of CD11c (anti-CD11c-FITC), MHC class II (anti-H-2b-PE), and CD86 (anti-CD86-PE) (all Abs were from BD Biosciences) and analyzed by FACS.

**NF-κB reporter activity**

Sixteen hours after stimulation, cell lysates were processed with luciferase substrate (Promega) to measure luciferase activity.

**Immunoblot of the signaling molecules**

To determine the influence of rmBPI on the *E. coli*-induced activation of the MAPK, RAW macrophages were stimulated with 0.1 MOI of *E. coli* in the presence or absence of 10 μg/ml rmBPI for various periods of time. Thereafter, cell lysates were prepared with modified radioimmuno-precipitated assay buffer containing 1 mM NaN$_3$, and 1 μg/ml each of leupeptin, pepstatin, and aprotonin (all from Sigma-Aldrich). To detect the activation of MAPK, 100 μg of total protein was separated on a SDS-PAGE. The phosphorylation of p38 and ERK1/2 was detected by a specific phospho-p38 (New England Biolabs) and specific phospho-ERK1/2 (Promega) Abs, respectively, and the ECL Plus Western Blotting reagent (GE). Equal loading of the gel was documented with Abs specific for total p38 (New England Biolabs) and ERK1/2 (Promega) Abs, respectively.

**Antibacterial activity of BPI**

Purified human BPI protein as well as rmBPI was analyzed in parallel for their antibacterial activity. These assays were performed in 96-well plates in a volume of 50 μl of the corresponding antibiotic concentration. 1/2 serial dilutions of the test sample were added to a suspension of the test bacteria, and the samples were incubated for 24 h at 37°C and subsequently plated on blood agar. After incubation at 37°C overnight, bacterial colonies were quantified.
To analyze the effect of rmBPI on the release of TNF into the supernatant from RAW macrophages, we first analyzed its effect on the activation of RAW macrophages by LPS. As expected, LPS was able to potently activate RAW macrophages as determined by the activation of the NF-κB luciferase reporter as well as poly(I:C) (Fig. 1A), revealing a single band of ~60 kDa. The specificity of the 60-kDa band was further corroborated by immunoblot using FLAG epitope tag and consecutive dialysis of the purified protein against PBS (pH 7.4). The protein content and the purity of rmBPI were evaluated by SDS-PAGE and Coomassie blue staining (Fig. 1A1), revealing a single band of 60 kDa. The specificity of the protein was confirmed by immunoblot using Abs specific for the FLAG epitope tag (Fig. 1B) or an antiserum specific for mouse BPI (Fig. 1C). Recombinant BPI was purified by fast protein liquid chromatography affinity using Abs specific for the FLAG tag (Fig. 1B) or an antiserum specific for mouse BPI (Fig. 1C).

Expression and purification of rmBPI

For the expression of rmBPI protein, the coding sequence of mouse BPI was cloned into the expression plasmid pCMV1-FLAG. Recombinant BPI-FLAG expression by transiently transfected 293T cells was confirmed by immunoblotting without and with prior immunoprecipitation using a FLAG-specific Ab (data not shown). Having established the expression of rmBPI in this system, we then generated three independent 293T cell clones stably expressing rmBPI. Recombinant BPI was purified by fast protein liquid chromatography affinity using Abs specific for the FLAG tag (Fig. 1B) or an antiserum specific for mouse BPI (Fig. 1C). Purification of rmBPI. Supernatants of 293T cells transfected with plasmids containing the open reading frame of mouse BPI and a N-terminal FLAG tag were purified by affinity chromatography using anti-FLAG M2 agarose beads. A, The eluted fractions 1–4 were dialyzed against PBS (pH 7.4), separated by SDS-PAGE, and analyzed by Coomassie blue staining after dialysis. B and C, An equivalent volume of a fraction containing eluted protein (lane 2) and a fraction that does not contain detectable amounts of protein (lane 1) were separated by SDS-PAGE and immunoblotted. BPI protein was revealed by using Abs against the FLAG-tag (B) or an antiserum specific for mouse BPI (C).

Statistics

Statistical significance was calculated with the Prism 3.0 GraphPad software using the Student t test. Values of p < 0.05 were considered as significant. For the calculation of the IC50 values of BPI, the Prism 3.0 GraphPad software was used.

Results

Statistical significance was calculated with the Prism 3.0 GraphPad software using the Student t test. Values of p < 0.05 were considered as significant. For the calculation of the IC50 values of BPI, the Prism 3.0 GraphPad software was used.

FIGURE 1. Purification of rmBPI. Supernatants of 293T cells transfected with plasmids containing the open reading frame of mouse BPI and a N-terminal FLAG tag were purified by affinity chromatography using anti-FLAG M2 agarose beads. A, The eluted fractions 1–4 were dialyzed against PBS (pH 7.4), separated by SDS-PAGE, and analyzed by Coomassie blue staining after dialysis. B and C, An equivalent volume of a fraction containing eluted protein (lane 2) and a fraction that does not contain detectable amounts of protein (lane 1) were separated by SDS-PAGE and immunoblotted. BPI protein was revealed by using Abs against the FLAG-tag (B) or an antiserum specific for mouse BPI (C).

FIGURE 2. Recombinant murine BPI inhibits the LPS-dependent activation of RAW macrophages. RAW-κB cells (1.6 × 10^6 cells/ml) were cultured and stimulated with 1 ng/ml LPS (white bars), 2 μM CpG (light gray bars), and 10 μg/ml peptidoglycan (PGN; dark gray bars), respectively, or left untreated (control). To analyze the function of rmBPI, the stimulated cells were cultured with graded amounts of rmBPI (10, 5, 1, 0.1, and 0.01 μg/ml) or without the addition of rmBPI. Sixteen hours later, the supernatants were collected and analyzed for TNF production (A), induction of inducible NO synthase activity (C and D), and IL-12 secretion (E and F). LPS-stimulated surface expression of CD86 by CD11c and MHC class II-positive cells was analyzed by FACS (B). Unstimulated and untreated BMDCs are depicted as controls. Four independent experiments were performed with similar results. n.d., Not detected; *, p < 0.05 and **, p < 0.005.

rmBPI inhibits the LPS-induced activation of macrophages

To determine the biological function of rmBPI, we first analyzed its effect on the activation of RAW macrophages by LPS. As expected, LPS was able to potently activate RAW-κB cells as determined by the activation of the NF-κB luciferase reporter as well as by the production and secretion of TNF (Fig. 2). In contrast, stimulation of the RAW-κB cells in the presence of rmBPI led to a dose-dependent inhibition of macrophage activation (Fig. 2). At the highest doses, rmBPI entirely antagonized the LPS-mediated activation of macrophages. This rmBPI-mediated inhibition of macrophage function was specific for LPS, because rmBPI did not inhibit the activation of RAW-κB cells by CpG DNA motifs or poly(I:C) (Fig. 2A). Essentially identical results were obtained

FIGURE 3. rmBPI inhibits LPS-mediated activation of BMDCs. BMDCs (2 × 10^6 cells/ml) were stimulated in the presence of either 10 ng/ml LPS (C) or 2 μM CpG (D). The effect of rmBPI on the activation of DCs was analyzed by coculture of the DCs with different amounts of rmBPI (10, 1, and 0.1 μg/ml). Sixteen hours later, the supernatants were collected and analyzed for TNF production (A), induction of inducible NO synthase activity (C and D), and IL-12 secretion (E and F). LPS-stimulated surface expression of CD86 by CD11c and MHC class II-positive cells was analyzed by FACS (B). Unstimulated and untreated BMDCs are depicted as controls. Four independent experiments were performed with similar results. n.d., Not detected; *, p < 0.05 and **, p < 0.005.
with rmBPI purified from three individual cell clones (data not shown).

To compare the LPS-neutralizing activity of mouse and human BPI, we performed the experiments described above for rmBPI with purified human BPI applying the same assay conditions. This approach revealed that the LPS-neutralizing activity of rmBPI is comparable to that of human BPI as determined by two different readout systems (NF-κB reporter assay: IC_{50} (rmBPI) = 4.9 ± 3.36 μg/ml vs IC_{50} (human BPI) = 1.5 ± 0.8 μg/ml; TNF suppression assay: IC_{50} (rmBPI) = 2.6 ± 1.6 μg/ml vs IC_{50} (human BPI) = 2.75 ± 2.01 μg/ml). From these experiments, we conclude that rmBPI is able to effectively neutralize the LPS-mediated activation of macrophages.

The LPS-mediated maturation of DCs is influenced by rmBPI

In an attempt to analyze whether rmBPI is able to block the LPS-induced maturation of mouse DCs, we generated BMDCs and cultured them in the presence of LPS or CpG as determined by the production of TNF (Fig. 3), IL-12 (Fig. 3, E and F), and NO (Fig. 3, C and D) as well as the surface expression of CD86 (Fig. 3B). The addition of rmBPI to the LPS-stimulated DCs led to a block in the maturation of DCs which was dependent on the amount of BPI used (Fig. 3, A–C and E), whereas the addition of rmBPI did not alter the activation of DCs mediated by CpG (Fig. 3, A, D, and F). These results along with the results obtained with macrophages demonstrate that rmBPI is able to robustly neutralize endotoxin and thereby prevent the activation of professional APCs (Fig. 3).

rmBPI inhibits the immunostimulatory capacity of Gram-negative bacteria

Having established the neutralizing activity of rmBPI on purified LPS derived from E. coli, we investigated whether rmBPI was also able to interfere with the immunostimulatory capacity of complex Gram-negative bacteria simultaneously engaging multiple TLRs. To this end, we analyzed macrophage function after stimulation with inactivated UV-treated Gram-negative bacteria (S. typhimurium, E. coli, P. aeruginosa, and N. meningitidis). As expected, all Gram-negative bacteria tested stimulated RAW-xB cells at low MOIs for luciferase reporter expression and for TNF production (Fig. 4). UV-inactivated Gram-positive bacteria (S. aureus, group

FIGURE 4. Recombinant murine BPI inhibits macrophage activation by Gram-negative bacteria. RAW-xB cells (1.6 × 10^5/ml) were stimulated in the presence of UV-inactivated Gram-negative bacteria in the presence or absence of rmBPI (10, 1, and 0.1). Sixteen hours after stimulation, the luciferase activity of each cell lysate and concentration of TNF in the corresponding supernatants were determined. The cells were stimulated with an equivalent of a MOI 10 of S. typhimurium (A), MOI 2 of E. coli (B), MOI 2 of P. aeruginosa (C), and MOI 3 of Neisseria spp. (D), respectively. As controls, RAW-xB cells were cultured in the presence of 10 μg/ml rmBPI (BPI) or left untreated (control). The data are representative of three independent experiments performed with each bacterium. n.d., Not detected; RLU, relative light unit. *, p < 0.05 and **, p < 0.005.

FIGURE 5. rmBPI does not interfere with macrophage stimulation by Gram-positive bacteria. RAW-xB cells (1.6 × 10^5 cells/ml) were stimulated in the presence of UV-inactivated Gram-positive bacteria in the presence or absence of rmBPI (10, 1, and 0.1 μg/ml). Sixteen hours after stimulation, the luciferase activity of each cell lysate and concentration of TNF in the corresponding supernatants were measured. The cells were stimulated with an equivalent of a MOI 20 of S. aureus (A), MOI 30 of group B streptococci (B), and MOI 25 of L. monocytogenes (C), respectively. As controls, RAW-xB cells were cultured in the presence of 10 μg/ml rmBPI (BPI) or left untreated (control). The data presented are representative of three independent experiments. n.d., Not detected; RLU, relative light unit.
B. The results showed that rmBPI interferes with macrophage activation by proliferating E. coli. RAW-κB cells (1.6 × 10^5 cells/ml) were incubated with E. coli (MOI of 0.1) for 3 and 3.5 h, either alone or with different concentrations of rmBPI (10 μg/ml, 1 μg/ml, 0.1 μg/ml). Thereafter, the supernatants were collected for measurement of bacterial growth. The bacteria were plated on Luria-Bertani plates and colonies were counted the next day (A). Furthermore, the secretion of TNF into the supernatant during the stimulation period was determined by ELISA (B). For the detection of the activity of MAPK, the cells were stimulated with E. coli (MOI 0.1) in the presence or absence of BPI (10 μg/ml) for 0, 1, 2, and 3 h. Thereafter, the cells were lysed and analyzed by immunoblot using specific Abs to detect total and phosphorylated ERK1/2 (C) and p38 (D). *p < 0.05 and **p < 0.005.

**Discussion**

Using an epitope tag and affinity chromatography, we have generated highly purified rmBPI protein. Mouse BPI is biologically active and is able to neutralize LPS and prevent the activation of APCs. In addition, rmBPI inhibits the immunostimulatory effects of Gram-negative bacteria, likely reflecting neutralization of LPS of Gram-negative bacteria without interfering with their replication, but does not prevent the stimulation of immune cells by Gram-positive bacteria.

To prevent the overwhelming toxic effects of LPS, the body is equipped with a large family of LPS-neutralizing proteins. For example, cathelicidin, secretory leukocyte protease inhibitor, and acyloxyacyl hydrolase (AOAH) have all been shown to inhibit LPS and block the induction of septic shock (27–29). These proteins use distinct molecular mechanisms to exert their functions. For example, AOAH cleaves the acyl chain from the lipid A part of LPS, which in turn inactivates the biological activity of LPS (30). In this article, we report that mouse BPI is able to neutralize the LPS stimulatory activity. The precise mechanism for this action is presently unknown. However, from the crystal structure of human BPI, it was predicted that hydrophobic pockets might be able to accommodate LPS (31). Binding of LPS to these hydrophobic pockets leads to an enhanced elimination of LPS from the system presumably by a macrophage-dependent phagocytic process (11). On the molecular level, it was demonstrated that human BPI binds to the lipid A portion of LPS with nanomolar affinity and thereby outcompeting other LPS-interactive proteins (e.g., LBP) and preventing the engagement of MD-2/TLR4 (32).
We have recently shown that mouse immature BMDCs express mRNA for BPI at low levels, which is strongly induced upon stimulation with LPS (15). In this study, we demonstrate that the addition of exogenous recombinant BPI inhibits the induction of DC maturation driven by LPS. Following activation of the maturation process, transcriptional activation of BPI may, in part, explain the reduced responsiveness of mature DCs to LPS (33). The differential expression of BPI by immature and mature DCs would ensure that immature DCs expressing low-level BPI first become activated before they start to neutralize the activation stimulus (LPS). Another LPS-neutralizing protein, AOAH, was also shown to be present in DCs and its transcription is inducible by LPS stimulation (30). Together, mouse BPI and AOAH may also contribute to the phenomenon of LPS tolerance, both in DCs and possibly in vivo.

mBPI is able to neutralize LPS from different Gram-negative species. The LPS structure of \( P. aeruginosa \) is highly acetylated and exhibits poor immunostimulatory activity through TLR4 in comparison to the classical LPS structure of \( E. coli \), \( S. typhimurium \), and \( N. Neisseria \) (34). Notably, BPI does not appear to discriminate among these structural variations in LPS, since neutralization was observed for \( P. aeruginosa \) as well as other Gram-negative organisms, although \( P. aeruginosa \) stimulation is not completely inhibited by rmBPI. The residual activation of the cells by \( P. aeruginosa \) might be due to the engagement of multiple TLR ligands by the bacteria. Therefore, the mechanism of LPS recognition by BPI appears to be distinct from the requirements for TLR4 activation by LPS subtypes. Most likely this is due to the recognition by BPI appears to be distinct from the requirements for TLR ligands by the bacteria. Therefore, the mechanism of LPS cells by \( P. aeruginosa \) discriminating among these structural variations in LPS, since murium species. The LPS structure of \( P. aeruginosa \) is highly acetylated in vivo. The phenomenon of LPS tolerance, both in DCs and possibly stimulation (30). Together, mouse BPI and AOAH may also contribute to the phenomenon of LPS tolerance, both in DCs and possibly in vivo.

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In summary, we report for the first time recombinant expression and affinity purification of mouse BPI using an eukaryotic expression system. This protein exerts strong LPS-neutralizing activity both on the isolated LPS level as well as on Gram-negative bacteria. Further characterization of the activity and mechanism of action of BPI and other LPS-neutralizing proteins will advance our understanding and ability to control the powerful immunostimulatory activity of bacterial LPS.

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Disclosures
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