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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 acid-oxyacyl hydrolase.

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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 monocyogenes (clinical isolate) were used.

Cells

293T HEK cells, RAW274.6 macrophages (American Type Culture Collection), and RAW macrophages stably transfected with a NK-βB luciferase reporter construct (RAW-βB) (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 cloned DNA was subjected to automated sequencing 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 (Invitrogen) and collection of the serum was performed by centrifugation (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).

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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

NO2− 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 cells 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 NaVO3 and 1 μg/ml each of leupeptin, pepstatin, and aprotinin (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 peptide concentration of interest. After incubation with different concentrations of either purified human BPI or rmBPI for 1 h at 37°C and subsequently plated on blood agar. After incubation at 37°C overnight, bacterial colonies were quantified.

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.
Expression and purification of rmBPI

For the expression of rmBPI, 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 epitope tag (B) or an antiserum specific for mouse BPI (C).

Results

Expression and purification of rmBPI

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 the production and secretion of TNF (Fig. 2A). In contrast, stimulation of the RAW-κB cells in the presence of rmBPI led to a dose-dependent inhibition of macrophage activation (Fig. 2A). 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

Statistics

Statistical significance was calculated with the Prism 3.0 GraphPad software using the Student’s t test. Values of p < 0.05 were considered as significant. For the calculation of the IC_{50} 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, NF-κB activation was determined by luciferase activity of the cell lysates (A). To analyze the effect of rmBPI on the release of TNF into the supernatant from RAW-κB cells, these cells were stimulated as indicated above and cocultured in case of LPS stimulation with different amounts of BPI (10, 5, 1, and 0.1 μg/ml). During CpG or peptidoglycan stimulation, RAW-κB were cultured in the presence (+) or absence (−) of 10 μg/ml rmBPI. In addition, the cells were left untreated (control) or cultured in the presence of 10 μg/ml rmBPI only (BPI). Sixteen hours after the stimulation, the release of TNF was determined by ELISA (B). One representative experiment of four independently performed is shown. n.d., Not detected; RLU, relative light unit. *, p < 0.05 and **, p < 0.005.

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 (E). 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 matured them in the presence of LPS or CpG as determined by the production of TNF (Fig. 3A), 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. 3A–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-κB cells at low MOIs for luciferase reporter expression and for TNF production (Fig. 4). UV-inactivated Gram-positive bacteria (S. aureus, group B streptococci, and L. monocytogenes) were also able to stimulate RAW-κB cells (Fig. 5). These results demonstrate that rmBPI is able to effectively neutralize the immunostimulatory activity of Gram-negative and Gram-positive bacteria.

FIGURE 4. Recombinant murine BPI inhibits macrophage activation by Gram-negative bacteria. RAW-κB 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-κB 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-κB 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-κB 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. B. B. B streptococci, and L. monocytogenes) potently stimulated RAW-κB cells, although higher MOIs were required in comparison to Gram-negative bacteria (Fig. 5). Our previous data showed that rmBPI could strongly antagonize LPS-mediated activation and maturation of macrophages and DCs. To determine the effect of rmBPI on the stimulation of macrophages with complex Gram-negative bacteria, we cocultured Gram-negative and Gram-positive bacteria with macrophages in the presence of graded amounts of rmBPI. Whereas exogenous rmBPI potently inhibited stimulatory activity of Gram-negative bacteria on RAW-κB macrophages, rmBPI did not antagonize the activity of Gram-positive bacteria to stimulate RAW-κB macrophages (Figs. 4 and 5). Notably, rmBPI potently neutralized the immunostimulatory activity of P. aeruginosa, demonstrating that rmBPI-mediated inhibition of macrophage function extends even to structurally diverse LPS isoforms as Pseudomonas LPS. Collectively, these data demonstrate that rmBPI inhibits endotoxic activity of all Gram-negative bacteria tested, likely reflecting neutralization of LPS.

Exogenous rmBPI inhibits endotoxic activity, but not growth of E. coli

Based on our data obtained with UV-treated Gram-negative bacteria, we sought to determine whether rmBPI has also the capacity to inhibit the activation of cells by proliferating bacteria. Because E. coli is a strong activator of RAW macrophages, we chose these bacteria as our model system. A 3- to 3.5-h infection with live E. coli at a MOI of 0.1 was sufficient to induce significant TNF release from RAW macrophages and led to the activation of the MAPK p38 as well as ERK1/2 (Fig. 6, B and C). Unlike human BPI (25), exogenous rmBPI had no significant effect on the proliferation of the bacteria (Fig. 6A), but potently inhibited TNF production by RAW macrophages (Fig. 6B). Furthermore, TLR4-mediated signal transduction after LPS stimulation was also inhibited in the presence of rmBPI as shown by reduced phosphorylation of p38 and ERK1/2 (Fig. 6C). These data demonstrate that rmBPI is able to neutralize the immunostimulatory capacity of proliferating E. coli yet it does not prevent bacterial growth.

Human, but not mouse, BPI, inhibits the growth of P. aeruginosa

Since we observed no antibacterial activity of rmBPI against E. coli, we tested its activity against another Gram-negative bacterium, P. aeruginosa. For human BPI, we recently demonstrated a strong and uniform antibacterial activity against various strains of P. aeruginosa that differed in their susceptibility to antibiotics (26). As expected, at concentrations of 10 μg/ml human BPI, we observed a strong antibacterial activity. In contrast, rmBPI tested under the same experimental conditions did not interfere with the bacterial growth at concentrations as high as 100 μg/ml (Fig. 7). From these data, we conclude that rmBPI lacks antimicrobial activity against P. aeruginosa.

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 acylxyacyl 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.

rmbPI 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 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 interaction of BPI with the lipid A region which is common in the LPS of all Gram-negative bacteria as it was demonstrated for human BPI (32, 35). Classical LPS is known as the strongest TLR-stimulating component derived from Gram-negative bacteria, which explains the pronounced inhibition of cell activation by these microorganisms in the presence of rmBPI (36). The residual NF-κB activity observed after the stimulation with Gram-negative bacteria in the presence of rmBPI (Fig. 4) in our study is most likely due to other TLR ligands such as flagellin and bacterial DNA, which cannot be neutralized by BPI.

The recombinant N-terminal fragment of human BPI was shown to neutralize LPS and LPS-mediated shock in animal models and exhibit bactericidal activity in infection models with Gram-negative bacteria (37, 38). Full-length mouse recombinant BPI neutralizes LPS both as an isolated stimulus as well as LPS on the surface of bacteria but does not interfere with the bacterial growth. Our results do not exclude the possibility that BPI has bactericidal properties; however, we were not able to demonstrate this in our experimental conditions. Because BPI is expressed and secreted in vivo, the combinatorial action of these molecules in vivo may lead to an overall enhancement of their microbicidal spectrum or potency (39). This was demonstrated for the enhanced antimicrobial activity of BPI with other defenses in vitro and the influence of other extracellular components (40).

Recently, it was shown that a human N-terminal fragment of BPI was able to bind to the Gram-positive bacterium Streptococcus pneumoniae as well as to pneumolysin, a pore-forming protein derived from these bacteria. This binding led to an enhanced immune response and thereby modestly enhanced the survival of mice lethally challenged with S. pneumoniae (41). In our study, we did not detect a major influence of recombinant full-length mouse BPI on group B Streptococci, a Gram-positive organism that does not secrete pneumolysin. We also observed that the immune stimulatory capacity of the Gram-positive organism analyzed in this study was generally very low.

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

The authors have no financial conflict of interest.

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