**Endotoxin-Induced Expression of Murine Bactericidal Permeability/Increasing Protein Is Mediated Exclusively by Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-β-Dependent Pathways**

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Endotoxin-Induced Expression of Murine Bactericidal Permeability/Increasing Protein Is Mediated Exclusively by Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-β-Dependent Pathways

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Antimicrobial effector proteins are a key mechanism for the innate immune system to combat pathogens once they infect the host. We report the identification and cloning of the mouse homologue of human bacterial permeability/increasing protein (BPI). Mouse BPI is constitutively expressed in lymphatic organs and tissues as well as in mouse testis. Upon stimulation with different TLR ligands, mouse BPI is strongly expressed in granulocytes and, surprisingly, in bone marrow-derived dendritic cells. Mouse BPI is most strongly induced by bacterial LPS through a signaling pathway that is completely dependent on TLR4-Toll/IL-1R domain-containing adaptor inducing IFN-β. Functional studies revealed that mouse BPI does have the potential to neutralize LPS and inhibits bacterial growth. Mouse BPI is expressed in granulocytes and bone marrow-derived dendritic cells, and the transcriptional activation is controlled by TLRs. The Journal of Immunology, 2006, 176: 522–528.

Recently, the ability of the innate immune system to induce immediate antimicrobial effector responses and control adaptive immune responses has come into special focus. During the innate immune response, granulocytes synthesize and secrete a repertoire of antimicrobial compounds that provide a first line of immune defense against invading pathogens (1). LPS, a complex glycolipid located in the outer membrane of Gram-negative bacteria is one of the most potent stimuli for the innate immune system (2). In mice and humans, a family of LPS binding proteins, including LPS binding protein (LBP), cholesteryl ester transfer protein, phospholipid transfer protein, and the human bactericidal permeability/increasing protein (BPI), has been described that either neutralize LPS or initiate LPS recognition by cells. Of all the naturally occurring mammalian LBPs, human BPI was shown to display the highest affinity for LPS despite the lack of an identifiable mouse homologue (3–5). The role of BPI during an immune response is 3-fold: 1) binding to LPS and consequent neutralization of LPS-mediated inflammatory responses, 2) direct antimicrobial effects toward Gram-negative bacteria by interaction with their inner and outer membranes, and 3) opsonization of bacteria for enhanced phagocytosis by macrophages (6). BPI is a 55- to 60-kDa glycoprotein that was originally detected and purified from azurophilic granules of human neutrophils. In addition, BPI can be found on the cell surface of human neutrophils and, to a lesser extent, in eosinophils (7). Human BPI forms a tandem barrel shape resembling a symmetrical bipartite structure that divides the protein into two functional domains: a cationic N-terminal region that confers the LPS neutralization as well as the antimicrobial function, and a C-terminal region for bacterial opsonization (8). Recently, a family of BPI-related proteins of unknown function with expression restricted to the oral, nasopharyngeal, and respiratory epithelia have been identified and termed palate, lung, and nasal epithelial clone, RY, and BPI-like (9–11).

Recognition of invading pathogens is mediated by the innate immune system through pattern recognition receptors that recognize conserved microbial structures not expressed by the host (pathogen-associated molecular patterns (PAMPs)) (12). Among the best-characterized pattern recognition receptors is the evolutionarily conserved family of TLRs. The mammalian TLR family currently consists of 11 members, and several of these receptors have been implicated in the recognition of PAMPs both in vivo and in vitro (13, 14). Microbial recognition leads to a robust innate immune response as well as the induction of an adaptive immune response (15). All mammalian TLRs are known to signal through MyD88, a cytoplasmic adaptor protein that consists of a N-terminal death domain and C-terminal Toll/IL-1 receptor homology (TIR) domain (16). MyD88 is recruited to the TIR domain of activated TLRs and through a homophilic association mediates a signaling cascade that results in the activation and nuclear translocation of NF-κB as well as activation of MAPKs (17).

TLR4, in concert with CD14 and MD-2, forms the LPS recognition complex on the cell surface (18–20). In addition to the principal signaling adaptor MyD88, LPS-TLR4 signals use another pathway that is independent of MyD88. Several alternative adaptors in the TLR4 signaling cascade have been described, including TIR domain-containing adaptor protein (also known as Mal) and TIR domain-containing adaptor-inducing IFN-β (TRIF; also known as TICAM-1). Although TIR domain-containing adaptor protein/Mal is used in TLR4 as well as TLR2 signaling (21, 22), TRIF/TICAM-1 is involved in the signal transduction of TLR4 and
TLR3. Recently, through analysis of cells derived from TRIF-deficient mice, it has become apparent that TRIF is indeed the adaptor responsible for signal transduction in the TLR4-dependent MyD88-independent pathway (23, 24).

Despite the fact that mice were not considered to express a homologue of human bacterial permeability/increasing protein (BPI), we report in this study the cloning of the orthologous gene from mouse neutrophils. Furthermore, we have analyzed the expression and induction of mouse BPI by various TLR ligands and performed functional studies to characterize the signaling components responsible for LPS-mediated induction as well as the role of mouse BPI as an antimicrobial effector molecule.

Materials and Methods

Mice and bacterial strain

MyD88<sup>−/−</sup> and TLR2<sup>−/−</sup> mice were a gift from S. Akira (Osaka University, Osaka, Japan). TRIF mutant mice were provided by B. Beutler (The Scripps Institute, La Jolla, CA). C3H/HeJ, C3H/HeN as well as C57BL/6 mice were purchased from Charles River. For in vitro infection, Salmonella enterica serovar typhimurium (S. typhimurium) NCTC 12023 was used.

Northern blot analysis and PCR

A 500-bp fragment from the C-terminal established sequence tag (EST; ENSMUSESTT00000001492) was first cloned into pGEM T easy vector using a cloning kit (Promega). The cloned fragment was released from the plasmid by restriction digest with NotI and purified by agarose gel electrophoresis and gel extraction. The labeling of the fragment was performed with Ready To Go DNA labeling beads (Pharmacia Biotech) by incorporation of [α-<sup>32</sup>P]dCTP during the labeling reaction. A multitissue mRNA blot (FirstChoice Northern Blot Mouse Blot II; Ambion) was probed with the labeled fragment. Hybridization was performed in ultrahigh sensitivity hybridization buffer, followed by serial washes with low and high stringency wash buffers as recommended by the manufacturer (Ambion). The blot was visualized by exposure on an x-ray film at −70°C.

Generation and stimulation of peritoneal exudate cells (PEC)

Thiglycolate-elicited PECs were isolated 16 h after injection as a source of granulocytes or 72 h after injection as a source of macrophages (25). For this purpose, the peritoneum of the mice was flushed aseptically with sterile PBS (pH 7.4) to isolate the exuded cells. PECs (5 × 10<sup>6</sup>/ml) were cultured in complete medium (Clicks/RPMI 1640 medium (Biochrom) supplemented with 5% FCS (Sigma-Aldrich), 2 mM t-glutamine, 10 mM HEPES, 100 μg/ml penicillin, 60 ng/ml streptomycin, 13 M NaHCO<sub>3</sub>, and 50 μM 2-ME) and stimulated with 100 ng/ml LPS, 10 μg/ml peptidoglycan, 10 μg/ml zymosan A (all from Sigma-Aldrich), and 10 μM CpG-DNA motifs (Alpha DNA) (26). Both the supernatant as well as the cells were collected at various time points for further analysis.

Isolation of lymphatic organs and tissue

C57BL/6 mice received injections i.p. with 100 μg of LPS derived from Escherichia coli (LPS; Sigma-Aldrich). At the indicated time points, thymus, heart, lung, liver, spleen, kidney, testis, intestine, lymph nodes, bone marrow, as well as peritoneal washout cells were isolated and snap-frozen in liquid nitrogen. The organs and tissues were kept at −80°C until total RNA was isolated as indicated below.

Quantification of mRNA expression by real-time PCR

Total RNA was isolated from the samples using TRIZol (Invitrogen Life Technologies). After DNAse treatment, a total of 1 μg of RNA was transcribed into cDNA using Moloney mouse leukemia virus reverse transcriptase RNase (H−; Promega) and oligo(dT). RNAse treatment and cDNA synthesis were conducted according to the manufacturer’s recommendations. The cDNA expression level was quantified by a real-time PCR using SYBR Green incorporation by a Light Cycler (Roche). Each 20 μl of reaction mixture contained the following ingredients: 1 μl of diluted cDNA, 1× platinum Taq reaction buffer, 4 mM MgCl<sub>2</sub>, 1 mM dNTP, 0.4 μM primer forward, 0.4 μM primer reverse, 0.5 ng/ml BSA, 5% DMSO, 0.5× SYBR Green, 1 U of platinum Taq polymerase (Invitrogen Life Technologies) by amplifying the cDNA for 50 cycles. Expression levels were normalized to the expression of the housekeeping gene porphobilinogen deaminase (PBG-D) (27). Primer pairs were as follows: mouse BPI forward primer, 5′-GGATCCGCTTGCAACCTCCTACG-3′, and reverse primer, 5′-GACACGGGCCGCGTACAGATG-3′; and mouse PBG-D forward primer, 5′-ATGTCGCTGAGCGGGCCG-3′, and reverse primer, 5′-CCAG GTTCTTGAGCTCGCAACCA-3′.

Cytokine production

Mouse TNF-α 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.

Cloning of murine BPI and generation of plasmids

The complete mouse BPI coding sequence was cloned from cDNA derived from LPS-stimulated granulocytes using the ESTs ENSMUSESTT00000001492 and ENSMUSESTT0000001051 from the ENSEMBL database (Sanger Institute), which contain the start and stop codons of the putative mouse BPI cDNA, respectively. A full-length product was obtained by a PCR with primer pairs amplifying the complete reading frame of mouse BPI. A preparative PCR was conducted with the Triple Master Mix (Eppendorf) and separated on an agarose gel. The 1.6-kb fragment with a gel extraction kit (Qiagen). The resulting fragment was cloned into pGEM T easy (Promega) and isolated from transformed bacteria by a plasmid extraction kit (Qiagen). The cloned cDNA was subjected to automated sequencing using the dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequence of mouse BPI is deposited at (www.ncbi.nlm.nih.gov/GenBank), and the accession number is AY648037.

Generation of dendritic cells (DC)

DCs were derived from bone marrow of isolated femur and tibia. Bone marrow was flushed from the bones and depleted of erythrocytes. The remaining bone marrow cells were cultured in Clicks/RPMI 1640 supplemented with 5% FCS, 10 mM HEPES, 10 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and GM-CSF. Medium was replaced every other day. On day 7, suspension cells were harvested as immature DCs, plated into 12-well plates and matured in the presence of the indicated amounts of LPS for 12, 24, or 48 h, respectively. Thereafter, supernatant was collected, and total RNA was prepared from the cells as described above.

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

The mouse BPI coding sequence was subcloned either as a complete sequence into a pcDNA3.1 expression plasmid in fusion with human IgG1/Fc or without its leader sequence into pSceTa2g' C harboring the human IgG1/Fc fragment (both expression constructs containing the IgG1/Fc were gifts from R. Medzhitov, Yale University, New Haven, CT). The plasmids were transiently transfected into 293 HEK cells using LipofectAMINE 2000 (Invitrogen Life Technologies). Forty-eight hours later, cell lysates as well as supernatants were harvested. In the case of stable expression, the constructs were transfected in RAW267.4 cells. After selection, single clones were isolated, and supernatant as well as cell lysates were collected. Recombinant mouse BPI-IgG1/Fc protein was purified from the supernatant by protein A/G plus agarose beads (Santa Cruz Biotechnology) and dissolved in SDS-PAGE sample buffer. Supernatant as well as cell lysate were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Thereafter, the blots were incubated with donkey anti-human IgG-HRP (Dianova). Immunoblots were visualized using the ECL Plus reagent (Amersham Biosciences).

NF-κB reporter activity

Transient transfections were performed with HEK 293 cells stably transfected with a NF-κB reporter construct as described previously (28). Briefly, 2 × 10<sup>5</sup> cells/well were plated into 6-well plates overnight at 37°C. The cells were then transfected with the LPS receptor complex (expression construct of 50 ng human CD14, 50 ng of human MD-2, as well as 15 ng of human TLR4) or a constitutively active TLR4 (500 ng of CD4/TLR4) construct was kept constant (500 ng), and stimulation of the cells by a standard sandwich ELISA technique using matched Ab pairs (BD Biosciences) according to the manufacturer’s recommendations.

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In all cases the total amount of DNA in each transfection was kept constant by adding pcDNA3.1 plasmid.

**Infection of RAW macrophages and assessment of intracellular growth of bacteria**

The infection of RAW macrophages as well as RAW cells expressing murine BPI and the assessment of intracellular growth of bacteria were conducted as described in detail previously (30). Briefly, bacteria were grown in Luria Bertoni broth and adjusted to an OD$_{600}$ = 0.2 with PBS. Fifty microliters of the bacterial suspension was adjusted to 0.45 ml of RPMI 1640 medium. Cells were infected with 500 μl of the bacterial suspension, which is an equivalent to a multiplicity of infection of 10. Thereafter, the infection was synchronized by centrifugation of the plates. The cells were pulsed with bacteria for 25 min at 37°C in 5% CO$_2$. Subsequently, extracellular bacteria were removed from the culture by discarding the supernatant and after three washes of the cells with PBS. The remaining bacteria attached to the cells were killed by adding medium containing 100 μg/ml gentamicin for 1 h, followed by medium containing 15 μg/ml gentamicin throughout the experiment.

The assessment of intracellular replication of bacteria was performed 2 and 16 h after infection. The supernatant was removed from the culture, and the cells were washed three times with Clicks medium (without serum). Finally, the cells were lysed with 0.1% Triton X-100, and serial dilutions of the lysates were plated onto Luria Bertoni agar to enumerate intracellular bacteria.

**Results**

**Cloning of mouse BPI coding sequence**

In an attempt to identify a mouse homologue of human BPI, a computer-based search of the mouse genome with the human BPI sequence was performed. Two overlapping ESTs of unknown function were identified in the mouse ENSEMBL database. The putative mouse ortholog of the human BPI gene was localized to mouse chromosome 2 in very close proximity to mouse LBP. This region displays synteny to the region of human chromosome 20 where the human BPI gene as well as the human LBP gene are located (31). From this sequence information we were able to clone a full-length cDNA encoding mouse BPI from LPS-stimulated granulocytes. Amino acid sequence alignment of mouse BPI with human BPI showed 54% sequence identity and 63% sequence similarity, as calculated with Genetics Computer Group program software (Fig. 1A).

**BPI expression in mouse tissues**

We first analyzed the tissue expression and distribution of mouse BPI by Northern blot under resting conditions. For this purpose, a mouse multitissue RNA blot was hybridized with a $^{32}$P-labeled cDNA probe consisting of 500 bp of the C-terminal coding sequence of mouse BPI. A positive hybridization signal corresponding to a 3-kb transcript was visualized in mouse testis; however, none of the other organs showed obvious BPI expression (Fig. 1B).

![FIGURE 1. Alignment and tissue distribution of mouse BPI. A, The N-terminal, barrel-shaped domain sequence of mouse BPI is aligned with the homologous human BPI sequence. The human sequence contains both the LPS neutralization domain as well as antibacterial activity. B, A mouse multiple tissue RNA blot was hybridized with a mouse BPI partial cDNA probe. RT-PCR analysis of total RNA from mouse testis (+) and elicited granulocytes (Gr.; C) as well as various lymphoid organs (D) using specific primers for mouse BPI cDNA synthesis was confirmed by PCR amplification of mouse hypoxanthine phosphoribosyltransferase (HPRT).](http://www.jimmunol.org/DownloadedFrom.Http://www.jimmunol.org/)

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Nevertheless, we were able to detect very weak, although constitutive, mRNA expression of mouse BPI in primary granulocytes (Fig. 1C) as well as in all lymphoid organs tested (Fig. 1D and data not shown).

Systemic LPS administration induces BPI in mouse organs

In an attempt to analyze whether systemic LPS administration would induce BPI mRNA in mouse organs, mice received injections i.p. with LPS. After 6, 12, and 24 h, total RNA from thymus, heart, lung, spleen, lymph node, small intestine, kidney, bone marrow, and PECs was isolated, and quantitative PCR for BPI was performed. Intraperitoneal administration of LPS induced BPI mRNA in PECs throughout the experiment and in kidney at 12 and 24 h after injection, respectively (Fig. 2). In addition, moderate BPI induction was detected in the lung at later time points. These results demonstrate that in response to LPS, mouse BPI is inducible with varying kinetics in different organs.

Transcriptional activation of BPI by TLR ligands

Under steady-state conditions, only weak mRNA expression of BPI could be detected by RT-PCR of thioglycolate-elicited granulocytes. Induction of BPI mRNA expression was observed after stimulation of these granulocytes with several TLR ligands (Fig. 3A). Qualitative comparison of the level of induction mediated by various PAMPs showed that LPS was the most potent transcriptional activator of mouse BPI (Fig. 3A). Real-time PCR was then used to quantify the mRNA levels. Twenty-four hours after LPS stimulation of granulocytes, a 65-fold relative increase in the mRNA level of mouse BPI was measured (Fig. 3B).

Because LPS was clearly the strongest stimulus for mouse granulocyte BPI mRNA expression, we used LPS for our additional experiments. To analyze the kinetics of the LPS-mediated BPI expression, we stimulated mouse granulocytes with LPS for increasing time intervals (Fig. 4, A and B). BPI was progressively induced throughout the stimulation period, with the highest BPI mRNA levels measured after 24 h of LPS stimulation (Fig. 4, A

FIGURE 2. Systemic LPS administration induces BPI expression. C57BL/6 mice received injections i.p. with 100 μg of LPS, and RNA was isolated from the lung ( ), kidney ( ), and peritoneal washout cells ( ) at the indicated time points. The expression level of BPI was analyzed by real-time PCR and compared with the expression of the housekeeping gene, PBG-D, to calculate relative induction. Data are the means of three independent experiments performed.

FIGURE 3. BPI expression in granulocytes is induced by a variety of bacterial stimuli. A, Peritoneal-elicited granulocytes were stimulated in the presence of 200 nM CpG DNA-motif (Cpg), 100 ng/ml LPS (LPS), 10 μg/ml Staphylococcus aureus bioparticles (S. aureus), 10 μg/ml peptidoglycan (PGN), or 10 μg/ml zymosan A (Zym. A) for 12 and 24 h or were left untreated (0). Thereafter, total RNA was isolated and transcribed into cDNA after DNase I treatment. The expression of BPI mRNA was analyzed by a specific PCR; cDNA synthesis was confirmed by hypoxanthine phosphoribosyltransferase (HPRT) PCR. B, Real-time PCR analysis of BPI expression; induction is calculated relative to the expression of PBG-D. n.d., not detected. The data given are the means of three independent experiments.

FIGURE 4. BPI expression by mouse granulocytes as well as mouse DCs. Peritoneal-elicited granulocytes (3 × 106 cells) were stimulated in the presence of 100 ng/ml LPS for the indicated time periods. A, BPI expression was determined by RT-PCR of total RNA and compared with hypoxanthine phosphoribosyltransferase (HPRT) PCR. +, The negative control; cDNA from mouse testis was used as positive control. B, Kinetic real-time PCR analysis of LPS-stimulated BPI expression relative to the housekeeping gene PBG-D. C, Peritoneal-elicited granulocytes were stimulated with decreasing amounts of LPS (100 μg/ml, 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, and 1 ng/ml, respectively) for 24 h. Thereafter, BPI expression was analyzed by real-time PCR as described above. D, BMDCs (3 × 105) were stimulated with 100 ng/ml LPS for the indicated time periods. BPI expression was analyzed by conventional PCR and compared with HPRT PCR. E, Real-time PCR analysis of BPI expression relative to PBG-D of LPS-stimulated BMDCs. The data are representative of three independent experiments performed with granulocytes and three performed with DCs.
and B). To determine the optimal LPS concentration for the induction of BPI mRNA, we stimulated granulocytes for 24 h with LPS concentrations ranging from 1 ng/ml to 100 μg/ml. At all concentrations tested, BPI mRNA expression could be induced, with the strongest induction observed from 100 ng/ml to 1 μg/ml (Fig. 4C). Therefore, all subsequent stimulation experiments were performed with 100 ng/ml LPS.

To determine whether the expression of BPI is restricted to granulocytes, we also analyzed macrophages. After LPS stimulation, neither macrophage cell lines (J774 and RAW 267.4) nor primary thioglycollate-elicited macrophages exhibited BPI mRNA expression by real-time PCR analysis (data not shown). Conversely, in bone marrow-derived DCs (BMDCs) the expression of BPI was transiently induced after stimulation with LPS (Fig. 4, D and E). Peak BPI induction was observed at 24 h, corresponding with the transition of DCs from an immature to a mature phenotype, and declined in association with full DC maturation. Taken together, these results show that the expression of BPI is inducible by LPS in granulocytes as well as in BMDCs.

**LPS-induced BPI expression is MyD88 independent and TRIF dependent**

To identify the receptor and downstream signaling components that mediate the transcriptional induction of BPI by LPS, granulocytes isolated from TLR4-deficient mice (C3H/HeJ) as well as TLR2-deficient, MyD88-deficient, and TRIF mutant mice were compared. As expected, LPS-mediated induction of BPI was strictly dependent on the presence of TLR4 and was independent of the presence of a functional TLR2 (Fig. 5, B, C, and F). Somewhat unexpectedly, BPI induction by LPS was independent of the presence of MyD88 (Fig. 5, D and G). To confirm that these granulocytes do not express a functional MyD88 molecule, supernatant from LPS-stimulated cells was analyzed for the secretion of TNF (which previously was shown to be completely dependent on MyD88 (32)). In contrast to the presence of BPI mRNA, TNF secretion was absent in LPS-stimulated MyD88-deficient granulocytes (Fig. 5, D and G, and data not shown). These results demonstrate that LPS-mediated induction of BPI is dependent on a functional TLR4, but is independent of the MyD88-mediated signaling pathway. Instead, transcription of BPI after LPS stimulation is completely dependent on the TRIF signaling molecule (Fig. 5H).

**Mouse BPI neutralizes LPS**

To elucidate the biological function of mouse BPI, we cloned its cDNA into an expression plasmid as a fusion protein with human IgG1/Fc. The secretion of this protein by transfected HEK 293 cells was documented by protein A agarose-purified supernatant subjected to a Western blot against the human Fc fusion partner (Fig. 6B). To test the hypothesis that mouse BPI binds and neutralizes LPS, HEK 293 cells stably expressing a NF-κB promoter/luciferase reporter construct were transiently transfected with the LPS receptor complex (TLR4, MD-2, and CD14) and stimulated with LPS. The NF-κB activation was measured by luciferase activity (Fig. 6A). In addition to the LPS receptor complex, the cells were transiently transfected with different concentrations of an expression construct of murine BPI. As depicted in Fig. 6A, murine BPI was able to inhibit LPS-induced NF-κB activation in a dose-dependent manner. This inhibition was specific, because the same concentration did not inhibit the activity of a constitutive active TLR4 (Fig. 6A). When the LPS concentration was titrated down, complete inhibition of LPS-induced NF-κB activation was observed, and at the highest LPS concentration the neutralizing activity of BPI could be overcome (Fig. 6C). Furthermore, the production of IL-8 from these LPS-stimulated cells was also inhibited in cotransfected HEK 293 cells expressing the LPS receptor and mouse BPI (Fig. 6D).

**Mouse BPI inhibits the growth of S. typhimurium**

Because RAW macrophages were found not to express endogenous BPI (data not shown), we established a RAW cell line stably expressing mouse BPI as a fusion protein with human IgG1/Fc. As depicted in Fig. 7A, a substantial amount of this protein was not secreted, but remained intracellularly. To analyze whether the expression of mouse BPI renders RAW macrophages more resistant
FIGURE 6. Mouse BPI inhibits LPS-mediated NF-κB activation. HEK 293 cells stably expressing an NF-κB reporter construct were transiently transfected with either the LPS receptor complex or a constitutively active TLR4 construct (CD4/TLR4), as indicated, and cotransfected with decreasing amounts of a mouse BPI/human IgG1/Fc fusion protein (500, 400, 300, 200, 100, and 50 ng/ml, respectively). Twenty-four hours after transfection, the cells were processed further. A, The cells were stimulated with 15 ng/ml LPS; control cells were not stimulated. Luciferase activity of cell lysates was measured 16 h later using a luminometer. B, The supernatants were collected and incubated with protein A-agarose. Finally, the samples were subjected to SDS-PAGE, and immunoblotting was conducted. The samples transfected with 500, 400, 300, 200, 100, and 50 ng/ml mouse BPI are shown. C, TLR4 receptor transfectants were cotransfected with 500 ng of a mouse BPI/human IgG1/Fc construct. Twenty-four hours after transfection, the cells were stimulated with decreasing amounts of LPS. Luciferase activity of cell lysates was measured 16 h later using a luminometer. The cells were transfected and stimulated as described in C (1 μg/ml, 100 ng/ml, 10 ng/ml, 10 μg/ml, and 10 μg/ml LPS are shown), and 16 h after stimulation, the IL-8 concentration was determined by a specific ELISA (D). As a control, CD4/TLR4 transfection in the absence (●) or the presence (○) of 500 ng of a mouse BPI-human IgG1/Fc construct was used (D). *, p < 0.05; **, p < 0.005 (determined by Student’s t test).

FIGURE 7. Mouse BPI inhibits the intracellular growth of S. typhimurium. RAW267.4 were stably transfected with mouse BPI/human IgG1/Fc, and expression was documented by immunoblot analysis of the cell lysate and the protein A-agarose-incubated supernatant (A). Parental RAW cells (●) as well as RAW macrophages expressing mouse BPI (○) were infected with S. typhimurium (multiplicity of infection, 10). Noninternalized bacteria were removed form the culture, and the number of intracellular bacteria was determined by plating serial dilutions of macrophage lysates on Luria Bertoni-agar. Intracellular replication is expressed as CFU at 2–16 h after infection (B). The data presented are representative of four independent experiments. *, p < 0.05 (determined by Student’s t test).

Discussion

We have identified a previously uncharacterized mouse homologue of human BPI and were able to clone it from LPS-stimulated granulocytes. In addition to granulocytes, BMDCs were found to be positive for BPI expression after LPS incubation. LPS-induced BPI expression was independent of the MyB88-dependent signaling pathway, but was dependent on the TRIF-dependent signaling pathway. Using an in vitro system, we have demonstrated that the major biological functions of mouse BPI during the innate immune response are the neutralization of LPS as well as the inhibition of bacterial growth.

LPS has two opposing, but not exclusive, effects on the host. After infection, LPS is recognized by receptors of the innate immune system to indicate the presence of an invading microbe. This subsequently leads to initiation of the adaptive immune response and definitive clearance of the infection (33). In contrast to this beneficial function during an immune response, overwhelming stimulation by LPS may provoke a fatal host inflammatory response, because isolated LPS from Gram-negative bacteria is sufficient to induce a state of septic shock (2). Therefore, although a mammalian host rapidly recognizes LPS as a foreign molecule, it also takes measures to neutralize the proinflammatory properties of LPS. It is known that human BPI displays a very high binding affinity toward LPS (34), leading to its neutralization, presumably through macrophage-dependent phagocytosis (35). We have shown in this study that the previously unidentified mouse homologue of human BPI is also able to neutralize LPS and to inhibit LPS-mediated activation and signal transduction by TLR4. Until recently, the expression of human BPI appeared to be restricted to azurophilic granules of granulocytes, where it is stored as a preformed protein; however, it has also been detected in eosinophil granules and most recently in keratinocytes after stimulation with eicosanoids (36). This strategic distribution might serve as a basis for two possible modes of action: first, by secretion and thereby neutralization of LPS in the extracellular space, and second by fusion to the endosomal pathway after phagocytosis to bind to LPS in the phagosome and act as an antimicrobial protein against Gram-negative bacteria (37). In addition to the expression in granulocytes, we were able to induce BPI mRNA in DCs, indicating that this cell type is capable not only of recognizing pathogens but also of producing products that directly combat the bacteria and neutralize LPS. Only recently, DCs were found to express mRNA for another LPS-neutralizing protein, acyloxyacyl hydrolase (38).
It has yet to be established whether DCs, like neutrophils, are also able to store premede BPI, or whether the production is strictly dependent on stimulation via TLRs. Only recently, our results were complemented by demonstrating the expression of mouse BPI at the protein level (39).

The transcription of BPI is inducible by its own ligand, suggesting the presence of an autocrine loop. By delaying the secretion of BPI, the host ensures that LPS first activates the immune system to initiate a definitive immune response (18, 19) before being neutralized to avoid overwhelming effector responses that are typically associated with the development of septic shock (2).

Transcriptional activation of BPI mediated by LPS is independent of MyD88 and is controlled by the TRIF signaling pathway as was previously described for several IFN regulatory factor 3-dependent genes (40). To our knowledge this is the first description of an antimicrobial protein that is regulated by the TLR4-TRIF signaling axis. In addition to LPS, other PAMPs, such as CpG DNA, that are known to activate signal transduction and transcription solely through MyD88 are also able to induce BPI mRNA in murine granulocytes (26). The relative contribution and interaction of the two pathways (MyD88-dependent and MyD88-independent) for the activation of BPI during an immune response are subjects for future investigation.

In summary, mouse BPI is expressed by granulocytes and BM-DCs of the innate immune system. This expression is most strongly inducible by LPS through a TLR4-TRIF-dependent pathway. Through this single effector molecule, both cell types may contribute to combating Gram-negative bacterial infection.

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Disclosures

The authors have no financial conflict of interest.

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