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Porcine beta defensin 2 attenuates inflammatory responses in IPEC-J2 cells against *Escherichia coli* via TLRs-NF-κB/MAPK signaling pathway

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Abstract

Background Porcine beta defensin 2 (pBD2) is one of the porcine beta defensins that has antibacterial activity, and plays an important role in the immunomodulatory activity that protects cells from pathogens. It has been reported that pBD2 plays their immunomodulatory functions related to the TLR4-NF-κB signal pathways. However, it is not completely clear how pBD2 reduces the inflammatory response caused by pathogens.

Results In this study, the effect of pBD2 on the expression of genes in the TLRs signaling pathway was investigated after IPEC-J2 cells were challenged with *E. coli*. The results showed that pBD2 decreased the expression of IL-8 induced by *E. coli* ($P < 0.05$), and pBD2 significantly decreased the expression of TLR4, TLR5 and TLR7 ($P < 0.05$), as well as the key downstream genes p38 and JNK which activated by *E. coli* ($P < 0.05$). In addition, pBD2 inhibited the p-p65, p-p38 and p-JNK which were up-regulated by *E. coli*.

Conclusions pBD2 could reduce the inflammatory response induced by *E. coli* perhaps by inhibiting the TLRs-TAK1-NF-κB/MAPK signaling pathway which was activated by *E. coli* in IPEC-J2 cells. Our study further reveals the immunomodulatory activity of recombinant pBD2 against *E. coli*, and provides insights into the molecular mechanisms that protect cells from *E. coli* infection.

Keywords Porcine beta defensin 2, Immunomodulatory function, Inflammatory responses, TLRs signaling pathway, *E. coli*

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Background

Defensins are an important class of antibacterial peptides, which are a kind of small molecular peptides that widely exist in animals, plants and microorganisms. They are an important component of the immune system and play a role against microbial invasion [1, 2]. They have the advantages of broad-spectrum antibacterial activity, low or selective cytotoxicity, and special mechanisms that are not easy to cause microbial resistance, which make them the ideal antibiotic substitutes [3]. Mammal defensins can be divided into three categories according to the positions of the three pairs of disulfide bonds: α-defensin, β-defensin and θ-defensin [4]. Only β-defensin has been discovered in pigs so far [5]. Porcine β defensin 1 (pBD1) was the first β defensin found in pigs [6], other pBDs were identified by bioinformatics approaches. pBD2 has the strongest bactericidal activity among the pBDs reported to date [7–9], which could inhibit bacteria such as *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. pBD2 also exhibits antiviral activity in vitro and in transgenic mice [10]. Furthermore, pBD2 has salt resistance, thermal stability, and a special antibacterial mechanism, which makes it an ideal antibiotic substitute.

In addition, pBD2 also has the immunomodulatory function, which protects the host from the pathogen. It was reported that pBD2 significantly reduced the inflammatory response in mice with DSS-induced acute colitis [11]. In addition, transgenic pigs with over-expressing pBD2 enhance resistance to *Actinobacillus pleuropneumoniae* and *Glasserella parasuis* infection respectively, and reduce the number of harmful bacteria and lesion damage [12, 13]. pBD2 significantly inhibited the inflammatory cytokines TNF-α, IL-1β, and IL-8 in piglets which were up-regulated by *E. coli* and also reduced the expression of TLR4 (Toll-like receptor 4) [14]. TLRs (Toll-like receptors), the most famous pattern-recognition receptors (PRRs), play a critical role in the activation of the immune system against infection. They could recognize the pathogens or the component of pathogens, for example, TLR4, TLR5 and TLR7 can recognize lipopolysaccharide (LPS), flagella and nucleic acid respectively [15–17]. Then activate nuclear factor-κB (NF-κB) or mitogen-activated protein kinases (MAPK) signaling pathways through myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor inducing interferon (TRIF). TLRs-NF-κB/MAPK signaling pathways are the key pathways that are involved in the inflammatory response caused by microbial pathogens [18–21]. It was reported that pBD2 significantly reduced *Salmonella*-induced inflammation in mice with over-expressing pBD2 via the TLR4-NF-κB pathway [22]. Our previous studies also indicated that pBD2 could reduce the inflammatory response caused by *E. coli* via the NF-κB pathway in IPEC-J2 cells [23]. However, it is not completely clear how pBD2 reduces the inflammatory response caused by *E. coli*.

In this study, we examined the changes of key genes related to TLRs-NF-κB/MAPK signaling pathways after IPEC-J2 cells were challenged with *E. coli*, and explored that pBD2 perhaps alleviates inflammatory responses induced by *E. coli* via TLRs-NF-κB/MAPK signaling pathway in IPEC-J2 cells including the TLR5 and TLR7 except for TLR4.

Materials and methods

Bacterial strains, cell line, and pBD2 preparation

E. coli ATCC 25,922 was purchased from the Beijing Ordinary Microbiology Strain Store Center (Beijing, China). The porcine jejunum epithelial cells (IPEC-J2) were gifted from Zhanyong Wei at the College of Veterinary Medicine, Henan Agricultural University.

E. coli ATCC 25,922 was cultured in Luria broth (LB) medium at 37℃ at 220 rpm and grown to the optical density (OD) at 600 nm was about 1 (the concentration of bacteria was about 1×10^9 CFU/mL) determined by a spectrophotometer (WFZ UV-2800 H, UNICO, Shanghai). After removing the LB medium by centrifugation, *E. coli* cells were resuspended in the 1640 medium for infection assays. pBD2 was expressed and purified by affinity chromatography based on the BL21(DE3) pLysS-pET30a-*pBD2* strains constructed in our laboratory [9]. Protein concentration was determined by the bicinchoninic acid (BCA) assay (CW Biotech, Beijing, China).

Cell treatment

IPEC-J2 cells were grown in 1640 medium supplemented with 10% fetal bovine serum (TianHang Biotechnology, Zhejiang, China) and 1% penicillin/streptomycin at 37℃ in an atmosphere of 5% CO_2. The cells were challenged with *E. coli* ATCC 25,922 (20 μl bacteria of 1×10^9 CFU/mL was added, MOI≈100) for 1 h when the cells grew to about 80% confluence in the 6-well plate, and the cultural solution was removed and washed with PBS for three times, then cells were collected after incubated for an additional 2 h, 4 h and 8 h respectively. In the experiments of studying the role of pBD2 against *E. coli*, pBD2 was added (the final concentrations were 0, 20, 40, and 80 μg/mL respectively) after IPEC-J2 cells were challenged with *E. coli* for 1 h, then cells were collected after incubated for an additional 2 h, 4 h and 8 h respectively.

RNA extraction, cDNA synthesis and qRT-PCR

RNA was extracted by Trizol (CW Biotech, Beijing, China) method [24], and the extracted RNA was reverse transcribed into cDNA according to the reverse transcription kit (Bao Bioengineering (Dalian) Co., Ltd). qRT-PCR assays were performed using SYBR Green Dye (QIAGEN, Dusseldorf, Germany) by a quantitative

Table 1 Primers used for qRT-PCR

Genes	Sequence (5′ → 3′)		GenBank number
TLR1	Forward	TTAGGAGACTCTTACGGG GAA	NM_001031775.1
	Reverse	ATTTACTGCGGTGCTGACTGA	
TLR2	Forward	TGCTGCAAGGTCAACTCTCT	NM_213761.1
	Reverse	CAGCAGGGTCACAAGACAGA	
TLR3	Forward	GCATTGCCTGGTTTGTTAG TTG	NM_001097444.1
	Reverse	TGTATCAAAAAGAATCACTG GGAG	
TLR4	Forward	ATATGGCAGAGGTGAAAG CAC	NM_001293316.1
	Reverse	GAAGGCAGAGATGAAAAG GGG	
TLR5	Forward	AGTTCCGGGGATTTTGTTTCA	NM_001123202.1
	Reverse	GCATAAGTAGGCATCGTATT TGTAT	
TLR6	Forward	CATCACCAGCCTCAAGCATTT	NM_213760.2
	Reverse	TTCAGTTGTGTCAAGTTGC CAA	
TLR7	Forward	ATAGCGAGCATCACTCCAGCC	NM_001097434.1
	Reverse	TAATCTGCTGCCTTCTGGTGC	
TLR8	Forward	CACTTTTTGCTCCTGACCTG	NM_214187.1
	Reverse	TTCCTGAAGTTGACGATTTG	
TLR9	Forward	ACAATGACATCCATAGCCG AGT	NM_213958.1
	Reverse	CAGATCGTTGCCGCTAAAGT	
JNK	Forward	GCTCTCCAGCACCCTTACAT	XM_021073087.1
	Reverse	GGTTCTCTCCTCCAAGTCCAT	
IκB-α	Forward	CACCAACCAGCCAGAAATCG	NM_001005150.1
	Reverse	CAGCACCCAAAGACACCA ACA	
NF-κB p65	Forward	CAACCCCTTCCAAGTTCCC ATAG	NM_001114281.1
	Reverse	CCGAGTTCCGATTCACCCG	
P38 MAPK	Forward	ATTCTCCGAGGTCTCAAGT	XM_013977842.2
	Reverse	GCCACATAGCCTGTCATT	
TAK1	Forward	GAGTCCATTGATGATGCCTT	NM_001244067.1
	Reverse	GTAGAGCCTGTTGCTGAG AAG	
MyD88	Forward	TGCCGTCGGATGGTAGTG	NM_001099923.1
	Reverse	ACAGTGATGAACCGCAGGAT	
TRIF	Forward	CCAGGAACCCGAGGAGATG	NM_001315738.2
	Reverse	AGGGCGAAGGGAGAGATTTG	
IRAK-1	Forward	CGGGCGGTGATGAGAAACA	XM_003135490.3
	Reverse	TTGGGCAGGAAGCCGTAGAC	
pBD2	Forward	ACCGCCTCCTCCTTGTAT	NM_214442.2
	Reverse	GGTGCCGATCTGTTTCAT	
IL-8	Forward	CTGGCTGTTGCCTTCTTG	NM_213867.1
	Reverse	TGTGGAATGCGTATTTATG	
GAPDH	Forward	ATGGTGAAGGTCGGAGTGAA	NM_001206359.1
	Reverse	CGTGGGTGGAATCATACTGG	

real-time PCR cycler (LightCycler 96, Roche, Basel, Switzerland). The cycling parameters included 95 ℃ for 30 s; followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 15 s. The results were analyzed using the $2^{-\Delta\Delta CT}$ method [25]. The primers of genes were designed using Primer Premier™ 5.0 software (Sigma–Aldrich, Saint Louis, MO, USA) and synthesized by Sangon Biotech (Shanghai) Co., Ltd (Table 1).

Western blotting

Total proteins were extracted with the Protein Extraction Kit (CW Biotech, Beijing, China) and the concentration of the total proteins was determined by the BCA kit (CW Biotech, Beijing, China). Total proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Burlington, MA, USA). The PVDF membrane was blocked with Tris buffer containing 0.05% Tween 20 and 5% fat-free milk (pH 7.2), then the PVDF membrane was incubated with primary antibodies (p-p65, p65, p-p38, p38, p-JNK, JNK and GAPDH; Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 in 5% milk overnight at 4 °C, then incubated with horseradish peroxidase-conjugated secondary antibodies (Proteintech Group, Wuhan, China) at a dilution of 1:5000 in 5% fat-free milk for 1 h after washed with TBST three times. Finally, proteins were detected using Ultra High Sensitivity ECL Kit (GLPBIO, Montclair, CA, USA) and photographed by the Amersham ImageQuant 800 Fluor (Cytiva, Washington, USA).

Statistical analyses

Data were displayed as the mean ± SD. Statistical analyses used one-way ANOVA and the Dunnett method was used to compare treatment means by SPSS Statistics 24 (IBM, Armonk, NY, USA). Statistical significance was defined as a $P<0.05$.

Results

pBD2 reduced the expression of inflammatory cytokines caused by *E. coli*

The expression of IL-8 increased significantly after IPEC-J2 cells were challenged with *E. coli* and incubated for an additional 4–8 h ($P<0.05$) (Fig. 1a), which suggested that *E. coli* caused the inflammatory responses. The different concentrations of recombinant pBD2 decreased the expression of IL-8 when IPEC-J2 cells were incubated with recombinant pBD2 for an additional 4–8 h after being infected with *E. coli* ($P<0.05$) (Fig. 2a), which indicated that pBD2 could reduce the inflammatory response caused by *E. coli*.

The expression of endogenous pBD2 was sharply increased after infection (at 0 h) and also increased after IPEC-J2 cells were incubated for an additional 4–8 h after infection ($P<0.05$) (Fig. 1b), which indicated the

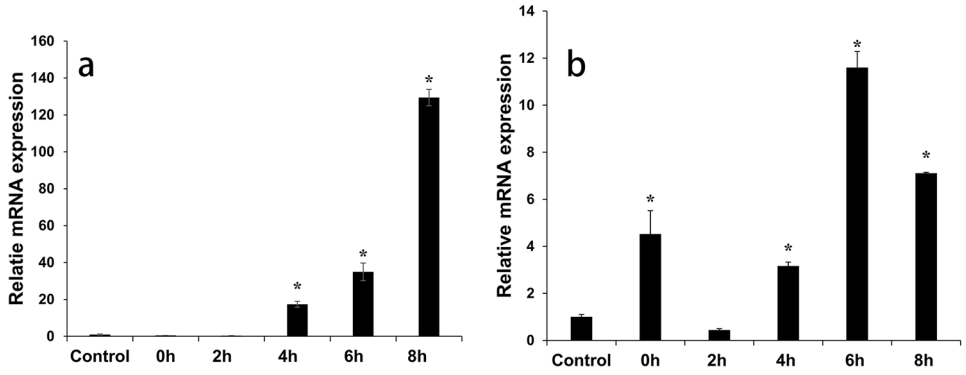

Fig. 1 The expression of IL-8 and pBD2 after cells were challenged with *E. coli*. IPEC-J2 cells were challenged with *E. coli* for 1 h when cells grew to 80% confluence, then incubated for an additional 0 h, 2 h, 4 h, and 8 h respectively. (**a**) The relative expression of IL-8. (**b**) The relative expression of pBD2. Data are the mean ± SD. * indicated that it was significantly difference compared with that in the control group ($P < 0.05$)

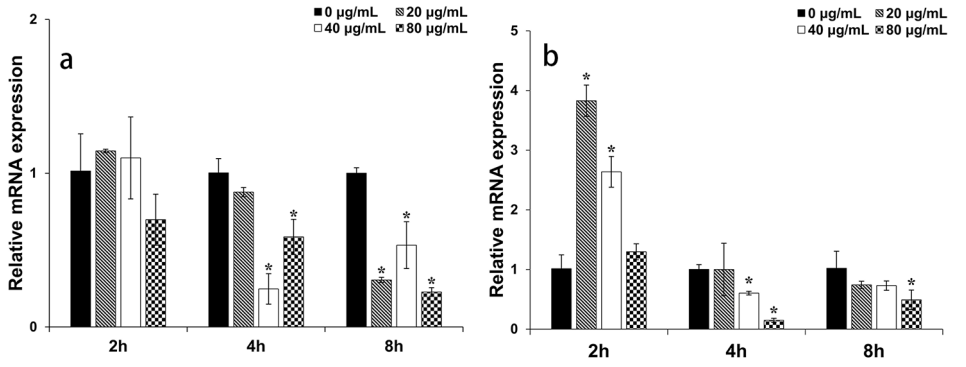

Fig. 2 pBD2 effected on the expression of IL-8 and endogenous pBD2 after cells were challenged with *E. coli*. IPEC-J2 cells were incubated with different concentrations of pBD2 (0, 20, 40, 80 μg/mL) for an additional 2 h, 4 h and 8 h respectively, after being challenged with *E. coli* for 1 h. (**a**) The relative expression of IL-8. (**b**) The relative expression of pBD2. Data are the mean ± SD. * indicated that it had a significant difference compared with that without pBD2 ($P < 0.05$)

endogenous pBD2 played an important role against infection. The added exogenous pBD2 increased the expression of endogenous pBD2 at 2 h after infection and decreased at 4–8 h (Fig. 2b), which were contrary to their expression after IPEC-J2 cells were challenged with *E. coli* without adding recombinant pBD2.

pBD2 influenced the expression of TLR4, TLR5 and TLR7 up-regulated by *E. coli*

The expression of TLR1-7 changed by *E. coli* was detected by qRT-PCR and shown in Fig. 3. The results showed that the expression of TLR4 at an additional 2–4 h, and TLR5 at 0 h and TLR7 at 2 h were significantly increased after IPEC-J2 cells were challenged with *E. coli* for 1 h ($P < 0.05$). In addition, the expression of TLR1, TLR2 and TLR3 was decreased at an additional 2–8 h ($P < 0.05$). TLR8 and TLR9 weren't detected and didn't show in the figure. These results indicated that *E. coli* activated TLR4, TLR5, and TLR7 in different degrees, which resulted in inflammatory responses.

pBD2 significantly reduced the expression of TLR4, TLR5 and TLR7 at 2 h which were up-regulated by *E. coli* ($P < 0.05$), and pBD2 also significantly reduced the expression of TLR4 and TLR5 at 4 h, and TLR 7 at 8 h (Fig. 4). The expression of TLR1, TLR2 and TLR5 were not detected at 8 h. These results indicated that pBD2 decreased the expression of TLR4, TLR5 and TLR7 in different degrees, which decreased the inflammatory responses.

pBD2 possibly reduced the inflammatory responses by TLRs-NF-κB/MAPK signaling pathway

To further identify the signaling pathways by which pBD2 reduced the inflammatory responses, the expression of key downstream genes in the TLRs-NF-κB/MAPK signaling pathway was detected by qRT-PCR. The results showed it significantly increased the expression of IRAK-1(Interleukin 1 receptor-associated kinase 1) at 2–8 h, TAK1 (TGF-β-activated kinase 1) at 2–4 h, p38 at 0 h, p65 at 4 h and JNK (c-Jun N-terminal kinase) at 4–8 h respectively after cells were challenged with *E.*

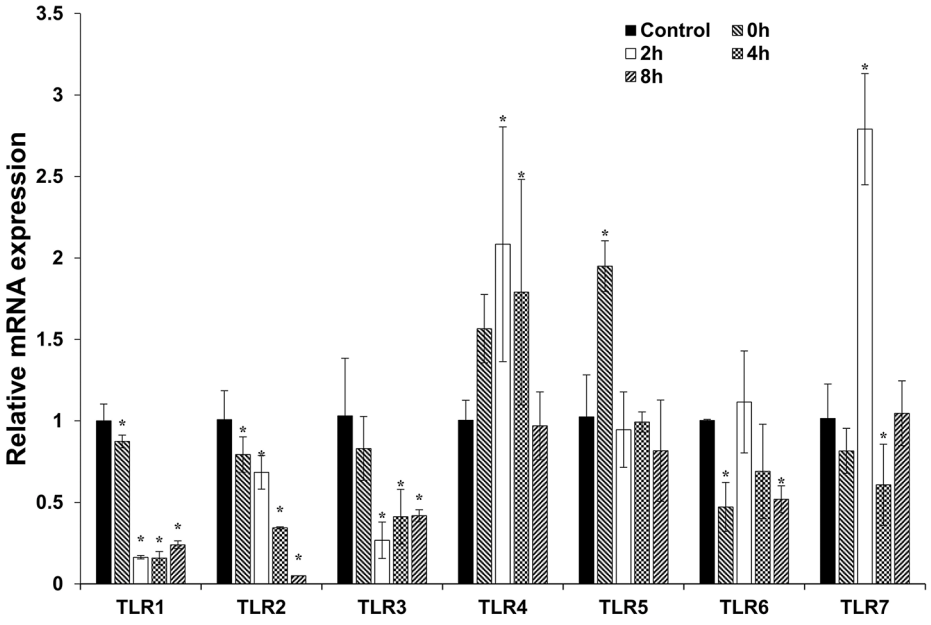

Fig. 3 The expression of TLRs after cells were challenged with *E. coli*. IPEC-J2 cells were challenged with *E. coli* for 1 h when cells grew to 80% confluence, then incubated for an additional 0 h, 2 h, 4 h, and 8 h respectively. Data are the mean ± SD. * indicated that it had a significant difference compared with that in the control group ($P < 0.05$)

coli ($P<0.05$) (Fig. 5). On the contrary, the expression of IκB-α (inhibitor of NF-κB-α) was significantly decreased at 0–8 h after infection. These results indicated that *E. coli* activated the key downstream genes in the TLRs-NF-κB/MAPK signaling pathway in different degrees, which resulted in inflammatory responses.

pBD2 reduced the expression of TAK1, p38 and JNK with different degrees which were up-regulated by *E. coli* (Fig. 6). pBD2 significantly decreased the expression of TAK1 and JNK at 2 h ($P<0.05$), and pBD2 significantly decreased the expression of TAK1, JNK and p38 at 4 h except for 20 μg/mL pBD2 for p38 and 40 μg/mL pBD2 for TAK1 ($P<0.05$). And pBD2 significantly decreased the expression of TAK1,JNK and p38 at 8 h for 80 μg/mL pBD2 ($P<0.05$). It was surprising that pBD2 didn't significantly influence the expression of p65 ($P>0.05$). In addition, pBD2 increased the expression of IRAK-1 at 2 h-8 h, and TRIF at 2–4 h ($P<0.05$). These results indicated that pBD2 attenuates inflammatory responses by decreasing the expression of the key downstream genes in the TLRs-NF-κB/MAPK signaling pathway in different degrees.

pBD2 reduced the phosphorylation of p65, p38 and JNK caused by *E. coli*

To further identify the signaling pathways, the phosphorylation of p65 (p-p65), p38 (p-p38) and JNK (p-JNK) were detected by western blotting (Fig. 7). The expression of p-p65, p-p38 and p-JNK significantly increased at 2 h after cells were challenged with *E. coli*, which had the same trend as that of mRNA. pBD2 significantly decreased the expression of p-p65 caused by *E. coli*, and pBD2 significantly decreased the expression of p-p38 and p-JNK except for at 20 μg/mL pBD2 after IPEC-J2 cells were challenged with *E. coli*. The results indicated that pBD2 could reduce the inflammatory response of IPEC-J2 cells via the NF-κB, JNK and p38 MAPK signaling pathways.

Discussion

pBD2 is one of the porcine beta defensins and plays an important role in immunomodulatory function in pigs. Our previous studies showed that pBD2 had good antibacterial activity, and played their immunomodulatory functions via NF-κB pathways [23, 26]. It is well known that TLRs-NF-κB/MAPK pathways are classic anti-infection pathways, which are involved in the inflammatory response after the cells are challenged with bacteria [27, 28]. However, it is not completely clear how pBD2 reduces the inflammatory response caused by bacteria via TLRs pathways. *E. coli* is a representative of Gram-negative bacteria and is one of the main causes of diarrhea in weaned piglets [29], which impacts on the growth and development of piglets [30, 31]. In this study, in order to further explore the mechanism of pBD2 against pathogens, we studied the effect of pBD2 on the genes related to TLRs signaling pathway after IPEC-J2 cells were challenged with *E. coli*.

The pro-inflammatory cytokines IL-8 is secreted by a variety of cells in response to a wide range of proinflammatory stimuli, and is used as a marker of inflammation

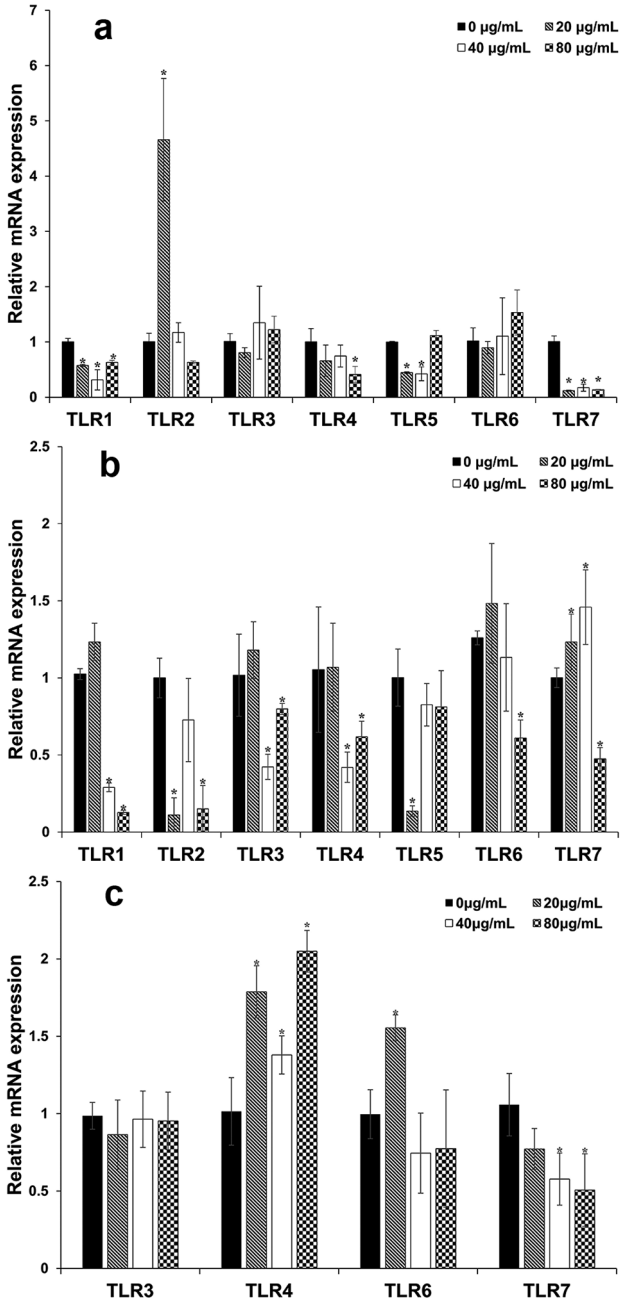

Fig. 4 pBD2 effected on the expression of TLRs after cells were challenged with *E. coli*. IPEC-J2 cells were incubated with different concentrations of pBD2 (0, 20, 40, 80 μg/mL) for an additional 2 h (**a**), 4 h (**b**) and 8 h (**c**) after IPEC-J2 cells were challenged with *E. coli* for 1 h, respectively. Data are the mean ± SD. * indicated that it had a significant difference compared with that without pBD2 ($P < 0.05$)

[26, 32]. In our previous study, the inflammatory cytokines IL-8, IL-6, TNF-α and IL-1α were all increased by *E. coli* at the mRNA and protein levels, and decreased by pBD2 after cells were treated with both pBD2 and *E. coli* [23]. In this study, IL-8 was selected as the symbol for inflammatory response. The expression of IL-8 was significantly increased after IPEC-J2 cells were challenged with *E. coli*, which indicated that *E. coli* induced the inflammatory response. pBD2 decreased the expression of IL-8, indicating that pBD2 could reduce the inflammatory response caused by *E. coli*. It was reported that Lipopolysaccharide (LPS) increased the expression of IL-8, IL-6, TNF-α and IL-1β at both mRNA and protein levels [33]. It was reported that pBD2 also reduced the expression of IL-8, IL-6 and TNF-α at mRNA and protein levels in mice which was up-regulated by dextran sodium sulfate (DDS) [11]. In addition, rhesus macaque θ-defensin 1 (RTD-1) decreased the expression of IL-8, IL-6 and IL-1β which was increased by *E. coli* at protein level after THP-1 cells were infected with *E. coli* [34]. These results are consistent with ours, which indicated that pBD2 could attenuate inflammatory responses induced by *E. coli.*

In this study, the expression of endogenous pBD2 was sharply increased after IPEC-J2 cells were infected by *E. coli*, and also increased at 4–8 h after infection. It was reported that expression of pBD2 significantly increased when the cells and pigs were infected by *E. coli* or LPS [35, 36]. These results indicated that endogenous pBD2 played an important role against *E. coli*, and are consistent with ours. The exogenous pBD2 increased the endogenous pBD2 at 2 h, and decreased endogenous pBD2 at 4 h after infection, which were contrary to their expression after IPEC-J2 cells were challenged with *E. coli* without adding recombinant pBD2. The results showed that both exogenous recombinant pBD2 and endogenous pBD2 play an important role against *E. coli*. The added exogenous pBD2 influenced the expression of endogenous pBD2, and implied that they have complementary roles, which need further study.

It is reported that the TLRs-NF-κB/MAPK signaling pathways are the key pathways that are involved in inflammatory response. The expression of TLR4, TLR5 and TLR7 was significantly increased after IPCE-J2 cells were challenged with *E. coli.* It was surprising that the expression of TLR1, TLR2 and TLR3 was decreased at an additional 2–8 h ($P<0.05$) after IPEC-J2 cells were challenged with *E. coli.* It was reported that the mRNA expression of TLR1, TLR3, and TLR7 was significantly increased, while the mRNA expression of TLR2 was significantly decreased, and the other TLR genes (i.e. TLR4, TLR5, TLR6, and TLR8) did not show any change in their expression levels after HT-29 cells were treated with *E. coli* Nissle 1917 supernatant compared with that in the control [37]. The decreased expression of TLR2 and increased expression of TLR7 were consistent with our results, while increased TLR1 and TLR3, as well as no change in expression of TLR4 and TLR5 were inconsistent with ours. It was reported that the mRNA expression was increased in TLR1, TLR5, TLR6, TLR7, TLR8 and

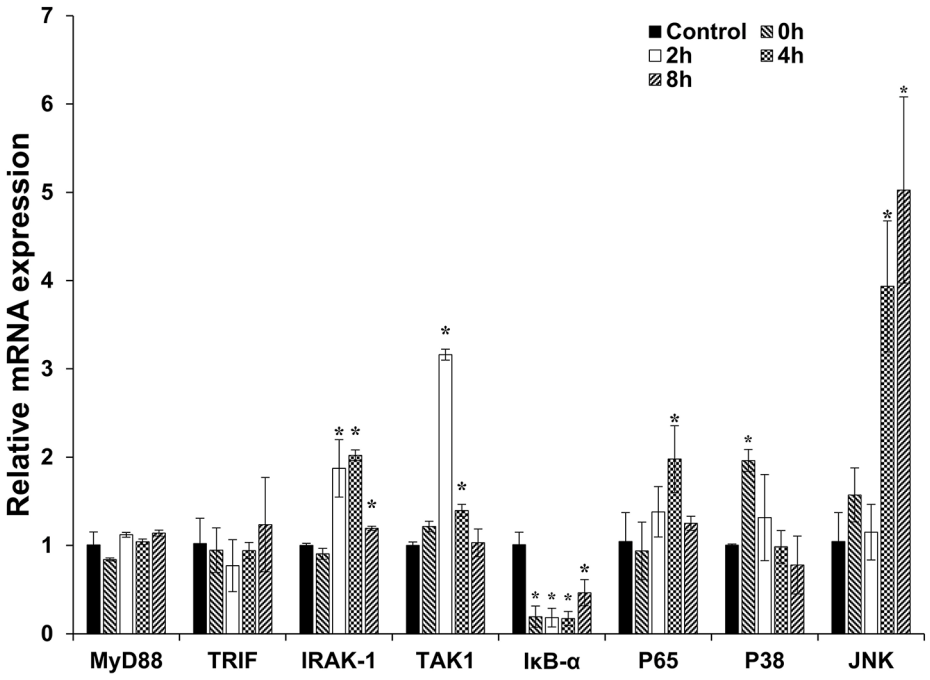

Fig. 5 The expression of key genes in TLRs-NF-κB/MAPK signaling pathway after cells were challenged with *E. coli*. IPEC-J2 cells were challenged with *E. coli* for 1 h when cells grew to 80% confluence, then incubated for an additional 0 h, 2 h, 4 h, and 8 h respectively. Data are the mean ± SD. * indicated that it had a significant difference compared with that in the control group ($P < 0.05$)

TLR9 when Caco2 cells (human colon adenocarcinoma cell line) were exposed to outer membrane vesicles of pathogen and non-pathogenic *E. coli* [38]. It was reported that ETEC (Enterotoxigenic *Escherichia coli*) induces TLR2, TLR5, TLR8 mRNA expression in jejunum tissues by small intestinal segment perfusion model in pigs, and induces TLR5 and TLR8 mRNA expression in IPEC-J2 cells at 2 h and 4 h after infection. Furthermore, non-pathogenic *E. coli* strain HB101 only induced mRNA expression of TLR8 in IPEC-J2 [39]. It was reported that the mRNA expression of TLR2, TLR4 and TLR9 was significantly increased after IPCE-J2 cells were challenged with ETEC [40]. The expression of TLR2, TLR4 at mRNA and protein levels were up-regulated by *E. coli* in the human umbilical cord blood-derived mesenchymal stem cells (MSC) [41]. These results are partly consistent with ours. Yang et al. reported that expression of TLR4 was significantly increased in the jejunum of piglets after weaned piglets were challenged with 5×10^{10} CFU/mL ETEC K88 [35]. ETEC increased the jejunal TLR4 expression at the mRNA and protein levels in pigs [42]. These results are consistent with ours. TLR5 recognizes bacterial flagellin and is up-regulated by *E. coli* [43]. TLR7 is usually activated by viruses including RNA [44, 45]. It is reported that *E. coli* secretes a large amount of RNA itself [46], which could be the reason why TLR7 is activated after IPEC-J2 cells were infected with *E. coli*, which needs further study. In addition, TLR5 and TLR4 were activated first, then TLR7, perhaps because the cells contact the flagella and LPS first, which need further study. pBD2 significantly reduced the up-regulated expression of TLR4 at 2 –4 h, TLR5 at 2 h and TLR7 at 2 h caused by *E. coli.* These TLRs play an important regulatory role in *E. coli* invasion. pBD2 inhibited the expression of TLR4, TLR5 and TLR7 perhaps indicating that pBD2 inhibited the immune response and decreased cell damage, which was conducive to the resistance to *E. coli* stimulation. It was surprising that pBD2 increased the expression of TLR7 at 4 h. It is possible because pBD2 killed the *E. coli* and made the RNA release, which activated TLR7 again, which needs further study. Furthermore, the expression of TLR1, TLR2 and TLR5 was not detected at 8 h, perhaps due to their expression being too low to be detected.

In this study, the expressions of IRAK-1, TAK1, p65, p38 and JNK were increased with different degrees when IPEC-J2 cells were challenged by *E. coli*. Activation of TLRs has been found to promote the actions of IRAK1, which affect downstream TAK1 [47, 48]. TAK1 is a key protein involved in host response to environmental stress signals, and TAK1 activates not only NF-κB, but also JNK and p38 in the MAPK pathway [49]. In addition, IκB-α is the key gene in the NF-κB signaling pathway that inhibits the activation of NF-κB [50], and the expressions of IκB-α significantly decreased, which was consistent with the up-regulated expression of p65. Guo et al. showed that the mRNA expression of p65 and the protein expression

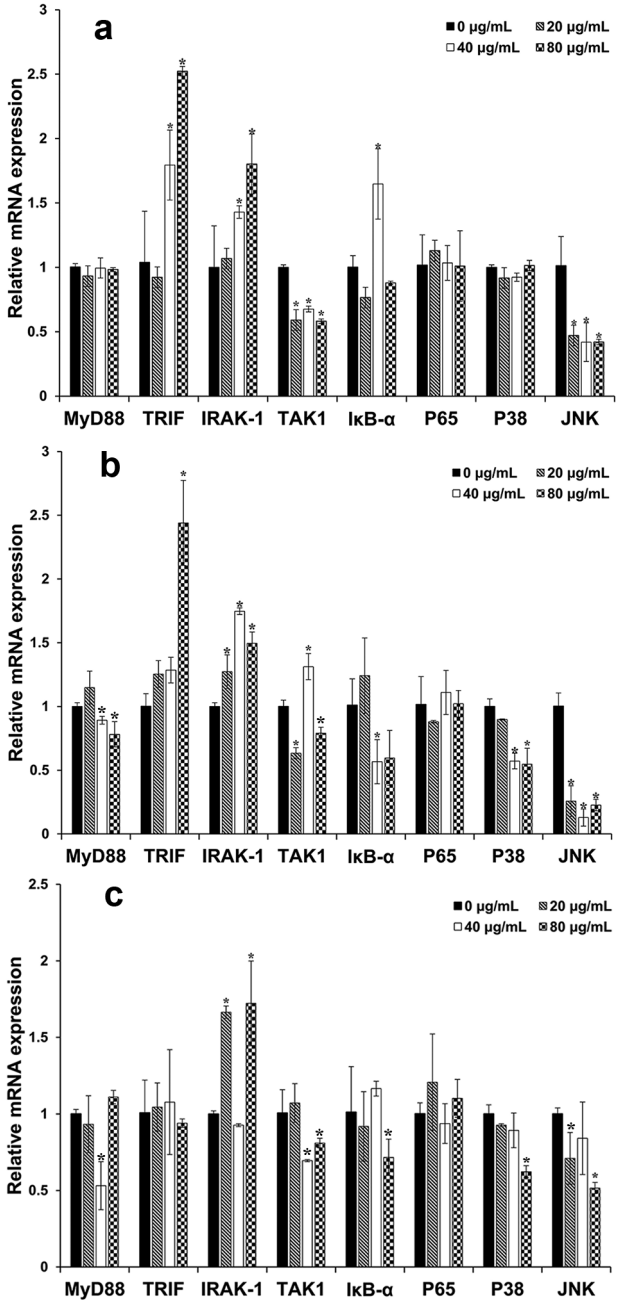

Fig. 6 pBD2 effected on the expression of key genes in TLRs-NF-κB/MAPK signaling pathway after cells were challenged with *E. coli*. IPEC-J2 cells were incubated with different concentrations of pBD2 (0, 20, 40, 80 μg/mL) for an additional 2 h (**a**), 4 h (**b**) and 8 h (**c**) after IPEC-J2 cells were challenged with *E. coli* for 1 h, respectively. Data are the mean ± SD. * indicated that it had a significant difference compared with that without pBD2 ($P < 0.05$)

of p-p65 significantly increased after IPEC-J2 cells were infected with *E. coli* [51]. It was also demonstrated that expression of p-p65, p-p38 and p-JNK was significantly increased at protein levels after IPEC-1 cells were infected with *E. coli* [52]. It was reported that the mRNA expression of p65, and p38 was significantly increased in sheep epithelial cells infected with *E. coli* F17 [53]. Our results are consistent with these results, suggesting that TLRs-NF-κB/MAPK signaling involved in inflammatory response were activated by *E. coli*.

pBD2 decreased the expression of TAK1, JNK and p38, while pBD2 didn't decrease the expression of p65 at mRNA level. The further result showed that pBD2 decreased the expression of p-p65, p-p38 and p-JNK which was up-regulated caused by *E. coli* at the protein level. The result indicated that pBD2 could inhibit the NF-κB pathway, but also the p38 and JNK pathways. Our result showed that pBD2 attenuated the inflammatory response through the TLR4,5,7-TAK1-NF-κB/MAPK signaling pathways. Those results were consistent with our previous study that showed that pBD2 inhibited the NF-κB pathway which was up-regulated by *E. coli* [23]. Huang et al. reported that pBD2 reduced LPS-induced cellular inflammation via the TLR4/NF-κB pathway [22]. pBD114 inhibited LPS-induced IPEC-J2 cells inflammation by down-regulating p-IκB-α, p-p65 and p-ERK1/2 at protein level [54]. β-defensin 118 (DEFB118) could alleviate ETEC-induced inflammation in IPEC-J2 cells through inhibition of the NF-κB pathway (DEFB118 inhibited the protein expression of p-65 and p-IκB-α) [55]. It was reported that Rhesus macaque θ-defensin RTD-1 inhibits proinflammatory cytokine secretion and gene expression by inhibiting the activation of NF-κB (p65 at mRNA level) and MAPK pathways (including p-38 and p-JNK at protein levels) [56]. The θ-defensin, retrocyclin-101 could inhibit TLR4-dependent signaling (including the p-p65, p-38 and p-JNK, which increased by LPS in mice) [57]. l-arginine diminished the LPS-induced expression of TLR4 and inhibited activation of TLR4-mediated p-65 and p-p38 in IPEC-J2 cells [33]. These researches were consistent with our results. Our results indicated that pBD2 alleviated the inflammatory response through the TLR4,5,7-TAK1-NF-κB/MAPK signaling pathways. These findings reveal new anti-infective mechanisms of pBD2 by which pBD2 reduces inflammatory response, providing a theoretical basis for the study and development of novel peptide drugs.

Conclusions

The effect of recombinant pBD2 on the TLRs-NF-κB/MAPK signal pathways was investigated in the IPEC-J2 cells after being challenged with *E. coli* in this study. The results revealed that pBD2 decreased the expression of IL-8 induced by *E. coli*, suggesting that pBD2 could inhibit the inflammatory response caused by *E. coli*. pBD2 influenced the expression of endogenous pBD2, and implied that they have complementary roles. pBD2 significantly decreased the expression of TLR4, TLR5 and TLR7, as well as the key downstream genes p38 and JNK which were up-regulated by *E. coli*. In addition, pBD2

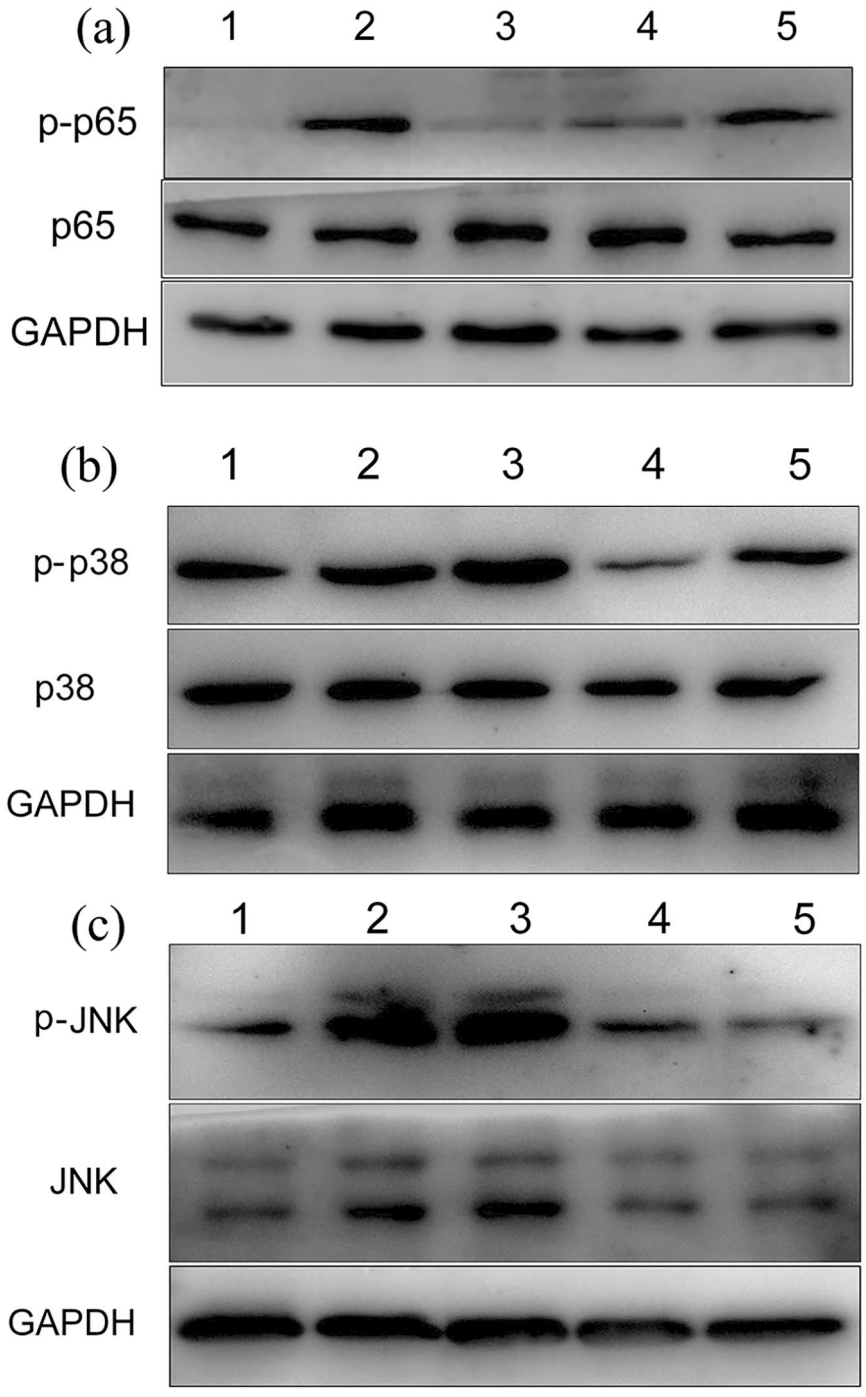

Fig. 7 pBD2 effected on the expression of p-p65, p-p38 and p-JNK after cells were challenged with *E. coli*. The protein expression of p-p65 (**a**), p-p38 (**b**) and p-JNK (**c**) were determined by western blot. 1 indicated the control. 2–5 indicated that IPEC-J2 cells were incubated with different concentrations of pBD2 (0, 20, 40, 80 μg/mL) for 2 h after cells were challenged with *E. coli* for 1 h, respectively

inhibited the p-p65, p-p38 and p-JNK which were up-regulated by *E. coli.* Collectively, the results indicated that pBD2 could reduce the inflammatory response of IPEC-J2 cells via the TLR4,5,7-TAK1-NF-κB/MAPK signaling pathway. Our study reveals the mechanism by which pBD2 reduces inflammatory response.

Abbreviations

pBD2	Porcine beta defensin 2
TLRs	Toll-like receptors
NF-κB	Nuclear factor-kappa B
IPEC-J2	Porcine intestinal epithelial cells
E. coli	Escherichia coli
MAPK	Mitogen-activated protein kinases
MyD88	Myeloid differentiation factor 88
TRIF	TIR domain-containing adaptor inducing interferon
IL-8	Interleukin-8
JNK	c-Jun N-terminal kinase
TAK1	TGF-β-activated kinase 1
IκB-α	Factor-kappa-B inhibitor alpha
IRAK-1	Interleukin-1 receptor-associated kinase 1

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
C.L. and K.W. designed the experiments plan. X.S. and K.Z. completed the cell experiment. M.G. performed the qRT-PCR and western bolt. H.C. and F.Z. analyzed the experimental data. C.L. and X.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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