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β -defensin 118 attenuates inflammation and injury of intestinal epithelial cells upon enterotoxigenic *Escherichia coli* challenge

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Abstract

Background: Antimicrobial peptides including various defensins have been attracting considerable research interest worldwide, as they have potential to substitute for antibiotics. Moreover, AMPs also have immunomodulatory activity. In this study, we explored the role and its potential mechanisms of β -defensin 118 (DEFB118) in alleviating inflammation and injury of IPEC-J2 cells (porcine jejunum epithelial cell line) upon the enterotoxigenic *Escherichia coli* (ETEC) challenge.

Results: The porcine jejunum epithelial cell line (IPEC-J2) pretreated with or without DEFB118 (25 μ g/mL) were challenged by ETEC (1×10^9 CFU) or culture medium. We showed that DEFB118 pretreatment significantly increased the cell viability ($P < 0.05$) and decreased the expressions of inflammatory cytokines such as the interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) in IPEC-J2 cells exposure to ETEC ($P < 0.05$). Interestingly, DEFB118 pretreatment significantly elevated the abundance of the major tight-junction protein zonula occludens-1 (ZO-1), but decreased the number of apoptotic cells upon ETEC challenge ($P < 0.05$). The expression of caspase 3, caspase 8, and caspase 9 were downregulated by DEFB118 in the IPEC-J2 cells exposure to ETEC ($P < 0.05$). Importantly, DEFB118 suppressed two critical inflammation-associated signaling proteins, nuclear factor-kappa-B inhibitor alpha (I κ B- α) and nuclear factor-kappaB (NF- κ B) in the ETEC-challenged IPEC-J2 cells.

Conclusions: DEFB118 can alleviate ETEC-induced inflammation in IPEC-J2 cells through inhibition of the NF- κ B signaling pathway, resulting in reduced secretion of inflammatory cytokines and decreased cell apoptosis. Therefore, DEFB118 can act as a novel anti-inflammatory agent.

Keywords: Antimicrobial peptide, ETEC, inflammation, IPEC-J2 cells, NF- κ B

Introduction

The intestinal epithelium not only acts as the major site for nutrient absorption but also acts as the primary physical barrier against a wide variety of endogenous and exogenous harmful substances in the gastrointestinal [1–3]. Disruption of the intestinal epithelium by

various bacterial pathogens may result in inflammation and severe diarrhea in neonatal animals [4, 5]. For instance, the enterotoxigenic *Escherichia coli* (ETEC) has been identified as the most critical bacterial causing post-weaning diarrhea (PWD) [6–8]. Colonization and proliferation of ETEC strains in the intestine produce a large number of enterotoxins that act on the small intestine and lead to the secretion of fluids and electrolytes, causing diarrhea [9, 10]. In last decades, antibiotics have been widely used to prevent PWD. However, long-term or overdose utilization of antibiotics may lead to the

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developing of drug resistance [11–13]. Therefore, novel avenues to prevent various bacteria-induced inflammation and intestinal epithelium disruption are urgently needed.

Previous studies indicated that the intestinal epithelium can also serve as a vital immune organ, and the intestinal epithelial cells can secrete a variety of bioactive substances (e.g. antimicrobial peptides) that play important roles in regulating immunity and intestinal health [14–17]. Defensins are diverse members of a large family of antimicrobial peptides, contributing to the antimicrobial action of granulocytes, mucosal host defence in the small intestine and epithelial host defence in the skin and elsewhere [18]. Previous studies indicated that defensins can be divided into α , β , and θ subclasses according to their disulfide bonding, genomic organization, and tissue distribution [19–22]. Amongst the three types of defensins, the β -defensins have been the most extensively studied to date. β -defensins are usually translated from characteristic two exon gene structures, the first of which encodes a prepropeptide while the mature peptide is encoded by the second exon, containing the six-cysteine motif [23]. Importantly, β -defensins were traditionally viewed as exclusively antimicrobial molecules, as their induction in response to diverse bacterial, viral, parasitic, and fungal infections was widely reported [24–26]. There are also reports showing that β -defensins can also inhibit inflammation. For instance, β -defensin 129 was reported to attenuate intestinal inflammation and epithelial atrophy in rat exposure to bacterial endotoxin [27]. Human β -defensin 114 regulates lipopolysaccharide (LPS)-mediated inflammation and protects sperm from motility loss [28].

β -defensin 118 (DEFB118) is a novel antimicrobial peptide that can obtain from caput and efferent ducts of epididymis [29]. Interestingly, DEFB118 can disrupt the membrane of *E. coli* and change their morphology of the bacterial surface [30]. Moreover, our previous study found that DEFB118 exhibited antimicrobial activity against both Gram-negative and Gram-positive bacteria [31]. Although DEFB118 has shown antimicrobial activity, the exact role of DEFB118 in regulating mucosal immunity and intestinal health are unknown. In the present study, we explored the role of DEFB118 in alleviating inflammation and injury of intestinal epithelial cells during exposure to ETEC. The mechanisms behind the DEFB118 regulated actions have also been partially investigated.

Materials and Methods

Strains and Vectors

The *E. coli* DH5 α and *E. coli* Orgami B (DE3) strains were purchased from TIANGEN (Beijing, China). The pET32a

(+) was purchased from Merck KGaA (Darmstadt, Germany). ETEC (O149: K91, K88ac) was purchased from China Veterinary Culture Collection Center (Beijing, China).

Plasmid Construction, Expression, and Purification of DEFB118

The target gene DEFB118 was synthesized and introduced *Eco* RI and *Not* I restriction sites at the 5' and 3' ends of the target gene by Tsingke Biological Technology Co., Ltd. (Chengdu, China). The DEFB118 fragment was cloned into the expression vector pET32a (+) after double enzymatic digestion by *Eco* RI and *Not* I (Japanese Takara). The resulting plasmid pET32a(+)-DEFB118 was transformed into *E. coli* Orgami B (DE3) and induced by 1.0 mM isopropyl β -D-1-thiogalactoside (IPTG). After incubation for 4 h at 28°C, the bacteria were collected by centrifugation at 8000 \times g for 20 min at 4°C and lysed by lysis buffer [500 mM NaCl, 20 mM Tris, 0.1% Triton X-100, 1 mM PMSE, Lysozyme 0.2 mg/mL, 10 U/mL DNase (pH 7.5)] for 30 min at 4°C. Then, schizolytic cells were sonicated (4 s pulse and 8 s interval; 30 cycles; Sonics-Vibra cell, USA). The supernatant of the cell lysate resulting from centrifugation at 15000 \times g for 30 min was applied to a Ni²⁺-NTA column (Sangon Biotech, Shanghai). After washing to baseline absorbance with Binding buffer (20 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 5 mM imidazole, pH 8.0), the column was washed with Elution Buffer (20 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 500 mM imidazole, pH 8.0) at a flow rate of 1 mL/min. The fractions were collected and dialyzed with sterile saline solution (0.09% [wt/vol] NaCl in distilled water). The purified DEFB118 was run on 12% SDS-PAGE. The rest was stored at -80°C after quantified with the BCA assay (Beyotime, China).

Cell culture and treatment

The porcine jejunum epithelial cell line (IPEC-J2) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM F12 medium supplemented with 10% FBS (Fetal bovine serum), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C with 5% CO₂ in a humidified atmosphere. In addition, the cells were seeded in plates once every 2–3 days to achieve 80% confluence. After incubated with antimicrobial peptide DEFB118 (25 $\mu\text{g}/\text{mL}$) for 12 h or BAY11-7082 (an inhibitor of I κ B- α phosphorylation and NF- κ B) for 2 h, then cells were challenged with 1×10^6 CFU/well ETEC for 1 h or 2.5 h (only for assessment of apoptosis). It is worth noting that when challenged with ETEC, the cells were cultured in DMEM F12 medium supplemented with 2% FBS (without any antibiotics).

Cell viability assay

MTT (Sigma, USA) was used to evaluate cell viability. Briefly, IPEC-J2 cells seeded in 96-well plates (Corning, USA) were incubated with 20 μ L MTT for 4 h immediately after treatment. Next, the culture medium containing MTT was aspirated, 150 μ L DMSO was added and oscillated at low speed for 10 min. Last, the optical density (OD) of the wells was read at 570 nm by a microplate reader (SpectraMax 190, Molecular Devices, California, USA). Cell viability (%) = $(OD_{\text{treatment group}} - OD_{\text{blank group}}) / (OD_{\text{control group}} - OD_{\text{blank group}}) \times 100$.

RNA extraction and RT-PCR

The total RNA was extracted from the IPEC-J2 cells by RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions. RNA concentration and purity were determined by the NanoDrop 2000 spectrophotometer at 260 and 280 nm (Thermo Fisher Scientific Inc., Waltham, MA, USA). And then cDNA was synthesized by a Reverse Transcriptase kit (Takara, Dalian, China). Quantitative PCR was performed by QuanStudio 6 Flex Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA) with a total of 10 μ L of assay solution containing 5 μ L SYBR Green mix (Takara), 0.2 μ L Rox, 3 μ L deionized H₂O, 1 μ L cDNA template, and 0.4 μ L each of forward and reverse primers (Sangon, China). The relative gene expressions compared with the housekeeping gene β -actin were calculated by $2^{-\Delta\Delta Ct}$ [32]. The primer sequences show in Table 1.

Assessment of apoptosis by flow cytometry

Apoptotic IPEC-J2 cells were detected by an Annexin V-PE/7-AAD Apoptosis Detection Kit (B&D Pharmingen, USA) or an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. Cells seeded in 12-well plates (Corning, USA) were harvested by 0.25% trypsin without EDTA after the treatment. After centrifuged at 350 \times g for 10 min, the cells were washed with ice-cold PBS, centrifuged again, and resuspended with 100 μ L 1 \times binding buffer. Next, 2 μ L PE Annexin V or AnnexinV-FITC and 2 μ L 7-ADD or PI were added into the cells and they were incubated for 15 min at room temperature in the dark. Finally, 400 μ L 1 \times Binding Buffer was added to the mixture and cell apoptosis was evaluated by CytoFlex flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Immunofluorescence

IPEC-J2 cells were seeded on coverslips treated with concentrated sulfuric acid placed in 12-well cell culture plates at a density of 2×10^5 cells/well and cultured to 80–90% confluence. Subsequently, the cells were treated

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Gene ^a	Primer sequence (5'-3')	Accession Number.
<i>IL-1β</i>	Forward: AAAGCCCAATTCAGGGACCCCTAC Reverse: CCATCACTTCCTTGGCGGGTT	NM_214055.1
<i>IL-6</i>	Forward: AGGGAATGTCGAGGCTGTGC Reverse: CCGGCATTTGTGGTGGGGTT	NM_214399.1
<i>TNF-α</i>	Forward: TTCGAGGTATCGGCCCCCA Reverse: GTGGGCGACGGGCTTATCTG	NM_214022.1
<i>CASP3</i>	Forward: GGAATGGCATGTCGATCTGGT Reverse: ACTGTCCGTCTCAATCCAC	NM_214131.1
<i>CASP8</i>	Forward: TCTCGGACTGGATGTGATT Reverse: TCTGAGGTTGCTGGTACAC	NM_001031779.2
<i>CASP9</i>	Forward: AATGCCGATTGGCTTACGT Reverse: CATTGCTTGGCAGTCAGGTT	XM_003127618.4
<i>β-actin</i>	Forward: TGGAACGGTGAAGGTGACAGC Reverse: GCTTTTGGGAAGGCAGGGACT	XM_003124280.5

^a *IL-1 β* interleukin-1 β , *IL-6* interleukin-6, *TNF- α* tumour necrosis factor- α , *CASP3* caspase 3, *CASP8* caspase 8, *CASP9* caspase 9, *β -actin*, beta-actin

with reagents (DEFB118 and BAY11-7082) and ETEC according to the experimental design. After washed with ice-cold PBS, cells were fixed with 4% paraformaldehyde 15 min at room temperature. After washed three times with PBS (pH 7.4) for 2 min each time, cells incubated overnight with the primary antibody at 4°C in the dark (rabbit anti-ZO-1; Novus; Cat no.: NBP1-85047; 1:200). Next, cells were washed three times with PBS (pH 7.4) for 2 min each time and incubated for 2 h at room temperature with the appropriate secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit immunoglobulin; CST; Cat no.:4412S; 1:1000). Finally, the cells were washed three times with PBS (pH 7.4) for 2 min each time and counterstained with DAPI (Sigma-Aldrich). cells were imaged using a confocal scanning microscope (NIKON ECLIPSE TI-SR)

Total protein extraction and western blot analysis

The total protein was extracted from the IPEC-J2 cells by cell lysis buffer for western blot (WB) analysis. After the protein concentration was determined by a BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China), the supernatants were diluted with 4 \times Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 10% β -mercaptoethanol and denatured at 98°C for 10 min. Then, equal amounts of proteins in boiled samples were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45 μ m polyvinylidene fluoride (PVDF) membranes (Merck Millipore Ltd., Tullagreen, Ireland). Next, the PVDF membranes were blocked with 5% non-fat dry milk at room temperature for 1 h. After being washed in TBS/T three times for 10 min each, the

membranes were incubated with specific primary antibodies [ZO-1 (Novus; Cat no.: NBP1-85047; 1:1000), p-NF- κ B p65 (CST; Cat no.:3033S; 1:1000), NF- κ B p65 (CST; Cat no.:6956S; 1:1000), I κ B α (CST; Cat no.:4814S; 1:1000), p-I κ B α (Invitrogen; Cat no.:MA5-15224; 1:1000), GAPDH (CST; Cat no.:2118S; 1:1000)] at 4°C for overnight under gentle agitation. After being washed in TBS/T three times for 10 min each, the membranes were incubated with the corresponding secondary antibodies [anti-rabbit IgG (CST; Cat no.7074S; 1:2500), anti-mouse IgG (CST; Cat no.7076S; 1:2500)] for 1 h at room temperature. Finally, after washing thrice with TBS/T, the PVDF membranes were treated with Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc.). The protein bands were photographed by the ChemiDoc™ MXRS+ Imager System (Bio-Rad Laboratories, Inc.). The intensity of the protein bands was quantified with Quantity One software (Bio-Rad Laboratories, Inc.), and the results were expressed as the abundance of the target protein relative to that of the reference protein (GAPDH).

Statistics analysis

All statistical analysis was performed using SPSS26.0 software. Data were expressed as the mean \pm standard error (SEM). Statistical analysis was carried out using Two-way analysis of variance (ANOVA) followed by LSD multiple comparison test. $P < 0.05$ was considered statistically significant. Image production using GraphPad Prism software (Version 8. GraphPad Software Inc., CA, USA).

Results

Expression and purification of DEFB118

As shown in Fig. 1, the cell extracts from *E. coli* Origami B (DE3) harboring the plasmid pET32a (+)-DEFB118 showed a clear band with molecular weight about 30 kDa. No bands were observed in the extract from the un-induced control strain or *E. coli* harboring the empty plasmids. The molecular weight of DEFB118 is consistent with the predicted size. The crude recombinant proteins were extracted from *E. coli* and then purified by Ni²⁺-NTA affinity chromatography. The result from SDS-PAGE verified successful purification, as only one clear band with molecular weight about 30 kDa was observed (Fig. 1).

Influences of ETEC challenge on the viability and inflammatory response of IPEC-J2 cells

To explore the influence of ETEC challenge on cell viability, the IPEC-J2 cells were treated with ETEC at different concentrations (0, 10⁵, 10⁶, 10⁷, 10⁸ CFU/well) for 3 h. As shown in Fig. 2A, the viability of the cells was significantly decreased upon ETEC challenge at a moderate or

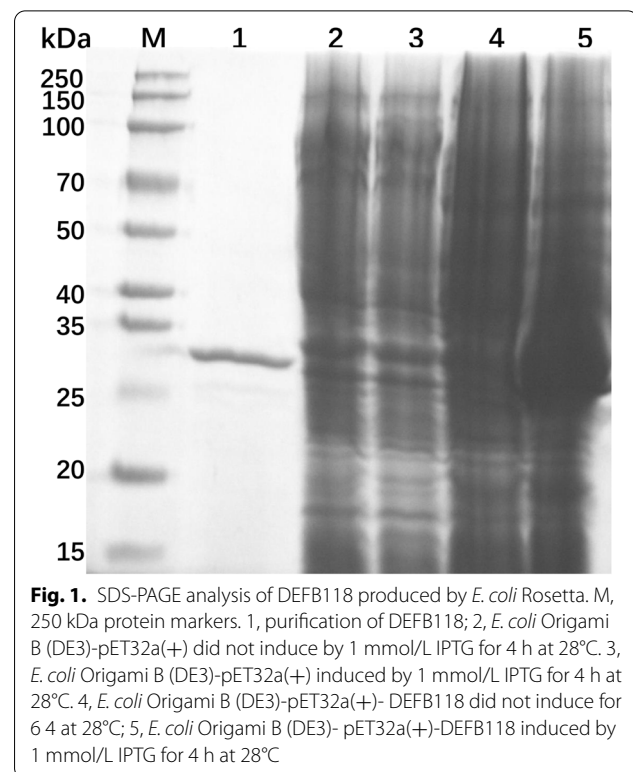
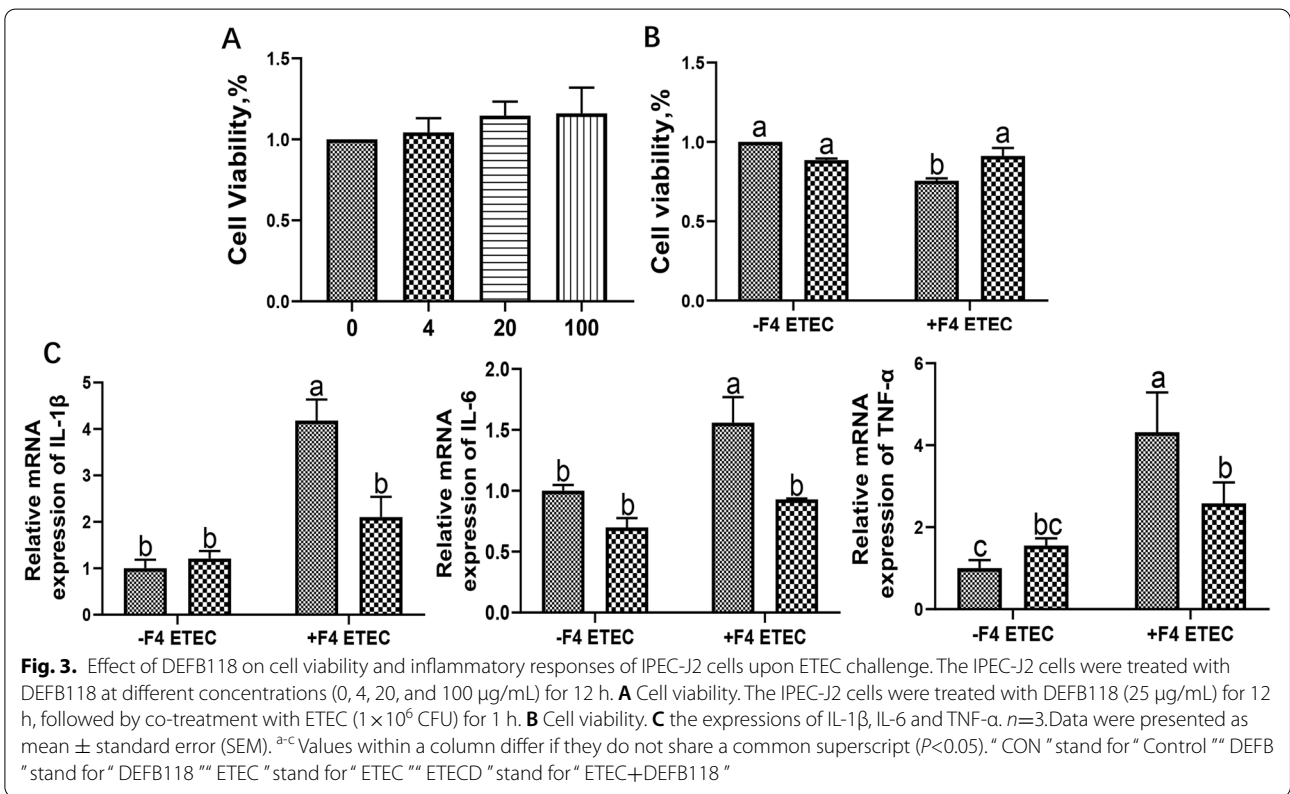
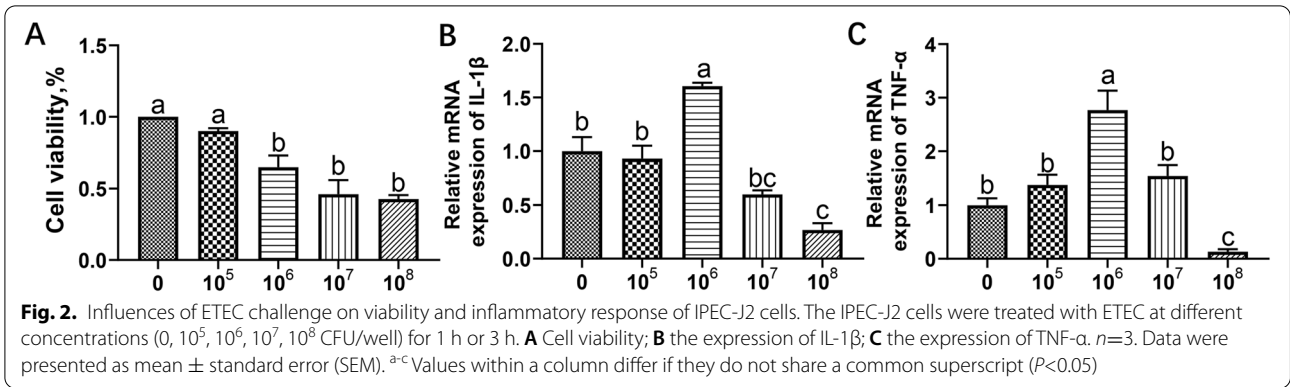


Fig. 1. SDS-PAGE analysis of DEFB118 produced by *E. coli* Rosetta. M, 250 kDa protein markers. 1, purification of DEFB118; 2, *E. coli* Origami B (DE3)-pET32a(+) did not induce by 1 mmol/L IPTG for 4 h at 28°C. 3, *E. coli* Origami B (DE3)-pET32a(+) induced by 1 mmol/L IPTG for 4 h at 28°C. 4, *E. coli* Origami B (DE3)-pET32a(+)-DEFB118 did not induce for 6 h at 28°C; 5, *E. coli* Origami B (DE3)-pET32a(+)-DEFB118 induced by 1 mmol/L IPTG for 4 h at 28°C

higher dose (10⁶, 10⁷, and 10⁸ CFU/well) ($P < 0.05$). However, there were no significant differences in cell viability among the three groups ($P > 0.05$). We also determined the influences of different doses of ETEC on the inflammatory response in the IPEC-J2 cells. As shown in Fig. 2B and C, the expression levels of inflammatory cytokines such as the IL-1 β and TNF- α were significantly elevated in the cells upon ETEC challenge at a dose of 10⁶ CFU/well ($P < 0.05$). As compared to this dose, a higher dose (10⁷ and 10⁸ CFU/well) significantly decreased their expression levels in the IPEC-J2 cells ($P < 0.05$). Therefore, a moderate dose (10⁶ CFU/well) was used for further construction of challenge model.

Effect of DEFB118 on cell viability and inflammatory responses of IPEC-J2 cells upon ETEC challenge

To explore the influence of DEFB118 on cell viability, the IPEC-J2 cells were treated with DEFB118 at different concentrations (0, 4, 20, and 100 μ g/mL) for 12 h. Results showed that treatment with the cells with DEFB118 ranging from 4 to 100 μ g/mL had no negative influence (toxic effect) on cell viability (Fig. 3A). Therefore, a moderate dose 25 μ g/mL was used for further studies. As shown in Fig. 3B, ETEC challenge decreased the viability of the IPEC-J2 cells; however, pretreatment of the cells with 25 μ g/mL DEFB118 significantly increased the cell viability upon ETEC challenge ($P < 0.05$). Moreover, ETEC



challenge significantly elevated the expression levels of inflammatory cytokines such as the IL-1β, IL-6, and TNF-α in IPEC-J2 cells (P<0.05). However, DEFB118 pretreatment significantly downregulated their expressions in the IPEC-J2 cells upon ETEC challenge (Fig. 3C).

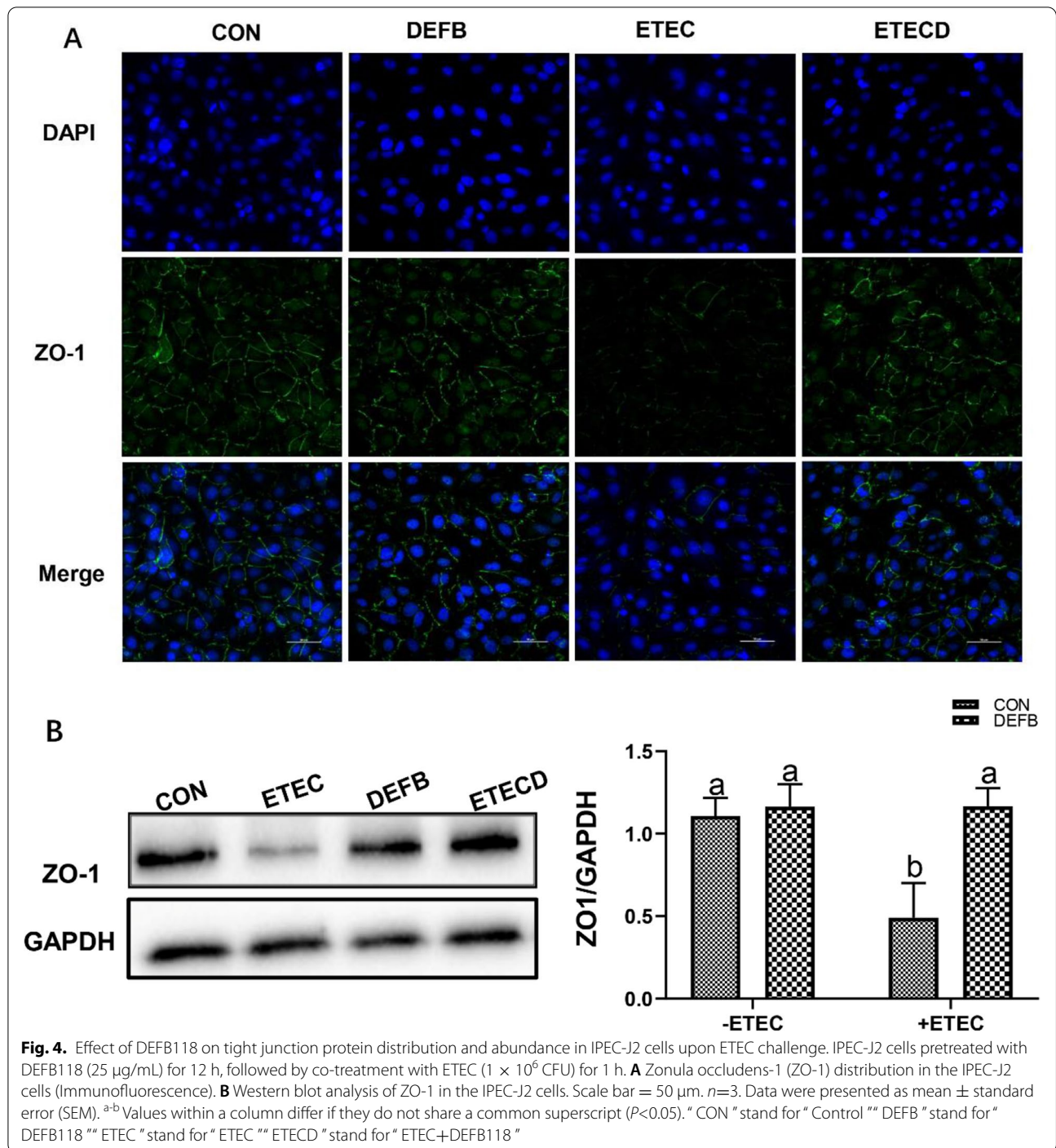
Effect of DEFB118 on tight junction protein abundance in IPEC-J2 cells upon ETEC challenge

As shown in Fig. 4A, there was less staining of the major tight junction protein ZO-1 in the ETEC-challenged cells. However, the staining of the ZO-1 was enhanced

by DEFB118 pretreatment in the ETEC-challenged cells. We also investigated the abundance of ZO-1 by using western blot assay. As shown in Fig. 4B, ETEC challenge decreased ZO-1 abundance in the IPEC-J2 cells; however, DEFB118 pretreatment significantly elevated its abundance in the ETEC-challenged cells (P<0.05).

Effect of DEFB118 on apoptosis of IPEC-J2 cells upon ETEC challenge

As shown in Fig. 5A and B, ETEC challenge increased the early and total apoptosis rate in the IPEC-J2 cells;



however, DEFB118 pretreatment significantly reduced the early and total apoptosis rate in the ETEC-challenged cells ($P<0.05$). ETEC challenge significantly elevated the expression levels of critical apoptosis-related genes such as caspase 3, caspase 8, and caspase 9 in the

cells (Fig. 5C). However, both the expressions of caspase 8 and caspase 9 were significantly downregulated by DEFB118 in the ETEC-challenged cells ($P<0.05$).

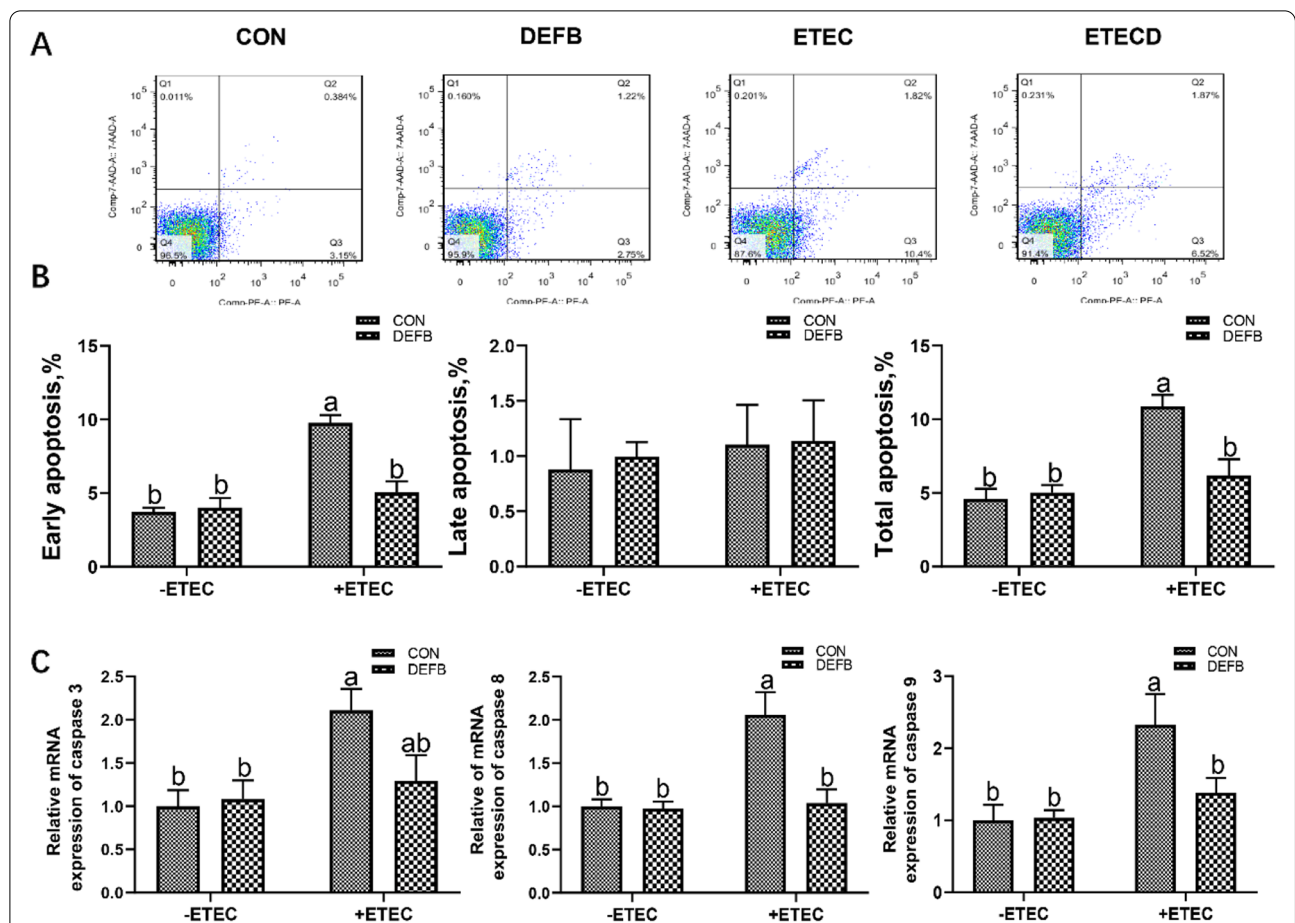


Fig. 5. Effect of DEFB118 on apoptosis of IPEC-J2 cells upon ETEC challenge.

IPEC-J2 cells pretreated with DEFB118 (25 µg/mL) for 12 h, followed by co-treatment with ETEC (1×10^6 CFU) for 1 h or 2.5 h. **A** Flow cytometry analysis of apoptotic cells (Annexin V-PE/7-AAD). **B** Quantification of apoptotic cells from flow cytometry data. **C** the expressions of Caspase 3, Caspase 8 and Caspase 9. $n=3$. Data were presented as mean \pm standard error (SEM). ^{a-b} Values within a column differ if they do not share a common superscript ($P<0.05$). "CON" stand for "Control" "DEFB" stand for "DEFB118" "ETEC" stand for "ETEC" "ETECD" stand for "ETEC+DEFB118"

DEFB118 suppressed ETEC-induced cell apoptosis and inflammatory response via suppressing the IκB-α/NF-κB signaling

NF-κB is the most critical transcription factor involved in various inflammatory signaling. So, we explore that if the DEFB118-modulated inflammatory response in IPEC-J2 cells were associated with the NF-κB signaling pathway. The results showed that both DEFB118 and BAY11-7082

significantly abolished the ETEC-induced inflammatory responses, indicated by decreases in cell apoptosis such as apoptosis rate, critical apoptosis-related genes (caspase 3, caspase 8, and caspase 9) and inflammatory cytokines such as the IL-1β, IL-6, and TNFα (Fig. 6). Moreover, both the DEFB118 and BAY11-7082 improved the abundance of ZO-1 in IPEC-J2 cells upon ETEC (Fig. 7A). Finally, we investigated the impacts of

(See figure on next page.)

Fig. 6. DEFB118 suppressed ETEC-induced cell apoptosis and inflammatory response via suppressing the IκB-α/NF-κB signaling. IPEC-J2 cells were pretreated with DEFB118 (25 µg/mL) for 12 h or with BAY11-7082 for 2 h, followed by co-treatment with ETEC (1×10^6 CFU) for 1 h or 2.5 h. **A** the expressions of IL-1β, IL-6 and TNF-α. **B** Flow cytometry analysis of apoptotic cells (Annexin V-FITC/PI). **C** Quantification of apoptotic cells from flow cytometry data. **D** the expressions of Caspase 3, Caspase 8 and Caspase 9. $n=3$. Data were presented as mean \pm standard error (SEM). ^{a-c} Values within a column differ if they do not share a common superscript ($P<0.05$). "CON" stand for "Control" "BAY" stand for "BAY11-7082" "DEFB" stand for "DEFB118" "ETEC" stand for "ETEC" "ETECB" stand for "BAY11-7082+DEFB118" "ETECD" stand for "ETEC+DEFB118"

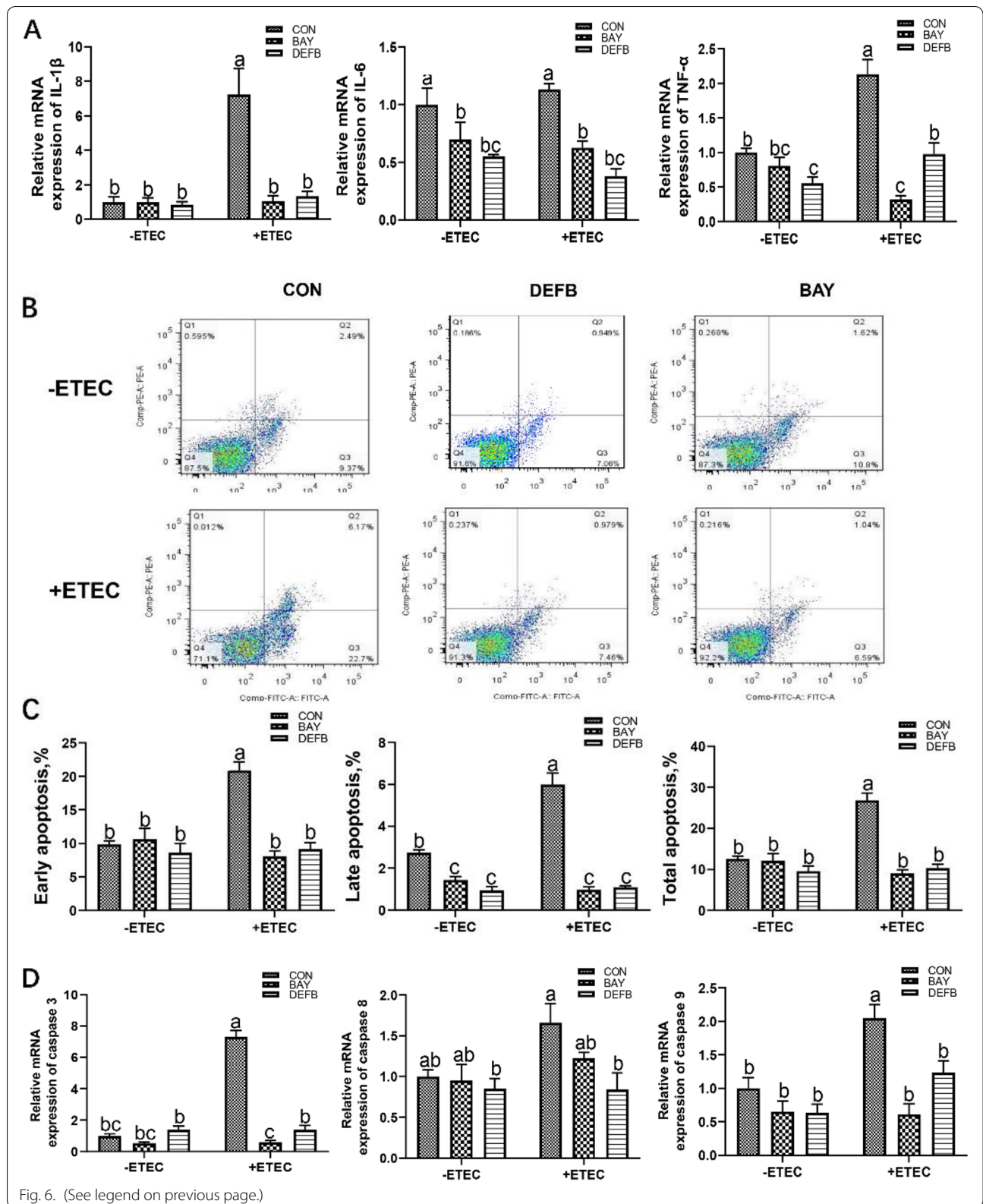


Fig. 6. (See legend on previous page.)

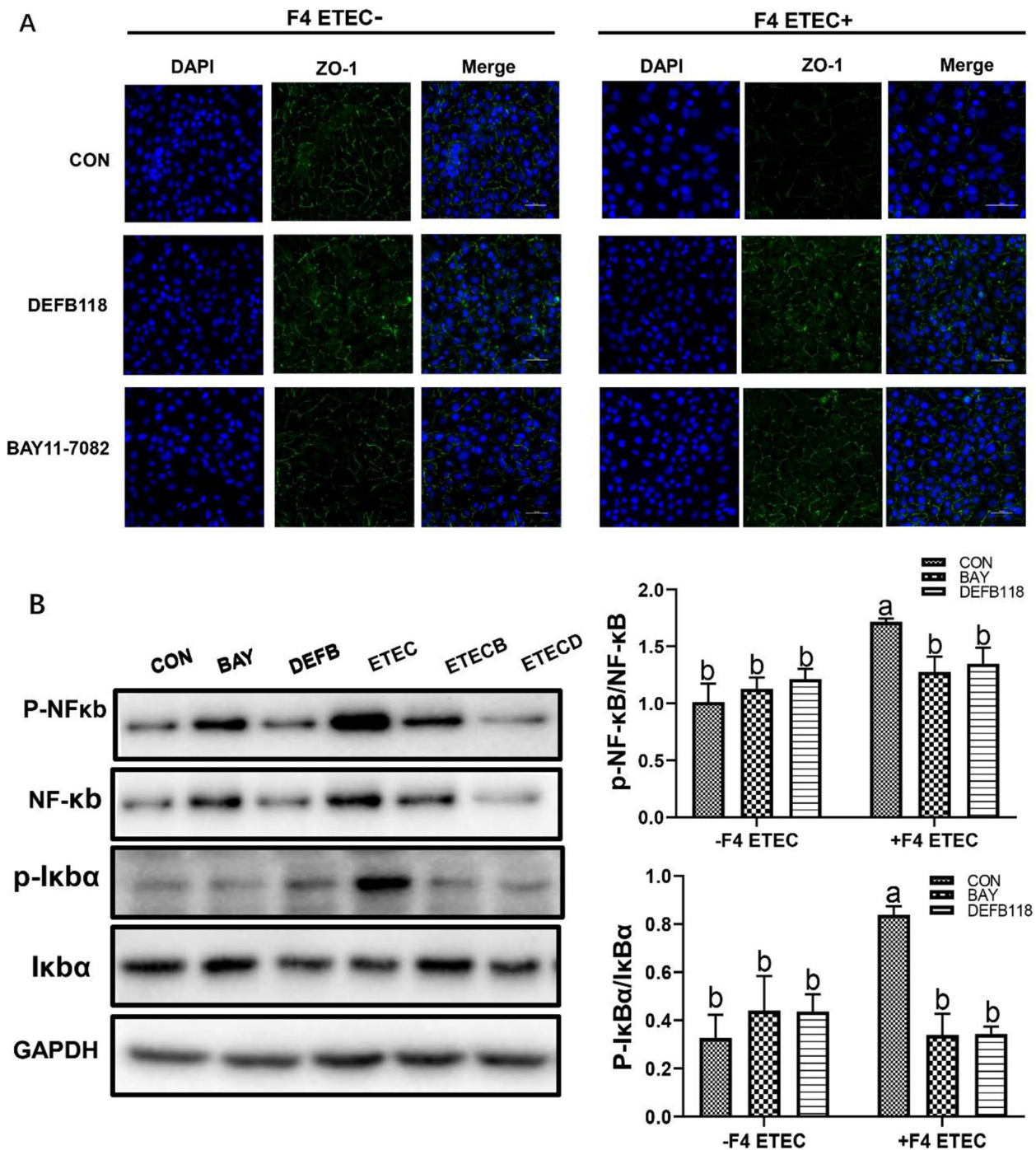


Fig. 7. DEFB118 suppressed ETEC-induced the jury of IPEC-J2 and phosphorylation of IκB-α and NF-κB. IPEC-J2 cells were pretreated with DEF118 (25 μg/mL) for 12 h or with BAY11-7082 for 2 h, followed by co-treatment with ETEC (1×10^6 CFU) for 1 h. **A** Zonula occludens-1 (ZO-1) distribution in the IPEC-J2 cells (immunofluorescence). **B** Western blot and quantitative analysis of phosphorylation of IκBα and NF-κB. Scale bar = 50 μm. $n=3$. Data were presented as mean \pm standard error (SEM). ^{a,b} Values within a column differ if they do not share a common superscript ($P<0.05$). "CON" stand for "Control" "BAY" stand for "BAY11-7082" "DEFB" stand for "DEFB118" "ETEC" stand for "ETEC" "ETECB" stand for "BAY11-7082+DEFB118" "ETECD" stand for "ETEC+DEFB118"

DEFB118 on the abundance of critical signaling proteins involved in the NF- κ B -induced inflammatory response. As shown in Fig. 7B, ETEC challenge acutely elevated the abundance of phosphorylated I κ B- α and NF- κ B; however, IPEC-J2 cells treated with DEFB118 and BAY11-7082 significantly decreased their phosphorylation.

Discussion

Enterotoxigenic *Escherichia coli* (ETEC) is one of the main pathogens that cause post-weaning diarrhea (PWD) [33, 34]. Post-weaning diarrhea is an acute and highly contagious disease in piglets and characterized by watery diarrhea, dehydration and even death [35, 36], resulting in significant economic loss to the global pig industry [37]. Among the different ETEC, those expressing the F4+ fimbrial antigen are the most prevalent form of ETEC infection [38]. These fimbriae mediate the adhesion of ETEC to the host epithelial cells, enabling colonization of the small intestine [39]. Subsequently, heat-labile (LT) and heat-stable (STa/b) enterotoxins are secreted, which induce severe diarrhea [40]. In addition, ETEC derived endotoxins (such as the lipopolysaccharide) can stimulate the release of a variety of proinflammatory cytokines and other soluble factors, leading to systemic inflammation [41]. IPEC - J2 cell line has typical epithelial cell characteristics, which is a permitted host of symbiotic bacteria and intestinal pathogens, and is an excellent model for studying the interaction between bacteria and pig intestinal epithelial cells [42, 43]. Previous studies indicated that LT enhanced adherence of ETEC to IPEC-J2 cells [44, 45], STa may play a major role in ETEC-induced cell proliferation, cell apoptosis, destroyed cell barriers in IPEC-J2 cell [46, 47]. In addition, LT could increase expression level of pro-inflammatory cytokines (IL-8 and TNF- α) by activating NF- κ B in HCT-8 cells [48], STb could induce intestinal barrier dysfunction in T84 cell [49–51], and induce apoptosis in intestinal epithelial cell lines (HRT-18 and IEC-18 cells) [52]. More studies are required to understand the effect of different virulence factors in pigs and other species. In the last decades, antibiotics have been widely used to treat the ETEC-induced diarrhea and inflammation in the swine industry. However, antibiotics, the most commonly applied control strategies, have been restricted in many countries due to the induction of antimicrobial resistance [53–55]. Therefore, novel avenues to prevent various bacteria-induced inflammation and intestinal epithelium disruption are urgently needed.

Previous studies have indicated that antimicrobial peptides (AMPs) are one of the most promising alternatives to antibiotics due to broad spectrum and a low propensity for developing resistance [56–66]. Moreover, AMPs can also act as an immunomodulator that plays a critical role in regulating the host innate immunity [67–69].

DEFB118 is a novel AMP, identified from epididymal epithelium and showed antibacterial activity against *E. coli* [31]. In the present study, we explored its protective effect on intestinal epithelial cells exposure to ETEC. We showed that DEFB118 can alleviate ETEC-induced inflammation in intestinal epithelial cells through inhibition of the NF- κ B signaling pathway, resulting in reduced secretion of inflammatory cytokines and decreased cell apoptosis.

Mucosal epithelial barrier is the first line of defense against the invasion of intestinal pathogenic microorganisms and toxins [70]. As an important part of the intestinal mucosal barrier, changes of tight junction protein can cause abnormal intestinal barrier function and affect the intestinal health [71]. Pathogenic microorganisms (such as pathogenic *Escherichia coli*, epidemic diarrhea virus, etc.) can cause the expression of tight junction protein in intestinal epithelial cells decrease and increase the permeability [49, 50, 72, 73]. It is well known that the defective intestinal TJ barrier allows paracellular permeation of luminal antigens which can initiate or propagate the inflammatory responses [74]. ZO-1 (zonula occludens-1) is an important tight junction protein [75]. In this study, we found that DEFB118 pretreatment restored the abundance of ZO-1 in IPEC-J2 cells induced by ETEC. Therefore, the protective effect of DEFB118 on the intestinal barrier may be partly explained by the increased abundance of ZO-1 protein.

Apoptosis is known as programmed cell death (PCD). It is a kind of suicidal behavior under physiological and pathological conditions, which occurs after cell death process activated by various intracellular and extracellular signals [76]. Caspase is a cysteine protease family, which plays a key role in the process of apoptosis. Caspase-3 is the most critical protease downstream of the apoptosis cascade. It plays a central role in controlling apoptosis and plays a key role in activating the specific morphological and physiological changes of apoptosis [77]. Caspase-3 mediates apoptosis through exogenous activation pathway and endogenous activation pathway [78]. Exogenous pathway is mainly mediated by the death receptor; the endogenous activation pathway is mediated by mitochondria [79]. Caspase 3 is activated by Caspase 8 in the death receptor pathway and Caspase 9 in the mitochondrial pathway [80]. Previous studies have shown that ETEC can induce apoptosis [81–83]. The present study suggest that ETEC causes an increase in the expressions of caspase-3, caspase-8, and caspase-9. At the same time, the number of IPEC-J2 cells with early apoptosis, late apoptosis, and total apoptosis was also increased. However, these changes could be counteracted by DEFB118 pretreatments, suggesting the protective role of DEFB118 against intestinal barrier damage. This may be due to

inhibition of caspase-3 gene expression by inhibiting caspase-8 gene expression in the death receptor pathway and caspase-9 gene expression in the mitochondrial pathway, thus inhibiting apoptosis induced by ETEC.

In order to further explore the anti-inflammatory mechanism of DEFB118, we used BAY11-7082 (an inhibitor of I κ B- α phosphorylation and NF- κ B) to investigate whether NF- κ B signaling pathway is involved. Nuclear factor kappa B (NF- κ B) is a multi-subunit nuclear transcription factor, which plays an important role in the regulation of many genes, including immune and inflammation, promoting or inhibiting the expression of chemotaxis and related apoptotic proteins, cell proliferation and tumorigenesis [84, 85]. When cells are in the resting state, NF- κ B is inactive due to the existence of I κ B- α . When cells are stimulated, I κ B- α phosphorylates and rapidly degrades, activating NF- κ B and transferring to nucleus. This translocation leads to the transcription and expression of inflammation-related genes [86, 87]. In agreement with previous studies [88, 89], elevated level of phosphorylated NF- κ B protein expression was documented in the IPEC-J2 cells induced by ETEC. However, the pretreatment of DEFB118 inhibited NF- κ B phosphorylation. Additionally, it also inhibited I κ B- α phosphorylation, which further downregulated NF- κ B activity. BAY11-7082 was described as an irreversible inhibitor of the NF- κ B pathway. It acts by inhibiting TNF- α -induced phosphorylation of I κ B- α , resulting in decreased NF- κ B and decreases expression of adhesion molecules [90]. And BAY11-7082 has been reported to display broad-spectrum anti-inflammatory activities and influence various physiological processes [91, 92]. In this study, we found that BAY11-7082 alleviate ETEC-induced inflammation in intestinal epithelial cells through inhibition of the NF- κ B signaling pathway, which results in suppressing of inflammatory cytokines secretion and cell apoptosis. Interestingly, the effect of BAY11-7082 pretreatment was similar to that of DEFB118 pretreatment.

Conclusion

DEFB118 can alleviate ETEC-induced inflammation in IPEC-J2 cells through inhibition of the NF- κ B signaling pathway, which results in suppressing of inflammatory cytokines secretion and cell apoptosis. The beneficial effect of DEFB118 will help for rational selection of novel anti-inflammatory agent for piglets

Abbreviations

IPEC-J2: the porcine jejunum epithelial cell line; FBS: Fetal bovine serum; DEFB118: defensin beta 118; ETEC: enterotoxigenic *Escherichia coli*; IL-1 β : interleukin-1 β ; IL-6: interleukin-6; TNF- α : tumor necrosis factor- α ; ZO-1: tight-junction protein zonula occludens-1; NF- κ B: nuclear factor-kappaB; I κ B- α : factor-kappa-B inhibitor alpha.

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Authors' contributions

QF and QL performed most of the experiments, QF wrote the main manuscript text and prepared Fig. 1, 2, 3, 4, 5, 6 and 7. DC, BY, YL, PZ, XM, ZH, JY, JL, HY and JH assisted in some studies and reviewed the manuscript. JH supervised all the studies and the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The datasets generated during and analyzed during the current study are not publicly available due to internal regulations but are available from the corresponding author on reasonable request.

Declarations

Competing of interests

The authors report no conflicts of interest in this work.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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