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Cathelicidin-BF suppresses intestinal inflammation by inhibiting the nuclear factor-κB signaling pathway and enhancing the phagocytosis of immune cells via STAT-1 in weanling piglets

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ABSTRACT

The severity of intestinal inflammation in mammals can be profoundly impacted by weaning stress. Cathelicidins protect intestinal homeostasis by not only directly killing bacteria but also immune regulators. Here, we investigated the effects of cathelicidin-BF (C-BF) derived from the snake venoms of *Bungarus fasciatus* on weaning stress and intestinal inflammation and examined the mechanisms by which C-BF modulates intestinal immune responses in weanling piglets. We found that C-BF treatment significantly increased performance and reduced the diarrheal index in weanling piglets. Serum IL-6, IL-22 and TNF-α production was decreased by C-BF treatment. We demonstrated that C-BF inhibited the expression of the inflammatory cytokines TNF-α, IL-6 and IL-8 but increased the expression of the anti-inflammatory cytokine IL-10 in the intestine. We also demonstrated that C-BF suppressed inflammation by down-regulating the nuclear factor-κB (NF-κB) signaling pathway in the intestine and in LPS-induced macrophages in vitro. However, C-BF significantly induced the phosphorylation of signal transducer and activator of transcription 1 (STAT-1) to enhance the phagocytosis of macrophages when inflammation was suppressed. In summary, our study demonstrated that C-BF suppressed intestinal inflammation by down-regulating the NF-κB signaling pathway and enhancing the phagocytosis of immune cells by activating STAT-1 during weaning.

1. Introduction

Infants and other mammalian neonates suffer during weaning, which is an indispensable stage for mammal [1,2]. Unfortunately, piglets are typically weaned at 3–4 weeks of age and then abruptly face nutritional, immunological and psychological disruptions [3–5]. Weaning stress causes intestinal mucosal inflammation and increases the expression of the inflammatory cytokines IL-6, IL-1β, TNF-α and IL-8 [6,7]. In addition, intestinal mast cells and corticotrophin-releasing factor signaling pathways are activated by weaning stress. Given the deficiency of adaptive immunity in neonates, maintaining intestinal homeostasis is a major issue for piglets during weaning, and this period is typically associated with severe diarrhea.

Antibiotics have been effectively used to prevent diarrhea or to improve performance in weanling piglets in past decades, but their widespread inappropriate use is common and serious in many countries [8]. Unfortunately, antibiotic resistance is becoming a terrible problem, leading to delayed administration of effective therapy, morbidity and mortality [9]. Moreover, antibiotic treatment during early life disrupts the balance of intestinal homeostasis by altering the expression of tight junction proteins, mucins, antimicrobial peptides and cytokines [10].

Cathelicidins are considered to be important and effective components of the innate host defense against microorganisms, and bacterial resistance is not easily developed [11–13]. Cathelicidins are able to directly kill or inactivate some antibiotic-resistant bacteria. Increasing evidence indicates that cathelicidins can protect the intestine via alternative mechanisms that are unrelated to their capacity to directly kill bacteria [13]. The cathelicidins display the capacity to recruit immune cells. For example, LL-37 attracts neutrophils, T cells, mast cells and macrophages [14,15]. In addition, the chemotactic effects of cathelicidin-related antimicrobial peptide (CRAMP) have been studied in Cramp$^{-/-}$ mice [16]. Cathelicidins display the capacity to control infections and inflammation and to promote macrophage recruitment. For instance, LL-37 was found to selectively decrease TNF-α and NO production in macrophages and to augment the secretion of the

Abbreviations: C-BF, cathelicidin-BF; STAT-1, signal transducer and activator of transcription 1; NF-κB, nuclear factor-κB; IL, interleukin; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; IFN, interferon; MCP-1, monocyte chemoattractant protein 1; LPS, lipopolysaccharide; TLR, toll-like receptors; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; VH, villus height; CD, crypt depth; H&E, hematoxylin and eosin; MFI, mean fluorescence intensity.

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chemotactic factors IL-8 and monocyte chemoattractant protein 1 (MCP-1) under certain conditions [17]. More recently, cathelicidins were found to interact with toll-like receptors (TLRs) and modulate T helper 17 cell maturation by interacting with DNA to activate TLRs [16]. This evidence indicates that cathelicidins function as potent immune regulators to maintain intestinal homeostasis.

Cathelicidin-BF (C-BF or BF-30) was derived from the snake venoms of the Chinese endemic genera *Bungarus fascia* and is considered to be a potent peptide antibiotic [18]. Cathelicidin-BF displayed effective antimicrobial activities against ciprofloxacin-resistant *Escherichia coli* and *Staphylococcus aureus* [19]. In addition, C-BF displayed higher antibacterial activity against enteric pathogens isolated from the feces of diarrheal weaned piglets than other cathelicidin peptides [12]. However, few studies have investigated the effects of C-BF on intestinal inflammation, let alone inflammation caused by weaning stress. In addition, the mechanisms by which C-BF regulates intestinal immunity remain unclear. In this study, we investigated the effects of C-BF on diarrhea and performance in weanling piglets and examined the changes in the morphology, inflammatory gene expression and signaling pathways of the porcine intestine. Importantly, we demonstrated that C-BF suppressed inflammation by reducing the phosphorylation of IκB-α and NF-κB in vivo and in vitro. Moreover, our results using a specific inhibitor of STAT-1 suggested that C-BF may enhance the phagocytosis of immune cells via a STAT-1-dependent mechanism.

2. Materials and methods

2.1. Materials

Cathelicidin-BF (KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF) was synthesized by GL Biochem (Shanghai, China) and the purity of the peptide was 98.57% as determined by analytical high performance liquid chromatography (HPLC). Lipopolysaccharide (LPS) from *E. coli* O111:B4 and FITC-dextran were purchased from Sigma (San Diego, CA, USA). Specific STAT-1 inhibitor fludarabine was purchased from Selleck Chemicals. ELISA kits for TNF-α (eBioscience, USA), IL-6 (R&D, USA), IL-8 (R&D), IL-10 (R&D), IL-22 and TGF-β (Raybiotech, USA) were used. Primary antibodies specific for β-actin (Abcam, USA), NF-κB (Santa Cruz, USA), phosphorylated NF-κB (Santa Cruz), IκB-α (Epitomics, USA), phosphorylated IκB-α (Epitomics), STAT-1 (Epitomics), and phosphorylated STAT-1 (Epitomics) were purchased.

2.2. Animals and sample collection

The animal protocols were approved by the Animal Care Committee of Zhejiang University. A total of 18 piglets (Duroc × Landrace × Yorkshire) were weaned at 21 d and were randomly assigned to two groups based on BW and origin; each group was separated into 3 pens containing 3 piglets per pen. The treatments were as follows: (1) the control group, which was intraperitoneally injected with normal saline, and (2) the C-BF group, which was intraperitoneally injected with C-BF (0.6 mg/kg body weight, calculated from the dosage of 5 mg/kg C-BF in mice). The experiment lasted 7 d. All piglets were fed the same diet without antibiotic supplementation according to the National Research Council requirements (NRC, 1998). All animals were provided with access to food and water ad libitum during the experimental period. The diarrheal index was measured according the method described by Bhandari et al. [20]. Fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea) was performed twice a day. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion efficiency (ADFI: ADG) were calculated. All piglets were sacrificed under anesthesia. Blood samples were collected from the anterior vena cava into tubes containing a coagulation accelerator. Serum was obtained after centrifugation at 3000 ×*g* for 15 min at 4 °C. Samples of the duodenum, the jejunum and the ileum were carefully collected and fixed in 10% PBS-buffered formalin for intestinal morphology. Additionally, samples of the duodenum, the jejunum, the ileum and the colon were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.3. Cytokine levels in serum

Cytokine production in serum was determined using specific porcine ELISA kits for TNF-α (eBioscience, USA), IL-6 (R&D, USA), IL-8 (R&D), IL-4 (R&D), IL-10 (R&D), IL-22 (Raybiotech, USA), IL-17 (Raybiotech) and TGF-β (Raybiotech) in this study. The samples were measured according the manufacturers' instructions.

2.4. Intestinal morphology

Intestinal morphological analysis was performed as described previously [21]. In brief, sections were dehydrated in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E). After images were acquired using a DM3000 microscope (Leica, Germany), villi and crypts were measured using Image-Pro software (MediaCybernetics, MD). A minimum of three villi from each pig was measured.

2.5. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA), and the yield and purity of the RNA extracts were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA). RNA integrity was inspected on a 1% agarose gel. The cDNA was generated via reverse transcription as described by Gao et al. [21]. Real-time PCR was performed using the StepOne Plus™ real-time PCR system (Applied Biosystems, USA). Primers for real-time PCR in this study are shown in Table 1. Each reaction included 5 μl of FastStart Universal SYBR green master mix (Roche, Switzerland), 0.5 μl of the forward primer (10 μM), 0.5 μl of the reverse primer (10 μM) and 4 μl of cDNA (diluted 10-fold). After denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 10 s and 60 °C for 35 s were performed. The melting curves were analyzed to evaluate the specificity of the PCR products. All samples were analyzed in triplicate, and the mean value was used. The 18S and GAPDH genes were used as housekeeping genes. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative mRNA expression levels.

Table 1
Primers used in this study.

Gene	Primer sequence (5′–3′)	Accession number
TNF-a	Forward: CCAATGGCAGAGTGGGTATG Reverse: TGAAGAGGACCTGGGAGTAG	NM_214022.1
IL-6	Forward: TGGCTACTGCCTTCCCTACC Reverse: CAGAGATTTTGCCGAGGATG	NM_001252429.1
IL-8	Forward: TTCGATGCCAGTGCATAAATA Reverse: CTGTACAACCTTCTGCACCCA	NM_213867.1
IL-22	Forward: GATGAGAGAGCGCTGCTACCTGG Reverse: GAAGGACGCCACCTCCTGCATGT	XM_001926156.1
IL-10	Forward: GCTGAAGACCCTCAGGCTGA Reverse: TTGCTCTTGTTTTCACAGGGC	HQ026020.1
TLR4	Forward: GCCATCGCTGCTAACATCATC CTCATACTCAAAGATACACCATCGG	NM_001113039.1
NF-κB	Forward: CTCGCACAAGGAGACATGAA Reverse: ACTCAGCCGGAAGGCATTAT	NM_001048232.1
18S	Forward: CCCACGGAATCGAGAAAGAG Reverse: TTGACGGAAGGGCACCA	AY265350.1
GAPDH	Forward: ACTCACTCTTCCACTTTTGATGCT Reverse: TGTTGCTGTAGCCAAATTCA	NM_001206359

2.6. Western blot

Western blot analysis was performed as described by Xihong Zhou et al. [22]. Sections of the intestine were ground in liquid nitrogen and then treated with lysis buffer. The protein supernatants were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane. After incubation for 1 h at room temperature in 5% skimmed milk powder, the membranes were incubated in the appropriate primary antibodies overnight at 4 °C, followed by incubation for 1 h in HRP-conjugated anti-rabbit or anti-mouse IgG. The Western blots were detected via ECL (Amersham Pharmacia Biotech, France). The signals were quantified using Scion Image software (Scion Corporation, USA). Primary antibodies specific for β-actin (Abcam, USA), NF-κB (Santa Cruz, USA), phosphorylated NF-κB (Santa Cruz), IκB-α (Epitomics, USA), phosphorylated IκB-α (Epitomics), STAT-1 (Epitomics), and phosphorylated STAT-1 (Epitomics) were used.

2.7. Macrophage culture

Porcine macrophages (3D4/2 cell line) were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10% FBS (Gibco, Australia). For each experiment, 5×10^6 cells were cultured in each well of 12-well cell culture plates. All plates were incubated at 37 °C in a humidified 5% CO_2 incubator. After 24 h of incubation, the plates were washed with D-Hanks to remove the non-adherent cells. The macrophages were treated with PBS alone, 20 μg/ml C-BF, 1 μg/ml LPS (Sigma, USA) or 20 μg/ml C-BF and 1 μg/ml LPS for 24 h. Then, the cells were washed with cold PBS and collected for RNA or protein extraction.

2.8. Flow cytometry

For testing the time curve of the FITC-dextran uptake, porcine macrophages were incubated in PBS alone or 20 μg/ml C-BF for 3 h, 6 h, 12 h, 24 h and 48 h at 37 °C. Porcine macrophages were incubated in PBS alone, 20 μg/ml C-BF, 10 μM fludarabine (Selleck, USA) or 20 μg/ml C-BF and 10 μM fludarabine for 24 h at 37 °C. After incubation, 1 mg/ml FITC-dextran (Sigma, USA) was added for 1 h. The cells were washed three times with PBS to remove excess FITC-dextran. The cellular uptake of FITC-dextran was measured and quantified via flow cytometry (BD FACScalibur, USA). The mean fluorescence intensity (MFI) of FITC-dextran was determined via FACS analysis.

2.9. Statistical analysis

The data were expressed as the means ± SEM. Statistical significance was determined via Student's t-test or one-way analysis of variance (ANOVA) using SPSS 16.0 software (SPSS Inc., USA). The differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Effects of C-BF on the performance, diarrheal index and serum cytokines of the weanling piglets

Average daily feed intake and average daily gain were significantly increased by C-BF ($P < 0.05$), but no difference in feed conversion efficiency (F:G) was observed (Fig. 1A and B). We found that C-BF significantly decreased the diarrheal index ($P < 0.05$), which is important for the health of weanling piglets (Fig. 1C). In addition, C-BF decreased the production of TNF-α, IL-6 and IL-22 but increased that of TGF-β compared with the control treatment (Fig. 1D, E, G and I). However, no difference in IL-8 or IL-10 was detected between the C-BF and control groups (Fig. 1F and H).

3.2. Effects of C-BF on small intestine morphology and intestinal inflammation in weanling piglets

Sections of the middle duodenum, the middle jejunum and the distal ileum were stained with H&E. Representative images are shown in Fig. 2. Compared with the control, C-BF significantly increased the villus height ($P < 0.01$) and the villus height: crypt depth ratio ($P < 0.01$) in the jejunum, and decreased the crypt depth ($P < 0.05$) and increased the villus height:crypt depth ratio in the ileum ($P < 0.05$) compared to the control treatment (Table 2). We investigated the relative mRNA expression levels of TNF-α, IL-6, IL-8, IL-10, TLR4 and NF-κB in different intestinal sections via real-time PCR (Fig. 3). The results demonstrated that C-BF reduced the relative mRNA expression levels of TNF-α and IL-8 in the duodenum, of TNF-α, IL-6 and IL-8 in the jejunum, and of IL-6 and IL-8 in the colon compared to the control treatment (Fig. 3A, B and C). However, the mRNA expression of IL-10 was significantly increased in the colon and the jejunum in the C-BF group compared to the control group (Fig. 3D). Additionally, the mRNA expression of NF-κB and TLR4 was only decreased in the jejunum in the C-BF group compared to the control group (Fig. 3E and F). The results for the ileum did not reveal any significant differences between the C-BF and control groups.

3.3. Effects of C-BF on NF-κB and STAT-1 signaling pathway members in the intestine

The protein expression of phosphorylated IκB-α (p-IκB-α), phosphorylated NF-κB (p-NF-κB), STAT-1 and phosphorylated STAT-1 (p-STAT-1) in the jejunum is shown in Fig. 4. The protein expression of IκB-α and NF-κB in the jejunum are not presented here due to the presence of multiple bands. The results demonstrated that C-BF suppressed the phosphorylation of NF-κB and IκB-α (Fig. 4B and C). Interestingly, C-BF dramatically activated the phosphorylation of STAT-1 (Fig. 4D), which has been associated with the phagocytosis of macrophages. Thus, we next examined whether C-BF suppressed inflammation and enhanced phagocytosis via STAT-1 in porcine macrophages in vitro.

3.4. Effects of C-BF on LPS-induced inflammation in macrophage in vitro

To determine the anti-inflammatory effects of C-BF on LPS-induced porcine macrophages, the relative expression of the inflammatory cytokines IL-6, IL-8 and IL-22 was evaluated via real-time PCR. Compared with the control group, higher mRNA relative expression levels of IL-6, IL-8 and IL-22 were detected in the LPS-treated group (Fig. 5A). However, the expression of IL-6, IL-8 and IL-22 was significantly lower in both the C-BF-treated group and C-BF- and LPS-treated group than in the LPS-treated group. No differences in the levels of inflammatory cytokines were detected between the C-BF-treated group and the C-BF- and LPS-treated group. Moreover, Western blot analysis revealed that LPS elevated the expression of p-IκB-α and p-NF-κB compared with the control treatment (Fig. 5B). The C-BF- and LPS-treated group displayed significantly decreased protein expression of p-IκB-α and p-NF-κB compared with the LPS-treated group (Fig. 5C). Interestingly, the p-STAT-1/STAT-1 ratio was significantly increased in the C-BF-, LPS- and C-BF- and LPS-treated groups compared with the control group. Moreover, the p-STAT-1/STAT-1 ratio in the C-BF and LPS-treated group was higher than that in the LPS-treated group (Fig. 5C).

3.5. Effects of C-BF on the phagocytosis of macrophages

A time curve of the FITC-dextran uptake assay was performed to evaluate the kinetic changes upon C-BF treated macrophages (Fig. 6A). The C-BF enhanced the phagocytosis of porcine macrophages at different time-points, especially at 24 h. To determine whether C-BF enhances phagocytosis by increasing the phosphorylation of STAT-1, a STAT-1 inhibitor (fludarabine) was used in our experiments.

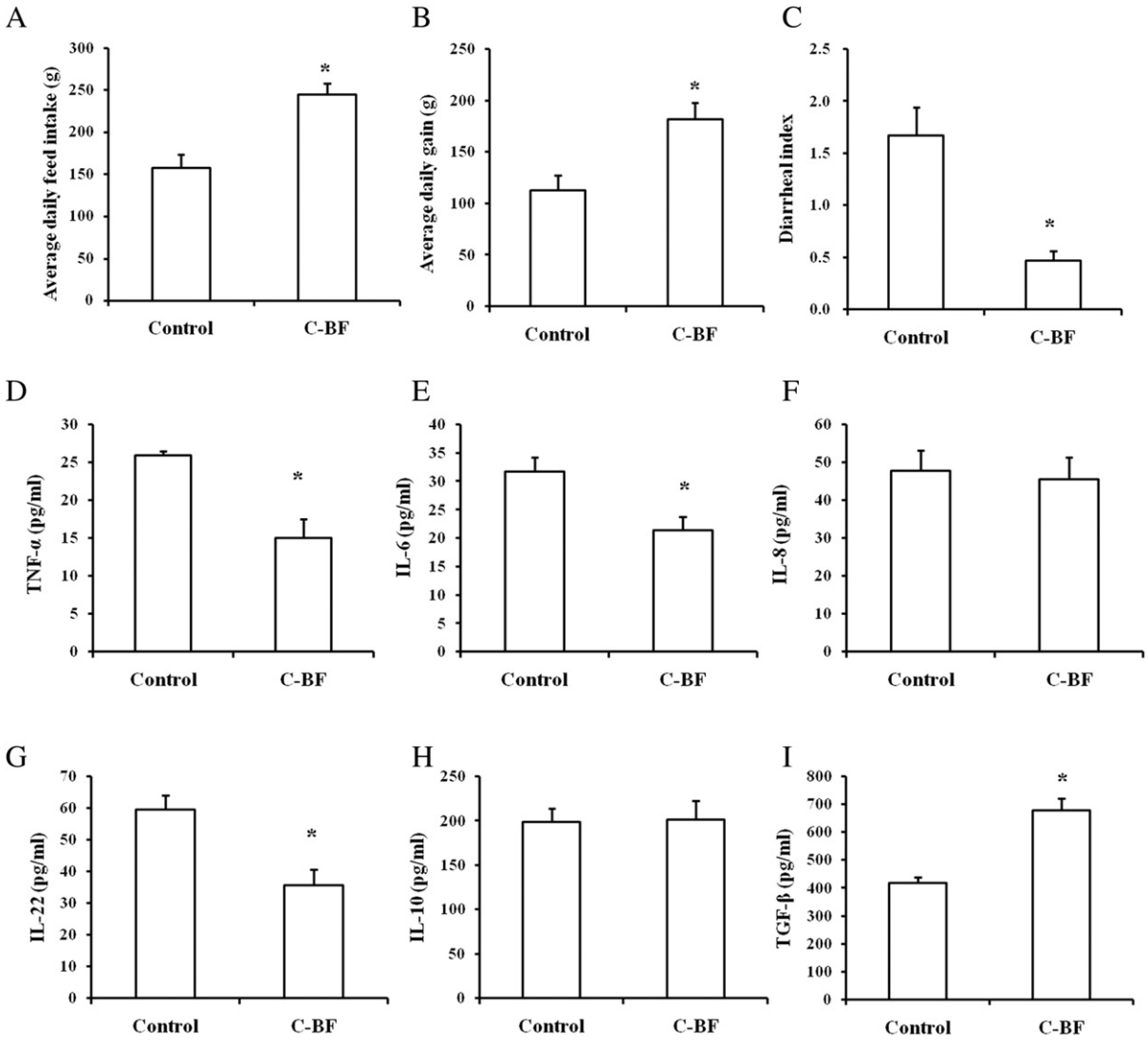

Fig. 1. Effects of C-BF on the performance, diarrhea index and serum cytokines of weanling piglets. Average daily feed intake (A) and average daily gain (B) were calculated. The diarrheal index (C) was measured according the fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea). The levels of TNF-α (D), IL-6 (E), IL-8 (F), IL-22 (G), IL-10 (H) and TGF-β (I) in serum were determined using specific porcine ELISA kits. The values for C-BF treatment are expressed as the means ± SEM (n = 9). The differences between the groups were evaluated via Student's t-test. $^{*}P < 0.05$ for C-BF compared with the control.

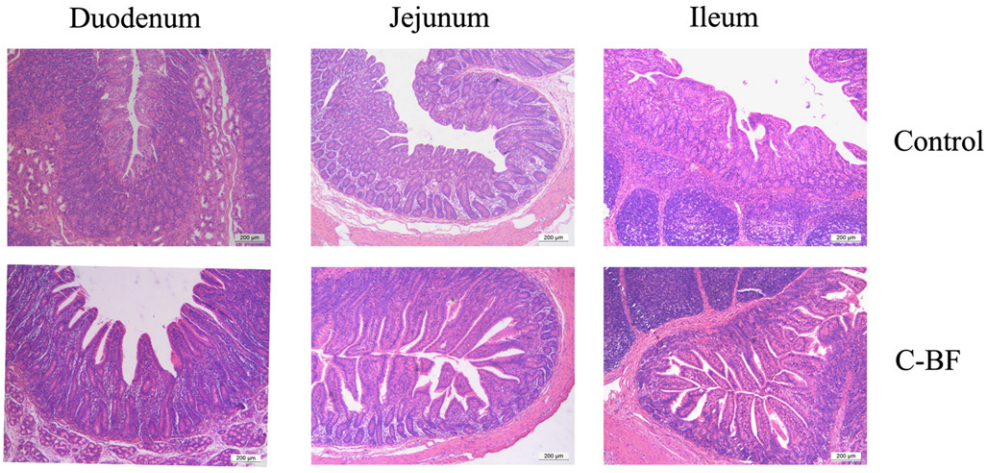

Fig. 2. C-BF improved the morphology of the small intestine. Sections of the duodenum, the jejunum and the ileum were stained with H&E. Representative images of the control and C-BF groups are shown at 40× magnification.

Table 2
Effects of C-BF on the intestinal morphology of weanling piglets[b].

Measurement[a]	Control	C-BF
Duodenum		
VH (μm)	274.92 ± 15.29	288.24 ± 27.45
CD (μm)	225.22 ± 20.60	205.34 ± 15.40
VH/CD	1.22 ± 0.10	1.41 ± 0.12
Jejunum		
VH (μm)	190.91 ± 15.46	300.93 ± 15.06 *
CD (μm)	150.8 ± 14.97	132.86 ± 6.43
VH/CD	1.33 ± 0.16	2.28 ± 0.11 *
Ileum		
VH (μm)	225.82 ± 18.47	248.12 ± 18.85
CD (μm)	208.76 ± 17.23	151.56 ± 15.58 *
VH/CD	1.14 ± 0.15	1.69 ± 0.13 *

[a] VH = villus height; CD = crypt depth.
[b] The data are presented as the means ± SEM (n = 6).
* $P < 0.05$.

Macrophages cultured in the presence of 20 μg/ml C-BF exhibited an increased uptake of FITC-dextran compared with the control cells (Fig. 6B). Accordingly, the mean fluorescence intensity (MFI) of the C-BF-treated group was higher than that of the control group (Fig. 6C). However, treatment with C-BF and fludarabine (C-BF + Flud) failed to increase the phagocytosis of FITC-dextran in macrophages. In addition, fludarabine dramatically inhibited the phosphorylation of STAT-1 (Fig. 6D). The fludarabine-treated group was not different from the C-BF + Flud-treated group. These results demonstrated that C-BF enhanced the phagocytosis capacity of macrophages via the activation of STAT-1.

4. Discussion

Weaning stress is typically associated with intestinal inflammation and is followed by diarrhea [6,23]. Although the specific receptors for cathelicidins on the surface of immune cells remain unclear, it is a fact that some cathelicidins function as immune regulators to reduce intestinal inflammation, thereby maintaining intestinal homeostasis [15,24,25]. Therefore, we hypothesized that C-BF, a novel member of the cathelicidin family, could decrease intestinal inflammation and reduce the diarrheal index in newly weaned piglets.

In this study, we observed improvements in terms of performance and the diarrheal index due to C-BF treatment. In addition, we determined that C-BF decreased the serum levels of IgG and pro-inflammatory cytokines, improved intestinal morphology and suppressed intestinal inflammation by down-regulating the NF-κB signaling pathway. Moreover, C-BF enhanced the phagocytosis of immune cells by activating STAT-1.

Although cathelicidins have been studied under many conditions, few studies have reported the effects of cathelicidins on diarrhea. Diarrhea causes reduced performance and increased mortality, and it is an indicator of animal welfare in terms of increased pathogen load and

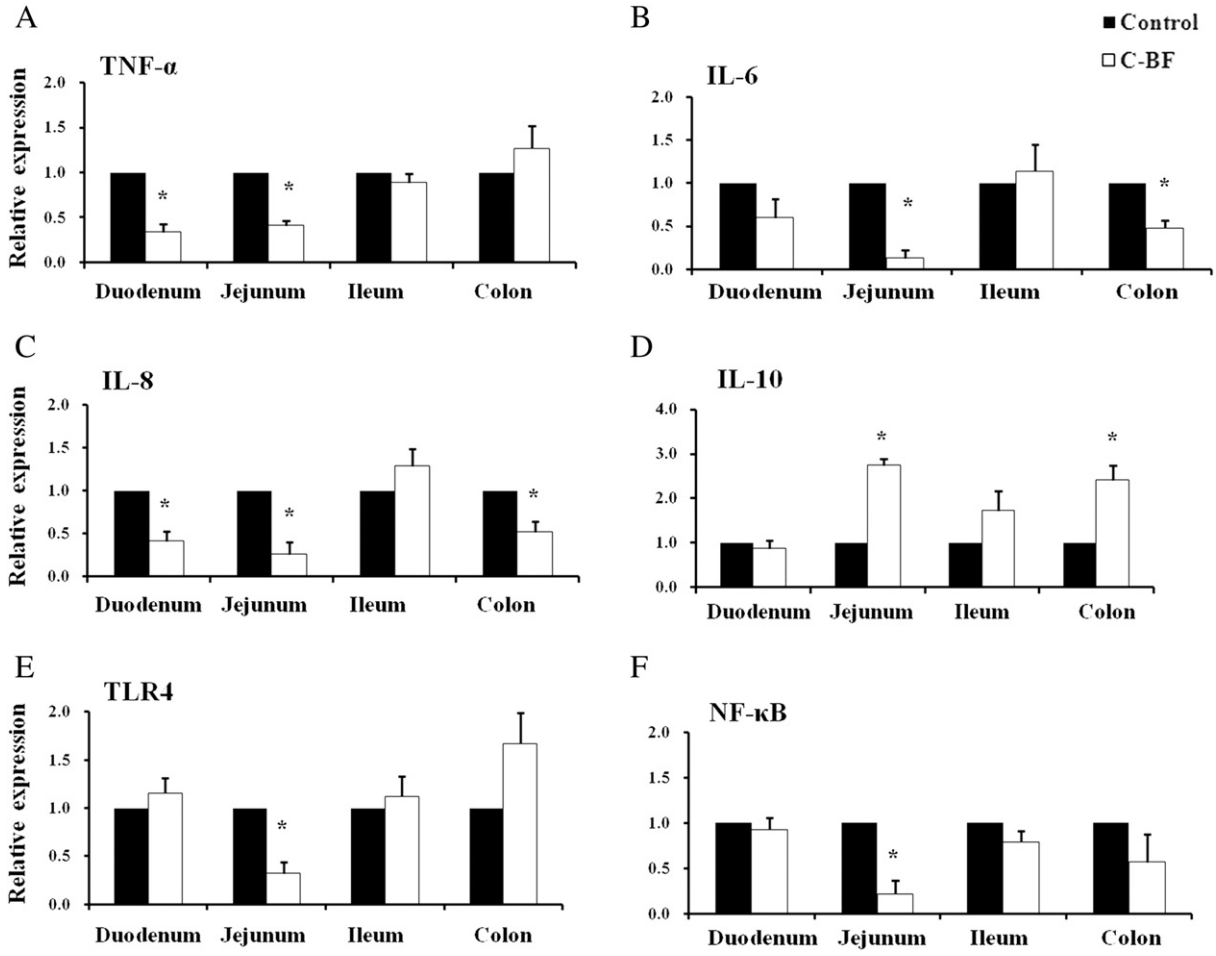

Fig. 3. Effects of C-BF on the relative mRNA expression levels of inflammatory genes in the intestine. The relative mRNA expression levels of TNF-α (A), IL-6 (B), IL-8 (C), IL-10 (D), TLR4 (E) and NF-κB (F) in the intestine were determined via real-time PCR. The 18S and GAPDH genes were used as housekeeping genes. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression levels relative to those of the control group. The values for C-BF treatment are expressed as the means ± SEM (n = 6). The differences between the groups were evaluated via Student's t-test. *$P < 0.05$ for C-BF compared with the control.

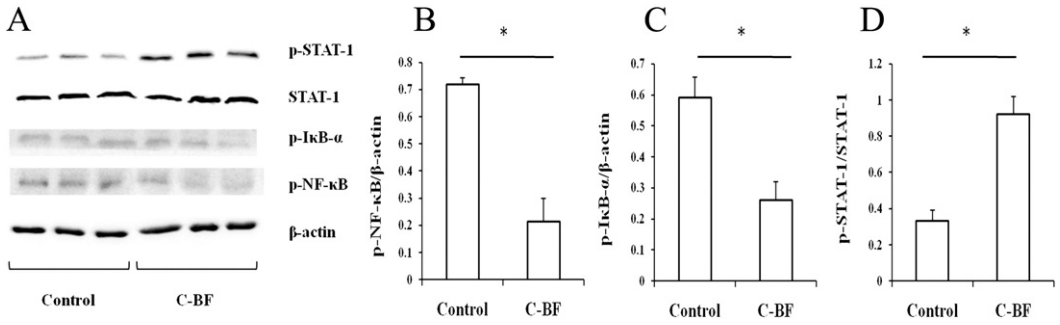

Fig. 4. Effects of C-BF on signaling pathways in the jejunum in weanling piglets. (A) Proteins involved in signaling pathways in the jejunum were analyzed via Western blot. β-actin (43 kDa) was used as a loading control. STAT-1 (91 kDa), p-STAT-1 (91 kDa), p-IκB-α (40 kDa), and p-NF-κB (65 kDa) were examined. The blots are representative of three piglets for each treatment. The relative protein expression levels are presented as the ratio of p-NF-κB/β-actin (B), p-IκB-α/β-actin (C) or p-STAT-1/STAT-1 (D) as calculated from three independent experiments. $^{*}P < 0.05$ for C-BF compared with the control.

odor and decreased hygiene in pens. Our data indicated that C-BF decreased the diarrheal index and increased ADG and ADFI in weanling piglets. These data suggested that C-BF ameliorated weaning stress. Then, we examined whole-body inflammation in weanling piglets. Cytokines in serum were indicators of whole-body immune responses. The serum levels of pro-inflammatory cytokines were increased during weaning in piglets due to bacterial invasion of the intestinal epithelium. However, we observed a decrease in the pro-inflammatory cytokines

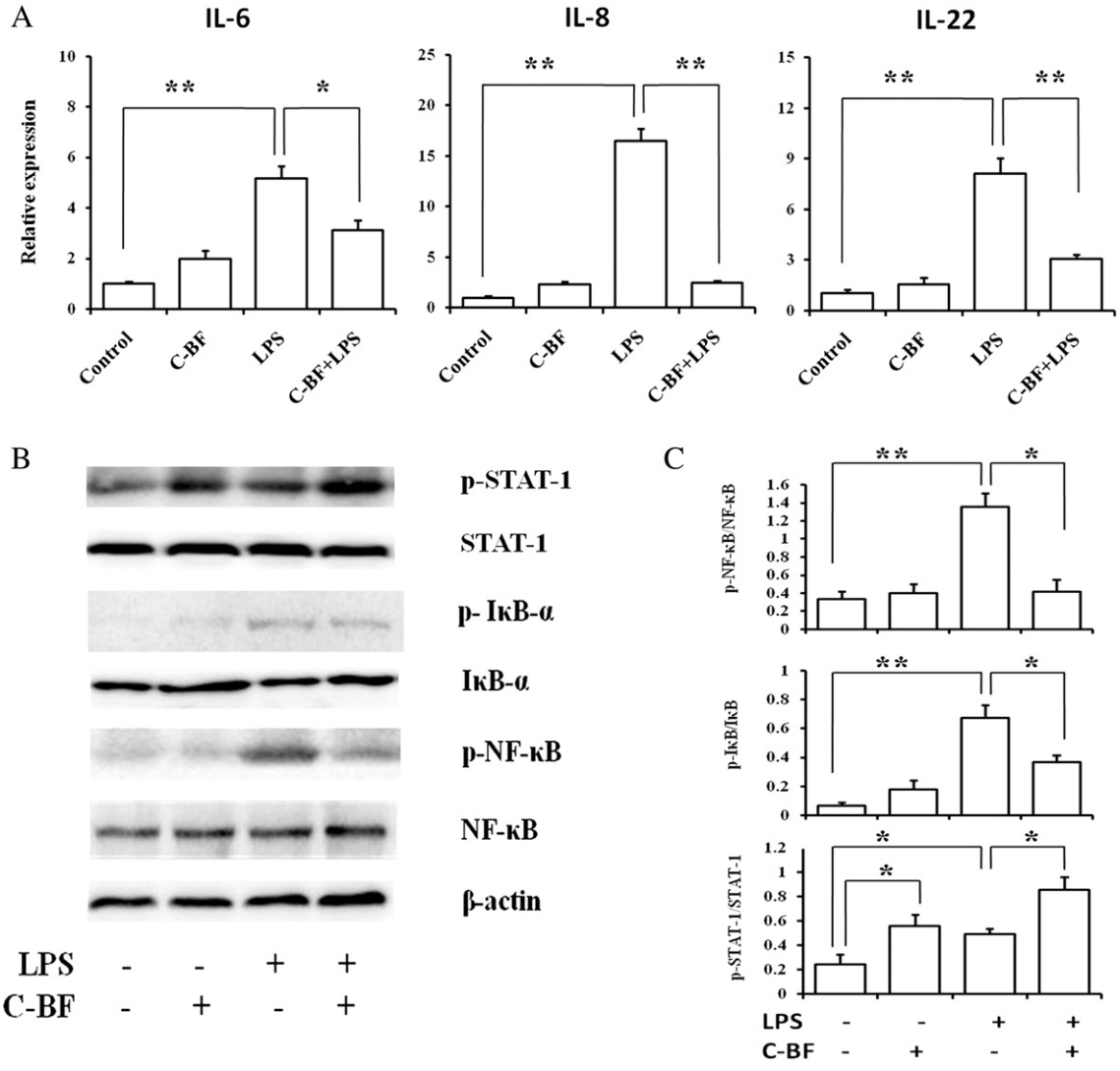

Fig. 5. C-BF suppressed inflammation in porcine macrophages. Porcine macrophages (3D4/2) were cultured with PBS alone (Control), 20 μg/ml C-BF (C-BF), 1 μg/ml LPS (LPS), or 20 μg/ml C-BF and 1 μg/ml LPS (C-BF + LPS) for 24 h. (A) The relative mRNA expression levels of IL-6, IL-8, and IL-22 were detected via real-time PCR (n = 6). (B) Representative Western blots for NF-κB, p-NF-κB, IκB-α, p-IκB-α, STAT-1, and p-STAT-1 after stimulation for 24 h. β-actin was used as a loading control. (C) The ratios of p-NF-κB/NF-κB, p-IκB-α/IκB-α, and p-STAT-1/STAT-1 were calculated from three independent experiments (n = 3). The data are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

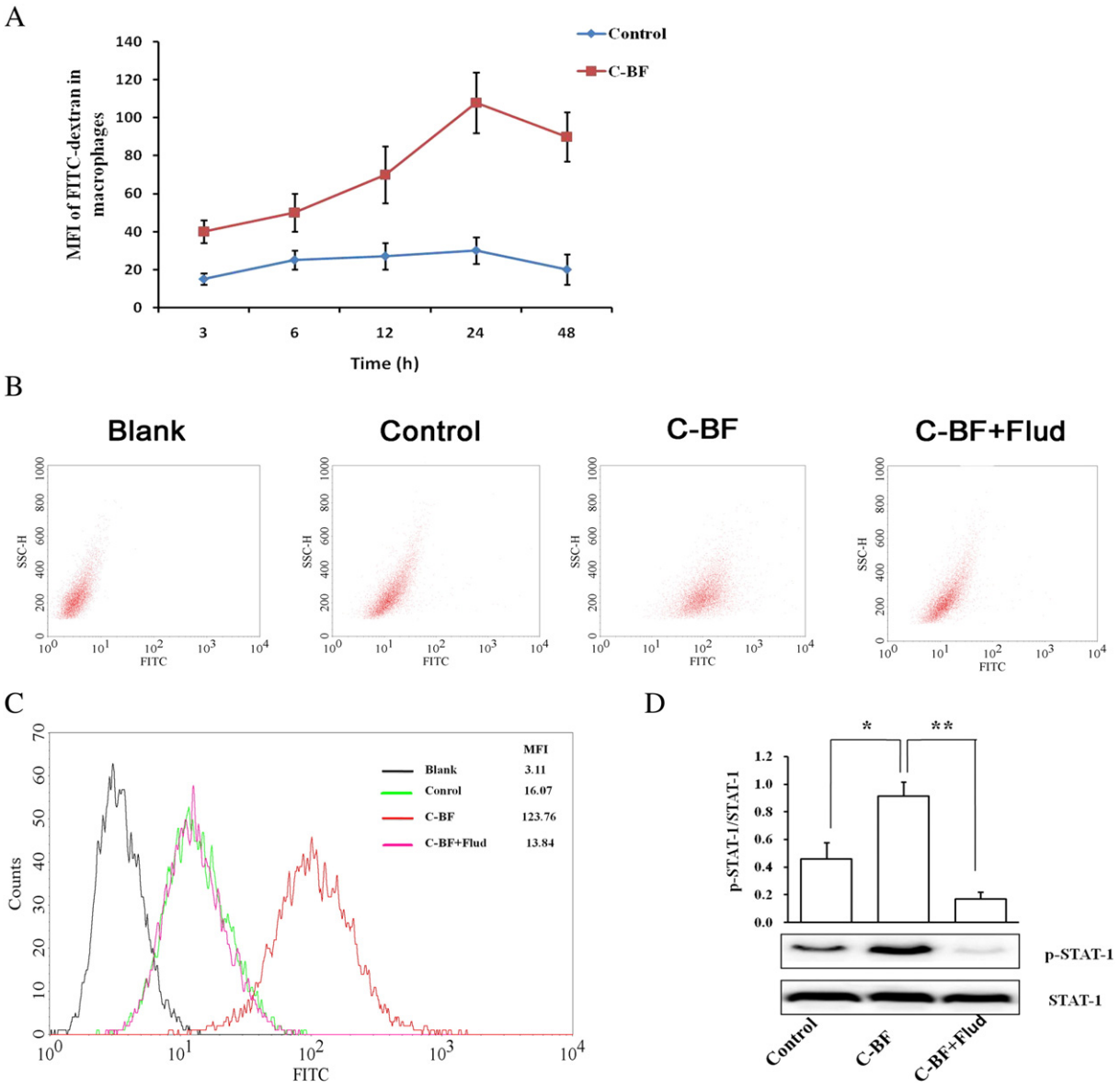

Fig. 6. C-BF enhanced phagocytosis in a STAT-1-dependent manner in porcine macrophages. (A) Time curve of the FITC-dextran uptake in C-BF treated macrophages. Macrophages were treated with PBS (Control), or 20 μg/ml C-BF (C-BF) for 3 h, 6 h, 12 h, 24 h, or 48 h before 1 mg/ml FITC-dextran incubation. Macrophages were treated with PBS alone, 20 μg/ml C-BF (C-BF) or 20 μg/ml C-BF and 10 μM fludarabine (C-BF + Flud) for 24 h before 1 mg/ml FITC-dextran incubation. (B) The cellular uptake of FITC-dextran into macrophages was measured and quantified via flow cytometry. (C) Representative histogram and MFI of intracellular FITC-dextran. (D) The protein expression of p-STAT-1 and STAT-1 in porcine macrophages after the specified treatment for 24 h. The data are representative of three independent experiments. The data are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

TNF-α, IL-6 and IL-22 and an increase in the anti-inflammatory cytokine TGF-β in the C-BF group compared to the control group. These data suggest that C-BF treatment may reduce whole-body inflammation during the weaning period.

The intestine is a complex biological system and is considered to be the largest immunological organ in mammals because it harbors 80% of all immune cells in the body [26]. Considering that intestinal immunity plays a crucial role in the inflammatory process during weaning, we examined the expression of inflammation-related and signaling pathway genes in the intestine. We did not find differences in the relative mRNA expression levels of TNF-α, IL-6, IL-8, IL-10, TLR4 or NF-κB in the ileum, which is considered as the primary immune tissue in the intestine. However, the findings of this study showed that C-BF reduced the mRNA expression of pro-inflammatory cytokines in other intestinal tissues, particularly the jejunum. The intestinal expression of pro-inflammatory cytokines is up-regulated by weaning in piglets [6,27]. In addition, we found that C-BF down-regulated the IκB-α–NF-κB signaling pathway in the jejunum, which has been reported as a classical pathway that is activated by inflammation or infection [28]. These findings were consistent with the results from our group for the effect of C-BF on an LPS-induced mouse model of sepsis, demonstrating that C-BF maintains intestinal integrity and reduces the expression of pro-inflammatory cytokines via the NF-κB signaling pathway (not published). NF-κB is a central regulator of the immune response and is a potential target for the development of anti-inflammatory agents. For example, anti-inflammatory peptide-6 (AIP6) suppressed inflammation by inhibiting NF-κB signaling in mice [29]. Accordingly, we also found that C-BF also suppressed LPS-inducing inflammation by inhibiting the IκB-α–NF-κB signaling pathway in porcine macrophages in vitro.

Interestingly, the transcription of IL-10 was increased by C-BF treatment. IL-10 is a potent immunosuppressive molecule that mediates the down-regulation of Th1 responses by inhibiting the production of pro-inflammatory cytokines. The crucial role of IL-10 in the intestine was established based on the finding that IL-10-deficient mice spontaneously developed enterocolitis [26,30–32]. In addition, IL-10 inhibits NF-κB activation in human monocytes [33]. Thus, these results suggest that

C-BF reduced intestinal inflammation by increasing IL-10 and subsequently inhibiting NF-κB activation in newly weaned piglets. Moreover, inhibiting inflammation of the intestinal mucosa exerted positive effects on intestinal integrity and epithelial function [34], and these effects might be responsible for the improvements in intestinal morphology observed following C-BF treatment in this study.

Given that IL-10 inhibits IFN-induced genes by suppressing the tyrosine phosphorylation of STAT-1 [26,33], we investigated the expression of STAT-1 and p-STAT-1 in vivo and in vitro. Interestingly, we found that C-BF increased the phosphorylation of STAT-1 in the jejunum of weanling piglets compared to the control treatment. Consistent with these results, we found that p-STAT-1 expression was increased in C-BF-treated porcine macrophages and that this increase was augmented in cells treated with C-BF and LPS. In contrast, the phosphorylation of STAT-1 has been suggested to contribute to macrophage activation and to promote inflammatory responses. For example, the phosphorylation of the serine 727 residue of STAT-1 is prolonged in HLA-B27-expressing monocyte-macrophage U937 cells after *Salmonella enteritidis* infection [35]. However, STAT-1 knockout mice were susceptible to Machupo virus, Ebola virus and Marburg virus infection [36,37]. In addition, sodium butyrate specifically activated STAT-1 gene expression and enhanced the IFN-α-induced phosphorylation and activation of the STAT-1 protein, which was useful for cancer treatment [38]. These reports indicated that the STAT-1 protein plays a dual role in the host immune defense against infection. Moreover, STAT-1-dependent and -independent signaling pathways respond to IFN-γ stimulation [39]. Microarray analysis of STAT-1-null macrophages indicated functional differences between STAT-1-dependent and -independent IFN-γ activities [40,41]. Interestingly, the cathelicidin peptide LL-37 suppressed IFN-γ activity, which was not associated with the inhibition of the canonical JAK1/2-STAT1 signaling pathway but rather was associated with the inhibition of the p38 MAPK and p65 NF-κB STAT-1-independent signaling pathways [42]. These findings partly agree with our results, which showed that C-BF reduced inflammatory cytokine production by inhibiting the p65 NF-κB STAT-1-independent signaling pathway and activating the STAT-1 signaling pathway. The specific suppressive effects of C-BF may result in the selective regulation of inflammatory responses rather than broad immunosuppression. These effects are very useful for treating mammalian hypoimmunity while maintaining a potent capacity to defend against bacterial infection.

The phagocytosis of immune cells is a key component of innate immunity and the host defense against infection. Given the deficiency of adaptive immunity in neonates, the phagocytosis of macrophages may play a very important role in clearing invasive bacteria in the intestinal mucosa during weaning. Given the role of STAT-1 in phagocytosis, we investigated whether the activation of STAT-1 by C-BF treatment affected the phagocytosis of macrophages. The results showed that C-BF enhanced the phagocytosis of porcine macrophages in vitro. These data were partly consistent with a previous report that cathelicidin peptide LL-37 promotes bacterial phagocytosis by human macrophages [43]. However, C-BF exerted no effect on the phagocytosis of porcine macrophages in the presence of a STAT-1 inhibitor (10 μM fludarabine). These results were consistent with those of Naruse et al., who demonstrated that STAT-1 activated bacterial phagocytosis in IL-10-deficient macrophages [44]. Therefore, these data suggested that C-BF enhances the phagocytosis of macrophages via the activation of STAT-1.

In conclusion, we have demonstrated that C-BF suppresses intestinal inflammation by down-regulating the NF-κB signaling pathway and by enhancing the phagocytosis of macrophages via the activation of STAT-1 during weaning. Our results indicated that C-BF or other cathelicidins selectively suppress inflammation and enhance specific functions of immune cells to synthetically maintain intestinal homeostasis. Moreover, our data imply that C-BF treatment may represent an effective therapeutic strategy not only for diarrhea induced by weaning stress but also for other intestinal diseases, such as inflammatory bowel disease and colon cancer.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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