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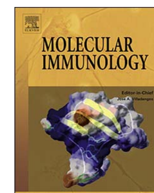

Cathelicidin modulates synthesis of Toll-like Receptors (TLRs) 4 and 9 in colonic epithelium

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ABSTRACT

Cathelicidin are innate antimicrobial peptides with broad immunomodulatory functions; however, their role in regulating intestinal defenses is not well characterized. This study aimed to investigate the role of cathelicidin modulating expression of Toll-like receptors (TLRs) 4 and 9 in colonic epithelium in response to bacterial patterns. We demonstrated herein that intestinal epithelial cells, when primed by bacterial lipopolysaccharide (LPS), responded to cathelicidin by increased transcription and protein synthesis of TLR4. This cathelicidin-induced response required the interaction of LPS-TLR4 and activation of MAPK signalling pathways. However, cathelicidin blocked TLR9 responses induced by TLR9 ligand CpG oligodeoxynucleotide (CpG ODN) in these colonic epithelial cells. Modulations of TLRs triggered by cathelicidin in intestinal epithelium occurred mainly in the apical compartment of intestinal cells. Activation of TLR4 by ligands in combination with cathelicidin promoted CXCL8 chemokine secretion and epithelial antimicrobial defenses against *Escherichia coli*. We concluded that cathelicidin selectively modulated synthesis of TLR4 and 9 in intestinal epithelium, but only when cells were exposed to virulence factors, mostly from apical surfaces. Enhanced TLR4 expression promoted by cathelicidin in intestinal epithelium may be crucial for controlling enteric infectious diseases.

1. Introduction

The intestinal epithelium regulates innate defenses in the gut by actively responding to signals from commensal and pathogenic organisms in both luminal and lamina propria (basal) compartments (Lee et al., 2006). These protective epithelial mechanisms include responses to enteric pathogenic bacteria, including gram-negative *Escherichia coli* and *Salmonella* spp., which cause infectious enterocolitis, often accompanied by diarrhea, septicemia and death. In response to pathogenic microbes, evolutionarily conserved Toll-like receptors (TLRs) expressed by intestinal epithelial cells are key to recognize pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009). Among bacterial virulence factors recognized by TLRs, lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, and non-methylated microbial CpG oligodeoxynucleotide (CpG ODN) are ligands for TLR4 and TLR9, respectively (Kawai and Akira, 2011). Upon ligand binding, these TLRs initiate downstream signalling in intestinal epithelium that promotes secretion of chemokines (a family of cytokines), including CXCL8 (Abreu, 2010). Chemokine CXCL8 is crucial to maintain intestinal homeostasis, as it promotes several biological functions, including recruitment of neutrophils and survival of intestinal cells (Le et al., 2004). Interestingly, colonic epithelial cells express only low levels of constitutive TLR4 and are poorly responsive to bacterial LPS during physiological states (Abreu et al., 2001; Suzuki et al., 2003). Mechanisms activated in the intestinal epithelium during infection, given the complexity and uniqueness of TLRs, remains of interest for understanding the pathophysiology of enteric infectious diseases.

Innate defenses in the colonic epithelium include cathelicidin, a cationic short peptide with antimicrobial activity against enteric pathogens (Kosciuczuk et al., 2012). In humans, LL-37/hCAP-18 is the only cathelicidin (Zanetti, 2005). Cathelicidin binds (electrostatic interactions) to negatively charged molecules, including DNA (Lande et al., 2007), glycosaminoglycans (Baranska-Rybak et al., 2006), mucin (Felgentreff et al., 2006), and the anionic lipid A portion of LPS (Nagaoka et al., 2001). Although cathelicidin has been associated with host innate responses, including chemotaxis, epithelial wound repair and activation of chemokine secretion (Bowdish et al., 2006), cross talk

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between cathelicidin and TLRs in the intestine has not been well characterized. In this study, naturally occurring and exogenous cathelicidin induce synthesis of TLR4, but abrogated TLR9, in the intestinal epithelium in response to pathogenic bacterial stimuli. Cathelicidin regulated secretion of CXCL8 in response to microbial ligands in the intestinal epithelium and enhanced antimicrobial defenses. This work advanced understanding of interactions between cathelicidin and TLRs in intestinal homeostasis and pathogenesis of infectious colitis.

2. Materials and methods

2.1. Cell cultures

Human adenocarcinoma colonic epithelial cell lines HT29 and T-84 (gifts from Dr. K. Chadee, University of Calgary), were maintained in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Burlington, ON, Canada) with 10% fetal bovine serum (Benchmark Gemini Bio-Products, Sacramento, CA, USA), 1 mM sodium pyruvate (Gibco, Life Technologies) and penicillin (100 U ml^{-1})/streptomycin (100 μg ml^{-1}; HyClone Thermo, Fisher Scientific, Whitby, ON, Canada) in a humidified environment of 95% air and 5% CO_2 at 37 °C.

2.2. Reagents

Sodium butyrate was purchased from Sigma-Aldrich (B5887-1G, St Louis, MO, USA). Synthetic cathelicidin LL-37 amide trifluoroacetate salt was purchased from Bachem (H-6224.0005, Torrance, CA, USA) and LL-37 scrambled peptides from Ana Spec (AS-63708, Fremont, CA, USA). LPS purified from *E. coli* was obtained from Sigma-Aldrich (L4516-1MG). Antagonist of TLR4, LPS-RS Ultrapure, and TLR9 ligand CpG ODN2395 were purchased from Invivogen (tlrl-prslps and tlrl-2395, San Diego, CA, USA). MAPK kinase inhibitors U0126 was purchased from Tocris Bioscience (109511-58-2, Ellisville, MO, USA) and PD98059 from Cell Signaling Technology (9900, Whitby, ON, Canada). Reagents were used according to manufacturer's recommendations.

2.3. Experimental design

Colonic epithelial cells were seeded in 24-well plates (Greiner Bio-One, Monroe, NC, USA) for gene expression assays and ELISA, in 6-well plates (Greiner Bio-One) for protein expression assays and, in 8-well chambers (Greiner Bio-One) for confocal immunofluorescence microscopy and cultured until they were 80–90% confluent. Cells were stimulated with butyrate or synthetic LL-37 (or scrambled LL-37) in association with TLR4 and TLR9 ligands, or pre-treated for 1 h with TLR4 antagonist LPS-RS or MAPK kinase inhibitors U0126 and PD98059. For studies of apical and basolateral surface TLR expression, T84 cells were grown on microporous filter inserts (0.4 μm pore side; ThinCert-24 well, Greiner Bio-One) (Lee et al., 2006). Doses were determined in preliminary studies using varying amounts of LPS (0.5–2 μg/mL) and CpG (1–20 μg/mL). Doses of 1 and 2 μg/mL LPS had the highest (albeit not significant) increase in *TLR4* mRNA expression (Fig. **S1A**), whereas *TLR9* mRNA expression was induced by CpG induced at doses from 1 to 20 μg/mL, with maximal induction at 10 μg/mL (Fig. S**1B**). The LL-37 concentrations used herein were within the range of *in vivo* concentrations in mucosal secretions (1–1000 μg/mL) (Schaller-Bals et al., 2002). Lack of cytotoxicity effects of LPS and ODN was confirmed by microscopical observation of the morphology and confluence of cell monolayers, complemented with dye exclusion assays (using Trypan blue dye). Moreover, LPS and ODN were used at concentrations reported as non cytotoxic by manufacturers.

2.4. qRT-PCR

Expression of *TLR4* and *TLR9* mRNA from colonic epithelial cells was quantified by real time reverse transcription polymerase chain reaction real time (qRT-PCR). Total RNA from non-stimulated and stimulated cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was prepared from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (iScript Reverse Transcription Supermix for qPCR; BioRad, Hercules, CA, USA). RNA quality was assessed by agarose gel electrophoresis. These analyses showed no significant RNA degradation and similar RNA yield across samples. Quality of resulting RNA and cDNA was assessed by the absorbance ratio (A_{260}/A_{280} ratio) (NanoVue Spectrophotometer, GE Healthcare Bio-Sciences) (Bustin et al., 2009), which was corroborated to be ~ 1.8–2.0 for each sample. The RNA preparations were screened for contaminating genomic DNA using a minus-reverse transcriptase control (i.e., a sample with all qRT-PCR reagents except reverse transcriptase). qRT-PCR was performed using a CFX-96 real time PCR system (BioRad, Hercules, CA, USA). Each reaction mixture contained 100 ηg of cDNA, 1X SsoAdvanced Universal SYBR Green Supermix (BioRad) and 0.5 μM of each specific primer, in a final volume of 10 μL. The primer used targeted human *TLR4* (PPH01795F), *TLR9* (PPH01809A), *cathelicidin antimicrobial peptide* (*CAMP*; PPH09430A) and, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; PPH00150F) (Table 1). These primers (330001, RT^2 qPCR Primer Assay, Qiagen) were verified for specificity and efficiency with amplification of a single product of the correct size with high PCR efficiency (> 90%). Reaction mixtures were incubated for 95 °C for 5 min, followed by denaturation for 5 s at 95 °C and combined annealing/extension for 10 s at 60 °C (total of 40 cycles). In all cases, treatments were tested in duplicate in at least three independent experiments. Negative controls for cDNA synthesis and PCR procedures were consistently included. Gene *GAPDH*, along with two other housekeeping genes, *hypoxanthine-guanine phosphoribosyltransferase 1* (*HGPRT1*) and *phosphoglycerate kinase 1* (*PGK1*), were optimized, and found to be invariable across different treatment groups (data not shown). In agreement, *GAPDH* has been reported to be the most desirable house-keeping gene for HT-29 cells (Jacobsen et al., 2014). Therefore, values of target mRNA were corrected relative to the normaliser *GAPDH*. Data were analysed using the $2^{-\Delta\Delta CT}$ methods (Bustin et al., 2009) and results reported as mean fold change of target transcription levels in stimulated groups versus an untreated control group (Bustin et al., 2009).

2.5. Protein detection by western blotting and ELISA

Non-stimulated and stimulated cells were lysed in RIPA buffer, and concentrations of proteins were determined with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). For this, 20 μg protein of each sample was mixed with 2X Laemmli sample buffer, denatured to 70 °C for 10 min, and separated on 4–15% MiniProtean TGX-stain free gels (BioRad). Subsequently, proteins were activated by UV-exposure for 5 min and then transferred onto a PVDF membrane using the *trans*-Blot Turbo Transfer System (BioRad). The membrane was blocked with 3% bovine serum albumin dissolved in Tris buffered saline plus 0.1% Tween 20 solution (TBST) for 1 h and probed with anti-TLR4 or anti-

Table 1
Details of primers for mRNA relative quantification by qRT-PCR.

Symbol	Catalog no:	RefSeq Accession no:[a]	Description
GAPDH	PPH00150F	NM_001256799	Glyceraldehyde-3-phosphate dehydrogenase
TLR4	PPH01795F	NM_003266	Toll-like receptor 4
TLR9	PPH01809A	NM_017442	Toll-like receptor 9
CAMP	PPH09430A	NM_004345	Cathelicidin antimicrobial peptide

[a] The RefSeq accession no. refers to the sequence used to design the RT^2 qPCR Primer Assay (Qiagen).

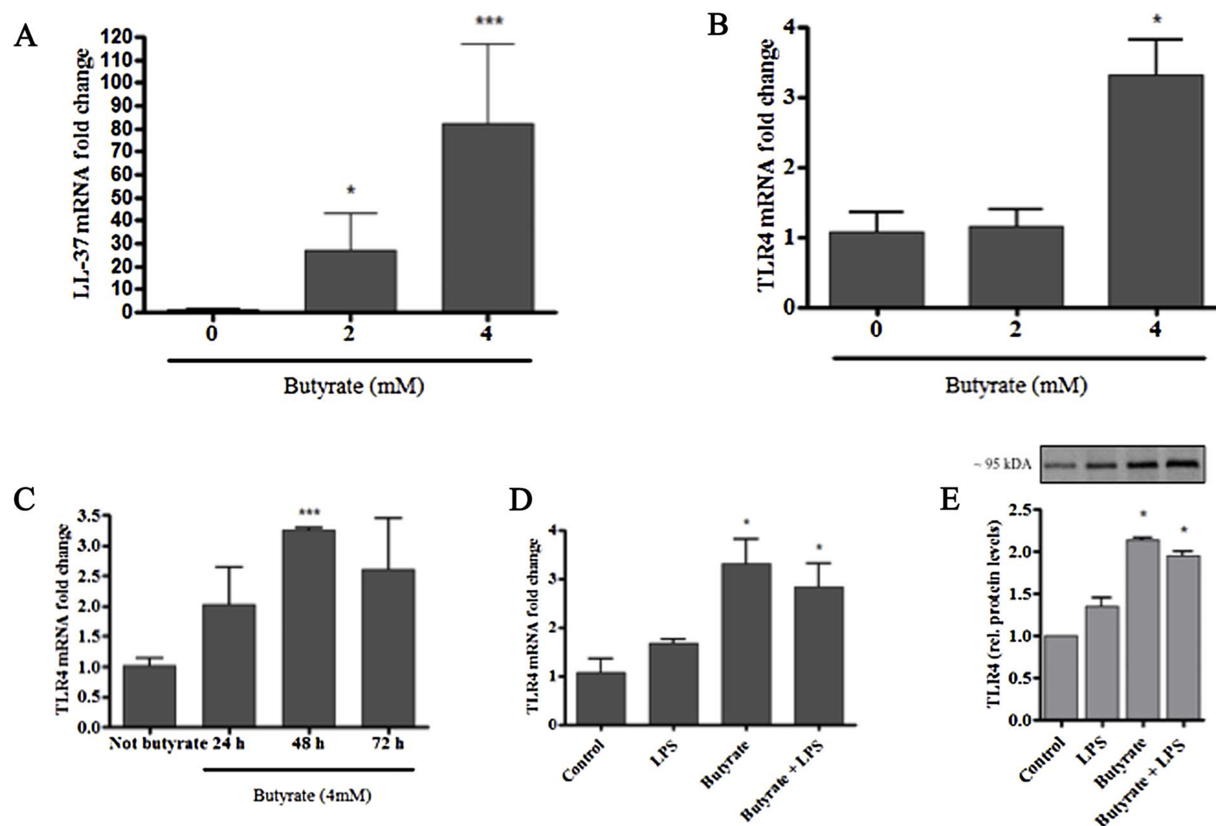

Fig. 1. Effects of butyrate on endogenous cathelicidin and TLR4 expression in human colonic epithelial cells. Colonic epithelial HT29 cells were treated with various concentrations of butyrate (2 and 4 mM) for 48 h (A and B), with 4 mM butyrate for the indicated intervals (24, 48 and 72 h; C) or were pre-treated with butyrate (4 mM) for 48 h, followed by TLR4 agonist LPS (1 μg/mL for 24 h; D and E). The mRNA relative expressions of LL-37 and TLR4 were assessed with qRT-PCR and concentrations of TLR4 protein were measured by western blotting. Representative blots from three independent experiments are shown above each bar. Results represented mean fold change of LL-37 or TLR4 concentrations in treated versus untreated cells (the latter served as the control).
$^*p < 0.05$ compared to untreated control.

TLR9 antibodies (ab22048 and ab134368 respectively from Abcam Inc., Cambridge, MA, USA; 1:400) at 4 °C overnight. After incubation with a horseradish-peroxidase-conjugate secondary anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:10000) during 1 h at room temperature (RT), blots were developed using the Clarity Western ECL Detection System (BioRad). Image captures and densitometric analyses were performed with the ChemiDoc MP Imaging system and ImageLab 4.0.1 software (BioRad), respectively. Normalization was done with reference to total lane protein. Results were reported as mean fold change of target expression in stimulated groups compared to an unstimulated control group.

For CXCL8 ELISA, supernatants were collected from non-stimulated and stimulated cells and levels of CXCL8 were determined using DuoSet ELISA kit (DY208, R & D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

2.6. Confocal immunofluorescence microscopy

The HT29 cells were grown in 8-well chambers and stimulated as discussed above; thereafter, they were rinsed with phosphate buffered saline (PBS) and fixed in cold acetone for 10 min. After rinsing in cold PBS plus Tween 0.05% (PBS-Tw; pH 7.2), cells were blocked with PBS-Tw, 1% bovine serum albumin, 10% donkey serum and 0.3 M glycine for 1 h at RT and rinsed with PBS-Tw. Cells were blotted with anti-TLR4 antibody (ab22048, Abcam Inc., Cambridge, MA, USA; 1:100) or anti-TLR9 antibody (HM2087, Hycult Biotech, Plymouth Meeting, PA, USA, 1:100) at 4 °C overnight and after rinsed with cold PBS-Tw. As secondary antibodies, human cells were blotted with Alexa Fluor 594-conjugated AffiniPure Donkey Anti-Mouse IgG (H + L; Jackson ImmunoResearch, West Grove, PA, USA). Secondary antibodies were diluted 1:1000 in PBS-Tw plus 1% bovine serum albumin and incubated for 1 h at RT. Preparations were rinsed in cold PBS-Tw and nuclei counterstained with 4′, 6-diamidino-2- phenylindole. Sections were rinsed with cold PBS-Tw and mounted with FluorSave reagent (Calbiochem, EMB Millipore, Etobicoke, ON, Canada). Slides were examined using a FluoView FV1000 confocal immunofluorescence microscope (Olympus, Toronto, ON, Canada). Quantitative statistical comparison of staining fluorescence intensity between groups was performed using Image Processing and Analysis in Java (Image J; NIH, Bethesda, MD, USA). Image files were processed as TIFF file formats and cell areas were selected to determine fluorescence index. Areas of image with similar numbers of cells and uniform background were randomly selected. Data were recorded as fold change in fluorescence intensity per unit area in a histogram (it represents three independent experiments). This work was done in the Live Cell Imaging Facility, Snyder Institute, University of Calgary.

2.7. Antimicrobial assay

The *E. coli* strain HB101 was grown on LB Miller broth (IBI Scientific, Peosta, IA, USA) in aerobiosis for 2 h at 37 °C with vigorous shaking (300 rpm). Then, HT29 cells seeded in 24-well plates and confluent (80–90%) were treated with LPS (1 μg/mL) or CpG (10 μg/mL), with or without synthetic LL-37 (20 μg/mL) for 2 h. These cells were challenged with *E. coli* at a multiplicity of infection (MOI) of 10:1 for 3 h at 37 °C with 5% CO_2, washed 3 times with PBS and lysed with 0.2% Triton X-100. Serial dilutions of the cell lysates were plated on LB agar (Difco, Sparks, MD, USA) and bacterial colonies were counted after

18 h of incubation at 37 °C. The number of *E. coli* was similarly quantified in culture supernatants. Total number of bacteria was calculated by a standard curve with known numbers of bacteria and verified by counting colonies in dilutions plated directly on LB agar after incubation for 18 h at 37 °C. Results were presented as percentage of internalized *E. coli* (in lysates) in treated groups compared to an untreated control group.

2.8. Statistical analyses

Normality of data was assessed using D'Agostino & Pearson omnibus' normality test. Results that were normally distributed were reported as means and standard errors of the means from at least three independent experiments. For comparison of differences between treated and untreated groups, a non-paired, two-tailed Student's *t*-test was used. All statistical analyses were performed with GraphPad 4.0.3 (Prism software, San Diego, CA, USA) and $p < 0.05$ was considered significant.

3. Results

3.1. Butyrate induced endogenous cathelicidin and TLR4 gene expression in colonic epithelium

Increasing doses of butyrate induced gene expression of *LL-37*, with maximal response to 4 mM for 48 h (Fig. 1A). To determine effects of butyrate in expression of *TLR4* and whether this response was modified after exposure to bacterial incitants, *TLR4* mRNA expression was quantified in colonic epithelial cells concurrently stimulated with butyrate and LPS (the latter is a TLR4 ligand virulence factor from Gram-negative pathogens). Butyrate alone significantly increased gene expression of *TLR4* in colonic epithelial cells, with a maximal increase in *TLR4* mRNA following treatment with 4 mM butyrate for 48 h (Figs. 1B and C). This effect of butyrate (with or without LPS) on *TLR4* expression exceeded the effect provoked by TLR4 ligand LPS only, with increased *TLR4* gene expression (Fig. 1D) and TLR4 protein synthesis (Fig. 1E).

3.2. Cathelicidin LL-37 promoted synthesis of TLR4 in LPS-primed colonic epithelium

Since butyrate promoted both endogenous cathelicidin and TLR4 in colonic epithelium (Hase et al., 2002), we investigated whether the effect on TLR4 could be attributed to the cathelicidin peptide. Colonic epithelial cells primed by LPS and exposed to synthetic LL-37 had increased *TLR4* gene expression (Fig. 2A). This upregulation of TLR4 in response to cathelicidin and LPS was translated into increased protein synthesis of the cell surface receptor (Fig. 2B). The effect of LL-37 promoting TLR4 depended specifically on the primary structure of the peptide, as there was no such effect with a scrambled LL-37 combined with LPS (Fig. 2C). This TLR4 response in response to a combination of LPS and LL-37 exceeded the response to LPS only (Fig. 2A and B). However, treatment with LL-37 peptides only did not affect *TLR4* (Fig. 2A). Direct involvement of TLR4 in this cathelicidin role was verified by inhibiting LPS-TLR4 interaction with a competitive TLR4 antagonist (LPS-RS), which blocked increases of *TLR4* mRNA in response to LPS and LL-37 (Fig. 2D). Upregulation of TLR4 synthesis after exposure to cathelicidin and LPS was corroborated by immunofluorescence studies that demonstrated increased immune staining of TLR4 in colonic epithelial cells (Fig. 3A and C).

3.3. Butyrate and cathelicidin peptides abrogated synthesis of TLR9 in colonic epithelium

To further ascertain the role of cathelicidin in modulating intestinal TLRs, colonic epithelial cells were stimulated with butyrate or synthetic cathelicidin in association with TLR9 ligand synthetic CpG DNA (CpG). Pre-treatment with butyrate inhibited *TLR9* gene overexpression promoted by TLR9 ligand CpG (Fig. 4A). Likewise, synthetic LL-37 cathelicidin abolished *TLR9* mRNA expression induced by CpG (Fig. 4B). Repression of TLR9 induction by butyrate and synthetic cathelicidin was confirmed by reduced protein expression of TLR9 in colonic epithelial cells by immunofluorescence images (Fig. 3B and D) and protein levels (Fig. 4C). There was no effect of LL-37 on *TLR9* when using scrambled LL-37 peptide instead of LL-37 (Fig. 4D). Butyrate and LL-37 only did not alter baseline expression of *TLR9* (Fig. 4A).

3.4. MAPK signalling was involved in cathelicidin-induced TLR expression in colonic epithelium

The MAPK cascade is involved in numerous cellular activities, including TLR activation (Kawai and Akira, 2011). Moreover, interaction of LL-37 with epithelial and leukocyte cells promote MAPK signalling (Tjabringa et al., 2003; Mookherjee et al., 2006). To gain mechanistic insights, transcriptional responses to TLR ligands and LL-37 were investigated in colonic epithelial cells pre-treated with MAPK kinase (MEK-1 and MEK-2) inhibitors (U0126 and PD98059). Both MEK-1 and MEK-2 inhibitors countered the *TLR4* overexpression induced by LL-37 in LPS-primed colonic epithelial cells (Fig. 5A). Likewise, MAPK kinase inhibitors (U0126/PD98059) abrogated CpG ligand induction (Fig. 5B).

3.5. TLR expression induction occurred in the apical compartment of colonic epithelial cells

Polarity of intestinal epithelial cells has a major role in colonic homeostasis, with various receptors asymmetrically distributed between apical and basolateral compartments (Lee et al., 2006). To characterize TLR distribution during stimulation of TLR ligands and cathelicidin, changes in TLR expression were determined after various treatments were applied to apical and/or basolateral surface domains. Simultaneous treatment with LPS and LL-37 at apical surface induced significant *TLR4* upregulation (Fig. 6A). However, there were no differences when co-stimulation (LPS and LL-37) was conducted at the basolateral surface, or when LPS was applied to apical domain and LL-37 to the basolateral domain (Fig. 6A). Upregulation of *TLR9* by its ligand CpG was also evident when the CpG treatment was done at the apical compartment (Fig. 6B). As expected, there were no differences between apical and basolateral treatments when CpG and LL-37 were applied concurrently (Fig. 6B).

3.6. Cathelicidin mediated TLR expression regulate CXCL8 secretion

TLRs upon stimulation by specific ligands (LPS for TLR4 and ODN for TLR9) initiate downstream signalling events that induce secretion of pro-inflammatory cytokines, including CXCL8 (Abreu et al., 2003). CXCL8 is determinant for intestinal homeostasis in terms of pro-survival signal (Maheshwari et al., 2004), epithelial restitution (Sturm et al., 2005) and neutrophil recruitment (de Oliveira et al., 2013). Colonic epithelial cells are an important source of CXCL8 secretion upon TLR receptor activation (Abreu et al., 2003). Therefore, we sought to analyse whether expression of TLRs were linked to secretion of CXCL8. Cathelicidin mediated up- and down-regulation of TLR4 and 9 in colonic epithelial cells, respectively, consistent with increased CXCL8 secretion in response to LPS and LL-37 (Fig. 7A) and reduced CXCL8 secretion in response to ODN and LL-37 (Fig. 7B). These results imply that cathelicidin in presence of specific bacterial virulent factors differentially regulate CXCL8 chemokine secretion, perhaps via modulation of TLR4 and TLR9 receptor expression.

3.7. Cathelicidin enhanced antimicrobial defense in intestinal epithelia through TLR activation

To investigate epithelial antimicrobial defenses induced by

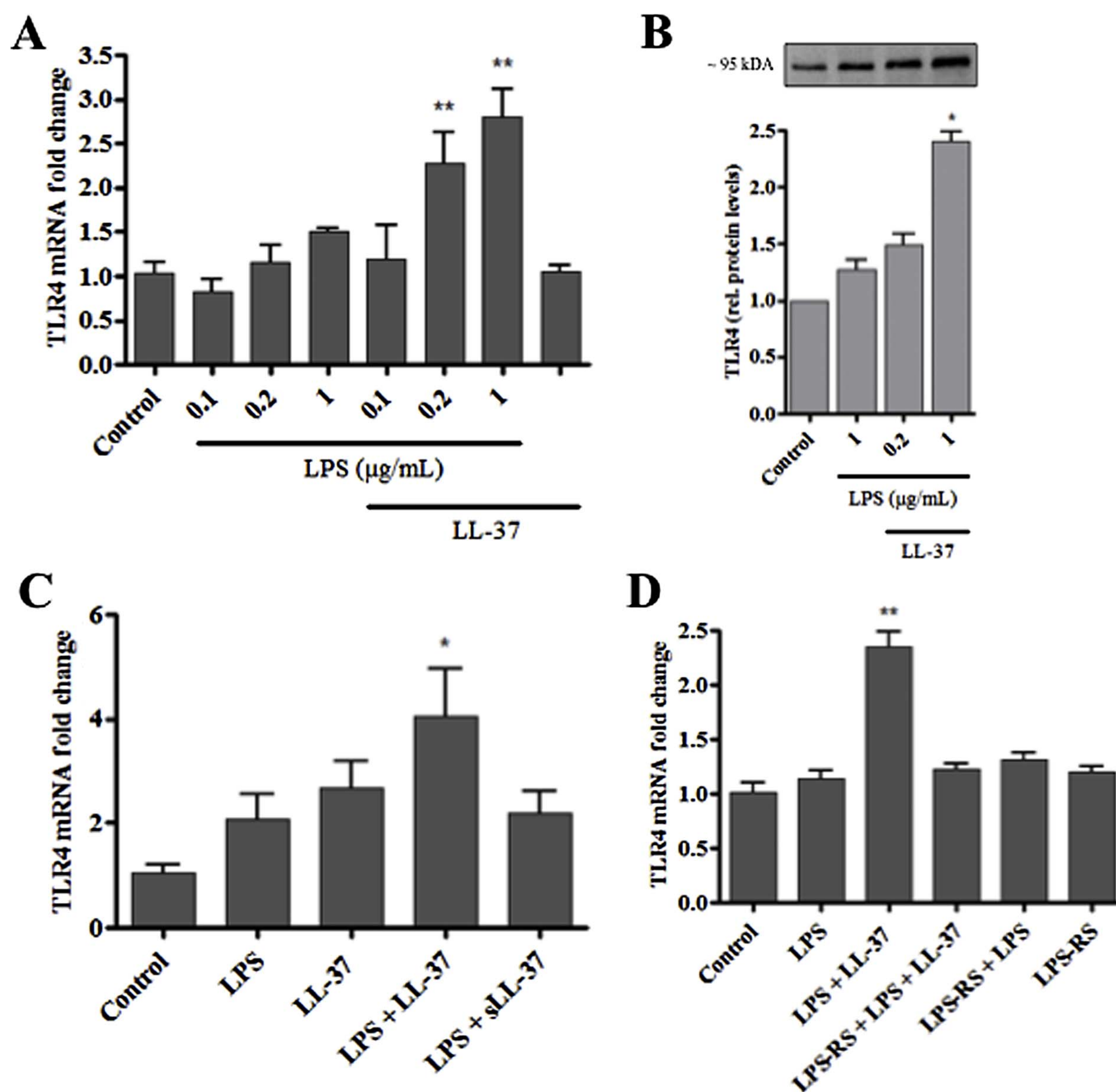

Fig. 2. Effects of synthetic cathelicidin and LPS on TLR4 intestinal expression. Colonic epithelial HT29 cells were stimulated with LPS (0.1–1 µg/mL for 4 h) ± cathelicidin LL-37 (20 µg/mL) (A and B) or scrambled LL-37 (sLL-37, 20 µg/mL; C) or were pre-treated with TLR4 antagonist LPS-RS ultrapure (10 µg/mL for 3 h), followed by LPS (0.2 µg/mL) ± LL-37 (20 µg/mL) for 4 h (D). Relative mRNA expression of TLR4 was assessed by qRT-PCR. Protein concentrations were measured by western blotting. Representative blots from three independent experiments are shown above each bar. Results represented mean fold change of TLR4 concentrations in treated versus untreated cells (the latter served as the control).
*$p < 0.05$ compared to untreated control.

cathelicidin, a bacterial internalization assay exposing colonic epithelial cells previously treated with TLR ligands and cathelicidin to *E. coli* was used. While synthetic cathelicidin or LPS only partially reduced the amount of bacteria that invaded intestinal epithelial cells, co-stimulation of cathelicidin with LPS caused significant (~50%) inhibition of bacterial internalization (Fig. 8A). Regarding TLR9, CpG per se or in combination with LL-37 similarly reduced the number of invading *E. coli* (Fig. 8A). Likewise, stimulation of colonic epithelial cells with cathelicidin and TLR ligands (LPS, CpG) reduced the number of bacteria surviving in the supernatant, with no significant differences among treatments (Fig. 8B).

4. Discussion

This study demonstrated that cathelicidin, either exogenous or endogenous (stimulated by butyrate), regulated TLR4 and TLR9 synthesis in the colonic epithelium in response to bacterial patterns. Furthermore, butyrate induced TLR4 expression even more than its natural ligand, LPS. This was consistent with butyrate-enhanced LPS-induced activation of TLR4 in epithelial cells by promotion of NF-κB responses (Lin et al., 2015). Furthermore, butyrate induced naturally occurring cathelicidin in colonic epithelium, as reported (Hase et al., 2002; Schauber et al., 2003). Thus, effects of butyrate on TLR4 were tentatively attributed to synthesis of endogenous cathelicidin, under either homeostatic conditions or an inflammatory milieu generated by bacterial LPS. To confirm the role of cathelicidin in modulating TLR4, we determined that synthetic LL-37 also induced expression and synthesis of TLR4 in colonic epithelium primed by bacterial LPS. Moreover, this induction was dependent on the primary cathelicidin peptide structure, suggesting a cathelicidin-specific effect. Furthermore, our experiments inhibiting LPS-TLR4 interaction with a competitive TLR4 antagonist confirmed that cathelicidin modulated TLR4 synthesis through an interaction with LPS and its innate TLR receptor. Consistent with these data, LL-37 potentiated lung epithelial responses by

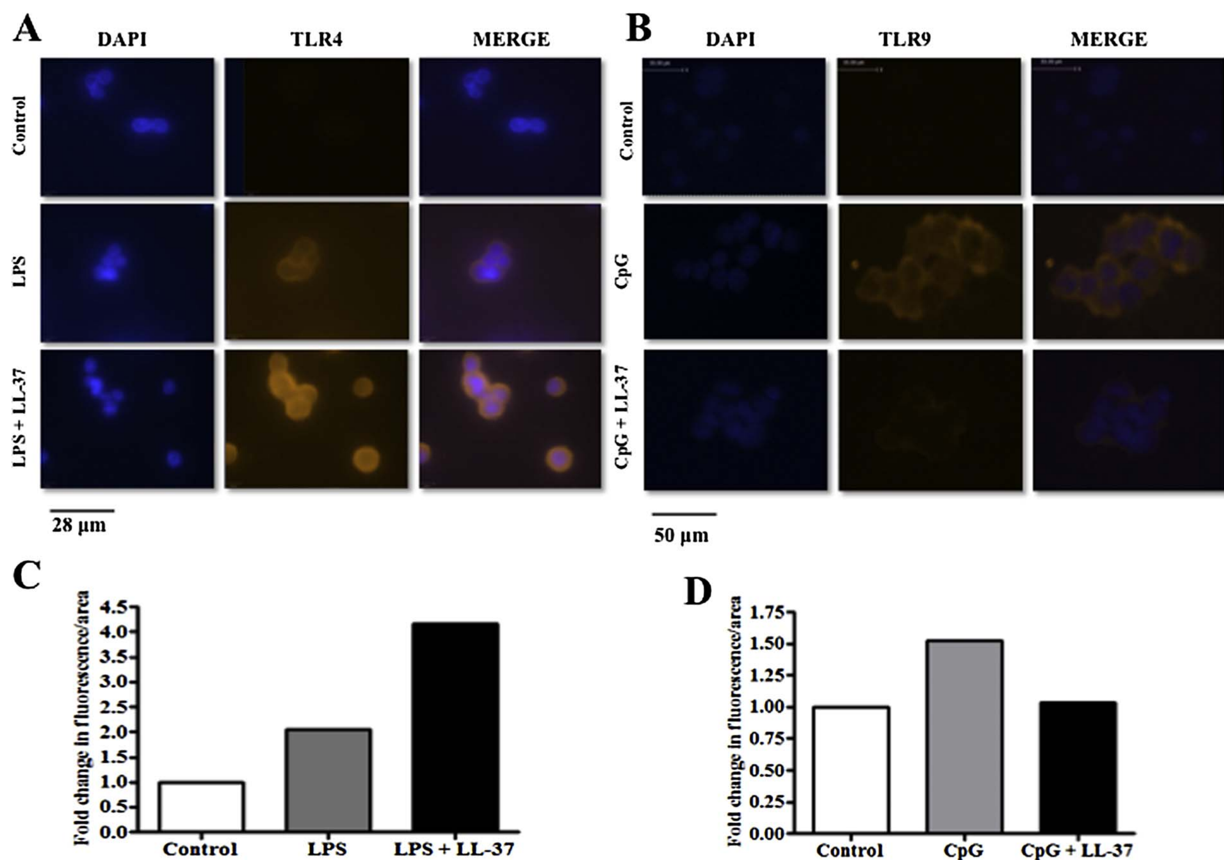

Fig. 3. Confocal cellular expression of TLR4 and TLR9 induced by TLR agonists and cathelicidin. Colonic epithelial HT29 cells were incubated with (A) TLR4 agonist LPS (1 μg/mL) ± LL-37 (5 μg/mL) or (B) TLR9 agonist CpG ODN (10 μg/mL) ± LL-37 (5 μg/mL) for 4 h and TLR4 or TLR9 were immune stained with an antibody against anti-TLR4 (yellow) or anti-TLR9 (yellow) and the nucleus with 4′, 6-diamidino-2-phenylindole (DAPI; blue). Scale bars = 28 μm (A) and 50 μm (B). Confocal images were quantified by measuring fluorescence intensity of TLR4 (C) or TLR9 (D) immuno staining within the cell. These images and graphs are from one of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increasing uptake of LPS and enabling its interaction with its receptor TLR4 (Shaykhiev et al., 2010). The interaction between cathelicidin and TLR4 in non-myeloid cells seems related to those observed for other host defense peptides, including defensins. For instance, primary synoviocytes challenged by *Pseudomonas aeruginosa* showed upregulated *TLR4* expression in association with increased amounts of secreted human beta defensin-2 (HBD-2) (Varoga et al., 2009a). Likewise, bacterial infection in cultured primary osteoblasts stimulated rapidly secretion of HBD-3 protein (after 6 h), which was significantly reduced when *TLR2* and *TLR4* genes were silenced (Varoga et al., 2009b). It is noted that the current effect of cathelicidin in colonic epithelial cells promoting TLR4 in response to LPS was differed from the cathelicidin effects in leukocytes. In this regard, myeloid cells stimulated by LPS in combination of cathelicidin had an LPS-neutralizing effect (Nagaoka et al., 2001) and inhibition of TLR4 responses (Mookherjee et al., 2006; Kandler et al., 2006). Furthermore, cathelicidin suppressed the interaction of LPS with LPS binding protein in monocytes, which mediated LPS transport, as a mechanism to inhibit release of pro-inflammatory cytokines (Mookherjee et al., 2006; Kandler et al., 2006). These differences were attributed to the intestinal epithelium being naturally hypo-responsive to TLR ligands. In this regard, our *in vitro* cell model, colonic adenocarcinoma epithelial (HT29) cells (Suzuki et al., 2003), and primary mature colonic epithelial cells from healthy humans and mice similarly show low and cytoplasmic expression of TLR4 (Meng et al., 2015). Likewise, reduced TLR4 expression has been reported in normal human colonic biopsies (Cario and Podolsky, 2000). This hypo responsive nature in the gut mucosa is perhaps a physiological mechanism to avoid exaggerated activation of cells by microbes present on intestinal surfaces (Melmed et al., 2003). Therefore, overexpression of epithelial TLR4 by LL-37 in the presence of LPS was regarded as a defensive mechanism in the intestinal epithelium in response to Gram-negative bacterial infection.

Epithelial TLR9 is a key receptor for recognition of microbial DNA and frequently studied by its response to synthetic CpG DNA (Kumagai et al., 2008). The capacity of cathelicidin to bind to DNA facilitates its delivery into endosomes (Lande et al., 2007) and activates TLR9 in dendritic cells and macrophages (Nakagawa and Gallo, 2015). Herein, exogenous cathelicidin or endogenous cathelicidin induced by butyrate blocked TLR9 responses induced by CpG in colonic epithelium. Similarly, cathelicidin inhibited DNA-driven innate immune responses and prevented assembly of functional signalling complexes in keratinocytes (Chiliveru et al., 2014). Cathelicidin internalized DNA nucleotides in endothelial cells, although this also suppressed production of pro-inflammatory mediators (Merkle et al., 2015). It is noteworthy that abrogation of the TLR9 response to its ligand by LL-37 could prevent TLR9-dependent exaggerated inflammatory responses during enterocolitis, either in response to microbial infection or circulating endogenous DNA-fragments. In agreement with this hypothesis, a major role for TLR9 in expression of cathelicidin in the colon was reported in murine models of colitis in which bacterial DNA promoted cathelicidin via TLR9-ERK signalling (Koon et al., 2011). Based on our current study, we inferred there was a regulatory mechanism by which LL-37 controlled TLR9 expression to prevent persistent cathelicidin responses via TLR9 ligands in the colon.

Intestinal epithelial cells are intrinsically polarized, with TLRs asymmetrically distributed between apical and basolateral membrane compartments. Thus, we studied the influence of the TLR location in the response to cathelicidin and bacterial incitants. Herein, modulation of

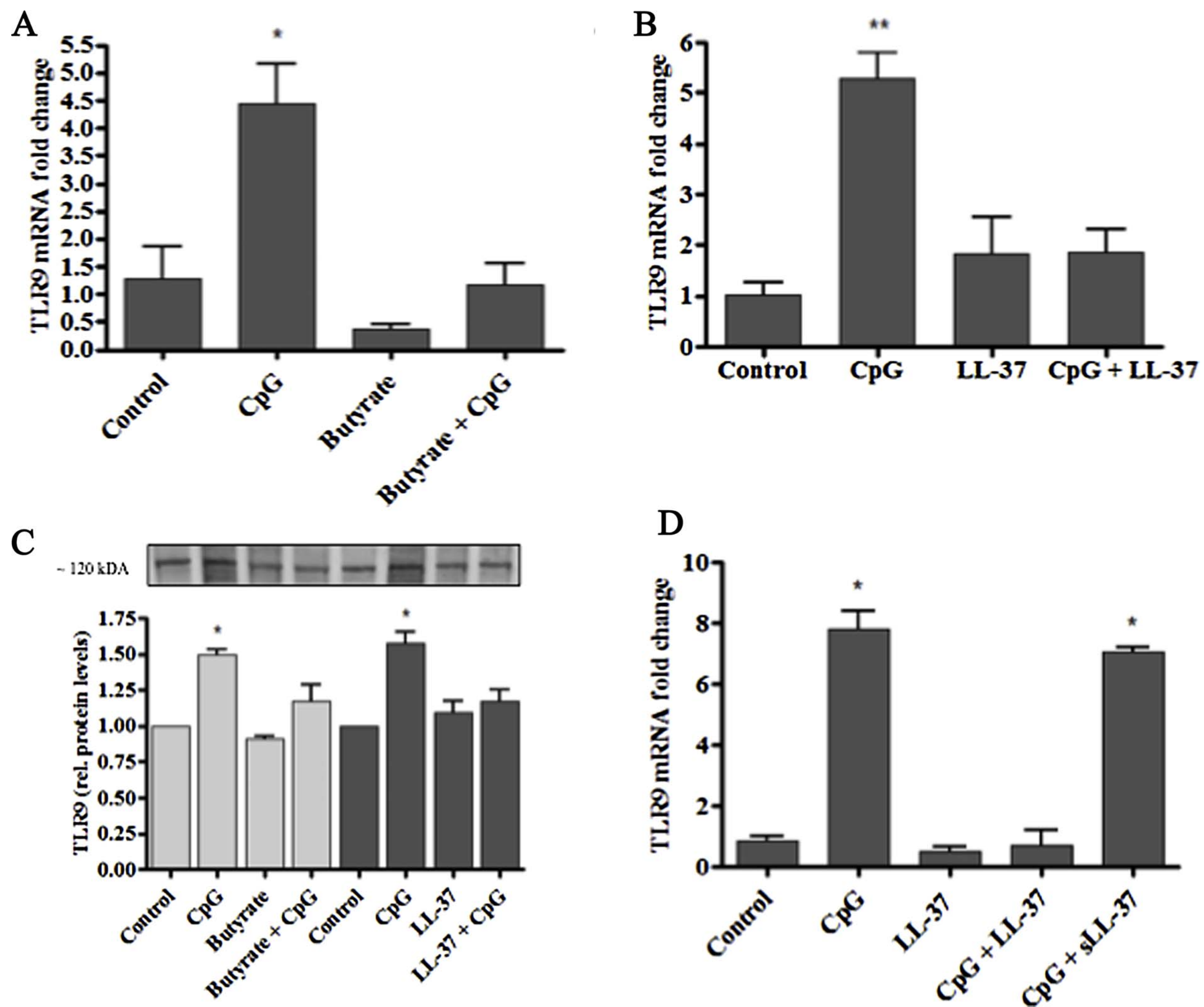

Fig. 4. Effects of cathelicidin inducer butyrate and synthetic cathelicidin on intestinal TLR9 expression. Colonic epithelial HT29 cells were pre-treated with butyrate (4 mM) for 48 h, followed by TLR9 agonist CpG ODN2395 (10 μg/mL for 6 h) (A and light grey bars in C) or were stimulated with CpG ODN2395 (10 μg/mL for 6 h) ± cathelicidin LL-37 (20 μg/mL) (B and dark grey bars in C) or scrambled LL-37 (sLL-37, 20 μg/mL) (**D**). Relative mRNA expression of TLR9 was assessed by qRT-PCR. Protein concentrations were measured by western blotting. Representative blots from three independent experiments are shown above each bar. Results represented mean fold change of TLR9 concentrations in treated versus untreated cells (the latter served as the control).
$^{*}p < 0.05$ compared to untreated control.

TLR4 and TLR9 by cathelicidin in colonic epithelial cells primed by LPS or by CpG agonist, respectively, mostly occurred through activation in the apical compartment. It has been established that TLR9 stimulation at the apical surface prevented NF-κB activation, whereas basolateral TLR9 signalling results in its activation (Lee et al., 2006). This was consistent with our finding that cathelicidin abrogated TLR9 responses when triggered in apical compartments.

In the present study, cathelicidin modulated TLR4 in the presence of LPS, mostly via MAPK signalling pathways. Likewise, TLR9 and CpG signalled through MAPK activation. This MAPK intracellular cascade was similar to a commonly described TLR signalling pathway initiated by recruitment of an adaptor molecule, mainly MyD88, and subsequent phosphorylation of MAPKs and activation of NF-kB (Kawai and Akira, 2011). In agreement, cathelicidin LL-37 induced vascular endothelial growth factor (VEGF) in human chondrocytes using similar pathways, TLRs and MAPKs (Khung et al., 2015; Kittaka et al., 2013). Moreover, inhibition of MAPK in chondrocytes abolished VEGF protein responses to Gram-positive and negative bacterial supernatants (Varoga et al., 2006).

TLRs are classic pattern recognition receptors that bind virulent factors from pathogens and initiate synthesis of pro-inflammatory cytokines (e.g. CXCL8). Therefore, irrespective of tissue type, levels of TLR expression have profound impacts on cytokine secretion. In line with that, in the current study, cathelicidin-regulated TLR4 and 9 expression was linked to CXCL8 secretion levels in colonic epithelial cells. In agreement, in studies involving pulmonary inflammation in response to bacterial endotoxins in rabbits, blocking TLR4 reduced CXCL8 expression in bronchoalveolar lavage fluid (Smith et al., 2008). Furthermore, neutrophils isolated from human blood upon exposure to cigarette smoke had enhanced CXCL8 secretion, associated with increased expression and activation of TLR9 (Mortaz et al., 2010). Our finding also suggested that cathelicidin promoted antibacterial activity in colonic epithelium exposed to bacteria. It is noted that LL-37 was tested in this study using normal cell culture media without fetal bovine serum, in agreement with previous studies of the role of cathelicidin on TLR signalling (Shaykhiev et al., 2010; Kandler et al., 2006). However, direct antimicrobial activity by cathelicidin has been controversial, owing to instability of the peptide under physiological conditions, including high salt and presence of serum protein (Kosciuczuk et al., 2012; Durr et al., 2006). Thus, antibacterial activity of LL-37 may also involve indirect mechanisms, including LPS priming and enhancement of certain chemokine, including CXCL8 with bactericidal activity (Bjorstad et al., 2005).

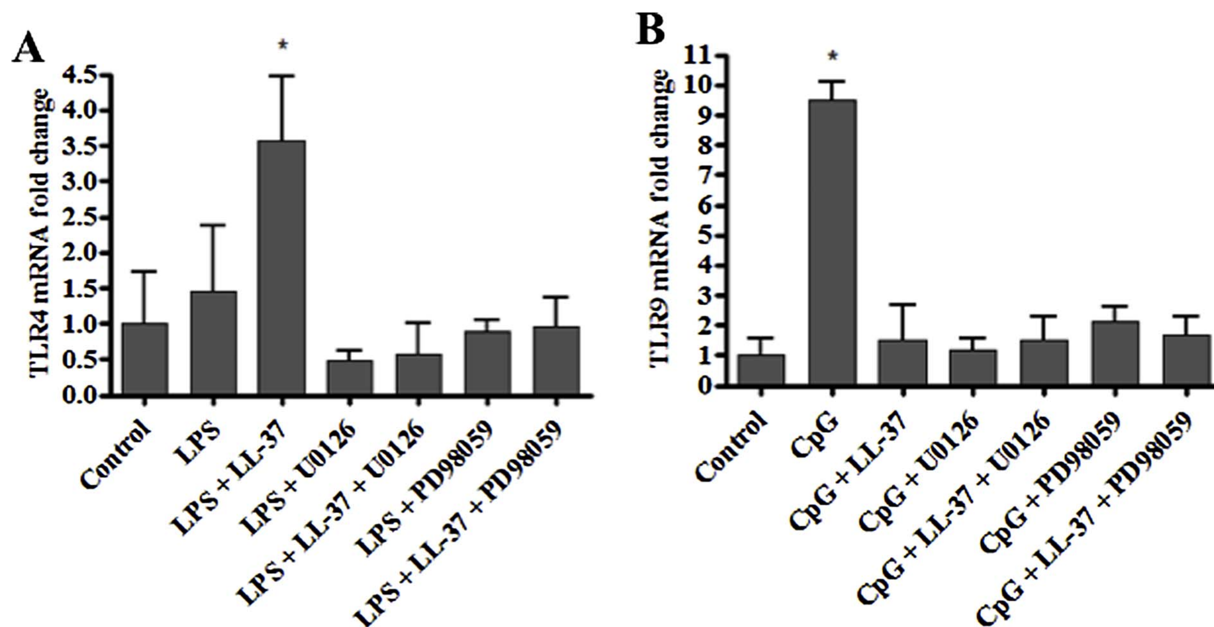

Fig. 5. Blocking of TLR ligands- and cathelicidin-induced TLR expression by MAPK kinase inhibitors. Colonic epithelial HT29 cells were pre-treated with MAPK inhibitors U0126 (10 μM) and PD98059 (20 μM) for 1 h, followed by LPS (1 μg/mL) ± LL-37 (20 μg/mL) (**A**) or CpG ODN2395 (10 μg/mL) ± LL-37 (20 μg/mL) (B) for 4 h. Relative mRNA expression of TLRs was assessed by qRT-PCR. Results represented mean fold change of TLR transcription levels in treated versus untreated cells (the latter served as the control).
$^{*}p < 0.05$ compared to untreated control.

5. Conclusions

The present work identified some intrinsic mechanisms by which cathelicidin promoted modulation of TLRs in colonic epithelium under infectious conditions. By promoting TLR4 in colonic epithelial cells challenged with microbial incitants, cathelicidin could revert a physiological hypo-responsive status to LPS and initiate appropriate host inflammatory responses (e.g. CXCL8 chemokine expression) to restore intestinal integrity. In addition, cathelicidin selectively abrogated TLR9 responses when cells were exposed to specific virulence factors. Activation of TLR4 by ligands in combination with cathelicidin promoted epithelial antimicrobial defenses against *E. coli*, maybe in part through CXCL8 chemokine secretion. Thus, we concluded that cathelicidin have key roles in intestinal homeostasis and inflammation, as they modulated the priming provided by TLR ligands and synthesis of TLR4 and 9 in the intestinal epithelium.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

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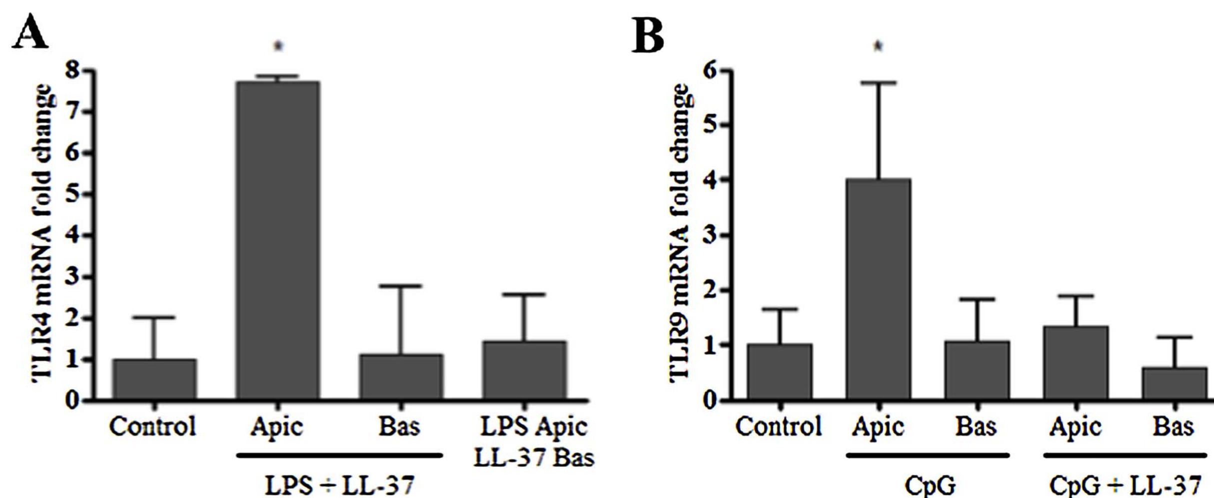

Fig. 6. TLR expression following apical and basolateral TLR ligands and cathelicidin stimulation. Colonic epithelial T84 cells grown on microporous filter inserts were stimulated for 4 h with LPS (1 μg/mL) and LL-37 (20 μg/mL), both applied at apical or basolateral compartments, or LPS at apical and LL-37 at basolateral surface (A) or CpG ODN2395 (10 μg/mL) ± LL-37 (20 μg/mL) at apical or basolateral compartment (B). Relative mRNA expression of TLRs was assessed by qRT-PCR. Results represented mean fold change of TLR transcription levels in treated versus untreated cells (the latter served as the control).
$^{*}p < 0.05$ compared to untreated control.

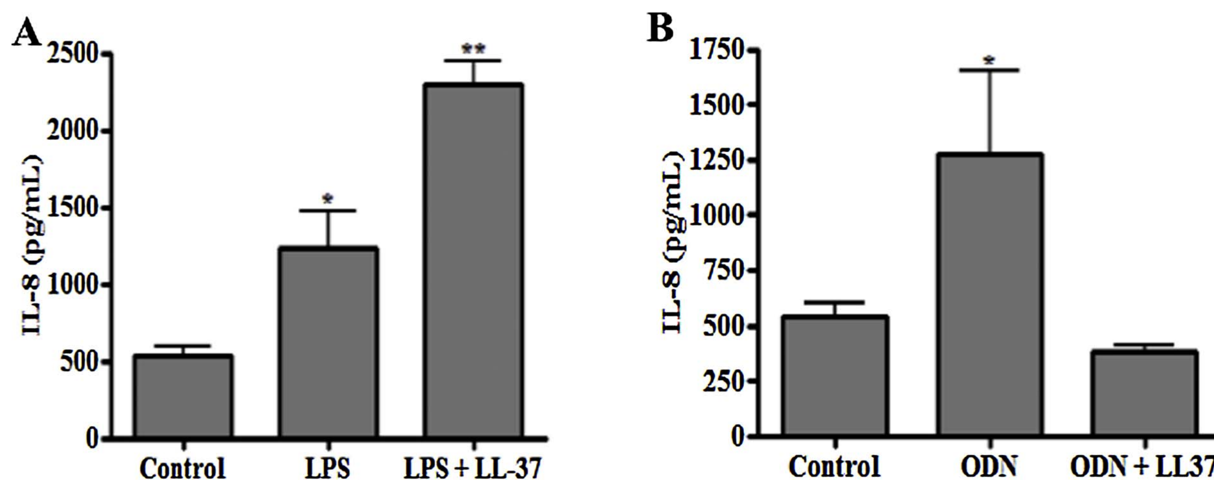

Fig. 7. Differential regulation of CXCL8 expression by colonic epithelial cells upon stimulation with cathelicidin in presence of virulent factors. Colonic epithelial HT29 cells were stimulated with LPS (1 μg/mL) ± LL-37 (20 μg/mL) (A) or ODN (1 μg/mL) ± LL-37 (20 μg/mL) (B) for 16 h. CXCL8 protein secretion was quantified in supernatant using ELISA. Results are represented as amount of protein secreted per unit volume of supernatant.
$^{*}p < 0.05$ and $^{**}p < 0.01$ compared to untreated control.

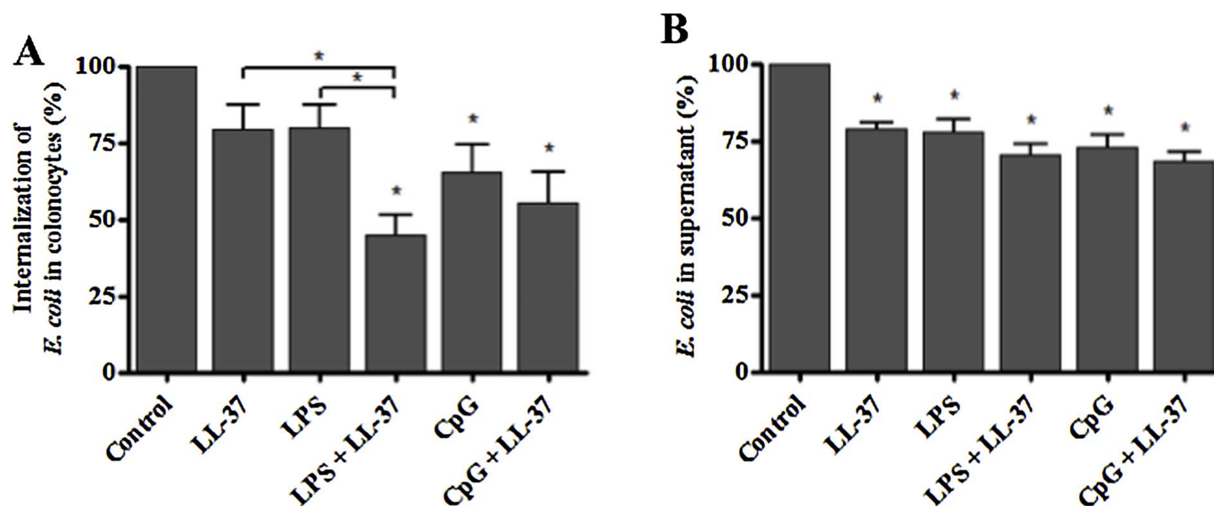

Fig. 8. Antimicrobial assay. Colonic epithelial HT29 cells were treated with LPS (1 μg/mL) or CpG ODN2395 (10 μg/mL) ± LL-37 (20 μg/mL) for 2 h, followed by a 3-h challenge with *E. coli* strain HB101 (MOI 10:1). Antimicrobial activities in cell lysates with 0.2% Triton X-100 (A) and supernatants (B) were analysed by bacterial counting. Amount of *E. coli* is percentage compared to untreated group.
$^{*}p < 0.05$ compared to untreated control or between treatments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.09.011.

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