Cell and Tissue Research (2019) 376:433–442
https://doi.org/10.1007/s00441-018-02984-7

REGULAR ARTICLE

Human cathelicidin improves colonic epithelial defenses against *Salmonella typhimurium* by modulating bacterial invasion, TLR4 and pro-inflammatory cytokines

Maia Marin[1,2] · Ravi Holani[3] · Graham A. D. Blyth[4] · Dominique Drouin[5] · Anselmo Odeón[2] · Eduardo R. Cobo[3]

Received: 15 August 2018 / Accepted: 6 December 2018 / Published online: 20 February 2019

Abstract

The intestinal mucosa contributes to frontline gut defenses by forming a barrier (physical and biochemical) and preventing the entry of pathogenic microbes. One innate role of the human colonic epithelium is to secrete cathelicidin, a peptide with broad antimicrobial and immunomodulatory functions. In this study, the effect of cathelicidin in the maintenance of epithelial integrity, Toll-like receptor recognition, bacterial invasion and initiation of inflammatory response against *Salmonella typhimurium* is investigated in cultured human colonic epithelium. We found exogenous human cathelicidin restores the epithelial integrity in *S. typhimurium*-infected colonic epithelial (T84) cells by mostly post-translational effects associated with reorganization of zonula occludens (ZO)-1 tight junction proteins. Endogenous cathelicidin prevents *S. typhimurium* internalization as shown in colonic epithelial cells genetically deficient in the only human cathelicidin, LL-37 (shLL-37). Moreover, supplementation of shLL-37 cells with synthetic LL-37 reduces the grade of *S. typhimurium* internalization in a dose-dependent manner. Mechanistically, shLL-37 cells have lower gene expression of TLR4 and pro-inflammatory cytokine IL-1β in response to *S. typhimurium*. Thus, human cathelicidin aids in the early colonic epithelial defenses against enteric *S. typhimurium* by preventing bacterial invasion and maintaining epithelial barrier integrity, likely to occur due to the production of sensing TLR4 and pro-inflammatory cytokines.

Keywords Cathelicidin · Intestinal epithelium · Innate immunity · Epithelial integrity · *Salmonella typhimurium* · Antimicrobial peptide

Highlights
- Cathelicidin restores *S. typhimurium* induced epithelial integrity degradation.
- Cathelicidin decreases *S. typhimurium* colonic epithelial invasion.
- Endogenous cathelicidin promotes TLR4 and IL-1β production in response to *S. typhimurium*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00441-018-02984-7) contains supplementary material, which is available to authorized users.

✉ Eduardo R. Cobo
ecobo@ucalgary.ca

[1] National Scientific and Technical Research Council (CONICET), Buenos Aires, Argentina

[2] Animal Production, Balcarce Experimental Station, National Institute of Agricultural Technology (INTA), Buenos Aires, Argentina

[3] Production Animal Health (PAH), Faculty of Veterinary Medicine, University of Calgary, HSC 2519, 3330 Hospital Dr NW, Calgary, AB T2N 4N1, Canada

[4] Department of Microbiology, Immunology & Infectious Diseases, Cumming School of Medicine, University of Calgary, Calgary, Canada

[5] Bachelor of Health Sciences, Cumming School of Medicine, University of Calgary, Calgary, Canada

Introduction

Salmonella typhimurium is an enteric pathogenic Gram-negative bacteria of both the small intestine and colon (Harish and Menezes 2015). Virulence of *S. typhimurium* is largely determined by an outer membrane consisting of lipopolysaccharides (LPS), which protects the bacteria from environmental challenges (e.g., acidic pH, antimicrobial peptides) (Bonnington and Kuehn 2016). *S. typhimurium* actively invades enterocytes and stimulates these non-phagocytic cells to internalize the bacteria. The intestinal epithelium actively participates in sensing and responding to luminal and invading *S. typhimurium* (Lee et al. 2006). Innate intestinal epithelial defenses are initiated through engagements of constitutively expressed evolutionarily conserved Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) and begin signaling events that result in the production of pro-inflammatory cytokines (Mogensen 2009). Epithelial signaling through TLR4 and TLR9 are of particular interest in *S. typhimurium* infection as they detect main components of the bacteria, LPS and unmethylated microbial CpG DNA, respectively (Kawai and Akira 2011).

Clinical symptoms of *S. typhimurium* intestinal infection include fever, abdominal cramps and principally, diarrhea, indicating dysfunctional epithelial integrity (Konig et al. 2016). Colonic epithelial integrity is maintained by tight junction proteins between epithelial cells. This epithelial paracellular barrier regulates a selective movement of solutes across the epithelium, proper absorption of water and restricts invasion of enteric luminal bacteria (Jung et al. 2015). Alterations in the epithelial tight junction integrity by *S. typhimurium* might facilitate bacterial penetration across the gut epithelium and the onset of colitis in the diarrhea pathophysiology (Kohler et al. 2007). Thus, protective epithelial innate mechanisms are key in preventing *S. typhimurium* infection and its pathogenic effects.

The first line of innate effectors in the intestine involves cathelicidin (Iimura et al. 2005). Colonic intestinal epithelial cells secrete cathelicidin, a cationic short peptide that contributes to host-microbial defenses, during the pathogenesis of infectious colitis (Cobo et al. 2017; Kosciuczuk et al. 2012; Marin et al. 2017; Zanetti 2005). Humans possess a single cathelicidin gene (hCAP-18/LL-37) that encodes the 37-amino acid residue C- terminal domain LL-37 (Zanetti 2005). Although cathelicidins were originally identified as broad-spectrum antimicrobials, there is emerging evidence that their contribution to innate gut defenses extends beyond direct lytic effect on bacteria. Immunomodulatory functions of cathelicidin have been reported in vitro, including recruitment of immune cells and regulation of epithelial barrier integrity (Iimura et al. 2005; Koon et al. 2011; Otte et al. 2009). The involvement of cathelicidin in *S. typhimurium* infection and the mechanisms of action remain unexplored despite the importance of understanding the pathogenesis of intestinal salmonellosis. Another key question is the extent to which immune effects of cathelicidins can be achieved by naturally occurring cathelicidins in comparison to the exogenous delivery of synthetic cathelicidin. Both stimulation of endogenous cathelicidins and the delivery of synthetic-derived peptides represent potential therapeutic roles for controlling infectious diarrhea. Thus, this work aims to determine the functionality of naturally occurring and exogenous human cathelicidin in the colonic epithelial integrity and inflammatory response against *S. typhimurium*.

Materials and methods

Colonic epithelial cells

Human adenocarcinoma colonic epithelial cell lines were used to examine the role of endogenous cathelicidins (HT29) (Hase et al. 2002) and tight junction proteins (T84) (Yamaura et al. 2016). HT29 cells have been used as a model to study the effects of antimicrobial peptides on innate immune expression and enteric bacterial invasiveness (Marin et al. 2017), whereas T84 cells provide an in vitro alternative for assessing the intestinal integrity given their known ability to form tight junctions (Dharmsathaphorn et al. 1984). Cells were maintained until passages 3–5 in Dulbecco's modified Eagle's medium (Gibco, Life Technologies) or Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich), respectively, with 10% fetal bovine serum (Benchmark Gemini Bio-Products), 1 mM sodium pyruvate (Gibco, Life Technologies) and penicillin (100 U ml^{-1})/streptomycin (100 μg ml^{-1}; HyClone Thermo Fisher Scientific) in a humidified environment of 95% O_2 and 5% CO_2 at 37 °C.

Silencing of endogenous LL-37 in colonic epithelial cells

HT29 cells genetically knocked down for endogenous cathelicidin (LL-37) were developed and maintained by our group (Holani et al., unpublished data). Briefly, HT29 cells were transfected with short hairpin (sh)-LL-37 pGFP-V-RS plasmid vector or scrambled non-targeting shRNA construct (ntLL-37) (TG314213; Origene) using Cell Line Nucleofector® Kit V (VCA-1003; Lonza). LL-37 knocked-down population was selected using puromycin (30 μg/mL) and fluorescence-activated cell sorting (FACS) against the green fluorescent protein (GFP). Cell populations (shLL-37 and ntLL-37) were tested for LL-37 expression knock-down using qPCR with human CAMP-specific primer (PPH09430A, Qiagen). The knock-down efficiency was assessed to be ~ 80–90% (Supplementary Fig. 1). Cells were

maintained by consistently culturing them in DMEM media supplemented with puromycin (15 μg/mL).

Salmonella typhimurium

A virulent and drug-resistant *S. typhimurium* definitive type (DT) 104 strain isolated from cattle and pathogenic for humans and animals (Poppe et al. 1998), was grown on LB-Miller broth (IBI Scientific) in aerobiosis for 2 h at 37 °C with vigorous shaking (300 rpm).

Experimental design

In epithelial integrity studies, T84 cells were grown in 8-well chambers (Greiner Bio-One) for immunofluorescent identification of ZO-1 and in 24-well plates (Greiner Bio-One) for determining tight junction protein gene expression. Cells cultured until 80–90% confluency were stimulated with synthetic LL-37 peptide (0–40 μg/mL) (H-6224.0005, Bachem) and/or challenged with *S. typhimurium* at a multiplicity of infection (MOI) of 5:1 (for 4 or 16 h). The concentration of LL-37 was within the range of in vivo concentrations found in mucosal secretions (2–3 μg/mL in adults and up to 20 μg/mL in infants) (Schaller-Bals et al. 2002).

For testing internalization of *S. typhimurium* and gene expression of TLRs and pro-inflammatory cytokines, HT29 cells (shLL-37 and ntLL-37) were seeded in 24-well plates (Greiner Bio-One). Cells (80–90% confluency) were pre-incubated with synthetic LL-37 (0–20 μg/mL; 1 h) (H-6224.0005, Bachem) and challenged with *S. typhimurium* (MOIs 1:1 or 5:1; for up to 24 h) at 37 °C with 95% O_2 and 5% CO_2.

Confocal immunofluorescence microscopy for tight junction ZO-1 protein

T84 cells 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 room temperature (RT) and rinsed with PBS-Tw. Cells were blotted with anti-zonula occludens (ZO)-1 monoclonal antibody (ZO1-1A12, Thermo Fisher Scientific; 1:100) at 4 °C overnight, rinsed with cold PBS-Tw and blotted with the secondary antibody Alexa Fluor 594-conjugated AffiniPure Donkey Anti-Mouse IgG (H + L; Jackson ImmunoResearch). The secondary antibodies were diluted 1:1000 in PBS-Tw plus 1% bovine serum albumin and incubated for 1 h at RT. Cells 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). Slides were examined using a wide-field immunofluorescence microscope (IX71 Olympus). Data were recorded in three independent experiments.

Immunofluorescence staining for *S. typhimurium*

HT29 cells were treated with *S. typhimurium* (MOI of 1) for 1 h, fixed with 4% PFA (15 min, RT), followed by permeabilization (or not) using 0.1% Triton X-100 (T8787; Sigma-Aldrich). Cells were blocked in PBS-Tw containing 10% donkey serum (017–000-021; Jackson ImmunoResearch), 1% bovine serum albumin (BSA, 9048-46-8; Amresco) and 0.3 M glycine (1 h, RT) and stained with primary anti-*Salmonella* spp. antibody (ab35156; abcam) diluted 1:20 in PBS (16 h, 4 °C). Cells were incubated with Alexa647-conjugated donkey anti-rabbit IgG (H + L) (711-605-152; Jackson ImmunoResearch) secondary antibody (1:200, 1 h, RT) followed by counterstaining for nuclei with 4′, 6-diamidino-2- phenylindole (DAPI, 62247; ThermoFisher Scientific, 1:1000, 30 min, RT). Slides were then examined using wide-field immunofluorescence microscope (IX71 Olympus).

Antimicrobial assay

The minimal inhibitory concentration (MIC) of LL-37 for *S. typhimurium* was determined in 96-well plates. *S. typhimurium* was grown in LB broth and diluted with fresh serum-free DMEM medium to a final concentration of 1×10^5 CFU per well. Bacterial cells were treated with different concentrations of LL-37 (up to 160 μg/mL) for variable time points (up to 4 h) and the OD_{600} measured. Data were represented as absolute values and compared with respect to corresponding time-dependent control group.

Cell invasion assay

HT29 cells (shLL-37 and ntLL-37) pre-treated with synthetic LL-37 and challenged with *S. typhimurium* were washed once with PBS and incubated with fresh media containing gentamicin (200 μg/mL) for the indicated timepoints to kill extracellular bacteria. Cells were then washed several times with PBS to remove extracellular bacteria and lysed with 0.2% Triton X-100. Serial dilutions of the cell lysates were plated on LB agar (Difco, Sparks) and bacterial colonies were counted after 18 h of incubation at 37 °C. The total number of bacteria was calculated by counting colonies in dilutions plated directly on LB agar after incubation for 18 h at 37 °C. Results were presented as a percentage of internalized *S. typhimurium* (in lysates) in treated groups compared to an untreated control group.

Gene expression of TLRs, pro-inflammatory cytokines and tight junction proteins

Transcriptional relative messenger gene (mRNA) expression of TLR4, TLR9, IL-1β and IL-18 in shLL-37 and ntLL-37 cells and tight junction proteins occludin (OCLN) and claudins 1 and 4 (CLDN1 and 4) in T84 was quantified by real-time RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was prepared from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (iScript Reverse Transcription Supermix for RT-qPCR; BioRad). The quality and quantity of resulting RNA and cDNA were determined using a NanoVue Spectrophotometer (GE Healthcare Bio-Sciences). The RNA preparations were screened for contaminating genomic DNA using a minus-reverse transcriptase control (i.e., a sample with all RT-PCR reagents except reverse transcriptase). RT-qPCR was performed using a CFX-96 real-time PCR system (BioRad). Each reaction mixture contained 100 ηg of cDNA, 1× SsoAdvanced Universal SYBR Green Supermix (BioRad) and 0.5 μM of each specific primer, in a final volume of 10 μL. Human primers for CAMP (PPH09430A), TLR4 (PPH01795F), TLR9 (PPH01809A), IL-1β (PPH00171C), IL-18 (PPH00580C), OCLN (PPH02571B), CLDN1 (PPH02779A), CLDN4 (PPH07330D) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (PPH00150F) were used. These primers (all from 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). Negative controls for cDNA synthesis and PCR procedures were consistently included. Values of target mRNA were corrected relative to the housekeeping gene GAPDH. Data were analyzed using the $2^{-\Delta\Delta CT}$ methods and results reported as mean fold change of target transcription levels in treated groups versus an untreated control group.

Statistical analyses

Normality was checked using the Shapiro-Wilk test (Royston 1982). Normally distributed (parametric) results are graphed as means and bars represent standard errors

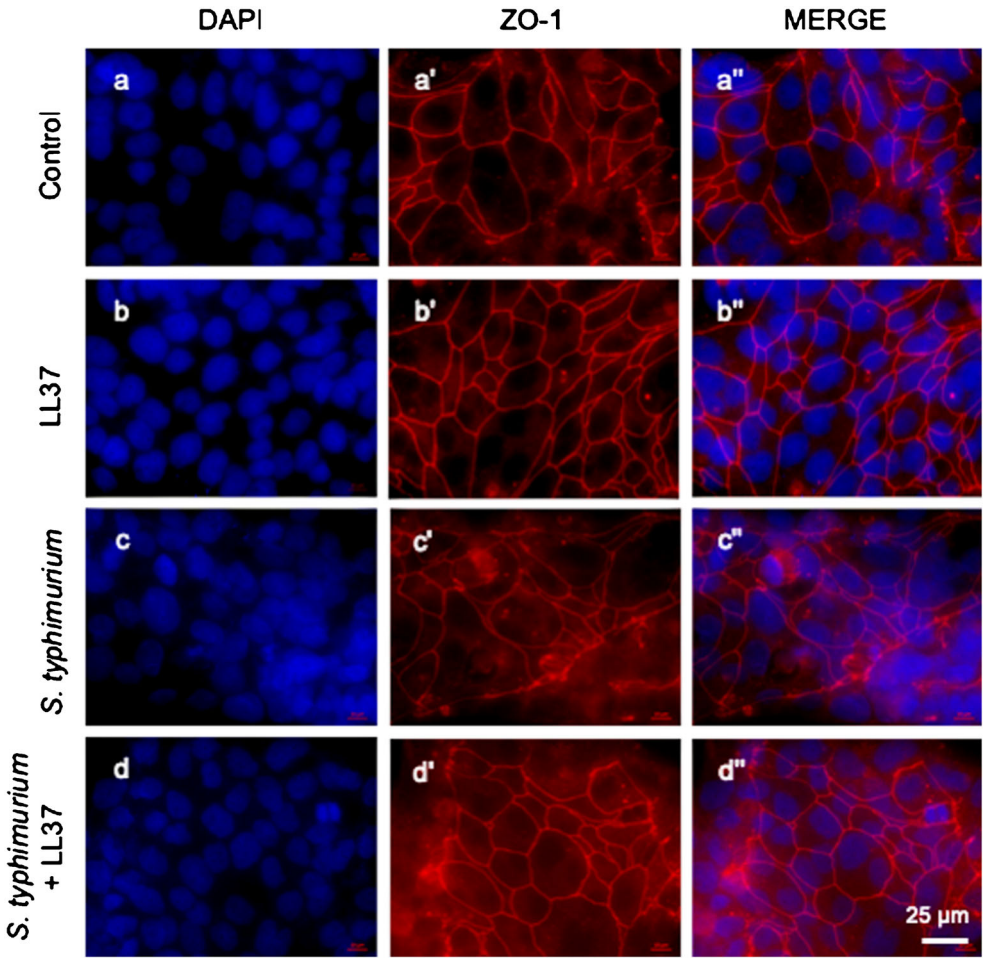

Fig. 1 Synthetic cathelicidin regulates tight junction ZO-1 proteins in colonic epithelium exposed to *S. typhimurium*. Colonic epithelial T84 cells were incubated with LL-37 (20 μg/mL; 1 h) ± *S. typhimurium* (MOI 5:1) for 4 h. The nucleus was stained with 4′, 6-diamidino-2-phenylindole (DAPI; blue) (a, b, c, d) and protein ZO-1 was immune stained with an antibody against ZO-1 (red) (a', b', c', d') to obtain merged images (a″, b″, c″, d″) (scale bar = 25 μm). Images are from one of three independent experiments

(SEM) of the mean from a minimum of three independent experiments, each one performed in duplicate or triplicate. For comparison of differences between treated and untreated groups, a non-paired, two-tailed Student's *t* test was used. *P* values of < 0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism software (GraphPad 5.0).

Results

Synthetic human cathelicidin improved epithelial integrity in *S. typhimurium*-infected colonic epithelium

To assess whether cathelicidin contributes to intestinal barrier maintenance, expression and distribution of main tight junctions, claudins, occludins and zonula occludens were assessed in polarized colonic (T84) cells after stimulation with synthetic LL-37. Immunolabeling studies showed the distribution of the ZO-1 protein in a chicken-wire pattern that represents an adequate tight junction formation (Fig. 1a, a′, a″). This pattern was unmodified by addition of synthetic LL-37 only (Fig. 1b, b′, b″). Exposure of T84 cells to *S. typhimurium* disrupted the ZO-1 protein distribution after 4 h (Fig. 1c, c′, c″). Treatment with synthetic LL-37 restored the ZO-1 organization in T84 cells infected with *S. typhimurium* (Fig. 1d, d′, d″). The ZO-1 distribution stimulated by exogenous cathelicidin was not associated with increased gene expression of tight junction proteins as no change was observed in the mRNA expression of OCLN, CLDN1 and CLDN4 upon LL-37 treatment (up to 40 μg/mL) (Fig. 2).

LL-37 prevented the invasion of *S. typhimurium* into the colonic epithelia

Adherence and invasion of *S. typhimurium* into the intestinal epithelium are essential mechanisms in the pathogenesis of diarrheic salmonellosis. LL-37 showed to contribute to maintaining the gut barrier (Fig. 1) and cathelicidin may have protective roles, either when naturally occurring or administrated exogenously. To further assess the role of cathelicidin in gut antimicrobial defenses, we tested how *S. typhimurium* invades into shLL-37 cells and complemented with increasing concentrations of synthetic LL-37. ShLL-37 cells showed higher levels of internalized *S. typhimurium* at 24 h compared with colonic epithelial cells expressing normal levels of endogenous cathelicidins (non-targeting, ntLL-37) (Fig. 3**a**). Pre-treatment of ntLL-37 cells with increasing doses of synthetic LL-37 (up to 20 μg/mL; 1 h) reduced the amount of internalized *S. typhimurium* to the lowest levels (> 80%) (Fig. 3a). The protective effect of synthetic LL-37 reducing the internalization of *S. typhimurium* was unnoticed in shLL-37 cells at later time points (24 h;

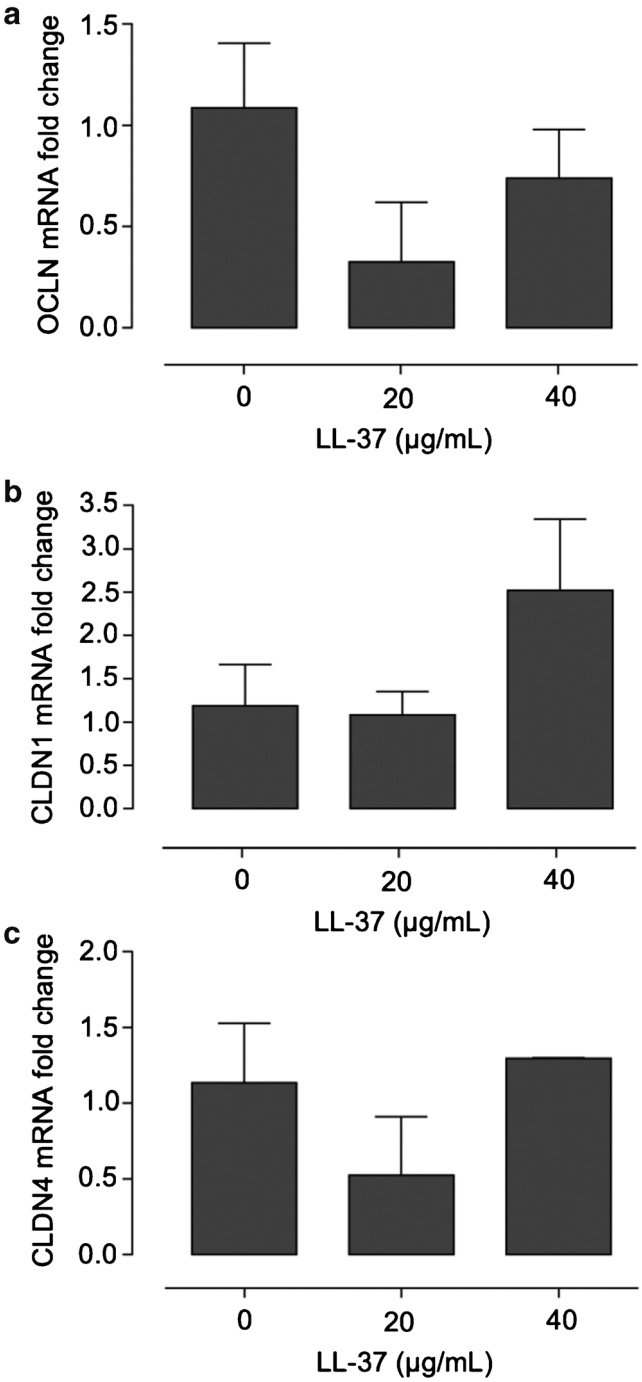

Fig. 2 Synthetic cathelicidin does not regulate the transcriptional gene expression of tight junction proteins in colonic epithelium. Relative mRNA gene expressions of (**a**) OCLN, (**b**) CLDN1 and (**c**) CLDN4 were determined by RT-qPCR in colonic epithelial T84 monolayers incubated with increasing concentrations of LL-37 (0–40 μg/mL) for up to 16 h. Means + the standard error (SEM) are shown ($n = 3$ independent experiments run in duplicate). Only significant comparisons ($^*P < 0.05$) with untreated cells are noted

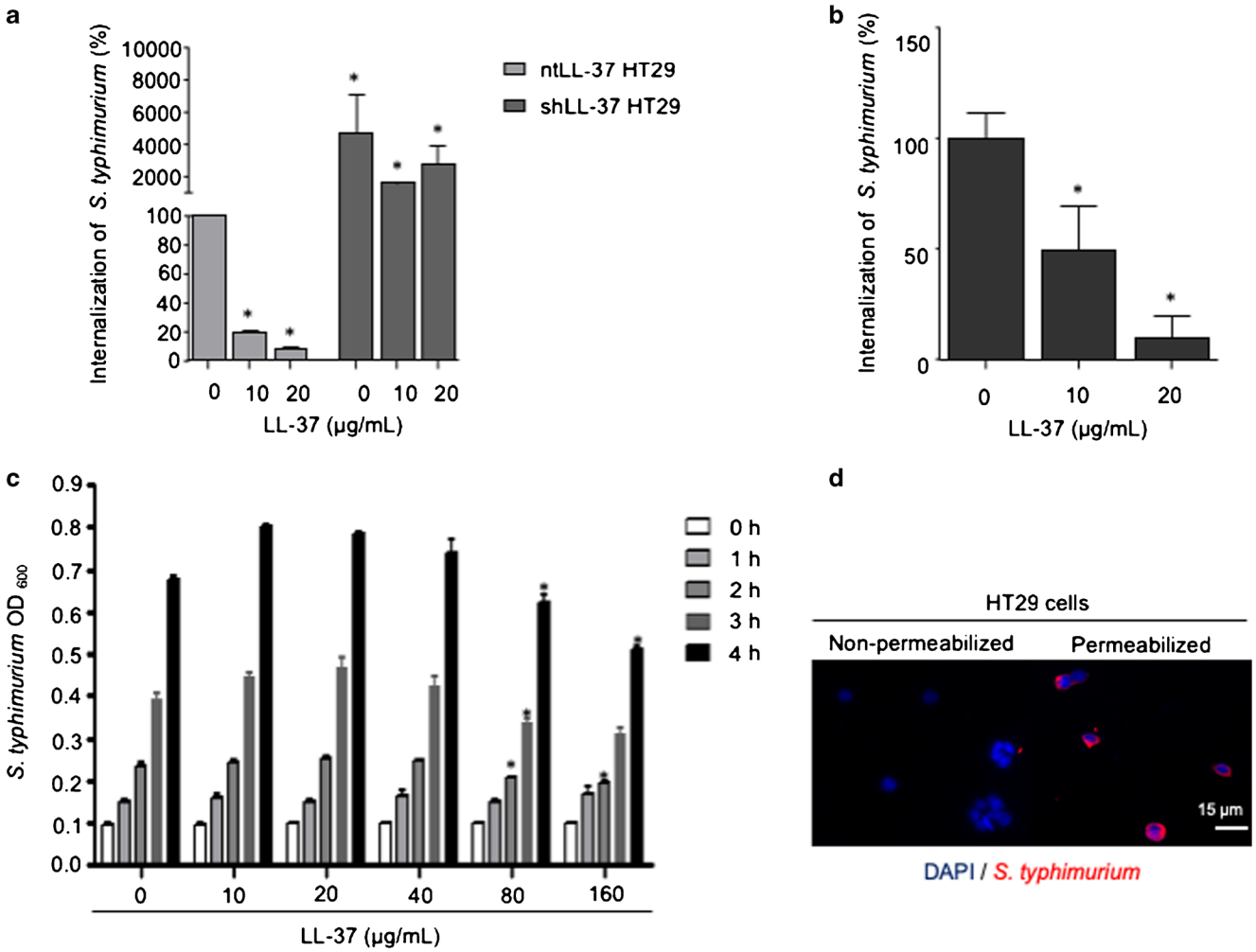

Fig. 3 *S. typhimurium* invasion in colonic epithelium genetically deficient in cathelicidin and stimulated with synthetic cathelicidin. Non-targeting (Nt) and genetically silenced (shLL-37) colonic epithelial HT29 cells were pre-treated with LL-37 (0–20 μg/mL; for 1 h), followed by challenge with *S. typhimurium* at MOI 5:1 for 24 h (**a**) or at MOI 1:1 for 16 h (**b**). Bacterial internalization was assessed by bacterial counting in cell lysates with 0.2% Triton X-100. For assessing direct killing, *S. typhimurium* was incubated with increasing concentrations of LL-37 in sera-free DMEM media and bacteria growth assessed by OD_{600} (**c**). Immunostaining for *S. typhimurium* in non-permeabilized and permeabilized infected HT29 cells (scale bar = 15 μm) (**d**). Only significant comparisons in the amount of *S. typhimurium* (*$P < 0.05$) compared with ntLL-37 untreated cells (**a**) or untreated shLL-37 cells (**b**) are noted

MOI 5:1) (Fig. 3a) but evident at an earlier time point and lower MOI (16 h; MOI 1:1; ~50–90% reduction) (Fig. 3b).

The function of synthetic LL-37 preventing bacterial invasion did not correspond to direct killing effects. Microbicidal activity of LL-37 against *S. typhimurium* in the first 2 h occurred at a peptide concentration of ≥ 80 μg/mL, above the concentrations used in our internalization studies (Fig. 3c). Internalization of *S. typhimurium* into HT29 cells was confirmed by immunofluorescent microscopy with *Salmonella*-specific antibodies, where bacteria were only detected in the cytoplasm of permeabilized cells (Fig. 3d). Thus, endogenous and exogenous cathelicidin prevents the invasion of *S. typhimurium* into the colonic epithelium.

Endogenous LL-37 is involved in TLR4 signaling and production of pro-inflammatory IL-1β during *S. typhimurium* infection

Mechanistically, it has been established that synthetic LL-37 selectively modulates TLR4 and TLR9 in the intestinal epithelium (Marin et al. 2017). To assess whether the immunomodulatory roles of cathelicidin in regulating *S. typhimurium* infection are attributed to endogenous or exogenous sources, TLR4, TLR9 and pro-inflammatory cytokines were studied in shLL-37 cells supplemented with synthetic cathelicidin. ShLL-37 cells responded to the *S. typhimurium* challenge with a lower gene expression of TLR4 compared with ntLL-37 cells (Fig. 4a). The levels of TLR4 gene expression were unmodified by the addition of synthetic LL-37, in both shLL-37

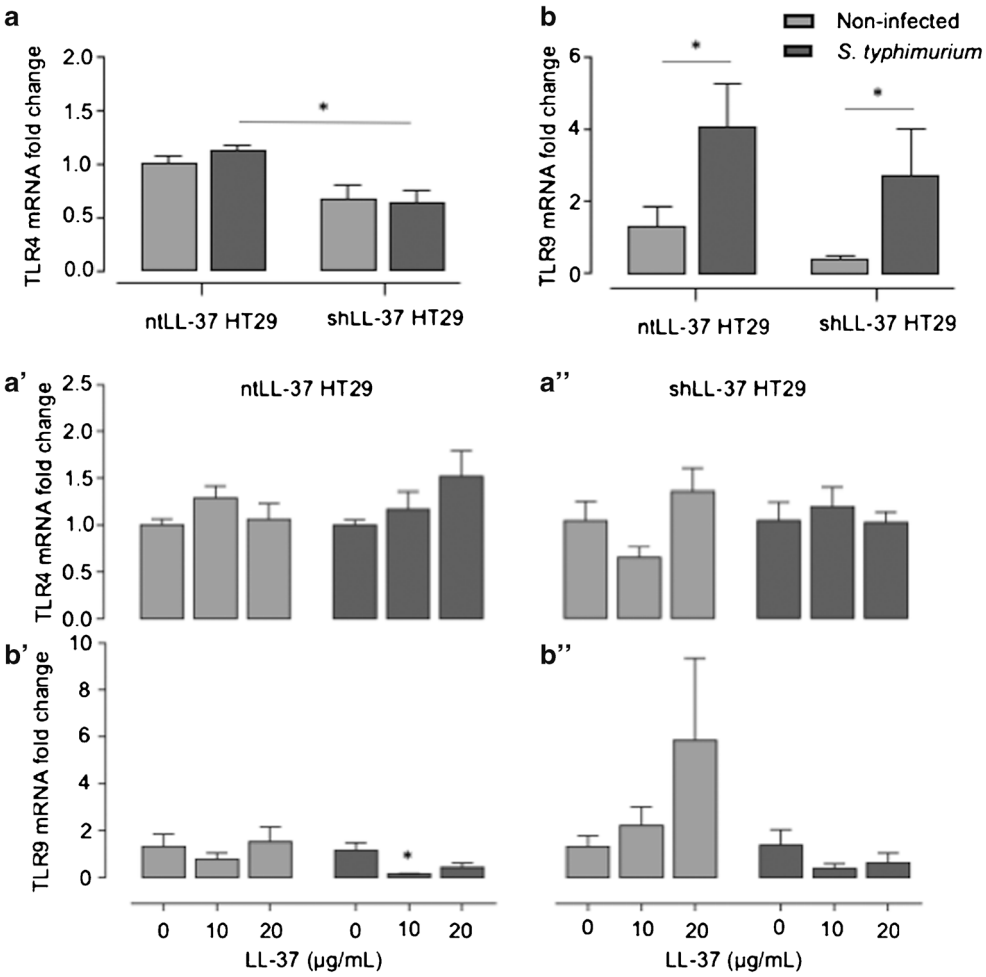

Fig. 4 Gene expression of TLR4 and TLR9 in colonic epithelium genetically deficient in cathelicidin, challenged by *S. typhimurium* and stimulated with synthetic cathelicidin. Relative mRNA gene expressions of TLR4 (a) and TLR9 (b) were determined by RT-qPCR in non-targeting (nt) and genetically silenced (shLL-37) colonic epithelial HT29 cells infected with *S. typhimurium* (MOI 5:1; 4 h). Cells were preincubated with synthetic LL-37 (0–20 μg/mL; 1 h) and challenged with *S. typhimurium* (MOI 5:1; 4 h) (a′ and a″ and b′ and b″). Means + the standard error (SEM) are shown ($n = 3$ independent experiments run in duplicate). Gene expression is relative to uninfected ntLL-37 HT29 cells (a, b) or to untreated cells of the same type (a′ and a″ and b′ and b″)

and ntLL-37 cells (Fig. 4a′, a″). The gene expression of TLR9 was increased after *S. typhimurium* infection, although the TLR9 upregulation was indistinct between shLL-37 and ntLL-37 cells (Fig. 4b). The levels of TLR9 mRNA expression were not modified by synthetic LL-37, except in infected ntLL-37 cells, where a downregulation was observed (Fig. 4b′, b″). These studies indicate that the endogenous synthesis of cathelicidin mostly contributes to the recognition of *S. typhimurium* via TLR4 signaling in colonic epithelial cells.

The NLRP3 inflammasome is a protein complex involved in IL-1β and IL-18 processing following exposure to both pathogen- and danger-associated molecular patterns. In order to examine if cathelicidin modulates the IL-1β/IL-18 response, we assessed gene expression of IL-1β and IL-18 after exposure to exogenous cathelicidins. Our studies show *S. typhimurium* increased gene expression of IL-1β in both shLL-37 and ntLL-37 cells (Fig. 5a). *S. typhimurium*-infected shLL-37 cells showed lower IL-1β mRNA expression levels in comparison to infected ntLL-37 cells (Fig. 5a). There was no difference in IL-1β gene expression in uninfected shLL-37 in comparison to ntLL-37 cells. The IL-1β gene response was not modified by the addition of synthetic LL-37 except in infected shLL-37 cells (Fig. 5a′, a″). Gene expression of IL-18 remained unmodified upon the *S. typhimurium* challenge in both shLL-37 and ntLL-37 cells, with exogenous cathelicidin providing no further effect (Fig. 5b, b′, b″). These results indicate a role of endogenous cathelicidins in the regulation of IL-1β gene expression by the colonic epithelium.

Discussion

S. typhimurium colitis is characterized by mucosal damage, erythema and edema and diarrhea (Santos 2014). Bacterial invasion and disruption of epithelial integrity are essential processes in *S. typhimurium* pathogenesis that require overcoming innate epithelial defenses. In the present work, human cathelicidin, LL-37, produced by the colonic epithelium or administrated as synthetic peptides, results in enhanced epithelial defenses against *S. typhimurium* and induction of pro-inflammatory cytokines. First, we demonstrated that colonic epithelial cells genetically deficient to

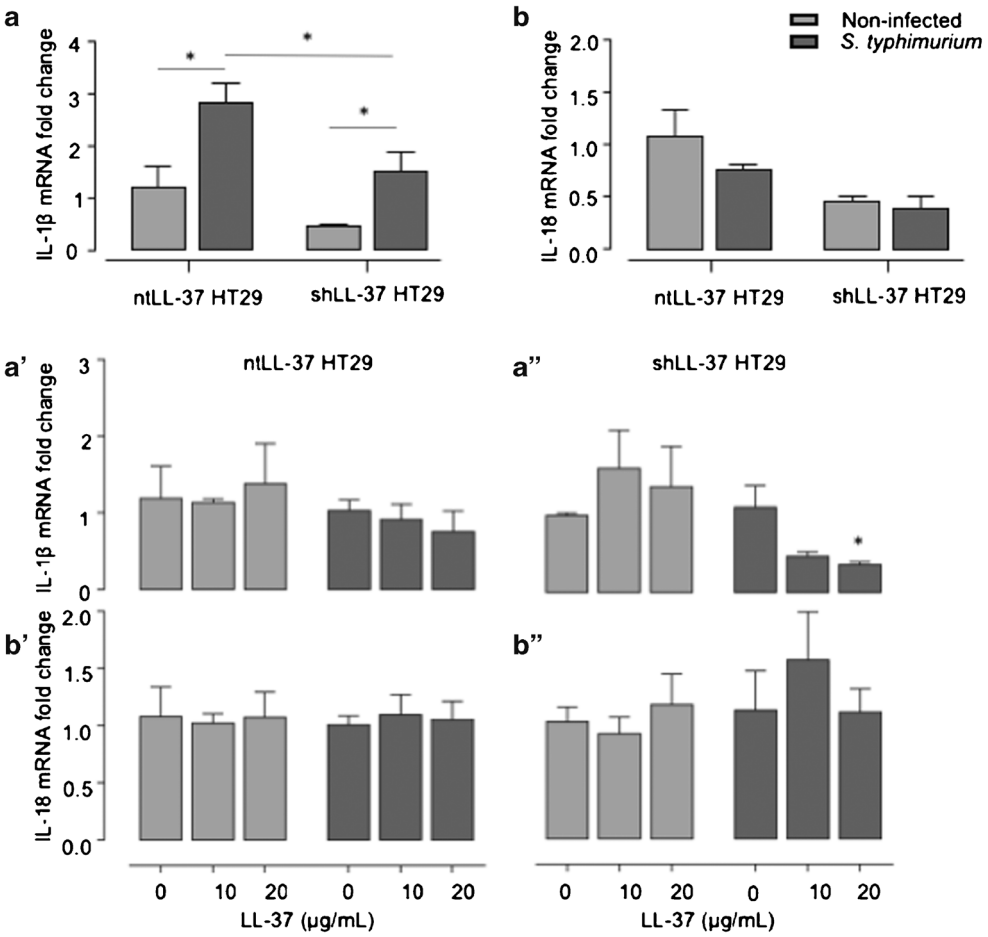

Fig. 5 Gene expression of IL-1β and IL-18 in colonic epithelium genetically deficient in cathelicidin, challenged by *S. typhimurium* and stimulated with synthetic cathelicidin. Relative mRNA gene expressions of IL-1β (a) and IL-18 (b) determined by RT-qPCR were assessed in non-targeting (Nt) and genetically silenced (shLL-37) colonic epithelial HT29 cells infected with *S. typhimurium* (MOI 5:1; 4 h). Cells were preincubated with synthetic LL-37 (0–20 μg/mL; 1 h) and challenged with *S. typhimurium* (MOI 5:1; 4 h) (a′ and a″ and b′ and b″). Means + the standard error (SEM) shown ($n = 3$ independent experiments run in duplicate). Gene expression is relative to uninfected ntLL-37 HT29 cells (a, b) or to untreated cells of the same type (a′ and a″ and b′ and b″)

produce endogenous cathelicidin have impaired ability to prevent the invasion of *S. typhimurium*. Strikingly, stimulation of colonic cells with synthetic LL-37 reduces the internalization of *S. typhimurium*, even when naturally occurring cathelicidin is lacking. Second, it is known *S. typhimurium* alters the gut functional barrier disturbing the scattering of tight junction complex proteins CLDN1, ZO-2 and E-cadherin and degrading ZO-1 (Kohler et al. 2007). We found that *S. typhimurium* rapidly disrupts tight junctional ZO-1 in colonic T84 cells and exogenous LL-37 re-establishes the chicken-wire pattern of tight junction proteins. Since the gene expression of tight junction proteins remains stable after supplementation with LL-37, post-translation effects and protein redistribution of tight junction proteins is likely to occur after treatment with synthetic cathelicidins. However, cathelicidin of other species may induce tight junction protein genes on certain colonic cell types, as cathelicidin derived from *Bungarus fascia* snakes (cathelicidin-WA) has been demonstrated to induce tight junction protein genes in enterocytes of pigs (Yi et al. 2016). Moreover, administration of cathelicidin-WA in piglets with clinical diarrhea improved intestinal morphology (increased villus and microvillus heights) and enhanced intestinal barrier function by increasing tight junction protein expression in intestinal epithelial cells (Yi et al. 2016).

Our work indicates that the immune stimulatory properties of cathelicidins are responsible for enhanced colonic epithelial defenses against *S. typhimurium*. Endogenous cathelicidin promotes the synthesis of TLR4 in colonic epithelial cells and its response to *S. typhimurium* (Fig. 4a). In agreement, synthetic LL-37, in synergy with bacterial LPS, induced activation of TLR4 whereas abrogated TLR9 responses to its ligand CpG DNA in the same colonic epithelial cell line (HT29) (Marin et al. 2017). By promoting TLR4, cathelicidin could revert the physiological hyporesponsive status of colonic epithelial cells during homeostasis (Meng et al. 2015) and initiate inflammatory responses (e.g., IL8 chemokine expression) in response to Gram-negative enteric pathogens.

Cytokines secreted by colonic epithelial cells and infiltrating leukocytes modulate the gut innate immune responses against *Salmonella* spp. (Nadeau et al. 2002). Increased expression of IL-1β and IL-18 from ileal CD8+ lymphocytes has been associated with protection against *S. typhimurium* (Bai et al. 2015). Our results found that endogenous cathelicidin contributes to colonic

IL-1β gene induction in response to *S. typhimurium* and may have a role in the inflammasome-mediated immune response to *S. typhimurium*. Inflammasome receptors activate caspase-1 in response to *S. typhimurium* together with endogenous signals to recruit ASC and caspase-1 for processing pro-IL-1β (Broz et al. 2010). In agreement, knockout mice lacking functional caspases or mice deficient in the end product of inflammasome activation (i.e., IL-1β and IL-18) have higher bacterial loads and succumb earlier upon infection with *S. typhimurium* (Broz et al. 2012; Raupach et al. 2006). Thus, cathelicidins, mostly those naturally occurring, might regulate TLR4 signaling and production of pro-inflammatory IL-1β during *Salmonella* spp. infection.

Immunomodulatory roles of cathelicidins could be complemented by direct microbicidal properties. Murine cathelin-related antimicrobial peptide (CRAMP) and cathelicidin-BF isolated from the venom of *Bungarus fasciatus* snakes have been shown to inhibit *S. typhimurium* growth (Rosenberger et al. 2004; Xia et al. 2015). A linear, 60-residue proline-rich C-terminal domain peptide (Bac7), of bovine origin and precursor of the cathelicidin family, has bactericidal killing properties against *Salmonella* spp., even in the presence of murine serum or plasma components (Benincasa et al. 2010; Young-Speirs et al. 2018). However, direct killing activity by cathelicidin in vivo in the gut remains inconclusive as cathelicidin stability may be influenced by physiological conditions, including high salt concentrations and the presence of serum proteins (Durr et al. 2006; Kosciuczuk et al. 2012). This work demonstrates that cathelicidins might have important immunomodulatory roles in gut defenses beyond bactericidal effects.

Antibiotics are currently critical in the treatment of invasive *Salmonella* spp. infections, although, use of conventional antibiotics is becoming unsustainable due to the emergence of clinical multi-drug resistant isolates of Salmonellae (Harish and Menezes 2015). For example, *S. typhimurium* ST313, multi-resistant to all commonly used antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamide and trimethoprim), has been found in human and poultry feces in Burkina Faso, Africa (Kagambega et al. 2018). Facing this problematic situation, cathelicidins are potential alternatives for controlling pathogenic microbes, including antibiotic-resistant microorganisms. Interestingly, the immunomodulatory functions of cathelicidins occur at much lower concentrations than the microbicidal concentrations. This likely results in antibacterial functions that are less susceptible to microbial resistance through accumulation of antibiotic resistance genes. Our finding that the human cathelicidin LL-37, endogenous and exogenous, contributes to controlling the invasion of *S. typhimurium* and maintaining the gut epithelial integrity by modulating TLR4 and pro-inflammatory IL-1β provides evidence of new roles of cathelicidins controlling enteric infections.

Acknowledgments MM was supported by the National Scientific and Technical Research Council (CONICET; Argentina) and DD by the Markin Undergraduate Student Research Program (USRP) in Health and Wellness (University of Calgary). We thank Dr. John Kastelic (University of Calgary, Faculty of Veterinary Medicine) for revising and editing the manuscript.

Funding information This research was supported by the Natural Sciences and Engineering Research Council (NSERC) Discovery Grant (RGPAS-2017- 507827), Alberta Agriculture and Forestry (2018F050R) and University of Calgary URGC SEM Faculty Seed Grant (no. 1041449).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Bai SP, Huang Y, Luo YH, Wang LL, Ding XM, Wang JP, Zeng QF, Zhang KY (2015) Effect of dietary nonphytate phosphorus content on ileal lymphocyte subpopulations and cytokine expression in the cecal tonsils and spleen of laying hens that were or were not orally inoculated with *Salmonella Typhimurium*. Am J Vet Res 76:710–718

Benincasa M, Pelillo C, Zorzet S, Garrovo C, Biffi S, Gennaro R, Scocchi M (2010) The proline-rich peptide Bac7(1-35) reduces mortality from *Salmonella typhimurium* in a mouse model of infection. BMC Microbiol 10:178

Bonnington KE, Kuehn MJ (2016) Outer membrane vesicle production facilitates LPS remodeling and outer membrane maintenance in *Salmonella* during environmental transitions. mBio 7(5). https://doi.org/10.1128/mBio.01532-16

Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM (2010) Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against *Salmonella*. J Exp Med 207:1745–1755

Broz P, Ohlson MB, Monack DM (2012) Innate immune response to *Salmonella typhimurium*, a model enteric pathogen. Gut Microbes 3:62–70

Cobo ER, Kissoon-Singh V, Moreau F, Holani R, Chadee K (2017) MUC2 mucin and butyrate contribute to the synthesis of the antimicrobial peptide cathelicidin in response to *Entamoeba histolytica*- and dextran sodium sulfate-induced colitis. Infect Immun 85(3). https://doi.org/10.1128/IAI.00905-16

Dharmsathaphorn K, McRoberts JA, Mandel KG, Tisdale LD, Masui H (1984) A human colonic tumor cell line that maintains vectorial electrolyte transport. Am J Phys 246:G204–G208

Durr UH, Sudheendra US, Ramamoorthy A (2006) LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta 1758:1408–1425

Harish BN, Menezes GA (2015) Determination of antimicrobial resistance in *Salmonella* spp. Methods Mol Biol 1225:47–61

Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF (2002) Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. Infect Immun 70:953–963

Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF (2005) Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. J Immunol 174:4901–4907

Jung K, Eyerly B, Annamalai T, Lu Z, Saif LJ (2015) Structural alteration of tight and adherens junctions in villous and crypt epithelium of the small and large intestine of conventional nursing piglets infected with porcine epidemic diarrhea virus. Vet Microbiol 177:373–378

Kagambega A, Lienemann T, Frye JG, Barro N, Haukka K (2018) Whole genome sequencing of multidrug-resistant *Salmonella enterica* serovar *Typhimurium* isolated from humans and poultry in Burkina Faso. Tropical Med Health 46:4

Kawai T, Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34:637–650

Kohler H, Sakaguchi T, Hurley BP, Kase BA, Reinecker HC, McCormick BA (2007) *Salmonella enterica* serovar *Typhimurium* regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. Am J Physiol Gastrointest Liver Physiol 293: G178–G187

Konig J, Wells J, Cani PD, Garcia-Rodenas CL, MacDonald T, Mercenier A, Whyte J, Troost F, Brummer RJ (2016) Human intestinal barrier function in health and disease. Clin Transl Gastroenterol 7:e196

Koon HW, Shih DQ, Chen J, Bakirtzi K, Hing TC, Law I, Ho S, Ichikawa R, Zhao D, Xu H, Gallo R, Dempsey P, Cheng G, Targan SR, Pothoulakis C (2011) Cathelicidin signaling via the toll-like receptor protects against colitis in mice. Gastroenterology 141(1852–1863): e1851–e1853

Kosciuczuk EM, Lisowski P, Jarczak J, Strzalkowska N, Jozwik A, Horbanczuk J, Krzyzewski J, Zwierzchowski L, Bagnicka E (2012) Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep 39:10957–10970

Lee CW, Bennouna S, Denkers EY (2006) Screening for *Toxoplasma gondii*-regulated transcriptional responses in lipopolysaccharide-activated macrophages. Infect Immun 74:1916–1923

Marin M, Holani R, Shah CB, Odeon A, Cobo ER (2017) Cathelicidin modulates synthesis of toll-like receptors (TLRs) 4 and 9 in colonic epithelium. Mol Immunol 91:249–258

Meng D, Zhu W, Shi HN, Lu L, Wijendran V, Xu W, Walker WA (2015) Toll-like receptor-4 in human and mouse colonic epithelium is developmentally regulated: a possible role in necrotizing enterocolitis. Pediatr Res 77:416–424

Mogensen TH (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 22:240–273 Table of Contents

Nadeau WJ, Pistole TG, McCormick BA (2002) Polymorphonuclear leukocyte migration across model intestinal epithelia enhances *Salmonella typhimurium* killing via the epithelial derived cytokine, IL-6. Microbes Infection / Institut Pasteur 4:1379–1387

Otte JM, Zdebik AE, Brand S, Chromik AM, Strauss S, Schmitz F, Steinstraesser L, Schmidt WE (2009) Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity. Regul Pept 156: 104–117

Poppe C, Smart N, Khakhria R, Johnson W, Spika J, Prescott J (1998) *Salmonella typhimurium* DT104: a virulent and drug-resistant pathogen. Canadian Vet J 39:559–565

Raupach B, Peuschel SK, Monack DM, Zychlinsky A (2006) Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar *Typhimurium* infection. Infect Immun 74:4922–4926

Rosenberger CM, Gallo RL, Finlay BB (2004) Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication. Proc Natl Acad Sci U S A 101: 2422–2427

Royston JP (1982) Algorithm AS 181: the W test for normality. J R Stat Soc Ser C (Applied Statistics) 31:176–180

Santos RL (2014) Pathobiology of salmonella, intestinal microbiota, and the host innate immune response. Front Immunol 5:252

Schaller-Bals S, Schulze A, Bals R (2002) Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. Am J Respir Crit Care Med 165:992–995

Xia X, Zhang L, Wang Y (2015) The antimicrobial peptide cathelicidin-BF could be a potential therapeutic for *Salmonella typhimurium* infection. Microbiol Res 171:45–51

Yamaura Y, Chapron BD, Wang Z, Himmelfarb J, Thummel KE (2016) Functional comparison of human colonic carcinoma cell lines and primary small intestinal epithelial cells for investigations of intestinal drug permeability and first-pass metabolism. Drug Metab Dispos 44:329–335

Yi H, Zhang L, Gan Z, Xiong H, Yu C, Du H, Wang Y (2016) High therapeutic efficacy of cathelicidin-WA against postweaning diarrhea via inhibiting inflammation and enhancing epithelial barrier in the intestine. Sci Rep 6:25679

Young-Speirs M, Drouin D, Cavalcante PA, Barkema HW, Cobo ER (2018) Host defense cathelicidins in cattle: types, production, bioactive functions and potential therapeutic and diagnostic applications. Int J Antimicrob Agents 51:813–821

Zanetti M (2005) The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol 7:179–196