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Enhancement of cholera vaccine-induced chemokine expression by short chain fatty acids in human intestinal epithelial cells

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심주리

ABSTRACT

Enhancement of cholera vaccine-induced chemokine expression by short chain fatty acids in human intestinal epithelial cells

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Objectives

Dukoral™ and Shanchol™ are currently available oral cholera vaccines, but both vaccines have some limitations in terms of vaccine efficacy such as low immunogenicity, short term protection and requirement of high doses. Oral cholera vaccines can activate intestinal epithelial cells which play a key role in the recruitment and activation of immune cells by producing chemokines. Short chain fatty acids (SCFAs) such as butyrate, acetate, and propionate have been known to modulate immune responses in the gut. However, the effect of SCFAs on the mucosal vaccine-induced immune responses has been poorly understood. The present study investigated whether SCFAs modulate the chemokine expression induced by a bivalent, killed whole cell, oral cholera vaccine, Shanchol™, in human intestinal epithelial cells.

Methods

The human intestinal epithelial cells, Caco-2 and HT-29, were stimulated with inactivated *Vibrio cholerae* or Shanchol™. In order to examine the effect of SCFAs on Shanchol™-induced chemokine production, Caco-2 cells and primary intestinal epithelial cells were treated with Shanchol™ in the presence of acetate, butyrate, or propionate. For the polarization of Caco-2 cells, the cells were cultured for 4 weeks in a transwell system, and then, the cells were apically or basolaterally stimulated with Shanchol™ and/or butyrate. To investigate the intracellular signaling events, Caco-2 cells were pre-treated with pertussis toxin, mepenzolate bromide, SB203580, PD98059, SP600125 and oxATP, respectively, and subsequently, the cells were stimulated with Shanchol™ and/or butyrate. In addition, Caco-2 cells were stimulated with Shanchol™ in the presence or absence of adenosine triphosphate (ATP) or trichostatin A (TSA). Chemokine production was determined using real-time RT-PCR and enzyme-linked immunosorbent assay (ELISA). ATP secretion in Caco-2 cells in response to Shanchol™ and/or butyrate was determined by ATP luminescence assay. After co-treatment with Shanchol™ and butyrate, the migration of immature dendritic cells (DCs) was examined using a transwell system.

Results

When Caco-2 and HT-29 cells were stimulated with Shanchol™, mRNA expression of several chemokines including CCL2, CCL5, CCL20 and CXCL10 substantially increased whereas the production of their proteins was negligibly induced. Interestingly, a significant increase of CCL20 secretion was observed when the cells were co-stimulated with Shanchol™ and butyrate, but neither acetate nor propionate did not produce such effect. Besides, co-treatment with Shanchol™ and butyrate

up-regulated only CCL20 expression, but not other chemokines. Furthermore, butyrate increased Shanchol™-induced CCL20 production in polarized epithelial cells and primary intestinal epithelial cells. Co-treatment with Shanchol™ and butyrate increased the expression of a butyrate receptor, GPR109A whereas CCL20 induction was suppressed by a specific inhibitor of GPR109A, indicating that the induction of CCL20 secretion is associated with GPR109A activation. TSA, an histone deacetylase (HDAC) inhibitor, enhanced Shanchol™-induced CCL20 production, suggesting that HDAC inhibition is involved in CCL20 production. In addition, the co-treatment with Shanchol™ and butyrate synergistically increased ATP levels in the culture supernatant and CCL20 secretion was decreased at the blockade of extracellular ATP receptor, P2X7, implying that ATP is also involved in the synergistic production of CCL20. The number of migrated immature DCs was increased when the cells were treated with the culture supernatant of Caco-2 cells co-treated with Shanchol™ and butyrate compared to that of Shanchol™-treated Caco-2 cells.

Conclusion

Collectively, the present study demonstrated that one of SCFAs, butyrate potently enhanced Shanchol™-induced CCL20 production in human intestinal epithelial cells via HDAC inhibition and ATP-P2X7 signaling by activating GPR109A, potentially contributing to the enhancement of the mucosal immune responses in the gut.

Keywords: Butyrate, Cholera vaccine, Chemokine, Mucosal immunity
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CONTENTS

Chapter I. Introduction

Cholera is an acute, non-inflammatory diarrheal disease that is a leading cause of life-threatening dehydration and shock [1]. It is endemic in many regions, in particular, Asia and Africa, and caused by ingestion of water or food contaminated with *Vibrio cholerae* [2]. *V. cholerae* is a Gram-negative, facultative anaerobic bacterium which colonizes human small intestine and secretes an enterotoxin called cholera toxin (CT) [3, 4]. CT is responsible for the fatal diarrhea and it is composed of five B subunits surrounding A subunit which is enzymatically active [5]. The B subunit pentamer binds to GM1 gangliosides in intestinal epithelial cells, and then the A subunit translocates into intracellular region to activate adenylate cyclase, which raises intracellular cyclic AMP level, leading to massive efflux of electrolytes and water into the lumen [6-8]. More than 200 serogroups of *V. cholerae* have been identified, but only O1 and O139 serogroups are related with epidemic cholera [9], and these serogroups produce CT, while other serogroups hardly produce CT [10]. The O1 serogroup is classified into two biotypes, classical and El Tor, and they can be further divided into three serotypes, Inaba, Ogawa and Hikojima [11].

Currently, two oral cholera vaccines are commercially available. Dukoral™ is formulated with heat- or formaldehyde-inactivated whole cells of *V. cholerae* O1 with recombinant cholera toxin B (CTB) subunit [12, 13]. Shanchol™ is a bivalent vaccine which comprises formaldehyde-inactivated *V. cholerae* O139 and heat- or formaldehyde-inactivated *V. cholerae* O1 without recombinant CTB subunit. Dukoral™ is found to be safe and stable with a shelf life of 3

years and induces strong anti-bacterial and anti-toxin immunity [14, 15]. Furthermore, Dukoral™ also provides cross-protection against cholera toxin produced by enterotoxigenic *Escherichia coli* (ETEC) through its CTB subunit [16]. However, it provides poor protection in children under 5 years of age [17], and requires a cold supply chain and high production cost [18]. Shanchol™ that has been licensed in Vietnam and India, is immunogenic across all populations in not only cholera endemic areas, but also other areas without historical exposure to cholera [19]. Although Shanchol™ does not require bicarbonate buffer and is less expensive than Dukoral™, it also showed a poor protective efficacy in children who are the most vulnerable to cholera [20].

Mucosal immune responses induced by oral cholera vaccine is mainly mediated by anti-toxic and anti-bacterial secretory IgA (sIgA) antibodies in the gut [21]. sIgA is the first line of protection in gut mucosa and enhances nonspecific defense via immune exclusion, neutralization of the toxins and antigen excretion [22]. The gastrointestinal tract is one of the mucosal induction sites for oral cholera vaccines. It has gut-associated lymphoid organ such as Peyer's patches, where immune responses are initiated and consist of various types of the innate or adaptive immune cells such as macrophages, DCs, T cells and B cells that play a role in mucosal immune system [23]. Following oral vaccination, antigens in the mucosal inductive site captured by DCs and presented to T cells, subsequently facilitating class switch and somatic mutation and differentiation of B cells to plasmablasts for antibody production. Finally, IgA-producing plasmablasts home to effector site and the antigen-specific

dimeric IgA is produced and transported to the lumen [22, 24].

The mucosal surface of gastrointestinal tract interacts with trillions of gut microbiota and intestinal epithelial cells, which directly recognize the external microbial components, are active participants in the mucosal defense system. Moreover, the intestinal epithelial cells are important for the communication system of mucosal immunity by producing mucosal mediators such as chemokines [25]. Chemokines play a central role in the communication system of mucosal immunity by regulating the patterns of chemotactic migration of leukocytes, including antigen-presenting cells [26]. Therefore, chemokines are associated with the regulation of adaptive immune responses by recruiting and activating the lymphocytes to the site of infection or vaccination. For example, CCL20, also known as macrophage inflammatory protein-3α (MIP-3α), binds to CC chemokine receptor-6 (CCR6) [27] and attracts immature DCs [28], memory T cells [29] and B cells [30] to the site of mucosal vaccination. Moreover, gut microbiota act as key contributors to intestinal homeostasis through the generation of metabolites by the fermentation of non-digestible carbohydrates [31]. The major end products of fermentation are short chain fatty acids (SCFAs) such as acetate, butyrate and propionate, which activate GPR41, GPR43, and GPR109A for the induction of protective immune responses by regulating chemokine production and neutrophil migration in the gut [32].

Previous reports have suggested that SCFAs are important immune modulators

in the communication between gut microbiota and host cells, and regulate intestinal immune responses by enteric pathogens. SCFAs can modulate immune cell functions either via inhibition of histone deacetylase (HDAC) activity regulating epigenetic modification, or the activation (GPCRs) [32-34]. Several reports have also demonstrated that butyrate is involved in energy metabolism [35], proliferation and apoptosis [36] of colonocytes contributing to intestinal homeostasis. Furthermore, SCFAs play critical roles in the enhancement of antimicrobial peptide production, epithelial integrity [37] and IgA production [38]. Moreover, the differentiation of regulatory T cells [33, 39], the generation of tolerogenic DCs [40] and the induction of anti-inflammatory responses [41, 42] are involved in SCFAs. However, the effect of SCFAs on vaccine induced-mucosal immune responses has not been clearly understood. The current study investigates whether SCFAs modulate oral cholera vaccine induced-chemokine expression in the human intestinal epithelial cells for the regulation of mucosal immune responses.

Chapter II. Materials and Methods

2.1. Reagents

A killed, whole-cell oral cholera vaccine Shanchol™ which contains heat- or formalin-inactivated *V. cholerae* O1 and formalin-inactivated *V. cholerae* O139 was purchased from Shantha biotechnics (Hyderabad, India). Pam2CSK4 was purchased from InvivoGen (San Diego, CA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, sodium acetate, sodium butyrate, sodium propionate, adenosine 5′-triphosphate (ATP), trichostatin A (TSA), mepenzolate bromide (MPN), oxATP and C646 were obtained from Sigma-Aldrich (St. Louis, MO, USA). A p38 kinase inhibitor, SB203580, an ERK inhibitor, PD98059, a JNK inhibitor, SP600125 were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Bacterial culture and preparation of inactivated bacteria

V. cholerae O1 Inaba (T19479), O1 Ogawa (X25049), and O139 (4260B) were kindly provided by Prof. Jan Holmgren (University of Gothenburg, Sweden). All bacterial strains were cultured in Brain Heart Infusion (BHI) broth at 37°C for 4 h with gentle shaking. In order to prepare the inactivation of *V. cholerae*, bacterial pellets were harvested by centrifugation for 5 min at 6,371×g washed with phosphate-buffered saline (PBS) and re-suspended in PBS. Heat-

inactivated bacteria were prepared by heating at 70°C for 2 h with gentle shaking. Formalin-inactivated *V. cholerae* was prepared by incubating bacterial suspension in 0.57 % formaldehyde for 5 h with stirring. After incubation, bacteria were extensively washed 8 times with PBS to remove residual formalin. To confirm the complete inactivation of *V. cholerae*, heat- or formalin-inactivated *V. cholerae* was plated on BHI agar plates for 24 h. The killed bacteria were re-suspended in PBS and adjusted to appropriate colony-forming unit (CFU)/ml which was based on optical density value at 600 nm absorbance.

2.3. Cell culture

Human intestinal epithelial cell lines, Caco-2 and HT-29, were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in complete Dulbecco's modified Eagle medium (DMEM), (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Burlington, ON, Canada), and 1% penicillin-streptomycin (HyClone). The cells were grown at 37ºC in a 5% CO_2-humidified incubator. For the polarization of cells, Caco-2 cells were cultured on 12-mm Transwell® with 0.4 μm pore polycarbonate membrane insert (Costar, Corning, NY, USA) up to 4 weeks. The polarization of the cells was confirmed by measuring trans-epithelial electrical resistance (> 400 Ω/cm^2) with EVOM2 (World Precision Instruments, Sarasota, FL, USA). Primary human intestinal epithelial cells including SNU-407 and SNU-61 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and maintained in complete Roswell Park Memorial

Institute (RPMI) 1640 (Hyclone) containing 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 5% CO_2-humidified incubator.

2.4. Preparation of human monocyte-derived DCs

The human peripheral blood was provided by Korean Red Cross after obtaining informed consent. Experiments using human blood were performed under approval of the Institutional Review Board at Seoul National University. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque Plus. $CD14^+$ monocytes were isolated from PBMCs using CD14 magnetic beads. The purified $CD14^+$ monocytes were suspended in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and plated in 60-mm cell culture dishes at 2×10^6 cells/ml in the presence of 10 ng/ml GM-CSF, and 10 ng/ml IL-4. Then, the monocytes were cultured for 6 days in the presence of GM-CSF and IL-4 to differentiate into immature DCs. The culture media supplemented with cytokines was changed every 3 days.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized from 3 μg of RNA using random hexamers (Roche, Basel, Switzerland) and reverse transcriptase (M-MLV RT) (Promega, Madison, WI,

USA). Amplification of cDNA was performed by PCR in a total volume of 20 μl containing EmeraldAmp PCR Master Mix (Takara Biomedical Inc, Osaka, Japan) and 10 pmole of human chemokine specific primers. Primer sequences for specific gene transcripts are listed in Table 1. PCR amplification was performed as follows: 32 or 35 cycles at 95 ℃ for 5 min, 95 ℃ for 40 sec, 60 ℃ for 40 sec, 72 ℃ for 40 sec, and 72 ℃ for 7 min for all chemokines used in this study, and 24 cycles at 95 ℃ for 5 min, 95 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 1 min, and 72 ℃ for 10 min for β-actin. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide or Safeview (Applied Biological Materials, Richmond, Canada) staining with a gel documentation system (Life Science Research, Hercules, CA, USA). Real-time RT-PCR was performed with Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA). PCR was performed to amplify cDNA in 20 μl of reaction volume with SYBR premix Ex Taq (perfect real time) (Takara Biomedical Inc.) and 10 pmole of human specific primers for chemokines or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed as follows: 1 cycle of 50℃ for 2 min and 95℃ for 10 min followed by 40 cycles of 95℃ for 15 sec, 60℃ for 1 min, and 1 cycle of 95 ℃ for 15 sec, 60℃ for 1 min, 95℃ for 30 sec and 60℃ for 15 sec. Relative expression levels were normalized to GAPDH by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences of genes used for RT-PCR analysis.

Primer	Forward primer	Reverse primer
CCL2	5'-TCCCCAGACACCCTGTTTTA-3'	5'-CAAAACATCCCAGGGGTAGA -3'
CCL3	5'-TGCAACCAGTTCTCTGCATC -3'	5'-ATTTCTGGACCCACTCCTCA -3'
CCL4	5'-CGCCTGCTGCTTTTCTTACA -3'	5'-AGCAGCTCAGTTCAGTTCCA -3'
CCL5	5'-GAAAGAACCGCCAAGTGTGT -3'	5'-GTAGAATCTGGGCCCTTCAA -3'
CCL20	5'-GCCAATGAAGGCTGTGACAT -3'	5'-AACCCCAGCAAGGTTCTTTC -3'
CCL25	5'-GTCCACACCCAAGGTGTCTT -3'	5'-TGTAGGGCGACGGTTTTATC -3'
CCL28	5'-GCTGATGGGGATTGTGACTT -3'	5'-GTTTCGTGTTTCCCCTGATG -3'
CXCL10	5'-GATGTTCTGACCCTGCTTCA -3'	5'-GAAAGAATTTGGGCCCCTTG -3'
GPR43	5'-CGGCCTCTGTATGGAGTGAT-3'	5'-CGGCCTCTGTATGGAGTGAT-3'
GPR109A	5'-GCTCCATCGGACTCACTAGC-3'	5'-GTCCCAACGCCTCACATAGT-3'
GAPDH	5'-AAGGTGAAGGTCGGAGTCAA-3'	5'-ATGACAAGCTTCCCGTTCTC-3'
β-actin	5'-GTGGGGCGCCCCAGGCACCA -3'	5'-CTCCTTAATGTCACGCACGATTTC -3'

2.6. Determination of chemokine production using enzyme-linked immunosorbent assay (ELISA)

Caco-2 or HT-29 cells (4×10^5 cells/ml) were cultured on 96-well culture plate until they were semi-confluent (70 - 80%). Subsequently, the cells were treated with indicated stimuli for 24 h. Then, the cell culture supernatants were collected and the production of chemokine was determined using Duoset ELISA kits (R&D Systems, Minneapolis, MN, USA) or ELISA kits (Biolegend, San Diego, USA) according to manufacturer's instructions.

2.7. Measurement of adenosine triphosphate (ATP) secretion

Caco-2 cells (4×10^5 cells/ml) were plated on 96-well culture plates for 24 h. Then, the cells were treated with Shanchol™ and/or butyrate for 2 h, then and the extracellular ATP concentration in the cell culture supernatants was determined with an ENLITIN® ATP Assay System (Promega) according to manufacturer's instructions.

2.8. Western blot analysis

Caco-2 cells (4×10^5 cells/ml) were plated for 24 h, and treated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for the indicated time periods. After the treatment, the cells were lysed with a lysis buffer (PRO-PREP™; iNtRON biotechnology, Gyeonggi-Do, Korea) containing 2 mM PMSF, 2 mM Na_3VO_4, 2 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein concentration was determined using BCA protein assay kit (Pierce, Rockford IL, USA). Subsequently, the equal amount of proteins was separated with 10% or 15% SDS-PAGE gel and electro-transferred onto a polyvinylidene

difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was probed with specific antibodies against MAP kinases including p38 kinase, ERK or JNK, followed by incubation with HRP-conjugated secondary antibodies. The immunoreaction was visualized using ECL reagents (Amersham Biosciences, Princeton, NJ, USA).

2.9. Determination of DC migration using trans-migration assay

Immature DCs (1×10^6 cells/ml) were added to the upper chamber of 24-well Transwell® with 5 μm pore polycarbonate membrane insert (Costar, Corning, NY, USA). Caco-2 cells (4×10^5 cells/ml) were plated in 6-well culture plates and stimulated with Shanchol™ and/or butyrate for 24 h. The culture supernatants (600 μl) were added to the lower chamber, and incubated at 37ºC for 2 h. Nonmigrating cells were removed from the upper chamber, and the cells on the surface of lower chamber were fixed with methanol for 30 min and then stained with 0.2% crystal violet for 1 h. Images of cells were obtained using a microscope, and the migrated cells in lower chamber were counted using trypan blue staining.

2.10. Statistical analysis

All data are expressed as mean values ± standard deviations (S.D.) from triplicates unless otherwise stated. Treatment groups were compared with an appropriate control group, and statistical analysis was determined using

unpaired two-tailed *t*-test with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant when $P < 0.05$.

Chapter III. Results

3.1. Inactivated *V. cholerae* strain induces the mRNA expression of chemokines in human intestinal epithelial cells

To elucidate whether heat- or formalin-inactivated *V. cholerae* strain induces chemokine expression in human intestinal epithelial cells, Caco-2 or HT-29 cells were stimulated with each strain of heat- or formalin-inactivated *V. cholerae*. As shown in Fig. 1, distinct patterns of chemokine production were observed in Caco-2 or HT-29 cells. Both heat and formalin-inactivated *V. cholerae* induced CCL2, CCL20 and CXCL10 mRNA expression in Caco-2 cells (Fig. 1A). Although heat- or formalin-inactivated *V. cholerae* did not increase CCL2 mRNA expression, up-regulated CCL5 mRNA expression was observed in HT-29 cells. Similar to Caco-2 cells, inactivated *V. cholerae*, except formalin-inactivated *V. cholerae* O139 provoked CCL20 mRNA expression in HT-29 cells (Fig. 1B). These results suggest that inactivated *V. cholerae* strain differently induced chemokine mRNA expression in human intestinal epithelial cells.

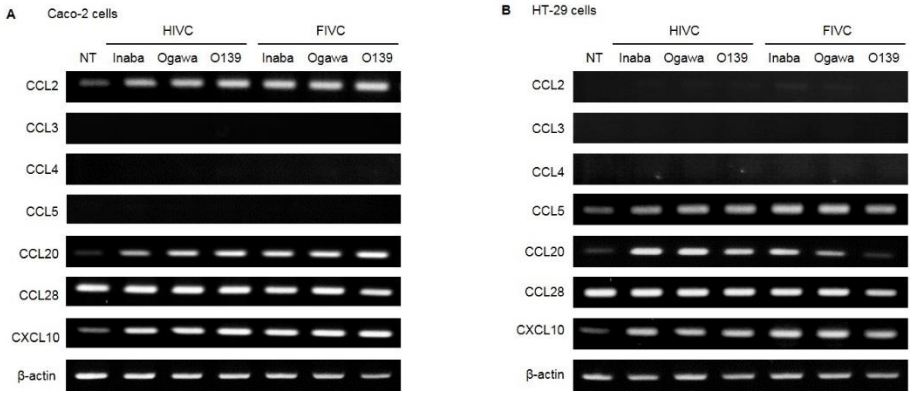

Figure 1. Inactivated *V. cholerae* induces chemokine mRNA expression in human intestinal epithelial cells. (A) Caco-2 cells (4×10^5 cells/ml) were stimulated with 10^8 CFU/ml of heat-or formalin-inactivated individual *V. cholerae* (HIVC and FIVC, respectively) strains (Inaba, Ogawa and O139) for 3 h. (B) HT-29 cells (4×10^5 cells/ml) were stimulated with 10^8 CFU/ml individual strains (Inaba, Ogawa and O139) of heat- or formalin-inactivated *V. cholerae* for 3 h. Then, total RNA was isolated and mRNA expression of chemokines was determined using RT-PCR. NT denotes non-treatment.

3.2. Shanchol™ dose-dependently induces mRNA expression of chemokines in human intestinal epithelial cells

A commercially-available oral cholera vaccine, Shanchol™ is a bivalent vaccine which is formulated with a mixture of heat- or formalin-inactivated whole cells of *V. cholerae* O1 Inaba, O1 Ogawa and O139 [38]. To examine whether Shanchol™ induces mRNA expression of chemokine in human intestinal epithelial cells as individual *V. cholerae* did, Caco-2 or HT-29 cells were treated with various concentrations (10^6 to 10^9 CFU/ml) of Shanchol™ for 3 h and chemokine mRNA expression was determined using RT-PCR. Both Caco-2 and HT-29 cells did not induce CCL3 or CCL4 mRNA expression in response to Shanchol™, Pam2CSK4 or EcLPS treatment. Fig. 2A and 2B illustrated that different patterns of chemokine mRNA expression were observed in Caco-2 and HT-29 cells. mRNA expression of CCL2, CCL5, CCL20, CCL25 and CXCL10 was dose-dependently induced by Shanchol™ in Caco-2 cells (Fig. 2A). However, mRNA expression of CCL5, CCL20, and CXCL10 was induced by Shanchol™ in HT-29 cells (Fig. 2B).

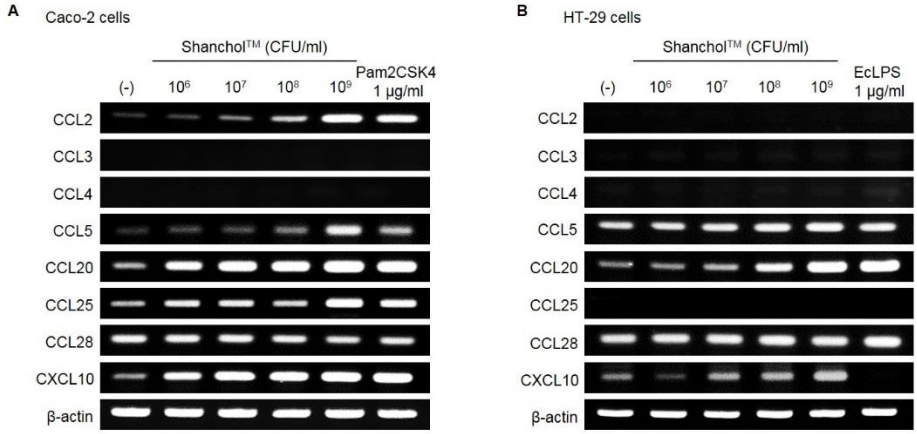

Figure 2. Shanchol™ dose-dependently up-regulates mRNA expression of chemokines in human intestinal epithelial cells. (A) Caco-2 cells (4×10^5 cells/ml) were stimulated with the various concentrations of Shanchol™ (10^6 to 10^9 CFU/ml) or Pam2CSK4 (1 μg/ml) for 3 h. (B) HT-29 cells (4×10^5 cells/ml) were stimulated with the various concentrations of Shanchol™ (10^6 to 10^9 CFU/ml) or *E. coli* LPS (EcLPS) (1 μg/ml) for 3 h. Then, total RNA was isolated and mRNA expression of chemokines was determined using RT-PCR.

3.3. Shanchol™ time-dependently induces chemokine mRNA expression after stimulation in human intestinal epithelial cells

Next, in order to investigate the time kinetics of Shanchol™-induced chemokine expression, Caco-2 or HT-29 cells were stimulated with Shanchol™ (10^8 CFU/ml) for the indicated time points. Fig. 3A indicates that mRNA expression of all chemokines except CCL5 was peaked at 3 to 6 h after stimulation and decreased at 12 h in Caco-2 cells. Interestingly, CCL5 mRNA expression was increased at 1 h after stimulation and decreased at 3 h in Caco-2 cells. Similar to Caco-2 cells, CCL5, CCL20 and CXCL10 mRNA expression was peaked at 3 to 6 h after stimulation and subsequently, decreased in HT-29 cells (Fig. 3B). Collectively, the results of Fig. 1 to Fig. 3 suggest that oral cholera vaccine Shanchol™ and individual strains of *V. cholerae* induces mRNA expression of chemokines in human intestinal epithelial cells.

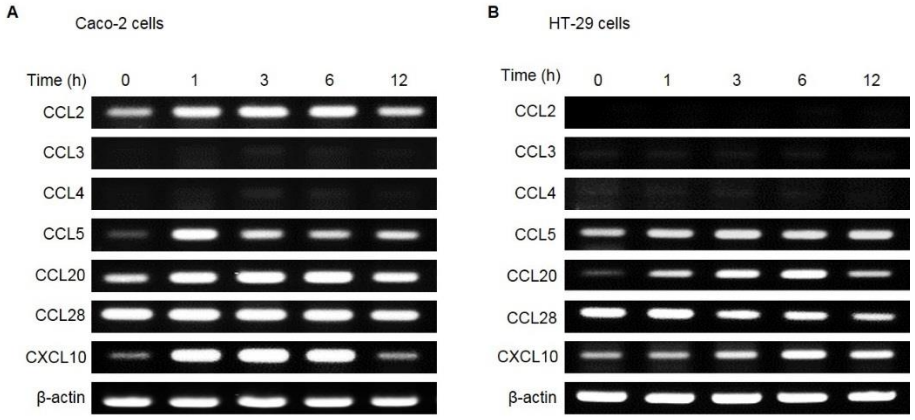

Figure 3. Shanchol™ time-dependently up-regulates mRNA expression of chemokines in human intestinal epithelial cells. (A) Caco-2 cells (4×10^5 cells/ml) and (B) HT-29 cells (4×10^5 cells/ml) were incubated with Shanchol™ (10^8 CFU/ml) for various time periods (0 to 12 h). Total RNA was extracted and mRNA expression of CCL2, CCL3, CCL4, CCL5, CCL20, CCL28 and CXCL10 was determined using RT-PCR.

3.4. Shanchol™ hardly induces chemokines secretion in human intestinal epithelial cells

To investigate whether Shanchol™ can induce chemokine secretion in human intestinal epithelial cells, Caco-2 or HT-29 cells were stimulated with various concentrations of Shanchol™ for 24 h and secretion of chemokines was measured using ELISA. As shown in Fig. 4, Shanchol™ did not induce CCL2, CCL5 or CXCL10 secretion in Caco-2 cells (Fig. 4A to 4C, respectively). Although Shanchol™ slightly induced CCL20 secretion in a dose-dependent manner, the protein level of CCL20 was negligible (< 50 pg/ml) (Fig. 4D). Fig. 5A and 5B indicated that CCL2 and CCL5 secretion was not induced by Shanchol™. In addition, Shanchol™ slightly induced CXCL10 and CCL20 secretion, but the level of chemokines was less than 100 pg/ml, indicating that Shanchol™ negligibly induced the secretion of chemokines in HT-29 cells (Fig. 5C and 5D). Taken together, these results suggest that Shanchol™ hardly induces chemokine secretion in human intestinal epithelial cells.

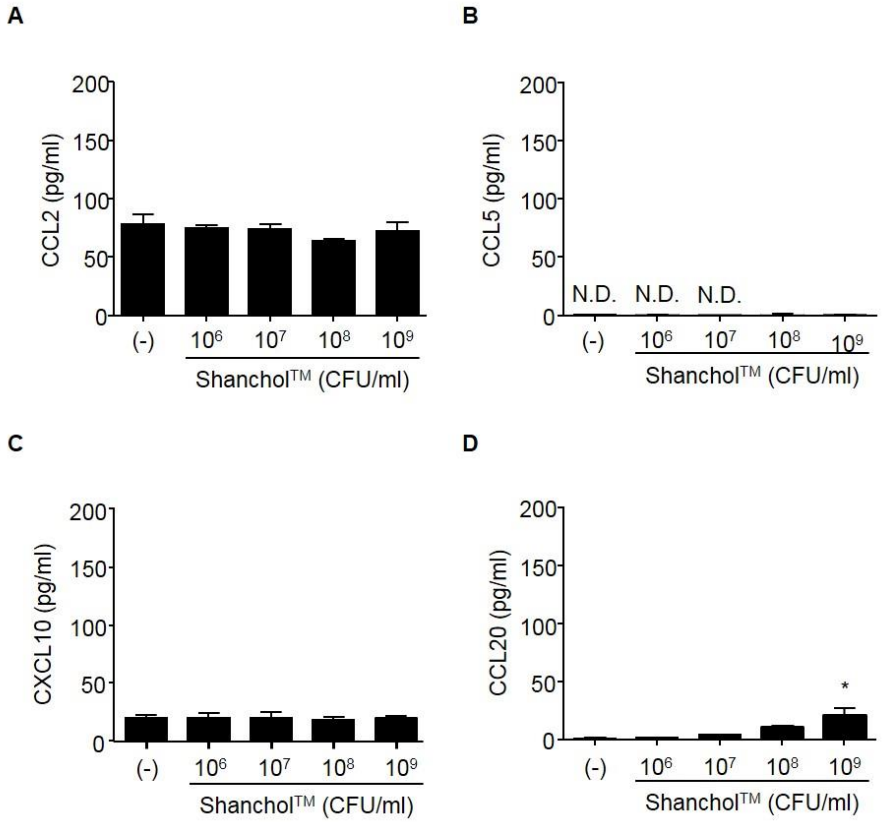

Figure 4. Shanchol™-induced chemokine secretion is negligible in Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were treated with the various concentrations of Shanchol™ (10^6 to 10^9 CFU/ml) for 24 h. Then, the cell culture supernatants were collected and CCL2 (A), CCL5 (B), CXCL10 (C), or CCL20 (D) was measured using ELISA. N.D. denotes not detected. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with control.

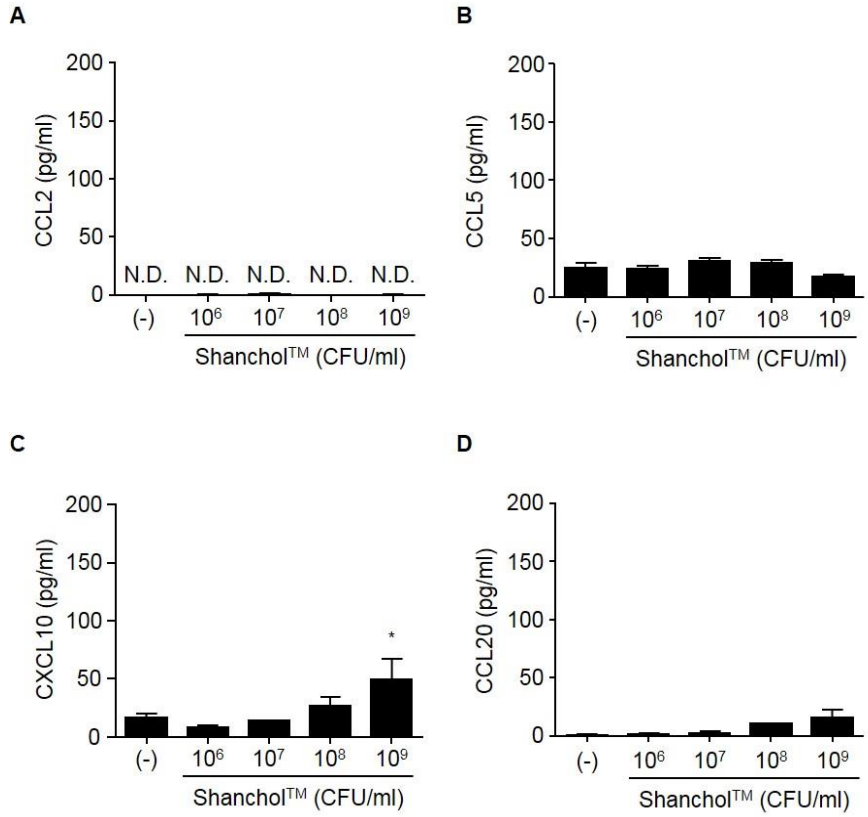

Figure 5. Shanchol™-induced chemokine secretion is negligible in HT-29 cells. HT-29 cells (4×10^5 cells/ml) were stimulated with the various concentrations of Shanchol™ (10^6 to 10^9 CFU/ml) for 24 h. Then, the cell culture supernatants were collected and CCL2 (A), CCL5 (B), CXCL10 (C), or CCL20 (D) was measured using ELISA. N.D. denotes not detected. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with control.

3.5. Shanchol™ potently induces CCL20 secretion in the presence of butyrate, but not propionate or acetate, in Caco-2 cells

It has been reported that microbiota associated metabolites such as SCFAs play a role in immuno-modulation in the gut [39]. In order to examine the effect of SCFAs on Shanchol™ -induced chemokine production in human intestinal epithelial cells, Caco-2 cells were treated with Shanchol™ in the presence of 10 mM of acetate, butyrate, or propionate for 24 h and chemokine secretion was determined using ELISA. Butyrate enhanced Shanchol™-induced CCL20 secretion. However, CCL2, CCL5 and CXCL10 secretion was not increased by Shanchol™ and butyrate co-treatment (Fig. 6A to 6D, respectively). As shown in Fig. 7A and 7B, butyrate potently increased Shanchol™-induced CCL20 production in a dose-dependent manner. However, Fig. 7C and 7D indicated that neither acetate nor propionate strongly up-regulated Shanchol™-induced CCL20 secretion at the same concentration rage used for butyrate. Furthermore, Shanchol™ up-regulated CCL20 mRNA expression, which was facilitated by co-stimulation with butyrate (Fig. 8C). However, mRNA expression of other chemokines including CCL2, CCL5 or CXCL10 mRNA expression was induced by Shanchol™ treatment but not Shanchol™ and butyrate co-treatment (Fig. 8A, 8B and 8D, respectively). Collectively, these results suggest that co-treatment with butyrate and Shanchol™ selectively augments CCL20 production in human intestinal epithelial cells.

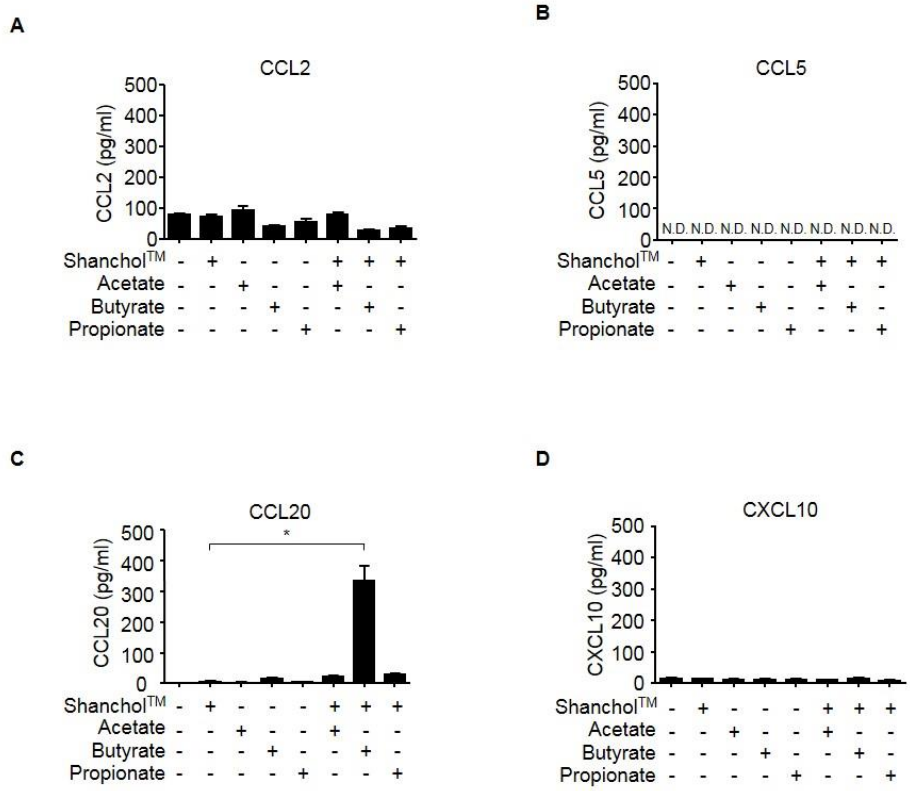

Figure 6. Shanchol™ substantially induces CCL20 secretion in the presence of butyrate in Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of acetate, butyrate or propionate at 10 mM. After the stimulation, the cell culture supernatants were collected and the secretion of CCL2 (A), CCL5 (B), CCL20 (C) and CXCL10 (D) was measured using ELISA. N.D. denotes not detected. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

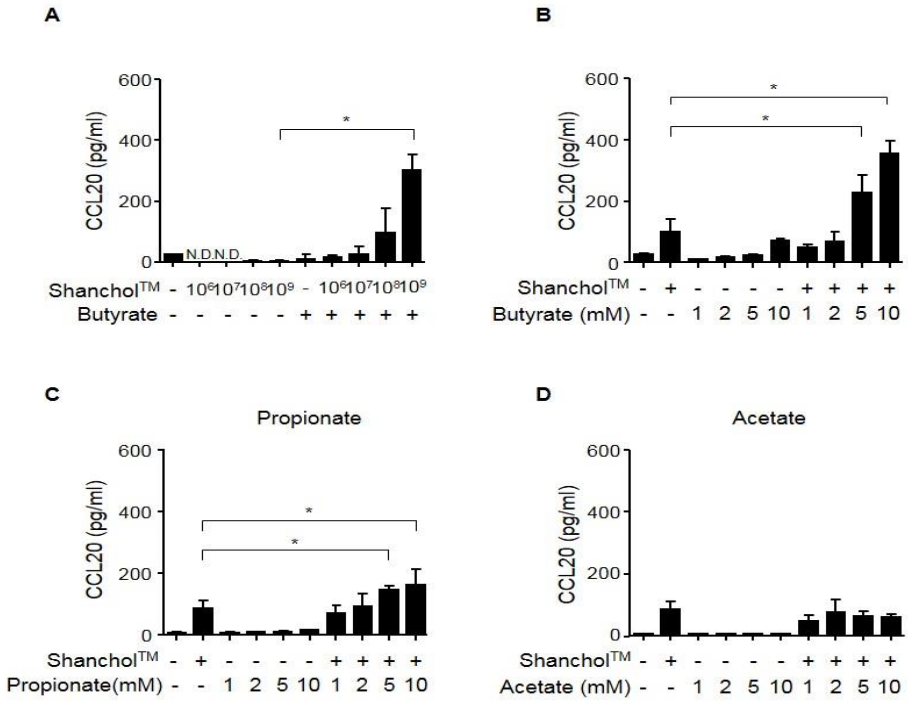

Figure 7. Butyrate potently increases Shanchol™-induced CCL20 secretion in a dose-dependent manner. Caco-2 cells (4×10^5 cells/ml) were incubated with various concentrations of Shanchol™ (10^6 to 10^9 CFU/ml) in the presence of butyrate (10 mM) (A), or with various concentrations of butyrate (1 to 10 mM) in the presence of Shanchol™ (10^9 CFU/ml) (B) for 24 h. Caco-2 cells were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of propionate (C) and acetate (D) at 1 to 10 mM for 24 h. After stimulation, the cell culture supernatants were collected and chemokine secretion was measured using ELISA. N.D. denotes not detected. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

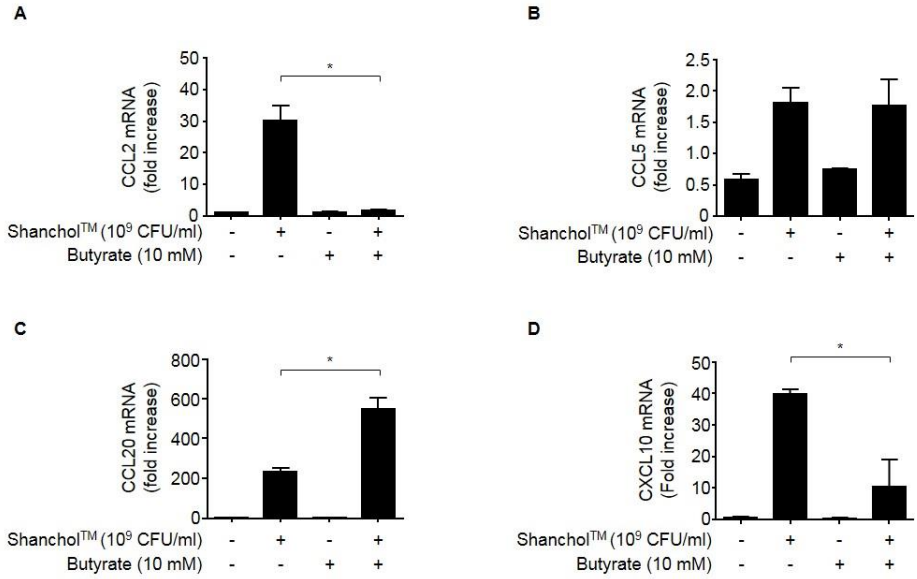

Figure 8. Butyrate facilitates Shanchol™-induced CCL20 mRNA expression in Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were co-stimulated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for 3 h. Total RNA was extracted and CCL2 (A), CCL5 (B), CCL20 (C) and CXCL10 (D) mRNA expression was determined using real-time RT-PCR. The relative expression of RT-PCR products for each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method. N.D. denotes not detected. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

3.6 Butyrate increases Shanchol™-induced CCL20 production in polarized epithelial cells and primary intestinal epithelial cells

It has been reported that Caco-2 cells can be polarized to enterocytes by culturing more than 3 weeks [40]. Thus, to elucidate whether butyrate can increase CCL20 production in the polarized Caco-2 cells, Caco-2 cells were cultured for 4 weeks till full polarization in a transwell system, and apically or basolaterally stimulated with Shanchol™ and/or butyrate for 24 h. Fig. 9A and 9B indicated that a significant CCL20 production was observed in the apical and basolateral compartments of Caco-2 cells upon apical treatment with Shanchol™ and/or butyrate. Comparing with apically secreted CCL20, the relatively high amounts of CCL20 production was detected in the basolateral compartment (Fig.9A and 9B). To further confirm whether primary intestinal epithelial cells induce CCL20 secretion, SNU-407 and SNU-61 cells derived from human intestinal epithelium were stimulated with Shanchol™ and/or butyrate for 24 h, and CCL20 secretion was determined using ELISA. CCL20 production was synergistically increased by co-treatment with Shanchol™ and butyrate, but CXCL10 production was not increased in human primary intestinal epithelial cells (Fig. 10A). Besides, SNU-61 cells potently increased CCL20 production in the presence of butyrate as shown in SNU-407 (Fig. 10B).

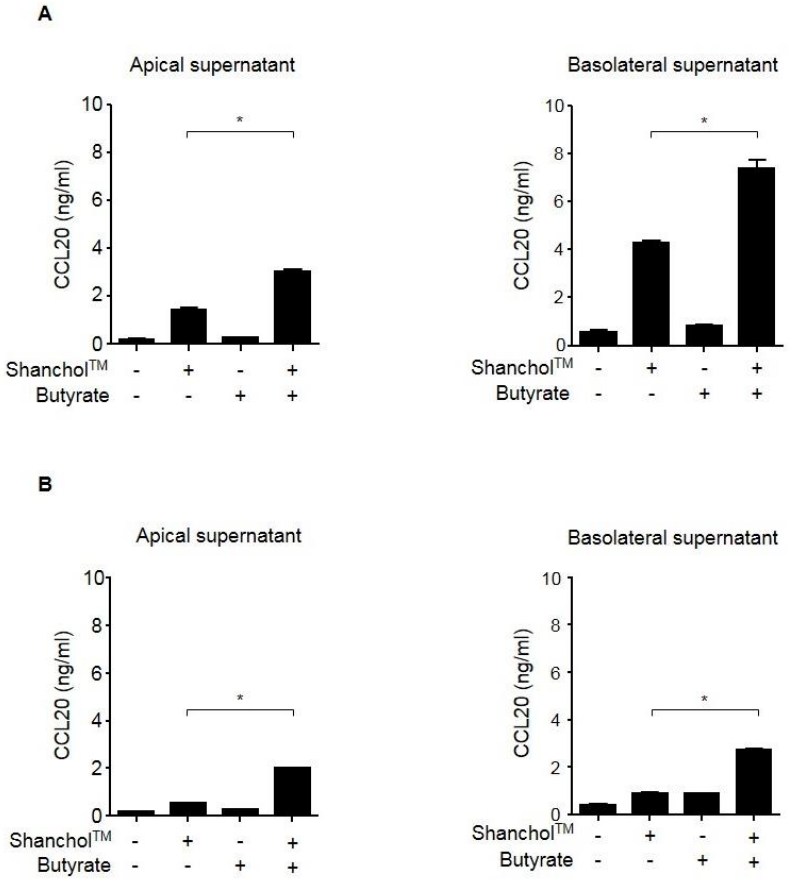

Figure 9. Butyrate enhances Shanchol™-induced CCL20 secretion in the polarized Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were plated on transwell for 4 weeks, and apically (A) or basolaterally (B) treated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for 24 h. Then, the cell culture supernatants from the apical and basolateral compartments were collected to determine CCL20 production using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

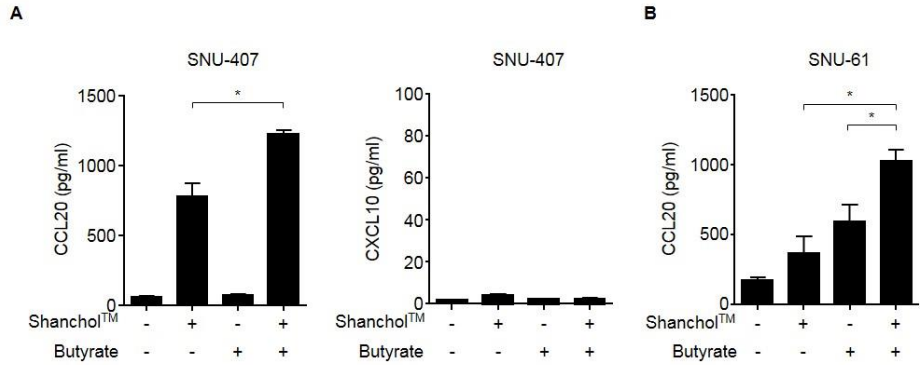

Figure 10. Butyrate enhances Shanchol™-induced CCL20 secretion in primary human intestinal epithelial cells. (A) SNU-407 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of 10 mM of butyrate for 24 h. (B) SNU-61 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for 24 h. After stimulation, the cell culture supernatants were collected, and CCL20 or CXCL10 secretion was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

3.7. The activation of GPR109A and MAPK is involved in the CCL20 secretion in Caco-2 cells

GPR43 and GPR109A are receptors recognizing butyrate and regulate various immune responses such as leukocyte migration and lymphocyte activation [41]. To determine whether GPR109A or GPR43 is involved in CCL20 production, mRNA expression of GPR109A and GPR43 was determined using RT-PCR. Fig. 11A indicated that mRNA expression of GPR109A was cooperatively increased by the co-treatment with Shanchol™ and butyrate, but mRNA expression of GPR43 was not up-regulated by the co-treatment with Shanchol™ and butyrate. Next, to examine whether GPR109A is involved in CCL20 production, the cells were pre-treated with a specific inhibitor of GPR109A (mepenzolate bromide) or a GPCR inhibitor (pertussis toxin) for 1 h and then, subsequently treated with Shanchol™ and/or butyrate for an additional 24 h. CCL20 production was significantly suppressed by the both GPR109A inhibitor and the GPCR inhibitor (Fig. 11B and 11C). To further investigate the intracellular signaling events, activation of p38 kinase, ERK and JNK in the CCL20 induction was examined using their specific inhibitors, SB203580, PD98059, and SP600125, respectively. CCL20 production was markedly inhibited by all of these inhibitors (Fig. 12A to 12C). Collectively, these results suggest that GPR109A and MAPK-mediated signaling is involved in CCL20 secretion in Caco-2 cells.

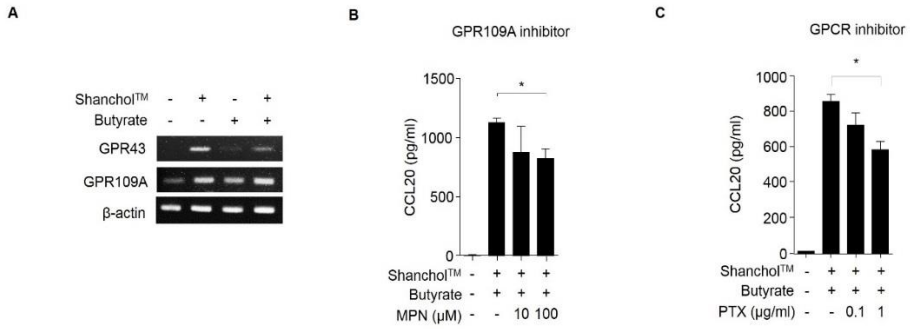

Figure 11. The activation of GPR109A is involved in the CCL20 secretion in Caco-2 cells. (A) Caco-2 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of butyrate (10 mM) for 3 h. Total RNA was extracted and, GPR43 and GPR109A mRNA expression was determined using RT-PCR. Caco-2 cells (4×10^5 cells/ml) were pre-treated with the indicated concentrations of a GPR109A inhibitor, mepenzolate bromide (MPN) (B), pertussis toxin (PTX) (C) for 1 h and subsequently, co-stimulated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for an additional 24 h. Then, the culture supernatants were collected and CCL20 secretion was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

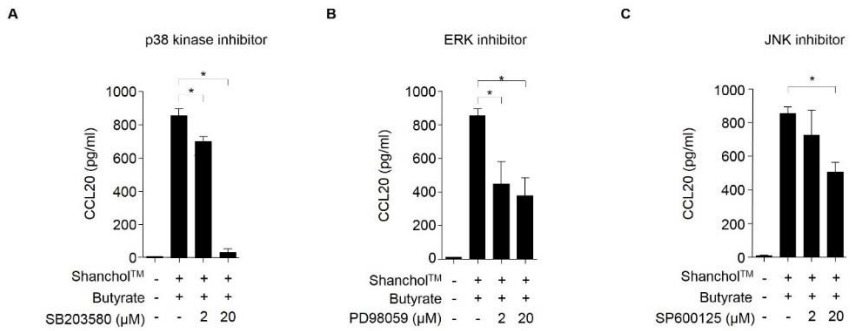

Figure 12. The activation of MAPKs is involved in the CCL20 secretion in Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were pre-treated with the indicated concentrations of a p38 kinase specific inhibitor, SB203580 (A), an ERK specific inhibitor, PD98059 (B) or a JNK specific inhibitor, SP600125 (C) for 1 h and subsequently, co-stimulated with Shanchol™ (10^9 CFU/ml) and/or butyrate (10 mM) for an additional 24 h. Then, the culture supernatants were collected and CCL20 secretion was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

3.8. ATP-P2X7 signaling is required for the CCL20 secretion in Caco-2 cells

Butyrate is well known as an energy source of colonocytes [29] and ATP enhances cytokine and chemokine production in intestinal epithelial cells [42]. To investigate whether butyrate can induce ATP secretion in Caco-2 cells, extracellular concentration of ATP was determined. As shown in Fig. 13A, extracellular ATP was increased by the co-treatment with Shanchol™ and butyrate. To examine whether extracellular ATP is involved in CCL20 production, Caco-2 cells were pre-treated with a P2X7 receptor antagonist (oxATP), then CCL20 secretion was measured by ELISA. Fig. 13B indicated that CCL20 secretion was dramatically suppressed by the pre-treatment with oxATP. To further confirm whether ATP is involved in CCL20 production, Caco-2 cells were treated with Shanchol™ in the presence or absence of ATP. Exogenously treated ATP significantly increased Shanchol™-induced CCL20 production, while it did not promote CXCL10 production (Fig. 14A and 14B). Collectively, these data suggest that butyrate-induced ATP secretion is crucial for the enhancement of Shanchol™-induced CCL20 production.

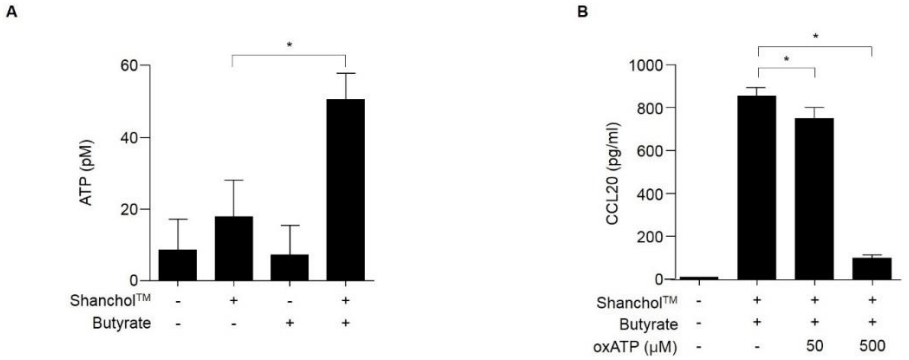

Figure 13. ATP-P2X7 signaling is required for CCL20 secretion in Caco-2 cells. (A) Caco-2 cells (4×10^5 cells/ml) were incubated with Shanchol™ (10^9 CFU/ml) in the presence or absence of butyrate (10 mM) for 2 h. Then, the cell culture supernatants were collected and extracellular ATP production was measured using an ATP activity assay kit. (B) Caco-2 cells (4×10^5 cells/ml) were pre-treated with oxATP (50 or 500 μM) for 1 h and subsequently, stimulated with Shanchol™ (10^9 CFU/ml) and butyrate (10 mM) for an additional 24 h. Then, the culture supernatants were collected, CCL20 secretion was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisks (*) indicates that $P < 0.05$ compared with an appropriate control.

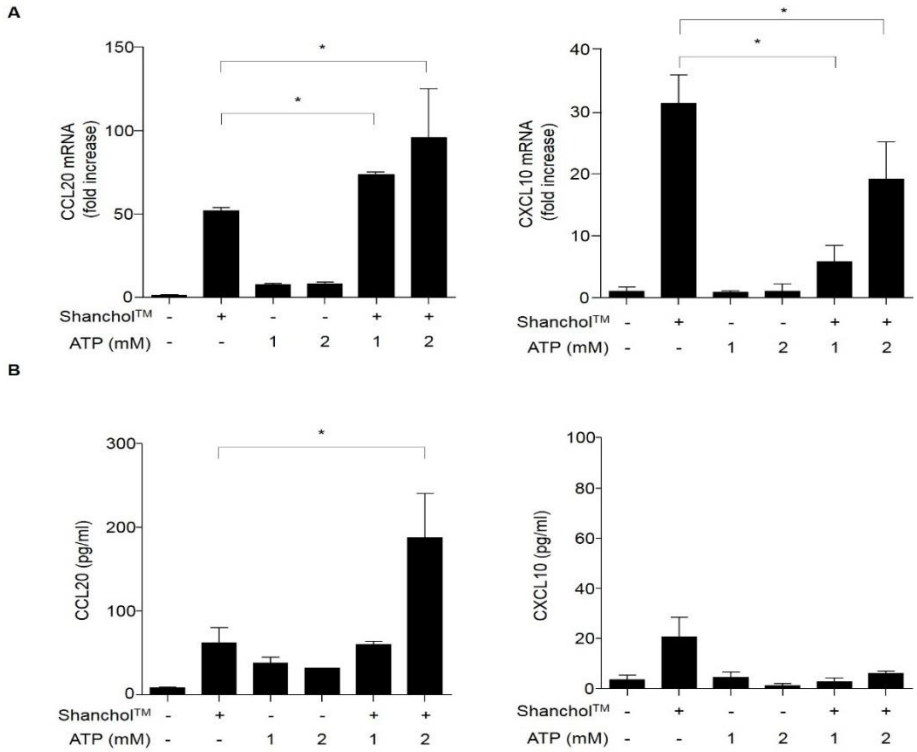

Figure 14. Treatment with ATP significantly increases Shanchol™-induced CCL20 production in Caco-2 cells. (A) Caco-2 cells (4×10^5 cells/ml) were stimulated indicated concentrations of ATP for 3 h. Total RNA was isolated, and CCL20 and CXCL10 mRNA expression was determined using real-time RT-PCR. The relative expression of RT-PCR products for each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method. (B) Caco-2 cells (4×10^5 cells/ml) were stimulated with Shanchol™ in the presence or absence of indicated concentration of ATP for 24 h. Then, the culture supernatants were collected and CCL20 or CXCL10 production were measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. The asterisks (*) indicates that $P < 0.05$ compared with an appropriate control.

3.9. HDAC inhibition is associated with the CCL20 production in Caco-2 cells

It has been reported that butyrate acts as an HDAC inhibitor [43] and regulates the transcription of chemokine or antimicrobial peptides genes in epithelial cells [44]. To investigate whether HDAC inhibition is associated with the synergistic production of CCL20, Caco-2 cells were treated with Shanchol™ in the presence of butyrate or TSA which is well known as an HDAC inhibitor [45]. As shown in Fig. 15A, CCL20 mRNA expression was enhanced by co-treatment with Shanchol™ and TSA. However, other chemokines such as CCL2 or CXCL10 were not upregulated in the presence of Shanchol™ and TSA (Fig. 15B and 15C). Taken together, these results suggest that HDAC inhibition is involved in synergistic induction of CCL20 production in Caco-2 cells.

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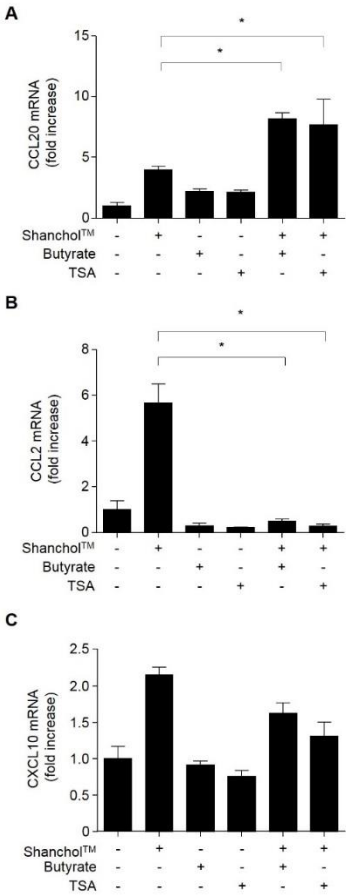

Figure 15. HDAC inhibition is associated with the CCL20 production in Caco-2 cells. Caco-2 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of butyrate (10 mM) or trichostatin A (TSA) (5 μM) for 3 h. Total RNA was isolated and CCL20 (A), CCL2 (B) and CXCL10 (C) mRNA expressions were determined using real-time RT-PCR. The relative expression of RT-PCR products for each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method. All results were expressed as mean ± S.D. of triplicate samples. The asterisks (*) indicates a statistical significance at $P < 0.05$ compared with an appropriate control.

3.10. Co-treatment with Shanchol™ and butyrate induces the chemotactic migration of immature DCs

It has been reported that CCL20 binds to the receptor CCR6 expressed on immature DCs which were able to migrate in response to recombinant hCCL20 [46]. To further examine whether butyrate enhances Shanchol™-induced chemotactic migration of DCs, Caco-2 cells were treated Shanchol™ in the presence of butyrate for 24 h, and culture supernatants of Caco-2 cells were collected and placed into lower chambers of transwell plates. Human immature DCs prepared from human peripheral blood monocytes were then added into the upper chambers for 2 h. The number of migrated immature DCs was increased when the cells were treated with the culture supernatant of Shanchol™ and butyrate co-treated Caco-2 cells compared to that of Shanchol™-treated Caco-2 cells (Fig. 16A and 16B). Taken together, these results suggest that migration of immature DCs was enhanced by Shanchol™ and butyrate co-treatment.

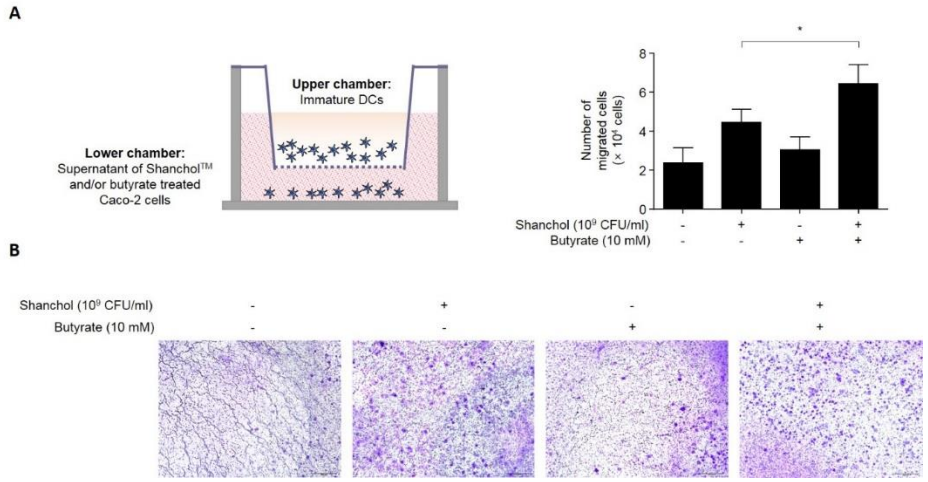

Figure 16. Co-treatment with Shanchol™ and butyrate induces the chemotactic migration of immature DCs. Caco-2 cells (4×10^5 cells/ml) were stimulated with Shanchol™ (10^9 CFU/ml) in the presence or absence of butyrate (10 mM) for 24 h. Then, the culture supernatants were put into the lower chamber of the transwell. Immature DCs prepared from human peripheral blood monocytes were put into the upper chamber followed by incubation for 2 h. The migrated cells in lower chamber were counted using trypan blue staining (A). The cells on the membrane facing the lower chamber were fixed with methanol for 30 min and then stained with 0.2 % crystal violet for 1 h. The image of cells was obtained using microscope (B). All results were expressed as mean ± S.D. of triplicate samples. The asterisk (*) indicate a statistical significance at $P < 0.05$ compared with an appropriate control. The scale bar is 200 μm.

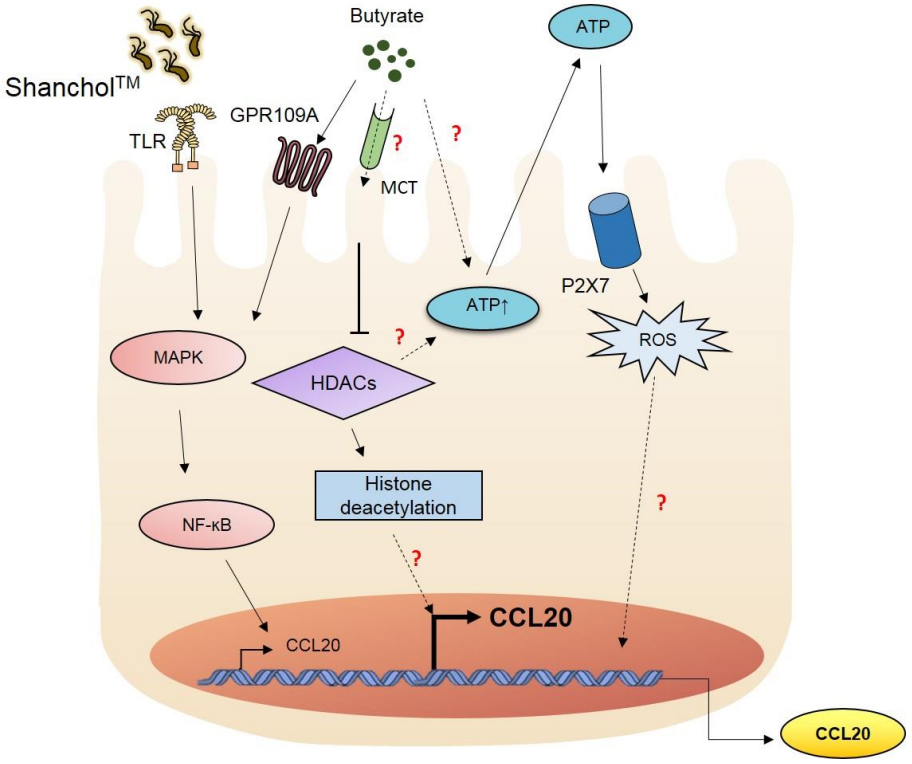

Figure 17. Schematic illustration of the proposed mechanism. An oral cholera vaccine, Shanchol™ potently induces mRNA expression of chemokines, but hardly induces chemokine secretion in Caco-2 cells. However, one of SCFAs, butyrate enhances Shanchol™ -induced CCL20 secretion via HDAC inhibition, ATP-P2X7 signaling through activating GPR109A, potentially, contributing to the enhancement of mucosal immune responses in the gut.

Chapter IV. Discussion

Although it has been reported that microbial metabolites, such as SCFAs play an important role in immuno-modulation of gastrointestinal tract, it has not been clearly showed that the effect of SCFAs on vaccine-induced mucosal immune responses in the gut. The present study demonstrated that one of SCFAs, butyrate potently enhanced cholera vaccine-induced CCL20 production in human intestinal epithelial cells. In accordance with our findings, several studies have evidently showed that butyrate regulates production of cytokines and chemokines in intestinal epithelial cells. Butyrate enhanced the IL-18 production in intestinal epithelium [45], and promoting epithelial integrity [46]. Moreover, butyrate altered IL-8 and monocyte chemotactic protein 1 (MCP-1) expression [47, 48] and it enhanced nucleotide-binding oligomerization domain-containing protein 2 (NOD2) expression through HDAC inhibition and further increased peptidoglycan (PGN) induced-IL-8 and growth-regulated protein alpha (GRO-α) [49] production in human intestinal epithelial cells. In contrast, it has been shown that butyrate inhibits pro-inflammatory cytokine production in macrophages and promotes anti-inflammatory responses [37] through the inhibition of NF-κB activation and IκBα degradation [36]. The present study suggests that butyrate promotes cholera vaccine-induced CCL20 production and might play a key role in the modulation of intestinal immune responses.

Chemokine production is important for the induction of mucosal immune responses following vaccination. Several studies have shown that mucosal

vaccination increases local chemokine production in gut mucosa attracting immune cells including IgA-producing plasma cells to site of vaccination [47, 48]. Recently, many studies have demonstrated that chemokines can be applicable as an adjuvant for several cancer vaccines, or DNA vaccines against simian immunodeficiency virus infection [49-51]. Among the chemokines, CCL20 binds to CCR6 [27] and attracts immature DCs, memory T cells and B cells [30] to the site of mucosal vaccination. It has been reported that DCs were decreased in Peyer's patches of CCR6 knockout mice, and humoral immune responses against orally administrated antigen were diminished in CCR6 knockout mice evidently indicating that CCL20-CCR6 axis facilitates the mucosal immune responses and induces adaptive immune responses in the small intestine [19]. Moreover, nasal administration with CCL20 increased DCs recruitment in nasal-associated lymphoid tissue and antigen-specific antibody responses were significantly elevated, suggesting that CCL20 can be considered as an effective vaccine adjuvant for mucosal vaccination [46]. In addition, another study reported that conjugation of DNA vaccine with CCL20 enhances the immunogenicity of the vaccine leading to an increase in antibody production [52]. Therefore, these findings can suggest the modulation of CCL20 production in the gut might be involved in vaccine-induced antibody responses.

The current study observed that only butyrate strongly increased Shanchol™-induced CCL20 production. Previous reports have suggested that carbon chain length of SCFAs is related to immuno-modulating potencies of SCFAs. [34, 53].

Acetate (C2), propionate (C3) and butyrate (C4) can activate G-protein coupled receptors such as GPR41 and GPR43, but only butyrate is a specific ligand for GPR109A [32]. The current study demonstrated that GPR109A is involved in the synergistic effect of CCL20 production in Caco-2 cells. Additionally, it has been shown that GPR41 and GPR43 were activated by similar ligands for SCFAs but with differing specificity for carbon chain length [34]. Furthermore, different chain lengths of SCFAs (C2-C6) varied in the induction of chemokine production according to their ability to induce histone acetylation [54]. Among SCFAs, butyrate has the most potent activity of histone acetylation by interfering with HDAC [54]. In addition, it has been reported that acetate has HDAC inhibitor activity at higher concentrations than propionate and butyrate [33]. Moreover, previous reports suggest that butyrate and propionate promoted regulatory T cell differentiation by increasing Foxp3 acetylation via HDAC inhibition, whereas acetate lacks significant HDAC inhibition activity [53]. Therefore, since other metabolites with differences between chain length of SCFAs results in differences in activating potency of G-protein coupled receptors and ability of epigenetic regulation, and consequently, differential immunomodulatory activities.

In this study, it was observed that butyrate or TSA selectively augmented Shanchol™-induced CCL20 production in gastrointestinal epithelial cells through HDAC inhibition. It corresponds well with previous reports demonstrating that butyrate increases CCL20 production at the transcriptional level via HDAC inhibition in gingival epithelial cells [55] and intestinal

epithelial cells [56]. However, the current study also found that butyrate or TSA downregulates other chemokines mRNA expression such as CCL2 or CXCL10 via HDAC inhibition in intestinal epithelial cells. A previous report has suggested that HDAC inhibition enhanced TLR-induced NF-κB reporter activation and TNF-α expression but abolished IL-8 and MCP-1 expression in HT-29 cells [57]. It can be assumed that inhibition of HDAC cannot upregulate all kinds of the chemokine expression at the transcriptional level.

In addition, butyrate-induced ATP secretion is a critical factor of synergistic effect on CCL20 secretion. It has been demonstrated that butyrate regulates energy metabolism in the gut and increases ATP production. Furthermore, when the epithelial cells were stressed, large amount of ATP is released to the extracellular space and activate purinergic signaling [58], modulating chemokine release [59, 60]. Previous reports suggest that extracellular ATP alters intestinal epithelial cells to inflammatory state, enhances chemokine secretion, and consequently increases T cell responses to flagellin [61]. Furthermore, it has been demonstrated that P2X7R is an important player in inflammation and apoptosis because it is able to release pro-inflammatory cytokines from immune cells in response to ATP [60, 62]. Although the relationship between HDAC inhibition and ATP production is not clearly elucidated in this study, HDAC inhibition might be involved in ATP production since it has been reported that HDAC inhibitor induces apoptosis through histone acetylation, and P2X7 receptors activation by ATP induces apoptosis in human epithelial cells [63, 64].

In summary, this study demonstrated that butyrate facilitates CCL20 production induced by cholera vaccine in human intestinal epithelial cells. Given the fact that CCL20 is an effective mucosal vaccine adjuvant since it induces chemotaxis of immature DCs, B cells and T cells, the current study provides an important insight into the application of butyrate as an enhancer of mucosal vaccine-induced immune responses. Further studies will be needed to investigate if butyrate enhances mucosal vaccine-induced antibody production or vaccine efficacy and how butyrate selectively regulates cholera vaccine-induced CCL20 production. A recent report has suggested that SCFAs activate B cell metabolism by increasing ATP production and consequently, boost antibody responses during enteric infection [38]. Therefore, butyrate may enhance the mucosal immune responses by oral cholera vaccine, consequently, promoting vaccine efficacy in the gut.

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국문초록

인간 장 상피세포에서 단쇄지방산에 의한 콜레라 백신 유도 케모카인 발현 증가

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1. 목 적

콜레라 백신은 듀코랄(Dukoral™)과 샨콜(Shanchol™)이 경구용 백신으로 승인/판매 되고 있으나, 효능 면에 있어서 장기 면역 효과가 떨어지는 점과 많은 양을 투여해야 한다는 단점이 있다. 콜레라 백신접종 초기에 백신에 의해 활성화된 장 점막 상피세포는 면역반응 유도에 필수적인 역할을 하는 면역세포들을 불러 모을 수 있는 케모카인을 발현할 수 있으며, 장내에 존재하는 미생물의 주요 대사체 중 하나인 단쇄지방산은 장 점막 상피세포의 장벽 기능 강화 등 장내 건강과 관련되어 있는 것으로 알려져 있다. 하지만, 현재까지 단쇄지방산과 점막 백신이 유도하는 면역 반응과의 관련성에 대해

서는 아직 잘 알려져 있지 않다. 따라서 본 연구는 단쇄지방산이 콜레라 백신이 유도하는 장내 케모카인 발현을 조절할 수 있는지에 대해 알아보았다.

2. 방 법

인간 장 점막 상피세포주인 HT-29와 Caco-2 세포에 상용 콜레라 백신 샨콜을 처리하여 케모카인의 발현양상을 역전사효소 중합연쇄반응(RT-PCR)과 효소결합 면역분석법(ELISA)를 이용하여 확인하였다. 주요 장내 대사체 중 하나인 단쇄지방산이 콜레라 백신에 의해 유도하는 케모카인 발현에 어떠한 영향을 주는지 알아보기 위해, Caco-2 세포에 콜레라 백신 샨콜과 단쇄지방산을 동시에 처리한 후, 케모카인의 mRNA 발현량을 Realtime PCR과 ELISA를 통해 측정하였다. 실제 인체 장 상피세포에서도 동일한 경향이 나타나는 지를 확인하기 위해 인간의 장 조직에서 분리한 상피세포인 SNU-407 세포와 SNU-61 세포에 샨콜과 단쇄지방산을 복합 처리 후 케모카인의 생산을 ELISA를 통해 확인하였다. 3주 이상 분극화시킨 완전분화 장 상피세포에서도 동일한 경향이 나타나는 지 확인하기 위해 transwell 시스템을 이용하여 Caco-2 세포를 4주 이상 배양하여, 분극화되었음을 확인한 후 샨콜과 단쇄지방산을 병용처리 한 뒤 케모카인의 분비량을 ELISA를 통해 측정하였다. 세포 내부 신호전달을 알아보기 위해 Caco-2 세포에 신호전달 관련 특이 억제제(MAPK 억제제, G 단백질 수용체 저해제, ATP 수용체 길항제)를 1시간 전처리 후 샨콜과 단쇄지방산을 병용 처리하여 분비된 케모카인의 양을 ELISA를 통해 분석하였다. 또한, 샨콜과 단쇄지방산을 병용 처리하였을 때 세포 밖으로 분비되는 ATP의 양을 측정하였으며, 단쇄지방산의 후성유전학적 신호 전달과 유사한 것으

로 알려진 histone deacetylase (HDAC) 저해제를 콜레라 백신과 복합 처리한 후 케모카인의 mRNA 발현량을 관찰하였다. 마지막으로, 샨콜과 단쇄지방산 병용 처리에 의해 유도되는 미성숙 수지상세포의 이동을 transwell을 이용하여 확인하였다.

3. 결 과

Caco-2 세포에서는 샨콜에 의해 CCL2, CCL20, CXCL10, 그리고 HT-29 세포에서는 CCL5, CCL20, CXCL10의 mRNA 발현이 강하게 유도되었으나, 대부분 케모카인의 단백질 발현은 증가 되지 않았으며, CCL20과 CXCL10만 단백질 수준에서 약하게 발현됨을 확인하였다. 또한 대부분의 케모카인은 단쇄지방산에 의해 mRNA와 단백질의 생성이 증가하지 않았지만 단쇄지방산 중 butyrate는 콜레라 백신 샨콜에 의해 유도되는 CCL20의 mRNA 발현과 단백질 발현을 강하게 증가시켰다. 이 현상은 샨콜의 농도가 증가할수록, 그리고 butyrate의 농도가 증가할수록 농도 의존적으로 나타나는 현상이었고, 실제 인체 유래 장 상피세포와 4주 이상 분극화된 Caco-2 세포에서도 동일한 경향을 나타내는 것을 확인하였다.
단쇄지방산의 주요 수용체로 알려진 G-단백질 수용체의 저해제, GPR109A 길향제, 그리고 MAPK 억제제 전처리에 의해 샨콜과 butyrate 병용 처리 시 유도하는 CCL20의 발현 증가가 부분적으로 억제됨에 따라 GPR109A 수용체와 하위 신호전달인 MAPK 신호전달이 CCL20 발현 증가에 관여함을 확인하였다. 또한, butyrate와 비슷하게 HDAC 저해제로 잘 알려진 트리코스타틴 A 역시 샨콜이 유도하는 CCL20의 발현을 눈에 띄게 증가시켰으나, 다른 케모카인의 발현은 유의적으로 감소시켰다. 마지막으로 샨콜과 butyrate

는 Caco-2 세포에 병용 처리 시 세포 외부로 분비되는 ATP의 양을 증가시키며, ATP 수용체 길항제를 전처리 시 CCL20 발현이 억제됨에 따라 세포 외부로 분비된 ATP가 P2X7 신호전달을 통해 CCL20 발현 증가에 관여함을 알 수 있었다. 마지막으로, 샨콜을 단독으로 처리한 배양 상층액에 비해 샨콜과 butyrate를 병용 처리한 배양 상층액을 미성숙 수지상세포에 처리 시 미성숙 수지상세포의 이동이 증가함을 확인하였다. 이를 종합해 보면, 단쇄지방산 중 butyrate는 Caco-2 세포에서 HDAC 저해와 세포 외부로 분비된 ATP-P2X7의 신호전달, 그리고 G-protein 수용체 중 GPR109A의 신호전달을 통해 콜레라 백신 샨콜이 유도하는 케모카인 중 CCL20 만을 특이적으로 강하게 증가시키며 이는 콜레라 백신이 장내에서 유도하는 점막 면역 반응을 더 증강시키는 데 관여할 것으로 예상된다.

주요어 : 콜레라 백신, 단쇄지방산, 케모카인, 점막면역, 장 점막 상피세포

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